Supporting Information

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SI Materials and Methods

Chicken GSE Library. The GSE cDNA library was provided by Elena Feinstein (Weizmann Institute of Science, Rehovot, Israel). It was constructed according to provided guidelines (1) but with some modifications. PolyA plus RNA was prepared from chick embryo fibroblast (CEF) cells (7-day embryos, around 35 population doublings). To achieve equal representation of 5′- and 3′- mRNA sequences in random fragment cDNA preparation, cDNA synthesis was carried out on fragmented mRNA using random oligonucleotides as primers. The resulting cDNA was ligated to the "universal linker" shown in Fig. S1*B*. cDNA fragments ranging from 100 to 400 bp were selected by agarose gel fractionation for further PCR amplification using the sense oligonucleotide of the universal linker. To achieve equal representation of transcripts of different abundance, the library was normalized (2). For this, aliquots of library DNA were denatured and reannealed for different time intervals. DNA from each time point was subjected to HAP (hydroxylapatite) chromatography. The aliquots of isolated ds- and ss-DNA were PCR amplified, blotted onto nylon membranes, and analyzed by Southern hybridization according to standard protocol for the relative abundance of different mRNA sequences. We used the chicken p53, myc, and β-actin cDNAs as reference probes for low, middle, and high abundance messages, respectively. The fraction that contained similar proportions of those cDNA sequences was used for library preparation. The pool of normalized cDNA fragments was digested with ClaI and ligated into the ClaI site of the RCASBP(A) vector. The complexity of the obtained library was $\approx 10^6$ clones, sufficient for isolation of cDNAs (GSEs) corresponding to most genes expressed.

Cells, Cell Culture, and Induction of Apoptosis. The immortalized CEF cell line DF-1 was obtained from the American Type Culture Collection (ATCC number CRL-12203) and cultured in Dulbecco's modified minimum essential medium (DMEM), supplemented with 2-mM glutamine, 100-U/ml penicillin and streptomycin, and 10% FCS (FCS, Life Technology). HeLa, 293, PC3, and CWR22R cells were cultured in DMEM supplemented with 2-mM glutamine, 100-U/ml penicillin and streptomycin, and 10% FBS (FBS, Life Technology). The purified stock of FAS agonistic antibody [hybridoma IPO-4 (3)] had a concentration of 1 mg/ml and was used at a final concentration of 1 μg/ml unless indicated otherwise. CHI (0.5 μg/ml) was used in all FAS treatment experiments involving human cells (it was not used with avian cells) by addition along with FAS antibody to a final concentration of 1 μM. CHI blocks protein synthesis, and at this concentration is presumed to counter the antiapoptotic effect of NF-κB activation during receptor-mediated apoptosis. We found that in HeLa cells, FAS-induced apoptosis was much more efficient in the presence of CHI (data not shown). Staurosporine was used at a concentration of 0.1 μM. The proteasomal inhibitor MG-132 was used at concentration of 10 μM (Calbiochem). The caspase inhibitors Z-VAD-FMK (Sigma, V116) and Z-IETD-FMK (Sigma, C1230) were used at concentration of 10 μM. Caspase 9 inhibitor Z-LEHD-FMK (Sigma, C1355) was used at concentration of 10 μM. Recombinant TRAIL was used at a final concentration of 0.1 μg/ml (a kind gift of Janet Houghton, Cleveland Clinic, Cleveland, OH). TNF-α was used at a final concentration of 0.5 μg/ml (Peprotech, cat # 300–01A). TRAIL and TNF-α were used in combination with CHI (0.5 μg/ml).

Vectors and Constructs. The RCASBP(A) and RCASBP(B) vectors (4) were a kind gift from Stephen H. Hughes (National Cancer Institute, Frederick MD). pBSfi I, a modified version of pBluescript in which the multicloning site is flanked by two SfiI sites, was a kind gift from Masa Aoki (The Scripps Research Institute, La Jolla, CA). RCASBP(A)SfiI and RCASBP(B)SfiI were prepared by inserting the SfiI adaptor with multiple cloning sites from pBSfi I into the ClaI site of the RCASBP(A) and (B) vectors. For expression of human FAS in chicken DF-1 cells, the full-length human FAS cDNA clone was obtained from Invitrogen. The FAS ORF was cloned into the pBSfi vector. It was then cut out with SfiI and directionally cloned into RCASBP(B)SfiI. For generation of the chicken GSE library, GSE inserts (see *Chicken GSE Library* preparation section, above) were cloned into the ClaI site of RCASBP(A). Recovered individual GSEs, as well as all other constructs, were prepared using the RCASBP(A)SfiI vector. Recovered individual GSEs were amplified by PCR using oligonucleotide primers specific for the corresponding genes, cloned into pBSfi I, and subsequently transferred into the RCASBP(A)SfiI vector. Sense and antisense constructs were identified by sequencing. All lentiviral constructs for overexpression experiments were prepared using the pLM-CMV-PL3 vector, a kind gift of Dr. Peter Chumakov (Cleveland Clinic, Cleveland, OH). The inserts were amplified by PCR with specific primers, digested with appropriate restriction enzymes, and cloned under the CMV promoter. All shRNA constructs were prepared using the pLSLP lentiviral vector containing the puromycin resistance gene. Expression of shRNA was driven by the H1 promoter.

