Figure S1. Wang et al

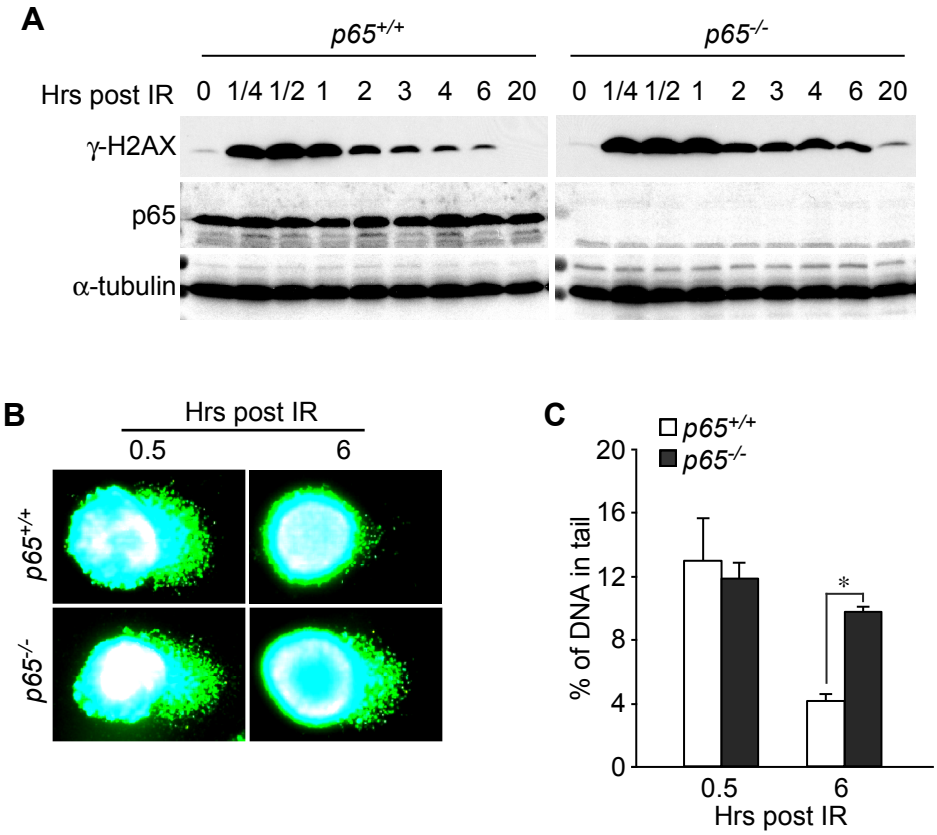


Figure S2. Wang et al

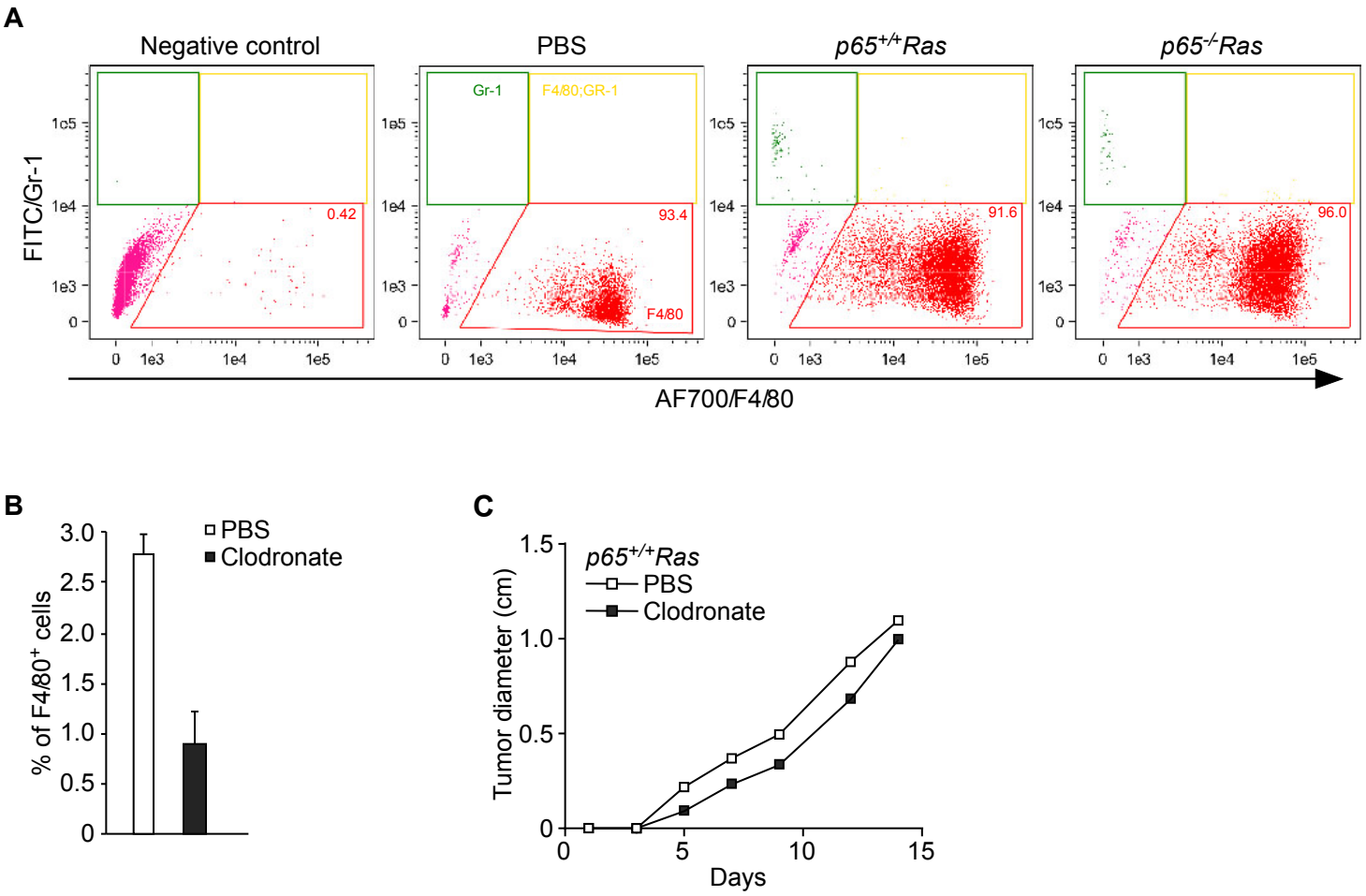


