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STEM CELLS AND REGENERATION

RESEARCH ARTICLE

Delta-like ligand 4-mediated Notch signaling controls proliferation of second heart field progenitor cells by regulating Fgf8 expression

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ABSTRACT

The role played by the Notch pathway in cardiac progenitor cell biology remains to be elucidated. Delta-like ligand 4 (Dll4), the arterial-specific Notch ligand, is expressed by second heart field (SHF) progenitors at time-points that are crucial in SHF biology. Dll4-mediated Notch signaling is required for maintaining an adequate pool of SHF progenitors, such that *Dll4* knockout results in a reduction in proliferation and an increase in apoptosis. A reduced SHF progenitor pool leads to an underdeveloped right ventricle (RV) and outflow tract (OFT). In its most severe form, there is severe RV hypoplasia and poorly developed OFT resulting in early embryonic lethality. In its milder form, the OFT is foreshortened and misaligned, resulting in a double outlet right ventricle. Dll4-mediated Notch signaling maintains Fgf8 expression by transcriptional regulation at the promoter level. Combined heterozygous knockout of *Dll4* and *Fgf8* demonstrates genetic synergy in OFT alignment. Exogenous supplemental Fgf8 rescues proliferation in *Dll4* mutants in *ex-vivo* culture. Our results establish a novel role for Dll4-mediated Notch signaling in SHF biology. More broadly, our model provides a platform for understanding oligogenic inheritance that results in clinically relevant OFT malformations.

KEY WORDS: Cardiac development, Delta-like ligand 4, Notch signaling, Outflow tract, Second heart field

INTRODUCTION

In the developing embryo, the heart forms from bilateral fields of two mesodermal cell progenitors in the lateral plate mesoderm, namely the first heart field (FHF) and second heart field (SHF) (Vincent and Buckingham, 2010; Lin et al., 2012). The FHF cells continually migrate to the midline and fuse to form a primitive heart tube (Lin et al., 2012). As the heart tube elongates, SHF cells are added to the arterial and venous poles. Following looping of the heart, the venous pole is placed dorsal to the arterial pole and ventricular septation ensues (Lin et al., 2012). Whereas the majority of the left ventricle (LV) is formed from FHF cells, the right ventricle (RV) is derived from cells that originated in the SHF. The outflow tract (OFT) is also exclusively derived from the SHF (Cai et al., 2003; Kelly et al., 2001; Mjaatvedt et al., 2001; Rochais et al., 2009; Waldo et al., 2001) and initially exits primarily from the RV. As more SHF cells are added and ventricular septation progresses, the OFT is divided by migrating neural crest cells that originated in the caudal end of the cranial neural crest. Simultaneously, the developing OFT is also aligned such that each semilunar valve exits from the respective ventricle and connects to the appropriate outflow vessel. Congenital heart disease (CHD) is the most common and most expensive birth defect in the United States, affecting ~40,000 live births per year (Hoffman and Kaplan, 2002). Given that both right and left ventricular outflow tracts originated from the same pool of progenitors and matured through a series of intricate spatially and temporally controlled molecular events, it is not surprising that OFT defects are seen in nearly 30% of all CHDs.

Notch signaling plays a crucial role in development, in general, and heart development, in particular (Luxán et al., 2016; MacGrogan et al., 2010; de la Pompa and Epstein, 2012). Animal studies have shown that endocardial Notch signaling regulates cell fate specification and tissue patterning in the early vertebrate heart to define chamber versus valve domains (Luxán et al., 2016). In addition, Notch also plays a role in epithelial-to-mesenchymal transformation and is thereby crucial for valve maturation (High et al., 2009; Luxán et al., 2016). During ventricular chamber development, Notch signaling initially regulates cardiomyocyte proliferation, and subsequently promotes trabecular patterning, maturation and compaction. It has been suggested that this switch may result from an alteration in the expression of, and affinity to, the two families of Notch ligands, namely Delta and Jagged. With particular reference to the OFT, Notch mutations have been implicated in clinical disorders, such as bicuspid aortic valve (McKellar et al., 2007; Garg, 2016) and aortic valve calcification (Garg, 2016). Mutations in the Notch ligand, jagged 1, have been implicated in Alagille syndrome (Li et al., 1997). Hes1, a transcriptional factor activated by Notch signaling, has been shown to play a crucial role in the deployment of SHF progenitor cells, such that loss of Hes1 leads to OFT defects (Rochais et al., 2009). Previous studies (High et al., 2009) have suggested a role for Notch (and its ligand jagged 1) in endothelial-to-mesenchymal transition within the endocardial cushions of the OFT as a potential mechanism underlying the clinical defects. However, what role Notch plays (and via what ligand) in SHF progenitor cell biology remains to be elucidated.

Delta like ligand 4 (Dll4) is a unique arterial endothelial-specific ligand of Notch (Duarte et al., 2004). Dll4 plays a distinctive, dosage-sensitive role in arterial maturation, such that haploinsufficiency results in vascular maturation arrest and embryonic lethality by embryonic day (E)10.5 in mice (Duarte et al., 2004). Mutant embryos

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show ventricular trabeculation abnormalities and a paucicellular OFT. However, the early lethality in these mutants precludes a detailed analysis of the specific role played by Dll4 in cardiac development. To that end, we used cardiac-specific Cre lines to ablate Dll4 expression in SHF progenitors to study its role in OFT development. Our data show that Dll4 is expressed in the early SHF progenitor cells and Dll4-mediated Notch signaling is crucially required to maintain SHF proliferation and an adequate pool of SHF progenitors for incorporation into the developing OFT. Loss of Dll4 in SHF leads to a spectrum of OFT abnormalities ranging from a shallow ventricular septal defect (VSD) with an overriding aorta, to an overt double outlet right ventricle (DORV) with aorta arising entirely from the RV. We further demonstrate that Dll4-mediated Notch signaling is required to maintain levels of key proteins in SHF biology, including Fgf8, and synergizes with Fgf8 to regulate SHF proliferation.

RESULTS

Dll4 is expressed at relevant sites and time-points during OFT formation in the developing heart

We began by evaluating Dll4 expression using multiple modalities in the developing heart at various embryonic stages and into the neonatal period (Fig. 1, Fig. S1). There was good correlation between Dll4 protein (immunofluorescence; IF) and transcript (*in situ* hybridization; ISH) expression. As a complementary technique, we used stable transgenic founder mouse lines in which the non-coding region (F2) in the third intron of *Dll4* drives a minimum promoter *lacZ* reporter (*F2-lacZ*) (Wythe et al., 2013). *lacZ* expression serves as a surrogate for *Dll4* expression in these animals. This enhancer element was identified specifically for activity in the arterial endothelial elements and, as such, we found that *lacZ* expression was strongly observed in arterial endothelial cells and OFT endocardium and faithfully phenocopied Dll4 protein expression in this regard, as previously reported (Wythe et al., 2013). Although expression was also observed in the ventricles and SHF progenitor cells, the level of expression was significantly lower compared with IF or ISH. This difference is likely because of lower levels of expression of this particular enhancer in these cells.

Dll4 expression was discernible in the pharyngeal mesodermal region as early as E8.5 (Fig. S1A-A″). Between E9.5-E11.5, at a time of intense SHF proliferation and incorporation into the developing heart, robust expression of Dll4 was observed in the area of the SHF and developing OFT. By E9.5, Dll4 was broadly expressed in the region of SHF progenitors in the pharyngeal mesoderm on both transverse (Fig. 1A,A′,B, Fig. S1B,B′) and sagittal (Fig. 1C,C′) sections. Dll4 expression in this region was confirmed by X-gal staining in *F2-lacZ* mice (Fig. 1D-F′, Fig. S1G,G′). Similarly, at E10.5, strong Dll4 expression could be demonstrated in the region of SHF progenitors by IF (Fig. 1G,G′,H,I,I′, Fig. S1C,C′), ISH (Fig. 1M,M′,N,O,O′) and X-gal staining (Fig. 1J,J′,K,L,L′). To specifically evaluate Dll4 expression in SHF cells, we lineage traced SHF by crossing *Mef2c-AHF-Cre* (Verzi et al., 2005) mice with *Rosa26-tdTomato* (*R26R, tdT*) mice and stained sections for Dll4. At E9 (Fig. 1R-R‴) and E10.5 (Fig. 1S-S‴), tdT-positive cells in the pharyngeal mesoderm co-expressed Dll4, confirming SHF expression. Complementarily, we co-stained sections with Islet1 (Isl1), which is specifically expressed by SHF progenitor cells at this time point. There was significant overlap between Islet1 and Dll4 expression in the pharyngeal mesoderm (Fig. 1P-P‴, Fig. S1Q-Q‴), confirming that Dll4 is indeed expressed by SHF progenitor cells. Double staining with the endothelial-specific marker, CD31 (Pecam1), and Dll4 showed that a distinct set of endothelial cells (presumably of arterial origin) in this region also expressed Dll4 (Fig. 1T-T‴). In contrast, airway epithelium marked by Nkx2-1 did not express Dll4 (Fig. S1R-R′). By E11.5, Dll4 expression was still seen in the pharyngeal mesodermal region of SHF cells (Fig. S1E,E′,I,I′), but was lost at later embryonic time-points (Fig. S1J). We then evaluated the expression of jagged 1, the other Notch ligand of relevance in OFT development (High et al., 2009), in SHF progenitors. Jagged 1 expression was barely detectable in Islet1-positive SHF progenitor cells in the pharyngeal mesoderm at E10.5 (Fig. 1Q-Q‴), whereas there was more robust expression seen in atrial and ventricular myocardium.

