Hand2 Is an Essential Regulator for Two Notch-Dependent Functions within the Embryonic Endocardium

Graphical Abstract

Highlights
- Hand2 endocardial ablation results in tricuspid atresia
- Hand2 mediates Notch and Ephrin signaling in the heart
- Hand2 modulates coronary development via regulation of Vegf signaling

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In Brief
VanDusen et al. demonstrate that the bHLH transcription factor Hand2 is a critical mediator of Notch signaling within the embryonic endocardium regulating trabeculation, septation, and coronary formation. Endocardial loss of Hand2 causes tricuspid atresia, multiple ventricular septa, and hypervascularized myocardium, establishing Hand2-dependent endocardial-myocardial signaling as essential for cardiac morphogenesis.
Hand2 Is an Essential Regulator for Two Notch-Dependent Functions within the Embryonic Endocardium

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SUMMARY

The basic-helix-loop-helix (bHLH) transcription factor Hand2 plays critical roles during cardiac morphogenesis via expression and function within myocardial, neural crest, and epicardial cell populations. Here, we show that Hand2 plays two essential Notch-dependent roles within the endocardium. Endocardial ablation of Hand2 results in failure to develop a patent tricuspid valve, intraventricular septum defects, and hypotrabeculated ventricles, which collectively resemble the human congenital defect tricuspid atresia. We show endocardial Hand2 to be an integral downstream component of a Notch endocardium-to-myocardium signaling pathway and a direct transcriptional effector of endocardial Notch signaling via direct transcriptional regulation of the secreted factor Neuregulin1 (Nrg1). Additionally, Hand2 participates in endocardium-to-endocardium-based cell signaling, with Hand2 mutant hearts displaying an increased density of coronary lumens. Molecular analyses further reveal dysregulation of several crucial components of Vegf signaling, including VegfA, Vegfr2, Nrp1, and Vegfr3. Thus, Hand2 functions as a crucial downstream transcriptional effector of endocardial Notch signaling during both cardiogenesis and coronary vasculogenesis.

INTRODUCTION

In the primitive heart, communication between the endocardium, which is the endothelium-like tissue that lines the heart, and the myocardium, which is the muscular heart tissue, is essential for processes central to normal cardiac morphogenesis, including trabeculation, chamber septation, and coronary vasculogenesis (Bruneau, 2003; Brutsaert, 2003). The transmembrane receptor Notch1 is integral to this communication, as endocardial Notch activates endocardial EphrinB2, which through unknown mechanisms activates expression of the secreted factor Neurgulin1 (Nrg1). Nrg1 initiates the trabeculation process, and Notch signaling subsequently activates expression of Bmp10 (Chen et al., 2004) through a separate pathway (Grego-Bessa et al., 2007). Bmp10 expression stimulates proper proliferation of trabecular, and possibly septal, myocardium. Disruption of Notch-mediated endocardial-myocardial communication results in congenital heart defects (CHDs), which are the most frequent human developmental anomalies (Hoffman, 1995).

Tricuspid atresia (TA) is a CHD characterized by the lack of a direct connection between the right atria (RA) and the right ventricle (RV), requiring both ventricular (VSD) and atrial (ASD) septal defects for embryonic survival (Anderson et al., 1979). Conditional ablation of the basic-helix-loop-helix (bHLH) factor Hand2 within the second heart field (SHF) via Mef2c-Cre results in TA (Tsuchihashi et al., 2011). Mef2c-Cre marks a pool of SHF progenitor cells that contribute to both the myocardium and endocardium (Tsuchihashi et al., 2011; Verzi et al., 2005), and SHF ablation of Hand2 causes TA via unknown mechanisms. As cardiomyocyte-specific ablation of Hand2 does not result in TA (Tsuchihashi et al., 2011), we hypothesized that loss of Hand2 function within the endocardium is causative of TA. Indeed, our data reveal that either endothelial or endocardial-specific deletion of Hand2 (H2CKO) using either Tie2-Cre or Nfatc1Cre, respectively, results in TA. These data show that Hand2 functions downstream of endocardial Notch to mediate endocardium-to-myocardium signaling via direct transcriptional regulation of the growth factor Neuregulin1 (Nrg1).

As cardiogenesis proceeds, development of the coronary vasculature allows for oxygenation of the thickening ventricular compact zone. Cardiac endothelial cells form the primitive coronary network by angiogenesis (Red-Horse et al., 2010; Wu et al., 2012), a process that extracardiac models demonstrate to be Notch dependent. Early embryonic lethality in Notch pathway mutants has precluded robust analysis of Notch signaling in
coronary development. Significantly, H2CKO mice survive long enough to assess the initiation of coronary vasculization, and our findings indicate that Hand2 modulates coronary development through regulation of multiple Vegf signaling components within the developing heart. Collectively, these data support a model whereby Notch signaling, via endocardial Hand2 function, regulates trabeculation, interventricular septum (IVS) formation, and coronary development.

