Supplementary materials

Materials: PDGF-BB, cell culture reagents, fetal bovine serum, freezing medium and antibiotics were purchased from Invitrogen (Grand Island, NY). [γ-^{32}P]ATP (specific activity 3,000Ci/mmol) was from NEN (Boston, MA). The Rp diastereoisomer of 8-bromoadenonosine 3',5'-cyclic monophosphothioate (Rp-8-Br-cAMPS) and Sp diastereoisomer of 3',5'-cyclic monophosphothioate (Sp-cAMPS) and microcystin were from Calbiochem (San Diego, CA). Type 1 collagenase was from Worthington Biochemical (Freehold, NJ). SDS/polyacrylamide gel electrophoresis and Western blot reagents were from Bio-Rad (Hercules, CA). PDE3A siRNA [sc-41593, which is a pool of a proprietary mixture of 3 target-specific 20-25 nt siRNAs (sequence not provided by the manufacturer) designed to knock down gene expression in mouse cells (Genetic locus: PDE3A (human) mapping to 11p15.1; Pde3a (mouse) mapping to 6 G1], and anti-MKP-1, -Cyclin-D1, -CREB, and -p21 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho ERK, anti-phospho Raf-1^{ser259}, anti-phospho Rb, anti-phospho p53^{Ser15}, anti-phospho CREB^{Ser133}, anti-phospho Akt ^{Ser473}, anti-Akt, anti-ERK, anti-Raf-1 antibodies were from Cell Signaling Technologies, Inc. (Beverly, MA). Anti-PDE3A and -PDE3B peptide antibodies were generated as detailed earlier (11, 16). Protease inhibitor tablets were from Roche Biochemicals. Sodium orthovanadate, insulin, BSA and all other reagents were from Sigma Chemicals (St. Louis, MO). PDE and PKA enzyme assay kits were from Amersham (Piscataway, NJ) and Upstate Biotechnologies Inc. (Lake Placid, NY), respectively. Adenoviruses engineered to express CREB and CREB mutants were kindly provided by Dr. Jane Reusch (University of Colorado Health Science Center, Denver, CO), and were purified by using an Adenovirus purification kit from Clonetech (Mountainview, CA).

ON-TARGET plus SMARTpool TRP53 siRNA (# L-040642-00 consisting of a proprietary mixture of four predesigned siRNAs targeting p53 and transfection reagents were purchased from Dharmacon (Lafayette, CO). The sequence of each p53 siRNA duplex that is included in the SMARTpool is as follows:

J-040642-09
sense: G.U.A.A.A.C.G.C.U.U.C.G.A.G.A.U.G.U.U.U.U,
antisense: 5'-P.A.A.C.A.U.C.U.C.G.A.A.G.C.G.U.U.U.A.C.U.U
J-0400642-10
sense: A.A.A.U.U.U.G.U.A.U.C.C.C.G.A.G.U.A.U.U.U
anti_sense: 5'-P.A.U.A.C.U.C.G.G.G.A.U.A.C.A.A.A.A.U.U.U.U.U
J-040642-11
sense: G.A.G.G.A.G.U.C.A.C.A.G.U.C.G.G.A.U.A.U.U
antisense: 5'-P.U.A.U.C.C.G.A.C.U.G.U.G.A.C.U.C.C.U.C.U.U
J-040642-12
sense: C.A.G.U.C.U.A.C.U.U.C.C.C.G.C.C.A.U.A.U.U
antisense: 5- P.U.A.U.G.G.C.G.G.G.A.A.G.U.A.G.A.C.U.G.U.U

Figure Legends

Supplementary Fig. 1A: Cell morphology of F1 and F8 generation PDE3A-WT and F8 PDE3A-KO VSMCs by Phase Contrast Microscopy: VSMCs were visualized using an Olympus DP1X51 inverted Phase contrast microscope. A representative picture from several different batches of cells demonstrates that VSMCs grown from male F1 generation 3A-KO mice were largely epithelioid in shape, whereas VSMCs grown from 3A-WT littermates and F8 3A-KO mice were largely spindle shaped.

