Supporting Information

Materials and Methods

Chemicals and Reagents. Cycloheximide (CHX) was purchased from Calbiochem. Lipopolysaccharide (LPS) was from InvivoGen (San Diego, CA). Recombinant human TNFα and TRAIL, Proteome Profiler Human Phospho-RTK Array kit, and human TNFα Quantikine HS ELISA kit were from the R&D Systems (Minneapolis, MN).

Antibodies. Goat anti-cIAP1 is a kind gift from Dr. John Silke (La Trobe University, Victoria, Australia). Rabbit antibodies for caspase-3, and -8, XIAP, TNFR1, TNFR2, TRAF2, TRADD, RIPK1, p65, p-p65 (S536), IkBα, p-IkBα (S32/36), NIK, p100/52, p105/50, PARP, cyclin D1, EGFR, Ron, Axl, c-Met, p-c-Met (Y1234/1235), EphA2, EphA4, EphB4, Cbl, c-FLIP, Src, p-Src(Y416), p-Src(Y529), Akt, p-Akt(S473), p70S6K, p-p70S6K(T389) and p-ERK1/2(T202/Y204), and Phospho-Tyrosine mouse monoclonal antibody (P-Tyr-100) were from the Cell Signaling Technology (Danvers, MA). Mouse monoclonal antibody for caspse-8 was from Assay Designs. Rabbit anti-LRIG1 antibody was from Abcam. Mouse monoclonal antibodies for cyclin D1, ERK1 and IkBα were from the BD Pharmingen. Mouse monoclonal antibodies for pan cIAP1/2 and cIAP2, neutralizing antibodies for TNFα, phycoerythrin-conjugated mouse monoclonal antibodies for human TNFR1 and isotype control mouse IgG1 were from the R&D Systems (Minneapolis, MN). Mouse monoclonal antibodies for PLCB4, TNFR1 c-Met and β-actin, and rabbit antibody for GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies for EphA4 and EphB4 were from Invitrogen.

Preparation of Conditioned Medium from MDA-MB-231 cells treated with SM-164. MDA-MB-231 cells were grown to confluence in 150 mm plates, washed with PBS, and then incubated with 15 ml of fresh medium containing 5 nM of SM-164 for 16 h. The cell-conditioned medium was collected, centrifuged at 1000 rpm for 10 minutes to remove cell debris, filtered through a 0.45 μm filter, and then stored at -20°C. The levels of TNFα in the conditioned medium were 12-37 pg/ml. The conditioned medium was used at 1:1 dilution with fresh medium in cell culture.

Microarray Analysis. Total RNA was isolated by RNA miniprep kit (Sigma). The Affymetrix human U133 Plus 2.0 chips were used for the hybridization. The cRNA probe, labeling, hybridization and the normalization of the gene expression levels from the raw microarray analysis was prepared by the University of Michigan Comprehensive Cancer Center Microarray Core facility. The expression levels

from 54675 probe sets were provided in log2 unit in an Excel spreadsheet. The data were read in the statistical program R(1) for additional analysis.

Duplicate expression levels of two resistant and two sensitive cell population were used to fit a linear model using the LIMMA package (2). The design matrix sets 0 for the resistant cell population and 1 for the sensitive cell population. The fitted model is further processed by the eBayes function to give empirical test statistics for each gene. The selection criteria select genes giving adjusted *p* values below 0.05 and yield a total number of 7200 genes from the total of 54675 probe sets. Among the 7200 genes, a second criterion was used to select genes which have one log unit difference in the average expression level between the resistant (T2 and T3) and sensitive (T6 and T8) groups. A set of 45 genes and two internal probes were obtained by these two selection procedures. Among these 45 genes, 10 genes were identified to be consistently differentially expressed by more than 1.5-fold in the resistant (T2 and T3) group relative to the sensitive (T6, T8) group and parental MDA-MB-231 cells. All the analyses were performed using R (version 2.8)(1) and Bioconductor (2.3.14) (3).

Real-time RT-PCR Probe/Primer Sets and siRNAs. These Taqman real-time RT-PCR primers/probe sets were used: BIRC3 (Hs00154109-m1), CYGB (HS00370476-m1), EXT1 (Hs00609162-m1), MSI2 (Hs00292670-m1), OXTR (Hs00168573-m1), SAA2 (Hs00754237_S1), TGM2 (Hs00190278-m1), and WT1 (Hs00240913-m1) (Applied Biosystems). ON-TARGETplus SMARTpool siRNAs for CYGB, EXT1, MSI2, OXTR, SAA2, TGM2 and WT1 were from Dhamarcon and the sequences are available from www.dharmacon.com.

Flow Cytometric Analysis of TNFα Receptor Expression. Cells were harvested by incubating in Hank's balanced salt solution (without Ca2+/Mg2+) containing 5 mM EDTA and gentle scraping. Following washing in Hank's solution, $5x10^5$ cells per condition were resuspended in 100 µl of Hank's solution containing 2% BSA and phycoerythrin-conjugated monoclonal antibodies specific for human TNFR1 or corresponding isotype control antibodies for 30 min on ice. After two washes with PBS, cells were analyzed using a FACS flow cytometry.

TNFα Reporter Assay. The human TNFα promoter reporter, which contains 0.8 kb of the human TNFα promoter, was obtained from the Panomics (Fremont, CA), and the pRL-SV40 vector, a renilla luciferase control reporter, was obtained from the Promega (Madison, WI). Cells cultured in 48-well plates were first transfected with LRIG1-specific or control siRNAs for 1 day, and then with the TNFα-Luc and pRL-SV40 plasmids using FUGENE 6 (Roche, Indianapolis, IN). The transfected cells were treated with 20 µM of Z-VAD with or without 10 nM of SM-164 for 20 hours before performing luciferase reporter assays using the Dual-Glo™ Luciferase Assay System (Promega).

Methods References

1. Team, R. D. C. (2010) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria
2. Smyth, G. K. (2005) Limma: linear models for microarray data. in *Bioinformatics and Computational Biology Solutions using R and Bioconductor* (Gentleman, R., Carey, V., Dudoit, S., Irizarry, R., and Huber, W. eds.), Springer, New York. pp 397--420
3. Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A. J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J. Y., and Zhang, J. (2004) *Genome Biol* **5**, R80

Supplemental Figure Legends

Fig. S1. Characterization of tumor cells isolated from the regrown MDA-MB-231 xenograft tumors after SM-164 treatment. (A) Cells were treated with SM-164 for 4 days and cell viability was evaluated by WST assay. Data are mean ± SD, and are representative of two independent experiments. (B) Cells were treated with AT-406 for 24 h and cell viability was evaluated by WST-based assay. Data are representative of two independent experiments.

Fig. S2. Characterization of MDA-Mb-231 tumor sublines (A) Cells were treated with 5 nM of SM-164 and 2 ng/ml of TNFα for 4 h, and cell lysates were immunoblotted to detect the indicated proteins. (B) Cells were treated with 5 nM of SM-164 for 16 h (parental) or 48 h (resistant sublines) for ELISA. Data are mean of two independent experiments. (C) TNFα mRNA expression was measured by quantitative RT-PCR. Data shown are mean ± SEM (n=3). (D) Cells were treated with 5 nM of SM-164 and 2 ng/ml of TNFα for 5 h, and TNF mRNA expression was measured by quantitative RT-PCR. Data shown are mean of two independent experiments. (E) Cells were incubated with the conditioned medium from MDA-MB-231 cells treated with SM-164, 2 ng/ml of TNFα or 1 ng/ml of TRAIL for 2 days. Cell viability was determined by WST assay. Data shown are representative of three independent experiments. P=MDA-MB-231 parental cells.

Fig. S3. Validation of differentially expressed genes identified by microarray analysis. (A) The mRNA levels were measured by quantitative RT-PCR. Data shown are the mean of two independent experiments. (B) Protein levels of PLCB4 were determined by immunoblotting. (C & D) Cells were transfected with the indicated siRNAs for 2 days, then treated with SM-164 for the indicated times. Cell viability was determined by WST assay. Data shown are representative of three independent experiments. Knockdown efficiency was determined by quantitative RT-PCR. Data were plotted as mean±SD of one experiment.