RESULTS

Endothelial/Endocardial Loss of Hand2 Causes Trabeculation and Septation Defects that Resemble Tricuspid Atresia

To test the hypothesis that loss of endocardial Hand2 results in TA, we intercrossed the endothelial cell-specific Tie2-Cre allele (Kisanuki et al., 2001) with mice carrying the Hand2 conditional allele (Hand2<sup>flx/flx</sup>). Tie2-Cre is expressed in all endothelial cells, including cells of the ventricular endocardium, coronary endothelium, and atrioventricular cushions (Kisanuki et al., 2001). Tie2-Cre expression initiates at embryonic day 8.5 (E8.5) (Kisanuki et al., 2001), which is concurrent with the onset of endocardial Hand2 expression (Barnes et al., 2011). Hand2<sup>flx/flx</sup>;R26<sup>LacZ</sup> females were mated with Tie2-Cre(+) males, and neonates were genotyped. No H2CKO mice were observed (n = 58; Table S1). We next set up timed matings and observed that Tie2-Cre(+)Hand2<sup>flx/flx</sup> embryos die by E14.5 (Table S1). Hand2 deletion within the endocardium is evident by in situ hybridization (ISH) at E10.5 (Figures 1A and 1B, arrows). Examination of Tie2-Cre H2CKOs in whole mount (Figures 1D and 1E) and in section histology (Figures 1G and 1H) reveals hypotrabeculation (arrow in Figure 1E) and a highly penetrant TA phenotype (Figure 1J, arrow). However, outflow tract (OFT) morphogenesis occurs normally in Tie2-Cre H2CKOs (Figures S1A–S1C).

While we also observe no defects in the yolk sac vasculature of Tie2-Cre H2CKOs (Figures S1D and S1E), Hand2 may play critical roles in vascular endothelium that could contribute to the observed embryonic lethality. Therefore, we used the endothelial-specific Nfatc1<sup>Cre</sup> allele (Wu et al., 2012) to generate H2CKOs (Figures 1C, 1F, and 1N). This Nfatc1<sup>Cre</sup> allele initiates expression throughout the endocardium at E9.0 (Wu et al., 2012), as opposed to the Nfatc1<sup>endCre</sup> allele, which labels only a subset of valve endothelial cells. Nfatc1<sup>Cre</sup> H2CKOs also show dramatic defects in trabeculation, malformed IVS, and atresia of the tricuspid valve (~70%; Figures 1F and 1N). Interestingly, in some cases, IVS malformations include large protrusions of myocardium that appear to indicate the formation of multiple IVSs (Figure 1N, arrows; Table S2). Robust expression of the septal/compact zone marker Hey2 and the septal marker lnx2 in these large protrusions confirm this observation, while expression of the trabecular marker Ant1 is excluded (Figures S2A–S2H). However, expression of the LV marker Hand1 is not observed beyond the leftmost septum (Figures S2I and S2J). Tbx5, which has been suggested to induce the IVS (Takeuchi et al., 2003), is also unchanged (Figures S2K and S2L). Together, these data confirm that conditional deletion of Hand2 within the endocardium is critical for normal trabeculation and septation. Given that these phenotypes are myocardial in nature, this suggests that endocardial Hand2 plays a crucial role in endocardium-to-myocardium signaling.

Hand2 Regulates Nrg1 Expression within the Endocardium

We next investigated changes in gene expression within E10.5 Tie2-Cre H2CKOs using ISH. Expression analysis of Fog2 was of interest, as Fog2-deficient mice also display TA (Svensson et al., 2000); however, no change in Fog2 expression was observed (Figures S3A and S3B). Deletion of Tgfrb2 from the endothelium results in DILV (Jiao et al., 2006). Analysis of Tgfrb2 expression revealed no differences between H2CKO and controls (Figures S3C and S3D).

It has been directly demonstrated that endocardial Notch signaling plays an essential role in orchestrating morphogenetic changes within the underlying myocardium, particularly during the process of trabeculation (Grego-Bessa et al., 2007). Therefore, we examined Notch pathway gene expression. We first analyzed expression of the Notch-regulated bHLH transcriptional repressors and potential Hand2 dimer partners, Hey1 and Hey2 (Figures S3E–S3H). Endocardial expression is not significantly changed. Next, we examined expression of the direct Notch1 target EphrinB2 (EphB2; Grego-Bessa et al., 2007). EphB2 ISH also shows no change in expression between H2CKOs and control embryos (Figures 2A–2D). However, epidermal growth factor family member Neuregulin1 (Nrg1) is markedly downregulated at E10.5 within the endocardium of H2CKOs (Figure 2F, arrow in Figure 2H) compared to control embryos (Figure 2E, arrow in Figure 2G). Nrg1 is known to be an essential mediator of trabeculation in the developing ventricles and is downregulated in Tie2-Cre Notch1CKOs, and EphB2 systemic null embryos (Grego-Bessa et al., 2007). The maintenance of EphB2 and loss of Nrg1 in H2CKOs suggests that Hand2 acts downstream of EphB2, but upstream of Nrg1, representing a novel step in Notch1 trabeculation and septation signaling.

