Supplemental Figure Legend

Figure S1. Potential roles of transcription factors in PUMA induction by regorafenib in CRC cells. (A) HCT116 cells were transfected with a control scrambled siRNA or siRNA against indicated genes. Expression of indicated genes at 24 hours after siRNA transfection was analyzed by RT-PCR. **(B)** Following siRNA transfection as in (A), HCT116 cells were treated with 40 μmol/L regorafenib for 24 hours. PUMA expression was analyzed by Western blotting. **(C)** The levels of p73, p-STAT1 (Y701), STAT1, p-FoxO3a (T32), and FoxO3a were analyzed by Western blotting at indicated time points in HCT116 cells treated with 40 μmol/L regorafenib. **(D)** HCT116 cells were transfected with the control or *FoxO3a* siRNA, and then treated with 40 μmol/L regorafenib for 24 hours. The levels of FoxO3a and PUMA were analyzed by Western blotting.

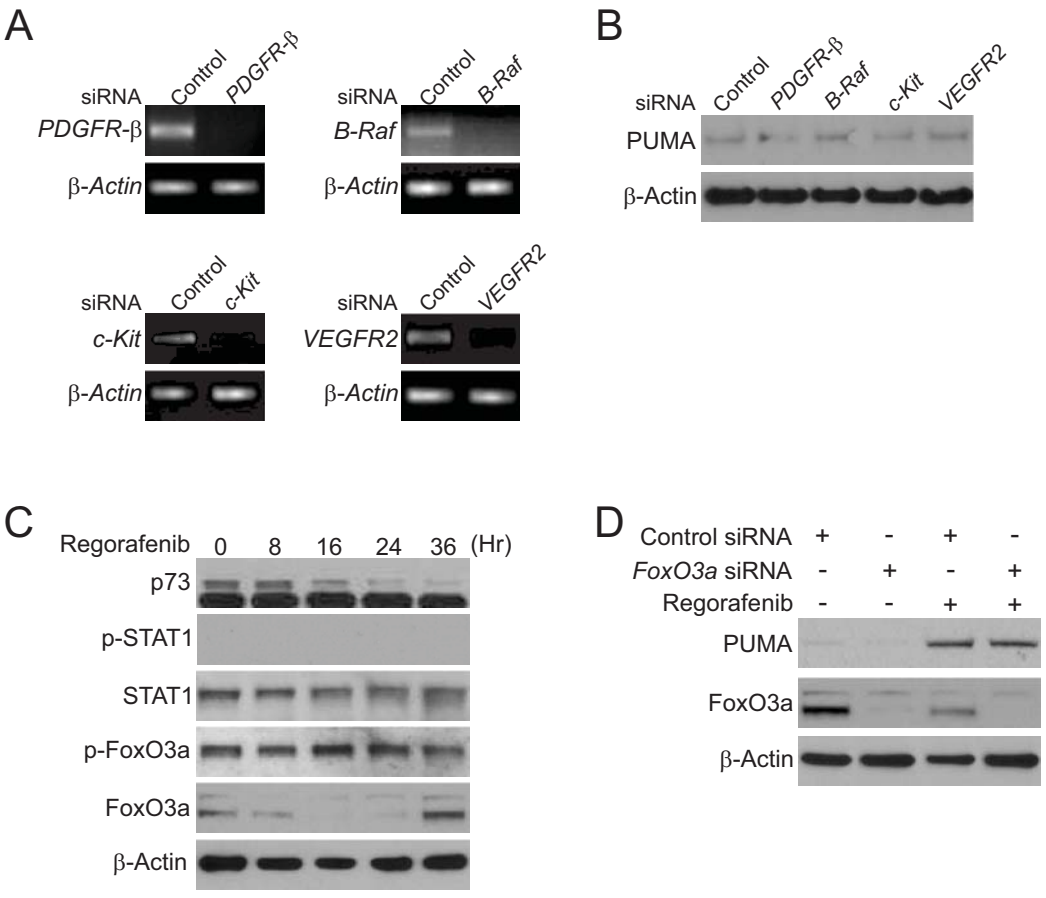
Figure S2. p65 mediates PUMA induction by regorafenib in CRC cells. (A) HCT116 cells were treated with regorafenib at indicated concentrations for 24 hours. The levels of p-p65 (S536) and β-actin were analyzed by Western blotting. **(B)** HCT116 cells were treated with 40 μmol/L regorafenib for the indicated times. The levels of p-p65 (S276, S468) were analyzed by Western blotting. **(C)** DLD1 cells were transfected with control or *p65* siRNA, and then treated with 40 μmol/L regorafenib for 24 hours. p65 and PUMA expression was analyzed by Western blotting. **(D)** WT and *p65*-KO MEFs were treated with 40 μmol/L regorafenib for 24 hours. PUMA expression was analyzed by Western blotting. **(E)** *Left*, schematic representation of the genomic structure of *PUMA* highlighting the *PUMA* promoter Fragments (Frag) A-E used in the luciferase experiment. *Right*, *p53*-KO HCT116 cells were transfected overnight with the indicated *PUMA* promoter luciferase reporters, and then treated with 40 μmol/L regorafenib for 16 hours. Reporter activities were normalized to the untreated control samples and plotted. **(F)** Schematic representation of the 5 putative κB sites in Fragment A of the *PUMA* promoter.

