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Notch Signaling in Vascular Smooth Muscle Cells

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Abstract

The Notch signaling pathway is a highly conserved pathway involved in cell fate determination in embryonic development and also functions in the regulation of physiological processes in several systems. It plays an especially important role in vascular development and physiology by influencing angiogenesis, vessel patterning, arterial/venous specification, and vascular smooth muscle biology. Aberrant or dysregulated Notch signaling is the cause of or a contributing factor to many vascular disorders, including inherited vascular diseases, such as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, associated with degeneration of the smooth muscle layer in cerebral arteries. Like most signaling pathways, the Notch signaling axis is influenced by complex interactions with mediators of other signaling pathways. This complexity is also compounded by different members of the Notch family having both overlapping and unique functions. Thus, it is vital to fully understand the roles and interactions of each Notch family member in order to effectively and specifically target their exact contributions to vascular disease. In this chapter, we will review the Notch signaling pathway in vascular smooth muscle cells as it relates to vascular development and human disease.

1. INTRODUCTION

The Notch signaling pathway is an evolutionarily conserved pathway that plays a critical role in cell fate decisions for numerous systems in vertebrates (Artavanis-Tsakonas, Rand, & Lake, 1999; Ehebauer, Hayward, & Arias, 2006). Over the past ~15 years, many studies have demonstrated the importance of Notch signaling in regulating vascular development and physiology, through control of endothelial cells and smooth muscle cells and their interactions with each other. The overall contribution of Notch signaling to the vasculature and especially the role of endothelial cell-derived Notch signaling has been well studied and previously covered in several exceptional reviews (Fouillade, Monet-Lepretre, Baron-Menguy, & Joutel, 2012; Gridley, 2007, 2010; Iso, Hamamori, & Kedes, 2003; Phng & Gerhardt, 2009; Roca & Adams, 2007; Rostama, Peterson, Vary, & Liaw, 2014). In this review, we will focus on the role of Notch signaling in vascular smooth muscle cells (VSMCs) and how it regulates the development and homeostasis of the vasculature, and how when deficient or perturbed can contribute to disease states.

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CONFLICT OF INTERESTS

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Originally discovered in *Drosophila* (and named for the "notched" wings that resulted from its mutation) (Morgan, 1917), the canonical Notch signaling pathway is relatively straightforward, consisting of a membrane-bound ligand and receptor engagement on neighboring cells leading to cleavage of the Notch intracellular domain (NICD) of the receptor, which shuttles to the nucleus and acts a transcriptional cofactor. This series of events allows for a signal to be passed directly from receptor activation to transcriptional modulation while bypassing the signaling intermediates and kinase cascades necessary for many other signaling pathways. This linear system coupled with its inherent feedback mechanisms allows for the creation of signal-sending: signal-receiving binary cell fate determinations in early developmental processes, such as those seen in the lateral specification of neural and epidermal cells in *Drosophila* (Heitzler & Simpson, 1991). However, beyond its role initially described in lower species, this basic signaling pathway has been adapted and utilized by nearly every cell type and organ system to enact a myriad of functions.

The cardiovascular system is the first organ system to develop in embryogenesis and is made up of the heart and an expansive network of vessels of different types and sizes (Patel-Hett & D'Amore, 2011). Early in development, angioblasts differentiate into endothelial cells and aggregate into a nascent vascular network in a process called vasculogenesis. This network is extended, pruned, and remodeled over the course of development to meet the needs of the developing embryo. To support the growing network of endothelial cell tubes, mural cells are recruited and differentiate into VSMCs or pericytes. These mural cells are vital to the maturation of vessels into functional arteries, veins, and capillaries. VSMCs and pericytes play countless roles in these vessels, from controlling blood flow to regulating permeability, and those roles shift depending upon the state of the vessel, embryological origin, and the environmental context. VSMCs are not "terminally differentiated" cells and are capable of adapting their activities in response to molecular and physiological cues. This ability of VSMCs to radically alter their functions in different contexts is known as VSMC phenotypic switching or plasticity. This concept has been well studied and reviewed (Alexander & Owens, 2012; Gomez & Owens, 2012; Owens, Kumar, & Wamhoff, 2004) and attributed to many of the activities of VSMCs in vascular diseases.

