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Notch signaling and cardiac repair

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Abstract

Notch signaling is critical for proper heart development and recently has been reported to participate in adult cardiac repair. Notch resides at the cell surface as a single pass transmembrane receptor, transits through the cytoplasm following activation, and acts as a transcription factor upon entering the nucleus. This dynamic and widespread cellular distribution allows for potential interactions with many signaling and binding partners. Notch displays temporal as well as spatial versatility, acting as a strong developmental signal, controlling cell fate determination and lineage commitment, and playing a pivotal role in embryonic and adult stem cell proliferation and differentiation. This review serves as an update of recent literature addressing Notch signaling in the heart, with attention to findings from non cardiac research that provide clues for further interpretation of how the Notch pathway influences cardiac biology. Specific areas of focus include Notch signaling in adult myocardium following pathologic injury, the role of Notch in cardiac progenitor cells with respect to differentiation and cardiac repair, crosstalk between Notch and other cardiac signaling pathways, and emerging aspects of noncanonical Notch signaling in heart.

Keywords

Notch; Cardioprotection; Progenitor; Regeneration; Crosstalk; Noncanonical

1. Introduction

Nearly one hundred years after the first description of Notch in fruit flies, scientific publications abound examining this deceptively simple pathway in almost every aspect of eukaryotic biology[1-9]. The mammalian Notch family includes four receptors (Notch1 – 4) and five canonical ligands (Jagged1, Jagged2 Delta1, Delta3, Delta4). Receptors and ligands share similar overall structure: a large extracellular domain, single pass transmembrane region, and an intracellular domain. Binding of the ligand and receptor extracellular domains on adjacent cells triggers receptor activation through two proteolytic cleavage events: ADAM/TACE catalyzes release of the extracellular ligand-bound region of the receptor, and presenilin, the active component of the gamma secretase complex, cleaves the transmembrane region. The untethered intracellular domain then enters the nucleus and

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binds CBF1/Su(H)/Lag-1 (CSL) transcription factor, displacing co-repressors and activating expression of downstream target genes, the best known of which are the Hes and Hes-related (Hesr) family of bHLH transcriptional repressor proteins. The Notch pathway participates in multiple cellular functions during development and adulthood, including cell-cell communication, maintaining tissue boundaries, cell fate determination, differentiation, tissue homeostasis and regeneration.

During cardiac development all Notch receptors and ligands participate in patterning and formation of the cardiac layers, as illustrated and described in great detail by de la Pompa and Epstein in their recent review of Notch signaling in cardiac development and disease [10]. All mammalian Notch receptors and four ligands (excluding Delta 3, which exhibits atypical signaling properties) have likewise been measured by quantitative PCR and immunohistochemistry in adult rat myocardium and failing human heart tissue, with Notch 3 and 4 identified as the predominant receptors in isolated adult rat cardiomyocytes [11]. Jagged 1 is expressed in adult mouse myocytes, intracellular Notch1 appears in developing and pathologically challenged adult mouse cardiomyocytes, while Delta4 ligand is detected in nonmyocyte mouse heart cells [12-15]. Furthermore, Notch 1 receptor is expressed in cardiac stem cells located in cardiac niches. Pharmacological ablation of Notch activity by gamma secretase inhibitors during the first week of postnatal life in mice leads to dilated cardiomyopathy in the adult heart, underscoring the importance of Notch1 during cardiomyogenesis [14].

Notch is critical for proper myocardial and outflow tract development. Recent evidence demonstrates that Notch and Jagged1 signal to each other in developing cardiac vasculature to create layers of smooth muscle around endothelium [16, 17]. Mutations in Notch receptors and ligands can perturb proper development and formation of the heart. Diseases impacting adult heart function arising from Notch receptor or ligand mutations include Alagille syndrome (Jagged1 [18, 19], Notch2 [20], CADASIL (Notch3, [21]) and aortic valve disorder (Notch1, [22, 23]). Several excellent reviews summarize the role of Notch in cardiac development and disease, and vasculogenesis following injury [24-31]. Activation of the Notch pathway in damaged adult myocardium has been linked to survival, proliferative and regenerative signaling [11-13, 15, 32, 33]. Collectively these studies indicate Notch as a potent formative and reparative signal in the mammalian heart.

