Title

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Journal

Circulation Research, 123(4)

ISSN

0009-7330

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Publication Date

2018-08-03

DOI

10.1161/circresaha.118.311339

Peer reviewed

OPEN

Tbx20 Is Required in Mid-Gestation Cardiomyocytes and Plays a Central Role in Atrial Development

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Rationale: **Mutations in the transcription factor *TBX20* (T-box 20) are associated with congenital heart disease. Germline ablation of *Tbx20* results in abnormal heart development and embryonic lethality by embryonic day 9.5. Because *Tbx20* is expressed in multiple cell lineages required for myocardial development, including pharyngeal endoderm, cardiogenic mesoderm, endocardium, and myocardium, the cell type–specific requirement for TBX20 in early myocardial development remains to be explored.**

Objective: **Here, we investigated roles of TBX20 in midgestation cardiomyocytes for heart development.**

Methods and Results: **Ablation of *Tbx20* from developing cardiomyocytes using a doxycycline inducible *cTnTCre* transgene led to embryonic lethality. The circumference of developing ventricular and atrial chambers, and in particular that of prospective left atrium, was significantly reduced in *Tbx20* conditional knockout mutants. Cell cycle analysis demonstrated reduced proliferation of *Tbx20* mutant cardiomyocytes and their arrest at the G1-S phase transition. Genome-wide transcriptome analysis of mutant cardiomyocytes revealed differential expression of multiple genes critical for cell cycle regulation. Moreover, atrial and ventricular gene programs seemed to be aberrantly regulated. Putative direct TBX20 targets were identified using TBX20 ChIP-Seq (chromatin immunoprecipitation with high throughput sequencing) from embryonic heart and included key cell cycle genes and atrial and ventricular specific genes. Notably, TBX20 bound a conserved enhancer for a gene key to atrial development and identity, *COUP-TFII/Nr2f2* (chicken ovalbumin upstream promoter transcription factor 2/ nuclear receptor subfamily 2, group F, member 2). This enhancer interacted with the *NR2F2* promoter in human cardiomyocytes and conferred atrial specific gene expression in a transgenic mouse in a TBX20-dependent manner.**

Conclusions: **Myocardial TBX20 directly regulates a subset of genes required for fetal cardiomyocyte proliferation, including those required for the G1-S transition. TBX20 also directly downregulates progenitor-specific genes and, in addition to regulating genes that specify chamber versus nonchamber myocardium, directly activates genes required for establishment or maintenance of atrial and ventricular identity. TBX20 plays a previously unappreciated key role in atrial development through direct regulation of an evolutionarily conserved *COUPT-FII* enhancer. (*Circ Res*. 2018;123:428-442. DOI: 10.1161/CIRCRESAHA.118.311339.)**

Key Words: cell lineage ■ epigenomics ■ heart defects, congenital ■ mutation ■ myocytes, cardiac

Mammalian heart development is orchestrated by a complex interplay of cardiac transcription factors, mutations in which are often associated with congenital heart defects.[1] The transcription factor *Tbx20* (T-box 20) is expressed in developing and adult cardiomyocytes, as well as pharyngeal endoderm, cardiac progenitors, endothelium, and endocardium.[2–4] Mutations in *TBX20* are associated with congenital heart defects, including septal defects and cardiomyopathies.[5–9]

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Original received March 15, 2018; revision received June 8, 2018; accepted June 13, 2018. In May 2018, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 13.37 days.

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The online-only Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/CIRCRESAHA.118.311339.

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Novelty and Significance

What Is Known?

- Mutations in the t-box transcription factor TBX20 (T-box 20) are associated with a diverse range of congenital heart defects including septal defects and left ventricular noncompaction.
- In mice, TBX20 is required for cardiogenesis and during embryonic development is expressed in multiple cell types, including cardiomyocytes, endocardial cells, and endodermal cells.
- The functions of TBX20 in developing cardiomyocytes and the mechanisms by which mutations in TBX20 contribute to congenital heart disease are not fully understood.

What New Information Does This Article Contribute?

- TBX20 is required during midgestation to drive multiple aspects of cardiomyocyte development.
- TBX20 directly activates genes required for myocyte proliferation and promotes G1 to S progression of the cardiomyocyte cell cycle.
- TBX20 has a key role in atrial development and identity.

Despite the recognition that TBX20 is a key factor driving heart development and function, its roles in embryonic cardiomyocytes have not been fully explored. Cardiomyocyte specific functions of TBX20 may underlie the association of TBX20 with congenital heart defects such as septal defects and left ventricular noncompaction. Using a cardiomyocyte-specific loss-of-function mouse model, we revealed cell-autonomous functions of TBX20 driving cardiomyocyte proliferation and chamber patterning, including in the left atria. The roles of this transcriptional regulator are more diverse and context dependent than previously appreciated.

Nonstandard Abbreviations and Acronyms

AVC	atrioventricular canal
cKO	conditional knock out mutant
EdU	5-ethynyl-2'-deoxyuridine
OFT	outflow tract
qPCR	quantitative polymerase chain reaction

After formation of the developing heart tube, the heart grows by addition of cardiac progenitor cells. In addition, cardiomyocyte proliferation between embryonic day (E) 9.5 to E12.5 makes major contributions to growth of chamber myocardium.[10] *Tbx20* global mutants exhibit decreased cardiomyocyte proliferation and arrest development at E9.5, with hypoplastic, unlooped hearts.[11–13] Initially, defective proliferation was attributed to ectopic expression of *Tbx2* throughout mutant hearts, which suppresses proliferation in cardiomyocytes.[11–14] However, combined loss of *Tbx20* and *Tbx2* does not rescue the hypoplastic heart phenotype, indicating that TBX20 regulates additional pathways to control cardiomyocyte proliferation, independent of *Tbx2*.[15] Although these studies demonstrated a key role for TBX20 in regulating cardiomyocyte proliferation, whether this requirement was cell autonomous has not been addressed, as TBX20 is expressed in multiple nonmyocardial cell lineages that are required for cardiomyocyte proliferation and development. Although recent studies have addressed roles of TBX20 in subsets of cellular lineages during heart development,[16,17] and in adult cardiomyocytes,[18,19] no study has yet addressed the function of TBX20 in embryonic cardiomyocytes that form the developing cardiac chambers. Thus, temporal and cell autonomous requirements for TBX20 in cardiomyocytes during heart formation remain to be explored. Additionally, a comprehensive view of direct downstream targets of TBX20 in fetal cardiomyocytes is lacking.

Here, using an inducible cardiomyocyte-specific Cre mouse line, we demonstrated that TBX20 is required within midgestation cardiomyocytes to drive multiple aspects of cardiomyocyte development. TBX20 directly activated genes required for myocyte proliferation, directly repressed progenitor-specific genes, and specified ventricular and atrial identity through both gene repression and activation. Notably, we uncovered a pivotal role for TBX20 in atrial development and identity, identifying the gene encoding the nuclear hormone receptor transcription factor COUP-TFII/NR2F2 (chicken ovalbumin upstream promoter transcription factor 2/nuclear receptor subfamily 2, group F, member 2) as a direct downstream target. A long-range enhancer for *COUP-TFII* bound by TBX20 was conserved between mouse and human and drove atrial specific expression in mouse embryos. Our work highlights myocyte autonomous requirements for TBX20 and comprehensively identifies gene networks directly regulated by TBX20 in this context. Additionally, we uncover transcriptional mechanisms regulating *COUP-TFII* expression and reveal a previously unappreciated key role for TBX20 in atrial development.

Methods

Genome-wide sequencing data have been made publicly available at the ArrayExpress database and can be accessed at https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-5596/.

Other data and analytical methods are available from the corresponding authors on reasonable request.

Mouse Strains and Experiments

Animal experiments were conducted according to protocols approved by Institutional Animal Care and Use Committee at University of California, San Diego or University of Chicago. Mice were maintained on BlackSwiss (NIHBL(S); Taconic Biosciences) background. *Tbx20*^{loxP}, *Tbx20-GFP*, *Tnnt2-rtTA;TetO-Cre*, and *Rosa26 flox-stop-flox tdTomato* (*R26-tdTom*) mouse lines were as described.[12,19–21] Cardiomyocyte-specific *Tbx20* conditional mutants (*Tbx20* conditional knockout mutant [*cKO*]; *Tnnt2-rtTA;TetO-Cre;Tbx20*$^{loxP/loxP}$) and controls (*control; Tnnt2-rtTA;TetO-Cre;Tbx20*$^{wt/loxp}$) were generated by breeding *Tnnt2-rtTA;TetO-Cre;Tbx20*$^{+/loxp}$ males with *Tbx20*$^{loxP/loxP}$ or *Tbx20*$^{loxP/loxP}$;R26$^{tdTom/tdTom}$ females. *Cre* expression was induced by doxycycline (MP Biomedicals; catalog number: 198955; 1 mg/mL) in water prepared fresh daily to pregnant females from E8.5 onward. All experiments were performed using somite stage and size-matched embryo pairs, images shown are representative examples of experiments with n≥3 biological replicates.

Quantification Experiments and Statistical Analysis

Cardiac chamber size was quantified by perimeter measurements in every fourth section (10 µm). 200 µL 5-Ethynyl-2'-deoxyuridine (EdU; Molecular probes; 3g/L) was injected intraperitoneally in

pregnant females 2 hours before embryo isolation. EdU incorporation was quantified using Volocity software. FlowJo software was used for cell cycle analysis on *tdTom*+ cardiomyocytes. Data are expressed as means±SEM, for n≥3 biological replicates (actual number of biological replicates for each experiment stated in figure legends). Mann–Whitney *U* test was used to compare 2 groups, reporting asymptotic 2-tailed significance *P* values. Cell cycle quantification and EdU incorporation counts were analyzed using negative binomial regression using SPSS25 software (Online Tables V through X). Post hoc tests for negative binomial regression were performed using the Bonferroni correction. $P<0.05$ was considered significant.