In VSMCs, the Notch receptors Notch2 and Notch3 and the ligand Jagged1 predominate (High et al., 2007; Tang et al., 2010). Notch1 and Notch4 are more highly expressed in endothelial cells, although Notch1 has been shown to play a role in VSMCs in the context of vascular injury (Li, Takeshita, et al., 2009) and in pericytes (Kofler, Cuervo, Uh, Murtomaki, & Kitajewski, 2015). Over the past few decades, extensive work has been performed to ascertain the role of these receptors and Notch signaling as a whole in VSMCs. While significant progress has been made and strong connections have linked the Notch pathway to vascular development, VSMC differentiation and phenotype, and vascular disease, there is still much that is not understood about the precise mechanisms involved (Fig. 1).

2. THE NOTCH SIGNALING PATHWAY

2.1 The Canonical Notch Signaling Pathway

In mammals, there are four Notch receptors (Notch1–4) and five ligands (Jagged1 and 2, Delta-like 1, 3, and 4) which are all Type I transmembrane proteins. Both the ligands and receptors have long extracellular domains (ECDs) made up of epidermal growth factor-like (EGF) repeats which serve as the binding platform for the receptor–ligand interaction. The receptor arrives at the cell membrane as a heterodimer after it is cleaved by furin at the site 1 (S1). After a receptor is bound by ligand, the negative regulatory region (NRR) is displaced, exposing the site 2 (S2) for cleavage by one of the ADAM metalloproteases (a disintegrin and metalloproteinase domain-containing protein), and the site 3 (S3) for cleavage by the γ-secretase complex, releasing the NICD fragment. After cleavage at the S3 site, the NICD's nuclear localization signal triggers translocation to the nucleus. The NICD acts as a transcriptional cofactor by forming a complex with the CSL (CBF1/SuH/Lag1) transcription factor named for its homologs in mammals, *Drosophila*, and *Caenorhabditis elegans*, though it is now designated RBPJ (recombination signal-binding protein for immunoglobulin kappa J region) in mammals. This complex binds to its target DNA sequence and to one of the three mastermind-like (MAML) proteins, which act as transcriptional activators. The formation of this complex displaces transcriptional repressors including SKIP (Ski-interacting protein), CIR (CBF1-interacting corepressor), KyoT2, and SHARP (SMRT- and HDAC-associated repressor) and histone deacetylases (HDACs) from RBPJ, leading to expression of Notch target genes (Borggrefe & Oswald, 2009). Among the most highly activated genes are a family of basic helix–loop–helix (bHLH) proteins hairy/enhancer of split (HES) and HES-related proteins (HEY/HRT/HERP) which act as transcriptional repressors.

Though the transduction of the Notch signal via NICD cleavage and nuclear translocation is well established, there are many opportunities along its path for regulation to occur that introduces signaling diversity. One such mechanism is the Fringe family of glycosyltransferases, Lunatic Fringe, Manic Fringe, and Radical Fringe (Moloney et al., 2000). Both Notch ligands and receptors have attached sugar moieties on the EGF repeats of the ECDs, and Fringe glycosyltransferases are able to elongate N- and O-linked glycans at certain positions during posttranslational processing in the Golgi. The length and position of these glycans can determine whether ligands and receptors interact between neighboring cells (*trans* activation) or within the same cell (*cis* inhibition) (LeBon, Lee, Sprinzak, Jafar-Nejad, & Elowitz, 2014). The glycosylation state can also influence the binding affinities for the different receptor–ligand pairs (Hicks et al., 2000; Stanley & Okajima, 2010). This influence on which receptor and ligand interact can then control ultimate effect of Notch signaling, as the different Notch ligands and Notch receptors are capable of producing different signaling outcomes. For instance, the ligands JAG1 and DLL4 have opposing effects on angiogenesis and their activity is regulated by their glycosylation (Benedito et al., 2009), and DLL4, but not DLL1, can induce pericyte differentiation in myoblasts (Cappellari et al., 2013). Also, the NICDs of the different Notch receptors can activate transcription of their target genes with varying efficacy depending on the orientation and number of RBPJ-binding sites in the promoter (Ong et al., 2006). Another potential

mechanism of Notch signal diversity is the control of the intensity and duration of the Notch signal. It has been demonstrated that some Notch target genes can be activated by low levels of active NICD, while others require high levels of activity (Ong et al., 2006). The number of NICD molecules present in the nucleus is dependent on both the influx of NICD from receptor activation and the turnover or degradation of NICD. The degradation of NICDs is regulated by the E3 ubiquitin ligases (Lai, 2002) which ubiquitinate NICDs in their PEST domain (rich in proline [P], glutamic acid [E], serine [S], and threonine [T]) and target them for degradation by the proteasome (Fig. 2).

