The scaffolding protein EBP50 promotes VSMC proliferation and neointima formation by regulating Skp2 and $p21^{cip1}$.

SUPPLEMENTAL MATERIAL

Supplemental Methods

Experimental animals and surgeries. Animal studies were performed in 10 weeks old wild type (WT) C57BL/6 mice and EBP50 knockout (KO) littermates. Mice were anesthetized using Ketamine (45 mg/kg) and Xylazine (5 mg/kg) i.p., shaved locally and washed with betadine. The left femoral artery was exposed and lidocaine was topically applied to induce vasodilatation. A guidant hi-torque guide wire 0.014" was inserted into the femoral artery and passed within the artery 3 times. The right femoral artery was used as a control uninjured artery. Sterile packaged autoclips were used to close the wound and the area was wiped with betadine. Femoral arteries were removed 2 weeks post surgery. All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Immunofluorescence and immunohistochemistry. Femoral arteries (both control and injured) were excised from WT and KO mice, fixed with 4% paraformaldehyde and embedded in OCT Tissue-Tek (Sakura Finetek). Sections (10 μm) were analyzed by immunohistochemistry. For CD31 and α-actin staining, sections were treated with 10% goat serum/ 0.5% TritonX-100 and incubated sequentially with rat anti-mouse CD31 (BD Pharmingen), Alexa546-conjugated anti-rat secondary antibody (Invitrogen) and FITC-conjugated anti-smooth muscle α-actin antibody (Sigma) for 1 h at RT. For EBP50 and Ki67 (celluar marker for proliferation) staining, sections were incubated in boiled Tris-EDTA buffer (10 μM Trizma base, 1.25 μM EDTA, 0.05% Tween 20, pH 9.0) for 10 min, blocked in 4% NGS, 1% BSA, 0.5% Triton X-100 for 20 min at room temperature and incubated with anti-EBP50 antibody (Thermo Pierce, 1:250 dilution) or Ki67 antibody (Labvision, 1:100 dilution) overnight. As a secondary antibody, anti-rabbit Alexa546-conjugated IgG (Invitrogen, 1:250 dilution for EBP50 and 1:200 for Ki67) was used.

For $p21^{cip1}$ and $p27^{kip1}$ staining, sections were boiled in citric acid buffer for 10 min and incubated with 0.3% H_2O_2 in 80% methanol for 30 min. Anti-$p21^{cip1}$ and anti-$p27^{kip1}$ antibodies (Santa Cruz, 1:100 dilution) were added in 1% goat serum and 1% BSA after blocking in 5% normal goat serum and 1% BSA in PBS for 30 min. As a secondary antibody, anti-mouse IgG HRP-conjugated (Cell signaling, 1:250

dilution) was used. After washing with PBS, sections were incubated with ABC reagent for 30 min and visualized with DAB kit (VECTOR).

Expression of $p21^{cip1}$ and PTEN in VSMC was determined as follows. Cells on glass coverslips were fixed with 2% paraformaldehyde and incubated with blocking buffer containing 5% goat serum and 0.2% Nonidet P-40 (NP-40), 0.01% sodium dodecyl sulfate (SDS) in PBS. A rabbit anti-$p21^{cip1}$ antibody (Santa Cruz) or anti-PTEN antibody (Santa Cruz) was applied at a dilution of 1:500 in the same buffer overnight at 4 ºC. Coverslips were washed with PBS, incubated with Alexa546-conjugated anti-rabbit secondary antibody (1:1000, Molecular Probes) and 4',6-diamidino- 2-phenylindole (DAPI, 0.1 μg/ml; Sigma) for 3 h and washed again. Coverslips were mounted for immunofluorescence microscopy and analyzed with an Olympus Fluoview confocal laser-scanning microscope with a x63 oil immersion objective. To quantify expression, images were acquired in five regions of the coverslip using an identical microscope setting. The focus was adjusted to obtain maximal intensity of the DAPI signal. Membrane expression of PTEN was imaged using total internal reflection fluorescence (TIRF) microscope (Nikon Ti-TIRF) equipped with 60x Oil TIRF objective (NA=1.49). Apoptosis in vessels and cultured cells was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling [TUNEL] assays using the "Click-it TUNEL Alexa Fluor594 Imaging Assay" (Invitrogen), according to the manufacturer's instructions. The fluorescence intensity of each image was determined with Image J software (National Institutes of Health).

Histomorphometric analysis. Frozen femoral artery sections (10 μm thick) from WT and KO mice were selected at 100 μm intervals and stained using an Elastic Stain Kit (Sigma). H&E staining of arteries sections were performed by the University of Pittsburgh Research Histological Services. Images were captured using a Leica DM5000B light microscope using a 40x objective. Images were analyzed with the Image J software (National Institutes of Health).

Primary VSMC culture and transfections. Primary VSMC were isolated from abdomen aortic explants and cultured in DMEM containing 10% FBS in 5% CO_2 at 37°C. All experiments were performed with cells between passage 3 and 15. Small interfering RNA (siRNA) for EBP50 and $p21^{cip1}$ were generated by Dhamarcon (Thermo Scientific) as follows. EBP50: 5'-GAAGGAGAGCAGCCGUGAAdTdT3' (sense) and 5'-UUCACGGCUGCUCUCCUUCdTdT-3' (antisense). $p21^{cip1}$: 5'-GACAAGAGGCCCAGUACUUdTdT3' (sense) and 5'-AAGUACUGGGCCUCUUGUCdTdT-3' (antisense). Accell Non-targeting siRNA (Dharmacon, Thermo Scientific) was used as a control siRNA. Cells were transfected with siRNAs (0.1 μM) using DharmaFECT Duo (3 μl/well of 12 well plates) transfection reagent (Dharmacon, Thermo Scientific) in DMEM with 1% FBS in the absence of antibiotic according to the manufacturer's instructions. Cells were used for the experiment 72 h after transfection.

