SUPPLEMENTAL MATERIAL

Detailed Methods

Generation and characterization of Mef2c-AHF-cre;R26^{mG}, Mef2c-AHF-cre;Alk3$^{f/f}$, Mef2c^{CRE};Alk3$^{f/f}$;R26^{mG} and control specimens

Mef2c-AHF-cre and floxed Alk3 mice have been described previously[1, 2] and were kindly provided by Dr. Brian Black and Dr. Yuji Mishina, respectively. As illustrated in Figure 3A, the mice were used to create Mef2c-AHF-cre;Alk3$^{f/f}$ conditional knockout mice, referred to as SHF-Alk3 cko mice in this paper. To trace the fate of cells derived from the SHF, the Mef2c-AHF-cre mouse was used in combination with the B6.129(Cg)-Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,dTomatLuo}$/J reporter mouse (Jackson Laboratory; stock no 007576) as reported[3]. In addition, male Mef2c^{CRE};Alk3$^{f/+}$ mice were mated with female Alk3$^{f/f}$;R26^{mT} mice to generate SHF-Alk3 ckos that also contain a GFP reporter allele (Mef2c^{CRE};Alk3$^{f/f}$;R26^{mG}). All experiments using animals were approved by the MUSC Institutional Animal Care and Use Committee and complied with federal and institutional guidelines.

Histological evaluation of mutant mice

Male Mef2c-AHF-cre;Alk3$^{f/+}$ mice were mated with female Alk3$^{f/f}$ or Alk3$^{f/f}$;R26^{mT} mice to collect SHF-Alk3 ckos, Mef2c^{CRE};Alk3$^{f/f}$;R26^{mG} ckos, and control embryos at stages ranging from 9-18ED with the presence of a vaginal plug defined as 0.5ED. Following sacrifice of time-pregnant dams, embryos were isolated in phosphate-buffered saline (PBS) and inspected using a dissecting microscope to confirm developmental stage. Embryos were then fixed overnight at 4°C in freshly dissolved paraformaldehyde (4% w/v in PBS), processed through a graded series of ethanol, cleared in toluene, embedded in Paraplast Plus (Fisher Brand, catnr: 23-021-400), serially sectioned (5μm), mounted on Superfrost/Plus microscope slides (Fisherbrand catnr: 12-550-15) and stored at room temperature. Hematoxylin/eosin staining was performed as previously described[4-6].

Immunofluorescent detection of antigens in mutant mice

To detect antigens of interest, sections were deparaffinized in xylene (2x five minutes). Following rehydration through a graded series of alcohols, slides were incubated in antigen unmasking solution (15 mL of Vector Biolabs unmasking solution, catnr H-3300, and 1600 mL dH_20) for five minutes in a pressure cooker (Fagor Splendid, item 918060616). Prior to primary antibody incubation, sections were treated with 1% bovine serum albumin (BSA; Sigma; catnr 9048-46-8) in PBS to decrease nonspecific binding. Immunofluorescence was performed with antibodies recognizing the following antigens: Islet1 (Isl1; DSHB 39.4D5), Myosin Heavy Chain (MF20; DSHB), pSMAD1,5,8 (pSMAD1/5/8; Millipore; catnr AB3848), α-Sarcomeric Actin (sAct; Sigma; catnr A2172), FilaminA (FLNA; Epitomics; catnr 2242-1), pSMAD3 (pSMAD3; Epitomics; catnr 1880-1), Crtl1 (DSHB; 9/30/8-A-4); HABP (Seikagaku; catnr 400763); Ki67 (Dako; catnr M7248), and EGFP (Abcam ab 13970). Secondary antibodies were obtained from Jackson Immunoresearch and included anti-rabbit TRITC (711-025-152), anti-rabbit FITC (711-095-152), anti-rabbit Cy5 (711-175-152), anti-mouse TRITC, anti-mouse FITC (715-095-151), anti-mouse Cy5 (715-175-150), anti-rat TRITC (715-025-150), anti-rat FITC (715-095-150), anti-rat Cy5 (711-175-150), and anti-chicken Cy5 (703-495-155). To detect apoptotic markers, the ApopTag fluorescein direct in situ kit (Millipore; catnr S7160) was used as indicated. Nuclei were visualized using DAPI (Invitrogen; Slowfade Gold Antifade Reagent with DAPI; catnr S36938) and fluorescence visualized using a Zeiss AxioImager II microscope.