Generation of Cyt b-Encoding cDNA in a Nuclear Code. The artificial (nuclear coded for cytoplasmic expression) Cyt b construct was synthesized by GENEART. The Cyt b gene sequence was optimized using Proprietary GeneOptimizer software, allowing optimal expression. The nuclear recoded Cyt b was supplied with a FLAG tag sequence at its C terminus. Briefly, synthesis of the artificial gene by GENEART involved generation of DNA oligonucleotides covering the entire gene sequence. The oligonucleotides are annealed to form a full-length contig, the gaps in which are filled by DNA polymerase followed by ligation and cloning into a GENEART-provided shuttle vector. Upon receiving the construct from GENEART, the insert was recloned into the pLM-CMV-PL3 lentiviral expression vector under the CMV promoter and sequenced to verify the sequence. In addition to full-length Cyt b, three derivatives were generated from the artificial Cyt b gene [see supporting information (SI) Fig. S2]: (*i*) Cyt $b^{508–1183}$ encoding the C-terminal portion, (*ii*) Cyt $b^{1–507}$ encoding the N-terminal portion, and (*iii*) Cyt $b^{856–982}$ encoding the 42 aa within the C-terminal portion of Cyt b that correspond to GSE F21. These derivatives were generated by PCR amplification and cloned into the pLM-CMV-PL3 lentiviral vector. The constructs were tested for the ability to drive expression of their respective proteins in transfected 293 cells and in transduced HeLa and DF-1 cells. Each construct gave rise to a protein of the expected size as visualized by Western blotting with both anti-FLAG and anti-Cyt b antibodies (see *Western Blot Analysis and Antibodies* section, below).

Retroviral and Lentiviral Transduction. All chicken retroviral transductions using RCASBP(A)- and (B)-based constructs were done as follows: plasmid DNA was transfected into DF-1 cells according to the standard Lipofectamin Plus (Invitrogen) pro-

tocol (10 μg of plasmid DNA per 10 cm plate; 50 μg per 15 cm plate). The virus was allowed to spread in the cell population for 4 days in the presence of 4-μg/ml polybrene. The culture supernatant was then collected and used to infect a fresh population of cells. For lentiviral transduction, human A293 kidney cells (BD Biosciences Clontech) were plated subconfluently on a 10-cm tissue culture plate. The next day, the cells were transfected with 3.3-μg lentiviral vector DNA, 3.3-μg pVSV-G expression plasmid and 3.3-μg packaging vector pCMVdeltaR8.2 (kind gifts of Dr. Peter Chumakov, Cleveland Clinic, Cleveland, OH) using Lipofectamine Plus (Invitrogen) according to the manufacturer's recommendations. The culture medium was changed 16 h after transfection and the virus-containing medium was collected either 48 or 72 h after transfection. The medium-containing virus was then used to infect target cells in the presence of polybrene (4 μg/ml, Sigma). Neither the RCASBP vectors nor the lentiviral vectors contained selectable markers. The efficiency of infection was monitored by RT-PCR or Western blot analysis with specific antibodies to expressed proteins. All shRNA constructs were designed using Dharmacon Web site software. Oligonucleotides corresponding to sense and antisense strands of the shRNA were ordered through Integrated DNA Technologies, Inc. 5′-phosphorylated shRNA oligos were PAGE-purified, annealed, and cloned into the pLSLP lentiviral vector (a kind gift of Dr. Peter Chumakov, Cleveland Clinic) under the H1 promoter. HeLa cells transduced with pLSLP-based shRNA constructs were selected with puromycin (1 μg/ml) for at least 3 days. Down-regulation of the targeted genes was assessed by RT-PCR with specific primers or by Western blotting with specific antibodies.