YFP-tagged and Flag-tagged EBP50, Flag-tagged Skp2 [1] (a generous gift from Dr. Michele Pagano, New York University School of Medicine), Myc-Akt (4 μg) were introduced in primary VSMC ($1x10^6$) by electroporation using an AMAXA electroporator and the Basic Nucleofect kit for primary smooth muscle cells (Lonza) . EBP50, constitutively active Myr-Akt (a generous gift from Dr. Daniel Altschuler, University of Pittsburgh School of Medicine) and Myc-$p21^{cip1}$ [2] (a generous gift from Dr. Richard Steinman, University of Pittsburgh School of Medicine) was transfected in CHO cells using Fugene6.
Lentiviral shRNA vectors (pLKO.1-puro) specific to mouse EBP50 (NM_012030) and GFP-expression control lentivirus (pLKO.1-puro-TurboGFP) were prepared by the Lentiviral Facility at the University of Pittsburgh Cancer Institute. The target sequences of the EBP50 shRNAs are: shEBP50 #1 GAGTTCTTCAAGAAGTGCAAA and shEBP50 #2 GACCGAATTGTGGAGGTCAAT. Aliquots of virus (titer ~10^6), plus 8 μg/mL of polybrene, were used to infect exponentially growing cells (1 x 10^5/mL). Fresh medium was supplemented at 24 hours after the infection. Cells were used 72 hours after infection.

Western blot analysis. Cells were lysated in urea lysis buffer (4M urea, 62.5mM TrisCL, 2% SDS, 1mM EDTA) containing a protease inhibitor cocktail. The cell lysates were resolved on 12% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane, which was then subjected to two sequential incubations with appropriate primary antibodies (1:500 dilution for EBP50, cyclin E, $p21^{CIP1}$, cyclin D1, cyclin D3, CDK2, CDK4 and 1:1000 dilution for SKP2 (all from from Santa Cruz); 1:1000 dilution for $p27^{KIP1}$, pAKT, AKT, Myc tag antibodies (from Cell Signaling); 1:5000 dilution for βactin (from Sigma)) and horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (1:2000 dilution, Cell signaling). Immunoreactivity was detected by incubation with Immune-Star ECL (Bio-Rad). Quantitation of band intensity was performed with the Image J software (National Institutes of Health).

Quantitative Real-Time PCR. Total RNA from VSMC was obtained by using the RNA easy micro kit (Qiagen, Valencia, CA) following the instructions of the manufacturer. One μg of total RNA was reverse transcribed in the following reaction buffer (Promega, Madison, WI): ImProm-II reverse transcriptase (1 μl), 1X reaction buffer, RNAse inhibitor (20 U), dNTP (10 mM each) and $Oligo(dT)_{15}$ primer (1 μM) at 42 ºC for 1 h. As a negative control, water was used instead of reverse transcriptase. cDNA was used to amplify $p21^{cip1}$ and GAPDH using specific primer sets (*$p21^{cip1}$*, forward 5'-ACGGTGGAACTTTGACTTCG-3' and reverse 5'-GAGTGCAAGACAGCGACAAG-3'; *$p27^{kip1}$*, forward 5'-GATACGAATGGCAGGAGGTG-3' and reverse 5'-TCTGACGAGTCAGGCATTTG-3'; *Skp2*, forward 5'-CCAACACCTCTCGCTCAG-3' and reverse 5'-CCAGGTTCTTCTTGCTGTCC -3'; *GAPDH*, forward 5'-CTCATGACCACAGTCCATGC-3' and reverse 5'-

ATGTAGGCCATGAGGTCCAC-3'). Quantitative gene expression analysis was performed on an ABI PRISM 7700 (Applied Biosystems, Warrington, UK) using SYBR Green technology. In 48-wells optical plates, 10 µl of SYBR Green master mix (Applied Biosystems, Warrington, UK) was added to 2 µl of cDNA and 300 nM forward and reverse primers in water. Following 10 min at 95 ºC, 40 cycles of 15 s at 95 °C, 30 s at 60 °C and 60 s at 72 ºC were applied. At the end of the run, samples were heated to 95 °C to check melting curve. The absence of genomic DNA contamination in the RNA preparations was confirmed by using total RNA samples that had not been subjected to reverse transcription. GAPDH was used as the standard housekeeping gene. Relative gene expression was normalized by GAPDH expression.

Proliferation Assays. Cells on 24-well plates were transfected as indicated and cultured until 70-80% confluent. Cells were incubated with 1 µCi/ml [^{3}H]thymidine in the culture media for 18 hours at 37 °C, rinsed with PBS and exposed to 10% trichloroacetic acid (TCA) for 10 min. TCA was removed and the cell monolayers dissolved in 1 N NaOH for the determination of radioactivity. For BrdU incorporation experiments, cells were grown on coverslips and incubated in the presence of 100 µM BrdU for 16 h. After fixation with 4% paraformaldehyde, cells were permeabilized in 0.5% Triton X-100/PBS for 30min and incubated with anti-BrdU antibody (1:100, Biodesign International) in blocking solution (10% promega RQ1 DNaseI, 1% BSA, 1% FBS in PBS) at 37ºC for 1 hour. Flag-Skp2 was visualized with anti-Flag antibody (Sigma, 1:1000) in the same buffer. Cells were rinsed in PBS, incubated with secondary antibody anti-sheep Alexa 594 (1:1000, Molecular Probes) or anti-rabbit Alexa 488 (1:1000, Molecular Probes) in PBS containing 1% BSA, 1% FBS.

