repiSalud
Repositorio Institucional en Salud

This is the peer reviewed version of the following article:

MacGrogan, D., Munch, J., & de la Pompa, J. L. (2018). Notch and interacting signalling pathways in cardiac development, disease, and regeneration. *Nature Reviews: Cardiology, 15*(11), 685-704. doi:10.1038/s41569-018-0100-2

which has been published in final form at: https://doi.org/10.1038/s41569-018-0100-2

NRCAR-17-245R1

Notch and interacting signalling pathways in cardiac development, disease, and regeneration

Working title: *Notch and other signals in cardiac development, disease and regeneration*

Donal MacGrogan[1,2], Juliane Münch[1,3,4] & José Luis de la Pompa[1,2,5]

[1]Intercellular Signalling in Cardiovascular Development and Disease Laboratory,
Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Melchor Fernández Almagro 3, E-28029 Madrid, SPAIN

[2] CIBER CV, Instituto de Salud Carlos III, Melchor Fernández Almagro 3, E-28029 Madrid, SPAIN

[3] Institute of Biochemistry and Biology, Potsdam University, Karl-Liebknecht-Straße 24-25, Potsdam D-14476, GERMANY

[4] Berlin School for Regenerative Therapies (BSRT), Charité-Universitätsmedizin, Berlin, GERMANY

[5]Corresponding author

-Donal MacGrogan is an associate investigator at CNIC. He made his PhD in INSERM, France, and has held postdoctoral positions at the University of Texas at San Antonio, Canji Inc. in San Diego and Memorial Sloan Kettering in New York, USA. He has been at CNIC since 2009. His research interest focuses on cardiac valves development and disease.

-Juliane Münch is a postdoctoral researcher at Potsdam University and the BSRT at Charité, Berlin, Germany. She made her PhD at CNIC in Madrid, Spain, studying the role of Notch signalling in zebrafish fin and heart regeneration. Her current research focuses on the specification of endocardial progenitors during early zebrafish development.

-José Luis de la Pompa is senior investigator at CNIC. He made his PhD in *Drosophila* developmental genetics at CSIC (Spain), postdoctoral in chicken development (EMBL, Germany) and mouse developmental genetics (Ontario Cancer Institute, Canada). CSIC investigator since 2003 and CNIC full professor and senior investigator since 2009. His research centers in the role of cell signalling in cardiac development and disease, and the genetics of CHD.

Abstract

Cardiogenesis is a complex developmental process involving multiple overlapping stages of cell fate specification, proliferation, differentiation and morphogenesis. Precise spatio-temporal coordination between the different cardiogenic processes is ensured by intercellular signalling crosstalk and tissue-tissue interactions. Notch is an intercellular signalling pathway critical for cell fate decisions during multicellular organismal development and is aptly positioned to coordinate the complex signalling crosstalk required for progressive lineage restriction during cardiogenesis. Here, we review Notch and crosstalk signalling, in the differentiation and patterning of the different cardiac tissues, and cardiac valve and ventricular chamber development. We examine how perturbation of Notch signalling activity is linked to congenital heart diseases affecting the neonate and adult and discuss studies that shed light on the role of Notch in heart regeneration and repair after injury.

Introduction

Notch is a highly conserved intercellular signalling pathway required for the development and tissue homeostasis of multicellular organisms. Notch signalling promotes or supresses the acquisition of cellular fates, regulates cell proliferation or death, and the activation of specific differentiation programmes, both in the embryo and in adult self-renewing tissues. In the last twenty years, a consistent set of data in animal models and humans has shown that Notch is crucial for cardiac development and disease and is involved in zebrafish heart regeneration. Notch participates in almost every aspect of cardiogenesis, including cardiac fate specification, patterning of the primitive heart tube, and morphogenesis of cardiac structures. The importance of Notch as a key developmental regulator in the heart is further underscored by the requirement of NOTCH function during human embryogenesis, as haplo-insufficiency or reduced dosage of core pathway components can result in congenital heart disease [1,2], or in developmental syndromes affecting the heart [3,4]. The study of Notch function in cardiac development has examined the role of individual pathway components by means of conditional gene inactivation/activation approaches in specific cell types and/or periods of development, to prevent early lethality caused by systemic gene inactivation. Despite the knowledge gained in the past years, how Notch intersects with other signalling pathways and the Notch-dependent genetic circuitry in the developing cardiovascular system, remain partially understood. In this review, we begin by describing the main elements of the Notch pathway. We then discuss data derived mainly from studies in mouse, with some contribution from avian and zebrafish data, of how Notch, acting in concert with other signalling pathways, regulates the processes of cardiac valve and chamber development and morphogenesis, and the simultaneous formation of the coronary vasculature, and how specific genetic alterations in NOTCH cause congenital heart disease in the form of valve disease and cardiomyopathy. We conclude by describing the role of Notch signalling in zebrafish heart regeneration and the studies carried out in the adult mouse heart to try to stimulate cardiac repair.

Key points box

-Vertebrate heart development is a complex multistep process that relies on the contribution of several cellular lineages in a spatio-temporal regulated manner

-Notch is a highly conserved local cell-cell signalling pathway required for proliferation, differentiation and tissue patterning in a variety of tissues, including the heart

-Notch signalling in the endocardium regulates cardiac specification, progenitor differentiation, valve primordium formation and morphogenesis, ventricular trabeculation and compaction, and coronary vessel development

-Notch coordinates cellular interactions during heart development by cross talking with other fundamental signalling pathways, including Wnt, Bmp and Nrg1/ErbB.

-Defective Notch signalling during heart development causes CHD affecting the neonate and adult

-Notch regulates cardiac regenerative processes taking place in zebrafish, providing an incentive for evaluating Notch-based cell therapies in human

---

Box 1: Overview of heart development:

a. Gastrulation and cardiac specification (E6.5-E7.0). Cardiac progenitors (red) migrate antero-laterally of the embryo after emerging from primitive streak. Cardiac crescent (E7.5), containing first heart field (FHF, green) progenitors. Heart-forming regions of the lateral plate mesoderm fuse at the embryo ventral midline and form a crescent-shaped structure demarcated by cardiac gene expression (red and yellow) extending upwards from the ventral midline towards the neural tube. **b.** Linear heart tube (E8.0-E8.5). As the embryo folds cardiac mesoderm fuses forming a linear myocardial tube lined by endothelial cells (endocardium). The heart tube accrues by addition of second heart field (SHF, yellow) cardiac precursors to both poles. **c.** Left, rightward looping heart (E8.5-E9.5). Right, looped heart, outflow tract (OFT) is formed at the arterial pole; regions at the outer curvatures proliferate extensively to form the future atrial and ventricular chambers (V) separated by the atrio-ventricular canal (AVC). **d**) Four-chamber heart (E10.5-E12.5). Endocardial cushion formation by epithelial-to-mesenchyme transition (EMT) of endocardial cells has given rise to atrio-ventricular (AV) valves (see **Figure 2a**). Cardiac chambers expand by proliferation. Proepicardium-derived cells contribute to AVC cushion mesenchyme. In the OFT, cushion cells form by endocardial EMT. At the same time, cardiac neural crest cells-derived mesenchyme (blue) contributes to arterial valves (**e**). **f.** Heart maturation (E13.5-onwards). Cardiac septation, morphogenesis of heart structures (valves and chambers), cardiac conduction system development, and formation of coronary vasculature. Abbreviations: a, atrium; la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle **(Box 1_Figure)**.

Box 2: Overview of the Notch pathway

The Notch receptors (Notch1–Notch4, in mammals) are type I transmembrane proteins with a conserved large extracellular domain, consisting or a variable number of EGF-like repeats required for binding. The Notch receptor is synthesized as a single polypeptide, which is directly cleaved after translation by a Furin-convertase in the Golgi. This first cleavage (S1) [5,6] is necessary to form the functional heterodimeric receptor that will be translocated to the membrane where the two domains remain associated by Ca^{2+}-dependent non-covalent bonds [7]. Notch binds to extracellular ligands of the DSL (Delta, Serrate and Lag2 domain) family of ligands, namely Delta and Jagged/Serrate ligands (Delta-like 1 (Dll1) [8], Dll3 [9] and Dll4 [10], or Jag 1 [11] and Jag2[12] expressed on neighboring cells. Similarly to the Notch receptor, the ligands are type I transmembrane proteins and also have an extracellular domain formed by EGF-like repeats [13]. The selectivity of Notch for its ligands is dependent on the activity of the Fringe family of glycosyltransferases, that elongate carbohydrates attached to the extracellular Notch EGF-like repeats [14]. Upon Notch glycosylation, Delta-Notch signalling is enhanced at the expense of Jag/Ser-Notch signalling [14,15]. Notch signalling is activated when the receptor-bound ligand is modified by the ubiquitin ligase Mind bomb 1 (Mib1) [16] in the signaling cell. This event is crucial for ligand endocytosis and the generation of a mechanical pull that produces a conformational change in the receptor, resulting in successive proteolytic

cleavages by ADAM metalloproteases (S2 cleavage) and γ-secretase/presenilin complex (S3 cleavage) [17] culminating in the release of the Notch intracellular domain (NICD), and its translocation to the nucleus of the signalling receiving cell. In the nucleus, NICD binds directly to the DNA-binding protein CSL (CBF1/RBPJ/Su(H)/Lag1), [18] and recruits the co-activator Mastermind-like [19,20]. The best characterized Notch targets are the Hairy/Enhancer of split (HES) and Hey families of basic helix–loop–helix (bHLH) transcription repressors [21] although several others have been described Moreover, the biological consequences of Notch pathway activation is highly context dependent [22], owing in part to its extensive interactions with several other signalling pathways [23] (**Box 2_Figure**).

Notch in cardiac specification

The vertebrate heart arises from bi-lateral cardiac mesoderm primordia at around E7.0 in mouse, having been specified at gastrulation by signals from the surrounding endoderm and ectoderm ([24,25]; **Box 1 Figure**). Wnt and bone morphogenic proteins (Bmps) are early inductive signals responsible for cardiogenesis, whereas later Wnt inhibition is required for progenitors to differentiate into cardiac derivatives [26,27]. The earliest characterised cardiac mesoderm marker is Mesp1 [28]. Single cell profiling of early cardiac progenitors indicate that Mesp1-labelled cardiac progenitors segregate rapidly from the epiblast into the distinct cardiovascular lineages, myocardium, endocardium and endothelium [29]. Notch1 is downregulated early in the cardiomyocte lineage and enriched in endocardial lineage [29], consistent with Notch anti-myogenic activity and functional requirement in endocardium.

Studies in *Xenopus* and mouse embryonic stem cells (mESCs) have helped clarify the early requirements of Notch in cardiac lineage restriction. Notch pathway components are expressed during gastrulation in *Xenopus* suggesting a specific role in specification of mesodermal derivatives including the heart. Thus, antagonising Notch signalling in cardiac mesoderm, promotes cardiac differentiation whereas ectopic Notch activation results in the opposite effect [30]. Notch activation at gastrulation, prior to cardiac specification, decreases the expression of cardiac mesodermal transcripts whereas temporally controlled suppression of Notch extends expression of mesodermal markers beyond their normal expression domain [31]. Transient upregulation of Notch signalling during gastrulation results in delayed formation of cardiac progenitors, whereas timed downregulation at the end of gastrulation de-represses cardiac field markers in cardiogenic mesoderm [32]. Collectively, these data indicate that Notch coordinates the timing of cardiac precursor development in cardiac mesoderm and restricts the ability of cells to contribute to the heart fields.

Consistent with this anti-cardiogenic effect, mESCs lacking *Rbpj* display increased cardiomyocyte differentiation in embryoid body (EB) cultures [33], whereas constitutive Notch activation in mESCs decreases their cardiomyogenic potential [34]. Stage-specific activation of Notch during *in vitro* mesodermal differentiation alters the lineage commitment of mesodermal progenitors. Thus, early Notch activation in mESC suppresses the emergence of $Flk1^+$ cardiovascular progenitors committed to the cardiac and haematopoietic lineages [35] and favours the commitment to the neuroectodermal lineage in the absence of added self-renewal and serum factors [36]. Later Notch activation in $Flk1^+$-committed progenitors blocks cardiomyocyte, endothelial and haematopoietic cell differentiation and promotes mural cell differentiation

[35]. Notch4 activation in Flk1$^+$ haematopoietic/vascular-fated progenitors at the hemangioblast stage of EB development induces cardiac gene expression and suppresses endothelial gene expression, suggesting that the cardiac lineage can be re-specified in endothelial/hematopoietc progenitors [37]. Transcriptome profiling revealed that Notch-mediated re-specification is partially mediated by activation of Bmp signalling and suppression of canonical Wnt signalling [37].

These studies highlight that the functional outcome of Notch signalling activation is highly dependent on developmental context [22] and could be leveraged for regenerative and therapeutic purposes.

Heart tube patterning, the AVC and cardiac conduction system

The heart tube develops from precursor populations of myocardial and endocardial cells characterised by their differential rate of proliferation and timing of differentiation. The first heart field (FHF) precursors, located in the cardiac crescent, differentiate earliest to give rise mostly to endocardium and myocardium of the primary heart tube, left ventricle and part of the ventricular septum (**Box 1a-c**). Mice with systemic inactivation of the Notch effector *Rbpj* die at E10.5 and have arrested looping morphogenesis [38-40], suggesting that early cardiac progenitors are specified but fail to differentiate. Thus, Notch signalling *in vivo* is not critical for the early cardiac cell fate decisions that lead to heart tube formation.

During the cardiac looping stage between E9.5 and E10.5, atrial and ventricular chambers develop on either side of a transient structure called the atrioventricular canal (AVC). Patterning of the AVC is necessary for cardiac chamber alignment, formation of the atrio-ventricular (AV) valves, the atrio-venticular node (AVN) and muscular components of the left ventricle. The AVN is the site of cardiac conduction after electric isolation of the atria and ventricles by the annulus fibrosus, which takes place by E13.5. Perturbation of AVC patterning results in congenital defects affecting AV valves and septa, and arrhythmias such as ventricular-preexcitation, the underlying cause of Wolf-Parkinson-White syndrome (WPWS) [41].

Highly conserved region-specific transcriptional networks regulate the morphology and electrophysiology of the AVC [42,43]. Bmp2 signalling and the T-box transcription factors Tbx2 and Tbx3 are uniquely expressed in the AVC [44]. Tbx2 maintains a primitive myocardial phenotype in the AVC and inner curvature of the heart [45] (**Figure 1a**). Tbx20 represses *Tbx2* in chamber myocardium and is required for chamber-specific gene expression upstream of Hey transcription factors [46,47] (**Figure 1a**). Notch target genes *Hey1* and *Hey2* are expressed in atrial and ventricular chambers and restrict the expression of *Bmp2* and *Tbx2* to the AVC, likely by suppressing *Bmp2* expression in chambers [48,49] (**Figure 1a**). Thus, in the E9.5 mouse heart, *Bmp2* expression is confined to AVC myocardium, while Dll4-Notch1 activity is widely distributed in AVC endocardium and more restricted in chamber endocardium (**Figure 1a,** and see below).

Cardiac-specific deletion of *Bmp2* results in a failure of AVC specification [50] (**Figure 1b**) while *Tbx2* mutants have altered patterning caused by ectopic expression of chamber-specific genes [51,52]. Myocardial-specific *Bmp2* inactivation disrupts AVC myocardium patterning and impairs EMT [50,53] (**Figure 1b**).Widespread expression of Bmp2 in ventricular myocardium does not affect chamber

myocardium patterning but leads to ectopic Notch1 activation throughout the endocardium, and conversion of chamber endocardium into AVC tissue able to undergo EMT [54] (**Figure 1b**).

Notch does not regulate myocardial *Hey2* expression, as *Hey2* transcription is not affected in *Rbpj* mutants [40,55], in agrrement with the lack of Notch activity in the embryonic myocardium [56-58]. In AVC endocardium however, *Hey1*, *Hey2* and *HeyL* are expressed between E9.5 and E11.5 [40,55,59] (**Figure 1a**). While ectopic Notch expression upregulates Hey gene expression [49,55,60], Hey genes are strongly downregulated in endocardium of *Rbpj* embryos, allowing ectopic Bmp2 expression throughout the endocardum of these mutants [55]. In contrast, *Bmp2* is ectopically expressed throughout the endocardium of *Rbpj* mutants (**Figure 1c)**, suggesting that Notch in the endocardium acts primarily through the Hey genes to repress Bmp2, while in the myocardium Hey-mediated repression of Bmp2 regulation is independent of Notch [55]. Ectopic Notch1 expression using the endothelial/endocardial driver *Tie2-Cre* [61] increases AVC EMT and leads to partial transformation of ventricular endocardial cells [55] (**Figure 1c**). Ectopic Notch1 expression driven by *Mesp1-Cre* [60] or *Nkx2.5-Cre* and *cTnT-Cre drivers* [55] leads to *Hey1* expression throughout the myocardium and suppression of *Tbx2* and *Bmp2* expression and expands chamber territory at the expense of the AVC, and EMT is impaired (**Figure 1c**).

Defective Notch signalling affects AVC remodelling and maturation and results in structural congenital heart disease causing abnormal cardiac conduction [62]. Thus, ectopic myocardial expression of a dominant-negative form of MAML (DNMAML) resulted in the formation of a hypoplastic compact AVN and inferior nodal extension, short PR intervals and defective AV nodal delay in mice [62]. In contrast, ectopic myocardial N1ICD expression produced epicardial and right-sided muscular accessory pathways and expansion of AV nodal tissue, resulting in post-natal mice displaying electrophysiological properties consistent with ventricular preexcitation and recapitulating human WPWS [62]. Notch-mediated effects giving rise to ventricular preexcitation may be mediated in part through canonical Wnt-β-catenin downregulation, although inhibition of canonical Wnt signalling is required but not sufficient for the development of ventricular pre-excitation [63], suggesting that additional pathways downstream of Notch might be implicated.

The role of Notch in AVN formation in the myocardium remains uncertain given the lack of evidence for endogenous Notch signalling activity in the early and mid-gestation myocardium. Moreover, the use of the *DNMAML* transgene may not be fully conclusive, given that other pathways necessary for cardiac development might be impacted. Further research is required to clarify the mechanisms by which Notch1 regulates Wnt-β-catenin signalling in AVC and promotes functional bypass tract formation and whether this mechanism is operative in WPWS.

SHF and OFT development

Heart development requires a second source of cardiac progenitors called second heart field (SHF), which are positioned medially to the FHF within pharyngeal mesoderm. These cells are more proliferative, undergo delayed differentiation and incorporate as a late addition into the arterial and venous poles of the

heart tube [64] **Box 1a-c**; [64,65]). They contribute myocardial, endocardial and mesenchymal cells [66], that give rise to the right ventricle (RV), outflow tract (OFT), branchial arches, atria and sinus venous (SV) [67-69] (**Box 1c**). Direct or indirect perturbation of SHF deployment leads to abnormal septation of the OFT and alignment of great arteries with the ventricular chambers. The SHF progenitors interact with other cellular lineages from inside and outside the heart proper including cardiac neural crest cells (NCCs), ectoderm-derived mesenchymal cells that originate from the dorsal neural tube, through complex inter-cellular interactions mediated by numerous signalling pathways including Notch, Wnt, Fgf, Bmp and Hedgehog signalling [70].

Notch signalling has pivotal roles in the formation of SHF-derived heart structures. Inactivating Notch1 in the SHF progenitor population using the *Isl1-Cre* driver line resulted in a hypoplastic right ventricle (HRV), which was associated with prolonged Isl1+ progenitor proliferation in the anterior pharyngeal mesoderm suggesting that Notch1 normally restricts SHF progenitor proliferation [71] (**Table 1**). Wnt/β-catenin functions downstream of Notch1 in Isl1 progenitors and β-catenin is stabilised in Notch1-inactivated cardiac precursors. Transcriptome profiling of these precursors uncovered Wnt pathway components and targets upregulated in these cells, consistent with a negative feedback loop. Moreover, effector genes involved in cell proliferation and differentiation (*Ndrg1, Bhlhb2* and *Fgfs*) were upregulated, whereas others like Islet-1 which functions in the SHF to promote cardiac differentiation, cardiogenic transcription factors (*Myocd,* and *Smyd1*), and SHF proliferative signalling pathways (*Shh*) were downregulated. Thus, Notch1 down-regulates Wnt/β-catenin signalling in Isl1 cells, to limit their expansion and promote their differentiation [71].