Fig. S4. Knockdown of LRIG1 attenuates SM-164-induced apoptosis in MDA-MB-231 and SKOV-3 cells. (A) Cells were transfected with siRNAs for 2 days, and then treated with 5 nM of SM-164 for 12 h; and SM-164-induced apoptosis was determined by Annexin V-PI staining followed by flow cytometry analysis. Data shown are mean ± SD of the Annexin V positive cells (n=3). (B) Cells were transfected with siRNAs for 2 days, and then harvested and stained with phycoerythrin-conjugated monoclonal antibodies specific for human TNFR1 or isotype-matched control antibody. Histograms with black lines depict negative controls stained with isotype-matched control antibody. Results shown are

representative of two experiments. (C&D) Cells were transfected with siRNAs for 2 days. The siRNA-transfected cells were treated with 5 nM of SM-164 for 4 h for immunoblotting (C) or 24 h for WST-based cell viability assay. Data shown are representative of two experiments (D).

Fig. S5. Knockdown of LRIG1 attenuates SM-164-induced apoptosis in a panel of SM-164-sensitive cell lines. (A) Cells were transfected with siRNA for 2 days, then treated with SM-164 for another 2 days. Cell viability was determined by WST assay. Data are representative of two independent experiments. (C) Cells were transfected with siRNAs for 2 days then treated with 5 nM of SM-164 for 16 h for immunoblotting.

Fig. S6. Characterization of Smac mimetic resistant sublines derived from SKOV-3 cell line. (A) Cells were treated with SM-164 for 24 hours, and the sensitivity of parental (SKOV-3/P**)** and SM-164 resistant derivative (SKOV-3/R) were evaluated by WST assay. Data are representative of three independent experiments. (B) Cells were treated with 10 nM of SM-164 for 16 h and TNFα production was detected by ELISA. Data shown are the mean of two independent experiments. (C) Cells were treated with the indicated concentrations of SM-164 for the indicated times and immunoblotting was performed.

Fig. S7. Depletion of LRIG1 had no significant effect on the activation of NF-kB upon SM-164 treatment in MDA-MB-231 cells. (A) Cells cultured in 48-well plates were first transfected with LRIG1-specific or control siRNAs for 1 day, and then with the TNFα-Luc and pRL-SV40 plasmids using FUGENE 6 (Roche, Indianapolis, IN). The transfected cells were treated with 20 μM of Z-VAD with or without 10 nM of SM-164 for 20 hours before collected for luciferase reporter assays. Data shown are mean ± SEM (n=3). (B) Cells were transfected with siRNAs for 2 days then treated with 5 nM of SM-164 for 16 h for immunoblotting. (C-E) Cells were transfected with the indicated siRNAs for two days, and then treated with 5 nM of SM-164, 2 ng/ml of TNFα or 5 μg/ml of LPS for 10 h. The expression of TNFα mRNA was measured by real time RT-PCR (C&D). Data are mean ± SEM (n=3). The efficient knockdown of the different components of NF-kB complex was confirmed by immunoblot (E).

Fig. S8. Multiple RTKs are up-regulated in MDA-MB-231 sublines resistant to Smac mimetics. (A) Whole cell lysates of MDA-MB-231 and T2 cells were incubated with the Human Phospho-RTK Array (R&D) to profile phospho-RTKs. (B) Phospho-Tyrosine profiling of MDA-MB-231 parental and sublines by immunoblotting using an antibody specific for phospho-Tyrosine (p-Y-100). (C) Protein profiling of MDA-MB-231 parental and sublines by immunoblotting.

Fig. S9. Knockdown of c-Met or Ron modestly enhanced the activity of SM-164 in T2 resistant cells. Cells were transfected with c-Met- or Ron-specific siRNAs for 2 days, then treated with 20 nM of SM-164 for 20 h for quantitative RT-PCR (A); or treated with 20 nM of SM-164 for 24 h for immunoblotting (B); or treated with 20 nM of SM-164 for 48 h for cell viability assay (C). Data shown are mean ± SEM (n=3). Asterisks indicate significant difference (p<0.05) between si control-transfected cells and si RTK-transfected cells.

Fig. S10. Synergy between TKIs and SM-164 in MDA-MB-231 resistant sublines. (A) T2 cells were treated with TKIs in the absence or presence of 20 nM of SM-164 for 2 days and cell viability was assessed by WST assay. Data are representative of two independent experiments. (B & C) T2 cells were treated with 20 nM of SM-164 and/or 1 μM of GSK or SKI for 20 h. TNFα mRNA expression and protein production was measured by quantitative RT-PCR (B) and TNFα ELISA (C), respectively. Data are mean of two independent experiments. (D) Cells were treated with SM-164 and/or the TKIs for 2 days and cell viability was determined by WST assay. Data shown are representative of three independent experiments.

Fig. S11. TKIs do not increase cell sensitivity to death receptor-mediated apoptosis. Cells were treated with recombinant human TNFα and TRAIL in the presence or absence of kinase inhibitors for two days and cell viability was assessed.

Fig. S12. Synergistic effect between SM-164 and GSK1363089 in T2 resistant tumors. (A) SCID mice bearing T2 resistant cells were treated with 5 mg/kg of SM-164 intravenously and/or 10 mg/kg of GSK orally for the indicated time points. Tumor lysates were immunoblotted to detect the indicated proteins. (D) Body weight change of mice in **Fig 6B**. Data shown are mean ± SEM for 6-8 mice.

Fig S1

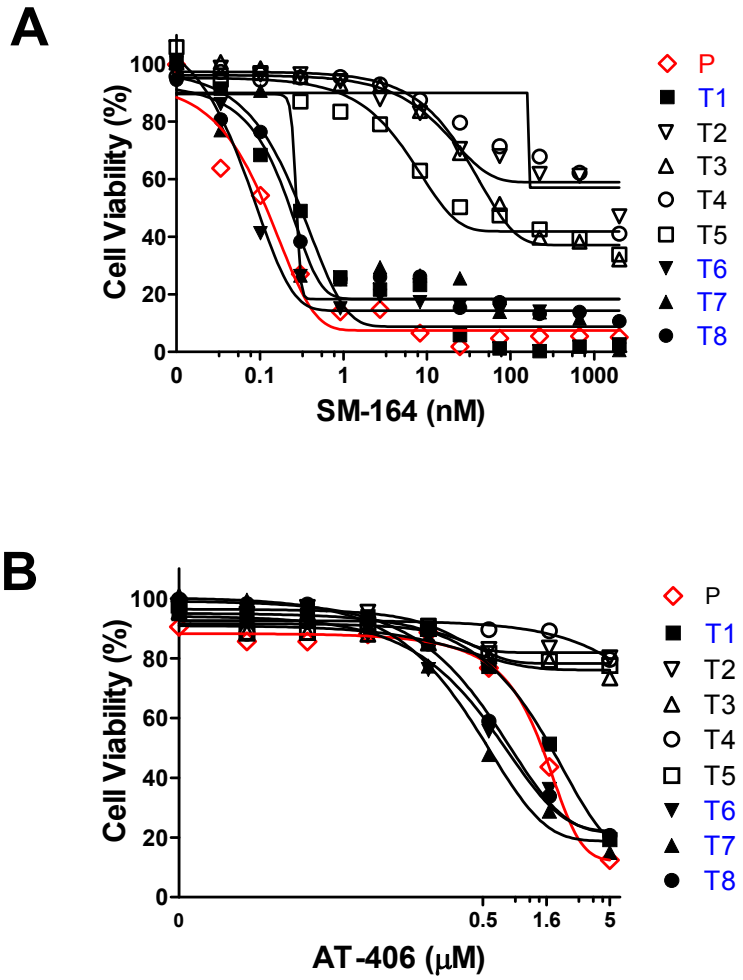

A
Cell Viability (%)
100
80
60
40
20
0
0
0.1
1
10
100
1000
SM-164 (nM)
P
T1
T2
T3
T4
T5
T6
T7
T8
B
Cell Viability (%)
100
80
60
40
20
0
0
0.5
1.6
5
AT-406 (μM)
P
T1
T2
T3
T4
T5
T6
T7
T8