For cell cycle profile, VSMC were grown to subconfluence in DMEM containing 10% FBS. Cells collected from 6 cm dishes were rinsed twice with PBS and fixed with 70% ethanol at 4 ºC overnight. Fixed cells were washed with PBS, pelleted, and resuspended in the staining PBS solution containing 50 µg/mL propidium iodide, 100 U/mL RNAse A, and 1 g/L glucose. The cell cycle profile and forward scatter were determined using a Becton Dickinson FACS Caliber, and data were analyzed using the ModFit LT 2.0 (Verity Software House, Inc.).

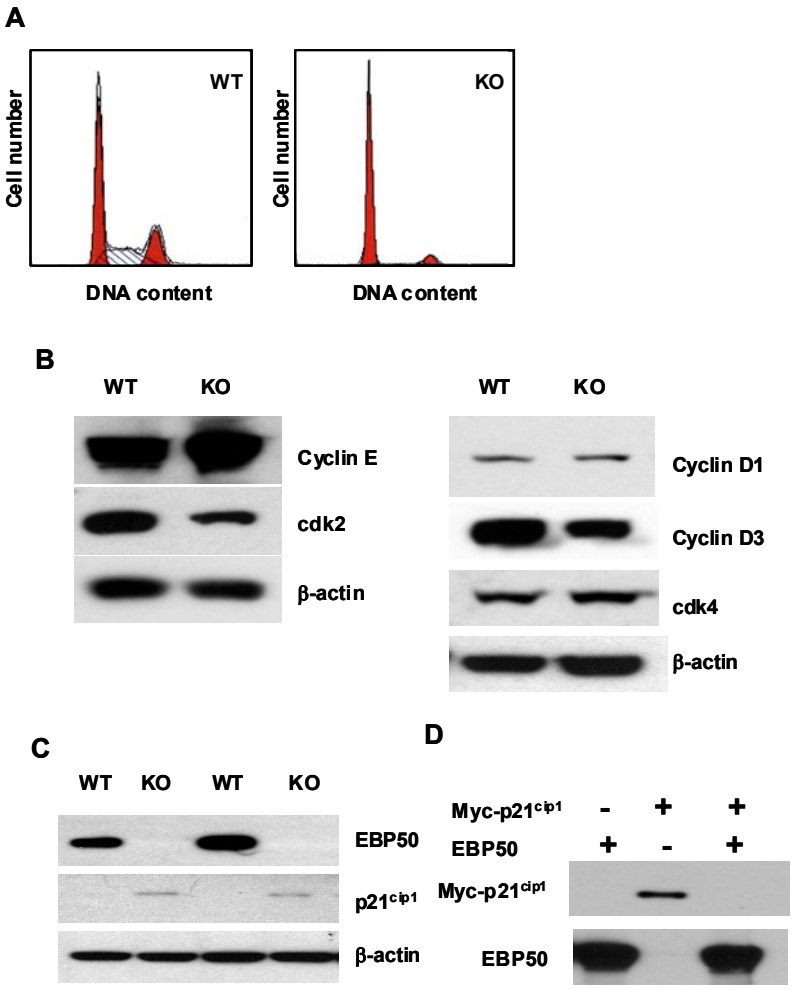
Statistical Analysis. Results from each experiment were averaged and expressed as mean ± S.E. Results were analyzed by ANOVA with Tukey's test or Student's t-test. Statistical calculations were performed with the GraphPad InStat3 software (GraphPad Software Inc., San Diego CA). P-values were considered statistically significant when lower than 0.05.

References

1. Ji P, Goldin L, Ren H, Sun D, Guardavaccaro D, Pagano M, Zhu L. Skp2 Contains a Novel Cyclin A Binding Domain That Directly Protects Cyclin A from Inhibition by $p27^{Kip1}$. *J Biol Chem.* 2006;281:24058-69. .

