Supplemental Materials
Molecular Biology of the Cell

Flusberg et al.

SUPPLEMENTARY INFORMATION

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Reversible resistance is observed using multiple assays and in multiple cell types. **A,** Plot showing the percentage of Annexin V-positive control and day 1 survivor cells following treatment with the indicated doses of TRAIL for 6h. **B-C,** Fold-change in resistance of survivor (day 1) and reset (day 7) cells, compared to control cells, in populations of MCF10A clonal cell lines (MCF10A Clone 2, Clone 15) **(B)** or in transformed cell populations derived from the MCF10A cell line (MCF10AT1, MCF10A-NeuT, MCF10AT1a, MCF10A-NeuN; (Debnath et al, 2003)) **(C)** treated with 50ng/ml TRAIL for 6h, measured by staining with antibodies to cleaved PARP. **D**, Fold-change in resistance of survivor (day 1) and reset (day 7) cells compared to control HMLEC, U251, HeLa, and MDA-MB-231 cells, measured using the methylene blue cell viability assay. Each cell line was treated (both initially and during the re-challenge) with a dose of TRAIL sufficient to kill 50-80% of the cell population (the actual dose varied with the initial sensitivity of the cell type and ranged from 50-500ng/ml TRAIL for 6-8hr). Error bars represent standard error of triplicate samples.

Figure S2. Maintaining the resistant state depends on protein synthesis and on the duration of TRAIL treatment in the presence of caspase inhibitors. **A,** Cell viability assay of MCF10A control and day 1 TRAIL-survivors treated with the indicated doses of TRAIL + CHX (2.5μg/ml) for 6h, normalized to the percentage of cells surviving treatment with CHX alone (which was equivalent to the percentage of untreated cells). **B,** Schematic (left panel) and cell viability plot (right panel) of MCF10A control cells or cells surviving a 6h treatment with TRAIL (50ng/ml) alone, TRAIL + caspase-8 inhibitor (C8i; 20μM), or C8i alone, that were then washed, replated, and tested on day 1 for subsequent sensitivity to the indicated doses of a 6h TRAIL treatment.

Figure S3. Q-PCR validation of microarray data and supervised hierarchical clustering of microarray samples. **A**, Q-PCR of transcript levels in RNA prepared from control, survivor, reset, and repeat cells. Transcript levels were normalized to GAPDH expression and plotted as mean fold-change relative to the control, +/- standard error from replicate samples. **B**, Cluster dendrogram showing supervised hierarchical clustering of duplicate control, survivor, reset, and repeat samples based on genes differentially expressed in survivor cells compared to control cells. Approximately Unbiased/ Bootstrapping Probability (AU/BP) values (%) are shown for each cluster branch, with 100% representing maximum confidence in cluster branching. Data were analyzed from an individual experiment to minimize batch error (Leek et al, 2010).

Figure S4. **Growth curves, cell cycle profile, and NF-κB activation dynamics in MCF10A cells treated with TRAIL, and effects of TNF-alpha and IL1R neutralizing antibody on resistance. A,** Growth curves for control, survivor (day 1), and reset (day 7) cells determined using absorbance measurements of cells labeled with methylene blue at the indicated timepoints and normalized to

absorbance at time 0. **B**, Cell cycle profiles of control, survivor (day 1), reset (day 7), and repeat (day 3) cells labeled for 30 minutes with EdU and analyzed for EdU incorporation and DNA content by flow cytometry. **C,** Percentage of MCF10A cells with nuclear NF-κB (p65) following treatment with the indicated doses of TRAIL (red) or TNFα (blue) for 1h. **D**, Nuclear: cytoplasmic ratio of NF-κB in MCF10A cells treated with TRAIL (50ng/ml) or TNFα (50ng/ml) for the indicated times. **E**, Q-PCR analysis of gene expression in survivor cells compared to control cells, and in survivor cells expressing the IκB-super-repressor (IκBsr-Surv) compared to control cells expressing the IκB-super-repressor (IκBsr-Cont), normalized to GAPDH levels (mean +/- standard error of replicate samples). **F**, ELISA of secreted CXCL1 (Gro-A) in 8h-conditioned medium from control, survivor and repeat cells +/- IκBsr expression. **G**, Sensitivity of MCF10A cells +/- a 6h TNFα (100ng/ml) pre-treatment followed by an overnight recovery, to the indicated doses of a 6h TRAIL treatment on day 1. 100% of cells survived the 6h TNFα pre-treatment but are referred to in the plot as "survivors" of TNF because the experimental setup is analogous to that for preparing TRAIL-survivors. **H**, Sensitivity of control MCF10A or survivors of the indicated 6h treatments (TRAIL (50ng/ml) alone, TRAIL + IL1R neutralizing antibody (10μg/ml), or IL1R neutralizing antibody alone) to a subsequent 6h treatment on day 1 with TRAIL (50ng/ml) alone, TRAIL + IL1R neutralizing antibody (10μg/ml), or IL1R neutralizing antibody alone. IL1R antibody treatments were added 30 minutes prior to addition of TRAIL. In **G-H**, cell survival was measured using the methylene blue viability assay.

Figure S5. FLIP shRNA does not sensitize survivor cells to TRAIL-mediated death.
A, Immunoblots of Bcl-2 family protein expression levels in control, survivor (day 1), and reset (day 7) cells. **B,** Immunoblot and quantitation of the levels of the two major isoforms of FLIP protein (FLIP-L and FLIP-S) in control, survivor (day 1), and reset (day 7) cells. **C,** Immunoblots of FLIP-L and TGM-2 protein in control, survivor (day 1), reset (day 7), and repeat (cells treated on day 1+2+3) cells. **D,** Immunoblot of FLIP-L and FLIP-S protein in control ("C") or day 1 survivor ("S") parental MCF10A cells ("No Construct"); or in control ("C") or survivor ("S") MCF10A cells derived from single-cell clones ("CL1, CL2, CL3") stably expressing shRNA constructs targeting FLIP ("FLIPsh") or nonspecific shRNA constructs ("Nonspecific"). **E,** Cell viability assay of control and survivor cells derived from parental MCF10A ("Control", blue lines; "Survivors", red lines) or from MCF10A cells expressing FLIP shRNA or nonspecific shRNA constructs (light blue and brown lines), treated with the indicated doses of TRAIL for 6h. **F**, ELISA of secreted IL1A and CXCL1 (Gro-A) in 8h-conditioned medium from control and day 1 survivor MCF10A cells +/- expression of IκBsr or FLIP shRNA.

Figure S6. **Effect of kinase inhibition and other co-drugging agents on TRAIL-sensitivity of control and survivor MCF10A cells.**
A, Cell viability plot of control and day 1 survivor cells treated with the indicated doses of the TGM-2 inhibitor cystamine +/- TRAIL (50ng/ml) for 6h. **B,** Cell viability plot of control and survivor cells treated for 6h with TRAIL (50ng/ml) + the indicated doses of MEK inhibitor (MEKi), PI3K inhibitor (PI3Ki), p38 inhibitor (p38i), or JNK inhibitor (JNKi). **C**, Percent apoptosis of control and day 1 survivor MCF10A cells treated for 6h with TRAIL +/- MEK inhibitor (PD98059, 20μM), EGFR inhibitor (Gefitinib, 10μM), or Bcl-2 inhibitor (ABT-737, 10μM), using an antibody to cleaved PARP. **D**, Sensitivity of control and day 1 survivor MCF10A cells to a 6h treatment with the indicated doses of TRAIL +/- MG-132. **E**, Sensitivity of control and day 1 survivor MCF10A cells to a 6h treatment with

the indicated doses of TRAIL +/- Bortezomib. **F,** Sensitivity of control and day 1 survivor MCF10A cells to a 6h treatment with the indicated of doses of TRAIL +/- etoposide (10μM). For **A-B** and **D-F**, cell survival was measured using the methylene blue viability assay.

Figure S7. **Cell surface expression of death receptors in control and survivor cells; DR5 and FADD expression using alternative cell lysis protocol or antibodies; and BH3 profiling of control, survivor, and reset cells. A-C**, Flow cytometry plots showing cell-surface receptor DR4/5 **(A),** DcR1/2 **(B),** or Fas-Receptor **(C)** expression in control (blue) and survivor cells (green), or labeled with an isotype control antibody (red). Individual plots are representative of two independent experiments. **D**, Immunoblot of DR5 in total cell lysates (right panel) or following DISC precipitation (left panel) in control and survivor cells stimulated with biotinylated-TRAIL (500ng/ml), using a different primary antibody (Cell Signaling Technologies) than that shown in Figure 7 (ProSci). For unstimulated (u/s) controls, biotinylated-TRAIL was added directly to cell lysates prior to pull-down. Total cell lysates were collected in RIPA buffer. **E**, Immunoblot and quantitation of total FADD and caspase-8 in total cell lysates. Caspase-8 lysates were collected in Triton lysis buffer as shown in Figure 7B. Error bars represent an average of measurements from at least three experiments. For measurement of FADD, lysates were collected in RIPA buffer, and under these conditions there is a reduction in total FADD expression in survivor cells compared to control and reset cells. **F**, BH3 profiling plots (Deng et al, 2007) for control, survivor, and reset cells, indicating the extent of mitochondrial depolarization following treatment with the indicated BH3 peptides or positive/negative controls (FCCP and DMSO, respectively), calculated from the area under the curve of a time-series measurement of JC-1 fluorescence.