Promotor Capture Chromosome Conformation Capture With High-Throughput Sequencing in Human Cardiomyocytes

Human cardiomyocyte cells were generated precisely as described before using induced pluripotent stem cell line 19101.[22] Promoter capture chromosome conformation capture with high-throughput sequencing was performed by combining in situ chromosome conformation capture with high-throughput sequencing with an oligo hybridization step as detailed in the Online Data Supplement.

Results

TBX20 Is Required in Cardiomyocytes During Cardiac Chamber Formation

TBX20 is expressed in cardiomyocytes throughout heart development.[2,11–13,23] Early lethality of systemic *Tbx20* mutants prevents analysis of its role in cardiomyocytes at later stages. To study roles of TBX20 in midgestation cardiomyocytes, we ablated *Tbx20* from cardiomyocytes after E8.5 using doxycycline inducible *Tnnt2-rtTA;TetO-Cre*[21] (Online Figure IA). After doxycycline administration, *R26-tdTomato* expression demonstrated efficient cardiomyocyte-specific Cre-mediated excision (Online Figure IB through ID). Significant ablation of *Tbx20* exon 2 was observed by quantitative polymerase chain reaction (qPCR) as early as E9.5 (Online Figure IE). Embryos with heterozygous cardiomyocyte-specific deletion of *Tbx20* were recovered at expected Mendelian ratios at all stages examined. However, by E14.5, embryos with homozygous cardiomyocyte-specific deletion of *Tbx20* (*Tbx20 cKOs*) were markedly reduced ($P=0.042$; Online Table I), suggesting an ongoing requirement for TBX20 in midgestation cardiomyocytes.

Analysis of gross external morphology revealed that *Tbx20 cKO* and wild-type control embryos were of similar size and shape at E11.5 and earlier stages (Figure 1). Histological examination revealed that at E9.5, *Tbx20 cKO* hearts were properly looped and visually indistinguishable from control hearts. However, at E10.5 and E11.5, overall heart size appeared reduced in *Tbx20 cKO* mutants. Most notably, left atrial size in mutant hearts was severely reduced (Figures 1 and 2). Circumferential measurements of cardiac chambers confirmed that left atrium and left ventricle were significantly smaller, whereas reduced sizes of right atrium and right ventricle were not statistically significant (Figure 2I). Moreover, *Tbx20 cKO* hearts had underdeveloped atrial and interventricular septa, and venous valves of *Tbx20 cKO* hearts were smaller at E10.5 and thinner at E11.5 when compared with controls (Figure 2). *Tbx20 cKO* outflow tracts (OFTs) appeared shorter at E10.5 and E11.5 (Figure 1J through 1X). Taken together, these results revealed that TBX20 was required in cardiomyocytes for multiple aspects of cardiac development, including cardiac chamber development and cardiac septation.

TBX20 Is Required for Cardiomyocyte Proliferation

The observation that loss of TBX20 in cardiomyocytes led to a hypoplastic heart and a concomitant decrease in chamber size led us to investigate cardiomyocyte proliferation. EdU incorporation was quantified in *Tnnt2-rtTA;TetO-Cre;R26*$^{+/tdTom}$ lineage traced cardiomyocytes at E9.5, E10.5, and E11.5 (Figure 3A through 3E). At all stages examined, cardiomyocyte proliferation was significantly decreased in *Tbx20* mutant hearts compared with cardiomyocytes of somite-matched control littermates (Online Tables IX and X). To understand whether proliferation was affected similarly in all chambers, EdU incorporation rates were quantified per compartment in E10.5 hearts. Proliferation of cardiomyocytes was decreased in developing chambers and OFT, with left atrial cardiomyocytes displaying the strongest reduction (Online Figure II; Online Table VI). In contrast, proliferation of nonmyocytes was comparable between mutants and controls (Online Figure II). Furthermore, reduced heart size was not associated with programmed cell death as indicated by comparable (low) levels of cleaved caspase 3 immunostaining in E10.5 mutant and control hearts (data not shown).

TBX20 Is Required for Cardiomyocyte G1 to S Phase Transition

Our phenotypic analysis, including quantification of chamber size and proliferation, were consistent with a role for *Tbx20* in cardiomyocyte cell division. To gain insight into the mechanisms of TBX20-mediated cardiomyocyte cell cycle regulation, we conducted an analysis of cardiomyocyte cell cycle progression (Online Tables XII and XIII). Fluorescence activated cell sorting analysis of EdU incorporation and DNA content demonstrated that a higher percentage of *Tbx20 cKO* cardiomyocytes were in G1 phase, whereas a lower percentage of *Tbx20 cKO* cardiomyocytes were in S phase relative to controls, suggesting defective G1 to S phase progression in *Tbx20* mutant cardiomyocytes (Figure 3F and 3G; Online Tables XII and XIII). These data suggested that TBX20 was required for cardiomyocyte cell cycle progression in cardiomyocytes.

TBX20 Regulates Pathways Associated With Cell Cycle and Cardiac Morphogenesis

To further understand genetic pathways regulated by TBX20 in cardiomyocytes, we performed RNA-Seq (RNA sequencing) on fluorescence activated cell sorting–purified cardiomyocytes from E11.5 *Tbx20 cKO* and somite stage–matched littermate control hearts (Online Figure IIIA). Of 1478 differentially expressed genes (fold change >1.2×; *P* adjusted <0.1), 816 were downregulated in *Tbx20 cKO* cardiomyocytes, and expression of 662 genes was increased compared with control cardiomyocytes (Online Table III).

Next, we performed Gene Ontology term enrichment analysis on differentially expressed genes. We grouped Gene Ontology terms in major categories, compared occurrence of these categories across gene sets, and observed a clear-cut difference between up- and downregulated genes in *Tbx20* mutant cardiomyocytes (Figure 4A). Downregulated genes were predominantly involved in cell cycle, contraction, and energy metabolism, whereas upregulated genes were primarily

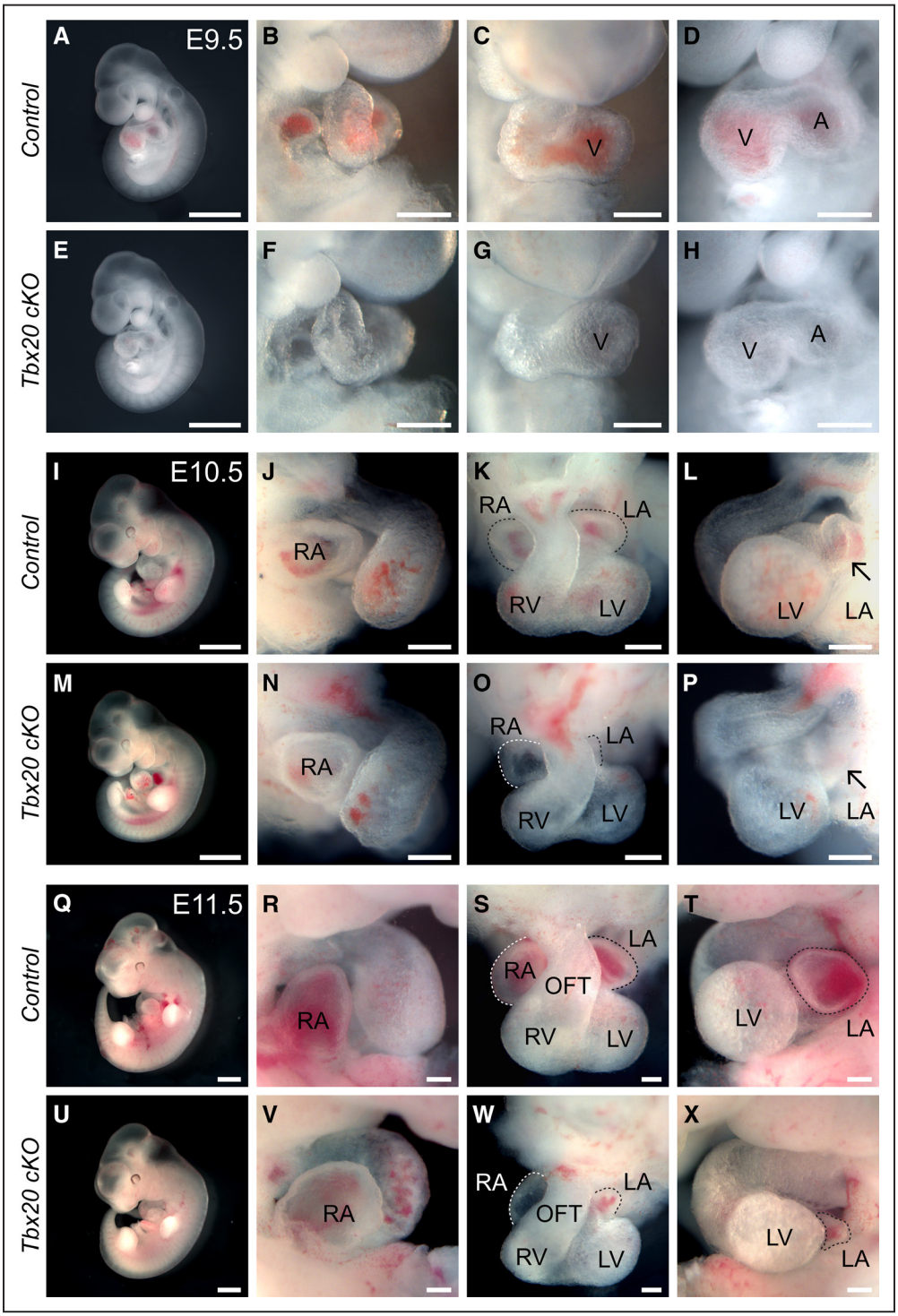

Figure 1. Cardiac morphology of *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{fl/fl}$ mutant embryos and *Tnnt2-rtTA; TetO-Cre; Tbx20* $^{+/fl}$ control littermates at E9.5 (A–H), E10.5 (I–P), and E11.5 (Q–X). From left to right in each row: whole embryo, right side view, ventral view, and left side view of hearts. A indicates atrium; cKO, conditional knock out mutant; LA, left atrium; LV, left ventricle; OFT, outflow tract; RA, right atrium; RV, right ventricle; Tbx20, T-box 20; and V, ventricle. Dotted line indicates outline of atria as labeled. Bars: **left**: 1 mm; other parts: 0.2 mm.

involved in general developmental pathways, neuronal function, and heart development.

