SUPPLEMENTARY INFORMATION

SUPPLEMENTARY MATERIAL AND METHODS

Primer sequences

The primers sets used were as follows: c-IAP2 (forward, 5′-GTTGGACTCAGGTGTTGGGA-3′; reverse, 5′-CAAGTACTCACACCTTGGAAACCA-3′), c-FLIP (forward, 5'-CATCCACAGAATAGACCTGAAGACAA-3'; reverse, 5'-GCTTGGAGAACATTCCTGTAACTTG-3'), EEFA1 (forward, 5'-CGTTACAACGGAAGTAAAATC-3'; reverse, 5'-CAGGATAATCACCTGAGC-3') and Actin (forward, 5′-TACCTCATGAAGATCCTCACC-3′; reverse, 5′-TTTCGTGGATGCCACAGGAC-3′).

shRNA sequences

The shRNA sequences utilized were:

p65_shRNA#2_F:

5'CACCGGACATATGAGACCTTCAAGATTCAAGAGATCTTGAAGGTCTCATATGTCC 3';

p65_shRNA#2_R:

5'AAAAGGACATATGAGACCTTCAAGATCTCTTGAATCTTGAAGGTCTCATATGTCC3';

p65_shRNA#3_F:

5’CACCGTGACAAGGTGCAGAAAGATTCAAGAGATCTTTCTGCACCTTGTCAC3’;

p65_shRNA#3_R:

5’AAAAGTGACAAGGTGCAGAAAGATCTCTTGAATCTTTCTGCACCTTGTCAC3’.

Annexin V/PI staining. Melanoma cells were treated with DMSO, TNFα, PLX4720, GSK'436/GSK'212 (Selleck Chemicals Houston, TX, USA) alone or in combination for 48 hours. Cells were harvested, washed in PBS and resuspended in 100 μl binding buffer (10 mM HEPES, 140 mM sodium chloride, 2.5 mM calcium chloride). Next, cells were stained with 5 μl annexin V-APC (BD Biosciences) and 5 μl PI solution (1 mg/ml) for 15 min before the addition of 400 μl binding buffer. Staining was measured by flow cytometry on a FACS Calibur (BD Biosciences). Data were analyzed using Flowjo software (Three Star, Inc., Ashland, OR, USA).

SUPPLEMENTARY FIGURE LEGENDS

Fig. S1. TNFα effects on apoptosis induced by ERK1/2 pathway inhibitors and etoposide. a) M238, M229 and A375 cells were treated with DMSO, TNFα (50 ng/ml), PLX4720 (5 μM for M238 and M229, 10 μM for A375) as indicated for 48 hours and analyzed for apoptosis by Annexin V/PI staining. **b)** M229 cells were treated with -/+ 50 ng/ml TNFα and -/+ 5 μM PLX4720 for 48 hours. Cells were harvested, fixed and stained with PI for cell cycle analysis. Representative traces from one of three independent experiments are shown. Percentages of cells from different growth phases are shown below the traces. **c)** M238 cells were treated with DMSO, etoposide (10 μM), PLX4720 (5 μM) and the combination of GSK'436 (180 nM) and GSK'212 (2 nM) alone or with TNFα (50 ng/ml) for 48 hours and stained with Annexin V/PI for apoptosis analysis. A375 cells were treated similarly except that 10 μM PLX4720 were used and cells were harvested after 72 hours. Average percentages of Annexin V/PI-positive cells from three independent experiments were shown for each treatment condition. Error bars represents standard deviation. * $p<0.05$; ** $p<0.01$.

Fig. S2. Temporal activation of caspase 3, 8 and 9 following RAF inhibition. a) M238 cells were treated with 5 μM PLX4720 for indicated times and analyzed for

caspase 3, 8, 9 expression by Western blot. Cleaved caspase 3, 8, 9 levels were quantitated and normalized against actin. Relative expression levels are indicated. Arrows point to the full length or cleaved products of caspase 3, 8 and 9. **b)** M238 cells were transfected with caspase 8 specific siRNAs for 72 hours and treated with 50 ng/ml TNFα and 5 μM PLX4720, as indicated for another 24 hours. Cells were lysed for Western blot analysis.

Fig. S3. Effects of NFκB or c-FLIP knockdown on cell death induced by chemo-drugs. a) M229 or **b)** A375 cells were treated with indicated siRNAs for 72 hours and then treated with DMSO, 10 μM etoposide or 10 μM cisplatin individually for a further 48 hours. Cells were harvested and stained with PI for cell cycle analysis.