The developing OFT, which is derived from SHF progenitors, also displayed strong Dll4 expression. At E9.5, both IF (Fig. 1B,B′,C,C′, Fig. S1B) and X-gal staining (Fig. 1E,E′,F,F′, Fig. S1G) confirmed Dll4 expression in the OFT. By E10.5, there was robust Dll4 expression in the OFT endocardium and a lower, yet detectable, expression in the OFT myocardium by all three modalities (Fig. 1H,H′,I,I′,K,K′,L,L′,N,N′,O,O′, Fig. S1H,H′). By E11.5, OFT expression was weaker (Fig. S1F,F′).

Atrial and ventricular endocardium also showed robust Dll4 expression up to E12.5. SHF-derived RV endocardium expressed Dll4 from E9.5 through E12.5 (Fig. 1A,A″,D,D″,G,G″,J,J″,M,M″, Fig. S1C,C″,E,E″,J,J′). At earlier time-points, Dll4 expression was present, but much less robust, in RV myocardium (Fig. 1A″,G″, Fig. S1A″), and myocardial expression was mostly lost by E11.5 (Fig. S1E″). Endocardial expression was reduced from E14.5 (Fig. S1K,L) and, by birth, Dll4 expression was observed only in coronary arterial elements (Fig. S1M,M′). Such a temporal variability in the expression of Notch ligands in the developing ventricle has been previously reported (de la Pompa and Epstein, 2012). In all the sections evaluated, Dll4 was also expressed in the dorsal aorta as expected, but not in the adjacent cardinal vein, confirming specificity of the signal observed (Fig. 1D,D′,E,G,G′, Fig. S1H).

Dll4 expression in SHF is required for appropriate development of SHF-derived RV and OFT

Global knockout of *Dll4* is embryonically lethal due to vascular maturation arrest (Duarte et al., 2004). No mutant survived past E10.5, with the majority dying even earlier. The few mutants that survived to E10.5 were severely underdeveloped and demonstrated arrested cardiac development and a very poorly developed OFT (Fig. S2A,A′), precluding detailed analysis of cardiac-specific effects. To circumvent this early mortality, we conditionally knocked out *Dll4* expression in SHF using specific Cre lines. We used both *Islet1-Cre* (more global SHF expression, Cai et al., 2003) and *Mef2c-AHF-Cre* (anterior SHF-specific expression, Verzi et al., 2005) lines. Efficient recombinase-mediated loss of Dll4 in the SHF was confirmed at E9.5 in *Mef2c-AHF-Cre,Dll4*$^{F/F}$ embryos, which showed loss of expression in the pharyngeal mesoderm (Fig. S2B,B′) and SHF-derived RV, but persistent robust expression in the LV (Fig. S2B,B″).

Depending on the time and extent of *Dll4* knockout, a spectrum of phenotypic defects was observed in mutant embryos. Homozygous deletion of *Dll4* in a more extensive population of cells (*Islet1-Cre,Dll4*$^{F/F}$) resulted in complete lack of RV and poorly developed hearts at E10.5 (Fig. S2C,C′). There was early embryonic lethality such that, by E10.5, only 13% of mutants (25% predicted by Mendelian inheritance) could be recovered. No live embryo was recovered by E14.5 (Fig. 2M). More restricted loss of expression in anterior SHF only (*Mef2c-AHF-Cre,Dll4*$^{F/F}$) also resulted in

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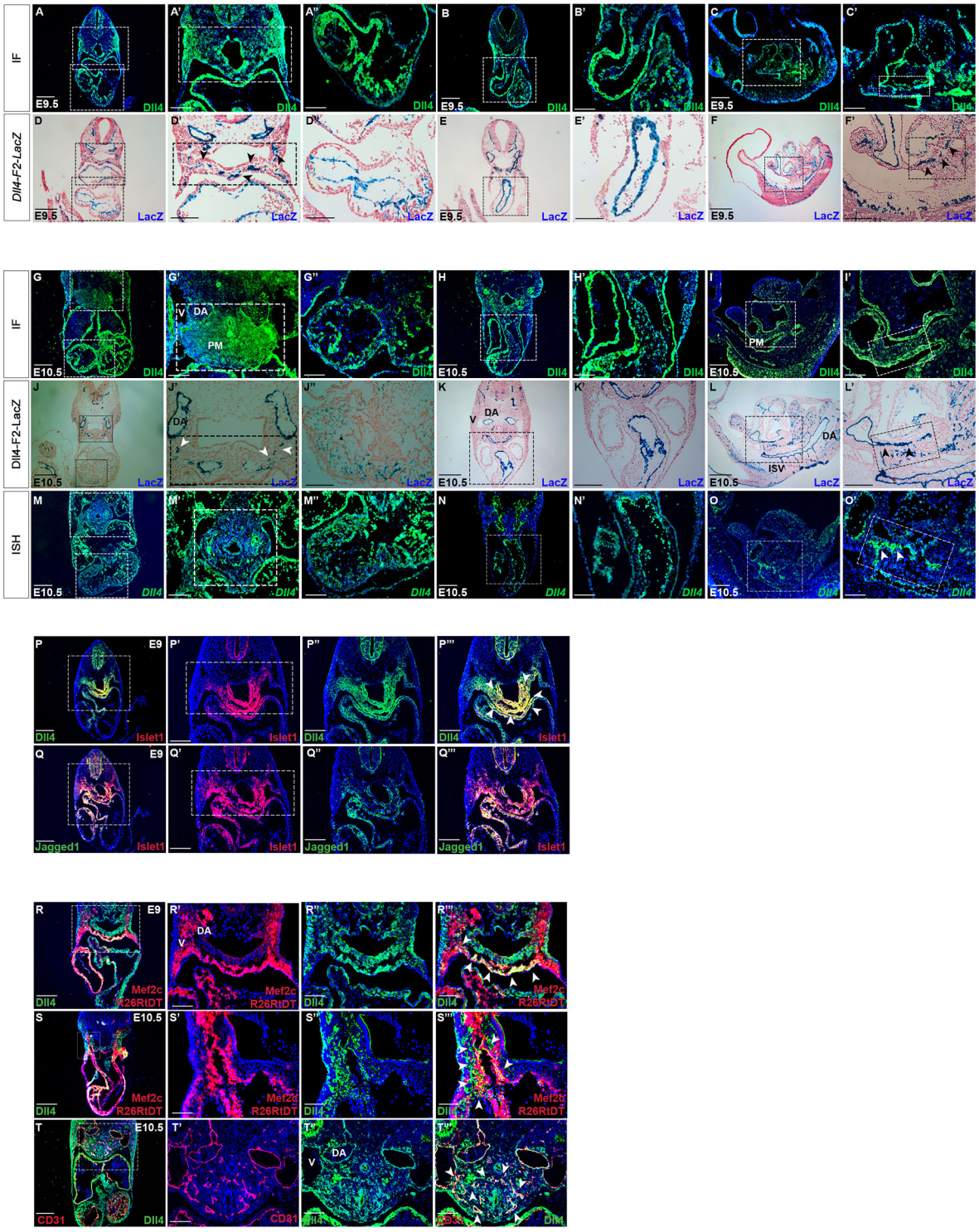

Fig. 1. See next page for legend.

recovery of fewer embryos at E14.5 than predicted (6% versus predicted 25%). All six mutants recovered live at E14.5 displayed DORV (Fig. 2A′,B′, Fig. S3B). These embryos had a large VSD (arrow in Fig. 2A′). The OFT was appropriately septated with distinct aortic and pulmonary valves (Fig. S3B,19-22 and 34-36). However, the aortic valve originated from the RV (A in Fig. 2B′) at the same level as the pulmonary valve (side-by-side orientation). The aortic valve connected to the aorta and the pulmonary valve to the pulmonary artery appropriately. This implies that *Mef2c-AHF-Cre*-mediated *Dll4* knockout impacts OFT alignment without any impact on septation. We interpret these findings to indicate that more extensive knockout of *Dll4* in SHF results in a severe reduction in SHF-derived RV and OFT resulting in early embryonic lethality, but anterior SHF-specific loss leads to less severe reduction in the size of these SHF-derived structures. The resultant shorter OFT is incapable of expanding to align itself

