SUPPLEMENTAL DATA

Supplementary figure legends

Supplementary figure 1

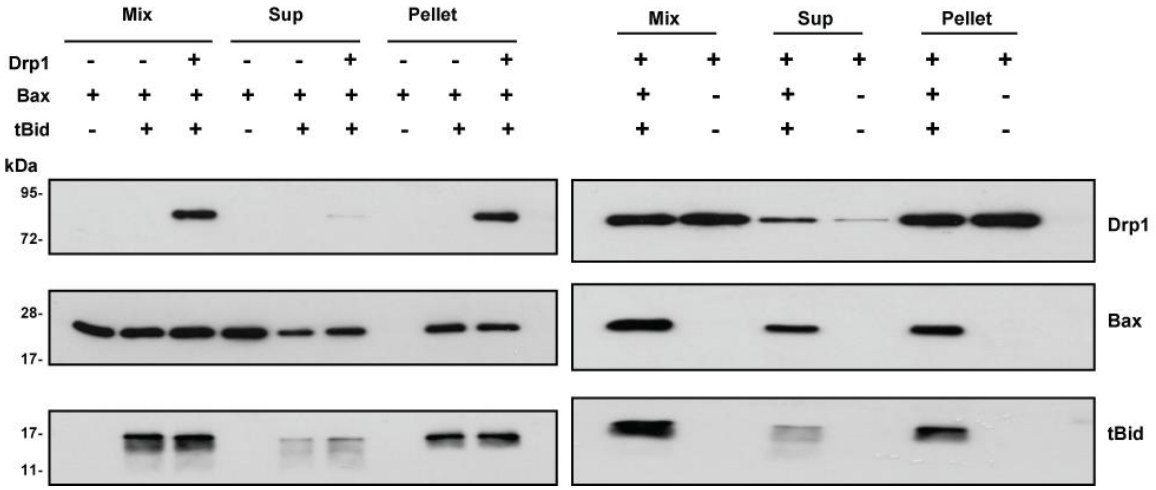


Figure S1: Binding of tBid, Drp1 and Bax to liposomes, related to Figure 2B

PC/PE/CL liposomes were incubated in the presence of 500 nM Drp1, 50 nM Bax and/or 10 nM tBid in 300 µl KCl buffer, as indicated in the figure. 50 µl of the suspension (called Mix) was removed for further immunoblotting and replaced by 50 µl KCl buffer. Liposomes were then ultracentrifuged and the pellet was resuspended in 300 µl KCl buffer. 50 µl of the Mix, Supernatant (Sup) and Pellet were analyzed by immunoblotting using antibodies to Bid, Bax or Drp1. This allowed a direct comparison of the proportion of the proteins that bound to liposomes or remained in suspension.

Supplementary figure 2

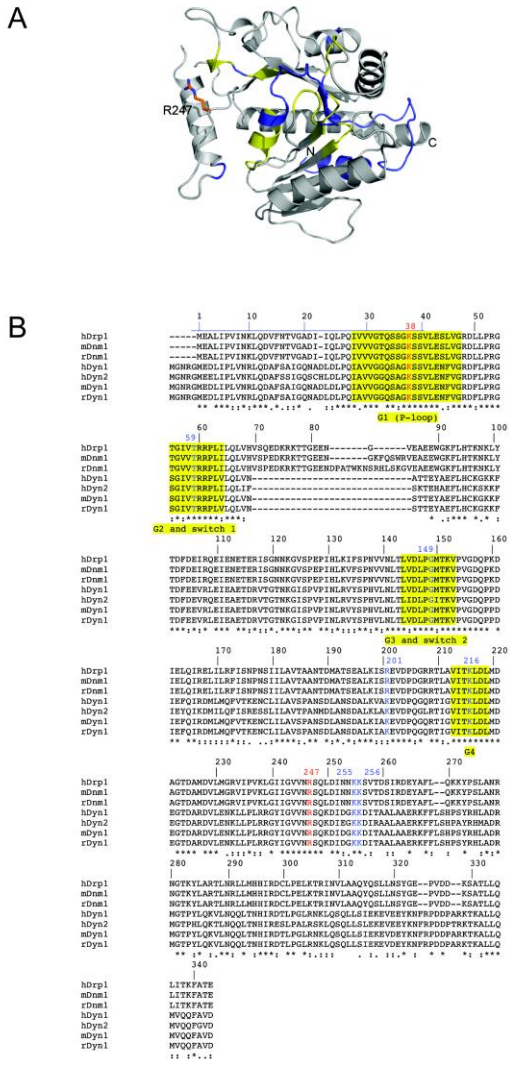


Figure S2. Arginine 247 of Drp1, related to Figure 2D-E

(A) Homology model of the hDrp1 GTPase domain (amino acids 1-313) based on the Dynamin-1 GTPase domain (2aka.pdb, sequence identity 57%) shown in cartoon representation. Regions involved in GTP-binding and hydrolysis are in yellow. Amino acids mutated in hDrp1 without a loss of capacity to stimulate Bax oligomerization are in blue; R247 is shown in red sticks. Models were prepared using the FFAS server (http://ffas.ljcrf.edu/) and PYMOL (DeLano scientific).