Figure S3 Wang et al

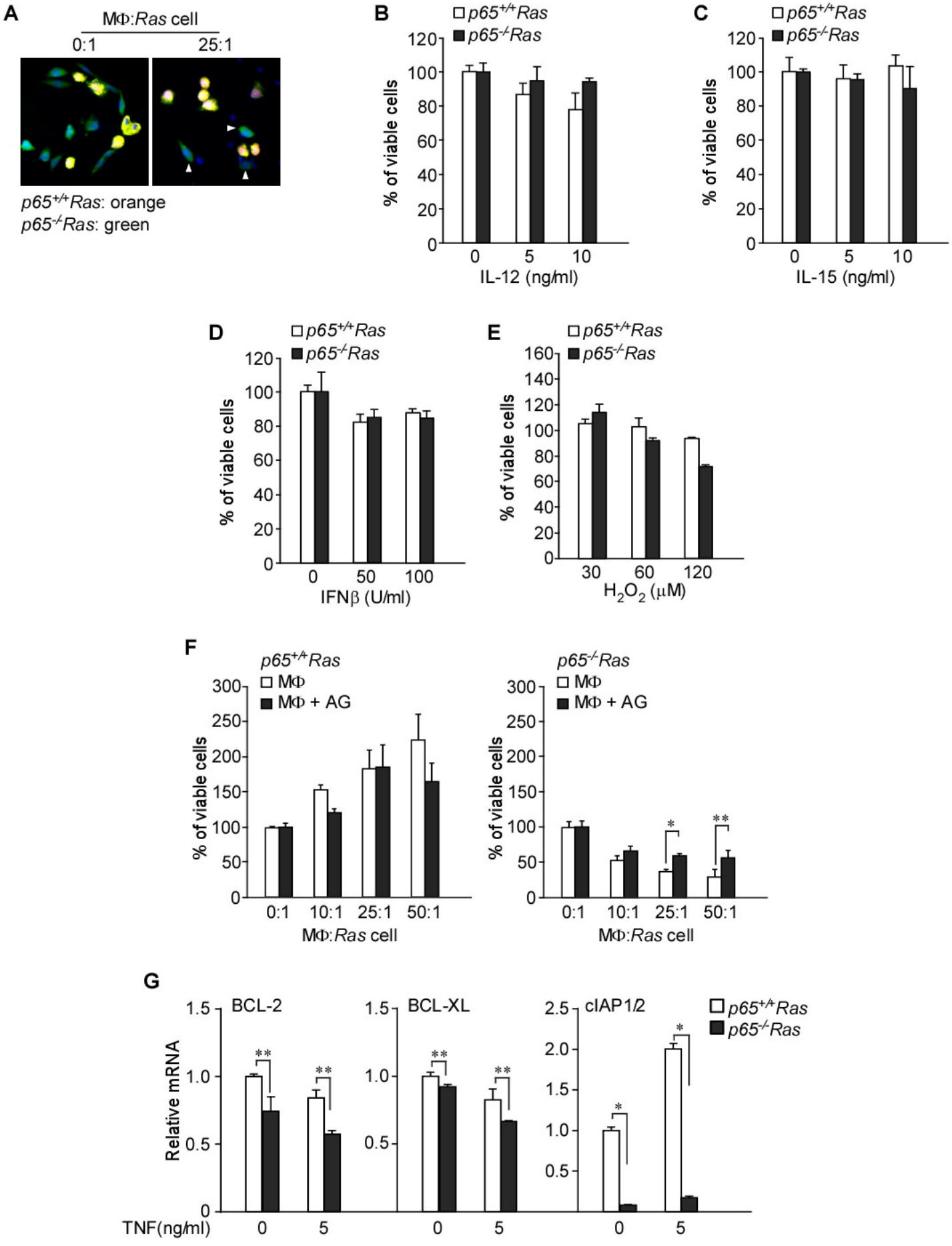
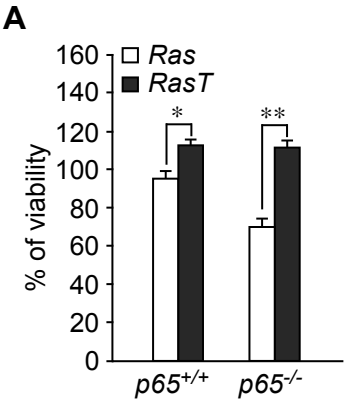


