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HAND transcription factors cooperatively specify the aorta and pulmonary trunk

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Abstract

Congenital heart defects (CHDs) affecting the cardiac outflow tract (OFT) constitute a significant cause of morbidity and mortality. The OFT develops from migratory cell populations which include the cardiac neural crest cells (cNCCs) and secondary heart field (SHF) derived myocardium and endocardium. The related transcription factors HAND1 and HAND2 have been implicated in human CHDs involving the OFT. Although *Hand1* is expressed within the OFT, *Hand1* NCC-specific conditional knockout mice (*H1CKOs*) are viable. Here we show that these *H1CKOs* present a low penetrance of OFT phenotypes, whereas SHF-specific *Hand1* ablation does not reveal any cardiac phenotypes. Further, HAND1 and HAND2 appear functionally redundant within the cNCCs, as a reduction/ablation of *Hand2* on an NCC-specific *H1CKO* background causes pronounced OFT defects. Double conditional *Hand1* and *Hand2* NCC

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Availability of data and materials
All data generated or analyzed in this study are included in this manuscript.

Declaration of competing interest
Authors have no conflict of interest regarding this work.

Ethics statement:
Animal work performed at Indiana School of Medicine (IUSM) was approved by the IUSM Animal Care and Use Committee (IACUC) under protocol 11326. Animal work performed at Lawrence Berkeley National Laboratory (LBNL) was reviewed and approved by the LBNL Animal Welfare Committee. IUSM mice were housed in room IB31. LBNL mice used for this study were housed at the Animal Care Facility (ACF) at LBNL. All mice were monitored daily for food and water intake, and animals were inspected weekly. LBNL mice were also inspected by the Chair of the Animal Welfare and Research Committee and the head of the animal facility in consultation with the veterinary staff on a weekly basis. The IUSM facilities and LBNL ACF is accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). LBNL transgenic mouse assays were performed in Mus musculus FVB background mice.

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knockouts exhibit persistent truncus arteriosus (PTA) with 100% penetrance. NCC lineage-tracing and *Sema3c in situ* mRNA expression reveal that *Sema3c*-expressing cells are mis-localized, resulting in a malformed septal bridge within the OFTs of *H1CKO;H2CKO* embryos. Interestingly, *Hand1* and *Hand2* also genetically interact within the SHF, as SHF *H1CKOs* on a heterozygous *Hand2* background exhibit Ventricular Septal Defects (VSDs) with incomplete penetrance. Previously, we identified a BMP, HAND2, and GATA-dependent *Hand1* OFT enhancer sufficient to drive reporter gene expression within the nascent OFT and aorta. Using these transcription inputs as a probe, we identify a novel *Hand2* OFT enhancer, suggesting that a conserved BMP-GATA dependent mechanism transcriptionally regulates both HAND factors. These findings support the hypothesis that HAND factors interpret BMP signaling within the cNCCs to cooperatively coordinate OFT morphogenesis.

1. Introduction

Congenital heart defects (CHDs) afflict roughly 1% of newborns according to the National Heart Lung and Blood Institute, and ultimately affect the quality of life of more than 1 million adults in the United States. (Cheng et al., 2012a) Of these affected individuals, approximately 25% require invasive treatment within the first year of life. (Mozaffarian et al., 2014) Owing to significant advances in cardiothoracic medicine, infant mortality rates from CHDs have fallen drastically in the past half-century. Unfortunately, CHDs occur with such frequency that they remain a significant cause of morbidity and mortality in both children and adults. Further progress in the prevention and treatment of CHDs hinges upon a complete understanding of their etiology.

A significant portion of CHDs can be attributed to dysfunction of the main developmental progenitors of the cardiac outflow tract (OFT), cardiac neural crest cells (cNCCs) and of the second heart field (SHF). (Mozaffarian et al., 2014) cNCCs and SHF cells are both migratory cell populations that originate outside of the primary heart-forming field. NCC subpopulations migrate from the dorsal neural tube throughout the developing embryo, whereupon they differentiate into distinct tissue types. Within the OFT, the cardiac NCCs, differentiate into smooth muscle and connective tissue to contribute to the aorta, pulmonary artery, and nascent ventricular septum. The SHF, a subpopulation of splanchnic mesoderm located anterior to the cardiac crescent, migrates into the arterial pole to contribute endocardial and myocardial cells to the growing heart. (Barnes et al., 2010b ; Harrison et al., 2005; Nakajima, 2010; Zaffran and Kelly, 2012) Within the OFT, the cNCCs coordinate both with a specific SHF subpopulation, known as the myocardial cuff (MC), and with adjacent endocardium, to divide the OFT into two separate blood vessels, and thereby sequester systemic and pulmonary circulation. Although a number of studies have identified genetic and environmental factors that affect OFT remodeling, and consequently cause CHDs, a failure to understand the local signaling cues and transcriptional mechanisms that regulate gene expression within the cNCCs after these cells have migrated into the OFT, the pleiotropic effects of crucial factors that have confounded studies of their tissue- and stage-specific cellular functions, and functional redundancy among mammalian genes that likewise mask their critical functions have hindered understanding of the transcriptional and

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extracellular signaling mechanisms that coordinately control cardiac NCC and MC morphogenesis.

The bHLH transcription factors HAND1 and HAND2 exhibit similar functional properties and have been proposed to perform redundant functions during development. (Barbosa et al., 2007; George and Firulli, 2019; McFadden et al., 2005; Vincentz et al., 2017) The two factors share dimer partners and bind to either of two consensus elements termed E-boxes (CANNTG) and D-boxes (CGNNTG). (Firulli et al., 2007; George and Firulli, 2019; Hollenberg et al., 1995) Studies in both chick (Srivastava et al., 1995) and mouse (McFadden et al., 2005) indicate that HAND1 and HAND2 may functionally compensate for each other's absence during early cardiogenesis. *Hand1* deletion within NCCs reveals gene dosage dependent craniofacial phenotypes in *Hand1* conditional knockouts that are also on a *Hand2* heterozygote background; however, cardiac defects were not investigated, and if present, are not severe enough to cause lethality (Barbosa et al., 2007).

HAND1 mutations have been observed in human patients with Tetralogy of Fallot (TOF; (Wang et al., 2017b; Wang et al., 2011a), with Double Outlet Right Ventricle (DORV; (Li et al., 2017), and with Ventricular Septal Defects (VSDs; (Cheng et al., 2012b), as well as, most recently, cardiac conduction system functional variation (Evans1 et al., 2016; Firulli et al., 2019; Sotoodehnia et al., 2010; van Setten et al., 2019; Vincentz et al., 2019). *HAND1* haploinsufficiency likely contributes to CHDs associated with 5q33 chromosomal deletions (Starkovich et al., 2016). Studies of children with CHDs such as TOF, pulmonary stenosis, and VSDs with DORV, have identified multiple *HAND2* missense mutations (Shen et al., 2010). Additionally, defects including VSDs, pulmonary atresia, coarctation of the aorta, and TOF, are associated with genomic duplications or deletions of chromosome 4q33, to which *HAND2* maps (Borochowitz et al., 1997; Byatt et al., 1997). Mouse genetic models indicate that the OFT CHDs associated with the human chromosomal disorder partial trisomy distal 4q (4q+) are caused by overexpression of *HAND2* (Tamura et al., 2013); however, the variable penetrance of CHDs associated with 4q+ suggest that unknown *HAND2* genetic modifiers influence the presentation of this disease. Increased *HAND2* mRNA expression is associated with TOF, whereas increased *HAND1* mRNA expression is associated with hypertrophic obstructive cardiomyopathy (Ritter et al., 1999). Collectively, these findings are consistent with a mechanism whereby either too much or too little HAND factor function is detrimental to cardiac growth and morphogenesis.

