Online data supplement

Materials and Methods

Gene targeting.

The allele *Tbx1^{Egfp}* was generated by homologous recombination in AB2.2 mouse embryonic stem (ES) cells, as shown in Fig. 1A. Briefly, an *Ires-Egfp* cassette was knocked into exon 5 of the *Tbx1* locus, in the same site that was previously used to generate the alleles *Tbx1^{Lacz}* [1] and *Tbx1^{Cre}* [2].

Mouse mutants and breeding.

All the experiments involving mice were done according to a protocol reviewed and approved by the Institutional Animal Care and Use Committee of Institute of Biosciences and Technology, in compliance with the USA Public Health Service Policy on Humane Care and Use of Laboratory Animals. The following mouse mutant lines have been described previously: *Tbx$^{LacZ/+}$* (also indicated as *Tbx1$^{+/-}$*) [1], *COET*[3], and *Mef2c-Cre* [4]. Mice were genotyped by PCR as described in the original reports.

Tissue culture, flow cytometry, cell sorting and differentiation.

Tbx1$^{Egfp/+}$ ES cells were cultured in undifferentiated state on γ-irradiated SNL76 feeder cells in DMEM (Gibco, 12100-046) supplemented with 15% fetal bovine serum (FBS, HyClone), 2-mercaptoethanol (Gibco, 21985-023) and Penicillin-Streptomycin-Glutamine (GPS, Gibco, 10378-016). For differentiation, cells were cultured at the concentration of $5x10^4$ cells/ml using the "hanging drop" method [5] in DMEM containing 20% FBS. After 2 days in hanging drops, the aggregates (that we refer to as embryoid bodies or EBs) were resuspended in 5 ml medium in bacteriological Petri dishes and cultured for additional 4-7.5 days in suspension.

We performed flow cytometric analysis using a two-laser instrument, FACScan (Beckton Dickinson). We carried out flow sorting of *in vitro* differentiated *Tbx1$^{Egfp/+}$* cells (day 2+4, 2+6.5 and 2+7.5 of differentiation, that is 2 days in hanging drop plus 4, 6.5, or 7.5 days in suspension in bacteriological Petri dish) using a triple-laser instrument (MoFlow, Cytomation, Fort Collins, CO). We seeded single *Tbx1-Egfp$^+$* cells from day 8.5 EBs into individual gelatin-coated wells of 96-well plates, and cultured the in DMEM containing 20% FBS for 2-3 weeks. Clones were expanded, stocked, and some of the cells were grown and subjected to a differentiation protocol, which is cultured in gelatin-coated 24 well plates in DMEM supplemented with 20% knockout™ serum replacement (Gibco, 10828-028) and GPS for 7-14 days. Then we carried out immunocytofluorescence staining with antibodies anti-cardiac troponin T (1:200, Lab Vision, MS-295-P) and anti-Platelet/Endothelial Cell Adhesion Molecule 1 (PECAM-1) (PharMingen, 550274, 1:200). RT-PCR was done with a primer pair for smooth muscle-myosin heavy chain (SM-MHC, see Tab. 1 for sequence). Undifferentiated clones were tested by RT-PCR for the expression of genes *Isl1*, *Nkx2.5*, *GATA-4* and *Foxa2* using the primer pairs listed on Tab. 1.

Quantitative expression analysis of genes during *in vitro* ES cell differentiation was carried out at EB day 0, 2, 4, 6, 8.5, and 9.5. For these analyses we used quantitative real time PCR with the following commercial primer pairs *Tbx1* (Applied Biosystems, Mm00448948_m1), *Isl1* (Mm00627860_m1), *Nkx2-5* (Mm00657783_m1), *Flk1* (Mm01222431_m1) and *T-Brachyury* (Mm01318252_m1).

Transfection and cell cycle analysis.