2.2 Interaction with Other Signaling Pathways and Noncanonical Signaling

In addition to the long understood canonical Notch pathway, there is increasing evidence that the Notch pathway can exert effects outside of its prominent role as a transcriptional cofactor (Andersen, Uosaki, Shenje, & Kwon, 2012; Ayaz & Osborne, 2014; Martinez Arias, Zecchini, & Brennan, 2002; Sanalkumar, Dhanesh, & James, 2010). An example of this is the ability of the NICD to bind active β-catenin, leading to its degradation by the lysosome (Kwon et al., 2011). Though non-canonical activities of Notch have been shown in many different cell types and systems, evidence for these activities in VSMCs is relatively sparse and the mechanisms not well defined (Wang, Prince, Mou, & Pollman, 2002). However, based on the ubiquity of noncanonical signaling in other contexts, it seems likely that some of the effects of Notch signaling in VSMCs are due to undiscovered noncanonical interactions.

Most of the observed noncanonical signaling involves interactions of Notch components with members of other signaling pathways, but the overlap of Notch signaling and other pathways is not limited to the non-canonical pathway. Signals from outside of the Notch pathway can regulate Notch expression and activity as well as alter the transcription factors recruited to Notch target genes. Notch signaling can also in turn regulate the expression and activity of other signaling pathways. Much of the diversity in Notch signaling outcomes can be attributed to its interaction with other signaling pathways. Here we will discuss some of the known interactions with other signaling pathways in VSMCs.

2.2.1 Platelet-Derived Growth Factor B—Platelet-derived growth factor B (PDGF-B) is a major mitogen and chemotractant in VSMCs that acts through its receptor, PDGFRβ (Donovan, Abraham, & Norman, 2013). PDGF signaling has been shown to interact with Notch signaling in VSMCs in two major capacities. First, Notch signaling has been shown to directly promote the transcription of PDGFRβ and enhance the signaling effects of PDGF-B (Donovan et al., 2013; Jin et al., 2008). This relationship is especially apparent in pericytes, as both PDGFRβ and Notch3 are considered markers of pericytes and their dysfunction in animal models and humans can produce similar phenotypes (Lee, 2013; Martignetti et al., 2013; Wang et al., 2014). Second, it has been shown that PDGF-B signaling can regulate the expression of Notch components (Baeten & Lilly, 2015; Campos, Wang, Pollman, & Gibbons, 2002). Overall, it is apparent that the PDGF and Notch signaling pathways are strongly intertwined, with significant functional overlap in pericytes and coregulation in VSMCs.

2.2.2 Transforming Growth Factor β—The transforming growth factor β (TGFβ) pathway is a vital contributor to the differentiation of VSMCs (Guo & Chen, 2012). The Notch and TGFβ signaling pathways cooperatively regulate the differentiation of VSMCs through direct interaction of NICD and SMAD2/3 (a transcription factor within the TGFβ pathway) and transcription of smooth muscle contractile genes (Tang et al., 2010). This cooperative induction of VSMC differentiation has also been shown in progenitor cell types (Grieskamp, Rudat, Ludtke, Norden, & Kispert, 2011; Kurpinski et al., 2010), and the direct interaction of NICD and SMAD3 as transcriptional activators has been shown in other systems (Blokzijl et al., 2003). Interestingly, it has also been shown that these two pathways can negatively regulate each other's expression. This is seen in fibroblasts where TGFβ signaling decreases expression of Notch3 (Kennard, Liu, & Lilly, 2008) and in VSMCs where Notch signaling promotes the transcription of miR145 which inhibits the TGFβ pathway by targeting the TGFβ receptor 2 (Boucher, Peterson, Urs, Zhang, & Liaw, 2011; Zhao et al., 2015). Taken together, the Notch and TGFβ pathways are able to both cooperatively promote transcription of their downstream genes and inhibit each other's expression, suggesting a very complex relationship and potentially a mechanism for negative feedback within each pathway.

2.2.3 Mitogen-Activated Protein Kinase—The mitogen-activated protein kinase (MAPK) pathway is one of the major signaling pathways controlling cell growth, survival, and many other processes and is a main transducer of growth factor signals (Pearson et al., 2001). In cultured VSMCs, Notch3 is capable of activating this pathway by phosphorylation of the MAPK component ERK (Baeten & Lilly, 2015; Wang, Campos, Prince, Mou, & Pollman, 2002), through a mechanism that is not yet known. Physical interaction of Notch and MAPK components has previously been shown in other models (Hodkinson et al., 2007). However, it may simply be a result of upregulation of growth factor ligands or receptors by Notch signaling, such as PDGFRβ. Regardless of how it is enacting this effect, it is clear that at least in some contexts Notch signaling is capable of promoting MAPK pathway activity.