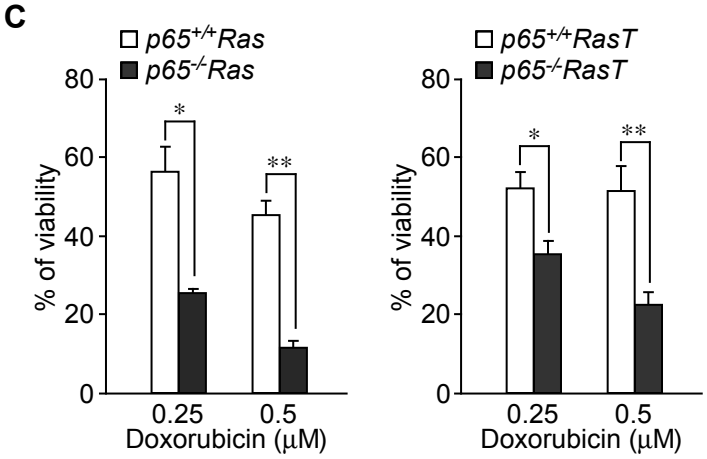
Figure S4. Wang et al



B

% of cell viability

SNP (μM)	*p65*$^{-/-}$*Ras*	*p65*$^{-/-}$*RasT*	p<
30	27.4 ± 6.4	112 ± 9.3	0.001
60	10.9 ± 2.2	76.5 ± 10.0	0.001
120	0 ± 1.6	51.5 ± 9.5	0.001