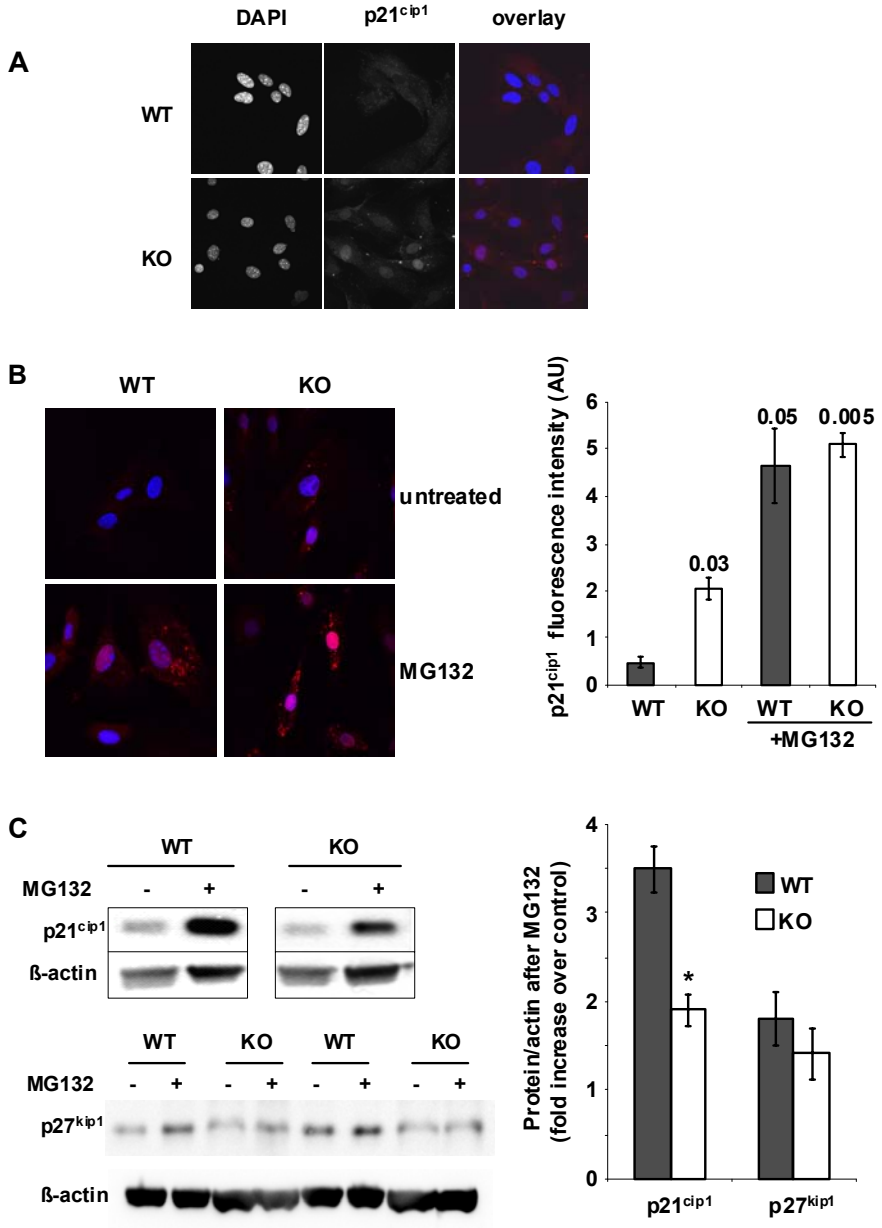
2. Ghanem L, Steinman RA. p21Waf1 inhibits granulocytic differentiation of 32Dcl3 cells. *Leuk Res.* 2006;30:1285-92.

Supplemental Figures



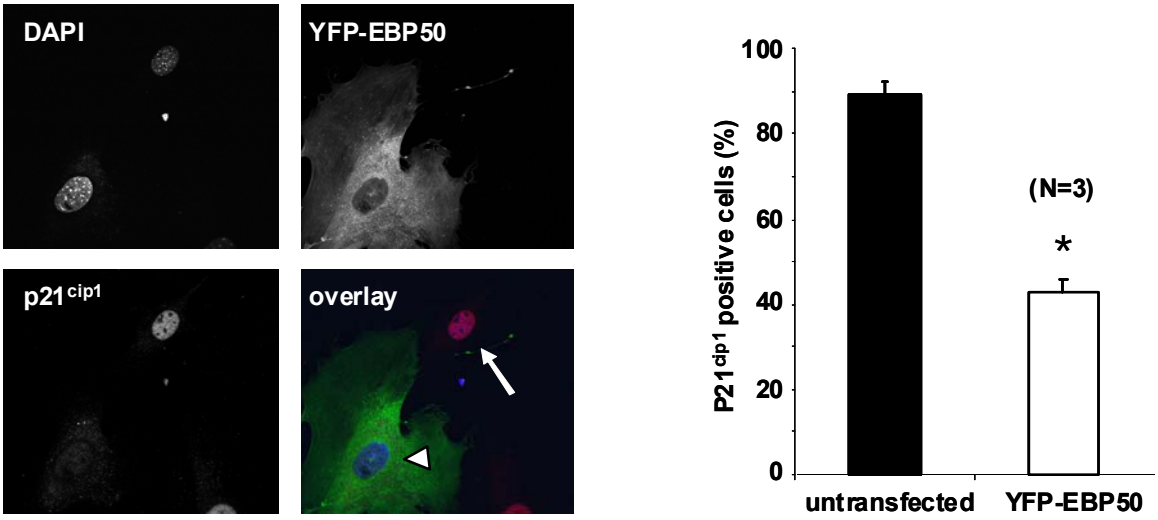
Supplemental Figure I. Effect of EBP50 on vascular smooth muscle cell cycle.

A. Cell cycle profile of WT (*left panel*) and KO (*right panel*) VSMC. **B.** Immunoblots for key G1-S transition molecules (cyclin E/cdk2 and cyclin D/ckd4, as indicated) in WT and KO VSMC. β-actin was used as loading control. Blots are representative of 2-4 independent experiments. **C.** Immunoblot for $p21^{cip1}$ in two different primary VSMC preparations from WT and KO mice. **D.** $p21^{cip1}$ and EBP50 expression in CHO cells. Cells were transfected with the plasmids for Myc-$p21^{cip1}$ and EBP50 as indicated. Total DNA in the transfections was equalized with empty plasmid (pcDNA3). Equal amount of proteins were analyzed. Blots are representative of two independent experiments.