As steady state expression levels of HAND1 and HAND2 appear to dictate their cellular function (Barbosa et al., 2007; Firulli et al., 2005; McFadden et al., 2005), unraveling the mechanisms by which *Hand1* and *Hand2* expression is regulated within these tissues, and, in turn, HAND factor dependent downstream targets, is necessary to understand their roles in heart development. To date, a *Hand2* right ventricle (RV)/OFT enhancer, directly regulated by GATA factors, that drives gene expression within the right ventricle (RV) and OFT myocardium has been identified (McFadden et al., 2000). Two separate *Hand1* transcriptional enhancers, one specific for the OFT and MC (Vincentz et al., 2016), and the other specific for the LV are also dependent on GATA *cis*-elements for their transcriptional activity (Vincentz et al., 2019; Vincentz et al., 2017). Indeed, a single nucleotide polymorphism (SNP) within one of two evolutionarily conserved GATA *cis*-elements

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located within the LV enhancer is associated with altered cardiac conduction in humans and human phenotypes are modeled in genetically engineered mice (Vincentz et al., 2019). Collectively, these data support the hypothesis that both HAND1 and HAND2 play essential roles in cardiogenesis.

In this study, we explore the molecular pathways and mechanism(s) by which HAND1 and HAND2 regulate proper development of the cNCC and MC components of the OFT. *Hand1* NCC conditional knockouts (*H1CKO*^{NCC}) exhibit a low penetrance of OFT phenotypes. Additionally, removing a single or both *Hand2* alleles within the NCC on a *H1CKO*^{NCC} background result in more serious and highly penetrant OFT defects that include persistent truncus arteriosus (PTA). SHF conditional *Hand1* knockout (*H1CKO*^{SHF}) and *Hand2* reveal genetic interactions within OFT myocardium. Like *Hand1* (George and Firulli, 2019; Vincentz et al., 2016), a novel *Hand2* OFT transcriptional enhancer includes transcriptional inputs for HAND2, GATA and SMAD1/5. Together these findings expand our understanding of how coordinate developmental events in distinct tissue populations (the cNCCs and MC) affect proper morphogenesis of the OFT and provide insight into the etiology of the CHDs defects associated with *HAND* factor mutations.

2. Materials and Methods.

Experimental Mice

Genotyping of the *Hand1*$^{tm1Eno/AbfiJ}$, (Firulli et al., 1998) *Hand1*^{f}, (McFadden et al., 2005) *Hand2*^{neo}, (Srivastava et al., 1997) *Hand2*^{f}, (Morikawa and Cserjesi, 2008) *Smad4*$^{tm2.1Cxd/J}$ (Yang et al., 2002) *Wnt1-Cre*, (Vincentz et al., 2013) *Tg(Mef2c-cre)2Blk*, (Verzi et al., 2005) and *Gt(ROSA)26*^{Sortm1Sor} (Soriano, 1999) and *Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo/J}$ (genotyped as per (Soriano, 1999)) alleles have been previously described. The mm1284 sequence was amplified from mouse genomic DNA (Clontech) and cloned into the *hsp68-lacZ* vector (Kothary et al., 1989; Visel et al., 2007). Pronuclear injection into fertilized eggs, implantation into pseudo pregnant mothers, and embryo dissection at embryonic day 11.5 (E11.5) was performed as described previously (Attanasio et al., 2013). Yolk sac DNA was used to assess the presence of the various alleles in embryos. Tail DNA was used to assess the presence of the various alleles in neonatal mice. All animal maintenance and procedures were performed in accordance with the Indiana University School of Medicine and Lawrence Berkeley National Laboratory Animal Care and use committees.

Embryo harvesting in situ hybridization & Xgal staining

Following dissection in cold 1X PBS, embryos of the indicated age were fixed in 4% paraformaldehyde at 4°C. Embryos were washed three times with wash buffer (2mM MgCl2; 0.01% deoxycholate; 0.02% NP-40; 100mM phosphate buffer, pH 7.3) for 30 min., then stained for 24 hrs. at room temperature with freshly made X-gal staining solution (0.8mg/mL X-gal; 4mM potassium ferrocyanide; 4mM potassium ferricyanide; 20mM Tris, pH 7.5 in wash buffer). The following day, embryos were rinsed three times in 1X PBS, then post-fixed in 4% paraformaldehyde. X-gal staining was performed as previously described (Barnes et al., 2010a). Nuclear Fast Red staining was performed as per manufacturer's instructions.

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Histology

Embryos (E10.5 and E16.5) were fixed in 4% paraformaldehyde, dehydrated, embedded, sectioned, and Hematoxylin and Eosin (H&E) or Sirus red & Fast Green stained as described. (Firulli et al., 2014; Firulli et al., 2019). All data was collected on a Leica DM5000 B compound wide field florescent microscope.

in situ hybridization was performed as described. (Vincentz et al., 2013; Vincentz et al., 2019) Antisense digoxygenin-labeled *Sema3c* and *Hand2* riboprobes were synthesized using T7, T3 or SP6 polymerases (Promega) and DIG-Labeling Mix (Roche) using a linearized plasmid template.

Bioinformatics

Histone marks and variants, chromatin accessibility, and transcription factor / chromatin regulator binding within 100 Kb of the Hand2 transcription start site was examined using the Toolkit for Cistrome Data Browser (http://dbtoolkit.cistrome.org). SMAD1/5, HAND2, and GATA4 tracks were loaded onto the UCSC mm10 genome browser and one region of ChIP-seq binding co-occupancy was identified. A DNA fragment containing this region of co-occupancy was then cloned into a lacZ reporter construct and used to generate transient transgenic embryos via previously reported methodology (Visel et al., 2007). VISTA Enhancer Browser-a database of tissue-specific human enhancers (Visel et al., 2007).

3. Results

A critical *Hand* factor genetic complement is crucial for morphogenesis of the developing OFT.

Hand1 is expressed in the developing OFT within both the cardiac NCC and MC, (Vincentz et al., 2011) Vincentz et al., 2016). Mutations in human *HAND1* are associated with OFT CHDs. (Wang et al., 2017a; Wang et al., 2011b) Surprisingly, $Hand1^{fx/fx}$;*Wnt1-Cre(+)* NCC-specific conditional knockouts (CKOs) have been reported to be viable and fertile. (Barbosa et al., 2007) This discrepancy indicates that current genetic understanding of HAND1 function within the OFT, based upon animal models, is incomplete. Moreover, evidence shows that *Hand2* expression shows a slight increase within *Hand1* systemic knockout embryos (Morikawa and Cserjesi, 2004) To explore the possibility that *Hand1* CKOs exhibits cardiac phenotypes that could be compensated by HAND2, we first tested whether *Hand1* function is required for OFT development within the developing MC and/or cNCCs.

To test whether HAND1 function is necessary within the MC, *Mef2c-AHF-Cre(+)* mice (Verzi et al., 2005) were employed to ablate *Hand1* function specifically within SHF derivatives (Fig. 1). $Hand1^{fx/+}$;*Mef2c-AHF-Cre(+)* mice were bred with $Hand1^{+/-}$ mice to generate $Hand1^{-/fx}$;*Mef2c-AHF-Cre(+)* embryos. Phenotypic analyses of E16.5 $Hand1^{-/fx}$;*Mef2c-AHF-Cre(+)* CKOs revealed no OFT abnormalities within $H1CKOs^{SHF}$ ($Hand1^{fx/fx}$;*Mef2c-AHF-Cre(+)*); Fig. 1B, F, and F'; n=5 all normal) compared with controls ($Hand1^{+/+}$;*Mef2c-AHF-Cre(+);Wnt1-Cre(+)*); Fig. 1A, E, and E'; n=6 all normal). We next looked carefully at E16.5 hearts from $H1CKOS^{NCC}$ (Fig. 1C, G, and G' n=9). One in three NCC-specific $H1CKOs^{NCC}$ ($Hand1^{fx/-}$;*Wnt1-Cre(+)*) displayed membranous VSDs (Fig.

