SUPPLEMENTAL METHODS

Preparation of conditioned media

p65$^{+/+}$*Ras* MEFs were cultured for 48h in DMEM with 10% heat inactivated FBS. The supernatant was then collected and centrifuged at 1000rpm for 5 minutes to precipitate any floating or dead cell and the clear fraction was then used.

Semi-quantitative real time PCR (qRT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen). SYBR green real time PCR reagents were obtained from Roche and used on a thermal cycler (ABI Biosystems). *Gapdh* was used as a control for normalizing gene amplification.

EMSAs and ChIPs

EMSAs and ChIPs were performed as described previously (Bakkar et al., 2012). For EMSAs, 20,000 cpm probes were incubated with nuclear extracts. For supershift assays, 4µg of p65 anti-sera (Millipore Cat. No. 06-418) and 4µg of p50 antisera (Santacruz, Cat. No. sc114) were used. For ChIPs, 2µg of p65 (Millipore, Cat. No. 17-10060) and pPol-II (serine-2 version) (Covance, Cat. No. MMS-129R) and isotype control IgG (Millipore, Cat. No. 17-10060) and IgM (Thermo-Pierce, Cat. No. 31172) were used respectively. DNA from the ChIPs was re-suspended in 50-100µl of water and 2-4µl were used for a qRT-PCR reaction. Results were normalized to input and expressed as fold enrichment over the isotype controls.

Transfections and infections

Transfections for shRNAs and luciferase vectors were performed using Lipofectamine 2000 as described by the manufacturer (Invitrogen). Selection of stable clones wherever necessary were made using puromycin selection. For the luciferase assays, cells were transfected in 12-well

plates and luciferase activity monitored as described previously (26). For retrovirus production, the pRS–muGDF15 shRNA or pRS-scramble vectors (Origene) were transfected along with a pCL-ECO plasmid using a calcium mediated gene transfection kit (Promega) into 293T cells as described previously (26). Infection of Ras-MEFs was carried out by applying 4-6 cfu/cell of virus in a total of 4ml of culture medium containing 4μg/ml of polybrene for 8 hours and stable clones isolated by puromycin selection.

CRISPR design and screening

Guide RNA sequences were identified using a publicly available resource developed by Feng Zhang's lab at Broad Institute of MIT and Harvard (http://crispr.mit.edu/). Sequences of length 866bp spanning exon 1 and half of exon 3 of the murine *p65* gene were selected for deletion. Guide sequences flanking the target site were then cloned into the pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid (Addgene) and transfected into KPC luciferase cells. Individual cell clones were grown and screened by qPCR for the deletion. A western blot was also performed on the selected clones to ensure total knockout of protein expression of p65 as well.

ELISA, immunoblots and immunohistochemistry

ELISA kits for mouse GDF-15 were purchased from MyBiosource (Cat. No. MBS2884063) and performed as described by the manufacturer. Samples were mainly conditioned media with 10% heat inactivated FBS. For immunoblots, whole cell extracts were made using RIPA buffer and phosphatase inhibitors (5μl/ml) and protein concentration estimated by the standard Bradford assay. 30-50μg of protein was used per assay. Primary bone marrow macrophages used in immunoblots were obtained from naïve bone marrow cells of C57BL/6 mice that were differentiated for 5-7 days with 100ng/ml recombinant M-CSF(R and D Systems) and then activated overnight with 100ng/mL LPS (Biolegend) and 2ng/ml INF-γ (5ng/ml) (Biolegend). In

the case of Raw 264.7 cells used for immunoblots, the cells were plated in adherent dishes with serum free RPMI overnight prior to time course treatment with rGDF-15. Antibodies used for western blots include p65 (Santa Cruz, 1:1000, Cat. No. sc109), pSMAD-2 (Cell Signaling Technology, 1:1000, Cat. No.3101), pSmad-3 (Millipore, 1:1000, Cat. No. 07-1389), Smad2/3 (Cell Signaling Technology, 1:100, Cat. No. 5678), pTAK1 (Cell Signaling Technology, 1:1000, Cat. No. 9339), TAK1 (Cell Signaling Technology, 1:1000, Cat. No. 5206), pp38 (Cell Signaling Technology, 1:1000 Cat. No. 4511), p38 (Santa Cruz, 1:1000 Cat. No. sc535), pIκB-α (Cell Signaling, 1:1000, Cat. No. 2859) and IκB-α (Santa Cruz, 1:1000, Cat. No. sc371). For immunoblots involving pIκB-α the cells were first treated with proteasome inhibitor MG132 (Selleckchem.com) (10μM) for 1h prior to treatment for varying lengths of time with rGDF-15. Control blots for the same cells treated with MG132 alone were run to verify that there were no changes in pIκB-α expression due to treatment with MG132. For immunohistochemistry, pancreatic tissue was fixed in 10% formalin overnight and paraffin embedded. The sectioned tissue was then stained using the Bond Rx (Leica) autostainer for Ki67 (Abcam, 1:200, Cat. No. ab1667), SMA (Abcam, 1:5000, Cat. No. ab5694), cleaved caspase-3 (Cell Signaling Technology, 1:800, Cat. No. 9664), and Gr-1 (Ly6G) (Biolegend 1:200, Clone RB6-8C5). The stained sections were imaged using a Vectra Multispectral Imaging System and quantified using the inForm software (Perkin-Elmer). For immunoflorescence, paraffin embedded pancreatic tissue was sectioned, deparaffinized and stained with FITC anti-mouse F4/80 (Biolegend, 1:250, Clone BM8) and PE-anti-mouse TNF-α (Biolegend, 1:250, Clone MP6-XT22). For staining patient PDAC tissue the same process of deparaffinization was carried out and antigen retrieval performed using Tris-EDTA buffer (pH 9.0) and stained with rabbit anti-NF-κB (phospho S536) antibody (Abcam, 1:250, Cat. No. ab86299) and mouse anti-human GDF-15 (Atlas Antibodies, 1:300, AMAb90687). Anti-rabbit Alexa fluor 568 and anti-mouse Alexa fluor 488 were used as counter stains. After staining, slides were mounted with DAPI containing mounting solution.