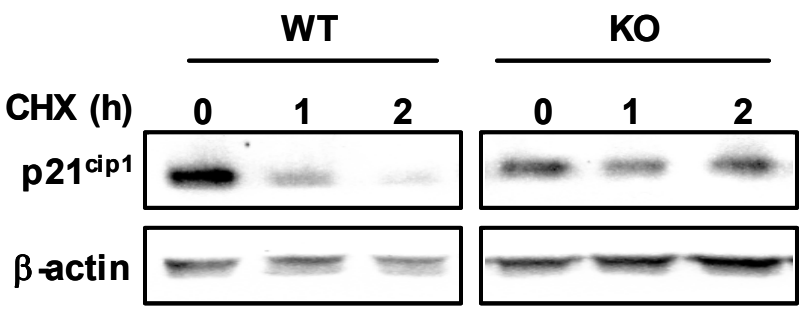


Supplemental Figure II. EBP50 and p21^{cip1} expression in VSMC. A. Primary VSMC from WT (*upper panels*) and KO (*lower panels*) mice were grown on coverslips in the presence of 10% FBS. Cells were fixed and immunostained for p21^{cip1} (in red). Nuclei were visualized with DAPI (in blue). Higher expression and nuclear localization of p21^{cip1} is evident in KO cells. **B.** *Left Panel*. Primary VSMC from WT (*left panels*) and KO (*right panels*) mice were grown on coverslips in the presence of 10% FBS. Cells were treated in the absence (*upper panels*) or the presence (*lower panels*) of the proteasome inhibitor MG132 (2 μM for 6 hours) fixed and immunostained for p21^{cip1} (in red). Nuclei were visualized with DAPI (in blue). *Right Panel.* Quantification of p21^{cip1} expression in primary WT (black bars) and KO

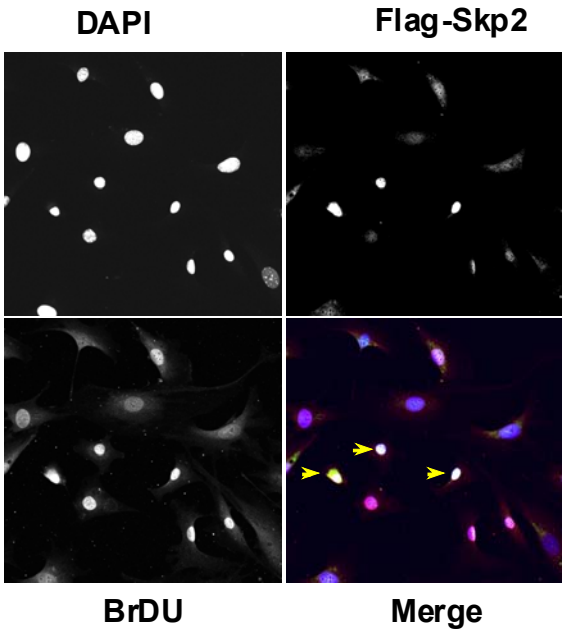
(white bars) VSMC. Graph shows the average fluorescence intensity ± standard error of $p21^{cip1}$ determined from sections. P values relative to untreated WT cells are shown above each bar. **C.** Primary VSMC from WT and KO mice were treated with MG132 (2 μM) for 6 h. Equal amounts of proteins were analyzed by immunoblotting for $p21^{cip1}$, $p27^{kip}1$ and β-actin (as loading control). Graph shows the quantitation of three independent experiments. Data are presented as mean (± standard error) of the $p21^{cip1}$ or $p27^{kip1}$ intensity (normalized with β-actin) relative to untreated cells. *, p=0.01.



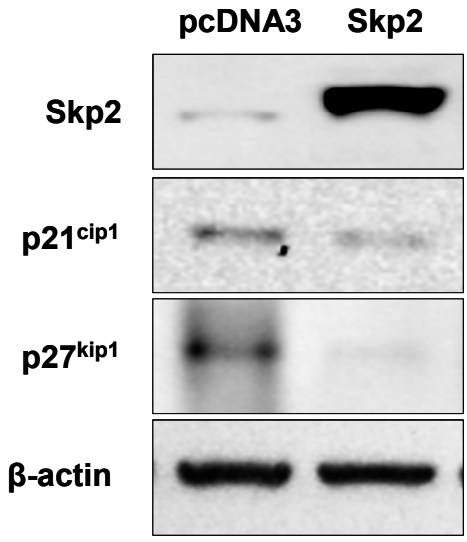
Supplemental Figure III. EBP50 and p21^{cip1} expression in VSMC. Primary VSMC from KO mice were electroporated with YFP-EBP50 (in green), fixed and stained for p21^{cip1} (in red). Nuclei were visualized with DAPI (in blue). The arrow indicates a cell expressing p21^{cip1} but not EBP50. The arrowhead indicates a cell expressing EBP50 but not p21^{cip1}. **C.** The percentage of p21^{cip1} positive non-transfected and YFP-EBP50-expressing cells was determined from three independent experiments. *, p=0.0001.



