Supporting Information (SI Appendix)

A set of NF-KB-regulated microRNAs induces acquired TRAIL resistance in lung cancer.

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probes used for qRT-PCRs are reported.

Table S3. List of Primers used in the study.

SI Experimental Procedures

Nanostring. The NanoString nCounter Human miRNA Expression Assay Kit (http://www.nanostring.com/) was used to profile more than 700 human and human-associated viral miRNAs. 100ng of total RNA was used as input for nCounter miRNA sample preparation reactions. All sample preparations were performed according to the manufacturer's instructions (NanoString Technologies). Preparation of small RNA samples involves the ligation of a specific DNA tag onto the 3' end of each mature miRNA. These tags are designed to normalize the T_m's of the miRNAs as well as to provide a unique identification for each miRNA species in the sample. Following the ligation reaction, excess tags and bridges were removed, and the resulting material was hybridized with a panel of miRNA:tag-specific nCounter capture and barcoded reporter probes. Hybridization reactions were performed with 5µl of the 5-fold diluted sample preparation reaction. All hybridization reactions were incubated at 64°C for a minimum of 18 h. Hybridized probes were purified using the nCounter Prep Station (NanoString Technologies) following the manufacturer's instructions to remove excess capture and reporter probes and to immobilize transcript-specific ternary complexes on a streptavidin-coated cartridge. Data collection was carried out on the nCounter Digital Analyzer (NanoString Technologies) to count individual fluorescent barcodes and quantify target RNA molecules

present in each sample. For each assay, a high density scan (600 fields of view) was performed. Nanostring data were normalized by using the Top-100 expressed miRNA normalization as implemented by the Nanostring nSolver software. P-values were calculated using the LIMMA package (Linear Models for Microarray Data) from the Bioconductor R project (1, 2) The heatmap was generated by the Hierarchical Clustering module of GenePattern software based on normalized miRNA expression data (3). Pearson correlation was used as a distance metric and pairwise complete-linkage as a clustering method for both miRNAs and samples.

Western blot analysis. Total proteins from NSCLC were extracted with radioimmunoprecipitation assay (RIPA) buffer (0.15mM NaCl, 0.05mM Tris-HCl, pH 7.5, 1% Triton, 0.1% SDS, 0.1% sodium deoxycholate and 1% Nonidet P40). Sample extracts (30-50 μg) were resolved on 4–20% SDS–polyacrylamide gels (PAGE) using a mini-gel apparatus (Bio-Rad Laboratories) and transferred to Hybond-C Extra nitrocellulose. Membranes were blocked for 1h with 3% Bovine Serum Albumin in Tris-buffered saline containing 0.05% Tween 20, incubated overnight with primary antibody, washed and incubated with secondary antibody and visualized by chemiluminescence. The following primary antibodies were used: TRAIL-receptors detection kit (ProSci), PARP-1, caspase-8, caspase-3, Mcl-1, FoxO3a, p-ERK1/2 , tot ERK1/2, TRAF-1, TRAF-2, NF-κB, p-NF-κB Ser536, IκB-α, p-IKK-α, tubulin, GAPDH (Cell Signaling), TRAF-7 (Imegenex), V5 (Invitrogen), NF-κB and cFLIP (Santa Cruz

Biotechnology).

MiRNA locked nucleic acid in situ hybridization of formalin fixed, paraffin-embedded tissue section. The detailed protocols are described, previously (4). The sequences of the probes containing the dispersed locked nucleic acid (LNA) modified bases with 5'-conjugated digoxigenin were: miR-21 (5') TCAACATCAGTCTGATAAGCTA; miR-30c -(5') GCTGAGAGTGTAGGATGTTTACA (3'); miR-100- (5') CACAAGTTCGGATCTACGGGTT3'. The probe cocktail and tissue miRNA were co-denatured at 60˚C for 5 minutes, followed by hybridization at 37 ˚C overnight and a stringency wash in 0.2X SSC and 2% bovine serum albumin at 4˚C for 10 minutes. The probe-target complexes were observed through the action of alkaline phosphatase on the chromogen nitroblue tetrazolium and bromochloroindolyl phosphate (NBT/BCIP). Negative controls included a probe which should yield a negative result in such tissues (scrambled miRNA). No counterstain was used, to facilitate co-labeling for caspase-3, caspase-8, FoxO3a and TRAF-7 proteins. After in situ hybridization with miRNAs, as previously described (4) the slides were analyzed for immunohistochemistry (IHC) using the optimal conditions for caspase-3 (1:100, cell conditioning for 30 minutes), caspase-8 (1:10, protease digestion for 4 minutes) FoxO3a (1:100, cell conditioning for 30 minutes), TRAF-7 (1:25, cell conditioning for 30 minutes). For the immunohistochemistry, Ultrasensitive Universal Fast Red or DAB from Ventana Medical Systems were used. The percentage of tumor cells expressing caspase-3, caspase-8, FoxO3a

and TRAF-7 and miR-21, miR-30c, and miR-100 was then analyzed with an emphasis on co-localization of the respective targets. Co-expression analysis was done with the Nuance system (Cambridge Research Institute) per the manufacturer's recommendations. Each core of the TMA was read blinded to the corresponding data. The score (0-100) was based on the signal intensity and % of + tumor cells – if a case had a score of 0-15, it was scored as negative. Any core with a score > 15 was scored as positive. The miRNA analysis was done by in situ hybridization and the protein analysis by immunohistochemistry

RNA extraction and Northern blotting. Total RNA was extracted with TRIzol solution (Invitrogen), according to the manifacturer's instructions and the integrity of RNA was assessed with an Agilent BioAnalizer 2100 (Agilent, Palo Alto, CA, USA). Northern blotting was performed as described previously (5). The oligonucleotides used as probes were the complementary sequences of the mature miRNA (miRNA registry):

mir-21: 5' TCAACATCAGTCTGATAAGCTA 3'

miR-30c: 5'GCTGAGAGTGTAGGATGTTTACA 3'

miR-100: 5' CACAAGTTCGGATCTACGGGTT 3'

Real-time PCR. The Real-time PCR was performed using a standard TaqMan PCR Kit protocol on an Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems). The 10 μl PCR reaction included 0.67 μl RT product, 1 μl TaqMan Universal PCR Master Mix (Applied

Biosystems), 0.2 mM TaqMan probe, 1.5 mM forward primer and 0.7 mM reverse primer. The reactions were incubated in a 96-well plate at 95 ˚C for 10 min, followed by 40 cycles of 95 ˚C for 15 s and 60 ˚C for 1 min. All reactions were run in triplicate. The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The comparative CT method for relative quantization of gene expression (Applied Biosystems) was used to determine miRNA and genes expression levels. The y-axis represents the $2^{(-\Delta CT)}$, or the relative expression of the different miRs and genes. MiRs expression was calculated relative to U44 and U48 rRNA (for microRNAs) and to GAPDH and b-actin (for genes). Experiments were carried out in triplicate for each data point, and data analysis was performed by using software (Bio-Rad).

Generation of Stable Clones with miR-21, miR-30c and miR-100 downregulation. H460R cells were stably infected with the Human anti-miR-21, anti-miR-30c, anti-miR-100 and the GFP gene under the control of two different promoters (System Biosciences) or an anti-miR control. Anti-miRs and control constructs were packaged with pPACKH1 Lentivector Packaging Plasmid mix (System Biosciences) in a HEK293TN packaging cell line. Viruses were concentrated using PEGit Virus Precipitation Solution, and titers were analyzed using the UltraRapid Lentiviral Titer Kit (System Biosciences). Infected cells were selected by FACS analysis (FACScalibur; BD Bioscience). Infection efficiency >90% was verified by fluorescent microscopy.