We next analyzed Bmp10 expression, as myocardially expressed Bmp10 is crucial for proper trabeculation and is independently downstream of Notch1 signaling (Chen et al., 2004; Grego-Bessa et al., 2007). BMP10 ISH at E10.5 suggests a subtle downregulation in H2CKOs (Figures S3I and S3J) while E12.5 ISH shows a near complete loss of Bmp10-expressing trabecular tissue within the RV but not LV (Figures 2I and 2J, arrows).

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To quantitatively assess Bmp10 expression, we dissected ventricles from E10.5 hearts and isolated RNA for quantitative RT-PCR (qRT-PCR). As expected, qRT-PCR analysis confirms that both Hand2 and Nrg1 are significantly downregulated in H2CKOs (Figure 2K) but Bmp10 myocardial expression is not altered (Figure 2K). Thus, the reduction of Bmp10 within the RV of E12.5 H2CKOs reflects the absence of RV trabeculation, as LV trabeculae express Bmp10 (Figure 2J).

Endocardial Hand2 Functions Downstream of Notch1 and the Direct Notch1 Target EfnB2

Upon interaction with one of its transmembrane ligands, the transmembrane receptor Notch1 is proteolytically cleaved to generate the Notch1 intracellular domain (N1ICD). N1ICD translocates to the nucleus, where it dimerizes with its partner RBPIJK to activate transcription of target genes. Previous studies have shown that deletion of Notch1 or RBΠJk results in hypotrabeculation due to loss of EfnB2 and Nrg1 (Grego-Bessa et al., 2007). To confirm that Hand2 lies within the Notch1 signaling pathway, we assayed Hand2 expression in E9.5 RBΠJk/−/− embryos. Whole mount analyses reveal a loss of endocardial Hand2, while expression within the OFT and pharyngeal mesenchyme is unaffected (Figures 3A and 3B). Sectioned embryos confirm a specific loss of Hand2 within mutant endocardium (Figures 3C and 3D), definitively establishing Hand2 as a Notch1 signaling effector.
During trabeculation, N1ICD/RBPJk directly transactivates EfnB2, which acts through its EphB2/EphB4 tyrosine kinase receptors to upregulate Nrg1 (Grego-Bessa et al., 2007). While our data clearly show Hand2 to be upstream of Nrg1, it was not clear if Hand2 lies downstream of EphrinB2 signaling or if Hand2 represents a parallel EfnB2-independent Notch signaling pathway. To address this question, we assayed Hand2 expression in E9.5 Tie2-Cre(+);EfnB2fx/fx embryos (Gerety and Anderson, 2002). ISH reveals that Hand2 is downregulated in endocardial cells of Tie2-Cre(+);EfnB2fx/fx embryos (Figure 3F, arrow), whereas Hand2 expression in pharyngeal mesenchyme and the proepicardial organ is unaffected when compared to control hearts (Figure 3E). qRT-PCR analysis at E10.5 confirms Hand2 downregulation within Tie2-Cre(+);EfnB2fx/fx-isolated ventricles (Figure 3G). Together, these data show that Hand2 is a Notch-dependent endocardial factor positioned between EfnB2 and Nrg1.

Hand2 Regulation of Nrg1 Is Direct via Interaction with the Nrg1 Promoter and Upstream Enhancer Sequences

As Hand2 encodes a transcription factor with a similar expression profile to Nrg1, we sought to determine if Hand2 regulation of Nrg1 is direct. An 850 bp region of the Nrg1 promoter has been identified as necessary for high Nrg1 transcriptional activity in vitro (Frensing et al., 2008). As Hand2 binds the consensus sequence CANNTG, termed an E-box, or alternatively CGNNTG, a D-box (Firulli et al., 2007), we searched this aligned promoter region for these conserved cis elements. Three were found within the 500 bp directly upstream of the Nrg1 translation start site (Figure 4A). To assess Hand2 interaction with this region of the Nrg1 promoter, we conducted chromatin immunoprecipitation (ChIP) assays in HeLa cells, employing a Myc-tagged Hand2 co-transfected with a plasmid encoding an untagged Hand2 dimerization partner, E12. Negative controls included Myc-Hand2 + E12 immunoprecipitated without a Myc, and pCS2+Myc samples immunoprecipitated with a Myc. Enrichment of the Nrg1 promoter region was assessed by PCR using primers corresponding to a 103 bp region of the human Nrg1 promoter. This amplicon shows robust enrichment within Myc-Hand2 + E12 immunoprecipitated samples (Figure 4B). To confirm that Hand2-E12 heterodimers are capable of binding the conserved consensus E/D-box sequences, double-stranded oligos corresponding to these sites were used in electrophoretic mobility shift assays (EMSA) with in vitro-translated Hand2 and E12 (Figure 4C). Hand2-E12 heterodimers specifically bind oligos corresponding to sites 1 and 3 (Figures 4D and 4E). No DNA binding is observed between Hand2-E12 complexes and site 2. To test if Hand2 is capable of transactivating the Nrg1 promoter, Hand2 and E12 expression constructs were transfected into HeLa cells along with a Nrg1 luciferase reporter containing the 1,000 bp directly upstream of the murine Nrg1 translation start site. This region contains multiple transcription start sites located between −395 and −425 (Frensing et al., 2008). Co-transfection of Hand2 and E12 resulted in 7-fold reporter activation. Subsequent assays utilizing truncated promoters (−500/0 bp and −500/−250 bp) demonstrated more robust activation
Hand2 is both necessary and sufficient for regulation of E12 when cotransfected with E12 (Figure 4G). Transactivation data from the 500 bp promoter revealed that E12 alone is not sufficient for activation, while Hand2 alone results in only modest (but significant) 3.5-fold transactivation. Consistent with EMSA results, cotransfection of Hand2 and E12 results in 18-fold transactivation alone or when cotransfected with E12 (Figure 4G).

