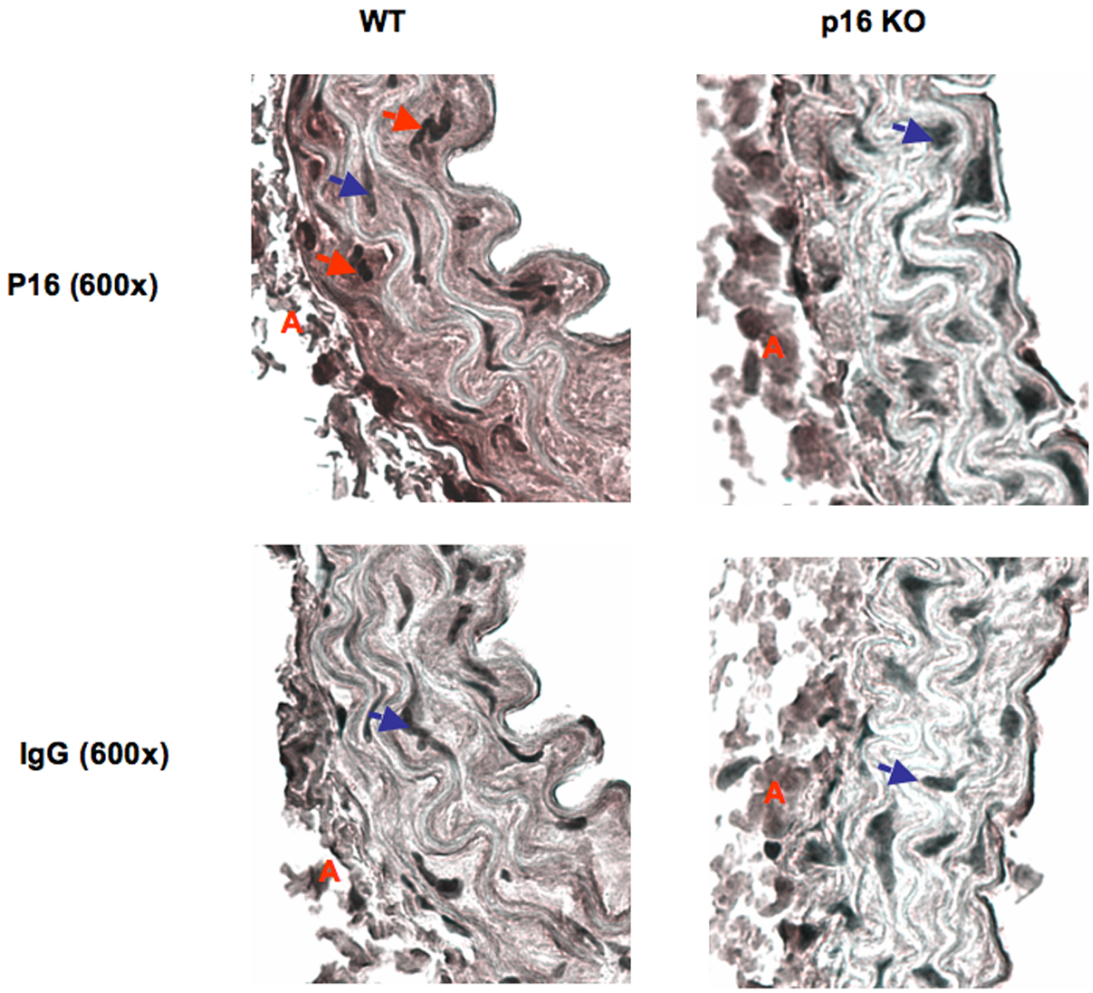