Human NF-κB Signaling Targets PCR Array. The overall procedure was conducted according to the manufacturer's instructions (SABiosciences). Briefly, total RNA was extracted from H460S/R and H292S/R. After generating cDNAs using a cDNA synthesis kit with 4ug of total RNAs, a PCR plate equipped with known probes for 84 NF-κB targets and housekeeping genes used as internal controls was incubated with cDNAs from H460S/R and H292S/R. The samples were then subjected to qRT-PCR analysis.

NF-κB activity. A pGL4.3-luc2p/NF-κB construct containing five copies of NF-κB response elements was purchased from Promega. The construct was transfected alone or with miR-21, miR-30c and miR-100, NF-κB siRNA or dominant active IκB-α plasmid into H460S/R or H292S/R cells. 24 hours later cells were incubated with TRAIL. 48hr after transfection NF-κB luciferase activity was tested with Dual Luciferase Assay (Promega) according to the manufacturer's instructions. Three independent experiments were performed in triplicate.

Soft agar colony formation assay. Briefly, 0.5×10^6/ml H460S/R and H292S/R cells were mixed with the agar matrix layer and added to the solidified base agar matrix layer. RPMI medium including 10% FBS with or without 50 ng/ml of TRAIL and Bay-117085 was added to the layers. After 7 days, the cell-matrix mixture was dissolved with a matrix solubilization buffer and colonies were quantified by standard MTS assay protocol. The absorbance of each sample was measured at 495 nm in a Multilabel Counter (Bio-Rad Laboratories).

***In vivo* studies.** Animal studies were performed according to institutional guidelines. 1×10^6 H460S and H460R cells were subcutaneously injected into the right flanks of 6-wk-old male nude mice (Charles River Breeding Laboratories). Treatments started 5 days after the injections by daily i.p. administration of TRAIL/Apo2 (10 mg/kg/day) or vehicle (PBS) for 8 days and/or Bay-117085 (5 mg/kg) twice per week for 2 weeks. Tumor size was assessed every 2 days by a digital caliper. Tumor volumes were determined by measuring the length (l) and the width (w) and calculating the volume ($V = lw^2/2$). 20 days after the injections, mice injected with H460R cells and treated with TRAIL were sacrificed due to significantly enlarged tumors. The other four groups of mice were sacrificed 27 days after inoculations. Animal experiments were conducted after approval of the Institutional animal care and use committee, Ohio State University.

Bioinformatics analysis. Bioinformatics analysis was performed by using these specific programs: Targetscan[1]**,** Pictar[2], RNhybrid [3].

1. http://www.targetscan.org/

2. http://pictar.bio.nyu.edu/

3. http://bibiserv.techfak.uni-bielefeld.de/

SI REFERENCES

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2. Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Statistical applications in genetics and molecular biology* 3:Article3.
3. Reich M, *et al.* (2006) GenePattern 2.0. *Nat Genet* 38(5):500-501.
4. Nuovo GJ, *et al.* (2009) A methodology for the combined in situ analyses of the precursor and mature forms of microRNAs and correlation with their putative targets. *Nature protocols* 4(1):107-115.
5. Calin GA, *et al.* (2002) Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proceedings of the National Academy of Sciences of the United States of America* 99(24):15524-15529.

SI Figures

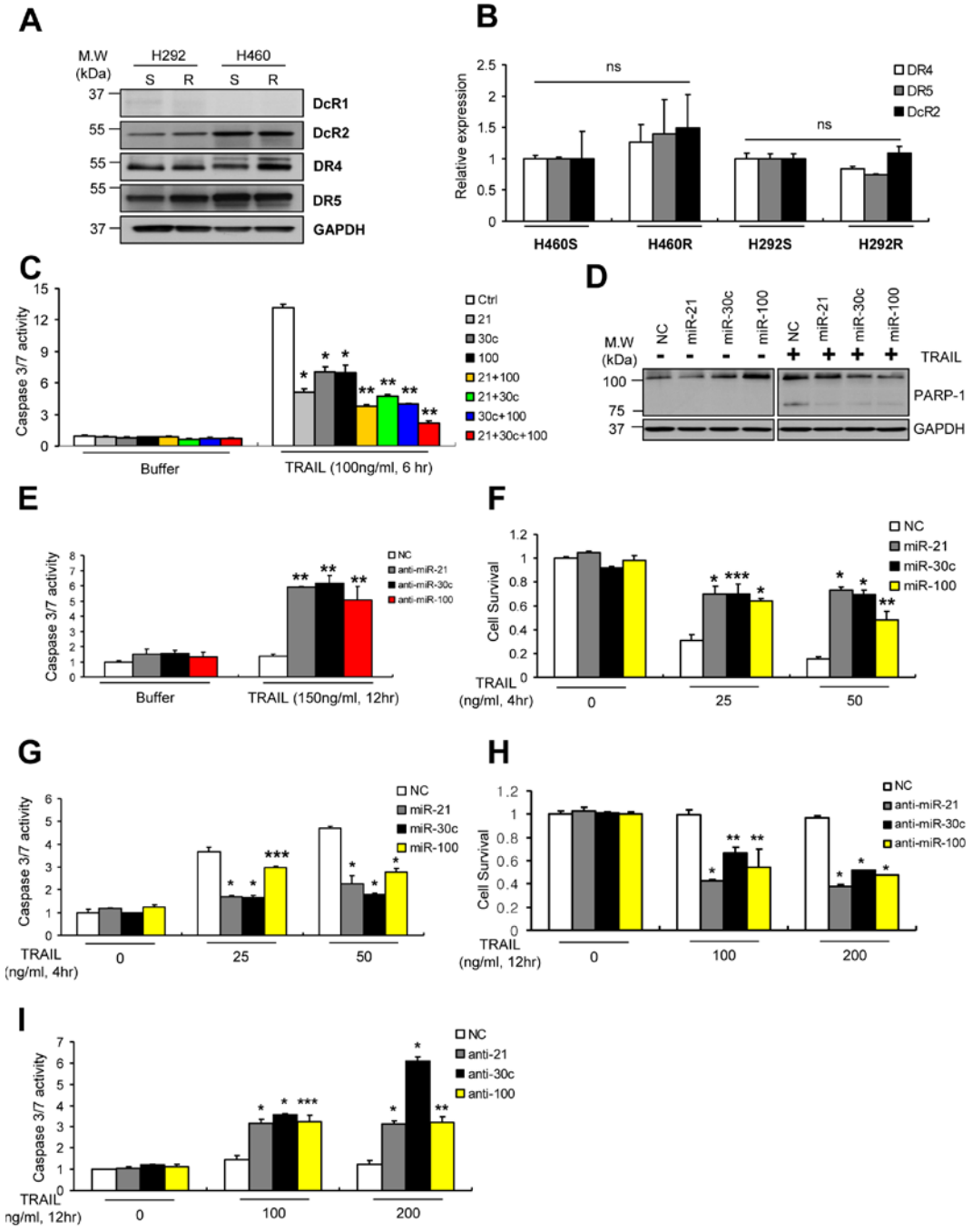


Fig. S1. MiR-21, miR-30c and miR-100 involvement in TRAIL resistance. **(*A*)** Western blot analysis of TRAIL receptors in H460S/R and H292S/R cells. **(*B*)** qRT-PCR analysis of

TRAIL receptors. All the values were normalized to GAPDH and the normalized values were re-normalized to the values from the sensitive cells. Bars indicate mean ±S.D (n=5). *p*-values were obtained by two tailed student t-test. **(*C*)** Decreased caspase -3/7 activity in TRAIL-sensitive H460 cells after miR-21, miR-30c, miR-100 or combinatorial overexpression of three miRNAs in response to TRAIL (100 ng/ml, 6 hr). Bars indicate mean ±SD (n=3) and the *p*-values were calculated by two-tailed student t-test (*$p < 0.005$, **$p < 0.001$). **(*D*)** PARP-1 cleavage in H460S cells after enforced expression of miR-21, miR-30c and miR-100. **(*E*)** Increased caspase-3/7 substrate activity in TRAIL resistant cells after miR-21, miR-30c and miR-100 knockdown by anti-miR oligonucleotides in response to TRAIL. **(*F* and *G*)** Increased survival rate (F) and decreased caspase -3/7 activity (G) in TRAIL-sensitive H292 cells (H292S) after miR-21, miR-30c and miR-100 overexpression. **(*H* and *I*)** Increased sensitivity of H292R cells in response to TRAIL after miR-21, miR-30c or miR-100 down-regulation as assessed by proliferation assay (H) and caspase-3/7 activity (I). Bars indicate mean ±SD (n=3) and the *p*-values were calculated by two-tailed student t-test (*$p < 0.01$, **$p < 0.02$, ***$p < 0.05$).