In situ hybridization

After sacrifice of time-pregnant dams, embryos were isolated in ice-cold phosphate-buffered saline (PBS) and dissected, fixed overnight in freshly dissolved 4%(w/v) paraformaldehyde (PFA) in PBS, and embedded in paraplast. In situ hybrization was performed essentially as previously described[7]. The embryos were sectioned at 10 µm, deparaffinized, rehydrated in a graded series of alcohol and incubated with 10 mg/ml proteinase K dissolved in PBS for 15 min at 37 °C. The proteinase K activity was blocked by rinsing the sections in 0.2% glycine in PBST (PBS+0.05%Tween-20) for 5 min. After rinsing in PBS, the sections were post-fixed for 10 min in 4% PFA and 0.2% glutaraldehyde in PBS, followed by rinsing in PBS. Pre-hybridization was performed for at least 1 h at 70°C in hybridization mix (50%formamide, 5×SSC (20×SSC; 3 M NaCl, 0.3 M tri-sodium citrate, pH 4.5), 1% blocking solution (Roche), 5 mM EDTA, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (Sigma; Steinheim, Germany), 0.5 mg/ml heparin (BD Biosciences; Erembodegem, Belgium), and 1 mg/ml yeast total RNA (Roche). A digoxigenin-labeled cDNA probe recognizing BMP2, 4, or 7 was added to the hybridization mix in a final concentration of 1 ng/ml. After overnight hybridization, the sections were rinsed with 2xSSC, followed by two washes with 50% formamide, 2×SSC, pH 4.5, at 65 °C, and a TNT rinse (0.1 M Tris–HCl, pH=7.5, 0.15 M NaCl, 0.05% Tween-20) at room temperature. Subsequently, the sections were incubated for 1 h in MABT-block (100 mM Maleic Acid, 150 mM NaCl, pH7.4, 0.05% Tween-20, 2% blocking solution), followed by 2 h incubation in MABT-block containing 100 mU/ml alkaline phosphatase-conjugated anti-DIG Fab fragments (Roche catnr: 1093274). After rinsing in TNT and subsequently in NTM (100 mM Tris pH9.0 100 mM NaCl, 50 mM $MgCl_2$), probe binding was visualized using nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Roche catnr: 1681451). Color development was stopped by rinsing in double-distilled water. The sections were dehydrated in a graded ethanol series, rinsed in xylene, and embedded in Entellan. Images were recorded using a Leica DFC320 camera mounted on an AxioPhot microscope (Zeiss).

Proliferative index of Dorsal Mesocardium

The splanchnic mesoderm situated between the pulmonary pit and foregut was assessed using 8 evenly spaced 5µm sections from 3 $Mef2c^{CRE};Alk3^{f/f};R26^{mG}$ and 4 $Mef2c^{CRE};Alk3^{f/+};R26^{mG}$ littermates at ED10 to determine the proportion of actively dividing SHF cells. Each section was co-labeled with GFP, Ki67 and Isl1. Average SHF proliferation was calculated by dividing the number of Isl1-positive;Ki67-positive or GFP-positive;Ki67-positive cells by, respectively, the total number of Isl1-positive or GFP-positive cells. Overall proliferation was determined by dividing number of Ki67-positive cells by total cell number. The number of GFP-positive cells expressing Isl1 was determined by dividing by number of GFP-positive;Isl1-positive cells by total number of GFP-positive cells. Significance was determined using a two-tailed student's t-test.