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1C, G, and G'; n=3/9), and one displayed aberrant origin of the right subclavian artery (AORSA; Fig. 1C; 1/9) whereas 6 out of 9 *H1CKO*^{NCC} hearts appeared phenotypically normal. To test for tissue interactions of *Hand1* within both SHF and NCC, we generated *H1CKO*$^{NCC/SHF}$ E16.5 embryos (Fig.1D, H, and H' n=4). We observed no increase in OFT defect severity or penetrance compared with what is observed in *H1CKO*^{NCC} hearts. These findings are summarized in Table 1. This data supports that *Hand1* loss-of-function within NCCs can cause OFT phenotypes with incomplete penetrance consistent with those observed in human patients with *HAND1* mutations, and that deletion of *Hand1* within the SHF presents no obvious cardiac phenotypes.

To investigate any HAND factor functional redundancy within the NCC, we next looked at hearts from combinations of *H1CKO*^{NCC} and *H2CKO*^{NCC} (Fig. 2). Hearts from double heterozygotes (*Hand1*$^{fx/+}$;*Hand2*$^{fx/+}$;*Wnt1-Cre(+)*) embryos (n=7) show normal development at E16.5 (Fig. 2A, F and F'). In contrast, double heterozygous *Hand1*$^{fx/-}$;*Hand2*$^{fx/-}$ embryos (n=7) all present with DORV and associated VSD (Fig. 2B, G, and G', black arrowheads). This difference between the double heterozygous genotypes likely reflects hypomorphic expression from one or both of the *Hand* conditional alleles. (McFadden et al., 2005; Morikawa et al., 2007) Embryos that contain a single *Hand2* allele, (n=9) show a spectrum of defects that include DORV with associated VSD (5/9), dilated/hypertrophic LV (1/9), bicuspid aortic valve (1/9) and three embryos showed normal phenotype (Fig. 2C, H, and H'). E16.5 embryos that contain only a single *Hand1* allele (n=9) exhibit DORV with associated VSD (9/9), interrupted aortic arch type B (3/9), and/or dilated/hypertrophic LV (4/9) (Fig. 2D, I, and I'). Finally analysis of *H1CKO*^{NCC};*H2CKO*^{NCC} E16.5 hearts (n=3) showed 100% penetrance of persistent truncus arteriosus (Fig 2E, J, and J', asterisk). Results are fully summarized in Table 2.

cNCC localization within the OFT is compromised within HAND loss-of-function hearts

OFT defects are often correlated with diminished NCC migration into the OFT. To completely characterize the phenotypes seen in *Hand1*;*Hand2* double NCC CKOs, we performed lineage trace analyses to monitor cNCC migration to the OFT in these double CKOs utilizing the *R26R*$^{mT/mG}$ allele, which switches from membrane-localized RFP tdTomato to a membrane-localized EGFP following Cre recombination. This allele enables visualization of NCC invasion of the OFT cushions at E12.5 (Fig. 3A-D). In contrast to *Hand1*$^{+/fx}$;*Hand2*$^{+/fx}$;*Wnt1-Cre(+)* controls (Fig. 3A) and *Hand1*$^{fx/-}$;*Hand2*$^{+/fx}$;*Wnt1-Cre(+)* or *Hand1*$^{+/fx}$;*Hand2*$^{fx/-}$;*Wnt1-Cre(+)* mutants (Fig. 3B and C), wherein two OFT cushions (OFTc) are clearly defined, the OFT cushions of *H1CKO*^{NCC};*H2CKO*^{NCC} hearts are clearly dysmorphic and cannot be individually identified, as would be expected in a PTA (Fig. 3D; n=4/4).

Although previous studies have revealed that NCC-specific *Hand2* CKOs display aortic arch defects, VSDs, and DORV (Holler et al., 2010), it is unclear whether impaired cNCC contribution to the OFT underlies these defects. Coordinate with cNCC migration into the OFT, the endothelial lining of the OFT undergoes endothelial-to-mesenchymal transition to form mesenchymal cells that colonize the OFT cushions (Sugishita et al., 2004). This process is critical for the growth of the OFT cushions, which fuse to form the septal bridge

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(Fig. 3E-H, shown at the white arrowheads) a precursor to the mature aortico-pulmonary septum. A signaling cue that is critical for the process of aortico-pulmonary septation is the class 3 semaphorin SEMA3C (Brown et al., 2001). SEMA3C signals through the transmembrane receptor Neuropilin 1 (NRP1; (Schwarz and Ruhrberg, 2010) and loss of *Sema3c* expression results in interruption of the aortic arch accompanied by PTA, thus regulating development and migration of cNCCs (Feiner et al., 2001). Cardiac NCC-derived SEMA3C signaling promotes septal bridge formation through regulation of endothelial-to-mesenchymal transition (endoMT) and, indirectly, cardiac NCC relocalization (Plein et al., 2015). *Sema3c* ISH within $H1CKO^{NCC};H2CKO^{NCC}$ mutants shows that *Sema3c*-expressing cells are mis-localized (asterisks), and suggests that the septal bridge is malformed within $H1CKO^{NCC};H2CKO^{NCC}$ mutant OFTs (Fig. 3D and H, arrowhead; n=5/5). Thus, the *Sema3c*-positive population of cardiac NCCs migrate to the OFT in these mutants, but interaction of the NCCs with the endocardium is likely disrupted such that endoMT and cNCC localization is abnormal. Conditional ablation of *Hand2* using the $Hand1^{eGFP\text{-}Cre}$ knock-in allele (one copy of *Hand1*), which recombines within both post-migratory cardiac NCCs and the MC, results in incompletely penetrant PTA (Barnes et al., 2011). ISH revealed that *Sema3c* expression is similarly mis-localized in $Hand1^{Cre}$;*Hand2* CKOs (Sup. Fig 1).

We next examined embryos in which SHF-specific *Hand1* function has been conditionally ablated and *Hand2* function has been reduced by half ($Hand1^{fx/fx}$;$Hand2^{fx/+}$;*Mef2c-AHF-Cre(+)*). Analyses of these embryos revealed a low penetrance of VSDs (Fig. 4, n=3/12; summarized in Table 3) and dilated coronary vessels within the ventricular free wall and septum. These coronary phenotypes are consistent with previous reports that *Hand2* is necessary for coronary vasculogenesis (Vandusen et al., 2014), but have not been reported with *HAND1*-associated clinical heart phenotypes. Cumulatively, these findings confirm that OFT morphogenesis cannot tolerate a collective reduction in HAND factor function beyond a critical threshold.

A conserved gene regulatory network regulates *Hand* factor expression in cardiac NCCs.

