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

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ABSTRACT

Rationale: Mutations in the transcription factor TBX20 are associated with congenital heart disease. Germline ablation of Tbx20 results in abnormal heart development and embryonic lethality by E9.5. As 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 mid-gestation 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 cKOs. 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 appeared to be aberrantly regulated. Putative direct TBX20 targets were identified using TBX20 ChIP-Seq 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-TFI/Nr2f2. 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 non-chamber 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 COUPTFI enhancer.

Keywords: Transcriptional regulation, heart development, congenital heart disease, cardiomyocyte differentiation, chamber specification.

Nonstandard Abbreviations and Acronyms:

| Abbreviation | Description |
|--------------|-------------|
| CHD          | congenital heart defects |
| FACS         | Fluorescence activated cell sorting |
| EdU          | 5-ethynyl-2'-deoxyuridine |
| GO           | gene ontology |
| TFBS         | transcription factor binding site |
| cKO          | conditional knock out mutant |
| AVC          | Atrioventricular canal |
| HiC          | Chromosome conformation capture with high-throughput sequencing |
INTRODUCTION

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 T-box 20 (Tbx20) 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 (CHD), including septal defects and cardiomyopathies (5–9).

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 non-myocardial 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, utilizing an inducible cardiomyocyte specific Cre mouse line, we demonstrated that Tbx20 is required within mid-gestation 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 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 UCSD or University of Chicago. Mice were maintained on BlackSwiss (NIHBL(S); Taconic Biosciences) background. Tbx20loxP, 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 cKO; Tnnt2-rtTA;TetO-Cre;Tbx20* 

\[\text{loxP/loxP}\] and controls (control; *Tnnt2-rtTA;TetO-Cre;Tbx20* 

\[\text{loxP/loxP}\] males with *Tbx20* 

\[\text{loxP/loxP}\] or *Tbx20* 

\[\text{loxP/loxP}\];*R26tdTom/tdTom* females. Cre expression was induced by Doxycycline (MP Biomedicals, cat# 198955; 1mg/mL) in water prepared fresh daily to pregnant females from E8.5 onwards. All experiments were performed using somite stage and size matched embryo pairs, images shown are representative examples of experiments with \(n \geq 3\) biological replicates.

**Quantification experiments and statistical analysis.**

Cardiac chamber size was quantified by perimeter measurements in every fourth section (10 \(\mu\text{m}\)). 200 \(\mu\text{l}\) 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 \(n \geq 3\) biological replicates (actual number of biological replicates for each experiment stated in figure legends). Mann-Whitney U test was used to compare two groups, reporting asymptotic 2-tailed significance P-values. Cell cycle quantification and EdU incorporation counts were analyzed using negative binomial (NB) regression using SPSS25 software (Online Tables V-X). Post hoc tests for NB regression were performed using the Bonferroni correction. \(P<0.05\) was considered significant.

**Promotor capture HiC in human cardiomyocytes.**

Human cardiomyocyte cells were generated precisely as described before using induced pluripotent stem cell (iPSC) line 19101 (22). Promoter capture Hi-C was performed by combining in situ Hi-C with an oligo hybridization step as detailed in supplemental methods.

**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 mid-gestation cardiomyocytes, we ablated *Tbx20* from cardiomyocytes after E8.5 using Doxycycline inducible *Tnnt2-rtTA;TetO-Cre* (21) (Online Figure IA). Following Doxycycline administration, *R26-tdTomato* expression demonstrated efficient cardiomyocyte specific Cre-mediated excision (Online Figure IB-D). Significant ablation of *Tbx20* exon 2 was observed by 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 mid-gestation 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 (Figure 1 and 2). Circumferential measurements of cardiac chambers confirmed that left atrium and left ventricle were significantly smaller, while reduced sizes of right atrium and right ventricle were not statistically significant (Figure 2I). Moreover, *Tbx20 cKO* hearts had under-developed atrial and interventricular septa, and venous valves of *Tbx20 cKO* hearts were smaller at E10.5 and thinner at E11.5 when compared to controls (Figure 2). *Tbx20 cKO* outflow tracts appeared shorter at E10.5 and E11.5 (Figure 1J-X). 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 Tnn2-rtTA;TetO-Cre;R26+/tdTom lineage traced cardiomyocytes at E9.5, E10.5 and E11.5 (Figure 3A-E). At all stages examined, cardiomyocyte proliferation was significantly decreased in Tbx20 mutant hearts compared to cardiomyocytes of somite-matched control littersmates (Online Tables IX, 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 outflow tract, with left atrial cardiomyocytes displaying the strongest reduction (Online Figure II; Online Table VI). In contrast, proliferation of non-myocytes 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, XIII). FACS analysis of Edu incorporation and DNA content demonstrated that a higher percentage of Tbx20 cKO cardiomyocytes were in G1 phase, while 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,G; Online Tables XII, 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 on FACS-purified cardiomyocytes from E11.5 Tbx20 cKO and somite stage matched littermate control hearts (Online Figure IIIA). Of 1478 differentially expressed genes (fold change > 1.2x, P adjusted < 0.1), 816 were downregulated in Tbx20 cKO cardiomyocytes, and expression of 662 genes was increased compared to control cardiomyocytes (Online Table III).