For cell cycle analysis, early passages clones were cultured to 80% confluency in 6-well plates in DMEM containing 15% fetal bovine serum. Then cells were starved with DMEM for 8 hours for synchronization, and transfected with a *Tbx1*-expressing plasmid. We used FuGENE® 6 transfection reagent (Roche) and 0, 1, or 3µg of DNA of *Tbx1*-expressing plasmid, normalized by 3, 2, 0µg, respectively, of DNA of empty plasmid for 24 hrs. The transfection efficiency was 54-56% as tested by co-transfection with a GFP expressing plasmid. Then, DMEM containing 15% FBS was added back for 24 hrs, followed by Propidium Iodide staining for cell cycle analysis using flow cytometry.

C2C12 mouse myoblast cells were cultured to 70-80% confluency in 6-well plates, and transfected with 0, 1, 3, or 5 µg of a *Tbx1-c-myc* -expressing vector DNA [6]. Twenty-four hours after transfection, cells were lysed, RNA was isolated using Trizol (Invitrogen, 15596-026) for real-time PCR analysis, and proteins were extracted after cell lysis with the RIPA buffer (1% NP40, 0.5% NaDoc, 0.1% SDS in PBS, protease inhibitor (Roche, 04 693 159 001)) for western blotting.

Co-Immunoprecipitation and western blotting.

C2C12 cells were transfected with *Tbx1-c-myc* cDNA plasmid and after 24 hrs cells were lysed in immunoprecipitation buffer (20 mM Tris·HCl, pH 8.0/0.5 M NaCl/5 mM $MgCl_2$/0.5% Triton X-100 and protease inhibitor (Roche)). For immunoprecipitation assays we used the ProFound Mammalian Co-Immunoprecipitation kit (Pierce, 23605) following manufacturer instructions. Briefly, after pre-clearing, lysates were incubated with gel-immobilized rabbit anti-Srf antibody (Santa Cruz Biotechnology, sc-335) or gel-immobilized rabbit IgG as control for 16 hrs at 4°C. Immunoprecipitates were washed with the immunoprecipitation buffer before elution. C2C12 cells were transfected with the *Tbx1*-c-myc expressing plasmid or empty vector using the PolyFect® transfection reagent (Qiagen) for 24 hours, followed by treatment with 3 µM of MG132 for 2 hrs. Then cells were washed before addition of fresh media for another 4 hrs. Cells were trypsinized, protein extracted and processed for western blotting. Western blotting was done using XCell II TM Blot Module (Invitrogen, EI9051) following manufacturer instructions. The primary antibodies were rabbit-anti-Srf (Santa Cruz Biotechnology, sc-335, 1:1,000), rabbit-anti-Tbx1 (Zymed, 34-9800, 1:500), mouse-anti-c-myc (Developmental Studies Hybridoma Bank, 9E10, 1:200) and monoclonal anti-α-Tubulin (Sigma, T9026, 1:1,000). The secondary antibodies were as follows: horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Thermo scientific, 31464, 1:10,000), HRP-conjugated anti-mouse antibody (GE Healthcare, NA931V, 1:10,000). The HRP-derived signal was detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, 34078).

Co-IP with mouse embryo material was carried out with the same procedure described above, except that nuclear extracts were derived from E9.5 *WT* or $Tbx1^{-/-}$ embryos. Extracts were immunoprecipitated with an anti-Srf antibody or mouse IgG (controls), and revealed by western blotting using an anti-Tbx1 antibody.

Immunofluorescence and immunohistochemistry.

For immunofluorescence, cryosections were briefly fixed in cold acetone, treated with 1XPBS/0.2% triton and then blocked in 2% bovine serum albumin (Vector Laboratories, SP-5050)/PBST (1XPBS/0.05% Tween20). Sections were incubated with the primary antibodies anti-Tbx1 (Zymed, 34-9800, 1:200) and anti-Isl1 (Developmental

Studies Hybridoma Bank, 39.4D5, 1:50) or anti-α-SMA (Sigma, Clone 14A, 1:200) in blocking solution at 4°C overnight and washed, followed by 1:500 Alexa fluor® 594 goat anti-rabbit secondary antibody (Invitrogen, A31632) and Alexa fluor® 488 donkey anti-mouse secondary antibody (Invitrogen, A21202) in blocking solution at RT for 30 mins. Sections were mounted with Vectashied® with DAPI (Vector Laboratory) and photographed under a Zeiss LSM510 laser scanning confocal microscope.