2.2.4 Wingless-Related Integration Site—The Wnt (wingless-related integration site) signaling pathway is an important developmental pathway that regulates smooth muscle proliferation, migration, and survival (Mill & George, 2012). The Wnt and Notch pathways have significant cross talk in many systems, by both direct effect on each other's pathway components and shared regulation of developmental processes (Andersen et al., 2012; Duncan et al., 2005; Espinosa, Ingles-Esteve, Aguilera, & Bigas, 2003; Fre et al., 2009; Hayward et al., 2005). These interactions have been shown to extend to VSMCs as well, as early mesoderm cells in chick embryos are pushed toward a VSMC lineage through cooperation of the Notch and Wnt pathway (Shin, Nagai, & Sheng, 2009). While evidence for Wnt/Notch pathway interaction in VSMCs is limited, the results in mesodermal smooth muscle progenitors coupled with the known cross talk between Notch and Wnt signaling in other systems suggest that these pathways cooperate to drive early VSMC differentiation.

3. NOTCH SIGNALING IN VSMC DEVELOPMENT

Notch signaling plays an essential role in the development of the cardiovascular system, from hematopoiesis to cardiac development to endothelial cell sprouting and vasculogenesis. In the development and differentiation of VSMCs, the functions of Notch signaling can be categorized into two main roles: construction of the vessel wall and arterial–venous specification.

3.1 Constructing a Vessel Wall

Following the initial stages of vasculogenesis, nascent vessels are composed of an endothelial tube surrounded by perivascular cells. For a vessel to mature into a conduit capable of withstanding increasing pressure and volume demands, it must recruit mural cells to encompass it and differentiate into one or more layers of vascular smooth muscle. This recruitment is largely driven by a PDGF-B gradient secreted by the endothelial cells (Hellstrom, Kalen, Lindahl, Abramsson, & Betsholtz, 1999). Once recruited to the nascent vessel, endothelial cell-derived Notch ligands can activate Notch signaling in these mural cells, which induces integrin adhesion to the endothelial basement membrane and initiates maturation and differentiation (Scheppke et al., 2012). We have learned a great deal about how endothelial-derived Notch signals affect VSMCs and mural cells from mutant mouse models and in vitro systems. In the great vessels of the outflow tract, cardiac neural crest progenitors give rise to the VSMCs within the vessel walls. With the complete abrogation of Notch signaling by “knocking in” of a dominant-negative MAML1 in cardiac neural crest cells, mice display various defects of the outflow tract and aortic arch associated with decreased differentiation and expression of smooth muscle markers (High et al., 2007). Mice with an endothelial deletion of Jagged1 also display decreased VSMC differentiation and expression of smooth muscle markers (High et al., 2008). Through a combination of in vitro coculture experiments (Liu, Kennard, & Lilly, 2009) and the cardiac neural crest-specific Jagged1 knockout mice (Manderfield et al., 2012), it was demonstrated that induction of Notch signaling in VSMCs by endothelial-expressed Jagged1 leads to the increased expression of both Notch3 and Jagged1. This allows for lateral induction of Notch signaling by homotypic VSMC–VSMC interactions through multiple layers of smooth muscle to promote VSMC differentiation after the initial signal is received from endothelial-derived Jagged1 (Hoglund & Majesky, 2012). This model has been further supported by smooth muscle deletion of Jagged1, RBPJ, and a smooth muscle deletion of Notch2 coupled with heterozygous deletion of Notch3 (Baeten et al., 2015; Feng, Krebs, & Gridley, 2010; Krebs, Norton, & Gridley, 2016). All three of these models develop patent ductus arteriosus (PDA) due to contractile deficits of the VSMCs and present decreased expression of smooth muscle markers. An interesting parallel exists between this model and one proposed from experiments performed by the Krasnow lab (Greif et al., 2012), where they demonstrate that the expression of PDGFRβ and ACTA2 (alpha smooth muscle actin) moves through layers of smooth muscle like a wave. They describe a major role for endothelial-derived PDGF-B in the migration, orientation, and proliferation of these smooth muscle cell layers and suggest that there may be another signaling pathway contributing to the radial pattern of construction. Indeed, Notch signaling would elegantly fit into this proposed model, as it is

known to induce PDGFRβ and ACTA2, and could be passed through the layers of smooth muscle via cell–cell contact (Jin et al., 2008; Noseda et al., 2006).

