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Supplemental Data
Granzyme A Cleaves a Mitochondrial Complex I Protein to Initiate Caspase-Independent Cell Death

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Supplemental Experimental Procedures

Cells, antibodies, and reagents

K562, HeLa, EL4 and 293T cells were from ATCC. Cells were grown in K10 medium (RPMI1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 2 mM HEPES, 100 units/ml of penicillin, 100 mg/ml of streptomycin) or MDEM supplemented with 10% fetal calf serum, 2 mM glutamine, 2 mM HEPES, 100 units/ml of penicillin, 100 mg/ml of streptomycin. CTL lines were generated from P14xGzmB$^{-/-}$ and P14xGzmA$^{-/-}$ mouse splenocytes activated with 1 μg/ml LCMV peptide gp33 (KAVYNFATC) for 1 hr, washed and cultured in K10 medium to which 25 IU/ml recombinant human IL-2 was added every other day beginning on day 2. Cell lines were used for experiments after 8-15 days of culture. Mice were backcrossed and maintained under specific pathogen-free conditions. Animal experiments were approved by the Harvard Medical School Animal Care and Use Committee. The following antibodies were used: NDUFS3, NDUFA9, NDUFB6, MTND6 and ATP5H (mouse monoclonals, Invitrogen, Carlsbad CA), Hsp70 and Hsp90 (BD PharMingen, San Diego CA); Hsp60 (Santa Cruz Biotechnology, Santa Cruz, CA); HA (rat mAb, R&D Systems, Minneapolis MN); α-tubulin (mouse mAb, Sigma-Aldrich, St Louis MO). Goat anti-GAPDH was from Abcam (Cambridge MA). Rabbit antiserum to SET peptide 3-16 and anti-GzmA (CB9) were produced as described (Beresford et al., 2001). GzmA antibody used for immunoblot was from AbD Serotec (Raleigh NC). Complex I Immunocapture Kit was from MitoScience (Eugene OR). Protein G agarose was from Roche Applied Science (Indianapolis IN). PI, *n*-dodecyl β-D-maltoside (lauryl maltoside), 3-[N,N-Dimethyl(3-myristoylaminopropyl) ammonio]propanesulfonate Amidosulfobetaine-14 (ASB 14), urea, thiourea, tributylphosphine, sodium pyruvate, uridine and ethidium bromide were from Sigma-Aldrich; HE, JC-1, Mitotracker Deep Red, MitoSox, annexin V-APC, and SilverQuest Silver Staining Kit, H_2DCFDA, ATP determination kit and Alexa Fluor 488 Labeling Kit were from Invitrogen; ReadyStrip IPG Strips, Bio-Lyte 3/10 ampholytes, ReadyPrep 2-D cleaning kit and iodoacetamide from Bio-Rad (Hercules CA); caspase inhibitor peptides, valinomycin, 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) and NADH from Calbiochem-Novabiochem (San Diego CA); Passive Lysis Buffer (Promega, Madison WI); Sephacryl S-300 from GE Healthcare (Piscataway NJ). The gp33 peptide was synthesized and purified at the Tufts Peptide facility. Recombinant human GzmA and inactive S-AGzmA, recombinant mouse GzmB and recombinant SET were expressed and purified as previously reported (Beresford et al., 1999; Beresford et al., 2001; Xia et al., 1998). PFN were purified from rat RNK-16 cells and cells were loaded with sublytic concentrations of PFN as described (Beresford et al., 1999). Midikros Hollow fiber membrane module was from Spectrum Laboratories, Inc (Rancho Dominguez, CA).

Human cytolytic granules were prepared from human lymphokine activated cells as described (Davis et al., 2003).