Replacement of Hand2 within EfnB2-Deficient Endocardium Results in an Improvement of Cardiac Trabeculation

EfnB2 mutant embryos display hypotrabeculation accompanied by loss of Hand2 and Nrg1 expression (Figure 4). Therefore, if Hand2 is both necessary and sufficient for regulation of Nrg1, we reasoned that Hand2 replacement in Nfatc1Cre EfnB2CKOs would restore Nrg1 expression and improve ventricular trabeculation. To test this, we generated the Cre-activatable Hand2 transgene CAG-CAT-Hand2 (CC-H2; Figure S4A). Ectopic expression of Hand2 in limb mesenchyme results in preaxial polydactyly (McFadden et al., 2002). As predicted, Prx1-Cre-mediated activation of CC-H2 within the limb results in polydactyly, indicating that the conditional transgene can be efficiently and specifically activated (Figure S4B). Similarly, Nfatc1Cre efficiently activates CC-H2 in the endocardium (Figure S4C). E13.5 Nfatc1Cre, CC-H2(+/+) embryos do not display any obvious cardiac phenotypes (data not shown). Subsequently, CC-H2(+/+); EfnB22tm females were then crossed with Nfatc1Cre; EfnB22tm males to generate Nfatc1Cre; CC-H2(+/+); EfnB2 CKOs. CC-H2(−/−); EfnB2 CKOs die by E11.5, with severe pericardial edema, hemorrhaging, and defects in cardiac looping and chamber development (Figure 5C). These phenotypes closely resemble the defects observed in Tie2-Cre EfnB2 CKOs (Gerety and Anderson, 2002), indicating that while EfnB2 function within extracardiac vasculature is likely important, loss of endocardial EfnB2 is sufficient to cause midgestation lethality. Hand2 ISH at E10.5 confirms robust endocardial Hand2 expression in controls (Figures 5E and 5F) and loss of Hand2 in EfnB2 CKOs (Figure 5G, arrow). The presence of the CC-H2 allele restores Hand2 expression within the EfnB2 CKOs (Figure 5H, arrow; n = 3). Nrg1 ISH reveals robust expression in controls (Figures 5I and 5J), and loss of Nrg1 expression in EfnB2 CKOs (Figure 5K, arrow). CC-H2(+); EfnB2 CKOs display a significant increase in Nrg1 expression (Figure 5L, arrow), while assessment of trabeculation by Bmp10 ISH reveals a marked improvement in Bmp10-expressing trabecular myocardium (Figure 5P, arrow) when compared to EfnB2 CKOs lacking the CC-H2 allele (Figure 5O, arrow). Expression analysis of isolated E10.5 ventricles by qRT-PCR demonstrates that while still well below control levels, Nrg1 expression in EfnB2 mutants that carry the CC-H2 allele (n = 4) is twice as high as in EfnB2 mutants lacking the CC-H2 allele (upregulated 112%). Bmp10 expression is increased by 79% with obvious improvement in trabeculation (Figure 5Q).