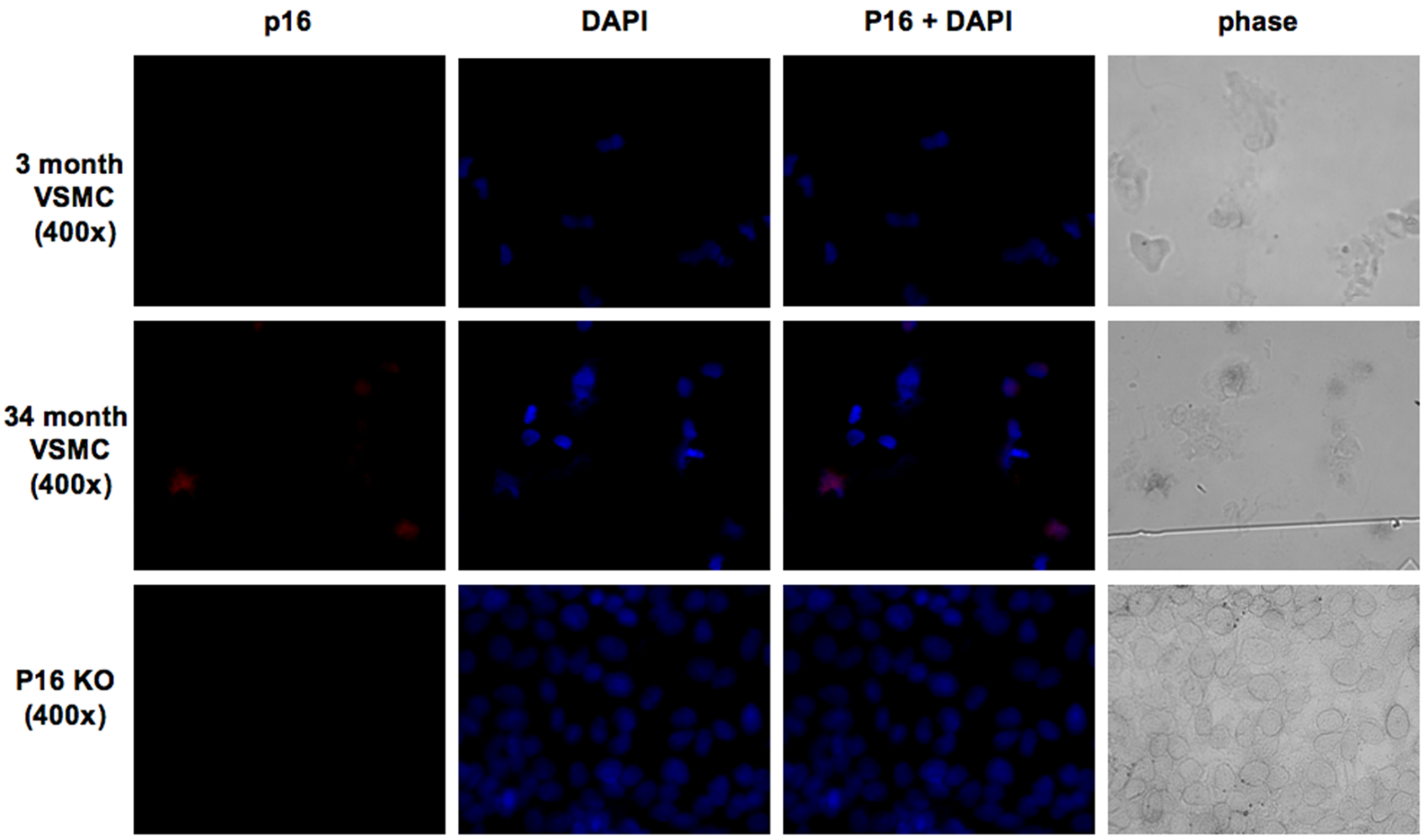