Images of these slides were taken in a Nikon e800 Fluorescent Microscope. For the Z-stack video images were taken on a Multispectral Confocal Microscope.

Xenograft tumor implantation and measurement

$1X10^5$ Ras MEF cells in 100µl of PBS was injected subcutaneously into SCID mice. In case of the experiment where *p65*$^{+/+}$Ras MEFs were injected to test the effect of neutralizing GDF-15 during tumor growth 1µg of GDF-15 Nab (Abgent, Cat. No.ALS11572)/ IgG control was added to the cell mixture prior to subcutaneous injection. Subsequent weekly injections used 20µg antibody per mouse in PBS delivered by tail vein injection. For the experiment with *p65*$^{-/-}$ Ras MEFs with rGDF-15 (MyBiosource), 1µg of protein was mixed in the cell suspension prior to sub-cutaneous injection. Subsequent weekly injections used 20µg protein per mouse in PBS delivered by tail vein injection. Tumor nodules were measured by touch and using calipers every other day starting at 7 days post injection.

Peritoneal cell analysis

Peritoneal cells were isolated 4-5 days after injecting $2X10^4$ cancer cells (*Ras*-MEFs and pancreatic cancers cells) in PBS intra-peritoneally into SCID mice. These cells were then stained with FITC anti-mouse F4/80 (Biolegend, Clone BM8) and PE anti-mouse TNF-α (Biolegend, Clone MP6-XT22). FACS was then used to analyze the cells. FlowSight2 software was used to analyze cells. The cells were first gated to obtain total number of F4/80$^+$ single cells that were in-focus. From these F4/80$^+$ cells, those that expressed TNF were subsequently gated out. Isotype controls were run using mice spleen cells to test specificity of F4/80 and TNF antibodies.

Cell growth analysis

Cell growth analysis was performed as described previously (10).

MTS assay

Colorimetric MTS assays were performed as recommended by the manufacturer (Promega). Similar to the assay performed earlier (27) 1000 KPC control and KPC $\Delta p65^{CRISPR}$ cells were plated in 96 well plates and cultured overnight. The cells were subsequently treated with different doses of cytokines. 48h later 20μl MTS reagent was added into each well and further incubated for 2h. The plate was then analyzed on a plate reader with the test wavelength of 490nM and reference wavelength at 650nM.

Clodronate depletion of macrophages and tumor growth

Age matched mice (6 weeks of age, weighing appx.16-17g) were injected with clodronate/ PBS liposomes (www.clodronateliposomes.com) intraperitoneally (250μl/20g) two days prior to tumor implantation. Subsequently, mice were injected subcutaneously with $1x10^6$ KPC shGDF-15 cells. Following tumor cell implantation, the mice were dosed biweekly with either clodronate or PBS liposomes (250μl/20g) for 3 weeks and tumor measurements were made starting 5 days post tumor cell injection by touch or use of vernier calipers every other day.

Prior to subcutaneous tumor implantation, on a separate cohort, a similar clodronate/PBS regimen was tried on orthotopically-implanted tumors within the mouse pancreas. Unfortunately the mice died due to intense dehydration and malnutrition four days into the experiment perhaps due to combined effects of an invasive surgical procedure used to implant tumor cells into the pancreas alongside a massive depletion in circulating monocytes by clodronate. Hence, a change in strategy was applied and we performed subcutaneous tumor injections that are non invasive.

Animal handling

C57BL/6 mice were obtained from Jackson Laboratory and SCID mice from Taconic Biosciences and housed in The Ohio State University Biomedical Research Tower Animal Vivarium under sterile conditions with constant temperature and humidity and fed a regular diet. All mice were bred and treated in accordance to the regulations of the Institutional Animal Care and Use Committee. All cell, protein and antibody injections for xenograft studies were made using PBS. Matrigel was used in case of the orthotopic tumor implants.

Bioinformatic analysis for RNA-seq

Gene sequences were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/genome/) and/or UCSC genome browser (http://genome.ucsc.edu/). Transcription factor binding site analysis was done using rVISTA (http://rvista.dcode.org/). For RNA sequencing analysis, the sequencing reads were first aligned to mm10 using STAR 2.4 (Spliced Transcripts Alignment to a Reference). Subsequently, HOMER (Hypergeometric Optimization of Motif EnRichment) (http://homer.salk.edu/homer/) software was used to create tag directories and quantify gene expression. HOMER uses the edgeR program to determine differential gene expression. Finally, ToppFun (Transcriptome, ontology, phenotype, proteome, and pharmacome annotations based gene list functional enrichment analysis) was used to detect functional enrichment of genes downregulated in the absence of p65 using Bonferroni multiple correction method and 0.05 significance cut-off as parameters.

