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Supplemental Data

Opa1-Mediated Cristae Opening Is Bax/Bak and BH3 Dependent, Required for Apoptosis, and Independent of Bak Oligomerization

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Table S1. Chemical inhibitors of cytochrome c release.
Results of a small screen for inhibitors of cytochrome c release from mouse liver mitochondria ("MOMP Inhibition"). Mitochondrial poisons tested included CCCP, rotenone, antimycin A, and cyclosporine A, none of which were found to inhibit MOMP.

Compound	Known Target(s)	MOMP Inhibition
AEBSF	serine proteases	-
z-VAD	caspases	-
Amastatin	metallo-aminopeptidases	-
Bestatin	metallo-aminopeptidases	-
DCIC	broad spectrum serine protease (including Rhomboid)	+++
DFP	serine proteases	-
E64	cysteine proteases	-
Leupeptin	ser/cys proteases	-
Pepstatin	aspartic proteases	-
Phenanthroline	metalloproteases	-
PMSF	serine proteases	-
TLCK	trypsin-like serine proteases	-
TPCK	chymotrypsin-like serine proteases	-
Calpastatin	calpain	-
Ucf101	Omi and possibly other serine proteases	+++
MG132	proteasomes	+++
MG115	proteasomes (and Calpain at higher concentrations)	+
γ-secretase inhibitor	γ-secretase, presenilin	-

Table S2. Summary of effects of MOMP inhibitors. *At very high concentrations (200 µM), MG132 did inhibit Opa1 complex disassembly.

Agent	Cytochrome c release	Bak oligomerization	Opa1 complex disassembly and cristae remodeling
Bcl-x_L	Blocked	Blocked	Blocked
Mcl1	Blocked	Blocked	Blocked
DCIC	Blocked	Blocked	Blocked
MG132	Blocked	Blocked	Unaffected*
Ucf101	Blocked	Blocked	Unaffected

Table S3. Summary of effects of MG132 on mitochondrial changes induced by addition of BH3 peptides or BH3-only proteins. Note that MG132 blocked cytochrome c release and Bak oligomerization, but not changes in Opa1 oligomerization and crista junction size.

Treatment	Cytochrome c	Bak	Opa1	Crista Junctions
None	Retained	Monomer	Complex	Wide
MG132	Retained	Monomer	Complex	Wide
BH3	Released	Oligomer	Monomer	Narrow
BH3 + MG132	Retained	Monomer	Monomer	Narrow

Figure S1. MOMP inhibitors blocked N/C-Bid-induced Bak oligomerization. (a) DCIC blocked Bak oligomerization. Mitochondria were treated with either 100 μM DCIC (lanes 4-6), 10 nM N/C-Bid (lanes 7-9) or both (lanes 10-12) or left untreated (lanes 1-3) for 30 minutes at 37º C. Pellets were suspended either in PBS (lanes 1, 4, 7 and 10), 10% DMSO in PBS (lanes 2, 5, 8 and 11) or 10 mM BMH in PBS (lanes 3, 6, 9 and 12) and incubated for 45 min. at 37º C. Samples were separated by 12% SDS-PAGE and Western blotted with anti-Bak antibody. The locations of Bak, cross-linked Bak and Bak oligomer are indicated. **(b) Bcl-x_L blocked Bak oligomerization**. Samples were prepared as in (a) except that 200 nM Bcl-$x_L\Delta C$ instead of DCIC was used. **(c, d)**. **MG132 and Ucf101 blocked Bak oligomerization**. Samples were prepared as before. 25 μM MG132 and 50 μM Ucf101 were used to inhibit Bak oligomerization. Data are representative of three separate experiments.