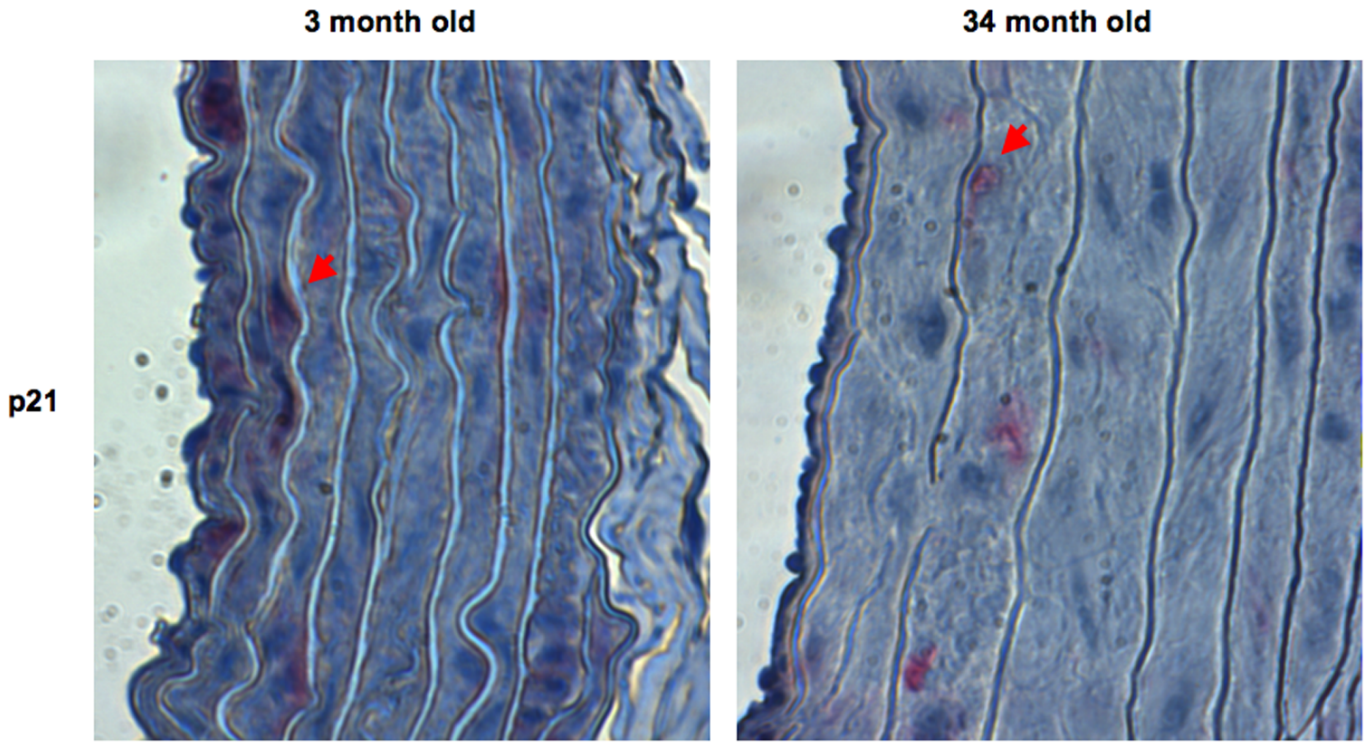
Supplementary Figure 1