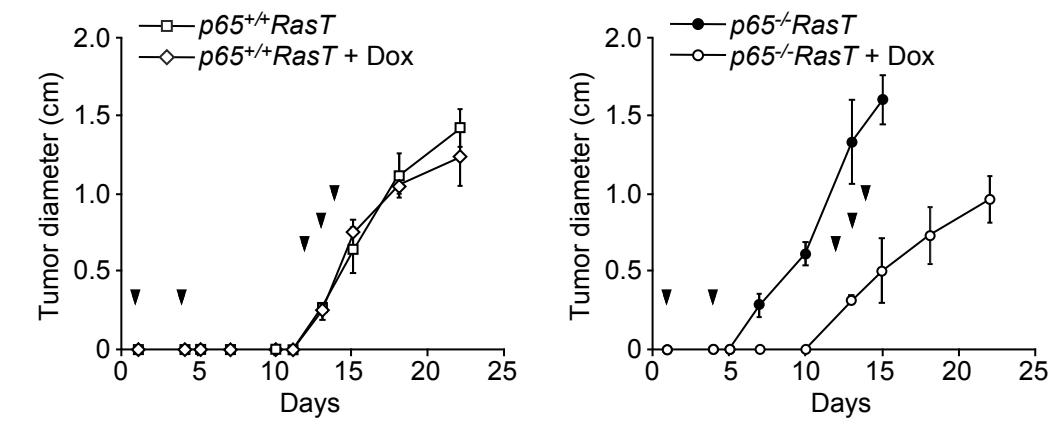


Figure S5. Wang et al

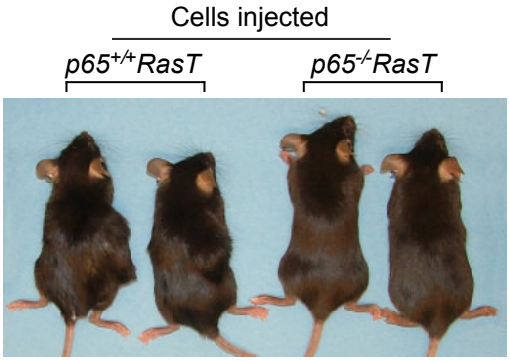


Figure S6. Wang et al

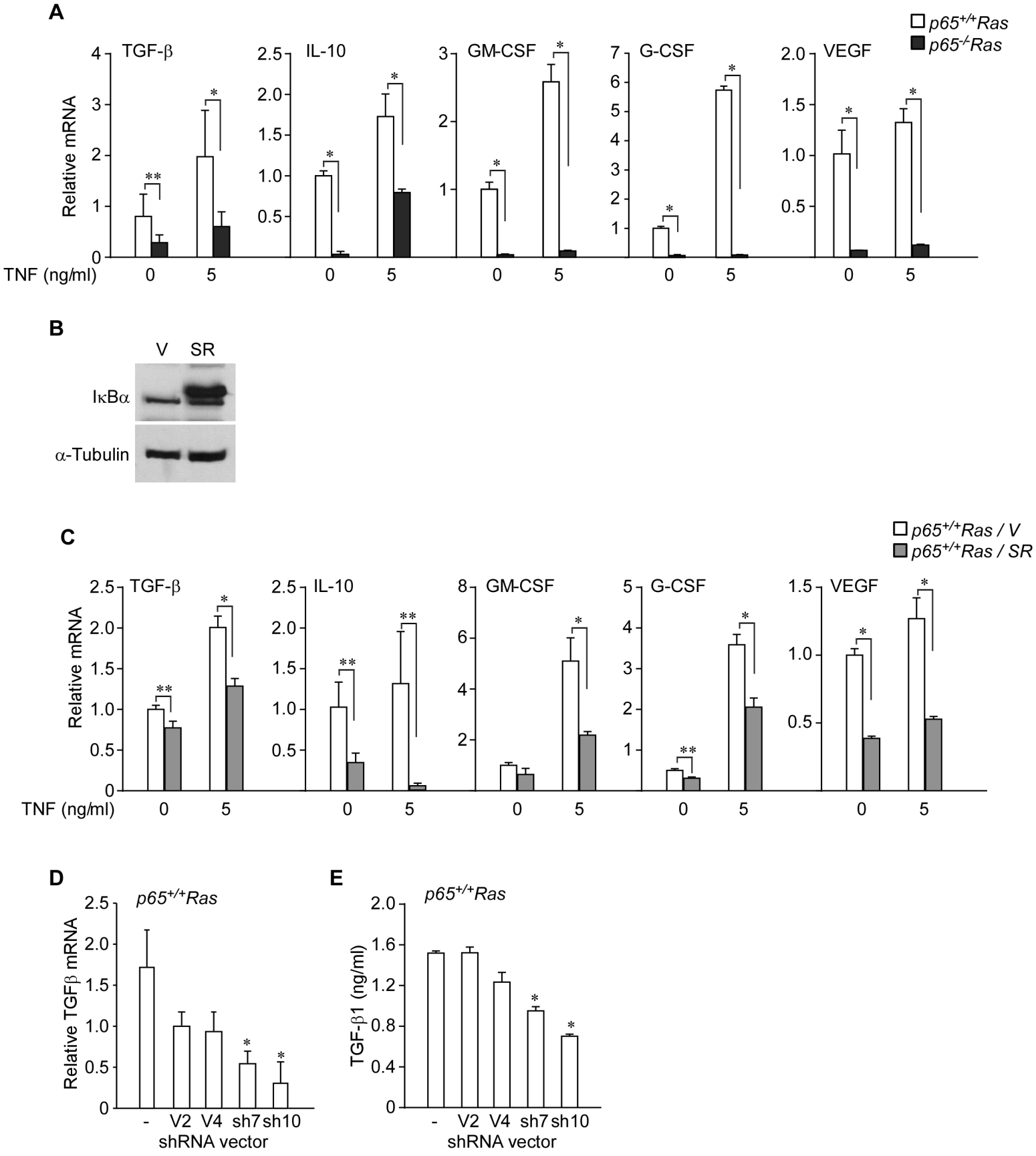
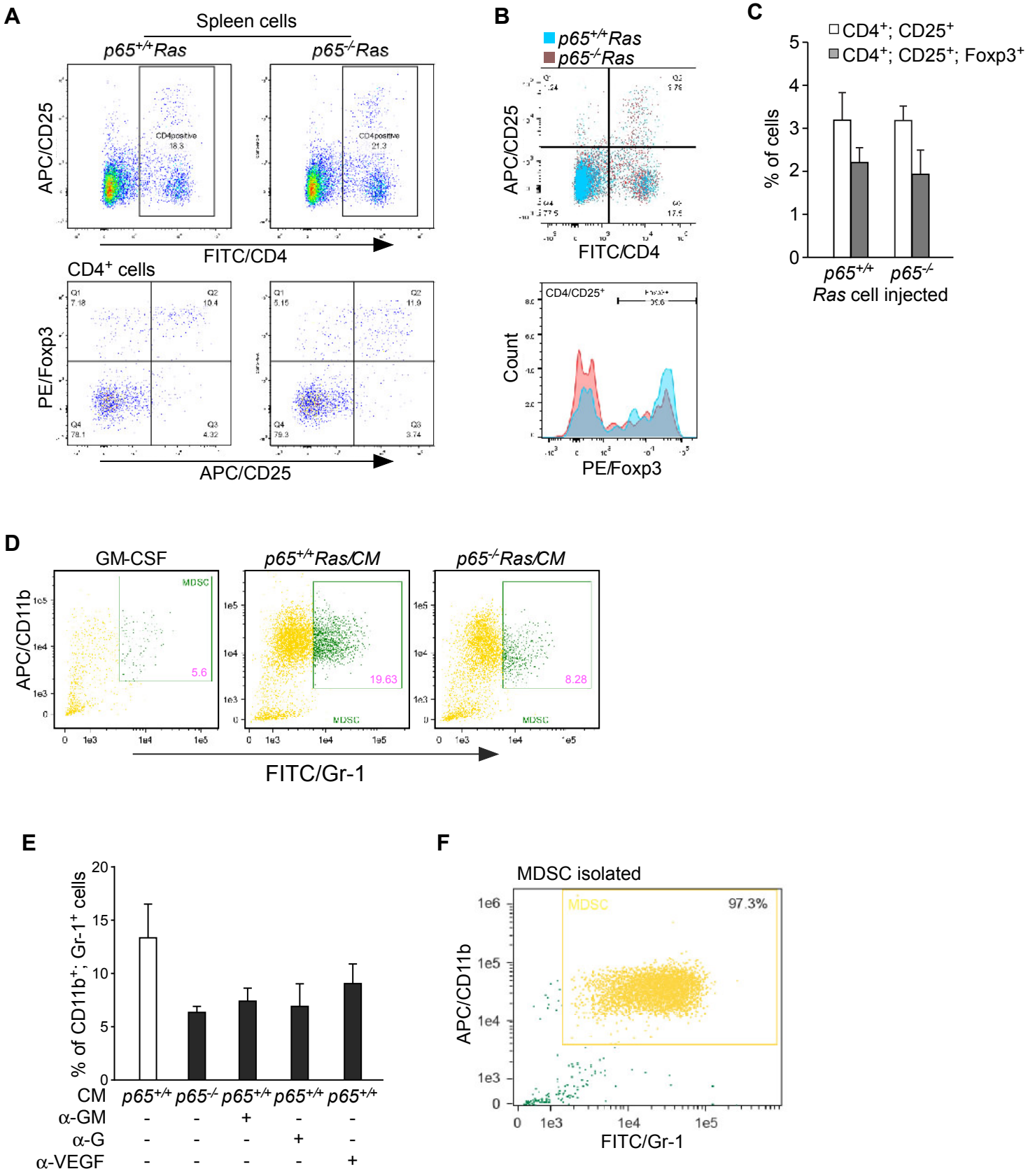