In agreement with this data, *Rbpj* deletion using the *Mesp1-Cre* driver line also results in HRV consistent with a SHF defect [72]. This phenotype was partially rescued by inactivating the canonical Wnt suppressor *Axin2*, confirming that Wnt/β-catenin functions downstream of Notch during right ventricle development [72]. Transcriptome analysis of in SHF progenitors from *Rbpj;Axin2* double mutant embryos identified upregulation of canonical Wnt/β-catenin components (*Wnt2, Dkk1* and *Lef1*), as well as direct regulation of cardiogenic transcription factors (*Nkx2.5, Isl1*) and the chromatin-remodelling factor *Baf60c.* and indirect regulation of *Gata4*, and *SRF* via Bmp4 signalling. Rbpj also directly influences Bmp2 and Bmp7, which control factors required for cardioblast formation (*Mef2c* and *Hand2*) independently of Wnt [72]. Collectively, these studies identify a regulatory hierarchy between Notch, Wnt and Bmp required for SHF progenitor cell differentiation.

In humans, inactivating mutations of Notch ligand *JAG1* or *NOTCH2* cause Alagille syndrome (**Table 1**), a complex liver disease with heart involvement including Tetralogy of Fallot (TOF), consisting of valvular or subvalvular pulmonary artery stenosis (PAS), ventricular septal defect (VSD), over-riding aorta (OA), and RV hypertrophy [3,4,73,74]. Loss-of-function *JAG1* mutations, or reduced copy number of *JAG1* and *NOTCH1* have also been identified in non-syndromic TOF [75,76]. Notch global and SHF-specific loss-of-function mouse models recapitulate human congenital heart disease phenotypes (**Table 1**; [77]). Mice deficient for the gamma-secretase component presenilin-1 exhibit VSD, double outlet right ventricle

(DORV), and PAS [78]. Double heterozygous *Jag1* and a *Notch2* hypomorph mutation caused HRV and, PAS, OA, and VSD resembling TOF [79]. Inactivation of *Jag1* or forced expression of a dominant-negative form of MAML (*DNMAML*) in SHF causes OFT alignment, deficient NCC contribution required for OFT septation and EMT defects, which were related to decreased Fgf8 and Bmp4 expression was decreased in the OFT. Accordingly, explant assays showed that EMT was severely impaired in *DNMAML* transgenic embryos but could be rescued by adding exogenous Fgf8 [80]. Thus, on the basis of Fgf8 requirement in SHF [81] it was deduced that Jag1/Notch signalling in the SHF stimulates Fgf8, which acts in the SHF to regulate Bmp4, which in turn signals to endothelium and cardiac NCCs [80].

Hes and Hey transcriptional repressors are likely mediators of Notch signalling in the SHF. Inactivation of Hes1 as a result of an integration site position effect results in OFT alignment defects including VSD and OA [70]. *Hes1* mutant embryos display reduced SHF proliferation, leading to the failure to extend the OFT,and subsequent alignment defects [70]. Moreover, *Hes1* mutants have defective NCC contribution and abnormal arterial valve development. Homozygous *Hey2* or double *Hey1;Hey2* or *Hey1;HeyL* mutants display similar defects (**Table 1**; [48,82-87]), indicating that Hes and Hey transcriptional repressors are likely mediators of Notch signalling in the SHF.

Collectively, these data illustrate how Notch functions as a signal integrator of tissue patterning during OFT development by regulating proliferation, differentiation and apoptosis. This role has important implications for understanding OFT developmental defects, which account for up to one third of congenital heart disease.

Early valve development

Cardiac valves ensure unidirectional circulation of blood flow through heart chambers and are essential for early embryonic survival and adult heart function. The AVC and OFT regions are the sites where the AV and arterial valves form respectively (**Box 1d**). Valves initially form from protrusions of cardiac jelly (CJ), an extracellular matrix (ECM) composed of proteoglycans and hyaluronan, located between the outer myocardium and inner endocardial layers of the primitive heart tube. The key players required for AVC identity (see *heart tube patterning* section) also regulate the production of ECM components forming the CJ in the endocardial cushions. Bmp2 promotes the expression of ECM proteins, as mice deficient in Bmp2 signalling have reduced ECM deposition in cushion forming regions of the heart [50,53] (**Figure 1c**). Moreover, Tbx2 downstream of Bmp2 is a positive regulator of ECM during cushion formation [88], whereas Tbx20 is a negative regulator of ECM remodelling [89].

Valve formation takes place in embryonic mouse at E9.5 in the AVC and a day later in the OFT when endocardial-derived mesenchymal cells invade the cardiac jelly by a process of epithelial to mesenchymal transition (EMT) in response to Bmp2/Bmp4 signalling from the myocardium [90] (**Figures 1a, 2a**). Notch is highly active in endocardium lining the AVC and OFT endocardial cushions [40,57,91], and disruption of *Notch1*, *Rbpj* or *Dll4* results in acellular endocardial cushions consequent to EMT impairment [40,91]. Dll4 is necessary and sufficient for Notch1 activation [91], resulting in direct upregulation of the zinc

finger transcription factors Snail1 and Snail2 [40,91] (**Figure 2a**). Snail1/2 transcription factors directly repress the *Cdh5* gene encoding VE-Cadherin adhesion molecule, which is required for cell-cell interaction in endothelium [40,92], causing the breakdown of endocardial cell-cell contacts and mesenchyme invasion of the cushions. Bmp2 signalling from myocardium is crucial for EMT [50,53] and requires Notch signalling for valve endocardium patterning and to fully activate the EMT process [54,55] (**Figure 2a**). Notch is required for Snail expression whereas Bmp2 regulates Snail activity by promoting its nuclear translocation, suggesting that both pathways converge to activate EMT [55]. Moreover, endocardial Notch1 signalling in the AVC induces the expression of Wnt4, which acts as a paracrine factor to upregulate Bmp2 expression in adjacent myocardium to induce EMT [93] (**Figure 2a**).

Studies in zebrafish have been very useful to study the role of Notch in valve formation, as the transparent fish larva allows live imaging analyses that cannot so far be performed in the mouse embryo. In addition, the usage of chemical inhibitors, antisense-morpholinos or specific mutants, enables to modulate blood flow and cardiac contraction, which are crucial mechanical signals during heart development. The zebrafish heart tube consists of two chambers, a single atrium and ventricle, and Notch is detected very early (28 hpf) throughout endocardial cells of the heart tube, as revealed by transgenic fluorescent Notch reporters [94]. This is in line with *notch1b* expression at 24 hpf, shortly after cardiac contraction is initiated [95]. Notch activation in the endocardium becomes restricted to the atrio-ventricular canal (AVC), a high-sheer-stress region, where the future valve leaflets will be formed at the boundary between atrium and ventricle [94,96,97]. Progressively, a subset of endocardial cells at the AVC acquire a protrusive morphology (56 hpf), evaginate into the cardiac jelly and present low expression of *klf2a*, a crucial flow-induced factor for cardiac valve development [97,98]. These evaginating cells can be distinguished from cells facing the ventricular lumen, which express high levels of *klf2a* at 80 hpf [98,99]. At this stage, Notch signalling is restricted to the luminal cells. In contrast, abluminal cells show strong activation of Wnt/β-catenin, as a fluorescent Wnt signalling reporter reveals [100], indicating a distinct role for Wnt and Notch signalling in two different subsets of endocardial cells during valve formation.

Functional studies indicated that during valve development Notch signalling regulates the differentiation of endocardial cells at the AVC, as a requirement for the patterning of this region in zebrafish [40,101]. Early Notch signalling inhibition (24–60 hpf) increases the number of differentiated cells showing a cuboidal shape [101]. However, later Notch signalling abrogation (36- 80hpf) results in disorganized leaflets [101]. Genetic overexpression of NICD in endothelial cells impairs valve leaflet formation [101], whereas transient overexpression of NICD causes hypertrophic cardiac valves, which is consistent with an EMT-promoting role for Notch during mouse valve development [40]. The differences in observations of these two studies [40,101] may arise from the overexpression and inhibition of Notch signalling throughout the embryo and at different temporal intervals. This may cause early developmental vascular defects [102] and thus, changes in the blood flow patterns and dynamics in the vasculature and the developing heart. In future studies, Notch modulation specifically in endocardial cells at the AVC should provide better insight into the concrete function of Notch during valve leaflet formation in zebrafish.

Wnt/β-catenin signalling is also required for cardiac valve development, as blockage of Wnt/β-catenin signalling prevents cushion formation [103]. Apc mutants, presenting elevated Wnt/β-catenin signalling, show concomitant expansion of *notch1b* expression and enlarged endocardial cushions, due to increased cell proliferation [103], and an augmented mobility of endocardial cells towards the cardiac jelly [100]. Moreover in this context, Wnt/β-catenin signalling controls the patterning of the AVC myocardium by inducing the expression of *bmp4* and *tbx2* [104].

These studies show that formation of the primitive valves relies on multiple essential signaling pathways, acting in a coordinated fashion from the myocardium and the endocardium to drive the EMT that will give rise to the primitive valve primordia. Recent zebrafish data reveal the importance of hemodynamic forces on the activation of these signaling pathways, and await to be extended to mammalian systems.

Valve morphogenesis, BAV and valve disease

In addition to endocardial-derived mesenchyme resulting from the EMT process, the arterial valve cushions are invaded distally by cardiac NCCs ([105]; **Box1,e**). Cardiac NCC cells migrate into the caudal pharyngeal arches and form the smooth muscle layer of the pharyngeal arches giving rise to the aortic arch arteries AAA, while another subset migrates into the distal OFT, condenses to form the aorticopulmonary septum and pattern the arterial leaflets [106]. Inactivation of Notch signalling in the cardiac NCCs using the *DNMAML* transgene does not cause the typical SHF-type defects described previously but results in AAA patterning defects, PAS and VSD **(Table 1)**. These malformations were associated with deficient smooth muscle layering in the AAA, despite normal patterning of the nascent endothelial tubes, suggesting that Notch is required cell-autonomously for cardiac NCC smooth muscle cell differentiation [107].

As EMT subsides, the interstitial mesenchymal cells proliferate and expand the endocardial cushions, a process that is essential for cardiac septation and valve development [108,109]. Notch signalling is required in endocardium to regulate proliferation, and remodelling in the valves in a non-cell autonomous fashion. Inhibition of Notch in the SHF using the *DNMAML* transgene results in dysmorphic and thickened arterial valve cusps and aortic regurgitation,,which was attributed to defective post-migrating cardiac NCC-mediated apoptosis of surrounding mesenchyme [110].

Targeted inactivation of specific elements of the pathway, including Jag1, Notch1 and Rbpj, in endocardium using a Nfatc1-cre driver line results in BAV and cardiac septation defects including VSD, OA and DORV (**Table 1;** [91,111]).

Moreover, Notch pathway mutants have abnormally thickened valves due to an overabundance of mesenchyme cells, which suggests that the mechanism of action of Notch in endocardium limits mesenchymal cell proliferation. Two mechanisms have been proposed to explain this phenotype. One suggests that Notch-Rbpj signalling regulates the remodelling of the leaflets by promoting TNFα - mediated apoptosis in interstitial cells. [111]. Alternatively, our own data suggests that Jag1 ligand signalling through Notch1 limits the extent of mesenchymal proliferation by activating the expression of heparin-binding

growth factor (Hbegf), which signals to Egfr, resulting in restricting Bmp/Smad1/5 signalling in mesenchyme [91]; (**Figure 2b**). These apparently opposing effects are difficult to reconcile and additional studies are required to confirm the significance of the findings for cardiac valve development and disease.

Box3: BAV

Bicuspid aortic valve (BAV, OMIM 109730) is the most common congenital heart defect affecting 0.5 to 2% of the population, in which the aortic valve consists of two instead of three leaflets [112,113]. The valve leaflets are normally remodelled from endocardial cushions, but failure to initiate EMT or fusion of two of the cardiac cushions due to defective cardiac NCC patterning of the OFT could potentially result in a BAV [114]. The resulting aortic valve stenosis or insufficiency is associated with altered haemodynamics, progressive fibrosis, leaflet thickening and calcification. BAV is mostly found in isolation but is often seen in syndromic disease (Williams or Turner´s syndrome) or associated with congenital lesions affecting the left side of the heart, including coarctation of the aorta and hypoplastic left heart syndrome. Here, the affected structures, left ventricle, aortic valve, and aorta, are abnormally narrowed or underdeveloped, creating an hindrance to systemic blood flow [115]. Patients with BAV are also often at increased risk of aneurysms of the ascending aorta, dissection and rupture, implicating predisposing genetic and haemodynamic factors [116,117]. Familial inheritance studies indicate that BAV is inherited and genetically heterogeneous [118] and linkage studies of BAV and have identified association to several loci [119-121].

Mutant *NOTCH1* alleles have been found in familial BAV (**Box 3**) in the context of calcific aortic valve disease (CAVD)[1], and isolated heart malformations [1,122], and in patients with sporadic BAV and/or left outflow malformations [121,123,124]. Other forms of left ventricular obstruction such as hypoplastic left heart have been associated mutations in the extracellular domain of NOTCH1 [121,125]. These studies imply that NOTCH has a dual role during aortic valve formation and post-natal homeostasis (**Figure 2c**). While the mechanism by which loss of Notch leads to BAV formation is unknown, loss of Notch function in CAVD has been related to the failure to repress, via Hey transcription factors, a pro-osteogenic gene program mediated by Runx2 [1]. Modelling of CAVD in heterozygous *Notch1*- or *Rbpj* -null mice fed a high fat diet indicates that Notch1-Rbpj signalling in aortic valves represses osteoblast-like calcification pathways mediated by Bmp2 [126,127] (**Figure 2d**).

Although murine models have provided crucial insights into the pathogenesis of valve disease they do not fully recapitulate human pathobiology. To better model CAVD, a study using induced pluripotent stem cell (iPSC) lines harbouring heterozygous nonsense mutations in *NOTCH1*, revealed that haemodynamic shear stress, which normally protects against calcification, cannot repress proinflammatory molecules or activate downstream antiosteogenic gene networks. This is consistent with the notion that anti-osteogenic shear stress effects are mediated, in part, by NOTCH1 [128]. Moreover, reduced NOTCH1 signalling disrupted several epigenetic marks, including H3K27ac at N1ICD-bound enhancers resulting in derepression of latent anti-inflammatory and pro-osteogenic gene networks [128].

NOTCH1 mutations have been found in only a fraction (5%) of BAV patients indicating that multifactorial causes including genetic and environmental factors are probably involved.Future research using multidisciplinary approaches combining developmental biology and systems biology will be essential to determine the underlying causes of BAV and CAVD [112].

Early ventricular chamber development: trabeculation

Trabeculation is the first sign of chamber development [129] and starts around E8.5 in mice, when cardiomyocytes begin to form protrusions towards the ventricular lumen, that ultimately will give rise to a complex meshwork [130] (**Figure 3a**). Trabeculae are formed by endocardial cells that overlie the myocardium; both cell types are separated by extracellular matrix. Trabeculation is dependent on the interaction between endocardium-myocardium and is essential for embryonic viability. Trabeculae are present throughout vertebrate hearts [130,131], their function is to increase the myocardial mass and facilitate oxygen and nutrient exchange in the heart muscle prior to coronary vascularization [130]. Trabecular cardiomyocytes proliferate very little and show a relatively mature sarcomeric structure in comparison with the myocytes of the outer compact layer, facilitating ventricular contractility during mid-gestation [132,133]. Rapid transmission of electrical impulse is favoured by Cx40 and Cx43 gap-junctions channels, strongly expressed in the trabecular myocardium, that will give rise to the Purkinje fibre network [134-136].

During the past several years a great deal of knowledge about the signalling pathways and transcription factors involved in trabeculation has been obtained through functional studies in mice, chicken and zebrafish, but the cellular processes leading to trabeculae formation are not well understood. Thus, how cellular polarity and oriented cell division impact on trabeculation, and how is this regulated, only now begins to be understood.

The early chamber myocardium has apico-basal polarity in both mice and zebrafish. Cell polarity is defined by the asymmetric localization of different proteins (ie: signalling receptors, adaptor proteins, cell fate determinants) and appears to be essential for oriented cell division (OCD) to occur during chamber development and trabeculae formation [137-139]. 3D analysis of cell division markers (ie: pericentrin and aurora kinase B) in mouse embryos reveals that the majority of the ventricular myocardium cells divide parallel to the ventricular surface, while very few cells divide in parallel to the trabeculae, suggesting that trabecular growth appears not to be driven by OCD [140]. Recent work shows that during mouse ventricular trabeculation some cardiomyocytes do divide perpendicularly to the ventricular lumen, and genetic disruption of the cell polarity complex results in abnormal mitotic spindle alignment, loss of polarized cardiomyocyte division, and impaired myocardial trabeculation [139]. In contrast, in zebrafish embryos, trabeculation occurs via a process of delamination, rather than OCD [141]. Thus, using a combination of clonal analysis and live cell imaging, it was shown that cardiomyocytes first extend luminal protrusions, and then constrict their abluminal surface and move their cell body into the trabecular layer. The neighbouring cardiomyocytes move into the space left behind to maintain a cohesive compact layer [141]. This view has been recently refined to suggest that cardiomyocytes undergo apical constriction prior to depolarization in order to

delaminate in a Nrg1/ErbB2-dependent manner, suggesting the idea of trabeculation as an EMT-like process [137]. How all these cellular processes and behaviours (OCD, EMT-like processes, migration and proliferation; **Figure 3a**) integrate to give rise to the trabecular network is the object of intense research.

Notch plays crucial roles in the endocardium-myocardium communication that underlies trabeculation. Notch1 activity is detected in the mouse ventricular endocardium prior to trabeculation, and at E9.5, is present in ventricular endocardial cells at the base of the forming trabeculae[58]. The ligands Dll4 and Jag1 are both expressed at this stage, Dll4 is found in the endocardium, particularly at the base of the forming trabeculae [56,58], while Jag1 is expressed in the cardiomyocytes forming the trabeculae, and more weakly in compact myocardium [56,142].

Systemic or endothelial deletion of *Rbpj* or *Notch1* causes ventricular hypoplasia and impairs trabeculation, leading to embryonic lethality at E10.5 [58] (**Table 1**). Functional and biochemical analyses showed that Notch is non-cell autonomously required for the expression and activity of Bmp10, expressed in trabecular myocardium and required for cardiomyocyte proliferation during trabeculation [143]. Two other pathways were affected in Notch mutants: Efnb2 [144] and Nrg1/ErbB [145], involved in cardiomyocyte differentiation. *Efnb2* is a direct transcriptional target of N1ICD/Rbpj [58] in the endocardium. *Nrg1* expression is endocardial cells is abrogated in Notch mutants. Nrg1 activates ErbB2 and ErbB4 receptors in nearby cardiomyocytes [145]. Expression analysis in the corresponding mutant embryos showed that Efnb2 functions upstream of Nrg1, and exogenous administration of Nrg1 in cultured *Rbpj* mutant embryos, rescues their cardiomyocyte differentiation defect [58]. Later work demonstrated that the expression of *Nrg1* is regulated by Hand2, a direct Notch target in the endocardium [146].

Dll4 inactivation in embryonic endocardium disrupts trabeculation (**Figure 4a**) while Jag1, expressed in the myocardium, is dispensable for this process [56]. Developmental analysis shows that *Efnb2*, *Nrg1* and *Bmp10* expression is strongly down-regulated in E9.5 endothelial/endocardial *Dll4* mutants at E9.5. Rna-seq expression profiling of E9.5 ventricles revealed that transcription of *Gpr126* was also strongly down-regulated in endocardial *Dll4* and *Notch1* mutants [56]. *Gpr126* encodes an orphan adhesion G protein-coupled receptor [147] whose inactivation in mice causes hypotrabeculation and ventricular wall thinning [148,149]. Experiments in zebrafish and human endothelial cells, and biochemical assays indicate that *Gpr126* is an important and likely direct Notch effector during trabeculation that may affect cardiomyocyte proliferation (and/or differentiation) through non-cell autonomous mechanisms [56]. Together, the data indicate that Dll4-Notch1 activity regulates the expression of genes encoding signals that connect ventricular endocardium and myocardium, and promote cardiomyocyte proliferation and differentiation during trabeculation ([56]; **Figure 4a**). Recent functional data from zebrafish have revealed the influence of mechanical stimuli in chamber development, as endocardial cells sense and respond to blood flow, and this is essential for *Notch1-Efnb2-Nrg1* pathway activity [94]. Recent elegant work in mice shows the interplay between Notch and Nrg1/ErbB2,4 signalling during trabeculation, whereby Notch1 signalling promotes ECM degradation, while Nrg1 promotes myocardial ECM synthesis necessary for trabecular rearrangement

and growth. Both signalling systems interconnect through Nrg1-mediated regulation of Vegfa, yet act antagonistically to establish trabecular architecture [150] .

These studies show that trabeculation is crucial for ventricular chamber development. The interplay between chamber endocardium and myocardium regulates ECM synthesis/degradation, and cardiomyocyte polarity and mitotic spindle orientation, both key for the initiation of trabeculation. It is important to emphasize the crucial patterning role played by the endocardium on the myocardium, in directing trabeculae formation and cardiomyocyte proliferation and differentiation. Fine spatio-temporally regulated endocardial Notch activity, acting in concert with several other pathways and effectors (Nrg1/ErbB2,4, EphrinB2, Hand2, Gpr126) is essential for trabeculation.