Fig. S4. The effect of TNFα on the expression of Bcl-2 family and IAPs family proteins. a) M238 cells were treated with -/+ 50 ng/ml TNFα and -/+ 5 μM PLX4720 for 6 or 24 hours before lysed for Western blot analysis on indicated proteins. **b**) M229 cells were treated with -/+ 50 ng/ml TNFα and -/+ 5 μM PLX4720 for 6 or 24 hours before lysis for Western blot analysis. Normalized c-FLIP/Actin band intensity ratios were shown below the corresponding blots. **c**) M229 cells were treated -/+ 50

ng/ml TNFα and -/+ 5 μM PLX4720 for 24 hours. Total RNAs were isolated from cells for qRT-PCR analysis on c-FLIP expression. ** $p<0.01$.

Fig. S5. Quantitation of c-FLIP up-regulation following TNFa and RAF inhibition with/without NFκB knockdown. a-d) Quantitation of relative band intensities for c-FLIP from Figure 3a, A375; Figure 3a, M238; Figure 3c, A375; and Figure 3c, M238, respectively. Shown is the average and standard deviation from two independent experiments. *, $p<0.05$.

Fig. S6. c-FLIP is required for TNFα-mediated protection against PLX4720 in A375 cells. A375 cells were treated -/+ 50 ng/ml TNFα and -/+ 10 μM PLX4720 for an additional 72 hours after siRNA transfection. Cells were then harvested for PI cell cycle analysis. Average percentages of sub-G1 cells from three independent experiments were shown for each knockdown condition. Error bars represent standard deviation. ** $p<0.01$.

Fig. S7. p65 expression in tumor samples. Three tumor samples for each shRNA construct (control shRNA, p65 shRNA#2, p65 shRNA#3) were recovered from sacrificed mice at the end point of the *in vivo* experiment (day 28). Tumor samples

were immediately lysed and analyzed for p65 expression by Western blot. Expression levels of p65 were quantitated and normalized against actin. Relative levels are indicated.

Fig. S8. Caspase 8 knockdown protects cells against PLX4720

Melanoma cells were treated with control or caspase 8 siRNAs for 72 hours and then treated with DMSO, 5 μM PLX4720 or 10 μM etoposide for another 48 hours. Cells were harvested for PI cell cycle analysis. Average percentages of sub-G1 cells from three independent experiments were shown. Error bars represent standard deviation. * $p<0.05$.