Figure S7. Wang et al



Supplemental Figure Legends

Figure S1 related to Figure 1. p65 is required to maintain genomic stability in primary cells but promotes tumor initiation in Ras expressing cells. (A) $p65^{+/+}$ or $p65^{-/-}$ MEFs (P4-P6) were subjected to X-irradiation (IR) and at indicated times, whole cell extracts were prepared and westerns performed probing for γ-H2AX. (B) Primary $p65^{+/+}$ and $p65^{-/-}$ cells were irradiated with X-ray and at indicated time points post irradiation, cells were loaded onto an agarose module and electophoresis was carried out under alkaline conditions (pH = 13). After electrophoresis, comet modules were stained with Sybr green and observed by fluorescence microscopy. (C) Tail length and percentage of tail DNA content representing DNA damage was calculated with Cometscore software, as described in the Supplemental Materials and Methods section. DNA damage index was derived from 3 independent experiments, with at least 50 nuclei counted per experiment. Data are a represented as mean ± SD. * $p < 0.0001$). (D) $p65^{+/+}$*Ras* and $p65^{-/-}$ *Ras* cells were subcutaneously injected into left flank of C57BL/6 nude mice. At indicated time points, tumor sizes were measured by a digital caliber. Tumor sizes were depicted as $(D_{long}+D_{short})/2$.

Figure S2 related to Figure 2. Clodronate mediated MΦ depletion. (A) Peritoneal cells were isolated from SCID mice injected with $p65^{+/+}$*Ras* or $p65^{-/-}$*Ras* cells, and stained with an Alexafluoror700 conjugated antibody for F4/80 (AF700/F4/80) along with an FITC conjugated antibody for Gr-1 (FITC/Gr-1). Data are representative of flow cytometry analysis from 2 independent experiments with at least 4 mice for each group. (B) Clodronate/liposome or PBS/liposome (control) were intravenously injected into FVB mice. Two days later, spleen cells were isolated and stained for F4/80. Following flow cytometry analysis, mean percentage of

F4/80 positive cells ± SD were represented, $p < 0.01$. (C) Macrophage depletion was carried out in SCID mice by tail vein injections of Clodronate/liposome (Clodronate) or PBS/Liposome (PBS) used as a control. Injections were repeated once per week. *p65*$^{+/+}$*Ras* cells were then injected subcutaneously and tumor growth was measured. Data are representative of two independent experiments ($p < 0.0001$).

Figure S3 related to Figure 3. *p65*$^{-/-}$*Ras* cells are sensitive to MΦ induced cell death. (A) Image of cell painting analysis as described for Figure 3A. (B - E) MTS viability analysis from *p65*$^{+/+}$ and *p65*$^{-/-}$ cells treated with IL-12 (B), IL-15 (C), IFN-β (D) and H_2O_2 (E). Data are representative of three independent experiments. (F) *p65*$^{+/+}$*Ras* and *p65*$^{-/-}$ *Ras* cells were co-cultured with increasing ratios of MΦ together with or without aminoguanidine. Viability was recorded with a trypan blue exclusion assay. ∗ $p < 0.04$, ∗∗ $p < 0.003$. (B – F), Data are represented as mean ± SD. (G) Real time RT-PCR analysis of BCL-2, BCL-XL and cIAP1/2 gene expression from RNA isolated from *p65*$^{+/+}$*Ras* or *p65*$^{-/-}$*Ras* cells before or after TNF treatment, ∗ $p < 0.0001$; ∗∗ $p < 0.01$.