2. Models of cardiac Notch activation and inhibition

Genetic and pharmacological models of Notch pathway activation or inhibition have revealed various roles for Notch in the developing and adult heart. Cardiomyocyte specific overexpression of the Notch intracellular domain (NICD) or silencing of Notch in embryonic cardiomyocytes both result in atrial and ventricular septal defects [32, 34, 35]. In adult heart, however, conditional activation of Notch is cardioprotective, while Notch blockade exacerbates damage following infarction [14, 32, 36]. Gain and loss-of-function studies of Notch in the developing epicardium reveal an important role for progenitors in this compartment during cardiovascular development and smooth muscle differentiation [37].

In their recent review, High and Epstein tabulate Notch mutant models and their associated cardiac defects [27]. Here we provide an updated list of genetically modified Notch mouse models described in the last three years to document further the role of Notch signaling in the heart (Table 1).

3. Notch in cardiac myocytes

Several recent studies have examined Notch expression and signaling in cardiomyocytes with respect to proliferation and survival [12, 13, 15, 32, 33]. Notch pathway activation has been documented in adult mammalian heart following infarction and hypertrophy [11, 12, 32, 38]. Overexpression of activated Notch in infarcted myocardium, whether through genetic engineering or transient overexpression, abrogates damage and promotes proliferative signaling in cardiac myocytes, whereas deletion of Notch signaling has the opposite effect; hearts in which Notch signaling is lacking or has been depleted display an exacerbated response to pathologic injury. Raya et al report Notch activation in cardiomyocytes following amputation of the ventricular apex in a zebrafish model of cardiac regeneration [39], and while mammalian heart lacks this level of cardiac repair, it is noteworthy that the Notch pathway is deployed in both systems in response to injury. Notch activity has been linked to proliferative signaling and cell cycle reentry in mammalian heart [12, 15]. Myocytes derived from mouse embryonic stem cells and neonatal rat ventricular myocytes overexpressing activated Notch exhibit shifts in cell cycle from G1 into S+G2+M, increased staining for Ki67 and upregulation of cyclinD1 expression and nuclear localization. In adult hearts, myocytes infected with adenovirus overexpressing activated Notch also express Ki67, suggesting that while Notch activity may not push differentiated mammalian cardiomyocytes through cytokinesis, it does support a proliferative and potentially regenerative program in these otherwise nondividing cells.

4. Notch in progenitors

Notch signaling during cardiac development is an ongoing focus of scientific investigation [23, 25-29, 40-46]. With the advent of cell-based therapy for the treatment of cardiac disease [47, 48], the role of Notch in cardiac lineage commitment in diverse stem and progenitor cell types has generated great interest [36]. Timing, dose and cellular context are crucial to the outcome of Notch signaling [49-52] during development and repair, leading to seemingly contradictory conclusions about Notch signaling and cardiac lineage commitment. Knockdown of Notch receptor in ESCs can either block or stimulate expression of cardiac lineage markers, depending on the stage of differentiation during which the signal is interrupted [53]. While overexpression of the intracellular Notch domain inhibits cardiomyogenesis in embryonic stem cells (ESCs) in one study [54], a more recent report shows that appropriately timed, forced expression of activated Notch can push mesoderm cells to a cardiac fate through the stimulation and inhibition of the BMP and Wnt pathways, respectively [55]. Similarly, Notch drives differentiation of Isl-1 cardiac progenitors in opposition to Wnt/ β -catenin dependent expansion. Interestingly Notch downregulates β -catenin activity via a noncanonical pathway wherein membrane associated Notch and Numb facilitate lysosomal degradation of activated β -catenin [56, 57].

Notch signaling has likewise been shown to play a critical role in adult cardiac stem cells populations. Adult ckit⁺ cardiac progenitor cells (CPCs) express Notch receptor surrounded by niche cells expressing the Jagged1 ligand. Activation of Notch in these cells stimulates proliferation and expression of Nkx2.5, driving commitment to the myocyte lineage while supporting a transient amplifying pool of very early myocytes. Pharmacological inhibition of Notch activity in infarcted hearts impedes endogenous CPC commitment to the myocyte lineage, thereby hindering repair through cardiomyogenesis [36]. Recent identification and characterization of Notch-activated epicardial-derived cells (NECs) [58, 59] further underscores the importance of Notch signaling in the cardiac injury response.