Identification and validation of tissue-specific enhancers is integral to establishing the gene regulatory networks that orchestrate OFT morphogenesis. One approach to isolate such enhancers is to identify conserved regions of DNA which developmentally important transcription factors co-occupy. Our previous findings demonstrate that the $Hand1^{PA/OFT}$ enhancer is activated by SMADs, HAND2, and GATA transcriptional inputs (Vincentz et al., 2016). BMP signaling is also established as being transcriptionally upstream of *Hand2* within the developing OFT (Holler et al., 2010; Liu et al., 2004) and reduced *Hand2* expression observed from *in situ* hybridization in $Smad4^{fx/fx}$; *Wnt1-Cre(+)* embryos further supports these findings (Fig. 5). Previous studies have identified a *Hand2* NCC enhancer sufficient to drive gene expression within the cranial NCCs, but not the cardiac NCCs (Charite et al., 2001). *Hand2* RV expression is GATA-dependent (McFadden et al., 2000). ChIP-seq datasets for HAND2 within the embryonic heart, (Laurent et al., 2017) GATA4 (from both heart and HL-1 cells; (He et al., 2014; He et al., 2011), GATA6 (mouse embryonic stem cells; (Wamaitha et al., 2015), and SMAD1/5 (mouse embryonic stem cells; Morikawa et al., 2016) are publicly available. As both *Hand1* and *Hand2* are well established to respond to BMP/TGFβ transcriptional regulation (Bonilla-Claudio et al.,

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2012; Howard et al., 2000; Vincentz et al., 2016; Wu and Howard, 2002), HAND factor, and/or GATA factor dependent expression in NCCs (Bonilla-Claudio et al., 2012; Vincentz et al., 2016), we used this regulatory relationship to search for novel *Hand2* enhancers that would express within the cNCC of the OFT. We next identified, using the publicly available ChIP-seq datasets, peaks of enrichment for HAND2/SMAD/GATA factor binding that occur within regions marked by histones associated with transcriptionally accessible chromatin, as revealed by ChIP-seq of these chromatin marks in NCCs (Minoux et al., 2017) an evolutionarily conserved *Hand2* enhancer that contained conserved *cis*-elements that matched HAND2/SMAD/GATA factor consensus sequences.

We first employed Cistrome (http://cistrome.org/db/#/) to load the aforementioned ChIP-seq tracks onto the UCSC Genome Browser (http://genome.ucsc.edu). We hypothesized that the SMAD/HAND2/GATA transcriptional synergy that drives $Hand1^{PA/OFT}$ enhancer expression (Vincentz et al., 2016) would also drive a *Hand2* autoregulatory mechanism within these tissues. Peaks of enrichment for HAND2, GATA4/6, and SMAD1/5 DNA occupancy overlap within a region, evolutionarily conserved among placentals, located 3' to the second exon of *Hand2* (Fig. 6A). This sequence also displays histone marks that indicate active transcription (Fig. 6A). We next examined ATAC-seq from E10.5 frontonasal process, maxilla, mandible, and 2nd pharyngeal arch (Minoux et al., 2017) E12.5 hearts (Zhou et al., 2017) and adult cardiomyocytes (Monroe et al., 2019) We observed a region of accessible chromatin 3' to *Hand2* and 5' to the *Hand2* downstream lncRNA, *Hdnr*. This peak of enrichment appeared in both NCC-derived and cardiac cells. (Yellow shading highlights the mandibular arch enhancer. (Charite et al., 2001)

This enhancer, denoted mm1284 (Fig. 6C), has been tested *in vivo* using a transient transgenic enhancer assay, and the results have been posted to the publicly available VISTA Cardiac Enhancer Browser (http://portal.nersc.gov/dna/RD/heart/index.html). Fitting our hypothesis, this enhancer's expression overlaps with endogenous *Hand2* expression within the OFT (Fig. 6D, white arrow). Reporter expression within the OFT is observed within the endocardial cushions (Fig. 6E, white arrow) in 5 of 7 transgene-positive embryos. Phylogenetic footprinting of this enhancer identified E-box, D-box, SMAD, and GATA *cis*-elements that exhibit conservation among mammals (Supp. Fig 2). Additionally, human ChIP-seq data sets for GATA4 in iPS-derived cardiomyocytes reveal a peak of enrichment that co-localizes with this conserved enhancer (Ang et al., 2016). These findings support that integration of mutant phenotype, gene expression, and DNA occupancy data can be used effectively to identify novel enhancers that are active specifically in the developing cNCCs.

4. Discussion

OFT defects in newborns constitute a significant cause of morbidity and mortality and are amongst the most highly encountered congenital defects in humans. Here we show that loss-of-function models of *Hand1* and *Hand2* indicate that these two highly related bHLH transcription factors genetically interact within both the cNCC and SHF. The data reveal OFT morphogenesis cannot tolerate a collective reduction of HAND factor function beyond a critical threshold. This critical threshold of HAND protein reflects both direct DNA occupancy on enhancer sequences of regulated genes as well as the reiteration of other

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bHLH factors that when unpartnered could exhibit deleterious gain of function effects such as what is observed in Saethre-Chotzen syndrome (Firulli et al., 2005). Given systemic knockout (Srivastava et al., 1997), as well as the NCC- (Holler et al., 2010) and SHF- (Tsuchihashi et al., 2011) conditional ablations of *Hand2* are sufficient to disrupt OFT morphogenesis with high penetrance, it is clear that HAND2 plays a more dominant role in this process. This could reflect actual direct transcriptional target efficiencies or simply reflect variation in expression levels. Although SHF *Hand1* ablation did not reveal any observable cardiac phenotypes alone (Fig. 1), NCC ablation of *Hand1* did reveal a low penetrance of OFT phenotype that include membranous VSDs and AORSA (Fig. 1C and G) revealing that less than a full complement of HAND factors can be deleterious to OFT morphogenesis. *Hand1* deletion within both the NCC and MC did not reveal any increase in phenotypic penetrance suggesting that HAND1 is not playing a significant role regulating the gene regulatory networks involved in modulating the cell-cell interactions of these OFT cell types. Interestingly, the observed $H1CKO^{NCC}$ OFT defects are not severe enough to be lethal, as $H1CKO^{NCC}$ are encountered at expected mendelian frequencies similar to as was reported (Barbosa et al., 2007). This is consistent with GWAS data showing disease numerous *HAND1* associated SNPs in humans linked to cardiac defects (Cheng et al., 2012b; Evans1 et al., 2016; Firulli et al., 2019; Li et al., 2017; Sotoodehnia et al., 2010; van Setten et al., 2019; Vincentz et al., 2019; Wang et al., 2017b; Wang et al., 2011a).

Complete loss of HAND factors within the NCC results in a full PTA with 100% penetrance (Fig. 2E and J) and indeed, when *Hand2* is knocked out using $Hand1^{eGFP\text{-}Cre}$ knock-in allele which recombines within both post-migratory cNCCs and MC, it also results in PTA at partial penetrance (Barnes et al., 2011). Within $H1CKO^{NCC};H2CKO^{NCC}$ mutants, the *Sema3c*-positive population of cNCCs migrate to the OFT, but interaction between these cNCCs and endocardium is disrupted such that endothelial-to-mesenchymal transition and cNCC localization is abnormal.

Although $H1CKOs^{SHF}$ OFTs are normal (Fig. 1), we do observe CHDs when *Hand1* is ablated within SHF derivatives on a *Hand2* heterozygous gene dosage (Fig. 4). These results suggest that reduction of SHF expressed *Hand2* sensitizes OFT morphogenesis to a loss of HAND1 function. Isolated membranous VSDs are observed in SHF-specific $H1CKOs^{SHF}$;*Hand2 het* mutants (Fig. 4), revealing a critical SHF HAND factor threshold in addition to the threshold established within the NCC (Fig. 2). The *Mef2c-AHF-Cre* driver recombines within both the myocardium and the endocardium of the OFT and right ventricle (Verzi et al., 2005). $Hand2^{fx/fx}$;*Mef2c-AHF-Cre(+)* CKOs present with PTA (Tsuchihashi et al., 2011). Although conditional ablation of *Hand2* within the endocardium is embryonic lethal, these *H2CKOs* do not exhibit OFT phenotypes (Vandusen et al., 2014). *Hand1* lineage does not mark the endocardium (Barnes et al., 2010a), and therefore, we conclude that HAND factor function within the endocardium is unlikely to be critical for OFT morphogenesis with the caveat that HAND2 loss-of function within both the NCC and endocardium collectively was not investigated.