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Fig. 1. Dll4 is expressed by SHF progenitor cells and SHF-derived structures in the developing heart. (A-O′) Representative images of E9.5 and E10.5 embryos are shown. Dll4 protein expression (IF) was studied in E9.5 transverse (A,B) and sagittal fixed-frozen sections (C). Dll4 is expressed in the pharyngeal mesoderm (PM) in the SHF progenitor cell region (A,C; higher magnification of upper boxed area of A and C in A′ and C′, respectively). Transverse sections demonstrate expression in RV endocardium and myocardium (A; higher magnification of lower boxed area of A in A″) and developing OFT (B; higher magnification of boxed area of B in B′). X-gal staining in *Dll4-F2-lacZ* embryos was used as a complementary method to assess Dll4 expression (D-F′). Comparable sections show staining in the PM (arrowheads in D′ and F′), developing RV (D; higher magnification of upper and lower boxed areas of D in D′ and D″, respectively) and OFT (E; higher magnification of boxed area of E in E′). E10.5 embryos also demonstrate a very similar pattern of Dll4 expression on IF (G-I′) and X-gal staining in *Dll4-F2-lacZ* embryos (J-L′). The dorsal aorta (DA in G′ and J′) expresses Dll4, whereas the adjacent cardinal vein (V) does not. *Dll4* transcript expression was evaluated using ISH (M-O′). (P-T‴) Comparable sections again show staining in PM, developing RV and OFT. Transverse sections were co-stained with Islet1 and Dll4 (P) or jagged 1 (Q) at E9. Higher magnification of the boxed areas in P and Q are shown as Islet1 expression (P′,Q′), Dll4 expression (P″), jagged 1 expression (Q″) and merged image (P‴,Q‴) to demonstrate the robust expression of Dll4 and the lack of expression of jagged 1 in the PM. Transverse sections were stained for Dll4 in Mef2c lineage traced embryos at E9 (R) and E10.5 (S). Higher magnification of the boxed areas in R and S are shown as Mef2c expression (R′,S′), Dll4 expression (R″,S″) and merged image (R‴,S‴) to demonstrate co-localization of Dll4 on SHF-expressing cells in the PM. Transverse sections of E10.5 embryos were co-stained for Dll4 and vascular endothelial marker (CD31) (T). Higher magnification of the boxed area in T is shown as CD31 expression (T′), Dll4 expression (T″) and merged image (T‴) to demonstrate co-localization of Dll4 and CD31 expression in (arterial) endothelial elements in the pharyngeal mesoderm. The boxed regions of A′, D′, G′, J′, M′, P′ and Q′ in transverse sections and C′, F′, I′, L′ and O′ in sagittal sections indicate the region occupied by SHF progenitor cells. DA, dorsal aorta; ISV, inter-somitic vessels. Scale bars: 50 µm (R′-S‴); 100 µm (A′-C′, G′-I′,M′-O′,P′-Q‴,R,T′-T‴); 150 µm (D′-F′,J′-L′); 250 µm (A-C,G-I,M-O,P,Q,S, T); 300 µm (D-F,J-L).

appropriately over the developing RV and LV, resulting in DORV. All of these conditional mutants showed cardiac inflow tract (venous pole) development that was appropriate for embryonic stage, implying that loss of Dll4 mediated by these Cre lines did not result in inflow tract defects. Similarly, lung development was also appropriate for age in all mutants examined.

We then examined mutant embryos in the *Mef2c-AHF-Cre* background at earlier time-points. At E9.5, the RV appeared to be slightly smaller in mutants (Fig. S2D,D′,E) and the OFT was foreshortened (Fig. S2F,F′,G). By E10.5, this difference was clearly evident on both whole-mount evaluation (Fig. S2H,H′) and sections (Fig. 2C,C′). In addition, the OFT was also much shorter and paucicellular (asterisk in Fig. 2H′ compared with Fig. 2H) in the mutants. We then proceeded to label the SHF-derived structures by breeding in the *Rosa26-lacZ* (*R26R,lacZ*) gene into our mutant crosses in order to quantitate this reduction. At E10, X-gal stained hearts were examined for the size of the *lacZ*-positive RV and OFT. Whole-mount examination confirmed that mutant hearts had a smaller RV (Fig. 2D,D′,F) and shorter narrower OFT (Fig. 2I,I′,K) compared with controls. Similarly, evaluation of sections showed that the mutant RV was 50% smaller than control RV (Fig. 2E,E′,G) indexed to the size of the respective LV. The OFT was also 50% shorter in length (Fig. 2J,J′,L).

Global heterozygous loss of Dll4 demonstrates incompletely penetrant haploinsufficiency resulting in vascular maturation defects and embryonic lethality. We therefore sought to evaluate whether heterozygous loss of Dll4 in these two Cre backgrounds had any phenotypic consequence. Of the 14 *Islet1-Cre,Dll4*$^{F/wt}$ embryos examined at E14.5, six (43%, Fig. 2M) demonstrated DORV (Fig. 2A″,B″, Fig. S3C) with a large VSD and aorta that arose from the RV. The aortic valve was appropriately located caudal to the pulmonary valve, implying that the sub-pulmonary conus was well developed in these mutants (Fig. S3C, 15-18 and 30-34). The remaining eight embryos had normal cardiac anatomy. In contrast, all 38 *Mef2c-AHF-Cre,Dll4*$^{F/wt}$ mice examined at E14.5 had normal anatomy. These mice were born alive and grew and reproduced normally. Our results indicate that Dll4 expression is required for appropriate development of SHF-derived RV and OFT. The observed phenotypes range from complete lack of RV and OFT following more extensive knockout, to a fully penetrant DORV following anterior SHF-specific knockout, or an incompletely penetrant DORV with partial loss and normal heart development in the setting of partial loss of Dll4 in a more restricted pool of SHF cells.

Dll4 expression is required for SHF cell proliferation to maintain an adequate progenitor cell pool

The observed mutant phenotypes suggest that there is a reduction in the number of SHF progenitor cells that are incorporated into the developing heart in *Dll4* mutants. One potential mechanism to explain this finding would be an inadequate pool of SHF progenitors available for incorporation. To directly test this hypothesis, we fate-mapped SHF cells (*Mef2c-AHF-Cre,R26R, lacZ*) in the pharyngeal mesodermal region. Both in transverse (Fig. 3A-C) and sagittal (Fig. 3D-F) sections, the area occupied by *lacZ*-positive SHF progenitor cells was significantly reduced in mutants (by 67% and 50%, respectively). This would imply that the reduction in SHF-derived structures in the heart is due to a reduction in the size of SHF progenitor pool. We studied SHF proliferation to explain this reduction in SHF progenitor pool. To this end, control and mutant embryos were stained for Islet1 to mark SHF cells and phosphorylated histone H3 (pHH3) to identify proliferating cells. Double-positive cells in the region of the pharyngeal mesoderm were counted in controls and mutants. At E9.5 (Fig. 3G-I), *Dll4* knockout resulted in a 51% reduction in proliferating SHF cells, whereas there was no change in proliferation of Islet1-negative non-SHF cells. This proliferation defect persisted to E10.5 (Fig. S4A-C), wherein a 72% reduction in proliferating SHF cells was observed. Given that SHF proliferation was impacted by E9.5, we studied apoptosis in SHF cells a day later by double staining for Islet1 and TUNEL. *Dll4* knockout resulted in a significant increase in SHF progenitor cell apoptosis at E10.5 (Fig. 3J-L). Taken together, these results indicate that Dll4 expression in SHF maintains SHF cell proliferation during the crucial time period between E9-E11 when these cells are being actively incorporated into the developing heart. Loss of Dll4 expression results in reduced proliferation of SHF cells and their subsequent apoptotic loss. These events lead to a significant reduction in the pool of progenitor cells available for incorporation into the developing heart, which in turn leads to a reduction in the size of SHF-derived RV and OFT, and the resultant phenotypes described above.

Dll4-mediated Notch signaling regulates Fgf8 expression in SHF

We sought to identify the molecular mechanisms that act downstream of Dll4-mediated Notch signaling to regulate SHF proliferation. We evaluated expression levels of various molecules with relevance to SHF biology. Fgf8 is a key regulator of SHF proliferation and maturation (Ilagan et al., 2006; Park et al., 2006; Fischer et al., 2002). We studied Fgf8 expression in SHF of control and mutant embryos at mRNA and protein level, by co-staining for