Supplementary Fig. 1B: Immnostaining of fixed VSMCs with Phalloidin Texas Red: 3A WT VSMCs, 3A-KO VSMCs (prepared from F1 and C57/B6 F8 generation), and 3B-KO VSMCs were grown on eight-well chamber slides. Cells were fixed with 4% ice-cold paraformaldehyde for 30 min, permeabilized with 0.2% Triton-X-100 in PBS for 5 min, blocked with 3% BSA in PBS for 1 hr, stained with Phalloidin-Texas Red (50 µg/ml) for 30 min, to stain F-actin, or with FITC α-actin (largely stains globular actin), rinsed x 3 with PBS, and visualized using a cooled CCD camera mounted on an Olympus DP 1X51 microscope. A representative image is shown. Similar results were obtained in several experiments.

Supplementary Fig. 2A: PDE3A and PDE3B proteins in cultured VSMCs: 3A-WT, 3B-WT, 3A-KO, and 3B-KO VSMCs were propagated in alpha MEM containing 10% FBS and antibiotics, and maintained in culture as described under materials and methods. VSMCs at 5^{th} passage were grown to confluence (8-10 days), incubated in serum-free medium (24h), and stimulated with insulin (100 nM) or PDGF-BB (10 ng/ml) for 5 min, or with insulin for 5 min followed by PDGF-BB for 5 min. Homogenate proteins (50 µg) were subjected to SDS/PAGE and immunoblot analysis with isoform specific antibodies, followed by detection with HP-conjugated anti-rabbit IGg and ECL, as described in materials and methods. A representative ECL image is shown. Similar results were obtained in multiple experiments performed at different times.

Supplementary Fig. 2B: Total PDE, PDE3, and PDE4 activities in VSMCs: Samples (5 µg proteins) from confluent, serum-starved, VSMCs were assayed for phosphodiesterase activities according to the manufacturer's protocol (Amersham), using 1 µM cAMP as substrate, without or with 1 µM cilostamide (PDE3 inhibitor) or 10 µM rolipram (PDE4 inhibitor). Results are mean ± SEM of values from three separate experiments performed in duplicate, and are expressed as percentage of total PDE activity in 3A-WT VSMCs, taken as 100%. *p< 0.05, 3A-KO vs. 3A-WT; **p< 0.05, 3B-KO vs. 3B-WT. Total PDE activities (pmoles/mg protein/min) are as follows: 3A-WT: 109.7 ±8.3; 3A-KO: 76.8±8.57; 3B WT: 164.7+1.1; 3B-KO: 116.8±1.00.

Supplementary Fig. 3: SpcAMPS blocks PDGF–induced ERK phosphorylation in 3B WT and 3B-KO VSMCs: Serum-starved VSMCs were incubated with saline or with SpcAMPS (100 µM) for 30 min, followed by treatment with PDGF (10 ng/ml) for 5 min. ERK phosphorylation was examined by Western blot analysis with anti-phosphoERK antibody as detailed in materials and methods. A representative ECL image is shown, with similar results in three separate experiments.

Supplementary Fig. 4: Lack of alterations in MKP-1 expression levels in PDE3B-KO VSMCs: Serum-starved 3B-WT and 3B-KO VSMCs were exposed to PDGF (10 ng /ml) or insulin (100 nM) or insulin-> PDGF as detailed in materials and methods. Equal amounts of proteins were subjected to immunoblot analysis with anti-MKP-1 antibody. A representative ECL image is shown. Similar results were observed in three separate experiments.