Next, we performed Gene Ontology (GO) term enrichment analysis on differentially expressed genes. We grouped GO terms in major categories, compared occurrence of these categories across gene sets and observed a clear-cut difference between up- and down-regulated genes in Tbx20 mutant cardiomyocytes (Figure 4A). Down-regulated genes were predominantly involved in cell cycle, contraction, and energy metabolism, whereas up-regulated genes were primarily involved in general developmental pathways, neuronal function, as well as heart development.

Downregulated genes within the cell cycle functional category included cell division cycle 6 (Cdc6), chromatin licensing and DNA replication factor 1 (Cdt1) and cyclin A2 (Ccna2). Cdc6 and Cdt1 are involved in the formation of the pre-replication complex that is necessary for DNA replication (24). Cdc6 and Ccna2 activate cyclin dependent kinase 2 (Cdk2), 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 mutant
cardiomyocytes displayed decreased G1 to S transition and provided support for the hypothesis that Tbx20 promotes cell cycle progression in mid-gestation cardiomyocytes.

Genes critical for cardiac development within the group of upregulated genes included Tbx2, Isl1, Fgf10, Hopx, Bmp2 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 mid-gestation cardiomyocytes, we made use of our TBX20 ChIP-Seq 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 co-factors, we scanned TBX20-binding regions associated with differentially expressed genes in Tbx20 mutant embryonic cardiomyocytes for over-representation of transcription factor-binding sites (TFBS). 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 and MADS-box TF (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 appeared downregulated in E9.5 and E11.5 Tbx20 cKO ventricles (Figure 5).

**TBX20 directly represses a cardiac progenitor gene program in cardiomyocytes.**

Amongst upregulated putative direct target genes were an intriguing number of key SHF genes, including the LIM/homeodomain transcription factor Islet1 (Isl1), fibroblast growth factor 10 (Fgf10), and HOP homeobox (Hopx). Marking subsets of cardiac 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 towards 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,C). *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-D). 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-L). We also assessed expression of the pan-cardiac marker *Nkx2-5* and found that its expression was comparable between control and mutants (Figure 6E,F). 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* cKOs.

At E11.5, *Tbx5* expression was markedly increased specifically in *Tbx20* cKO atria compared to controls (Figure 6D,E). Embryos lacking *Tbx5* have abnormal heart tube formation and hypoplastic atria, whereas over-expression 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* cKOs, 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 examined expression patterns of other key genes regionally expressed in developing heart. *Bone morphogenetic protein 2 (Bmp2)* is expressed within atrioventricular canal (AVC) myocardium and outflow tract 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* cKOs, *Bmp2* expression within AVC was unaltered, but its expression domain was aberrantly extended into atrial myocardium (Figure 6F,G). 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* cKOs. However, aberrant upregulation of *Bmp10* was observed in right atria of mutants (Figure 6H,I). Ectopic *Bmp10* expression in mutant atria did not result in a significant increase in overall *Bmp10* mRNA levels when qPCR analyses 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 non-atrial genes, including *Bmp2* and *Bmp10*, in atrial cardiomyocytes.
Upregulation of *Tbx5, Bmp2* or *Bmp10* in atria did not appear 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 hybridization, we did not observe differences in *Pitx2* expression levels or pattern in E9.5 or E11.5 mutants (Online Figure IVN-Q 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 cKOs 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,D). Expression of the atrial gene *MLC2a* did not appear to be affected in Tbx20 cKOs relative to controls (Figure 7F). Reduced *COUP-TFII* and *Hey1* expression suggested perturbation of atrial identity in Tbx20 cKOs.

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 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 dataset 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 (Online Figure V). We next selected genes differentially expressed in Tbx20 cKO cardiomyocytes, and found that amongst 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.

**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
two TBX20-binding sites by ChIP-Seq in mouse embryonic hearts, one of which was also identified in adult heart (Figure 8A and (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 utilized an in vivo mouse transgenic reporter assay. One of the regions tested resulted in consistent, robust reporter gene expression 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 COUP-TFII 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-expressing atrial cardiomyocytes in 6 out of 7 transgenic embryos, with the remaining embryo having very 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 wildtype 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 Hi-C in iPSC-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 mid-gestation 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 appears 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

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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 anatomical 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 a number of genes required to effect cardiomyocyte proliferation, including Mycn, Erbb2, and Cdc6. Recently, Tbx20 mutations were associated with left ventricular non-compaction (LVNC) and decreased proliferation in hIPSc derived cardiomyocytes, potentially via downregulation of the TGFB inhibitor PRDM16 (9). In our study, PRDM16 was downregulated in embryonic cardiomyocytes upon loss of Tbx20, and we identified four Tbx20 binding sites within the PRDM16 gene body in embryonic heart, providing further support for a direct regulatory role for Tbx20 in development of LVNC. 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, while non-chamber myocardium of the AVC, OFT and IFT 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, non-chamber 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 cKOs. 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 to 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 (SHF) 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 owing to the lower sensitivity of the RNA in situ assay.