Cell Viability and Apoptosis Assays. Methylene-blue staining of cells remaining attached to the plate after induction of apoptosis was performed as an initial assessment of cell viability. Floating cells were removed from plates and the remaining attached cells were fixed with methanol for 15 min and stained with methylene-blue (1% solution in PBS) for 1 h. Plates were then rinsed with water to remove excess dye and allowed to dry completely. Cell numbers were quantitated by methylene-blue elution and spectrophotometric measurement at 630 nm using a Beckman Coulter DU800 instrument. The dye was eluted from dried plates with 0.1 N HCl at room temperature. For caspase-activation assays, HeLa, PC3, and CWR22R cells were plated in 96-well plates at 7,000 cells per well in DMEM/10% FCS and treated 24 h after plating with lentiviral isolates (where indicated, in the range from undiluted to 1:64). Dilutions were made with complete medium plus polybrene (4 μg/ml). Lentiviral isolates were incubated with cells for 4 h and were then replaced with 200-μl complete medium containing the fluorogenic substrates DEVD-AMC (7-Amino-4-methylcoumarin, *N*-CBZ-L-aspartyl-L-glutamyl-L-valyl-l-aspartic acid amide, 50 nM, Promega) to measure caspase 3/7 activation or LEHD-AMC (7-Amino-4-methylcoumarin, N-acetyl-L-leucyl-Lglutamyl-L-histidinyl-L-aspartic acid amide, 50 nM, Promega) to measure caspase 9/6 activation. DEVDase activity was measured using a fluorogenic assay kit (CellTiterBlue, Promega) after 48 h of treatment by recording fluorescence at 569/590 nm using a Labsystems Fluoroskan Ascent plate reader. For cell cycle analysis during apoptosis, cells attached to the plate as well as those in the supernatant were analyzed. Attached cells were trypsinized and collected in 1% FBS in PBS then mixed with the supernatant containing floating cells. The combined cells were centrifuged, resuspended in 0.3 ml of PBS and fixed by being slowly dropped into 5-ml cold 70% ethanol while vortexing, followed by incubation at 4°C for 30 min. To stain nuclei with propidium iodide (PI), the fixed cells were collected by centrifugation, resuspended in an appropriate volume (to give $\approx$2–5 $\times$ 10^5 cells/ml) of PBS containing 100-μg/ml RNase A and 40-μg/ml PI and then incubated at 37°C for 30 min. The stained cells were filtered through a cell strainer cap (BD Falcon) before FACS analysis on Beckton Dickinson FACSCalibur machine.

Oligonucleotides. Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (all were PAGE-purified, those indicated were 5′-phosphorylated).

For amplification of human FAS from the Invitrogen clone for further cloning, the following primers were used:

hFAS-5′Xba: 5′-CCG TCT AGA GAT TGC TCA ACA ACC ATG CTGGGC A-3′

hFAS-3′Sal: 5′-CCG GTC GAC CTA GAC CAA ACT TTG GAT TTCATT T-3′.

For generation of Cyt b derivatives (C-terminal portion, N-terminal portion and GSE) from the full-length artificial (nuclear recoded) Cyt b construct, the following primers were used for amplification:

FLAG-Cyt b-Xba-5′.

5′-ccg ccg tct aga gcc acc atg gat tac aag gat gac gac gat aag atg gga ggc tac tcag tag aca gt-3′

Cyt b-Sal-3′: 5′-ccg ccg gtc gac tca att tga gta ttt tgt ttt caa tta-3′

FLAG-Cytb-GSE-5′: 5′- CC GCC ACC ATG GAT TAC AAG GATGAC GAC GAT AAG TGG CGC TCC ATC CCC AAC-3′

Cytb-GSE-3′: 5′- C TCA TGG GTC ATG GGG GAG-3′

Gene-specific primers used for amplification of recovered GSEs are available upon request.

Western Blot Analysis and Antibodies. Cells were lysed in Nonidet P-40 lysis buffer (50-mM Tris, pH 8.0; 150-mM NaCl; 1% Nonidet P-40) containing protease inhibitor mixture at a 500-fold dilution (Sigma) for 30 min on ice. The lysates were centrifuged at 14,000 $\times$ *g* at 4°C and the supernatant was collected and used for Western blot analysis. The protein concentration was measured using DC Protein Assay kit (Bio-Rad). Equal amounts of protein were electrophoresed on gradient 4 to 20% precast gels (Novex). For immunoblotting, the proteins were transferred to a PVDF membrane (Perkin-Elmer Life Sciences) by electroblotting and the membranes were blocked in 5% milk in Tris-buffered saline with Tween 20 [TBST; 20-mM Tris-Cl (pH 8.0), 150-mM NaCl, 0.1% Tween 20], probed with the indicated primary antibody in TBST, and visualized by enhanced chemiluminescence (Perkin-Elmer Life Sciences). All primary antibodies were used at a dilution of 1:200. The goat anti-rabbit and anti-mouse HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) were used at a dilution of 1:10,000. The immunoblots were stripped [4% SDS, 62.5-mM Tris-HCl (pH 6.8), 100 mM 2-mercaptoethanol] at 55°C for 45 min before reprobing. The following primary antibodies were used: anti-Cyt b rabbit polyclonal P-49 raised against the C-terminal half of the protein was described earlier (5), anti-FLAG M2 mouse monoclonal (Sigma), anti-caspase 8 SC-56070 mouse monoclonal IgG (Santa Cruz Biotechnology), anti-PARP H-250 rabbit polyclonal (Santa Cruz Biotechnology), anti-GRP75 C-19 goat polyclonal (Santa Cruz Biotechnology), anti-PCNA F-2 mouse monoclonal (Santa Cruz Biotechnology), anti-cytochrome *c* mouse monoclonal (BD PharMingen, catalogue number 556433), anti-hFAS C-20 rabbit polyclonal (Santa Cruz Biotechnology), anti-β-actin HRP-conjugated (Sigma A3854), and anti-OxPhos compl IV mouse monoclonal (Molecular Probes, 20E8)

