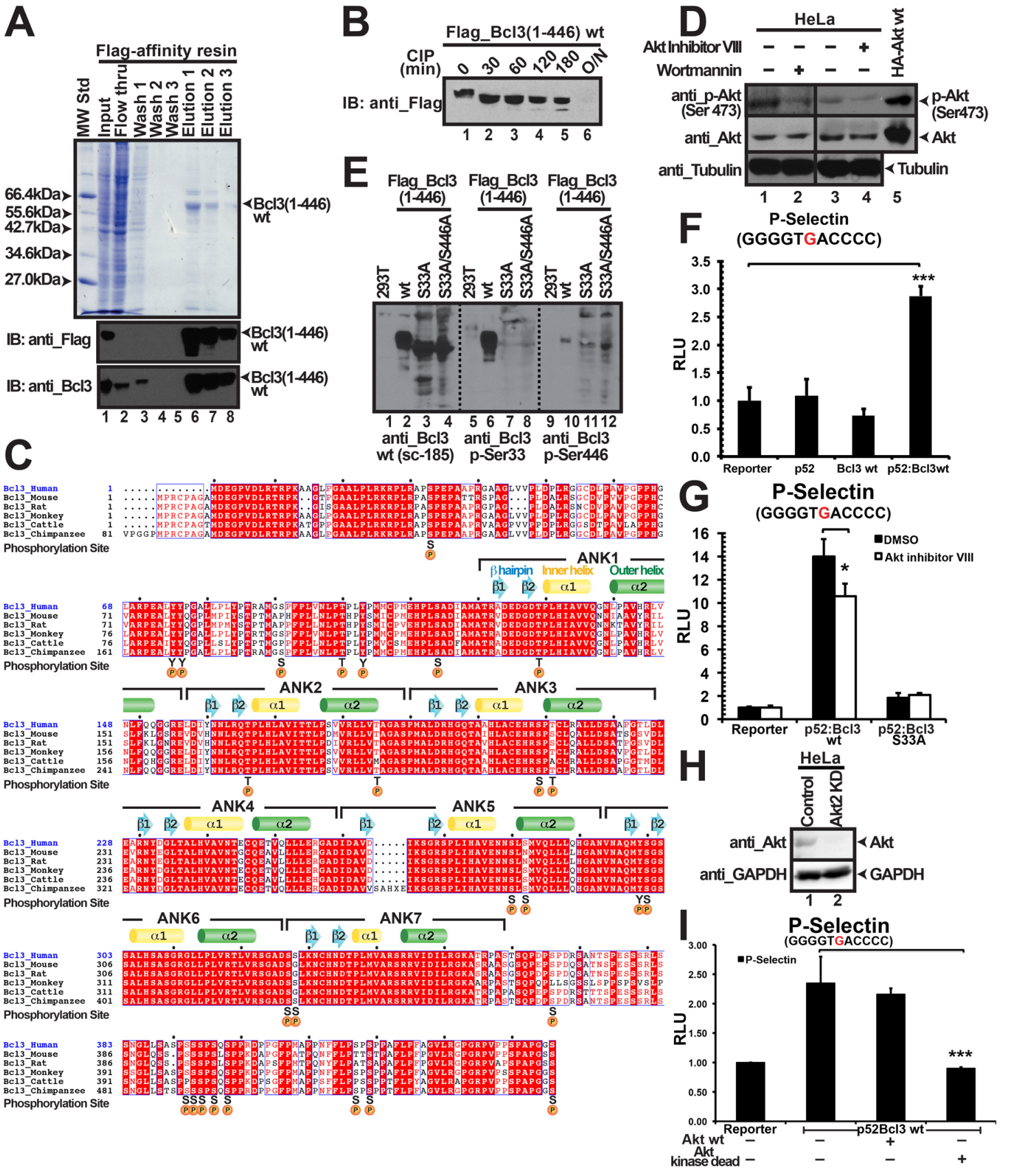

A
Flag-affinity resin
MW Std
Input
Flow thru
Wash 1
Wash 2
Wash 3
Elution 1
Elution 2
Elution 3
66.4kDa
55.6kDa
42.7kDa
34.6kDa
27.0kDa
Bcl3(1-446) wt
IB: anti_Flag
IB: anti_Bcl3
1 2 3 4 5 6 7 8
B
Flag_Bcl3(1-446) wt
CIP (min)
0 30 60 120 180 O/N
IB: anti_Flag
1 2 3 4 5 6
D
HeLa
Akt Inhibitor VIII
Wortmannin
HA-Akt wt
anti_p-Akt (Ser 473)
anti_Akt
anti_Tubulin
p-Akt (Ser473)
Akt
Tubulin
1 2 3 4 5
E
Flag_Bcl3 (1-446)
293T
wt
S33A
S33A/S446A
anti_Bcl3 wt (sc-185)
anti_Bcl3 p-Ser33
anti_Bcl3 p-Ser446
F
P-Selectin (GGGGTGACCCC)
RLU
Reporter
p52
Bcl3 wt
p52:Bcl3wt
***
C
Bcl3_Human
Bcl3_Mouse
Bcl3_Rat
Bcl3_Monkey
Bcl3_Cattle
Bcl3_Chimpanzee
Phosphorylation Site
ANK1
ANK2
ANK3
ANK4
ANK5
ANK6
ANK7
β hairpin
Inner helix
Outer helix
G
P-Selectin (GGGGTGACCCC)
DMSO
Akt inhibitor VIII
RLU
Reporter
p52:Bcl3 wt
p52:Bcl3 S33A
H
HeLa
Control
Akt2 KD
anti_Akt
anti_GAPDH
Akt
GAPDH
I
P-Selectin (GGGGTGACCCC)
P-Selectin
RLU
Reporter
p52Bcl3 wt
Akt wt
Akt kinase dead