(B) Sequence alignment of Drp1 and dynamins 1 and 2. The protein sequences of the GTPase domains of human, mouse and rat Drp1 (respectively hDrp1, mDnm1 and rDnm1), human dynamins 1 and 2 (hDyn1 and hDyn2) and mouse and rat dynamins 1 (mDyn1 and rDyn1) were aligned using the T-Coffee software (www.ebi.ac.uk/t-coffee/). Numbering corresponds to the sequence of hDrp1. GTP-binding motifs are outlined in yellow. Deletion of the N-terminal segment of hDrp1 or point mutation of residues important for GTP binding and hydrolysis (in blue) did not reduce the capacity of hDrp1 to promote tBid-induced Bax oligomerization, unlike mutation of R247 (in red).

Supplementary figure 3

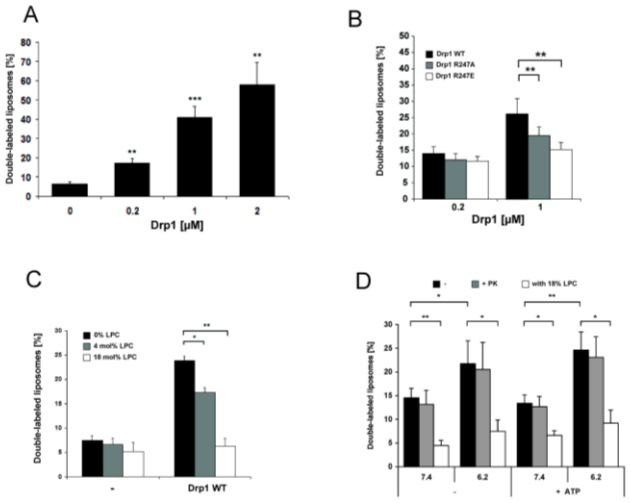


Figure S3: Membrane hemifusion assessed by FACS, related to Figure 4

(A) Single-labeled PC/PE/CL liposomes containing either Rhodamine-PE or Bodipy-FL C5 HPC were mixed and incubated with 2 mM ATP and increasing concentrations of Drp1. Liposomes were then treated with proteinase K before analysis by flow cytometry. *: $p < 0.02$; **: $p < 0.006$; ***: $p < 0.0005$ (Values are means of more than three experiments ± SEM).

(B) Effects of Drp1 WT (black bars), Drp1 R247A (grey bars) or Drp1 R247E (white bars) on liposome hemifusion. Values are means of more than four independent experiments ± SEM; *: $p < 0.02$; **: $p < 0.01$.

(C) PC/PE/CL liposomes containing 0% (black bars), 4 mol% (grey bars) or 18 mol% (white bars) LPC were assayed for Drp1-induced membrane hemifusion. Values are means of four independent experiments ± SEM; *: $p < 0.04$; **: $p < 0.01$.

(D) Membrane hemifusion induced by cytochrome c. Single-labeled PC/PE/CL liposomes were incubated with cytochrome c (0.25 mg/ml), in the absence or presence of ATP (2.5 mM), at pH 6.2 or 7.4. At pH 6.2, cytochrome c induced a significant increase in the fraction of double-labeled liposomes, suggesting membrane hemifusion (black bars). The fraction of double-labeled liposomes was not reduced after incubation with proteinase K (grey bars). In the presence of 18% LPC, cytochrome c did not induce liposome hemifusion (white bars). Values are means of three or more independent experiments ± SEM; *: $p < 0.04$; **: $p < 0.007$.

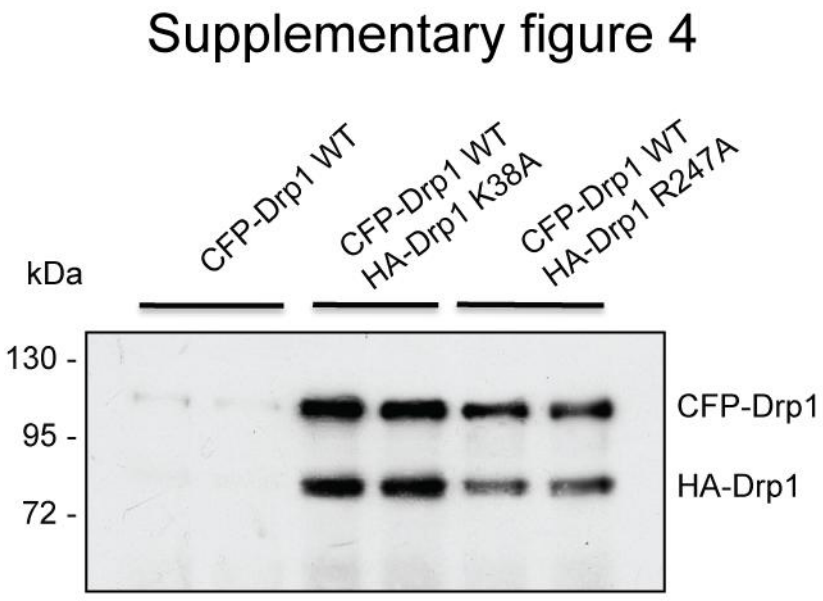


Figure S4: Drp1 R247A and Drp1 WT interact in HeLa cells, related to Figure 5B

HeLa cells were transfected with equal amounts of plasmids encoding HA-Drp1 R247A and CFP-tagged Drp1 WT or HA-Drp1 K38A and CFP-tagged Drp1 WT or with CFP-Drp1 WT alone. 48 hours after transfection, mitochondria were isolated, lysed in PBS pH 7.5, 2% CHAPS and HA-Drp1 was immunoprecipitated using an anti-HA antibody. The immunoprecipitate was analyzed by immunoblotting using an antibody directed against Drp1. Drp1 K38A was used as a positive control. This dominant negative mutant displays an impaired GTPase activity and is known to heterodimerize with endogenous Drp1.

