Congenital Heart Disease

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REVIEW

Variants and Molecular Mechanism of NOTCH1 in Congenital Heart Disease

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ABSTRACT: Congenital heart disease (CHD) is the most common birth defect, with 34% of cases attributed to genetic variants. NOTCH1, a multi-domain transmembrane protein, regulates heart development by controlling the differentiation and migration of myocardial mesoderm cells, and different variants are present in different types of CHD. In this review, we aim to provide a detailed description of NOTCH1 structural domains and their functions, highlighting *NOTCH1* variants in CHD and the molecular mechanisms through which they contribute to CHD occurrence. NOTCH1 has two main domains, the NOTCH extracellular domain (NECD) and the NOTCH intracellular domain (NICD). NECD facilitates ligand binding and NICD formation, while the NICD functions as a transcription factor, forming complexes with co-factors in the nucleus to initiate gene transcription. Among the NOTCH1 variants associated with CHD occurrence, most are loss-of-function variants. Moreover, most of the variants are located in the EGF-like domain. The molecular mechanism behind the *NOTCH1* variant-associated CHD occurrence appears to be either due to a loss-of-function or missense variant. In the loss-of-function mutations, *NOTCH1* haploinsufficiency is noted and directly reduces the NICD production, causing CHD occurrence. In the less common case of missense variant, only a mild NOTCH1 malfunction is observed, but insufficient to directly lead to CHD occurrence. However, when a missense variant is combined with a risk factor, such as exposure to an environmental toxin, the cumulative effect can lead to CHD. Understanding the genetic and molecular mechanisms linking *NOTCH1* variants to CHD is crucial for improving clinical management and patient quality of life.

KEYWORDS: NOTCH1; congenital heart disease; mutation; loss-of function variant; haploinsufficiency

1 Introduction

The incidence of congenital heart disease (CHD) has increased in recent decades, making it the most common birth defect. While the exact cause of CHD is often unknown, risk factors include contracting rubella, diabetes, alcohol consumption during pregnancy, some medications, smoking and genetics [1,2]. In recent years, early prenatal detection and advances in surgical

techniques have improved survival rates among CHD individuals [1,3]. With the widespread application of whole-exome sequencing (WES) technology, numerous CHD-related pathogenic mutations have been identified, including variants in genes such as *NKX2.5*, *GATA4*, *NOTCH1*, *TBX1*, and *TBX20* [1,4]. Notably, of these genes, *NOTCH1* mutations account for 6% of CHD cases, highlighting its significance for further exploration.

The NOTCH signaling pathway involves multiple receptor-ligand interactions. The receptors consist of NOTCH1, NOTCH2, NOTCH3, and NOTCH4, while the ligands are divided into two families: the Jagged family (JAG1 and JAG2) and the Delta-like family (Delta1, Delta3, and Delta4). The NOTCH genes are transcribed in the nucleus and translated into precursor NOTCH proteins, such as pre-NOTCH1 (Fig. 1). These precursor proteins are transported to the Golgi apparatus, where they undergo S1 cleavage by a furin-like convertase to form a mature heterodimer transmembrane receptor that is transported to the cell membrane [5]. Upon ligand interaction, the receptor undergoes a conformational change, exposing its S2 site, which is then cleaved by metalloproteases, such as ADAM10 [6,7]. This cleavage releases the NOTCH extracellular domain (NECD) and leaves behind the remaining receptor, NOTCH extracellular truncation (NEXT) (Fig. 1) [8].

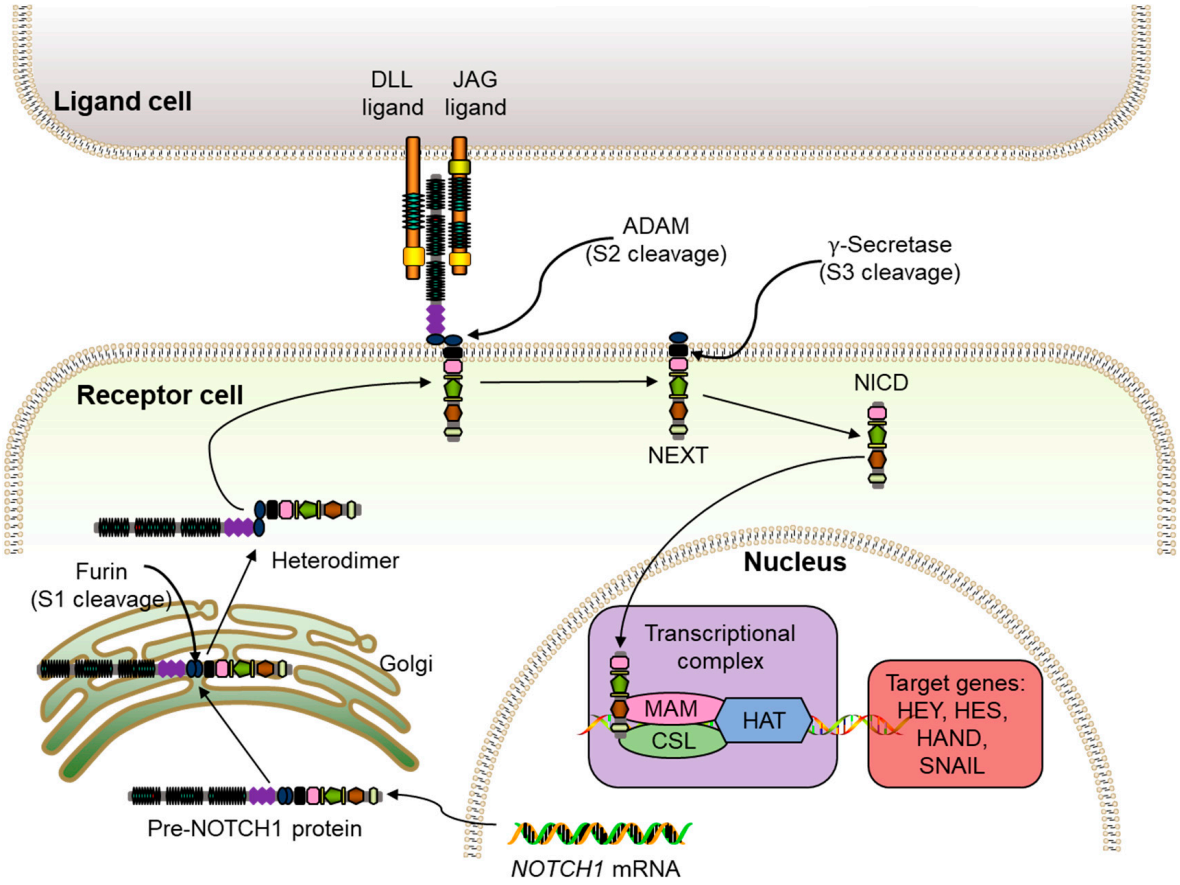

Figure 1: Schematic map of the NOTCH signaling pathway. Pre-NOTCH1 protein, the precursor NOTCH protein; DLL, delta-like ligand; ADAM, ADAM metalloproteases group; NICD, NOTCH intracellular domain; NEXT, NOTCH extracellular truncation; MAM, transcriptional coactivator mastermind; CSL, a nuclear effector (CBF1/RBP-J, Su(H), Lag1); HAT, histone acetyltransferase.