Although proliferation of both atria was significantly reduced in Tbx20 cKOs, 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 cKOs.

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 cKOs, 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).

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DISCLOSURE
The authors declare that no conflict of interest exists.
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FIGURE LEGENDS

Figure 1. Cardiac morphology of Tnnt2-rtTA; TetO-Cre; Tbx20^WT^ mutant embryos and Tnnt2-rtTA; TetO-Cre; Tbx20^+/−^ 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: atrium; V: ventricle; RV: right ventricle; LV: left ventricle; RA: right atrium; LA: left atrium; OFT: outflow tract. Dotted line indicates outline of atria as labeled. Scale bars: left panels 1mm; other panels 0.2mm.

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, left atrium, right ventricle and left ventricle in Tbx20 cKO and control hearts. *, P<0.05 Mann-Whitney U test; n = 3 biological replicates. RA: right atrium; LA: left atrium; RV: right ventricle; LV: left ventricle; AU: arbitrary units. Scale bars: 0.2mm (A-H).

Figure 3. Proliferation and cell cycle analysis in control and Tbx20 mutant cardiomyocytes. A-D, EdU incorporation (green) along with tdTomato (red) for Tnnt2-rtTA;TetO-Cre lineage traced cardiomyocytes and DAPI (blue) for nuclei. E, Quantification of EdU incorporation in lineage traced cardiomyocytes. F, FACS 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^/−^ (mutant, outer circle) and Tnnt2-rtTA;TetO-Cre;Tbx20^+/−^ (control, inner circle) littermates. E,G: n=3 biological replicates; *, P < 0.05 based on negative binomial regression analysis. Scale bars: 0.2mm (A-D).

Figure 4. Gene ontology analysis of differentially expressed genes in E11.5 Tbx20 cKO cardiomyocytes versus 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 down-regulated genes. B, Intersection of RNA-Seq and TBX20-GFP ChIP-Seq 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.

Figure 5. TBX20 regulates cardiomyocyte proliferation genes. A, qPCR analysis of 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 E9.5 and E11.5. C, Erbb2 expression is reduced in ventricles of Tbx20 cKO at E9.5 and E11.5. Scale bars 0.2mm.

Figure 6. TBX20 regulates second heart field and cardiac development genes. A, qPCR analysis of Isl1, Bmp2 and Bmp10 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,C, Proximal border of high Isl1 expression (arrow) is expanded from distal outflow tract towards right ventricle at E11.5 in Tbx20 cKO heart. D,E, Bmp5 expression is enhanced in Tbx20 cKO atria (ventral view). F,G, Bmp2 expression is expanded to atria in Tbx20 cKO heart (arrows). H,I, Bmp10 is overexpressed in right atrium of Tbx20 cKO heart (arrow); B-E,H,I ventral view; F,G dorsal view; Scale bars 0.2mm.

Figure 7. TBX20 regulates chamber identity genes. A, COUP-TFII expression is downregulated in Tbx20 cKO hearts compared to 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, qPCR analysis 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 analysis of Mlc2v gene expression in E9.5 and E11.5 hearts.

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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). Scale bars 0.2 mm.

**Figure 8.** TBX20 binds and regulates an enhancer upstream of COUP-TFI that drives expression in atrial cardiomyocytes. 

**A**, TBX20-GFP ChIP-Seq of E11.5 mouse hearts in COUP-TFI genomic region. 

**B**, Magnification of enhancer 1 and schematic representation of reporter construct. 

**C**, COUP-TFI enh1::lacZ embryo showing reporter gene expression in developing atrial myocardium (arrow) and venous inflow region (arrowhead). 

**D**, Section analysis of COUP-TFI enh1::lacZ transgenic embryo demonstrates X-gal staining in developing atrial myocardium (arrows) and caval vein (arrowheads). 

**E**, COUP-TFI enh1-mutTBE::lacZ embryo showing loss of staining in atrial region, and sustained expression in venous inflow region (arrowhead). 

**F**, COUP-TFI 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 and Fgf10. In Atria, TBX20 contributes to atrial specification by suppressing ventricular and atrioventricular canal genes Bmp2 and Bmp10, while activating COUP-TFI 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. Scale bars: 0.2mm, except for left panels in C, E: 1mm.
NOVELTY AND SIGNIFICANCE

What Is Known?

- Mutations in the t-box transcription factor TBX20 are associated with a diverse range of congenital heart defects including septal defects and left ventricular non-compaction.