Figure S1. Bcl3 is a phosphorylated protein and targeted by Akt. Related to Figure 1

(A) Full-length Flag-tagged Bcl3(1-446) was expressed and purified from HEK 293T cells using Flag affinity chromatography. 12.5% SDS-PAGE analysis of eluates from the Flag affinity resin. Coomssie staining showing purity of Flag-Bcl3 (Top) and Western blots (WB) using both Flag and Bcl3 antibodies showing identity of the protein. (B) CIP dephosphorylation of Flag-Bcl3(1-446). 3 μg of purified Flag-tagged Bcl3 was treated with CIP at room temperature for indicated times followed by anti-Flag WB analysis. (C) Sequence alignment of Bcl3. 27 newly identified phosphorylation sites are indicated bellow the sequences; the secondary-structural elements of ARD are shown above the sequences; black dots above the sequences indicate every tenth residue in the sequence of human Bcl3. (D) Both wortmannin and Akt Inhibitor VIII inhibited Akt kinase activation in HeLa cells as marked by reduction of activation loop residue Ser473 phosphorylation. HeLa cells were left untreated (lane 1 and 3) or treated with 0.5 μM wortmannin (lane 2) or 5 μM Akt Inhibitor VIII (lane 4) for 48 hours. Cell extracts were subjected to anti-p-Akt(Ser473) and anti-Akt WB analysis. HA-tagged wt Akt was transiently transfected into HeLa cells used as a control for Akt expression and constitutive activation (lane 5). (E) WB showing the specificity of the phosphor-Bcl3 antibodies. p-Ser33 antibody only recognized wt Bcl3 expressed in HEK 293T cells, but not S33A or S33A/S446A mutant. (F) p52 or Bcl3 alone failed to activate luciferase reporter driven by the P-Selectin promoter. Co-expression of p52 and Bcl3 however activated the reporter. (G) Akt inhibitor VIII decreased wt Bcl3's transcriptional activity on P-Selectin reporter; however, it has no effect on S33A mutant. (H) Efficiency of shRNA mediated Akt2 knock down (KD) in HeLa cells. (I) Overexpressed kinase dead Akt, but not wt Akt, abolished Bcl3's transcriptional activity from the P-Selectin luciferase reporter. All luciferase reporter data were analyzed from three independent experiments performed in triplicate. *$p<0.05$, ***$p<0.005$. Error bar represent SD.

