Supporting Information

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SI Materials and Methods

For ChIP assay, embryonic hearts at embryonic day (E)9.5–E9.75 were divided to 4-parts, outflow tract (OFT), ventricles, atrioventricular canal (AVC), atria at the posterior boundary of endocardial cushion (EC) in OFT, anterior and posterior boundaries of EC. Dissected tissues were quick frozen in liquid nitrogen and stored at −80 °C until embryo and yolk sac DNA was genotyped. ChIP experiments were performed as described (1). Tbx2 polyclonal antibody was used same one as described in the main text. Extracts were prepared from 4-parts of embryonic cardiac tissues as described in the main text.

The following PCR primers against the 5′ Has2 promoter region were used: mHas2ChIPF6 (5′-ACTTAAGGGGGTCTTAACAAGTGAG-3′) and Has2ChIPR6 (5′-ACTGCCTGAGGAGAGGTATCAG-3′).

As a control, primers to an unrelated region of Has2 promoter region were used: mHas2ChIPCF1 (5′-CCACATCTGTGCTTTATGTCTACAA-3′) and mHas2 ChIPCR1 (5′-AAAAATAATTGGGTTGAAAAGAACC-3′).

The following PCR primers against the 5′ *TGFβ2* promoter region were used: m TGFβ2ChIPF1 (5′-CCTTCTCTCTTTCTCTCCCACTC -3′) and m TGFβ2 ChIPR1 (5′-AGACAGTGACTACTGCAAATTCCTC -3′).

As a control, primers to an unrelated region of *TGFβ2* promoter region were used: mTGFβ2ChIPCF4 (5′-TAAACTAGGTCCTGTTATCCCCTCT -3′) and mTGFβ2ChIPCR4 (5′-ATGTCTAATGCCTAACTGGACTTTG -3′).

1. Cai CL, et al. (2005) T-box genes coordinate regional rates of proliferation and regional specification during cardiogenesis. *Development* 132:2475–2487.

