Integration of Bmp and Wnt signaling by Hopx specifies commitment of cardiomyoblasts

Rajan Jain[1],[#], **Deqiang Li**[1],[#], **Mudit Gupta**[1], **Lauren J. Manderfield**[1], **Jamie L. Ifkovits**[1],[‡], **Qiaohong Wang**[1], **Feiyan Liu**[1], **Ying Liu**[1], **Andrey Poleshko**[1], **Arun Padmanabhan**[1],[+], **Jeffrey C. Raum**[2], **Li Li**[1], **Edward E. Morrisey**[1], **Min Min Lu**[1], **Kyoung-Jae Won**[2], and **Jonathan A. Epstein**[1],[*]

[1]Department of Cell and Developmental Biology, Penn Cardiovascular Institute, Institute of Regenerative Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

[2]Department of Genetics, Institute for Diabetes, Obesity, and Metabolism, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

Abstract

Cardiac progenitor cells are multipotent and give rise to cardiac endothelium, smooth muscle, and cardiomyocytes. Here, we define and characterize the cardiomyoblast intermediate that is committed to the cardiomyocyte fate, and we characterize the niche signals that regulate commitment. Cardiomyoblasts express Hopx, which functions to coordinate local Bmp signals to inhibit the Wnt pathway, thus promoting cardiomyogenesis. Hopx integrates Bmp and Wnt signaling by physically interacting with activated Smads and repressing Wnt genes. The identification of the committed cardiomyoblast that retains proliferative potential will inform cardiac regenerative therapeutics. In addition, Bmp signals characterize adult stem cell niches in other tissues where Hopx-mediated inhibition of Wnt is likely to contribute to stem cell quiescence and to explain the role of Hopx as a tumor suppressor.

Lineage analyses during cardiac development in the chick and mouse over the last two decades have demonstrated that at least two pools of progenitor cells contribute to the heart (1). Cardiac progenitor cells (CPCs) derived from the cardiac crescent, or the first heart field (FHF), express *Nkx2-5* and contribute to a primitive heart tube. After subsequent looping of the heart tube, additional progenitor cells are added to the arterial and venous poles of the heart from the second heart field (SHF). SHF cells arise just medial and posterior to the FHF

[*]Correspondence to: Jonathan A. Epstein, Smilow Center for Translational Research, 09-105, 3400 Civic Center Blvd., Philadelphia, PA 19104, USA, Phone: 215-898-8731, Fax: 215-898-9871, epsteinj@upenn.edu.
[#]These authors contributed equally to this work.
[‡]Present address: GSK, 1250 S. Collegeville Road, Mail Code: 12-L16E, Collegeville, PA 19426
[†]Massachusetts General Hospital, 55 Fruit St, Boston, MA 02114

in the cardiac crescent, and populate the pharyngeal arches and dorsomedial mesoderm prior to migration into the heart proper (2, 3).

Studies modeling cardiac development using differentiation of ES-derived embryoid bodies (EBs), have demonstrated that SHF progenitors, marked by *Islet1* (*Isl1*) expression (4), are multipotent, with potential for differentiation into cardiomyocyte, endothelial, or smooth muscle lineages (5–7). Wnt signaling is necessary to promote and expand multipotent CPCs (8–10), and subsequent differentiation of cardiac myocytes is influenced by Bone morphogenetic protein (Bmp) signaling and Wnt inhibition (11, 12). However, the implications of these studies for *in vivo* cardiogenesis are unknown. The characteristics of an embryonic CPC niche are poorly described and the degree to which CPCs remain uncommitted during mid and late gestation has been unclear. For example, recent reports using inducible lineage tracing of early cardiac progenitors, marked by Mesp1 expression, suggest the existence of a relatively small pool of multipotent progenitors *in vivo* and at least some of these become committed at very early stages, perhaps soon after gastrulation (13, 14). Efforts to fully characterize the signaling pathways active during cell fate decisions *in vivo* have been hampered, at least in part, by the lack of specific markers of lineage commitment.

Here, we report that CPCs committed to the myocyte lineage can be prospectively identified prior to the expression of sarcomere genes based on Hopx expression, an atypical homeodomain protein expressed during early cardiac development and in multiple stem cell populations (15–19). Akin to an erythroblast in hematopoietic differentiation, we have termed these committed CPCs "cardiomyoblasts." We show that SHF-derived cardiomyoblasts are specified in the distal outflow tract (OFT) within a zone of high Bmp and low Wnt signaling. Finally, we show that Hopx not only marks commitment, but that it also promotes myogenesis by interacting with an activated-Smad complex to repress Wnt.

Cardiomyoblasts are defined by Hopx expression

At early stages of cardiac development, *Nkx2-5* and *Isl1* mark populations of CPCs (2, 4), and *Hopx* expression initiates shortly after *Nkx2-5* in precardiac mesoderm (15). We used a knock-in allele in which Hopx is epitope tagged and GFP is expressed in Hopx^{+} cells (18) to determine that Hopx is expressed in a subset of CPCs at E8.0 and E8.5 in the FHF and SHF, respectively (Fig. 1, A and B). As SHF progenitors enter the distal outflow tract (OFT), Isl1 expression is gradually extinguished and Hopx expression initiates providing a restricted region of co-expression in the distal OFT (Fig. 1B). Fate-mapping using *Nkx2-5*[Cre/+] and cre-dependent reporter mice indicates that essentially the entire late-gestation heart derives from *Nkx2-5*[+] precursors, including myocytes, smooth muscle, endothelium, and epicardium (Fig. 1C). Isl1^{+} cells also give rise to smooth muscle, endothelium, myocytes and epicardium, but some of the left ventricle myocardium and atria, derived from the FHF, is not labeled in *Isl1* fate-mapping experiments (Fig. 1D). Lineage tracing experiments using a *Hopx*[Cre/+] allele, in which we inserted cre following an internal ribosomal entry sequence (IRES) so as to avoid perturbing *Hopx* expression (20), demonstrate labeling in all four cardiac chambers (Fig. 1E and fig. S1, A and B). However, in contrast to *Nkx2-5* and *Isl1*, *Hopx*-derivatives within the heart are entirely restricted to cardiac myocytes (Fig. 1E and

fig. S2). Some cardiac fibroblasts derive from *Nkx2-5* and *Isl1*-expressing precursors, but *Hopx*^{+} cells do not give rise to fibroblasts in the heart (fig. S2). Most cardiac myocytes derive from *Hopx*[+] precursors, although some specialized myocytes surrounding the pulmonary veins and within the interatrial septum are not derived from Hopx^{+} cells (fig. S3). Analysis of E9.5 *Nkx2-5*[Cre/+]*; R26*[Tom/+]*; Hopx*[3XFlag/+] embryos reveals that all Hopx[+] cardiomyocytes at this time point derive from *Nkx2-5*[+] precursors (Fig. 1F). Flow cytometry analysis of dissociated post-natal day 2 (P2) hearts, expressing a reporter allele, confirms the multi-lineage contribution of *Nkx2-5* and *Isl1*, in contrast to *Hopx*^{+} cells (fig. S4). We do not detect Hopx in any non-myocyte cell types within the heart, consistent with our lineage tracing data (fig. S5).