For Immunohistochemistry, we fixed embryos in 4% Paraformaldehyde/PBS, dehydrated and embedded them in paraffin for histological sections. For antigen retrieval, we boiled sections in sodium citrate buffer (10mM, PH6.0) for 10 mins. After peroxidase blocking (3% H_2O_2 in PBS), sections were blocked in 2% goat serum/PBS and incubated with primary antibodies to α-sarcomeric actin (monoclonal, Sigma, A2172, 1:200) anti-cardiac troponin T (1:200, Lab Vision, MS-295-P), MF20 (hybridoma bank, 1:50) or Srf (Santa Cruz Biotechnology, sc-335, 1:200) in blocking solution overnight at 4ºC. Then sections were treated with biotinylated anti-mouse (1:200) or anti-rabbit (1:200) secondary antibodies (Vector Laboratories) at RT for 1 hr, followed by treatment with Vectastain Elite ABC reagent (avidin−horseradish peroxidase; Vector Laboratories). Horseradish Peroxidase (HRP) activity was revealed using the DAB kit (Vector laboratories). For the alkaline phosphatase-conjugated anti-α-smooth muscle actin (α-SMA) antibody (Sigma, Clone 14A), sections were blocked in 1% sheep serum/PBS and incubated with anti-α-SMA antibody (1:100 in 1% sheep serum/PBS) at 4 °C overnight. NBT/BCIP (Roche) was used to visualize the alkaline phosphatase activity. Sections were dehydrated, counter-stained with NFR as needed, mounted with Permount and examined under a Zeiss light microscope.