Supplementary Figure 1
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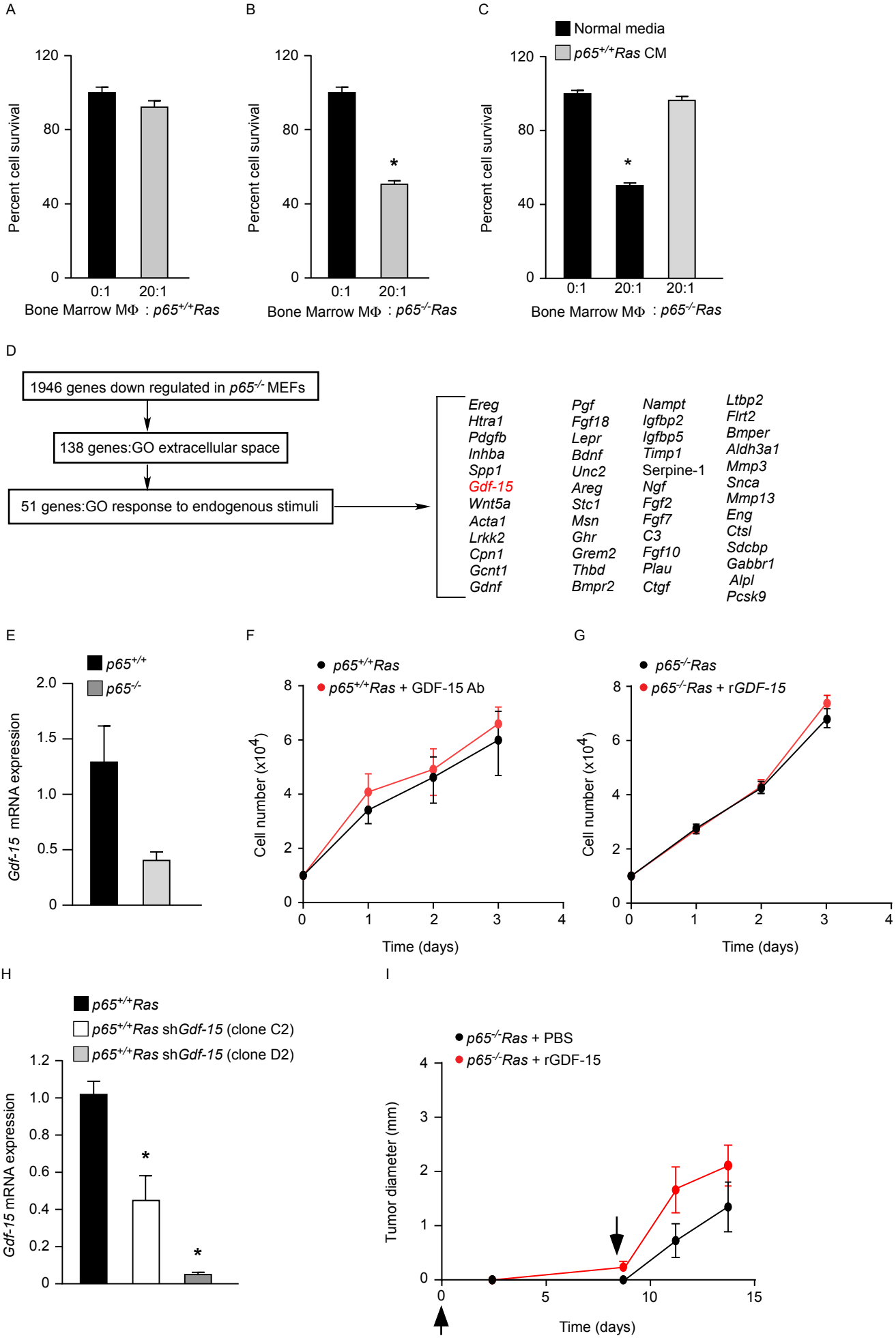

Suppl. Figure 1. Identifying GDF-15, a secreted factor capable of overcoming macrophage mediated killing of tumor cells and promoting tumor development in vivo.

(A-B) Bone marrow macrophages (BMM) were co cultured with *p65*$^{+/+}$ and *p65*$^{-/-}$*Ras* MEFs respectively and cell survival analyzed by trypan blue exclusion and normalized to untreated *p65*$^{+/+}$ and *p65*$^{-/-}$*Ras* MEFs respectively (n=6, mean ± SEM *p≤0.05, Student's *t* test). (C) *p65*$^{-/-}$ *Ras* MEFs were co-cultured with BMMs either with normal media or conditioned media from *p65*$^{+/+}$*Ras* MEF cultures. Graph represents cell survival scored by trypan blue exclusion similar to A-B (n=6, mean ± SEM *p≤0.05, 1-way ANOVA). (D) Flowchart representing analysis of RNA-Seq. data and identification of *Gdf-15* as a potential gene of interest. (E) Validation of RNA seq. results in MEFs by qRT-PCR showing average gene expression (normalized to *Gapdh*) (mean ± SEM, Student's *t* test). (F) Measuring growth of *p65*$^{+/+}$*Ras* MEFs treated with 625ng/ml of GDF-15 Ab by trypan blue exclusion method at designated time points. Data represent mean ± SEM from two separate experiments, each performed in triplicates (2-way ANOVA). (G) Measuring growth of *p65*$^{-/-}$*Ras* MEFs treated with 5ng/ml of rGDF-15 similar to (F). Data represent mean ± SEM from two separate experiments, each performed in triplicate (2-way ANOVA). (H) qRT-PCR to verify knockdown of GDF-15 in *p65*$^{+/+}$ *Ras* MEFs transduced with shRNA for *Gdf-15*. Data represent average gene expression from two selected single clones (normalized to *Gapdh*) compared to control *p65*$^{+/+}$ *Ras* MEFs (*p≤0.05, 1-way ANOVA). (I) *p65*$^{-/-}$*Ras* MEFs (1 x10^{6}) were injected subcutaneous in SCID mice (n=10). Arrowheads indicate time points at which each cohort of mice (n=5) were injected with rGDF-15 (20μg/mouse) or PBS as control. Tumor sizes were measured at indicated time points (SPSS Repeated Measures, General Linear Model).