Fig S2

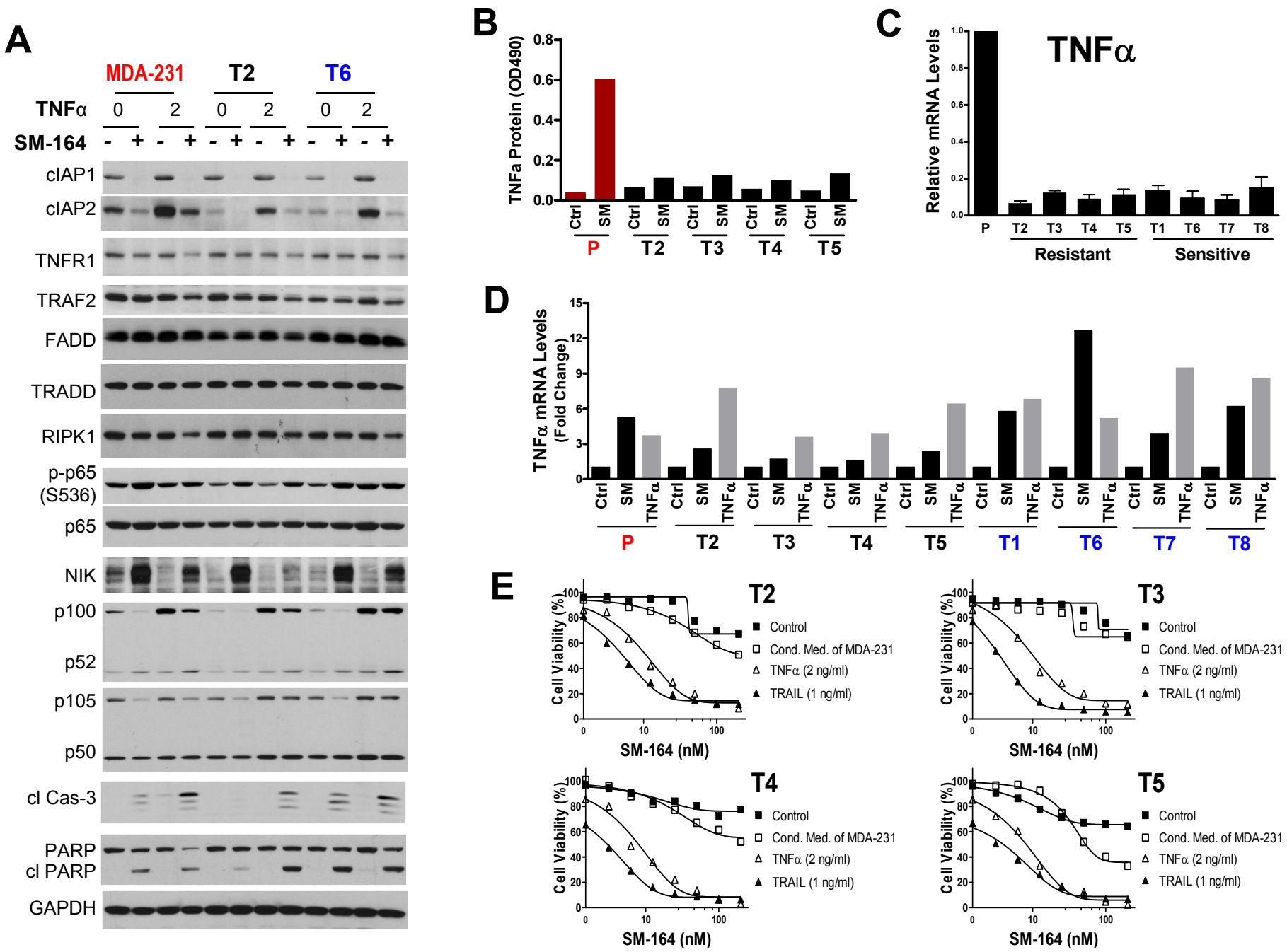

A
MDA-231
T2
T6
TNFα
0
2
SM-164
cIAP1
cIAP2
TNFR1
TRAF2
FADD
TRADD
RIPK1
p-p65 (S536)
p65
NIK
p100
p52
p105
p50
cl Cas-3
PARP
cl PARP
GAPDH
B
TNFa Protein (OD490)
Ctrl
SM
P
T2
T3
T4
T5
C
TNFα
Relative mRNA Levels
P
T2
T3
T4
T5
T1
T6
T7
T8
Resistant
Sensitive
D
TNFα mRNA Levels (Fold Change)
TNFα
P
T2
T3
T4
T5
T1
T6
T7
T8
E
T2
T3
T4
T5
Control
Cond. Med. of MDA-231
TNFα (2 ng/ml)
TRAIL (1 ng/ml)
Cell Viability (%)
SM-164 (nM)

Fig S3

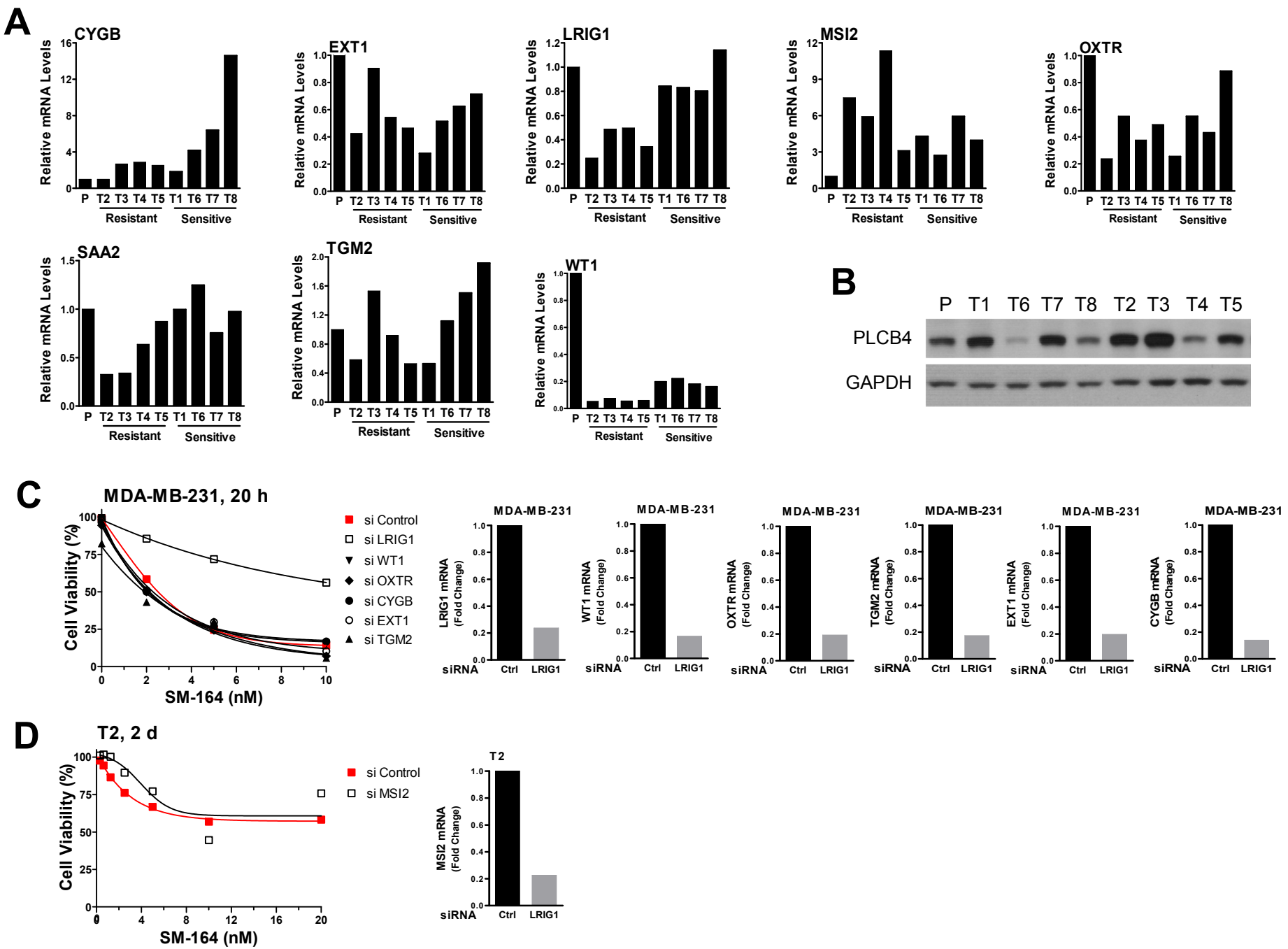

A
CYGB
EXT1
LRIG1
MSI2
OXTR
SAA2
TGM2
WT1
Relative mRNA Levels
P T2 T3 T4 T5 T1 T6 T7 T8
Resistant
Sensitive
B
P T1 T6 T7 T8 T2 T3 T4 T5
PLCB4
GAPDH
C
MDA-MB-231, 20 h
Cell Viability (%)
SM-164 (nM)
si Control
si LRIG1
si WT1
si OXTR
si CYGB
si EXT1
si TGM2
MDA-MB-231
LRIG1 mRNA (Fold Change)
WT1 mRNA (Fold Change)
OXTR mRNA (Fold Change)
TGM2 mRNA (Fold Change)
EXT1 mRNA (Fold Change)
CYGB mRNA (Fold Change)
siRNA
Ctrl
LRIG1
D
T2, 2 d
si Control
si MSI2
SM-164 (nM)
T2
MSI2 mRNA (Fold Change)