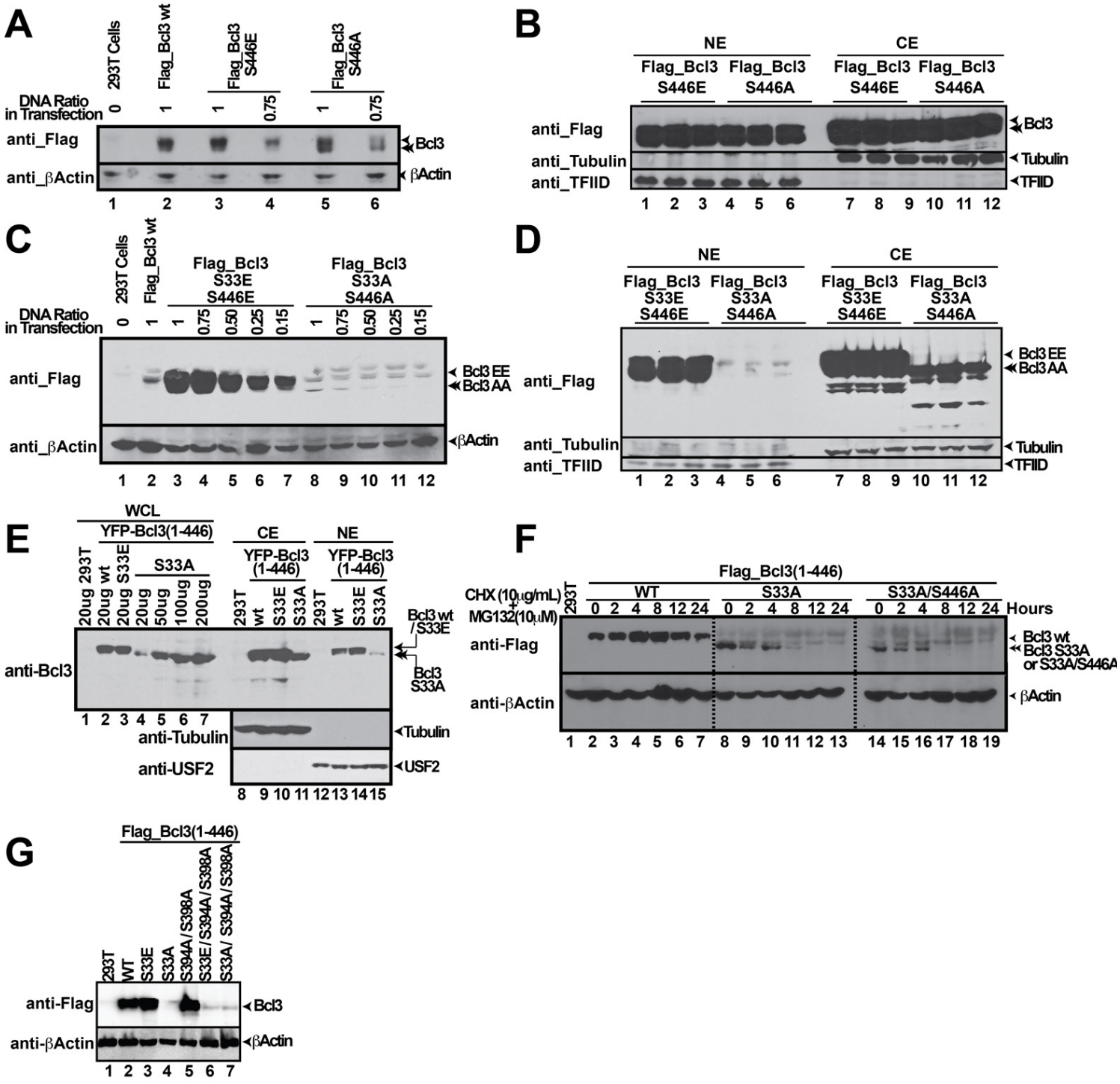

A
293T Cells
Flag_Bcl3 wt
Flag_Bcl3 S446E
Flag_Bcl3 S446A
DNA Ratio in Transfection
0
1
1
0.75
1
0.75
anti_Flag
anti_βActin
Bcl3
βActin
1 2 3 4 5 6
B
NE
CE
Flag_Bcl3 S446E
Flag_Bcl3 S446A
Flag_Bcl3 S446E
Flag_Bcl3 S446A
anti_Flag
anti_Tubulin
anti_TFIID
Bcl3
Tubulin
TFIID
1 2 3 4 5 6 7 8 9 10 11 12
C
293T Cells
Flag_Bcl3 wt
Flag_Bcl3 S33E S446E
Flag_Bcl3 S33A S446A
DNA Ratio in Transfection
0
1
1 0.75 0.50 0.25 0.15
1 0.75 0.50 0.25 0.15
anti_Flag
anti_βActin
Bcl3 EE
Bcl3 AA
βActin
1 2 3 4 5 6 7 8 9 10 11 12
D
NE
CE
Flag_Bcl3 S33E S446E
Flag_Bcl3 S33A S446A
Flag_Bcl3 S33E S446E
Flag_Bcl3 S33A S446A
anti_Flag
anti_Tubulin
anti_TFIID
Bcl3 EE
Bcl3 AA
Tubulin
TFIID
1 2 3 4 5 6 7 8 9 10 11 12
E
WCL
YFP-Bcl3(1-446)
CE
NE
YFP-Bcl3 (1-446)
YFP-Bcl3 (1-446)
293T
20ug wt
20ug S33E
S33A
20ug
50ug
100ug
200ug
293T
wt
S33E
S33A
293T
wt
S33E
S33A
anti-Bcl3
Bcl3 wt /S33E
Bcl3 S33A
1 2 3 4 5 6 7
anti-Tubulin
Tubulin
anti-USF2
USF2
8 9 10 11 12 13 14 15
F
Flag_Bcl3(1-446)
CHX (10μg/mL) + MG132(10μM)
293T
WT
S33A
S33A/S446A
0 2 4 8 12 24 0 2 4 8 12 24 0 2 4 8 12 24 Hours
anti-Flag
anti-βActin
Bcl3 wt
Bcl3 S33A or S33A/S446A
βActin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
G
Flag_Bcl3(1-446)
293T
WT
S33E
S33A
S394A / S398A
S33E / S394A / S398A
S33A / S394A / S398A
anti-Flag
anti-βActin
Bcl3
βActin
1 2 3 4 5 6 7

Figure S2. Protein Expressions, Cellular Localizations and Stabilities of Bcl3 Mutants. Related to Figure 2

WB monitoring total (A) and sub-compartment (B) expression wt Bcl3 wt, S446E and S446A. (C) Comparison of expression levels of wt Bcl3, S33E/S446E (EE) and S33A/S446A (AA) mutant. Different amounts of DNA were used for the transfection in HEK 293T cells in order to monitor the expression levels. (D) Nuclear and cytoplasmic expressions of Bcl3 EE and AA mutants. (E) YFP-tagged Bcl3 expression and cellular localization. (F) Flag-tagged Bcl3 wt, S33A, and AA mutants were transiently transfected into HEK 293T cells followed by CHX and MG132 treatments for indicated times. S33A and AA mutants were partially stabilized by MG132 treatment. (G) Mutations of GSK3β mediated phosphorylation, S394A and S398A, did not stabilize Bcl3 when expressed along with S33 mutations.