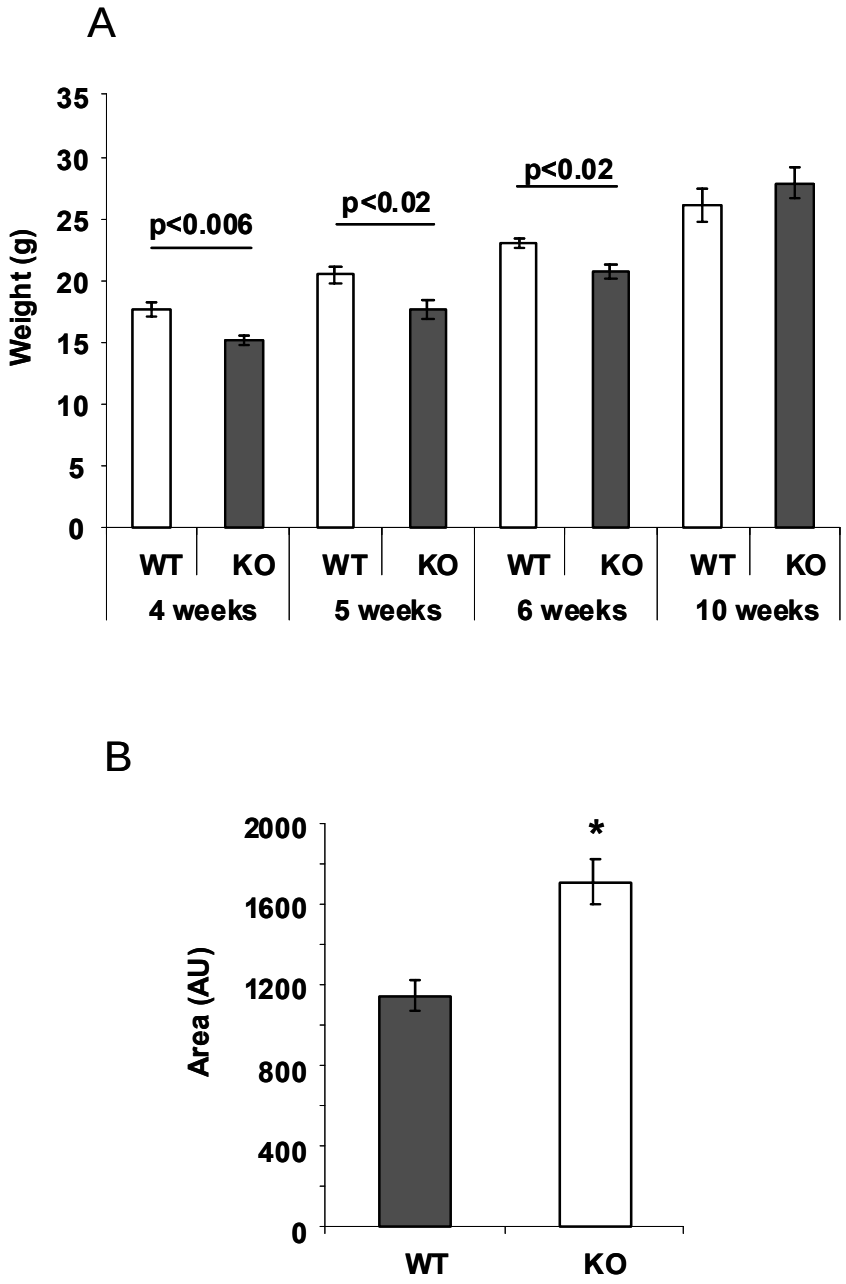
Supplemental Figure IV. EBP50 regulates p21^{cip1} stability. Primary VSMC from WT and KO mice maintained in 10% FBS were treated with cyclohexamide (10 μg/ml) or MG132 (2 μM) for the indicated times. Cell lysates were analyzed by immunoblotting for p21^{cip1} and β-actin (as loading control).



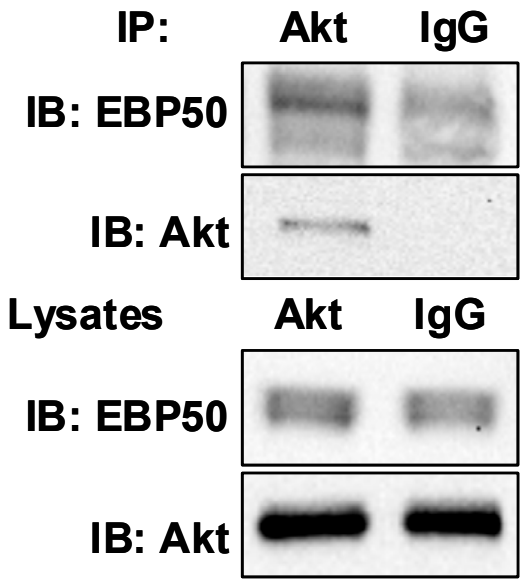
Supplemental Figure V. Primary VSMC from KO mice were electroporated with Flag-Skp2, cultured for 16 h in the presence of BrDU, fixed and stained for Flag (in green) and BrDU (in red). Nuclei were visualized with DAPI (in blue). Nuclei positive for DAPI, Skp2 and BrDU (indicated by the arrows) appear in white in the merged image.