Figure S4 related to Figure 4. *p65*$^{-/-}$*RasT* cells are Resistant to TNF-α and NO induced cell death. (A and B) MTS assay results comparing viability of *p65*$^{+/+}$ and *p65*$^{-/-}$ cells before (*Ras*) and after (*RasT*) reconstitution from tumor when cultured in the presence of a pro-apoptotic cytokine, TNF, ∗ $p = 0.534$, ∗∗ $p < 0.003$ (A) or the NO donor SNP (B). (C) MTS assay results comparing viability of *p65*$^{+/+}$ and *p65*$^{-/-}$ cells before (*Ras*) and after (*RasT*) reconstitution from tumors when cultured in the presence of doxorubincin (Dox). Results are graphed as mean ± SD. (D and E) *p65*$^{+/+}$*RasT* and *p65*$^{-/-}$*RasT* cells were injected into SCID mice, and at indicated times

(denoted by black triangles) mice were injected with 2mg/kg of Doxorubicin (Dox). Tumor size were measured and presented as in (A). (B) $p = 0.366$, (C) $p < 0.0001$. (A-C) All data are represented as mean ± SD.

Figure S5 related to Figure 5. *p65*$^{-/-}$*RasT* cells are incompetent in forming tumors in C57BL/6 mice. *p65*$^{+/+}$*RasT* and *p65*$^{-/-}$*RasT* cells were injected subcutaneously into C57/BL/6 mice. Picture was taken at 20 days post injection.

Figure S6 related to Figure 6. Lentivirus mediated knock down of TGF-β. (A) Real time RT-PCR analysis of TGF-β, IL-10, GM-CSF, G-CSF and VEGF gene expression from RNA isolated from *p65*$^{+/+}$*Ras* or *p65*$^{-/-}$*Ras* cells before or after TNF treatment, ** $p = 0.041$, * $p < 0.001$. (B) Western blotting analysis of IκBα expression from protein extracts of *p65*$^{+/+}$*Ras* cells infected with either pBabe-puro (V) or pBabe-puro expressing IκBα Super suppressor (SR). (C) RNA was isolated from *p65*$^{+/+}$*Ras* cells infected with either pBabe-puro (V) or pBabe-puro expressing IκBα-SR with or without TNF treatment. Real time RT-PCR were then performed probing for TGF-β, IL-10, GM-CSF, G-CSF and VEGF, ** $p < 0.05$, * $p < 0.005$. (D) pGIPZ lentivirus express GFP (V) only or shRNA against TGF-β (shTGF), were utilized to infect *p65*$^{+/+}$*Ras* cells. Single cell selection was performed on these cells. Real time RT PCR was performed on RNA extracted from *p65*$^{+/+}$*Ras* cells or selected clones, V2, V4, shTGF7, and shTGF10, to confirm knock down of TGF-β. Results are represented as mean ± SD. * $p < 0.001$. (E) The same as (D), except ELISA was performed on conditioned media from isolated clones, * $p < 0.001$.

Figure S7 related to Figure 7. *p65*$^{+/+}$*Ras* conditioned media induces MDSC production (A) *p65*$^{+/+}$*Ras* and *p65*$^{-/-}$*Ras* cells were injected subcutaneously into C57/BL/6 mice. After 9 days, spleen cells were isolated from these mice and subsequently stained for CD4 (FITC), CD25 (APC), and Foxp3 (PE), and cells were then analyzed by flow cytometry. (B) The same cells as in (A) were gated for CD4^{+}/CD25^{+} and then FoxP3 a histogram overlay was plotted. (C) Graph from the mean percentage values of CD4^{+}/CD25^{+} or Tregs ± SD from (A) derived from 5 mice. Data are representative of at least two experiments from a minimum of 4 mice in each group. (D) Bone marrow cells were isolated from C57BL/6 mice and co-cultured with *p65*$^{+/+}$*Ras* and *p65*$^{-/-}$*Ras* cell conditioned media (*p65*$^{+/+}$*Ras*/CM and *p65*$^{-/-}$*Ras*/CM). After 6 - 7 days, cells were harvested, stained for CD11b and Gr-1, and subsequently analyzed by FACS. (E) Bone marrow cells from C57BL/6 mice were co-cultured with *p65*$^{+/+}$*Ras* cell conditioned media (CM). At the same time, monoclonal antibodies against GM-CSF (α-GM), G-CSF (α-G) and VEGF (α-V) were added, and cells were subsequently stained for CD11b and GR-1 and analyzed by flow cytometry. (F) *p65*$^{+/+}$*Ras* cells were injected subcutaneously into C57/BL/6 mice. After 10 – 16 days, spleen cells were isolated from these mice and MDSC were purified with an MDSC isolation kit. A small portion (1 x10^{6}) of the isolated cells were again stained with antibodies again CD11b and Gr-1 and further analyzed by flow cytometry.

Video S1 related to Figure 2. Clodronate injection cause serious side effects in SCID mice. Each group of 5 mice were injected with either Clodronate/liposome (Clodronate) or PBS/liposome (PBS) once per week. The video shows the declining activity of mice that had survived 2 injections of clodronate treatment. Data are representative of 2 independent experiments with at least 10 mice used per experiment.