Successful engraftment and differentiation of adoptively transferred cells remains a significant hurdle in cell-based therapies. Elevated Notch signaling in hematopoietic stem

and progenitor cells can enhance *ex vivo* expansion and accelerate engraftment of transplanted cord blood progenitors [60, 61]. Likewise, Notch1 has been shown to improve the reparative capacity of bone marrow derived cells transplanted into infarcted myocardium, such that cells from mice with a global hemizygous deletion for Notch1 are compromised in their ability to reduce infarct size and restore function to the recipient infarcted heart, whereas overexpression of the Notch1 intracellular domain improves reparative capacity [38].

In noncardiac stem cells, Notch has been shown to play an important role in stem cell homeostasis, namely regulating the balance between stem cell quiescence, renewal and differentiation. Stem cell quiescence, not to be confused with full growth arrest, is critical for maintenance of a regenerative stem cell pool. Paradoxically, Notch signaling can promote both quiescence and proliferation. Within a hematopoietic stem cell niche, quiescent stem cells reside in a hypoxic environment and cycle very slowly or remain in G0. These long-term hematopoietic stem cells (LT-HSC) self renew to maintain and provide a stem cell pool capable of reconstitution over the lifetime of an organism. Butler et al propose that Notch signaling in endothelial cells is a critical part of the “angiocrine” environment provided by the bone marrow vascular niche supporting proliferation and replenishment of LT-HSCs [62]. Additionally, Notch signaling is implicated in the mechanism underlying pleiotropin-mediated expansion of HSCs [63]. Stem cell homeostasis in *Drosophila* adult intestinal stem cells is maintained through differential levels of Notch signaling whereby a higher threshold is required for cells to commit than that required for terminal differentiation [64].

The role of Notch in skeletal muscle development and adult stem cells is well established [65-71]. Recent evidence indicates that a temporary pulse of Notch activity is necessary for terminal differentiation of muscle progenitors in developing chick skeletal muscle [72] in contrast to prior studies in which Notch was reported to oppose myogenesis [73]. Two simultaneous publications using mouse models of satellite cell targeted deletion of RBPJk reveal a critical role for Notch signaling in maintaining quiescence and capacity for self renewal within the adult skeletal muscle stem cell niche [74, 75]. Satellite cells in which canonical Notch signaling is ablated exit quiescence and differentiate without passing through cell division, thereby depleting the stem cell pool. Notch mediates quiescence in mammary epithelial and breast cancer cell lines through crosstalk with p63, a p-53 related transcription factor important in cardiogenesis [76-78]. Here again, Notch signal dose is critical to outcome: high Notch activity in 3D cultures of mammary epithelial cells results in p63 mediated growth arrest and decreased cell-to-matrix adhesion, whereas lower activity levels promotes hyperproliferation [49]. Interestingly, hearts of p63 null embryos exhibit multiple cardiac defects, including dilated cardiomyopathy, myofibrillar disorganization, mitochondrial swelling and weak Ca⁺⁺ signaling, in addition to fewer isl1 expressing progenitor cells. Knockdown of p63 in ESCs prevents formation of beating cardiomyocytes in embryoid bodies and reduces expression of cardiac lineage and sarcomeric markers, while rescue by Gata6 expression or addition of Activin A designates p63 as a cardiogenic endodermal induction signal. Although a direct link between Notch and quiescence has not been established in the postnatal heart, the question arises whether Notch regulates quiescence in the adult cardiac stem cell niche, possibly through crosstalk with p63.

In summary, Notch signaling is a potent mediator of stem cell quiescence, proliferation and differentiation, depending on the context in which the signal is active. Clearly timing, dose, cell type and localization are critical to the outcome of Notch signaling in cardiac stem cells and progenitors, highlighting the need for deeper understanding of this versatile pathway to harness its full potential in the development of regenerative therapies for cardiac disease.