To confirm the fate of the earliest Hopx^{+} expressing cells we used a tamoxifen-inducible *Hopx* allele (*Hopx*[ERCre/+]) (19). Low doses of tamoxifen induction in *Hopx*[ERCre/+]*; R26*[Tom/+] embryos reveal that single E8.25 *Hopx*[+] cardiomyoblasts expand to form clusters of myocytes by E18.5 (Fig. 2, A and B) and Hopx[+] cardiomyoblasts express markers of proliferation at early embryonic time points (fig. S6). In addition, inducing cre recombinase activity with a single dose of tamoxifen at E8.25, a time point correlating with Hopx expression within a subset of *Nkx2-5*[+] progenitors, demonstrates lineage-labeled myocytes primarily in the left ventricle and both atria at E18.5 (Fig. 2C). However, similar lineage tracing of E9.25 Hopx^{+} cells demonstrate that cardiomyoblasts expressing Hopx at this later time point contribute to myocytes in both ventricles and both atria (Fig. 2D). Taken together, these results establish that cardiac Hopx-expression identifies a pool of progenitors committed entirely to the myocyte lineage (e.g. cardiomyoblasts) and that *Hopx*[+] cardiomyoblasts expand during cardiogenesis. In addition, our results suggest that commitment of FHF and SHF CPCs occurs at distinct time points during cardiogenesis.

Hopx promotes myogenesis by inhibiting Wnt signaling

Hopx is expressed early during cardiomyocyte differentiation, and precedes that of troponin T (Tnnt2) in both the FHF and SHF (Fig. 3, A and B). In order to examine the function of Hopx during cardiomyogenesis, we overexpressed Hopx at various time points of embryonic stem (ES) cell differentiation. *Hopx* is normally detectable by day 5, before *Tnnt2* and *Mhy6* are expressed by maturing EBs (fig. S7A). Using established protocols (11, 21), we differentiated ES cells into EBs and expressed Hopx starting on day 4. Flow cytometry analyses of the resulting cultures at day 7 and day 11 indicate that precocious expression of Hopx induces a significant increase in the number of Tnnt2^{+} cells (Fig. 4A). When Hopx is induced only 1 day later, at day 5, no statistically significant increase in cardiomyogenesis is observed (Fig. 4A).

We performed reciprocal loss-of-function experiments by generating embryonic stem cells (ESCs) from *Hopx*^{−/−} and littermate *Hopx*^{+/−} blastocysts and differentiating them into cardiac cell types (20). *Axin2* and *Nkx2-5* are each expressed at comparable levels when we compared *Hopx*^{−/−} and *Hopx*^{+/−} EBs at days 3 and 4.75 (fig. S7B). However, there is a marked reduction of Tnnt2^{+} cells and beating foci upon differentiation of *Hopx*^{−/−} EBs compared to *Hopx*^{+/−} EBs (Fig. 4B). We performed microarrays from *Hopx*^{−/−} and *Hopx*^{+/−} EBs (day 8, n=3), and multiple myocyte related genes are down-regulated in *Hopx*^{−/−} EBs

compared to $Hopx^{+/-}$ control EBs [table S1, e.g. *Mhy6* -30.3X, *Myh7* -30.3X, *Mybpc3* -13.0X, *Ttn* -7.0X, *Tnnt2* -5.5X, *Nkx2-5* -3.984X, false discovery rate (FDR) cutoff = 10%]. Gene ontology analysis of the top 3000 genes that are down-regulated confirmed enrichment for families of genes related to heart development and myogenesis (Fig. 4C). Although sarcomere genes are expressed in $Hopx^{-/-}$ hearts, the onset of Tnnt2 expression is delayed in SHF myoblasts as they enter the OFT, as evidenced by a lack of Tnnt2 expression in Nkx2-5[+] precursors within the distal OFT of $Hopx^{-/-}$ versus $Hopx^{+/+}$ hearts at E9.5 (Fig. 4D). The eventual expression of sarcomere genes in *Hopx* mutants suggests that redundant pathways exist for activation of the myogenic program.

In order to further define the role of Hopx during myogenesis, we defined the genomic regions occupied by Hopx by performing chromatin-immunoprecipitation followed by massively paralleled sequencing (ChIP-seq). Although Hopx does not bind to DNA directly (22) it can interact with co-repressor complexes to inhibit gene expression. We performed ChIP-seq analysis of pooled chromatin derived from ~35 E9.5 microdissected murine embryonic hearts (table S2). KEGG analysis of the genes associated with the strongest 3000 peaks suggested that Hopx occupancy was enriched in genomic regions proximal to Wnt family member genes (p=3.9×10^{-4}), and the Wnt signaling pathway was the top hit using PANTHER analysis (p=1.2×10^{-3}).

We independently validated Hopx occupancy close to several Wnt ligand transcriptional start sites by ChIP-qPCR (Fig. 5A). Consistent with the known function of Hopx as a transcriptional repressor (15), many of these ligands, including *Wnt2, Wnt5b*, and *Wnt6*, are expressed at higher levels at day 8 of differentiation in $Hopx^{-/-}$ EBs compared to $Hopx^{+/-}$ EBs (Fig. 5B). *Axin2* and *Isl1*, both target genes of canonical Wnt signaling (23, 24), are expressed at significantly higher levels in E9.5 $Hopx^{-/-}$ hearts as compared to littermate controls (Fig. 5C). Immunohistochemistry of E9.5-10.5 control OFTs reveals that the distal OFT is a transition zone in which Axin2 and Isl1 expression diminish while Hopx expression is activated (Fig. 5D). In $Hopx^{-/-}$ embryos, Wnt signaling, as represented by Axin2 and Isl1 expression, is expanded into the proximal OFT compared to littermate controls (Fig. 5E). Overexpression of Hopx in EBs reduces *Axin2* expression, whereas a mutant form of Hopx that does not effectively interact with Smad4 (discussed further below) does not have this effect (fig. S7C). These data suggest that Hopx represses Wnt signaling during cardiogenesis.