Notch-Dependent Hand2 Function Also Regulates Coronary Angiogenesis

The above analyses demonstrate that endocardial Hand2 plays a crucial role in myocardial morphogenesis. Myocardial trabeculation and compaction are intimately linked to endothelial cell behavior during development of the coronary vasculature by a complex signaling network that includes Vegf, Fgf, Tgf-β, and Notch components (Smart et al., 2005). Furthermore, early
embryonic lethality in genetic models of dysfunctional Notch signaling has precluded an in vivo assessment of the role of Notch signaling in formation of the coronary arteries. As we have established Hand2 as an integral component of endocardial Notch signaling, and as coronary endothelium is derived at least in part from the endocardium (Wu et al., 2012), the survival of H2CKOs to E14.5 allows us a unique opportunity to assess Notch function in early coronary development. Analysis of hearts at E13.5, 1 day after initiation of intramyocardial coronary formation (Tian et al., 2013), shows that Nfatc1Cre H2CKO hearts (Figures 6B and 6D, arrows) exhibit an increased density of primitive coronary vessels when compared to control ventricles (Figures 6A and 6C). Subsequent analyses revealed a comparable degree of hypervascularization in Tie2-Cre H2CKOs (data not shown; Table S2). To ascertain the mechanism underlying this hypervascularization, we analyzed genes associated with vascular development in E10.5 and E13.5 Nfatc1Cre H2CKO isolated ventricles by qRT-PCR (n = 4; Figures 6E and 6F, respectively). As expected, expression levels of Hand2 and Nrg1 are down at both time points. Analysis of vascular markers at E10.5 reveals dysregulation of select components of Vegf signaling (Figure 6E). VegfR2 is downregulated by 30%, and its
coreceptor Nrp1 is downregulated by 14%. VegfD, which encodes a Vegfr3-specific ligand, is downregulated by 25%. By E13.5, expression levels of Vegfr2 and VegfD have recovered (Figure 6F), whereas Nrp1 remains downregulated. Surprisingly, expression of Vegfa, which encodes the primary Vegf ligand that regulates vascular development (Ferrara et al., 1996), is upregulated by over 200%. Expression of Vegfr3 is upregulated by 120%, and expression of Dll4, which encodes a notch ligand regulated by Vegfrs (Wythe et al., 2013), is upregulated by approximately 85%. Differential regulation of several additional key vascular factors was also observed at E13.5. The venous markers CoupfII and Ephb4 are downregulated by 21% and 24% respectively, while the arterial marker Efnb2 is downregulated by 33%. Surprisingly, Sox18 and Lyve1, which encode...
factors typically associated with endothelium of the lymphatic system, were upregulated by 45% and 73% respectively. To determine what cardiac cell populations were upregulating Lyve-1, we conducted Lyve-1 immunostaining at E10.5 (Figures 6G and 6H) and E13.5 (Figures 6I–6N) in control and H2CKO hearts. Sections from E13.5 hearts were costained with VegfR2 to mark cardiac endothelium (see Table S3 for a glossary of terminology pertaining to endothelial cell populations). E10.5 immunostaining reveals that Lyve-1 is expressed robustly within endocardium of both control and H2CKO hearts. In contrast, by E13.5, Lyve-1 immunostaining in control hearts is restricted to a population of peripheral cardiac macrophages (Figure 6I; Pinto et al., 2012), whereas robust Lyve-1 immunostaining persists in the ventricular endocardium of H2CKOs (Figures 6J and 6L). However, while Lyve-1 still robustly marks H2CKO ventricular endocardium (Figure 6N, white arrow), Lyve-1 expression does not mark the expanded coronary endothelium of H2CKOs (Figure 6N, yellow arrow).

As previously mentioned, qRT-PCR demonstrates differential expression of several Vegf signaling components. We therefore investigated, using ChIP, ChIP sequencing (ChIP-seq), EMSA, and luciferase reporter assays, the possibility that Hand2 directly regulates at least a subset of these components (Figure 7A). Given that our results establish Hand2 as a downstream effector of Notch signaling, and Notch also regulates expression of VegfRs within endothelial cells (Herbert and Stainier, 2011), we first addressed the possibility that Hand2 directly regulates VegfR3 expression. Using the previously described HeLa ChIP samples, we show that immunoprecipitation of Hand2-Myc/E12-transfected cell lysates results in selective enrichment of a region within the VegfR3 promoter that has been previously reported to have regulatory activity (Figure 7B; Shawber et al., 2012).
Furthermore, by EMSA, we show that Hand2 E12 heterodimers specifically bind an oligo corresponding to one of the two E-boxes within this human promoter region (Figure 7C), which consists of the 500 bp upstream of the VegfR3 translation start site. Finally, a luciferase reporter containing the homologous 500 bp mouse promoter was significantly repressed by Hand2 E12 heterodimers (p ≤ 0.05; Figure 7D).

Collectively, these data indicate that Hand2 may directly repress VegfR3 expression within cardiac endothelium.

To detect additional Hand2 targets, we utilized a Hand2 ChIP-seq data set that was generated for an alternate study (Osterwalder et al., 2014). This ChIP-seq data set employed a Hand2-3xFLAG knockin allele. E10.5 Hand2-expressing tissues, including the heart, were collected and used for an immunoprecipitation of Hand2-bound regions of genomic DNA. This data set was cross-referenced with our H2CKO gene expression data, where in addition to increased VegfR3, we observed decreased expression of the VegfR2 cofactor Nrp1. Analysis of Hand2-3xFLAG ChIP-seq data revealed a region of high enrichment 356 kb upstream of the Nrp1 coding region (Figure 7E; Table S4). An approximately 3 kb region containing this potential enhancer was cloned upstream of a luciferase reporter driven by the thymidine kinase (TK) minimal promoter. When cotransfected into HeLa cells with Hand2 and E12, a significant 5-fold transactivation was observed (Figure 7F).

Analysis of VegfR3 and Nrp1 expression by ISH reveals expression within cardiac endothelium (Figures S5A and S5B), although differential expression between controls and H2CKOs is not detectable by this nonquantitative assay.

Given that VegfA is upregulated by over 200% in E13.5 H2CKOs, we analyzed the Hand2-3xFLAG ChIP-seq data set for evidence of direct regulation. No prominent peaks were
detected within the VegfA locus. Indeed, ISH demonstrates that VegfA expression is not detectable within endocardium. However, VegfA is strongly expressed within the myocardial compact zone, with low levels of expression observed within trabeculae (Figure 7G). In H2CKOs, the difference in expression levels between compact and trabecular myocardium is visibly less distinct, with high VegfA expressing regions appearing to extend beyond the compact zone (Figure 7H).