Table S1. Primer sequences and PCR conditions:

TGFβ1	forward	5’ - TGCTGCCTTCGCCCTCTTTAC
	reverse	5’ - AAGCGGAAGCTTCGGGATTT
VEGF1	forward	5’ - AATGCTCCCCCAATCCTCACT
	reverse	5’ - TTCACCCTGACCCCCTAAATG
IL-10	forward	5’ - TAAGGCTGGCCACACTTGAGA
	reverse	5’ - CGGCTGGGGGATGACAGTAG
GAPDH	forward	5’ - AGCCTCGTCCCGTAGACAAAA
	reverse	5’ - GCCTTGACTGTGCCGTTGAAT
GM-CSF	forward	5’ - ACGGGGCAATTTCACCAAAC
	reverse	5’ - GGCCTGGGCTTCCTCATTTT
G-CSF	forward	5’ - TCCCTGGAGCAAGTGAGGAA
	reverse	5’ - CCCACTGTGGAGCTGGCTTA
CIAP1/2	forward	5’ - TCCAGGAAGTCACGCACAGA
	reverse	5’ - CAGGGAGGGGAAGGGACAAG
BCL-2	forward	5’ - CACCCCTGGTGGACAACATC
	reverse	5’ - TTTGTTTGGGGCAGGTTTGTC
BCL-XL	forward	5’ - ACTGTGCGTGGAAAGCGTAGA
	reverse	5’ - GAGCCCAGCAGAACCACACC
All Real Time PCR		95ºC, 10’ then (95ºC, 30’’; 60ºC, 30’’; 72ºC, 30’’; Repeat 40 cycles)

Supplemental Experimental Procedures

Materials

Antibodies to p65 were from Rockland Immunochemicals Inc. (Gilbertsville, PA), Pan-Ras was from Oncogene Research products (San Diego, CA); TNF, IL-2, IL-12, IFN-β, IL15 and IFN-γ was purchased from Roche Biochemicals (Indianapolis, IN), X-GAL and sodium nitroprusside (SNP) from Sigma (St. Louis, MO). Clodronate/liposomes and PBS liposomes are from clodronateliposomes.com (Haarlem, The Netherlands).

Mice handling and tumor formation

SCID, C57BL/6 and C57BL/6 nude mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Animals were housed at The Ohio State University BRT Animal Facility under sterile conditions with constant temperature and humidity and fed a standard diet. All treatments of mice were in accordance with the guidelines of the Institutional Animal Care and Use Committee. For tumorigenesis assays, all cells were trypsinized, washed and resuspended in saline. All injections were performed with 0.1ml PBS containing appropriate cell numbers for injection subcutaneously into the right flank or both flanks of each mouse. Tumor growth was monitored daily with a digital caliber.

Growth analysis

For growth analysis, 10^4 cells were plated in triplicate into 24 well plate and the cells were trypsinized and counted every day.

Preparation of MEFs and cell culture

MEFs were prepared and 3T3 analysis was performed as previously described (Wang et al., 2009). For reconstitution of MEFs from isolated tumors, mice were sacrificed under sterile conditions and tumors were isolated, minced into small pieces, and subsequently treated with collagenase solution for 30 min at 37ºC. Resulting cell suspension were passed through a 100mm cell strainer. After removing Red blood cells (RBC) with RBC lysis buffer (Biolegend), cells were washed with PBS and then plated into 100mm tissue culture dish. Cultured cells were subsequently selected with hygromycin for 2 weeks to purify MEFs by removing host cells.

Mouse macrophage isolation

Eight-week old mice were intra peritoneally injected with Brewer's modified thioglycolate medium (Sigma). Four days following injection, mice were euthanized and infiltrated cells were washed out from peritonea with DMEM containing 10% heat inactivated FBS. After removing red blood cells with RBC lysis buffer (Biolegend), cells were washed with PBS and cultured in a 100mm dish in a humidified incubator at 37ºC with 5%CO_2 for 3h in full media. Then, cultured dishes were washed twice with serum free DMEM followed by trypsinization for 5-10 min at 37ºC with 5% CO_2. Trypsinized dishes were washed three more times with serum free DMEM. These steps efficiently removed the non-adherent and loosely adherent cells and significantly enriched strongly adherent macrophages (>90%). To harvest, macrophages were lightly scraped into 10ml PBS. After centrifugation, harvested macrophages were resuspend in full media at appropriate concentrations.

Retrovirus preparation and infection

pBabe-hygro was used for expressing human H-RAS^{G12V}, which was generously provided by **G. Leone** (Human Cancer Genetics, Ohio State University). The pBabe-LacZ vector was constructed by subcloning the LacZ gene fragment into the SnaB I site of pBabe-puro. Retroviruses were prepared by calcium mediated gene transfection kit (Promega, Madison WI) as described (Wang et al., 2009). Infection of MEFs was performed by applying 4-5cfu/cell viruses in a total volume of 4ml full culture medium containing 4μg/ml polybrene for 8h.