Fig. S1

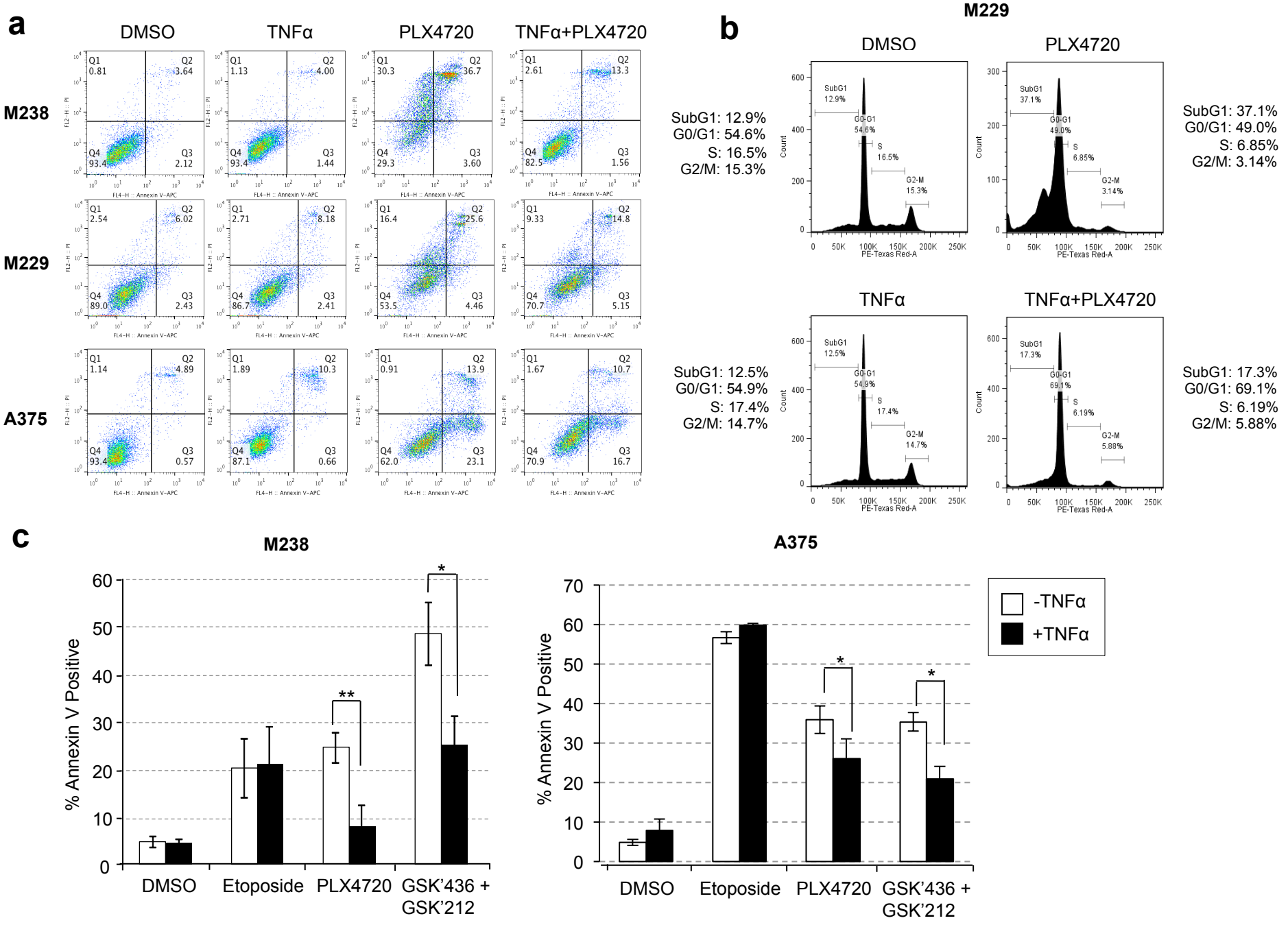

a
DMSO
TNFα
PLX4720
TNFα+PLX4720
M238
M229
A375
b
M229
DMSO
PLX4720
TNFα
TNFα+PLX4720
SubG1: 12.9%
G0/G1: 54.6%
S: 16.5%
G2/M: 15.3%
SubG1: 37.1%
G0/G1: 49.0%
S: 6.85%
G2/M: 3.14%
SubG1: 12.5%
G0/G1: 54.9%
S: 17.4%
G2/M: 14.7%
SubG1: 17.3%
G0/G1: 69.1%
S: 6.19%
G2/M: 5.88%
PE-Texas Red-A
Count
c
M238
A375
% Annexin V Positive
-TNFα
+TNFα
DMSO
Etoposide
PLX4720
GSK'436 + GSK'212