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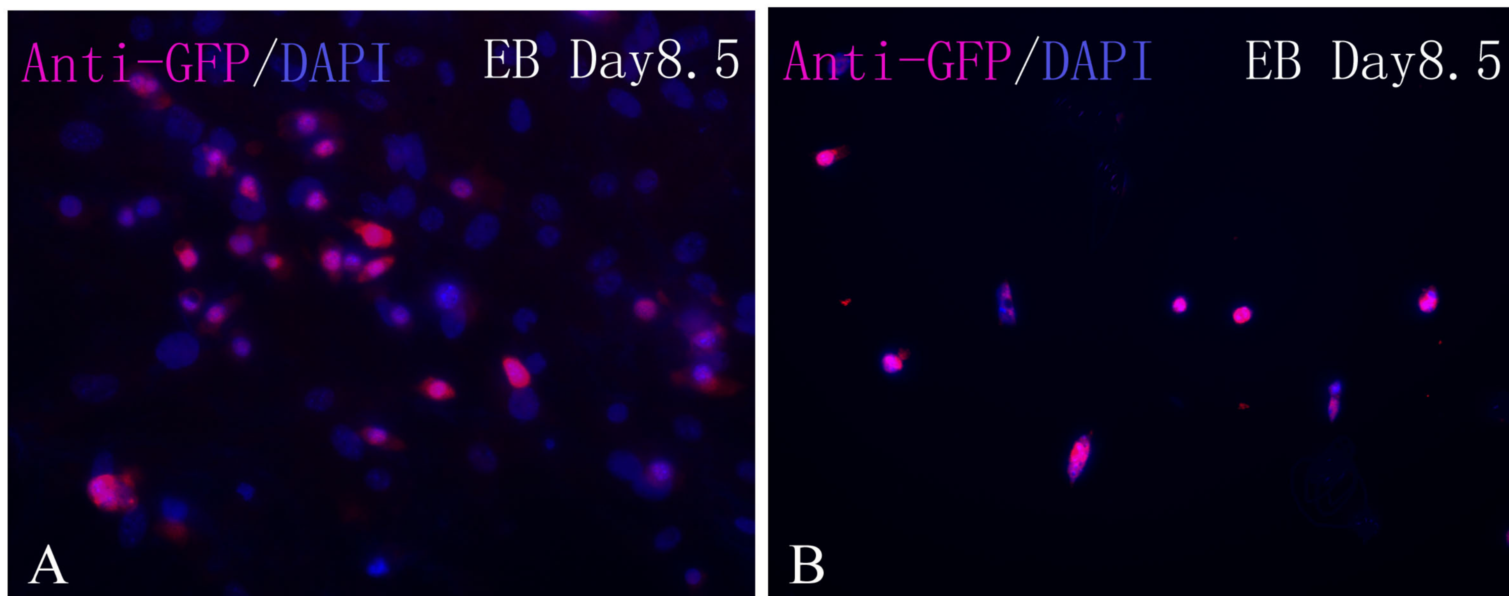
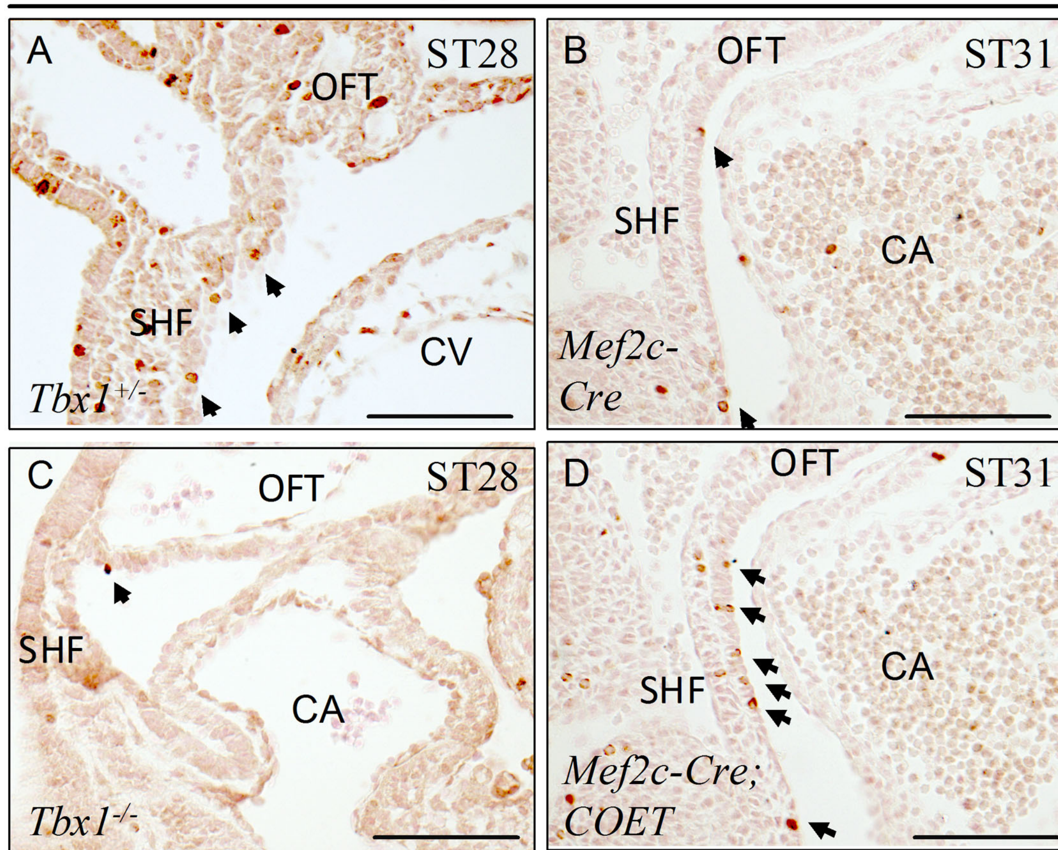
Baldini_Fig_S1