SUPPLEMENTAL TABLES

Table S1. Summary of microarray differential gene expression data.
Excel spreadsheet containing a summary of the lists of differentially expressed genes from the different analyses of the microarray dataset. Worksheet 1 ("RankProd"): differentially-expressed genes from the RankProd analysis of experiments 1-3, listing the top genes upregulated and downregulated in Survivors vs. Control and Repeat vs. Control. Worksheet 2 ("LIMMA IκBsr Exp"): from the LIMMA analysis of experiment 4, listing the differentially-expressed genes in Survivors vs. Control and the degree of differential expression of these genes in IκBsr-Survivors vs. IκBsr-Control. Worksheet 3 (LIMMA Combined Dataset- IκBsr"): from the LIMMA analysis of the combined dataset (experiments 1-4), listing the genes upregulated in Survivors vs. Control and the degree of upregulation of these genes in IκBsr-Survivors vs. IκBsr-Control. This list was used to determine the genes whose upregulation in survivor cells was inhibited, partially inhibited, or not inhibited by expression of the IκB super-repressor in Table S5; "inhibited", "partial", or "not inhibited" is listed beside each gene. Worksheets 4-5: differentially-expressed genes from the LIMMA analysis of experiments 1-4. Worksheet 4 ("LIMMA Combined Dataset") lists the upregulated and downregulated genes in Survivors vs. Control and Repeat vs. Control. Worksheet 5 ("LIMMA Combined Dataset (2))" lists the upregulated and downregulated genes in Survivors vs. Control and the extent of up- or downregulation in Repeat vs. Control, Reset vs. Control, and IκBsr-Survivors vs. IκBsr-Control. For all analyses, the significance cut-off used for differential expression was 1.5-fold change, 5% FDR (adj. P-value).
(Table S1 in "Table_S1_DifferentialExpressionSummaries.xls" file)

Table S2. PCA loadings.
Microarray data was subjected to Principal Components Analysis (PCA) as described in Materials & Methods. Listed in the first worksheet ("Eigenvectors") are the eigenvectors and the corresponding eigenvalues (loadings) for the first two principal components (PC1 and PC2) that accounted for the greatest amount of variance in the data (~74%). Genes with the highest eigenvalues (absolute value ≥ 4.0) for PC1 and PC2 are listed in the second worksheet ("Top Loadings").
(Table S2 in "Table_S2_PrincipalComponents.xls" file)

Table S3. Gene Ontology: Categories of genes upregulated in survivor cells

Functional Annotation Clustering	Enrichment Score	# of Genes
Secreted/Extracellular/Glycoprotein	7.7	55
Inflammation/ Wound Response	6.8	24
Peptidase Inhibitor Activity	5.9	13
Epidermal Differentiation	3.9	18
Cell Adhesion/ ECM	3.6	19
Cell Migration/ Chemotaxis (positive)	2.9	19
Calcium Binding/ Signaling	2.5	15
Response to Stress (Oxidative or Nutrient-Related)	1.6	20
Chromatin Remodeling/ Assembly	1.2	9
Anti-Apoptosis	1.0	10

Genes determined to be significantly upregulated in survivor cells were subjected to Gene Ontology (GO) functional analysis using DAVID. Redundant categories were removed or combined at the user's discretion. An enrichment score of ≥ 1 is considered to be significant (Huang da et al, 2009).

Table S4. Subset of genes upregulated in survivor cells classified by enriched Gene Ontology functional categories.

Inflammation/ Wounding	Epidermal Differentiation	Migration, Chemotaxis (positive)	Cell Adhesion	Anti-apoptosis	Chromatin Assembly
CXCL1	KLK7	LAMA3	FLRT3	CFLAR	HIST2H2AA3
OLR1	KRT6B	SERPINE2	MTSS1	SERPINB2	HIST1H2BD
S100A8	S100A7	PDPN	OLR1	TGM2	HIST1H1C
PDPN	KLK5	MMP9	PDPN	HSPB1	HIST1H2BK
CFB	SPRR2F	CXCL1	IL32	IL1B	HIST2H2AC
C3	LAMB3	TYMP	MFGE8	HSPA5	
S100A9	COL17A1	SAA2	SIRPA	IFI6	
SAA4	LAMA3	CCL20	NRCAM	IL1A	
TPM1	COL7A1	SAA1	LPXN	SOD2	
SOD2	KRT17	S100A9	COL17A1	BIRC3	
TFRC	SPRR2D	IL1B	ITGA5		
ADM	KRT16	NRCAM	ITGAV		
SAA2	SPRR1A	S100P	MSLN		
CCL20	SPRR2A	ITGA5	CLDN1		
SAA1	SPRR1B	DNER	LAMC2		
ITGA5	TGM1	SEMA3C	COL8A1		
CD59	LAMC2	MTSS1	SAA2		
SERPINE1	DCN	HSPB1	SAA1		
AOX1		SOD2	TGM2		
SERPINA3			IL1B		
SERPINB2			TPM1		
IL1B					
LOX					
IL1A					
KYNU					
S100A7					
IL32					
TAPBP					
HIST1H2BK					
TAP1					
TNIP1					
HLA-H					
SUSD2					
SEMA3C					
CTSC					
IFI6					
GBP2					

A subset of genes upregulated in survivor cells are listed by GO category. Some genes appear in more than one category. For full lists see Table S1.

Table S5. Effect of IκB super-repressor on gene regulation in survivors.

Effect of IκB-repressor on survivor gene upregulation	Percent of Genes
Inhibited (NF-κB-dependent genes)	58%
Partially Inhibited (partial NF-κB-dependence)	11%
Not Inhibited (NF-κB-independent genes)	31%

Starting with the list of genes upregulated in survivor cells (vs. control cells; minimum 1.5-fold change, 5% FDR), we compared the extent of upregulation with that of IκBsr-expressing survivors vs. IκBsr-expressing control cells for the individual genes. The table lists the percentage of genes upregulated in survivors whose upregulation was either completely inhibited, partially inhibited, or not at all inhibited in IκBsr-expressing survivor cells (see Materials & Methods). These represent NF-κB-dependent, partially NF-κB-dependent, and NF-κB-independent genes, respectively.

Table S6. Subset of genes upregulated in survivors by functional category and extent of upregulation in repeat and IκBsr-expressing survivor cells.

Inflammation/ Wounding	***Up in Repeat***	***Up in IκBsr-Survivors***	**Epidermal Differentiation**	***Up in Repeat***	***Up in IκBsr-Survivors***
CXCL1	+	-	KLK7	-	+
OLR1	(+)	-	KRT6B	(+)	**partial**
S100A8	(+)	-	S100A7	-	-
PDPN	-	-	KLK5	(+)	+
CFB	(+)	-	SPRR2F	-	**partial**
C3	(+)	-	LAMB3	(+)	-
S100A9	(+)	-	COL17A1	(+)	-
SAA4	-	-	LAMA3	(+)	-
TPM1	-	+	COL7A1	-	-
SOD2	+	-	KRT17	-	**partial**
TFRC	(+)	+	SPRR2D	-	**partial**
ADM	-	-	KRT16	-	+
SAA2	-	-	SPRR1A	-	+
CCL20	+	-	SPRR2A	-	**partial**
SAA1	(+)	**partial**	SPRR1B	-	-
ITGA5	-	-	TGM1	-	+
CD59	(+)	-	LAMC2	(+)	-
SERPINE1	(+)	**partial**	DCN	(+)	**partial**
AOX1	+	-			
SERPINA3	-	**partial**	**Migration, Chemotaxis (positive)**	***Up in Repeat***	***Up in IκBsr-Survivors***
SERPINB2	(+)	-	LAMA3	(+)	-
IL1B	(+)	-	SERPINE2	-	-
LOX	-	+	PDPN	-	-
IL1A	+	partial	MMP9	-	-
KYNU	(+)	-	CXCL1	+	-
S100A7	-	-	TYMP	-	**partial**
IL32	+	-	SAA2	-	-
TAPBP	-	-	CCL20	+	-
HIST1H2BK	-	+	SAA1	(+)	**partial**
TAP1	(+)	-	S100A9	(+)	-
TNIP1	(+)	-	IL1B	(+)	-
HLA-H	-	-	NRCAM	+	-
SUSD2	-	-	S100P	-	-
SEMA3C	-	+	ITGA5	-	-
CTSC	-	-	DNER	-	-
IFI6	(+)	-	SEMA3C	-	+
GBP2	-	+	MTSS1	(+)	-
Cell Adhesion	***Up in Repeat***	***Up in IκBsr-Survivors***	HSPB1	-	+
FLRT3	+	-	SOD2	+	-
MTSS1	(+)	-			
IL1B	(+)	-			
TPM1	-	+			