Fig. S2

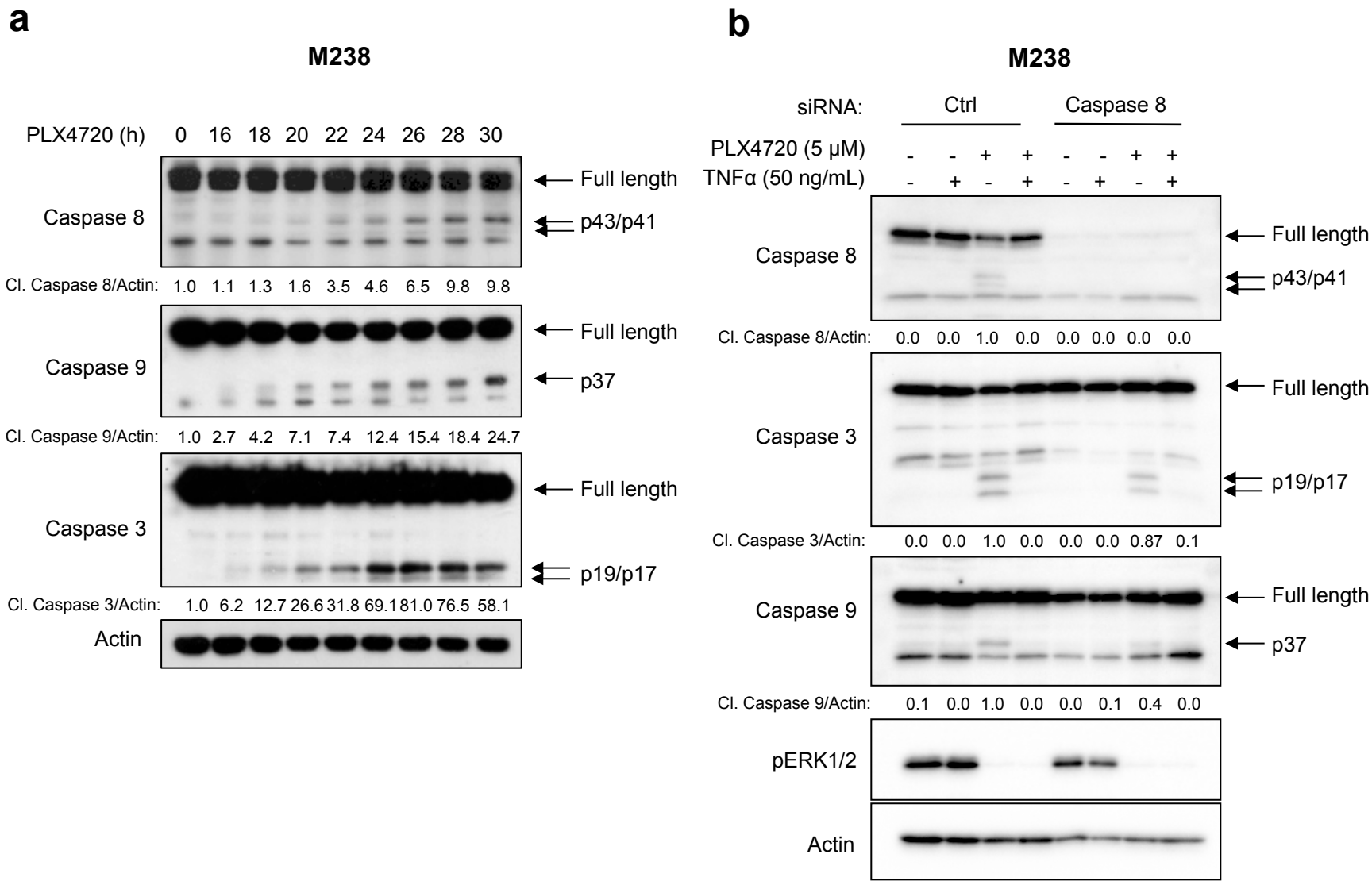

a
M238
PLX4720 (h) 0 16 18 20 22 24 26 28 30
Caspase 8
Full length
p43/p41
Cl. Caspase 8/Actin: 1.0 1.1 1.3 1.6 3.5 4.6 6.5 9.8 9.8
Caspase 9
Full length
p37
Cl. Caspase 9/Actin: 1.0 2.7 4.2 7.1 7.4 12.4 15.4 18.4 24.7
Caspase 3
Full length
p19/p17
Cl. Caspase 3/Actin: 1.0 6.2 12.7 26.6 31.8 69.1 81.0 76.5 58.1
Actin
b
M238
siRNA: Ctrl Caspase 8
PLX4720 (5 μM) - - + + - - + +
TNFα (50 ng/mL) - + - + - + - +
Caspase 8
Full length
p43/p41
Cl. Caspase 8/Actin: 0.0 0.0 1.0 0.0 0.0 0.0 0.0 0.0
Caspase 3
Full length
p19/p17
Cl. Caspase 3/Actin: 0.0 0.0 1.0 0.0 0.0 0.0 0.87 0.1
Caspase 9
Full length
p37
Cl. Caspase 9/Actin: 0.1 0.0 1.0 0.0 0.0 0.1 0.4 0.0
pERK1/2
Actin