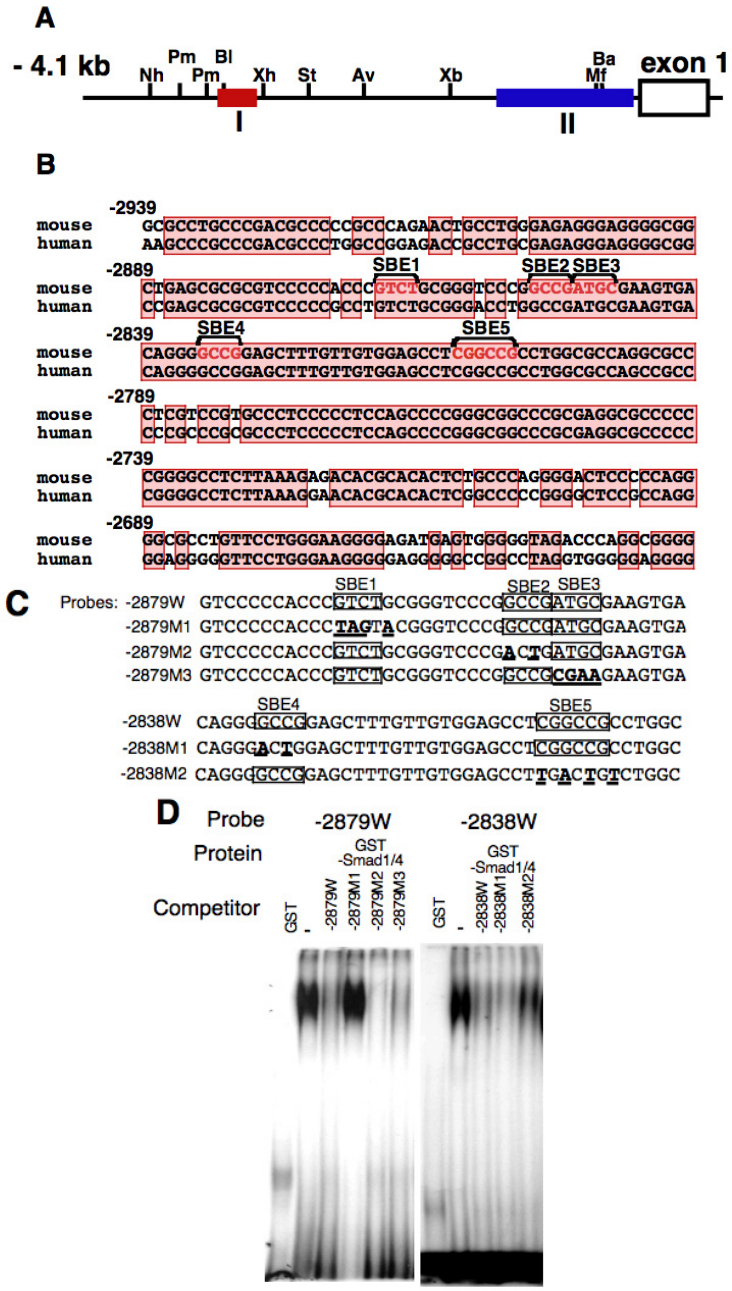

Fig. S1. *Tbx2* enhancer composed of multiple Smad binding sites. (*A*) Schematic representation of 5′ flanking regions of mouse *Tbx2*. Two regions are conserved between human and mouse sequences (I and II). (*B*) Comparison of the mouse *Tbx2* distal enhancer with human sequence. Five Smad binding sites present in the distal conserved region at −2939 to −2602. (*C*) Smad1 and 4 bind to −2879/−2839 and −2839/−2800 fragments. Electrophoretic mobility shift assay were performed with Smad1 and four proteins translated in vitro and two ^{32}P end-labeled oligonucleotides containing Smad binding sites located at −2879 to −2839 and −2839 to −2800 of *Tbx2* distal element. (*D*) Specificity of Smads binding was confirmed by competition with unlabeled WT competitor mutant Smad binding sites oligonucleotides at 200× the labeled WT probe. Each oligonucleotide sequence was shown (*C*). The consensus Smad binding sites within fragments −2879/−2839 and −2838/−2800 was analyzed by electrophoretic mobility shift assay, as described by Cai et al. (1). GST-Smad proteins were induced by IPTG in bacterial cells and purified using glutathione-Sepharose beads. Annealed oligonucleotide probes were ^{32}P end-labeled with T4 polynucleotide kinase, purified over a G25 spin column; ^{32}P-labeled probe (1×10^5 cpm) and then incubated with 1 μg of GST fusion proteins in binding buffer for 30 min at room temperature. Competition experiments were performed to test for specificity of DNA binding by using unlabeled WT and mutated duplexed oligonucleotide as competitors in 200-fold excess. The DNA-protein complex was separated by electrophoresis on a 6% nondenaturing polyacrylamide gel in 0.5× TBE.

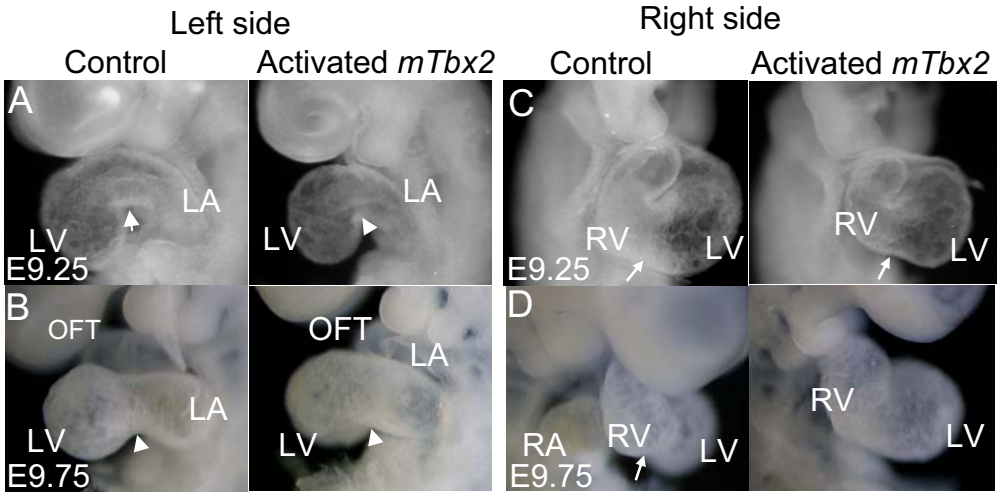

Fig. S2. Cardiac malformation progressed rapidly within only 0.5 day in *mTbx2*-misexpressing hearts. (*A–D*) Enlarged images highlight embryonic hearts at E9.25–E10.5. Left view of embryos (*A* and *B*), and right view of embryos (*C* and *D*). There is no obvious difference between control littermates and activated *Tbx2* embryos at E9.25 (*A* and *C*). At E9.75 (*B* and *D*), relative to control littermates, activated *Tbx2* embryos exhibited cardiac dilatation. Arrows indicate intraventricular sulcus (IVS). Arrowheads indicate AVC.