Cell Fractionation and Isolation of Mitochondria. Mitochondrial and cytoplasmic cell fractions were prepared as described in detail elsewhere (Benchtop Mitochondria Isolation Protocol, Mitosciences, Inc.). In brief, cells were plated on 10-cm plates and treated as described. At the end of the experiment, the floating apoptotic cells were collected from the media and the remaining

attached cells were collected by trypsinization. The floating and attached cells were pooled, washed several times with PBS, and incubated in 1-ml hypotonic buffer supplemented with protease inhibitors for 15 min (10-mM NaCl, 1.5-mM $MgCl_2$, 10-mM Tris-HCl, pH 7.5, Protease Inhibitor Mixture, Sigma). Cell membranes were disrupted by mechanical shearing (2-ml Dounce homogenizer), and 400 μl of Sucrose 2.5× MS Buffer was added (2.5× MS Buffer: 525-mM Mannitol, 175-mM Sucrose, 125-mM Tris-HCl, pH 7.5, 2.5 mM EDTA, pH 7.5). The cellular lysate containing mitochondria was separated from cell debris and nuclei by centrifugation at 500 × *g*. The supernatant was then subjected to sucrose gradient centrifugation at 30,000 × *g*. The resulting mitochondrial pellet was resuspended in RIPA buffer supplemented with protease inhibitors and the supernatant containing the cytoplasmic fraction was directly loaded onto SDS/PAGE gels for Western blot analysis. All reactions were performed on ice and all centrifugation steps were performed at 4°C. Protein concentrations were determined for equal loading.

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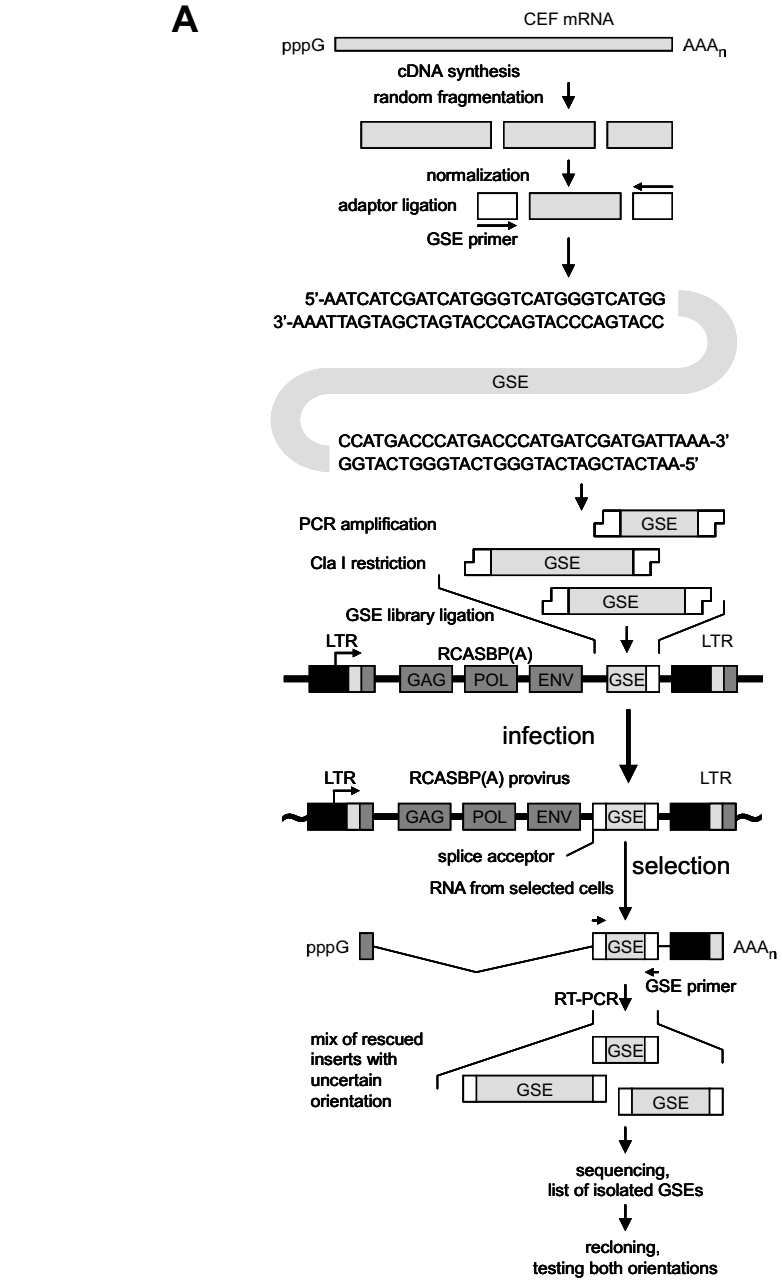