Following S2 cleavage, a γ-secretase cleavage site is exposed [8]. This third cleavage generates the NOTCH1 intracellular domain (NICD) [9], which then translocates to the nucleus to bind nuclear effectors, such as CSL (CBF1/RBP-J, Su(H), Lag1) to transform it from a repressor to a transcriptional activator. This NICD-CSL binding recruits coactivators like Mastermind (MAM)/Lag-3 to form the NICD-CSL-MAM complex, which recruits the ARC-L/MED complexes, a positive transcription regulator [10], histone ubiquitin ligases (Bre1) [11], and histone acetyltransferases (HAT) [12] to promote gene expression (Fig. 1). Overall, the NOTCH1 signaling cascade governs cellular processes such as proliferation, apoptosis, and differentiation, with each linked to specific structural domains of NOTCH1. Therefore, this review will focus on the domains of NOTCH1 and elaborate on the functions of each domain.

During cardiac embryogenesis, NOTCH1 is expressed in the outflow tract (OFT), atrioventricular canal (AVC), trabecular ventricular wall, and epicardium, but is absent in the dense myocardial layers of the atria and ventricles [13]. The NOTCH1 signaling pathway is critical for cardiac development, influencing aortic and pulmonary valve formation and large blood vessel maintenance [13–15]. In adults, NOTCH1 plays a role in cardiac repair by limiting myocardial hypertrophy, promoting precursor cell proliferation, improving cardiomyocyte survival, and reducing fibrosis [16]. Furthermore, following myocardial infraction, enhanced NOTCH1 activity has been to increase survival rates, improve cardiac function, and mitigate fibrosis via anti-apoptotic and pro-angiogenic mechanisms [17].

During cardiac development, NOTCH1 plays a role in promoting epithelial-to-mesenchymal transition (EMT), with the targeted deletion of *Notch1* or its nuclear partner (RBP-J/CBF1/Su(H)) in mice resulting in EMT impairment, leading to endocardial collapse and the absence of cushion cells [18]. These findings demonstrate the critical role of NOTCH1 in cardiac development and its association with various clinical CHD phenotypes [19,20]. NOTCH1 is highly intolerant to variation, as reflected by its low residual variation intolerance score (0.33%) [21].

NOTCH1 variants are frequently associated with CHD. In 2005, a *NOTCH1* variant was linked with an aortic valve malformation for the first time [22]. Furthermore, the *NOTCH1* locus is the most frequent site of genetic variants that cause non-syndromic tetralogy of Fallot (TOF), accounting for 4.5% of TOF cases (37/829) [23]. In familial valve diseases and left ventricular outflow tract obstruction (LVOTO), *NOTCH1* mutations represent 5.9% of cases, with an additional 16% involving variants of uncertain significance [24,25]. Among CHD patients, *NOTCH1* variants are considered the most significant of *de novo* mutations identified [26]. In addition to CHD, *NOTCH1* mutations are implicated in various cancers, including T-cell acute lymphoblastic leukemia (T-ALL), where 62–66% of adults exhibit such mutations [27,28]. However, this review will focus on *NOTCH1* variants in CHD.

Herein, a comprehensive view of the mechanisms by which *NOTCH1* variants mediate CHD development is explored to provide insight into the role of NOTCH1 in heart development and disease.

2 NOTCH1 Structure and Function

The NOTCH receptor is a type I single channel transmembrane protein consisting of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain [13].

In this section, the structures and functions of NECD and NEXT, the membrane bound fragment that is generated following S2 cleavage, are examined.

2.1 NECD Structure and Function

The NECD portion of NOTCH1 receptor is 209 kDa and contains 36 epidermal growth factor-like (EGF) repeats that serve as the ligand-binding domain and a negative regulatory region (NRR) domain (Fig. 2A) [29].

EGF repeats. Each region of the repeated EGF is comprised of a distinctive six cysteine motif consisting of 30–40 amino acid residues from three internal disulfide bridges [30]. These domains interacted with a specific ligand and a conformational change occurs leading to dimerization and subsequent proteolytic cleavages, initiating signal transduction. When binding the delta-like 4 (DLL4) ligand, the EGF11 and EGF12 regions with NECD interacts with the DSL (Delta/Serrate/Lag-2) and MNNL (N-terminus of Notch ligands) ligand domains, respectively [31]. However, when binding JAG, the EGF8 and EGF 11 regions of the NECD interact with the EGF 3 and DSL domains of the ligand, respectively [32].