Cloning of *NDUFS3*

The multiple cloning site in pcDNA5/FRT (Invitrogen) was modified to contain the coding sequence for the HA epitope tag between the Not I and Xho I restriction endonuclease sites using complementary oligonucleotides: HA forward, 5'p-GGCCGCTATCCATATGACGTTCCAGATTACGCTTAGC-3' and HA reverse, 5'p-TCGAGCTAAGCGTAATCTGGAACGTCATATGGATAGC-3'. The *NDUFS3* cDNA was obtained from Origene technologies and cloned into the Eco RV and Not I sites of the HA-containing pcDNA5/FRT by PCR amplification using *NDUFS3* specific primers: forward 5'-ATGCGATATCATGGCGGCGGCGGCGGTAGCC-3' and reverse 5'-GCTAGCGGCCGCCCTTGGCATCAGGCTTCTTGTCT-3'. Similarly, NDUFS3 was cloned into the Eco RI and Xho I restriction endonuclease sites of pET-26b(+) (Novagen) using *NDUFS3* specific forward (5'-GATCGAATTCGATGGCGGCGGCGGCGGTAGCC-3') and reverse (5'-GATCCTCTGAGCTTGGCATCAGGCTTCTTGTCT-3') primers to produce an in-frame fusion of NDUFS3 with the His-tag provided from the pET26b(+) vector. The *NDUFS3-HA* tag was subcloned from the pcDNA5/FRT vector described above into the Nhe I and Xba I sites in pcDNA3.1-hygro(-) (Invitrogen). The Granzyme A resistant form of *NDUFS, K56A-NDUFS3*, was made by introducing two point mutations in codon 56 to substitute Ala for Lys. This was accomplished using the Quik-Change® II site-directed mutagenesis kit (Stratagene, La Jolla CA) and the mutant forward (5'-CCACGGAATGATGTGGCCCAC**GC**GCAGCTCTCAGCTTTTGGAG-3') and reverse (5'-CTCCAAAAGCTGAGAGCTGC**GC**GTGGGCCACATCATTCCGTGG-3') primers (mutation in bold).

Electron microscopy and immunoelectron microscopy

Samples were fixed for 2 hr in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. For transmission electron microscopy, samples were embedded in resin and ultrathin sections were analyzed. For immunoelectron microscopy, ultrathin frozen sections were immunogold-labeled. Both transmission electron microscopy and immunoelectron microscopy were performed at the Electron Microscopy Facility, Department of Cell Biology, Harvard Medical School. Detailed protocols are available at: http://www.cellbio.med.harvard.edu/research_facilities/conventional_electron_microscopy/#Methods.

Intracellular ROS production and dissipation of $\Delta\Psi_m$

ROS production was monitored by adding 2 μM HE just before flow cytometry analysis to cells treated for 1 hr (or indicated time) at 37^0C. Changes in $\Delta\Psi_m$ were monitored with the potentiometric dye JC-1 using the Mitochondrial Membrane Potential Detection Kit (BioCarta, San Diego CA). As a control, cells were cultured overnight following UV irradiation (200 μJ/cm^2 x 5 min). Flow cytometry analyses were performed using a Becton Dickinson FACSCalibur flow cytometer and Cellquest Pro software.

Isolated mitochondria assays

Mitochondria and S100 supernatants were freshly prepared from mouse liver as described (Susin et al., 2000). Mitochondria (0.5 mg/ml protein) were incubated with or without S100 supernatant (1 mg/ml) plus Gzms at indicated concentrations in a final volume of 60 μl of mitochondrial buffer (220 mM sucrose, 68 mM mannitol, 10 mM KCl, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 0.5 mM EGTA, 5 mM succinate, 2 mM rotenone, 10 mM HEPES, pH 7.2) for 30 min at 37^0C. Mitochondria were stained with 150 μM HE for flow cytometry as above. To determine whether Hsps were contained in mitochondrial preparations, mitochondria were washed at 4^oC several times with 0.2 M $HCO3^-$ pH 10 or mitochondrial buffer.

Mitochondrial import assay was performed as reported (Young et al., 2003) with slight modification. Purified mouse liver mitochondria (0.75 mg/ml in 250 mM sucrose, 80 mM KOAc, 20 mM HEPES, pH 7.5, 5 mM MgOAc) were pre-incubated at room temperature for 20 min with or without the indicated concentration of valinomycin before adding 1 μM SET-Alexa488 or 2 μM GzmA-Alexa488 for an additional 20 min at RT. Treated mitochondria were washed in the same buffer before incubation with 2.5 μg/ml trypsin for 10 min at 4^0C. The reaction was stopped with 1 mM PMSF. After 2 more washes with buffer, Alexa488 fluorescence was evaluated by flow cytometry.