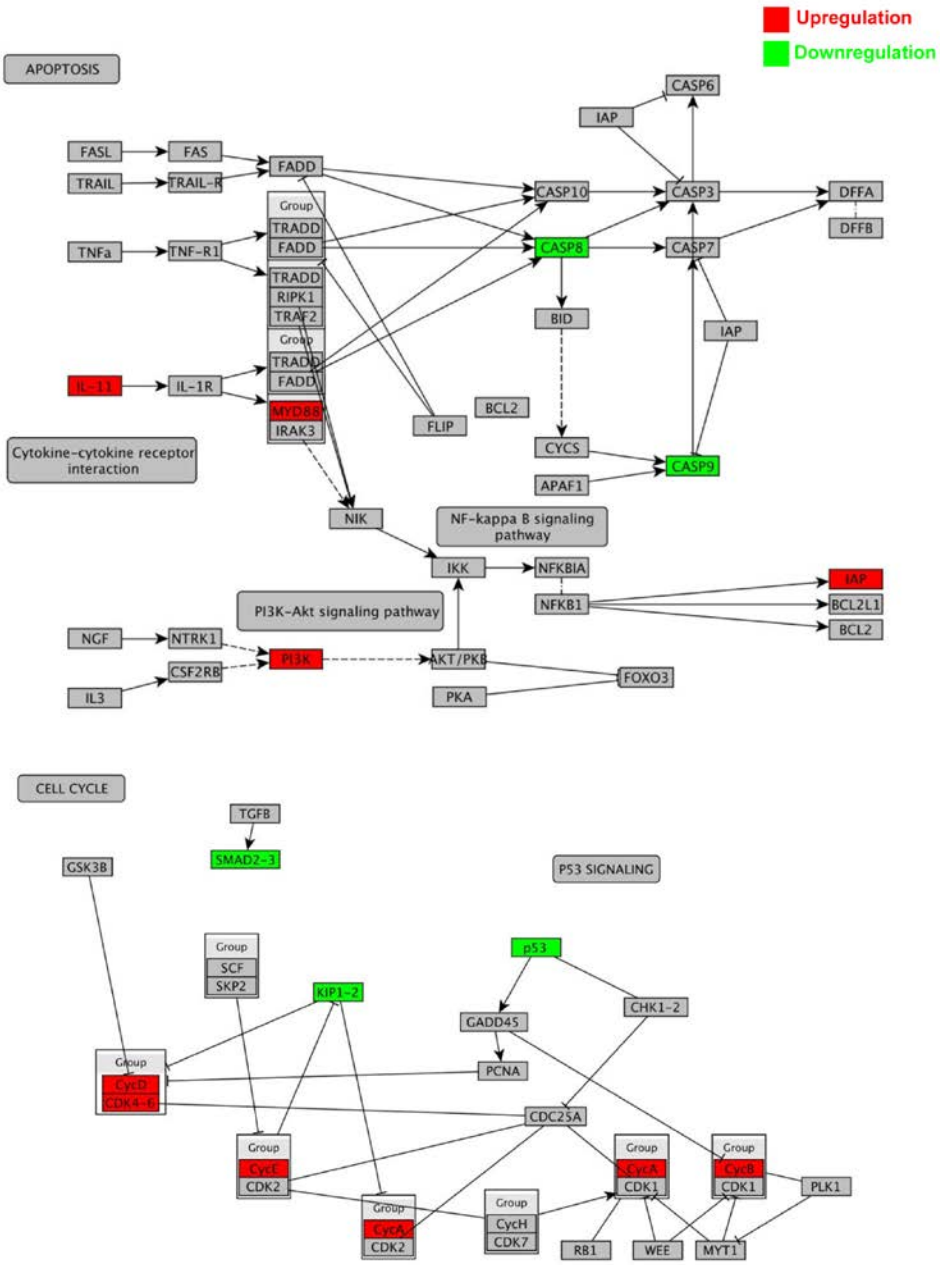
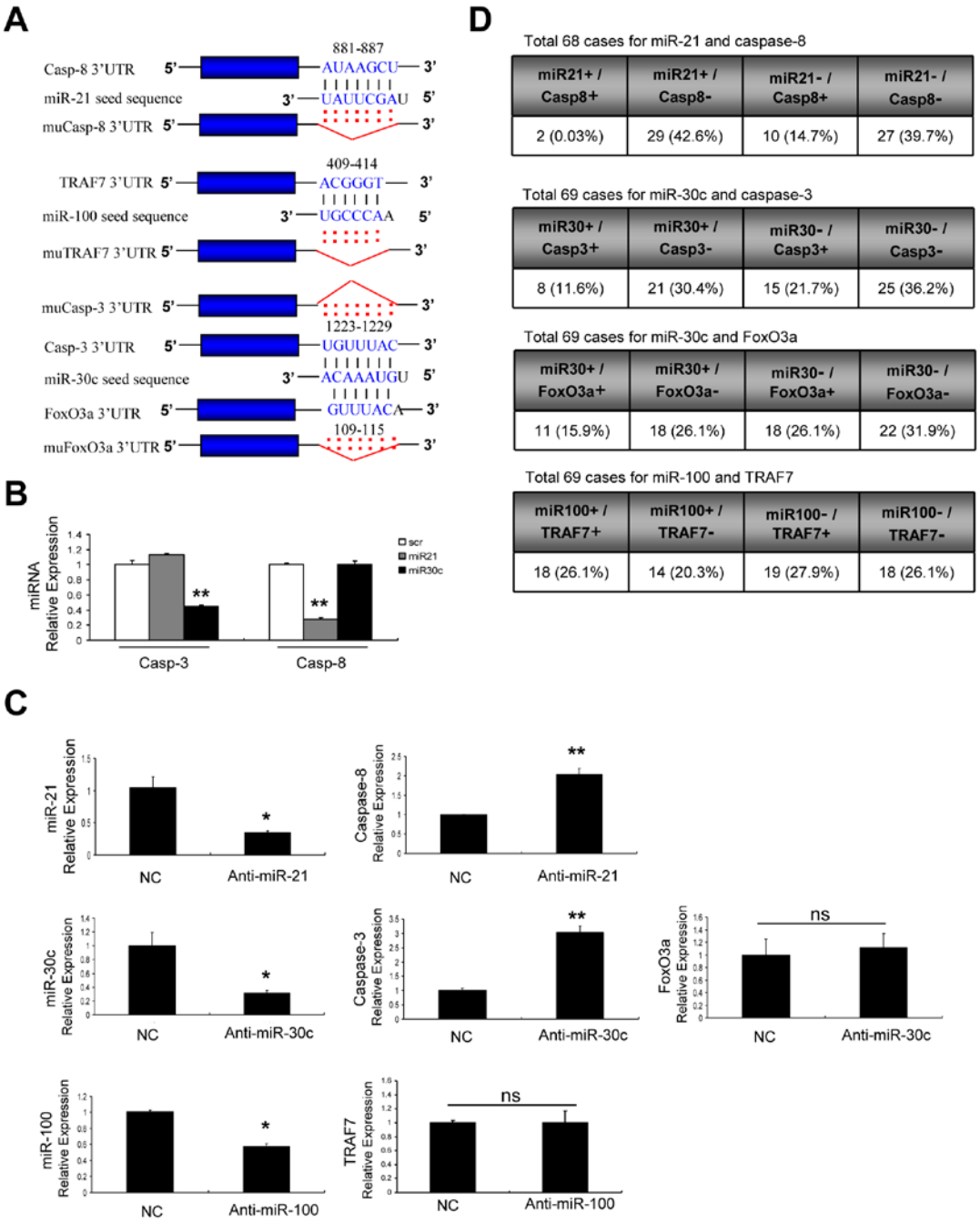


Fig. S2. Gene Affymetrix microarray of H460S and H460R cells showing increased expression of anti-apoptotic genes compared to H460S cells.