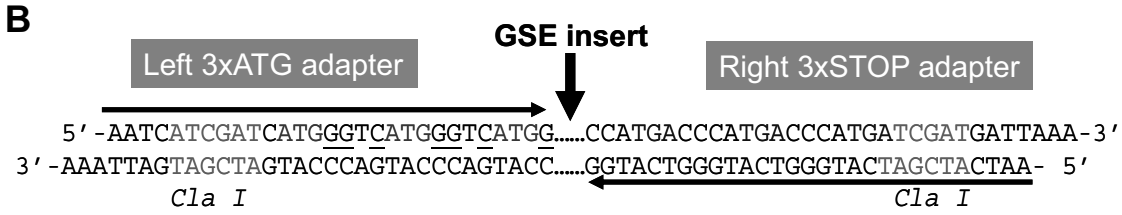

Fig. S1. (*A*) Scheme of GSE library generation in RCASBP(A) vector (details in text). (*B*) Structure of GSE adaptors, carrying three start ATG codons, three stop TGA codons, and Kozak sequence (underlined).

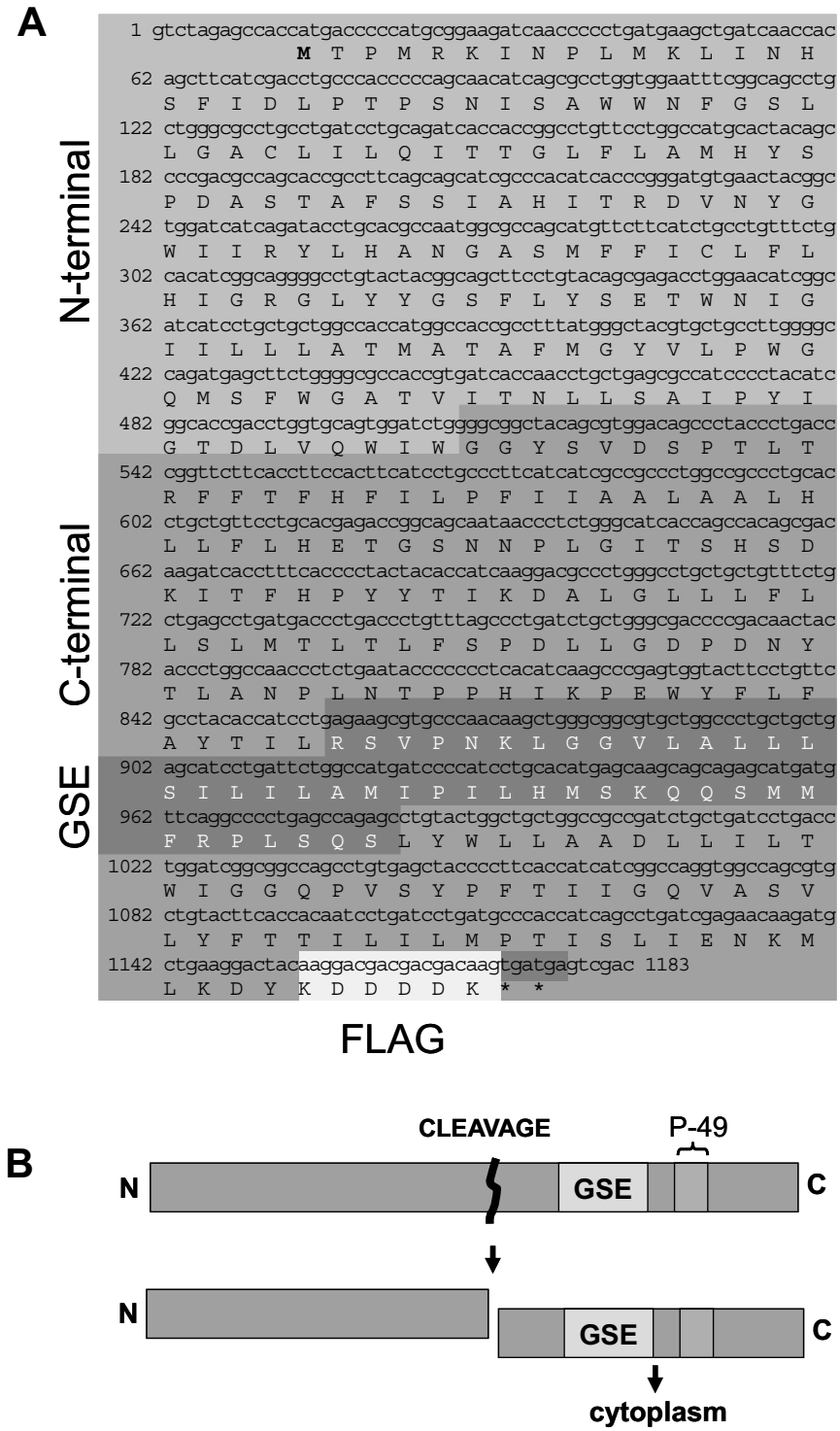

Fig. S2. (*A*) Scheme of generated artificial (nuclear recoded) Cyt b construct showing its humanized DNA sequence based on the protein amino acid content. This construct was also supplied with FLAG sequence at the C-terminal. GSE-, C-terminal, and N-terminal parts are shown. White box indicates a cluster of overlapping imperfect caspase-recognition sites. (*B*) According to our results, the C-terminal part of Cyt b is cleaved and released into the cytoplasm. The positions of the GSE sequence and the P-49 antibody epitope are indicated.