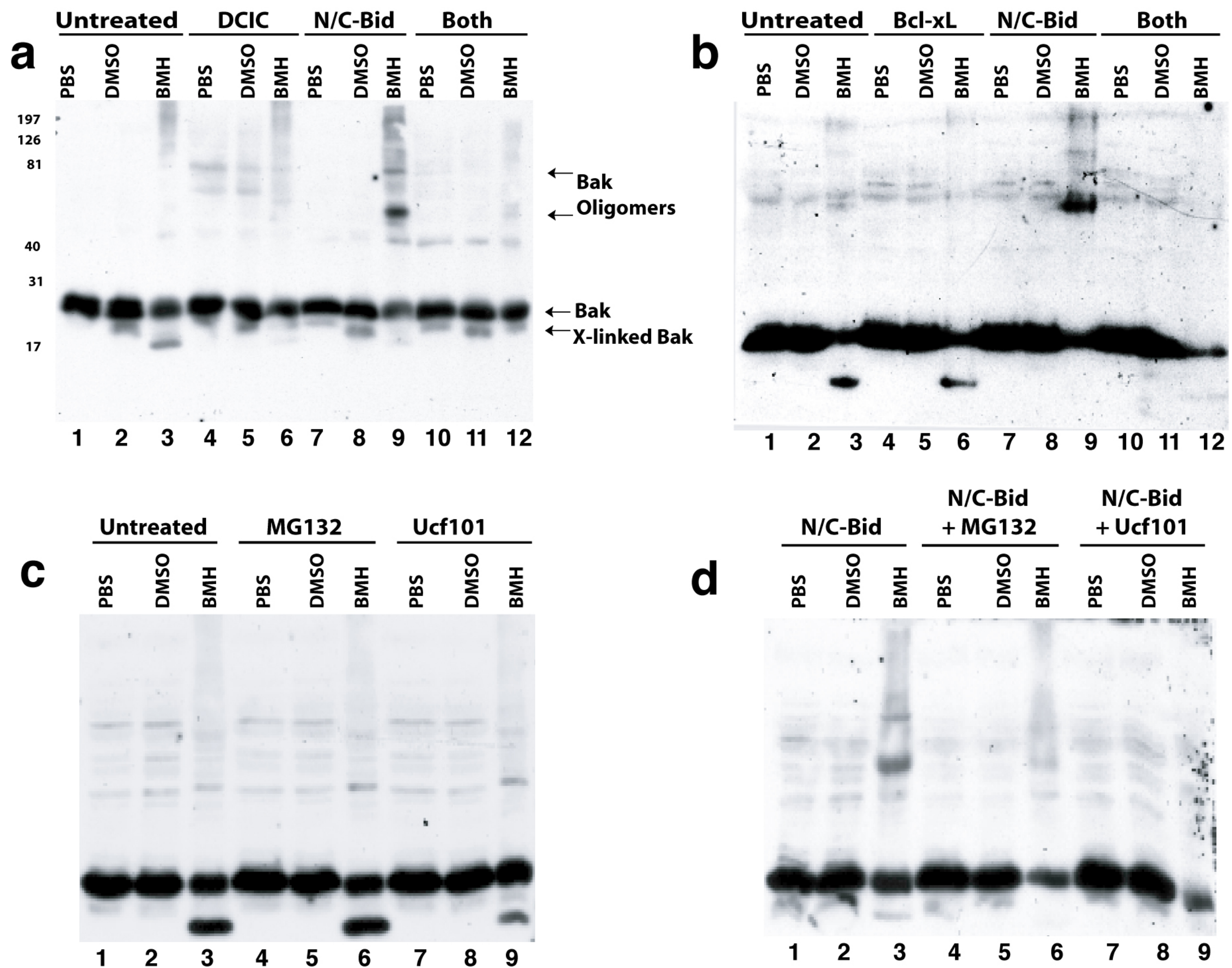
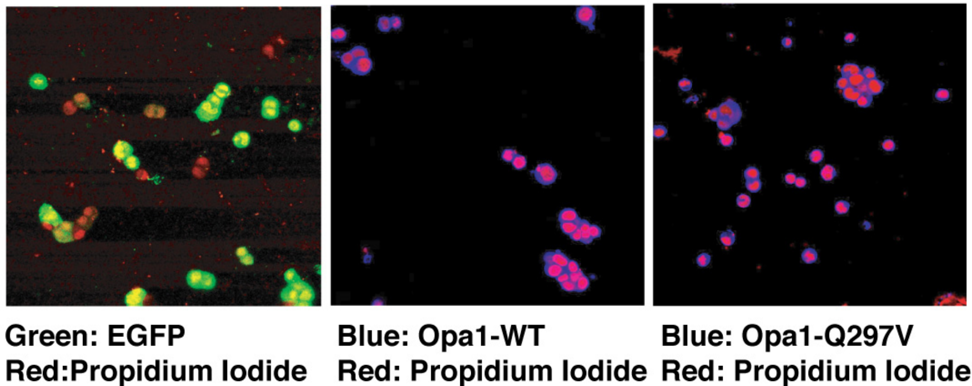