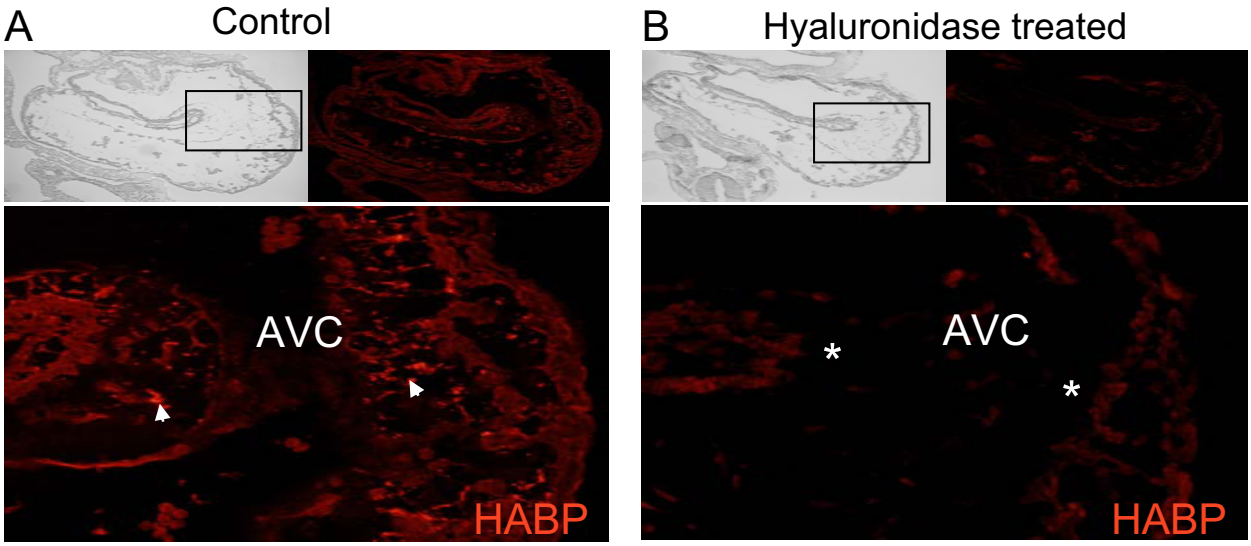

Fig. S3. Visualization of hyaluronan (HA) with HA-binding protein (BP) allowed for confirmation of HA specific binding activity of HABP. Sectioned E9.75 embryonic hearts showed deposition of HA between the endocardium (En) and myocardium (My) in the AVC (arrowheads; *A*). HABP-positive staining was reduced by hyaluronidase treatment for 2 h (asterisks; *B*).