5. Notch signaling and crosstalk

Notch crosstalk with other cellular signaling pathways has been extensively characterized in the context of cancer and development and to some extent in vascular, stem cell and cardiac systems [79-93]. Zhang et al propose an elaborate signaling network in confluent endothelial cells whereby Angiopoietin-1/Tie-2 induction of PI3K/Akt inhibits GSK3B and stabilizes B-catenin, which forms a complex with NICD/RBPJk to enhance expression of DLL4, resulting in vascular quiescence [94]. In cardiac valve development, Notch drives the endothelial-to-mesenchymal transition by increasing nitric oxide production through transcriptional upregulation of soluble guanylyl cyclase heterodimer, which constitutes the nitric oxide receptor, and Activin A, which activates PI3K and phosphorylation of eNOS [95]. Notch and PI3K signaling both mediate expansion of hematopoietic stem cells (HSCs) downstream of pleiotropin [63] while Notch and PI3K/Akt pathways work together to promote megakaryocyte development [96].

Likewise, Notch participates in crosstalk with various pathways in the developing and adult heart [35]. Activation of the PI3K/Akt pathway in cardiomyocytes via hepatocyte growth factor (HGF) or insulin has been shown to increase Notch signaling, and conversely forced expression of activated Notch in cardiomyocytes increases Akt activity [12]. Similarly, HGF/c-Met signaling in human vascular smooth muscle cells, which contributes to vascular calcification, may be mediated through increased Akt and Notch3 activation [97]. Notch crosstalk with growth factors and integrins has been described in neural progenitor cells [98, 99] but as yet remains uncharacterized in cardiac progenitors. Noncanonical wnt signaling promotes cardiomyogenesis in bone marrow cells [100, 101] and cooperative signaling between Notch and noncanonical wnts has been reported in cardiac specification of human circulating endothelial progenitor cells in a neonatal cardiomyocyte coculture system [102]. Wnt16, which may serve as a biomarker for fetal cardiac heart disease [103], controls hematopoietic stem cell specification in zebrafish via Notch pathway activation [104]. While a direct connection between Wnt16 and Notch in cardiac specification has not been established, crosstalk between these pathways may occur during cardiogenesis or repair.

Crosstalk between Notch and the mTOR pathways is documented in the cancer [82, 86, 105-112] and stem cell literature [113, 114] but with the exception of one study [115] remains largely unexplored in the cardiac setting. Nonetheless, further research elucidating the roles of both pathways in heart will undoubtedly reveal more mechanistic connections between Notch and mTOR in cardiac development and disease.

6. Non-canonical Notch

In canonical Notch signaling, DSL (Delta, Serrate, LAG-2 family) ligand activation of the Notch receptor on a neighboring cell triggers proteolytic cleavage and subsequent translocation of the receptor intracellular domain to the nucleus, where binding to the canonical CSL target converts it from a transcriptional repressor to an activator of Notch target genes. Exceptions to this “rule” surface with increasing frequency in the scientific literature as novel modes of Notch interaction are discovered [116, 117]. In addition to the conventional mammalian Delta and Jagged family ligands, several membrane associated and secreted proteins have been shown to modify Notch signaling [118-120], contributing to the subtlety and complexity with which Notch regulates numerous cell functions. For example, Delta-like 3 ligand (DLL3) does not activate the Notch receptor in trans but inhibits signal when located in cis with the receptor in late endocytic vesicles, targeting the receptor for degradation. Mutations in DLL3 cause somite segmentation defects in mice and underlie spondylocostal dysostosis (SCD) in humans [121]. In *Drosophila*, the Wry gene product acts as a non-DSL Notch ligand. Weary mutants exhibit a cardiomyopathic phenotype that can

be rescued by cardiac specific overexpression of Notch family proteins, while overexpression of Weary rescues a similar phenotype induced by siRNA depletion of Notch ligands in *Drosophila* [122].

Endocytic processing of Notch receptors and ligands has been described in development and regulates pathway activity [123, 124]. Ligand-independent activation of Notch receptor in the late endosome depends on the interplay of Deltex, Kurtz and Shrub proteins in the ESCRTIII complex and subsequent ubiquitylation status of Notch [125], while specific trafficking of Notch and Delta to Sara endosomes contributes to asymmetric division in sensory organ precursors in *Drosophila* and may be relevant to mammalian stem cell systems [126, 127]. Hypoxia inducible factor 1 α , (Hif1 α) known to enhance Notch activity during hypoxia [128], stabilizes and activates Notch in a ligand-independent manner in early endosomes of *Drosophila* blood crystal cells during development and in hypoxic conditions [129]. A direct role for endocytic processing of Notch family proteins in adult myocardium and cardiac stem cells remains to be explored, however endocytic degradation of Notch receptor by UV-resistance associated gene (UVRAG), a tumor suppressor known to regulate autophagy and vesicle trafficking, directs proper organ rotation during development in *Drosophila*. Levels of activated Notch are also elevated in cells from patients with heterotaxy caused by mutant UVRAG [130]. Although a direct link between Notch, endocytosis and autophagy has not been established in heart, a correlative connection has been reported. Cardiac levels of activated Notch are decreased in a model of alcohol-induced cardiomyopathy, along with Akt, mTOR and STAT3 signaling, while autophagy marker LC3B II increases, suggestive of interaction between Notch, survival signaling and cardiac autophagy. More rigorous cellular analysis will be required to demonstrate a mechanistic association among these pathways in heart [115]. Given the importance of hypoxia and autophagy [131] in cardiac systems, the likelihood exists that endocytic ligand-independent Notch signaling plays a role in these processes in the heart.