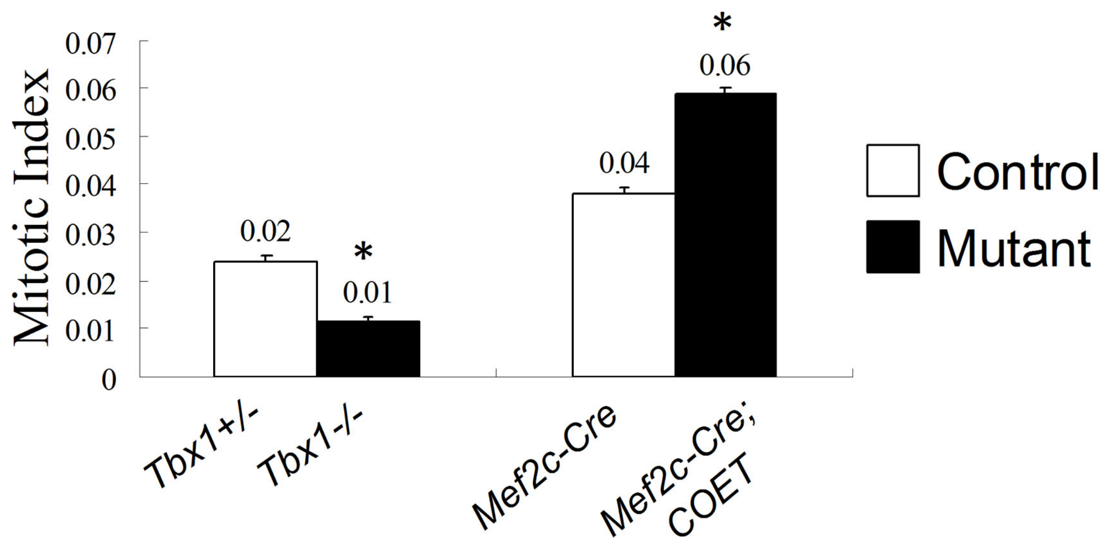


Supplementary Figure 1.

Confirmation of GFP expression by immunofluorescence with an anti-GFP antibody before (A) and after (B) flow sorting.