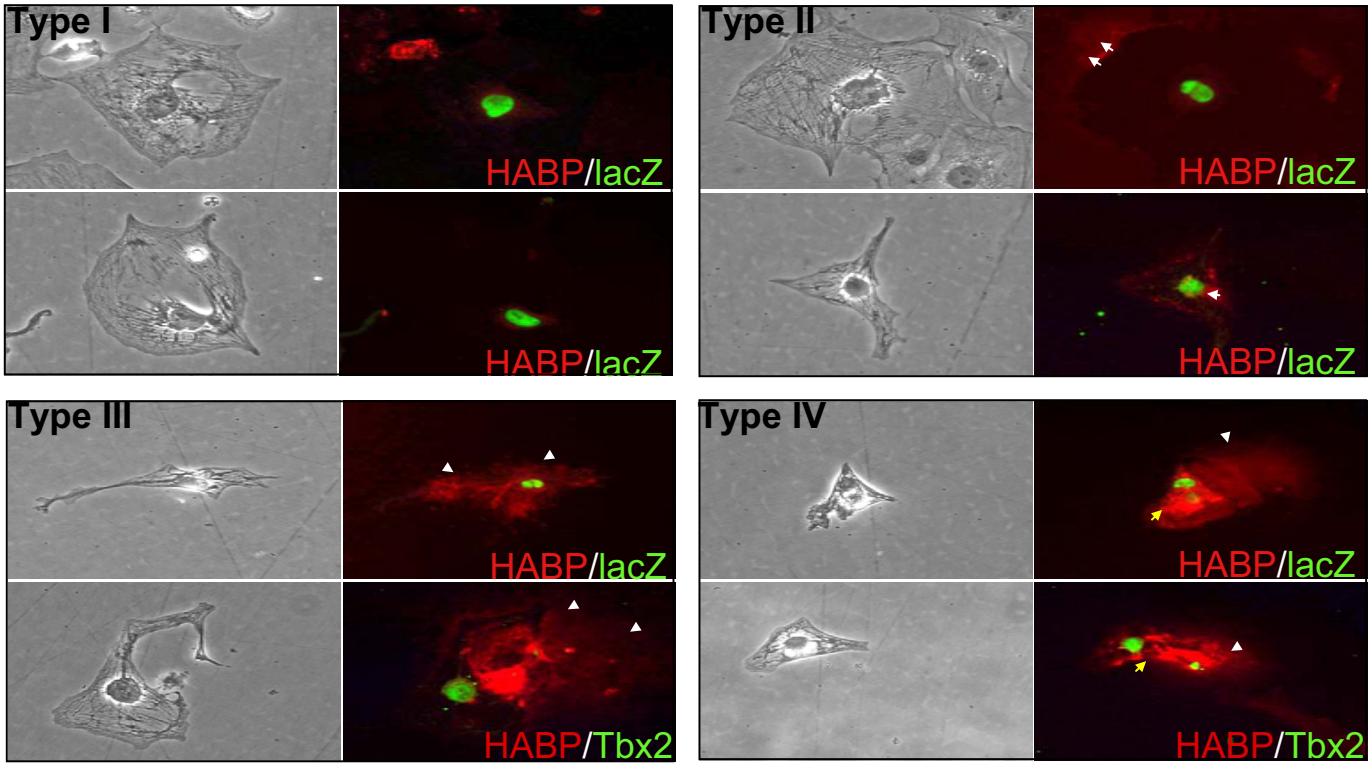

Fig. S4. Classification of the cardiomyocytes expressing HA synthesis. Cardiac cells were isolated from 4-parts of embryonic cardiac tissues and cultured on gelatinized dishes for 4 days. Cardiomyocytes were identified by *lacZ* or *mTbx2* expression and classified by the relative amount of HA secretion. Type I cardiomyocytes, no secretion of HA; Type II, little amount of HA around the cells; Type III, greater HA is secreted around the cells; Type IV, much greater HA is secreted around the cells and also deposited in the cells.