Supplementary Figure 2
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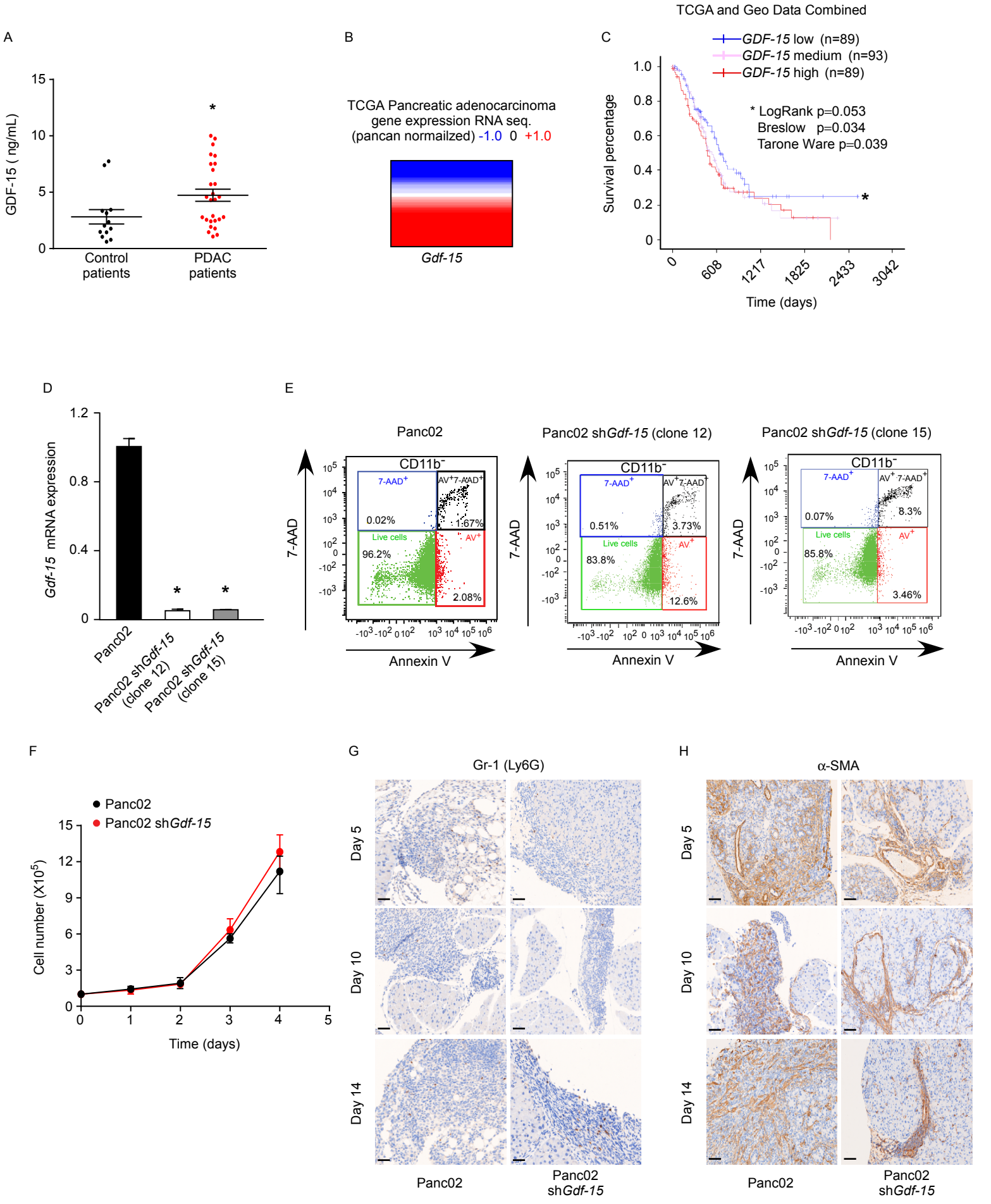
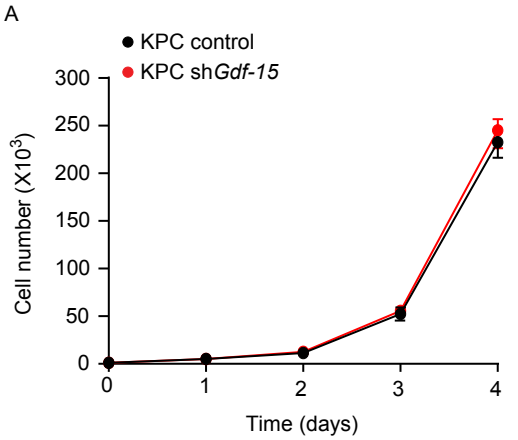


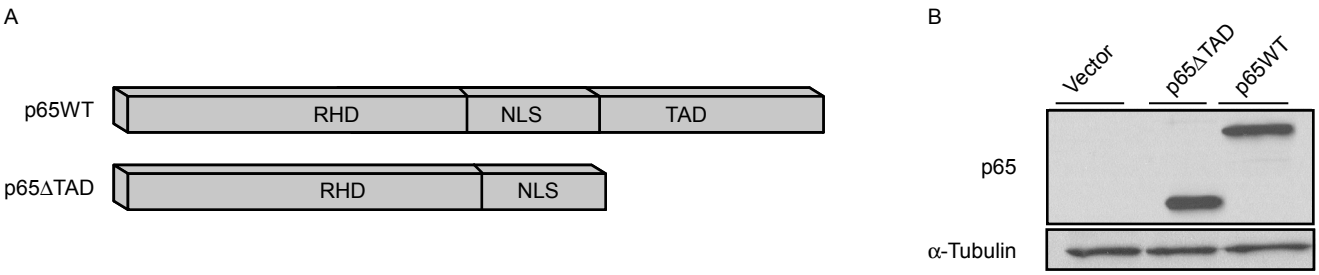
Suppl. Figure 2. GDF-15 affects pancreatic cancer development.