Fig S4

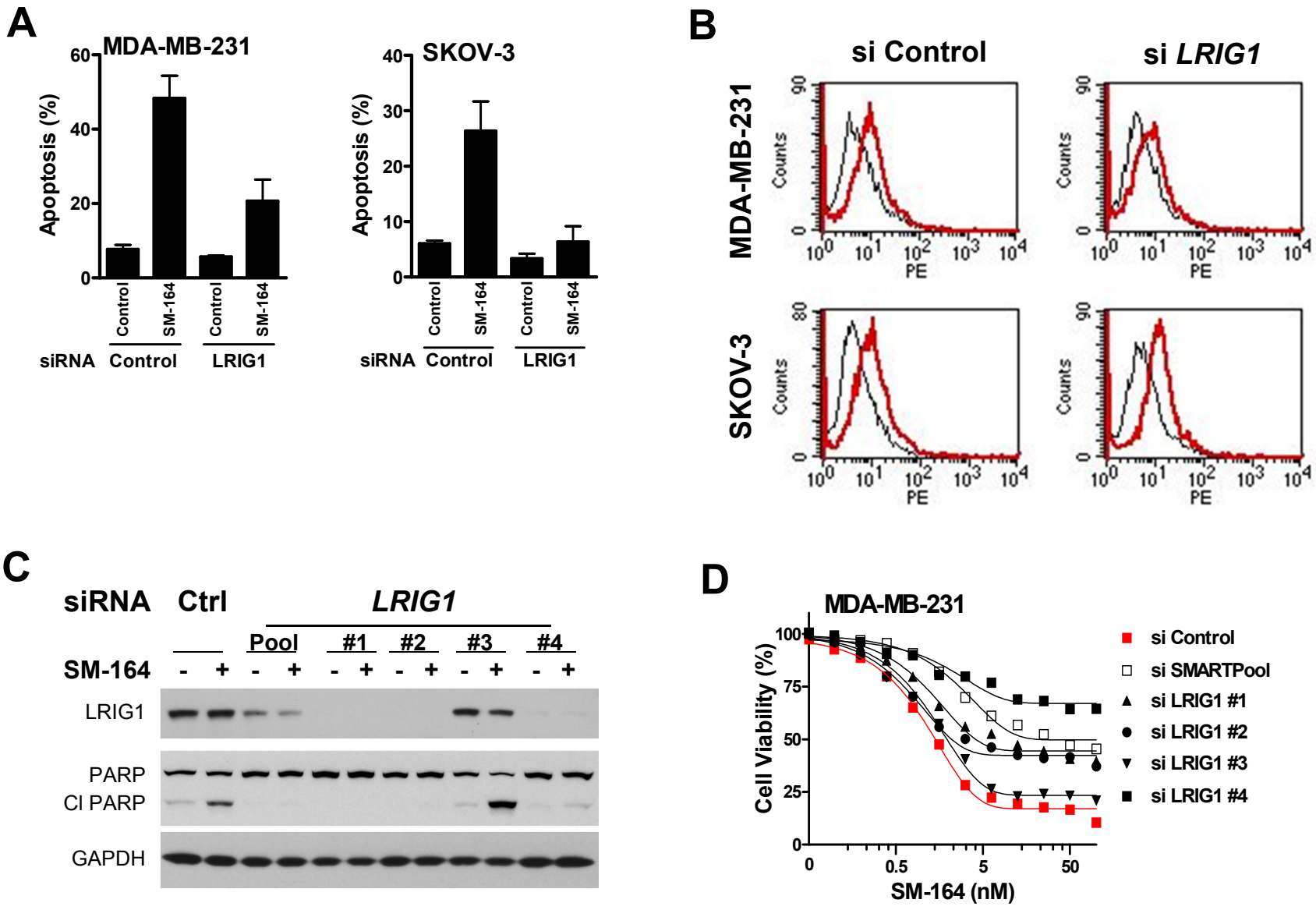

A
MDA-MB-231
Apoptosis (%)
60
40
20
0
Control
SM-164
Control
SM-164
siRNA Control
LRIG1
SKOV-3
Apoptosis (%)
40
30
20
10
0
Control
SM-164
Control
SM-164
siRNA Control
LRIG1
B
si Control
si LRIG1
MDA-MB-231
SKOV-3
Counts
PE
C
siRNA Ctrl
LRIG1
Pool
#1
#2
#3
#4
SM-164 - + - + - + - + - + - +
LRIG1
PARP
Cl PARP
GAPDH
D
MDA-MB-231
Cell Viability (%)
100
75
50
25
0
0
0.5
5
50
SM-164 (nM)
si Control
si SMARTPool
si LRIG1 #1
si LRIG1 #2
si LRIG1 #3
si LRIG1 #4

Fig S5

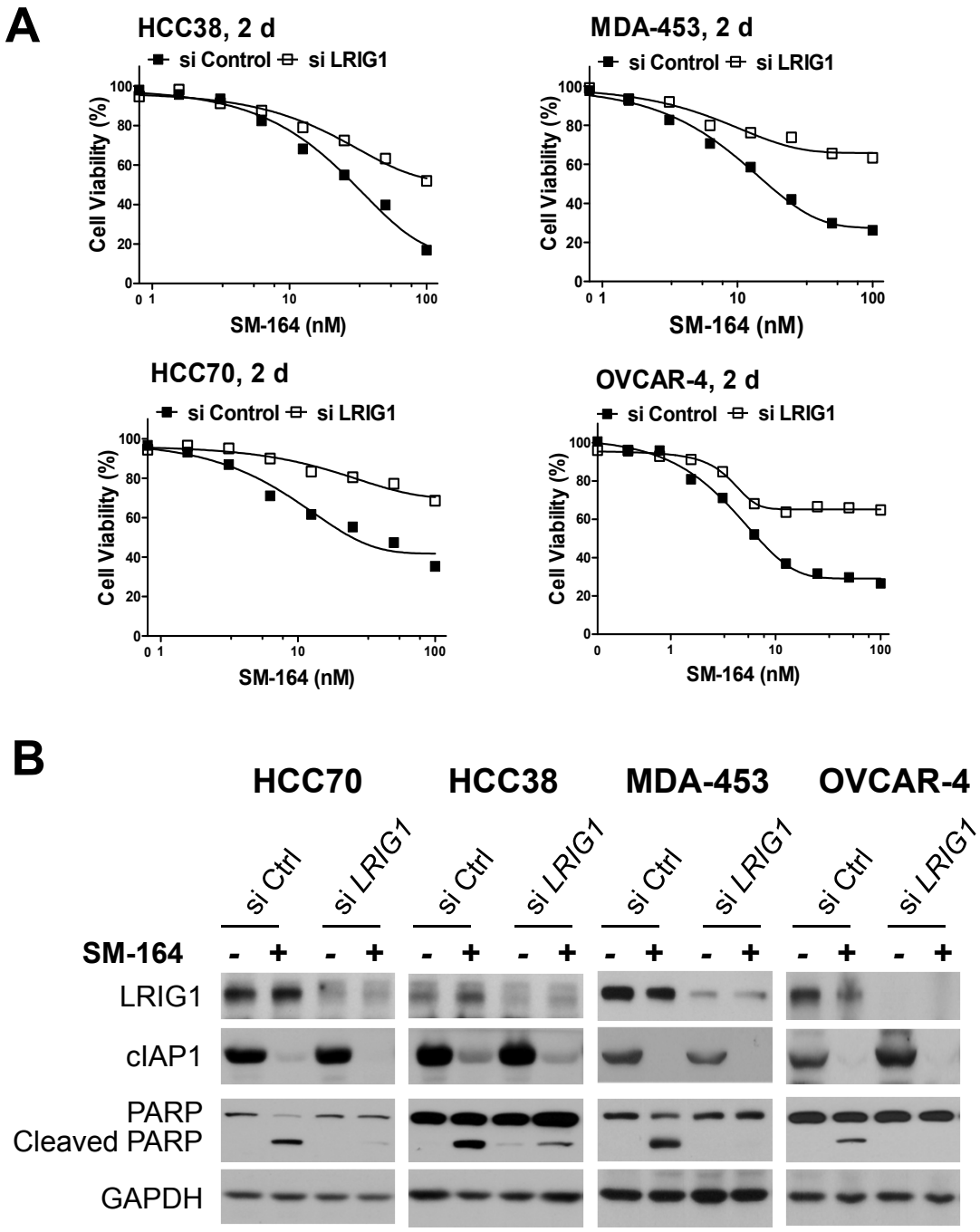

A
HCC38, 2 d
si Control
si LRIG1
Cell Viability (%)
SM-164 (nM)
MDA-453, 2 d
HCC70, 2 d
OVCAR-4, 2 d
B
HCC70
HCC38
MDA-453
OVCAR-4
si Ctrl
si LRIG1
SM-164
LRIG1
cIAP1
PARP
Cleaved PARP
GAPDH