During ES cell differentiation into cardiomyocytes, Hopx also functions to repress Wnt. KEGG analysis of the up-regulated genes in $Hopx^{-/-}$ versus $Hopx^{+/-}$ day 8 EB microarrays confirms overrepresentation of the Wnt signaling pathway in $Hopx^{-/-}$ cells (p=1.4×10^{-3}). EBs lacking *Hopx* show elevation of *Axin2* and significant impairment of sarcomere gene expression at day 8 of differentiation (Fig. 5F). Addition of XAV939, a potent inhibitor of Wnt signaling, at day 5 of differentiation to $Hopx^{-/-}$ EBs restores *Axin2* to control levels and partially rescues sarcomere gene expression. *Nkx2-5* is expressed by CPCs and its expression markedly increases over the course of cardiac differentiation of EBs (21). Differentiating $Hopx^{-/-}$ EBs, however, fail to up-regulate *Nkx2-5* normally. Inhibition of Wnt in $Hopx^{-/-}$ EBs rescues *Nkx2-5* to levels found in controls (Fig. 5F). Microarray analysis (table S3, n=3) confirms that expression of multiple sarcomere genes is rescued

upon Wnt inhibition (Fig. 5G, e.g. *Myh6, Myh7, Myl7, Myl3, Tnnc1, Actn2, Actc1*). Taken together, these data suggest that Hopx repression of Wnt signaling promotes cardiomyogenesis.

Hopx interacts with Smad4

We purified Hopx-containing protein complexes from E9.5 *Hopx*[3XFlag/+] murine hearts and identified protein components by mass spectrometry. Numerous members of the Mi-2/NuRD (nucleosome remodeling deacetylase) complex were identified (e.g. Hdac1, Hdac2, Rbbp4/7, MTA 1/2/3, and MBD3), consistent with the known association of Hopx with Hdac2 (22). In addition, Smad4 was identified as a Hopx-interacting protein. This finding is of particular interest because *Bmp4*, phospho-Smad1/5/8 and, to a lesser extent, *Bmp2* are expressed in the OFT at E9.5, as we confirmed (Fig. 6A and fig. S8A). We confirmed the interaction between Hopx and Smad4 by co-immunoprecipitation of both factors overexpressed in 293Tx cells in the presence of increasing concentrations of recombinant Bmp4 and performing co-immunoprecipitation experiments. An interaction between Hopx and Smad4 that is dependent upon the presence of Bmp4 is detectable (Fig. 6B). Further, we confirmed that endogenous Hopx interacts with an activated Smad complex (Smad4 and phospho-Smad1/5/8) in co-immunoprecipitation experiments from E9.5-10 whole embryo lysates (Fig. 6C and fig. S8B).

We have previously determined the structure of Hopx by NMR spectroscopy demonstrating a helix-turn-helix motif (22). Residues within the first and second alpha helix that are located close to one another were shown to be important for Hopx-mediated transcriptional repression, as were a distinct cluster of residues at the carboxyl terminus (shown in green, Fig. 6D). We mutated amino acids in the first and second helix and at the carboxyl terminus and assayed whether the mutants could interact with Smad4 using an *in situ* proximity ligation assay (Fig. 6E–I). We confirmed expression of Hopx constructs (fig. S8, C and D), and specificity of the proximity ligation assay (fig. S8E). Consistent with the co-immunoprecipitation experiments (Fig. 6B), the Smad4 interaction with Hopx is enhanced by Bmp4 (Fig. 6E and F). The Smad4-Hopx interaction is attenuated by mutation of residues at the carboxyl terminus of Hopx, but not by mutations in helix 1 or 2 (Fig. 6G–I). Consistent with a physical interaction between Hopx and Smad4, we detect enrichment of Smad4 occupancy by ChIP at Wnt ligand loci that are also occupied by Hopx (fig. S8F).

Hopx integrates Bmp and Wnt signaling

The data presented thus far indicate that Hopx expression defines a cardiomyoblast, and that Hopx modulates cardiomyogenesis by repressing Wnt signaling. Further, Hopx can interact with an activated Smad complex. In murine EBs, myogenesis requires inhibition of Wnt and is promoted by activation of Bmp (9, 11, 25–27). Hence, we sought to determine if Hopx functions to integrate Bmp signaling with Wnt repression.

First, we confirmed that *Bmp4* and *Bmp2* levels are unchanged in *Hopx*^{−/−} embryonic hearts by *in situ* hybridization and qRT-PCR (Fig. 7A). Protein expression and nuclear localization of Smad4 and phosphorylated Smad1/5/8 is also unchanged (Fig. 7A). Once in the nucleus, the active Smad complex functions to enhance transcription of Bmp target genes such as

Msx1. Control E9.5 cardiac explants respond to exogenous Bmp4 by up-regulating *Msx1* in a dose-dependent fashion (Fig. 7B). A similar response is seen in *Hopx*$^{-/-}$ explants, indicating an intact Bmp response system (Fig. 7B).

Bmp signaling can result in repression of Wnt activity in various tissues, although the mechanism has been unclear (28–31). Addition of recombinant Bmp4 decreases *Axin2* expression in *Hopx*$^{+/+}$ cardiac explants compared to vehicle-treated controls (Fig. 7C, white bars). However, *Axin2* expression in *Hopx*$^{-/-}$ explants is relatively unresponsive to Bmp4 (Fig. 7C, black bars). We also tested whether Hopx participates in Bmp-mediated Wnt inhibition during cardiac differentiation of EBs (Fig. 7D). Bmp4 treatment of *Hopx*$^{+/-}$ EBs starting at day 5 results in a 60% decrease in *Axin2* by day 10 of differentiation compared to vehicle treated EBs (white bars, Fig. 7D). However, Bmp4 minimally affects *Axin2* expression in *Hopx*$^{-/-}$ EBs (black bars, Fig. 7D). These data suggest that Hopx is required for Bmp-mediated repression of Wnt signaling during cardiogenesis.

Discussion

Here, we have shown that cardiac precursor cells that express *Hopx* are irreversibly committed to the myocyte lineage, thereby defining progenitor cells that we call cardiomyoblasts. *Hopx* expression not only marks cardiomyoblasts, but it also functions to enhance cardiomyogenesis by linking Bmp signaling with repression of Wnt. A subset of committed myoblasts derived from the FHF express *Hcn4,* which is also expressed by endothelial cells during cardiac development (32, 33). However, the lack of a definitive marker of a cardiomyoblast has hampered a detailed analysis of cardiomyocyte commitment. Our studies provide such a marker, and reveal that commitment of FHF and SHF CPCs occurs at distinct time points and in different locations during cardiogenesis. In the SHF, Isl1^{+}, Wnt-activated CPCs stream into the OFT from the surrounding mesoderm, where they encounter local Bmp4 signals. CPCs then express Hopx, down-regulate Wnt, and become committed to the myocyte fate. Thus, the distal OFT is a "zone of commitment" in the developing heart (Fig. 7E).