Fig. S3

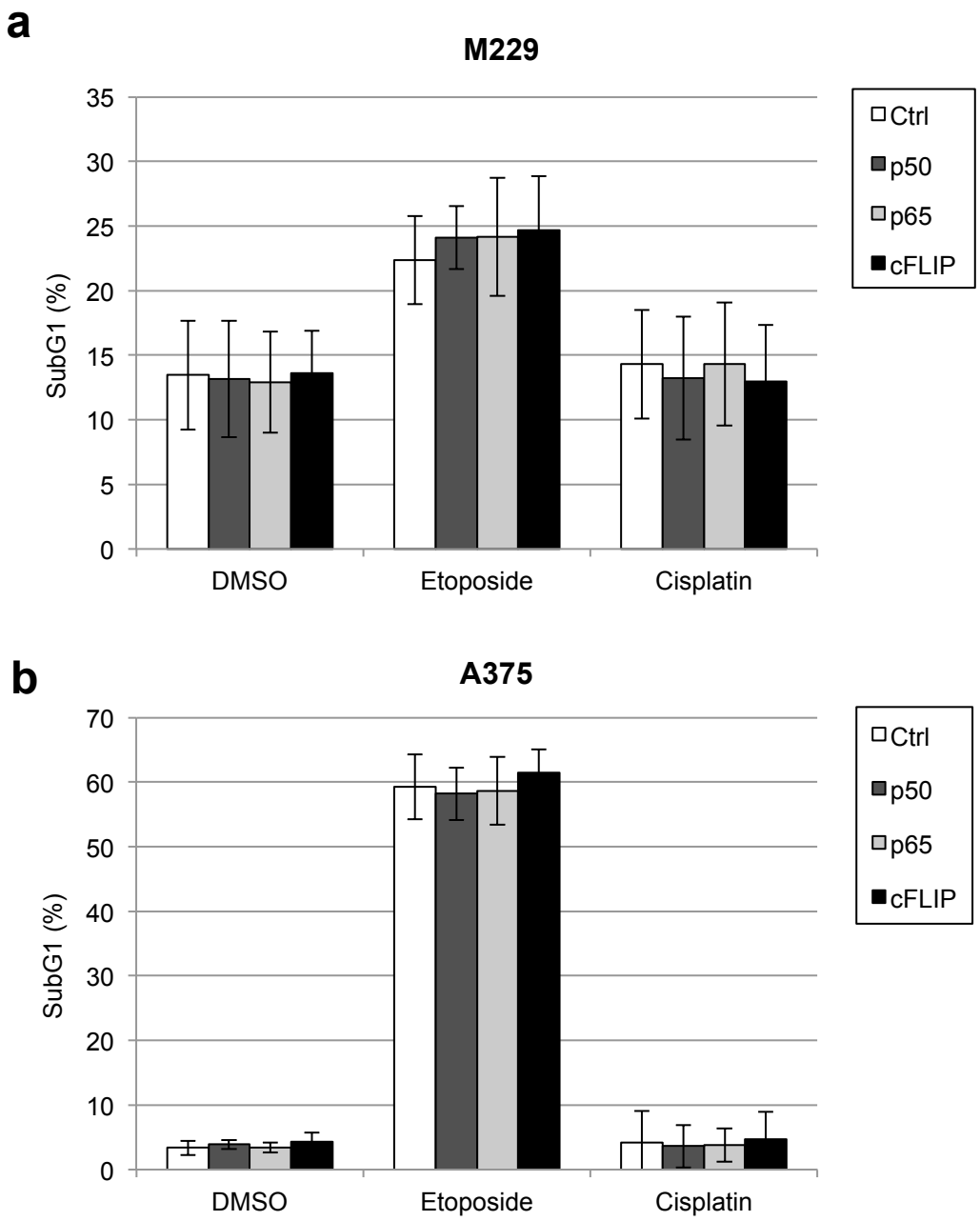

a
M229
SubG1 (%)
35
30
25
20
15
10
5
0
DMSO
Etoposide
Cisplatin
Ctrl
p50
p65
cFLIP
b
A375
SubG1 (%)
70
60
50
40
30
20
10
0
DMSO
Etoposide
Cisplatin
Ctrl
p50
p65
cFLIP