Supplementary figure 5

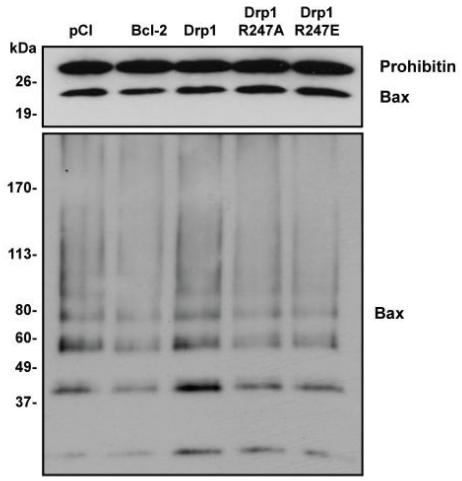


Figure S5: Bax oligomerization assessed by cross-linking. Related to figure 6E

HeLa cells were transfected with an empty pCI vector or with vectors encoding Bcl-2, Drp1 WT or Drp1 R247A/E. 72 h later cells were treated with 3 μM ActD for 4 h before isolation of mitochondria, addition of cross-linkers, and Bax immunostaining. This blot is representative of six independent experiments. The amount of Bax attached to mitochondria before addition of the cross-linkers is shown in the upper blot, together with Prohibitin used as a loading control.

Experimental procedures

Preparation of cytosolic extracts. Rat tissues were homogenized with a Dounce homogenizer in 35 mM PIPES buffer pH 7.4 containing 35 mM KOH, 5 mM $MgCl_2$, 1 mM EGTA, 0.5 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 1 mM DTT and 0.5 mM ATP. The homogenate was then centrifuged at 2'000 g, 4°C for 10 min and twice 1 h at 100'000 g at 4°C.

HeLa cells were resuspended in MB buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Hepes pH 7.4), broken by at least 10 passages through a 25G1 0.5 × 25 needle fitted on a 5-ml syringe and centrifuged at 100'000 g for 30 min at 4°C.

Recombinant proteins. Recombinant His-tagged full length Bax and caspase-8-cleaved Bid (tBid) were purified as described previously (Desagher et al., 1999; Eskes et al., 1998). C-terminal 6xHis-tagged wild-type and mutant recombinant Drp1 (Isoform 3, Uniprot KB/Swiss-Prot O00429) were produced in *Escherichia coli* Rosetta cells. Cells were grown in LB media containing 30 μg/ml kanamycin at 37°C to reach an OD_{600} of 0.6, incubated in the presence of 0.2 mM isopropyl β-D-thiogalactopyranoside and harvested by centrifugation. The pellets were suspended in 25 mM Hepes pH 7.4, 1 mM PMSF/benzamidine, 5 mM ß-mercaptoethanol, 300 mM KCl, 1 mM $MgCl_2$, 10% (v/v) glycerol, 25 mM imidazole, DNase (1 μg/ml), and lysozyme (10 μg/ml), and incubated on ice for 30 min. The cells were then broken with a French press and centrifuged at 20'000 g for 15 min. The supernatants were affinity purified on a Ni-NTA agarose resin (Qiagen). The protein was eluted with 150 mM imidazole and dialyzed with 25 mM

Hepes-KOH pH 7.5, 125 mM KCl, 1 mM PMSF/benzamidine, 1 mM DTT and 10% (v/v) glycerol.

Partial purification of Bax-activating factors. Cytosolic extracts were loaded on an S Sepharose Fast Flow column (Amersham Pharmacia) and the activity was recovered in the flow-through which was concentrated using an Amicon membrane before further processing. Bax-activating factors were further purified by size exclusion chromatography performed with a Superose 6 column (Amersham Pharmacia). A 120 ml run in 25 mM Hepes-KOH pH 7.4, 200 mM KCl, 0.1 mM PMSF, 0.1 mM benzamidine, 1 mM DTT was performed and 2 ml fractions were recovered. Pools of 4 consecutive fractions were made and tested in the '2 step' Bax oligomerization assay.

Preparation of salt-extracted mitochondrial proteins. Mitochondria from rat liver were purified as described by Eskes et al., (1998) and were incubated in KCl buffer (500 mM KCl, 4 mM $MgCl_2$, 5 mM KH_2PO_4, 10 mM Hepes-KOH pH 7.4) at 4°C for 30 min before centrifugation at 10'000 g. The supernatant was dialysed in 125 mM KCl buffer.