Table S6 (Continued)					
Cell Adhesion	***Up in Repeat***	***Up in IκBsr-Survivors***	**Anti-apoptosis**	***Up in Repeat***	***Up in IκBsr-Survivors***
OLR1	**(+)**	-	CFLAR (cFLIP)	-	+
PDPN	-	-	SERPINB2	**(+)**	-
IL32	+	-	TGM2	-	+
MFGE8	**(+)**	+	HSPB1 (hsp27)	-	+
SIRPA	-	+	IL1B	**(+)**	-
NRCAM	+	-	HSPA5	-	-
LPXN	**(+)**		IFI6	**(+)**	-
Col17A1	**(+)**	-	IL1A	+	**partial**
LAMB3	**(+)**	-	SOD2	+	-
LAMA3	**(+)**	-	BIRC3	**(+)**	-
COL7A1	-	-			
ITGA5	-	-	**Chromatin Assembly**	***Up in Repeat***	***Up in IκBsr-Survivors***
ITGAV	-	-	HIST2H2AA3	**(+)**	+
MSLN	-	+	HIST1H2BD	-	+
CLDN1	-	+	HIST1H1C	-	+
LAMC2	**(+)**	-	HIST1H2BK	-	+
COL8A1	**(+)**	-	HIST2H2AC	-	+
SAA2	-	-			
TGM2	-	+			

A subset of genes upregulated in survivor cells are listed by category (see also Table S4). Genes that are also upregulated significantly in IκBsr-expressing survivor cells are indicated by a "+"; genes not upregulated in these cells are indicated by a "-". "Partial" indicates a partial (but significant) upregulation of the gene in IκBsr-expressing survivor cells compared with the upregulation observed in parental survivors. Genes upregulated significantly in repeat cells in all analyses are indicated by a "+"; "(+)" in parentheses indicates that the gene was upregulated in repeat cells in at least one analysis of a subset of the data, but not necessarily in the final analysis (that was used to generate the list of approximately 200 genes upregulated in survivor cells and 55 genes upregulated in repeat cells). Note that very few genes are upregulated significantly in both repeat and IκBsr-expressing survivor cells.

Table S7. Genes upregulated in survivors, IκBsr-survivors, and repeat cells.

Gene Name	Anti-apoptosis	Up in repeat cells
TGM2	+	(Protein only)
SPRR1A		(+)
HIST2H2AA3		(+)
CLDN1		
KRT16		
KLK7		
MAFB		+
SAT1		+
LEPREL1		(+)
RGS2		
LOX		
C17orf91		
HIST1H2BD		
GBP2		
MFGE8		(+)
SIRPA		
ROS1		+
KLK5		(+)
MSLN		
CYP1B1		(+)
TFRC		(+)
PALLD		
GRINA		
PLOD2		(+)
SLC22A5		
MVP		
CYB5R1		
PRSS8		
SEMA3C		
TIMP2		
NRBP2		
OPLAH		
ALOX15B		(+)
S100A4		
HIST1H1C		(+)
KANK4		
TGM1		
YPEL5		
TPM1		
MMP7		
HSPB1	+	
ATP9A		
HIST2H2AC		
PLD5		(+)
ATP2B4		
CFLAR	+	(below signif. cutoff)

Table S7 (ctd.)

Forty-six genes upregulated in survivors that are fully upregulated in IκBsr-expressing survivor cells are listed under "gene name". Of these, 3 genes are classified "anti-apoptosis" by Gene Ontology analysis (indicated by a "+"). Genes from this list that were upregulated significantly in repeat cells in all analyses (including the analysis used to generate the final list of genes upregulated in survivor and repeat cells) are indicated by a "+" in the third column ; "(+)" in parentheses indicates that the gene was upregulated in repeat cells in at least one analysis of a subset of the data, but not in the final analysis.

SUPPLEMENTAL METHODS

Cell culture and generation of stable cell lines. HeLa cells were cultured as described (Albeck et al, 2008). MDA-MB-231 cells were cultured in RPMI (MediaTech, Inc.), supplemented with 10% FBS. HMLEC primary human mammary epithelial cells were obtained from R. Weinberg, Massachusetts Institute of Technology, and were cultured in DMEM/F12 media containing EGF, insulin, hydrocortisone, and penicillin/streptomycin as described (Elenbaas et al, 2001). U251 cells were a gift from T. Bagci, K. Shah lab, MGH, Boston, USA, and were maintained in DMEM supplemented with L-glutamine, penicillin/streptomycin, and 10% FBS. Single-cell clones of MCF10A and HCT116 cell lines were generated by serial dilution followed by expansion and selection of clones with properties similar to the parental population. Previously established transformed derivatives of MCF10A cells were obtained from V. Quaranta (Vanderbilt, USA) and were generated and cultured as described (Bargmann et al, 1986; Debnath et al, 2003; Santner et al, 2001). The MCF10A-AT set of cell lines was generated by transducing MCF10A cells with the *ras* oncogene followed by transplantation into mice and serial passage of xenograft tumors. MCF10A-AT1 cells, which arose from tumors that were passaged only once *in vivo*, are weakly tumorigenic whereas MCF10A-AT1a cells, which underwent multiple passages *in vivo*, are highly tumorigenic and invasive (Santner et al, 2001). The related MCF10A-NeuN and NeuT lines were transduced with the Her2/Neu oncogene and are transformed but not tumorigenic in mice (Bargmann et al, 1986; Debnath et al, 2003). MCF10A cells stably expressing FLIP shRNA (with GFP expressed off of a separate promoter) or a nonspecific shRNA construct were generated using retroviral infection, selection with puromycin, and sorting for GFP-positive cells using flow cytometry. GFP-positive single-cell clones generated by serial dilution were tested by western blot for reduced FLIP expression in untreated cells, and for lack of FLIP upregulation in day 1 survivors of TRAIL treatment (50ng/ml for 6h).

Materials. Cycloheximide, cystamine, and etoposide were obtained from Sigma-Aldrich; MEK inhibitor PD98059, p38 inhibitor SB203580, and JNK inhibitor SP600125 were obtained from EMD Biosciences. shRNA targeting both the short and long isoforms of FLIP (CAGAATAGACCTGAAGACAAA) and a nonspecific shRNA sequence were obtained from Open Biosystems.

Challenge-Recover ("Survivors") Experiment. "Survivors" were defined as cells that had recovered for one day following the initial treatment and exhibited reversible/transient resistance; "Reset" were defined as cells that recovered sensitivity after 6-7 days. One day prior to each re-challenge, control, survivor, and reset cells were plated at equivalent densities into dishes appropriate for the experimental design. Cells were washed directly prior to re-challenge to remove residual dead cells which may have been carried over during the replating of survivor cells. During the recovery period, cells were maintained in normal growth media and were split regularly to avoid confluency. For cell lines other than MCF10A or treatments other than TRAIL, the treatment dose and time for the initial challenge were adjusted in order to achieve maximal cell death while sparing at least 10% survivors, and the same dose used in the first challenge was applied for the re-challenge. "Repeat" cells were cells that were treated with TRAIL (50ng/ml for 6 hours) on 2-3 subsequent days following the initial TRAIL treatment. After each 6h treatment, "repeat" cells were replenished with fresh growth media, and were passaged when necessary to avoid confluency.

Data analysis for cell viability (cell count) assay. For the methylene blue assay, absorbance 620-405 has been shown to relate linearly to cell number (Oliver et al, 1989), and we first determined the cell densities (for each cell type) that fell within the linear range of the assay. Fraction of cells surviving (%) = (Abs620-Abs405 Treated) / (Abs620-Abs405 Control) * 100 (representing the fraction of surviving cells in a treated sample relative to an untreated control). Methylene blue labels both live and dead cells, and functions as a count of viable cells when the dead cells are successfully washed off prior to staining. We compared results obtained using the methylene blue assay with those from several other viability and apoptosis assays and found that the data were well-correlated.