Downregulated genes within the cell cycle functional category included *Cdc6* (cell division cycle 6), *Cdt1* (chromatin licensing and DNA replication factor 1), and *Ccna2* (cyclin A2). CDC6 and CDT1 are involved in the formation of the prereplication complex that is necessary for DNA replication.[24] CDC6 and CCNA2 activate CDK2 (cyclin-dependent kinase 2), which is required during G1 phase of the cell cycle for onset of chromosomal DNA replication in mammalian cells.[25,26] Downregulation of *Cdc6*, *Cdt1*, and *Ccna2* in *Tbx20 cKO* cardiomyocytes was consistent with our observation that

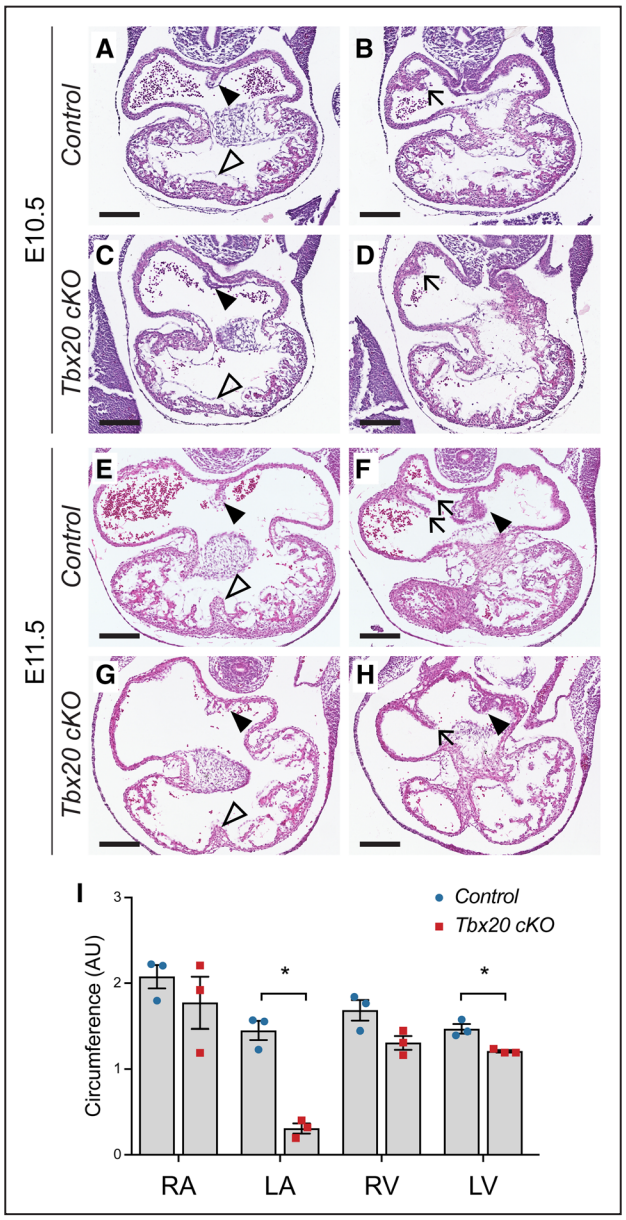

Figure 2. Analysis of cardiac phenotypes and quantification of chamber size. A–H, Hematoxylin and eosin–stained sections of *Tbx20 cKO* and control hearts at E10.5 (**A–D**) and E11.5 (**E–H**). Filled arrowhead: primary atrial septum; open arrowhead: interventricular septum; arrow: venous valves. **I**, Circumference measurement of right atrium (RA), left atrium (LA), right ventricle (RV), and left ventricle (LV) in *Tbx20 cKO* and control hearts. **P*<0.05 Mann–Whitney *U* test; n=3 biological replicates. AU indicates arbitrary units; cKO, conditional knock out mutant; and Tbx20, T-box 20. Bars: 0.2 mm (**A–H**).

mutant cardiomyocytes displayed decreased G1 to S transition and provided support for the hypothesis that TBX20 promotes cell cycle progression in midgestation cardiomyocytes.

Genes critical for cardiac development within the group of upregulated genes included *Tbx2*, *Isl1* (LIM/homeodomain transcription factor Islet1), *Fgf10* (fibroblast growth factor 10), *Hopx*, *Bmp2* (bone morphogenetic protein 2), and *Bmp10*, indicating that TBX20 was essential for directly or indirectly regulating genes encoding transcription factor and signaling pathways critical for cardiac morphogenesis.

Intersection of RNA-Seq and TBX20 ChIP-Seq Reveals Critical Direct Targets of TBX20

TBX20 has a dual role as both a transcriptional activator and a repressor.[27] In adult heart, each of these functions regulates genes with specialized and distinct molecular roles. To identify putative direct targets of TBX20 in midgestation cardiomyocytes, we made use of our TBX20 ChIP-Seq (chromatin immunoprecipitation with high throughput sequencing) analysis in E11.5 hearts and attributed TBX20-bound sites to the nearest expressed gene in E11.5 control or *Tbx20* cKO cardiomyocytes. Next, we overlaid our RNA-Seq data with that of our TBX20 ChIP-Seq analysis in E11.5 hearts[17] and identified 548 genes that were differentially expressed in *Tbx20* cKO cardiomyocytes and were marked by one or more TBX20 binding events in the vicinity of the gene (Figure 4B). Functional analysis of these putative direct TBX20 targets revealed that TBX20 activated and repressed genes fall into distinct categories (Online Figure III). Most notably, TBX20 directly activated cardiac muscle development and function genes and directly repressed other developmental pathways.

Motif Analysis

To investigate potential TBX20 DNA-binding cofactors, we scanned TBX20-binding regions associated with differentially expressed genes in *Tbx20* mutant embryonic cardiomyocytes for over-representation of transcription factor-binding sites. With this analysis, we found that TBX20-bound regions were enriched for DNA-binding motifs of T-box transcription factors, GATA type zinc fingers, basic leucine zipper domains (bZIP), TEA domain TF (transcriptional enhancer activator domain transcription factors) and MADS-box TF (MCM1, agamous, deficiens, serum response factor-Box domain transcription factors; Figure 4C). Notably, we did not observe significant differences in type of motifs found between upregulated and downregulated genes (Online Table IV). These results indicated that TBX20 cooperated with multiple TFs to stimulate or repress expression of target genes but did not provide an explanation as to why some genes were repressed and others were activated.

TBX20 Directly Activates Genes Required for Cardiomyocyte Proliferation

Decreased proliferation and interrupted cell cycle in *Tbx20 cKO* mutant hearts suggested downregulation of genes important for cell cycle regulation in cardiomyocytes. In keeping with this, identified putative direct targets of TBX20 downregulated in *Tbx20 cKO* myocytes included *Cdc6*, a gene that regulates G1-S cell cycle progression. Putative direct downregulated targets also included *Mycn* and *Erbb2*, each required for myocardial proliferation and essential for heart development.[28,29] Using qPCR, we confirmed downregulation of *Cdc6* and *Mycn* at E9.5, whereas *Erbb2* was not significantly downregulated in E9.5 or E11.5 *Tbx20 cKO* hearts (Figure 5A). Using in situ hybridization, both *Mycn* and *Erbb2* expression seemed downregulated in E9.5 and E11.5 *Tbx20 cKO* ventricles (Figure 5).