- 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 mid-gestation 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 non-compaction. 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.
FIGURE 3

A. EdU tdTom DAPI
B. Tbx20 cKO
C. E9.5
D. E11.5
E. % EdU+ CM

F. Control Tbx20 cKO

G. Cell cycle phases (% of cells)

Control Tbx20 cKO

G1 82.1
S 77.2
G2-M 16.7

Tbx20 cKO (n=4)
(n=5)
Tbx20 Is Required in Mid-Gestation Cardiomyocytes and Plays a Central Role in Atrial Development

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Supplemental material

Supplemental methods

Histology
Embryos were isolated in cold PBS and tissue was fixed in 4% paraformaldehyde at 4°C overnight. Tissue was embedded in paraplast for histology or in 20% Sucrose:OCT (1:1) for immunostaining (1) using Cleaved caspase-3 (Cell Signaling; 9664; 1:200) and ACTN2 (Sigma Aldrich; A7811; 1:200) antibodies. Secondary antibody control images are not included in the manuscript as these commercially available antibodies were previously validated in our lab and are broadly applied for immunostaining within the field. EdU incorporation was detected using Click-iT® EdU (Molecular probes; C10337).

Cardiomyocyte isolation
Hearts were harvested and dissociated as described previously (2) using enzymatic digestion by collagenase (10mg/ml; Worthington) and dispase (10mg/mL, Invitrogen). Live Tnt2-rtTA;TetO-Cre;R26-ttdTom lineage traced cells were sorted on Influx Cell sorter (BD Biosciences) and collected in TRIzol reagent (Ambion) for RNA extraction.

RNA extraction, RNA-Seq and qRT-PCR
RNA was extracted from ~10,000 FACS-sorted E11.5 cardiomyocytes using TRIzol. TruSeq mRNA stranded libraries (Illumina) were generated from 25 ng total RNA (four Tbx20 cKO and four control biological replicates). RNA-Seq data is available at https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-5596/. TBX20-GFP ChIP-Seq data have been previously published and are available at https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-3967/ (2). After cDNA generation by SuperScript VILO (invitrogen), qRT-PCR was performed using FastStart SYBR Green Master Mix (Roche) on a Bio-Rad CFX96 Real-Time PCR system.

RNA in situ hybridization
Embryos were collected in PBS, fixed overnight in 4% paraformaldehyde, and dehydrated using methanol. Whole-mount in situ hybridization was performed as previously described with probes targeting RNA of the following genes: mouse Isl1, Mycn, Erbb2, Bmp2, Bmp10, Tbx5, Gja5, Nkx2.5, COUP-TFII, Hey1, Hey2, Irx4, MLC2a and MLC2v (3,4).

COUP-TFII in vivo transgenic reporter assays.
TBX20 bound regions near COUP-TFII (Chr7: 77,641,240-77,642,549; COUP-TFII Enh1) Chr7: 77,411,862 – 77,412,563; no enhancer activity) were cloned into Hsp68-LacZ (5). To generate COUP-TFII Enh1-mutTBE::lacZ reporter mice, the conserved T-box binding site sequence TTTTCCCCACTC was mutated to AAAAATAAAAAAA. Mouse in vivo transgenic reporter assays were performed by pronuclear injection as reported before (6). At least 8 transgenic embryos were analyzed for each construct, and representative examples are shown. Embryos were harvested at E11.5 and subjected to X-gal staining.
**RNA-Seq analysis**

Illumina sequencing reads were aligned to the mouse genome (mm9) with STAR 2.4.0j (7). The number of reads in RefSeq genes (8) was computed. Raw read counts were quantile normalized and transformed using the `voom` function of the `limma` software package (3.26.8) (9). A design containing wild-type and mutant conditions, along with the batch information (Supplemental Table 2) was given to `limma` to identify differentially expressed genes. An adjusted P-value of 0.1 and minimum fold-change of 1.2 were used as cutoffs.

**Gene ontology analysis**

The height of the bars indicate the difference of Gene Ontology (10) enrichment for each category between up- and down-regulated genes. The enrichment in each category was calculated as number of enriched Gene Ontology terms in a given category (>1.5 fold-difference, adjusted P-value < 0.01) X mean fold-enrichment of terms in the category. The terms in each category were manually assigned.

**Transcription factor motif discovery**

Overrepresented transcription factor binding motifs in (subsets of) TBX20-GFP ChIP-Seq peaks were identified using the `findmotifsgenome.pl` script in HOMER v4.6 (11).