Markers of lineage commitment, and the signals that modulate lineage decisions, are likely to inform regenerative and stem cell approaches for cardiac disease. In the hematopoietic system, detailed understanding of these processes has allowed for definitive identification of various progenitor cells of the blood lineages, leading to the development of important therapies for human diseases (e.g. erythropoietin and granulocyte-macrophage stimulating factor). The ability to identify committed, but undifferentiated cardiomyocyte precursors may facilitate development of cardiac regenerative therapies including those using ES and iPS cells (34).

Reciprocal signaling between Bmp and Wnt has been recognized in multiple progenitor populations (28–31, 35). However, the mechanisms that coordinate these pathways in progenitor cell niches have remained elusive. Our current work raises the possibility that Hopx-mediated integration of Bmp signaling to repress Wnt may be active in other progenitor populations. For example, Hopx is expressed by +4 stem cells in the intestine (19) where niche Bmp signals repress Wnt (28, 36). Recent work by the Fuchs laboratory

also suggests that balance between Bmp and Wnt signaling influences the fate of hair follicle cells as they differentiate into various progeny lineages (35) and Hopx may play a role in this process (18). *HOPX* is a tumor suppressor gene implicated in colorectal and other cancers (37, 38). Hijacking of developmental pathways is emerging as a potent mechanism of carcinogenesis (39). Loss of Hopx in cancer stem cells could result in uncoupling of niche-mediated Bmp signaling and quiescence through loss of Wnt repression.

Finally, although *Hopx*-deficiency leads to thinned myocardium and cardiac rupture in a portion of embryos, cardiomyogenesis is not altogether blocked. Inhibition of Wnt signaling in SHF cardiomyoblasts is delayed, but not completely prevented and some $Hopx^{-/-}$ mice live to adulthood. This suggests that, not surprisingly, alternative mechanisms reinforce Wnt repression independent of Hopx (40). Further insights into the mechanisms that coordinate signaling in the niche are likely to inform our ability to harness the potential of regenerative medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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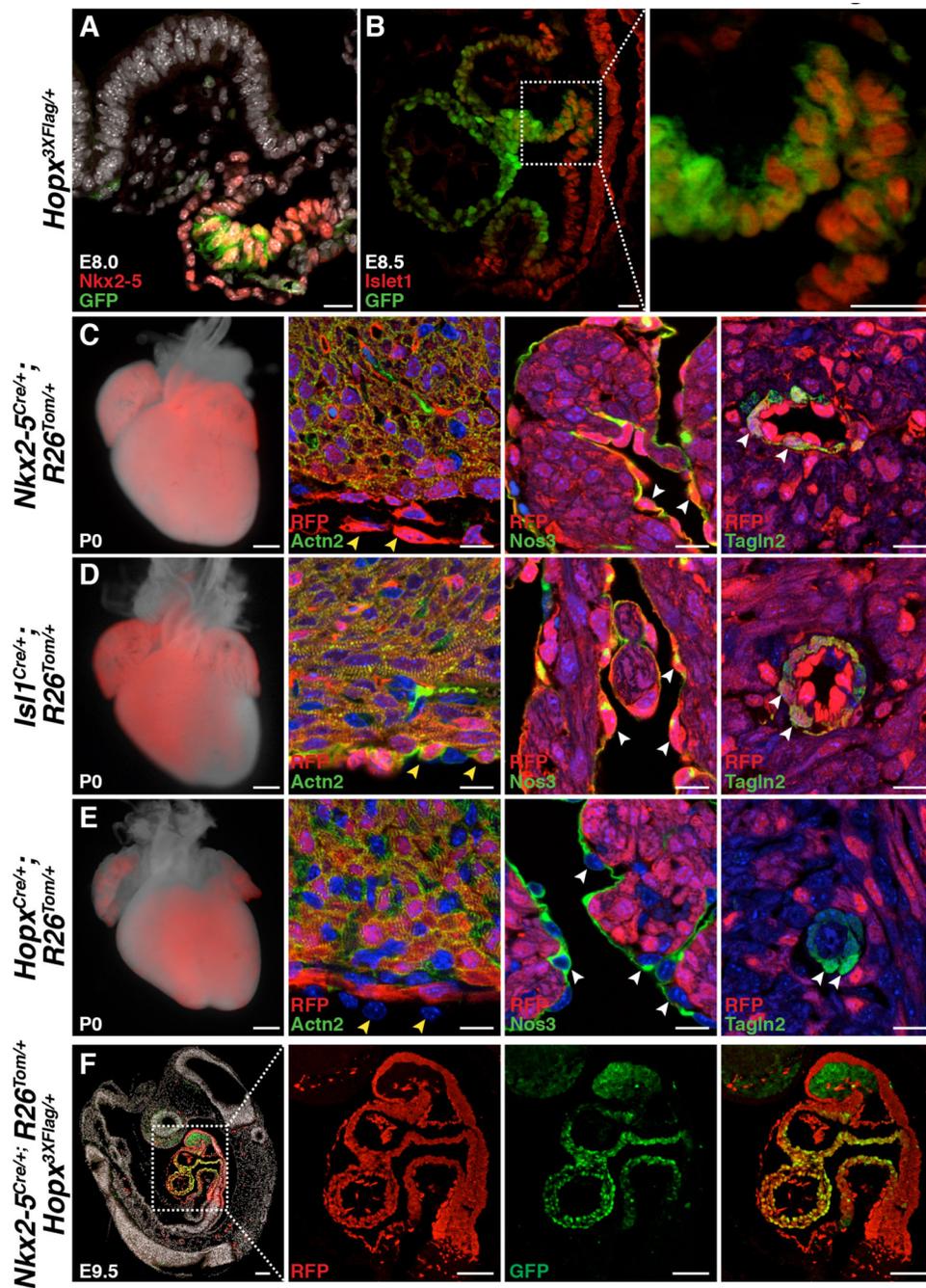
Fig. 1. Prospective identification of cardiomyoblasts