Box 4: LVNC

Left ventricular non-compaction (LVNC, OMIM 601493) is a cardiomyopathy of complex aetiology that is characterized by the presence of abnormally persistent trabeculae with deep recesses in the ventricular wall [151,152]. LVNC can be viewed as a congenital heart disease, as it is caused by the intrauterine arrest of myocardial compaction [153]. LVNC was included in 2006 in the list of genetic cardiomyopathies by the American Heart Association [154] and it is the most recently classified cardiomyopathy [155]. LVNC prevalence ranges from 0.05% to 0.3% of the general population [156-158]. Echocardiography has been until recently the reference standard to diagnose LVNC, and in the myocardium a thin outer compact layer, and a much thicker non-compacted inner layer formed by the trabecular meshwork with deep endocardial spaces is observed. A ratio >2 of non-compacted to compacted myocardium (non-compaction index) at systole is characteristic of LVNC [151]. CMRI is also being used to more accurately diagnose LVNC [159].

The clinical manifestations of LVNC are highly variable, and it can range from being asymptomatic to occur with depressed systolic function that may evolve into end-stage heart failure, or be associated with lethal arrhythmias, sudden cardiac death, or embolic events [151,155,157], and all this influence treatment choices. The genetic basis of LVNC is heterogenous, and is most frequently inherited in an autosomal-dominant fashion [160]. Mutations in genes encoding sarcomere proteins [161] have been shown to cause LVNC, although in some cases, LVNC may be part of a syndrome, as it occurs with *MYH7* mutations [162]. Genes encoding cytoskeletal [163], sodium channel [164] and nuclear-membrane proteins [165] have been causally related to LVNC. We have shown that inactivating mutations in the *MIB1* ubiquitin ligase gene have been identified in families with isolated LVNC, relating defective NOTCH signalling with LVNC[2]. It is important to point out that several subtypes of LVNC have been defined on the basis of showing features related to other cardiomyopathies (HCM, DCM), in addition to LVNC characteristics [155]. Thus, it is crucial to define well the genetic substrate of LVNC and its wide phenotypic spectrum, in order to contribute to more efficient diagnose of patient and improved stratification.

Late ventricular chamber development: compaction

At around E13.5 in mice (6-7 weeks of gestation in humans) and lasting well after birth, trabeculae become compressed within the ventricular wall through the process of compaction, contributing to thicken the myocardium, leaving a smooth endocardial surface [130] (**Figure 3b**). Recent elegant lineage tracing work in mice by Tian et al. has shown that Nppa$^+$ cardiomyocytes form the endocardial, and Hey2$^+$ cardiomyocytes form the epicardial zones of the postnatal ventricular wall. Disrupting the Hey2$^+$ cell contribution results in persistence of trabeculae in the postnatal heart [166]. These data suggest that compaction may occur through proliferative expansion of the compact myocardium in epicardial-to-endocardial direction (from outside in), increasing the volume of trabecular myocardium, which subsequently compress the intertrabecular spaces, facilitating trabecular coalescence [166] (**Figure 3b**). We favour this hypothesis, as our proliferation analysis suggest that compact myocardium-derived cardiomyocytes become part of the compacting trabeculae (unpublished data). Lineage tracing studies also identified a mosaic region in the ventricular wall termed "hybrid myocardial zone", composed of Nppa$^+$ trabecular cardiomyocytes mixed with unlabelled cells from compact myocardium [166] (**Figure 3b**). Thus, the compacting ventricular wall is formed by the subendocardial inner myocardial wall or trabecular myocardium, a medial hybrid myocardial zone or intermediate myocardium (see also below and [56]), and an outer compact myocardium, or outer myocardial wall (**Figure 3b**). As the ventricular wall expands by proliferation of the compact myocardium and the trabeculae coalesce during compaction, a hypoxic environment facilitates the invasion of coronary vessels to nourish the increasingly thickened compact myocardium of the ventricular wall, and at the same time trapped endocardial cells transform into vascular endothelial cells to form the vascular supply for the newly compacted myocardium [167] (**Figure 3b**, see also ***coronary vessels*** **section**).

The ligand Jag1 is expressed in trabecular myocardium during compaction and activates Notch1 in the endocardium lining the trabeculae [2,168] (**Figure 4b**). In the myocardium, Jag1 is a substrate of the ubiquitin ligase Mib1 that regulates its activity and recycling (**Box 2**). Inactivation of *Mib1* using an early-acting myocardial Cre driver (*cTnT-Cre)* leads to a phenotype strongly reminiscent of LVNC (**Box 4**). Fetal or adult hearts of *Mib1^{flox};cTnT-Cre* mutants show a thin-wall myocardium, and large non-compacted trabeculae protruding towards the expanded ventricular lumen [2] (**Figure 4b**). Image analysis of these mice revealed a non-compaction index higher than 2, typical of human LVNC [169]. The developmental nature of this phenotype was shown by the expansion in myocardial-*Mib1* mutants of primitive compact myocardium markers to the large trabeculae, and the concomitant loss of trabecular markers, indicating disruption of trabecular patterning and impaired maturation. Gene profiling by RNA-seq showed that the expression of a high number of genes encoding cardiac development and disease genes, and sarcomere proteins was impaired in *Mib1$^{flox/flox}$;cTnT-Cre* mutant embryos. These results indicate that ventricular chamber maturation is impaired after myocardial *Mib1* deletion, and demonstrate the requirement of endocardium-myocardium signalling for compaction (**Figure 4b**).

Sequencing of human *MIB1* in a cohort of 100 LVNC patients revealed two germline mutations, V943F and R530X, that segregated in autosomal-dominant fashion with the disease. *In silico* modelling

suggested that MIB1 monomers form a head-to-tail homodimer that interacts with JAG1 [2]. Both V943F and R530X mutant forms of MIB1 can dimerize *in vitro* with wild type monomers, although in the case of the R530X mutation this is unlikely to occur *in vivo,* due to the degradation of the mutant protein by non-sense-mediated decay [2]. In both cases, LVNC would likely result from MIB1 haploinsufficiency [2].

We hypothesized that the cardiac phenotypes of myocardial-specific *Mib1* and *Jag1* mutants would be similar, based on the idea that Jag1 was the only Mib1 substrate in the myocardium. However, myocardial-*Jag1* mutants show signs of dilated cardiomyopathy with systolic dysfunction, but not LVNC. A second myocardial ligand, Jag2 is expressed at later stages of chamber development, supporting the possibility that Jag2 could be another substrate for Mib1 in the myocardium [56]. *Jag2* deletion in the myocardium disrupts chamber maturation, and combined *Jag1* and *Jag2* myocardial inactivation causes a phenotype very much alike to LVNC [56] (**Table 1**).

Manic Fringe (MFng) is expressed in the endocardium during trabeculation, where is progressively downregulated, and upregulated in the endothelium of coronary vessels. *In vitro* data suggest that MFng might modulate the ligand-specific Notch signalling response [56]. During compaction, the attenuation of *MFng* in the endocardium may allow Jag1 and Jag2 to trigger Notch1 signalling in the endocardium, as the severe LVNC phenotype caused by endothelial-MFng gain-of-function indicates ([56] and **Table 1**). Interestingly, molecular characterization of the severe LVNC phenotype caused by endothelial *MFng* overexpression or *Jag1* and *Jag2* combined myocardial disruption, showed impaired chamber patterning, so that in the E16.5 ventricle, compact myocardium markers expand to the base of the large non-compacted trabeculae, while trabecular markers are only expressed at the tip of trabeculae, revealing a population of trabecular cardiomyocytes (*Hey2*$^-$, *Cx40*$^-$, *Bmp10*$^-$) that we termed intermediate myocardium [56] that may be the same than that later identified in the lineage tracing study of Tian et al [166]. These observations are worth extending to try to understand the mechanism of compaction.

Our current working model about the role of Notch in compaction suggests that the endocardial expression of *MFng* determines the temporal specificity of Notch1 towards its ligands during ventricular chamber development. Thus, in the early ventricle undergoing trabeculation, Dll4-Notch1 signalling promotes cardiomyocyte proliferation and differentiation (Figure 2b). The downregulation of *MFn*g (and *Dll4*) in the endocardium allows myocardial-to-endocardial signalling by Jag1 and Jag2, to sustain cardiomyocyte proliferation, chamber patterning and maturation (**Figure 3b**). In developing coronary vessels, Dll4 signals to the Notch1 receptor (MFng-modified) to promote coronary development and sustain the compacting myocardium (**Figure 4b**). During this process, both Jag1 and Jag2 are Mib1 substrates in the myocardium (**Figure 4b**) [56].

These data demonstrate the developmental nature of cardiomyopathies like LVNC, and are informative about the process of trabecular coalescence during compaction. In addition, these studies reveal that tightly regulated sequential activation of Notch activity in the endocardium is crucial for the proliferative expansion of the ventricular wall, and the integration of the trabecular network in the outer myocardial wall or compact myocardium. The identification of inactivating mutations in *MIB1* in LVNC

pedigrees, confirms the relevance of these experimental studies for gaining knowledge on the genetic bases of this disease, and its relation with other cardiomyopathies.

Coronary vessels

Coronary vessels supply oxygen and nutrients to the myocardium and arise once the metabolic needs of the early avascular heart are no longer met by simple oxygen and nutrient diffusion. Myocardial growth requires enhanced oxygen delivery, triggering an influx of endothelial cells that undergo vasculogenesis to form a capillary plexus [170]. A fully compacted and irrigated ventricular wall is the result of complex morphogenetic processes that conclude well after birth. Reduced vasculogenesis as well as defects in compaction during ventricular wall formation are the causes of human cardiomyopathies, such as LVNC. Chick-quail chimera experiments [171] and more recent lineage-tracing approaches indicate that the embryonic vascular coronary endothelium derives from multiple cellular sources including sinus venosus, endocardium and proepicardium [170,172,173]. At around E11.5 in mouse, the primitive coronary plexus giving rise to the coronaries invades the sub-epicardial layer on the heart surface by angiogenic sprouting from the SV [170] and enters the myocardium to differentiate into arteries, whereas veins remain at the heart surface [172]. The SV sprouting model proposed for coronary artery development prompted the suggestion that coronary arteries are re-specified from sub-epicardial veins [170]. However, our recent data indicate that the Notch ligands Jag1 and Dll4 and the activated Notch1 receptor are expressed in the SV endothelium, leading to the proposition that nascent subepicardial vessels have a mixed arterial/venous identity that is progressively defined during arterial differentiation through reiterated Notch signalling (Travisano et al., MS submitted).

Ventricular wall endocardium constitutes a second source of coronary endothelium and contributes to most ventricular septum arteries [167,173]. Endocardium is incorporated into the myocardial wall during compaction by trabecular coalescence [167] (**Figure 3b**). Likely, SV endothelium and ventricular endocardium have similar developmental origins [174], explaining the functional compensation between SV and ventricular endocardium that occurs during coronary endothelial deployment [175]. Our recent studies indicate that Jag1 and Dll4 antagonism in the SV endocardium regulates capillary sprouting and defines the size and shape of the primary coronary plexus. Later in development, Jag1 and Dll4 cooperate to promote arterial-venous remodelling and maturation of the primary coronary plexus (Travisano et a., MS submitted).

A third potential source of endothelial progenitors is the pro-epicardium but the reported contribution of the proepicardium to the coronary endothelial lineage varies from 10% to 20% [176,177] to minimal contribution [178]. The proepicardium also gives rise to epicardium and epicardial derived cells (EPDCs), which provide a source of trophic signals for the myocardium and a cellular source for the coronary vasculature and the fibrous skeleton of the heart [179]. Notch signalling represses Bmp2 in the pro-epicardium and is required for cardiac inflow muscularisation [168] and also acts upstream of TGF-β

signalling and PDGFR-β expression to regulate the SMC differentiation of EPDCs. [180] Notch and TGF-β signalling cooperate in this process while PDGF signalling plays an antagonising role [180].

In sum, coronary development is a complex process and the genes and pathways involved are only beginning to be deciphered. Understanding how Notch and other pathways are involved in the cellular and molecular mechanisms that control coronary artery development and their coordination with compaction, will have important implications for determining the causes of cardiac pathophysiology.

Notch in zebrafish heart regeneration

Organ regeneration and repair often involve the reactivation of cellular signals and pathways occurring during developmental processes and Notch signalling is highly implicated in diverse regenerative processes of different organs including the caudal fin, the spinal cord, and the brain [181-186]. Thus, many studies in mouse and zebrafish have addressed the function of Notch signalling during heart repair and regeneration. In zebrafish three different approaches have been used: the genetic ablation of ventricular cardiomyocytes in the developing zebrafish heart, which involves the transdifferentiation of atrial to ventricular cardiomyocytes [187], a partial resection of the adult ventricle [188] and the induction of a cryo-induced injury of the ventricle in adult zebrafish [189-191]. Cardiac insult in zebrafish causes an early infiltration of inflammatory cells, followed by the deposition of fibrotic tissue, which is especially prominent after a cryoinjury and resembles processes occurring upon myocardial infarction in mammals. However, in the zebrafish heart this fibrotic tissue dissolves and is replaced by new cardiomyocytes, which possess the ability to dedifferentiate and strongly proliferate, allowing the complete cardiac [189-193].

Notch signalling is activated after cardiac injury [194], including the expression of various Notch receptors (*notch1a*, *notch1b*, *notch2*, *notch3*), ligands (*deltaC*, Dll4) and the Notch signalling modulator *lunatic fringe* (*lfng*) [194-196]. Genetic cardiomyocyte ablation in the embryonic heart causes strong activation of the Notch pathway in the atrial endocardium (**Figure 5a**), whereas in the injured adult heart early at 1 day post injury (dpi), Notch pathway genes are expressed by endocardial cells all-over the ventricle. Gene expression then becomes restricted to endocardial and epicardial cells in proximity to the injury site when regeneration proceeds (3 dpi, 7dpi, **Figure 5a**) [194-196].

To address the function of Notch signalling in cardiac regeneration in the zebrafish, genetic and chemical approaches were used. These include the heatshock-induced expression of a dominant negative (DN) form of the murine mastermind-like protein to prevent Notch-target gene expression, and of the intracellular domain of the notch1 receptor (NICD) to over-activate the pathway [195,196].

Notch signalling inhibition impairs regeneration of the ventricular myocardium and causes a persistent deposition of fibrous and collagenous tissue [195,196], showing the absolute requirement of Notch pathway activation for complete cardiac regeneration in zebrafish. Stimulation of the pathway by heatshock-induced expression of NICD upon ventricular resection or cryoinjury, also impairs cardiac regeneration [195,196]. This suggests a role for Notch signalling in regenerative processes in the injured ventricle, with an absolute need for a well-adjusted regulation. Indeed, various functions of Notch signalling, including cell-autonomous

and non-cell-autonomous roles, during cardiac regeneration, have been reported (**Figure 5b**). Notch inhibition affects the expression of developmental and injury-induced endocardial/endothelial genes (*efnb2a*, *id1*, *klf2b*, *heg*, *vegfc*) in the injured ventricle [195]. This is accompanied by changes in endocardial cell morphology and maturation at the injury site in Notch-inhibited cryoinjured hearts (**Figure 5b-I**), including an increased number of filopodia-showing endocardial cells, an effect that is inverted by the over-activation of the pathway [195]. This suggests a similar function of Notch in the endocardium at the injury site to its role during angiogenesis [197,198]. In addition, in the cryoinjured heart, Notch signalling negatively regulates the inflammatory response. Abolished pathway activation leads to aberrant expression of inflammatory genes (*tnsfrsf9a*, *arg2*, *ptgs2b*) and to the increased and prolonged presence of *mpeg1*$^+$ and *l-plastin*$^+$ inflammatory cells (**Figure 5b-II**) [195]. These observations maybe also attributed to Notch´s function in regulating endocardial maturation.

Notch receptor expression has not been reported in cardiomyocytes of the injured zebrafish hearts. Due to the non-cell-autonomous role of endocardial Notch signalling during cardiac development [58], special attention has been paid on a potential implication of Notch for the regenerating myocardium.
Notch pathway inhibition interferes with cardiomyocyte proliferation in three models of cardiac injury in the embryo and adult zebrafish [187,195,196]. Studies in the embryonic heart upon nitroreductase-induced ablation of ventricular cardiomyocytes, showed that the activation of Notch signalling in atrial endocardial cells is necessary for the transdifferentiation of atrial to ventricular cardiomyocytes for cardiac regeneration [187]. In the adult zebrafish heart Notch pathway inhibition leads to an increased expression of sarcomeric genes (*mylk3*, *acta1*, *tcap*) [195], pointing to a reduction in cardiomyocyte dedifferentiation in the ventricle. In this line, in cryoinjured hearts, the over-activation of the pathway augments cardiomyocyte proliferation. Moreover, an increased number of cardiomyocytes, showing expression of developmental genes (*hand2* and *nkx2.5*), accumulate at the border of the injury site, and complete restoration of the ventricular myocardium is prevented upon persistent Notch signalling activation [195]. These results suggest that endocardial Notch signalling regulation is crucial for the dedifferentiation and proliferation of wound-adjacent cardiomyocytes and thus the regeneration of the ventricular myocardium (**Figure 5b-III**). Nevertheless, which downstream signals of endocardial Notch activation allow its non-cell-autonomous regulation of myocardial regeneration in the adult and embryonic heart still requires further investigation.

Our study suggests Notch-mediated downregulation of the secreted molecule plasminogen activator inhibitor 1 (Pai1, Serpine1) as one important requirement [195]. *Serpine1* is strongly expressed in endocardial cells in the injured mouse heart [199] and becomes similarly activated as early as 24 hpi in the zebrafish ventricle. However, in zebrafish *serpine1*-expression is strongly reduced at the time point of high cardiomyocyte proliferation (7 dpi) and earlier inhibition of Serpine1 augments cardiomyocyte proliferation, indicating its role as an important, secreted, Notch-regulated factor, involved in myocardial regeneration [195].

Altogether, by using different experimental models of cardiac injury, studies in zebrafish have discovered widespread functions for endocardial and epicardial Notch signalling for regulating endocardial

morphology and maturation, inflammatory signals and nevertheless cardiomyocyte dedifferentiation and proliferation, showing the potential of Notch signalling in modulating regenerative processes also in the infarcted mammalian heart.

Notch in mammalian heart repair

An important goal of cell-based therapy in human is the replacement of functional myocardium by cell transplantation. However, this strategy has been limited as only low numbers of highly enriched populations of differentiated cardiomyocytes can be obtained routinely. One approach has taken advantage of the ability of Notch to redirect the hemangioblast towards the cardiovascular lineage [200] (see *cardiac specification* section). It was found that infarcted mice that received a Notch4-redirected cardiovascular cell graft (consisting of >60% cardiomyocytes) showed greater functional improvement in transplant assays, than those transplanted with an uninduced-hematopoietic/vascular cell graft [200], suggesting that cardiovascular progenitors have better regenerative potential than vascular progenitors in cardiac infarction models.

Another strategy is to extend the proliferative potential of immature cardiomyocytes and/or delay their differentiation. Indeed, regeneration of the myocardium in zebrafish and mouse is related to the capacity of cardiomyocytes to proliferate, rather than differentiation of resident cardiac stem cells [192,201,202]. In mouse neonate the ability of myocardial cells to repair injury is associated with continued proliferation of cardiomyocytes up to 7 days after birth [203]. Several approaches have taken advantage of activating Notch signalling to expand neonatal cardiomyocytes cell number *in vitro*. Inhibiting Notch signalling in immature neonatal cardiomyocytes blocks proliferation and induces apoptosis, suggesting that Notch may be manipulated to promote cell division in early cardiomyocytes [204]. Notch activation mediated by Jag1 or constitutive expression of Notch1 (N1ICD) [204] or Notch2 (N2ICD) [205] stimulates proliferation and promotes cardiomycyte expansion *in vitro*, indicating indicate that might be possible to extend the proliferative window of early cardiomyocytes, through endogenous Notch signalling activation, providing a conceptual basis for future cardiac cell-based therapies.

A third approach has taken advantage of pro-survival and anti-apoptotic roles of Notch, to the effect that ectopic activation of Notch signalling in the myocardium has been shown to limit the extent of cardiac pathological remodelling under conditions of chronic cardiac stress induced by myocardial infarction. Notch and hepatocyte growth factor crosstalk can activate survival mechanisms in cardiomyocytes via activation of the c-Met and Akt kinase survival pathways [206]. Ectopic N1ICD expression by transgene adenoviral transfer [206], or inducible conditional overexpression of a myocardial-specific Notch1 transgene, or intramyocardial delivery of a Notch1 activating antibody [207], improve cardiac functional performance, minimizes fibrosis and promotes cardiomyocyte survival, therefore implicating Notch signalling in a cardioprotective role following cardiac injury.

