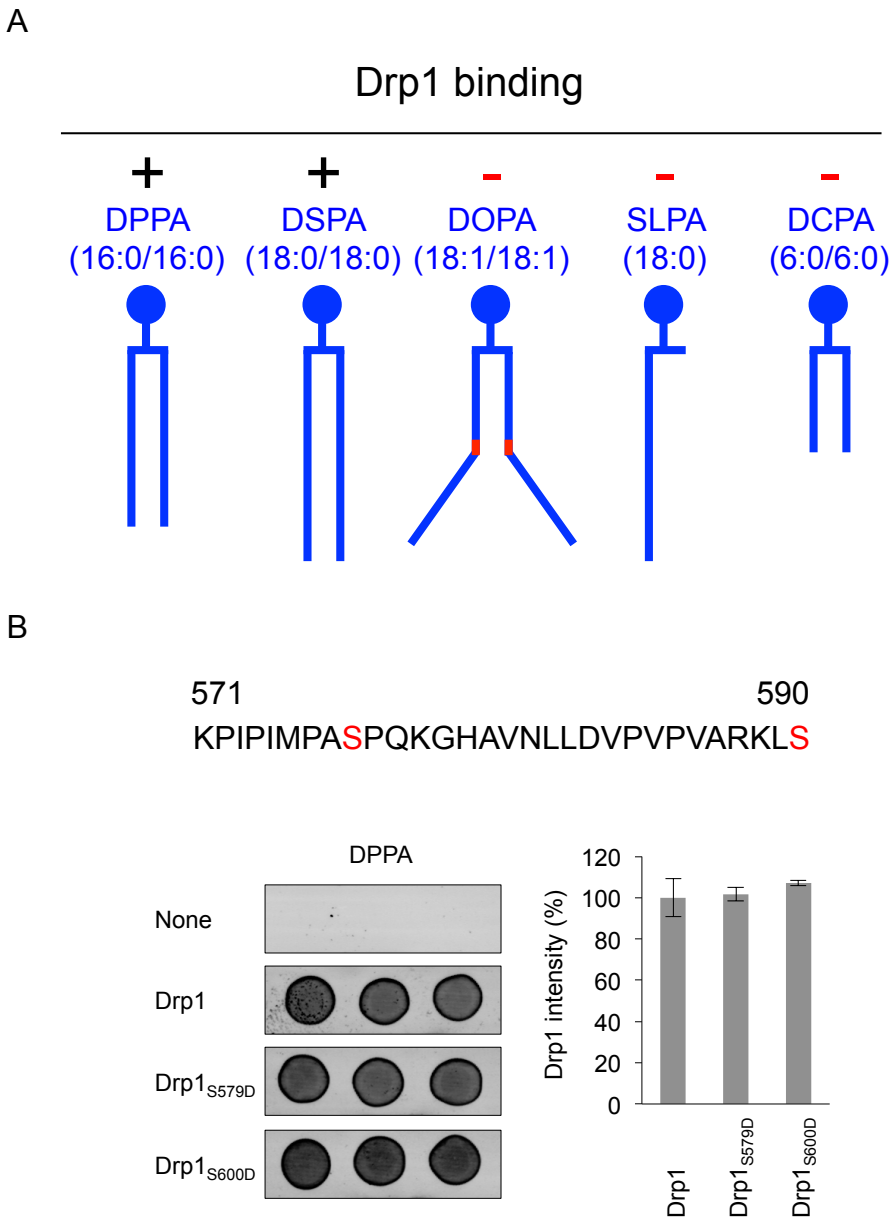

Figure S1. Drp1 binds saturated PA by recognizing the headgroup, the saturation, number, and length of the acyl chains, Related to Figure 1. (A) Summary of Drp1-PA interactions. Drp1 preferentially binds saturated (DPPA and DSPA), compared to unsaturated (DOPA), saturated lysoPA (SLPA), and saturated PA with short acyl chains (DCPA). (B) Phosphomimetic mutations do not affect the interaction of Drp1 with saturated PA. Two phosphorylation sites in Drp1, S579 and S600, are shown in red. His_6-Drp1, His_6-Drp1S_{579D}, or His_6-Drp1$_{S600D}$ was incubated with PVDF membranes spotted with saturated PA (DPPA). Drp1-DPPA interactions were detected using anti-Drp1 antibodies. Mean ± SEM. (n = 3).