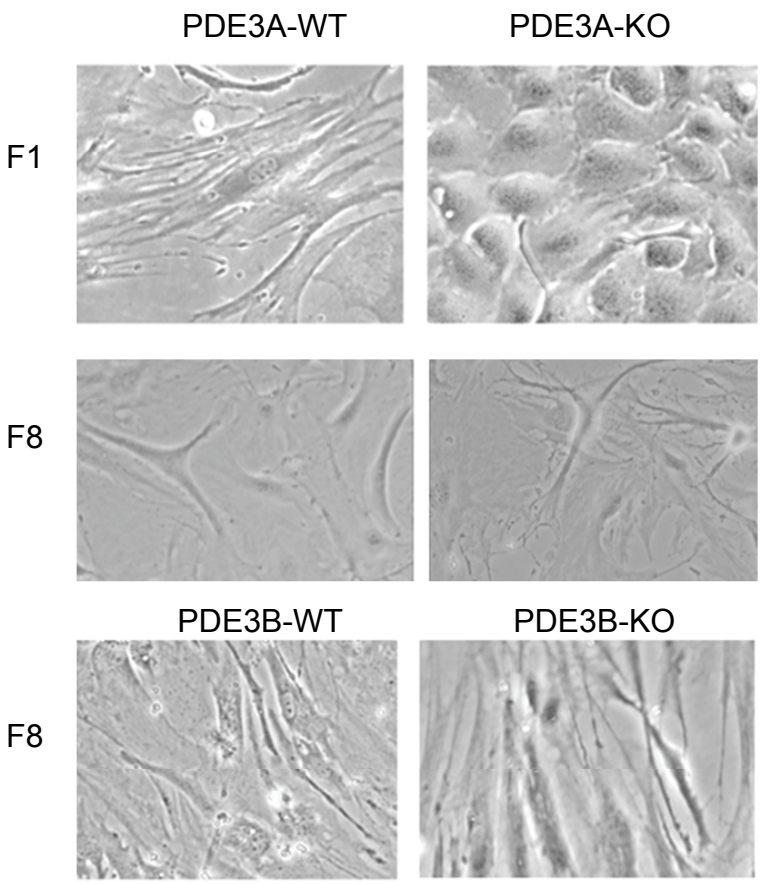
Supplementary Fig. 5: Alterations in levels of cell cycle regulatory proteins and their phosphorylation status in 3A-KO VSMCs: Serum-starved 3A-WT and 3A-KO VSMCs were incubated for 24 h without or with PDGF-BB (10 ng/ml). Equal amounts of homogenate samples (50 µg proteins) were analyzed by Western blots for Cyclin-D1, p21, Rb, phosphoRb, p53, phosphop53 and β-actin (loading control), and by densitometric quantification, presented here. **Top Panel:** ECL images from four separate experiments were scanned, and protein levels of Cyclin-D1, p21, Rb, and p53 were quantified as arbitrary densitometric units relative to β-actin. The densitometric ratio of 3A-WT basal (no additions) was set at 1, and the remaining values were calculated relative to 3A-WT basal. Data are reported as means +/- SE, n=4. *p< 0.05 vs. 3A-WT basal; **p< 0.05 vs. respective 3A-WT. **Bottom Panel:** ECL signal images from 4 independent experiments were scanned, and the

densitometric ratios (in arbitrary densitometric units) between phosphoRb (pRb) and Rb as well as phosphop53 (pp53) and p53 were quantified. The phosphoprotein/protein ratio of 3A-WT basal (no additions) was set at 1 and the remaining ratios were calculated relative to 3A-WT basal. Data are reported as means +/- SE, n=4. *p< 0.05 vs. 3A-WT basal; **p< 0.05 vs. 3A-WT basal and PDGF-treated.