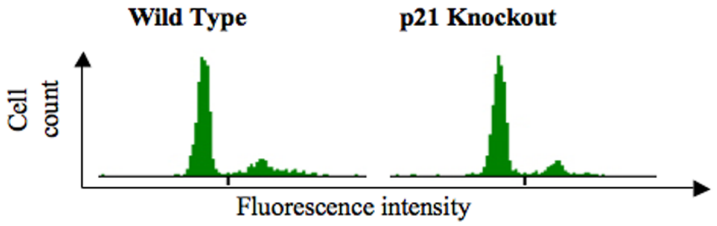
Supplementary Figure 2



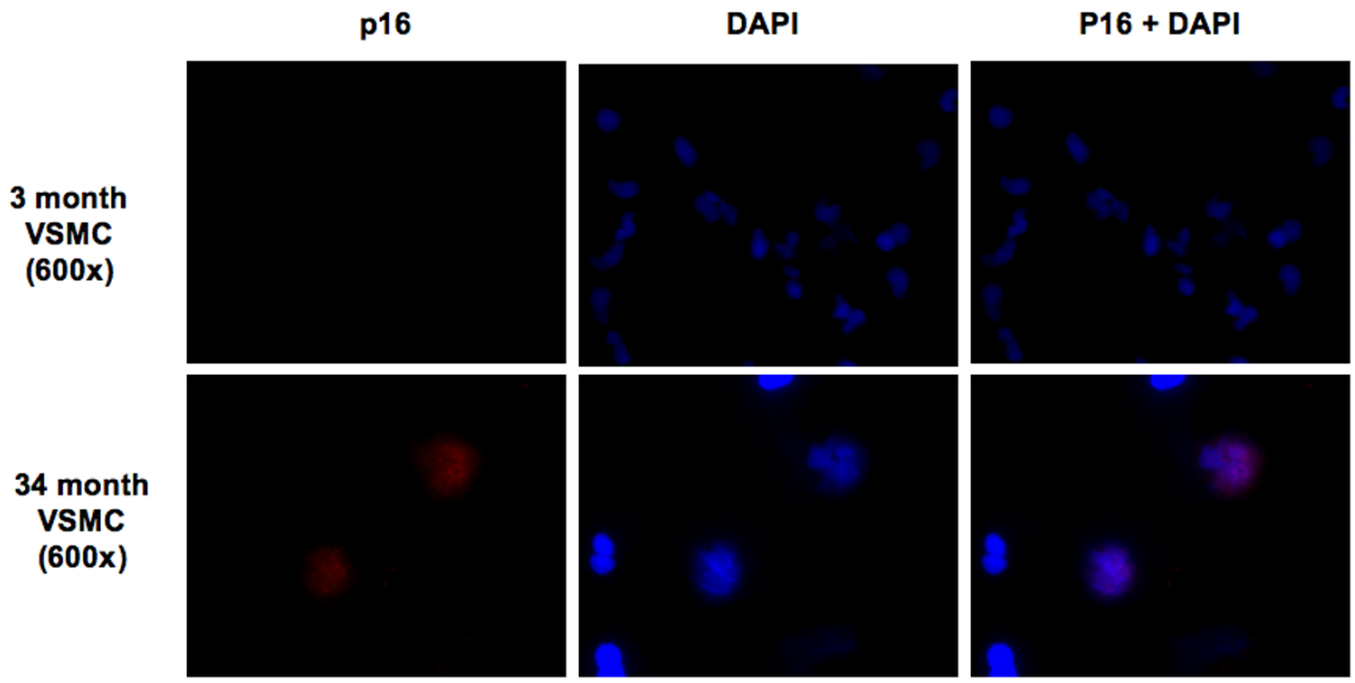
Supplementary Figure 3A