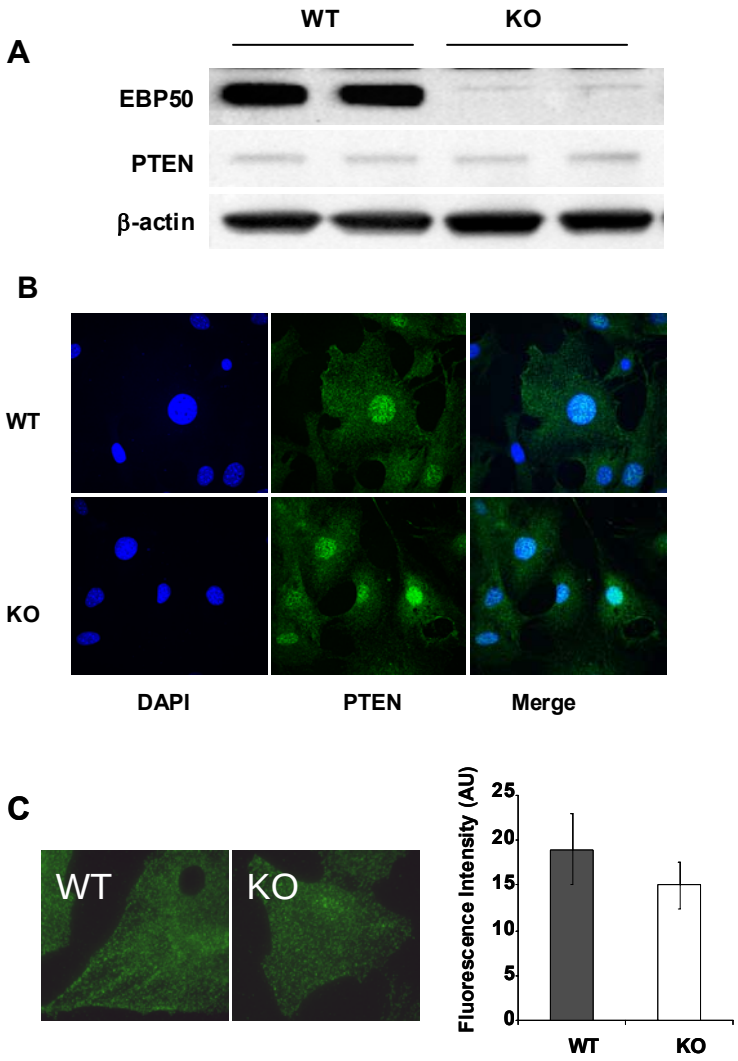
Supplemental Figure VI. Primary KO VSMC were electroporated with Skp2 and cultured for 2 days. Equal amounts of proteins were analyzed by immunoblotting for Skp2, $p21^{cip1}$ and $p27^{kip1}$, as indicated. β-actin was used as loading control.



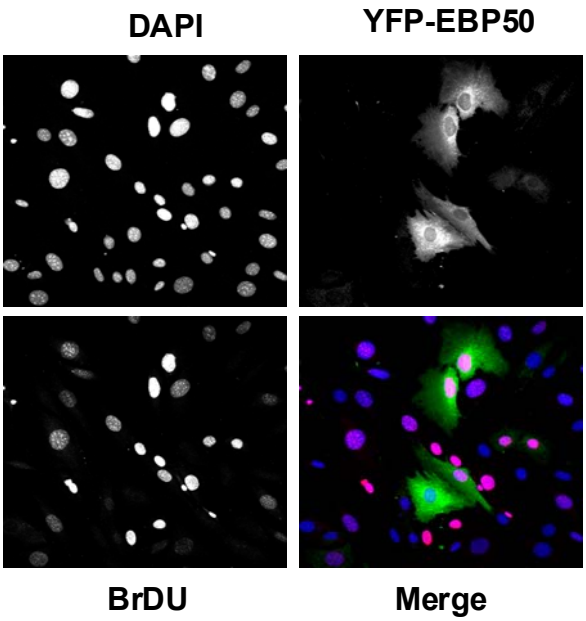
Supplemental Figure VII. A. Weight of WT and KO mice at the indicated ages. P values are indicated. N=5. **B.** Area (in arbitrary units) of WT and KO VSMC. *, $p<0.006$, N=4.



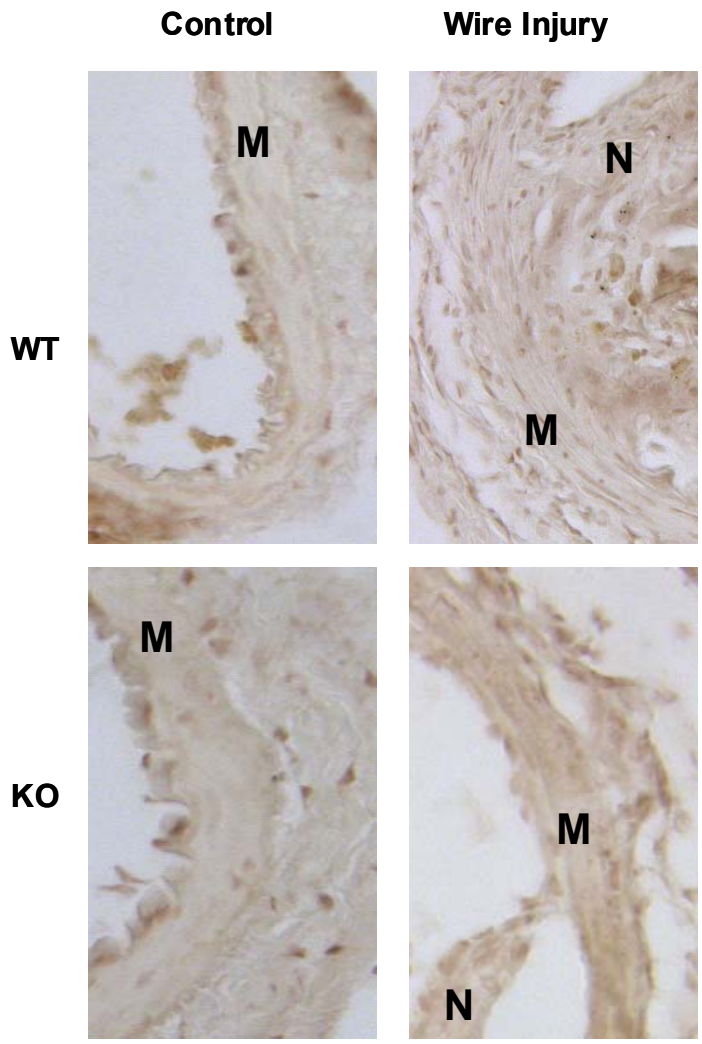
Supplemental Figure VIII. Primary VSMC were lysed, and immunoprecipitation experiments were performed with mouse anti-Akt or mouse IgG antibodies followed by SDS-PAGE and immunoblotting with rabbit anti-Akt or anti-EBP50 antibodies.