**DISCUSSION**

Loss of Hand2 within the SHF cardiac progenitors that give rise to both endocardial and myocardial lineages has been associated with TA. In this study, we show that loss of Hand2 specifically in the endocardium, a cell population in which robust Hand2 expression has previously gone uninvestigated, can cell-autonomously generate TA. These results underscore the importance of effective endocardial-myocardial signaling during early cardiac morphogenesis. Mechanistically, our data show that endocardial Hand2 sits downstream of Notch1 and EfnB2 and is itself directly upstream of Nrg1, being necessary and sufficient for Nrg1 expression in vivo. Notch signaling is a well-established component of endocardium-to-myocardium communication. Hand2 endocardial roles include regulation of trabeculation, positioning of the IVS, and IVS morphogenesis. Indeed, endocardial H2CKOs present multiple septa that are marked by Irx2 and compact zone/septal marker Hey2, while excluding trabecular marker Anf (Figure S2). These ectopic right-sided septa do not express Hand1 or Tbx5 at their left side base (Figures S2I–S2L), suggesting that these septa form via a noncanonical mechanism from RV cardiomyocytes.

These data demonstrate that endocardial Hand2 regulates the specification of trabecular and septal myocardium from primitive myocardium. Given the striking similarities between the cardiac defects of EfnB2 knockouts (Figures 3F and 3G), which do not express Nrg1, and the reported phenotypes of Nrg1 knockouts (Kramer et al., 1996), the most logical conclusion is that loss of Nrg1 expression is the root cause of the hypotrabeculation, septal, and TA phenotypes observed in H2CKOs.

We show that trabeculation and Nrg1 expression within EfnB2 CKO mice is improved by Hand2 replacement via the CC-H2 transgene (Figure 5). Hand2 is necessary for normal expression of Nrg1 (Figure 2), and we demonstrate that Hand2 is sufficient for partial restoration of Nrg1 expression levels in EfnB2 CKOs. While trabeculation is markedly improved in CC-H2(+) EfnB2 CKOs, these embryos still display hallmarks of cardiovascular failure such as pericardial edema and hemorrhage. The incomplete restoration of normal cardiac phenotype is not surprising given that restoration of Nrg1 expression is incomplete, and EfnB2 signaling likely has a much broader range of endocardial downstream targets than just Hand2. Our observation that ~37% of Nrg1 expression remains in Tie2-Cre H2CKOs (Figure 2K) while less than 10% remains in Tie2-Cre EfnB2 CKOs (Figure 3G) indicates that EfnB2 also influences Nrg1 expression via Hand2-independent inputs.

While it is clear that endocardial loss of Hand2 impairs the Notch-dependent processes of trabeculation and septal development, it is less clear how this impairment results in TA. It is possible that multiple morphogenetic inputs contribute to the TA observed in H2CKOs. Previous studies have concluded that TA occurs when the atrial connection to the AV canal expands rightward but the ventricular inner curvature fails to remodel (Kim et al., 2001). Given the dramatic myocardial defects observed in H2CKOs, Hand2-dependent remodeling of AV canal myocardium could be involved; however, histological analysis of H2CKOs suggests a simpler model wherein the septum shifts rightward in H2CKOs and interferes with development of the primitive right AV canal, thus resulting in TA. Indeed, we show that Tie2-Cre H2CKOs have a smaller RV, which is not due to increased cell death within SHF progenitors, increased cell death within the RV, or decreased proliferation within the RV (Figures S6A–S6D). Furthermore, analysis of ventricle areas reveals that Tie2-Cre H2CKOs have a smaller RV and larger LV but no change in total area (Figure S6E), thus supporting histological observations of a rightward-shifted septum. In extreme rightward septal shifts, AV cushion maturation is not hindered but the tricuspid valve forms above the LV, thus resulting in DILV. Finally, Hand2 ablation within the developing OFT and AV valve mesenchyme does not alter septation or valve morphogenesis and does not result in TA (VanDusen et al., 2014). In total, these data demonstrate a cell-autonomous Hand2-dependent role of the endocardium in the etiology of TA/DILV.

In addition to the dramatic defects in myocardial morphogenesis observed in endocardial H2CKOs, histological analyses at E13.5 also reveal significant vascular phenotypes. H2CKOs display precocious and disorganized development of the coronary vascular plexus. Analysis of vascular related gene expression in E10.5 and E13.5 H2CKOs surprisingly reveals that major components of cardiac Vegf signaling are dysregulated. VegfR2 and Vegfd are downregulated at E10.5, while Vegfr3 and Vegfa are significantly upregulated by E13.5. Additionally, Vegfr2 coreceptor Nrp1 is downregulated at both E10.5 and E13.5, while expression of the notch ligand Dll4 is increased at E13.5, an indication of enhanced Vegf signaling (Figures 6E and 6F). Furthermore, qRT-PCR and immunostaining reveals elevated expression of the receptor Lyve-1 within endocardium of E13.5 H2CKOs. Analysis at E10.5 demonstrates that Lyve-1 is initially expressed throughout the early endocardium and is subsequently downregulated as the endocardium matures, such that by E13.5, expression is no longer detectable within ventricular endocardium but is observed only within cardiac macrophages. Given the early endocardial expression of Lyve-1, this persistence within H2CKOs most likely represents a defect in endocardial maturation, rather than ectopic activation of the lymphatic gene program.