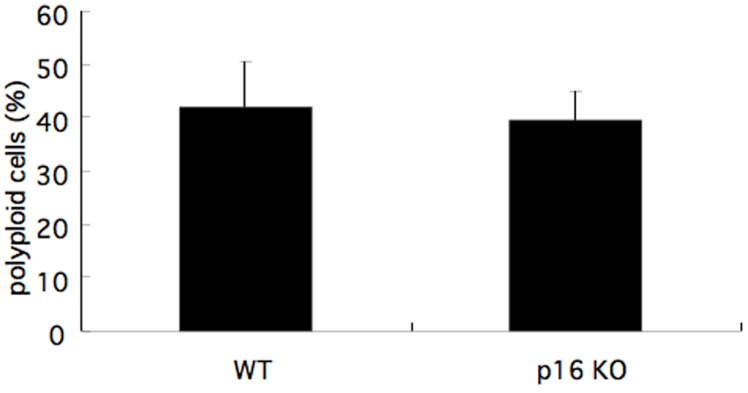
Supplementary Figure 3B



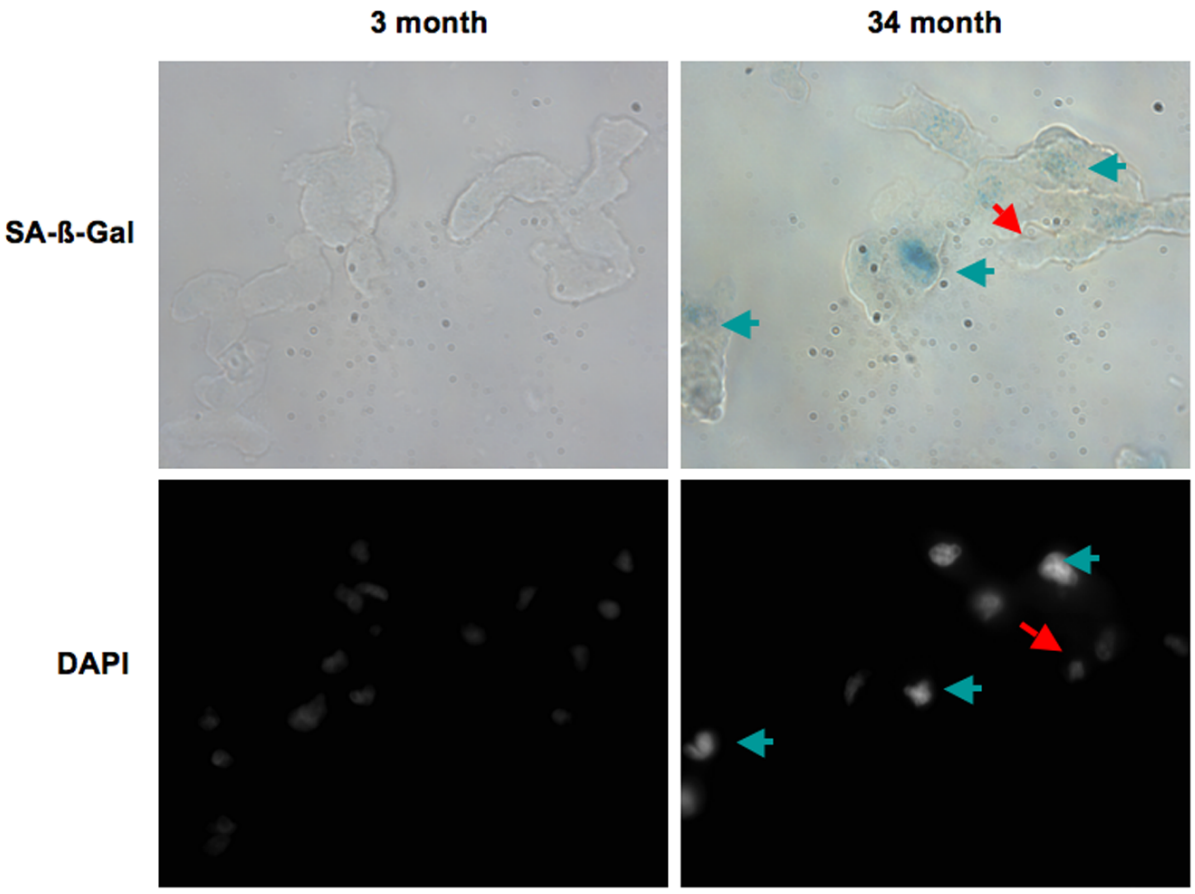
Supplementary Figure 2 (cont.)