The physiological relevance of Notch in the murine regenerative studies carried out thus far has to be considered from the point of view that Notch is not normally active in the myocardium during

development, nor reactivated in the myocardium in situations involving heart injury in the juvenile or adult heart, so that gene attenuation strategies cannot be considered. This implies that only gene therapy approaches leading to ectopic Notch activation strategies, similar to the ones described above, appear to be the only alternative to try to stimulate cardiomyocyte proliferation after cardiac injury.

The importance of the role of Notch activation in endocardium has been largely overlooked primarily because regenerative and repair studies have focused on the damage to the myocardium. However, mouse developmental studies to this date have shown that activation of the pathway takes place in endocardium, whereas the outcome of Notch activation can affect the myocardium indirectly through non-cell autonomus mechanisms (ie: the release of a diffusible factor). Future studies should therefore explore the role of Notch in adult endocardium, and its potential as a cellular source for cardiac disease and repair processes.

Conclusions

Heart development is a complex morphogenetic process that depends on the precise spatial and temporal coordination of many different signalling pathways. Here, we have reviewed data describing how Notch signalling functions reiteratively during cardiac specification, proliferation, differentiation and patterning, and morphogenesis of the valves and chambers. Notch often acts in concert with other signalling pathways, especially Wnt and Bmp, to regulate these processes. The available data indicate that: (1) during cardiac specification, Notch prevents early cardiac differentiation, and can re-specify the haematopoietic/vascular lineage to a cardiac fate, via activation of Bmp and inhibition of Wnt. This property may be useful for regenerative and therapeutic purposes; (2) in the AVC, myocardial Bmp2 and endocardial Notch cooperate to pattern the AVC endocardium. Endocardial Dll4-Notch1 signaling acts in concert with Wnt4 and myocardial Bmp2, to promote EMT via Snail transcription factors activation; (3) in early OFT formation, Notch acts in SHF progenitors upstream of Wnt and Bmp to arrest proliferation and promote differentiation. Later on, Notch promotes EMT upstream of Fgf8 and Bmp4; (4) NOTCH signaling defects in SHF cardiac progenitors leads to syndromic and non-syndromic CHD (Alagille, TOF, BAV); (5) during the proliferative phase of valve development, endocardial Jag1-Notch1 restricts Bmp-pSmad1/5-mediated mesenchymal proliferation by promoting HbEgf-Egfr signalling; (6) *NOTCH1* mutations cause BAV and predispose to CAVD. In the healthy valve, Notch signalling inhibits a pro-inflammatory and osteogenic gene program, mediated in part by Bmp signalling; (7) during chamber development, endocardial Mib1-Dll4-Notch1 signalling promotes trabecular proliferation and differentiation activating a downstream circuitry connecting endocardium and myocardium and, during ventricular wall maturation Mib1-Jag1&Jag2-Notch1 regulate myocardial patterning, maturation and compaction. *MIB1* mutations cause familial LVNC inherited in an autosomal dominant fashion; (8) compaction depends on the formation of coronary vessels that originate from SV and ventricular endocardium. The role of Notch in the establishment of coronary arterio-venous identities remains to be shown; (9) during zebrafish heart regeneration, endocardial Notch signalling, in part through serpine-1, mediates fibrotic tissue deposition and cardiomyocyte proliferation.

In addition to having fundamental biological significance for our understanding of both Notch and signalling crosstalk mechanisms operating in cardiac development, these data shed light on the origins of congenital heart disease.

Perspectives

To progress beyond the current situation, we need multidisciplinary approaches combining genetics, developmental studies, disease modelling and systems biology to determine how Notch integrates with cardiovascular genome networks in normal and diseased states. Next generation sequencing of exome and whole genomes combined with studies of disease families and population-based studies are now required to identify these genes and pathways. This goal further entails sophisticated systems biology approaches integrating epigenomic, transcriptomic and proteomic studies to measure the molecular states induced by Notch perturbation, determine interactions using computational methods (interactome) and establish platforms for mathematical modelling. High-throughput studies from smaller amounts of starting biological material through miniaturization and amplification schemes have begun to explore the mechanisms. These integrated approaches should reveal commonalities and singularities of Notch signaling in the heart and pave the way to targeted molecular therapy, pharmacogenomics, disease modelling, and cellular regenerative medicine, in order to monitor and treat heart disease. For example, how Notch integrates into the gene regulatory networks underpinning valve morphogenesis and disease is under intense investigation. Similarly, the detailed cellular behaviors occuring during formation of the early ventricular wall and the interplay among the multiple cell types and signaling pathways involved, only now begin to be understood through analysis of data obtained in a variety of experimental systems. How studies on ventricular wall development and maturation may illuminate our understanding of cardiomyopaties, and help us to decipher the underlying genetic complexity by modelling in mice the novel findings obtained through exome of GWAS analysis only now begins to reveal its full potential.

ACKNOWLEDGMENTS

The authors thank present and past members of the laboratory for their contribution to this review and apologize for the omission of studies not discussed or cited because of space limitations.

FUNDING

J.L.dlP is funded by grants SAF2016-78370-R, CB16/11/00399 (CIBER CV), and RD16/0011/0021 (TERCEL) from the Ministerio de Ciencia, Innovación y Universidades and grants from the Fundación BBVA (Ref.: BIO14_298) and Fundación La Marató (Ref.: 20153431). The cost of this publication was supported in part with funds from the ERDF. The CNIC is supported by the Ministerio de Ciencia, Innovación y Universidades and the Pro CNIC Foundation, and is a Severo Ochoa Center of Excellence (SEV-2015-0505).

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Figure legends

Figure 1. Notch is required for cardiac patterning, EMT and valve morphogenesis

a) *Left*, gene network establishing chamber versus valve domains in the developing heart at E9.5. In the myocardium, Tbx20 drives *Hey1* and *Hey2* and represses *Tbx2* expression in chamber myocardium. Hey1 and Hey2 confine *Bmp2* expression to AVC myocardium (dark green). *Tbx2* and *Tbx3* (dark green) are activated by Bmp2 and restricted to valve territory by Tbx20 and Hey. In the endocardium, Dll4/Notch represses *Bmp2* in the endocardium, upstream of Hey1 and HeyL in AVC and Hey2 in chambers. *Right*, E9.5 wild type heart. Dll4 and N1ICD expression (red) in AVC endocardium, in the endocardium at the base of forming trabeculae, and in the atrium. Bmp2 expression (dark green) is restricted to the AVC myocardium. Ventricular and atrial myocardium are coloured in light green and in yellow, respectively. The extracellular matrix is coloured in beige. at, atria; avc, atrio-ventricular canal; left ventricle. **b**) *left*, *Bmp2* inactivation (in *Bmp2^{flox};Nkx2.5-Cre* mutants) disrupts AVC myocardium specification and Notch1 activity is strongly reduced in AVC. *Right,* Bmp2 overactivation throughout the myocardium (in *R26Bmp2^{GOF};Nkx2.5-Cre* mice) specifies chamber endocardium as valve through ectopic Notch activation in chamber endocardium, and ectopic EMT occurs in ventricles. **c**) *left*, Notch inactivation (in *Rbpj* mutants) leads to ectopic Bmp2 expression in the endocardium (dark green), while myocardial Bmp2 expression appears unaffected. *Right,* Notch overactivation (red) in the endothelium and endocardium (*R26N1ICD;Tie2-Cre* mice) does not affect myocardial patterning but AVC EMT is increased. *Bottom*, Notch activation in the myocardium (*R26N1ICD;cTnT-Cre* mice) impairs AVC patterning and EMT is severely attenuated.

Figure 2. Endocardial Notch is required for valve development and homeostasis.

a) *Left*, gene network triggering EMT. Wnt4 from endocardium activates *Bmp2/4* in myocardium, which converges with Dll4-Notch/RBPJ to activate Snail1/2 that repress *Cdh5* and initiate EMT. Twist1 and Twist2 are also required downstream of Bmp2/4 and Notch/RBPJ respectively. *Right*, view of OFT and AVC endocardial cushions at E9.5-E10.5 where EMT takes place and valves form. Myocardium, green; endocardium, purple; extracellular matrix, beige. Migratory cardiac NCC mesenchyme invades the distal OFT region and interacts with endocardial mesenchyme to pattern the arterial valves. **b**) Endocardial Jag1 is required for post-EMT valve proliferation after E11.5. In wild-type valves, Jag1-Notch1 signalling in endocardium (pink) restricts mesenchyme cell proliferation by downregulating Bmp/pSmad1/5 signalling via Hbegf. Post-EMT *Jag1^{flox};Nkx2.5-Cre* mutants are characterized by dysmorphic and thickened valve leaflets and have reduced *Hbegf* expression resulting in increased Bmp/p-Smad1/5 signalling and uncontrolled mesenchyme proliferation. **c**) dual NOTCH function in aortic valve development and homeostasis. NOTCH ensures proper tricuspid aortic valve morphogenesis by suppressing BAV formation while post-natally NOTCH suppresses calcification in both TAV and BAV to maintain homeostasis. **d**) Mechanism of CAVD caused by interaction of the hypercholesterolemic diet and Notch haploinsufficiency.

(Right) In the normal situation, physiological Notch signalling blocks osteoblast cell fate and matrix mineralization by maintaining Hey1 expression, and Hey1 represses the pro-osteogenic gene program regulated by Bmp2, pSmad1/5/8, Runx2, and Osterix. (Left) In the pathological situation hyper-lipidemia and macrophage foam cell accumulation activates a pro-inflammatory signalling program in valve tissue leading to formation of osteoblastic-like cells. Due to reduced Hey1 expression and derepression of the osteogenic gene program these processes are accelerated in valve leaflets of *RbpjKO*/+ and *Notch1KO*/+ mice, and in patients with *NOTCH1* mutations. Panels **a, b** and **d** were adapted with permission from REFS. [91,127],208.

Figure 3. Ventricular chamber development: trabeculation and compaction.

a) Cartoons depicting the initial stages of trabeculation beginning at E8.5, when the heart tube is formed by an inner endocardium and a slowly proliferative epithelial myocardium. At E9.0, the first myocardial protrusions covered by endocardium, bulging towards the ventricular lumen, appear. Various cellular mechanisms, including oriented cell division (OCD) may account for this. At E9.5-E10.5, a growing trabecular network is forming by OCD, cardiomyocyte migration, EMT-like, and proliferation. A rapidly dividing outer compact myocardium layer (CM), and a slowly dividing inner trabecular myocardium (TM) are well defined. **b**) At E11.5, trabeculae form a complex network, and at around E13.5, compaction begins, and trabeculae integrate into the CM. In parallel, coronary vessels begin to form to nourish the thickening CM. At E15.5, the TM or inner myocardial wall (IMW), the intermediate myocardium (IM) or hybrid myocardial zone (HMZ), and the CM or outer myocardial wall can be distinguished. For details see [56,166]. Compaction continues postnatally, and approximately concludes at P28 in mice (see [166,167]).

Figure 4. Endocardial Notch activity is required for ventricular chamber development.

a) *Left*, *top*, detail of a section of the left ventricle of an E9.5 wild-type embryo stained with antibodies against sarcomeric myosin (MF20, grey) to delineate the myocardium and CD31 (red) to mark the endocardium. Nuclei are counterstained with Dapi (blue). The arrowheads point to the forming trabeculae. Bottom, section of the ventricle of a *Dll4*$^{flox/flox}$*;Tie2-Cre* E9.5 mutant showing reduced trabeculae and endocardium dettached from myocardium. The arrowheads point to the forming trabeculae. Scale bar, 100 μm. Right, cartoon depicting a magnification of the E9.5 left ventricle. In endocardial cells at the base of the trabeculae (red circle), the ligand Dll4 binds to the Fringe-modified Notch1 receptor in the adjacent cell. At this stage, Notch activity is required for proliferation and differentiation of the cardiomyocytes within the developing trabeculae (dark green). Jag1 does not seem to play a role in activation of Fringe-modified Notch1 receptor. For details see [56]. (**b**) *Left,* E16.5 heart sections stained with endomcuin (green) and cTnT antibodies (red) to identify chamber endocardium and myocardium. The wild type heart shows a thick cTnT-positive compact myocardium and compacting trabeculae in comparison with the thin compact myocardium and the large trabeculae of the *Mib1*$^{flox/flox}$*;cTnT-Cre* mutant. The yellow bar indicates the thickness of compact myocardium. Scale bar, 100 μm. Right, cartoon depicting a magnification of the

E15.5 compacting ventricle. The myocardial ligands Jag1 and Jag2, regulated by Mib1, activate Notch1 throughout the endocardium to promote chamber maturation. In developing coronary vessels, Dll4 activates the Fringe-modified Notch1 receptor. Both ventricular myocardium maturation and coronary vessel development are required for compaction. For details see [56]. Adapted and reproduced with permission from REFS. [56,208].

Figure 5. Notch signalling activation in the injured zebrafish heart

a) Schematics, showing Notch activation (red) in the atrial endocardium upon cardiomyocyte ablation in the embryonic heart at 4 dpf and Notch activation (red) in the endocardium and epicardium in the adult injured heart. Representations of the yellow insets (I, II, III) are shown in **b**). **b**) Schematics, indicating functions of endocardial Notch signalling (red arrows) in the adult injured heart. I) Notch signalling promotes endocardial maturation at the injury site. II) Notch signalling negatively regulates inflammatory cell infiltration at the injury site. III) Notch regulates cardiomyocyte proliferation.

References

1 Garg, V. *et al.* Mutations in NOTCH1 cause aortic valve disease. *Nature* **437**, 270-274 (2005).
2 Luxan, G. *et al.* Mutations in the NOTCH pathway regulator MIB1 cause left ventricular noncompaction cardiomyopathy. *Nat Med* **19**, 193-201, doi:10.1038/nm.3046 (2013).
3 Li, L. *et al.* Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nature genetics* **16**, 243-251. (1997).
4 Oda, T. *et al.* Mutations in the human Jagged1 gene are responsible for Alagille syndrome. *Nature genetics* **16**, 235-242. lineage decision. (1997).
5 Blaumueller, C. M., Qi, H., Zagouras, P. & Artavanis-Tsakonas, S. Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. *Cell* **90**, 281-291, doi:S0092-8674(00)80336-0 [pii] (1997).
6 Logeat, F. *et al.* The Notch1 receptor is cleaved constitutively by a furin-like convertase. *Proc Natl Acad Sci U S A* **95**, 8108-8112. (1998).
7 Rand, M. D. *et al.* Calcium depletion dissociates and activates heterodimeric notch receptors. *Molecular and cellular biology* **20**, 1825-1835 (2000).
8 Bettenhausen, B., Hrabe de Angelis, M., Simon, D., Guenet, J. L. & Gossler, A. Transient and restricted expression during mouse embryogenesis of Dll1, a murine gene closely related to Drosophila Delta. *Development* **121**, 2407-2418 (1995).
9 Dunwoodie, S. L., Henrique, D., Harrison, S. M. & Beddington, R. S. Mouse Dll3: a novel divergent Delta gene which may complement the function of other Delta homologues during early pattern formation in the mouse embryo. *Development* **124**, 3065-3076 (1997).
10 Shutter, J. R. *et al.* Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes & development* **14**, 1313-1318. (2000).
11 Lindsell, C. E., Shawber, C. J., Boulter, J. & Weinmaster, G. Jagged: a mammalian ligand that activates Notch1. *Cell* **80**, 909-917 (1995).
12 Shawber, C., Boulter, J., Lindsell, C. E. & Weinmaster, G. Jagged2: a serrate-like gene expressed during rat embryogenesis. *Dev Biol* **180**, 370-376 (1996).
13 Chillakuri, C. R. *et al.* Structural analysis uncovers lipid-binding properties of Notch ligands. *Cell Rep* **5**, 861-867, doi:10.1016/j.celrep.2013.10.029 (2013).
14 Panin, V. M., Papayannopoulos, V., Wilson, R. & Irvine, K. D. Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-912 (1997).
15 Yang, L. T. *et al.* Fringe glycosyltransferases differentially modulate Notch1 proteolysis induced by Delta1 and Jagged1. *Mol Biol Cell* **16**, 927-942, doi:E04-07-0614 [pii]
10.1091/mbc.E04-07-0614 (2005).
16 Itoh, M. *et al.* Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev Cell* **4**, 67-82 (2003).
17 Musse, A. A., Meloty-Kapella, L. & Weinmaster, G. Notch ligand endocytosis: mechanistic basis of signaling activity. *Semin Cell Dev Biol* **23**, 429-436, doi:10.1016/j.semcdb.2012.01.011 (2012).
18 Jarriault, S. *et al.* Signalling downstream of activated mammalian Notch [see comments]. *Nature* **377**, 355-358 (1995).
19 Borggrefe, T. & Oswald, F. The Notch signaling pathway: transcriptional regulation at Notch target genes. *Cell Mol Life Sci* **66**, 1631-1646, doi:10.1007/s00018-009-8668-7 (2009).
20 Kovall, R. A. & Blacklow, S. C. Mechanistic insights into Notch receptor signaling from structural and biochemical studies. *Curr Top Dev Biol* **92**, 31-71, doi:S0070-2153(10)92002-4 [pii]
10.1016/S0070-2153(10)92002-4 (2010).
21 Fischer, A. & Gessler, M. Delta-Notch--and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors. *Nucleic acids research* **35**, 4583-4596, doi:10.1093/nar/gkm477 (2007).
22 Bray, S. J. Notch signalling in context. *Nat Rev Mol Cell Biol* **17**, 722-735, doi:10.1038/nrm.2016.94 (2016).

23 Guruharsha, K. G., Kankel, M. W. & Artavanis-Tsakonas, S. The Notch signalling system: recent insights into the complexity of a conserved pathway. *Nat Rev Genet* **13**, 654-666, doi:10.1038/nrg3272 (2012).

24 Abu-Issa, R. & Kirby, M. L. Heart field: from mesoderm to heart tube. *Annu Rev Cell Dev Biol* **23**, 45-68, doi:10.1146/annurev.cellbio.23.090506.123331 (2007).

25 Evans, S. M., Yelon, D., Conlon, F. L. & Kirby, M. L. Myocardial lineage development. *Circ Res* **107**, 1428-1444, doi:10.1161/CIRCRESAHA.110.227405 (2010).

26 Noseda, M., Peterkin, T., Simoes, F. C., Patient, R. & Schneider, M. D. Cardiopoietic factors: extracellular signals for cardiac lineage commitment. *Circ Res* **108**, 129-152, doi:10.1161/CIRCRESAHA.110.223792 (2011).

27 Vincent, S. D. & Buckingham, M. E. How to make a heart: the origin and regulation of cardiac progenitor cells. *Curr Top Dev Biol* **90**, 1-41, doi:10.1016/S0070-2153(10)90001-X (2010).

28 Saga, Y. *et al.* MesP1: a novel basic helix-loop-helix protein expressed in the nascent mesodermal cells during mouse gastrulation. *Development* **122**, 2769-2778. (1996).

29 Lescroart, F. *et al.* Defining the earliest step of cardiovascular lineage segregation by single-cell RNA-seq. *Science (New York, N.Y.)* **359**, 1177-1181, doi:10.1126/science.aao4174 (2018).

30 Rones, M. S., McLaughlin, K. A., Raffin, M. & Mercola, M. Serrate and Notch specify cell fates in the heart field by suppressing cardiomyogenesis. *Development* **127**, 3865-3876. (2000).

31 Contakos, S. P., Gaydos, C. M., Pfeil, E. C. & McLaughlin, K. A. Subdividing the embryo: a role for Notch signaling during germ layer patterning in Xenopus laevis. *Dev Biol* **288**, 294-307, doi:10.1016/j.ydbio.2005.09.015 (2005).

32 Miazga, C. M. & McLaughlin, K. A. Coordinating the timing of cardiac precursor development during gastrulation: a new role for Notch signaling. *Dev Biol* **333**, 285-296, doi:10.1016/j.ydbio.2009.06.040 (2009).

33 Schroeder, T. *et al.* Recombination signal sequence-binding protein Jkappa alters mesodermal cell fate decisions by suppressing cardiomyogenesis. *Proc Natl Acad Sci U S A* **100**, 4018-4023, doi:10.1073/pnas.0438008100
0438008100 [pii] (2003).

34 Nemir, M., Croquelois, A., Pedrazzini, T. & Radtke, F. Induction of cardiogenesis in embryonic stem cells via downregulation of Notch1 signaling. *Circ Res* **98**, 1471-1478 (2006).

35 Schroeder, T. *et al.* Activated Notch1 alters differentiation of embryonic stem cells into mesodermal cell lineages at multiple stages of development. *Mechanisms of development* **123**, 570-579, doi:S0925-4773(06)00062-1 [pii]
10.1016/j.mod.2006.05.002 (2006).