TBX20 Directly Represses a Cardiac Progenitor Gene Program in Cardiomyocytes

Among upregulated putative direct target genes were an intriguing number of key second heart field genes, including the *Isl1*, *Fgf10*, and *Hopx* (HOP homeobox). Marking subsets of cardiac

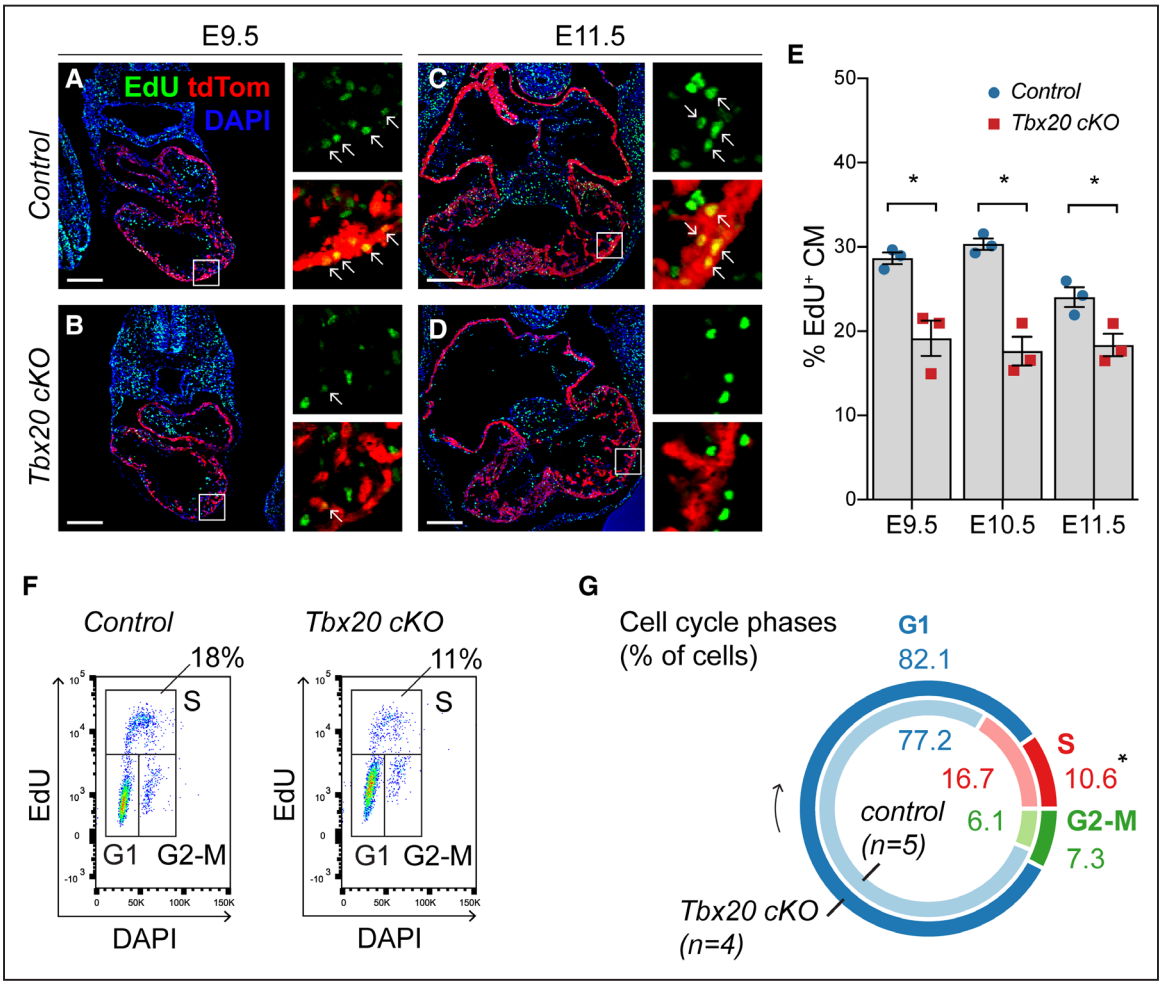

Figure 3. Proliferation and cell cycle analysis in control and *Tbx20* (T-box 20) mutant cardiomyocytes. A–D, 5-Ethynyl-2'-deoxyuridine (EdU) incorporation (green) along with tdTomato (red) for *Tnnt2-rtTA;TetO-Cre* lineage-traced cardiomyocytes and DAPI (4',6-diamidine-2'-phenylindole dihydrochloride; blue) for nuclei. **E**, Quantification of EdU incorporation in lineage-traced cardiomyocytes. **F**, Fluorescence activated cell sorting cell cycle analysis using EdU incorporation and DNA content (DAPI) in *Tbx20 cKO* and control lineage–traced cardiomyocytes. Percentage of cells in S phase is labeled. **G**, Quantification of cardiomyocytes per cell cycle stage in *Tnnt2-rtTA;TetO-Cre;Tbx20*$^{fl/fl}$ (mutant, outer circle) and *Tnnt2-rtTA;TetO-Cre;Tbx20*$^{+/fl}$ (control, inner circle) littermates. **E** and **G**: n=3 biological replicates; *, $P<0.05$ based on negative binomial regression analysis. Bars: 0.2 mm (**A–D**). cKO indicates conditional knock out mutant; and Tbx20, T-box 20.

progenitor cells, *Isl1*, *Fgf10*, and *Hopx* are essential for proliferation, survival, and migration of undifferentiated cardiomyocyte progenitors and are downregulated as progenitors enter the heart and differentiate toward cardiomyocytes.[30–32] To validate sustained expression of a cardiac progenitor gene program in cardiomyocytes after ablation of *Tbx20*, RNA in situ hybridization was performed for *Isl1* and *Fgf10*. At E11.5, where *Isl1* expression is typically restricted to the distal OFT, in *Tbx20 cKO* mutants, *Isl1* was expanded into the proximal OFT and right ventricle (Figure 6B and 6C). *Isl1* upregulation could also be detected by qPCR at this stage (Figure 6A). In contrast, and despite significant increased RNA levels by RNA-Seq, *Fgf10* transcript levels did not achieve sufficient levels in mutant and control hearts to be detectable by RNA in situ (Online Figure IVA through IVD). However, *Fgf10* was significantly upregulated by qPCR in E11.5 *Tbx20* cKO hearts (Online Figure IVM). Collectively, these data suggested that TBX20 directly represses a subset of cardiac progenitor genes, thereby promoting further differentiation and maturation of cardiomyocytes.

Cardiac Chamber Formation Is Properly Initiated in Tbx20 cKO Hearts

After heart tube formation and cardiac looping, cardiac chambers form at the outer curvature of the looped heart. Global *Tbx20* mutants display gross defects in initiation of chamber formation. We assessed whether chamber-specific differentiation patterns were initiated in *Tbx20 cKO* hearts by analyzing expression of molecular markers for chamber myocardial differentiation, including potential direct targets *Gja5* and *Tbx5*. At early stages examined, E9.5 and E10.5, expression of *Gja5* and *Tbx5* was not markedly changed between controls and mutants (Online Figure IVE through IVL). We also assessed expression of the pan-cardiac marker *Nkx2-5* and found that its expression was comparable between control and mutants (Figure 6E and 6F). The transcriptional repressor *Tbx2* that can suppress chamber-specific gene expression was upregulated in *Tbx20* cKO hearts at E9.5 (Online Figure IVM). Together, these data suggested that some, but not all, aspects of cardiac chamber formation were properly initiated in *Tbx20 cKO*s.

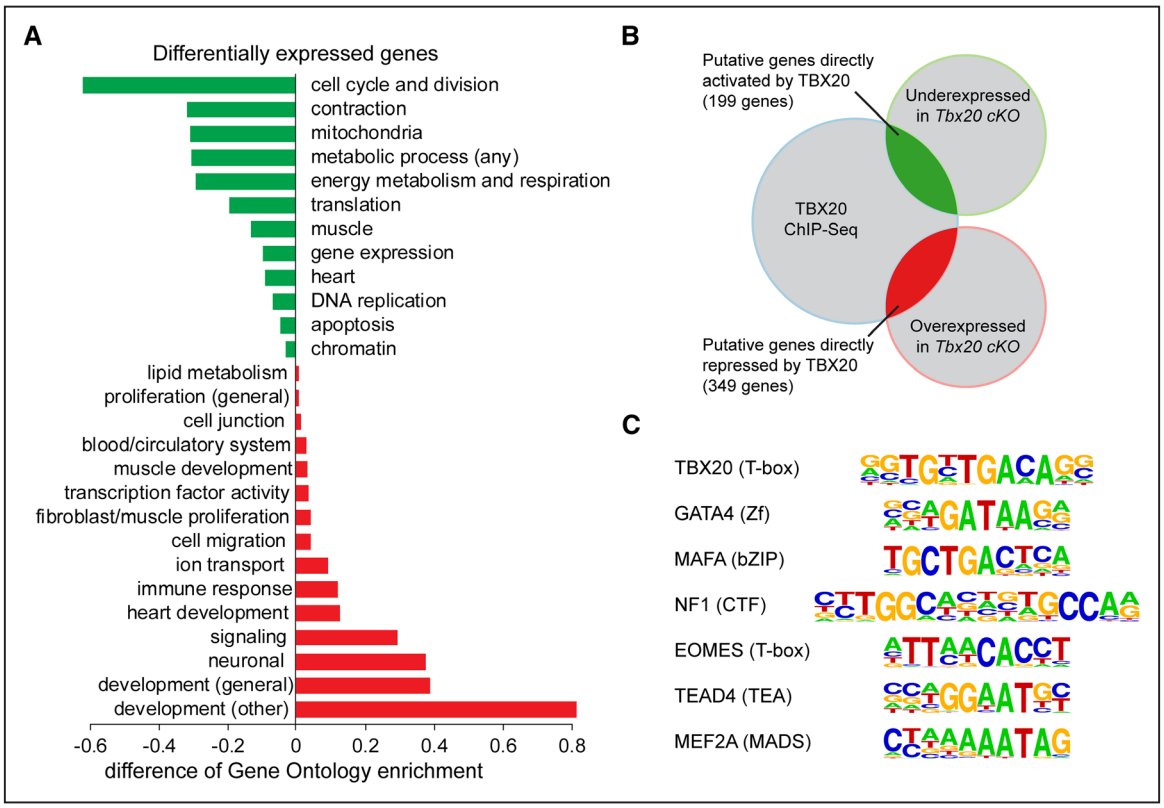

Figure 4. Gene ontology analysis of differentially expressed genes in embryonic day (E) 11.5 *Tbx20 cKO* cardiomyocytes vs control littermates. **A**, Gene ontology analysis of differentially expressed genes in *Tbx20 cKO* cardiomyocytes clustered into functional categories (green: underexpressed; red: overexpressed). Length of bars indicate the difference of Gene Ontology enrichment for each category between up- and downregulated genes. **B**, Intersection of RNA-Seq (RNA sequencing) and TBX20 (T-box 20)-GFP (green fluorescent protein) ChIP-Seq (chromatin immunoprecipitation with high throughput sequencing) in E11.5 hearts reveals putative direct targets of TBX20 in embryonic cardiomyocytes. **C**, Top overrepresented TF motifs in TBX20 ChIP-Seq peaks associated with differentially expressed genes. cKO indicates conditional knock out mutant.