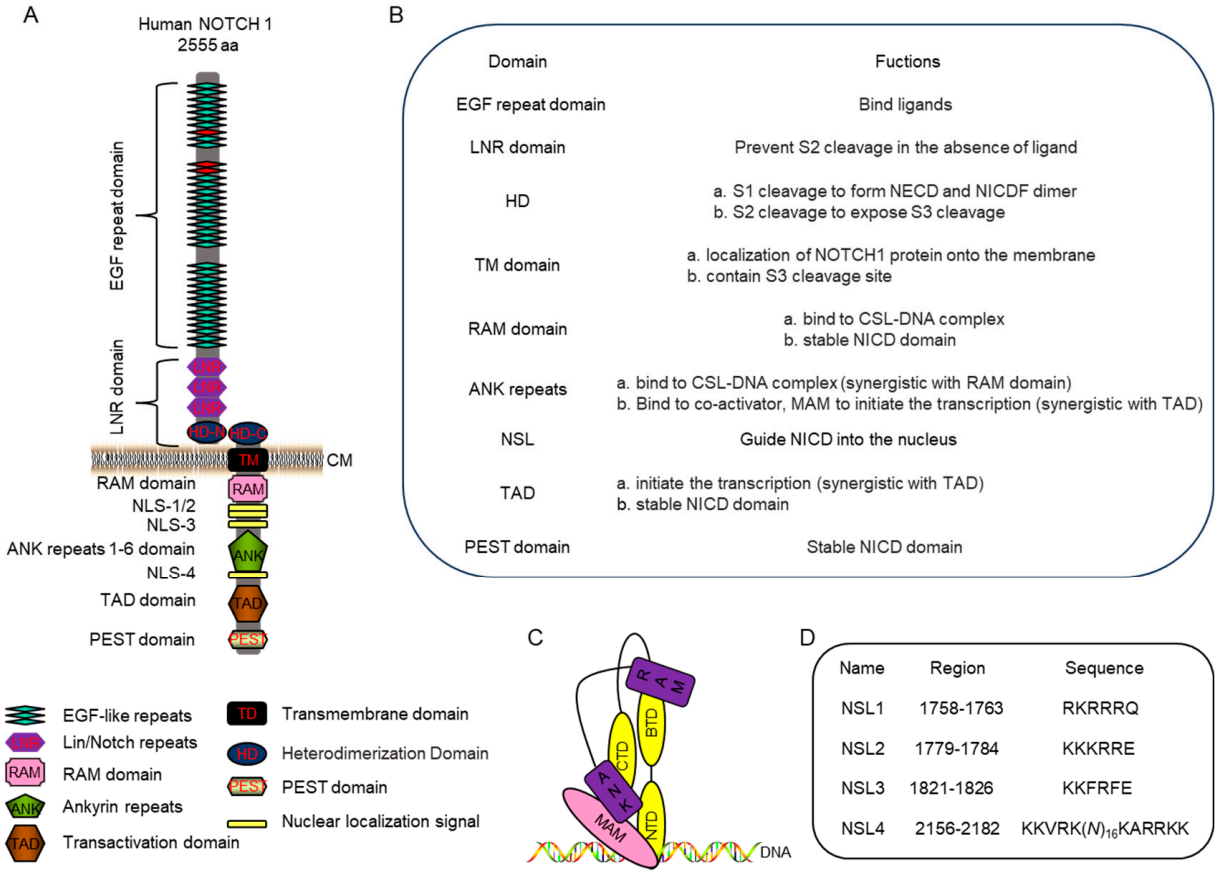

Figure 2: NOTCH1 structure and function. (**A**): Detailed structure of NOTCH1. The red EGF domain, including EGF8, EGF11 and 12, interact with ligands. CM, cell membrane. (**B**): The function of each structure listed in 2A. (**C**): The CSL-NICD-MAM complex with the RAM domain and ANK repeats of NICD (purple) interacting with BTD, NTD and CTD of CSL (yellow) to recruit MAM (pink). MAM, Mastermind; NTD, N-terminal domain; BTD, beta-trefoil domain; and CTD, C-terminal domain. (**D**): Putative NLS in NOTCH1 protein.

Another important aspect that influences ligand binding is post-translational modifications within the EGF-like domain, specifically *O*-glycosylation that adds an *O*-fucose or *O*-glucose to a serine or threonine residues within a domain. In *O*-fucosylation, EGF 2/3/5/6/8/9/12/16/18/20/21/23/24/26/27/30/31/32/35/36 have fucose sites, with the specific location of *O*-fucosylation having different effects, such as a fucose addition at EGF9 affecting receptor trafficking [33,34]. In a patient with a global developmental delay and contained heart development disease, including severe coarctation of the aorta and ventricular septal defect (VSD), a large decrease at EGF9 and the complete absence at EGF12 were observed [35]. When NOTCH1 binds its ligands, its *O*-fucosylated EGF8 and 12 regions directly interact with the EGF3 and C2 domains of JAG1, while EGF11 and 12 interact with the DSL domain on DLL4 [31,32,36]. Moreover, the addition of *O*-fucose to residue Thr466 in EGF12 and residue Ser435 in EGF11 has been shown to stabilize the interaction between NOTCH1 and DLL4 [31]. NOTCH signal activation also requires calcium-dependent phospholipid binding in the case of JAG, but in the case of DLL, calcium ions did not directly contribute to activation, but did provide DLL an increased rigidity that potentiated the interaction [31].

Unlike an *O*-fucose modification that affects ligand binding, an *O*-glucose modification enhances NOTCH signaling by increasing receptor susceptibility to proteolytic processing [34]. The consensus sequence for *O*-glucosylation is C-X-S-X-P/A-C, with serine being the only hydroxyamino acid that is modified with *O*-glucose on an EGF region. These modifications vary across cell types and affect Notch signaling activation differently depending on the modification site [34]. Overall, EGF1–36 plays a role in ligand binding and signaling initiation (Fig. 2B).

NRR domain. Close to the cellular membrane is the NRR, which is comprised of three Lin12/Notch repeats (LNRs) and a heterodimerization domain (HD) that acts as a regulatory switch for Notch signaling (Fig. 2). Each LNR repeat contains 40 residues, with three conserved aspartate (D) or asparagine (N) residues, and three disulfide bonds that pair in a characteristic pattern, cys (I)—cys (V)—cys (II)– cys (IV)—cys (III)–cys (VI) [37]. Without the receptor-ligand interaction, the LNRs maintain the receptor in a resting conformation to prevent S2 cleavage [8], but activate NOTCH in the presence of ligand [29,38]. While adjacent LNR domains can also affect the structural domains, their main function is to modulate cleavage in the presence or absence of ligands (Fig. 2B) [39].

The second portion of the NRR, which is the HD region, contains the S1 cleavage site (cleavage site: 1659-1670-GGRRRR′ELDPMD) that is targeted by a furin-like protease during protein maturation [5]. After cleavage, its N-terminal part (HD-N) terminates the NECD region, while the C-terminal (HD-C) begins the NEXT region and both form a heterodimerization (Fig. 2) [5]. If furin cleavage is disrupted, ligand-dependent signaling through the well-characterized mediator of Notch signal transduction will be abolished [40]. Moreover, the S2 cleavage site (1716-1724-YKIEAVQSE) is located in the HD-C, with cleavage at this site required to expose the S3 site (1705-1720: ALASLG′SLNIPYKIEA) [8]. Thus, the HD plays an important role in heterodimerization formation and modulates S1 and S2 cleavage (Fig. 2B).