36 Lowell, S., Benchoua, A., Heavey, B. & Smith, A. G. Notch promotes neural lineage entry by pluripotent embryonic stem cells. *PLoS Biol* **4**, e121, doi:05-PLBI-RA-0564R4 [pii]
10.1371/journal.pbio.0040121 (2006).

37 Chen, V. C., Stull, R., Joo, D., Cheng, X. & Keller, G. Notch signaling respecifies the hemangioblast to a cardiac fate. *Nat Biotechnol* **26**, 1169-1178, doi:nbt.1497 [pii]
10.1038/nbt.1497 (2008).

38 Oka, C. *et al.* Disruption of the mouse RBP-J kappa gene results in early embryonic death. *Development* **121**, 3291-3301 (1995).

39 Souilhol, C., Cormier, S., Tanigaki, K., Babinet, C. & Cohen-Tannoudji, M. RBP-Jkappa-dependent notch signaling is dispensable for mouse early embryonic development. *Molecular and cellular biology* **26**, 4769-4774, doi:10.1128/MCB.00319-06 (2006).

40 Timmerman, L. A. *et al.* Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. *Genes & development* **18**, 99-115 (2004).

41 Munger, T. M. *et al.* A population study of the natural history of Wolff-Parkinson-White syndrome in Olmsted County, Minnesota, 1953-1989. *Circulation* **87**, 866-873 (1993).

42 de la Pompa, J. L. & Epstein, J. A. Coordinating tissue interactions: notch signaling in cardiac development and disease. *Dev Cell* **22**, 244-254, doi:10.1016/j.devcel.2012.01.014 (2012).

43 Munshi, N. V. Gene regulatory networks in cardiac conduction system development. *Circ Res* **110**, 1525-1537, doi:10.1161/CIRCRESAHA.111.260026 (2012).

44 Yamada, M., Revelli, J. P., Eichele, G., Barron, M. & Schwartz, R. J. Expression of chick Tbx-2, Tbx-3, and Tbx-5 genes during early heart development: evidence for BMP2 induction of Tbx2. *Dev Biol* **228**, 95-105, doi:10.1006/dbio.2000.9927 (2000).

45 Christoffels, V. M. *et al.* T-box transcription factor Tbx2 represses differentiation and formation of the cardiac chambers. *Dev Dyn* **229**, 763-770, doi:10.1002/dvdy.10487 (2004).
46 Singh, M. K. *et al.* Tbx20 is essential for cardiac chamber differentiation and repression of Tbx2. *Development* **132**, 2697-2707, doi:10.1242/dev.01854 (2005).
47 Stennard, F. A. *et al.* Murine T-box transcription factor Tbx20 acts as a repressor during heart development, and is essential for adult heart integrity, function and adaptation. *Development* **132**, 2451-2462, doi:10.1242/dev.01799 (2005).
48 Kokubo, H., Tomita-Miyagawa, S., Hamada, Y. & Saga, Y. Hesr1 and Hesr2 regulate atrioventricular boundary formation in the developing heart through the repression of Tbx2. *Development* **134**, 747-755 (2007).
49 Rutenberg, J. B. *et al.* Developmental patterning of the cardiac atrioventricular canal by Notch and Hairy-related transcription factors. *Development* **133**, 4381-4390, doi:10.1242/dev.02607 (2006).
50 Ma, L., Lu, M. F., Schwartz, R. J. & Martin, J. F. Bmp2 is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning. *Development* **132**, 5601-5611 (2005).
51 Aanhaanen, W. T. *et al.* The Tbx2+ primary myocardium of the atrioventricular canal forms the atrioventricular node and the base of the left ventricle. *Circ Res* **104**, 1267-1274, doi:CIRCRESAHA.108.192450 [pii]
10.1161/CIRCRESAHA.108.192450 (2009).
52 Harrelson, Z. *et al.* Tbx2 is essential for patterning the atrioventricular canal and for morphogenesis of the outflow tract during heart development. *Development* **131**, 5041-5052, doi:10.1242/dev.01378
131/20/5041 [pii] (2004).
53 Rivera-Feliciano, J. & Tabin, C. J. Bmp2 instructs cardiac progenitors to form the heart-valve-inducing field. *Dev Biol* **295**, 580-588 (2006).
54 Papoutsi, T., Luna-Zurita, L., Prados, B., Zaffran, S. & de la Pompa, J. L. Bmp2 and Notch cooperate to pattern the embryonic endocardium. *Development*, doi:10.1242/dev.163378 (2018).
55 Luna-Zurita, L. *et al.* Integration of a Notch-dependent mesenchymal gene program and Bmp2-driven cell invasiveness regulates murine cardiac valve formation. *J Clin Invest* **120**, 3493-3507, doi:42666 [pii]
10.1172/JCI42666 (2010).
56 D'Amato, G. *et al.* Sequential Notch activation regulates ventricular chamber development. *Nature cell biology* **18**, 7-20, doi:10.1038/ncb3280 (2016).
57 Del Monte, G., Grego-Bessa, J., Gonzalez-Rajal, A., Bolos, V. & De La Pompa, J. L. Monitoring Notch1 activity in development: Evidence for a feedback regulatory loop. *Dev Dyn* **236**, 2594-2614 (2007).
58 Grego-Bessa, J. *et al.* Notch signaling is essential for ventricular chamber development. *Dev Cell* **12**, 415-429, doi:S1534-5807(06)00600-9 [pii]
10.1016/j.devcel.2006.12.011 (2007).
59 Fischer, A. *et al.* Combined loss of Hey1 and HeyL causes congenital heart defects because of impaired epithelial to mesenchymal transition. *Circ Res* **100**, 856-863, doi:01.RES.0000260913.95642.3b [pii]
10.1161/01.RES.0000260913.95642.3b (2007).
60 Watanabe, Y. *et al.* Activation of Notch1 signaling in cardiogenic mesoderm induces abnormal heart morphogenesis in mouse. *Development* **133**, 1625-1634 (2006).
61 Kisanuki, Y. Y. *et al.* Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev Biol* **230**, 230-242, doi:10.1006/dbio.2000.0106 (2001).
62 Rentschler, S. *et al.* Notch signaling regulates murine atrioventricular conduction and the formation of accessory pathways. *J Clin Invest* **121**, 525-533, doi:44470 [pii]
10.1172/JCI44470 (2011).
63 Gillers, B. S. *et al.* Canonical wnt signaling regulates atrioventricular junction programming and electrophysiological properties. *Circ Res* **116**, 398-406, doi:10.1161/CIRCRESAHA.116.304731 (2015).
64 Kelly, R. G. The second heart field. *Curr Top Dev Biol* **100**, 33-65, doi:10.1016/B978-0-12-387786-4.00002-6 (2012).

65 Dyer, L. A. & Kirby, M. L. The role of secondary heart field in cardiac development. *Dev Biol* **336**, 137-144, doi:10.1016/j.ydbio.2009.10.009 (2009).
66 Meilhac, S. M., Lescroart, F., Blanpain, C. & Buckingham, M. E. Cardiac cell lineages that form the heart. *Cold Spring Harb Perspect Med* **4**, a013888, doi:10.1101/cshperspect.a013888 (2014).
67 Ma, Q., Zhou, B. & Pu, W. T. Reassessment of Isl1 and Nkx2-5 cardiac fate maps using a Gata4-based reporter of Cre activity. *Dev Biol* **323**, 98-104, doi:S0012-1606(08)01139-1 [pii]
10.1016/j.ydbio.2008.08.013 (2008).
68 Verzi, M. P., McCulley, D. J., De Val, S., Dodou, E. & Black, B. L. The right ventricle, outflow tract, and ventricular septum comprise a restricted expression domain within the secondary/anterior heart field. *Dev Biol* **287**, 134-145, doi:10.1016/j.ydbio.2005.08.041 (2005).
69 Zaffran, S., Kelly, R. G., Meilhac, S. M., Buckingham, M. E. & Brown, N. A. Right ventricular myocardium derives from the anterior heart field. *Circ Res* **95**, 261-268, doi:10.1161/01.RES.0000136815.73623.BE
01.RES.0000136815.73623.BE [pii] (2004).
70 Rochais, F. *et al.* Hes1 is expressed in the second heart field and is required for outflow tract development. *PloS one* **4**, e6267, doi:10.1371/journal.pone.0006267 (2009).
71 Kwon, C. *et al.* A regulatory pathway involving Notch1/beta-catenin/Isl1 determines cardiac progenitor cell fate. *Nature cell biology* **11**, 951-957, doi:10.1038/ncb1906 (2009).
72 Klaus, A. *et al.* Wnt/beta-catenin and Bmp signals control distinct sets of transcription factors in cardiac progenitor cells. *Proc Natl Acad Sci U S A* **109**, 10921-10926, doi:10.1073/pnas.1121236109 (2012).
73 McDaniell, R. *et al.* NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway. *Am J Hum Genet* **79**, 169-173 (2006).
74 McElhinney, D. B. *et al.* Analysis of cardiovascular phenotype and genotype-phenotype correlation in individuals with a JAG1 mutation and/or Alagille syndrome. *Circulation* **106**, 2567-2574 (2002).
75 Eldadah, Z. A. *et al.* Familial Tetralogy of Fallot caused by mutation in the jagged1 gene. *Hum Mol Genet* **10**, 163-169 (2001).
76 Greenway, S. C. *et al.* De novo copy number variants identify new genes and loci in isolated sporadic tetralogy of Fallot. *Nature genetics* **41**, 931-935, doi:ng.415 [pii]
10.1038/ng.415 (2009).
77 Neeb, Z., Lajiness, J. D., Bolanis, E. & Conway, S. J. Cardiac outflow tract anomalies. *Wiley Interdiscip Rev Dev Biol* **2**, 499-530, doi:10.1002/wdev.98 (2013).
78 Nakajima, M., Moriizumi, E., Koseki, H. & Shirasawa, T. Presenilin 1 is essential for cardiac morphogenesis. *Dev Dyn* **230**, 795-799, doi:10.1002/dvdy.20098 (2004).
79 McCright, B., Lozier, J. & Gridley, T. A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. *Development* **129**, 1075-1082 (2002).
80 High, F. A. *et al.* Murine Jagged1/Notch signaling in the second heart field orchestrates Fgf8 expression and tissue-tissue interactions during outflow tract development. *J Clin Invest* **119**, 1986-1996, doi:38922 [pii]
10.1172/JCI38922 (2009).
81 Ilagan, R. *et al.* Fgf8 is required for anterior heart field development. *Development* **133**, 2435-2445, doi:10.1242/dev.02408 (2006).
82 Donovan, J., Kordylewska, A., Jan, Y. N. & Utset, M. F. Tetralogy of fallot and other congenital heart defects in Hey2 mutant mice. *Curr Biol* **12**, 1605-1610 (2002).
83 Fischer, A., Schumacher, N., Maier, M., Sendtner, M. & Gessler, M. The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. *Genes & development* **18**, 901-911 (2004).
84 Gessler, M. *et al.* Mouse gridlock: no aortic coarctation or deficiency, but fatal cardiac defects in Hey2 -/- mice. *Curr Biol* **12**, 1601-1604 (2002).
85 Kokubo, H., Miyagawa-Tomita, S., Nakazawa, M., Saga, Y. & Johnson, R. L. Mouse hesr1 and hesr2 genes are redundantly required to mediate Notch signaling in the developing cardiovascular system. *Dev Biol* **278**, 301-309 (2005).
86 Sakata, Y. *et al.* Ventricular septal defect and cardiomyopathy in mice lacking the transcription factor CHF1/Hey2. *Proc Natl Acad Sci U S A* **99**, 16197-16202, doi:10.1073/pnas.252648999
252648999 [pii] (2002).

87 Sakata, Y. *et al.* The spectrum of cardiovascular anomalies in CHF1/Hey2 deficient mice reveals roles in endocardial cushion, myocardial and vascular maturation. *J Mol Cell Cardiol* **40**, 267-273, doi:S0022-2828(05)00286-5 [pii]
10.1016/j.yjmcc.2005.09.006 (2006).
88 Shirai, M., Imanaka-Yoshida, K., Schneider, M. D., Schwartz, R. J. & Morisaki, T. T-box 2, a mediator of Bmp-Smad signaling, induced hyaluronan synthase 2 and Tgfbeta2 expression and endocardial cushion formation. *Proc Natl Acad Sci U S A* **106**, 18604-18609, doi:10.1073/pnas.0900635106 (2009).
89 Shelton, E. L. & Yutzey, K. E. Tbx20 regulation of endocardial cushion cell proliferation and extracellular matrix gene expression. *Dev Biol* **302**, 376-388, doi:S0012-1606(06)01264-4 [pii]
10.1016/j.ydbio.2006.09.047 (2007).
90 Armstrong, E. J. & Bischoff, J. Heart valve development: endothelial cell signaling and differentiation. *Circ Res* **95**, 459-470 (2004).
91 MacGrogan, D. *et al.* Sequential Ligand-Dependent Notch Signaling Activation Regulates Valve Primordium Formation and Morphogenesis. *Circ Res* **118**, 1480-1497, doi:10.1161/CIRCRESAHA.115.308077 (2016).
92 Niessen, K. *et al.* Slug is a direct Notch target required for initiation of cardiac cushion cellularization. *The Journal of cell biology* **182**, 315-325, doi:jcb.200710067 [pii]
10.1083/jcb.200710067 (2008).
93 Wang, Y. *et al.* Endocardial to myocardial notch-wnt-bmp axis regulates early heart valve development. *PloS one* **8**, e60244, doi:10.1371/journal.pone.0060244 (2013).
94 Samsa, L. A. *et al.* Cardiac contraction activates endocardial Notch signaling to modulate chamber maturation in zebrafish. *Development* **142**, 4080-4091, doi:10.1242/dev.125724 (2015).
95 Walsh, E. C. & Stainier, D. Y. UDP-glucose dehydrogenase required for cardiac valve formation in zebrafish. *Science (New York, N.Y.)* **293**, 1670-1673. (2001).
96 Heidersbach, A. *et al.* microRNA-1 regulates sarcomere formation and suppresses smooth muscle gene expression in the mammalian heart. *Elife* **2**, e01323, doi:10.7554/eLife.01323 (2013).
97 Vermot, J. *et al.* Reversing blood flows act through klf2a to ensure normal valvulogenesis in the developing heart. *PLoS Biol* **7**, e1000246, doi:10.1371/journal.pbio.1000246 (2009).
98 Steed, E. *et al.* klf2a couples mechanotransduction and zebrafish valve morphogenesis through fibronectin synthesis. *Nat Commun* **7**, 11646, doi:10.1038/ncomms11646 (2016).
99 Donat, S. *et al.* Heg1 and Ccm1/2 proteins control endocardial mechanosensitivity during zebrafish valvulogenesis. *Elife* **7**, doi:10.7554/eLife.28939 (2018).
100 Pestel, J. *et al.* Real-time 3D visualization of cellular rearrangements during cardiac valve formation. *Development* **143**, 2217-2227, doi:10.1242/dev.133272 (2016).
101 Beis, D. *et al.* Genetic and cellular analyses of zebrafish atrioventricular cushion and valve development. *Development* **132**, 4193-4204 (2005).
102 Gridley, T. Notch signaling in the vasculature. *Curr Top Dev Biol* **92**, 277-309, doi:S0070-2153(10)92009-7 [pii]
10.1016/S0070-2153(10)92009-7 (2010).
103 Hurlstone, A. F. *et al.* The Wnt/beta-catenin pathway regulates cardiac valve formation. *Nature* **425**, 633-637. (2003).
104 Verhoeven, M. C., Haase, C., Christoffels, V. M., Weidinger, G. & Bakkers, J. Wnt signaling regulates atrioventricular canal formation upstream of BMP and Tbx2. *Birth Defects Res A Clin Mol Teratol* **91**, 435-440, doi:10.1002/bdra.20804 (2011).
105 Plein, A., Fantin, A. & Ruhrberg, C. Neural crest cells in cardiovascular development. *Curr Top Dev Biol* **111**, 183-200, doi:10.1016/bs.ctdb.2014.11.006 (2015).
106 Phillips, H. M. *et al.* Neural crest cells are required for correct positioning of the developing outflow cushions and pattern the arterial valve leaflets. *Cardiovascular research* **99**, 452-460, doi:10.1093/cvr/cvt132 (2013).
107 High, F. & Epstein, J. A. Signalling pathways regulating cardiac neural crest migration and differentiation. *Novartis Found Symp* **283**, 152-161; discussion 161-154, 238-141 (2007).
108 MacGrogan, D. *et al.* How to make a heart valve: from embryonic development to bioengineering of living valve substitutes. *Cold Spring Harb Perspect Med* **4**, a013912, doi:10.1101/cshperspect.a013912 (2014).

109 Lin, C. Y. *et al.* The secondary heart field is a new site of calcineurin/Nfatc1 signaling for semilunar valve development. *J Mol Cell Cardiol* **52**, 1096-1102, doi:10.1016/j.yjmcc.2012.01.013 (2012).
110 Jain, R. *et al.* Cardiac neural crest orchestrates remodeling and functional maturation of mouse semilunar valves. *J Clin Invest* **121**, 422-430, doi:44244 [pii]
10.1172/JCI44244 (2011).
111 Wang, Y. *et al.* Notch-Tnf signalling is required for development and homeostasis of arterial valves. *Eur Heart J* **38**, 675-686, doi:10.1093/eurheartj/ehv520 (2017).
112 Prakash, S. K. *et al.* A roadmap to investigate the genetic basis of bicuspid aortic valve and its complications: insights from the International BAVCon (Bicuspid Aortic Valve Consortium). *J Am Coll Cardiol* **64**, 832-839, doi:10.1016/j.jacc.2014.04.073 (2014).
113 Siu, S. C. & Silversides, C. K. Bicuspid aortic valve disease. *J Am Coll Cardiol* **55**, 2789-2800, doi:S0735-1097(10)01433-6 [pii]
10.1016/j.jacc.2009.12.068 (2010).
114 Fernandez, B. *et al.* Bicuspid aortic valves with different spatial orientations of the leaflets are distinct etiological entities. *J Am Coll Cardiol* **54**, 2312-2318, doi:S0735-1097(09)03051-4 [pii]
10.1016/j.jacc.2009.07.044 (2009).
115 Aboulhosn, J. & Child, J. S. Left ventricular outflow obstruction: subaortic stenosis, bicuspid aortic valve, supravalvar aortic stenosis, and coarctation of the aorta. *Circulation* **114**, 2412-2422, doi:10.1161/CIRCULATIONAHA.105.592089 (2006).
116 Michelena, H. I. *et al.* Bicuspid aortic valve: identifying knowledge gaps and rising to the challenge from the International Bicuspid Aortic Valve Consortium (BAVCon). *Circulation* **129**, 2691-2704, doi:10.1161/CIRCULATIONAHA.113.007851 (2014).
117 Tadros, T. M., Klein, M. D. & Shapira, O. M. Ascending aortic dilatation associated with bicuspid aortic valve: pathophysiology, molecular biology, and clinical implications. *Circulation* **119**, 880-890, doi:10.1161/CIRCULATIONAHA.108.795401 (2009).
118 Cripe, L., Andelfinger, G., Martin, L. J., Shooner, K. & Benson, D. W. Bicuspid aortic valve is heritable. *J Am Coll Cardiol* **44**, 138-143, doi:10.1016/j.jacc.2004.03.050
S0735109704007739 [pii] (2004).
119 Hinton, R. B. *et al.* Hypoplastic left heart syndrome links to chromosomes 10q and 6q and is genetically related to bicuspid aortic valve. *J Am Coll Cardiol* **53**, 1065-1071, doi:10.1016/j.jacc.2008.12.023 (2009).
120 Martin, L. J. *et al.* Evidence in favor of linkage to human chromosomal regions 18q, 5q and 13q for bicuspid aortic valve and associated cardiovascular malformations. *Hum Genet* **121**, 275-284, doi:10.1007/s00439-006-0316-9 (2007).
121 McBride, K. L. *et al.* NOTCH1 mutations in individuals with left ventricular outflow tract malformations reduce ligand-induced signaling. *Hum Mol Genet* **17**, 2886-2893, doi:ddn187 [pii]
10.1093/hmg/ddn187 (2008).
122 Foffa, I. *et al.* Sequencing of NOTCH1, GATA5, TGFBR1 and TGFBR2 genes in familial cases of bicuspid aortic valve. *BMC Med Genet* **14**, 44, doi:10.1186/1471-2350-14-44 (2013).
123 McKellar, S. H. *et al.* Novel NOTCH1 mutations in patients with bicuspid aortic valve disease and thoracic aortic aneurysms. *J Thorac Cardiovasc Surg* **134**, 290-296, doi:S0022-5223(07)00599-5 [pii]
10.1016/j.jtcvs.2007.02.041 (2007).
124 Mohamed, S. A. *et al.* Novel missense mutations (p.T596M and p.P1797H) in NOTCH1 in patients with bicuspid aortic valve. *Biochemical and biophysical research communications* **345**, 1460-1465, doi:S0006-291X(06)01086-2 [pii]
10.1016/j.bbrc.2006.05.046 (2006).
125 Kerstjens-Frederikse, W. S. *et al.* Cardiovascular malformations caused by NOTCH1 mutations do not keep left: data on 428 probands with left-sided CHD and their families. *Genet Med* **18**, 914-923, doi:10.1038/gim.2015.193 (2016).
126 Nigam, V. & Srivastava, D. Notch1 represses osteogenic pathways in aortic valve cells. *J Mol Cell Cardiol* **47**, 828-834, doi:S0022-2828(09)00329-0 [pii]
10.1016/j.yjmcc.2009.08.008 (2009).