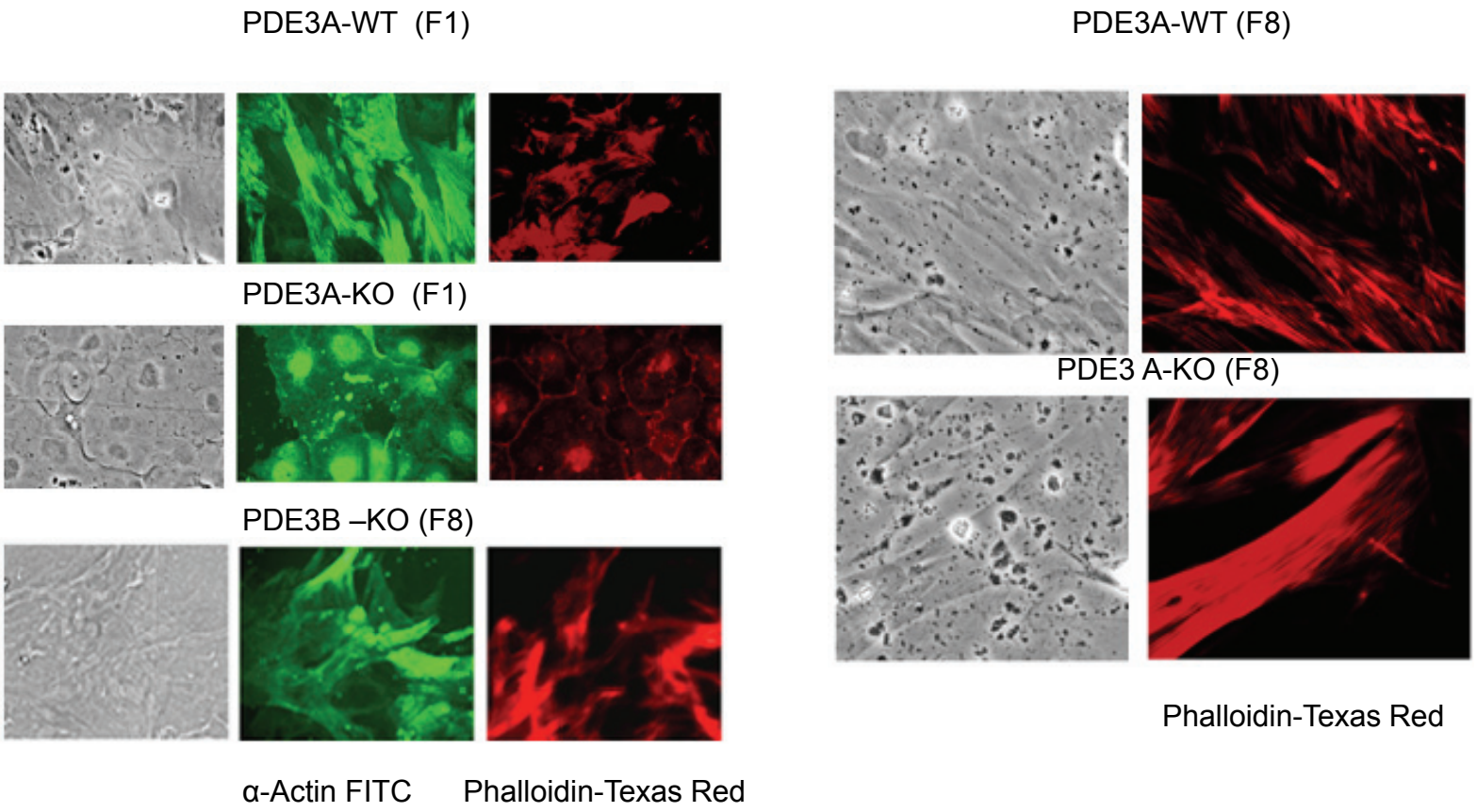
Supplementary Fig. 6: p53 antisense RNA blocks elevations in p21 and MKP-1 without affecting Cyclin-D1 or Rb phosphorylation in 3A-KO VSMCs: Subconfluent VSMCs were transfected with 100 nM TRP53 siRNA smart pool or 100 nM scrambled (Sc SiRNA) siRNA, using Dharmacon transfection medium 1. After 48 h, VSMCs, in serum-free medium, were incubated without or with PDGF for 24 h. Samples of homogenates (50 µg proteins) were analyzed by Western blots for cell cycle regulatory proteins and by densitometric quantification, and presented here. (Note: To detect p53 protein, present at low levels in 3A-WT VSMCs, and to demonstrate that TRP53 siRNA depleted p53 in 3A-KO VSMCs, 100 µg proteins were analyzed by Western blots for p53.) **SF6A:** ECL images from five separate experiments were scanned, and amounts of Cyclin-D1, MKP1, p21, and p53 were quantified as arbitrary densitometric units relative to β-actin. The densitometric ratio of 3A-WT basal (no additions) was set at 1 and the remaining values were calculated relative to 3A-WT Basal. Results are means ± SE, n=5. *p< 0.05 vs. 3A-WT basal; **p< 0.05 vs. respective 3A-WT; ***p<0.05 vs. respective 3A-KO**. SF6B**: Linear ECL images from four independent experiments were scanned, Rb protein (top) and phosphorylation of Rb (pRB, bottom) was quantified as arbitrary densitometric units relative to β-actin and Rb protein, respectively . The phosphoprotein/protein ratio of 3A-WT basal (no additions) was set at 1 and the remaining values were calculated relative to 3A-WT basal. Results are means ± SE, n=4. *p< 0.05 vs. 3A-WT basal; **p< 0.05 vs. respective 3A-WT.