D

Total 68 cases for miR-21 and caspase-8

miR21+ / Casp8+	miR21+ / Casp8-	miR21- / Casp8+	miR21- / Casp8-
2 (0.03%)	29 (42.6%)	10 (14.7%)	27 (39.7%)

Total 69 cases for miR-30c and caspase-3

miR30+ / Casp3+	miR30+ / Casp3-	miR30- / Casp3+	miR30- / Casp3-
8 (11.6%)	21 (30.4%)	15 (21.7%)	25 (36.2%)

Total 69 cases for miR-30c and FoxO3a

miR30+ / FoxO3a+	miR30+ / FoxO3a-	miR30- / FoxO3a+	miR30- / FoxO3a-
11 (15.9%)	18 (26.1%)	18 (26.1%)	22 (31.9%)

Total 69 cases for miR-100 and TRAF7

miR100+ / TRAF7+	miR100+ / TRAF7-	miR100- / TRAF7+	miR100- / TRAF7-
18 (26.1%)	14 (20.3%)	19 (27.9%)	18 (26.1%)

Fig. S3. *Caspase-8, caspase-3, FoxO3a* and *TRAF-7* 3'UTRs are direct targets of miR-21, miR-30c and miR-100. **(*A*)** MiRNA seed sequences in the 3'UTRs of the target genes. *Caspase-8* 3'UTR presents one miR-21 binding site (nucleotides 881-887); *caspase-3* 3' UTR

has one miR-30c binding site (nt 1223-1229); *FoxO3a* 3' UTR has one miR-30c binding site (nt109-115); *TRAF-7* 3'UTR presents one miR-100 binding site (nt 409-414). In the figure the alignment of the seed regions of miRNAs with the respective target 3'UTR is shown. The sites of target mutagenesis are indicated in red. **:** = deleted nucleotides. **(*B*)** qRT-PCR showing caspase-3 and caspase-8 downregulation after miR-21 and miR-30c enforced expression. **(*C*)** qRT-PCR showing the expression of the miRNAs and their target genes in H460R cells transfected with anti-miR-21, anti-miR30c and anti-miR-100. Error bars indicate mean ±SD (n=3) and the *p*-values were calculated by two-tailed student t-test (*$p < 0.01$, **$p < 0.005$). **(*D*)** Co-expression analysis of miR-21, miR-30c and miR-100 and target genes. The tables report the inverse relation between miRNAs and the target gene in 69 lung tumors. Each core of the TMA was read blinded to the corresponding data. The score (0-100) was based on the signal intensity and % of + tumor cells – if a case had a score of 0-15, it was scored as negative. Any core with a score > 15 was scored as positive. The miRNA analysis was done by in situ hybridization and the protein analysis by immunohistochemistry.

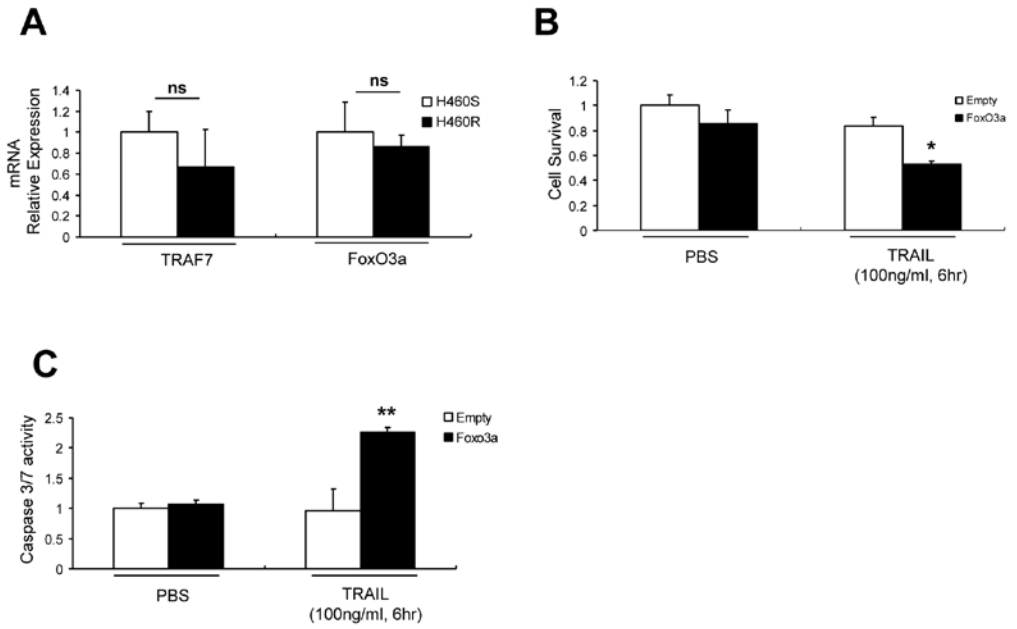


Fig. S4. TRAF-7 and FoxO3a overexpression sensitizes TRAIL-resistant cells to TRAIL-induced cell death. (*A*) mRNA levels of FoxO3a and TRAF-7 are not significantly changed in H460S and H460R. **(*B*)** MTS assay showing decreased proliferation in H460R after FoxO3a enforced expression in H460R cells. **(*C*)** Caspase-3/7 assay showing increased apoptosis after FoxO3a enforced expression in H460R cells. Bars indicate mean ±SD (n=3) and the *p*-values were calculated by two-tailed student t-test (*$p < 0.01$, **$p < 0.005$).