(**A**) A subset of E8.0 Nkx2-5$^+$ cells in the FHF precardiac mesoderm express Hopx. (**B**) A subset of E8.5 Isl1$^+$ SHF cells in the outflow tract express Hopx (inset highlights distal outflow tract). (**C,D**) *Nkx2-5*$^+$ (C) and *Isl1*$^+$ (D) cells give rise to myocytes (Actn2$^+$), epicardium (yellow arrowheads), endothelium (Nos3$^+$, white arrowheads), and smooth muscle (Tagln2$^+$, white arrowheads) at P0. (**E**) *Hopx*$^+$ cells give rise to myocytes, not epicardium (yellow arrowheads), endothelium (white arrowheads), or smooth muscle (white arrowheads) at P0. (**F**) Hopx$^+$ cells (GFP$^+$) derive from *Nkx2-5*$^+$ cells (RFP$^+$) (E9.5, sagittal

section). Scale bars: 500 μm (C–E, whole mount), 100 μm (F), 25 μm (A,B), 10 μm (C–E, histology).
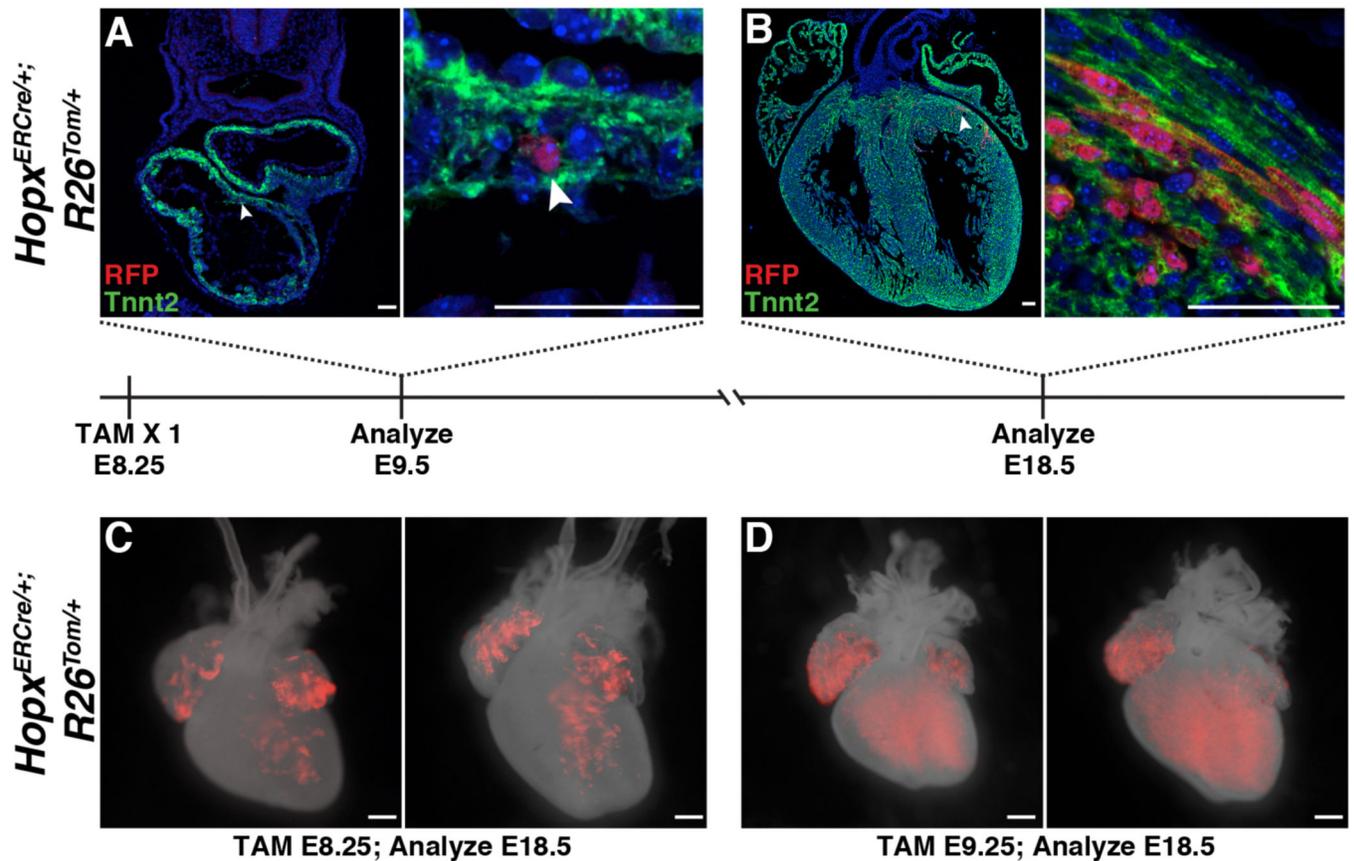
Fig. 2. Cardiomyoblasts expand during cardiogenesis