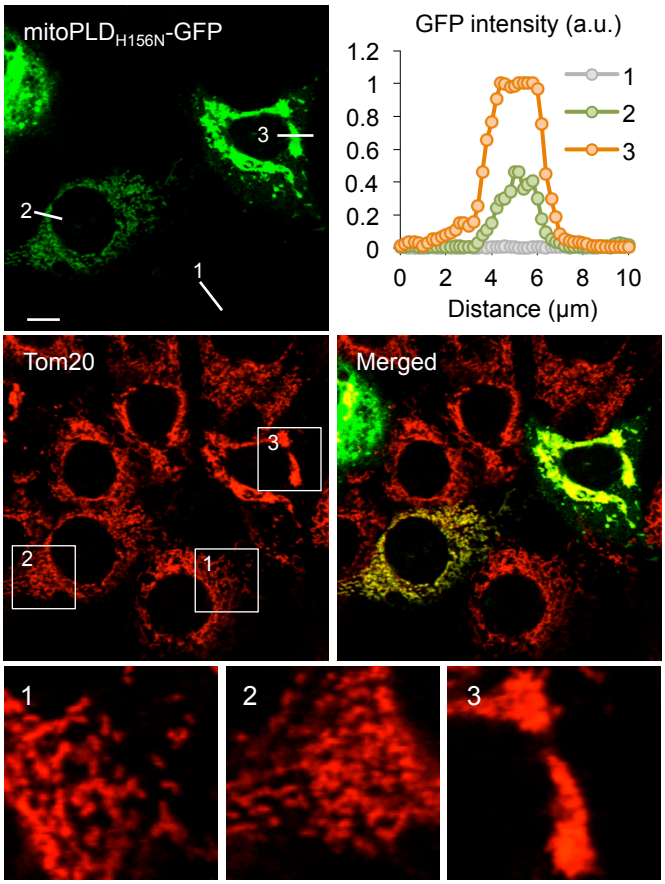

Figure S2. MitoPLD$_{H156N}$-GFP does not elongate mitochondria, Related to Figure 2. WT MEFs were transfected with catalytically inactive MitoPLD$_{H156N}$-GFP. We quantified GFP intensity along the lines #1–3. Mitochondrial shape was visualized using immunofluorescence with anti-Tom20 antibodies. The boxed regions are enlargements. Bar, 10 μm.