In summary, the main function of the sequence of NECD is to bind a specific ligand and ensure that the subsequent cleavage events in the extracellular segment are performed to generate the NEXT intermediate.

2.2 NEXT Structure and Function

The NEXT intermediate of NOTCH1 contains HD-C, which is located at the N-terminal, a transmembrane domain (TD), which anchors the protein to the membrane, and a segment that will become the NICD (Fig. 2). The NICD contains an RBP-J association module (RAM) domain, ankyrin (ANK) repeats, nuclear localization signal (NLS), transcriptional activation domain (TAD) and PEST, a sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T) [6,8]. The regions on NICD have different functions, with the ANK repeat and TAD domain shown to be required for T-cell leukemogenesis, while the RAM and PEST domain are nonessential [41]. NICD overexpression can lead to complex defects in heart morphology, including the abnormal maturation of myocardial cells due to changes in gene expression [42]. In some cases, NICD can inhibit myoblast differentiation by binding to myocyte enhancer factor 2C (MEF2C), a regulatory factor that is associated with development in various tissues, and inhibit its transcription [43]. Thus, to more fully characterize the function of NICD, we will discuss the function of each domain in NICD.

RAM domain. RAM contains an RBP-J associated sequence and a conserved Trp-Xaa-Pro (WXP) tripeptide that has a high affinity for the β-trefoil domain of CSL [44]. Upon NOTCH signal transduction, NICD is released and enters the nucleus, where it binds CSL, a transcriptional repressor (Fig. 2C) [44]. While NICD, specifically the RAM domain, has been considered crucial in transcriptional activation, one study has showed that deleting the RAM domain only partially reduce NOTCH signaling activity as the ANK region was more pivotal in transcription [45]. Additionally, RAM is essential for NICD stability with ubiquitination of RAM by NEDD4-binding protein 1 (N4BP1) mediating NICD degradation [46]. Therefore, RAM has two functions, one is to interact with RBP-J, and the other is to regulate NICD stability (Fig. 2B).

ANK repeats. ANK repeats, consisting of two or more tandem repeats, are one of the most frequently observed amino acid motifs in protein databases, and form curved solenoid structures that are associated with protein-lipid, protein-sugar, and protein-protein interactions [47]. Each ANK repeat has a highly conserve TPLH (Thr-Pro-Leu-His) tetrapeptide that plays an important role in stability [48]. In NOTCH1, the ANK region contains six repeats. The ANK repeats bind the N-terminal and C-terminal domains of CSL to create a high-affinity binding site for MAM, to form a ternary complex (NICD-CSL-MAM complex) that is necessary for activate transcription [44]. The NICD-CSL-MAM complex formation induces a substantial conformational change within CSL that converts it from a repressor to an activator [49]. A deletion of any of the ANK repeats would completely reduce NOTCH signaling activity [45]. In *Caenorhabditis elegans*, the ANK repeats were shown to not only be involved in RBP-J interactions, but to also act as an autonomous transactivation domain [50]. Moreover, while the RAM region is the primary determinant of NICD-CSL complex stability, the ANK region largely directs the binding of the co-activator MAM to CSL [51]. Therefore, ANK and RAM act synergistically to promote NOTCH1 transcriptional activity (Fig. 2B).

NSL domain. A NSL domain consists of a short peptide segment, with the sequence K-R/K-X-R/K (X is any amino acid), that guides NICD into the nucleus [52]. NOTCH1 sequencing analysis has identified four putative NLS segments within NICD, the first three NLSs are monopartite, with the first and second closely resembling the RAM domain. The fourth NLS is bipartite given its location between the ANK and TAD domains (Fig. 2A,D) [53,54]. When examining the role of each region, NLS1 and

NLS2 were found to be non-essential for NICD nuclear import, while NLS3 and NSL4 were required, with NSL3 showing a clear independence from NLS4 [53]. Furthermore, the deletion of NLS3 and NLS4 has been shown to negatively affect nuclear localization, further confirming the importance of these regions [55]. Additionally, while the specific NLS amino acid sequence is important for nuclear localization, its neighboring amino acids also impact the nuclear localization. In a cancer mouse model, the phosphorylation of serine residue 2152 (2147-2153: K-A-R-K-P-S-T) within the NSL4 region by all three Pim family kinases was shown to be necessary for nuclear localization and signal transduction [56]. In addition to the NSL regions directing nuclear localization, one study examining mice identified S/T-P-S/T domains (2122-2124: T-P-T, 2126-2128: S-P-T) that can also direct nuclear localization of NICD [57]. Overall, the main function of NLS domains within NICD is to guide nuclear entry.

TAD. Located between the ANK repeats and the PEST domains is TAD, which stimulates activation of an associated gene at the promotor (Fig. 2A). After the NICD-CSL-MAM complex is formed, chromosome modifying proteins are recruited, including histone ubiquitin ligase and HAT, to open the chromosome and initiate activate transcription [12]. However, TAD alone has been shown to be insufficient to fully activate NOTCH1 transcriptional activity, and relies on the presence of the ANK repeats to enhance transcriptional activity [41]. Furthermore, TAD also promote the phosphorylation and proteolytic turnover of NICD, which is essential for Notch transcription [10]. At the 3′-terminal of TAD, there is a glutamine-rich OPA sequence that is conserved between drosophila and mammalian [41], but its role has not been researched. Therefore, TAD collaborates with ANK to activate NOTCH1 transcriptional activity and plays a role in maintaining NICD stability.

PEST domain. The PEST domain is a polypeptide sequences that aids in modulating NICD degradation via a ubiquitin-proteasome pathway [58], and modulates by post-transcriptional modification [59]. In one study, a point mutation in the conserved Ser residues within the PEST domain prevented hyperphosphorylation of PEST and stabilized ICD. The PEST domain also interacts with FBW7, an E3 ligase, to ubiquitinate and degrade NICD [60]. In this study, they showed that introducing a T2512A substitution at a potential phosphorylation site abolished the interaction between NICD and FBW7 and enhanced NOTCH1 pathway signaling. Furthermore, when PEST is ubiquitinated, NOTCH signaling is reduced [61]. An overexpression of the NOTCH1 C-terminal, which contains PEST, does not activate NOTCH signaling, and an alteration in PEST can significantly contribute to neoplastic transformation [62]. Therefore, PEST has a lesser role in transactivation, with its primary function being NOTCH1 stability and turnover.