(**A,B**) E8.25 *Hopx^{ERCre/+}; R26^{Tom/+}* embryos were induced with tamoxifen and harvested at E9.5 (A) and E18.5 (B). Individual *Hopx*-derived cells are labeled at E9.5 (A, B area highlighted by arrowhead is magnified on right). At E18.5, clusters of myocytes are identified (B). (**C,D**) *Hopx^{ERCre/+}; R26^{Tom/+}* embryos were induced with tamoxifen at E8.25 (C) or E9.25 (D) and analyzed at E18.5 (n ≥ 2 litters per time point, 2 examples at each time point shown). Scale bars: 500 μm (C,D) and 50 μm (A,B).
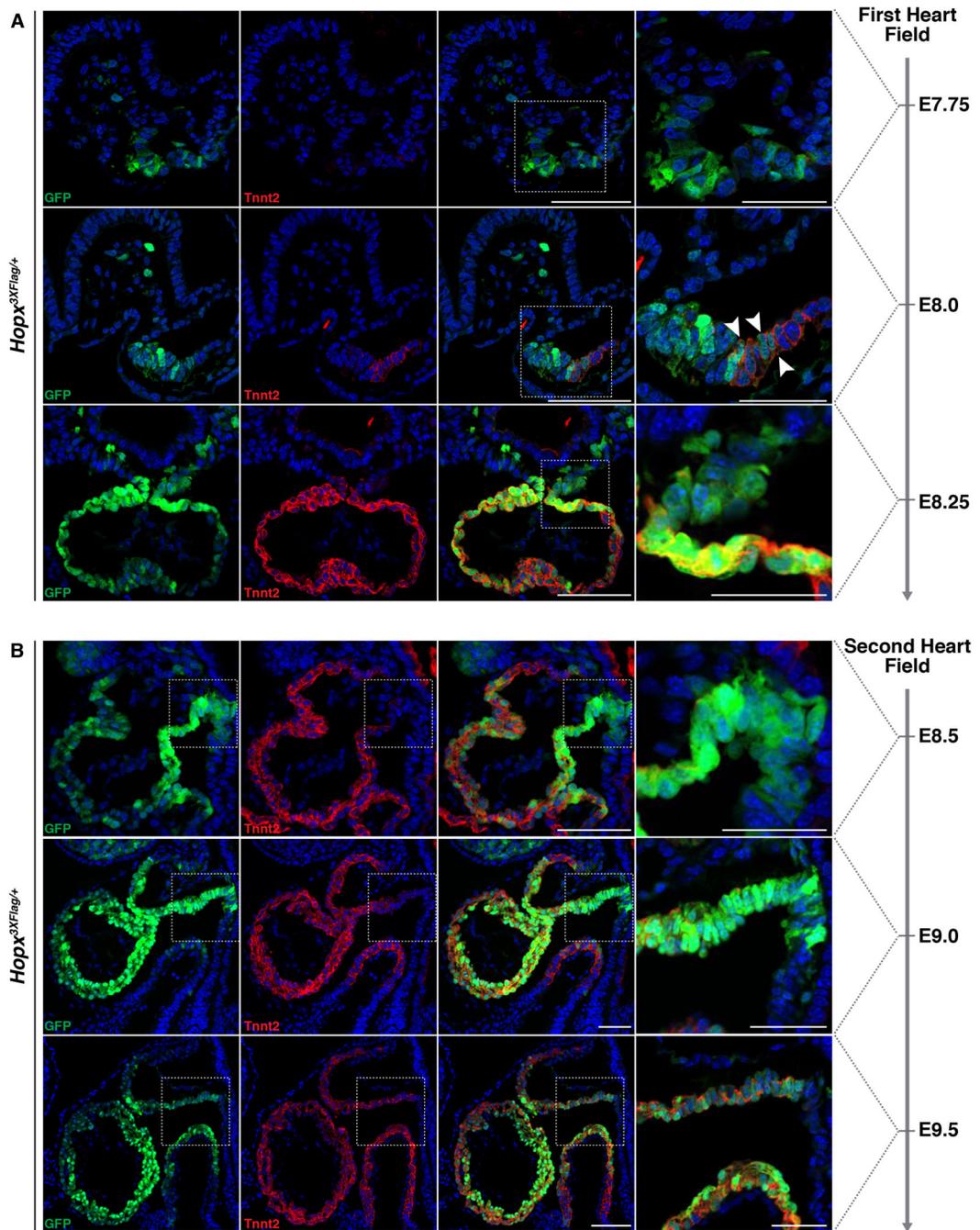
Fig. 3. Hopx expression precedes troponin expression

(**A**) Hopx expression precedes Tnnt2 expression in the precardiac mesoderm/FHF at early time points during cardiac development. Hopx[+], Tnnt2^{+} cells are identified a few hours later (white arrowheads). (**B**). The distal outflow tract and SHF mesoderm harbor Hopx[+], Tnnt2^{−} cells at early time points during cardiac development. Scale bars: 100 μm except right most panels/insets which are 50 μm.
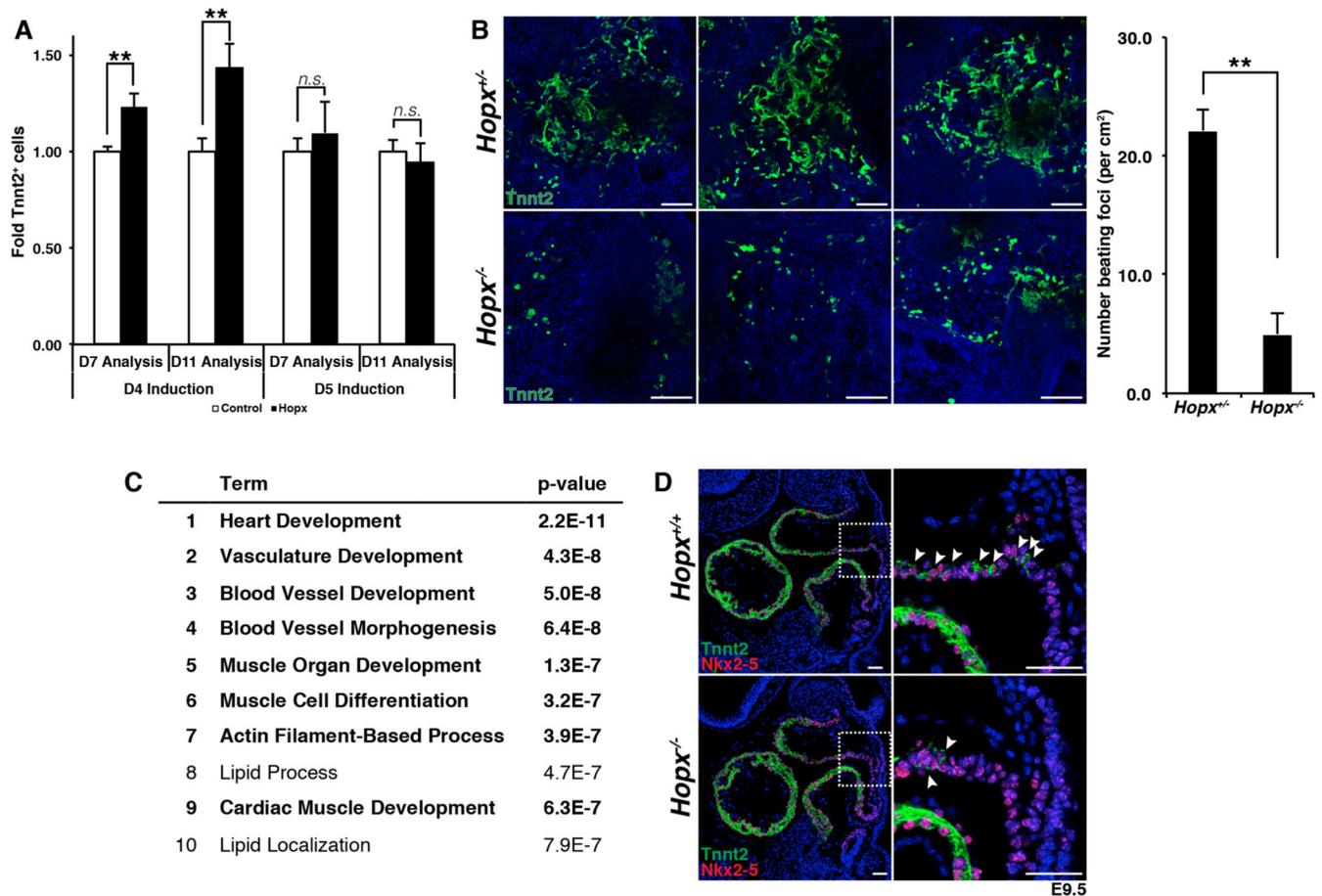
Fig. 4. Hopx promotes myogenesis