Live cell imaging for ROS production

HeLa cells, plated the night before at $5x10^4$ cells/well on collagen-coated cover slides (VWR) in 6 well plates, were stained for 1 h at 37^0C with 25 nM MitoTracker Deep Red plus 2.5 μM MitoSOX Red mitochondrial superoxide indicator in DMEM complete medium. After three washes in complete medium, the cells were incubated in complete medium for 30 min at 37^0C. Just before imaging, cover slides were washed three times in warm HBSS and incubated in a heated imaging chamber containing 1 ml of loading buffer with either PFN alone or PFN plus 0.4 μM GzmA. Fluorescence was observed immediately in a time-lapse manner using a Zeiss laser-scanning fluorescent microscope and SlideBook 4.0 OS X digital imaging software.

2-D Gel electrophoresis

Samples were treated with 2-D gel compatible lysis and loading buffer (1% ASB 14, 7 M urea, 2 M thiourea, 2 mM tributylphosphine and 0.2% ampholytes). The samples were precipitated with TCA (ReadyPrep 2-D cleaning kit) to remove all non-protein contamination. 50 μg of protein per sample were resolved by 2-D gel electrophoresis. The first dimension was performed using 17 cm non linear ReadyStrip IPG Strips pH 3-10. After 24 hr rehydration and loading, the samples were focused for 1 hr at 250 V, then 1 hr at 500 V followed for 10 hr at 10000 V using the PROTEAN IEF Cell from Bio-Rad (Hercules CA). The IPG Strips were equilibrated for 10 min in 10 ml equilibration buffer I (6 M urea, 375 mM TrisHCl, pH 7.4, 2% SDS, 2% glycerol, 2% DTT) then for 10 min in 10 ml equilibration buffer II (6 M urea, 375 mM TrisHCl, pH 7.4, 2% SDS, 2% glycerol, 2% DTT, 2% iodoacetamide) before the second dimension analysis on a 20 by 20 cm, 12% polyacrylamide gel. The gels were silver stained and analyzed using the 2-D gel analysis software Protetix 2D from Nonlinear Dynamics (Newcastle, UK). Spots

were picked, digested with trypsin in gel then extracted from the gel before fractionation on a nano-scale reverse-phase HPLC and analyzed by electrospray ionization and LTQ linear ion-trap mass spectrometry at the Taplin Biological Mass Spectrometry Facility, Harvard Medical School. Data were analyzed using the SeQuest data base search program, filtered for tryptic peptides using a cross correlation value of 1.5 at position +1, 1.5 at position +2 and 3 at position +3, associated with a delta correlation value greater than 0.1.

Complex I purification

Complex I was purified as reported by (Sazanov et al., 2000; Carroll et al., 2003). The positive fractions from the S-300 gel filtration column were pooled and diluted in PBS to a final concentration of 0.1 mM lauryl maltoside (below the critical micellar concentration of 0.16 mM) and the diluted sample was then concentrated using Midikros Hollow fiber membrane module by cross flow filtration according to the manufacturer (Spectrum Laboratories**)**. Complex I was also affinity-purified using the MS101 complex I immunocapture kit from Mitoscience according to the manufacturer's protocol. In this case, the excess of lauryl maltoside was removed by TCA precipitation.

Complex I activity assay

Complex I activity was monitored by measuring the conversion of NADH into NAD^+, as reported in (Yamaguchi et al., 1998), with some modifications. 5-10 μg of mitochondria were incubated in 200 μl Complex I buffer (50 mM potassium phosphate, pH 6.5, 5 mM $MgCl_2$, 2 mM KCN, 0.13 mM NADH) at 37°C in the presence or absence of GzmA or catalytically inactive S-AGzmA, and complex I activity was measured after 15 min by OD_{340}, (ε_{340} NADH = 6.22 mM^{-1}).

Generation of pseudo ρ^0 cells

Pseudo ρ^0 cells were generated as described (Kaminski et al., 2007). K562 were cultured in K10 medium containing 250 ng/ml ethidium bromide, 50 μg/ml uridine and 110 mg/ml sodium pyruvate for 3 wk. MtDNA was quantified by real time PCR on the BioRad iCycler using the Syber® Green PCR master mix (Applied Biosystems, Foster City, CA) and the mitochondrial-specific primers; mito sense 5'-GACGTTAGGTCAAGGTGTAG-3' and mito antisense 5'-CAACTAAGCACTCTACTCTC-3'. All measurements were normalized to GAPDH; GAPDH sense 5'-GACCCCTTCATTGACCTCAAC, and GAPDH antisense 5'-CTTCTCCATGGTGGTGAAGA-3'. Each assay was performed in triplicate.