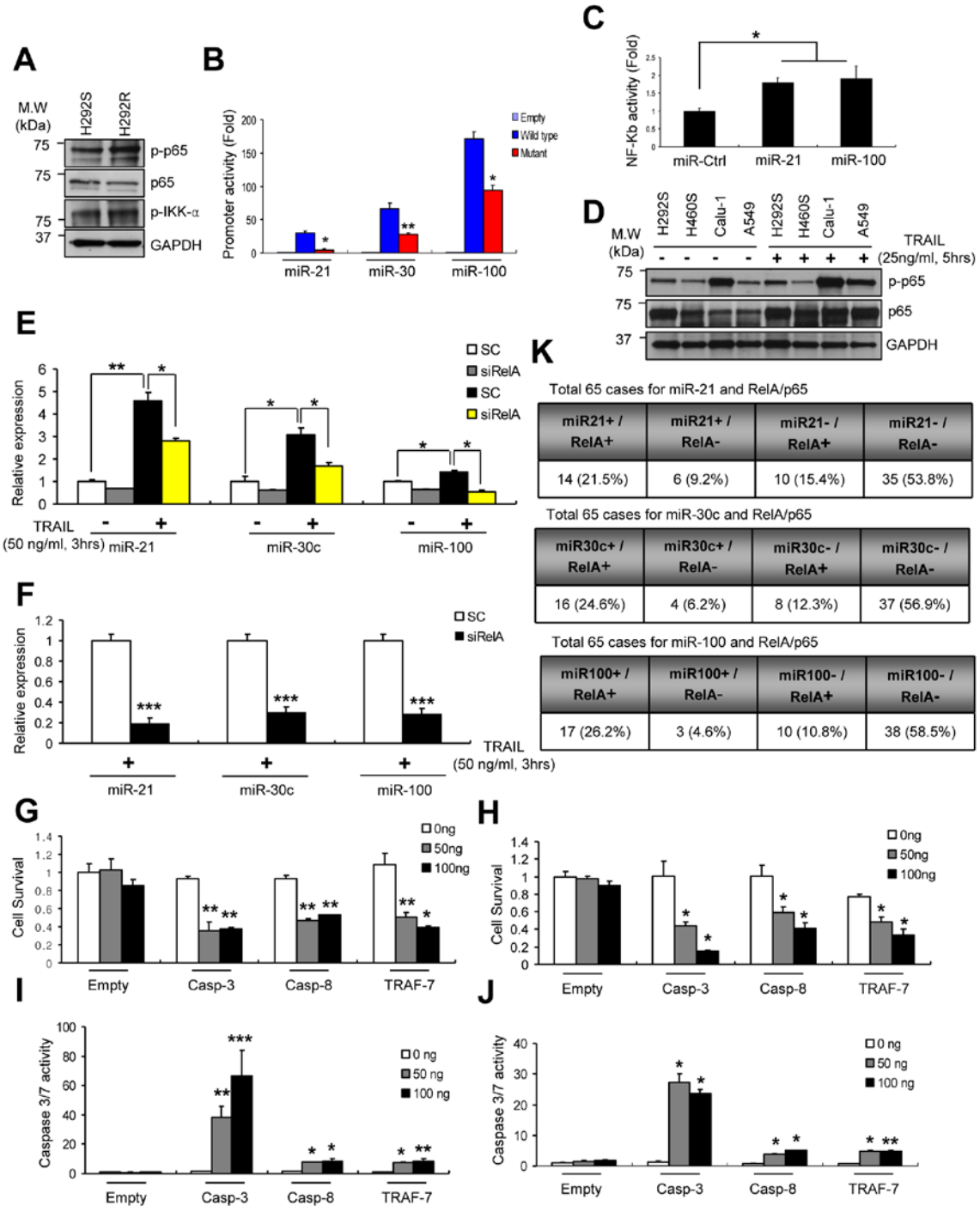


Fig. S5. NF-κB-mediated regulation of miR-21, -30c and -100 *in vitro* and *in vivo*. (*A*) NF-κB activation in H292R after TRAIL treatment. ***(B)*** NF-κB activity on miR-21, miR-30c and miR-100 promoters. The promoter regions of miR-21, miR-30c or miR-100, containing NF-κB

binding sites were sub-cloned into a promoterless vector. Deleted mutants were generated from the wild type constructs by standard mutagenesis (Agilent Technology). The wild type or mutant constructs were transfected into H460R. After 48 hrs, promoter activity was assessed by measuring firefly luciferase activity. Error bars represent mean ±SD (n=6) and p-values were obtained by two-tailed student t-test (*p<0.001 and **p<0.005). **(*C*)** MiR-21 and miR-100 activate NF-κB in H460R cells. pGL4.32-luc2-luciferase NF-κB vector was co-transfected with a scrambled miRNA, miR-21 or miR-100 in H460R cells. After 48 hrs, cells were subjected to a luciferase assay to measure NF-κB activity. Error bars represent mean ±SD (n=3) and *p*-values were calculated by two-tailed student t-test (*p<0.05). **(*D*)** Comparison of NF-κB phosphorylation in TRAIL-sensitive and -resistant cells. H292S, H460S, Calu-1 and A549 cells were stimulated with TRAIL for 5hrs. **(*E* and *F*)** qRT-PCR showing reduced levels of miR-21, -30c and -100 after NF-κB silencing in Calu-1 (E) and A549 (F) cell. Bars indicate mean ±SD (n=3) and the *p*-values were calculated by two-tailed student t-test (*p < 0.02, **p < 0.005 and ***p < 0.01). **(*G* and *H*)** MTS assay showing decreased proliferation in A549 (G) and Calu-1 (H) cells after caspase-8, caspase-3 and TRAF-7 overexpression in response to TRAIL-treatment. **(*I* and *J*)** Caspase -3/7 activity increased after caspase-8, caspase-3 and TRAF-7 overexpression in A549 (I) and Calu-1 (J) cells. Bars indicate mean ±SD (n=3) and the *p*-values were calculated by two-tailed student t-test (*p < 0.01, **p < 0.02, ***p < 0.05). **(*K*)** Tables

showing the direct relation between miR-21, miR-30c and miR-100 and NF-κB in 69 lung tumors. Each core of the TMA was read blinded to the corresponding data.

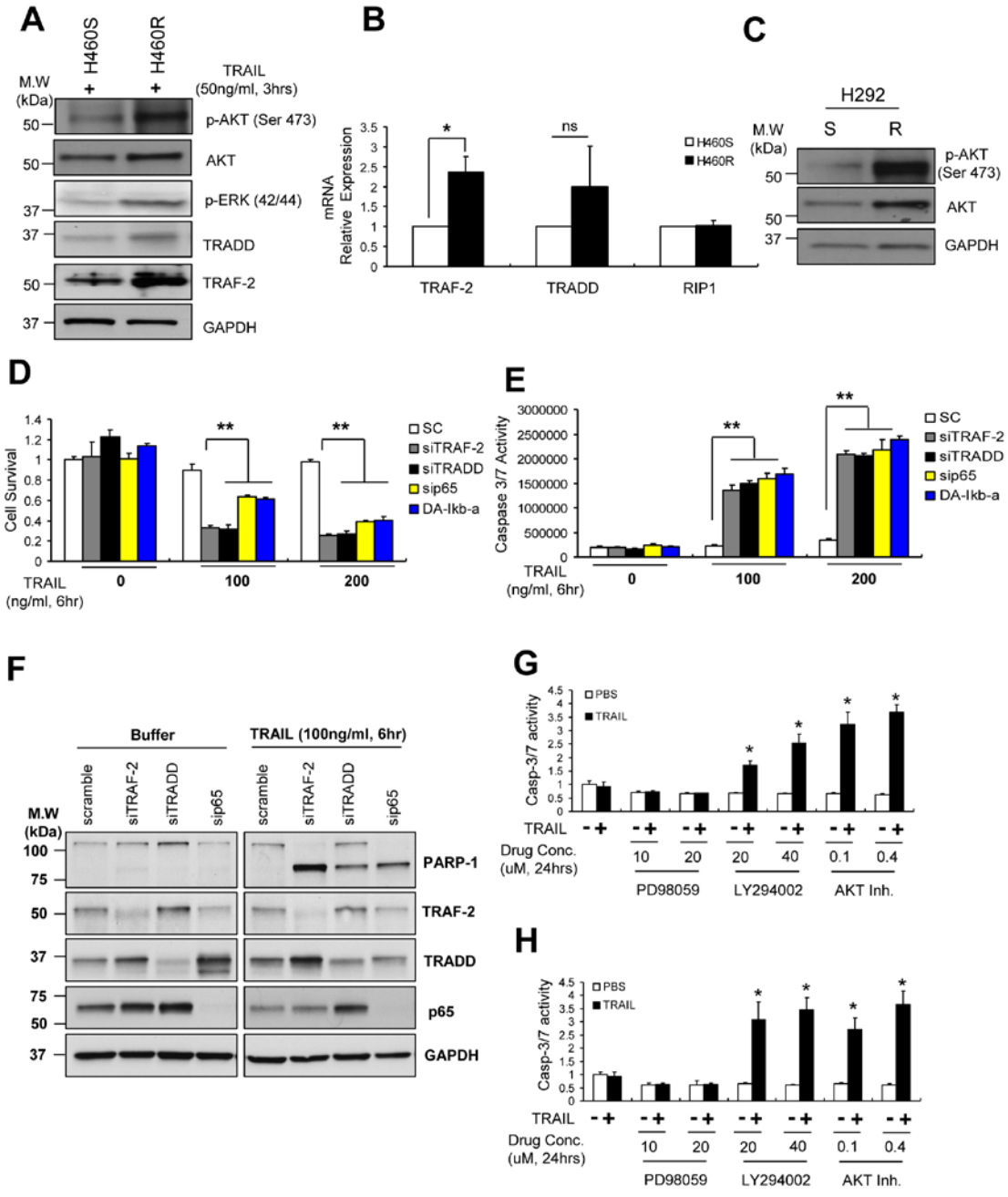


Fig. S6. Silencing of upstream activators of NF-κB sensitizes H460R cells to TRAIL-induced cell death. (***A***) Western blot analysis showing upregulation of p-AKT, p-ERK1/2,