Asterisks denote mutated nucleotides. **(G)** HCT116 cells were treated as (B). The levels of p-IκB (S22/23) and IκB were analyzed by Western blotting at indicated time points.

Figure S3. PUMA-dependent chemosensitization by regorafenib in CRC cells. (A) WT HCT116 cells were treated with 20 μmol/L regorafenib, 25 μmol/L cisplatin, or their combination for 24 hours. PUMA expression was analyzed by Western blotting. **(B)** WT and *PUMA*-KO HCT116 cells were treated as (A) for 48 hours. Apoptosis was determined by nuclear staining with Hoechst 33258. **(C)** WT and *PUMA*-KO HCT116 cells were treated for 48 hours with 20 μmol/L regorafenib, alone or in combination with 5 μmol/L gefitinib. Apoptosis was determined as in (B). **(D)** WT and *PUMA*-KO HCT116 cells were treated for 48 hours with 20 μmol/L regorafenib, alone or in combination with 1 μmol/L UCN-01. Apoptosis was determined as in (B). Results in (B)-(D) were expressed as means $\pm$ SD of 3 independent experiments. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

Figure S4. PUMA-dependent chemosensitization by regorafenib in xenograft tumors. (A) Mice with WT or *PUMA*-KO HCT116 xenograft tumors were treated with 15 mg/kg regorafenib daily by oral gavage, 25 mg/kg 5-FU every other day by i.p. injection, or their combination for 10 consecutive days. Representative tumors at the end of the experiment are shown. **(B)** Mice with WT or *PUMA*-KO HCT116 xenograft tumors were treated with 15 mg/kg regorafenib daily, 25 mg/kg 5-FU every other day, or their combination as in (A) for 4 consecutive days. Paraffin-embedded sections were analyzed by TUNEL staining. TUNEL-positive cells were counted and plotted. Representative TUNEL staining pictures are shown. **(C)** Tissue sections from (B) were analyzed by active caspase 3 staining. Representative staining pictures are shown. Arrows, example cells with positive staining; scale bars, 25 μm.

Figure S1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Control
PDGFR-β
PDGFR-β
β-Actin
siRNA
Control
B-Raf
B-Raf
β-Actin
siRNA
Control
c-Kit
c-Kit
β-Actin
siRNA
Control
VEGFR2
VEGFR2
β-Actin
B
siRNA
Control
PDGFR-β
B-Raf
c-Kit
VEGFR2
PUMA
β-Actin
C
Regorafenib
0
8
16
24
36
(Hr)
p73
p-STAT1
STAT1
p-FoxO3a
FoxO3a
β-Actin
D
Control siRNA
FoxO3a siRNA
Regorafenib
PUMA
FoxO3a
β-Actin