(A) ELISA for GDF-15 on plasma from 13 control non-PDAC and 27 PDAC patients (mean ± SEM) (*p≤0.05, Student's *t* test). (B) Gene expression of GDF-15 from 183 PDAC patients obtained from RNA seq. (pan-cancer normalized), from the TCGA database. (C) Kaplan-Meir survival curve for 271 patients with PDAC based on the level of *Gdf-15* expression combined from TCGA and a published study on the Geo Database. The blue line represents PDAC patients with low *Gdf-15* expression, while the white line represents patients with medium *Gdf-15* expression and the red line indicates patients with high GDF-15 expression (* compares GDF-15 high and low groups). (D) qRT-PCR was performed to verify knockdown of *Gdf-15* in Panc02 cells transfected with shRNA for *Gdf-15*. Data represent average gene expression ± SEM from two selected single clones (normalized to *Gapdh*) compared to control Panc02 cells (*p≤0.05, 1-way ANOVA). (E) Representative flow cytometry plots for results from cell viability assays involving co-cultures of control Panc02 cells with *Gdf-15* knockdown Panc02 clones as described in Figure 2B. $CD11b^-$ cells were then gated based on 7-AAD and AV apoptosis markers. (F) Cell growth analysis of control Panc02 cells and *Gdf-15* knockdown Panc02 cells (clone 12) using trypan blue exclusion method. Data represent mean ± SEM from two individual experiments each performed in triplicates (2-way ANOVA) (G) Immunohistochemistry staining for granulocyte infiltration on sections from control and *Gdf-15* knockdown orthotopic pancreatic tumors. All images were taken at 20X magnification. Scale bar denotes 15 microns. (H) α-SMA staining for stromal activation on sections from orthotopic pancreatic tumors from control and *Gdf-15* knock down Pan02 cells. All images were taken at 20X magnification. Scale bar denotes 15 microns.



Suppl. Figure 3. Comparison of ex-vivo growth KPC control and KPC Gdf-15 knockdown cells.

(A) Cell growth analysis of control KPC control cells and *Gdf-15* knockdown KPC cells (clone 1) using trypan blue exclusion method. Data represent mean ± SEM from two individual experiments each performed in triplicates (2-way ANOVA).



Suppl. Figure 4. NF-κB is direct transcriptional regulator of GDF-15.

(A) Schematic representing p65-full length (p65WT) and ΔTAD reconstituted in *p65*$^{-/-}$MEFs. (B) Western blot from *p65*$^{-/-}$MEFs infected with virus encoding p65 and p65ΔTAD, with empty vector as control. α-tubulin serves as a loading control.

Supplementary Figure 5
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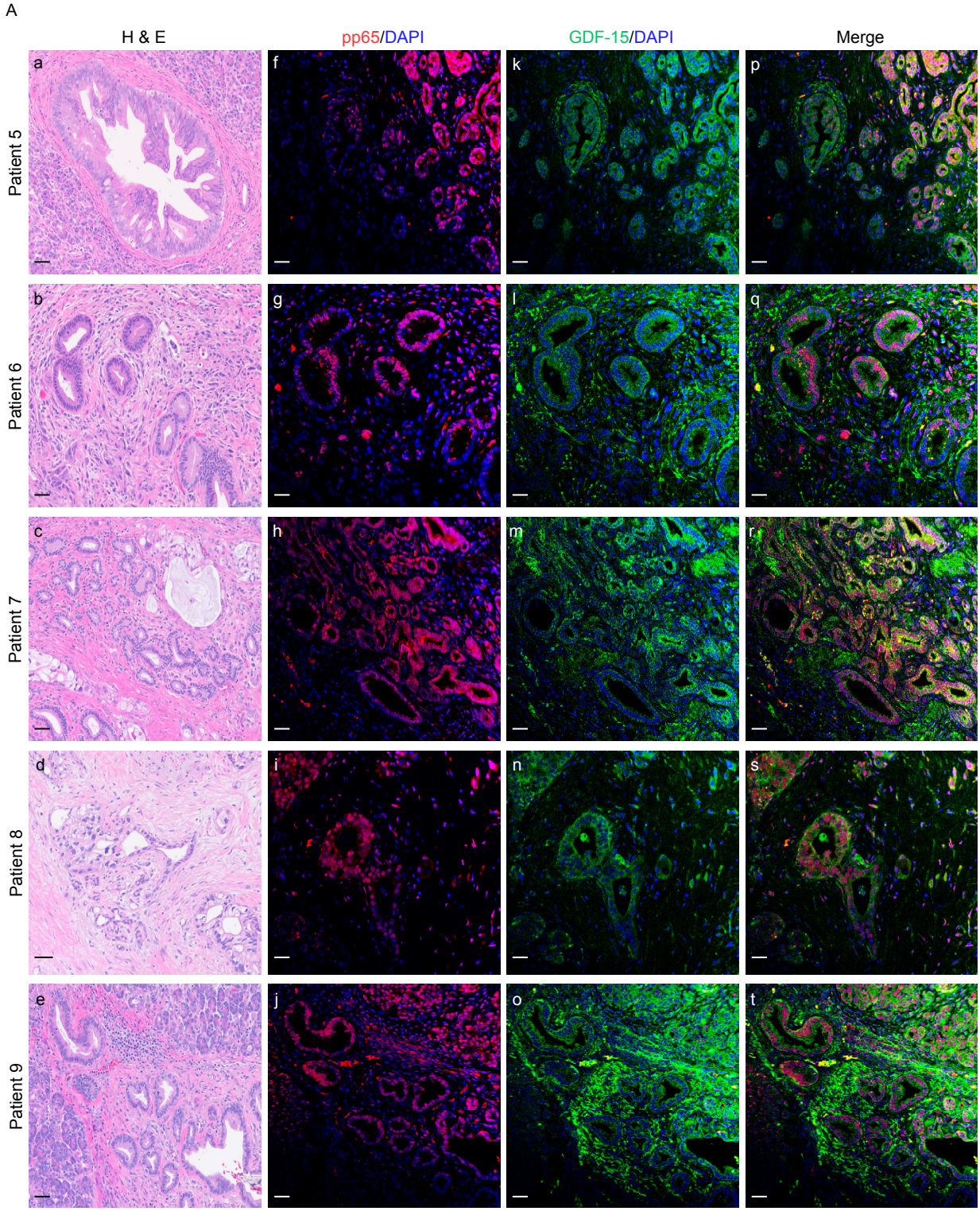