An interesting finding from comparing the knockout phenotypes of mice deficient in Notch2 and Notch3 is their unique roles in different vascular beds. Notch2 is mainly expressed in the outflow tract and other large-caliber vessels, while Notch3 is expressed in all mural cells (High et al., 2007; Joutel et al., 2000). Likewise, in large-caliber vessels, Notch2 signaling is indispensable, with loss of Notch3 only acting to amplify defects in Notch2-null mice (McCright et al., 2001; Wang et al., 2012). This demonstrates that while there may be some functional redundancy of Notch2/3 in large-caliber vessels, Notch3 cannot completely replace Notch2 function. However, in pericytes and VSMCs in smaller caliber vessels where Notch2 expression is significantly diminished, Notch3 plays a major role, especially in the cerebral vasculature (Henshall et al., 2015; Liu et al., 2010; Volz et al., 2015; Wang et al., 2014).

3.2 Arterial–Venous Specification

Another important role for Notch signaling in VSMCs in the developing vasculature is the specialization of vessels into arteries and veins. Arteries and veins have distinct structural properties and express distinct protein markers that contribute to their unique functions in the cardiovascular system (Wang, Chen, & Anderson, 1998). In endothelial cells, Notch signaling regulates arterial specification in cooperation with VEGF (Hirashima, 2009; Kim et al., 2008). Similarly, Notch3 regulates arterial specification in VSMCs. In a mouse model with global knockout of Notch3, arteries acquired a more venous phenotype, with enlarged vessels, thinner and disorganized smooth muscle layers, and less festooned elastic lamina (Domenga et al., 2004). They did not see these changes in the outflow tract or aorta where Notch2 is highly expressed, likely suggesting a functional redundancy for Notch2 and Notch3 in this context. The expression of the arterial marker smoothelin and a LacZ reporter driven by an arterial-specific promoter element was also reduced in these Notch3-null mice, showing both structural and morphological defects in arterial specification. In zebrafish, Notch3 expression was found to be restricted to arteries, and inhibition of Notch signaling in zebrafish leads to decreased arterial specification and similar phenotypes compared to the mouse model (Lawson et al., 2001).

4. NOTCH SIGNALING AND VSMC PHENOTYPE

The ability of VSMCs to display various phenotypes and enact disparate functions depending on their cellular context, known as phenotypic switching or plasticity, is central to their changing roles from early developmental vasculogenesis to vascular homeostasis and remodeling in adult animals. Notch signaling has been closely tied to the many possible phenotypic endpoints of VSMCs. While there are still disparities in the literature about some of the phenotypic effects of Notch signaling in VSMCs, we are starting to obtain a clearer picture of how Notch activation drives VSMCs to different endpoints and the contributions from the different Notch receptors.

4.1 Differentiation

A “differentiated” VSMC is generally described as a quiescent cell expressing contractile proteins (such as ACTA2, smooth muscle myosin heavy chain [MYH11], transgelin [SM22α], and calponin [CNN1]) and primed to provide contractile force to constrict (or dilate) the vessel in which it resides in response to external cues. This is the state necessary for mature vessels to maintain physiological homeostasis. Notch signaling has been tied to regulation of VSMC differentiation and expression of contractile proteins for some time. While initial studies in vitro and in injury models showed that Notch-activated genes of the HRT (hairy-related transcription factors) family could repress myocardin-induced contractile protein expression (Doi et al., 2005; Proweller, Pear, & Parmacek, 2005; Yoshida et al., 2003), there were also several studies, showing that Notch activity promoted VSMC differentiation and contractile marker expression (Doi et al., 2006; Domenga et al., 2004; High et al., 2007). Although the discrepancy has not been conclusively explained, it is likely that the observed repression of contractile genes by the HRT family is negative feedback, as this family of repressors interferes with the transcription of many Notch target genes, including the HRT genes themselves (King et al., 2006; Nakagawa et al., 2000; Takebayashi et al., 1994). Notch activity has since been shown to directly promote the transcription of ACTA2 (Noseda et al., 2006), and Notch-induced transcription of ACTA2 was repressed by HRT transcription factors (Tang, Urs, & Liaw, 2008). Overall, the preponderance of data supports the hypothesis that Notch signaling in VSMCs promotes contractile differentiation (Baeten et al., 2015; Boscolo et al., 2011; Boucher, Gridley, & Liaw, 2012; Doi et al., 2006; Domenga et al., 2004; High et al., 2008, 2007; Lin & Lilly, 2014b; Liu et al., 2010; Meng, Zhang, Lee, & Wang, 2013; Noseda et al., 2006; Tang et al., 2010; Wang et al., 2012), which fits with our understanding of its role in development of the vessel wall and response to contact-induced signaling with endothelial cells (Baeten et al., 2015; Gaengel, Genove, Armulik, & Betsholtz, 2009; High et al., 2008; Hoglund & Majesky, 2012; Lilly & Kennard, 2009; Lin & Lilly, 2014a, 2014b; Liu et al., 2009; Manderfield et al., 2012; Regan & Majesky, 2009; Wang et al., 2012).