AMIRA 3D reconstruction of Dorsal Mesocardium

To generate a 3D rendering of venous pole Isl1-positive and GFP-positive SHF cells in $Mef2c^{CRE};Alk3^{f/f};R26^{mG}$ and $Mef2c^{CRE};Alk3^{f/+};R26^{mG}$ specimens, twenty 5µm serial sections of the dorsal mesocardium were stained using antibodies recognizing Isl1 and GFP. Both Isl1 and GFP expression was recorded using a set exposure time on a Zeiss AxioImager II microscope. Images were imported into AMIRA software and, based on Isl1 or GFP expression, segmentation was generated using a uniform threshold. 3D renderings of venous pole SHF cells were generated using SHF-Alk3 cko and control littermates as well. Twelve 5µm serial sections of the dorsal mesocardium from 3 SHF-Alk3 ckos and 3 $Alk3^{f/f}$ control littermates were stained for Isl1. Isl1 expression was recorded using a set exposure time on a Zeiss AxioImager II microscope, after which AMIRA software was used to create threshold segmentation and calculate voxel size.

AV cushion volume and proliferation

Morphology and volume of the AV cushions was assessed using images of hematoxylin/eosin-stained 5μm serial sections from the entire AV junction of three SHF-Alk3 cko specimens and three control littermates at ED11, 12 and 13.5. Datasets were imported into AMIRA to create 3D-renderings and to determine volume. Statistical significance was determined via two-tailed student's t-test. Proliferation was assessed using six 5μm sections, separated by an average of 19μm, from the major AV cushions of three SHF-Alk3 cko specimens and three control littermates at ED11, 12 and 13.5. Number of cells expressing Ki67 was divided by total number of AV cushion cells and significance determined by two-tailed student's t-test.