Figure S2

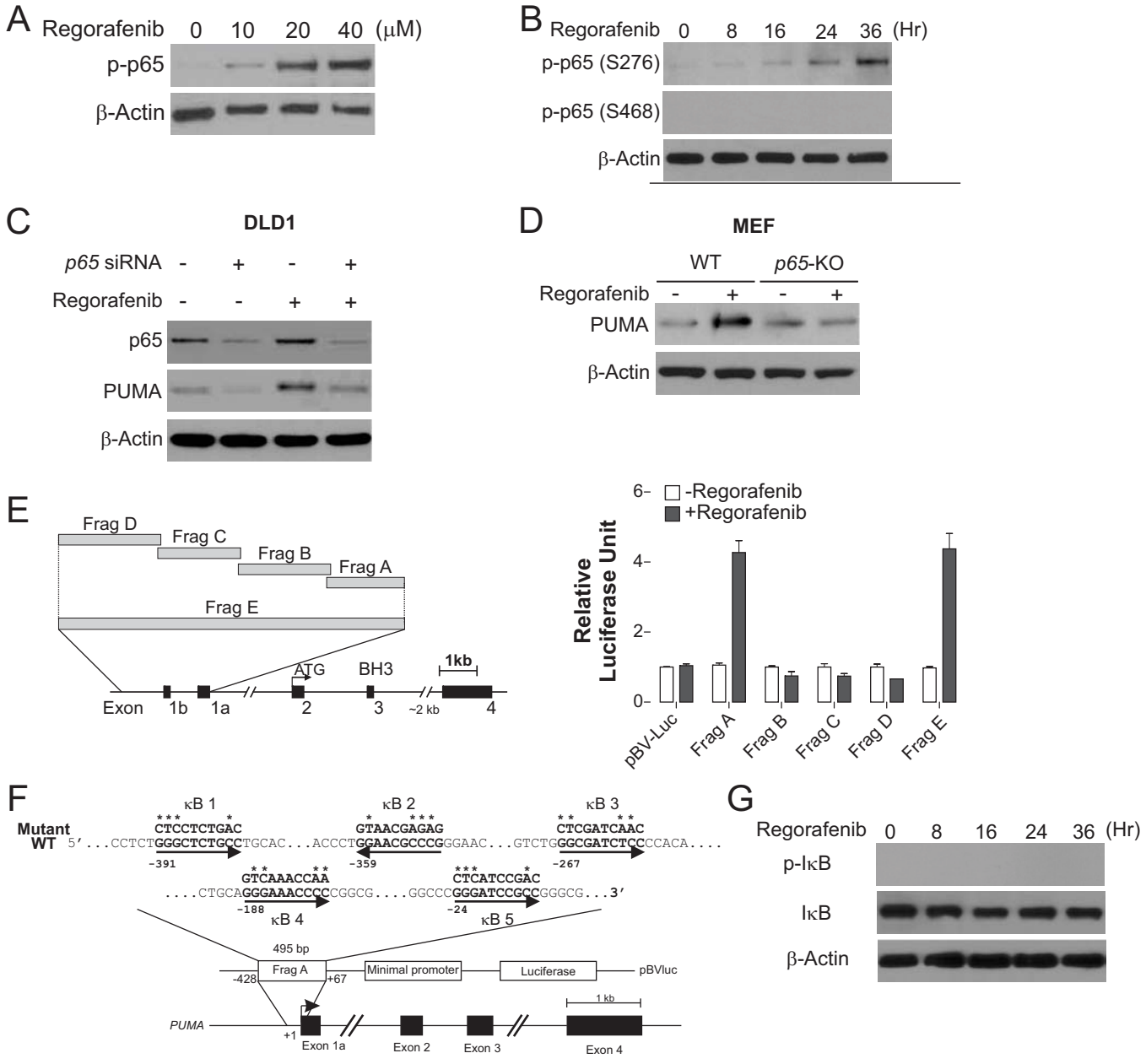

A
Regorafenib 0 10 20 40 (μM)
p-p65
β-Actin
B
Regorafenib 0 8 16 24 36 (Hr)
p-p65 (S276)
p-p65 (S468)
β-Actin
C
DLD1
p65 siRNA - + - +
Regorafenib - - + +
p65
PUMA
β-Actin
D
MEF
WT
p65-KO
Regorafenib - + - +
PUMA
β-Actin
E
Frag D
Frag C
Frag B
Frag A
Frag E
ATG
BH3
1kb
Exon 1b 1a 2 3 ~2 kb 4
-Regorafenib
+Regorafenib
Relative Luciferase Unit
6
4
2
0
pBV-Luc
Frag A
Frag B
Frag C
Frag D
Frag E
F
κB 1
κB 2
κB 3
Mutant
WT 5′...CCTCTGGGCTCTGCCTGCAC...ACCCTGGAACGCCCGGGAAC...GTCTGGGCGATCTCCCCACA....
CTCCTCTGAC
GTAACGAGAG
CTCGATCAAC
-391
-359
-267
GTCAAACCAA
CTCATCCGAC
....CTGCAGGGAAACCCCCGGCG....GGCCCGGGATCCGGCGGGCG...3′
-188
-24
κB 4
κB 5
495 bp
Frag A
-428
+67
Minimal promoter
Luciferase
pBVluc
1 kb
PUMA
+1
Exon 1a
Exon 2
Exon 3
Exon 4
G
Regorafenib 0 8 16 24 36 (Hr)
p-IκB
IκB
β-Actin