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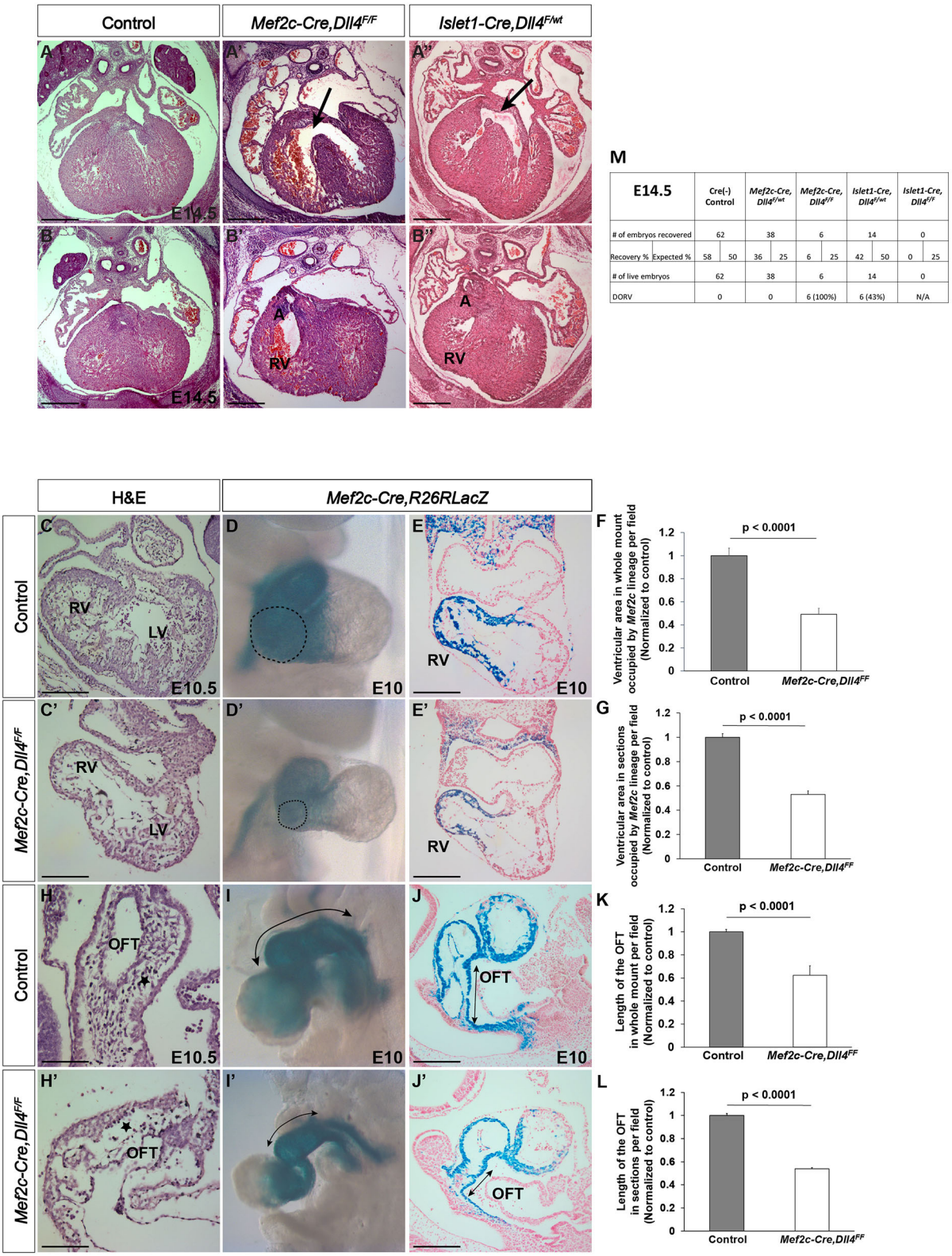

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E14.5	Cre(-) Control		Mef2c-Cre, Dll4F/wt		Mef2c-Cre, Dll4F/F		Islet1-Cre, Dll4F/wt		Islet1-Cre, Dll4F/F	
# of embryos recovered	62		38		6		14		0	
Recovery % Expected %	58	50	36	25	6	25	42	50	0	25
# of live embryos	62		38		6		14		0	
DORV	0		0		6 (100%)		6 (43%)		N/A	

Fig. 2. See next page for legend.

Mef2c and Islet1, respectively. Fgf8 mRNA and protein levels were markedly reduced in the pharyngeal mesoderm of mutant embryos (without significant change in areas outside SHF territory such as the neural tube) at both E9.5 (Fig. 4A-B‴,E-F‴) and E10.5 (Fig. S5A-B‴). mRNA levels of Fgf10, another important molecule in SHF maturation (Watanabe et al., 2012), were also significantly reduced in SHF (Fig. 4C-D‴), but not in the atrial wall (non-SHF-derived tissue). Hand2 is an important specification marker of RV myocardium (Tsuchihashi et al., 2011), and this also showed markedly reduced expression in RV of mutants (Fig. 4G,G′,H,H′).

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Fig. 2. Dll4 expression in SHF is required for appropriate development of SHF-derived RV and OFT. (A-L) Dll4 expression was conditionally knocked out in SHF progenitor cells using *Islet1*- or *Mef2c*-mediated Cre expression. H&E stained transverse sections of E14.5 embryos show a normally developed heart in Cre-negative littermate controls (A,B). Dll4 homozygous knockout driven by *Mef2c-AHF-Cre* (*Mef2c-Cre,Dll4*$^{F/F}$) (A′,B′) and *Dll4* heterozygous knockout driven by *Islet1-Cre* (*Islet1-Cre,Dll4*$^{F/wt}$) (A″,B″) show a large VSD (arrow in A′ and A″) and DORV (A in B′ and B″). H&E stained transverse sections of E10.5 control (C,H) and *Mef2c-Cre,Dll4*$^{F/F}$ mutant (C′,H′) embryos demonstrate hypoplastic RV and a foreshortened and paucicellular OFT (asterisk in H′) in mutants. SHF-derived structures were identified in developing heart by lineage tracing using the *R26R,lacZ* mice crossed into the *Mef2c-AHF-Cre* line. X-gal stained whole-mount, transverse and sagittal sections of E10 control (D,E,I,J) and mutant (D′,E′,I′,J′) embryos confirm hypoplastic RV and shorter and narrower OFT in mutants compared with controls. Dashed circle in D,D′ indicates area of the right ventricle; double-sided arrows in I-J′ indicate the OFT length for measurements. Area (mean±s.e.m.) of the *lacZ*-positive RV in whole-mount embryos (six control and seven mutant; F) and *lacZ*-positive ventricular wall within the entire ventricular wall (57 control and 50 mutant sections; G) was measured and normalized to control embryo in transverse sections. This shows a 50% reduction in size of SHF-derived RV in mutants (P<0.0001 by unpaired two-tailed *t*-test) by both methods. Length (mean±s.e.m.) of *lacZ*-positive OFT in whole mount embryos (six control and seven mutant; K) and *lacZ*-positive OFT normalized to control embryo in sagittal sections (10 control and nine mutant; L) was measured. This shows a 40-50% reduction in SHF-derived OFT length in mutants (P<0.0001 by unpaired two-tailed *t*-test). M indicates the number and phenotypes of embryos recovered amongst the different genotypes shown. The number of embryos recovered, the percentage recovery and the expected percentage recovery are based on Mendelian inheritance. A, aortic valve; RV, right ventricle; LV, left ventricle; OFT, outflow tract. Whole-mount magnification: ×30 (D,D′,I,I′). Scale bars: 150 µm (C,C′,H,H′,E,E′,J,J′); 300 µm (A-B″).

There was no change in the very low basal expression level in the LV (non-SHF-derived, Fig. 4G″,H″). There was no difference in the expression levels of molecules in other pathways of relevance in SHF biology, such as Bmp4 (Liu et al., 2004) or Mlc2v (Myl2; Franco et al., 1999) (Fig. S5C-F). In order to evaluate potential rotation abnormality, we stained for the sub-pulmonary rotation marker Sema3C. The extent of Sema3C expression in the developing OFT and its regionalization was not impacted in the mutants compared with controls (Fig. S5G-N). These results would imply that the primary molecular defect underlying the DORV phenotype observed in our mutants is reduced SHF progenitor cell proliferation and incorporation into the developing OFT. Alternative mechanisms, such as defective OFT rotation, although possible, appear less likely to be the primary defect.

We chose to further pursue Fgf8 regulation given its important role in SHF biology, in particular its role in SHF progenitor cell proliferation (Ilagan et al., 2006; Park et al., 2006). Ligand binding to Notch receptors results in proteolytic cleavage and release of the Notch intracellular domain (NICD). NICD binds to downstream molecules and assembles a transcriptional machinery comprised of proteins such as RBPjk (Rbpj) and master-mind-like (MAML). This complex activates transcription of Notch target genes within the nucleus. TGGGAA is the putative consensus binding sequence for RBPjk, the essential transcription factor in Notch signaling (Del Bianco et al., 2010; Castel et al., 2013). We studied the mouse chromosome 10 upstream of the *Fgf8* transcriptional start site and identified two putative RBPjk binding sites 989 and 4410 bases upstream of 5′UTR (Fig. 5A). We cloned 1 kb regions around these binding sites as well as a control 1 kb region not including either site into a promoter-less luciferase expression vector. It has been previously demonstrated that the third large intron of the *Fgf8* gene has significant enhancer activity (Gemel et al., 1999). We therefore also evaluated this intron and identified the consensus sequence for RBPjk binding within this intron. We cloned two 1 kb regions of this intron, one with and one without this binding site. These were sub-cloned into an enhancerless luciferase expression vector. We then performed a luciferase assay in two different cell lines using two different methods to quench basal Notch activity. 293T cells were treated with DAPT, a γ-secretase inhibitor, and then transfected with the various luciferase expression vectors with and without the NICD expression vector to induce Notch activity. Luciferase expression was increased 2.5-fold from baseline only when the promoter 1 construct was co-transfected with NICD, indicating that Notch signaling regulates Fgf8 expression at the promoter level (Fig. 5B). Similarly, HeLa cells also showed a 2.4-fold increase in luciferase expression when promoter 1 construct was co-transfected with NICD (Fig. S6A). Using another inhibitor of Notch protein assembly to quench basal activity, SAHM1, we were able to reproduce a 2-fold increase in luciferase expression in 293T cells with promoter 1 construct (Fig. S6B). To confirm the specificity of the binding sequence in promoter 1, we created two site-directed mutant clones of promoter 1 (Fig. 5A), both of which lost luciferase activity, confirming the veracity of the putative binding site (Fig. 5C).