Figure S2. Efficiency of 293T transformation on fibronectin-coated plates. 293T cells were pre-seeded so that at the time of transfection, they were 90-100% confluent. For 60-mm plates, we typically used 16-24 µg of DNA and 20-40 µl of Lipofectamine. 16-24 hours post-transfection, we replated cells. **a.** 293T cells were transfected with either pIRES2-EGFP (left panels) , pcDNA-Opa1-Flag (middle panels) and pcDNA-Opa1 (Q297V)-Flag (right panels) and fixed in 3.7% formaldehyde 44 hours after transfection. All cells were stained with anti-Flag monoclonal antibody and goat anti-mouse-IgG–Alexafluor 647 secondary antibody (Invitrogen). For detail, see Experimental Procedures. **b.** Cells positive for EGF expression and flag-tagged-Opa1 expression were counted. Error bars represent standard deviations from counts done with three fields of cells. Results are representative of three independent experiments.

a



b

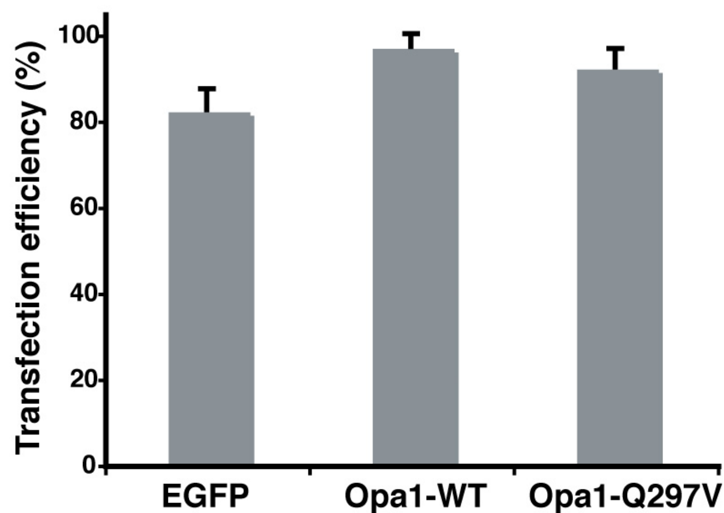


Figure S3. Over-expression of Opa1-Q297V mutant protects 293T cells from death. 293T cells were transfected either with EGFP, wt-Opa1 or Q297V-Opa1 were treated. 24 hour post-transfection, cells were replated and further incubated for 20 hours. Then either 5 μM STS or DMSO were applied for 24 hours. Trypan blue-positive cells were counted, and averages and standard deviations are plotted. Results are representative of three separate experiments.

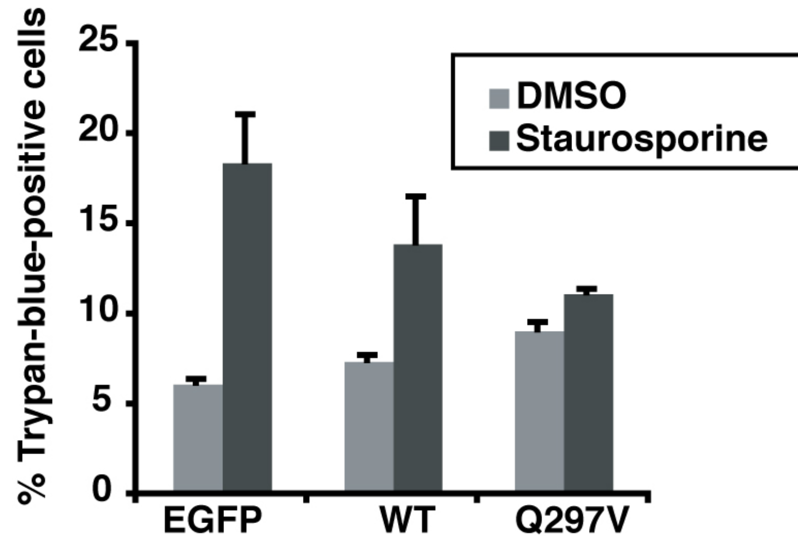


Figure S4. Time-course of Bax activation by Chariot-delivered BimS. 293T cells transfected with pcDNA-Opa1-wt (middle column), pcDNA-Opa1-Q/V (right column) or transfected with empty vector (left column) were examined 0, 4, 8, and 12 hours post-chariot delivery of BimS (first four rows) or 12 hours post-chariot delivery of GST (bottom row). Cells were fixed with formaldehyde, and nuclei were stained red with propidium iodide while activated Bax (6A7 antibody) was stained green as described in Experimental Procedures. Data are representative of three separate experiments.