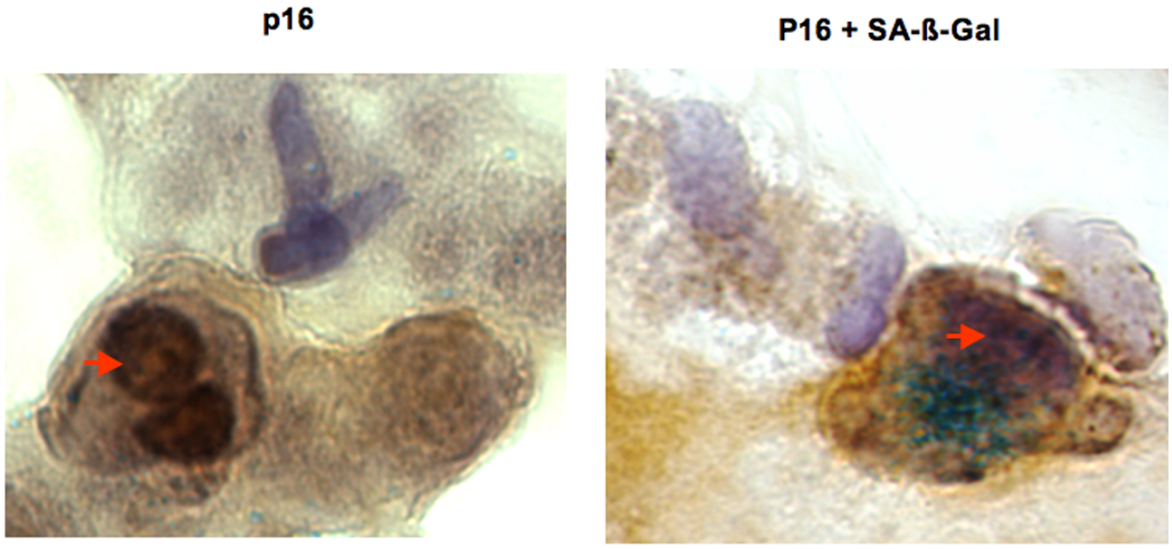
Supplementary Figure 3C



Supplementary Figure 4



Supplementary Figure 5



Supplementary Material

Experimental Procedures

Senescence-associated β-galactosidase (SA-β-gal) staining

Aortic tissues were embedded in Tissue-Tek Optimal Cutting Temperature Compound (Sakura, Tokyo, Japan, cat# 4583), frozen in liquid nitrogen and immediately stored at -80 ^{0}C until sectioning. Sections were cut at a thickness of 5 μm using a Microm HM500 OM cryostat (Microm, Walldorf, Germany). Slides were then subjected to SA-β-gal staining according to Campisi *et al*. (Dimri et al., 1995). Briefly, staining was carried out with fresh senescence-associated β-Gal (SA-β-Gal) stain solution which consists of the following reagents: 1mg 5-bromo-4-chloro-3-indolyl P3-D-galactoside (X-Gal from American Bioanalytical, Natick, MA, cat# AB02400), 40 mM citric acid (American Bioanalytical, Natick, MA cat# AB02365), 40 mM sodium phosphate monobasic (American Bioanalytical, Natick, MA, cat# AB2025), 40 mM sodium phosphate dibasic (American Bioanalytical, Natick, MA, cat# AB2060), 5 mM potassium ferrocyanide (Sigma, St. Louis, MO, cat# 455989), 5 mM potassium ferricyanide (Sigma, St. Louis, MO, cat# 455946), 150 mM $NaCl_2$ (American Bioanalytical, Natick, MA, cat# AB1915), and 2 mM $MgCl_2$ adjusted to pH 6.0 (American Bioanalytical, Natick, MA, cat#AB01310). After staining, slides were rinsed with phosphate buffered saline (PBS).

Immunohistochemistry staining for p21

3 month and 34 month-old rat aortas were dissected and fixed in 4% paraformaldehyde (J.T. Baker, Phillipsbure, NJ, cat# S898-07) solution for 24 hours at 4 °C. Aortas were embedded with paraffin and cut at a thickness of 5 μm. After de-paraffinization, rehydration, and high temperature antigen retrieval (by placing sections in 10 mM Citric Acid pH 6 and heating at a 700W microwave for 2 minutes for 3 cycles, with 2 minute intervals between each time, followed by cooling down for 20 minutes), paraffin sections were blocked with 10% normal goat serum (Vector Laboratories, Inc. Burlingame, CA, cat# S-1000) for 30 min at room temperature, and then incubated overnight at 4 °C with rabbit polyclonal anti-p21 antibody diluted 1:300 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, cat# sc-471). The staining was revealed using goat anti-rabbit biotinylated

secondary antibody (Vector Laboratories, Inc. Burlingame, CA, cat# BA-1000) at a dilution of 1:100 with an incubation time of 30 min at room temperature. After a wash with PBS, sections were incubated with ABC-AP reagent (Vector Laboratories, Inc. Burlingame, CA, cat# AK-5000) for 30 min at room temperature. Vector Red alkaline phosphatase substrate (Vector Laboratories, Inc. Burlingame, CA, cat# SK-5100) was used to develop the red positive signal by incubation for 20 minutes. Sections were counterstained with Gill 3 Hematoxylin (Thermo Shandon, Pittsburg, PA, cat #6775009).