**Promotor capture HiC in human cardiomyocytes.**

Human cardiomyocyte cells were generated precisely as described (12) using the induced pluripotent stem cell (iPSC) line 19101 which was generated as part of that study. Briefly, 5 million cardiomyocyte cells were crosslinked in 1% formaldehyde for 10 minutes at room temperature and then the reaction was quenched with 0.2M glycine and cells were pelleted and snap frozen in liquid nitrogen. Cell pellets were then processed using MboI as described (13) with the only modification that NEBNext adapters were used (NEB E7335S) instead of Illumina. Specifically, following the A-tailing step, T1 beads (Life Technologies 65602) containing the Hi-C fragments were resuspended in 38 μl of 1X NEB Quick Ligation reaction buffer (NEB, M2200) and 2 μl of NEB DNA Quick Ligase (NEB, M2200) was added plus 10 μl of an NEB adapter from the NEBNext kit. The reaction was mixed by pipetting, incubated at room temperature for 15 minutes and then 3 μl of USER enzyme (from the kit) was added and incubated an additional 15 minutes at 37 degrees C. The adapter-ligated Hi-C library was then cleaned as described (13). Hi-C libraries were amplified directly off of T1 beads (Life Technologies 65602) using 3 μl of beads in each 50 ul PCR reaction as follows: combine 3 μl Hi-C beads, 2 μl barcode primer (NEBNext kit), 2 μl universal primer (NEBNext kit), 25 μl Q5 Hot Start High-Fidelity 2X Master Mix (NEB M0494S) and 18 μl water. The PCR conditions were 98°C for 30 sec., followed by 6 cycles of 98°C for 10 sec., 56°C for 10 sec., 72°C for 10 sec, then 72°C for 10 minutes and final hold at 4°C. Enough PCR reactions were set up to use all of the beads (typically 16 reactions). PCR reactions were then pooled and a double Ampure bead purification was carried out to isolate fragments in the range of 300bp-600bp, after which the Hi-C library was eluted off the beads using 200 μl of water.
The promoter capture step was performed as described (14) with the following minor changes: 500 ng of the in situ Hi-C library was hybridized to 120-nucleotide custom RNA oligomers (Custom Array) targeting two to three MboI fragments per human RefSeq promoter. Post-capture PCR (eight amplification cycles) was performed on the DNA bound to the beads via biotinylated RNA using the same PCR conditions and cycling parameters as above. Final libraries were purified using Ampure beads as before and paired-end sequenced (100 bp, HiSeq4500, Illumina).

Promoter Capture in situ Hi-C data analysis
Alignment of 100 bp paired-end reads to hg19 was performed independently for each mate using bowtie2-2.2.3 with the --local option. Reads with mapping quality lower than 10 were discarded. Mates were paired by name with a custom script. Data from all individuals were pooled. HOMER v4.7.2 (11) was used to call interactions with P < 1e-5 using -res 2000 and -superRes 10000 to bin reads. Interactions were mapped to RefSeq mRNA transcription start sites based on alignment to hg19.

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Supplemental tables

**Online Table I.** Observed genotype distribution of live embryos recovered from matings between *Tnnt2-rtTA;TetO-Cre;Tbx20^{+/-loxP}* males and *Tbx20^{loxP/loxP}* females. Expected number in parentheses. Chi-square tests were performed, *P*<0.05 considered significant (*).

| Stage | No Cre             | Tnnt2-rtTA;TetO-Cre |
|-------|--------------------|---------------------|
|       | *Tbx20^{+/-loxP}*  | *Tbx20^{loxP/loxP}*  |
|       | *Tbx20^{+/-loxP}*  | *Tbx20^{loxP/loxP}*  |
|       | 27 (27.5)          | 20 (20.75)          |
| E9.5  | 32 (27.5)          | 31 (27.5)           |
| P=0.35|                   |                     |
| E10.5 | 27 (33)            | 33 (33)             |
|       | 42 (33)            | 30 (33)             |
| P=0.28|                   |                     |
| E11.5 | 149 (133)          | 128 (133)           |
|       | 124 (133)          | 132 (133)           |
| P=0.44|                   |                     |
| E12.5 | 7 (6.25)           | 8 (6.25)            |
|       | 3 (6.25)           | 7 (6.25)            |
| P=0.50|                   |                     |
| E13.5 | 14 (10)            | 8 (10)              |
|       | 13 (10)            | 5 (10)              |
| P=0.14|                   |                     |
| E14.5 | 9 (4.25)           | 3 (4.25)            |
|       | 4 (4.25)           | 1 (4.25)            |
| P=0.042|                  |                     |

**Online Table II.** Batch information for all RNA-Seq samples based on the date of cell sorting.

| Sample   | Genotype                         | Condition | Batch |
|----------|----------------------------------|-----------|-------|
| Tbx20-KO1| *Tnnt2-rtTA; TetO-Cre;Tbx20^{+/-loxTom}* | ko        | A     |
| Tbx20-KO2| *Tnnt2-rtTA; TetO-Cre;Tbx20^{+/-loxTom}* | ko        | B     |
| Tbx20-KO3| *Tnnt2-rtTA; TetO-Cre;Tbx20^{+/-loxTom}* | ko        | C     |
| Tbx20-KO4| *Tnnt2-rtTA; TetO-Cre;Tbx20^{+/-loxTom}* | ko        | C     |
| WT1      | *Tnnt2-rtTA; TetO-Cre;Tbx20^{+/-loxTom}* | wt        | C     |
| WT2      | *Tnnt2-rtTA; TetO-Cre;Tbx20^{+/-loxTom}* | wt        | C     |
| WT3      | *Tnnt2-rtTA; TetO-Cre;Tbx20^{+/-loxTom}* | wt        | C     |
| WT4      | *Tnnt2-rtTA; TetO-Cre;Tbx20^{+/-loxTom}* | wt        | B     |

**Online Table III.** RNA-Seq of E11.5 *Tbx20 cKO* and control FACS purified cardiomyocytes. (Excel file)

**Online Table IV.** Motif analysis within TBX20-GFP ChIP-Seq peaks associated with differentially expressed genes (Excel file)
Online Table V. Edu+ cardiomyocytes in different compartments at E10.5. Total numbers of counted cells are presented, Range of Edu+ cardiomyocyte percentages measured in single biological replicate are presented in brackets.