Fig S6

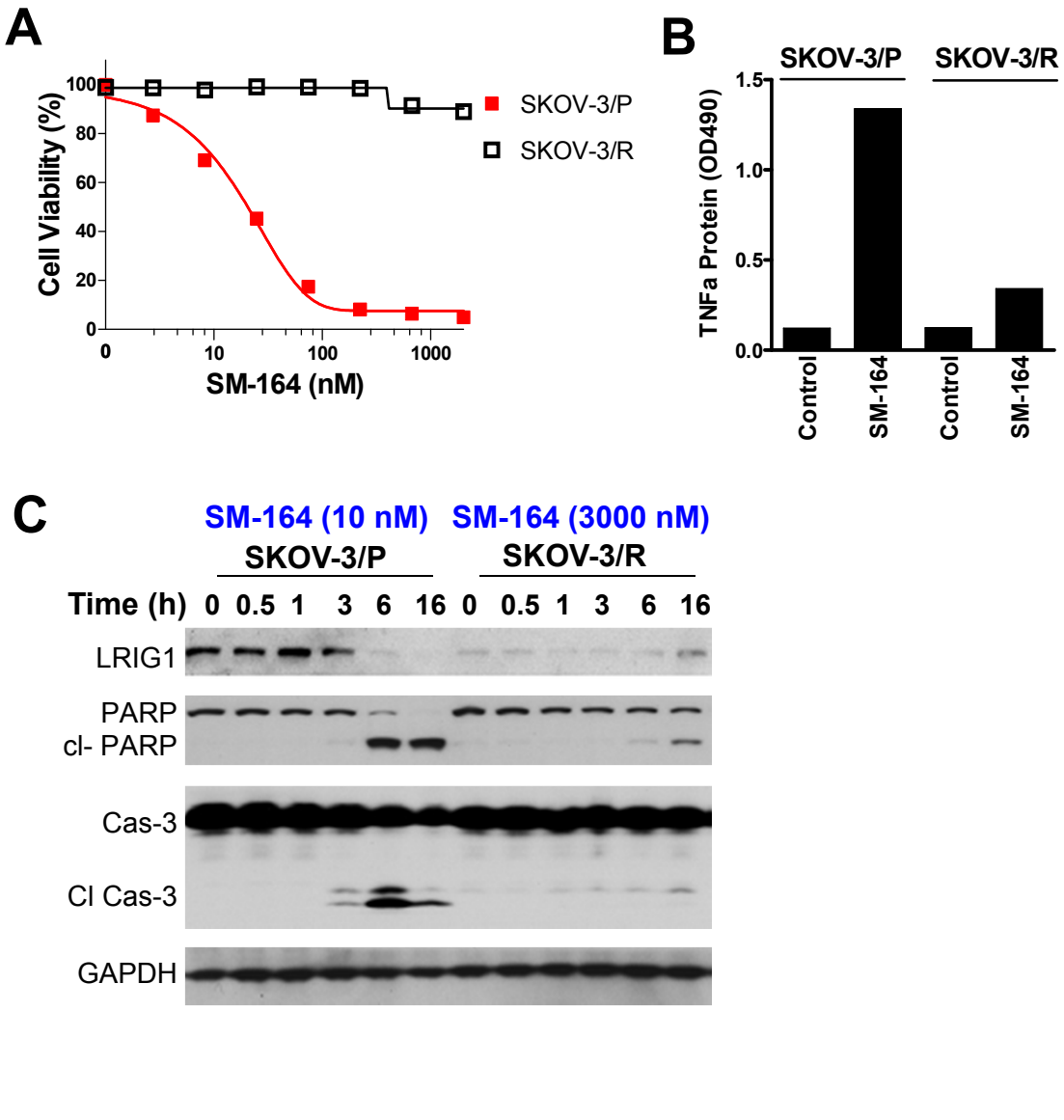

A
Cell Viability (%)
100
80
60
40
20
0
0
10
100
1000
SM-164 (nM)
SKOV-3/P
SKOV-3/R
B
SKOV-3/P
SKOV-3/R
TNFa Protein (OD490)
1.5
1.0
0.5
0.0
Control
SM-164
Control
SM-164
C
SM-164 (10 nM)
SM-164 (3000 nM)
SKOV-3/P
SKOV-3/R
Time (h) 0 0.5 1 3 6 16 0 0.5 1 3 6 16
LRIG1
PARP
cl- PARP
Cas-3
Cl Cas-3
GAPDH

Fig S7

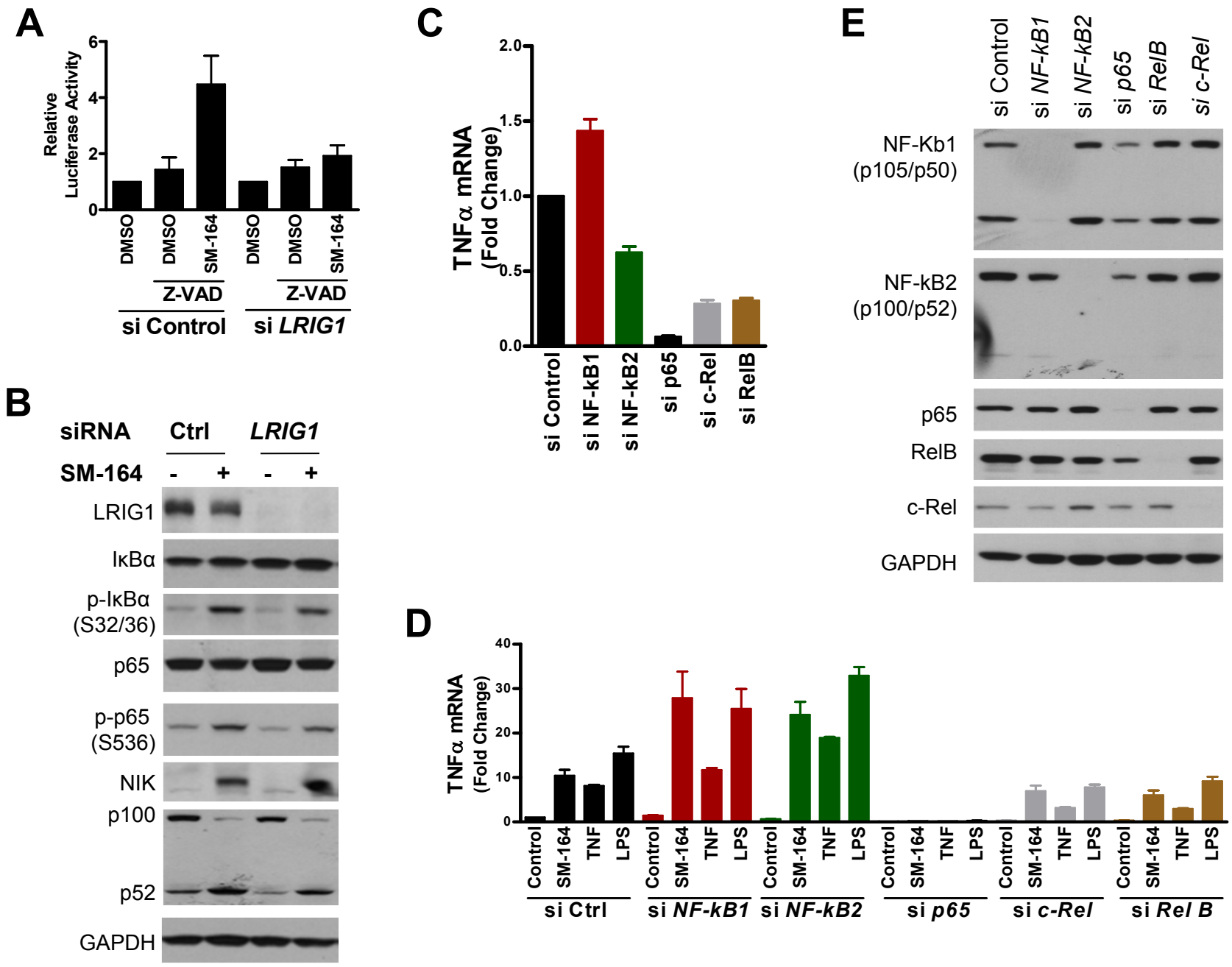

A
Relative Luciferase Activity
DMSO
DMSO
SM-164
DMSO
DMSO
SM-164
Z-VAD
Z-VAD
si Control
si LRIG1
B
siRNA
Ctrl
LRIG1
SM-164
LRIG1
IκBα
p-IκBα (S32/36)
p65
p-p65 (S536)
NIK
p100
p52
GAPDH
C
TNFα mRNA (Fold Change)
si Control
si NF-kB1
si NF-kB2
si p65
si c-Rel
si RelB
D
TNFα mRNA (Fold Change)
Control
SM-164
TNF
LPS
si Ctrl
si NF-kB1
si NF-kB2
si p65
si c-Rel
si Rel B
E
si Control
si NF-kB1
si NF-kB2
si p65
si RelB
si c-Rel
NF-Kb1 (p105/p50)
NF-kB2 (p100/p52)
p65
RelB
c-Rel
GAPDH