Supplementary Figures

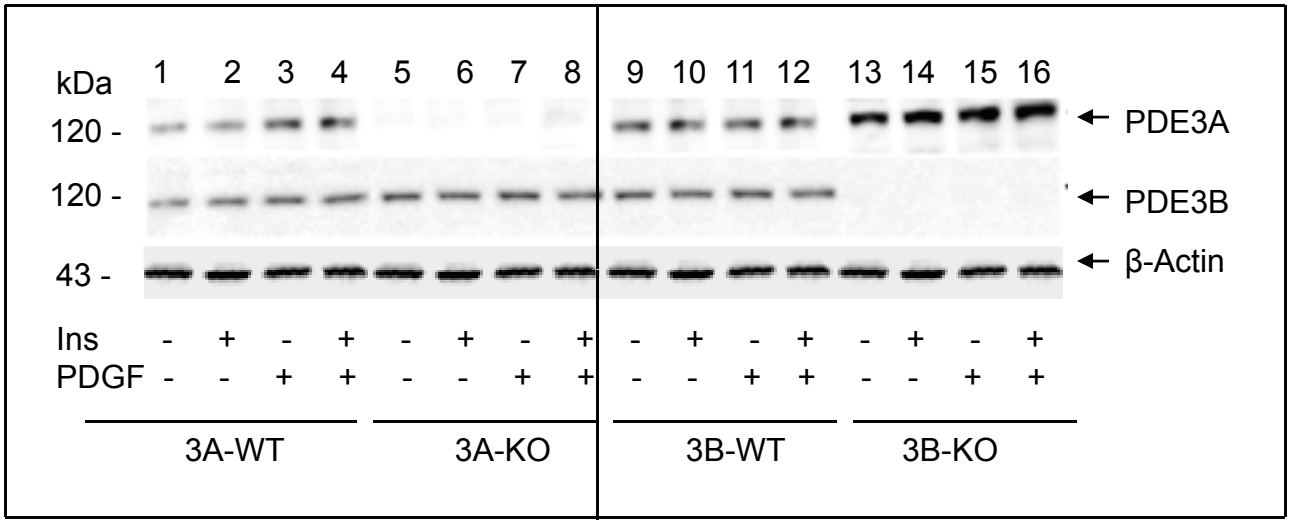
Supplementary Fig.1A



Supplementary Fig.1B



Supplementary Fig.2A