The $Hand1^{PA/OFT}$ enhancer is directly regulated by SMAD/HAND2/GATA transcriptional inputs (Vincentz et al., 2016). Utilizing co-occupancy data of these factors within the *Hand2* locus, we predicted a *Hand2* cNCC enhancer that exhibits cNCC *cis*-regulatory activity

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consistent with *Hand2* mRNA expression (Fig. 6). The finding that both *Hand* genes are likely co-regulated by a trio of transcriptional inputs that allows cNCC co-expression is perhaps not surprising but is confirmation that HAND1 and HAND2 lie directly downstream within the BMP and GATA gene regulatory networks of the OFT. Previous findings show that *Hand1* cranial NCC expression is HAND2, BMP, and GATA dependent whereas all *Hand2* NCC expression is independent of HAND1 but completely dependent on BMP signaling and GATA (Bonilla-Claudio et al., 2012; Howard et al., 2000; McFadden et al., 2000; Vincentz et al., 2016; Wu and Howard, 2002). This suggests subtle differences in NCC gene regulatory networks where in cranial populations BMP and GATA upregulate HAND2, which is followed by BMP, GATA and HAND2 directly regulating HAND1. In cNCC *Hand1* expression is not HAND2 dependent indicating that post-migratory NCC display overlapping but subtly unique transcriptional networks that likely convey the specific transcriptional information for normal development. Collectively, these findings provide evidence that HAND factors are a component of these overlapping yet unique transcriptional inputs that interpret BMP-mediated extracellular signaling cues and GATA transcriptional cues to effect aorticopulmonary septogenesis and OFT morphogenesis within the cNCC.

5. Conclusion

This study helps to resolve why mutations in human *HAND1*, which are associated with CHDs affecting the OFT, are not directly phenocopied by mutations in mouse. *Hand1*. SHF loss-of function, on its own, does not cause OFT defects; however, *Hand1* NCC deletion does present with low penetrance CHDs. *Hand1* loss-of function phenotypes are enhanced by reduction of *Hand2* gene dosage in both SHF and NCC cell populations and NCC transcriptional enhancers of both Hand1 and Hand2 have similar transcriptional inputs and through comparison of cranial and cardiac NCC regulation display unique features that help define the difference in the post-migratory fates of these distinct cell populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- HAND1 and HAND2 genetically interact within the cardiac neural crest
- HAND1 and HAND2 double mutants reveal a mis-organization of cardiac neural crest
- *In silico* prediction of a *Hand2* cardiac neural crest enhancer based on an identified *Hand1* enhancer shows conservation in upstream transcriptional regulation inputs.

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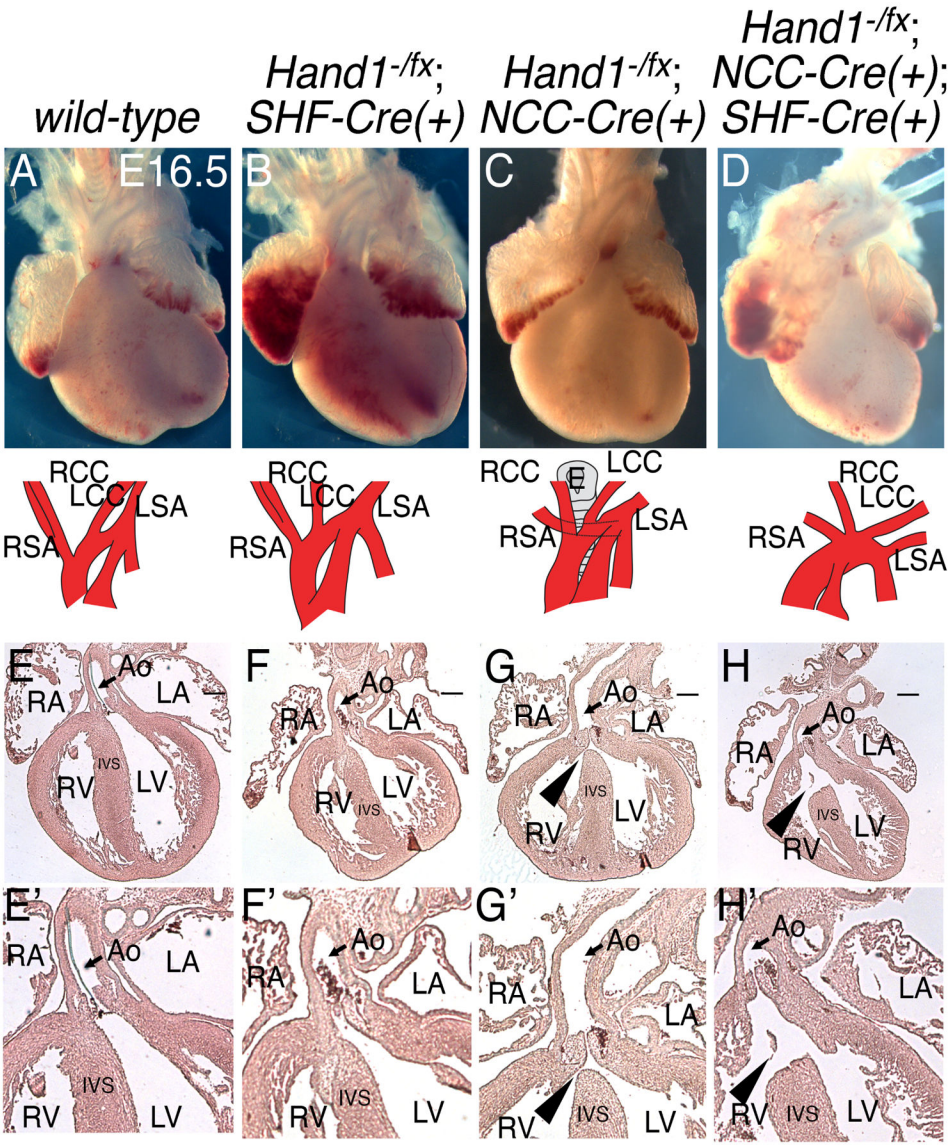

Figure 1. *Hand1* neural crest cell loss-of-function leads to a low penetrance of ventricular septal defects.

Whole mount preparations (A-D) and hematoxylin and eosin-stained (E-H) E16.5 hearts and aortic arches in which SHF- (B and F), NCC- (C and G), or both SHF and NCC-specific (D and H) *Hand1* has been conditionally ablated reveals rare aortic arch patterning defects (C), and a low penetrance of VSDs (G and H, arrowheads) in NCC *Hand1* conditional knockouts. Aortic arches are schematized in A-D. Scale bars = 250 mm. Arrowhead, VSD; Asterisk, PTA; Ao, aorta; LA, left atrium; LCC, left common carotid; LSA, left subclavian artery; LV, left ventricle; RA, right atrium; RCC, right common carotid; RSA, right subclavian artery; RV, right ventricle. (E'-H') Higher magnification images of E-H to better show structures.