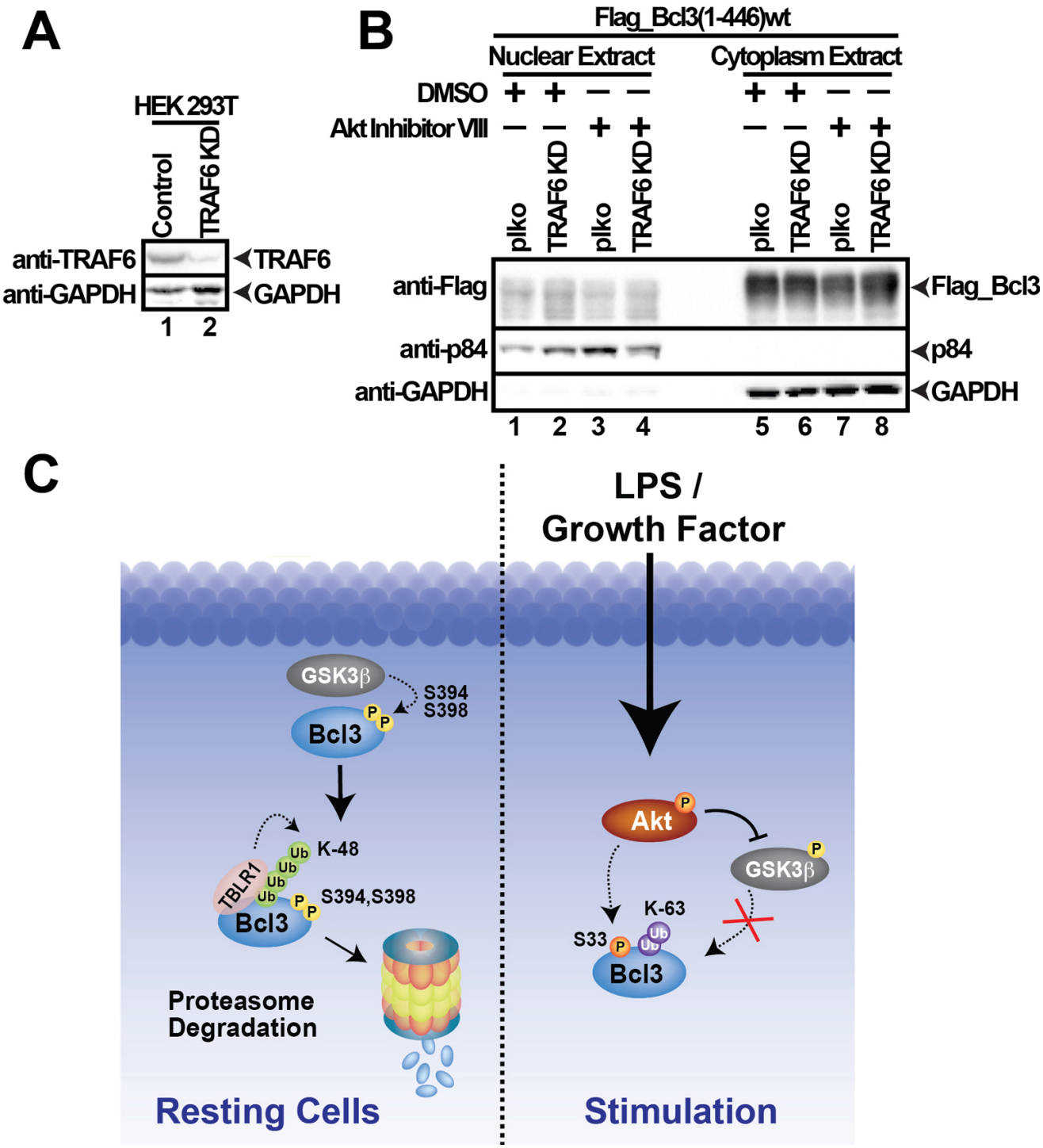

A
HEK 293T
Control
TRAF6 KD
anti-TRAF6
anti-GAPDH
TRAF6
GAPDH
1 2
B
Flag_Bcl3(1-446)wt
Nuclear Extract
Cytoplasm Extract
DMSO
Akt Inhibitor VIII
plko
TRAF6 KD
anti-Flag
anti-p84
anti-GAPDH
Flag_Bcl3
p84
GAPDH
1 2 3 4 5 6 7 8
C
LPS /
Growth Factor
GSK3β
S394
S398
Bcl3
K-48
Ub
TBLR1
S394,S398
Proteasome
Degradation
Resting Cells
Akt
P
K-63
S33
Stimulation

Figure S3. Model of Bcl3 Stabilization in Stimulating Cells. Related to Figure 1, 2 and 3

(A) Efficiency of TRAF6 KD in HEK 293T cells monitored by WB using TRAF6 specific antibody. (B) In TRAF6 KD HEK 293T cells, nuclear localization of transiently expressed Flag_Bcl3 was not affeted upon Akt Inhibitor VIII treatment. (C) In resting cells, Bcl3 is phosphorylated at Ser394 and Ser398 by GSK3β and this leads to its basal degradation. Upon stimulation, Akt gets activated which will in turn stabilize Bcl3 by directly phosphorylate Bcl3 at Ser33; and also phosphorylate GSK3β resulting in the deactivation of GSK3β.