Genetic synergy between Dll4-mediated Notch and Fgf8 signals in SHF proliferation

Our results thus far indicate that Dll4-mediated Notch signaling in SHF regulates Fgf8 expression to maintain SHF proliferation. Loss of Dll4 leads to loss of Fgf8 expression and a reduction in SHF proliferation and progenitor pool of cells. To confirm that Fgf8 was the key downstream molecular pathway that impacted SHF proliferation, we evaluated whether replenishing Fgf8 could rescue the loss in SHF proliferation in an *in vitro* model system. We dissected the thoracic area of E9.5 embryos and cultured them for 8 h *in vitro* in the presence of increasing doses of exogenous recombinant Fgf8. Cultured ‘organs’ were then sectioned and stained for Islet1 and pHH3 to discern the degree of SHF proliferation. Exogenous Fgf8 supplementation led to a small, statistically insignificant, increase in double-positive proliferating SHF cells in the pharyngeal area of thoracic organs from control embryos (Fig. 5D-D″,G, Fig. S6C-D″). Thoracic organs from mutant embryos exhibited a greater than 4-fold reduction in SHF proliferation compared with controls under baseline culture conditions (Fig. 5E-E″,G). Supplementation with Fgf8 led to a dose-dependent and significant increase in SHF proliferation in mutant organs (Fig. 5F-F″,G, Fig. S6E-E″). At 100 ng/µl of exogenous Fgf8, there was no difference in the number of proliferating SHF cells in mutant organs compared with controls. These data further support the hypothesis that the reduction in SHF proliferation observed with loss of Dll4 expression is primarily due to loss of Fgf8 expression.

We then studied genetic synergy between these two pathways *in vivo*. We hypothesized that because Dll4 and Fgf8 pathways impacted SHF proliferation, compound partial loss of both of these proteins would have a more penetrant SHF phenotype. *Mef2c-AHF-Cre,Dll4*$^{F/wt}$ embryos displayed normally developed hearts at E14.5 (Fig. 5I,I′,M). Partial loss of Fgf8 in SHF (*Mef2c-AHF-Cre,Fgf8*$^{F/wt}$) resulted in an incompletely penetrant phenotype. Of the seven embryos evaluated, only one (14%) demonstrated mal-alignment of the aortic valve (Fig. 5K,K′). In two (28%) other mice, a very shallow VSD with a normal OFT was encountered, and the remainder of the embryos were normal (Fig. 5J,J′,M). In contrast, 10 of the 12 (83%) embryos with concomitant partial loss of both proteins

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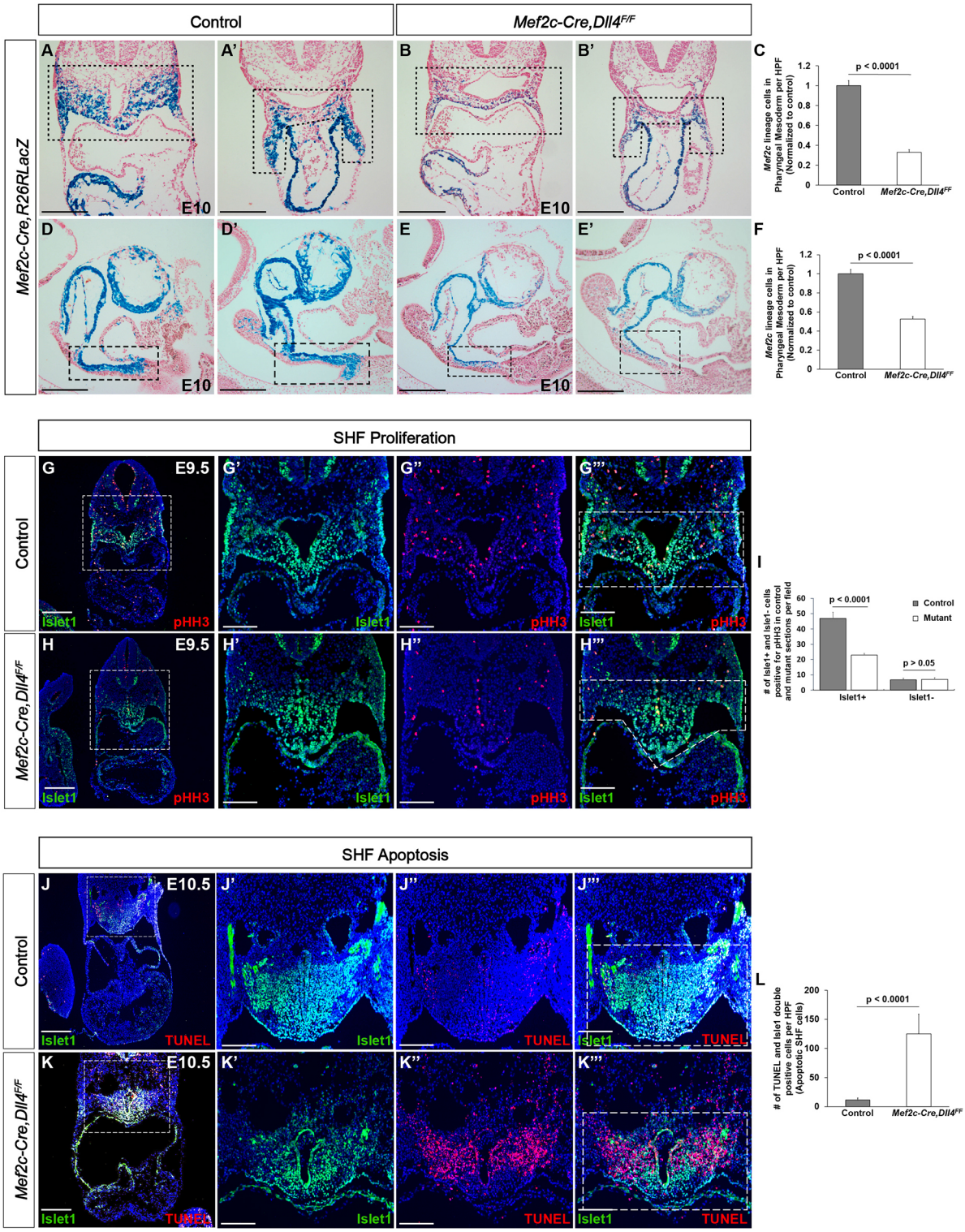

Fig. 3. See next page for legend.

(double heterozygous *Mef2c-AHF-Cre,Dll4*$^{F/wt}$*,Fgf8*$^{F/wt}$) displayed DORV (Fig. 5L,L′,M) confirming genetic synergy between these two pathways. There was a gradation in the severity of the phenotypes observed. The VSD in *Fgf8* heterozygotes was very shallow and there was only a slight displacement of the aortic valve towards the RV. The double heterozygotes showed deeper VSD and the aortic valve was more prominently overriding the ventricular septum, reminiscent of the clinically encountered tetralogy-type DORV. The aortic valve was still more caudal in location compared with the pulmonary valve in these mutants. In contrast, the *Dll4* knockout embryos had an even larger VSD and the aortic valve was completely displaced over the RV and located at the same level as the pulmonary valve (Fig 2B′ compared with Fig 5L′).

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Fig. 3. Dll4 expression is required for SHF cell proliferation to maintain an adequate progenitor cell pool. (A-F) SHF cells were lineage traced by crossing the *R26R,lacZ* mice into *Mef2c-Cre* line. Transverse (A,A′) and sagittal (D,D′) sections of control and corresponding transverse (B,B′) and sagittal (E,E′) section of *Mef2c-AHF-Cre,Dll4*$^{F/F}$ mutant E10 embryos were X-gal stained. The *lacZ*-positive area (mean±s.e.m.) within the pharyngeal mesodermal region (boxed) was measured in transverse sections (38 control and 55 mutant) and normalized to control embryos (C). Mutants demonstrated a 67% reduction in the SHF cell progenitor pool size compared with the controls (P<0.0001; unpaired two-tailed *t*-test). The *lacZ*-positive area (mean±s.e.m.) in the SHF region (boxed) was measured in sagittal sections (109 control and 89 mutant) and normalized to control embryos (F). Mutants demonstrated a 50% reduction in the SHF cell progenitor pool size compared with the controls (P<0.0001; two-tailed *t*-test). (G-I) Transverse sections of E9.5 control (G) and *Mef2c-AHF-Cre,Dll4*$^{F/F}$ mutant (H) embryos were co-stained for Islet1 and pHH3 expression to study SHF proliferation. Higher magnification of the boxed areas in G and H are shown as Islet1 expression (G′,H′), pHH3 expression (G″,H″) and merged images (G‴,H‴). Islet1 and pHH3 double-positive cells and cells positive for pHH3 but negative for Islet1 were counted separately in 21 control and 23 mutant fields within the boxed regions of G‴ and H‴ (I; mean±s.e.m.) showing a 51% reduction in proliferating SHF cells in mutants compared with controls (P<0.0001; two-tailed *t*-test), whereas there was no difference in proliferating non-SHF cells (P>0.05). (J-L) Transverse sections of E10.5 control (J) and *Mef2c-AHF-Cre,Dll4*$^{F/F}$ mutant (K) embryos were co-stained for Islet1 and TUNEL expression to study SHF apoptosis. Higher magnification of the boxed areas in J and K are shown as Islet1 expression (J′,K′), TUNEL expression (J″,K″) and merged images (J‴,K‴). Double-positive cells were counted in 21 control and 21 mutant fields each within the boxed region of J‴ and K‴ (L; mean±s.e.m.) showing an 11-fold increase in apoptosis in SHF in mutants compared with controls (P<0.0001; two-tailed *t*-test). Scale bars: 100 µm (G′-H‴,J′-K‴); 150 µm (A-B′,D-E′); 250 µm (G,H,J,K).