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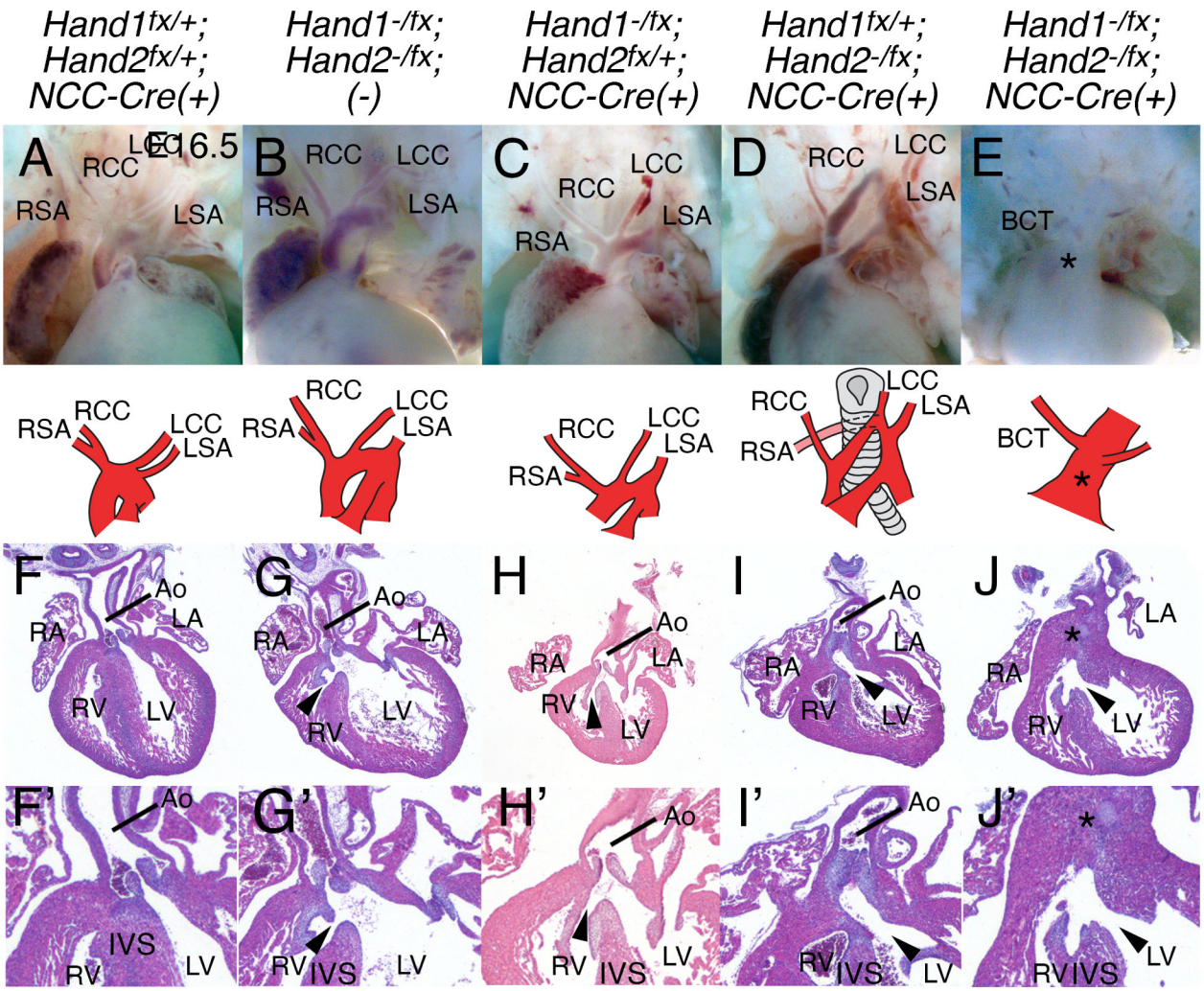

Figure 2. *Hand1* and *Hand2* genetically interact in the cardiac NCCs.

Whole mount preparations (A-E) and hematoxylin and eosin-stained (F-J) E16.5 hearts and aortic arches in which NCC-specific *Hand1* and/or *Hand2* has been conditionally ablated. Aortic arches are schematized in A-E. Arrowhead, VSD; Asterisk, PTA; Ao, aorta; BCT, brachiocephalic trunk; LA, left atrium; LCC, left common carotid; LSA, left subclavian artery; LV, left ventricle; RA, right atrium; RCC, right common carotid; RSA, right subclavian artery; RV, right ventricle. (F'-J') higher magnification images of F-J to better show structures.

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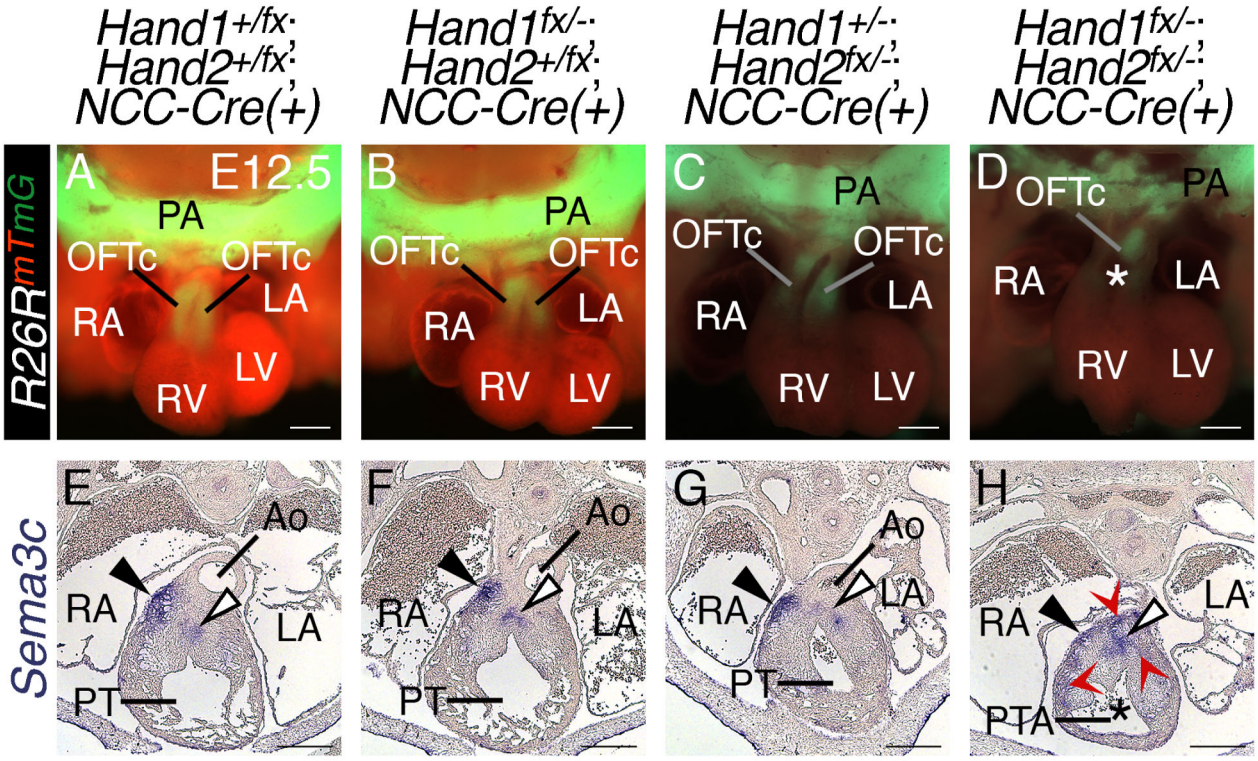

Figure 3. *Hand1* and *Hand2* regulate cardiac NCCs localization in the developing OFT.