Immunoprecipitation

Cells were lysed in PBS containing 1% lauryl maltoside plus complete protease inhibitor cocktail for 2 h at 4°C. The post-nuclear supernatant were incubated overnight at 4°C with either anti-HA agarose beads or with GzmA (CB9) antibody bound to protein G-agarose beads. For Hsp co-IP, incubation was done in the presence or absence of 10 mM ATP. Beads were washed 3-5 times with lysis buffer before eluting with 2 ml 0.2 M glycine HCl, pH 2.5. The eluate was neutralized and then concentrated using a Centricon filter (10kDa cutoff) (Millipore, Billerica MA) and analyzed by immunoblot.

ATP determination
Cells (10^6) grown under aerobic conditions were washed in HBSS and cell pellets were lysed using Passive Lysis Buffer and 3 freeze-thaw cycles. ATP levels were measured using the ATP determination kit according to the manufacturer's protocol.

Cytotoxicity assays
EL4 target cells ($2x10^6$ in 1 ml K10) were incubated with 0.5 μg/ml of LCMV gp33 peptide for 1 hr in the presence of 100 μCi/ml of $Na_2{}^{51}CrO_4$ at 37^0C. After washing, $2x10^3$ target cells in triplicate microtiter wells were incubated with the indicated effector cells at indicated effector:target ratios in 150 μl at 37^oC for 6 hr. After centrifugation at 760xg for 5 min, 50 μl of supernatant was counted on a TopCount (Packard Instrument Company). Percent specific lysis was defined as [(cpm – spontaneous release)/(total release - spontaneous release)]x100.

Statistical analysis
Comparisons between experimental conditions were by student's 2-sided t-test, except for the analysis of specific lysis of transduced target cells by CTLs, which was analyzed by the Wilcoxon rank-sum test.