DISCUSSION

Our study evaluates the biological role of Dll4 expression in SHF progenitors and demonstrates that Dll4 expression is required for progenitor cells to proliferate and ensure the availability of an adequate pool of cells for incorporation into the developing heart. Such a pro-proliferative role for Dll4 has been suggested in other progenitor beds as well. Dll4 is expressed by retinal progenitor cells and serves as the major Notch ligand to expand the progenitor pool (Luo et al., 2012). Dll4 is also expressed in a subset of neural progenitors in the spinal cord and its expression is required for inter-neuronal subset specification (Rocha et al., 2009). Dll4 signaling is required to ensure early commitment to T cell lineage and to maintain an adequate pool of T cell progenitors (Hozumi et al., 2008; Billiard et al., 2012; Yu et al., 2015). In the context of cardiomyocytes, following initial cardiomyocyte specification, endocardial Dll4-Notch1 signaling promotes cardiomyocyte proliferation, whereas subsequent patterning requires downregulation of Dll4 expression later in gestation (D'Amato et al., 2016). Thus, Dll4 serves as the primary Notch ligand that expands cells immediately following their early commitment to ensure that an adequate pool of cells is available for differentiation into their ultimate cell fate.

Notch receptor and ligands are expressed widely at different time-points and are thought to play an important role in heart development. We show that during early time-points of heart development (E8.5-E10.5), Dll4, but not jagged 1, is expressed by SHF progenitor cells. As SHF cells mature to form the OFT and RV, they continue to express Dll4. Previous studies have shown that the expression of myocardial cell-specific factors in the developing cardiomyocyte suppresses Dll4 expression (D'Amato et al., 2016). Our evaluation confirms these findings, demonstrating that, by E11.5, Dll4 expression is lost in the myocardium and is primarily restricted to the endocardium. D'Amato et al. (2016) showed by RNA analysis that, as early as E9.5, Dll4 mRNA is restricted to the endocardium alone. Our protein analysis suggests continued expression, albeit weak, in the myocardium up to E11.5. This discrepancy may relate to experimental differences, or may represent residual protein translated from mRNA expressed earlier in development. Our current study also provides additional insights into the role of Notch signaling in OFT development. High et al. utilized dominant-negative mastermind-like protein to knock out signaling by all Notch receptors in the SHF (High et al., 2009). They observed similar cardiac phenotypes including DORV, VSD and, occasionally, common arterial trunk. Using jagged 1 knockout, they demonstrate that Notch signaling regulates endothelial-to-mesenchymal transition and maturation between E12.5-E13.5 within an adequately formed OFT, a process they elegantly recapitulate *in vitro*. Their study did not evaluate a particular role for Notch in SHF progenitor cell biology. Our results demonstrate that, during early stages of cardiogenesis, Notch signaling is primarily mediated by Dll4 and plays a distinct and novel role in maintaining SHF proliferation. Loss of Dll4 results in reduced pool of SHF cells leading to a foreshortened OFT, which also results in a fully penetrant DORV phenotype, as observed by High et al. (2009). Our results also show that Fgf8 is the primary mediator of Notch signaling in SHF, similar to the observations of High et al. However, we did not notice any septation defects in *Dll4* mutants unlike the common arterial trunk phenotype observed by High et al., implying that Dll4 and jagged 1 mediated Notch signaling pathways likely diverge at some downstream level (High et al., 2009). The results from these two studies would imply that Notch signaling is crucial in OFT development, but is orchestrated by different ligands at different time-points. The earlier effect mediated by Dll4 primarily regulates SHF proliferation, whereas the later role mediated by jagged 1 regulates more specific maturation effects, such as endothelial-to-mesenchymal transition. Such a differential effect of Notch signaling has also been shown in the context of cardiomyocyte development (D'Amato et al., 2016).

Neural crest-specific knockout of *Notch* primarily resulted in defects in pharyngeal arch patterning and pulmonary artery stenosis, and rarely VSD (High et al., 2007). There were no OFT alignment defects reported. Taken together with previous observations, our results would imply that SHF-expressed Dll4 signals via SHF-expressed Notch receptors to mediate SHF progenitor cell biology. Such signaling by SHF cells into other SHF cells has been described in the context of Fgf8, wherein Fgf8 secreted by SHF acts on Fgfr also expressed by SHF cells (Park et al., 2008). Dll4 and Notch have generally been thought to interact in trans, such that membrane-bound Dll4 on one cell interacts with Notch expressed on the adjacent cell. Whether Dll4-Notch signaling in SHF also represents trans interaction or cis interaction remains to be elucidated.

Our data also show that Dll4-mediated Notch signaling regulates Fgf8 expression. The importance of Fgf8 pathway in heart development is well established (Frank et al., 2002; Macatee et al., 2003; Park et al., 2006, 2008). Generally, it is believed that the first enhancer segment located in the third intron of *Fgf8* serves as the primary regulator of Fgf8 expression. We show here that Notch signaling regulates Fgf8 expression at the promoter level. Given the importance of Fgf8 in SHF biology, two distinct sites of regulation allow for redundancy and the ability to further modify expression through multiple mechanisms. At around E9 in mice, as SHF progenitors are actively proliferating, Dll4 regulates proliferation through multiple pathways. Fgf8 levels begin to fall by E9.5, and concomitantly, there is reduction in SHF proliferation. Our data would therefore suggest that the primary mechanism by which Dll4 regulates SHF proliferation is via Fgf8 expression. As we

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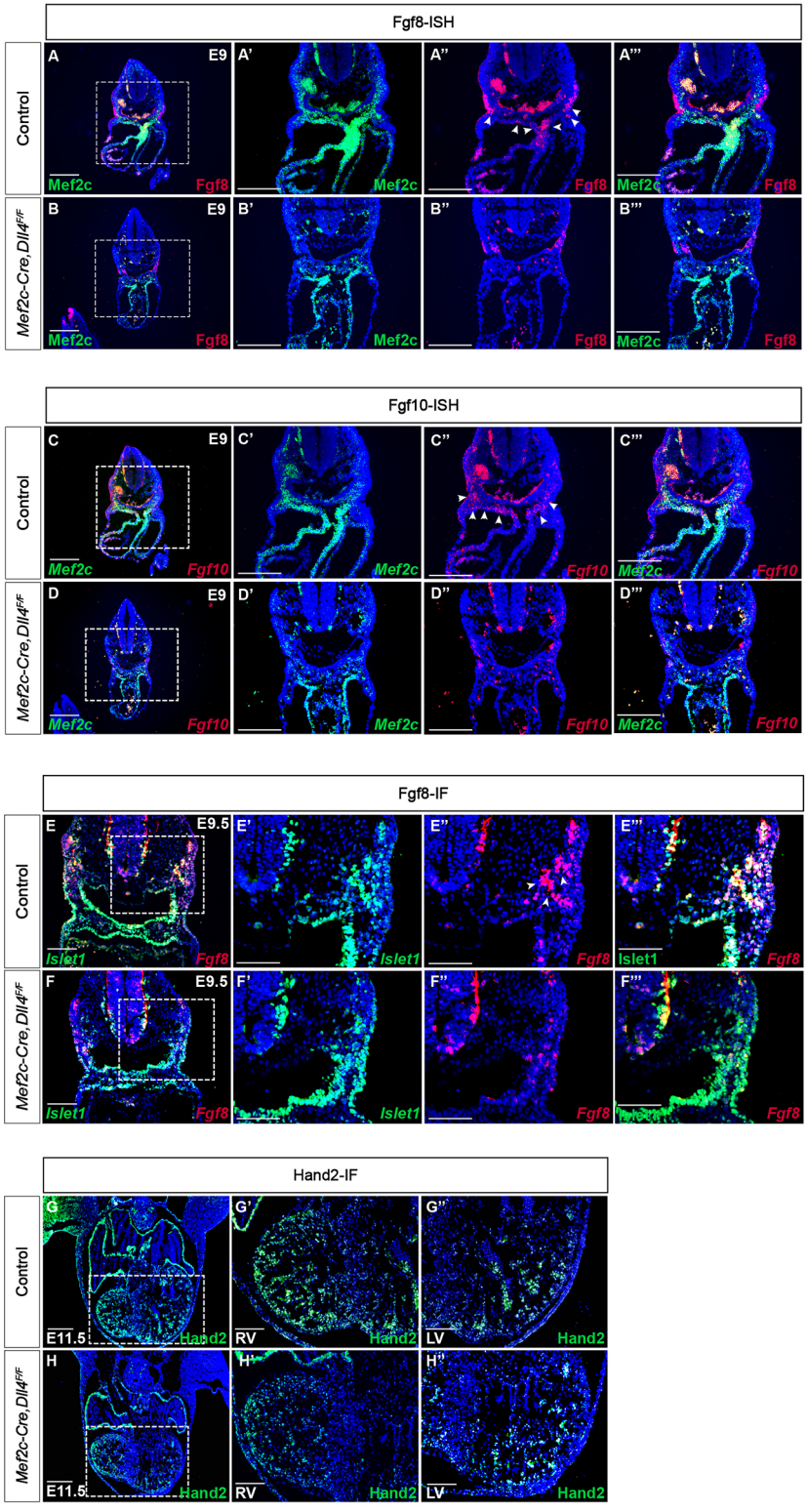