TRADD and TRAF-2 in H460R cells. (***B*)** qRT-PCR showing up-regulation of TRAF-2, TRADD and RIP1 mRNAs in H460R cells. (***C*)** Western blot analysis showing increased expression of p-AKT and total AKT in H292R cells. **(*D* and *E*)** Proliferation (D) and caspase-3/7 (E) assays showing increased sensitivity to TRAIL-induced apoptosis in H460R cells after TRAF-2, TRADD and NF-κB silencing or overexpression of dominant active (DA)-IκB-α. (***F*)** Increased PARP-1 cleavage in H460R cells following TRAF-2, TRADD and NF-κB silencing. The results were verified by performing each western blot three times. Bars indicates mean ±SD (n=4) and *p*-values were calculated by ANOVA test (**p*<0.01, ***p*<0.005). **(*G-H*)** AKT inhibition increases caspase -3/7 activity in H460R (G) and H292R (H) cells. Cells were treated with MEK inhibitor (PD98059, Cell Signaling Technology), PI3K-AKT inhibitor (LY294002, Sigma-Aldrich) and direct AKT-1/-2 inhibitor (AKT inh., Sigma-Aldrich) at the indicated concentrations for 24 hrs. Bars indicate mean ±SD (n=5) and the *p*-values were calculated by two-tailed student t-test (**p* < 0.005).

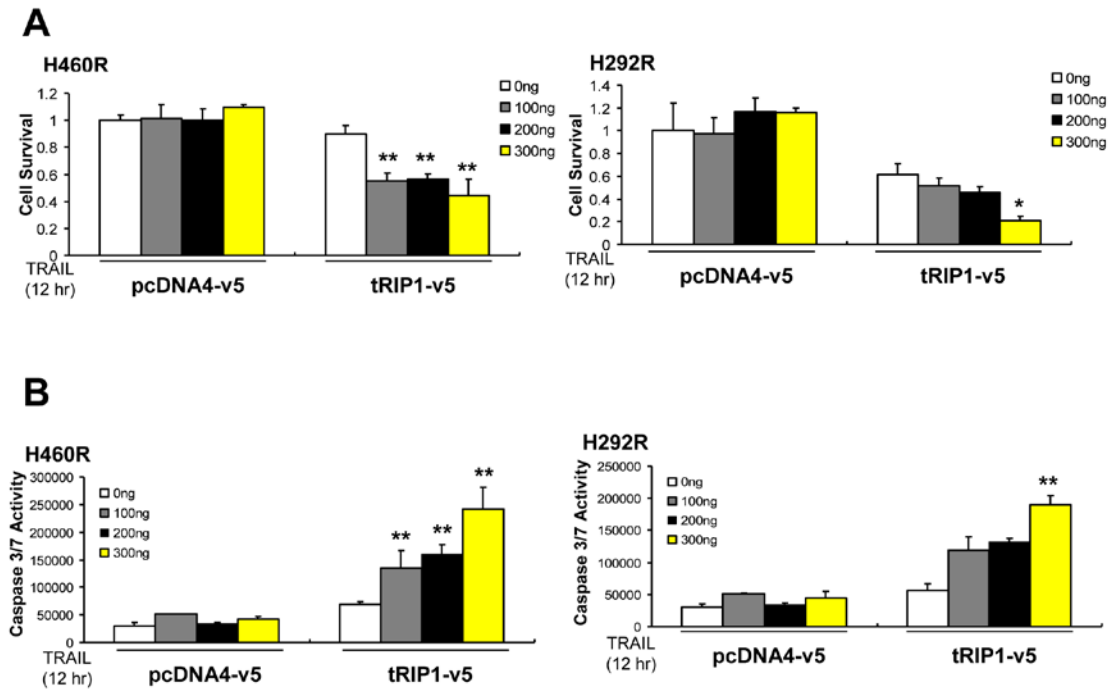


Fig. S7. Truncated RIP1 sensitizes cells with acquired TRAIL-resistance to TRAIL-induced cell death. (*A* and *B*) H460R and H292R cells were transfected with pcDNA4-tRIP1-V5 for 24 hrs. After transfection cells were exposed to TRAIL for 12 hrs and then cell proliferation and apoptosis were analyzed by MTS **(A)** and casapse-3/7 assay **(B)**, respectively. Error bars indicate mean ±SD (n=4) and *p*-values were calculated by ANOVA test (*$p<0.01$, **$p<0.001$)