Supplemental Figure 1

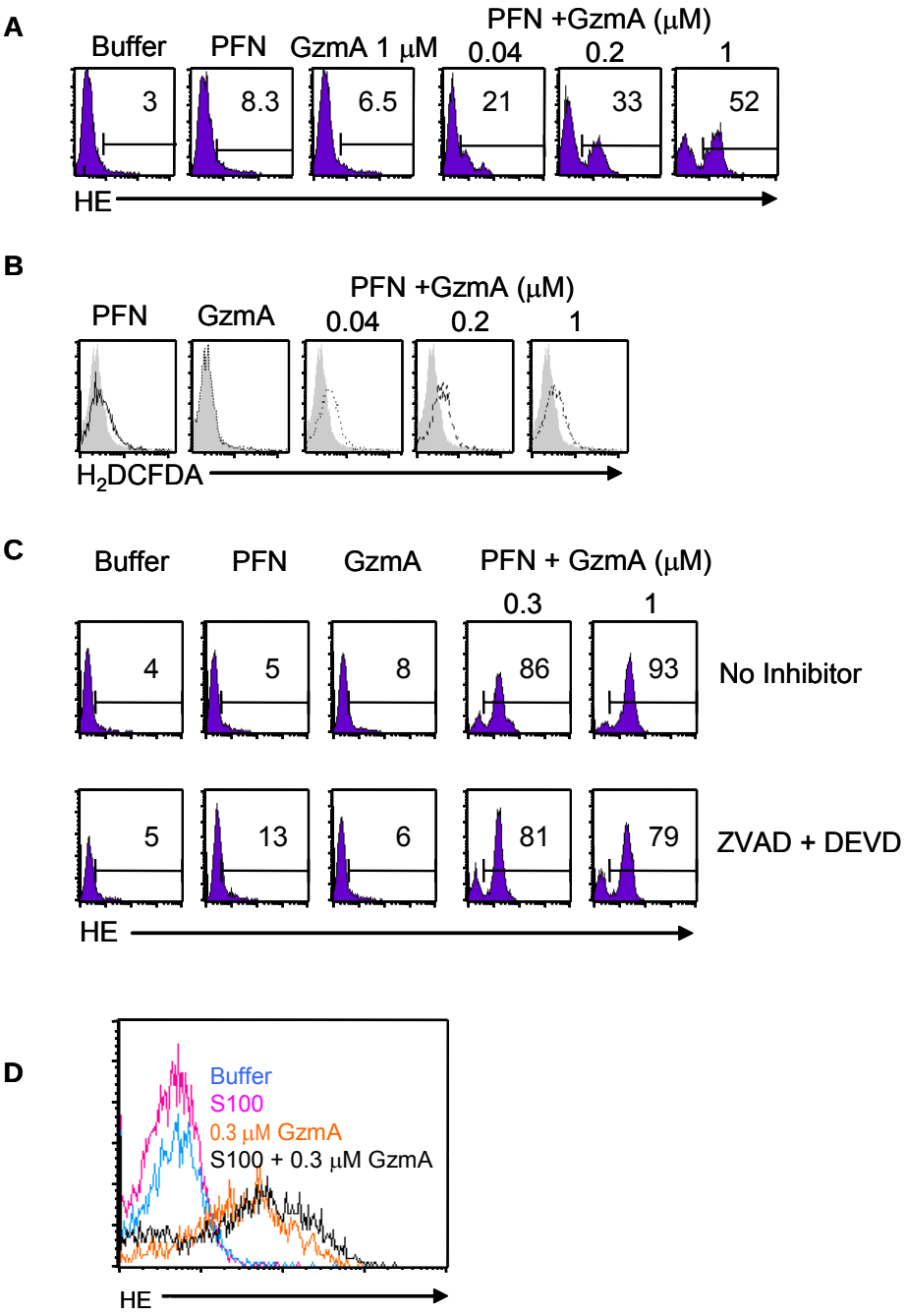

Figure S1. GzmA induces a caspase-independent increase in ROS by directly targeting the mitochondria

(A) GzmA loading of K562 cells with PFN induces a dose-dependent increase of ROS. K562 cells were treated with buffer, a sublytic dose of PFN, GzmA alone or PFN plus increasing GzmA concentrations and stained for ROS with HE.
(B) Cells were treated as in (A) and ROS was measured by H_2DCFDA staining. Grey histograms are buffer-treated cells and white histograms are cells treated as indicated.
(C) PFN loading of GzmA in K562 induces a caspase-independent increase in ROS. K562 cells, preincubated or not with 100 μM ZVAD and DEVD as pan-caspase inhibitors (not shown), were treated with PFN and/or GzmA and stained for ROS. Caspase inhibition did not inhibit ROS production.
(D) GzmA treatment of purified mitochondria induces a rapid increase in ROS. Freshly purified mouse liver mitochondria were either untreated or treated with GzmA in the presence or absence of cytosolic S100 fraction and stained for ROS with HE. On its own, GzmA induced ROS. Results shown are representative of at least 3 independent experiments.