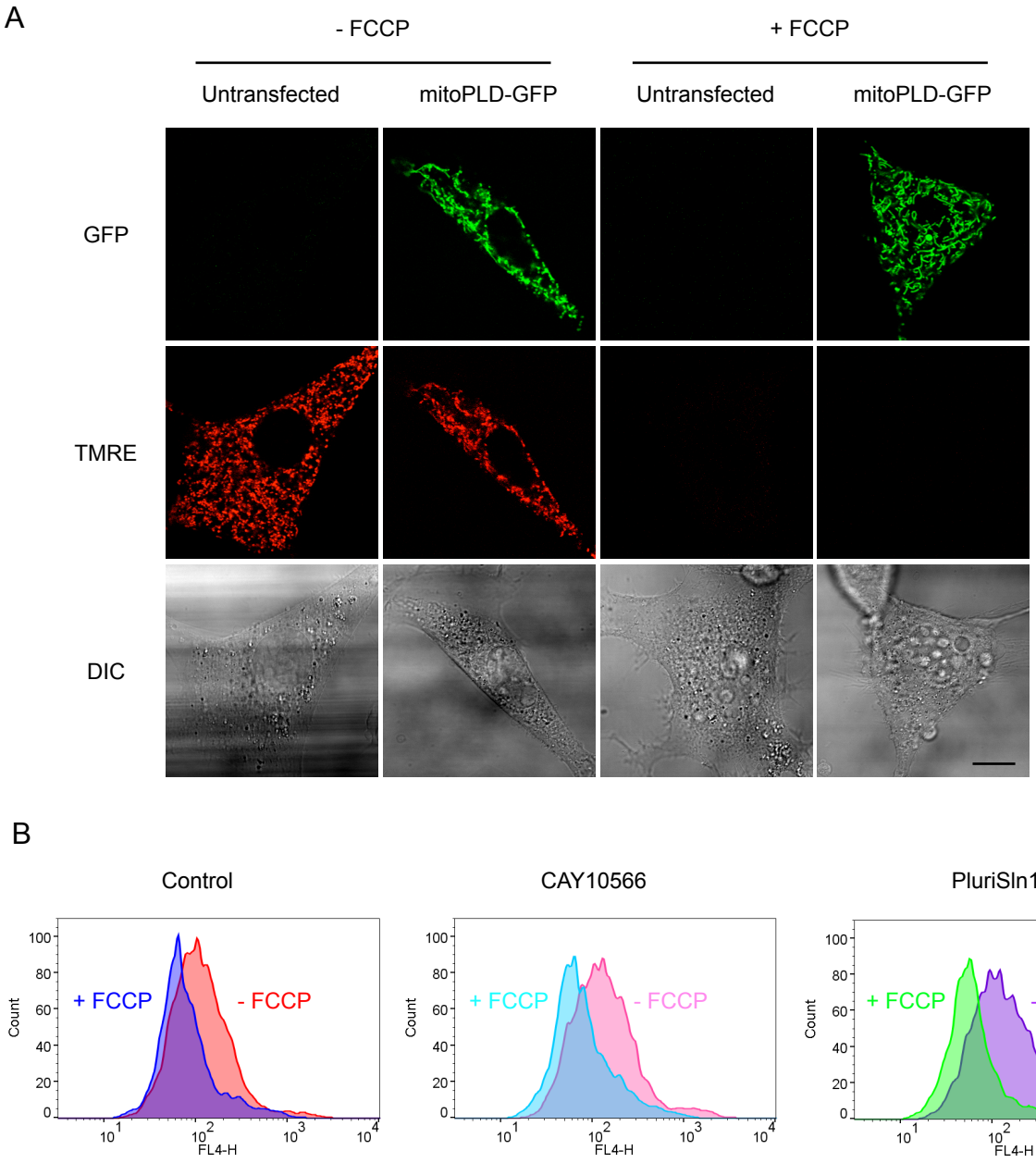

Figure S3. FCCP depolarizes mitochondria in cells expressing MitoPLD-GFP or treated with SCD1 inhibitors, Related to Figure 2 and 3. (A) WT MEFs were transfected with MitoPLD-GFP. The cells were incubated in the presence or absence of 10 µM FCCP for 30 min and then stained with 7 nM tetramethylrhodamine ethyl ester (TMRE) for 30 min. Confocal microscopy shows that FCCP decreases TMRE fluorescence similarly in untransfected control cells and MitoPLD-GFP-expressing cells. Bar, 10 µm. (B) After treatment with the SCD1 inhibitors (100 nM CAY10566 (6 hr) or 20 µM PluriSln1 (4 hr)), WT MEFs were incubated in the presence or absence of 10 µM FCCP for 30 min. The cells were then stained with the membrane potential-sensitive dye MitoLite NIR (AAT Bioquest) according to the manufacturer's instructions. MitoLite NIR fluorescence was measured by flow cytometry (FACSCalibur) in the FL4 channel (Ex/Em = 633/661 nm). The data were analyzed using the FlowJo software. FCCP decreased the membrane potential similarly in untreated control cells and SCD1 inhibitor-treated cells.