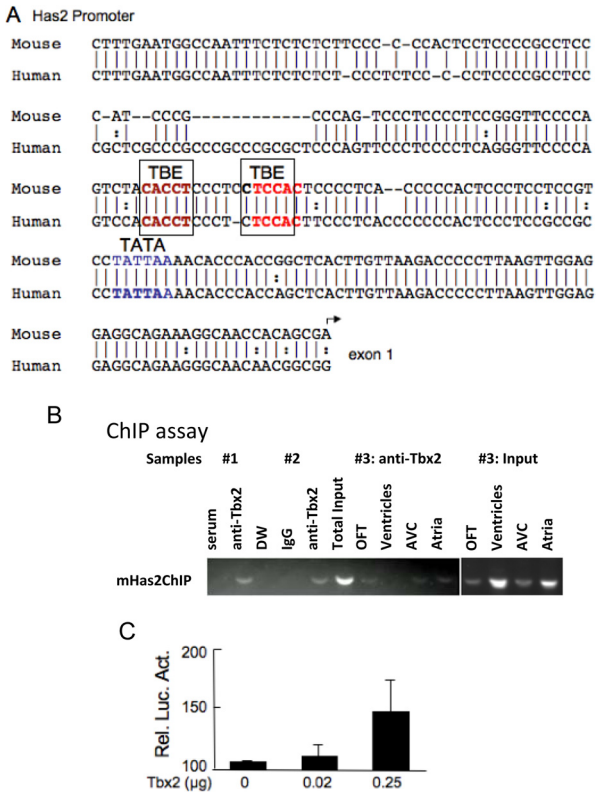

Fig. S5. Additional evidence to support *Has2*, as a downstream Tbx2 gene target. (*A*) Comparison of the human and mouse *Has2* promoter sequences showed well conserved T-box consensus sites. (*B*) ChIP analysis using three independent samples; two extracts from whole embryonic hearts and one extract from separated embryonic cardiac tissues at E9.5–E9.75 revealed specific binding of Tbx2 to region containing conserved T-box sites. Whereas binding was seen in extracts from embryonic OFT, AVC, and atria, it was not seen in extracts from control embryonic ventricles where *Has2* expression was not observed by whole-mount in situ hybridization (Fig. 4*B*). No recruitment was found with control serum, DW, and IgG. (*C*) Cotransfection of *Tbx2* expression vector with *Has2* promoter-luciferase constructs into HEK293 cells exhibited a significant increase in relative luciferase activity in response to Tbx2.

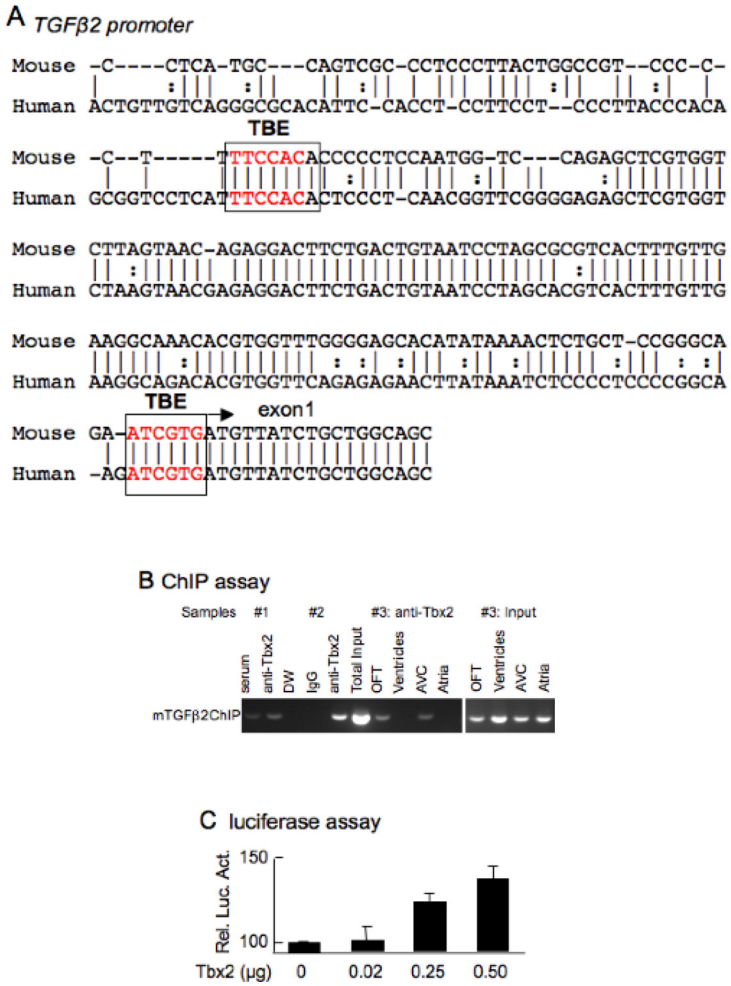

Fig. S6. Additional evidence to support *Tgfβ2*, as a direct gene target of *Tbx2*. (*A*) Comparison of the human and mouse sequences showing conserved T-box consensus sites within the *Tgfβ2* promoter. (*B*) ChIP analysis using three independent samples; two extracts from whole embryonic hearts and one extract from separated embryonic cardiac tissues at E9.5–E9.75 revealed specific binding of Tbx2 to region containing conserved T-box sites. Binding was observed only in extracts from embryonic OFT and AVC, not seen in extracts from embryonic ventricle and atria where *Tgfβ2* expression was not observed by whole-mount in situ hybridization (Fig. 5*B*). No recruitment was found with serum, DW, and IgG. (*C*) Cotransfection of *Tbx2* expression vector with *Tgfβ2* promoter-luciferase constructs into HEK293 cells demonstrated a significant increase of the *Tgfβ2* promoter luciferase activity in response to Tbx2.