Of the genes we observed to be differentially expressed in E13.5 H2CKOs, both the Dll4 and Nrp1 loci contain prominent Hand2 Chip-seq peaks (Table S4). Nrp1 encodes an isoform-specific VegfA receptor that acts in concert with Vegfr2. Interestingly, an RNA subtractive hybridization screen previously identified Nrp1 as being downregulated in Hand2 systemic null embryos (Yamagishi et al., 2000). Our data indicate that Nrp1 is an endocardial target of Hand2, although regulation may also occur in additional tissues where these factors are coexpressed (Figure S5A). Cotransfection of Hand2 and E12 with a luciferase reporter containing the potential upstream
enhancer region yielded over 5-fold transactivation, further supporting direct regulation of Nrp1 by Hand2. In contrast, luciferase reporter assays failed to demonstrate Hand2-mediated repression via the enriched Dll4 upstream region, indicating that the increased Dll4 expression in H2CKOs may be secondary to changes in expression of Vegf receptors, which are known to regulate Dll4 (Wythe et al., 2013). However, our data do not rule out Hand2 direct regulation of Dll4 by alternate undiscovered enhancers. Expression of VegfR3 is upregulated in E13.5 H2CKOs by over 100%. VegfR3 is capable of functioning as both a homodimer and VegfR3-VegfR2 heterodimer, and distinct functions have been associated with different interactions (Dixielius et al., 2003). VegfR3 plays crucial roles in angiogenic sprouting and development of the lymphatic system (Benedito et al., 2012), and we show that VegfR3 is expressed within at least a subset of the endocardium (Figure S5B, arrow). While no high-ranking ChIP-seq peaks were observed within the VegfR3 locus, cardiac endothelium represents only a small portion of the total Hand2 expressing tissue that was utilized, and so sensitivity may be a limiting factor of this assay. Our HeLa ChIP results indicate that Hand2 interacts with the VegfR3 promoter, while transactivation assays correlate with H2CKO expression data, indicating that Hand2 may repress VegfR3 transcription. In addition to VegfR3, we observe a 45% increase in Sox18 expression at E13.5. As Sox18 specifically marks endothelium of the coronary vasculature at this time point (Figure S5C), this increase most likely reflects the hypervascularization phenotype.

Similar to several extracardiac angiogenic models of Notch signaling obstruction (Benedito et al., 2012; Tammela et al., 2008), we show that endocardial Hand2 ablation results in a hypervascularization phenotype featuring the formation of an excessive number of new vessels. Furthermore, we show that this phenotype is accompanied by broad dysregulation of Vegf signaling. Homeostasis of Vegf signaling is crucial during embryonic development, as mice heterozygous for a VegfA null mutation die at E9.5, while an increase in Vegfa expression (∼3-fold) results in lethality at approximately E13.5 (Miquerol et al., 2000). In the present study, we demonstrate that expression of Vegfa is expanded in H2CKOs. This could reflect aberrant specification of trabecular myocardium or secondary pathological effects of compromised cardiac function. Vegfa is the most differentially regulated gene that we observe, while Vegfr2, VegfR3, and Nrp1 are also dysregulated within H2CKOs. Given the complex interactions that take place between these molecules, disrupted receptor stoichiometry and upregulated Vegfa, a growth factor that is well known for its proangiogenic qualities, most likely account for the observed coronary phenotype. These data not only provide insight into a second novel function of Hand2 within the endocardium but also reveal a wider role of Notch signaling during coronary vessel development. Coronary heart disease is a major cause of mortality in developed nations, being responsible for approximately one of every six deaths in the United States (Go et al., 2013). Consequently, further assessment of Hand2’s regulatory role in Vegf signaling during coronary vascularization, as well as potential adult homeostatic roles of Hand2-dependent Notch signaling, is an interesting avenue of future investigations.