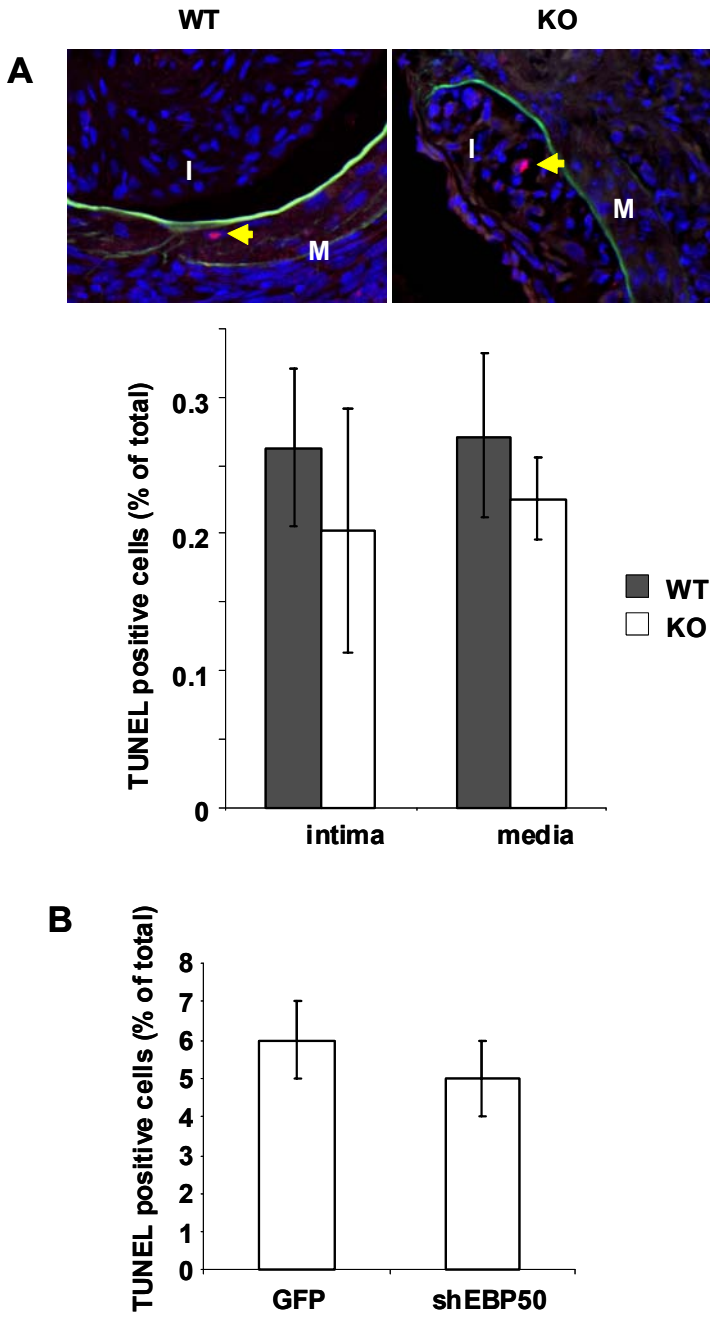
Supplemental Figure IX. PTEN expression. A. PTEN expression in primary VSMC from WT and KO mice was analyzed by immunoblotting. β-actin was used as loading control. **B and C.** Primary VSMC from KO mice were fixed and stained for PTEN (in green). Nuclei were visualized with DAPI (in blue). Cells were analyzed by confocal microscopy (B) and TIRF microscopy (C). Graph shows the fluorescence intensity of PTEN in WT and KO cells from TIRF images.



Supplementary Figure X. Primary WT VSMC were electroporated with YFP-EBP50 (in green), cultured for 16 h in the presence of BrDU, fixed and stained for BrDU (in red). Nuclei were visualized with DAPI (in blue).



Supplemental Figure XI. Expression of $p27^{kip1}$ in mouse femoral arteries. Sections from uninjured femoral artery (*left panels*) or 2 weeks after wire injury (*middle panels*) from WT (*upper panels*) and KO (*lower panels*) mice were stained for $p27^{kip1}$ (in brown). N indicates the neointima and M the media. The expression of $p27^{kip1}$ is evident in femoral arteries from both mouse strains in uninjured vessels and two weeks after injury.



Supplemental Figure XII. Apoptosis in WT and KO vessels and VSMC. A. *Top Panel.* TUNEL staining (in red) of sections from injured femoral arteries of WT (*left panel*) and KO (*right panel*) mice. Nuclei are stained in blue and the elastic lamina autofluorescence is in green. TUNEL positive cells are indicated by yellow arrows. M indicates media and I the intima. *Bottom Panel.* Quantitation of the percentage of TUNEL positive cells over total cells in the intima and media of WT (gray bars) and KO (white bars) mice. N=4. **B.** TUNEL staining of WT VSMC infected with control GFP or shRNA for EBP50. Cells were serum starved for 24 h. N=3.