Alkaline Comet assay

The FLARE-Comet assay kit was purchased from Trevigen (Gaitherburg, MD). Early passage (P4 - P6) primary murine fibroblasts were irradiated with 4-8Gy of X-ray. Irradiated cells were subjected to alkaline comet assay at different time points post irradiation. In brief, cells were embedded on a comet assay module and lysed as recommended by the manufacturer. Lysed comet modules were directly subjected to alkaline electrophoresis as suggested by the manufacturer without FGP enzyme treatment. After electrophoresis, those modules were neutralized with 0.4M Tris at pH7.4, stained with Sybr green. Multiple pictures of fields containing at least 50 comets were acquired under fluorescence microscopy. Comet tail length and percentage of DNA content in comet tails were calculated with Cometscore software (www.tritekcorp.com). The DNA damage index was calculated with a formula as DNA damage index = comet tail length × % of tail DNA content /10000.

MTS assay

MTS reagent was obtained from Promega (Madison WI), and colorimetric MTS assays were performed as recommended by the manufacturer. Briefly, 1000 Ras transformed cells were plated in 96 well plates and cultured overnight. The cells were subsequently treated with different doses of cytokines or chemotherapeutic reagents in triplicate. 48h later 20µl MTS reagent was added into each well and the plate was further incubated for 2 h. The plate was then analyzed on a plate reader with the test wavelength of 490nM and reference wavelength at 650nM.

Cytotoxic T-Cells (CTL) assay

$p65^{+/+}$Ras or *$p65^{-/-}$Ras* cells were either irradiated with 50Gy X-ray or treated with 1mg/ml mitomycin C. X-ray irradiated cells were subcutaneously injected into C57BL/6 mice. Two weeks post injection, cells were isolated from spleen, lymph nodes, and then treated with RBC lysis buffer (Biolegend). Then cells were washed with PBS and subsequently cultured in RPMI1640 containing 10% heat inactivated fetal bovine serum and 50µM mercaptoethanol in 24 well plates containing mitomycin C treated Ras cells. The next day, IL-2 was added to 10 units/ml. Four days later, the expanded CTL were harvested by centrifugation and added to tumor cell lines in a 96 well plates with 5-10 units/ml IL-2. 36 to 48h later, plates were trypsinized and viable cells were counted by trypan blue exclusion assay.

In vitro MDSC production

Bone marrow cells were isolated from 7-10 week old C57BL/6 mice and cultured with RPMI1640 + 10% Heat inactivated FBS. Next day, floating cells were harvested by

centrifugation and then co-cultured with either an experimentally decided number of *p65*$^{+/+}$*Ras* or *p65*$^{-/-}$*Ras* cells or conditioned media derived from 50% of cultured *p65*$^{+/+}$*Ras* or *p65*$^{-/-}$*Ras* cells. After 6 – 7 days, cells were harvested by pooling of both floating and adherent cells and further stained with fluorescent antibodies against CD11b and Gr-1. For antibody neutralization analysis, 25% of conditioned media were used with antibodies against GM-CSF (Biolegend, San Diego, CA), G-CSF (Ebioscience, San Diego, CA) or VEGF (biolegend).

FACS analysis

FITC rat anti mouse Ly-6G (Gr-1), APC rat anti mouse CD11b were purchases from Biolegend (San Diego, CA), and Treg staining kits were purchased from Ebioscience (San Diego, CA). Spleen cells were isolated from C57BL/6 mice 7-9 days after injection with either *p65*$^{+/+}$Ras or *p65*$^{-/-}$Ras cells. Those isolated cells were stained for either Gr-1, CD11b for MDSC or CD4, CD25 and Foxp3 for Treg with protocols recommended by the manufacturer. These stained cells were then analyzed by LSR II or Flowsight 2 (imaging) flow cytometry (Amnis, Seattle, WA).

Immunohistochemistry, immunoblotting, and ELISA

Frozen sections (5μm) were prepared from Ras tumors with similar size. For immunohistochemistry, a Vectastain kit was used from Vector Laboratories (Burlingame, CA). The frozen sections were stained according to a protocol supplied by the manufacturer with an anti p65 antibody. For immunofluorescence staining, frozen sections were prepared and stained as previously described (Wang et al., 2009) with a rat anti mouse F4/80 antibody followed by FITC-Anti-Rat IgG at the dilution of 1:250 (EBioscience; San Diego, CA). After staining, slides were mounted with a DAPI containing mounting solution. Whole cell extract preparation and

western blotting were performed as previously described (Wang et al., 2009). TGF-β Elisa kit was purchased from Biolegend. RPMI 1640 without serum was used to culture an equal number of cells for 48 hours, then supernatants were collected and assays were performed as recommended by the manufacturer.

Semi-quantitative real time RT-PCR

Total RNA was extracted with Trizol reagent from Invitrogen (Carlsbad, CA). Sybr green semi-quantitative real time RT-PCR reagents were obtained from Bio-Rad (Hercules, CA). The amplification efficiency was calculated with different folds of diluted samples as a standard. Copy numbers were calculated with the amplification efficiency derived from a standard curve and the sample application was normalized to GAPDH. For primers sequences, please refer to table S1.

LacZ Genomic Stability Assay

Genomic stability assays dependent on LacZ expression were performed as earlier described (Wang et al., 2009).

Supplemental Reference

Wang, J., Jacob, N.K., Ladner, K.J., Beg, A., Perko, J.D., Tanner, S.M., Liyanarachchi, S., Fishel, R., and Guttridge, D.C. (2009). RelA/p65 functions to maintain cellular senescence by regulating genomic stability and DNA repair. EMBO Rep *10*, 1272-1278.