Supplemental References

1. Mishina Y, Hanks MC, Miura S, Tallquist MD, Behringer RR. Generation of bmpr/alk3 conditional knockout mice. *Genesis*. 2002;32:69-72
2. Verzi MP, McCulley DJ, De Val S, Dodou E, Black BL. The right ventricle, outflow tract, and ventricular septum comprise a restricted expression domain within the secondary/anterior heart field. *Dev Biol*. 2005;287:134-145
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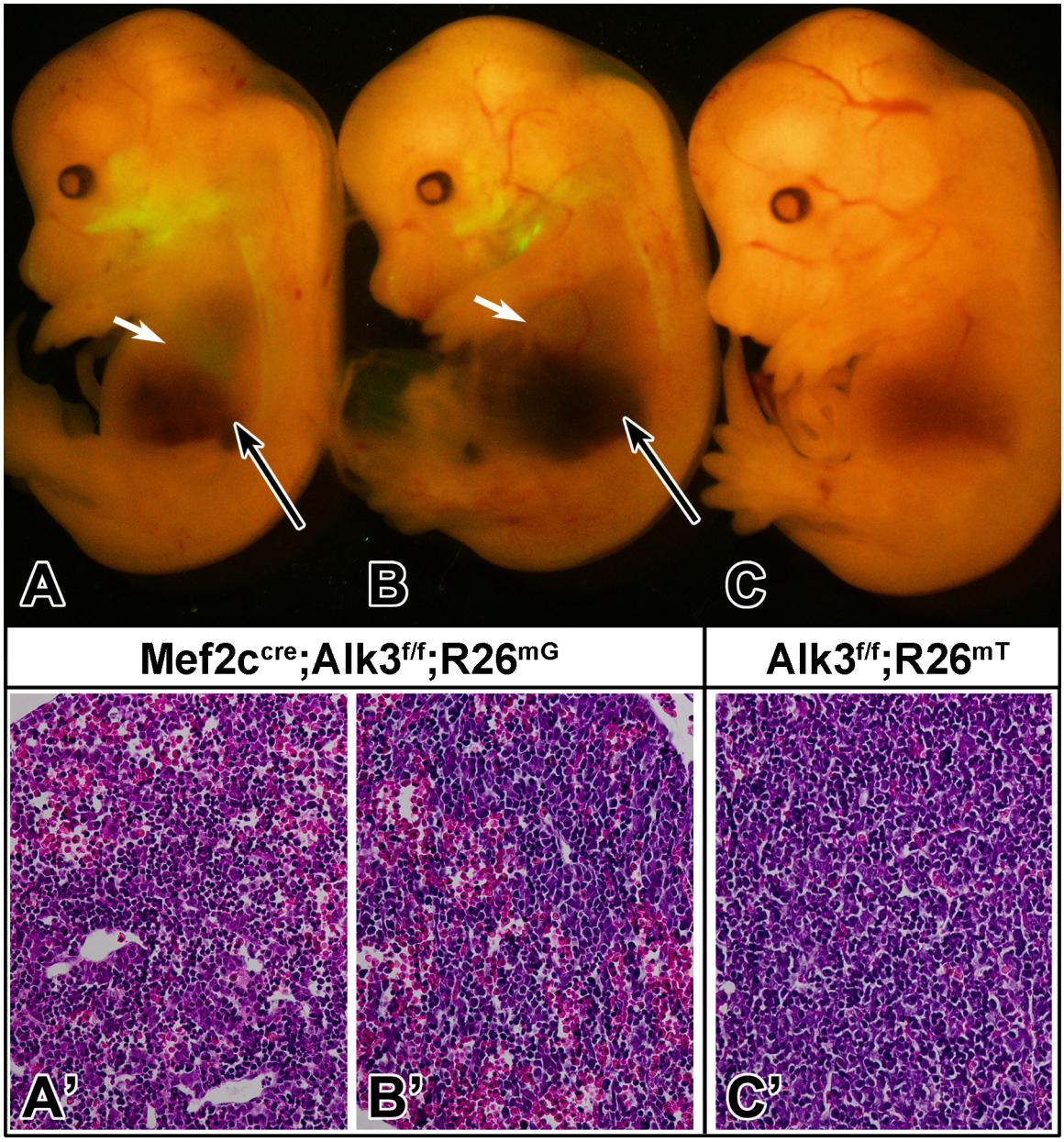
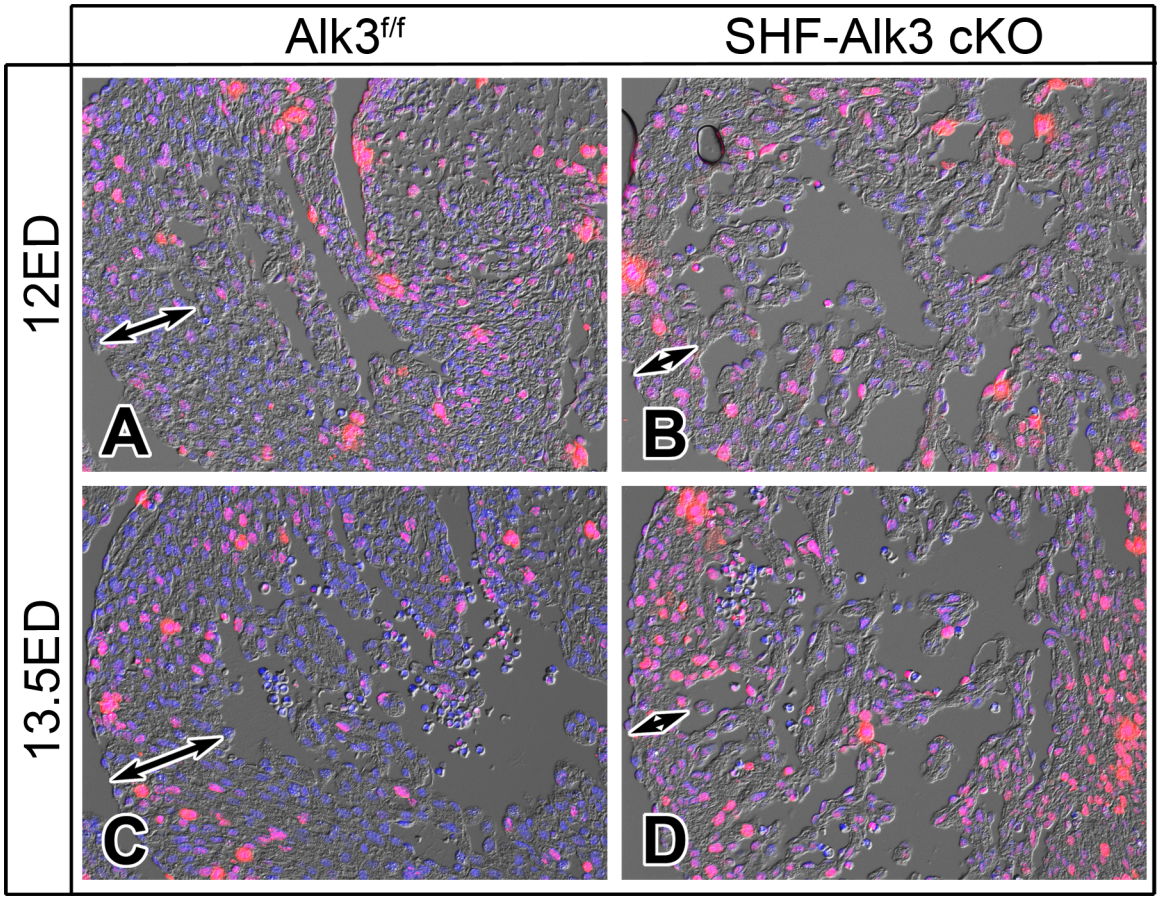
4. Waller BR, 3rd, Wessels A. Cardiac morphogenesis and dysmorphogenesis. An immunohistochemical approach. *Methods Mol Biol*. 2000;135:151-161
5. Snarr BS, O'Neal JL, Chintalapudi MR, Wirrig EE, Phelps AL, Kubalak SW, Wessels A. Isl1 expression at the venous pole identifies a novel role for the second heart field in cardiac development. *Circ Res*. 2007;101:971-974
6. Snarr BS, Wirrig EE, Phelps AL, Trusk TC, Wessels A. A spatiotemporal evaluation of the contribution of the dorsal mesenchymal protrusion to cardiac development. *Dev Dyn*. 2007;236:1287-1294
7. Kruithof BP, van Wijk B, Somi S, Kruithof-de Julio M, Perez Pomares JM, Weesie F, Wessels A, Moorman AF, van den Hoff MJ. Bmp and fgf regulate the differentiation of multipotential pericardial mesoderm into the myocardial or epicardial lineage. *Dev Biol*. 2006;295:507-522