Baldini_Fig_S2

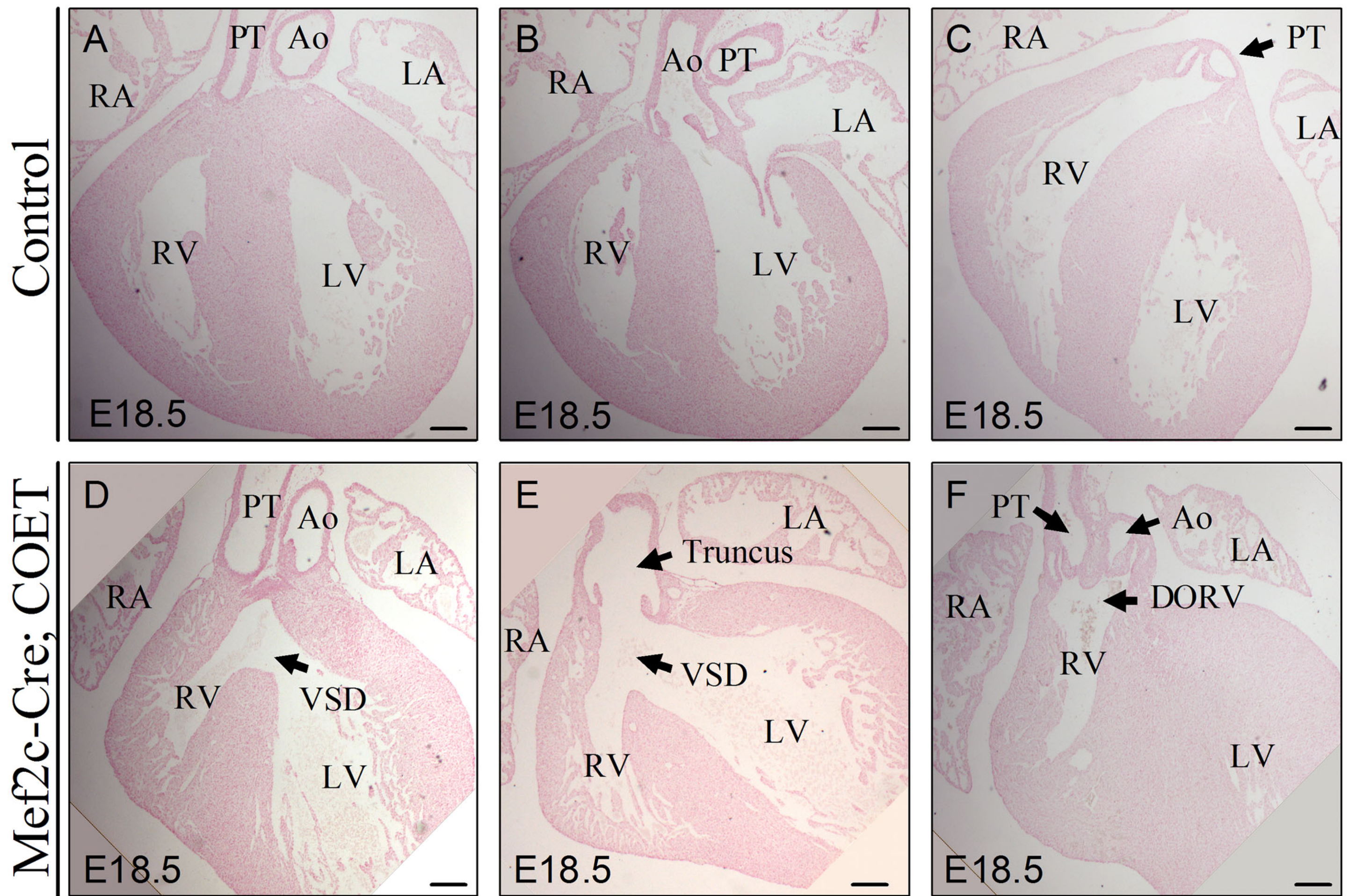




Supplementary Figure 2. Proliferation of SHF cells in *Tbx1* loss and gain of function embryos at E9.5.

(A-D) Phospho-Histone H3 immunohistochemistry shows reduced proliferation in the SHF of *Tbx1*$^{-/-}$ embryos (ST28) and increased proliferation in *Mef2c-Cre; COET* embryos (ST31). Arrows indicate phospho-Histone H3 positive cells in the SHF. (E) Mitotic Index (MI) shows statistical significance between control (*Tbx1*$^{+/-}$ or *Mef2c-Cre*) and mutants (*Tbx1*$^{-/-}$ or *Mef2c-Cre; COET*) ($p<0.05$; n=3). Approximately 500 cells were scored per embryos, and 2 embryos were scored for each experimental point. OFT: outflow tract; SHF: second heart field; CA: common atrium. Scale bar: 100 μm.