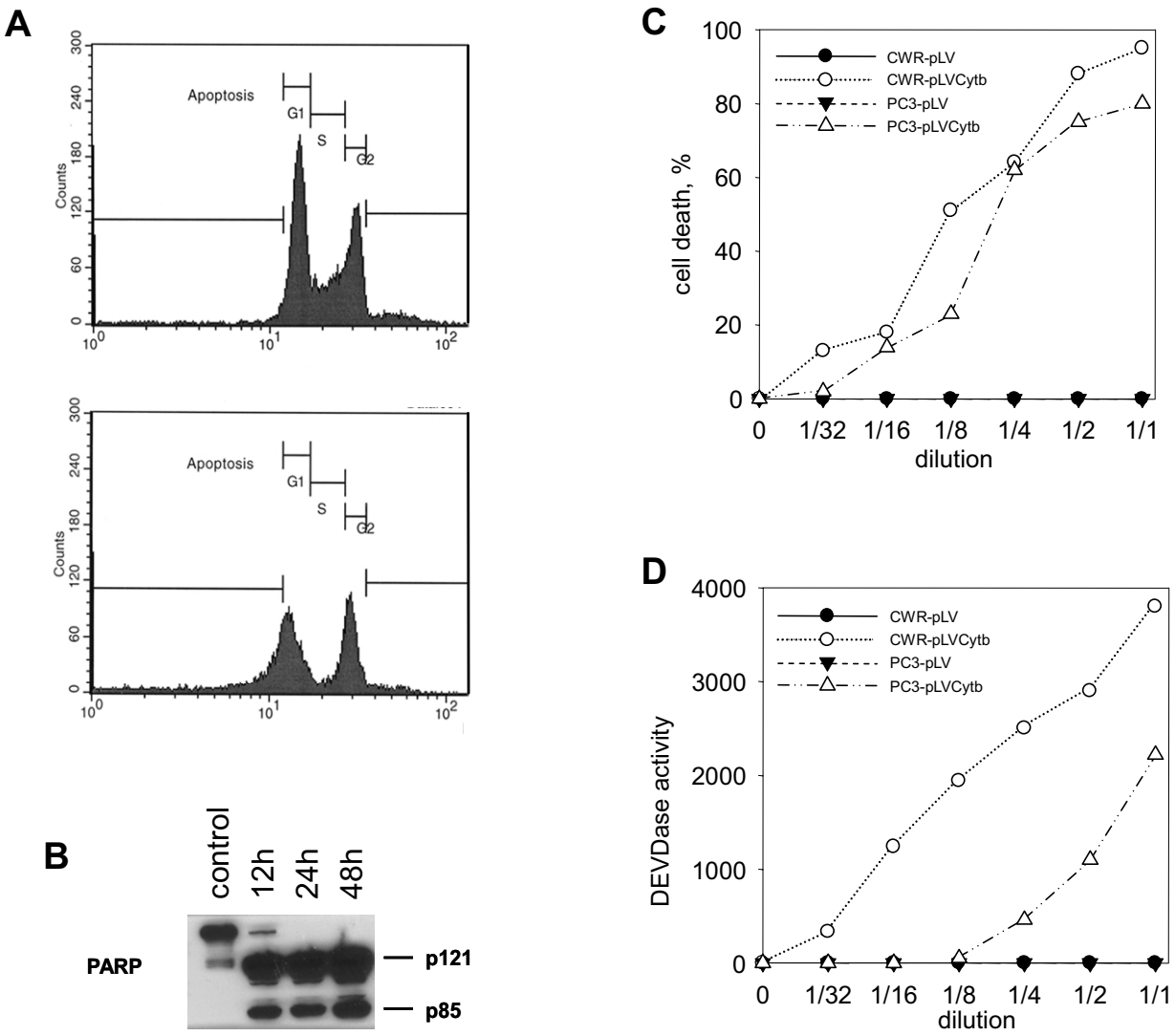

Fig. S3. (*A*) FACS analysis of the cell cycle of HeLa cells overexpressing Cyt b. Cells were collected in 48 h after infection with lentiviral constructs, and their DNA content was measured by staining with propidium iodide. Cyt b expression induces apoptosis as judged by the appearance of a population of cells with subG1 DNA content. (*B*) Western blot analysis of apoptosis-specific PARP cleavage during initiation of cell death by Cyt b. (*C*) Cyt b induces caspase activation when overexpressed in the cytoplasm of PC3 and CWR22R cells. The cells were treated with lentiviral supernatants in the range from undiluted to 1:64, and cell death was measured by methylene-blue staining of surviving cells 48 h after infection. (*D*) Cyt b induces caspase-dependent apoptosis when overexpressed in the cytoplasm of PC3 and CWR22R cells. The cells were treated with lentiviral supernatants in the range from undiluted to 1:64. At 48 h after infection the supernatant was replaced with DEVD-AMC to measure caspase 3/7 activation.