Supplemental Figure 2

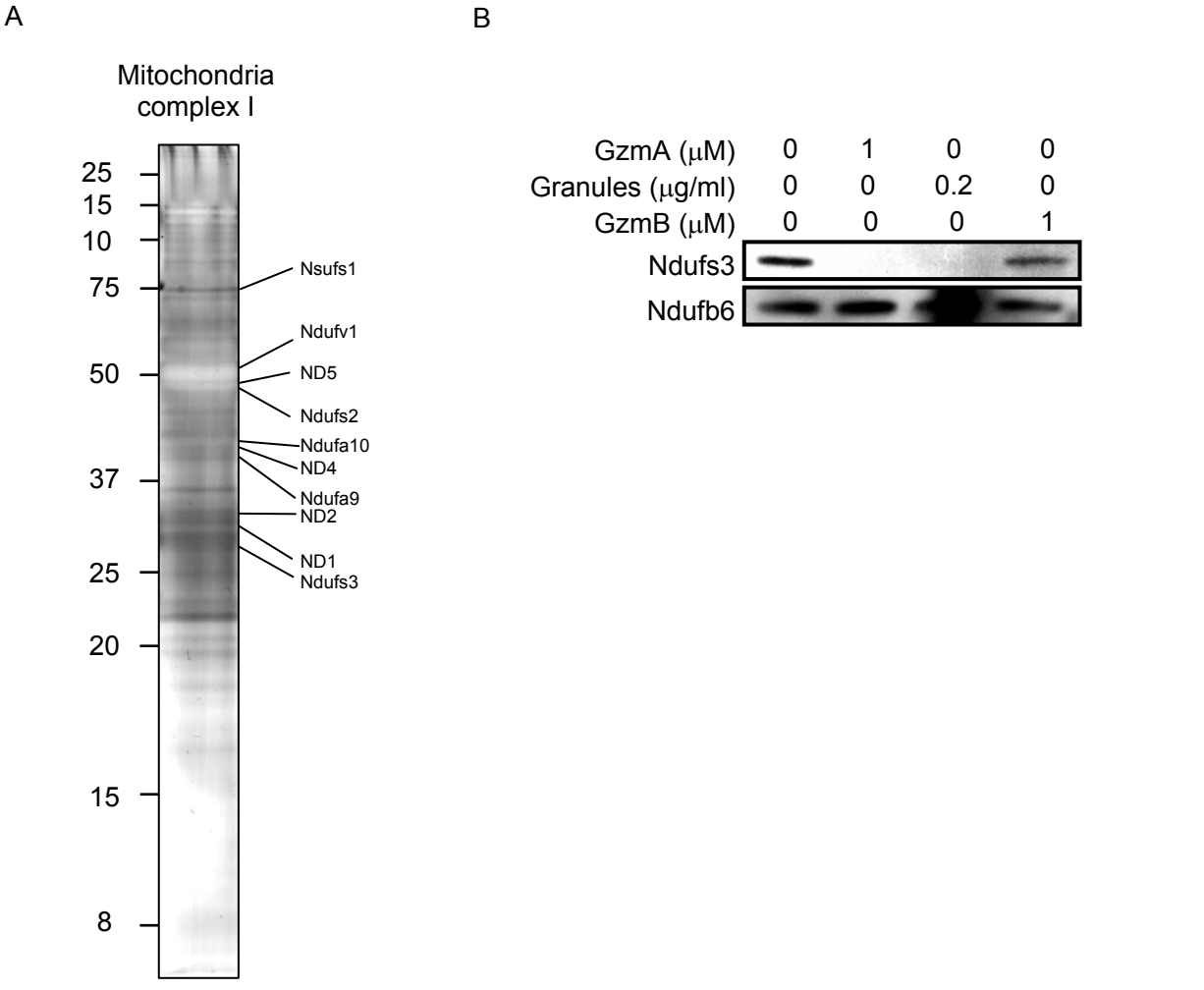

Figure S2. Ndufs3 in purified ETC complex I is targeted by purified rat CTL granules and recombinant human GzmA

(A) Mouse liver mitochondrial complex I was purified by immunocapture and analyzed by gel electrophoresis and silver stain. Annotation on the right identifies the most prominent complex I subunits.

(B) Mouse Ndufs3 is cleaved by human and rodent GzmA. Complex I was treated with purified rat CTL granules or recombinant human GzmA or GzmB. Nudfs3, but not Ndufb6, was cleaved by both sources of GzmA, but not by GzmB.

Supplemental Figure 3

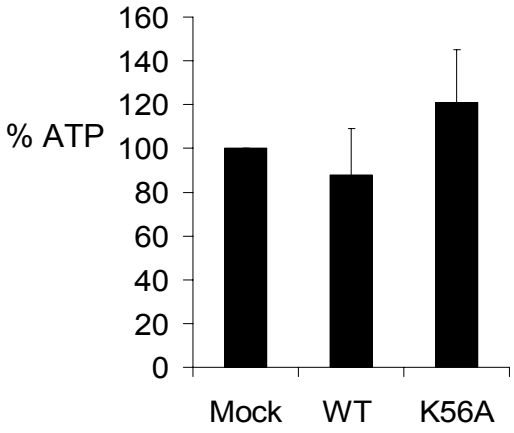

Figure S3. Over-expression of K56A-NDUFS3 doesn't alter cellular ATP levels

EL4 cells stably transfected with empty vector, WT NDUFS3 or K56A-NDUFS3 expression vectors were grown in normal aerobic conditions and ATP content was measured and compared to ATP levels in mock transfected cells.

Supplemental Figure 4

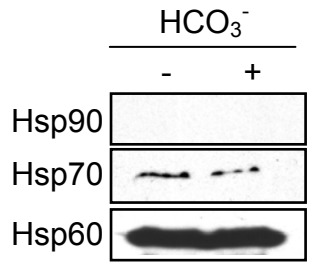

Figure S4. Hsp70 adheres to isolated mitochondria
Purified mouse liver mitochondria were washed or not with bicarbonate ($HCO3^-$) and the presence of Hsps in the mitochondrial pellet assessed by immunoblot. Hsp70 was detected in isolated mitochondria, but levels decreased after bicarbonate washing, suggesting that some Hsp70 was loosely adhered to the mitochondrial outer membrane, but some was either internalized or more firmly adherent. By contrast, no Hsp90 was detected and matrix Hsp60 was detected, but was not affected by washing.