In summary, of the NOTCH1 components, NECD primarily facilitates ligand binding and proper extracellular cleavage, while NEXT contains NICD that undergoes nuclear localization, promotes transcriptional activation, and modulates its stability.

3 *NOTCH1* Variants

NOTCH1 is highly intolerant of both loss-of-function (LOF) variants, including stop-gain, frameshift, splicing variants, micro-deletion, and missense variation, with a pLI of 1 and a missense z score of 4.48 on the Exome Aggregation Consortium (ExAC) [23]. *NOTCH1* variants have been implicated in both sporadic and hereditary CHD cases [24,25,63,64] and pathogenic *NOTCH1* variants have been found in approximately 6% of CHD patients, with a higher prevalence in

LVOTO and TOF [25]. Currently, a total of 216 *NOTCH1* variants have reported related to CHD (Fig. 3 and Supplementary Table).

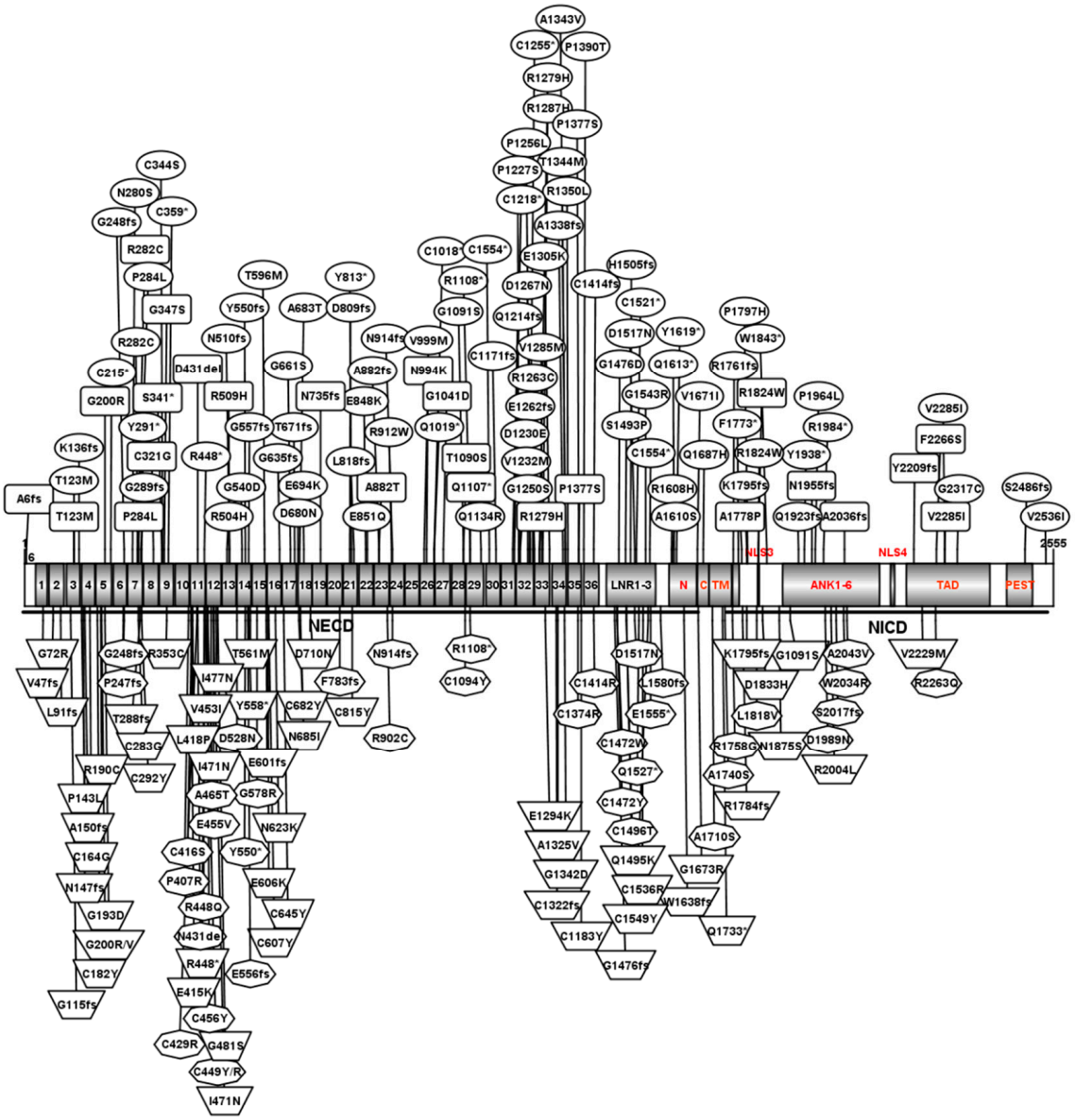

Figure 3: *NOTCH1* variants associated with cardiac disease. Variants associated with LVOTO individuals (oval), TOF individuals (trapezoid), AOS individuals (octagon) and other individuals (rounded rectangle) are indicated. C359*, a truncated variant with the cysteine at position 359 changed into a stop codon. N914–1G>A, splicing variant at amino acid N914. D809fs, a frameshift variant at position 809 that contains a frameshift and splice site variant. 1–36, EGF–domain 1–36 repeats; LNR1–3, the LNR1–3 domain; N, the heterodimerization domain located the N-terminal part of the NECD region; C, the heterodimerization domain located the C-terminal begins the NEXT region; TM, transmembrane domain; NLS3 and NLS4, nuclear localization signals 3 and 4, respectively; ANK1–6, the ANK 1–6 domain; TAD, the transcriptional activation domain; PEST, the PEST domain. This figure was prepared using IBS (https://ibs.renlab.org/) based on protein structure.