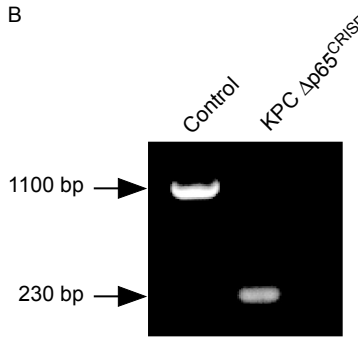

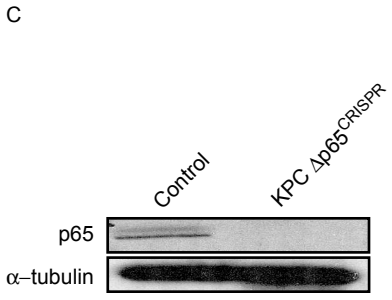

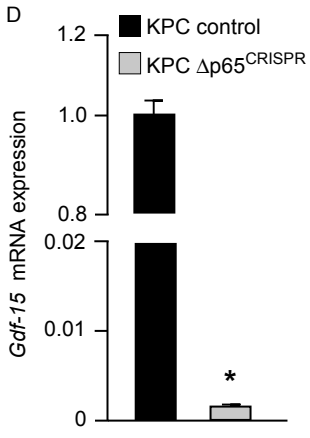

Suppl. Figure 5. NF-κB regulates GDF-15 expression in KPC cells.

(A) Representative images for five patient samples with histologically confirmed PDAC. (a-e) H&E images; (f-j) immunofluorescence staining for phospho-p65 (pp65) (nuclear stain) and nuclei counterstained with DAPI; (k-o) immunofluorescence staining for GDF-15 (cytoplasmic stain) and nuclei counterstained with DAPI; (p-t) merged images of pp65, GDF-15, and DAPI staining. All images were taken at 20X magnification. For H& E images scale bar denotes 15 microns. For fluorescent images scale bar denotes 100 microns. (B) Agarose gel image representing deletion in p65 in KPCΔp65^{CRISPR} clone compared to control KPC cells. (C) Western blot to ensure protein level knockout of p65 in KPCΔp65^{CRISPR} clone shown Suppl. Figure 5B. (C) *Gdf-15* gene expression analysis by qRT-PCR in KPCΔp65^{CRISPR} clone shown previously Suppl. Fig 5A and 5B. Data represent average gene expression ± SEM (normalized to *Gapdh*), compared to control KPC cells (*p≤0.05, Student's *t* test).