Supplemental Figure 5

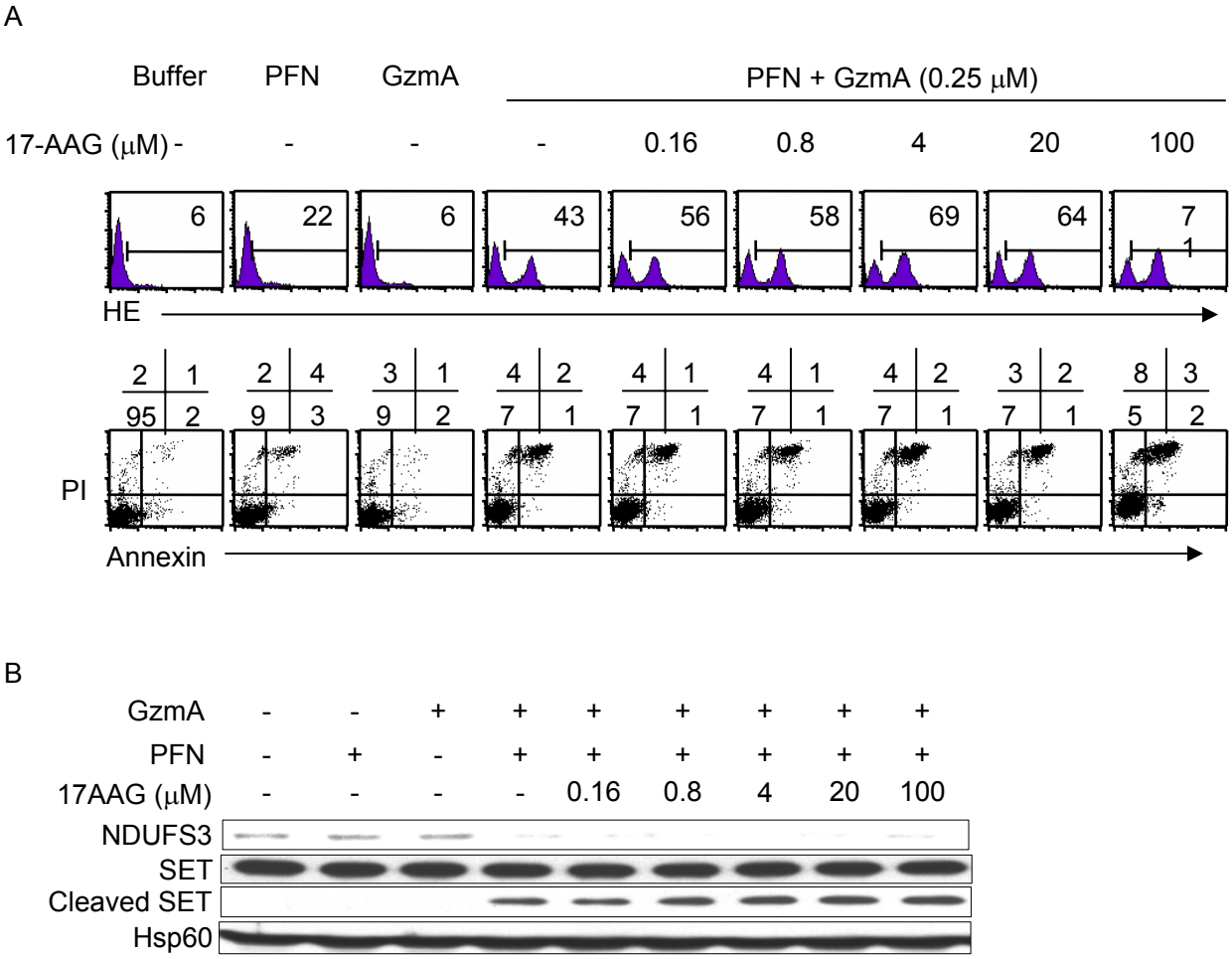

Figure S5. 17-AAG increases GzmA-induced ROS, cell death and SET cleavage. (A) K562 cells, preincubated or not with the Hsp90 inhibitor 17-AAG as indicated, were treated with PFN and/or GzmA and stained for ROS (top panel) and cell death by annexin-V +PI (bottom panel). (B) K562 cells were treated as in A, and SET cleavage was followed by immunoblot.