Fig S8

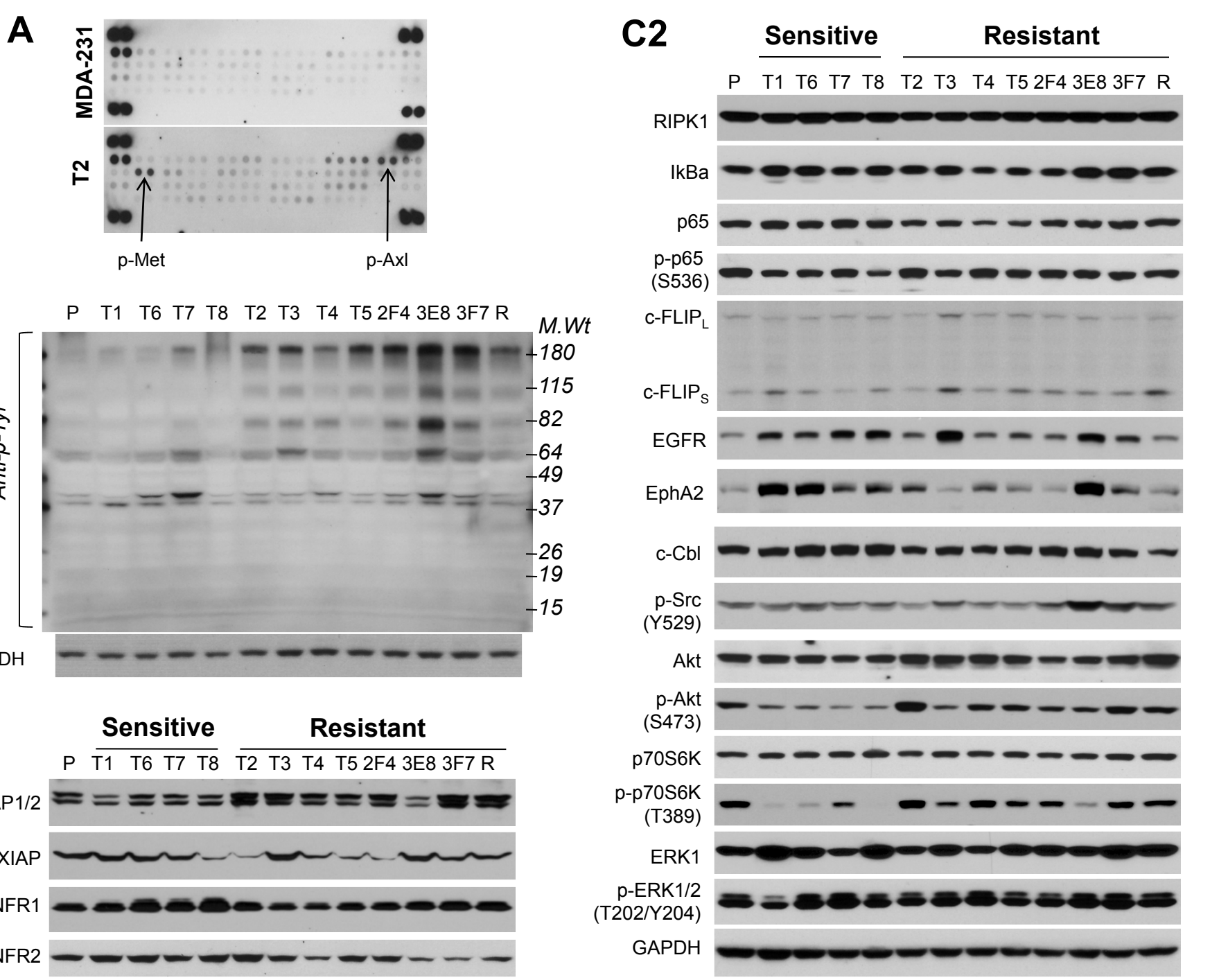

A
MDA-231
T2
p-Met
p-Axl
B
P T1 T6 T7 T8 T2 T3 T4 T5 2F4 3E8 3F7 R
M.Wt
180
115
82
64
49
37
26
19
15
Anti-p-Tyr
GAPDH
C1
Sensitive
Resistant
P T1 T6 T7 T8 T2 T3 T4 T5 2F4 3E8 3F7 R
cIAP1/2
XIAP
TNFR1
TNFR2
C2
Sensitive
Resistant
P T1 T6 T7 T8 T2 T3 T4 T5 2F4 3E8 3F7 R
RIPK1
IkBa
p65
p-p65 (S536)
c-FLIP$_L$
c-FLIP$_S$
EGFR
EphA2
c-Cbl
p-Src (Y529)
Akt
p-Akt (S473)
p70S6K
p-p70S6K (T389)
ERK1
p-ERK1/2 (T202/Y204)
GAPDH