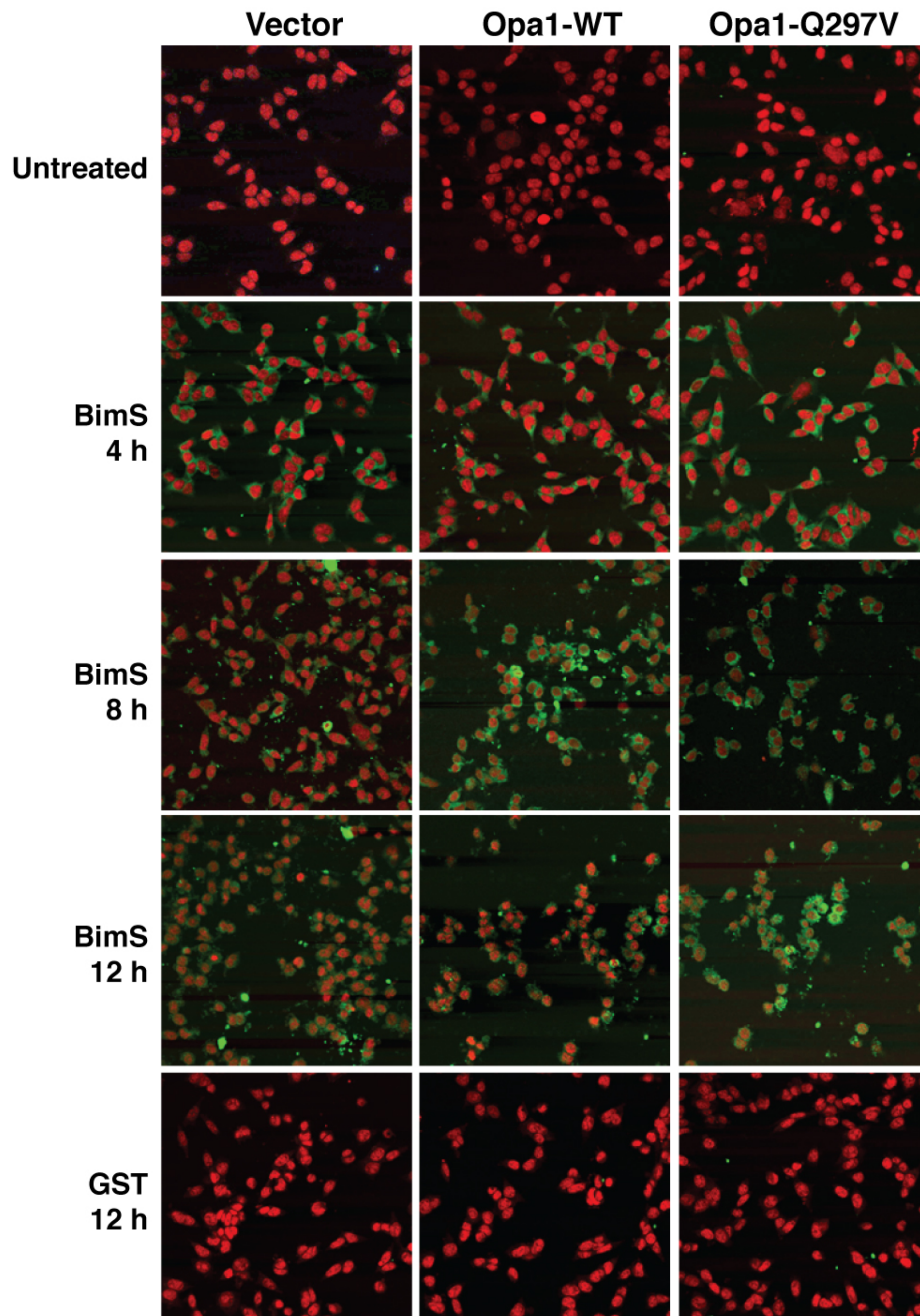


Figure S5. Expression of Opa1-Q297V blocks apoptosis but not the rapid activation of Bax. a. Graphic representation of Bax activation time course in Figure S4. **b. TUNEL assay for apoptosis vs. Bax activation staining.** 293T cells transfected with vector alone (pcDNA) or vector containing cDNA for Opa1-Q297V mutant were chariot-loaded with BimS as in Figure S4a and stained for DNA fragmentation (TUNEL) and activated Bax (antibody 6A7) after 12 h. Data are representative of three independent experiments. Error bars represent standard deviation from counts done with three different fields in each of two separate wells.