| Location  | Edu+ cKO N=3 | Ctrl N=3 | Total Ctrl N=3 | Edu- cKO Total  |
|-----------|--------------|----------|----------------|-----------------|
| AV cushion| 33.5% (28.5%-39.7%) | 39.1% (38.2%-40%) | 35.8% (34.1%-37.5%) | 1850 1093 2545 |
| IVS       | 20.1% (18.9%-22.9%) | 27.4% (26.1%-28.3%) | 27.6% (26.1%-28.3%) | 275 654 1549 |
| LA        | 9.8% (7.7%-12.7%) | 29.8% (28.8%-30.5%) | 29.8% (28.8%-30.5%) | 162 177 2771 |
| OFT       | 14.8% (11.9%-16.8%) | 24.5% (21.9%-26.3%) | 24.5% (21.9%-26.3%) | 423 456 1406 |
| RA        | 21.3% (18.4%-24.6%) | 29.9% (28.1%-31.2%) | 29.9% (28.1%-31.2%) | 913 1684 3950 |
| RV        | 17.6% (14.1%-20.6%) | 34.1% (31.5%-35.8%) | 34.1% (31.5%-35.8%) | 962 2865 5538 |

Online Table VI. Post hoc analysis of Edu+ cardiomyocytes in different compartments at E10.5. df, degrees of freedom.

| Pairwise Comparisons | Mean Difference (Control-cKO) EdU+ cells (%) | Std. Error | df | Bonferroni Sig. | 95% Wald Confidence Interval for Difference |
|----------------------|---------------------------------------------|-------------|----|----------------|-------------------------------------------|
| AV cushion           | 5.38                                        | 3.076       | 1  | 1.000          | -4.98                                     | 15.74                                     |
| IVS                  | 7.26                                        | 0.918       | 1  | 0.000          | 4.17                                      | 10.35                                     |
| LA                   | 20.06                                       | 1.358       | 1  | 0.000          | 15.49                                     | 24.64                                     |
| OFT                  | 9.65                                        | 1.323       | 1  | 0.000          | 5.19                                      | 14.10                                     |
| RA                   | 8.66                                        | 1.834       | 1  | 0.000          | 2.48                                      | 14.84                                     |
| RV                   | 16.6                                        | 2.131       | 1  | 0.000          | 9.43                                      | 23.78                                     |
**Online Table VII.** Distribution of cell counts in different cell cycle phases.

| Group                     | Cell Cycle Phase | Total |
|---------------------------|-----------------|-------|
|                           | G1   | S       | G2M  |
| Lineage traced            |      |         |      |
| Control N=5               | 7555 | 1634    | 599  | 9788 |
| % within Tracing          | 77.2%| 16.7%   | 6.1% | 100.0% |
| Range                     | 74.9%-82.5%    | 13.6%-18.0% | 3.9%-8.4% |
| Tbx20 cKO N=4             | 5310 | 686     | 470  | 6466 |
| % within Tracing          | 82.1%| 10.6%   | 7.3% | 100.0% |
| Range                     | 81.3%-84.1%    | 6.9%-12.4% | 6.1%-9.2% |
| Non Lineage               |      |         |      |
| Control N=5               | 13087| 3976    | 828  | 17891 |
| % within Tracing          | 73.1%| 22.2%   | 4.6% | 100.0% |
| Range                     | 71.2%-75.4%    | 18.1%-23.2% | 2.5%-7.8% |
| Tbx20 cKO N=4             | 12823| 3789    | 764  | 17376 |
| % within Tracing          | 73.8%| 21.8%   | 4.4% | 100.0% |
| Range                     | 70.3%-83.6%    | 11.8%-24.6% | 3.6%-5.1% |

**Online Table VIII.** Post hoc analysis of Cell cycle of lineage traced cardiomyocytes.

| Mean Difference (Control-cKO) EdU+ cells (%) | Std. Error | df | Bonferroni Sig. | 95% Wald Confidence Interval for Difference |
|---------------------------------------------|------------|----|-----------------|-------------------------------------------|
| G1                                          | -4.95      | 1.447 | 1    | 0.009 | -9.20 | -0.71 |
| S                                           | 6.09       | 1.321 | 1    | 0.000 | 2.21  | 9.97  |
| G2M                                         | -1.21      | 0.797 | 1    | 1.000 | -3.55 | 1.13  |
### Online Table IX. Numbers of Edu+ cardiomyocytes at different timepoints. Total numbers of counted cells are presented, Range of Edu+ cardiomyocyte percentages measured in single biological replicate are presented in brackets