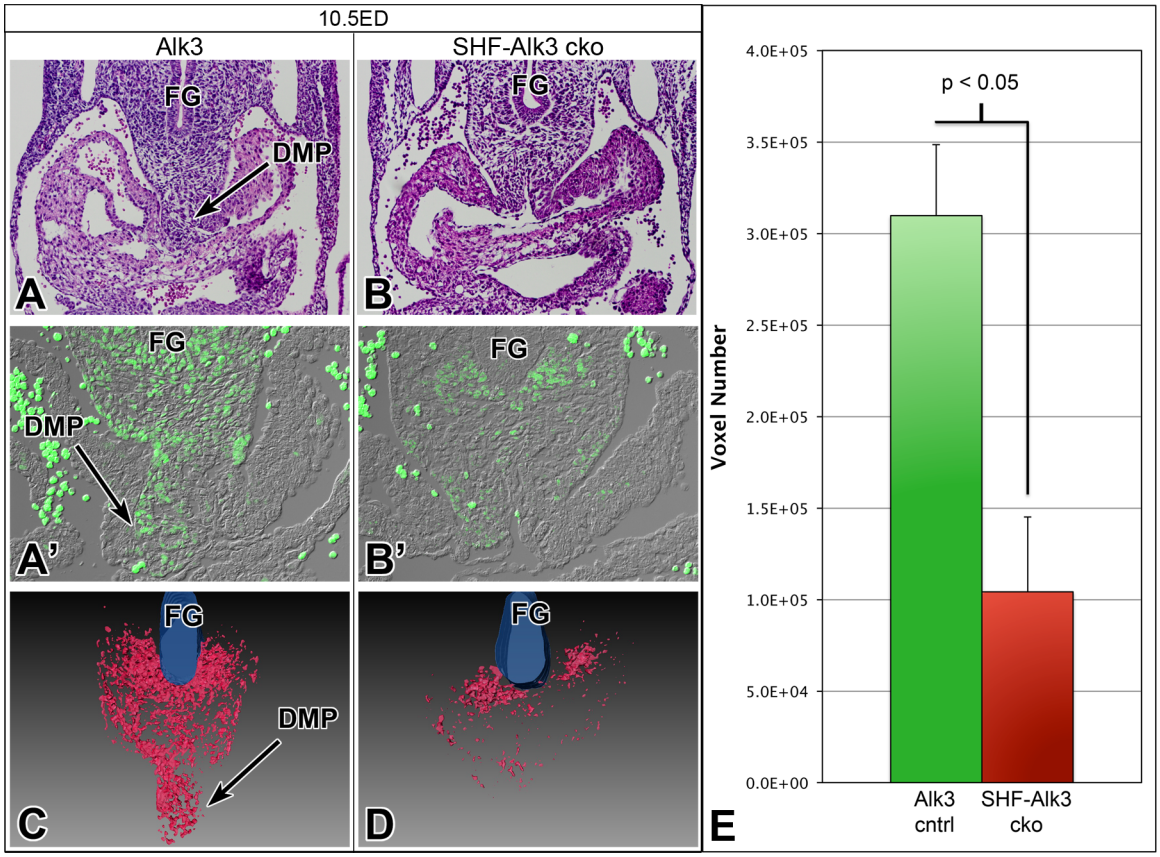
Supplemental Figures and Legends



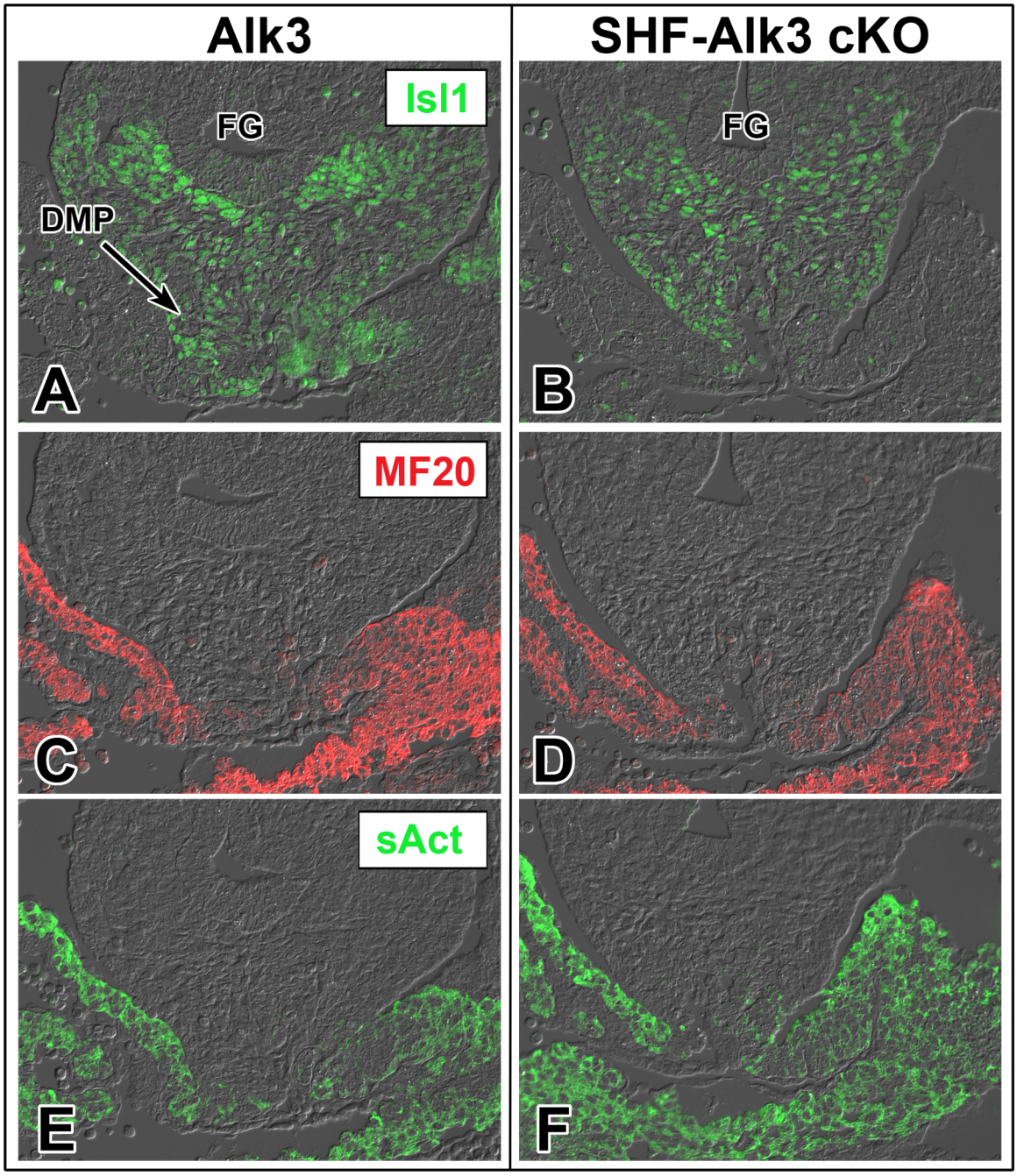
Online Figure I. External and histological features of the liver in SHF-Alk3 ckos. Upon macroscopic inspection, the livers of Mef2c^{CRE};Alk3$^{f/f}$;R26^{mG} mutants (black arrow, A, B) appear darker and enlarged when compared to those of Alk3$^{f/f}$;R26^{mG} control littermates (C). Upon histological examination, the livers from knockouts (A', B') demonstrate sinusoidal dilation and increased numbers of red blood cells, signs consistent with congestive hepatopathy. GFP expression can be observed in the heart of Mef2c^{CRE};Alk3$^{f/f}$;R26^{mG} mutants (white arrow, A, B).