Fig. 4. Dll4 expression in SHF cells is required to maintain expression of key SHF-related proteins. (A-B‴) Transverse sections were evaluated for *Mef2c* and *Fgf8* transcript expression at E9 in control (A) and *Mef2c-AHF-Cre,Dll4$^{F/F}$* mutant (B) by RNAscope. Higher magnification of the boxed areas in A and B are shown as *Mef2c* expression (A′,B′), *Fgf8* expression (A″,B″) and merged images (A‴,B‴) to demonstrate the reduced expression of *Fgf8* transcripts in the mutants compared with the controls in the pharyngeal mesoderm (PM). (C-D‴) Similarly, transverse sections were evaluated for *Mef2c* and *Fgf10* transcript expression at E9 in control (C) and *Mef2c-AHF-Cre,Dll4$^{F/F}$* mutant (D). Higher magnification of the boxed areas in C and D are shown as *Mef2c* expression (C′,D′), *Fgf10* expression (C″,D″) and merged images (C‴,D‴) to demonstrate that the PM in mutants has decreased expression of *Fgf10* transcripts. (E-F‴) Transverse sections of control (E) and *Mef2c-AHF-Cre,Dll4$^{F/F}$* mutant (F) E9.5 embryos were co-stained for Islet1 and Fgf8 protein expression. Higher magnification of the boxed areas in E and F are shown as Islet1 expression (E′,F′), Fgf8 expression (E″,F″) and merged images (E‴,F‴), showing reduced expression of Fgf8 in the SHF region. (G-H″) Transverse sections of control (G) and *Mef2c-AHF-Cre,Dll4$^{F/F}$* mutant (H) E11.5 embryos were stained for Hand2 protein expression. Higher magnification of the boxed areas in G and H show the RV and LV in control (G′,G″) and mutant (H′,H″) embryos. Hand2 expression is lost in the mutant RV compared with controls. There is no change in the low basal level expression seen in LV. Scale bars: 50 µm (E′-F‴); 100 µm (A′-D‴,E,F,G′-H″); 250 µm (A-D,G-H).

have shown, other molecules such as Fgf10 are also reduced when Dll4 expression is lost. These changes in other molecules may, in part, explain some of the earlier reduction observed in SHF proliferation. This may also underlie the observation that the phenotype seen in homozygous Dll4 knockout is more penetrant and severe than the phenotype in *Dll4* and *Fgf8* double heterozygotes.

Notch pathway mutations have been implicated in a variety of CHD. Mutations in the Notch ligand, *Jag1*, is thought to be causative in Alagille syndrome, which is characterized by biliary malformations, pulmonary artery stenosis and, rarely, OFT defects. Recently, heterozygous deleterious mutations in *Dll4* have been implicated in Adams-Oliver syndrome. This is a rare genetic disease characterized by aplasia cutis congenita, terminal transverse limb

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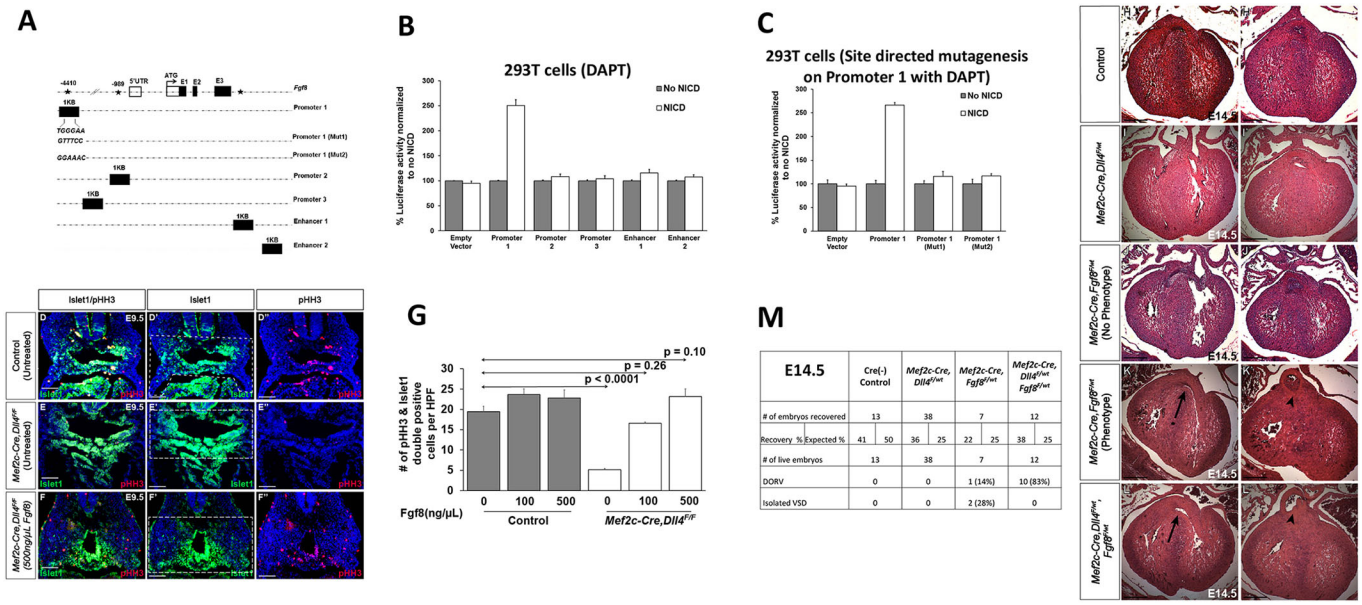

E14.5	Cre(-) Control		Mef2c-Cre, Dll4F/wt		Mef2c-Cre, Fgf8F/wt		Mef2c-Cre, Dll4F/wt Fgf8F/wt	
# of embryos recovered	13		38		7		12	
Recovery % Expected %	41	50	36	25	22	25	38	25
# of live embryos	13		38		7		12	
DORV	0		0		1 (14%)		10 (83%)	
Isolated VSD	0		0		2 (28%)		0	

Fig. 5. Dll4-mediated notch signaling regulates Fgf8 expression in SHF. (A) Schematic of the mouse chromosome 10 around the region of the *Fgf8* gene (E, exon). Putative RBPjk binding sites are indicated with asterisks. Constructs cloned for luciferase assay are shown as black boxes. Promoter 3 and Enhancer 2 were used as negative controls. (B) 293T cells were treated with DAPT to quench basal Notch activity. They were then transfected with various luciferase expression vectors with (empty bars) or without (solid bars) the NICD expression vector. Luciferase activity was measured in triplicate wells (mean±s.e.m.) 24 h later with eight experimental repeats. (C) The experiment was then repeated in triplicate after mutating the putative RBPjk binding site of Promoter 1. Mutation of putative binding sites led to loss of luciferase activity. (D-F″) Thoracic regions were dissected in control (D-D″) and *Mef2c-AHF-Cre,Dll4*$^{F/F}$ mutant (E-F″) embryos at E9.5 and cultured *in vitro*. Mutant organs were cultured with (F-F″) or without (E-E″) exogenous recombinant Fgf8. Sections were then co-stained for Islet1 and pHH3 expression to study SHF proliferation. Representative images are shown as Islet1 expression (D′,E′,F′), pHH3 expression (D″,E″,F″) and merged images (D,E,F). (G) Double-positive cells were counted in multiple fields (23 untreated control, 23 Fgf8 100 ng/µl control, 40 Fgf8 500 ng/µl control, 37 Fgf8-untreated mutant, 7 Fgf8 100 ng/µL mutant and 14 Fgf8 500 ng/µl mutant sections; mean±s.e.m.) showing a significant reduction in SHF proliferation in mutant organs compared with control (P<0.0001 between Fgf8-untreated control and mutant, P>0.05 between Fgf8-untreated controls and Fgf8-treated mutants by two-tailed *t*-tests). For quantification purposes, the boxed regions in D′, E′ and F′ were used as the area occupied by SHF progenitor cells. Exogenous Fgf8 supplementation had no significant impact on control embryos, but fully rescued proliferation defects seen in mutant embryos. (H-L′) Compound heterozygotes were analyzed by H&E staining of transverse sections of E14.5 embryos to demonstrate genetic synergy between Dll4-mediated Notch and Fgf8 signaling in SHF maturation. Cre-negative control embryos showed fully septated ventricles (H) and an aortic valve normally aligned over the left ventricle (H′). Heterozygous knockdown of Dll4 driven by *Mef2c-AHF-Cre* (*Mef2c-Cre,Dll4*$^{F/wt}$) also demonstrated a normal phenotype (I,I′). Heterozygous knockdown of *Fgf8* driven by *Mef2c-AHF-Cre* (*Mef2c-Cre,Fgf8*$^{F/wt}$) showed a low incomplete penetrance of cardiac defects. The majority of the embryos showed a normal phenotype (J,J′). A shallow VSD (arrow in K) and a slightly mal-aligned aorta mildly over-riding the ventricular septum (arrowhead in K′) was seen in 14% of the embryos. Double heterozygous knockdown of *Dll4* and *Fgf8* driven by *Mef2c-AHF-Cre* (*Mef2c-Cre,Dll4*$^{F/wt}$*,Fgf8*$^{F/wt}$) showed high penetrance of DORV, with 83% of the embryos studied showing VSD (arrow in L) and a prominent over-riding of aorta with greater than 50% aorta arising from the RV (arrowhead in L′). (M) Table indicates number and phenotypes of embryos recovered amongst the different genotypes shown. The number of embryos recovered, the percentage recovery and the expected percentage recovery are based on Mendelian inheritance. Scale bars: 50 µm (D-F″); 300 µm (H-L′).