Immunostaining and microscopy. For cell morphology images, cells seeded in glass-bottom chamber slides were fixed with 4% PFA, permeabilized in Cytoskeleton Buffer (CB) containing 0.2% Triton-X-100 as described (Small, 1981), and labeled with Alexa-488-Phalloidin and Hoechst 33342 (Invitrogen). For NF-κB images, cells were fixed with 4% PFA, permeabilized for 10 minutes with 100% methanol, and labeled with antibodies to p65-NF-κB (Santa Cruz). Detection was performed using Alexa-488-labeled secondary antibodies (Invitrogen), and cells were imaged on a Deltavision microscope (Applied Precision, Inc.) using a 60x lens. For quantification of NF-κB staining, cells grown in 96-well optical plates and treated as indicated were fixed with 4% PFA, permeabilized in 100% methanol, blocked with Odyssey Blocking Buffer (Li-COR) and labeled with antibodies to p65-NF-κB (Santa Cruz), followed by detection with Alexa-fluor 488- or 647-conjugated secondary antibodies. Nuclei were labeled with Hoechst 33342 and cytoplasm was labeled with Whole Cell Blue dye (Cellomics). Images were scanned on a CellWorX station (Applied Precision, Inc.) and the NF-κB nuclear:cytoplastic ratio (for at least 100 cells) was calculated using ImageRail software (Millard et al, 2011).

Microarray data normalization and differential expression analysis. Raw data was log-transformed and quantile-normalized using the Bioconductor Lumi package in R. In cases of multiple probes for the same gene, the probe with the highest value for that gene was used for the analysis. Differential expression was determined using both the Rank Product (RankProd, Bioconductor) (Hong et al, 2006), or Linear Models for Microarray Data (LIMMA, Bioconductor) algorithms; all samples were analyzed for differential expression relative to the control sample. Analyses were performed for each individual experiment, as well as on a pooled dataset from all of the experiments combined. For lists of differentially expressed genes, significance cutoffs were chosen at 1.5 minimum fold-change and 5% False Discovery Rate (FDR) (i.e. 5% expected proportion of false-positives), which is equivalent to an adjusted p-value < 0.05 (Pawitan et al, 2005). Analysis performed on different subsets of experiments or using the different algorithms generated lists with different numbers of significantly differentially expressed genes, but the overall trends, as well as the most highly differentially expressed genes, were the same across analyses.

Functional analysis of microarray data. Venn Diagrams were generated from the RankProd-derived list of differentially expressed genes (applied to the combined dataset of control, survivors, repeat, and reset samples) using Venny (Oliveros, 2007); similar results were obtained using the LIMMA-derived data. Principal Components Analysis (PCA) was performed using both Bioconductor and MultiExperiment Viewer (Mar et al, 2011) on the normalized data from an individual experiment (to minimize batch effects across experiments (Leek et al, 2010)). Supervised Hierarchical Clustering was performed in Spotfire (cluster method: ward; distance metric: correlation) based on genes differentially expressed in survivor cells compared to control cells, also using data from an individual experiment.

Functional Annotation Clustering of Gene Ontology terms was performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da et al, 2009) on the list of differentially expressed genes generated by the LIMMA analysis of the complete dataset. For determination of NF-κB-dependent gene upregulation in survivor cells, we compared whether genes upregulated in survivors vs. control cells were also upregulated in IκB super-repressor-expressing survivor vs. control cells. NF-κB-dependent genes were defined as those that were not significantly upregulated in IκBsr-expressing survivor vs. control cells (< 1.5-fold increase or adjP > 0.05), or whose fold-increase was at least 2.5x less than the increase in wild-type survivors vs. controls. Partially NF-κB-dependent genes were defined as those that were significantly upregulated in IκBsr-expressing survivor vs. control cells, but whose fold-increase was between 1.5- 2.5x less than the increase in wild-type survivors vs. controls.

q-RT-PCR. Selected genes that exhibited differential expression by gene expression microarray analysis were confirmed by q-RT-PCR of duplicate RNA samples. RNA was reverse-transcribed using a RETROscript® kit (Ambion). The cDNA generated was amplified by Real-Time PCR using SYBR® Green reagent (Applied Biosystems). PCR was performed using an Eppendorf Reaplex MasterCycler ep Gradient S (40 cycles). Signals were normalized to GAPDH levels. The following forward (F) and reverse (R) primers (purchased from Integrated DNA Technologies) spanning the exon of the gene were determined for the appropriate splice variants:

CFLAR_F	GAC AGA GCT TCT TCG AGA CAC
CFLAR_R	GCT CGG GCA TAC AGG CAA AT
PI3_F	CAC GGG AGT TCC TGT TAA AGG
PI3_R	GAG CCA GGC TTA GTG GAG ACT
SOD2_F	CCT CAC ATC AAC GCG CAG AT
SOD2_R	CGT TCA GGT TGT TCA CGT AGG
IL1A_F	GAA GAG ACG GTT GAG TTT AAG CC
IL1A_R	CAG GAA GCT AAA AGG TGC TGA
SERPINB2_F	GAA GGG TAG TTA TCC TGA TGC G
SERPINB2_R	GCA CAT TCT AGG AAG TCT ACT GC
TGM2_F	AGC GTT CCT CTT TGC ATC CTC
TGM2_R	GTA GCT GTT GAT AAC TGG CTC C
MFGE8_F	TTT GGC TCT GTC CAG TTT GTG
MFGE8_R	AGT TCG CAC TGT CAT TAC TGT AG
AURKA_F	TTC AGG ACC TGT TAA GGC TAC A
AURKA_R	ATT TGA AGG ACA CAA GAC CCG

Cell cycle measurements and cell proliferation analysis. Cells incubated with 10μM EdU for 30 minutes at 37°C were fixed and labeled with Alexa Fluor® 488 azide and Click-iT™ EdU CellCycle 633-red according to the manufacturer's protocol (Click-iT® EdU Cell Proliferation Assay, Invitrogen). Cell cycle was analyzed by two-color flow cytometry on a FACSCalibur Instrument (BD Biosciences)

using the green and red lasers. Percent cells in G1, S, and G2/M were calculated using FlowJo. For cell proliferation curves, cells were seeded at low density in 96-well plates and the number of cells in wells fixed at 24, 48, and 72h was calculated relative to the number of cells at time 0, using the methylene blue assay.

Cell surface receptor analysis. Cells were washed with PBS and dissociated with enzyme-free, PBS-based cell dissociation buffer (Invitrogen) and collected on ice in PBS containing 10% fetal bovine serum (FACS buffer). $2x10^5$ cells at a concentration of $1x10^6$ cells/ml in FACS buffer were incubated with a specific death receptor antibody (2μg/ml) or with the corresponding IgG isotype control antibody for 1h on ice, washed twice at 4°C with PBS, and then incubated with Alexa-488 or -647-conjugated secondary antibodies for 1h on ice. In some samples, cells were also labeled with 1μM Sytox Green (Molecular Probes) to assess cell viability. Labeled cells were analyzed by flow cytometry on a FACSCalibur (BD Biosciences).

Antibodies used for cell-surface receptor analysis. RNAi-validated antibodies to DR4 (Clone 4H6) and DR5 (Clone 3H3) were kindly provided by A. Ashkenazi and colleagues at Genentech; similar results were obtained with DR4 antibody MAB347 (R&D) and DR5 antibody ab18366 (Abcam). Antibodies to DcR1 and DcR2 were from Enzo Life Sciences (HS301 and HS402; (Walczak & Haas, 2008)); anti-FasR antibody (Apo-1-3) was from Alexis Biochemicals. Similar results were obtained in cells labeled with a FITC-conjugated Fas antibody (R&D). IgG1 or IgG3 (Santa Cruz) were used as isotype controls. Experiments were performed using duplicate samples and were repeated at least twice.

Antibodies and immunoblot analysis. The following primary antibodies were used for immunoblot analysis: DR4 (ProSci), DR5 (ProSci or Cell Signaling Technologies #3696), FADD (BD Biosciences), Caspase-8 (Clone 1C12, Cell Signaling Technologies), Bid (Atlas Antibodies), Caspase-3 (ActiveMotif), FLIP (clone NF6, Alexis Biochemicals), TGM-2 (Thermo Scientific), Bcl-2 (Santa Cruz Biotechnology), Bcl-xL (Cell Signaling Technologies), Mcl-1 (Cell Signaling Technologies), Bax (Santa Cruz Biotechnology), Bak (Upstate Biotechnology), Bim (Cell Signaling Technologies), Bid (Atlas Antibodies), Bad (Santa Cruz Biotechnology), Puma (Cell Signaling Technologies). Detection was performed using secondary antibodies conjugated with IRDye 680CW or IRDye 800CW (Li-COR) and a Li-COR Odyssey scanner. GAPDH or Actin was used as a loading control. Quantitation was performed using Odyssey software; integrated pixel intensity was calculated for uniformly-sized rectangular regions framing individual bands, and background correction was performed by subtracting the integrated pixel intensity for equally-sized regions within the same lane.