At E11.5, *Tbx5* expression was markedly increased specifically in *Tbx20 cKO* atria compared with controls (Figure 6D and 6E). Embryos lacking *Tbx5* have abnormal heart tube formation and hypoplastic atria, whereas overexpression of *Tbx5* inhibits ventricular maturation.[33,34] In chicken embryos, *Tbx5* overexpression inhibits myocyte proliferation.[35] Therefore, overexpression of *Tbx5* in *Tbx20 cKO* atria might contribute to reduced atrial proliferation in *Tbx20 cKO*s, highlighting an important role for TBX20 in regulating atrial gene expression.

TBX20 Directly Represses Both Atrioventricular Canal and Ventricular Specific Genes Within Atria to Establish an Atrial Gene Program

To further explore roles TBX20 might have in regulating compartment-specific gene expression, we assessed expression patterns of other key genes regionally expressed in developing heart. *Bmp2* is expressed within atrioventricular canal (AVC) myocardium and OFT and is critical for early AVC development and cardiac cushion formation.[36,37] BMP2 activates *Tbx2* in AVC myocardium to repress a chamber myocardial phenotype and induce cushion development.[38] In E11.5 *Tbx20 cKO*s, *Bmp2* expression within AVC was unaltered, but its expression domain was aberrantly extended into atrial myocardium (Figure 6F and 6G). Increased *Bmp2* expression in E11.5 *Tbx20 cKO* cardiomyocytes was confirmed by qPCR (Figure 6A). *Bmp10* is a critical gene for trabeculation and growth of the ventricular wall.[39] Expression levels of *Bmp10* were not affected in ventricles of *Tbx20 cKO*s. However, aberrant upregulation of *Bmp10* was observed in right atria of mutants (Figure 6H and 6I). Ectopic *Bmp10* expression in mutant atria did not result in a significant increase in overall *Bmp10* mRNA levels when qPCR were performed on RNA from total cardiomyocytes purified from E11.5 heart (Figure 6A). However, *Bmp10* mRNA levels were found to be significantly increased in mutants right atria relative to controls when RNA was specifically extracted from isolated E11.5 right atrial tissue (Figure 6A). Although previous studies have associated upregulation of *Bmp10* with hypertrabeculated ventricles,[40] potential effects of *Bmp10* upregulation in right atrium have not been described. Together, these findings suggested that TBX20 might regulate atrial cardiomyocyte development by directly repressing nonatrial genes, including *Bmp2* and *Bmp10*, in atrial cardiomyocytes.

Upregulation of *Tbx5*, *Bmp2*, or *Bmp10* in atria did not seem to explain why left atrial proliferation was more severely affected than right atrial proliferation. To investigate potential pathways accounting for increased severity of the left atrial phenotype, we investigated expression of *Pitx2*, a major regulator of left–right asymmetry in the heart.[41] Our ChIP-Seq data suggested that *Pitx2* might be a direct TBX20 target. PITX2 inhibits left atrial proliferation, with mutants showing right atrial isomerism.[42,43] Using in situ

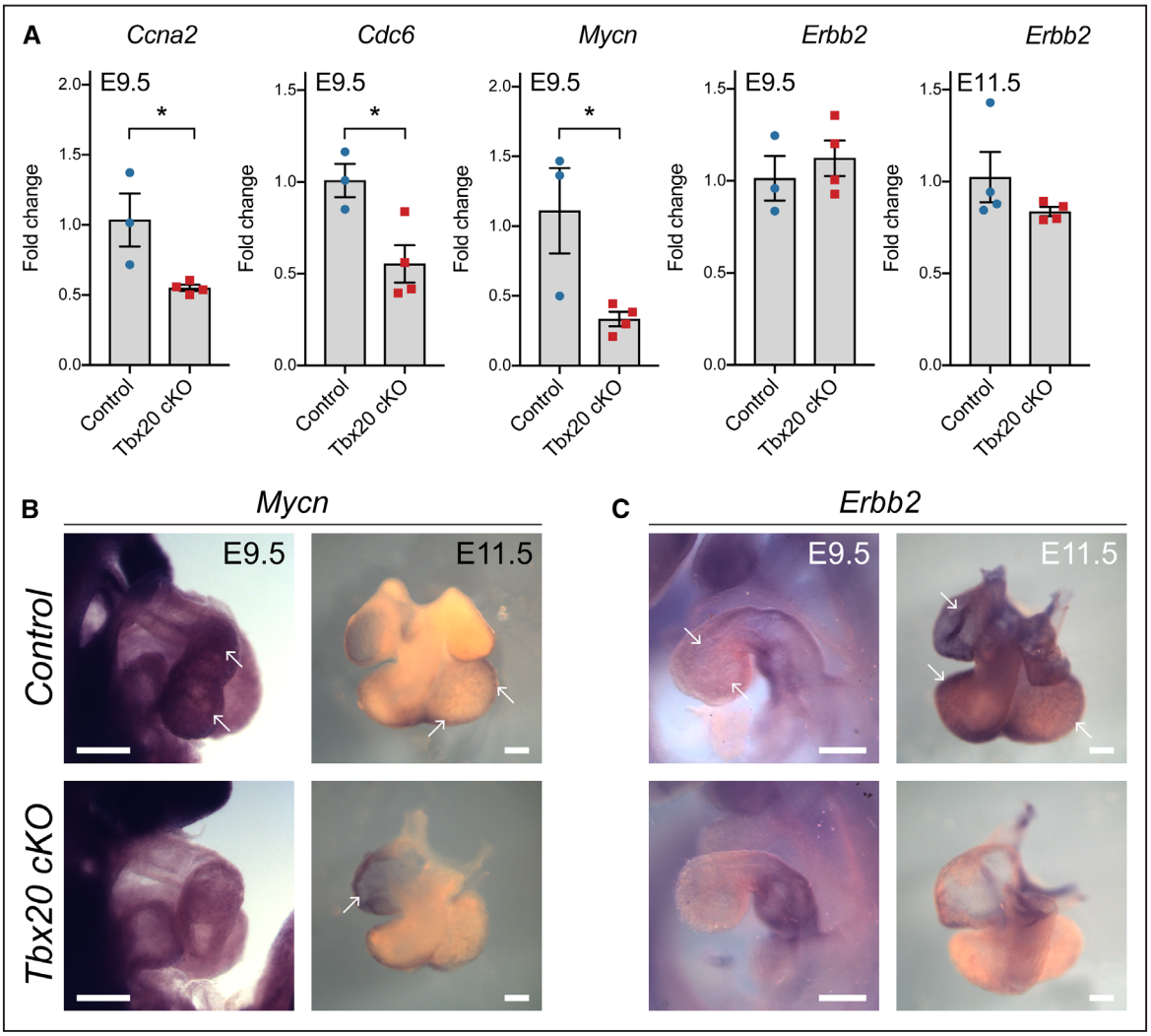

Figure 5. TBX20 (T-box 20) regulates cardiomyocyte proliferation genes. A, Quantitative polymerase chain reactionof genes associated with cell cycle and proliferation in control and *Tbx20 cKO* hearts (n=3–4 biological replicates; **P*<0.05 Mann–Whitney *U* test). **B**, *Mycn* expression is reduced in ventricles and OFT of *Tbx20 cKO* at embryonic day (E) 9.5 and E11.5. **C**, *Erbb2* expression is reduced in ventricles of *Tbx20 cKO* at E9.5 and E11.5. Bars: 0.2 mm. cKO indicates conditional knock out mutant.

hybridization, we did not observe differences in *Pitx2* expression levels or pattern in E9.5 or E11.5 mutants (Online Figure IVN through IVQ and data not shown). These observations indicated that left–right differences in *Tbx20 cKO* hearts occurred independently of alterations in *Pitx2* mRNA expression.

TBX20 Directly Activates Atrial and Ventricular Specific Genes to Establish Atrial and Ventricular Identity

From our genome-wide transcriptome and ChIP-Seq analysis, we identified multiple putative direct downstream targets of TBX20 downregulated in cKOs that have critical roles in establishing chamber identity, including *COUP-TFII*, *Hey1*, *Hey2*, and *Irx4*.[44–46] In developing human and mouse heart, *COUP-TFII* is abundantly expressed in atria and determines atrial identity by activating atrial markers and by repressing ventricular markers.[46,47] Using qPCR, we confirmed that *COUP-TFII* was downregulated in *Tbx20 cKO* hearts at E9.5 and E11.5 (Figure 7A). In situ hybridization indicated that *COUP-TFII* expression in atria was similar between *Tbx20 cKO*s and controls at E9.5 (Figure 7B). However, at E10.5 and E11.5, *COUP-TFII* expression was greatly reduced in *Tbx20 cKO* atria relative to controls. The atrial specific gene *Hey1* was absent or reduced in mutant atria at E9.5 (Figure 7C and 7D). Expression of the atrial gene *MLC2a* did not seem to be affected in *Tbx20 cKO*s relative to controls (Figure 7F). Reduced *COUP-TFII* and *Hey1* expression suggested perturbation of atrial identity in *Tbx20 cKO*s.