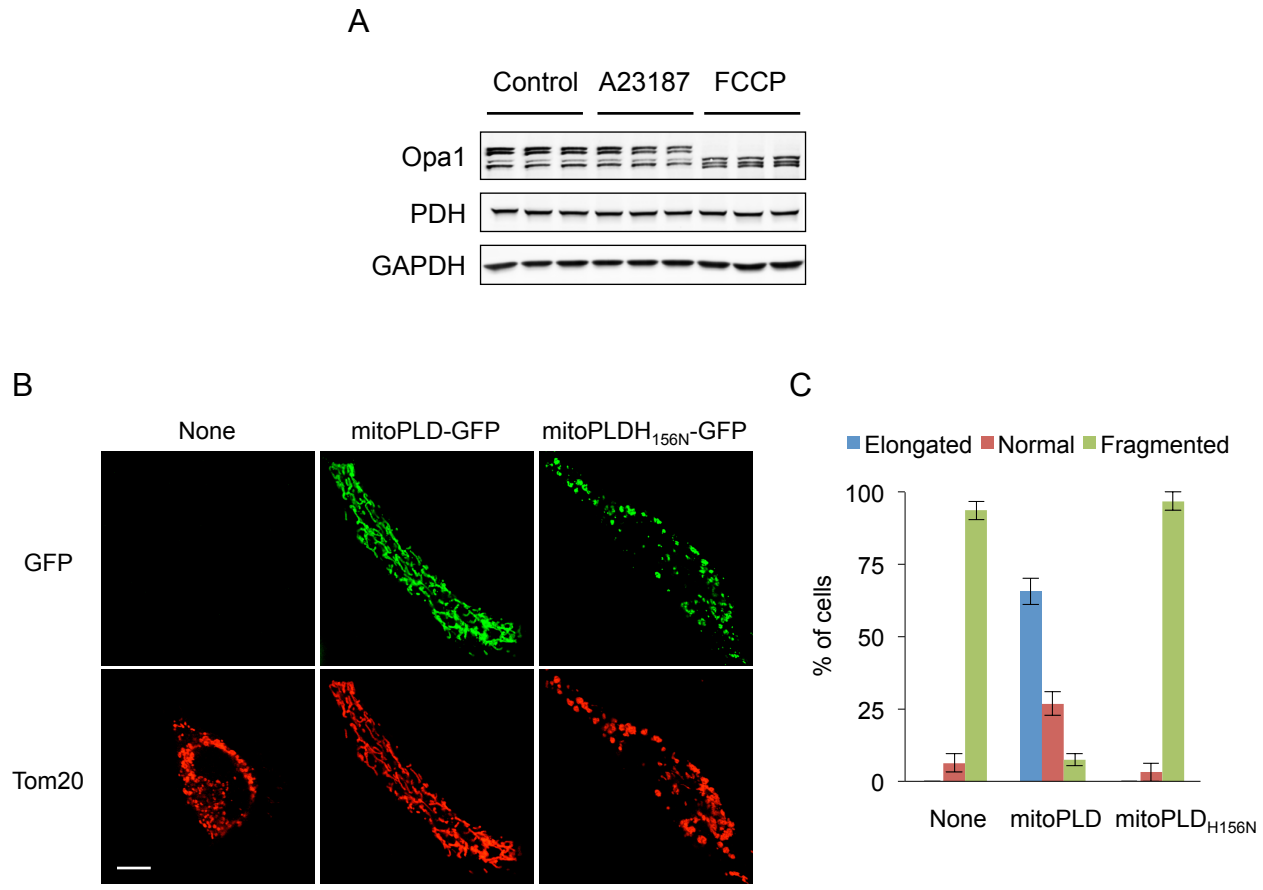

Figure S4. MitoPLD decreases the mitochondrial division induced by 4Br-A23187, Related to Figure 2. (A) HeLa cells were treated with 10 μM 4Br-A23187 for 10 min or 10 μM FCCP for 30 min and analyzed via Western blotting using antibodies specific for Opa1, pyruvate dehydrogenase (PDH) and GAPDH. While FCCP induced the cleavage of Opa1, 4Br-A23187 did not. Three independent samples were analyzed. (B) HeLa cells were transfected overnight with MitoPLD-GFP or MitoPLD$_{H156N}$-GFP and treated with 10 μM 4Br-A23187 for 10 min. The cells were subjected to immunofluorescence microscopy with antibodies specific for the mitochondrial protein Tom20. 4Br-A23187 induced mitochondrial fragmentation in untransfected cells and MitoPLD$_{H156N}$-GFP-expressing cells. In contrast, MitoPLD-GFP-expressing cells were partially resistant to 4Br-A23187. Bar, 10 μm. (C) Quantification of mitochondrial morphology after 4Br-A23187 treatment in cells expressing MitoPLD-GFP or MitoPLDH$_{156N}$-GFP. Mean ± SD (n = 3, 90 cells).

Supplemental Experimental Procedures

Plasmids

Mouse full-length Drp1 (isoform 3, Uniprot) and its GTPase, stalk, and variable domains were PCR amplified from the mouse cDNA (Open Biosystems, ID: 6400313) and cloned into the XhoI site of pET15b. Plasmids carrying MitoPLD-GFP, MitoPLD$_{H156N}$-GFP HA-lipin, and pHA-lipin$_{D712A}$ have been described previously (Huang et al., 2011). We PCR amplified SCD1 from Ultimate ORF (IOH6261) and cloned into the pcDNA3.1. The FLAG-tagged cytoplasmic domain of MitoPLD that lacks the transmembrane segment (TM) (FLAG-mitoPLDΔTM) was PCR amplified from the MitoPLD-GFP plasmid and cloned into the XhoI of pET15b. All of the plasmids were confirmed by DNA sequencing.