Size exclusion chromatography. Liposomes or mitochondria (250 μg) isolated from HeLa cells were lysed in 2% CHAPS and the insoluble material was removed by centrifugation (100'000 g for 30 min). The supernatant was loaded on a sephacryl S200 column (Amersham Pharmacia). A 130 ml run in 25 mM Hepes pH 7.5, 200 mM NaCl, 1% CHAPS, 2 mM DTT was performed and 2 ml fractions were collected. Proteins

present in each fraction were precipitated with trichloroacetic acid. The distribution of Bax or Drp1 was analyzed by immunoblotting.

Construction of expression plasmids for Drp1 mutants. The N-terminal deletion construct of Drp1 was made by PCR amplification of the Drp1 ORF cloned in pET29a using specific primers (5'-GGA ATT CCA TAT GAG CTC AGT GCT AGA AAG C-3' and 5'-CCG CTC GAG CCA AAG ATG AGT CTC CCG GAT-3') followed by ligation of the restriction enzyme digested PCR product (NdeI/XhoI) into prelinearised pET29a.

Site directed mutagenesis was performed using a QuickChange Site-Directed Mutagenesis kit (Stratagene) according to manufacturer's instructions. The primers used for the site directed mutations were:

Drp1 K38A (5'-GCAGAGCAGCGGAGCGAGCTCAGTGCTAGAAAGC-3'and 5'GCTTTCTAGCACTGAGCTCGCTCCGCTGCTGCTCTGC-3') Drp1 T59A (5'-GGT ACT GGA ATT GTC GCC CGG AGA CCT CTC-3' and 5'-GAG AGG TCT CCG GGC GAC AAT TCC AGT ACC-3'), Drp1 G149A (5'-CTT GTG GAT TTG CCA GCA ATG ACC AAG GTG CCT G-3' and 5'-CAG GCA CCT TGG TCA TTG CTG GCA AAT CCA CAA G-3'), Drp1 K216A (5'-CCT AGC TGT AAT CAC TGC ACT TGA TCT CAT GGA TG-3' and 5'-CAT CCA TGA GAT CAA GTG CAG TGA TTA CAG CTA GG-3'), Drp1 R201A (5'-CAC TTA AAA TTT CAG CAG AGG TAG ATC CAG-3' and 5'-CTG GAT CTA CCT CTG CTG AAA TTT TAA GTG-3'), Drp1 R247A (5'-GGA GTA GTT AAC GCG AGC CAG CTA GA-3' and 5'-TCT AGC TGG CTC GCG TTA ACT ACT CC-3'), Drp1 R247E (5'-ATT GGA GTA GTT AAC GAG AGC CAG

CTA GAT AT-3' and 5'-ATA TCT AGC TGG CTC TCG TTA ACT ACT CCA AT-3'), Drp1 K255A (5'-GCT AGA TAT TAA CAA CGC GAA GAG TGT AAC TG-3' and 5'-CAG TTA CAC TCT TCG CGT TGT TAA TAT CTA GC-3'), Drp1 K256A (5'-CAT ATT AAC AAC AAG GCG AGT GTA ACT GAT T-3' and 5'-AAT CAG TTA CAC TCG CCT TGT TGT TAA TAT G-3'), Drp1 R255G (5'-GCT AGA TAT TAA CAA CGA GAA GAG TGT AAC-3' and 5'-CAG TTA CAC TCT TCT CGT TGT TAA TAT CTA GC-3') and Drp1 R256G (5'-CAT ATT AAC AAC AAG GAG AGT GTA ACT GAT T-3' and 5'-GCT AGA TAT TAA CAA CGC GAA GAG TGT AAC TG-3'). The constructs were sequenced to verify the mutations.

Composition of KCl buffer. Unless otherwise stated the KCl buffer was made of 125 mM KCl, 2 mM $MgCl_2$, 5 mM KH_2PO_4, 10 mM Hepes, pH 7.4

In vitro Bax oligomerization assay. Bax oligomerization was assayed as described previously (Lucken-Ardjomande et al., 2008) with some modifications. Unless otherwise stated, liposomes (840 µM lipids composed of 54 mol% PC, 26 mol% CL and 20 mol% PE) were resuspended in 200 µl KCl buffer before addition of ATP and proteins, respecting the following order: cytosolic extracts, Drp1, then ATP (2.5 mM), Bax (50 nM) and tBid (10 nM). The suspension was incubated at 30°C for 30 min without agitation. Liposomes were then ultracentrifuged at 100'000 g for 30 min at 15°C, resuspended in KCl buffer and incubated with trypsin (0.17 mg/ml) for 2 h at 30°C. Reactions were stopped with trypsin inhibitor (1.4 mg/ml, Sigma). Bax resistant to

trypsin digestion was analyzed by immunoblotting using a Bax antibody from BD-Pharmingen.

A ‘2 step’ Bax activation assay was used to monitor purification of Bax activating proteins from brain cytosolic extracts. Fractions from the S Sepharose (1 mg) or size exclusion chromatography (100 µg) were incubated with liposomes for 1 h under rotary shaking at room temperature, centrifuged at 100’000 g for 30 min at 15°C and resuspended by sonication in 200 µl KCl buffer. ATP (2.5 mM), recombinant Bax (50 nM) and tBid (10 nM) were sequentially added to the proteoliposomes before incubation at 30°C for 30 min. Liposomes and Bax were then processed as described above.