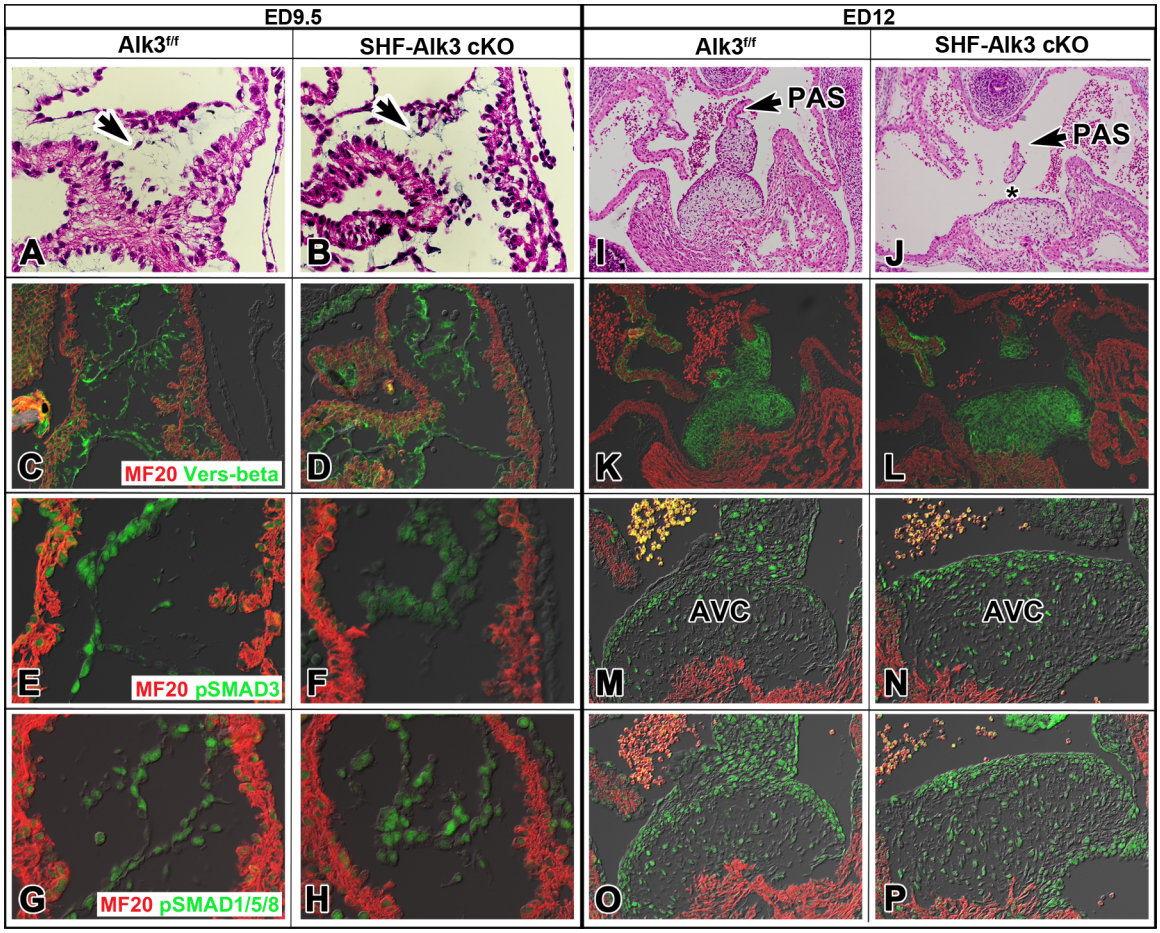
Online Figure II. The level of ventricular myocyte proliferation in SHF-Alk3 cko and control specimens does not show significant differences. Although the ventricular myocardium appears thin in SHF-Alk3 ckos (arrow, B, D) when compared to control littermates (arrow, A, C), the level of proliferation of ventricular myocytes (pHH3, red) at ED12 and ED13.5 does not appear to be reduced in the conditional Alk3 knockout mice.