List of PCR primers

ID	Sequence
5-Xho+mDrp1WT	AAACTCGAGGAGGCGCTGATCCCGGTCAT
3-Xho+mDrp1WT-R	AAACTCGAGTCACCAAAGATGAGTCTCGC
5-Xho+mDrp1 Stalk	AAACTCGAGGATGATAAAAGTGCTACTTTAC
3-Xho+mDrp1 Stalk	AAACTCGAGTCACTCGGATTCAGTCAGAAGGTC
5-fusion mDrp1 Stalk	CAATAATATAGAGGAACAGCGAGATTGTGAGG
3-fusion mDrp1 Stalk	CCTCACAATCTCGCTGTTCCTCTATATTATTG
5-Xho+mDrp1Variable	AAACTCGAGGATGCCTGTGGGCTAATGAACA
3-Xho+stop+mDrp1Var	AAACTCGAGTCACAGTTTTCTTGCAACTGGAACT
5-Xho+mDrp1 GTPase	AAACTCGAGGCGGACATCATCCAGCTGCC
3-Xho+stop+mDrp1G	AAACTCGAGTCAATCTCTGATATGATGCATAAGT
5-Xho+hMitoPLD ΔTM	GCCGCGCGGCAGCCATATGCTCGAGCGCTGGCTGCGGTCCAGGCGGCGGC
3-Xho+3xFLAG	TTTGTTAGCAGCCGGATCCTCGAGCTACTTGTCATCGTCATCCTTGTAG
5-KZ+hSCD1	CACCGCCACCATGCCGGCCCACTTGCTGCAGGA
3-hSCD1	TCAGCCACTCTTGTAGTTTCCA

Protein expression and purification

Rosetta 2(DE3) pLysS competent cells (Novagen) were transfected with pET15b vectors carrying Drp1, its domains, or FLAG-mitoPLDΔTM. These cells were grown in LB containing ampicillin and chloramphenicol overnight at 37°C. We diluted 1 ml of the culture into 1 L of the same growth medium and continued to grow the culture for 3–5 hours at 37°C. The culture was cooled down on ice, and 0.1 mM of IPTG was added. The expression of His$_6$-Drp1 was induced overnight at 16°C. The bacteria were washed twice with PBS by centrifugation at 4000 rpm for 15 min and harvested and