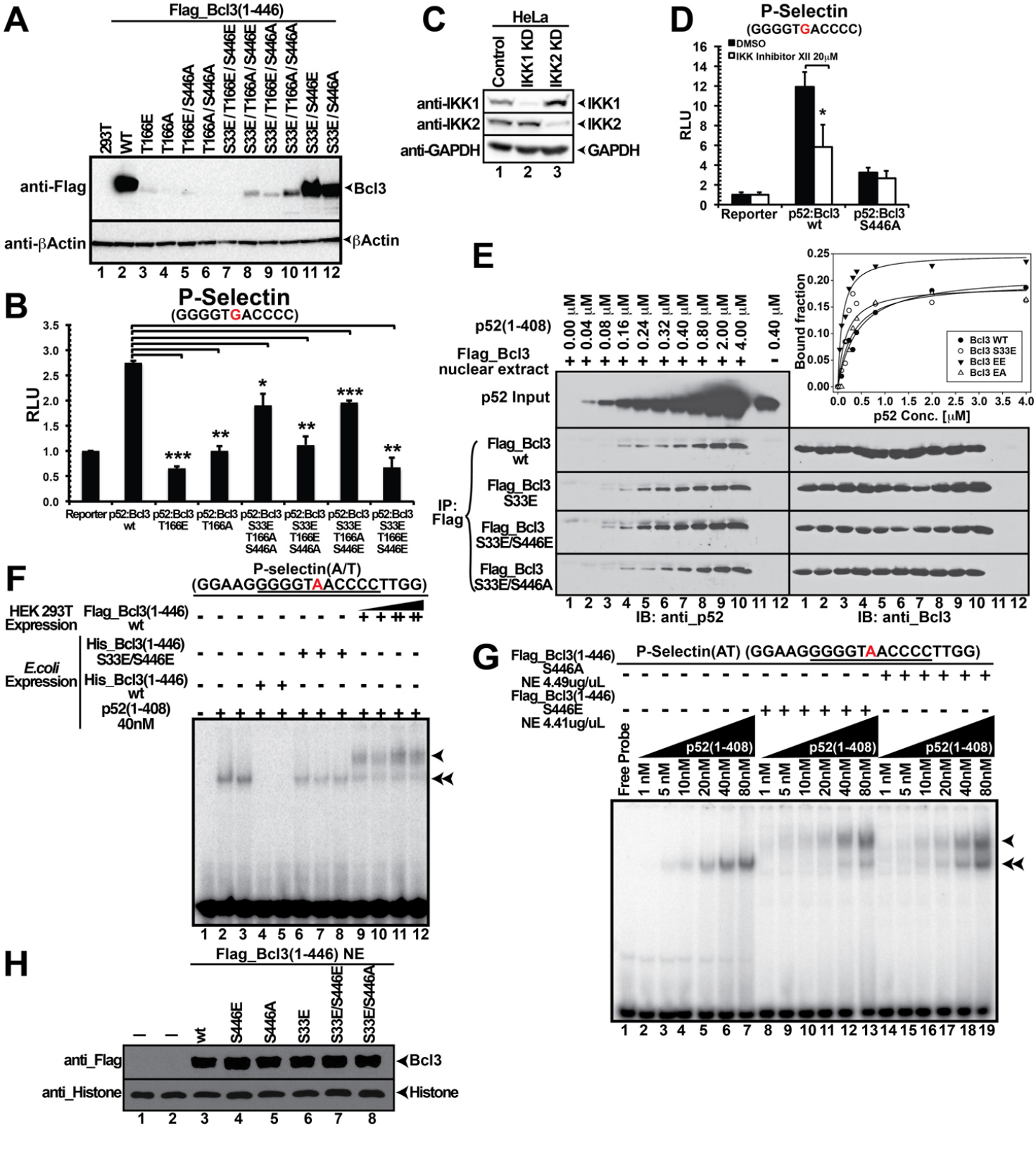

A
Flag_Bcl3(1-446)
293T
WT
T166E
T166A
T166E / S446A
T166A / S446A
S33E / T166E / S446E
S33E / T166A / S446E
S33E / T166E / S446A
S33E / T166A / S446A
S33E / S446E
S33E / S446A
anti-Flag
Bcl3
anti-βActin
βActin
1 2 3 4 5 6 7 8 9 10 11 12
B
P-Selectin
(GGGGTGACCCC)
RLU
Reporter
p52:Bcl3 wt
p52:Bcl3 T166E
p52:Bcl3 T166A
p52:Bcl3 S33E T166A S446A
p52:Bcl3 S33E T166E S446A
p52:Bcl3 S33E T166A S446E
p52:Bcl3 S33E T166E S446E
C
HeLa
Control
IKK1 KD
IKK2 KD
anti-IKK1
anti-IKK2
anti-GAPDH
IKK1
IKK2
GAPDH
1 2 3
D
P-Selectin
(GGGGTGACCCC)
DMSO
IKK Inhibitor XII 20μM
RLU
Reporter
p52:Bcl3 wt
p52:Bcl3 S446A
E
p52(1-408)
0.00 μM
0.04 μM
0.08 μM
0.16 μM
0.24 μM
0.32 μM
0.40 μM
0.80 μM
2.00 μM
4.00 μM
0.40 μM
Flag_Bcl3 nuclear extract
p52 Input
IP: Flag
Flag_Bcl3 wt
Flag_Bcl3 S33E
Flag_Bcl3 S33E/S446E
Flag_Bcl3 S33E/S446A
Bound fraction
p52 Conc. [μM]
Bcl3 WT
Bcl3 S33E
Bcl3 EE
Bcl3 EA
1 2 3 4 5 6 7 8 9 10 11 12
IB: anti_p52
IB: anti_Bcl3
F
P-selectin(A/T)
(GGAAGGGGGTAACCCCTTGG)
HEK 293T Expression
Flag_Bcl3(1-446) wt
E.coli Expression
His_Bcl3(1-446) S33E/S446E
His_Bcl3(1-446) wt
p52(1-408) 40nM
1 2 3 4 5 6 7 8 9 10 11 12
G
Flag_Bcl3(1-446) S446A NE 4.49ug/uL
Flag_Bcl3(1-446) S446E NE 4.41ug/uL
P-Selectin(AT) (GGAAGGGGGTAACCCCTTGG)
Free Probe
p52(1-408)
1 nM
5 nM
10nM
20nM
40nM
80nM
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
H
Flag_Bcl3(1-446) NE
wt
S446E
S446A
S33E
S33E/S446E
S33E/S446A
anti_Flag
Bcl3
anti_Histone
Histone
1 2 3 4 5 6 7 8