Fig. S4

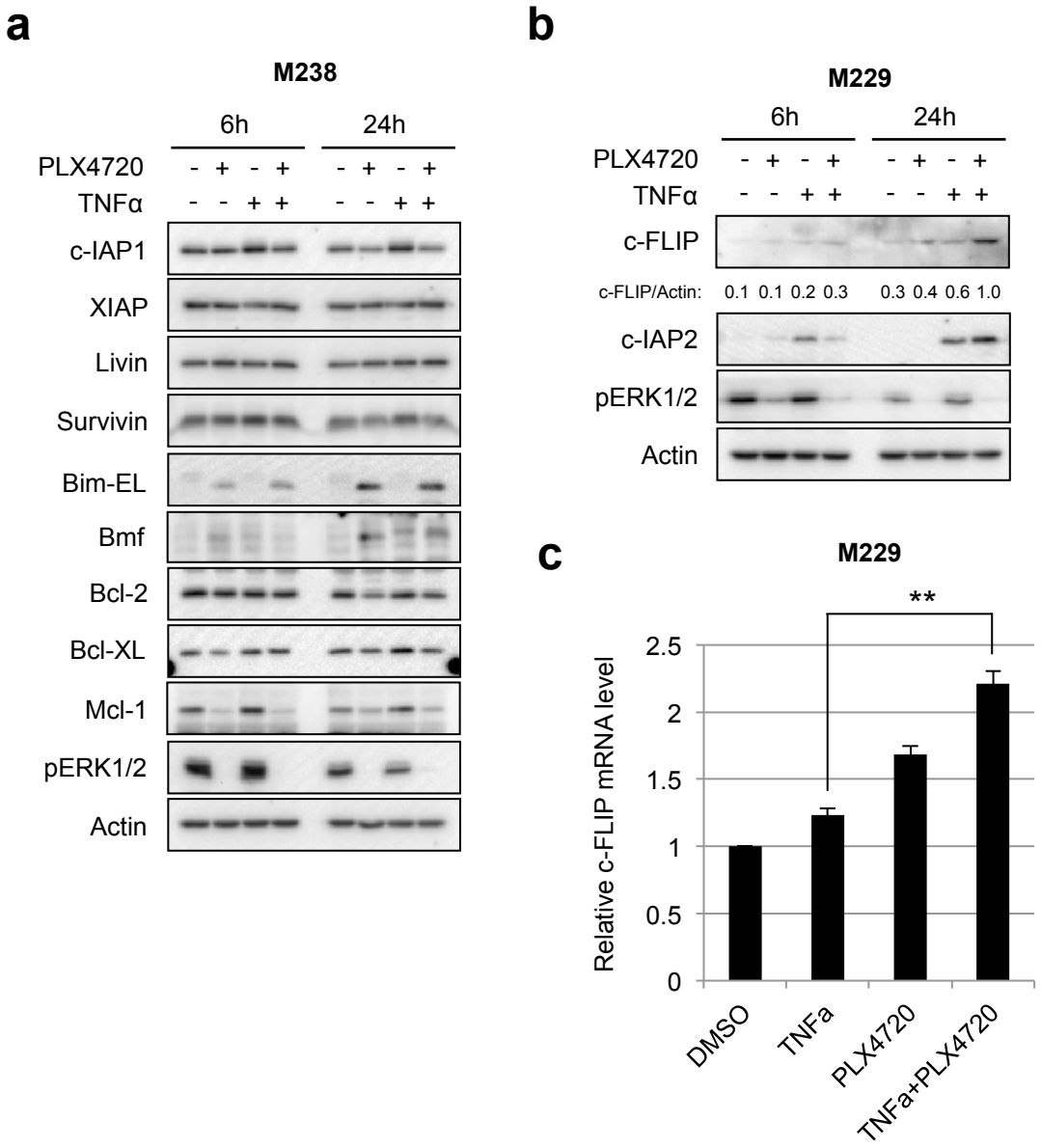

a
M238
6h
24h
PLX4720
TNFα
c-IAP1
XIAP
Livin
Survivin
Bim-EL
Bmf
Bcl-2
Bcl-XL
Mcl-1
pERK1/2
Actin
b
M229
6h
24h
PLX4720
TNFα
c-FLIP
c-FLIP/Actin: 0.1 0.1 0.2 0.3 0.3 0.4 0.6 1.0
c-IAP2
pERK1/2
Actin
c
M229
**
Relative c-FLIP mRNA level
2.5
2
1.5
1
0.5
0
DMSO
TNFa
PLX4720
TNFa+PLX4720