froze at -80°C. The frozen cell pellets were resuspended in 40 ml of lysis buffer (10 mM imidazole, 1 mM $MgCl_2$, 500 mM NaCl, 2 mM β-mercaptoethanol, 20 mM Hepes, pH 7.4) and sonicated on ice for 10 × 5 sec at setting 5 and then 10 × 5 sec at setting 2 using a Fisher Scientific Sonic dismembrator model 100. The cell homogenate was centrifuged at 4100 rpm for 15 min at 4°C, and the supernatant was further clarified at 15,000 rpm for 15 min at 4°C. After being passed through a membrane filter with a pore size of 0.45 μm, the lysate was incubated with 2 ml of pre-washed 50% Ni-NTA beads (His-Bind Resin, Novagen) overnight at 4°C and placed in a 15-ml column (Poly-prep chromatography column, Bio-Rad). The beads were washed with 3 ml of lysis buffer, 15 ml of lysis buffer, 3 ml of wash buffer (40 mM imidazole, 1 mM $MgCl_2$, 500 mM NaCl, 2 mM β-mercaptoethanol, 20 mM Hepes, pH 7.4), and 15 ml of wash buffer. The His_6-tagged recombinant proteins were eluted from the column using 3 × 0.9 ml of elution buffer (250 mM imidazole, 1 mM $MgCl_2$, 500 mM NaCl, 2 mM β-mercaptoethanol, 20 mM Hepes, pH 7.4) and collected it into nine 300-μl fractions. After confirming the purification of proteins using SDS-PAGE and Coomassie Brilliant Blue staining of each fraction, the peak fractions (typically fractions #3 and 4) were collected and diluted into 15 ml of lysis buffer without imidazole. The residual imidazole was removed by passing the solution three times through an Amicon Ultra Centrifugation Filter (a 50K filer for full-length Drp1 and a 10K filter for the Drp1 domains and FLAG-mitoPLDΔTM) according to the manufacturer's instructions. The purified proteins were mixed with final 20% DMSO, snap frozen in liquid nitrogen, and stored at -80°C.

Liposome floatation assay

POPC (850457), DPPA (830855), DOPA (840875), palmitoyl lysoPA (857123), dihexanoyl PA (830841), DPPC (850355), DOPC (850375), DPPE (850705), DOPE (850725), DPPG (840455), DOPG (840475), DPPS (840037), DOPS (840035), TOCL (710335), and rhomamine-DPPE (810158) were purchased from Avanti Polar Lipids. TPCL (L-C160) was purchased from Echelon Biosciences. Unless otherwise noted, the lipids were mixed in a POPC:rhodamine-DPPE:variable lipids ratio of 84:1:15 (% mol). The lipids were dried under a flow of nitrogen gas for 5 min and additionally dried in a SpeedVac overnight. The lipid film was resuspended at a concentration of 10 mM in 100 mM NaCl and 20 mM MES (pH 7.0), voltexed for 1 hour, and subjected to freeze-thaw cycles 5 times using dry ice and a 42°C heat block. Unilamellar liposomes were generated via extrusion through a nanopore membrane with a pore size of 400 nm; this process was repeated 21 times. The liposomes (5 mM lipids) were mixed with His_6-Drp1 (5 μM) (final volume: 200 μl) and incubated at 4°C with gentle mixing for 1 hour. The Drp1-liposome mixture was diluted in 1.728 M sucrose and 20 mM MES (pH

7.0) (final volume: 1.25 ml) and placed at the bottom of tubes. We then overlaid 2.9 ml of 1.25 M sucrose /20 mM MES (pH7.0) and 0.85 ml of 0.25 M sucrose/20 mM MES (pH7.0). The sucrose gradient was centrifuged at 55,000 for 2 hours at 4°C in an SW55Ti (Beckman). fFive fractions (0.5, 0.75, 1.75, 1.75, and 0.25 ml) were collected from the top. The majority of liposomes were floated to the top two fractions, and each fraction (equal volume) was analyzed using SDS-PAGE and silver staining. The stained gels were scanned, and the band intensity was quantified using NIH Image J. The liposomes were detected based on the fluorescence intensity of rhodamine-PE. The amounts of Drp1 and liposomes were normalized relative to the volume of each fraction.