| Labeling | Timepoint | E9.5  | E10.5 | E11.5 | Total  |
|----------|-----------|-------|-------|-------|--------|
| Edu+     | cKO       | N=3   |       |       |        |
|          |           |       | 1350  | 4549  | 8465   | 14364  |
|          |           |       | 18.3% | 18.1% | 18.2%  |        |
|          |           |       | (15%- 21.6%) | (15.4%- 20.9%) | (16.5%- 16.5%) |        |
| Control  | N=3       |       | 2942  | 10282 | 16349  | 29573  |
|          |           |       | 27.3% | 29.3% | 21.9%  |        |
|          |           |       | (29.7%-28.9%) | (31.6%-30.4%) | (26%-24.2%) |        |
| Total    |           |       | 4292  | 14831 | 24814  | 43937  |
| Edu-     | cKO       |       | 6037  | 20559 | 38087  | 64683  |
|          | control   |       | 7225  | 23543 | 51341  | 82109  |
| Total    |           |       | 13262 | 44102 | 89428  | 146792 |
| Total cKO|           |       | 7387  | 25108 | 46552  | 79047  |
| Control  |           |       | 10167 | 33825 | 67690  | 111682 |
| Total    |           |       | 17554 | 58933 | 114242 | 190729 |

### Online Table X. Post hoc analysis of Edu+ CMs at different timepoints.

| Pairwise Comparisons | Mean Difference (control-cKO) EdU+ cells (%) | Std. Error | df | Bonferroni Sig. | 95% Wald Confidence Interval for Difference |
|----------------------|---------------------------------------------|------------|----|----------------|-------------------------------------------|
|                      |                                              |            |    |                | Lower         | Upper         |
| E9.5                 | 9.95                                        | 1.931      | 1  | 0.000          | 4.28          | 15.62         |
| E10.5                | 12.67                                       | 1.488      | 1  | 0.000          | 8.30          | 17.04         |
| E11.5                | 5.7                                         | 1.440      | 1  | 0.001          | 1.47          | 9.92          |
Online Figure I. *Tnnt2-rtTA; TetO-Cre* ablates *Tbx20* efficiently in mid-gestation cardiomyocytes. A, *Tnnt2-rtTA; TetO-Cre* expression is induced from E8.5 onwards by providing Doxycycline in drinking water to pregnant females. B, *tdTomato* fluorescence on sections of E9.5 *Tnnt2-rtTA; TetO-Cre; R26*+/tdTom hearts indicates cre activity throughout the primitive heart tube following induction at E8.5. C, At E11.5, *tdTomato* fluorescence marks the vast majority of alpha sarcomeric actinin (SAA) expressing cardiomyocytes, whereas cardiac cushion cells are not lineage traced. D, High magnification of left ventricular free wall (E11.5) showing co-localization of *tdTomato* fluorescence and SAA staining, and absence of *tdTomato* fluorescence in SAA negative epicardial cells (arrows) and endocardial cells (arrowheads). E, quantification of *Tbx20* expression levels in E9.5 hearts, E11.5 hearts and in E11.5 FACS purified cardiomyocytes. Scalebars 200 μm (A,B), 20 μm (C). *, P<0.05 Mann-Whitney U test; n=4-5 biological replicates.
Online Figure II. Quantification of proliferation in E10.5 cardiomyocytes in different compartments. EdU incorporation was quantified in Tnnt2-rtTA;TetO-Cre lineage traced control and Tbx20 cKO cardiomyocytes (CM), except for AVC where non-cardiomyocytes were assayed. EdU incorporation was also quantified in atrioventricular cushion (AVC) cells as internal control. *, $P<0.05$ by negative binomial regression analysis; $n=3$ biological replicates. LA: left atrium; RA: right atrium; LV: left ventricle; RV: right ventricle; OFT: outflow tract; AVC: non-cardiomyocytes within atrioventricular cushion.
Online Figure III. Flowchart of RNA-Seq experiment and analysis, and gene ontology analysis of intersection of RNA-Seq and TBX20-GFP ChIP-Seq in E11.5 hearts. A, To identify potential TBX20 direct targets that were differentially expressed in Tbx20 cKO hearts, lineage traced cardiomyocytes (CM) were FACS purified from E11.5 Tbx20 cKO hearts and stage matched controls (n=4 for each condition). RNA was isolated and quality checked using Bioanalyzer (RNA integrity value > 9) prior to library generation. Differentially expressed genes were called as described in supplementary methods, using a 1.2x fold change and adjusted p-value of 0.1 as threshold. Gene ontology analysis was then performed to identify biological processes that were most enriched upon differentially expressed genes. Predicted direct targets based on TBX20-GFP ChIP-Seq were selected and candidate direct target genes that might underlie the mutant phenotype were selected for further validation using qRT-PCR, in situ hybridization and analysis of enhancer activity. B, Gene ontology analysis of differentially expressed genes in Tbx20 cKO cardiomyocytes that are predicted direct targets based on TBX20-GFP ChIP-Seq in E11.5 hearts clustered into functional categories (green: underexpressed; red: overexpressed). Length of bars indicate the difference of Gene Ontology enrichment for each category between up- and down-regulated genes.
**Online Figure IV.** Expression of Fgf10, Gja5, Nkx2-5, Tbx5, Tbx2 and Pitx2 in control and Tbx20 cKO mutant hearts. A-D, Changes in Fgf10 expression in E9.5 heart could not be detected in Tbx20 cKO compared to controls. E,G, Gja5 expression marks developing chambers, and is similar between Tbx20 cKO and control hearts. F,H, Nkx2-5 expression is similar between Tbx20 cKO and control hearts. I-L, Tbx5 expression in E9.5 control and Tbx20 cKO hearts was similar. M, qPCR assessment of Fgf10 and Tbx2 expression in Tbx20 cKO hearts and controls at indicated stages (n=3-4 biological replicates; *, P<0.05 Mann-Whitney U test). N-Q, Pitx2 expression was comparable between control and Tbx20 cKO hearts at E11.5. Scalebars 0.2mm.
Online Figure V. TBX20 and COUP-TFII share a group of target genes. Intersection of TBX20 ChIP-Seq from E11.5 heart, COUP-TFII ChIP-Seq from E14.5 atria and RNA-Seq of Tbx20 cKO mutant and control hearts reveals a pool of genes that are putatively regulated by TBX20, COUP-TFII or through regulatory interactions between these. Several key target genes are highlighted, underlined genes are differentially expressed in the (COUP-TFII negative) ventricles, genes marked by an asterisk were marked by TBX20 and COUP-TFII binding to the same genomic region.
Online Figure VI. Mouse COUP-TFII enhancer bound by TBX20 is conserved between mouse and human, interacts with NR2F2 (COUP-TFII) promoter in human cardiomyocytes and displays enhancer signature in human fetal hearts. A) Interaction map from NR2F2 in human cardiomyocytes. B) Alignment of mouse TBX20 binding regions (arrows) with human COUP-TFII (NR2F2) genomic region and epigenome tracks from human fetal hearts including DNAse hypersensitivity and histone modifications.
Long In Vivo Checklist