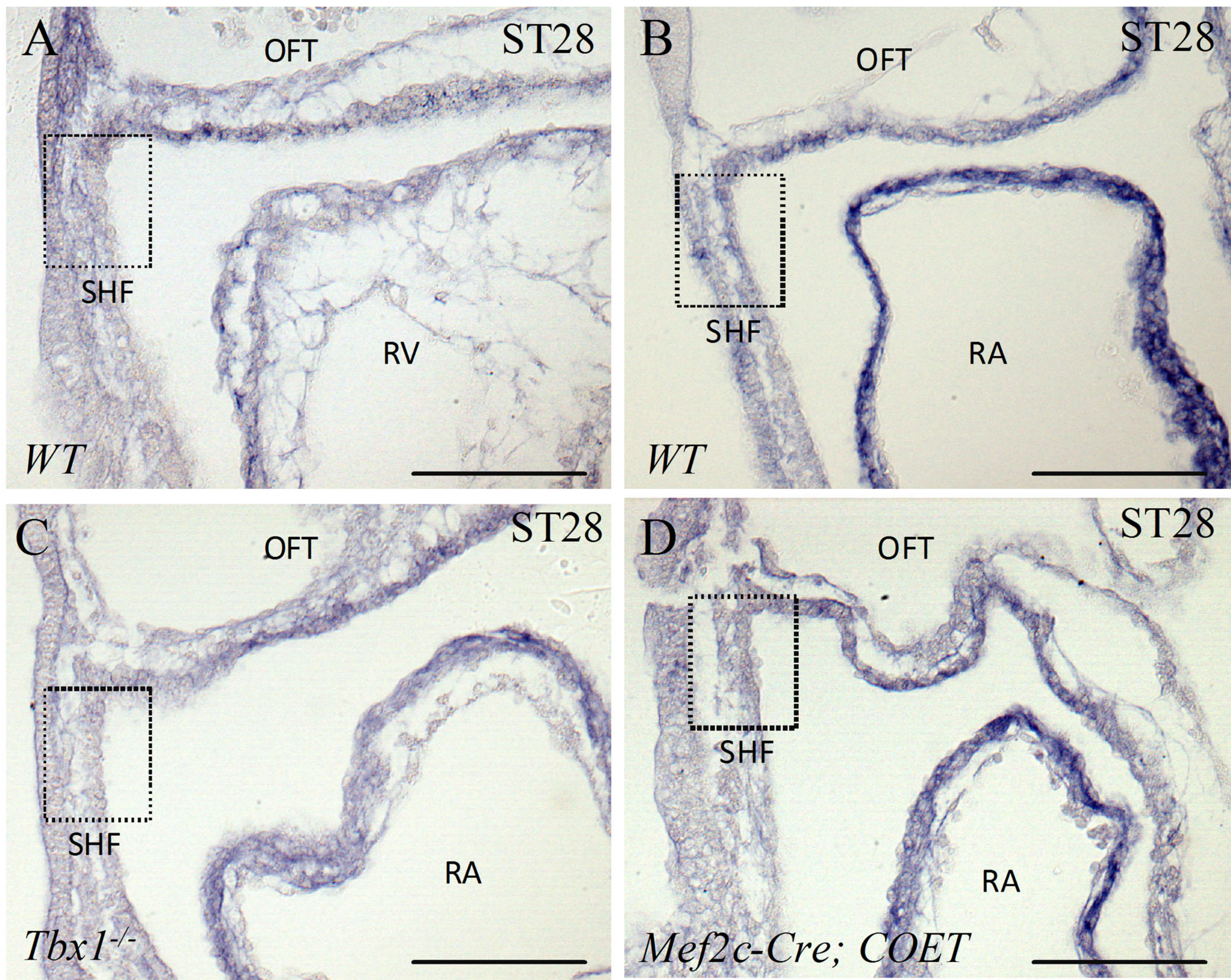
Baldini_Fig. S3



Supplementary Figure 3. Developmental defects of SHF-derived structures of the heart of *Mef2c-Cre; COET* mutants at E18.5.

(A-C) Heart sections of control embryos; (D) heart section of a mutant embryo with small right ventricle (RV) and a ventricular septal defect (VSD); (E) heart section of a mutant embryo with small RV, VSD and truncus arteriosus; (F) heart section of a mutant embryo with small RV and double outlet right ventricle (DORV). RV: right ventricle; LV: left ventricle; RA: right atrium; LA: left atrium; PT: pulmonary trunk; Ao: aorta. All sections were coronal. Scale bar: 100 µm.

Baldini_Fig_S4



Supplementary Figure 4. Similar Srf gene expression in *Tbx1* loss and gain of function mouse models at E9.5.

In situ hybridization with an Srf probe on control (A-B), *Tbx1*$^{-/-}$ (C), and *Mef2c-Cre; COET* (D) embryos. There is no detectable difference among genotypes.OFT: outflow tract; SHF: second heart field; RA: right atrium. Scale bar: 100 μm.