Lipid dot blot assay

The lipids were suspended at 50 μM in a chloroform:methanol:water ratio of 1:1:0.1. 2 μl of lipid solution (100 pmol) was spotted onto PVDF membranes and dried for 1 hour at room temperature. The PVDF membranes were rehydrated and blocked in PBS containing 3% fatty acid free BSA and 1% Tween-20 for 1 hour at room temperature. After washing in PBS containing 0.05% Tween-20 (PBST), the membranes were incubated with 1 μg/ml His_6-Drp1 in 2 ml of binding buffer (100 mM NaCl and 10 mM sodium phosphate, pH 7.0) overnight at 4°C with gentle mixing. The membranes were washed in PBST three times for 10 min and incubated with anti-Drp1 antibodies (BD 611113) in PBST containing 3% BSA for 1 hour at room temperature. The immunocomplexes were visualized using fluorescently labeled secondary antibodies and Pharos (Bio-Rad). NIH Image J was used to quantify the fluorescent signals.

Lipid competition assay

To examine competition between DPPA spotted onto PVDF membranes and phospholipids in a detergent-containing solution, lipid-spotted PVDF membranes were incuabated with 1 μg/ml His_6-Drp1 and 100 μM competitor lipids in a binding buffer containing 0.1% Tween-20 overnight at 4°C. After washes in PBST, we processed the PDVF membranes, and Drp1 was detected, as described above for the lipid dot blot assay.

Immunofluorescence microscopy

WT and Drp1KO MEFs were cultured in IMDM medium as previously described (Kageyama et al., 2012). The MEFs were plated onto an 8-well chambered coverglass and transfected the next day with plasmids using Lipofectamin 3000 according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were fixed with pre-warmed 4% PFA in PBS for 20 min at room

temperature (Kageyama et al., 2014). The cells were permeabilized with PBS containing 0.1% Triton X-100 for 8 min at room temperature. The cells were incubated with antibodies against Tom20 (Santa Cruz Biotechnology, sc-11415), Drp1 (BD, 611113), and/or HA (12CA5) in PBS containing 0.5% BSA, followed by the appropriate secondary antibodies. The samples were viewed using a Zeiss LSM510-Meta 2 laser scanning confocal microscope (Kageyama et al., 2014). Image analysis was performed using NIH Image J software. To determine the effect of inhibition of SCD1 on mitochondrial shape and dynamics, the cells were first incubated with CAY10566 (100 nM for 5.5 hours) or PluriSln1 (20 μM for 3.5 hours). Next, the cells were further incubated in the presence or absence of FCCP (10 μM) for 30 min. As controls, we added equivalent amounts of the DMSO solvent. The cells were subjected to immunofluorescence microscopy.

GTPase assay

The GTPase activity of His_6-Drp1 was measured as described previously (Macdonald et al., 2014). We generated liposomes as described in the liposome floatation assay except that rhodamine-PE was omitted. 0.1 μM Drp1 was pre-incubated in the presence or absence of liposomes (150 μM lipids) in 150 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, and 20 mM MES (pH 7.0). 1 mM GTP was added to Drp1-liposome mixture (50 μl) and incubated for 15 min at 37°C. 25-μl samples were mixed with 100 μl of malachite green solution (Echelon, K-1500) at room temperature for 20 min, and the absorbance at OD_{620} was measured.