Fig S9

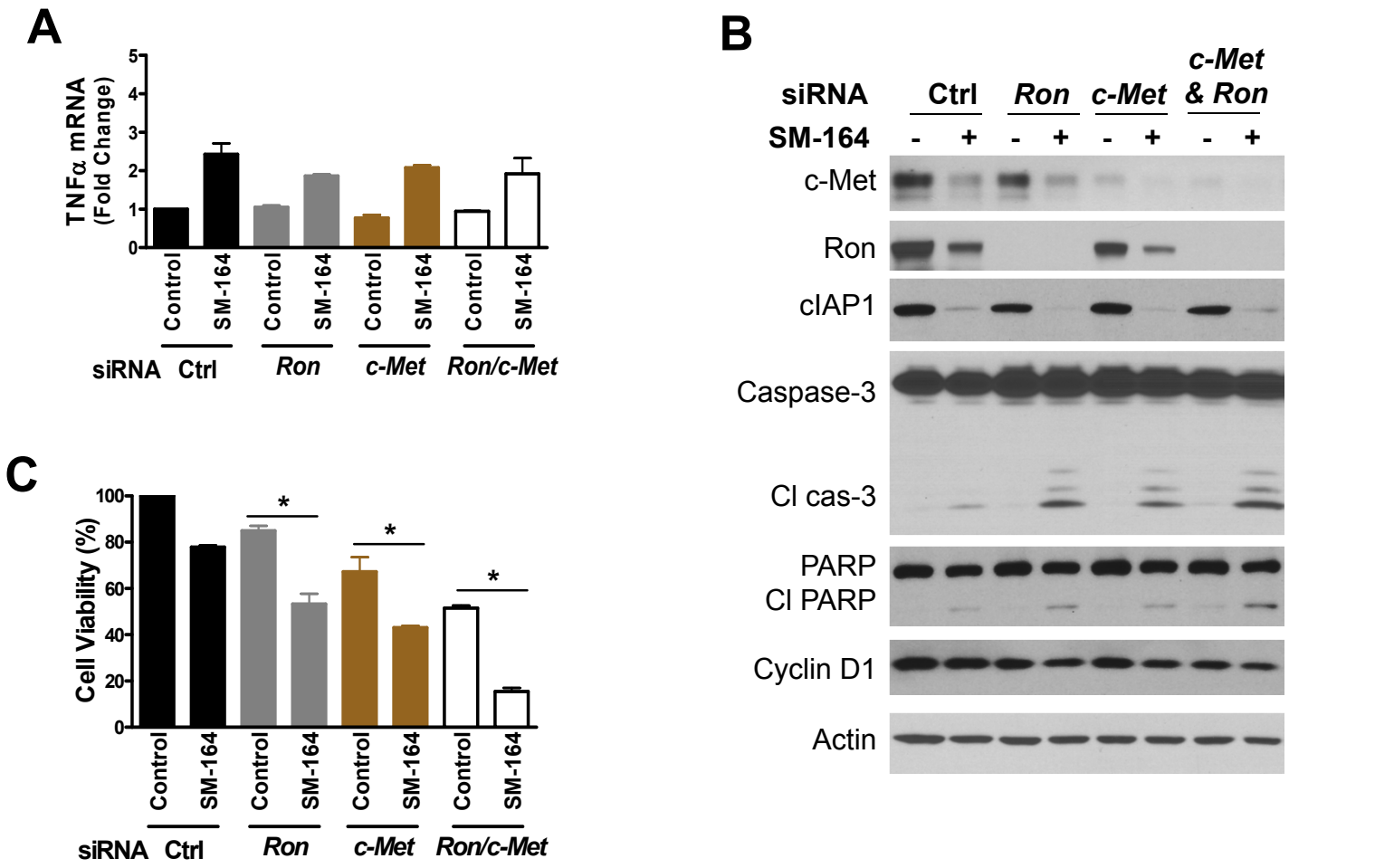

A
TNFα mRNA (Fold Change)
0
1
2
3
4
5
Control
SM-164
siRNA Ctrl
Ron
c-Met
Ron/c-Met
B
siRNA
Ctrl
Ron
c-Met
c-Met & Ron
SM-164
- + - + - + - +
c-Met
Ron
cIAP1
Caspase-3
Cl cas-3
PARP
Cl PARP
Cyclin D1
Actin
C
Cell Viability (%)
0
20
40
60
80
100
*
*
*
Control
SM-164
siRNA Ctrl
Ron
c-Met
Ron/c-Met

Fig S10

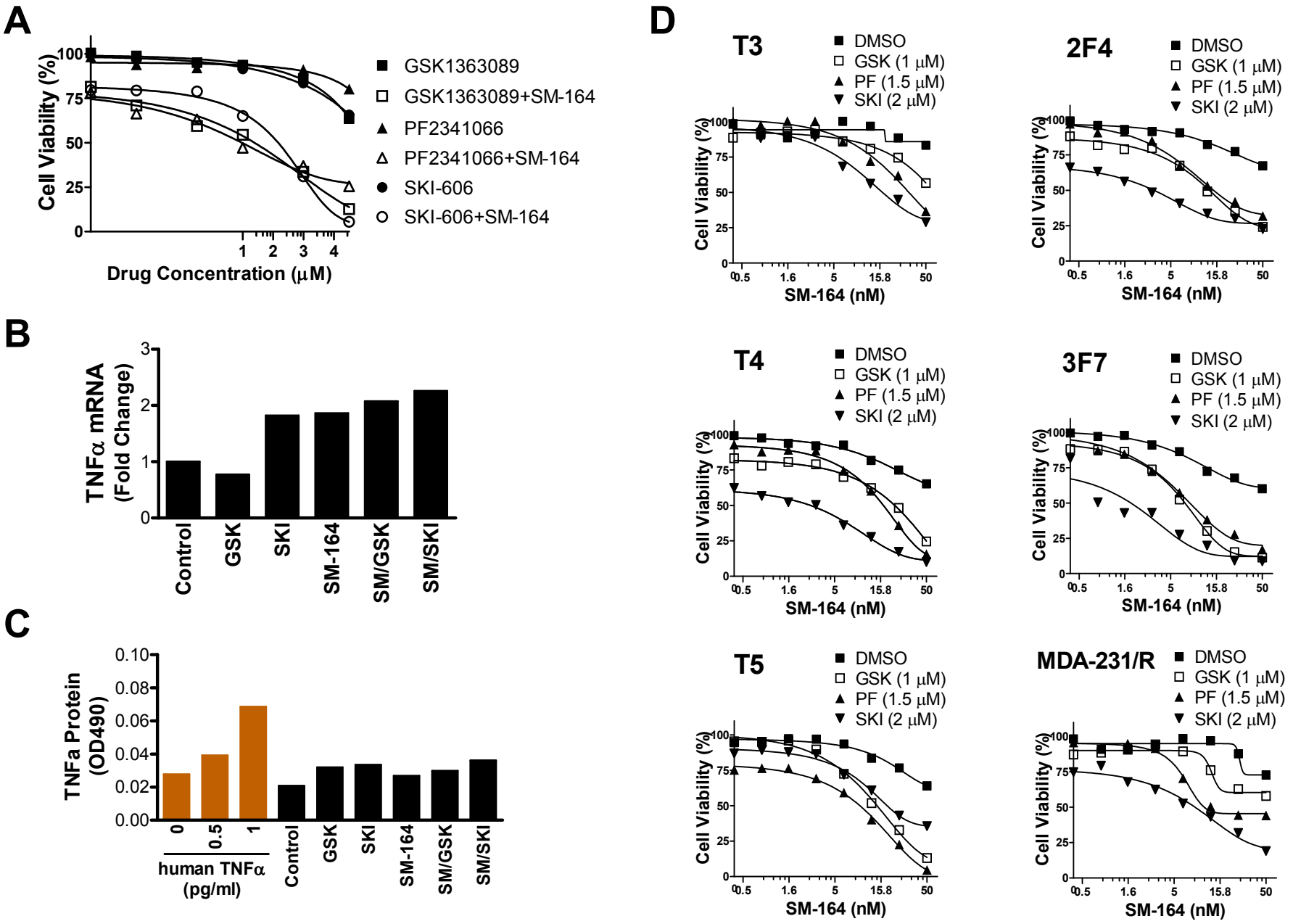
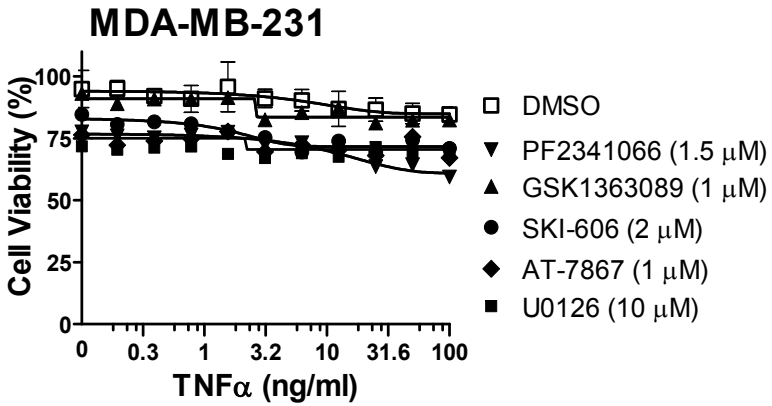
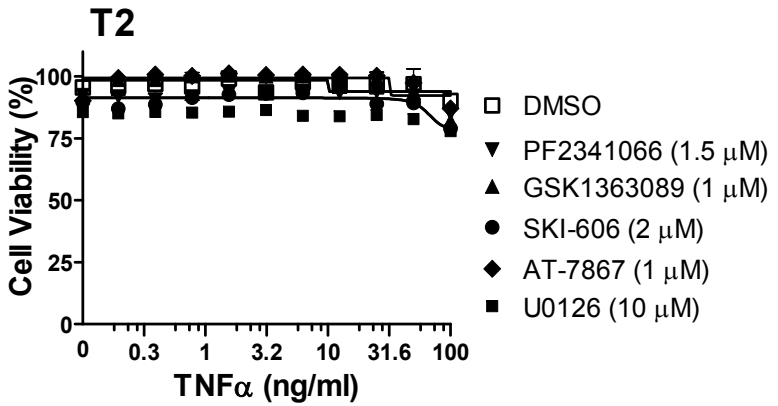
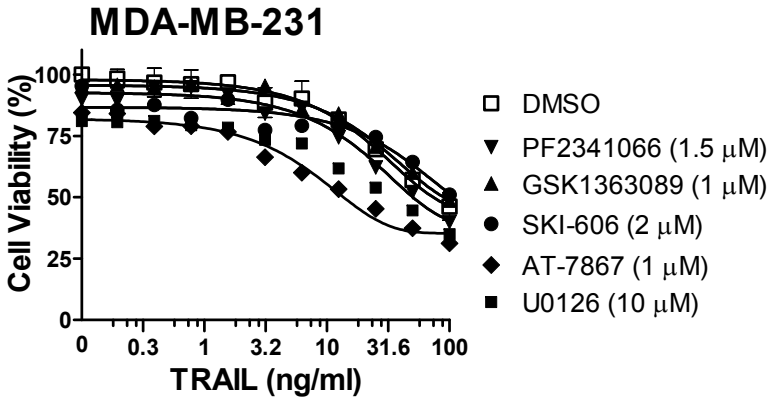