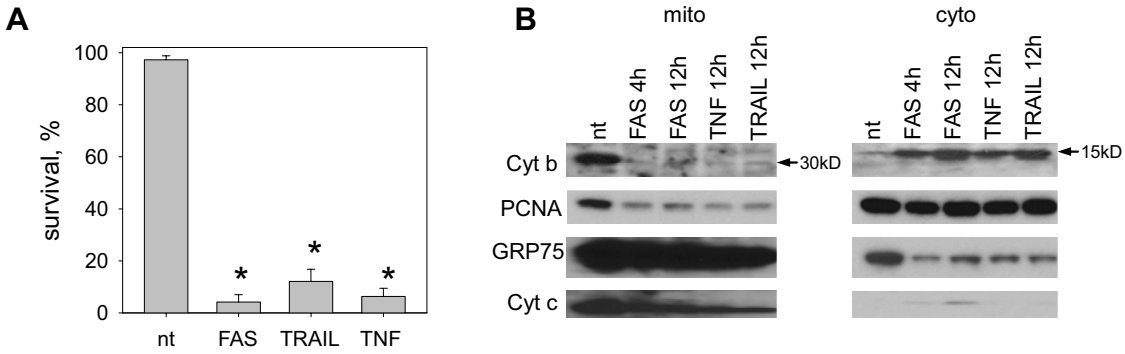

Fig. S4. Cyt b cleavage and translocation to the cytoplasm is a common property of death receptor-mediated apoptosis induced by different ligands. (*A*) HeLa cells were treated with FAS, TRAIL, or TNFα (all in combination with 1-μg/ml cycloheximide) as described in *SI Materials and Methods*. Survival was measured by methylene-blue staining 24 h after treatment. Bars represent averages of three independent experiments, with the error bars indicating standard deviations. (*B*) All three receptor-mediated apoptotic ligands induce cleavage and translocation of Cyt b to cytoplasm. Mitochondrial and cytoplasmic fractions were prepared from HeLa cells, untreated (nt) or incubated with FAS, TRAIL, or TNFα at the indicated times of treatment. Fractions were analyzed by Western blotting for Cyt b (left panel: full-length, 30 kDa; right panel: cleaved, 15 kDa), GRP75 (mitochondrial marker), PCNA (cytoplasmic marker), and Cyt c. Asterisks indicate high statistical significance ($P < 0.05$).