BH3 Profiling. BH3 profiling was performed as described (Deng et al, 2007; Ryan et al, 2010). Briefly, cells were trypsinized, washed in PBS, re-suspended in T-EB buffer, permeabilized lightly with 0.005% digitonin and labeled with 1μM JC-1, a marker for mitochondrial membrane potential, in the presence of 5mM beta-mercaptoethanol and 10μg/ml oligomycin. Cells were then treated in triplicate with the indicated BH3 peptides or with DMSO (negative control) or FCCP (positive control for loss of mitochondrial membrane potential). Fluorescence was read on a Tecan Safire2 with Ex 545 ± 20nM and Em 590 ± 20nM; Emission at 590nM was read every 5 minutes for 180 minutes, and the area under the curve was calculated for the readings between 50-180min. Depolarization was indicated by a decrease in red fluorescence (emission at 590nM), and percent depolarization using a particular BH3 peptide was defined as the percentage of the difference in depolarization between the positive and negative controls.

SUPPLEMENTAL REFERENCES

Albeck JG, Burke JM, Aldridge BB, Zhang M, Lauffenburger DA, Sorger PK (2008) Quantitative analysis of pathways controlling extrinsic apoptosis in single cells. *Mol Cell* **30:** 11-25

Bargmann CI, Hung MC, Weinberg RA (1986) Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185. *Cell* **45:** 649-657

Debnath J, Muthuswamy SK, Brugge JS (2003) Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods* **30:** 256-268

Deng J, Carlson N, Takeyama K, Dal Cin P, Shipp M, Letai A (2007) BH3 profiling identifies three distinct classes of apoptotic blocks to predict response to ABT-737 and conventional chemotherapeutic agents. *Cancer Cell* **12:** 171-185

Elenbaas B, Spirio L, Koerner F, Fleming MD, Zimonjic DB, Donaher JL, Popescu NC, Hahn WC, Weinberg RA (2001) Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. *Genes Dev* **15:** 50-65

Hong F, Breitling R, McEntee CW, Wittner BS, Nemhauser JL, Chory J (2006) RankProd: a bioconductor package for detecting differentially expressed genes in meta-analysis. *Bioinformatics* **22:** 2825-2827

Huang da W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4:** 44-57

Leek JT, Scharpf RB, Bravo HC, Simcha D, Langmead B, Johnson WE, Geman D, Baggerly K, Irizarry RA (2010) Tackling the widespread and critical impact of batch effects in high-throughput data. *Nat Rev Genet* **11:** 733-739

Mar JC, Wells CA, Quackenbush J (2011) Defining an informativeness metric for clustering gene expression data. *Bioinformatics* **27:** 1094-1100

Millard BL, Niepel M, Menden MP, Muhlich JL, Sorger PK (2011) Adaptive informatics for multifactorial and high-content biological data. *Nat Methods*

Oliver MH, Harrison NK, Bishop JE, Cole PJ, Laurent GJ (1989) A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. *J Cell Sci* **92 (Pt 3):** 513-518

Oliveros JC. (2007) VENNY. An interactive tool for comparing lists with Venn Diagrams.

Pawitan Y, Michiels S, Koscielny S, Gusnanto A, Ploner A (2005) False discovery rate, sensitivity and sample size for microarray studies. *Bioinformatics* **21:** 3017-3024

Ryan JA, Brunelle JK, Letai A (2010) Heightened mitochondrial priming is the basis for apoptotic hypersensitivity of CD4+ CD8+ thymocytes. *Proceedings of the National Academy of Sciences of the United States of America* **107:** 12895-12900

Santner SJ, Dawson PJ, Tait L, Soule HD, Eliason J, Mohamed AN, Wolman SR, Heppner GH, Miller FR (2001) Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. *Breast Cancer Res Treat* **65:** 101-110

Small JV (1981) Organization of actin in the leading edge of cultured cells: influence of osmium tetroxide and dehydration on the ultrastructure of actin meshworks. *J Cell Biol* **91:** 695-705

Walczak H, Haas TL (2008) Biochemical analysis of the native TRAIL death-inducing signaling complex. *Methods Mol Biol* **414:** 221-239

Figure S1

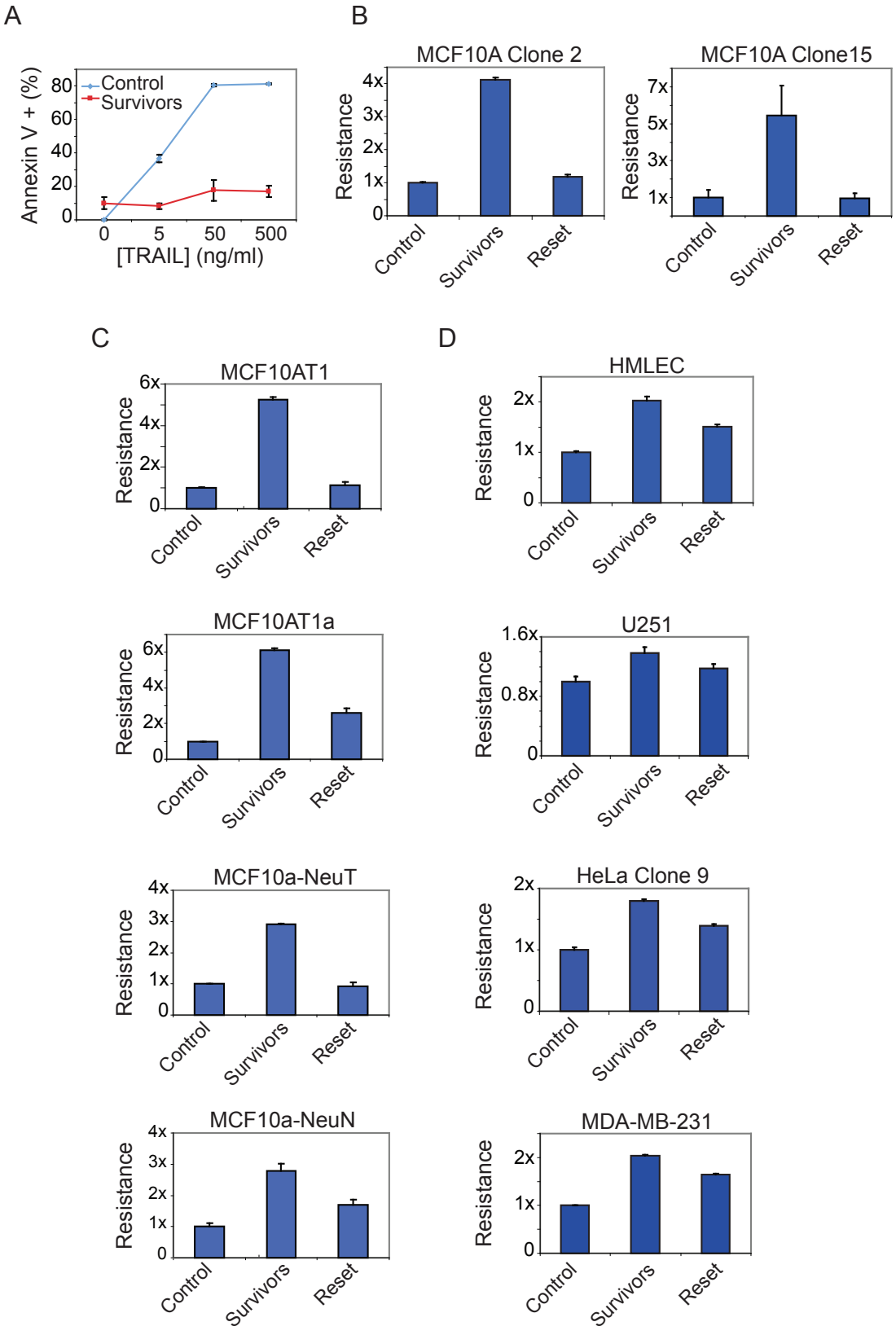
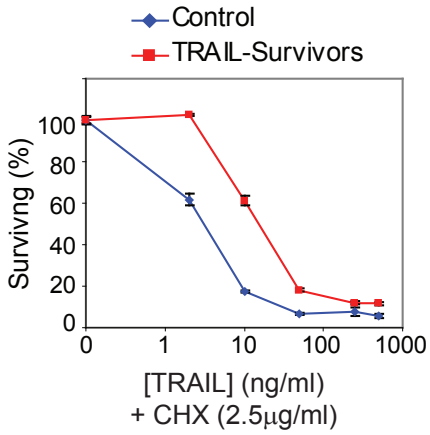
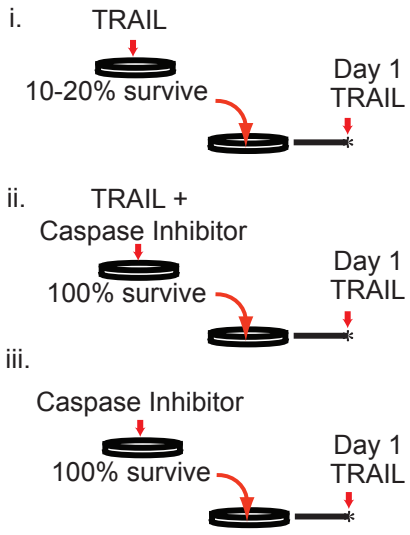