Aggregation of liposomes. Liposomes were incubated in 100 µl KCl buffer in the presence of 2.5 mM ATP and the WT or mutant Drp1 proteins for 30 min at 30°C. 700 µl KCl buffer were then added to the liposome suspension before determination of the turbidity at 450 nm. Values represent the difference between OD_{450} of the liposome suspension incubated in the presence of proteins and OD_{450} in the absence of proteins. Statistical significance was assessed using Student’s *t*-test (unpaired, two-tailed).

Hemifusion analyzed by FACS. PC/PE/CL liposomes containing Rhodamine-PE (0.2%, Invitrogen) or Bodipy FL C5 HPC (0.4%, Invitrogen) were prepared and mixed in a 1:1 ratio. Liposomes (2.75 mg/ml) were then incubated without or with ATP (2.5 mM), Drp1 proteins (0.2, 1 or 2 µM) for 30 min at room temperature. Samples were then analyzed using a FACScalibur system. Statistical significance was assessed using paired Student’s *t*-tests.

Drp1 GTPase activity. The GTPase activity of WT and mutant Drp1 was assayed using a colorimetric assay as described previously (Leonard et al., 2005). Briefly, 1.5 μM Drp1 was added to 1.25 mM GTP for 30 min at 30°C in 200 μl KCl buffer, without phosphate. 5 μl of the solution were then added to 200 μl Malachite Green stock solution (500 μM Malachite Green, Sigma, 10 mM ammonium molybdate in 1 N HCl) in microtiter wells and the absorbance at 650 nm was determined using a microplate reader.

Cell culture, cell transfection and apoptosis induction. HeLa cells (European Collection for Cell Cultures) were grown in DME supplemented with 10% FCS, 100 u/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine, and maintained in 5% CO_2 at 37°C. HeLa cells were transfected with various plasmids encoding Drp1, Drp1 mutants or Bcl-2 using calcium phosphate except for the experiments aiming at determining levels of Bax oligomerization for which a very high transfection efficiency was required. For this, we used the Microporator system (Digital Bio, Axon lab) which allows to achieve transfection efficiencies > 80% for HeLa cells.

Apoptosis was induced with a UV Stratalinker 2400 apparatus (60 mJ/cm^2, Stratagene) or with Actinomycin D (3 μM, Sigma). Apoptotic cells were labeled with Annexin V (BD Biosystems) and counted by flow cytometry as described previously (Lucken-Ardjomande et al., 2008).

Immunoblotting and immunocytochemistry. Samples were separated in polyacrylamide gels and transferred to Hybond-P membranes (Amersham Pharmacia).

Immunoblotting was performed with the following antibodies: Bax (BD-Pharmingen, 554104 for immunoblotting), Bid (R&D systems, AF 860), Drp1 (BD-Biosciences), Prohibitin (Thermoscientific, Ab-1, MS261), GAPDH (Abcam, Ab8245), hFis1 (Alexis, ALX-210-907), mHsp70 (MA3-028, Thermoscientific), Tom 20 (FL-145, Santa-Cruz Biotechnology), Bcl-XL (B22630, BD transduction laboratories), Hsp90 (a kind gift of Dr O. Toft, Mayo Clinic), Actin (AC-40 Sigma).

Immunocytochemistry for cytochrome c was performed with a monoclonal antibody from BD-Pharmingen.

Determination of Bax oligomerization during apoptosis using the trypsin assay. Mitochondria were isolated from control and apoptotic cells as described previously (Parone et al., 2006). Briefly**,** HeLa cells were resuspended in MB buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Hepes-KOH, pH 7.4), broken by five passages through a 25G1 0.5 × 25 needle fitted on a 2-ml syringe and the suspension was centrifuged at 10'000 g for 10 min at 4°C.

Mitochondria (4 μg/μl in KCl buffer) were treated with trypsin (0.17 mg/ml) for 90 min. Reaction was stopped with trypsin inhibitor (1.4 mg/ml, Sigma) and the fraction of Bax resistant to trypsin digestion was analyzed by immunoblotting using a Bax antibody from BD-Pharmingen.

Determination of Bax oligomerization by cross-linking. Mitochondria were purified from control and apoptotic cells and incubated in the presence of BS^3 (Bis-sulfosuccinimidyl-suberate) and DSS (Disuccinimidyl suberate) at a concentration of 2

mM for 30 min before addition of Tris-HCl, pH 8 to stop the reaction. Mitochondria were lysed before analysis of Bax by immunoblotting.

Statistical analysis

Data are presented as means +/- s.e.m unless otherwise stated. Statistical significance was assessed using unpaired, two-tailed Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

References

Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., and Martinou, J.C. (1999). Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. J Cell Biol *144*, 891-901.

Eskes, R., Antonsson, B., Osen-Sand, A., Montessuit, S., Richter, C., Sadoul, R., Mazzei, G., Nichols, A., and Martinou, J.-C. (1998). Bax-induced cytochrome c release from mitochondria is independent of the permeability transition pore but highly dependent on Mg2+ ions. J Cell Biol *143*, 217-224.