In myocardial knockouts of *COUP-TFII*, ventricular genes *Hey2*, *Irx4*, and *MLC2v* are upregulated in mutant atria at E14.5.[46] We examined their expression in *Tbx20 cKOs* (Figure 7C) and found that *MLC2v* was ectopically expressed in right atrium, although overall transcript levels in E9.5 or E11.5 hearts were not significantly altered as measured by qPCR. At E11.5, although *Hey2* and *Irx4* were not upregulated in *Tbx20 cKO* atria, *Hey2* and *Irx4* were reduced in *Tbx20 cKO* ventricles, which was confirmed by qPCR. *Hey2* and *Irx4* are important for regulation of a

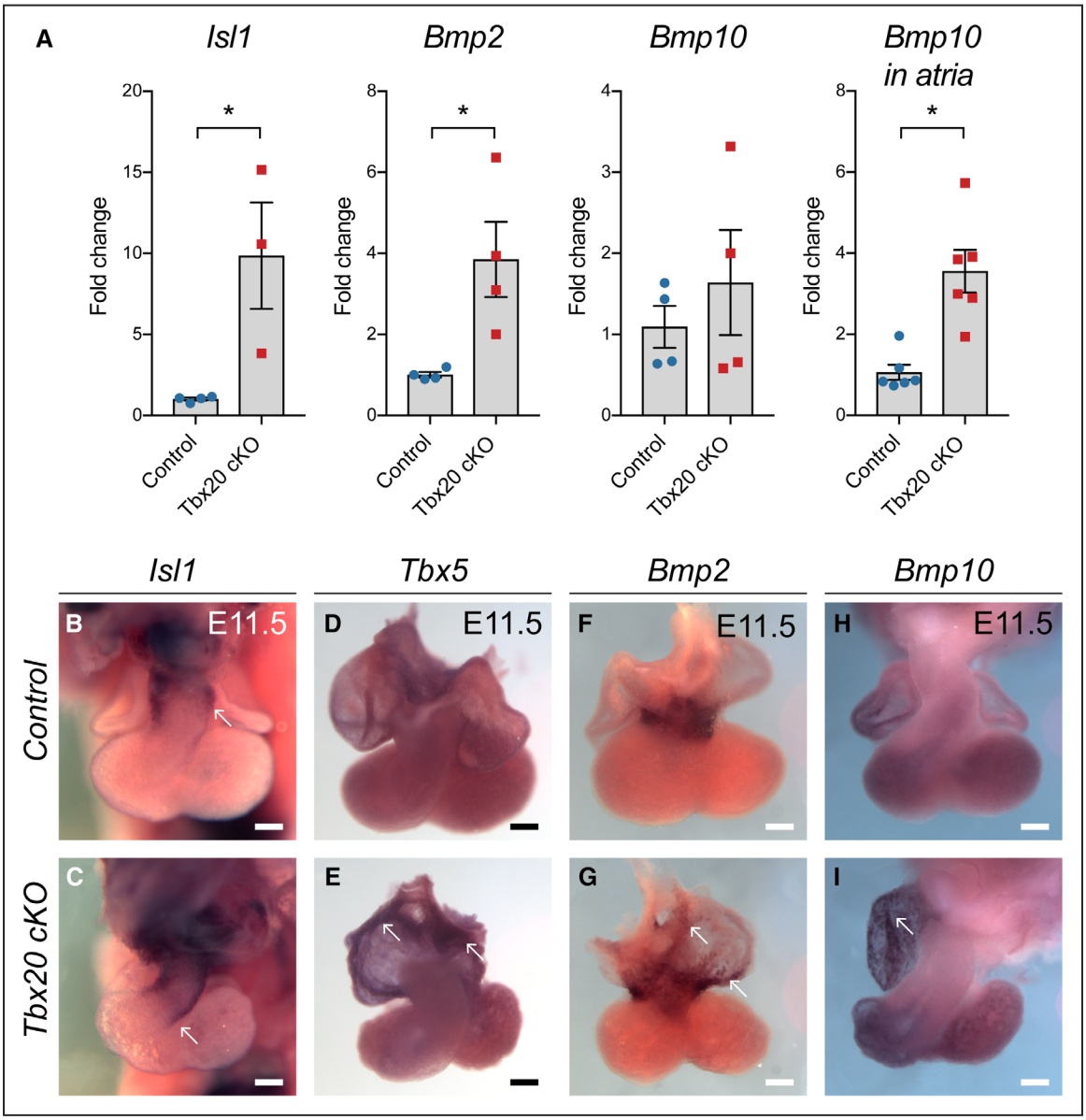

Figure 6. TBX20 (T-box 20) regulates second heart field and cardiac development genes. A, Quantitative polymerase chain reaction of *Isl1* (LIM/homeodomain transcription factor Islet1), *Bmp2* (bone morphogenetic protein 2), and *Bmp10* (bone morphogenetic protein 10) in control and *Tbx20 cKO* cardiomyocytes (n=3–4 biological replicates; **P*<0.05 Mann–Whitney *U* test) and BMP10 in control and *Tbx20 cKO* right atrial tissue (right; n=6; *, *P*<0.05) **B** and **C**, Proximal border of high *Isl1* expression (arrow) is expanded from distal outflow tract toward right ventricle at E11.5 in *Tbx20 cKO* heart. **D** and **E**, *Tbx5* expression is enhanced in *Tbx20* cKO atria (ventral view). **F** and **G**, *Bmp2* expression is expanded to atria in *Tbx20 cKO* heart (arrows). **H** and **I**, *Bmp10* is overexpressed in right atrium of *Tbx20 cKO* heart (arrow); **B–E**, **H**, and **I**, ventral view; **F** and **G**, dorsal view. Bars: 0.2 mm. cKO indicates conditional knock out mutant; and Tbx20, T-box 20.

ventricular specific program.[48,49] In summary, these results demonstrated that TBX20 plays a critical role in establishing atrial and ventricular identity, potentially by direct regulation of genes required to execute atrial and ventricular gene programs.

TBX20 and COUP-TFII May Cooperate in Target Gene Regulation

To explore a potential regulatory interaction between TBX20 and COUP-TFII, we compared putative direct targets of COUP-TFII and TBX20, using a previously published data set of COUP-TFII ChIP-Seq in embryonic atria.[46] This analysis indicated minimal overlap between TBX20 and COUP-TFII binding (289 out of 5110 TBX20 ChIP-Seq peaks). These regions are candidate enhancers that may be regulated by cooperative binding of TBX20 and COUP-TFII. Intriguingly, however, we noted considerable overlap between putative direct TBX20 and COUP-TFII target genes, indicating that TBX20 and COUP-TFII may act on distinct enhancers to achieve regulation of shared downstream target genes (Onine Figure V). We next selected genes differentially expressed in *Tbx20 cKO* cardiomyocytes and found that among shared target genes were both upregulated and downregulated genes. Together, these data provide further insights into potential regulatory interactions between TBX20 and COUP-TFII during heart development.