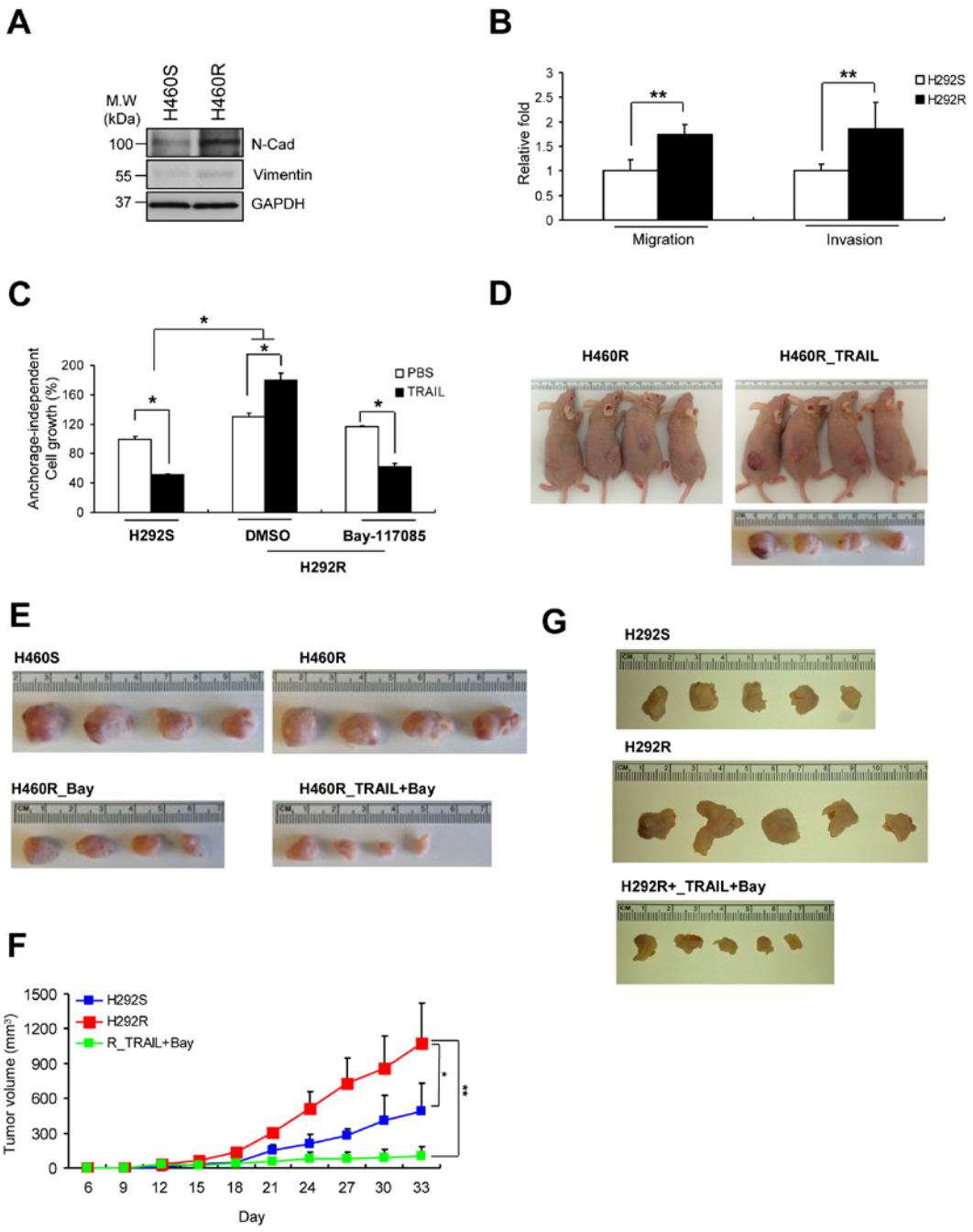


Fig. S8. Cells with acquired TRAIL resistance are more tumorigenic compared to the parental cells. (***A***) Western blot showing EMT markers in H460S and H460R cells. **(*B*)** Enhanced migration and invasion capacity of H292R cells. Bars show mean ±SD (n=4) and *p*-

values were calculated by paired student t-test (**p<0.01). **(*C*)** Anchorage independent growth assay of H292R cells in response to TRAIL and the NF-κB inhibitor, Bay 117085 treatments. Bars show mean ±SD (n=3) and p-values were calculated by paired student t-test (*p<0.01). **(*D*)** Comparison of tumor engraftments of H460R cells in nude mice treated with PBS or TRAIL 20 days after injections. TRAIL treatment induces a drastic increase in tumor volumes. **(*E*)** Comparison of tumor engraftments of nude mice injected with H460S and H460R treated with PBS, Bay-117085 alone or a combination of Bay-117085 plus TRAIL 27 days after the injections. **(*F*)** Comparison of tumor xenografts of nude mice treated with PBS or TRAIL+ Bay-117085 after inoculation with H292S or H292R cells. Combinatory treatment of NF-κB inhibitor and TRAIL drastically reduced tumor volumes. **(*G*)** Growth curve of engrafted tumors in nude mice injected with H292S and H292R cells. Combination of TRAIL and the NF-κB inhibitor Bay-117085 reduced tumor volumes as compared with a control group treated with PBS and DMSO only. Error bars show mean ±SD (n=5) and p-values were calculated by paired student t-test (*p=0.008, **p=0.002).

SI TABLES

A

Gene	Fold difference (H460R/ H460S)	*p*-value	Gene	Fold difference (H460R/ H460S)	*p*-value
ADM	4.63	0.048118	CXCL9	-1.12	0.746105
AGT	3.28	0.09602	EGFR	2.58	0.002418
AKT1	-1.11	0.276424	EGR2	1.07	0.645877
ALDH3A2	1.15	0.415261	F3	-25.58	0.002762
BCL2A1	11	0.010072	F8	1.7	0.03382
BCL2L1	3.36	0.004741	FAS	1.63	0.040778
BIRC2	8.3	0.002303	FASLG	-1.54	0.27992
BIRC3	1.01	0.910926	GADD45B	1.38	0.015121
C3	21.63	0.001173	ICAM1	17.63	0.00001
CCL11	1.48	0.323488	IFNB1	13.29	0.004441
CCL2	-3.83	0.114289	IFNG	2.69	0.17709
CCL22	2.28	0.36472	IL12B	-1.11	0.895393
CCL5	4.83	0.001856	IL15	1.48	0.15052
CCND1	1.67	0.014074	IL1A	9.73	0.000005
CCR5	-1.02	0.654614	IL1B	-1.25	0.283957
CD40	-1.61	0.015632	IL1R2	-1.34	0.061942
CD69	-8.52	0.088244	IL1RN	2.01	0.275901
CD80	-1.1	0.660674	IL2	1.8	0.312223
CD83	-2.04	0.031974	IL2RA	2.48	0.264841
CDKN1A	1.64	0.015706	IL4	1.38	0.4302
CFB	10.27	0.000484	IL6	23.82	0.00007
CSF1	1.84	0.000745	IL8	2.62	0.005148
CSF2	1.03	0.85063	INS	1.22	0.306009
CSF2RB	1.89	0.303937	IRF1	2.07	0.003517
CSF3	6.74	0.007002	LTA	-1.84	0.372589
CXCL1	1.94	0.009895	LTB	2.52	0.086104
CXCL10	1.14	0.698567	MAP2K6	1.7	0.000002
CXCL2	2.18	0.000186	MMP9	-1.16	0.965454
MYC	1.7	0.004772	SELE	1.36	0.220026
MYD88	2.12	0.02729	SELP	1.47	0.42933
NCOA3	2.2	0.002865	SNAP25	2.25	0.018132
NFKB1	1.46	0.014763	SOD2	1.29	0.246468
NFKB2	1.95	0.01377	STAT1	1.86	0.074462
NFKBIA	2.63	0.023518	STAT3	1.52	0.035552
NQO1	-1.1	0.74778	STAT5B	1.17	0.341276
NR4A2	-6.08	0.00134	TNF	2.23	0.196026
PDGFB	1.19	0.57371	TNFRSF1B	-5.11	0.005167
PLAU	1.8	0.003655	TNFSF10	1.65	0.125082
PTGS2	-1.94	0.027328	TP53	1.07	0.634745
REL	-1.02	0.884562	TRAF2	-1.31	0.402014
RELA	1.63	0.065139	VCAM1	4.81	0.11117
RELB	2	0.030101	XIAP	4.52	0.006631

B

Gene	Fold difference (H292R/ H292S)	*p* -value	Gene	Fold difference (H292R/ H292S)	*p* -value
ADM	8.89	0.01222	CXCL9	1.03	0.598592
AGT	1.81	0.072771	EGFR	2.21	0.05829
AKT1	−1.2	0.383227	EGR2	2.24	0.019504
ALDH3A2	−1.75	0.157979	F3	−3.73	0.033866
BCL2A1	6.7	0.000651	F8	3.4	0.024478
BCL2L1	2.24	0.102607	FAS	1.38	0.445185
BIRC2	11.45	0.025292	FASLG	2.45	0.16091
BIRC3	6	0.015584	GADD45B	6.36	0.000539
C3	−3.7	0.026632	ICAM1	1.28	0.478011
CCL11	1.2	0.796196	IFNB1	−2.32	0.098606
CCL2	−3.16	0.579781	IFNG	3.71	0.020502
CCL22	−1.03	0.931913	IL12B	1.21	0.418285
CCL5	−2.94	0.003016	IL15	1.02	0.739571
CCND1	2.3	0.189254	IL1A	−1.93	0.008834
CCR5	2.29	0.267038	IL1B	1.11	0.932234
CD40	−1.01	0.790632	IL1R2	−11.04	0.158389
CD69	−1.64	0.321076	IL1RN	2.21	0.253921
CD80	−1.1	0.57875	IL2	−1.08	0.90112
CD83	−4.46	0.000088	IL2RA	1.39	0.097979
CDKN1A	2.06	0.116563	IL4	−1.38	0.467844
CFB	1.16	0.880538	IL6	−1.22	0.736038
CSF1	−1.15	0.547919	IL8	19.4	0.302996
CSF2	−1.02	0.650727	INS	−1.23	0.106259
CSF2RB	−1	0.913172	IRF1	1.39	0.43055
CSF3	−1.96	0.207222	LTA	1.75	0.019004
CXCL1	−1.22	0.74478	LTB	−1.24	0.61284
CXCL10	−2.35	0.006705	MAP2K6	−1.06	0.75625
CXCL2	−1.07	0.697314	MMP9	−2.34	0.001223
MYC	2.78	0.009061	SELE	2.29	0.210315
MYD88	−6.5	0.188344	SELP	−2.77	0.154945
NCOA3	−1.5	0.092776	SNAP25	−1.09	0.744753
NFKB1	1.08	0.952182	SOD2	−1.73	0.015688
NFKB2	3.57	0.027728	STAT1	−1.63	0.009272
NFKBIA	−1.42	0.353373	STAT3	1.13	0.60073
NQO1	−4.72	0.072026	STAT5B	1	0.884366
NR4A2	−1.6	0.495621	TNF	−1.58	0.595658
PDGFB	1.99	0.021367	TNFRSF1B	1.13	0.915608
PLAU	2.09	0.267793	TNFSF10	1.13	0.833388
PTGS2	1.26	0.637073	TP53	2.75	0.043257
REL	−1.47	0.026354	TRAF2	1.85	0.003822
RELA	1.88	0.103387	VCAM1	−3.62	0.040747
RELB	1.18	0.845516	XIAP	3.55	0.002672