4.2 Proliferation

The proliferation of VSMCs is negatively tied to their differentiation status, so much so that they are often discussed as polar opposites, where mitotically active cells are considered “dedifferentiated” or “synthetic” and differentiated VSMCs are assumed to be quiescent, though there is likely a range of intermediate phenotypes between these extremes (Owens et al., 2004). Since Notch signaling in VSMCs promotes contractile differentiation, one would assume cells with active Notch signaling would not be actively proliferating. However, the initial data suggested the opposite, with several papers showing that Notch signaling promoted proliferation in vitro (Campos et al., 2002; Havrda, Johnson, O’Neill, & Liaw, 2006; Sweeney et al., 2004) and in the context of vascular injury in vivo (Li, Takeshita, et al., 2009; Wang, Campos, et al., 2002). In 2013, findings from Lucy Liaw’s lab demonstrated that the Notch2 receptor specifically inhibited proliferation via cell-cycle arrest and Notch2 was localized in non-proliferating regions of injured vessels, suggesting a receptor-specific regulation of VSMC proliferation (Boucher et al., 2013). Indeed, the previous data demonstrating promotion of proliferation were largely focused on the activity of Notch1 and/or Notch3 (Havrda et al., 2006; Li, Takeshita, et al., 2009; Sweeney et al.,

2004; Wang, Campos, et al., 2002). Delving further into receptor-specific roles, our own lab demonstrated that manipulation of Notch2 and Notch3 had opposite effects on PDGF-B-dependent proliferation, and that PDGF-B treatment decreased expression of Notch2, but increased expression of Notch3 (Baeten & Lilly, 2015). Interestingly, while Notch2 and Notch3 have disparate roles in regulating VSMC proliferation, they both promote contractile differentiation (Baeten et al., 2015). Together these results indicate that regulation of the expression of the different Notch receptors in VSMCs could have a large impact on phenotypic outcome.

4.3 Survival

The role of Notch signaling in VSMC survival and apoptosis has drawn significant attention due to the human disease CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), which is involves the loss of VSMCs and pericytes from cerebral vessels and is caused by mutation of the Notch3 receptor (which will be discussed further in the next section). Notch signaling has been implicated in suppression of apoptosis in a number of cell types (Dror et al., 2007; Jehn, Bielke, Pear, & Osborne, 1999; Meurette et al., 2009; Rosati et al., 2009), especially in many forms of cancer (Dang, 2012). This role as an inhibitor or apoptosis and promoter of cell survival appears to be expressed in VSMCs and pericytes as well (Arboleda-Velasquez et al., 2014; Baeten & Lilly, 2015; Henshall et al., 2015; Li, Takeshita, et al., 2009; Liu et al., 2010; Sweeney et al., 2004; Wang, Campos, et al., 2002; Wang et al., 2014; Wang, Prince, et al., 2002). Interestingly, the Notch receptors responsible for promotion of cell survival in mural cells are also those implicated in the promotion of proliferation, with Notch1 and Notch3 promoting survival, but with no known role for Notch2 (Baeten & Lilly, 2015; Henshall et al., 2015; Li, Takeshita, et al., 2009; Liu et al., 2010; Sweeney et al., 2004; Wang, Campos, et al., 2002; Wang et al., 2014; Wang, Prince, et al., 2002). Direct comparison of the effects of Notch2 and Notch3 manipulation in vitro on VSMC survival revealed that Notch3 promoted survival, but Notch2 had no effect (Baeten & Lilly, 2015). Notch3 activity correlates with MAPK/ERK pathway activation and with survival gene expression, which could be abrogated by inhibition of the MAPK/ERK pathway. This suggests that Notch3 may be promoting VSMC survival through a noncanonical association with the MAPK pathway, which had previously been suggested (Wang, Prince, et al., 2002). Overall, Notch activity promotes survival in VSMCs, but this effect is receptor specific and involves a noncanonical interaction with the MAPK pathway.