3.1 NOTCH1 Variants in LVOT

The LVOTO defect is associated with structural malformations on the left side of the heart, including hypoplastic left heart syndrome (HLHS), aortic coarctation (CoA), aortic stenosis (AS), and bicuspid aortic valve (BAV) [65]. In LVOTO individuals, *NOTCH1* mutations represent 5.9% of cases, with an additional 16% involving variants of uncertain significance [25]. Among reported variants, we have searched the NCBI database and found 92 *NOTCH1* variants associated with LVOTO patients (Supplementary Table). Among them, 62 variants (out of 92 total variants) are located in the EGF-like domain. EGF-like repeats are short peptides with a distinctive motif of six cysteines with three internal disulfide bridges, and make up the most important domain, accounting for about 60% of the animo acids in NOTCH1 protein (1426/2555) (Fig. 3) [30]. If a mutation site is located at a cysteine residue, disulfide bond formation will be disrupted leading to protein structural instability, S1 clearance failure, and the formation of fewer NICDs, affecting the transmission of NOTCH signals. The missense mutation, c.G1820;p.C607Y, which is located in EGF16, can disrupt conserved disulfide bonds and impair S1 cleavage, thereby reducing NICD formation [23]. Therefore, we classify cysteine variants in the EGF-like domain as LOF mutations that are pathogenic. In LVOTO, a total of 92 *NOTCH1* variants were reported (Fig. 3, Supplementary Table), of which 44 were classified as LOF mutations and 62 variants were located in the EGF-like domain (Fig. 3, Table 1).

Table 1: Number of *NOTCH1* variants in different type of CHD.

Disease Type	Total Variants	Loss of Function					All Variants in EGF-Like Repeat	Missense Variants
		Stop-Gain Variants	Frameshifts	Micro-Deletions	C in EGF-Like Repeats	n		
LVOTO	92	18	21	4	1	44/92	62/92	48
TOF	57	3	12	2	9	26/57	41/57	31
AOS	42	4	7	3	8	22/42	22/42	18
Other CHD	25	2	5	0	1	8/25	18/25	18
Total	216	27	45	9	19	100/216	143/216	115

C, Cys, cysteines; LVOTO, left ventricular outflow tract obstruction; TOF, Tetralogy of Fallot; AOS, Adams–Oliver syndrome; Other CHD, CHD not assigned to the phenotypes in LVO, TOF and AOS. The number of total variants = number of stop-gain + number of frameshift + number of micro-deletion + number of C in EGF-like repeats + number of missense variant. n represents the total number of the loss of function.

3.2 NOTCH1 Variants in TOF

TOF is one of the most common and complex CHD forms, with a prevalence of approximately 1 in every 3000 live births, accounting for 4.5% of newborns with CHD [64]. TOF is considered a malformation of the cardiac outflow tract, which includes four specific structural features: VSD, right ventricular outflow tract stenosis, right ventricular hypertrophy, and aortic override. Genetic factors are the main cause of TOF, with 20% of patients having pathological copy number variants (CNVs) or significant chromosomal abnormalities [66]. In TOF patient, *NOTCH1* variants account for at least 4.5% of TOF cases (37/829) [23,67]. Among reported variants, a total of 57 *NOTCH1* variants were reported in TOF patients (Fig. 3, Supplementary Table). In TOF individuals, 26 variants (out of 57 total variants) were LOF mutations and 21 variants (out of 57 total variants) were located in the EGF-like domain (Fig. 3, Table 1).

3.3 NOTCH1 Variants in AOS

NOTCH1 is an important candidate gene in AOS, a rare disorder featuring congenital scalp defects and terminal limb anomalies, that is often accompanied by CHD, including atrial septal defect (ASD), VSD, aortic valve stenosis, pulmonary valve stenosis and TOF. *NOTCH1* variants account for 10% of AOS cases, particularly those with cardiovascular abnormalities [68]. Among reported variants, a total of 42 *NOTCH1* variants were reported in AOS patients (Fig. 3, Supplementary Table). In these AOS individuals, 22 variants (total variants were 42) were LOF mutations, and 22 variants (out of 42 total variants) were located within the EGF-like domain (Fig. 3, Table 1).

3.4 NOTCH1 Variants in Other CHDs

NOTCH1 variants have also been associated with other CHDs not mentioned above, including VSD, ASD, outflow tract and coronary artery abnormalities. In ASD and mitral stenosis, a splice variant, c.2207+1G>T:pN735fsX*, was identified [69]; while in VSD and tricuspid atresia, a frameshift variant, c.13_14dupCT:p.A6fs*28, was observed [70]. A frameshift-deletion variant, c.6105Cdel:p.A2036fs3*, was observed in probands with an outflow tract defect, such as double-outlet right ventricle (DORV) and right ventricular dilation [71]. Additionally, a stop-gain variant, c.1023C>A:p.S341*, was found in a patient with coronary artery abnormalities [72]. Up to now, a total of 25 *NOTCH1* variants were reported in other CHD patients (Fig. 3, Supplementary Table). Among them, 8 variants (out of 25 total variants) were LOF mutations, and 18 variants (out of 25 total variants) were located in EGF-like domain (Fig. 3, Table 1).

Overall, a total of 216 *NOTCH1* variants were reported, and most of the *NOTCH1* variants appeared to be predominantly located in the EGF-like domain (143/216), with many being LOF variants (100/216).

4 NOTCH1 Mechanistic Links to CHD

In a computational study performed in 2010, the probability that NOTCH1 function is susceptible to haploinsufficiency was predicted to be 0.957 [73]. Moreover, *NOTCH1* and its family members have been found to be downregulated in both *NOTCH1* mutant and non-mutant CHD samples [23,74,75]. Therefore, *NOTCH1* haploinsufficiency is a significant factor in CHD occurrence.

In induced pluripotent stem cells (iPSCs) that were reprogrammed from fibroblast patients with valve disease, both a stop-gain variant, c.C3322T:p.R1108X*1108, and a frameshift variant, c.C4515del:p.H1505fs74*, can result in *NOTCH1* haploinsufficiency [22,75]. In a transgenic mouse model, Notch1-p.V1744G, which is analogous to human p.V1754G, and located within the intramembranous processing site, was shown to significantly reduce NOTCH1 processing [76]. This study also found that homozygous Notch1 knockout mice ($Notch1^{-/-}$) and homozygous of Notch1-p.V1744G mice displayed that same phenotype, including development retardation, embryonic lethality before e12.5 days, and a distended pericardial sac, but with a beating heart. Meanwhile, our group aimed to investigate the relationship between NOTCH1 variants and TOF. We constructed two variant transgenic mice, both located at the C-amino acid of the EGF-like domain, and generated fewer NICDs in the homozygous and heterozygous mice. The experimental results showed that, homozygous mice with these two mutation sites had the same phenotype as mice with *Notch1* knockout (lethality before e12.5 days, with the presence of a pericardial

sac and a beating heart). Heterozygotes were born with high permeability CHD, such as VSD, overriding aorta, and right ventricular hypoplasia (data unpublished). Therefore, *NOTCH1* variants can lead to a decrease in NICD, affecting NOTCH1 functioning and disrupting normal embryonic development, causing various types of CHD (Fig. 4).