Supplementary Figure 6
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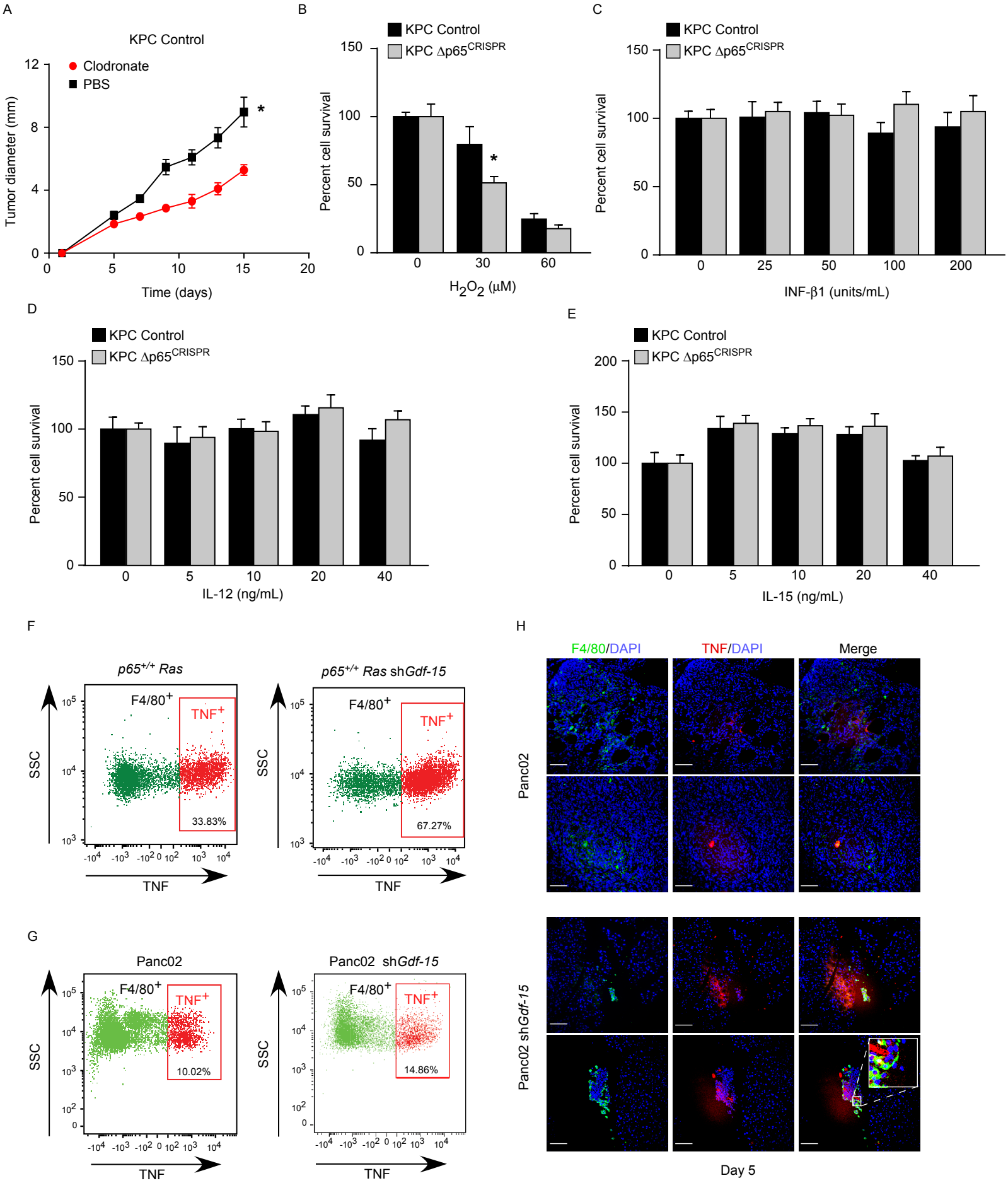
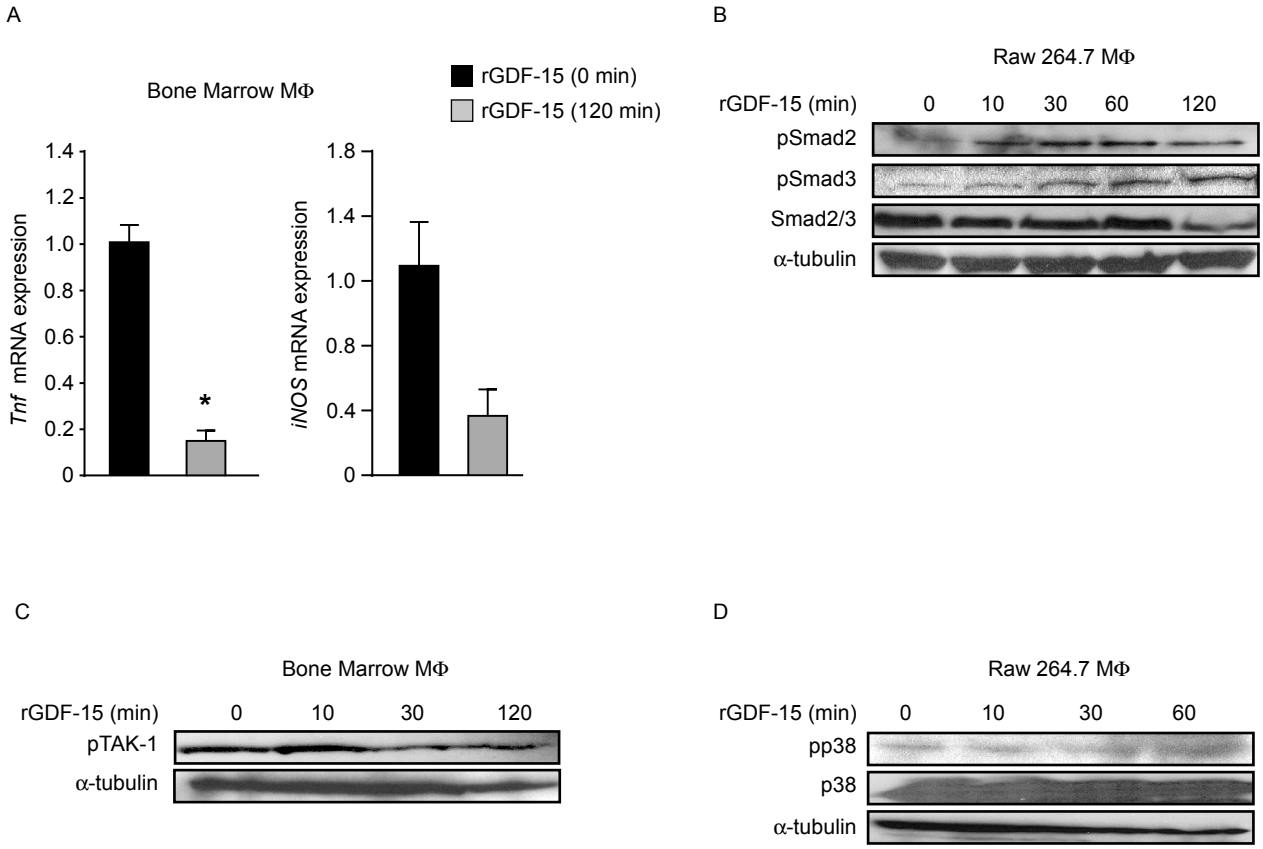


Suppl. Figure 6. GDF-15 suppresses macrophage cytotoxic activity by inhibiting the production of TNF.