A
Annexin V + (%)
80
60
40
20
0
Control
Survivors
0
5
50
500
[TRAIL] (ng/ml)
B
MCF10A Clone 2
Resistance
4x
3x
2x
1x
0
Control
Survivors
Reset
MCF10A Clone15
Resistance
7x
5x
3x
1x
Control
Survivors
Reset
C
MCF10AT1
Resistance
6x
4x
2x
0
Control
Survivors
Reset
MCF10AT1a
Resistance
6x
4x
2x
0
Control
Survivors
Reset
MCF10a-NeuT
Resistance
4x
3x
2x
1x
0
Control
Survivors
Reset
MCF10a-NeuN
Resistance
4x
3x
2x
1x
0
Control
Survivors
Reset
D
HMLEC
Resistance
2x
1x
0
Control
Survivors
Reset
U251
Resistance
1.6x
0.8x
0
Control
Survivors
Reset
HeLa Clone 9
Resistance
2x
1x
0
Control
Survivors
Reset
MDA-MB-231
Resistance
2x
1x
0
Control
Survivors
Reset

Figure S2

A


Control
TRAIL-Survivors
Survivng (%)
100
80
60
40
20
0
0
1
10
100
1000
[TRAIL] (ng/ml)
+ CHX (2.5μg/ml)

B


i.
TRAIL
10-20% survive
Day 1
TRAIL
ii.
TRAIL +
Caspase Inhibitor
100% survive
Day 1
TRAIL
iii.
Caspase Inhibitor
100% survive
Day 1
TRAIL

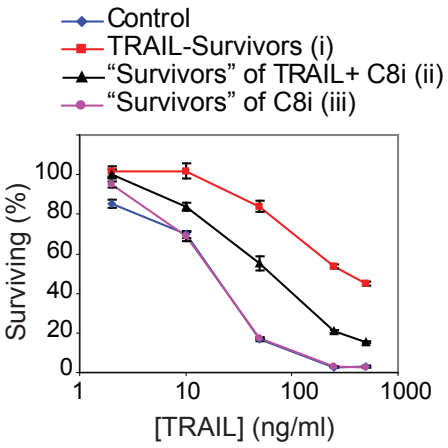
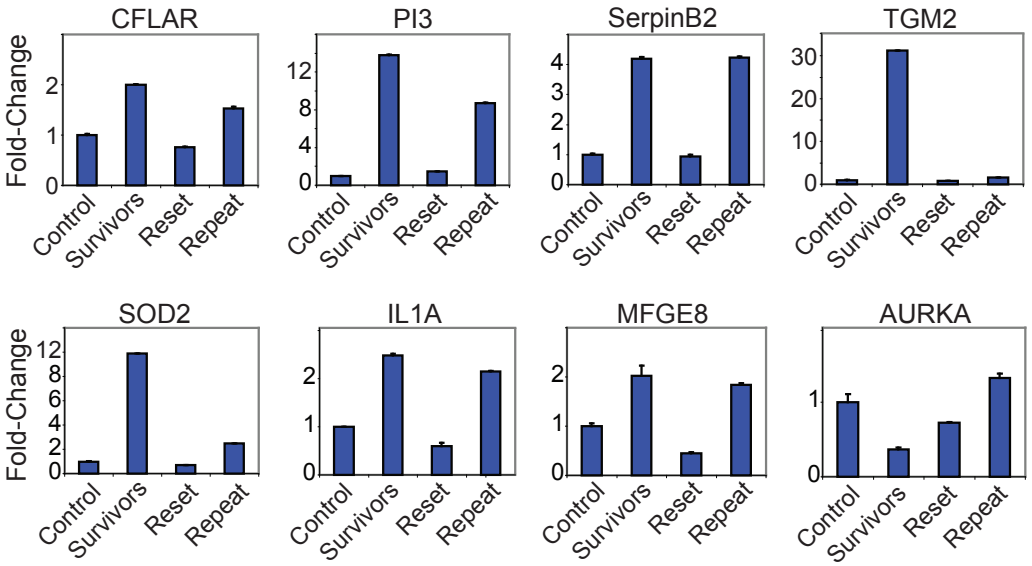

Control
TRAIL-Survivors (i)
"Survivors" of TRAIL+ C8i (ii)
"Survivors" of C8i (iii)
Surviving (%)
100
80
60
40
20
0
1
10
100
1000
[TRAIL] (ng/ml)

Figure S3

A


CFLAR
PI3
SerpinB2
TGM2
SOD2
IL1A
MFGE8
AURKA
Fold-Change
Control
Survivors
Reset
Repeat

B

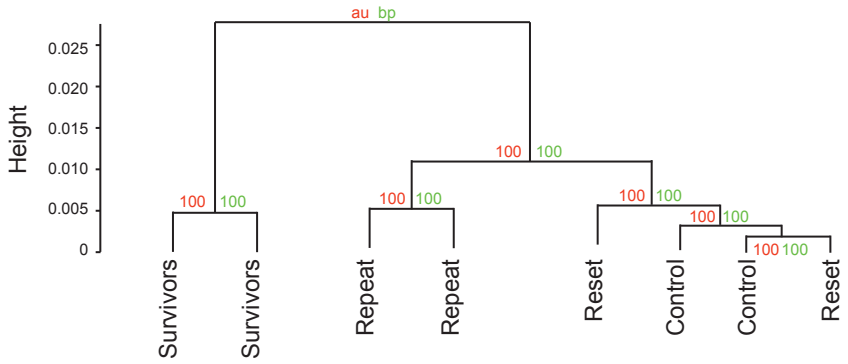

au bp
Height
0.025
0.020
0.015
0.010
0.005
0
100
Survivors
Survivors
Repeat
Repeat
Reset
Control
Control
Reset