defects and cutis marmorata. CHD is encountered in about 20% of these patients, and includes VSDs or DORV/tetralogy-type defects (Meester et al., 2015; Nagasaka et al., 2017). In a large study of whole-genome sequencing or targeted resequencing of the *Dll4* gene with a custom enrichment panel in independent families with Adams-Oliver syndrome, nine heterozygous mutations in *Dll4* were identified, including two nonsense and seven missense variants (Meester et al., 2015). All of these mutations resulted in loss of Dll4 function. Similar to these clinical reports, heterozygous loss of *Dll4* in the *Islet1-Cre* background in our study resulted in a ∼40% incidence of DORV/tetralogy-type defects. Thus, our study is the first demonstration of the molecular basis underlying the clinical CHD finding in this syndrome.

De novo mutations in single genes have been shown to contribute to approximately 10% of all severe CHD (Zaidi et al., 2013), implying that the majority of CHD lack an identifiable monogenic etiology. Interaction between mutations in two distinct genes can potentiate or suppress the impact of these mutations in isolation. There is growing evidence to suggest that such complex and co-existing oligogenic mutations may underlie a larger proportion of CHD (Akhirome et al., 2017; Granados-Riveron et al., 2012; Jin et al., 2017). It is conceivable that heterozygous mutations may be inherited from parents who could be silent carriers, but the convergence of these mutations in the offspring would result in CHD not observed in either parent. In a recent study by Gifford et al., compound heterozygosity in *MKL2* (*MRTFB*), *MYH7* and *NKX2-5* genes inherited by the offspring of clinically unaffected parents (who carried only one or two of the mutations) resulted in non-compaction cardiomyopathy (Gifford et al., 2019). Similarly, double heterozygous mutations in the dynein family of proteins have been implicated in heterotaxy (Li et al., 2016). With particular reference to tetralogy-type defects, Topf et al. sequenced 12 genes implicated in the SHF transcription network in 93 non-syndromic tetralogy patients (Topf et al., 2014). Concomitant heterozygous mutations in *HAND2* and *FOXC1* were found to be functionally significant in their cohort of patients. Our data showing genetic synergy between Dll4 and Fgf8 pathways serves as a potential model to study compound heterozygosity in DORV. Although the more severe phenotype observed in *Islet1-Cre*-mediated *Dll4* mutants may be due to more widespread gene loss, it could also have resulted from

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compound heterozygosity, given that the *Islet1-Cre* line we used is a knock-in and, therefore, a functional *Isl1* heterozygote. The incomplete penetrance of phenotypic defects in heterozygous mice may also be leveraged to study the impact of other environmental teratogenic events in a genetically permissive background. Thus, our mouse models have broad relevance for further evaluating the impact of genetic mutations in OFT anomalies.

The spectrum of phenotypic defects observed in our mutants also bears resemblance to the DORV spectrum seen in the clinical setting. The most severe form of defect seen with *Dll4* homozygous loss in either Cre background is not viable and, as such, could explain the lack of *Dll4* homozygous mutations in the clinical setting. The milder forms of defects seen with heterozygous loss of *Dll4* in the *Islet1-Cre* background or the *Dll4/Fgf8* double heterozygotes in the *Mef2c-AHF-Cre* background are highly reminiscent of the tetralogy-type DORV or tetralogy of Fallot encountered in children. This would suggest that one molecular mechanism underlying DORV/tetralogy is a later and more regional loss of proliferative signals in SHF. This allows SHF-derived structures to develop early in gestation; however, the RV and, in particular, OFT are hypoplastic, resulting in OFT mal-alignment. The degree of mal-alignment would vary between over-riding the septum (as in tetralogy) to originating primarily from RV with aortic-mitral discontinuity (as seen in DORV). The variability in the thickness of the conus in the OFT that we observed is also frequently encountered in clinical DORV/tetralogy and has relevance in the surgical approach to correct these lesions. Whether and how these subtle phenotypic variations impact long-term outcomes in children remains to be elucidated.

In summary, Dll4-mediated Notch signaling plays a crucial role in early SHF progenitor cell proliferation, primarily via regulation of Fgf8 expression. Dll4 expression is required to maintain an adequate pool of SHF cells that contribute to the RV and OFT in the developing heart. Loss of Dll4 results in a spectrum of OFT abnormalities. In their most severe forms, there is extreme cardiac under-development and early embryonic lethality. Milder forms represent clinically relevant CHD and, apart from providing a molecular mechanism for such clinical phenotypes, also provide a platform to study more complex oligogenic inheritance patterns.

MATERIALS AND METHODS

Mice

All animal experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee of the University of Southern California. *Islet1-Cre* (Cai et al., 2003) and *Mef2c-AHF-Cre* (Verzi et al., 2005) mice have been previously described. In both Cre lines, the *Cre* gene was maintained on the paternal side to eliminate risk of germline transmission. $Dll4^{F/F}$ mice were generated in the Duarte lab and previously reported (Benedito and Duarte, 2005; Duarte et al., 2004; Koch et al., 2008). $Fgf8^{F/F}$ mice were received from the Moon lab and have also been previously reported (Park et al., 2006). *Dll4-F2-lacZ* mice were a kind gift from Joshua Wythe (Wythe et al., 2013). Embryos were dissected at appropriate time-points and genotyped by polymerase chain reaction (PCR) using specific primers listed in Table S2.

Tissue analysis and histology

The antibodies used for IF are listed in Table S1. Standard validation techniques included deletion of primary or secondary antibody or use of blocking peptide to validate antibody specificity, as appropriate. The *Dll4* probe used for ISH has been previously described (Benedito and Duarte, 2005). *Fgf8*, *Fgf10* and *Mef2c* ISH were undertaken using the RNAscope protocol (Advanced Cell Diagnostics). To assess proliferation in SHF, sections were stained with Islet1 to label SHF and pHH3 to label proliferating cells. Double-positive cells in multiple high-power fields were counted and compared between control and mutant sections. Similarly, to assess apoptosis, sections were stained with Islet1 and TUNEL and double-positive cells in multiple high-power fields were counted and compared. The area in sections positive for β-galactosidase staining was analyzed using ImageJ and normalized to the control. In all cases experiments were repeated in multiple sections of multiple embryos from different litters with littermate controls. Two-tailed unpaired Student's t-test was used to compare significant differences (P-value <0.05).

Thoracic organ and cell culture

Embryos were harvested at E9.5. The thoracic region of the embryo was dissected by removing the head up to the level of pharynx and the lower trunk below the level of the thorax. Thoracic organs were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and varying doses of recombinant Fgf8 (Novus Biologicals, NBP2-35033) for 8 h. The organs were then cryoembedded. Proliferation in SHF was evaluated in sections of cultured control and mutant organs by double-staining for Islet1 and pHH3 as detailed above. For cell culture, commercially available cell lines (Table S1) were authenticated, lack of contamination confirmed, and cultured in same medium as above.

Fgf8 promoter and enhancer analysis

We cloned 1 kb segments encompassing the 6 bp putative binding site of RBPjk in the *Fgf8* promoter and enhancer regions using the primers shown in Table S2. The PCR products were cloned into a promoterless (Promega, E1771) or an enhancerless (Promega, E1761) luciferase vector as appropriate. 293T or HeLa cells were cultured in the presence of Notch inhibitors (DAPT 30 ng/µl, MilliporeSigma, D5942-5MG, or SAHM1 20 ng/µl, MilliporeSigma, 491002-1MG) to quench endogenous Notch activity. Cells were co-transfected with the luciferase construct and NICD expression vector 3XFlagNICD1 (Addgene plasmid #20183). Luminescence was measured using a standard luminometer 24 h later.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: P.D.Z., A.M., P.S.G., A.D., H.M.S., R.K.S.; Methodology: P.D.Z., J.L., O.T., J.C., A.M., P.S.G., A.D., H.M.S., R.K.S.; Validation: P.D.Z., R.K.S.; Formal analysis: P.D.Z., J.L., O.T., J.C., R.K.S.; Investigation: P.D.Z., J.L., O.T., J.C., R.K.S.; Resources: R.K.S.; Data curation: P.D.Z., R.K.S.; Writing - original draft: P.D.Z.; Writing - review & editing: P.D.Z., J.L., O.T., A.M., P.S.G., A.D., H.M.S., R.K.S.; Visualization: P.D.Z., H.M.S., R.K.S.; Supervision: R.K.S.; Project administration: R.K.S.; Funding acquisition: R.K.S.

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