Fig. S5

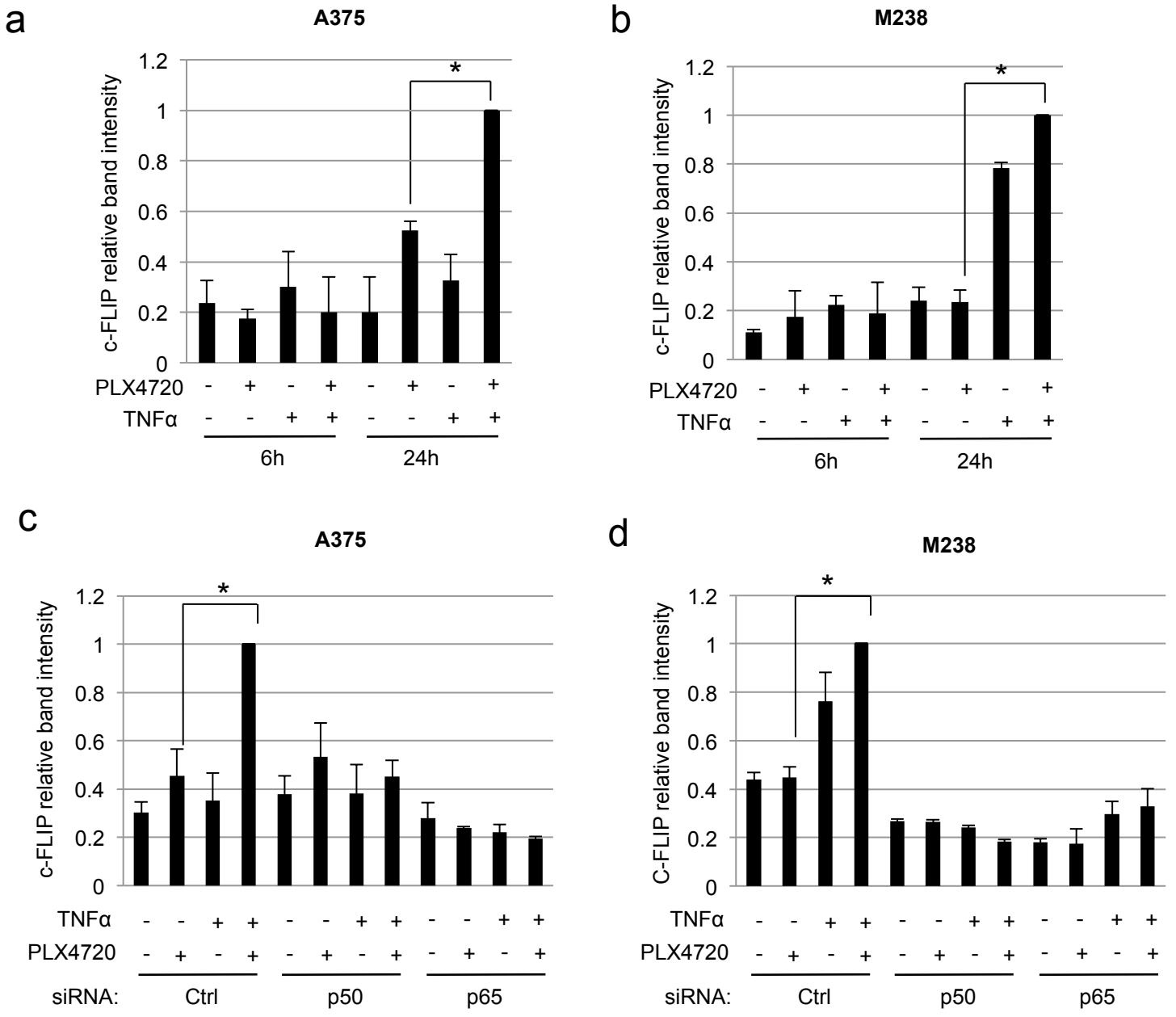

a
A375
c-FLIP relative band intensity
1.2
1
0.8
0.6
0.4
0.2
0
*
PLX4720 - + - + - + - +
TNFα - - + + - - + +
6h
24h
b
M238
c-FLIP relative band intensity
1.2
1
0.8
0.6
0.4
0.2
0
*
PLX4720 - + - + - + - +
TNFα - - + + - - + +
6h
24h
c
A375
c-FLIP relative band intensity
1.2
1
0.8
0.6
0.4
0.2
0
*
TNFα - - + + - - + + - - + +
PLX4720 - + - + - + - + - + - +
siRNA: Ctrl p50 p65
d
M238
C-FLIP relative band intensity
1.2
1
0.8
0.6
0.4
0.2
0
*
TNFα - - + + - - + + - - + +
PLX4720 - + - + - + - + - + - +
siRNA: Ctrl p50 p65

Fig. S6

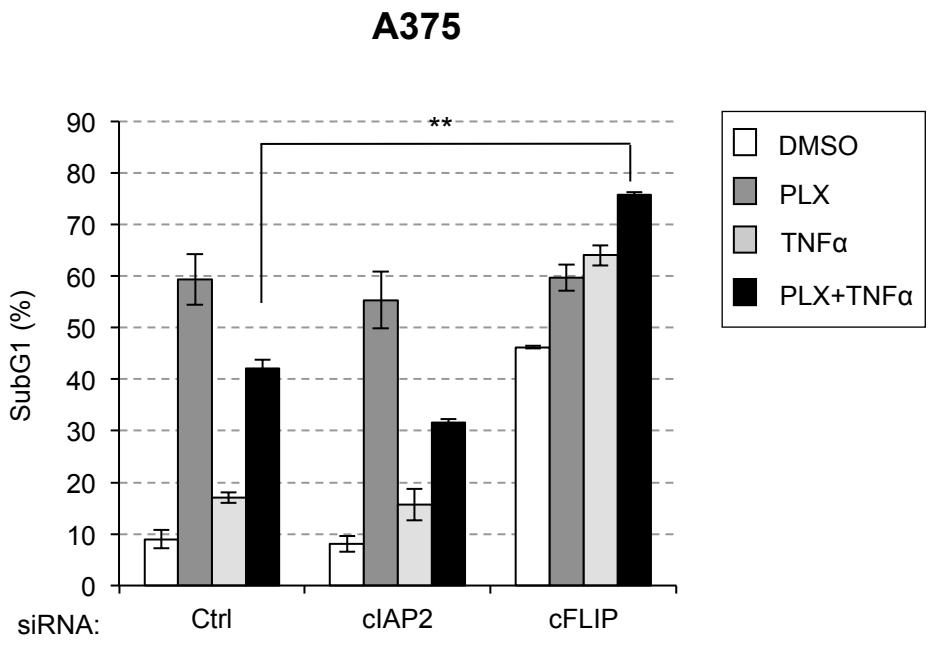

A375
SubG1 (%)
90
80
70
60
50
40
30
20
10
0
**
DMSO
PLX
TNFα
PLX+TNFα
siRNA:
Ctrl
cIAP2
cFLIP