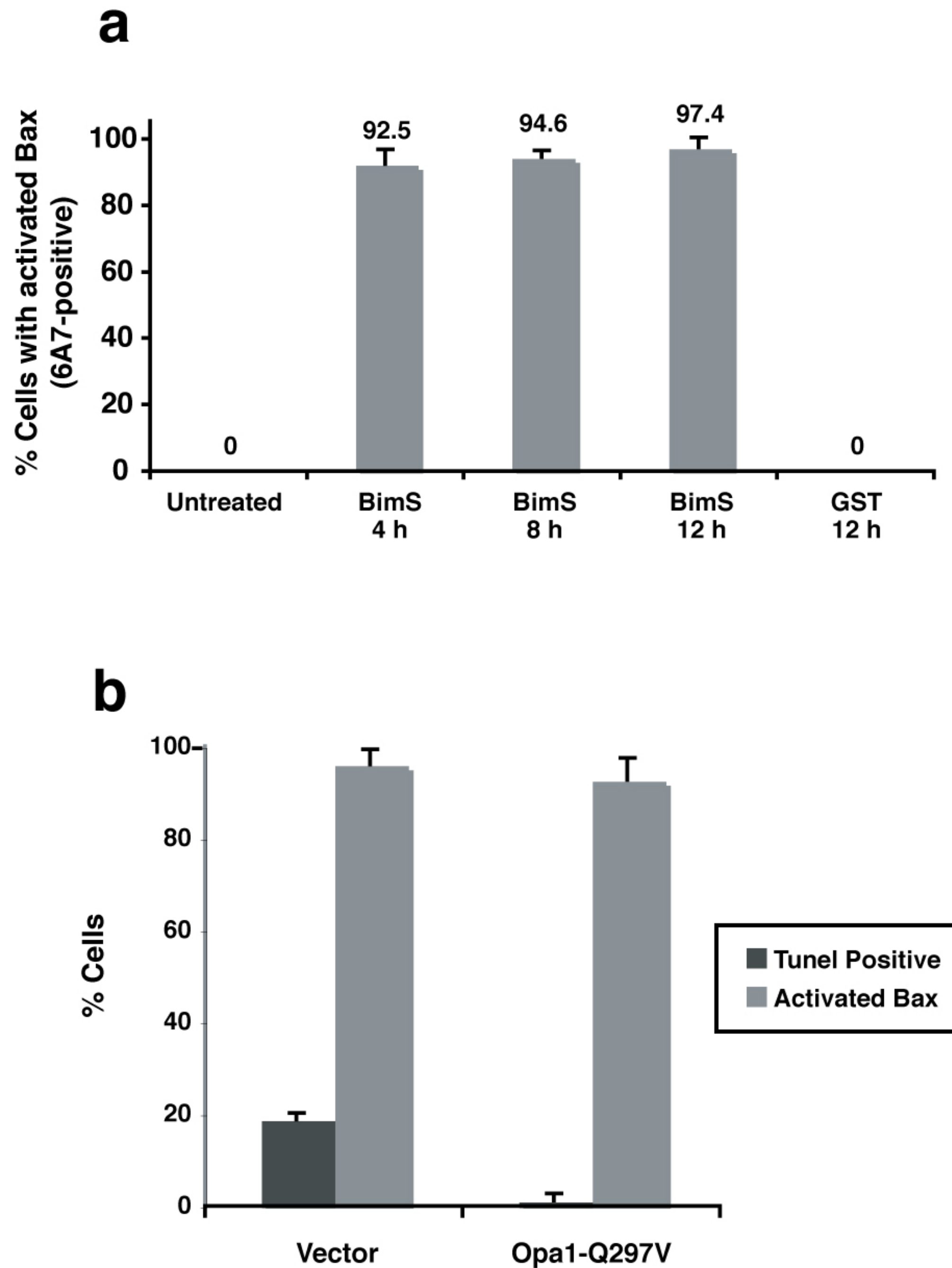


Figure S6. Example results from screen of Opa1 point mutants for altered self-binding in an in vitro assay. GST-Opa1 was incubated with radiolabeled in vitro-translated Opa1 WT, Opa1-T302N, Opa1-Q297V, or Crm1 (an unrelated protein) for 30 min in PBS containing 125 mM NaCl. GST-Opa1 WT was pulled down with glutathione-Sepharose beads, and the radiolabeled proteins were analyzed by SDS-PAGE and compared as shown with the input samples of radiolabeled proteins. Note: regions containing the bands of interest from different parts of an autoradiogram were assembled to make this figure. In all, Opa1 K255A, G295E, G295S, G300E, Q297A, Q297V, Q310A, Q310V, T302N, K301A (all variant 1), and Opa1 WT variant 8 were analyzed for binding to themselves and WT Opa1 variant 1. In this in vitro binding screen, only Q297V displayed altered interactions: enhanced binding to GST-fusions of itself and other forms of Opa1; this mutant was selected for further analysis.