127 Nus, M. *et al.* Diet-Induced Aortic Valve Disease in Mice Haploinsufficient for the Notch Pathway Effector RBPJK/CSL. *Arterioscler Thromb Vasc Biol* **31**, 1580-1588, doi:ATVBAHA.111.227561 [pii]
10.1161/ATVBAHA.111.227561 (2011).

128 Theodoris, C. V. *et al.* Human disease modeling reveals integrated transcriptional and epigenetic mechanisms of NOTCH1 haploinsufficiency. *Cell* **160**, 1072-1086, doi:10.1016/j.cell.2015.02.035 (2015).

129 Sedmera, D., Pexieder, T., Hu, N. & Clark, E. B. Developmental changes in the myocardial architecture of the chick. *Anat Rec* **248**, 421-432 (1997).

130 Sedmera, D., Pexieder, T., Vuillemin, M., Thompson, R. P. & Anderson, R. H. Developmental patterning of the myocardium. *Anat Rec* **258**, 319-337, doi:10.1002/(SICI)1097-0185(20000401)258:4<319::AID-AR1>3.0.CO;2-O [pii] (2000).

131 Hu, N., Sedmera, D., Yost, H. J. & Clark, E. B. Structure and function of the developing zebrafish heart. *Anat Rec* **260**, 148-157. (2000).

132 Sedmera, D. *et al.* Spatiotemporal pattern of commitment to slowed proliferation in the embryonic mouse heart indicates progressive differentiation of the cardiac conduction system. *The anatomical record. Part A, Discoveries in molecular, cellular, and evolutionary biology* **274**, 773-777, doi:10.1002/ar.a.10085 (2003).

133 Park, D. S. *et al.* Pocket proteins critically regulate cell cycle exit of the trabecular myocardium and the ventricular conduction system. *Biology Open* **2**, 968-978, doi:10.1242/bio.20135785 (2013).

134 Rentschler, S. *et al.* Visualization and functional characterization of the developing murine cardiac conduction system. *Development* **128**, 1785-1792 (2001).

135 Moorman, A. F. & Christoffels, V. M. Cardiac chamber formation: development, genes, and evolution. *Physiological reviews* **83**, 1223-1267 (2003).

136 Christoffels, V. M. & Moorman, A. F. Development of the cardiac conduction system: why are some regions of the heart more arrhythmogenic than others? *Circ Arrhythm Electrophysiol* **2**, 195-207, doi:2/2/195 [pii]
10.1161/CIRCEP.108.829341 (2009).

137 Jimenez-Amilburu, V. *et al.* In Vivo Visualization of Cardiomyocyte Apicobasal Polarity Reveals Epithelial to Mesenchymal-like Transition during Cardiac Trabeculation. *Cell Rep* **17**, 2687-2699, doi:10.1016/j.celrep.2016.11.023 (2016).

138 Li, J. *et al.* Single-Cell Lineage Tracing Reveals that Oriented Cell Division Contributes to Trabecular Morphogenesis and Regional Specification. *Cell Rep* **15**, 158-170, doi:10.1016/j.celrep.2016.03.012 (2016).

139 Passer, D., van de Vrugt, A., Atmanli, A. & Domian, I. J. Atypical Protein Kinase C-Dependent Polarized Cell Division Is Required for Myocardial Trabeculation. *Cell Rep* **14**, 1662-1672, doi:10.1016/j.celrep.2016.01.030 (2016).

140 Le Garrec, J. F. *et al.* Quantitative analysis of polarity in 3D reveals local cell coordination in the embryonic mouse heart. *Development* **140**, 395-404, doi:10.1242/dev.087940 (2013).

141 Staudt, D. W. *et al.* High-resolution imaging of cardiomyocyte behavior reveals two distinct steps in ventricular trabeculation. *Development* **141**, 585-593, doi:10.1242/dev.098632 (2014).

142 de Luxan, G., D'Amato, G., MacGrogan, D. & de la Pompa, J. L. Endocardial Notch Signaling in Cardiac Development and Disease. *Circ Res*, doi:10.1161/CIRCRESAHA.115.305350 (2015).

143 Chen, H. *et al.* BMP10 is essential for maintaining cardiac growth during murine cardiogenesis. *Development* **131**, 2219-2231, doi:10.1242/dev.01094 (2004).

144 Gerety, S. S. & Anderson, D. J. Cardiovascular ephrinB2 function is essential for embryonic angiogenesis. *Development* **129**, 1397-1410 (2002).

145 Meyer, D. & Birchmeier, C. Multiple essential functions of neuregulin in development. *Nature* **378**, 386-390. (1995).

146 VanDusen, N. J. *et al.* Hand2 is an essential regulator for two Notch-dependent functions within the embryonic endocardium. *Cell Rep* **9**, 2071-2083, doi:10.1016/j.celrep.2014.11.021 (2014).

147 Bjarnadottir, T. K., Fredriksson, R. & Schioth, H. B. The adhesion GPCRs: a unique family of G protein-coupled receptors with important roles in both central and peripheral tissues. *Cell Mol Life Sci* **64**, 2104-2119, doi:10.1007/s00018-007-7067-1 (2007).

148 Waller-Evans, H. *et al.* The orphan adhesion-GPCR GPR126 is required for embryonic development in the mouse. *PloS one* **5**, e14047, doi:10.1371/journal.pone.0014047 (2010).
149 Patra, C. *et al.* Organ-specific function of adhesion G protein-coupled receptor GPR126 is domain-dependent. *Proc Natl Acad Sci U S A* **110**, 16898-16903, doi:10.1073/pnas.1304837110 (2013).
150 Del Monte-Nieto, G. *et al.* Control of cardiac jelly dynamics by NOTCH1 and NRG1 defines the building plan for trabeculation. *Nature* **557**, 439-445, doi:10.1038/s41586-018-0110-6 (2018).
151 Jenni, R., Oechslin, E. N. & van der Loo, B. Isolated ventricular non-compaction of the myocardium in adults. *Heart* **93**, 11-15, doi:10.1136/hrt.2005.082271 (2007).
152 Oechslin, E. & Jenni, R. Left ventricular non-compaction revisited: a distinct phenotype with genetic heterogeneity? *Eur Heart J* **32**, 1446-1456, doi:ehq508 [pii]
10.1093/eurheartj/ehq508 (2011).
153 Chin, T. K., Perloff, J. K., Williams, R. G., Jue, K. & Mohrmann, R. Isolated noncompaction of left ventricular myocardium. A study of eight cases. *Circulation* **82**, 507-513 (1990).
154 Maron, B. J. *et al.* Contemporary definitions and classification of the cardiomyopathies: an American Heart Association Scientific Statement from the Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups; and Council on Epidemiology and Prevention. *Circulation* **113**, 1807-1816, doi:10.1161/CIRCULATIONAHA.106.174287 (2006).
155 Towbin, J. A., Lorts, A. & Jefferies, J. L. Left ventricular non-compaction cardiomyopathy. *Lancet* **386**, 813-825, doi:10.1016/S0140-6736(14)61282-4 (2015).
156 Ritter, M. *et al.* Isolated noncompaction of the myocardium in adults. *Mayo Clin Proc* **72**, 26-31, doi:10.1016/S0025-6196(11)64725-3 (1997).
157 Oechslin, E. N., Attenhofer Jost, C. H., Rojas, J. R., Kaufmann, P. A. & Jenni, R. Long-term follow-up of 34 adults with isolated left ventricular noncompaction: a distinct cardiomyopathy with poor prognosis. *J Am Coll Cardiol* **36**, 493-500 (2000).
158 Stollberger, C. & Finsterer, J. Value of cardiac magnetic resonance imaging in the diagnosis of left ventricular hypertrabeculation/noncompaction. *J Cardiovasc Magn Reson* **6**, 959-960; author reply 961-952 (2004).
159 Petersen, S. E. *et al.* Left ventricular non-compaction: insights from cardiovascular magnetic resonance imaging. *J Am Coll Cardiol* **46**, 101-105, doi:10.1016/j.jacc.2005.03.045 (2005).
160 Captur, G. & Nihoyannopoulos, P. Left ventricular non-compaction: genetic heterogeneity, diagnosis and clinical course. *Int J Cardiol* **140**, 145-153, doi:10.1016/j.ijcard.2009.07.003 (2010).
161 Klaassen, S. *et al.* Mutations in sarcomere protein genes in left ventricular noncompaction. *Circulation* **117**, 2893-2901, doi:10.1161/CIRCULATIONAHA.107.746164 (2008).
162 Postma, A. V. *et al.* Mutations in the sarcomere gene MYH7 in Ebstein anomaly. *Circ Cardiovasc Genet* **4**, 43-50, doi:10.1161/CIRCGENETICS.110.957985 (2011).
163 Ichida, F. *et al.* Novel gene mutations in patients with left ventricular noncompaction or Barth syndrome. *Circulation* **103**, 1256-1263 (2001).
164 Shan, L. *et al.* SCN5A variants in Japanese patients with left ventricular noncompaction and arrhythmia. *Mol Genet Metab* **93**, 468-474 (2008).
165 Hermida-Prieto, M. *et al.* Familial dilated cardiomyopathy and isolated left ventricular noncompaction associated with lamin A/C gene mutations. *Am J Cardiol* **94**, 50-54, doi:10.1016/j.amjcard.2004.03.029 (2004).
166 Tian, X. *et al.* Identification of a hybrid myocardial zone in the mammalian heart after birth. *Nat Commun* **8**, 87, doi:10.1038/s41467-017-00118-1 (2017).
167 Tian, X. *et al.* Vessel formation. De novo formation of a distinct coronary vascular population in neonatal heart. *Science (New York, N.Y.)* **345**, 90-94, doi:10.1126/science.1251487 (2014).
168 Del Monte, G. *et al.* Differential Notch Signaling in the Epicardium Is Required for Cardiac Inflow Development and Coronary Vessel Morphogenesis. *Circ Res* **108**, 824-836, doi:CIRCRESAHA.110.229062 [pii]
10.1161/CIRCRESAHA.110.229062 (2011).

169 Jenni, R., Oechslin, E., Schneider, J., Attenhofer Jost, C. & Kaufmann, P. A. Echocardiographic and pathoanatomical characteristics of isolated left ventricular non-compaction: a step towards classification as a distinct cardiomyopathy. *Heart* **86**, 666-671 (2001).

170 Red-Horse, K., Ueno, H., Weissman, I. L. & Krasnow, M. A. Coronary arteries form by developmental reprogramming of venous cells. *Nature* **464**, 549-553, doi:nature08873 [pii]
10.1038/nature08873 (2010).

171 Perez-Pomares, J. M. *et al.* Origin of coronary endothelial cells from epicardial mesothelium in avian embryos. *The International journal of developmental biology* **46**, 1005-1013 (2002).

172 Tian, X. *et al.* Subepicardial endothelial cells invade the embryonic ventricle wall to form coronary arteries. *Cell Res* **23**, 1075-1090, doi:10.1038/cr.2013.83 (2013).

173 Wu, B. *et al.* Endocardial cells form the coronary arteries by angiogenesis through myocardial-endocardial VEGF signaling. *Cell* **151**, 1083-1096, doi:10.1016/j.cell.2012.10.023 (2012).

174 Zhang, H. *et al.* Genetic lineage tracing identifies endocardial origin of liver vasculature. *Nature genetics* **48**, 537-543, doi:10.1038/ng.3536 (2016).

175 Sharma, B. *et al.* Alternative Progenitor Cells Compensate to Rebuild the Coronary Vasculature in Elabela- and Apj-Deficient Hearts. *Dev Cell* **42**, 655-666 e653, doi:10.1016/j.devcel.2017.08.008 (2017).

176 Cano, E. *et al.* Extracardiac septum transversum/proepicardial endothelial cells pattern embryonic coronary arterio-venous connections. *Proc Natl Acad Sci U S A* **113**, 656-661, doi:10.1073/pnas.1509834113 (2016).

177 Katz, T. C. *et al.* Distinct compartments of the proepicardial organ give rise to coronary vascular endothelial cells. *Dev Cell* **22**, 639-650, doi:10.1016/j.devcel.2012.01.012 (2012).

178 Zhou, B. & Pu, W. T. Genetic Cre-loxP assessment of epicardial cell fate using Wt1-driven Cre alleles. *Circ Res* **111**, e276-280, doi:10.1161/CIRCRESAHA.112.275784 (2012).

179 Riley, P. R. An epicardial floor plan for building and rebuilding the mammalian heart. *Curr Top Dev Biol* **100**, 233-251, doi:10.1016/B978-0-12-387786-4.00007-5 (2012).

180 Grieskamp, T., Rudat, C., Ludtke, T. H., Norden, J. & Kispert, A. Notch signaling regulates smooth muscle differentiation of epicardium-derived cells. *Circ Res* **108**, 813-823, doi:CIRCRESAHA.110.228809 [pii]
10.1161/CIRCRESAHA.110.228809 (2011).

181 Munch, J., Gonzalez-Rajal, A. & de la Pompa, J. L. Notch regulates blastema proliferation and prevents differentiation during adult zebrafish fin regeneration. *Development* **140**, 1402-1411, doi:10.1242/dev.087346 (2013).

182 de Oliveira-Carlos, V., Ganz, J., Hans, S., Kaslin, J. & Brand, M. Notch receptor expression in neurogenic regions of the adult zebrafish brain. *PloS one* **8**, e73384, doi:10.1371/journal.pone.0073384 (2013).

183 Grotek, B., Wehner, D. & Weidinger, G. Notch signaling coordinates cellular proliferation with differentiation during zebrafish fin regeneration. *Development* **140**, 1412-1423, doi:10.1242/dev.087452 (2013).

184 Beck, C. W., Christen, B. & Slack, J. M. Molecular pathways needed for regeneration of spinal cord and muscle in a vertebrate. *Dev Cell* **5**, 429-439 (2003).

185 Dias, T. B., Yang, Y. J., Ogai, K., Becker, T. & Becker, C. G. Notch signaling controls generation of motor neurons in the lesioned spinal cord of adult zebrafish. *J Neurosci* **32**, 3245-3252, doi:10.1523/JNEUROSCI.6398-11.2012 (2012).

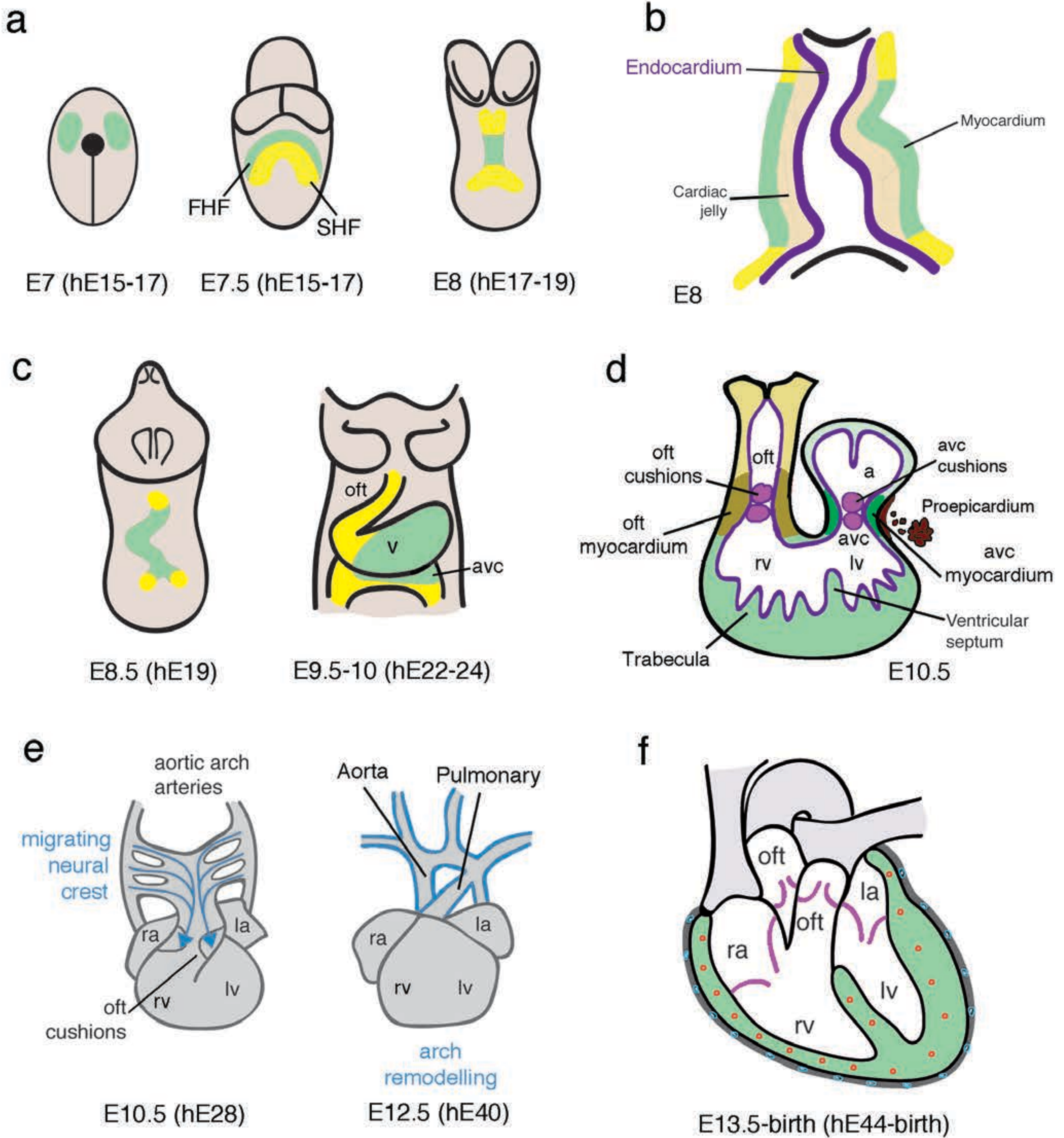
186 Wan, J., Ramachandran, R. & Goldman, D. HB-EGF is necessary and sufficient for Muller glia dedifferentiation and retina regeneration. *Dev Cell* **22**, 334-347, doi:10.1016/j.devcel.2011.11.020 (2012).

187 Zhang, R. *et al.* In vivo cardiac reprogramming contributes to zebrafish heart regeneration. *Nature* **498**, 497-501, doi:10.1038/nature12322 (2013).