Figure S4. Analysis of Bcl3 Phosphorylation Sites by IKK. Related to Figure 4

(A) Expression of Flag-tagged full-length wt Bcl3 and Thr166 mutants in HEK 293T cells. (B) Both T166A and T166G mutations were all defective in luciferase reporter activation. (C) WB showing efficiency of IKK1 and IKK2 KD in HeLa cells using IKK1- and IKK2-specific antibodies. KD of one kinase did not affect the expression of the other kinase. (D) IKK inhibitor XII decreased wt Bcl3's transcriptional activity on P-Selectin reporter; however, it has no effect on S446A mutant. The data was analyzed from three independent experiments performed in triplicate. *$p<0.05$, **$p<0.01$, ***$p<0.005$. Error bars represent SD. (E) Flag-IP demonstrating binding of recombinant p52 (1-408) to different forms of Flag-Bcl3 isolated from the nuclear extracts. Bound p52 (detected by WB using anti-p52 antibody) was plotted against total p52 input at various concentrations. These results show that Bcl3 EE bound to p52 homodimer better than the other Bcl3 proteins. (F) Bacterial expressed wt Bcl3 removed p52 homodimer from κB DNA (lane 4-5). Bacterial expressed Bcl3 S33E/S446E (EE) mutant failed to form ternary complex with recombinant p52 protein and κB DNA in EMSA (lane 6-8); however, the HEK 293T-derived wt Bcl3 formed the stable Bcl3:p52:DNA ternary complex (lane 9-12). (G) EMSA showing HEK 293T-derived Bcl3 S446E formed ternary complexes with recombinant p52 on κB DNA more efficiently than S446A. Flag-tagged full-length Bcl3 nuclear extracts, recombinant p52 and His-tagged full-length Bcl3 protein and [γ-^{32}P]-ATP labeled P-Selectin κB DNA was used in the assay. In all EMSAs, single arrow head indicates Bcl3:p52(or p50):DNA ternary complex; double arrow head indicates p52(or p50):DNA binary complex**.** (H) WB showing similar levels of wt Bcl3 and mutants in the nucleus.