4.4 Extracellular Matrix Synthesis

VSMCs and their precursors are predominant contributors to the production of the extracellular matrix (ECM) that comprises the basement membrane and supports tensile strength and elasticity in the vessel (Armulik, Genove, & Betsholtz, 2011; Underwood, Bean, & Whitelock, 1998; Wagenseil & Mecham, 2009). The ability of Notch signaling to promote ECM synthesis has been demonstrated in fibroblasts, where it induces collagen production (Dees et al., 2011; Lilly & Kennard, 2009), and in renal podocytes, where inhibition of Notch signaling reduced expression of collagen IV and laminin, but increased expression of MMP-2 and MMP-9 (Yao, Wang, Zhang, Chi, & Gao, 2015). This relationship is maintained in VSMCs, where coculture studies identified that endothelial-derived Notch

signaling in VSMCs promotes collagen synthesis and other markers of a synthetic phenotype (Lilly & Kennard, 2009; Lin & Lilly, 2014b). This may be a recapitulation of the developmental program in early vessel development, wherein Notch and other synthetic signaling pathways (such as TGFβ) induce ECM synthesis in perivascular cells to produce the framework of the vessel wall.

4.5 Migration

The migration of VSMCs is primarily important in two situations: recruitment of mural cells to the developing vessel and in response to vessel injury (Hellstrom et al., 1999). The main chemotractant for VSMCs is PDGF-B secreted by endothelial cells as well as platelets and macrophages (Hellstrom et al., 1999; Shimokado et al., 1985). Because Notch signaling has been shown to increase expression of the PDGF receptor β (Jin et al., 2008), it would follow that active Notch signaling in VSMCs would increase migration in response to PDGF-B. Indeed, much of the work done on Notch and VSMC migration has shown an induction of migratory ability. Similar to their roles in proliferation, Notch1 and Notch3 were shown to promote VSMC migration in vitro (Sweeney et al., 2004) and in a mouse injury model Notch1 was shown to be important for migration, proliferation, and formation of the neointimal layer (Li, Takeshita, et al., 2009). The Notch-responsive gene HEY2 was also shown to promote proliferation, migration, and neointimal formation (Sakata et al., 2004), and Notch signaling has been shown to regulate expression of MMP-2 and MMP-9, which are important in the matrix remodeling necessary for migration through the vessel wall rich in ECM (Delbosc et al., 2008). Notch signaling has also been shown to regulate cellular adhesions between cells via N-cadherin (Li et al., 2011; Lindner et al., 2001) and to the surrounding matrix via integrins (Scheppke et al., 2012). These results support the idea that Notch signaling is important for recruitment of mural cells to the vessel wall in response to a PDGF-B gradient, and maintaining their position via cellular adhesions.

5. NOTCH SIGNALING IN VASCULAR DISEASE

The various roles of Notch signaling in regulating smooth muscle development and phenotypic modulation make it an obvious player in many vascular diseases associated with VSMCs. These include both inherited genetic diseases and ailments brought on by defective physiological homeostasis (Table 1).

5.1 Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy

Perhaps the most studied Notch-related disease in VSMCs, CADASIL, is caused by mutations in the Notch3 gene through a mechanism we still do not fully understand (Joutel et al., 1996). This disorder presents in the patient as migraines, mood disorders, ischemic attacks, cognitive impairment, and stroke and is marked by lesions within the cerebral and systemic vasculature characterized by accumulation of granular osmiophilic material (GOM) at smooth muscle basement membrane and progressive loss of VSMCs (Chabriat et al., 2009; Joutel et al., 2000, 1997). These GOM deposits appear to be comprised of oligomerized Notch3 ECD, and the mutations found in human patients lead to a gain or loss of a cysteine residue within the EGF-like repeats of the ECD (Joutel et al., 1997). It is not