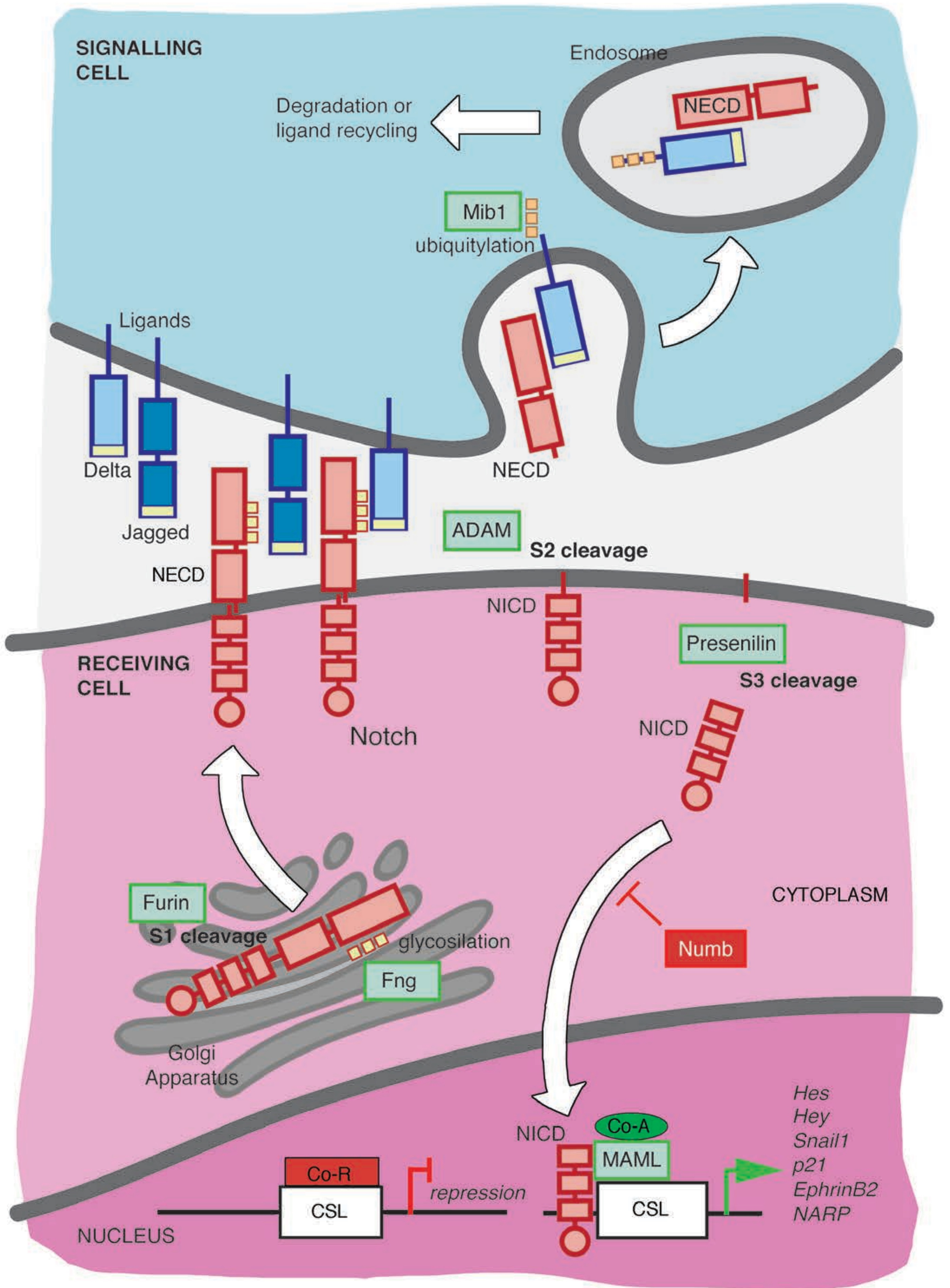
188 Poss, K. D., Wilson, L. G. & Keating, M. T. Heart regeneration in zebrafish. *Science (New York, N.Y.)* **298**, 2188-2190 (2002).

189 Gonzalez-Rosa, J. M., Martin, V., Peralta, M., Torres, M. & Mercader, N. Extensive scar formation and regression during heart regeneration after cryoinjury in zebrafish. *Development* **138**, 1663-1674, doi:dev.060897 [pii]
10.1242/dev.060897 (2011).

190 Schnabel, K., Wu, C. C., Kurth, T. & Weidinger, G. Regeneration of cryoinjury induced necrotic heart lesions in zebrafish is associated with epicardial activation and cardiomyocyte proliferation. *PloS one* **6**, e18503, doi:10.1371/journal.pone.0018503 (2011).
191 Chablais, F., Veit, J., Rainer, G. & Jazwinska, A. The zebrafish heart regenerates after cryoinjury-induced myocardial infarction. *BMC Dev Biol* **11**, 21, doi:1471-213X-11-21 [pii]
10.1186/1471-213X-11-21 (2011).
192 Jopling, C. *et al.* Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature* **464**, 606-609, doi:nature08899 [pii]
10.1038/nature08899 (2010).
193 Lepilina, A. *et al.* A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. *Cell* **127**, 607-619 (2006).
194 Raya, A. *et al.* Activation of Notch signaling pathway precedes heart regeneration in zebrafish. *Proc Natl Acad Sci U S A* **100 Suppl 1**, 11889-11895 (2003).
195 Munch, J., Grivas, D., Gonzalez-Rajal, A., Torregrosa-Carrion, R. & de la Pompa, J. L. Notch signalling restricts inflammation and serpine1 expression in the dynamic endocardium of the regenerating zebrafish heart. *Development* **144**, 1425-1440, doi:10.1242/dev.143362 (2017).
196 Zhao, L. *et al.* Notch signaling regulates cardiomyocyte proliferation during zebrafish heart regeneration. *Proc Natl Acad Sci U S A* **111**, 1403-1408, doi:10.1073/pnas.1311705111 (2014).
197 Hellstrom, M. *et al.* Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature* **445**, 776-780 (2007).
198 Suchting, S. *et al.* The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. *Proc Natl Acad Sci U S A* **104**, 3225-3230, doi:0611177104 [pii]
10.1073/pnas.0611177104 (2007).
199 Darehzereshki, A. *et al.* Differential regenerative capacity of neonatal mouse hearts after cryoinjury. *Dev Biol* **399**, 91-99, doi:10.1016/j.ydbio.2014.12.018 (2015).
200 Adler, E. D. *et al.* The cardiomyocyte lineage is critical for optimization of stem cell therapy in a mouse model of myocardial infarction. *FASEB J* **24**, 1073-1081, doi:10.1096/fj.09-135426 (2010).
201 Kikuchi, K. *et al.* Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes. *Nature* **464**, 601-605, doi:nature08804 [pii]
10.1038/nature08804 (2010).
202 Senyo, S. E. *et al.* Mammalian heart renewal by pre-existing cardiomyocytes. *Nature* **493**, 433-436, doi:10.1038/nature11682 (2013).
203 Porrello, E. R. *et al.* Transient regenerative potential of the neonatal mouse heart. *Science (New York, N.Y.)* **331**, 1078-1080, doi:331/6020/1078 [pii]
10.1126/science.1200708 (2011).
204 Collesi, C., Zentilin, L., Sinagra, G. & Giacca, M. Notch1 signaling stimulates proliferation of immature cardiomyocytes. *The Journal of cell biology* **183**, 117-128, doi:jcb.200806091 [pii]
10.1083/jcb.200806091 (2008).
205 Campa, V. M. *et al.* Notch activates cell cycle reentry and progression in quiescent cardiomyocytes. *The Journal of cell biology* **183**, 129-141, doi:jcb.200806104 [pii]
10.1083/jcb.200806104 (2008).
206 Gude, N. A. *et al.* Activation of Notch-mediated protective signaling in the myocardium. *Circ Res* **102**, 1025-1035, doi:CIRCRESAHA.107.164749 [pii]
10.1161/CIRCRESAHA.107.164749 (2008).
207 Kratsios, P. *et al.* Distinct roles for cell-autonomous Notch signaling in cardiomyocytes of the embryonic and adult heart. *Circ Res* **106**, 559-572, doi:10.1161/CIRCRESAHA.109.203034 (2010).
208 Luxan, G., D'Amato, G., MacGrogan, D. & de la Pompa, J. L. Endocardial Notch Signaling in Cardiac Development and Disease. *Circ Res* **118**, e1-e18, doi:10.1161/CIRCRESAHA.115.305350 (2016).



Box1_Figure_MacGrogan



Box2_Figure_MacGrogan et al

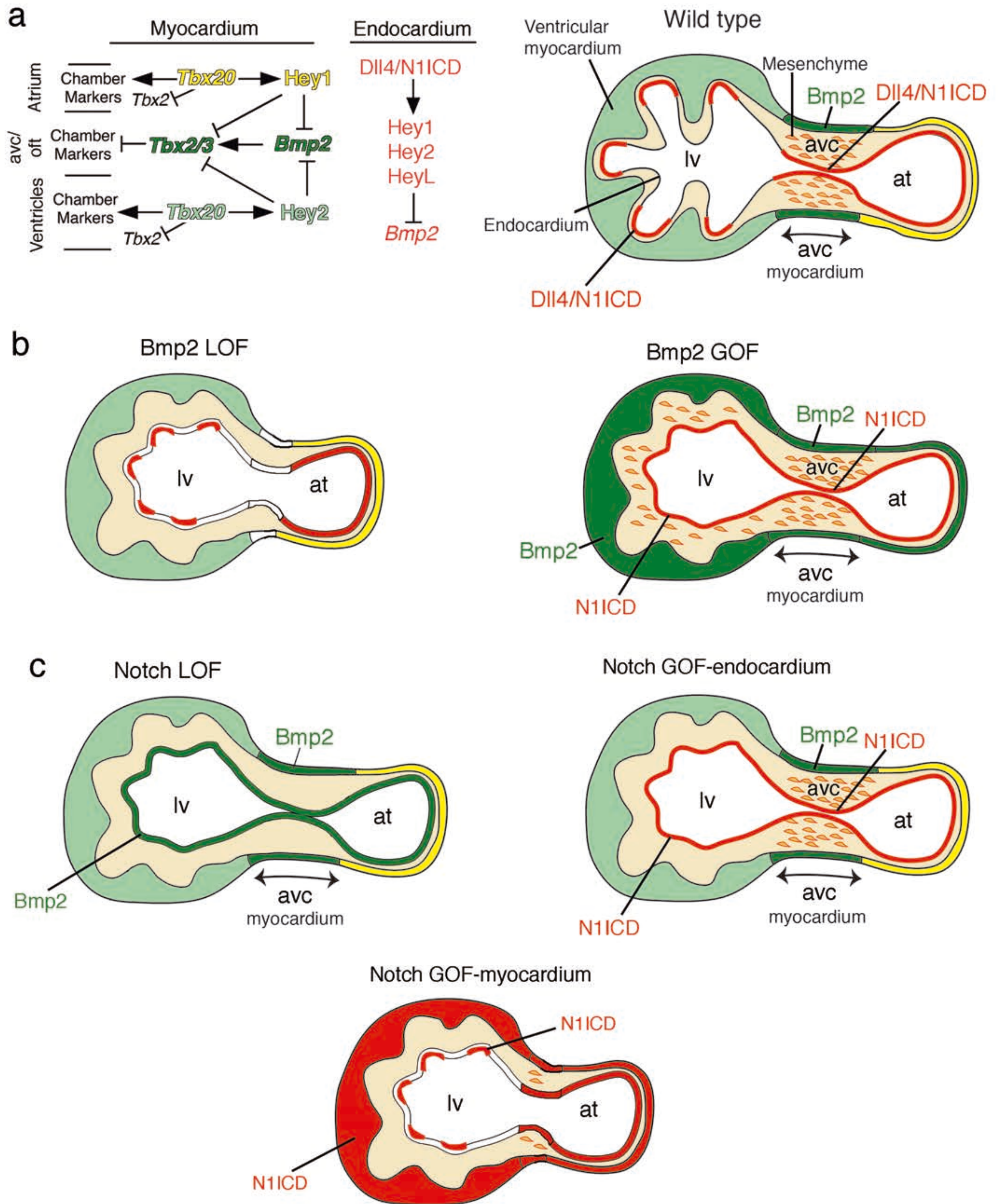


Figure 1_MacGrogan

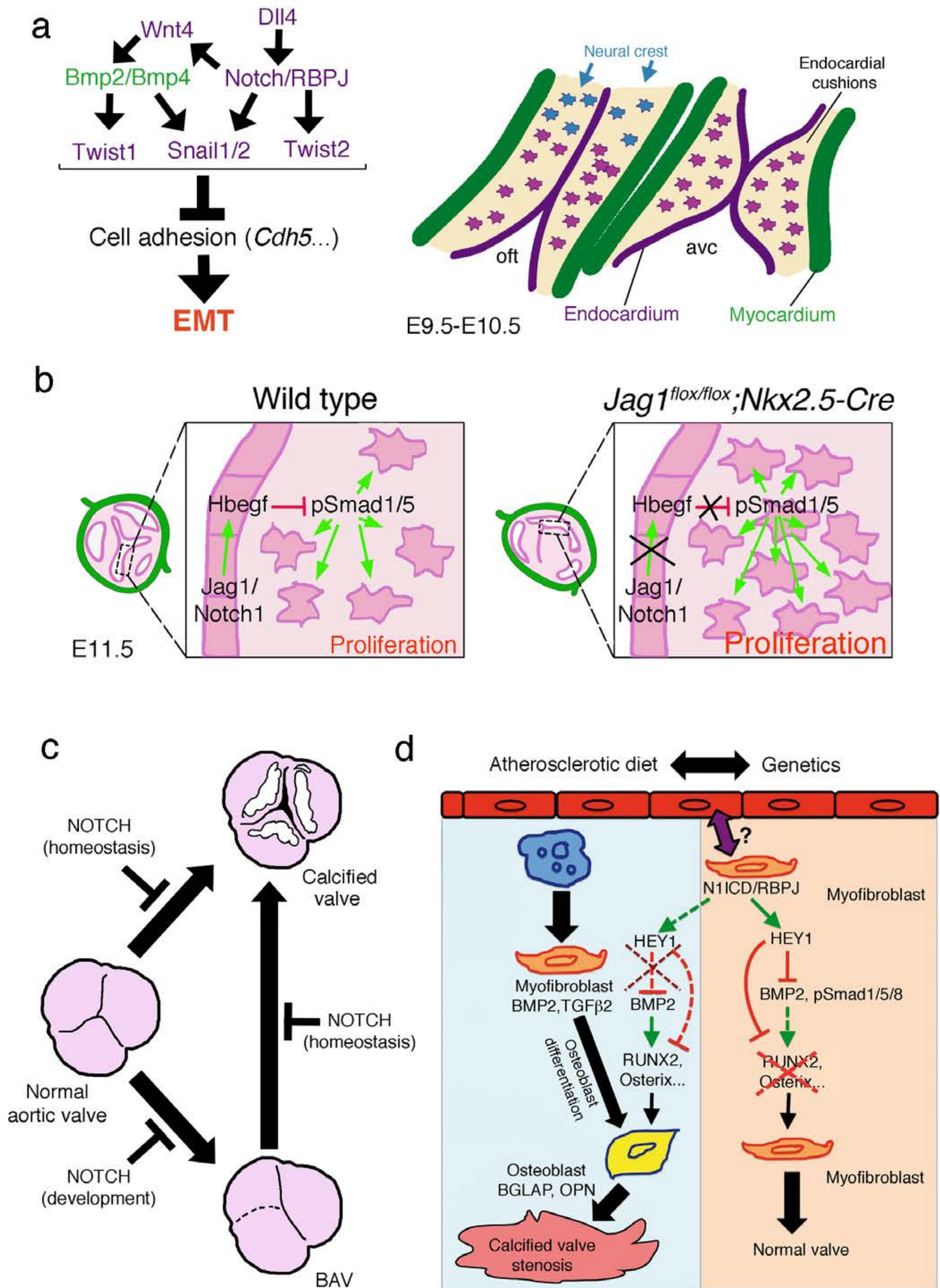


Figure 2_MacGrogan

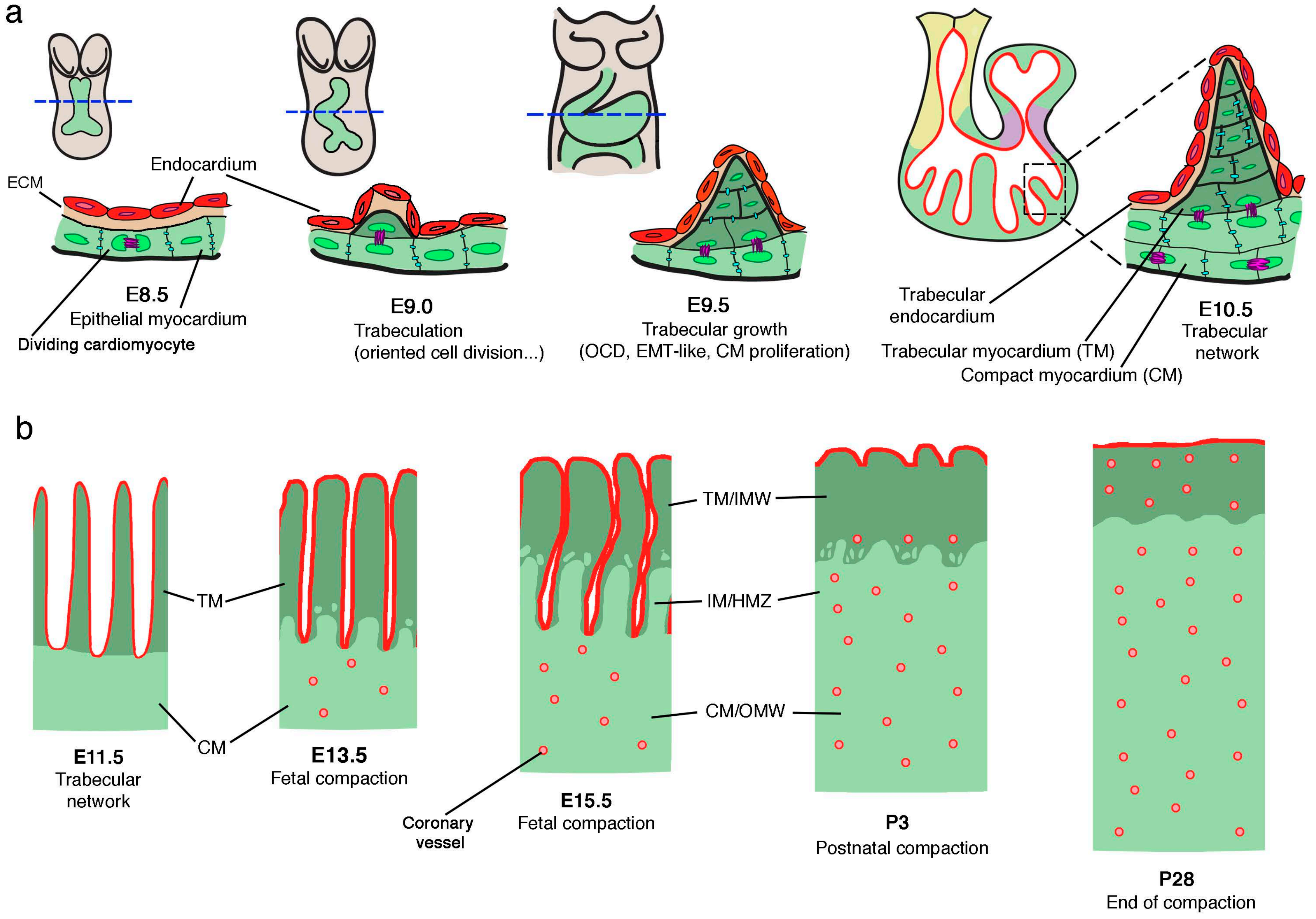


Figure 3_MacGrogan

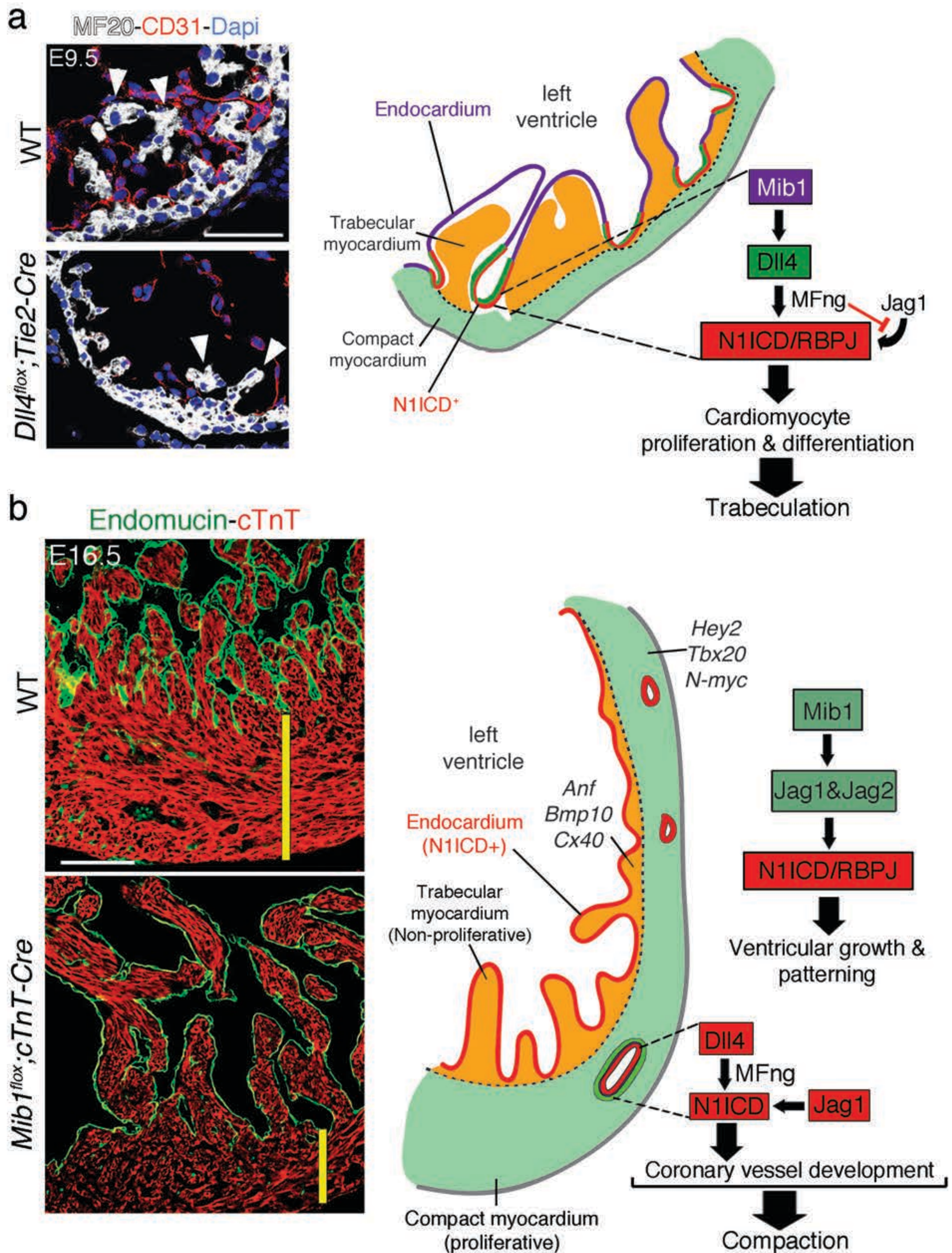


Figure 4_MacGrogan et al

Supplementary Table 1. Mutations in NOTCH pathway elements affecting heart development in mice and humans
pathway elements going from the signalling cell (Mib1, the ligands) to the nucleus of the receiving cells (Notch receptors, RBPJ and target transcription factors).