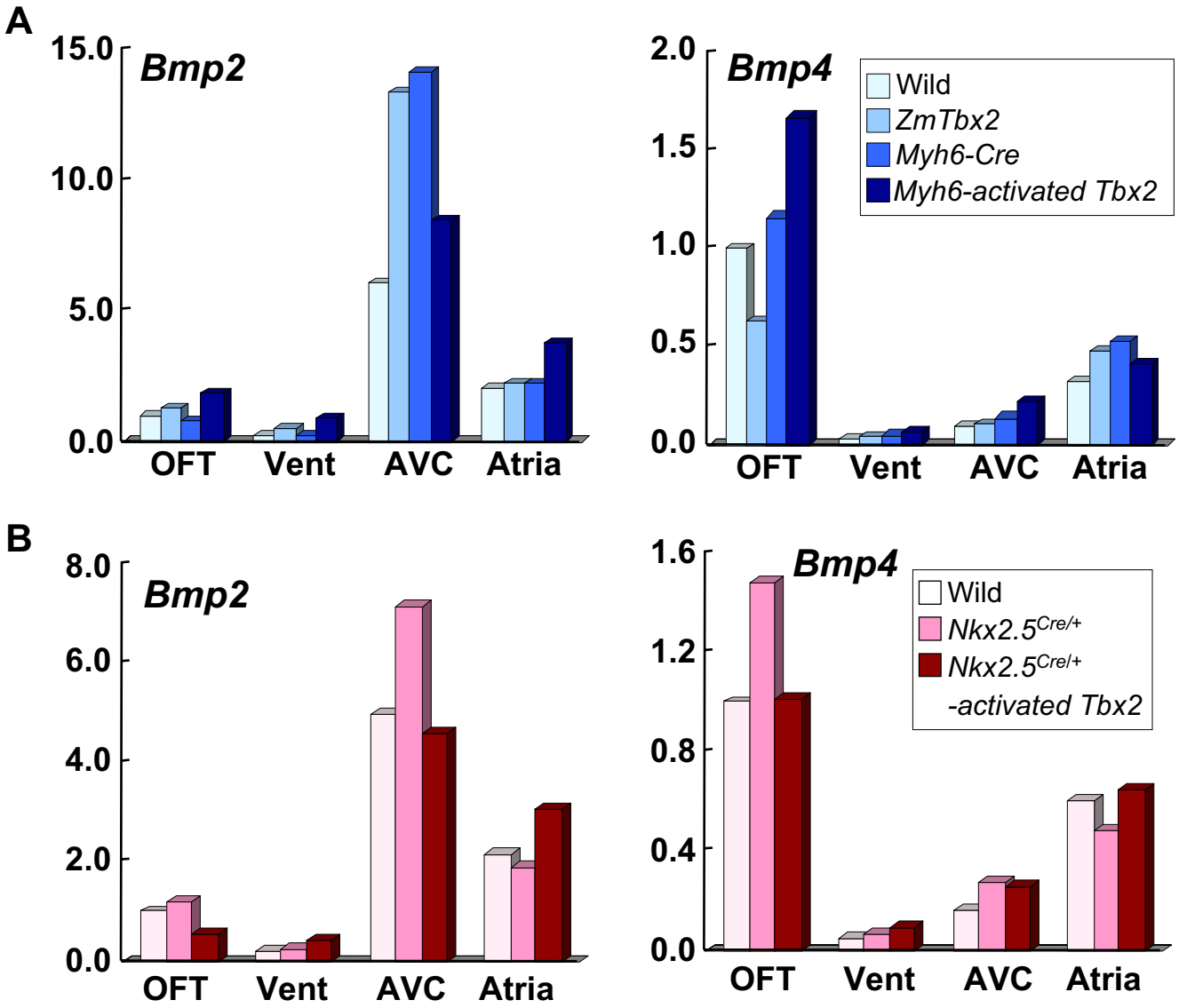

Fig. S7. Bmp2/4 were not up-regulated in the activated *Tbx2* embryonic hearts. (*A*) Real-time PCR analysis of dissected hearts from *ZmTbx2* : *Myh6-Cre* embryos at E9.75. (*B*) Real-time PCR analysis of dissected hearts from *ZmTbx2* : *Nkx2.5*$^{Cre/+}$ embryos at E9.5.