(**A**) Precocious expression of Hopx in CPCs at day 4 of EB differentiation results in more Tnnt2$^+$ cells measured by flow cytometry. (**B**) Differentiation of $Hopx^{-/-}$ EBs results in fewer Tnnt2$^+$ cells and fewer beating foci compared to $Hopx^{+/-}$ EBs (day 10). Images from 3 different replicates are shown. (**C**) Gene ontology analysis from microarrays done with triplicate samples of $Hopx^{-/-}$ vs. $Hopx^{+/-}$ EBs (top 3000 genes down-regulated, ranked by fold change, FDR cutoff = 10%, day 8, GOTERM_BP_FAT). (**D**) Paucity of Tnnt2$^+$ cells (arrowheads) in the distal outflow tract $Hopx^{-/-}$ compared to $Hopx^{+/+}$ embryos (E9.5). ** p<0.05. Scale bars: 500 μm (B), 50 μm (D).
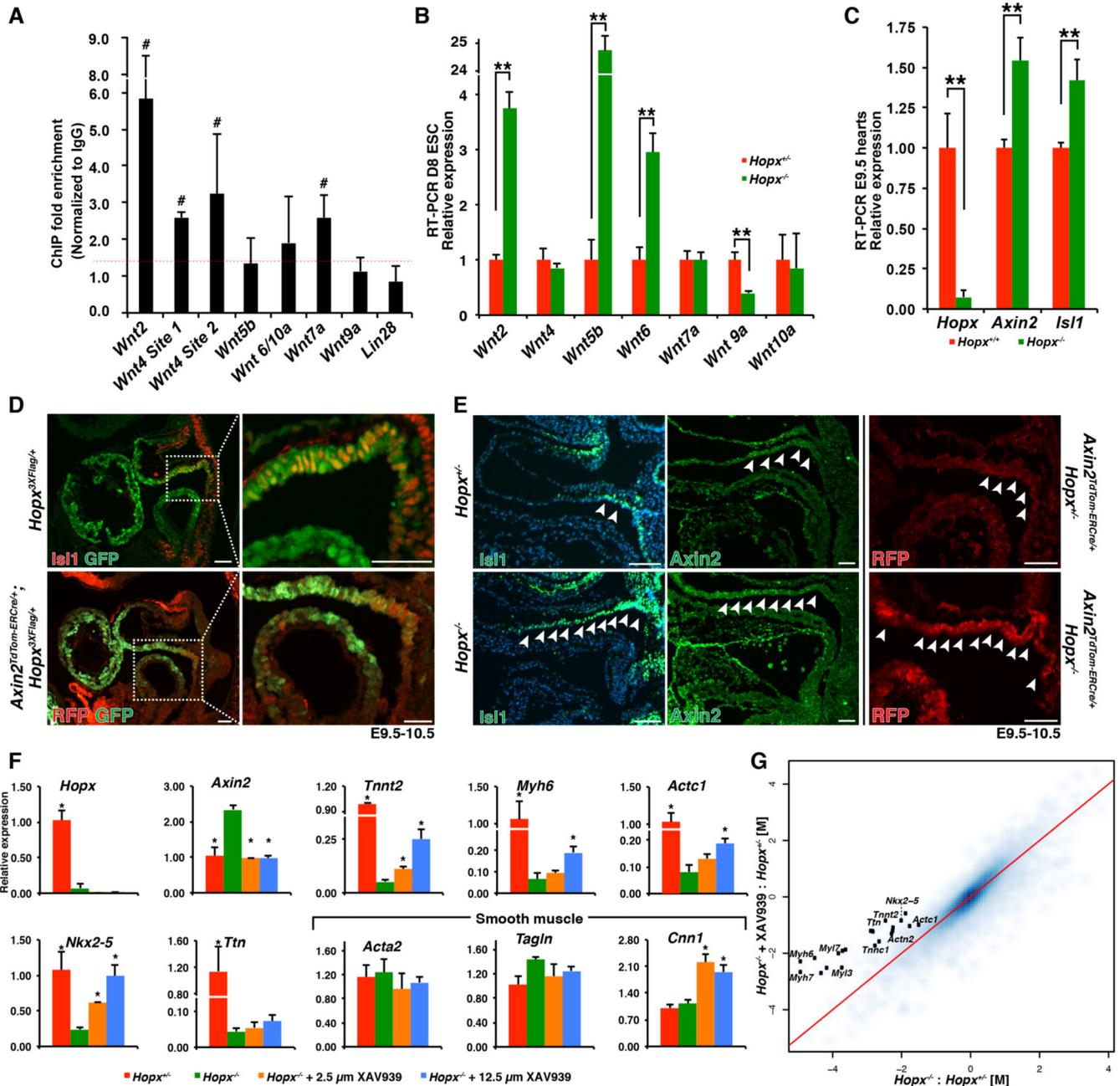
Fig. 5. Myogenesis requires inhibition of Wnt signaling

(**A**) ChIP-qPCR from E9.5 hearts. *Wnt2, Wnt4* sites 1 and 2, and *Wnt7a* demonstrate greater than 1.4X enrichment (red dashed line) over IgG in all replicates (denoted by #, n 3 replicates). (**B**) Expression analysis of *Hopx*$^{-/-}$ vs. *Hopx*$^{+/-}$ EBs (day 8). (**C**) qRT-PCR from littermate E9.5 microdissected hearts. (**D**) Isl1 (upper panels) and Axin2 (lower panels, RFP expression reflects *Axin2* in an *Axin2*$^{TdTom-ERCre/+}$ embryo, sagittal sections) are each coexpressed with Hopx in the cardiac OFT. (**E**) Isl1 and Axin2 expression is expanded in *Hopx*$^{-/-}$ (arrowheads point to Isl1$^+$ and Axin2$^+$ cells; middle panels show Axin2 IHC (green), right panels show RFP IHC in *Axin2*$^{TdTom-ERCre/+}$ embryos). (**F**) qRT-PCR