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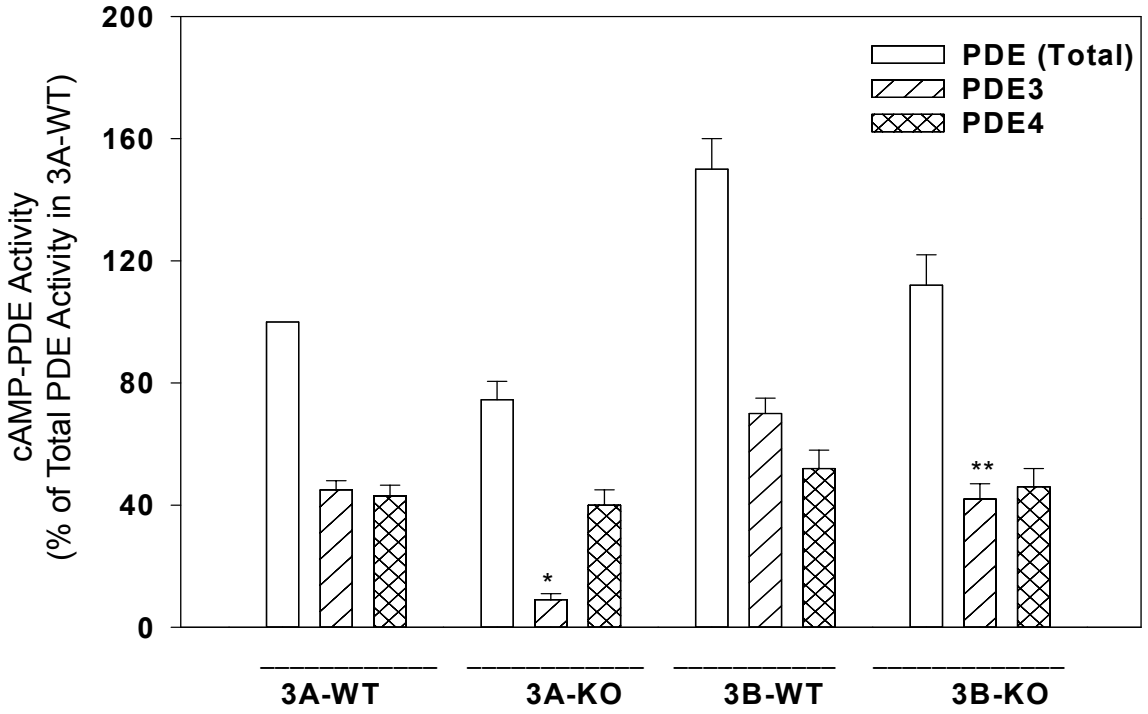
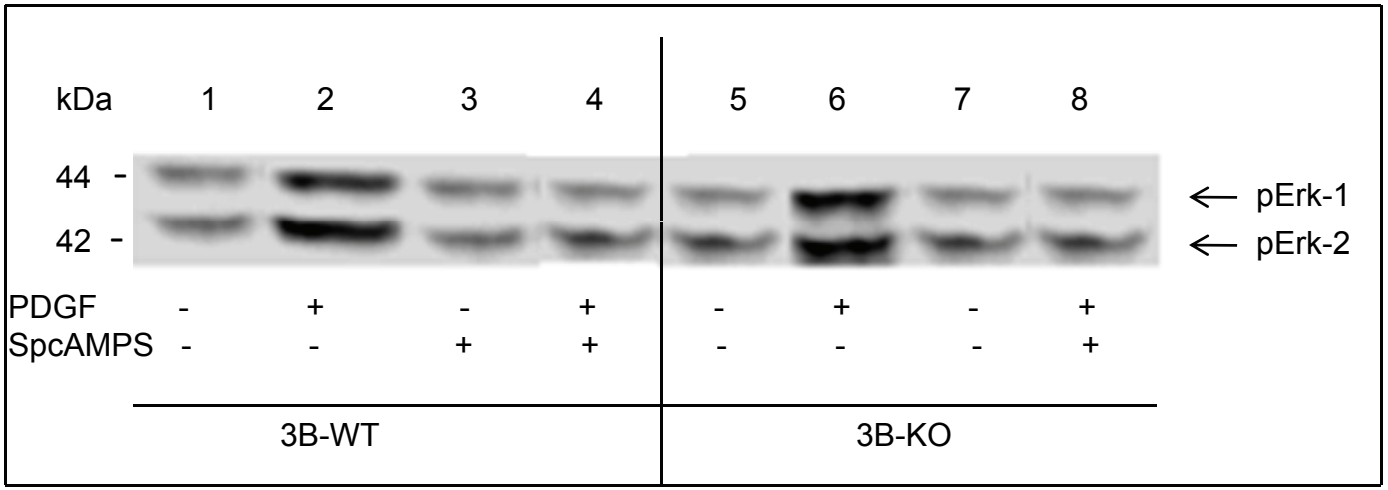
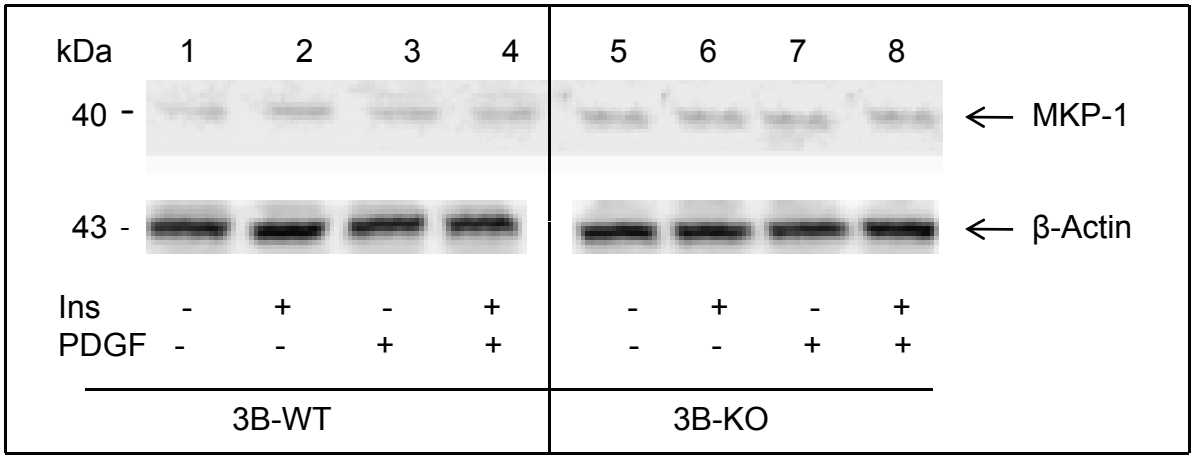