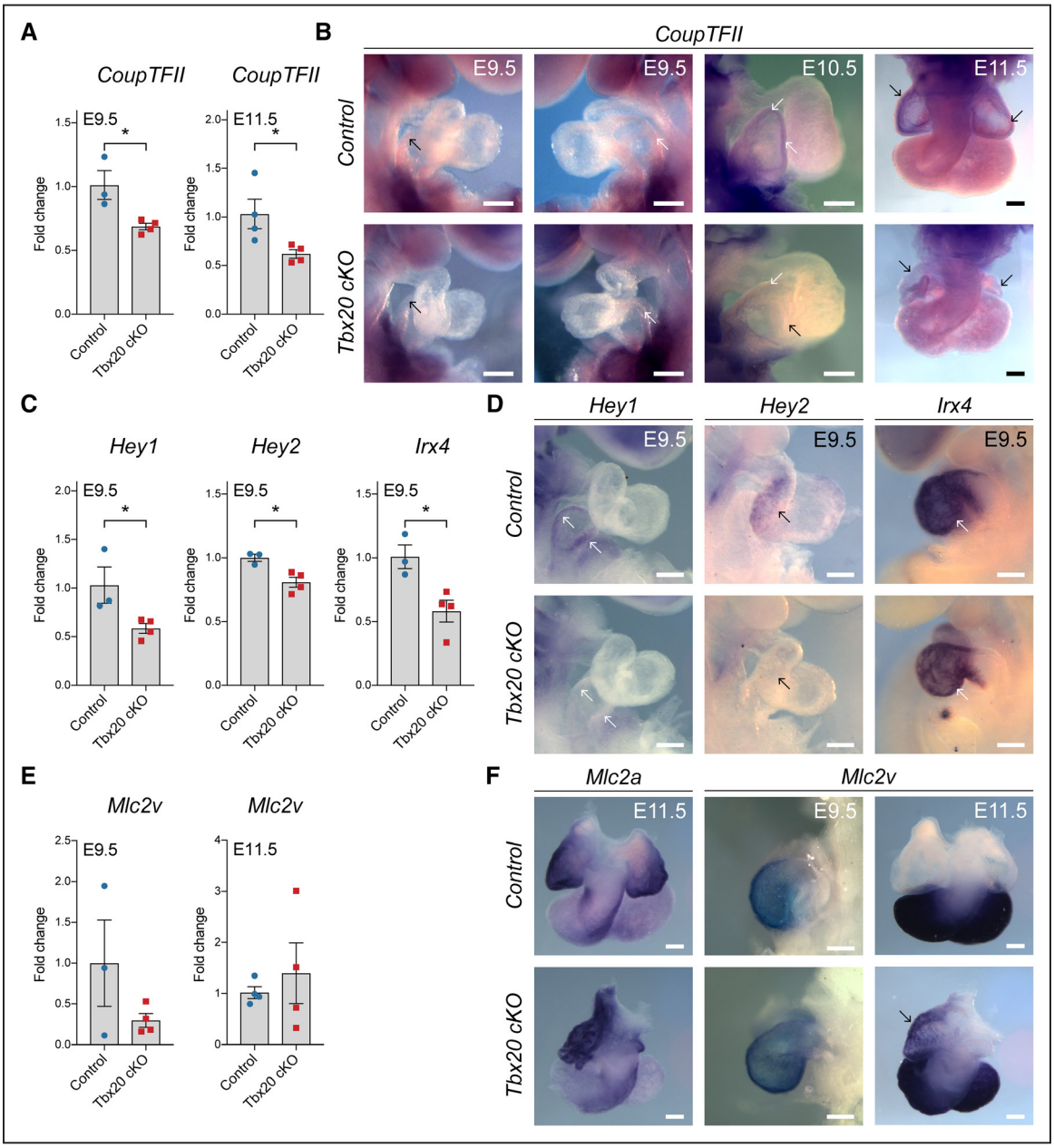

Figure 7. TBX20 (T-box 20) regulates chamber identity genes. A, *COUP-TFII* (chicken ovalbumin upstream promoter transcription factor 2) expression is downregulated in *Tbx20 cKO* hearts compared with control (n=3–4 biological replicates; $P<0.05$ Mann–Whitney *U* test). **B**, In situ hybridization for *COUP-TFII* at E9.5, E10.5, and E11.5 **C**, Quantitative polymerase chain reaction (qPCR) of chamber identity genes in control and *Tbx20 cKO* cardiomyocytes (n=3–4 biological replicates; **P*<0.05 Mann–Whitney *U* test). **D**, *Hey1* expression is downregulated in atria (**left**), and *Hey2* (**middle**) and *Irx4* (**right**) are downregulated in ventricles of E9.5 *Tbx20 cKO* mutant. **E,** qPCR of *Mlc2v* gene expression in E9.5 and E11.5 hearts (n=3–4 biological replicates; not significant Mann–Whitney *U* test). **F**, In situ hybridization shows comparable Mlc2a expression at E11.5 (**left**), comparable Mlc2v expression at E9.5 (**middle**), and ectopic Mlc2v expression in atria of Tbx20 cKO hearts at E11.5 (arrow, **right**). Bars: 0.2 mm. cKO indicates conditional knock out mutant.

A COUP-TFII Enhancer Bound by TBX20 Drives Transgene Expression In Vivo

Because *COUP-TFII* was significantly downregulated in *Tbx20 cKO* cardiomyocytes, and as this reduced expression was likely to contribute to cardiac defects in our mutant mice, we further investigated direct regulation of *COUP-TFII* by TBX20. Scanning the *COUP-TFII* regulatory landscape, we identified 2 TBX20-binding sites by ChIP-Seq in mouse embryonic hearts, one of which was also identified in adult heart (Figure 8A and the study by Shen et al[19]). Both sites were evolutionarily conserved and marked by enhancer-associated histone modifications including H3K4-methylation and H3K27-acetylation, as well as P300 binding in embryonic mouse hearts, suggestive that these regions correspond to cardiac enhancers.[50] By inspection of the Human Epigenome Roadmap data,[51] we noticed that orthologous human regions corresponding to these candidate enhancers also harbor epigenetic marks that are hallmarks of enhancers in human fetal heart samples (Online Figure VI). To directly test the enhancer properties of these candidate regions, we used an in vivo mouse transgenic reporter assay. One of the regions tested resulted in consistent, robust reporter gene expression

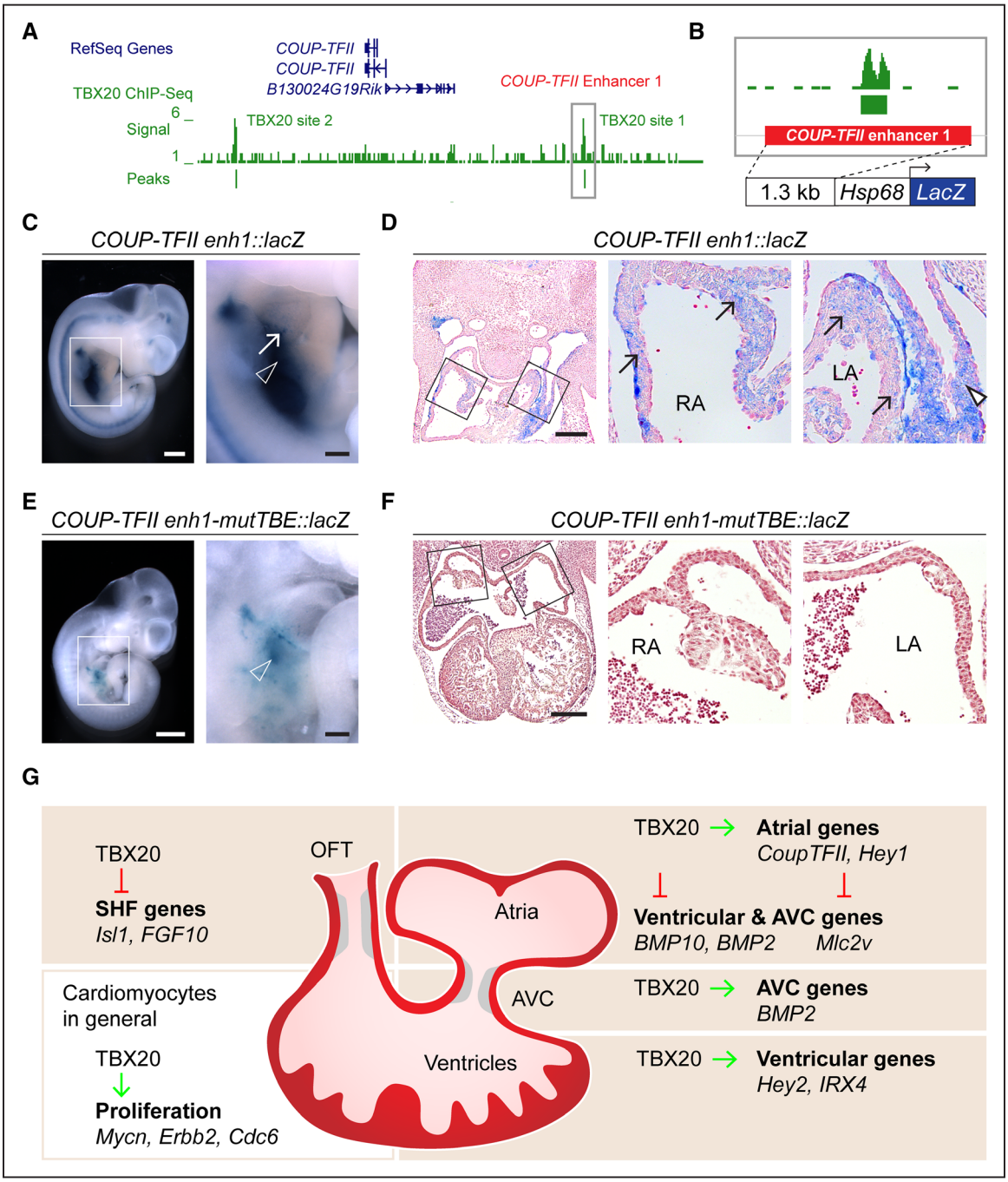

Figure 8. TBX20 (T-box 20) binds and regulates an enhancer upstream of *COUP-TFII* (chicken ovalbumin upstream promoter transcription factor 2) that drives expression in atrial cardiomyocytes. A, TBX20-GFP (green fluorescent protein) ChIP-Seq (chromatin immunoprecipitation with high throughput sequencing) of E11.5 mouse hearts in *COUP-TFII* genomic region. **B**, Magnification of enhancer 1 and schematic representation of reporter construct. **C**, *COUP-TFII enh1::lacZ* embryo showing reporter gene expression in developing atrial myocardium (arrow) and venous inflow region (arrowhead). **D**, Section analysis of *COUP-TFII enh1::lacZ* transgenic embryo demonstrates X-gal staining in developing atrial myocardium (arrows) and caval vein (arrowheads). **E**, *COUP-TFII enh1-mutTBE::lacZ* embryo showing loss of staining in atrial region and sustained expression in venous inflow region (arrowhead). **F**, *COUP-TFII enh1-mutTBE::lacZ* transgenic embryo demonstrates absence of X-gal staining in developing atrial myocardium (arrows). **G**, Overview of suggested regulatory pathways by which TBX20 determines chamber identity and cardiomyocyte development based on the current and previous studies. In outflow tract, TBX20 suppresses expression of second heart field (SHF) genes, including *Isl1* (LIM/homeodomain transcription factor Islet1) and *Fgf10* (fibroblast growth factor 10). In atria, TBX20 contributes to atrial specification by suppressing ventricular and atrioventricular canal genes *Bmp2* (bone morphogenetic protein 2) and *Bmp10* (bone morphogenetic protein 10), while activating *COUP-TFII* and *Hey1* expression. In atrioventricular canal, TBX20 activates *Bmp2* expression. In ventricles, TBX20 activates expression of *Hey2* and *Irx4*. Furthermore, TBX20 regulates cardiomyocyte proliferation, via the activation of *Mycn*, *Erbb2*, and *Cdc6*. Bars: 0.2 mm, except for **left** figures in **C** and **E**: 1 mm.