analyses of $Hopx^{+/-}$ day 8 EBs (red), or $Hopx^{-/-}$ EBs (green) with either 2.5μM (orange) or 12.5μM (blue) XAV939 (*p<0.05 in comparison to $Hopx^{-/-}$). (**G**) Comparison of the log2-transformed fold change (M) of genes differentially expressed in $Hopx^{-/-}$ vs. $Hopx^{+/-}$ EBs (x-axis) versus $Hopx^{-/-}$ + 12.5 μm XAV939 vs. $Hopx^{+/-}$ (y-axis) (n=3 samples). Sarcomere-related genes annotated with black dots. Genes above the red line are partially normalized by Wnt inhibition. ** p<0.05, Scale bars 50 μm.
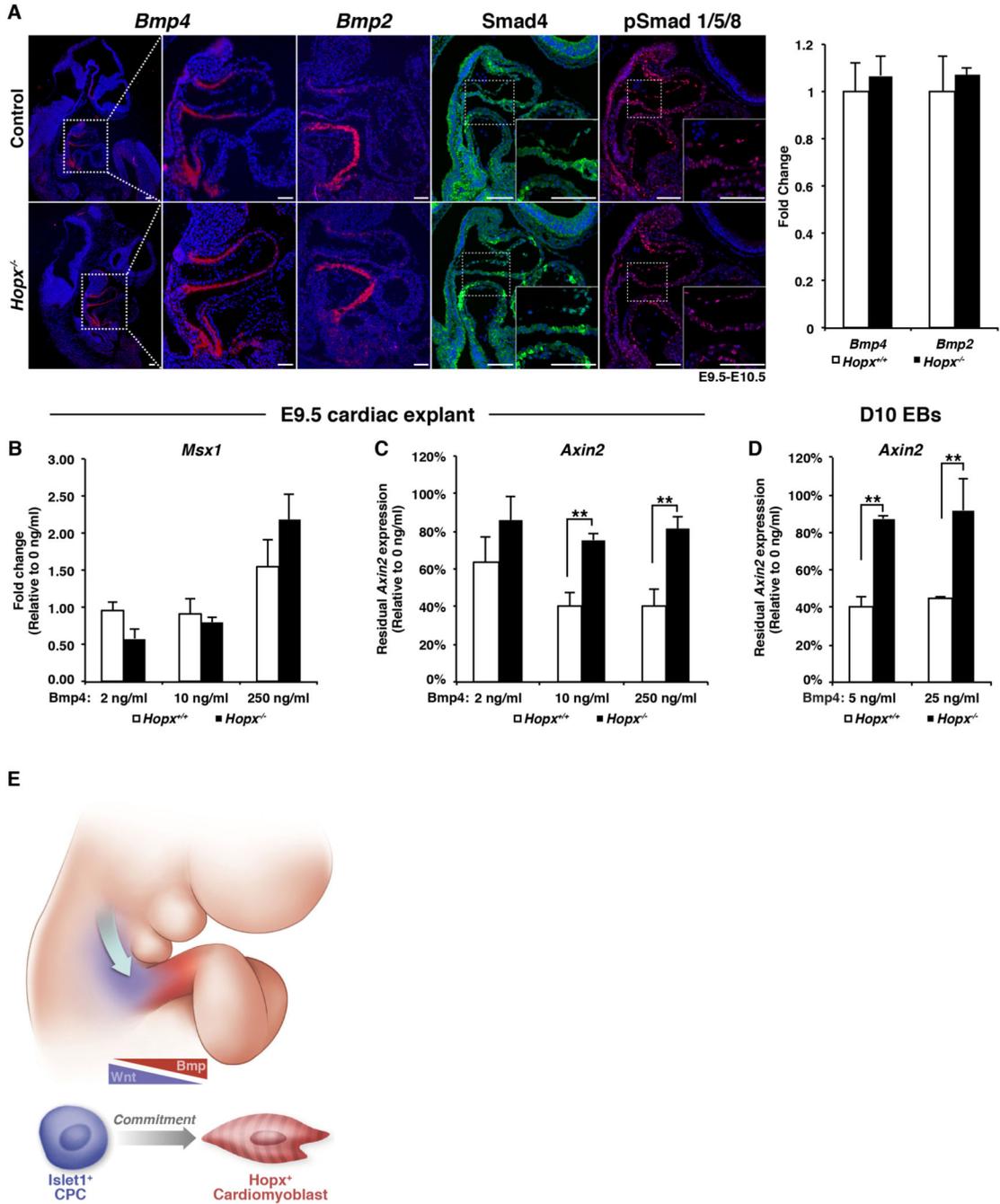
Fig. 6. Hopx interacts with an activated Smad complex
(**A**) *Bmp4* expression in control E9.5 heart (RNAscope *in situ* hybridization, brown; sagittal section). Inset focuses on outflow and inflow tracts. (**B**) Hopx co-immunoprecipitates with Smad4 in 293Tx cells in a Bmp4 dependent manner (0, 2, 10, 25 ng/ml Bmp4, 7.5% input). (**C**). Hopx co-immunoprecipitates with Smad4 and phospho-Smad1/5/8 *in vivo* (E9.5-E10 tissue lysates 3 independent examples of each genotype shown, 7.5% input). (**D**) NMR structure of Hopx (22) and schema of mutant constructs. (**E–I**) Proximity ligation assay using transfected 293Tx cells. Presence of Bmp4 and transfected plasmids are indicated. Best representative images from n=3 experiments shown. Scale bars: 250 μm (A, top) and 50 μm (A bottom, E–I).

Fig. 7. Bmp signaling represses Wnt

(**A**) Expression of *Bmp4, Bmp2* (*in situ* hybridization), Smad4, phospho-Smad1/5/8 (IHC) in control and *Hopx*$^{-/-}$ E9.5-10.5 embryonic hearts, and qRT-PCR of E9.5 hearts (right panel). (**B,C**) qRT-PCR of E9.5 hearts after culture in increasing concentrations of Bmp4. (**D**) qRT-PCR of day 10 *Hopx*$^{+/-}$ and *Hopx*$^{-/-}$ EBs after differentiation with Bmp4. *Hopx*$^{-/-}$ explants and EBs failed to repress *Axin2* as effectively as controls in the presence of Bmp4 (n 3 for each experiment in B–D). (**E**) Model: A "zone-of-commitment" in the developing OFT. Wnt-activated, Isl1$^+$ CPCs stream into the OFT and are exposed to Bmp signaling.

Hopx$^+$ cardiomyoblasts are committed to the myocyte lineage. ** p<0.05, Scale bars: 100 μm.