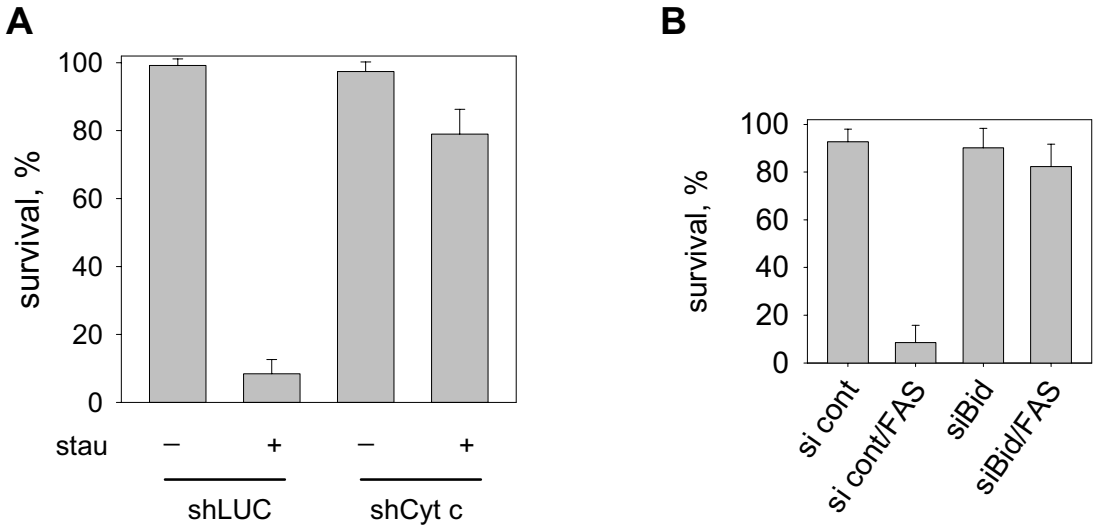

Fig. S5. (*A*) Down-regulation of Cyt c blocks staurosporine-induced apoptosis. HeLa cells were infected with lentiviruses directing expression of shCyt c or shLuc (negative control) and left untreated (nt) or treated with staurosporine (stau, 0.1 μM). Cell survival was measured on day 3 after treatment (*B*) siRNA-mediated knockdown of Bid completely blocks FAS apoptosis in HeLa cells. siRNA against Bid (a mixture of five synthetic gene-specific siRNAs) and control scrambled siRNA was obtained from Dharmacon and applied to the cells according to manufacturer's recommendations. Three days later cells were treated with FAS agonistic antibodies and cell survival was measured 24 h later by methylene-blue staining. The quantification data represent averages of three independent experiments, with the error bars indicating standard deviations. Asterisks indicate high statistical significance ($P < 0.05$).

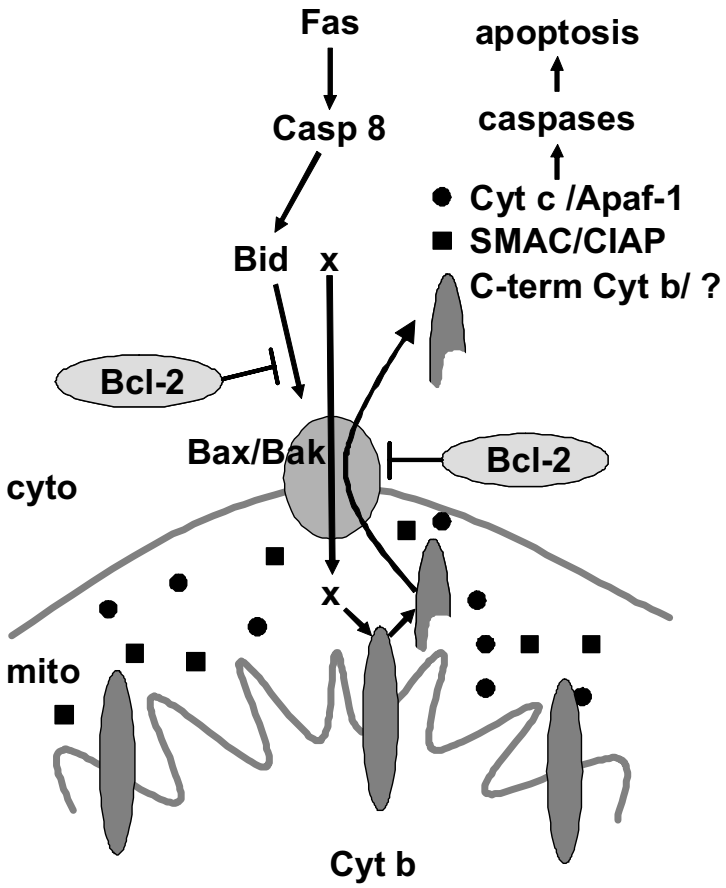

Fig. S6. Scheme of Cyt b action in FAS-induced apoptosis. FAS engagement at DISC induces a signal leading to activation of Bid and mitochondrial Bax/Bak pore opening. In addition, a second signal originates in the cytoplasm, which results in Cyt b inducing caspase-dependent apoptosis and is downstream of proapoptotic factors released from mitochondria (details in text).

Table S1. List of isolated GSEs

GSE	Length, bp	Chicken gene name	Similarity to human gene, %	Known relation to apoptosis	Known relation to FAS apoptosis	References
F21	112	Cyt b	93	No	No	1
F25	175	cFLIP	60	Yes	Yes	2,3
F6	164	KIR/NKB	33	Yes	Yes	4
F3	95	PKA	91	Yes	Yes	5
F23	122	GRP-E	75	Yes	No	6
F22	95	Moesin	66	Yes	Yes	7
F32	125	KEKE	72	No	No	—
F5	202	CGI-94	64	No	No	—
F11	182	Gi:24308185	82	No	No	—
F9	256	Fibronectin	82	No	No	—
F15	167	Actin	87	No	No	—

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