in multiple embryonic regions, including venous inflow area and atria in 4 out of 5 transgenic embryos (Figure 8C). Section analysis further revealed that enhancer 1 consistently drove reporter gene expression in atrial cardiomyocytes (4 out of 5 transgenic embryos), including venous valve myocardium, recapitulating endogenous cardiac *COUPTF-II* expression. In contrast, no expression was observed in ventricular myocardium, with the exception of a small patch of myocardial cells in

the right ventricle of a single transgenic embryo (not shown). To further confirm that expression of this *COUP-TFII* enhancer in atrial myocardium was directly regulated by TBX20, we mutated a conserved TBX20 binding site within this enhancer and found that expression in atrial cardiomyocytes was largely abolished in transgenic embryos (no detectable *LacZ* [beta-galactosidase]-expressing atrial cardiomyocytes in 6 out of 7 transgenic embryos, with the remaining embryo having few scattered lacZ-expressing atrial cardiomyocytes; Figure 8D), while reporter gene expression outside the heart was observed in a pattern similar to that of the wild-type enhancer (4 out of 7 embryos; Figure 8D). We next established that this enhancer was functionally connected with *COUP-TFII*. We performed a promoter-based capture chromosome conformation capture with high-throughput sequencing in induced pluripotent stem cell–derived human cardiomyocytes to identify long-range physical interactions between genes and enhancers. We observed that this enhancer directly loops and contacts the *COUP-TFII* promoter, 140 kb away, confirming that this is a *COUP-TFII* enhancer (Online Figure VI). Taken together, these results linked TBX20 binding to an evolutionary conserved enhancer that regulates *COUP-TFII* expression in developing atrial cardiomyocytes, uncovering a mechanism by which *COUP-TFII* expression is TBX20 dependent. As discussed further below, decreased *COUP-TFII* expression is likely to contribute to several aspects of observed *Tbx20 cKO* phenotypes.

Discussion

Using global transcriptome analysis combined with embryonic heart ChIP-Seq, we identified previously unrecognized critical gene targets and cell autonomous functions of TBX20 in midgestation cardiomyocytes, illuminating a major role for TBX20 in establishing ventricular versus atrial identity and a particularly critical role in left atrial growth.

Mutations in *TBX20* are associated with interventricular septal defects and atrioventricular septal defects.[5,8,52] In previous studies, we showed that TBX20 is required in endothelial lineages for interatrial and interventricular septation, via regulation of the extracellular matrix proteoglycan versican.[17] Here, we show that TBX20 is also required in cardiomyocytes for development of the atrial and interventricular septa, potentially via the regulation of proliferation in these structures. Thus, TBX20 seems to be required in multiple cellular lineages for cardiac septation.

Cell proliferation on the outer curvature of the heart between E9.5 and E12.5 makes major contributions to growth of chamber myocardium.[10] *Tbx20* global mutants exhibit decreased cardiomyocyte proliferation and arrest development at E9.5, with severely hypoplastic, unlooped hearts.[11–13] Here, ablation of *Tbx20* in developing cardiomyocytes led to failure of cardiac chamber expansion and septal defects, associated with reduced proliferation in *Tbx20 cKO* cardiomyocytes. These results demonstrated for the first time a cell autonomous requirement for TBX20 in embryonic cardiomyocyte proliferation. Intriguingly, overexpression of TBX20 induces cardiomyocyte proliferation in adult cardiomyocytes.[18]

Previous work in global *Tbx20* mutants indicated ectopic *Tbx2* expression might contribute to proliferation defects.[11–13] However, studies with compound *Tbx2*;*Tbx20* mutants show that additional pathways exist by which TBX20 regulates cardiomyocyte proliferation, independent of *Tbx2*.[15] Here, intersection of RNA-Seq data and ChIP-Seq data gave new insights into additional mechanisms by which TBX20 cell autonomously regulates myocyte proliferation. Genes that were differentially expressed, with TBX20 binding near promoter or *cis*-regulatory elements, were considered putative direct targets. However, we cannot formally rule out that gene expression changes are the consequence of anatomic changes in *Tbx20 cKO* hearts or reflect indirect regulation by TBX20. Further proof that genes identified here putative direct downstream targets of TBX20 would require experiments testing enhancer activity and dependence on TBX20 in vivo. Our data suggested that TBX20 directly activates many genes required to effect cardiomyocyte proliferation, including *Mycn*, *Erbb2*, and *Cdc6*. Recently, *TBX20* mutations were associated with left ventricular noncompaction and decreased proliferation in human induced pluripotent stem cell–derived cardiomyocytes, potentially via downregulation of the TGFB (transforming growth factor beta) inhibitor PRDM16.[9] In our study, PRDM16 was downregulated in embryonic cardiomyocytes on loss of TBX20, and we identified 4 TBX20-binding sites within the PRDM16 gene body in embryonic heart, providing further support for a direct regulatory role for TBX20 in development of left ventricular noncompaction. Thus, our data shed new light on pathways by which TBX20 directly and cell autonomously regulates cardiomyocyte proliferation.

Cardiac chamber formation is marked by activation of a cardiomyocyte differentiation gene program in developing chamber myocardium, whreas nonchamber myocardium of the AVC, OFT, and inflow tract retains more primitive characteristics.[53] Multiple *T-box* genes play important roles in different aspects of this process. Previous studies using global *Tbx20* mutants and in vitro assays have indicated that TBX20 and TBX5 cooperate with NKX2.5 and GATA4 to promote chamber differentiation via activating *Nppa* and *Gja5* expression.[3,23,33,54,55] In addition, within chambers, TBX20 represses non–chamber-specific genes, such as *Tbx2*.[11–13,15] In AVC, TBX2 represses chamber-specific gene expression to maintain the less differentiated, nonchamber myocardial fate. *Tbx2* expression in AVC is activated by BMP2.[36,37,56] Here, we provided evidence to suggest that TBX20 directly suppressed *Bmp2* expression in developing atrial cardiomyocytes, resulting in ectopic *Bmp2* expression in *Tbx20 cKO*s. Ectopic *Bmp2* expression in *Tbx20 cKO* atria may also result from reduced expression of putative TBX20 direct targets *Hey1* and *Hey2*, as *Hey1* and *Hey2* restrict expression of *Bmp2* and *Tbx2* to the AVC.[57] Altogether, our studies demonstrated that TBX20 cell autonomously promotes chamber myocardial fate by suppression of an AVC gene program in chamber myocardium.

RNA-Seq of *Tbx20 cKO* cardiomyocytes revealed increased expression of cardiac progenitor markers *Isl1*, *Fgf10*, and *Hopx* compared with littermate controls.[30,32,58] Notably, these genes were also predicted direct targets of TBX20 in embryonic hearts. *Isl1* is necessary for a subset of undifferentiated cardiac progenitors of the second heart field to proliferate,

survive, and migrate.[30] *Isl1* is downregulated in OFT when cardiac progenitors enter the heart and differentiate. TBX20 has been shown to directly repress *Isl1* in E8.5 myocardium.[12] Our studies demonstrated an ongoing requirement for TBX20 to repress *Isl1* in E11.5 cardiomyocytes. *Fgf10* overexpression in E11.5 *Tbx20 cKO* cardiomyocytes as measured by RNA-Seq and qPCR could not be confirmed by whole mount RNA in situ studies, perhaps because of the lower sensitivity of the RNA in situ assay.

Although proliferation of both atria was significantly reduced in *Tbx20 cKO*s, left atrial proliferation was more drastically affected than right atrial proliferation. We examined *Pitx2* expression but found no difference in expression that could explain this phenotype. Left atrial hypoplasia along with other cardiac defects was found in a stillborn baby with a 15q26.2 deletion that includes *COUP-TFII*.[59] Patients with similar 15q26.2 deletion but intact *COUP-TFII* do not show cardiac defects. Moreover, a mouse *COUP-TFII* hypomorphic mutant exhibits left atrial hypoplasia,[60] and our *Tbx20 cKO* mutants display significant reductions in *COUP-TFII* expression. Therefore, *COUP-TFII* insufficiency may underlie the left atrial hypoplasia in *Tbx20 cKO*s.

Atrial and ventricular chambers have unique roles in effecting blood circulation.[61–63] Intersection of *Tbx20 cKO* RNA-Seq and TBX20-GFP ChIP-Seq data illuminated important cell autonomous roles and mechanisms by which TBX20 sets up both atrial and ventricular identity (Figure 8G). Notably, *COUP-TFII* was a direct target of TBX20. COUP-TFII is an orphan nuclear receptor essential for establishment and maintenance of atrial identity.[46] During heart development, *COUP-TFII* is selectively expressed in atrial, not ventricular, myocardium.[64] Cardiomyocyte loss of *COUP-TFII* leads to reduced atrial gene expression and ventricularization of atria.[46] In keeping with this, in *Tbx20 cKO*s, reduced expression of *COUP-TFII* in both atria was accompanied by reduced expression of the atrial gene *Hey1*, and ectopic atrial expression of the ventricular marker *Mlc2v*. Previous in vitro studies have described factors regulating *COUP-TFII* expression in other contexts.[65–67] Our work has identified TBX20 as a direct regulator of *COUP-TFII* during cardiogenesis in vivo. In addition to its critical role in atrial development and identity, our studies provide evidence indicating that TBX20 establishes ventricular identity by direct regulation of *Hey2* and *Irx4* in developing ventricular myocytes.[48,49]

Acknowledgments

Confocal microscopy was performed at the University of California, San Diego School of Medicine Microscopy Core Facility, which is supported by the grant P30 NS047101.

Sources of Funding

This work was supported by the American Heart Association (13POST16480012) and the Netherlands Organization for Scientific Research (NWO, 825.10.016) to C.J. Boogerd, National Institutes of Health grants HL119967, HL114010, HL123857, HL128075, and HL118758 (to M.A. Nobrega) and HL123747, HL117649, and HL074066 (to S.M. Evans). J. Bogomolovas is supported by the European Commission's Marie Skłodowska-Curie Individual Fellowship (Titin Signals, 656636).

Disclosures

None.

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