Supplementary Fig. 3



Supplementary Fig.4



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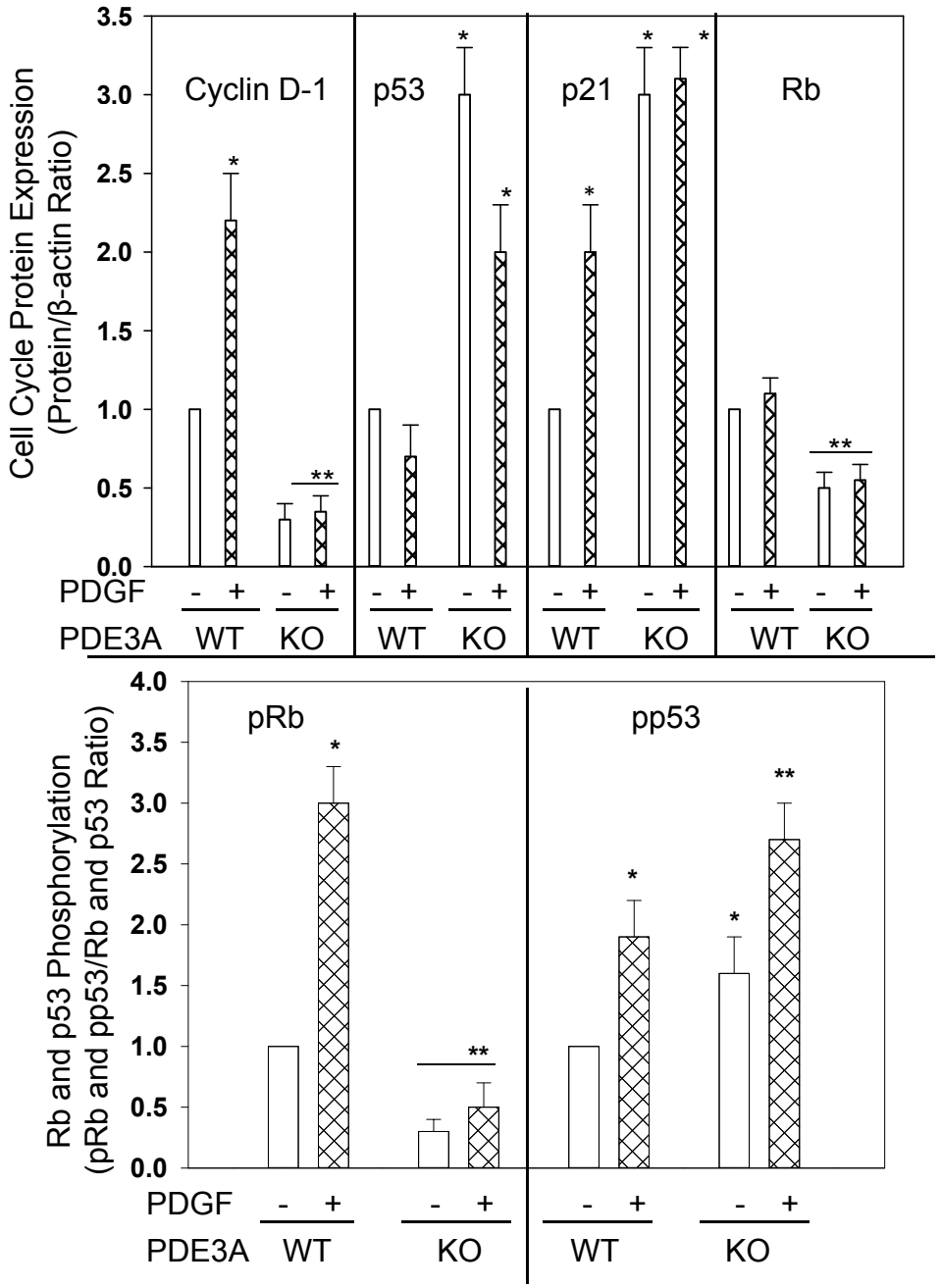
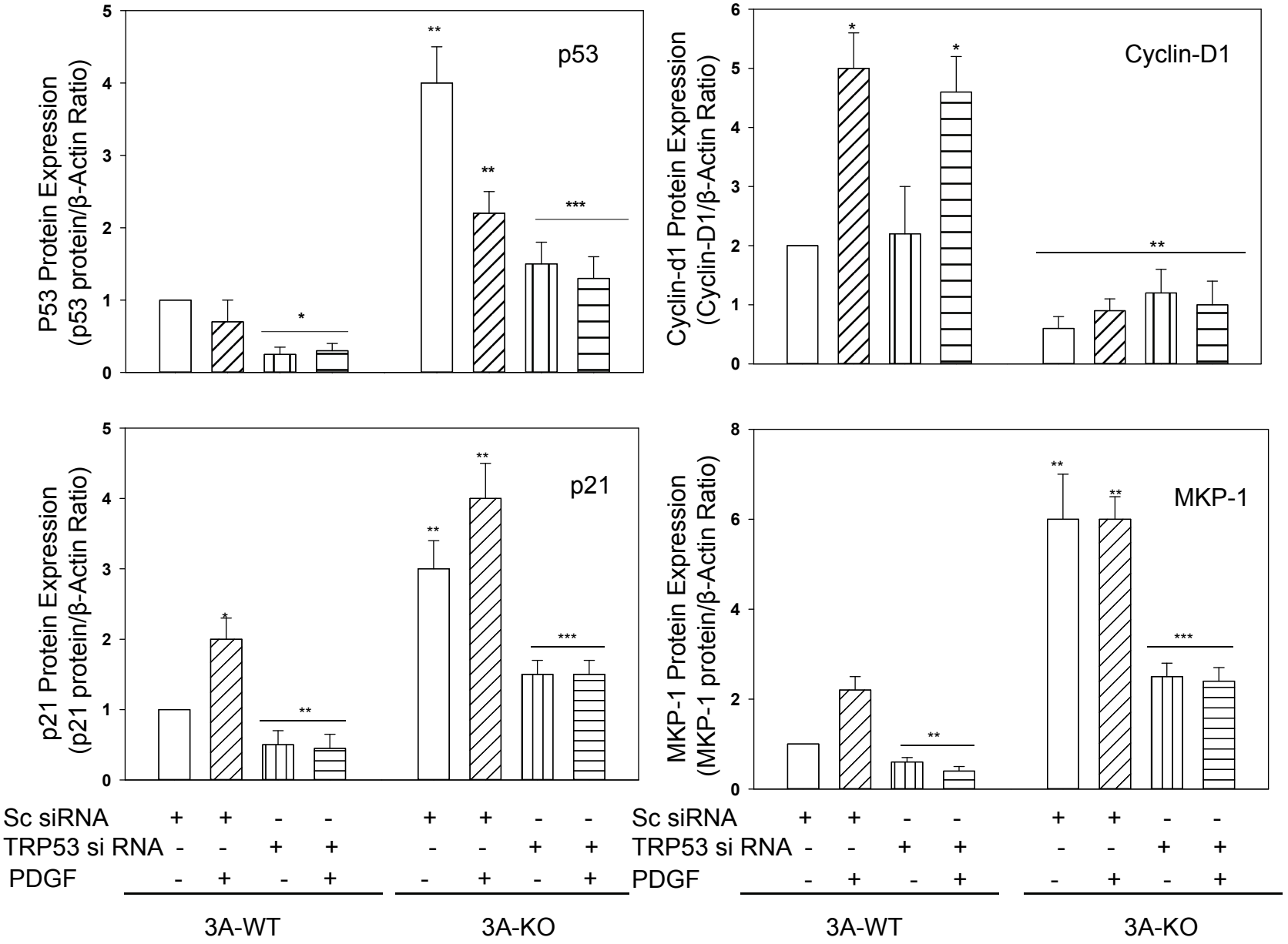


Supplementary Fig.6A



Supplementary Fig.6B