Immunohistochemistry staining for p16^{INK4a}

Staining was carried out in paraformaldehyde fixed tissue sections as well as in cryosections, yielding similar results. Cryosections were as described above for SA-β-Gal staining. For paraformaldehyde fixed sections; 3 month, 24 month and 34 month-old rat aortas were dissected, fixed, and stained as described for p21 immunohistochemistry with some changes. After blocking the samples with 10% normal goat serum (Vector Laboratories, Inc. Burlingame, CA, cat# S-1000) for 30 min at room temperature sections were incubated overnight at 4°C with mouse anti–rat p16^{INK4a} monoclonal antibody at a dilution of 1:100 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, cat # sc-1661). The staining was revealed using two alternative methods, including Vector Red alkaline phosphatase or Vector DAB substrate (Figure 1A and Supplementary Figure 1), as indicated in the figure legends. The Vector Red is more sensitive, and it yields a higher background. For Vector Red, goat anti-mouse biotinylated secondary antibody (Vector Laboratories, Inc, Burlingame, CA, cat # BA-9200) was used at a dilution of 1:200 with an incubation time of 30 min at room temperature. After a wash with PBS, sections were incubated with ABC-AP reagent (Vector Laboratories, Inc, Burlingame, CA, cat# AK-5000) for 30 min at room temperature, and a subsequent incubation for 20 min with Vector Red alkaline phosphatase substrate (Vector Laboratories, Inc, Burlingame, CA, cat# SK-5100) led to red positive signal. Alternatively, the staining was revealed using goat anti-mouse biotinylated secondary antibody (Vector Laboratories, Inc, Burlingame, CA; cat # BA-9200) at a dilution of 1:200 with an incubation time of 1 hour at 37 °C. After a wash with PBS, sections were incubated with ABC reagent (Vector Laboratories, Inc, Burlingame, CA, Cat# PK-6100) for 30 min at 37 °C and Vector DAB substrate

(Vector Laboratories, Inc, Burlingame, CA, cat# SK-4100) was used to develop the brown positive signal by incubation for 2 minutes. Sections were counterstained with Gill 3 Hematoxylin (Thermo Shandon, Pittsburg, PA, cat #6775009). In each case, appropriate controls were examined, including a reaction with IgG as a primary antibody and confirmation of negative staining in cells known to lack $p16^{INK4a}$.

Supplementary Figure 1

Validation of the immunohistochemistry method used to stain aortic tissue sections with anti-p16^{INK4a}. Aortic tissue sections derived from control wild type (WT) or p16^{INK4a} knockout mice (p16^{INK4a} KO) (Sharpless et al., 2001) that were strain (FVB)-, sex (females)- and age (seventeen week old)-matched were subjected to immunostaining with anti-p16^{INK4a} and Vector DAB substrate as described under Experimental Procedures. At this age group, over 40% of the smooth muscle cells are polyploid. Staining with anti-IgG was routinely used as a control. Shown are images at 600x magnification. Sections were also stained briefly with hematoxylin (violet/grey background). Blue arrows point to negatively stained cells and the red to p16^{INK4a}-positive cells that did not appear in the KO cells within the smooth muscle layer. It has been our experience that the adventitia (denoted with A) tends to display non-specific staining with a variety of antibodies.