Immunoprecipitation

HEK293T cells were transfected with plasmids carrying GFP or MitoPLD-GFP using Lipofectamin 3000 on 6-cm dishes. 24 hours after transfection, the plates were washed with ice-cold PBS and placed on ice. We added 0.25 ml of ice-cold RIPA buffer (1.0% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, pH 7.5) containing protease inhibitor cocktail (Roche, 11873580001), and we collected the cells using a cell scraper. The cells were homogenized by gently pipetting on ice for 15 min. The homogenates were clarified at 13,000 rpm for 5 min at 4°C in a microfuge twice. 0.2 ml of the supernatants were incubated with 10 μl of GFP-Trap (ChromoTek) for 3 hours at 4°C. The beads were washed with RIPA buffer containing the protease inhibitor cocktail three times. The clarified cell lysates and immunoprecipitated fractions were analyzed by SDS-PAGE and immunoblotting with antibodies to Drp1 (BD, 611113), Opa1 (BD, 612607), Mfn1 (Abcam, ab57602), Mfn2 (Sigma, M6319), GFP, Tom20 (Abcam, ab11033), and GAPDH (Life Technologies, AM4300).

To examine the interactions between endogenous MitoPLD and Drp1, testes were harvested from two wildtype C57BL/6J mice and snap-frozen in liquid nitrogen. The testes were incubated at -80°C for 10 min and thawed in 1 ml of RIPA buffer containing the protease inhibitor cocktail. The samples were homogenized using a Dounce homogenizer and then sonicated on ice for 2 × 5 seconds at setting 2, using a Fisher Scientific Sonic dismembrator, Model 100. The homogenates were clarified at 10,000 rpm for 5 min at 4 °C. 500 µl of the supernatants were incubated with 5 µl of anti-MitoPLD antibodies (MBL, M207-3) and 10 µl of pre-washed 50% protein A beads for three hours at 4 °C. The beads were washed three times with RIPA buffer containing the protease inhibitor cocktail. The clarified lysates and immunoprecipitated fractions were analyzed by SDS-PAGE and immunoblotting with antibodies to MitoPLD (MBL, M207-3), Drp1 (BD, 611113), Tom20 (Abcam, ab11033), and GAPDH (Life Technologies, AM4300). To analyze MitoPLD, dithiothreitol was omitted from the SDS-PAGE sample buffer to separate bands of MitoPLD and IgG light chains. For Drp1, Tom20 and GAPDH, 25 mg/ml dithiothreitol was included in the SDS-PAGE sample buffer.

In vitro binding assay

We incubated 85 µl of buffer A (100 mM NaCl, 1 mM $MgCl_2$, 0.05% Tween-20, and 50 mM Hepes, pH 7.4) containing 1 µM Drp1 and 6.7 µM FLAG-mitoPLDΔTM with 5 µl anti-Drp1 antibodies (NOVUS Biologicals 110-55288) and 10 µl of pre-washed 50% protein A beads overnight at 4°C. The beads were washed in buffer A three times. The protein mixture and bound fractions were analyzed using SDS-PAGE and immunoblotting with antibodies to Drp1 (BD, 611113) and FLAG (Sigma, F2555).

Supplemental References

Huang, H., Gao, Q., Peng, X., Choi, S.Y., Sarma, K., Ren, H., Morris, A.J., and Frohman, M.A. (2011). piRNA-associated germline nuage formation and spermatogenesis require MitoPLD profusogenic mitochondrial-surface lipid signaling. Developmental cell *20*, 376-387.

Kageyama, Y., Hoshijima, M., Seo, K., Bedja, D., Sysa-Shah, P., Andrabi, S.A., Chen, W., Hoke, A., Dawson, V.L., Dawson, T.M.*, et al.* (2014). Parkin-independent mitophagy requires Drp1 and maintains the integrity of mammalian heart and brain. The EMBO journal *33*, 2798-2813.

Kageyama, Y., Zhang, Z., Roda, R., Fukaya, M., Wakabayashi, J., Wakabayashi, N., Kensler, T.W., Reddy, P.H., Iijima, M., and Sesaki, H. (2012). Mitochondrial division ensures the survival of postmitotic neurons by suppressing oxidative damage. The Journal of cell biology *197*, 535-551.

Macdonald, P.J., Stepanyants, N., Mehrotra, N., Mears, J.A., Qi, X., Sesaki, H., and Ramachandran, R. (2014). A dimeric equilibrium intermediate nucleates Drp1 reassembly on mitochondrial membranes for fission. Molecular biology of the cell *25*, 1905-1915.