Figure S4

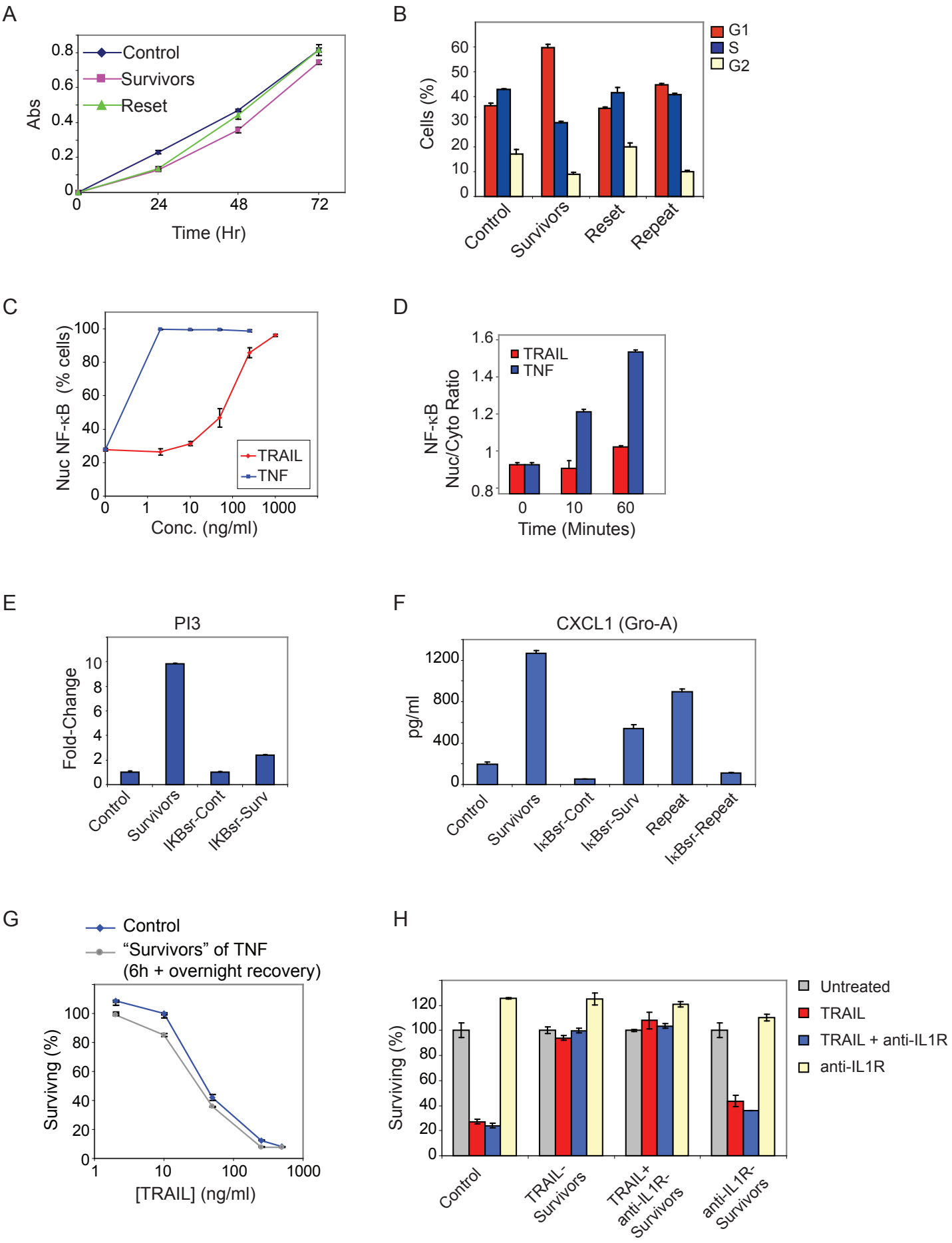

A
Control
Survivors
Reset
Abs
Time (Hr)
B
G1
S
G2
Cells (%)
Control
Survivors
Reset
Repeat
C
Nuc NF-κB (% cells)
TRAIL
TNF
Conc. (ng/ml)
D
TRAIL
TNF
NF-κB Nuc/Cyto Ratio
Time (Minutes)
E
PI3
Fold-Change
Control
Survivors
IKBsr-Cont
IKBsr-Surv
F
CXCL1 (Gro-A)
pg/ml
Control
Survivors
IκBsr-Cont
IκBsr-Surv
Repeat
IκBsr-Repeat
G
Control
"Survivors" of TNF
(6h + overnight recovery)
Survivng (%)
[TRAIL] (ng/ml)
H
Untreated
TRAIL
TRAIL + anti-IL1R
anti-IL1R
Surviving (%)
Control
TRAIL-
Survivors
TRAIL+
anti-IL1R-
Survivors
anti-IL1R-
Survivors

Figure S5

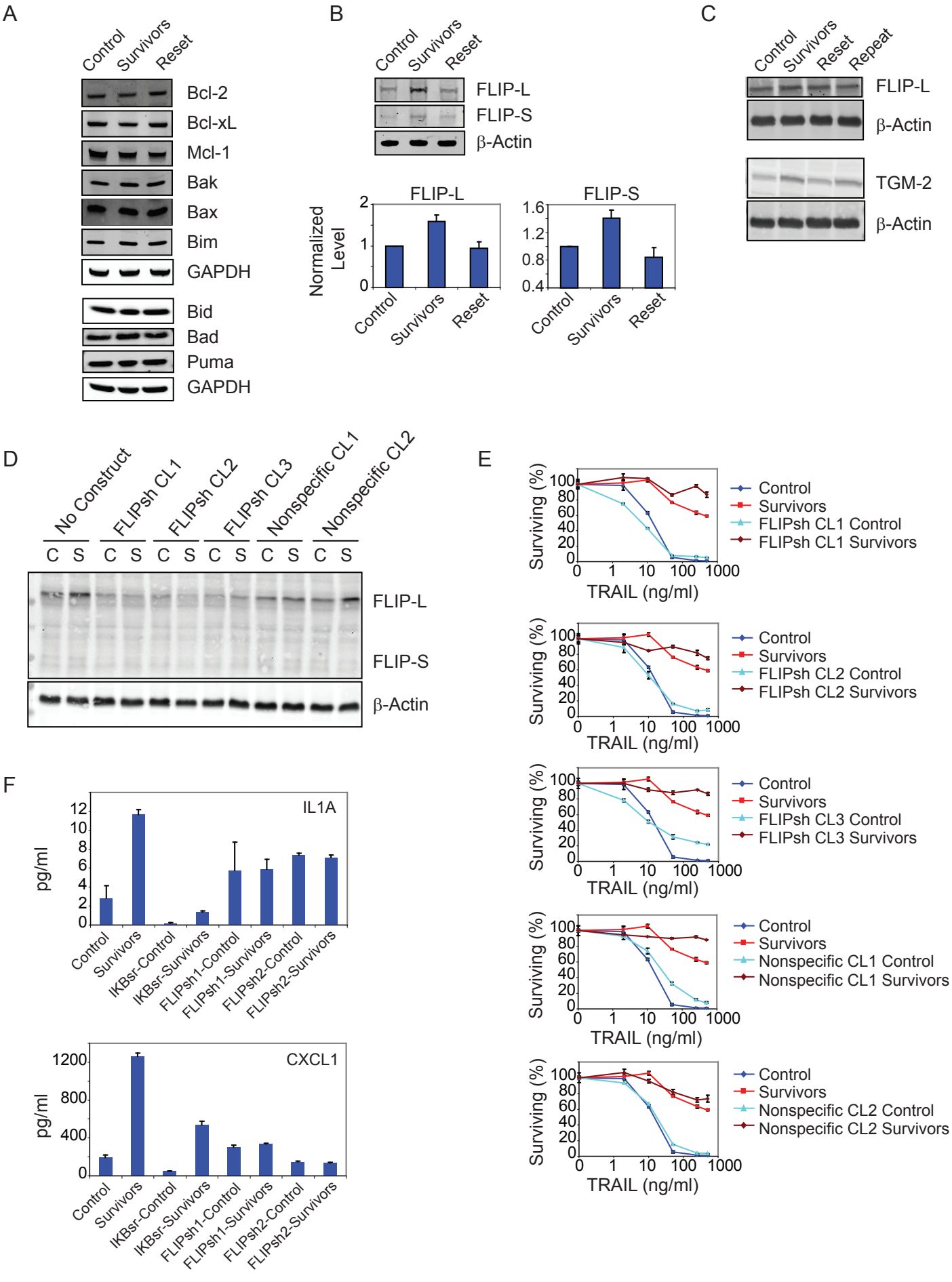

A
Control
Survivors
Reset
Bcl-2
Bcl-xL
Mcl-1
Bak
Bax
Bim
GAPDH
Bid
Bad
Puma
GAPDH
B
Control
Survivors
Reset
FLIP-L
FLIP-S
β-Actin
FLIP-L
FLIP-S
Normalized Level
C
Control
Survivors
Reset
Repeat
FLIP-L
β-Actin
TGM-2
β-Actin
D
No Construct
FLIPsh CL1
FLIPsh CL2
FLIPsh CL3
Nonspecific CL1
Nonspecific CL2
C S C S C S C S C S C S
FLIP-L
FLIP-S
β-Actin
E
Surviving (%)
TRAIL (ng/ml)
Control
Survivors
FLIPsh CL1 Control
FLIPsh CL1 Survivors
FLIPsh CL2 Control
FLIPsh CL2 Survivors
FLIPsh CL3 Control
FLIPsh CL3 Survivors
Nonspecific CL1 Control
Nonspecific CL1 Survivors
Nonspecific CL2 Control
Nonspecific CL2 Survivors
F
IL1A
pg/ml
CXCL1
Control
Survivors
IKBsr-Control
IKBsr-Survivors
FLIPsh1-Control
FLIPsh1-Survivors
FLIPsh2-Control
FLIPsh2-Survivors