Supplementary Figure 2

Immunohistochemistry of isolated aortic vascular smooth muscle cells (VSMC) stained with anti-p16^{INK4a}. VSMC were isolated from the aortas as described in (Jones and Ravid, 2004), cytospun on slides (approximately 50,000 cells per slide), fixed, and then stained with anti-p16^{INK4a} antibody, using the Vector Red alkaline phosphatase method (see Supplementary Material). Our previous study demonstrated that based on nuclear fluorescence quantitation of 4',6-diamindino-2-phenylindole (DAPI)-stained cells, it is possible to distinguish between diploid and polyploid cells (Nagata et al., 2005). No p16^{INK4a} staining was observed in 3 month-old rat aortas. Some of the polyploid VSMC in the old aorta displayed staining for p16^{INK4a}, compared to no staining in smaller-size (less-intense DAPI) cells from 3 month-old rats. U2OS cells which are devoid of p16^{INK4a} (p16^{INK4a} KO) (Park et al., 2002) were used as a negative control. As indicated, images shown are at 400x or 600x magnification at phase or fluorescence microscopy.

Supplementary Figure 3

A. Immunohistochemistry of aortic sections with anti-p21. Staining was carried out as described under Supplementary Material. In contrast to $p16^{INK4a}$, p21 is noted in both, young and old rat aortas in a small fraction of VSMC. **B. Ploidy analysis of VSMC derived from p21 knockout mice.** Aortic tissues derived from control wild type or p21 knockout mice (Jackson Laboratories) that were strain (C57BL-6)-, sex (male)- and age (fifteen week old)-matched were subjected to cell isolation followed by ploidy analysis by flow cytometry as we described in (Jones and Ravid, 2004). No significant difference was observed in the percentage of polyploid cells in both samples. Shown is a representative histogram, out of 3 mice analyzed. **C. Ploidy analysis of VSMC derived from $p16^{INK4a}$ knockout mice.** Aortic tissue sections derived from control wild type (WT) or $p16^{INK4a}$ knockout mice ($p16^{INK4a}$ KO) (Sharpless et al., 2001) that were strain (FVB)-, sex (females)- and age (seventeen week old)-matched were analyzed for polyploidy in the vascular smooth muscle cell layers as described for Figure 1B. Shown are average percentages +/- standard deviations for 3 mice analyzed in each group.

Supplementary Figure 4

SA-β-gal activity assay in isolated aortic vascular smooth muscle cells. VSMC were isolated from the rat aortas as described in (Jones and Ravid, 2004), cytospun on slides (50,000 cells per slide), fixed, and then stained using the SA-β-gal activity assay (as described in Supplementary Material). Upon cytospinning, the cells appear flat with a tendency to aggregate, and staining with DAPI (1 μg/ml for 10 min at room temperature) displays the diploid nuclei (weaker fluorescence) as well as polyploid ones (more intense fluorescence, calibrated as we described in (Nagata et al., 2005)). No SA-β-gal staining was observed in 3 month-old rat aortas. Blue arrows point to SA-β-gal staining in VSMC derived from 34 month-old rat aortas and to the respective polyploid nuclei. Note smaller cells with lower intensity DAPI staining in 3 month-old rat VSMC. The red arrow points to a representative diploid cell in the preparation of 34 month-old aorta, showing no SA-β-gal activity. Results are representative of three preparations per rat, using 3 young and 2 old rats. The upper panels show cells using phase contrast microscopy (Olympus

microscope; 100x objective) and the lower panels display the respective cell nuclei using DAPI fluorescence.

Supplementary Figure 5

Isolated aortic VSMC subjected to immunohistochemistry with anti-p16^{INK4a} and to SA-β-gal activity assay. VSMC were isolated from 34 month-old aortas as described in (Jones and Ravid, 2004), cytospun onto slides, fixed, stained using the SA-β-gal activity assay (blue) (as in Figure 2) followed by staining with anti-p16^{INK4a} (brown; stained as in Figure 1). Cells were also stained briefly with hematoxylin (violet background). The orange arrows point to the nucleus of a p16^{INK4a}-positive cell. Two thirds of the p16^{INK4a}-postive cells were also positive for SA-β-gal (a representative cell is depicted on the right panel).