Online Figure III. The size of the SHF DMP-precursor population is reduced in SHF-Alk3 ckos. When compared to Alk3$^{f/f}$ control littermates (A, A'), the DMP fails to properly form in SHF-Alk3 ckos (B, B). Based upon the expression of Isl1 (green, A', B'), 3D-renderings of 3 control (C) and 3 SHF-Alk3 cko (D) specimens at ED10.5 were generated using AMIRA software. Using these datasets, the volume of the SHF DMP-precursor population as determined by Isl1 expression was calculated. When compared to control littermates, SHF contribution to the venous pole is reduced in SHF-Alk3 ckos (E).



Online Figure IV. Perturbation of DMP development in SHF-Alk3 ckos does not result from premature myocardial differentiation of SHF precursors. Immunohistochemical staining for the presence of gene products generally associated with differentiated cardiomyocytes shows no difference in the level of expression of these genes in the SHF DMP-precursor population of SHF-Alk3 ckos (B, D, F) when compared to control littermates (A, C, E). In both groups, MF20 (C, D) and sarcomeric actin (sAct, E, F) are expressed in the myocardial mesocardial reflections but are not significantly expressed within the Isl1-positive DMP-precursor population. FG, foregut



Online Figure V. Deletion of Alk3 from the SHF does not delay epithelial-to-mesenchymal transition in the AV cushions, nor does it alter the expression of markers for BMP/TGFβ signaling. Histological analysis of the developing AV cushions in SHF-Alk3 cko and control specimens at ED9.5 shows no difference in the immigration of mesenchymal cells into the AV cushions (arrows, A and B). Furthermore, both groups demonstrate similar levels of expression of versican β (C, D, K, L). The expression levels of pSMAD3 (E, F, M, N) and pSMAD1/5/8 (G, H, O, P) are comparable in the AV cushions of both SHF-Alk3 cko and control specimens, consistent with active BMP/TGFβ signaling in these tissues. PAS, primary atrial septum; AVC, atrioventricular cushion