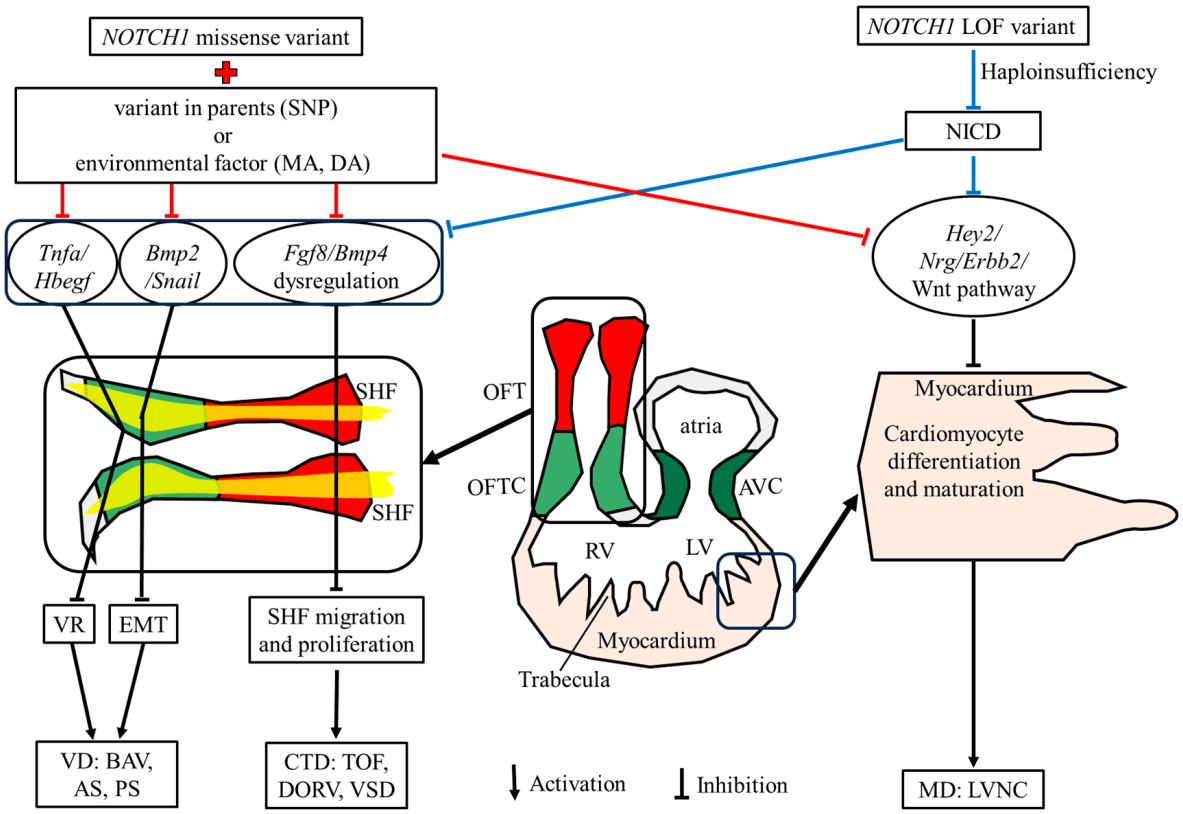

Figure 4: Heart development in embryos at 10.5 days and the two molecular mechanism contributing *NOTCH1* variant associated CHD occurrence. OFT, outflow tract; OFTC, OFT cushion; RV, right ventricular; LV, left ventricular; AVC, atrioventricular cushion; CTD, conotruncal defects; MD, myocardial defect; VD, valve defect; NICD, NOTCH intracellular domain, TOF, Tetralogy of Fallot; DORV, double-outlet right ventricle; VSD, ventricular septal defects; AS, aortic stenosis; BAV, bicuspid aortic valve; PS, pulmonary stenosis; LVNC, left ventricular non-compaction; EMT, epithelial-to-mesenchymal transition; LOF, loss-of-function. VR, valve remodeling; MA, maternal alcohol; DA, drug abuse; Red backgrounds indicate OFT; Yellow backgrounds indicate second heart field; and light green and dark green backgrounds indicate OFT and atrial-ventricular cushions, respectively.

NOTCH1 variants associated with LOF variants, including stop-gain, frameshift, splicing, micro-deletion and cysteine variants, have been shown to be commonly located within the EGF-like domain, to reduce NICD function, and can result in haploinsufficiency. In stop-gain and frame shift variants, the conversion of a coding amino acid to a premature stop codon generates a truncation or haploinsufficiency. Splicing variants that are located at the junction of a 5′ or 3′ exon can cause exon skipping or intron-containing transcripts due to aberrant pre-messenger RNA splicing. This aberrant splicing can result in a premature truncation or generation of another unknown protein. In a microdeletion, the *NOTCH1* gene, which is located at 9q.34, is completely or partially removed,

leading to a haploinsufficiency. A cysteine variant in the EGF-like domain affects the disulfide bonds formation and can cause S1 cleavage failure, reducing NICD production. The mentioned above variants make up 100 (out of 216 total variants) of all CHD associated mutations.

In another transgenic *Notch1* mouse model, a substitution in the ANK-repeat domain at the Arg in position 1974 was introduced, and while NICD formation was not affected, sequence-paired site binding was impaired [77]. Moreover, homozygous mice with this substitution had a normal lifespan and did not develop any T-cell abnormalities or CHD, with both defects noted in *Notch1* knockout; however, they did show an increased sensitivity to dextran sulfate sodium (DSS), which induced colitis and highly penetrant with severe VSD. This study indicated that a missense *NOTCH1* variant could maintain normal target activation functioning, but increase CHD susceptibility when combined with environmental toxins. Taken together, these finding suggest that rare variants found in both unaffected and affected individuals, such as *de novo* missense variants alone, are, on their own, insufficient to cause CHD. However, when combined with a affected environmental (toxic exposures, such as drug abuse, maternal alcohol, consumption) or inherited variants with unaffected parents (e.g., SNP), the risk of CHD occurrence in the offspring increases (Fig. 4).