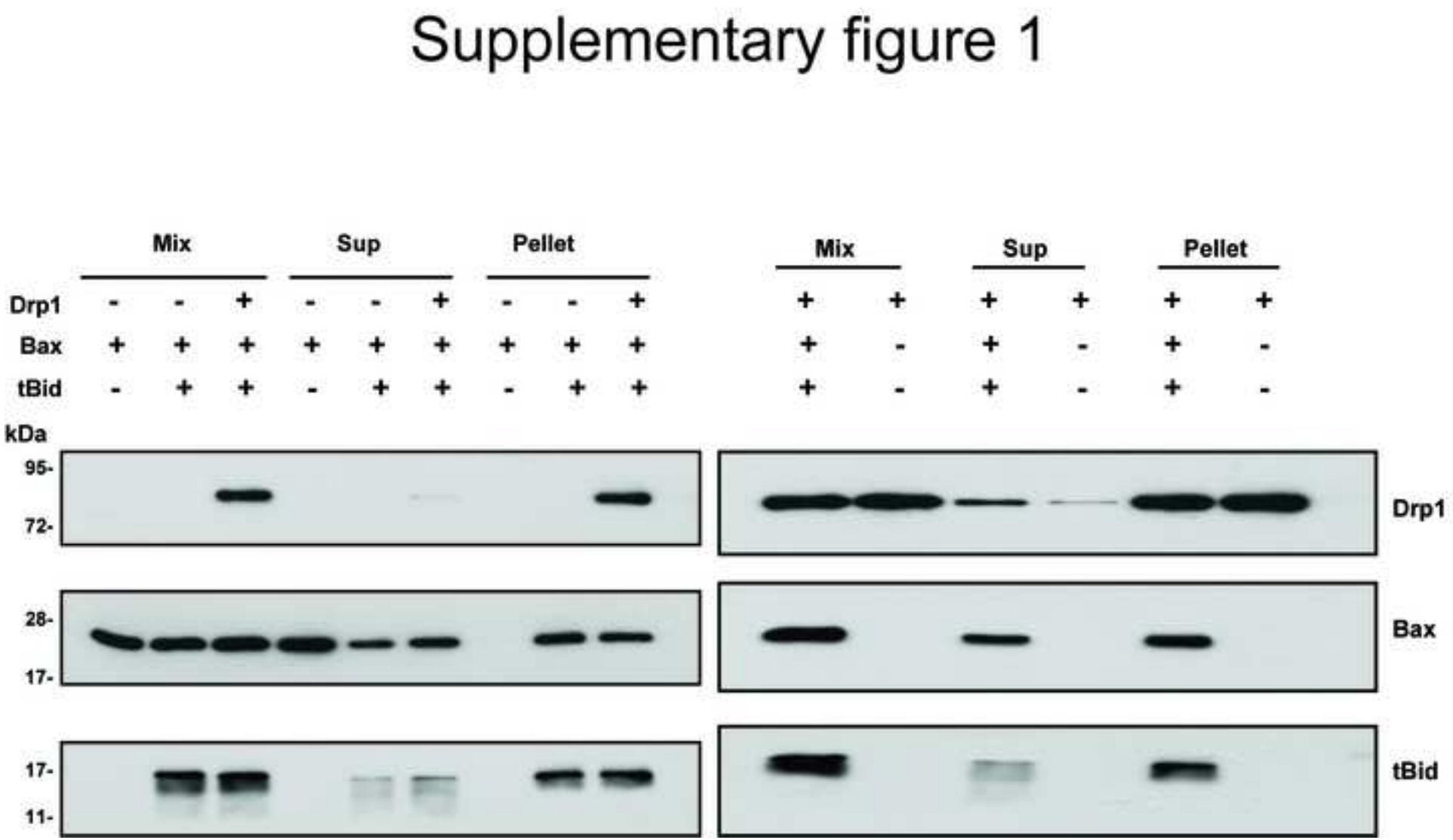
Leonard, M., Song, B.D., Ramachandran, R., and Schmid, S.L. (2005). Robust colorimetric assays fo dynamin's basal and stimulated GTPase activities. Methods Enzymol *404*, 490-503.

Lucken-Ardjomande, S., Montessuit, S., and Martinou, J.C. (2008). Bax activation and stress-induced apoptosis delayed by the accumulation of cholesterol in mitochondrial membranes. Cell Death Differ *15*, 484-493.

Parone, P.A., James, D.I., Da Cruz, S., Mattenberger, Y., Donze, O., Barja, F., and Martinou, J.C. (2006). Inhibiting the mitochondrial fission machinery does not prevent Bax/Bak-dependent apoptosis. Mol Cell Biol *26*, 7397-7408.

Roucou, X., Montessuit, S., Antonsson, B., and Martinou, J.C. (2002). Bax oligomerization in mitochondrial membranes requires tBid (caspase-8-cleaved Bid) and a mitochondrial protein. Biochem J *368*, 915-921.