(A) $1x10^6$ KPC control cells were subcutaneously injected into C57BL/6 mice and treated either with clodronate liposomes (250μg/20mg) or control PBS liposomes (250μg/20mg) twice a week and tumor sizes measured (mean ± SEM, *p≤0.05, SPSS Repeated Measures, General Linear Model). (B-E) MTS assay to test the effects of increasing concentrations of H_2O_2, INF-β1, IL-12 and IL-15 on KPC control and KPC $\Delta p65^{CRISPR}$ cells. Data represents the mean from two independent experiments each done in triplicates (*p≤0.05, 2-way ANOVA). (F-G) Representative flow cytometry plots for results macrophage isolations done after intraperitoneal injections of *p65*$^{+/+}$*Ras* MEFs and *Gdf-15* knockdown *Ras* MEFs and Panc02and *Gdf-15* knockdown Panc02 cells respectively. TNF^+ cells were gated from the total $F4/80^+$ cells isolated. (H) Orthotopic pancreatic tumors from mice injected with either Panc02 or Panc02 cells containing a knockdown for *Gdf-15*. Tumors harvested 5 days post injection were stained by immunoflorescence for F4/80 and TNF. All images were taken at 20X magnification. Scale bar denotes 100 microns.



Suppl. Figure 7. GDF-15 signals in macrophages to suppress NF-κB signaling via TAK1.

(A) Bone marrow macrophages (BMMs) were treated with 5ng/ml of rGDF-15 for 2h and then *Tnf* and *iNOS* expression was quantitated by qRT-PCR (*p≤0.05, Student's *t* test). (B) Western blot was performed on cell lysates from Raw 264.7 macrophages treated with 5ng/ml rGDF-15 to probe for pSMAD2, pSMAD3. On a separate blot, the same cell lysates were probed for total SMAD2/3. In both cases α-tubulin served as a loading control. (C) Western blot was performed on cell lysates from BMMs treated with 5ng/ml of rGDF-15 to probe for phospho-TAK1 activation. α-tubulin was used as a loading control. (D) Western was blot performed on cell lysates from Raw 264.7 macrophages treated with 5ng/ml of rGDF-15 to probe for pp38 and p38. α-tubulin was used as a loading control.

Supplementary Table 1

Database	Accession	Score	Mass	Num.of significant matches	Num.of significant sequences	em PAI	Description	Relative Abundance
SwissProt_ID	*GDF15_MOUSE*	*3424*	*33718*	*215*	*8*	*5.53*	*Growth/differentiation factor 15,OS=Mus musculus*	*85.20801233*
SwissProt_ID	*ANGL4_MOUSE*	*453*	*45966*	*11*	*4*	*0.52*	*Angiopoietin-related protein,OS=Mus musculus*	*8.012326656*
SwissProt_ID	*K2C8_MOUSE*	*91*	*54531*	*3*	*3*	*0.19*	*Keratin,type II,OS=Mus musculus*	*2.927580894*
SwissProt_ID	*K2C1B_MOUSE*	*75*	*61379*	*2*	*2*	*0.11*	*Keratin,type II,OS=Mus musculus*	*1.694915254*
SwissProt_ID	*K1C15_MOUSE*	*50*	*49278*	*2*	*2*	*0.14*	*Keratin,type II,OS=Mus musculus*	*2.157164869*
					Sum of Empai	*6.49*		

Suppl. Table-1. rGDF-15 is free of TGF-β contamination.

Table showing the results from a mass spectrometry analysis indicating the purity of commercially available mouse rGDF-15 used in this study.

Supplementary Table 2

Primer	Sequence	T_m (°C)
mu GDF-15 qRT-PCR	F- ggcggctctcaactgaggtt R- agggtcgctgttcaggcatt	60
mu TNF qRT-PCR	F- cccaaagggatgagaagttccc R- cctggtatgagatagcaaatcgg	55
mu iNOS qRT-PCR	F- ggtggtgacaagcacatttgg R- tgggacagcttctggtcgat	60
mu GAPDH	F- agcctcgtcccgtagacaaaa R- gccttgactgtgccgttgaat	60
mu GDF-15 ChIP (p65 and pPolI)	F- ccggtgttctggtttcttct R- ttcaggggcctagtgatgtc	60
mu TNF site1 ChIP	F- ggggagaagtgactccactg R- tgctcccaagtgagttttcc	60
mu TNF site2 ChIP	F- cgcagtcaagatatggcaga R- gggggtaatggatgagtatg	60

mu iNOS site1 ChIP	F- ttgaggccacacacttttg R- tgacagtgttaggggaaaagg	60
mu iNOS site2 ChIP	F- atggccttgcatgaggatac R- gcagcagccatcaggtatt	60
For all qRT-PCRs the cycling conditions were: 95°C for 10min, 94°C for 15 sec, 60°C for 30 sec and 72°C for 20 sec (data collection)(40cycles). This is followed by melt curve analysis. For all muTNF qRT-PCR the cycling conditions were: 95°C for 10min, 94°C for 15 sec, 55°C for 30 sec and 72°C for 20 sec (data collection)(40cycles). This is followed by melt curve analysis.		

Suppl. Table-2. Primers and qRT-PCR cycling conditions.

Table listing the primers and reaction conditions used for gene amplification by qRT-PCR in this study.