Table S1. Different gene expression in acquired TRAIL-resistant cells compared to parental sensitive cells. Relative expression values of the genes expressed in H460R (A) and

H292R (B) cells were divided by the relative expression values of those in control cells. The fold difference and *p*-value were obtained by RT^2 profiler PCR Array Data analysis software (SABiosciences) from three independent experiments.

Table S2. Probes for qRT-PCR (Taqman Assay)

Gene Name (Alias)	Assay ID
TNFRSF10A	Hs00269492_m1
TNFRSF10B	Hs00366278_m1
TNFRSF10C	Hs00182570_m1
TNFRSF10D	Hs04187502_m1
Caspase-8	Hs01018151_m1*
Caspase-3	Hs00234387_m1*
Foxo3	Hs00921424_m1
TRAF-1	Hs01090170_m1
TRAF-2	Hs00184192_m1
TRAF-7	Hs00260228_m1
TRADD	Hs00182558_m1
RIPK1	Hs00169407_m1
hsa-miR-21	397
hsa-miR-30c	419
hsa-miR-100	437

Table S2. List of probes used for qRT-PCR. Gene names and Identification numbers of the probes used for qRT-PCRs are reported.

Primer Name	Sequence (5'-3')	Usage
DR4 Forward	ATGGCGCCACCACCAGCTAGAGTAC	DR4 sequencing in H292S/R and H460S/R cells
DR4 Reverse	TCACTCCAAGGACACGGCAGAGCC	DR4 sequencing in H292S/R and H460S/R cells
DR5 Forward	ATGGAACAACGGGGACAGAACGCCC	DR5 sequencing in H292S/R and H460S/R cells
DR5 Reverse	TTAGGACATGGCAGAGTCTGCATTAC	DR5 sequencing in H292S/R and H460S/R cells
Caspase-8 3'utr Forward	AAAACTTGTCTTCCCTTCTGATTGATGG	Luciferase assay for caspase-8 with miR-21
Caspase-8 3'utr Reverse	ATCAAATTATATTTTATTCTATTAAAA	Luciferase assay for caspase-8 with miR-21
Caspase-3 3'utr Forward	TCTAGAAGGGCGCCATCGCCAAGTAAGAAA	Luciferase assay for caspase-3 with miR-30
Caspase-3 3'utr Reverase	TCTAGACCCGTGAAATGTCATACTGACAG	Luciferase assay for caspase-3 with miR-30
FoxO3a Forward	TCTAGAAATGTTGTTGGTTTGAACGTGGGGAACTTCAC	Luciferase assay for FoxO3a with miR-30
FoxO3a Reverse	TCTAGAGCTCACCACCCTGTACAAGGAAGATTTTGCATA	Luciferase assay for FoxO3a with miR-30
Caspase-8 3'utr Forward	ATGATTTTTACAAGTTTTAACTCCCCAAAC	mutagenesis of 3'utr of miR-21
Caspase-8 3'utr Reverse	AGGCATAAAGCAAGTTTGGGGATTAAAACTTG	mutagenesis of 3'utr of miR-21
Caspase-3 3'utr Forward	GCAAAATTCTTAAGTATGTTATTTTCTGTTGAAATCAAAGGAAAATAGTAATGTTTTAT	mutagenesis of 3'utr of miR-30
Caspase-3 3'utr Reverase	AGTATAAAACATTACTATTTTCCTTTGATTTCAACAGAAAATAACATACTTAAGAATTT	mutagenesis of 3'utr of miR-30
FoxO3a Forward	CAAAGGAAAATAGTAATGTTTTATACTGAAAG	mutagenesis of 3'utr of miR-30
FoxO3a Reverse	CCTATGTGCTCATAGGTCTTTTTCTTTCAGTATAAAAC	mutagenesis of 3'utr of miR-30
TRAF7 Forward	GCACATCAAATGCCCCCACTCCAAGTGCACGTTCATCG	mutagenesis of 3'utr of miR-100
TRAF7 Reverse	TGTCCTGGTTCCCGATGAACGTGCACTTGGAGTGGGGGC	mutagenesis of 3'utr of miR-100
mIR21 Forward	ATTGGAGTGGATGGGTTCTGCC	CHIP assay
miR21 Reverse	AAGTATGTCAGTGCAAAGTATGG	CHIP assay
miR30c Forward	TTATTTCAGCTGTAGACTTGCC	CHIP assay
miR30c Reverse	GGCGATATCATTTCTCTGTAAC	CHIP assay
miR100 Forward	TATTCCGAGACTTCACATTCC	CHIP assay
miR100 Reverse	GGAACCATTATGTTTACTTC	CHIP assay
Cleaved RIP Forward	GGATTGTGTGGCAGTACCTTCAAG	cloning into pcDNA4-v5
Cleaved RIP Reverse	GTTCTGGCTGACGTAAATCAAG	cloning into pcDNA4-v5
miR21 5'utr for NF-Kb Forward	CACTTCAAGCATTATAGATGATCCTC	Luciferase assay for NF-kB activity
miR21 5'utr for NF-Kb Reverse	ATTCCACCCTAAGAAAACACAAGG	Luciferase assay for NF-kB activity
miR30c 5'utr for NF-Kb Forward	GGAGGCTCAGATACCCCATTTTACAG	Luciferase assay for NF-kB activity
miR30c 5'utr for NF-Kb Reverse	GTGGGTTAGAGCCTGGGCGCT	Luciferase assay for NF-kB activity
miR100 5'utr for NF-Kb Forward	CATGCCAGGTAATCACTGCCCAATTC	Luciferase assay for NF-kB activity
miR100 5'utr for NF-Kb Reverse	CCTAAAAATTTGCAATACTTGAGGAAGCG	Luciferase assay for NF-kB activity
Caspase-8 For	GACTTCAGCAGAAATCTTTATG	cloning into pcDNA4-v5
Caspase-8 Rev	ATCAGAAGGGAAGACAAGTTTT	cloning into pcDNA4-v5
miR-21 deleted mutant - For	AGCCGGGAAATTCCCTTTTCCAACCTGTATC	mutagenesis of PGL-30b-miR-21 5UTR
miR-21 deleted mutant - Rev	CAGGTTGGAAAAGGGAATTTCCCGGCTTGG	mutagenesis of PGL-30b-miR-21 5UTR
miR-30 deleted mutant - For	TATAAAGCCAAGTGAACTCTCCCCAGGCTAA	mutagenesis of PGL-30b-miR-30 5UTR
miR-30 deleted mutant - Rev	TGAAAAATTAGCCTGGGGAGAGTTCACTTGGC	mutagenesis of PGL-30b-miR-30 5UTR
miR-100 deleted mutant - For	AAGGTCCAAGCGTAGCCAATTCCAGGAAAG	mutagenesis of PGL-30b-miR-100 5UTR
miR-100 deleted mutant - Rev	ACAAATCTTTCCTGGAATTGGCTACGCTTGGAC	mutagenesis of PGL-30b-miR-100 5UTR

Table S3. List of Primers used in the study.