Figure S3

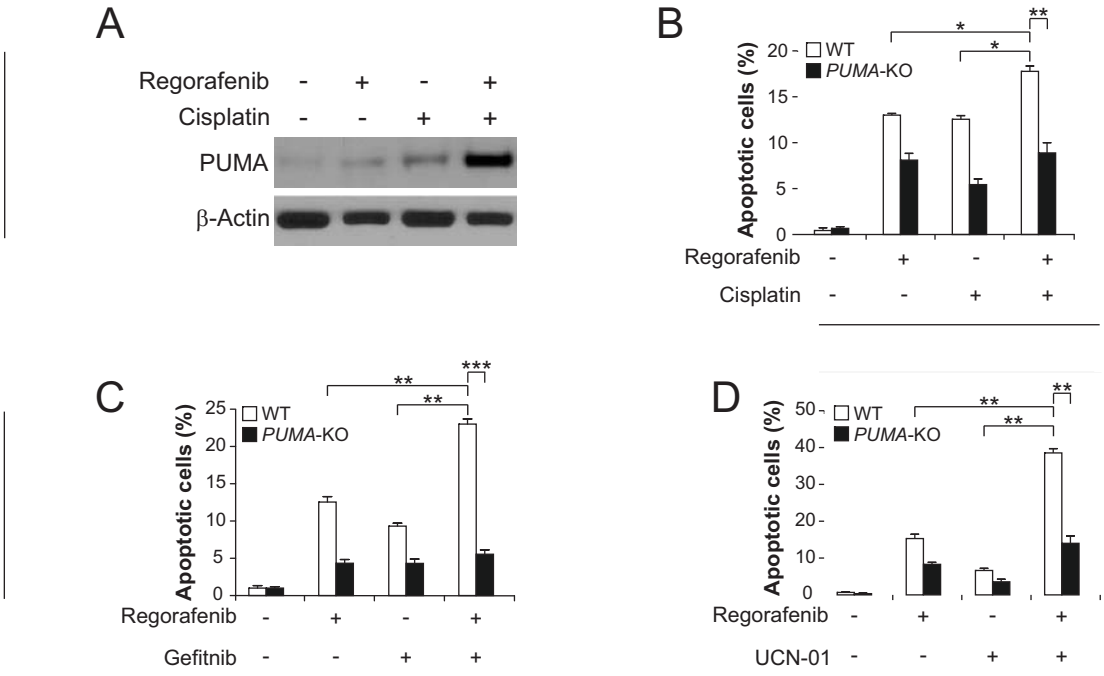
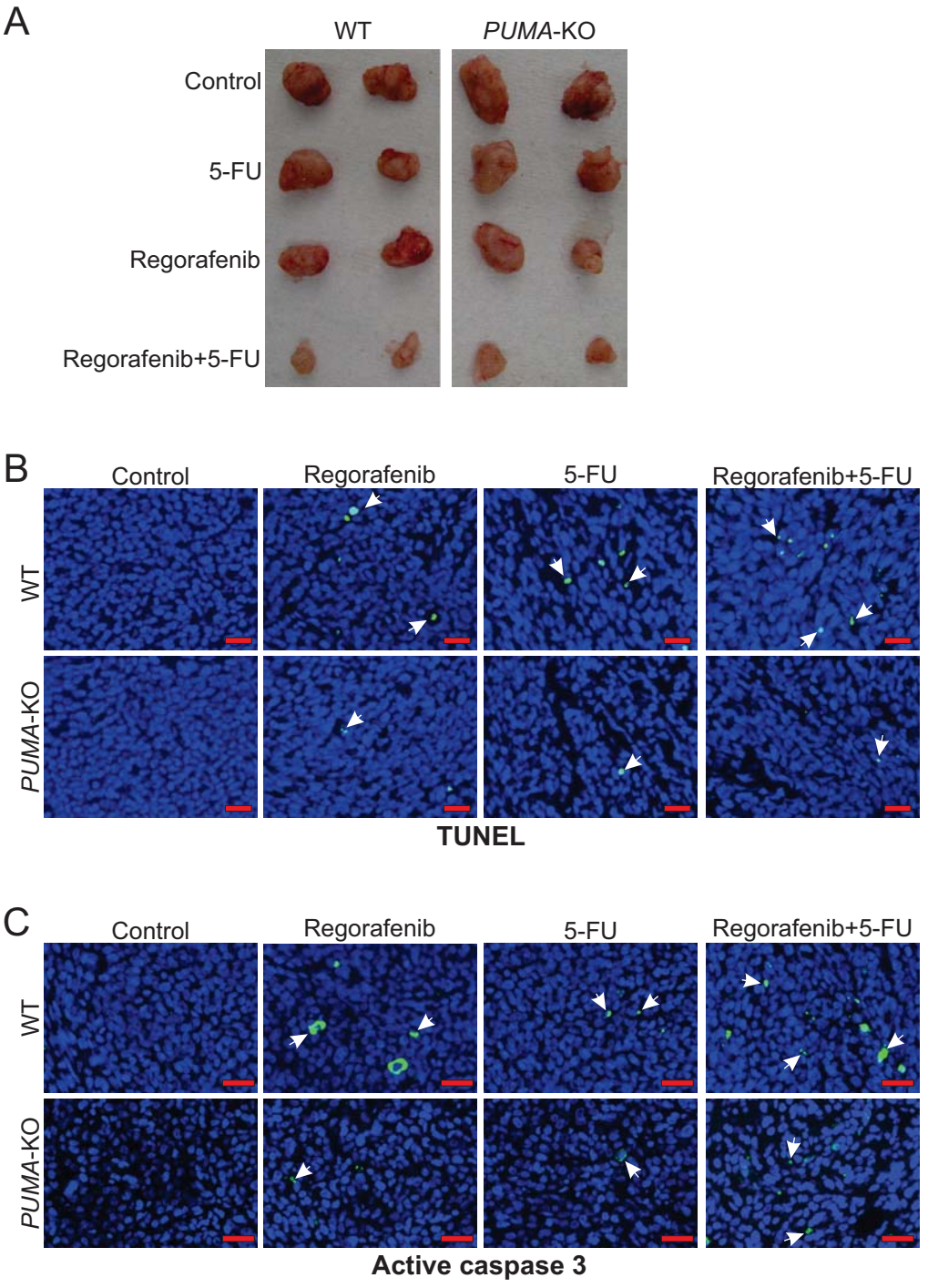

A
Regorafenib - + - +
Cisplatin - - + +
PUMA
β-Actin
B
Apoptotic cells (%)
WT
PUMA-KO
Regorafenib - + - +
Cisplatin - - + +
C
Apoptotic cells (%)
WT
PUMA-KO
Regorafenib - + - +
Gefitnib - - + +
D
Apoptotic cells (%)
WT
PUMA-KO
Regorafenib - + - +
UCN-01 - - + +

Figure S4


A
WT
PUMA-KO
Control
5-FU
Regorafenib
Regorafenib+5-FU
B
Control
Regorafenib
5-FU
Regorafenib+5-FU
WT
PUMA-KO
TUNEL
C
Control
Regorafenib
5-FU
Regorafenib+5-FU
WT
PUMA-KO
Active caspase 3