Supplemental Figure 1



Supplemental Figure 2

Supplementary figure 2

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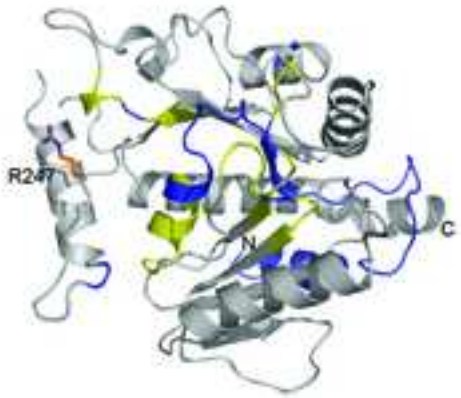

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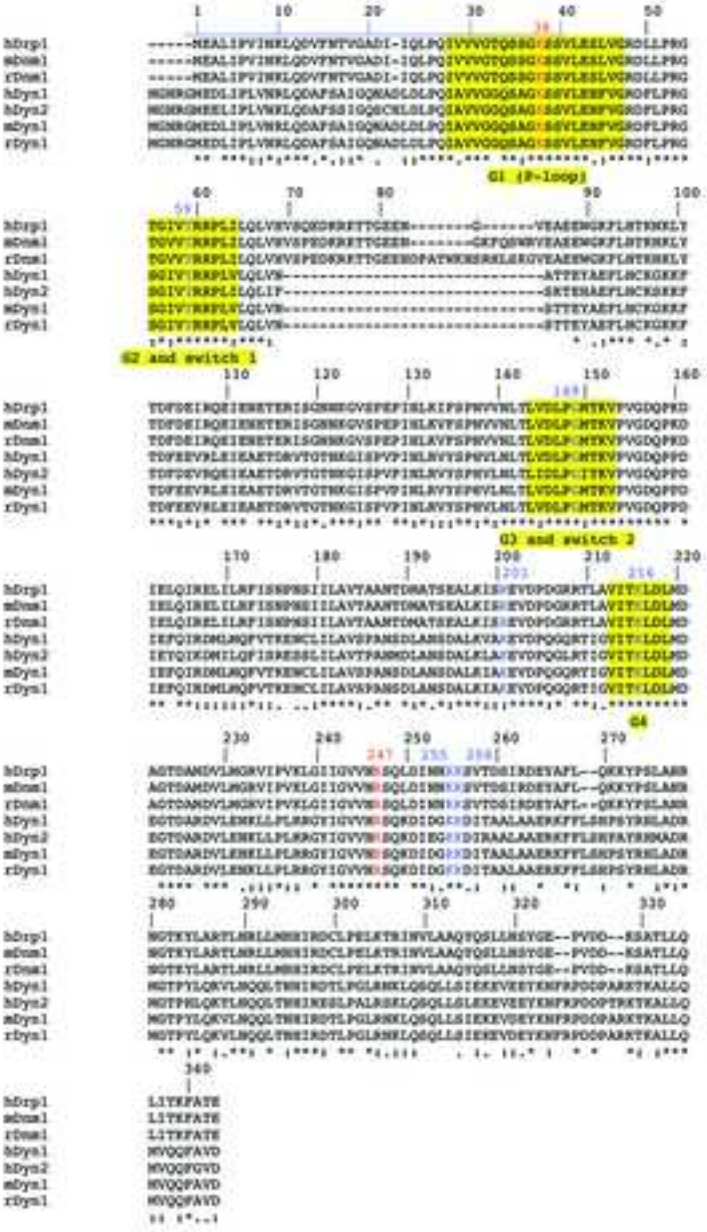
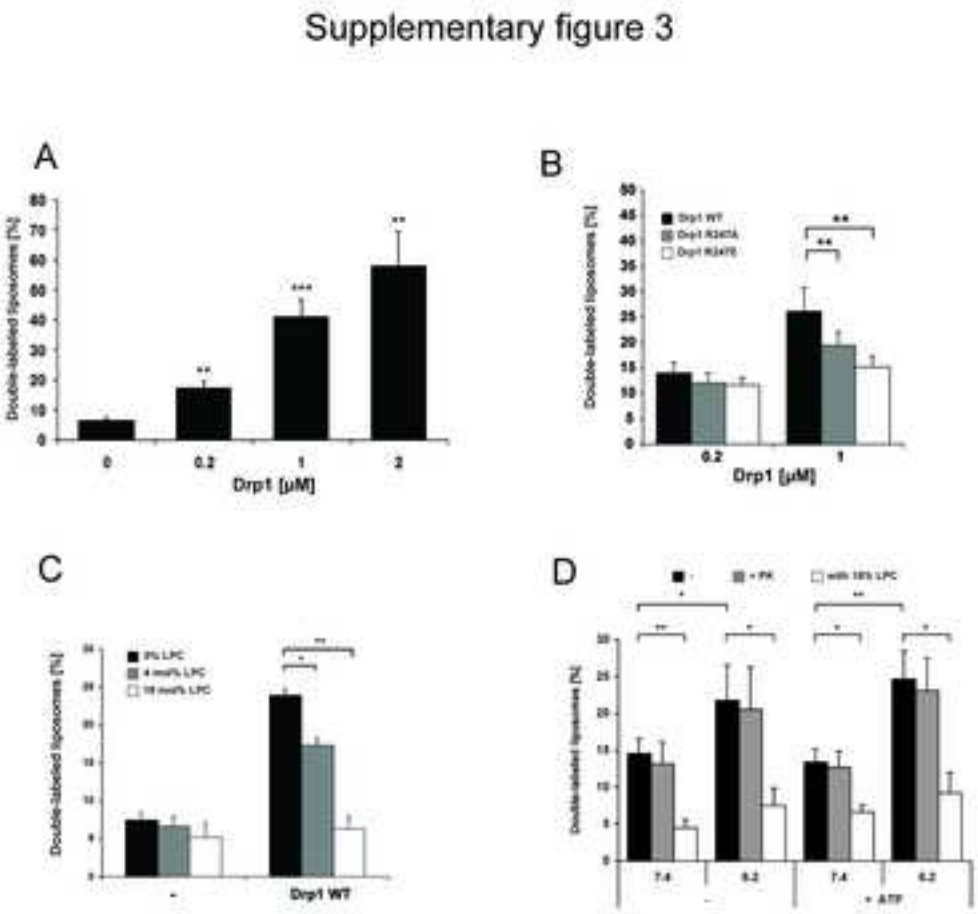
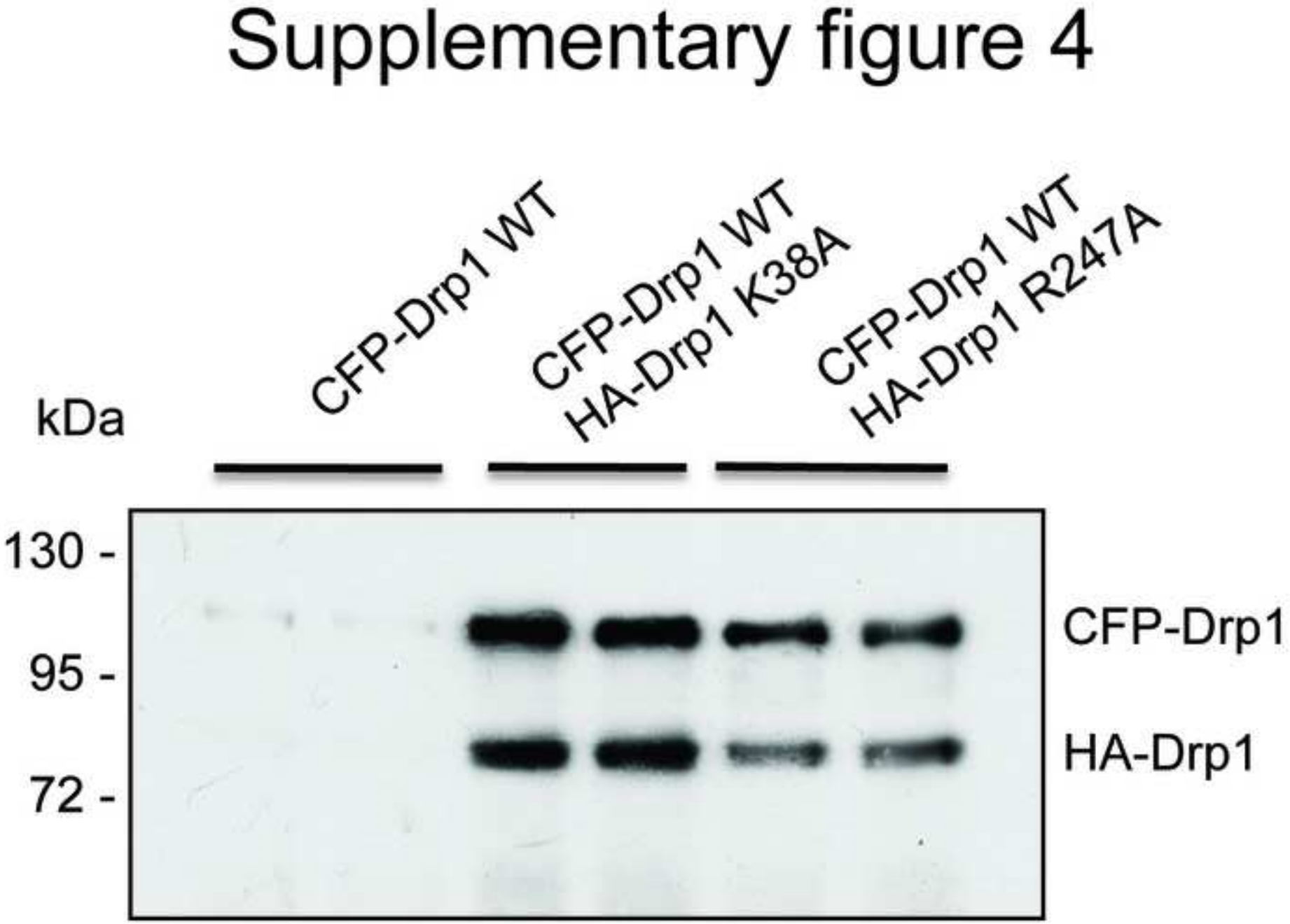


Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5

Supplementary figure 5