**EXPERIMENTAL PROCEDURES**

**Mice and Genotyping**

Tie2-Cre(+) and Nfatc1-Cre(+) mice were crossed with Hand2−/− mice to generate Tie2-Cre(+)Hand2−/− and Nfatc1-Cre(+)Hand2−/− males. These males were then crossed with Hand2lox/lox;ROSAs2R reporter mice (lacZ or eYFP) to generate conditional null Hand2 embryos. Tie2-Cre(+) females were crossed with Efnb2lox/lox males to generate Tie2-Cre(+)Efnb2lox/lox males. These males were then crossed with Efnb2lox/lox females to generate conditional null Efnb2 embryos. The cre-activatable transgene CAG-CAT-Hand2 was constructed by replacing the Myc- Twist cDNA of CAG-CAT-Twist (Conneyer et al., 2006) with the murine Myc-Hand2 cDNA. This construct was used for microinjection to establish a transgenic line. For genotyping information, see Supplemental Experimental Procedures. Mouse maintenance and experimentation was performed according to protocols approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

**Hand2-3xFLAG ChIP-Seq**

The Hand2 ChIP-seq data set was generated as part of a previous study using homozygous Hand2-3xFLAG embryos, which express a 3xFLAG epitope-tagged Hand2 protein (Osterwalder et al., 2014). Briefly, Hand2-expressing tissues (heart, limbs, and pharyngeal arches) from 150 Hand2-3xFLAG E10.5 embryos were dissected and pooled. Samples were crosslinked in 1% formaldehyde for 5 min and ChIP was performed using FLAG antibodies (Sigma F1804). ChIP-seq reads were mapped to the mouse genome (NCBI37/mm9) and peaks and peaks were detected using MACS (version 1.3.7.1) for the HAND2-3xFLAG and input control samples. Statistically validated peaks were sorted according to fold enrichment and number of reads.

**Section RNA In Situ Hybridization**

Antisense digoxigenin labeled riboprobes were transcribed with T7, SP6, or T3 (Roche). Section ISH was performed as previously described (Vincentz et al., 2008). The CDNA for a Fogo riboprobe was kindly supplied by Eric Svensson, Tbx20 by Simon Conway, Anf and In2 by Vincent Christoffels, TgifII by Heleny Sucov, and Efnb2 by Hai Wang. All data reflect assessment in n ≥ 3 embryos for ISH and immunostaining and n ≥ 4 for qRT-PCR analyses.

**Quantitative RT-PCR**

Total RNA was isolated from E10.5 or E13.5 ventricles using the High Pure RNA Isolation Kit (Roche). This mRNA served as a template to generate cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). For qRT-PCR, cDNA was amplified using TaqMan Probe-Based Gene Expression Assays (Applied Biosystems). Relative gene expression was determined after normalization to GAPDH. The Student’s t test was used to detect significant differences between sample groups; asterisks denote p values ≤ 0.05. Error bars represent SE.

**EMSA, Luciferase Assays, and ChIP**

Hand2 and E12 were in vitro translated using the Promega Reticulocyte Lysate System. A total of 5 μl of translated protein was incubated in binding buffer for 30 min at 25 °C with radiolabeled oligos corresponding to E-boxes and D-boxes within the Nrg1 and VegfR2 loci (Filunetti et al., 2007). Transcription factor/oligo complexes were run out on a nondenaturing 6% polyacrylamide gel and assessed on a phosphomager. For luciferase assays, HeLa cells were transfected using X-tremeGENE HP (Roche) at a ratio of 3:1 with Nrg1-500bp+pGL4.10 (luciferase), VegfR3-500bp+pGL4.10 (luciferase), pGL4.73 (SV40-renal) or pGL4.74 (TK-renal), Pcs2+Myc-Hand2, Pcs2+Myc-E12, pcDNA3.1Myc-JBasic-Hand2 (kindly provided by Eric Olson), or pcDNA3.1 and cultured for 48 hr. After harvest and processing, luciferase and renilla activities were measured for significant difference from pcDNA control (p value ≤ 0.05); error bars represent SE. For construction of Npr1−/−−−−358−−−−355 kb and Dll4−−−−23−−−−20 kb luciferase reporters, genomic regions containing a Hand2-3xFLAG ChIP-seq peak were PCR amplified and cloned into TK-pGL4.10. For ChIP of the Npr1 promoter, HeLa cells were again transfected using X-tremeGENE HP at a ratio
of 3:1. After culturing for 48 hr, cells were processed as previously described (Barnes et al., 2011). Briefly, equal amounts of sheered chromatin were immuno-precipitated overnight at 4°C with 50 μl of α-Myc-conjugated agarose beads (Sigma) or beads without antibody for a negative control. After reverting cross-links, eluted immunoprecipitated DNA was phenol chloroform extracted, re-suspended in dH2O, and used for subsequent PCR reactions. After 37 cycles of PCR amplification, product was analyzed on an agarose gel. ChiP transfection constructs included Pcs2+Myc-Hand2, Pcs2+E12, and Pcs2+Myc. See Supplemental Experimental Procedures for oligo information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at https://dx.doi.org/10.1016/j.celrep.2014.11.021.

AUTHOR CONTRIBUTIONS

N.J.V. wrote the manuscript, conceived of experiments, and carried out experiments. J.C. carried out experiments and critically read the manuscript. J.W.V. and B.A.F. interpreted data, provided reagents, and critically read the manuscript. R.Z., M.O., and J.L.R. conceived of and carried out Hand2-3xFLAG ChiP-seq. B.Z. aided in experimental planning and provided Nfatc1Crk mice. J.G.B. and J.L.P. assayed Hand2 expression in RBPJK−/− mice. W.S. interpreted data and evaluated the manuscript. A.B.F. wrote and edited the manuscript and conceived of experiments.

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REFERENCES