A
Cell Viability (%)
100
75
50
25
0
1 2 3 4
Drug Concentration (μM)
GSK1363089
GSK1363089+SM-164
PF2341066
PF2341066+SM-164
SKI-606
SKI-606+SM-164
B
TNFα mRNA (Fold Change)
3
2
1
0
Control
GSK
SKI
SM-164
SM/GSK
SM/SKI
C
TNFa Protein (OD490)
0.10
0.08
0.06
0.04
0.02
0.00
0
0.5
1
human TNFα (pg/ml)
Control
GSK
SKI
SM-164
SM/GSK
SM/SKI
D
T3
T4
T5
2F4
3F7
MDA-231/R
DMSO
GSK (1 μM)
PF (1.5 μM)
SKI (2 μM)
Cell Viability (%)
100
75
50
25
0
0.5
1.6
5
15.8
50
SM-164 (nM)

Fig S11


MDA-MB-231
Cell Viability (%)
100
75
50
25
0
0
0.3
1
3.2
10
31.6
100
TNFα (ng/ml)
DMSO
PF2341066 (1.5 μM)
GSK1363089 (1 μM)
SKI-606 (2 μM)
AT-7867 (1 μM)
U0126 (10 μM)


T2
Cell Viability (%)
100
75
50
25
0
0
0.3
1
3.2
10
31.6
100
TNFα (ng/ml)
DMSO
PF2341066 (1.5 μM)
GSK1363089 (1 μM)
SKI-606 (2 μM)
AT-7867 (1 μM)
U0126 (10 μM)


MDA-MB-231
Cell Viability (%)
100
75
50
25
0
0
0.3
1
3.2
10
31.6
100
TRAIL (ng/ml)
DMSO
PF2341066 (1.5 μM)
GSK1363089 (1 μM)
SKI-606 (2 μM)
AT-7867 (1 μM)
U0126 (10 μM)

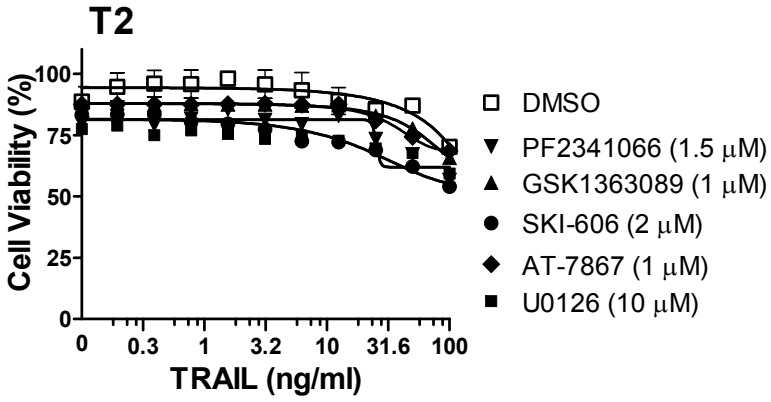
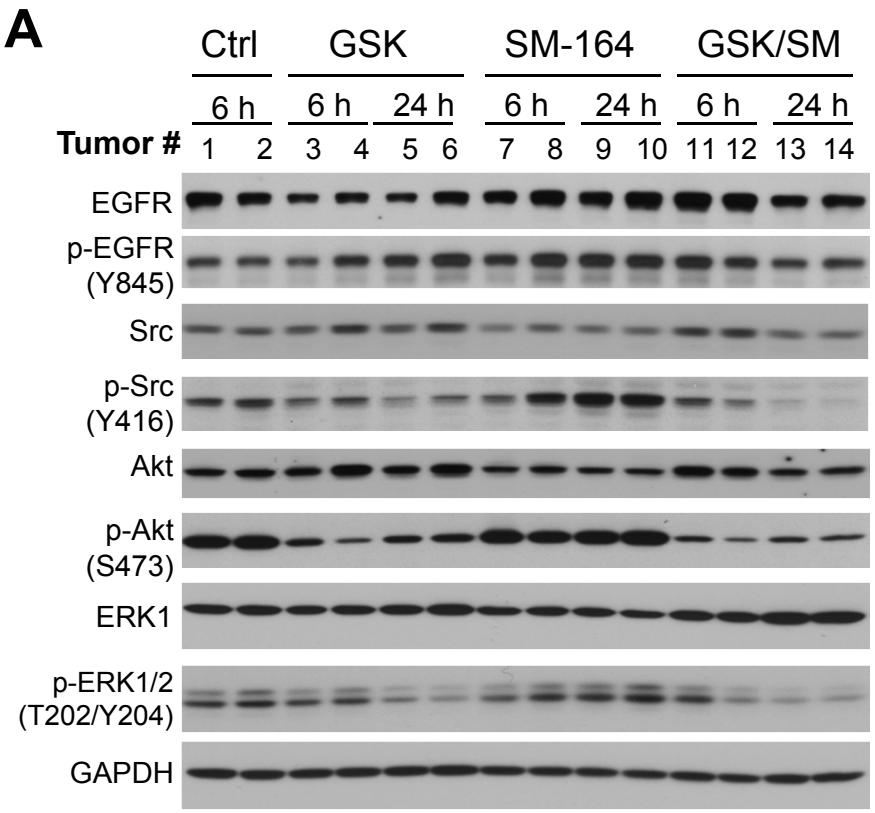

T2
Cell Viability (%)
100
75
50
25
0
0
0.3
1
3.2
10
31.6
100
TRAIL (ng/ml)
DMSO
PF2341066 (1.5 μM)
GSK1363089 (1 μM)
SKI-606 (2 μM)
AT-7867 (1 μM)
U0126 (10 μM)

Fig S12


A
Ctrl
GSK
SM-164
GSK/SM
6 h
6 h
24 h
6 h
24 h
6 h
24 h
Tumor #
1 2 3 4 5 6 7 8 9 10 11 12 13 14
EGFR
p-EGFR (Y845)
Src
p-Src (Y416)
Akt
p-Akt (S473)
ERK1
p-ERK1/2 (T202/Y204)
GAPDH

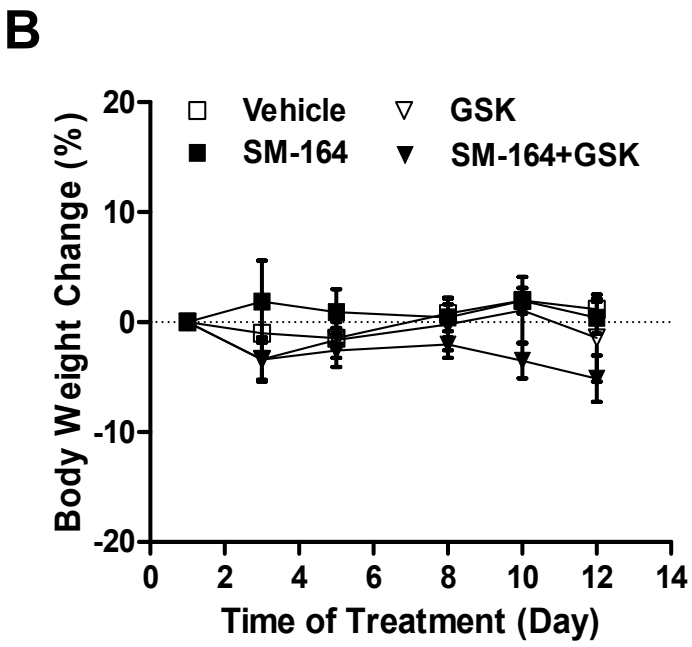

B
Vehicle
GSK
SM-164
SM-164+GSK
Body Weight Change (%)
20
10
0
-10
-20
0 2 4 6 8 10 12 14
Time of Treatment (Day)

Table S1. List of differentially expressed genes in the T2 and T3 resistant sublines compared with the T6 and T8 sensitive sublines. Genes listed have consistently increased or decreased expression ≥1.5-fold in resistant group (T2 and T3 sublines) versus sensitive group (T6 and T8 sublines) ($p < 0.05$).

Symbol	Gene Name	Fold Change
C6orf155	chromosome 6 open reading frame 155	-1.88
CYGB	cytoglobin	-2.50
EXT1	exostoses (multiple) 1	-1.72
LRIG1	leucine-rich repeats and immunoglobulin-like domains 1	-2.01
MSI2	musashi homolog 2 (Drosophila)	2.37
OXTR	oxytocin receptor	-2.00
PLCB4	phospholipase C, beta 4	5.13
SAA2	serum amyloid A2	-3.19
TGM2	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	-1.71
WT1	Wilms tumor 1	-1.82