Figure S6

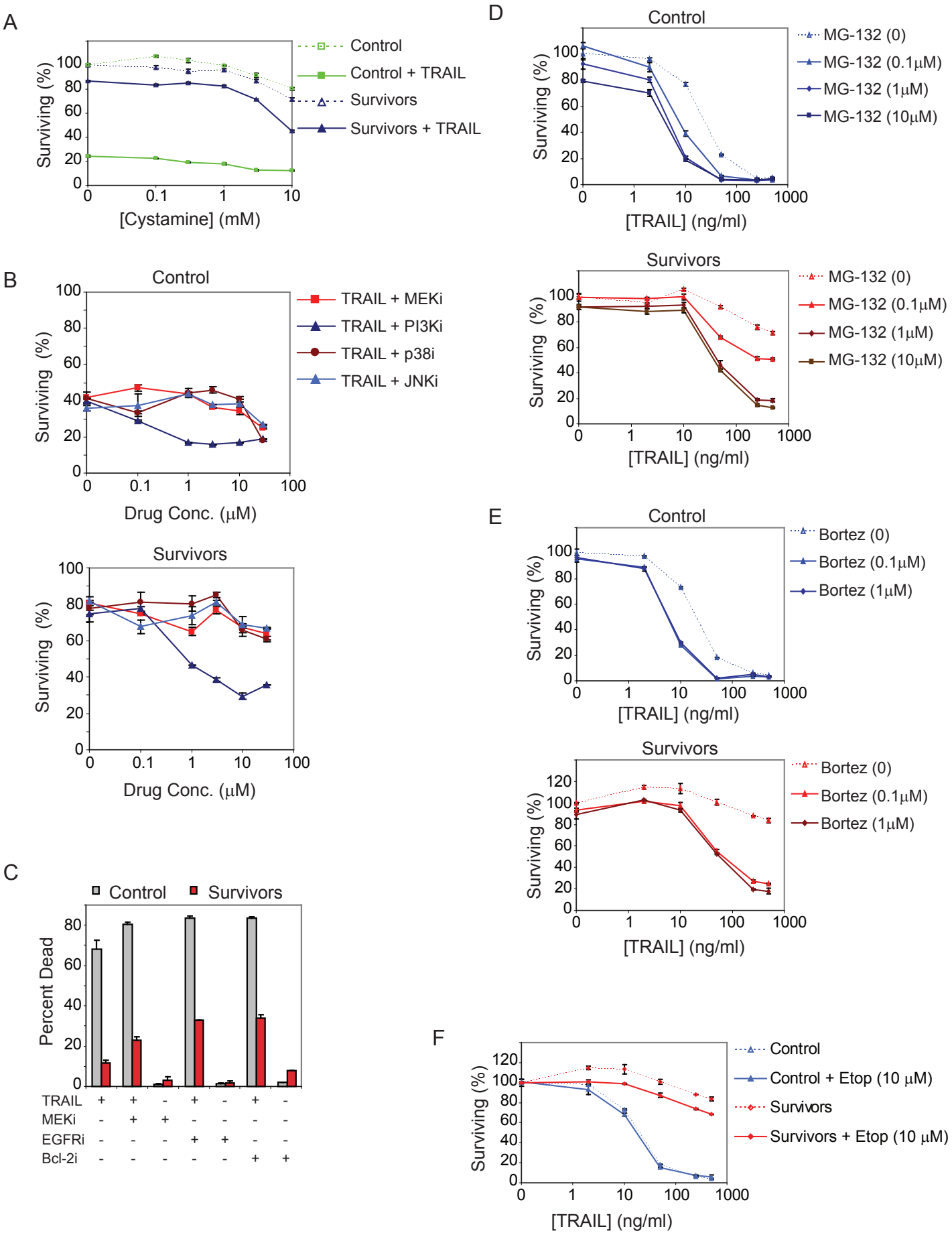
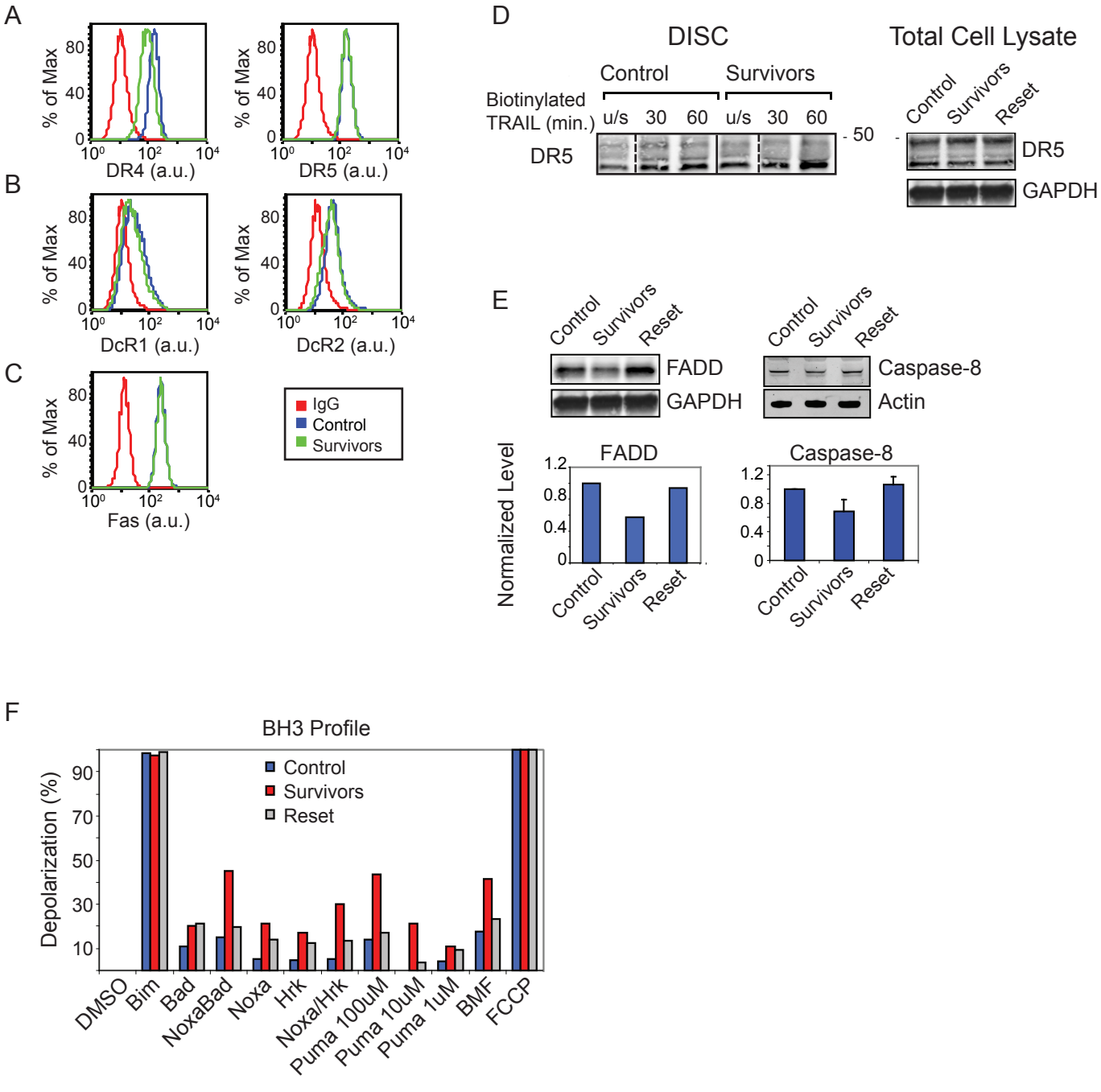

A
Surviving (%)
[Cystamine] (mM)
Control
Control + TRAIL
Survivors
Survivors + TRAIL
B
Control
Survivors
Surviving (%)
Drug Conc. (μM)
TRAIL + MEKi
TRAIL + PI3Ki
TRAIL + p38i
TRAIL + JNKi
C
Control
Survivors
Percent Dead
TRAIL
MEKi
EGFRi
Bcl-2i
D
Control
Survivors
[TRAIL] (ng/ml)
MG-132 (0)
MG-132 (0.1μM)
MG-132 (1μM)
MG-132 (10μM)
E
Control
Survivors
[TRAIL] (ng/ml)
Bortez (0)
Bortez (0.1μM)
Bortez (1μM)
F
Control
Control + Etop (10 μM)
Survivors
Survivors + Etop (10 μM)
[TRAIL] (ng/ml)

Figure S7


A
% of Max
DR4 (a.u.)
DR5 (a.u.)
B
DcR1 (a.u.)
DcR2 (a.u.)
C
Fas (a.u.)
IgG
Control
Survivors
D
DISC
Total Cell Lysate
Control
Survivors
Biotinylated TRAIL (min.)
u/s 30 60 u/s 30 60
DR5
GAPDH
Reset
E
FADD
Caspase-8
Actin
Normalized Level
F
BH3 Profile
Depolarization (%)
Reset
DMSO
Bim
Bad
NoxaBad
Noxa
Hrk
Noxa/Hrk
Puma 100uM
Puma 10uM
Puma 1uM
BMF
FCCP