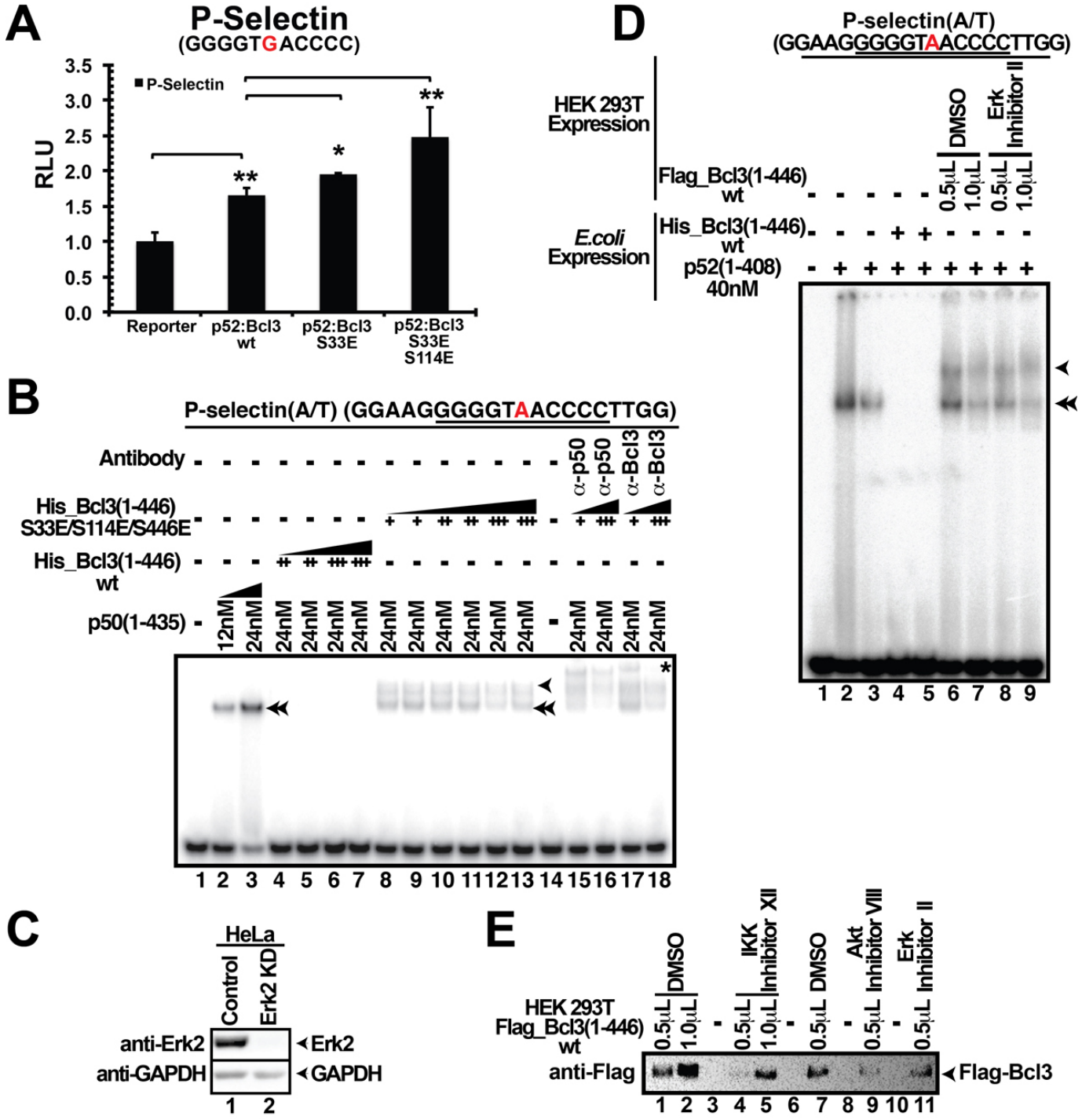
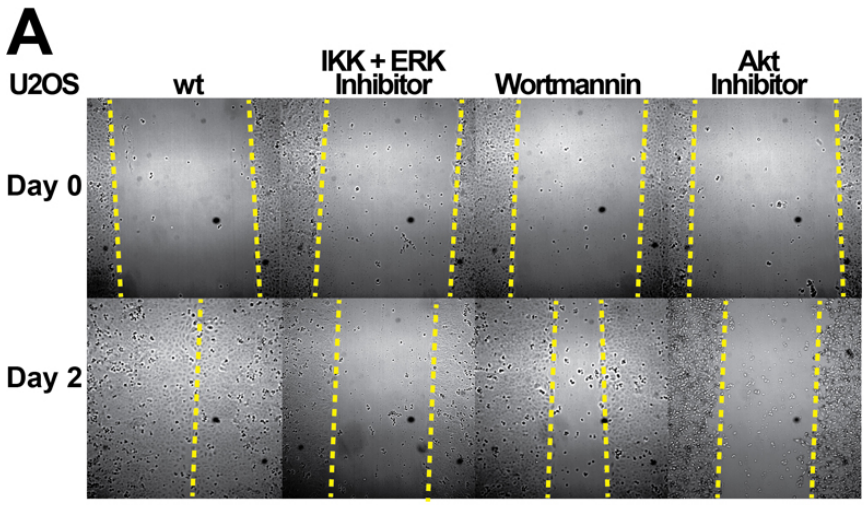
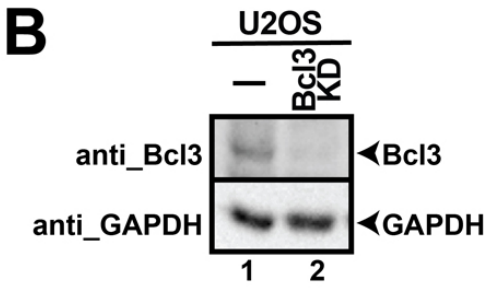

A
P-Selectin
(GGGGTGACCCC)
P-Selectin
RLU
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
*
**
Reporter
p52:Bcl3 wt
p52:Bcl3 S33E
p52:Bcl3 S33E S114E
B
P-selectin(A/T) (GGAAGGGGGTAACCCCTTGG)
Antibody
α-p50
α-p50
α-Bcl3
α-Bcl3
His_Bcl3(1-446) S33E/S114E/S446E
His_Bcl3(1-446) wt
p50(1-435)
12nM
24nM
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
C
HeLa
Control
Erk2 KD
anti-Erk2
anti-GAPDH
Erk2
GAPDH
1 2
D
P-selectin(A/T)
(GGAAGGGGGTAACCCCTTGG)
HEK 293T Expression
Flag_Bcl3(1-446) wt
DMSO
Erk Inhibitor II
0.5μL
1.0μL
E.coli Expression
His_Bcl3(1-446) wt
p52(1-408) 40nM
1 2 3 4 5 6 7 8 9
E
HEK 293T
Flag_Bcl3(1-446) wt
DMSO
IKK Inhibitor XII
Akt Inhibitor VIII
Erk Inhibitor II
0.5μL
1.0μL
anti-Flag
Flag-Bcl3
1 2 3 4 5 6 7 8 9 10 11