Crosstalk between noncanonical Notch signaling and known survival pathways is further implicated in cardiac ischemic preconditioning. Yang et al hypothesize that Notch participates in ischemic preconditioning through cross-talk with PI3K/Akt and NF- κ B [132]. Notch intracellular domain is thought to trigger the PI3K/Akt cascade in T-cell leukemia and the Itchy mouse autoimmune model through direct interaction at the inner cell membrane with p56lck, recruiting PI3K and activating Akt [133-135]. That p56lck is also known to mediate ischemic preconditioning in adult myocardium [136, 137] sets the stage to propose a mechanism for protective crosstalk between Notch and p56lck following cardiac damage.

Another site for protective Notch crosstalk is the mitochondrion, where noncanonical Notch signaling has recently been described. Perumalsamy et al dissect the mitochondrial and molecular interactions between cytoplasmic Notch intracellular domain (NIC), Bax, mitochondria, Akt and Mitofusins (Mfns) in HeLa cells to establish a protective NIC-Akt-Mfn cascade independent of canonical nuclear Notch signaling [138]. Additionally, N-terminal trimming of NIC by a novel mitochondrial intermediate peptidase (MIPEP) further indicates a regulatory role for mitochondria in Notch signaling [139]. Studies investigating Notch signaling in cardiac mitochondria have yet to emerge, nonetheless, given reports in other cell types, the mitochondrial network may serve as a new subcellular compartment to investigate noncanonical Notch signaling in heart.

Clearly Notch exerts many of its effects through noncanonical mechanisms in multiple cellular locations, as illustrated in Figure 1. Uncovering the role of these interactions in cardiac myocytes and progenitors will further reveal how Notch impacts cardiac function and repair, and how the pathway can be manipulated for therapeutic purposes in heart disease.

7. Conclusions

In conclusion, in addition to its previously well established role in cardiac development and disease, the Notch pathway is emerging as an important and complex signaling cascade in adult heart. Key outstanding questions regarding Notch signaling in cardiac repair are multiple and include 1) How does Notch signaling participate in cardiac repair in resident cardiac cell types (myocytes, vasculature, cardiac fibroblasts, stem cells) and in stem cells recruited from the circulation following pathological challenge? 2) How can the Notch pathway be exploited for therapeutic purposes in the treatment of heart disease? Genetic models of cardiac gain and loss of Notch function provide mechanistic explanations for how this pathway impacts cardiac formation and repair. That Notch signaling supports protective and proliferative signaling in adult myocardium following injury highlights its potential as a therapeutic target for treatment of heart disease. As a key regulator of stem cell proliferation, commitment and differentiation, Notch is also an attractive genetic engineering candidate for enhancing proliferation and engraftment of cells used in adoptive transfer applications. Understanding the precise timing and dose of signal required to optimize restoration of damaged myocardial tissue using Notch modified cells is key to making the most effective use of this promising reagent. As novel modes and subcellular locations of Notch signaling are revealed, consideration of how Notch modulates cell signaling through noncanonical interactions and crosstalk with multiple molecular partners is necessary to appreciate the complexity and potential of the pathway. Future research of Notch signaling in heart will continue to build on findings in non-cardiac systems to elucidate these important pathway connections in the cardiac context.

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Highlights

- Activation of Notch promotes myocardial repair.
- Notch signaling controls cardiac progenitor specification and differentiation.
- Notch crosstalks with survival pathways in heart.
- Noncanonical Notch signaling may participate in cardiac repair.