NOTCH1 is the one of the High-Confidence Genes in Conotruncal Cardiac Defects by Gene Burden Analyses [78]. Using a fate-mapped mouse model, Notch signal abnormality (*Dll4* or *Notch1* knockout mouse model) has been found to reduce SHF proliferation via *Fgf8* and *Bmp4* [79]. *Notch1*$^{+/-}$ heterozygous mice exhibited an inhibition of Notch signal transmission, and a reduction in second cardiac cell proliferation and migration, through *Fgf8* or *Bmp4*. This can lead to conotruncal defects, such as TOF, DORV and VSD [79]. Furthermore, *Notch1* or *RBP-J* deletion affects outflow tract clockwise rotation and septation [80]. During OFT development, the rotation and alignment of OFT rely on the accurate proliferation and migration of anterior SHF [81]. Therefore, in *NOTCH1* variants, NOTCH1 malfunction can lead to the abnormal proliferation and migration of SHF and cause conotruncal defects, such as TOF, DORV, and VSD (Fig. 4).

During early valve development, cardiomyocytes of the conotruncal cushions recruit NCCs into conotruncal cushions, where they promote EMT and play essential roles in aorticopulmonary septation [81]. NOTCH pathways activate the transcription activity of *Snail* and subsequently repress *VE-cadherin* expression, which is a prerequisite for the EMT [82]. NOTCH signals also activate *Bmp2* expression and promote the proliferation of mesenchymal cells (MCs) during endocardial cushion formation [82]. Additionally, NOTCH1 induces the apoptosis and proliferation of valve interstitial cells via *Tnfa* and *Hbegf*, respectively, which controls the cardiac valve remodeling process [82]. *NOTCH1* loss-of-function (LOF) mutant embryos severely impaired the EMT and valve remodeling process, and disruption of the NOTCH signaling pathway can lead to valve defects, such as thickened valve leaflets, PS, AS, and BAV (Fig. 4). β β

The genetic landscape indicated that NOTCH1 is associated with both aorta and aorta valve disease, as well as cardiomyopathy, such as HLHS [83]. In human induced pluripotent stem cells (iPSCs), *NOTCH1* is essential for ventricular-like cardiomyocyte differentiation and proliferation through regulating the cell fate of cardiac mesoderms and modulating the cell cycle via the Wnt pathway; notably, insufficient *Notch1* functionality results in HLHS [84,85]. In mouse models, *Notch1* malfunction can also affect cardiomyocyte differentiation and proliferation during early myocardium formation via *Hey1/Nrg/Erbb2* [86]. Furthermore, inhibiting NOTCH signals can reduce the regenerative ability of ventricular myocardia [87]. Therefore, *NOTCH1* variants can lead

to *NOTCH1* malfunctions and cause myocardial defects at the cardiac mesoderm stage, such as left ventricular non-compaction (LVNC) (Fig. 4).

5 Conclusions

NOTCH1 encodes a protein with two major domains: NECD and NICD. The NECD is comprised of an EGF-like domain that contains 1–36 repeats, and a NRR domain. The EGF-like repeats function in ligand binding and activate the NOTCH signal. The NRR domain harbors the S1 and S2 cleavage sites and negatively regulates NOTCH1 stability to ensure the correct triple cleavage of the extracellular segment to form NICD. NICD, which contains RAM, ANK, TAD, and PEST domains, functions as a transcription factor by entering the nucleus to promote transcription by forming the NICD-CSL-MAM complex, which recruits HAT to form transcription complexes and initiate gene transcription.

After examining *NOTCH1* variants associated with CHD, they were found to have a high prevalence in the EGF-like domain. Additionally, LOF variants make up 44 (out of 92 total variants) variants in LVOTO, 26 (out of 57 total variants) in TOF, 22 (out of 42 total variants) in AOS, and 8 (out of 25 total variants) in other types of CHD. The two possible molecular mechanisms underlying NOTCH1-associated CHD occurrence included LOF variants, which directly reduce NICD production, or a missense variant, which causes a mild NOTCH1 mal-function, but is insufficient to CHD occurrence. In the case of a missense variant, other factors, such as toxic exposure, are required to cumulatively promote CHD occurrence.

6 Limitations and Prospects

With the development of larger-scale genetic sequencing platforms, an increasing number of NOTCH1 variants have been discovered in CHD. However, several limitations remain.

1. The *NOTCH1* variants have not yet been clinically used for improved methods for CHD diagnosis and management.
2. To date, there is no validated animal model that accurately mimics the CHD phenotypes caused by *NOTCH1* variants.
3. Large and more diverse clinical studies are needed to develop and validate the relationship between *NOTCH1* variants and CHD, especially TOF.

Future research avenues:

1. As sequencing technologies advance and clinical sample sizes increase, it will become possible to develop improved diagnostic and management methods for CHD.
2. With current genetic testing, screening can aid in reducing or delaying incidence rates, improve the quality of life of variant carriers.
3. A deeper understanding of the functional impact of *NOTCH1* mutations may allow their integration into reproductive medicine, potentially reducing the occurrence of severe CHD through the pre-implantation genetic diagnosis and screening of embryos.

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Abbreviation

CHD	Congenital heart disease
NECD	NOTCH extracellular domain
NICD	NOTCH intracellular domain
NEXT	NOTCH extracellular truncation
WES	Whole-exome sequencing
EMT	Epithelial-to-mesenchymal transition
TOF	Tetralogy of Fallot
LVOTO	Left ventricular outflow tract obstruction
T-ALL	T-cell acute lymphoblastic leukemia
HAT	Histone acetyltransferase
EGF	Epidermal growth factor-like
NRR	Negative regulatory region
VSD	Ventricular septal defect
LNRs	Lin12/Notch repeats
HD	Heterodimerization domain
TD	Transmembrane domain
NLS	Nuclear localization signal
RAM	RBP-J association module
ANK	Ankyrin
TAD	Transcriptional activation domain
MEF2C	Myocyte enhancer factor 2C
LOF	Loss-of-function
ExAC	Exome Aggregation Consortium
HLHS	Hypoplastic left heart syndrome
CoA	Aortic coarctation
AS	Aortic stenosis
BAV	Bicuspid aortic valve
ASD	Atrial septal defect
CNVs	Copy number variants
iPSCs	Induced pluripotent stem cells
DSS	Dextran sulfate sodium
LVNC	Left ventricular non-compaction
PS	Pulmonary stenosis
DORV	Double-outlet right ventricle

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