Figure S5. Erk2 Phosphorylates Bcl3 and Converts it from an NF-κB Inhibitor to a Transcription Coactivator. Related to Figure 5

(A) Bcl3 S114E mutant enhanced transcriptional activity indicated by luciferase reporter assay. The data was analyzed from three independent experiments performed in triplicate. *$p<0.05$, **$p<0.01$. Error bars represent SD. (B) Bacterial expressed Bcl3 S33E/S114E/S446E (EEE) triple mutant, but not wt Bcl3, formed ternary complex with recombinant p50 protein and κB DNA as shown by EMSA. (C) WB showing efficiency of Erk2 KD in HeLa cells. (D) Bcl3 isolated from Erk inhibitor II treated HEK 293T cells failed abolish Bcl3's ability to form ternary complex formation. In comparison, Bcl3 derived from DMSO treated cells also formed the ternary complex served as a control. In all EMSA, single arrow head indicates Bcl3:p52(or p50):DNA ternary complex; double arrow head indicates p52(or p50):DNA binary complex; (*) indicates the super-shifted band of the ternary complex in the presence of specific antibody. (E) Flag-Bcl3 expression level in different inhibitor treated HEK 293T cells used in EMSAs.


A
U2OS
wt
IKK + ERK Inhibitor
Wortmannin
Akt Inhibitor
Day 0
Day 2


B
U2OS
—
Bcl3 KD
anti_Bcl3
Bcl3
anti_GAPDH
GAPDH
1
2

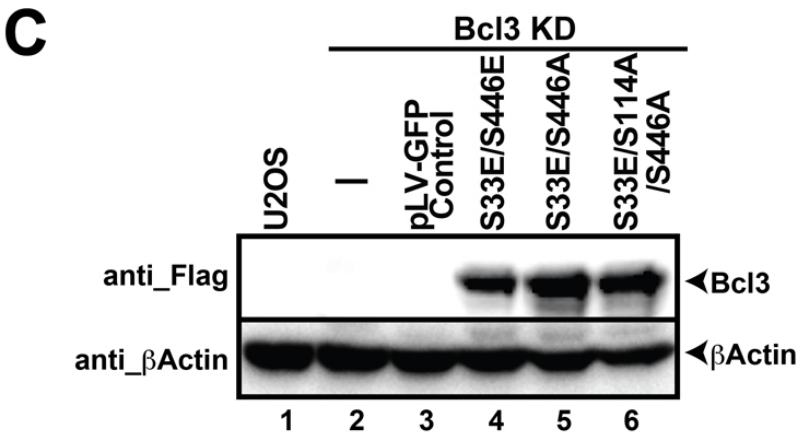

C
Bcl3 KD
U2OS
—
pLV-GFP Control
S33E/S446E
S33E/S446A
S33E/S114A /S446A
anti_Flag
Bcl3
anti_βActin
βActin
1
2
3
4
5
6

Figure S6. Bcl3 Phosphorylation is Required for its Cellular Functions. Related to Figure 6
(A) U2OS cells showed migration defects when treated with Akt, IKK and Erk inhibitors. The yellow dotted lines indicate the edge of the scratch showing the width of scratches. (B) WB using anti-Bcl3 antibody showing reduced level of endogenous Bcl3 in U2OS cells upon CRISPR-Cas9 KD. (C) Different mutants were expressed to a similar level in Bcl3 KD U2OS cells.

Table S1. Bcl3 phosphorylation residues identified in mass spectrometric analyses. Related to Figure 1, 2, 4, 5 and 6

Flag_Bcl3 (HEK 293T)		Flag_Bcl3+IKK Inhibitor (HEK 293T)		IKK1 *In Vitro* Kinase Assay		ERK2 *In Vitro* Kinase Assay	
Phospho-Residue	Confidence Level	Phospho-Residue	Confidence Level	Phospho-Residue	Confidence Level	Phospho-Residue	Confidence Level
		T10	96				
S33	99	S33	99	S33	99		
Y75	99	Y75	99				
Y76	99	Y76	99				
S91	99						
T100	99	T100	99			T100	99
Y103	99	Y103	99			Y103	99
S114	99	S114	99			S114	99
						T121	65.7
T129	20-40	T129	99			T129	99
T166	99			T166	96		
T185	79.6	T185	93				
		T199	99				
S209	96.6	S209	99				
T211	96.5	T211	99				
				S219	99		
						Y232	71.3
						T236	71.3
		T244	99				
S280	99	S280	99			S280	94.9
S282	99	S282	99			S282	99
Y299	99	Y299	99			Y299	78.1
S300	44	S300	99				
S327	99	S327	99	S327	99		
S328	99	S328	99	S328	99		
				T355	99		
S366	99	S366	99				
S392	16	S392	72-98				
S393	16	S393	72-93				
S394	16	S394	66-95				
S396	16	S396	66-95	S396	99		
S398	82.5	S398	72-95	S398	98		
S417	99						
S419	99	S419	71				
				S440	99		
S446	99			S446	99		

Blue:	**Not fully conserved**
yellow:	**Present in both Flag_Bcl3 from HEK 293T cells and IKK in vitro kinase assay; but absent in IKK inhibitor in 293T cells**