Wnt1-Cre;R26R^{mtmg} lineage mapping *Control* (A), *H1CKO*, *Hand2* heterozygous (B and C) and *H1CKO;H2CKO* E12.5 day embryos (D). two outflow tract cushions (OFTc) are visible in control and *H1CKO; H2* heterozygous hearts; however, only a single cushion is visible in the double knockout hearts. The class 3 semaphorin SEMA3C promotes septal bridge formation (white arrowhead). *Sema3c in situ* hybridization in *Control* (E) and *H1CKO; H2* heterozygous hearts display *Sema3c* expression consistent with controls (F and G). *Hand1*$^{fx/-}$;*Hand2*$^{fx/-}$;*Wnt1-Cre(+)* mutants; however, exhibit mis-localized *Sema3c*-expressing cNCCs (H, red arrow heads). As *Sema3c* marks the septal bridge, this structure appears malformed within double CKOs. All double CKOs present with persistent truncus arteriosus (PTA, n=5/5). pharyngeal arch, PA; Myocardial cuff, denoted by black arrowhead; aorta, Ao; pulmonary trunk, PT; left atrium, LA; right atrium, RA; left ventricle, LV; right ventricle, RV; white asterisk, PTA

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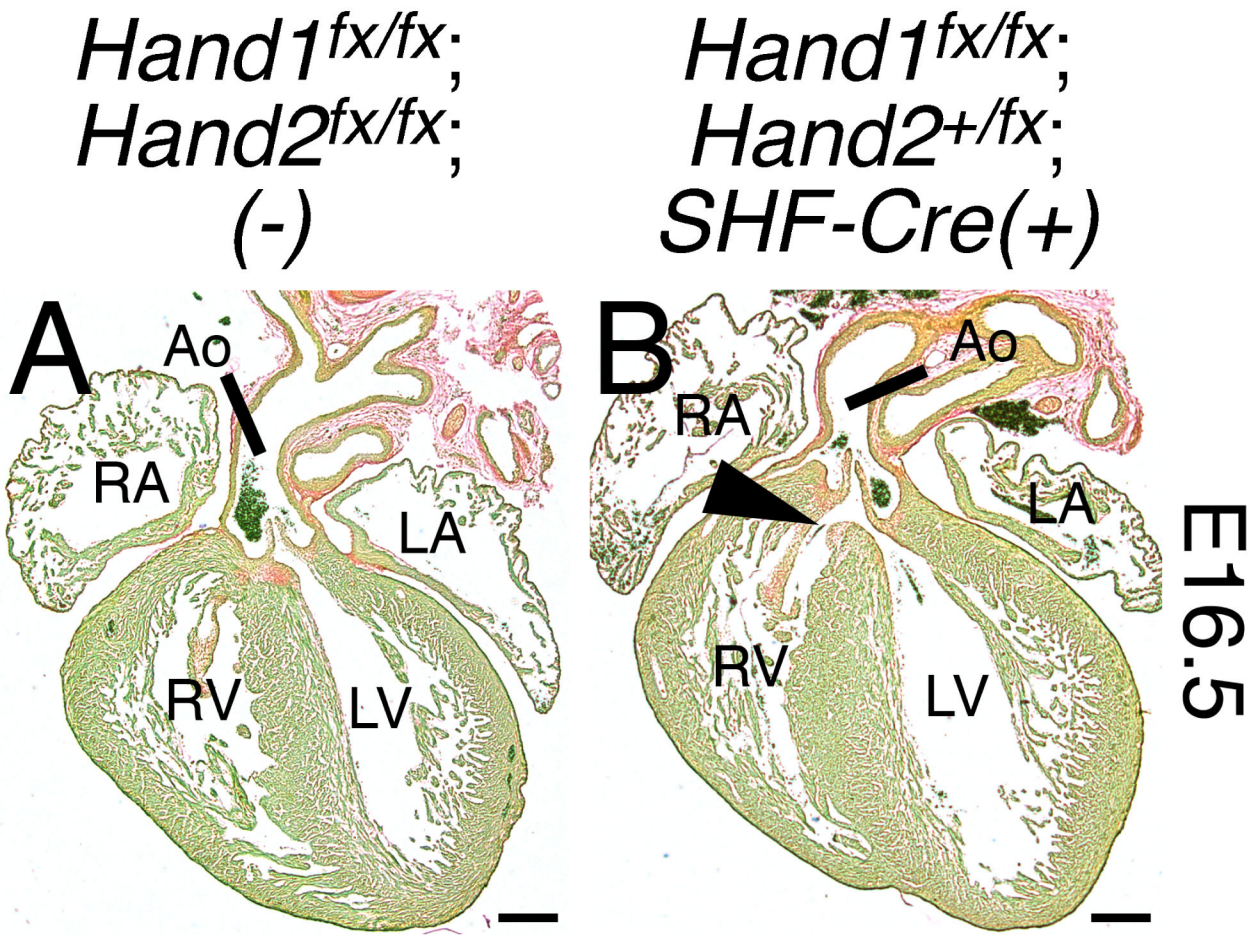

Figure 4. *Hand1;Hand2* genetic interaction in the second heart field.

E16.5 hearts in which *Hand1* has been ablated and *Hand2* function has been reduced by half within the SHF (*Hand1*$^{fx/fx}$;*Hand2*$^{fx/+}$;*Mef2c-AHF-Cre(+)*) display a low penetrance of VSDs (B, arrowhead; summarized in Table 3). Coronal sections have been stained with Sirius Red and Fast Green. Scale bars = 250 μm. Ao, aorta; LA, left atrium; RA, right atrium; LV, left ventricle; RV, right ventricle.

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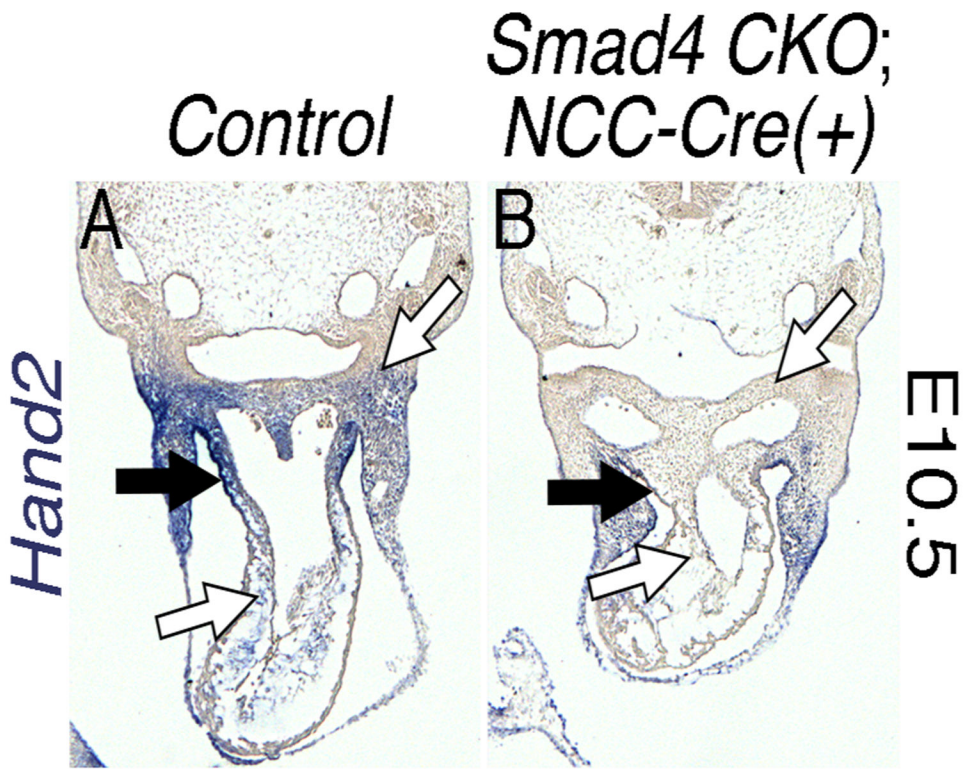

Figure 5. *Hand2* expression is lost in both the cardiac neural crest cells and the myocardial cuff of Smad4 conditional knockouts.

In situ hybridization shows that *Hand2* expression is lost in both the cNCCs (B, white arrows) and MC (B, black arrows) of NCC-specific (*Wnt1-Cre*) *Smad4* CKOs (n=3/3).