Figure 1. Graphic representation of Notch signaling networks

Canonical Notch signaling following trans ligand activation is depicted as proteolytic cleavage of the Notch receptor extracellular domain (NECD, burgundy) by ADAM/TACE (light green bolt) followed by cleavage within the plasma membrane by γ -secretase (red bolt) to release the intracellular domain (NIC, magenta). NIC translocates to the nucleus (green arrows) and binds the canonical nuclear target CSL to permit expression of downstream targets. Alternatively, intact or ADAM/TACE cleaved receptor can be endocytosed and NIC released by γ -secretase action within the endosomal membrane. NIC generated in this fashion may translocate to the nucleus, participate in Akt (orange heart) mediated protective signaling or be targeted for degradation (brown arrow, magenta fragments). Mitochondrial peptidase (MIPEP, light green Pac Man symbol) trims the N-terminus of NIC to generate Δ NIC, the function of which remains unknown. NIC also works together with Akt and mitofusins (Mfns, blue box) to prevent mitochondrial localization of proapoptotic protein Bax (brown cloud). Crosstalk between Notch and Akt can occur through receptor tyrosine kinase (RTK, light brown) mediated activation of PI3K (yellow) or binding of p56lck (purple) to NIC and activation of PI3K/Akt.

Table 1

Recent Notch mouse expression models and their cardiovascular phenotypes.

Construct	On/Off	Phenotype	Ref
α MyHC-CRre;MAML1 ^{DN}	Off E8.5	Mild RV thinning, normal function, poor response to infarction.	[32]
Nkx2.5-Cre;MAML1 ^{DN}	Off E7.0	Perinatal lethality, ASD, VSD, hypoplastic RV	[32]
α MyHC-Cre;NICD1	On E8.5	Perinatal lethality	[32]
α MyHC-mERCremER;NICD1	On conditionally	cardioprotective	[32]
Notch1 Pseudoligand	Injectable	Cardioprotective, supports neovascularization	[32]
Notch1 adenovirus	Intramyocardial injection	Cardioprotective	[12]
Gamma secretase inhibitor	perinatal injection	Reversible dilated myopathy	[14]
Cre-Lox RPBK-K deletion	Adult	Aortic valve disease like changes	[140]
MLC2v-Cre;Notch1 ^{lox/lox}	Off E8.0	Normal baseline function, increased mortality with pressure overload	[13]
Isl1-Cre;Notch1 ^{lox/lox}	Off E9.5	Expansion of Isl1 CPCs	[57]
Notch1+/-	Development	Exacerbated loss of cardiac function after infarction, poor neovascularization, reduced proliferation and survival of MSCs	[38]
α MyHC-CRE;Notch1 ^{lox/lox}	Off E8.5	No phenotype, possible compensation by other Notch receptors	[38]
Tbx18-Cre;RBPJK ^{lox/lox}	Off E9.5	Abnormal coronary vasculature	[37]
Tbx18-Cre;ROSA26 ^{NICD/+}	On E9.5	Embryonic lethal E14.5. Disrupted epicardial development, premature smooth muscle differentiation.	[37]
Tie2-Cre;N1ICD	On E8.5	Heart dysmorphogenesis	[35]
Nkx2.5-Cre;N1ICD	On E7.0	Heart dilation, thin ventricular myocardium, ill defined chamber boundaries	[35]
cTnNT-Cre;N1ICD	On E7.5	Poorly defined cardiac regions	[35]
Wt1-Cre;Notch1 ^{lox/lox}	Off E11.5	Embryonic lethal, pericardial hemorrhage, defective coronary vasculature,	[141]

Construct	On/Off	Phenotype	Ref
		thinning of compact myocardium	
WT-1Cre;ROSA26 ^{N1ICD/+}	On E11.5	Embryonic lethal E15.5, pericardial bleeding, thinning of subepicardium and compact myocardium	[141]
Wnt1-Cre;ROSA ^{Notch}	On E8.0	Embryonic lethal by E18.5, outflow tract malformations, VSD.	[142]
Wnt1-Cre;RBPJk ^{flox/flox}	Off E8.0	Perinatal lethality, outflow tract malalignment	[142]
Wnt1-Cre;Notch1 ^{flox/flox}	Off E8.0	Perinatal lethality, outflow tract malformation, VSD	[142]