Fig. S7

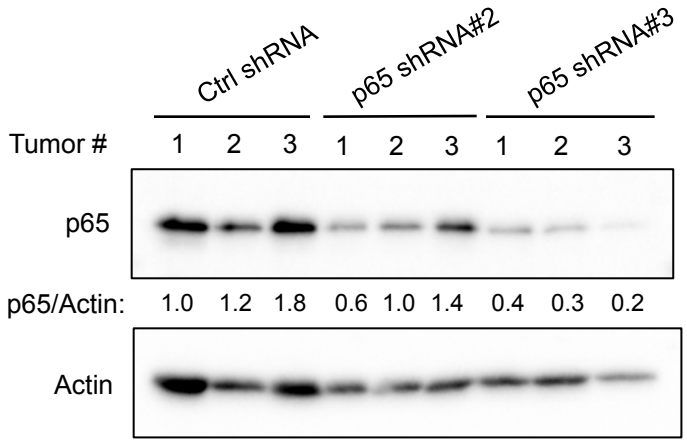
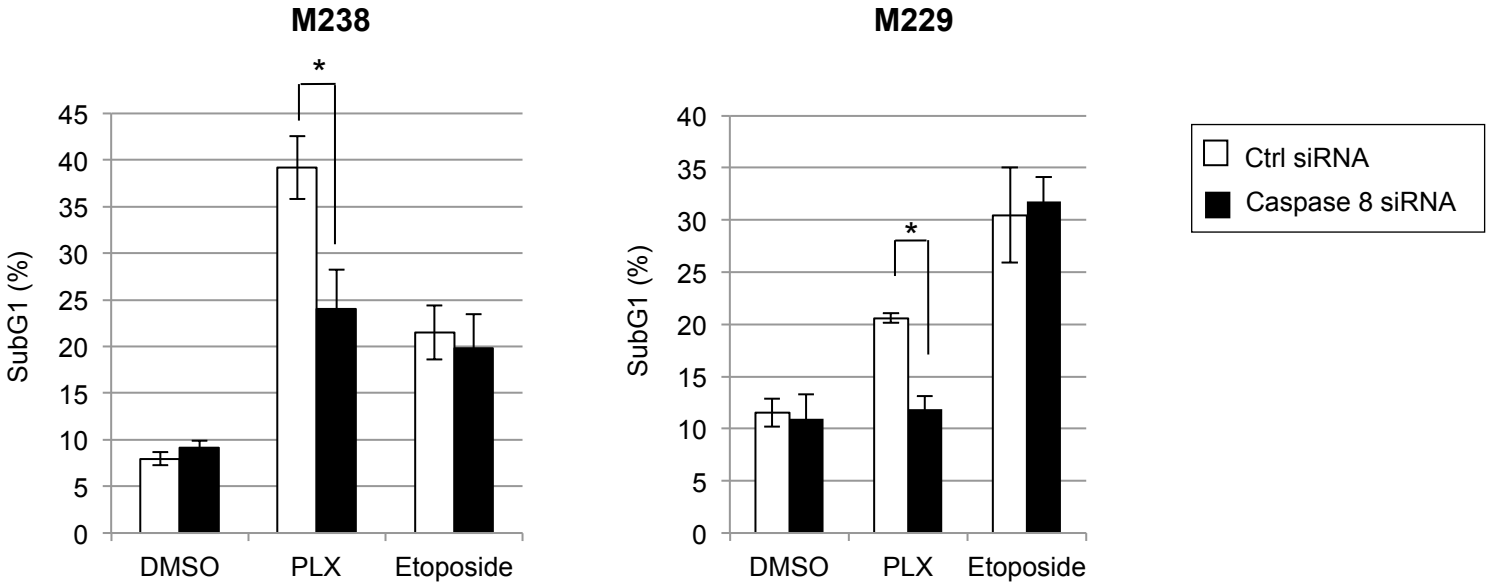

Ctrl shRNA
p65 shRNA#2
p65 shRNA#3
Tumor #
1 2 3 1 2 3 1 2 3
p65
p65/Actin:
1.0 1.2 1.8 0.6 1.0 1.4 0.4 0.3 0.2
Actin

Fig. S8


M238
M229
SubG1 (%)
DMSO
PLX
Etoposide
Ctrl siRNA
Caspase 8 siRNA
*