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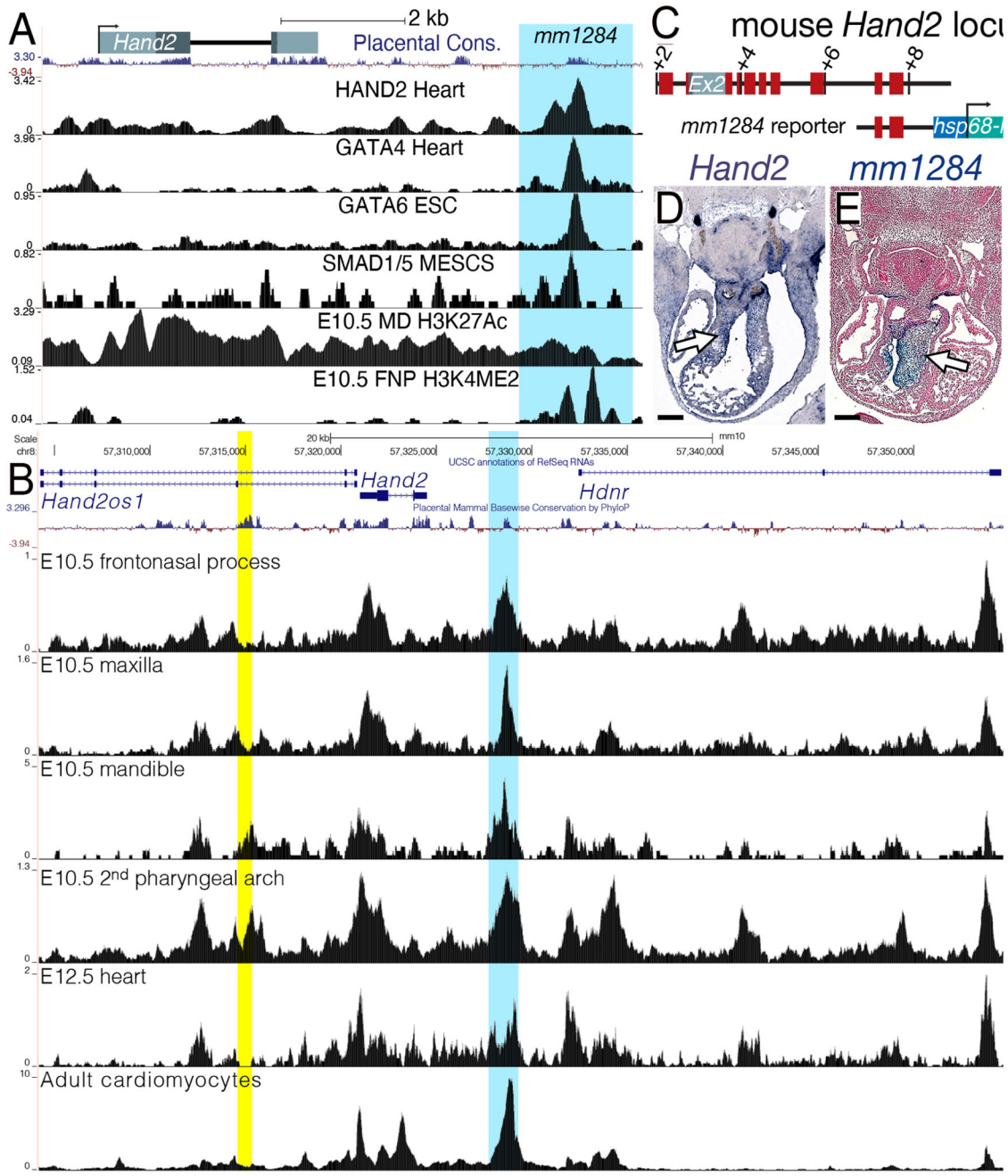

Figure 6. Identification of an endocardial cushion-specific *Hand2* enhancer.

A) Schematic of the mouse *Hand2* locus. Peaks of enrichment for HAND2, GATA4/6, and SMAD1/5 overlap with a region, evolutionarily conserved among placentals, that displays histone marks for active transcription in NCCs (H3K4ME2 in the E10.5 fronto-nasal processes (FNP) and H3K27Ac in the mandibular pharyngeal arch (MD). This *mm1284* sequence is highlighted in blue. B) ATAC-seq from E10.5 frontonasal process, maxilla, mandible, and 2nd pharyngeal arch (Minoux et al., 2017), E12.5 hearts (Zhou et al., 2017), and adult cardiomyocytes (Monroe et al., 2019) (Yellow shading highlights the *Hand2* mandibular arch enhancer. (Charite et al., 2001) C) Schematic of the *mm1284+hsp68-lacZ*

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reporter construct. Red blocks denote regions of significant sequence conservation D) *Hand2* section *in situ* hybridization showing expression in the E11.5 OFT cushions (white arrow). E) Section of an X-gal stained E11.5 *mm1284+hsp68-lacZ* transgenic embryo showing expression in the OFT cushions (white arrow). n = 5/7. Scale bars = 250 μm.

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Table 1.

Congenital heart defects in OFT *Hand1* mutants.

Genotype	n	AoRSA	membranous VSD	ASD	Phenotypically Normal
Hand1$^{+/+}$; *Wnt1-Cre(+); Mef2c-AHF-Cre(+)*	6	0 (0%)	0 (0%)	0 (0%)	6 (100%)
Hand1$^{-/fx}$; *Mef2c-AHF-Cre(+)*	5	0 (0%)	0 (0%)	0 (0%)	5 (100%)
Hand1$^{-/fx}$; *Wnt1-Cre(+)*	9	1 (11.1%)	3 (33.3%)	0 (0%)	6 (66.6%)
Hand1$^{-/fx}$; *Wnt1-Cre(+); Mef2c-AHF-Cre(+)*	4	0 (0%)	2 (50.0%)	1 (25.0%)	2 (50.0%)

AoRSA, aberrant origin of the right subclavian artery; VSD, ventricular septal defect; ASD, atrial septal defect

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Table 2.

OFT defects in *HAND* factor mutants.

Genotype	n	PTA + VSD	IAA-B	AoRSA	DORV + VSD	Dilated and/or hypertrophic LV	Bicuspid Ao and PT valves	Phenotypically Normal
Hand1$^{+/fx}$; *Hand2*$^{+/fx}$; *Wnt1-Cre(+)*	7	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	7 (100%)
Hand1$^{fx/-}$; *Hand2*$^{fx/-}$; *(−)*	7	0 (0%)	0 (0%)	0 (0%)	7 (100%)	7 (100%)	0 (0%)	0 (0%)
Hand1$^{fx/-}$; *Hand2*$^{fx/-}$; *Wnt1-Cre(+)*	9	0 (0%)	0 (0%)	0 (0%)	5 (55.6%)	1 (11.1%)	1* (11.1%)	3 (33.3%)
Hand1$^{+/fx}$; *Hand2*$^{fx/-}$; *Wnt1-Cre(+)*	9	0 (0%)	3 (33.3%)	6 (66.7%)	9 (100%)	4 (44.4%)	0 (0%)	0 (0%)
Hand1$^{fx/-}$; *Hand2*$^{fx/-}$; *Wnt1-Cre(+)*	3	3 (100%)	0 (0%)	0 (0%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)

AoRSA, aberrant origin of the right subclavian artery; DORV, double outlet right ventricle; IAA-B, interrupted aortic arch type-B; PTA, persistent truncus arteriosus; VSD, ventricular septal defect.

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Table 3.

OFT defects in *HAND* factor SHF mutants.

Genotype	n	membranous VSD	Phenotypically Normal
Hand1$^{fx/fx}$; Hand2$^{+/fx}$; (−)	3	0 (0%)	3 (100%)
Hand1$^{+/fx}$; Hand2$^{+/fx}$;SHF-Cre(+)	8	0 (0%)	8 (100%)
Hand1$^{fx/fx}$;Hand2$^{+/fx}$;SHF-Cre(+)	12	3 (25%)	9 (75%)

VSD, ventricular septal defect.

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