Mouse model	Phenotype	Defective endocardial signalling	Human gene	Cardiovascular disease	Mechanism of human disease	Target genes and pathways	REF.
Mib1$^{flox/flox}$;cTnT-Cre	LVNC	Yes	*MIB1*	LVNC	HI DN	ND	[1]
Dll4$^{-/-}$	Reduced atrial and ventricular chamber size, defective trabeculation, AV malformations Dominant lethal (E10.5)	Yes	*DLL4*	NA	NA	ND	[2,3]
Dll4$^{flox/flox}$;Tie2-Cre	Defective trabeculation, homozygous lethal	Yes	*DLL4*	NA	NA	*Gpr126*	[4]
Dll4$^{flox/flox}$;Nfatc1-Cre	Defective trabeculation, homozygous lethal	Yes	*DLL4*	NA	NA	*Gpr126*	[4]
Jag1$^{-/-}$	Pericardial oedema, homozygous lethal (E11.5)	ND	*JAG1*	AGS (heterozygous mutations)	HI	ND	[5-7]
Jag1$^{flox/flox}$;cTnT-Cre	Cardiomyopathy. Ventricular dysfunction	Yes	*JAG1*	NA	HI	*Gpr126*	[4]
Jag1$^{+/-}$;Notch2$^{+/del1}$	RVHP,AVSD,VSD, PAS. Perinatal lethality	ND	*JAG1; NOTCH2*	AGS	HI	ND	[5,6]
Jag1$^{flox/flox}$;Cdh5-Cre^{ERT2}	TOF, valve calcification	Yes	*JAG1*	AGS	HI	*Adamts9*	[8]
Jag1$^{flox/flox}$;Nfatc1-Cre	Hypoplastic EC, homozygous lethal (E11.5)	Yes	*JAG1*	NA	ND	*Wnt4, Bmp2*	[9]
Jag1$^{flox/flox}$;Mef2c-AHF-Cre	AA abnormalities, VSD, ASD, homozygous neonatal lethal	Yes	*JAG1*	NA	ND	ND	[10]
Jag1$^{flox/flox}$;Islet1-Cre	AA abnormalities, VSD, ASD, homozygous neonatal lethal	Yes	*JAG1*	NA	NA	*Fgf8,Bmp4*	[10]
Jag1$^{flox/flox}$;Nkx2.5-Cre	Dysplastic valves, BAV	Yes	*JAG1*	NA	NA	*Hbegf,* Cxcr4, Bmp	[11]
Jag2$^{flox/flox}$;cTnT-Cre	Cardiomyopathy, ventricular dysfunction	Yes	*JAG2*	NA	NA	*Gpr126*	[4]
Jag1$^{flox/flox}$;Jag2$^{flox/flox}$;cTnT-Cre	LVNC	Yes	*JAG1; JAG2*	NA	HI	*Gpr126*	[4]
Mfng$^{-/-}$;Lfng$^{-/-}$;Rfng$^{-/-}$	Defective coronary vessel development	Yes	*MFNG; LFNG; RFNG*	NA	NA	*Efnb2*	[4]
Notch1$^{+/-}$	Valve calcification	Yes	*NOTCH1*	CAVD, BAV, TOF	HI	*Hey1, Hey2, Runx2*	[12]
Notch1$^{-/-}$	Hypoplastic EC and impaired trabeculation. Homozygous lethal (E10.5)	Yes	*NOTCH1*	NA	NA	*Snail1, Tgfb2, Efnb2*	[13,14]
Notch1$^{flox/flox}$;Islet1-Cre	HRV, SHF expansion (E9.5)	Yes	*NOTCH1*	NA	NA	WNT–β–catenin	[15]
Notch1$^{flox/flox}$;Tie2-Cre	Hypoplastic EC and impaired trabeculation. Homozygous lethal (E10.5)	Yes	*NOTCH1*	NA	NA	*Efnb2*	[14]
Notch1$^{flox/flox}$;Nfatc1pan-Cre	Hypoplastic EC. Homozygous lethal (E12.5)	Yes	*NOTCH1*	NA	NA	WNT, BMP	[9]

$Notch1^{flox/flox}$;*Nfatc1en-Cre*	Dysplastic valves, BAV	Yes	*NOTCH1*	NA	NA	*Hbegf*	[11]
$Notch1^{flox/flox}$;*Cdh5-*Cre^{ERT2}	Dysplastic valves, BAV	Yes	*NOTCH1*	NA	NA	*Hbegf*	[11]
$Notch1^{flox/flox}$;*Wt1-Cre*	Reduced coronary plexus, endothelial cysts. Reduced compact myocardium (E13.5)	ND	*NOTCH1*	NA	NA	*Efnb2, Raldh2*	[16]

Mouse model	**Phenotype**	**Defective endocardial signalling**	**Human gene**	**Cardiovascular disease**	**Mechanism of human disease**	**Target genes and pathways**	**REF.**
$Notch2^{del1/+}$	Hypomorphic allele, AGS phenotype in $Jag1^{+/dDSL}$;$Notch2^{+/del1}$ double heterozygous. Homozygous lethal (E10.5)	ND	*NOTCH2*	AGS	HI	ND	[17-20]
$Notch2^{flox/ex}$;*Pax3-Cre*	Narrow aortas and pulmonary arteries	ND	*NOTCH2*	AGS (PAS)	HI	ND	[21]
$Notch2^{flox/ex}$;*Tag1-Cre*	Narrow aortas and pulmonary arteries. Neonatal lethality	ND	*NOTCH2*	AGS (PAS)	HI	ND	[21]
$Psen1^{-/-}$	VSD, DORV,PAS (E12.5–E15.5)	Yes	*PSEN1*	Dilated cardiomyopathy	NA	ND	[22-24]
$Psen1^{-/-}$; $Psen2^{-/-}$	Defective cardiac looping. Homozygous lethal (E10.5)	ND	*PSEN1, PSEN2*	NA	NA	ND	[25]
$Rbpj^{-/-}$	Defective cardiac looping, hypoplastic EC and impaired trabeculation. Homozygous lethal (E10.5)	Yes	*RBPJ*	NA	NA	*Snail1, Tgfb2, Efnb2*	[13,14]
$Rbpj^{+/-}$	Aortic valve sclerosis, stenosis	Yes	*RBPJ*	Calcific aortic valve disease	HI	*Hey1, Runx2*	[26]
$Rbpj^{flox/flox}$;*Tie2-Cre*	Impaired trabeculation. Homozygous lethal (E10.5)	Yes	*RBPJ*	NA	NA	*Efnb2*	[14]
$Rbpj^{flox/flox}$;*Dermo 1-Cre*	Ventricular septal defect	Yes	*RBPJ*	NA	NA	ND	[27]
$Rbpjk^{flox/flox}$;*Mesp 1-Cre*	HRV (E9.75)	Yes	*RBPJ*	NA	NA	WNT–βc-atenin, BMP	[28]
$Rbpj^{flox/flox}$;*Tbx18-Cre*	Reduced coronary arteriogenesis. Postnatal death	ND	*RBPJ*	NA	NA	TGFß	[29]
$Rbpj^{flox/flox}$; *Nfatc1en-Cre*	Dysplastic valves, BAV	Yes	*RBPJ*	NA	NA	*Hbegf,* Cxcr4, Bmp	[11]
$Hey2^{-/-}$	VSD, ASD, PAS, TOF, TVA, AVV dysfunction Cardiomyopathy (PN See at the bottom of the Table 4 weeks)	Yes	*HEY2*	Brugada syndrome	NA	ND	[30-35]
$Hey1^{-/-}$;$Hey2^{-/-}$	Defective cardiac looping, hypoplastic EC and impaired trabeculation. Homozygous postnatal lethality	Yes	*HEY1, HEY2*	NA	NA	*Tbx2, Bmp2*	[36,37]
$Hey1^{-/-}$;$HeyL^{-/-}$	VSD, AVV defects. Homozygous perinatal lethality	Yes	*HEY1, HEY3*	NA	NA	ND	[38]
$Hes1^{-/-}$	VSD and OA. Homozygous lethal (E18.5)	Yes	*HES1*	NA	NA	ND	[39,40]
DNMAML;Pax3-Cre (NLOF)	OFT, AA abnormalities, VSD Postnatal lethality	ND	*MAML*	NA	NA	ND	[41]
DNMAML;Wnt1-Cre (NLOF)	OFT, AA abnormalities. Postnatal lethality	ND	*MAML*	NA	NA	ND	[41]

DNMAML;Islet1-Cre (NLOF)	OFT, AA abnormalities, VSD, TVA. Neonatal lethality	Yes* OK	*MAML*	NA	NA	*Fgf8, Bmp4*	[10]
DNMAML;Mef2c-AHF-Cre (NLOF)	OFT, AA abnormalities, VSD. Neonatal lethality	Yes* OK	*MAML*	NA	NA	ND	[10]
MFng$^{GOF/GOF}$;Tie2-Cre (NLOF)	LVNC	Yes	*MFNG*	NA	NA	*Gpr126*	[4]
Numb$^{flox/flox}$;Numbl$^{flox/flox}$;Nkx2.5-Cre (NGOF)	OFT septation defect, DORV, AVSD. Embryonic lethal (E15.5–18.5)	Yes* OK	*NUMB, NUMBL*	NA	NA	ND	[42]
N1ICD ME;Mesp1-Cre (NGOF)	Impaired ventricular myocardial differentiation, ectopic trabeculation in AVC. Homozygous lethal (E10.5)	Yes*	*NOTCH1*	NA	NA	*Wnt2, Bmp6, Tnni, Jag1*	[43]
R26N1ICD;Tie2-Cre (NGOF)	Expansion of valve features to ventricles, ectopic partial EMT in ventricles. Homozygous lethal (E10.5)	Yes	*NOTCH1*	NA	NA	*Tgfß2, Snail1, Snail2, Twist2, Hey1, Hey2, HeyL*	[44]
R26N1ICD;Nkx2-5-Cre (NGOF)	Hypoplastic EC and thin chamber myocardium, defective trabeculation. Embryonic lethal (E11.0)	Yes	*NOTCH1*	NA	NA	*Tgfß2, Snail1, Snail2, Twist2, Hey1, Hey2,HeyL*	[44]
R26N1ICD;cTnT-Cre (NGOF)	Hypoplastic EC and delayed trabeculation. Embryonic lethal (E11.5)	No	*NOTCH1*	NA	NA	*Hey1-HeyL*	[44]
R26N1ICD;Wt1-Cre	Endothelial cysts (E14.5). Reduced compact myocardium	No	*NOTCH1*	NA	NA	*Raldh2*	[16]
R26N1ICD;Tbx18-Cre	Epicardial protrusions (E14.5). Reduced compact myocardium	No	*NOTCH1*	NA	NA	TGFβ	[29]
Hey1 ME;Mesp1-Cre (HGOF)	Reduced AVC extension. Embryonic lethal (E11.5)	Yes	*HEY1*	NA	NA	*Bmp2, Tbx2*	[34]
Hey2 ME;Mesp1-Cre (HGOF)	Absent AVC, no EC, defective trabeculation. Embryonic lethal (E10.5)	Yes	*HEY2*	NA	NA	*Bmp2, Tbx2*	[34]

*Hypothetical. Abbreviations: AA, aortic arch; ASD, atrial septum defect; AGS, Alagille syndrome; AV, atrioventricular; AVSD, atrioventricular septum defect; AVV, atrioventricular valve; BAV, bicuspid aortic valve; CAVD, calcific aortic valve disease; DN, dominant negative; DORV, double outlet right ventricle; EC; endocardial cushion; HGOF, Hey gain-of-function; HI, haploinsufficiency; LVNC, left ventricular non-compaction; NA, not applicable; ND, not determined; NGOF, Notch gain-of-function; NLOF, Notch loss-of-function; OA, overriding aorta; PAS, pulmonary artery stenosis; RVHP, right ventricular hypoplasia; TOF, tetralogy of Fallot; TVA, tricuspid valve atresia; VSD, ventricular septal defect.

References

1. Luxan, G. *et al.* Mutations in the NOTCH pathway regulator MIB1 cause left ventricular noncompaction cardiomyopathy. Nat Med. 19, 193-201 (2013).
2. Duarte, A. *et al.* Dosage-sensitive requirement for mouse Dll4 in artery development. Genes Dev. 18, 2474-2478 (2004).
3. Krebs, L. T. *et al.* Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. Genes Dev. 18, 2469-2473 (2004).
4. D'Amato, G. *et al.* Sequential Notch activation regulates ventricular chamber development. Nat Cell Biol. 18, 7-20 (2016).
5. Li, L. *et al.* Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nat Genet. 16, 243-251. (1997).
6. Oda, T. *et al.* Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nat Genet. 16, 235-242. lineage decision. (1997).
7. Xue, Y. *et al.* Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. Hum Mol Genet. 8, 723-730. (1999).
8. Hofmann, J. J. *et al.* Endothelial deletion of murine Jag1 leads to valve calcification and congenital heart defects associated with Alagille syndrome. Development. 139, 4449-4460 (2012).
9. Wang, Y. *et al.* Endocardial to myocardial notch-wnt-bmp axis regulates early heart valve development. PLoS One. 8, e60244 (2013).
10. High, F. A. *et al.* Murine Jagged1/Notch signaling in the second heart field orchestrates Fgf8 expression and tissue-tissue interactions during outflow tract development. J Clin Invest. 119, 1986-1996 (2009).
11. MacGrogan, D. *et al.* Sequential Ligand-Dependent Notch Signaling Activation Regulates Valve Primordium Formation and Morphogenesis. Circ Res. 118, 1480-1497 (2016).
12. Nigam, V. & Srivastava, D. Notch1 represses osteogenic pathways in aortic valve cells. J Mol Cell Cardiol. 47, 828-834 (2009).
13. Timmerman, L. A. *et al.* Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. Genes Dev. 18, 99-115 (2004).
14. Grego-Bessa, J. *et al.* Notch signaling is essential for ventricular chamber development. Dev Cell. 12, 415-429 (2007).
15. Kwon, C. *et al.* A regulatory pathway involving Notch1/beta-catenin/Isl1 determines cardiac progenitor cell fate. Nat Cell Biol. 11, 951-957 (2009).
16. Del Monte, G. *et al.* Differential Notch Signaling in the Epicardium Is Required for Cardiac Inflow Development and Coronary Vessel Morphogenesis. Circ Res. 108, 824-836 (2011).
17. McCright, B. *et al.* Defects in development of the kidney, heart and eye vasculature in mice homozygous for a hypomorphic Notch2 mutation. Development. 128, 491-502. (2001).
18. McCright, B., Lozier, J. & Gridley, T. A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. Development. 129, 1075-1082 (2002).
19. McDaniell, R. *et al.* NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway. Am J Hum Genet. 79, 169-173 (2006).
20. Kamath, B. M. *et al.* NOTCH2 mutations in Alagille syndrome. J Med Genet. 49, 138-144 (2012).
21. Varadkar, P. *et al.* Notch2 is required for the proliferation of cardiac neural crest-derived smooth muscle cells. Dev Dyn. 237, 1144-1152 (2008).
22. Nakajima, M., Moriizumi, E., Koseki, H. & Shirasawa, T. Presenilin 1 is essential for cardiac morphogenesis. Dev Dyn. 230, 795-799 (2004).
23. Shen, J. *et al.* Skeletal and CNS defects in Presenilin-1-deficient mice. Cell. 89, 629-639 (1997).
24. Li, D. *et al.* Mutations of presenilin genes in dilated cardiomyopathy and heart failure. Am J Hum Genet. 79, 1030-1039 (2006).
25. Donoviel, D. B. *et al.* Mice lacking both presenilin genes exhibit early embryonic patterning defects. Genes Dev. 13, 2801-2810 (1999).
26. Nus, M. *et al.* Diet-Induced Aortic Valve Disease in Mice Haploinsufficient for the Notch Pathway Effector RBPJK/CSL. Arterioscler Thromb Vasc Biol. 31, 1580-1588 (2011).
27. Morimoto, M. *et al.* Canonical Notch signaling in the developing lung is required for determination of arterial smooth muscle cells and selection of Clara versus ciliated cell fate. J Cell Sci. 123, 213-224 (2010).
28. Klaus, A. *et al.* Wnt/beta-catenin and Bmp signals control distinct sets of transcription factors in cardiac progenitor cells. Proc Natl Acad Sci U S A. 109, 10921-10926 (2012).
29. Grieskamp, T., Rudat, C., Ludtke, T. H., Norden, J. & Kispert, A. Notch signaling regulates smooth muscle differentiation of epicardium-derived cells. Circ Res. 108, 813-823 (2011).
30. Donovan, J., Kordylewska, A., Jan, Y. N. & Utset, M. F. Tetralogy of fallot and other congenital heart defects in Hey2 mutant mice. Curr Biol. 12, 1605-1610 (2002).

31. Gessler, M. *et al.* Mouse gridlock: no aortic coarctation or deficiency, but fatal cardiac defects in Hey2 -/- mice. Curr Biol. 12, 1601-1604 (2002).
32. Sakata, Y. *et al.* Ventricular septal defect and cardiomyopathy in mice lacking the transcription factor CHF1/Hey2. Proc Natl Acad Sci U S A. 99, 16197-16202 (2002).
33. Sakata, Y. *et al.* The spectrum of cardiovascular anomalies in CHF1/Hey2 deficient mice reveals roles in endocardial cushion, myocardial and vascular maturation. J Mol Cell Cardiol. 40, 267-273 (2006).
34. Kokubo, H., Tomita-Miyagawa, S., Hamada, Y. & Saga, Y. Hesr1 and Hesr2 regulate atrioventricular boundary formation in the developing heart through the repression of Tbx2. Development. 134, 747-755 (2007).
35. Veerman, C. C. *et al.* The Brugada Syndrome Susceptibility Gene HEY2 Modulates Cardiac Transmural Ion Channel Patterning and Electrical Heterogeneity. Circ Res. 121, 537-548 (2017).
36. Fischer, A., Schumacher, N., Maier, M., Sendtner, M. & Gessler, M. The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. Genes Dev. 18, 901-911 (2004).
37. Kokubo, H., Miyagawa-Tomita, S., Nakazawa, M., Saga, Y. & Johnson, R. L. Mouse hesr1 and hesr2 genes are redundantly required to mediate Notch signaling in the developing cardiovascular system. Dev Biol. 278, 301-309 (2005).
38. Fischer, A. *et al.* Combined loss of Hey1 and HeyL causes congenital heart defects because of impaired epithelial to mesenchymal transition. Circ Res. 100, 856-863 (2007).
39. Ishibashi, M. *et al.* Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. Genes Dev. 9, 3136-3148 (1995).
40. Rochais, F. *et al.* Hes1 is expressed in the second heart field and is required for outflow tract development. PLoS One. 4, e6267 (2009).
41. High, F. A. *et al.* An essential role for Notch in neural crest during cardiovascular development and smooth muscle differentiation. J Clin Invest. 117, 353-363 (2007).
42. Zhao, C. *et al.* Numb family proteins are essential for cardiac morphogenesis and progenitor differentiation. Development. 141, 281-295 (2014).
43. Watanabe, Y. *et al.* Activation of Notch1 signaling in cardiogenic mesoderm induces abnormal heart morphogenesis in mouse. Development. 133, 1625-1634 (2006).
44. Luna-Zurita, L. *et al.* Integration of a Notch-dependent mesenchymal gene program and Bmp2-driven cell invasiveness regulates murine cardiac valve formation. J Clin Invest. 120, 3493-3507 (2010).