Circulation Research - Preclinical Animal Testing: A detailed checklist has been developed as a prerequisite for every publication involving preclinical studies in animal models. Checklist items must be clearly described in the manuscript; if the answer to a question is "No", an explanation should be provided both within the manuscript text and on the following screen. If this information (checklist items and/or explanations) cannot be included in the main manuscript because of space limitations, please include it in an online supplement. If the manuscript is accepted, this checklist will be published as an online supplement. See the explanatory editorial for further information.

This study involves use of animal models: Yes

Study Design

The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. Yes

An overall study timeline is provided. Yes

The protocol was prospectively written N/A

The primary and secondary endpoints are specified N/A

For primary endpoints, a description is provided as to how the type I error multiplicity issue was addressed (e.g., correction for multiple comparisons was or was not used and why). (Note: correction for multiple comparisons is not necessary if the study was exploratory or hypothesis-generating in nature). N/A

A description of the control group is provided including whether it matched the treated groups. N/A

Inclusion and Exclusion criteria

Inclusion and exclusion criteria for enrollment into the study were defined and are reported in the manuscript. N/A

These criteria were set a priori (before commencing the study). N/A

Randomization

Animals were randomly assigned to the experimental groups. If random assignment was not used, adequate explanation has been provided. N/A

Type and methods of randomization have been described. N/A

Allocation concealment was used. N/A

Methods used for allocation concealment have been reported. N/A

Blinding

Blinding procedures with regard to masking of group/treatment assignment from the experimenter were used and are described. The rationale for nonblinding of the experimenter has been provided, if such was not performed. N/A

Blinding procedures with regard to masking of group assignment during outcome assessment were used and are described. N/A

If blinding was not performed, the rationale for nonblinding of the person(s) analyzing outcome has been provided. N/A

Sample size and power calculations
Formal sample size and power calculations were conducted before commencing the study based on a priori determined outcome(s) and treatment effect(s), and the data are reported.

If formal sample size and power calculation was not conducted, a rationale has been provided.

Data Reporting

Baseline characteristics (species, sex, age, strain, chow, bedding, and source) of animals are reported.

The number of animals in each group that were randomized, tested, and excluded and that died is reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided is provided for all experimental groups.

Baseline data on assessed outcome(s) for all experimental groups are reported.

Details on important adverse events and death of animals during the course of the experiment are reported for all experimental groups.

Numeric data on outcomes are provided in the text or in a tabular format in the main article or as supplementary tables, in addition to the figures.

To the extent possible, data are reported as dot plots as opposed to bar graphs, especially for small sample size groups.

In the online Supplemental Material, methods are described in sufficient detail to enable full replication of the study.

Statistical methods

The statistical methods used for each data set are described.

For each statistical test, the effect size with its standard error and \( P \) value is presented. Authors are encouraged to provide 95\% confidence intervals for important comparisons.

Central tendency and dispersion of the data are examined, particularly for small data sets.

Nonparametric tests are used for data that are not normally distributed.

Two-sided \( P \) values are used.

In studies that are not exploratory or hypothesis-generating in nature, corrections for multiple hypotheses testing and multiple comparisons are performed.

In “negative” studies or null findings, the probability of a type II error is reported.

Experimental details, ethics, and funding statements

Details on experimentation including formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring are described.

Both male and female animals have been used. If not, the reason/justification is provided.

Statements on approval by ethics boards and ethical conduct of studies are provided.

Statements on funding and conflicts of interests are provided.