clear what exactly causes the disease progression of CADASIL, whether it is impaired function of Notch3, aberrant effects of GOM deposition, or a combination of both. Mouse models expressing Notch3 receptors containing disease causing mutations have had mixed results in recreating CADASIL disease states. A knockin model of a CADASIL Notch3 mutation did not develop disease phenotypes or GOM deposits (Lundkvist et al., 2005), but another mouse with ectopically overexpressed mutant Notch3 protein did produce GOM deposits and mirrored many of the CADASIL disease symptoms (Joutel et al., 2010). Notch3-null mutant mice are viable and fertile and do not show any overt symptoms associated with CADASIL (Krebs et al., 2003), though they do exhibit some disruption of the VSMCs in cerebral arteries (Henshall et al., 2015). It has also been shown that hypomorphic mutations of Notch3 in humans do not cause CADASIL (Rutten et al., 2013). These results would seem to suggest that it is the gained aberrant function of GOM deposits that causes CADASIL phenotypes. However, a homozygous-null Notch3 mutation has been shown to produce a recessive early-onset arteriopathy and cavitating leukoencephalopathy very similar to CADASIL without the deposition of GOM (Pippucci et al., 2015), and a mouse model with combined deficiency of Notch1 and Notch3 causes pericyte dysfunction and models some phenotypes of CADASIL (Kofler et al., 2015). While it is still difficult at this point to definitively rule out any of the possible explanations, it seems likely that impaired Notch3 signaling plays at least some role in the CADASIL phenotypes, based on the known function of Notch3 in regulating cell survival in VSMCs and pericytes (Baeten & Lilly, 2015; Liu et al., 2010).

5.2 Patent Ductus Arteriosus

PDA results from the failure of the ductus arteriosus (DA), which diverts blood around the fetal lungs, to close shortly after the first breath and inflation of the lungs (Schneider & Moore, 2006). There are several signaling pathways involved in regulating the timing of this closure, but ultimately, it relies on the VSMCs of the DA to contract and functionally close the vessel. Because of this, defects in contractile proteins or VSMC differentiation often result in PDA (Guo et al., 2007; Huang et al., 2008; Zhu et al., 2006). Since Notch signaling promotes VSMC differentiation, it is not surprising that certain mouse models of Notch deficiency also present with the PDA phenotype (Baeten et al., 2015; Feng et al., 2010; Krebs et al., 2016). This connection is also seen in human disease, where a subset of patients afflicted with Alagille, Adams–Oliver, or Hajdu–Cheney syndromes, all caused by mutation of Notch family members, present with PDA (Battelino, Writzl, Bratanic, Irving, & Novljan, 2016; Li et al., 1997; Stittrich et al., 2014).

5.3 Alagille Syndrome

Alagille syndrome is an autosomal dominant disorder caused by loss of Notch2 or Jagged1 and is characterized by many developmental defects, including those of the liver, heart, skeleton, eye, face, and kidney (Li et al., 1997; McDaniell et al., 2006). While many of these defects are caused by the roles of Notch signaling in other cell types, there are some interesting vascular phenotypes that could be related to dysfunctional Notch signaling in VSMCs. In addition to the PDA previously discussed, Alagille syndrome patients often present with defects of the cardiac outflow tract and great vessels (McElhinney et al., 2002). These defects are similarly modeled in mice with abrogated Notch signaling in the cardiac

neural crest lineage that gives rise to the VSMCs of the outflow tract and great vessels (High et al., 2007).

5.4 Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is characterized by a persistent elevation of pulmonary arterial pressure, pulmonary arterial remodeling, and right ventricular hypertrophy (Crosswhite & Sun, 2014). This pulmonary arterial remodeling presents as increased VSMC proliferation, survival, and expression of contractile proteins such as ACTA2. Notch signaling has been proposed to regulate the pathogenesis of PAH, as elevated Notch3 expression is one of the hallmarks of the disease (Crosswhite & Sun, 2014; Geraci et al., 2001), and this would fit with the proliferation, survival, and differentiation roles that have been shown for Notch3. Two Notch3 mutations were identified in cases of childhood PAH, and in vitro experiments using the mutant proteins demonstrated increased proliferation (Chida et al., 2014). This role for Notch3 in PAH is further confirmed by the discovery that Notch3-null mice do not develop PAH in a hypoxia-induced PAH mouse model and that treatment with the Notch inhibitor DAPT (Li, Zhang, et al., 2009), or with a soluble Jagged1 ligand, which functions as a Notch antagonist, could prevent disease progression (Xiao, Gong, & Wang, 2013).

5.5 Infantile Myofibromatosis

Infantile myofibromatosis is an unusual mesenchymal malignancy that is seen at or near birth and typically spontaneously regresses in early childhood (Hatzidaki et al., 2001). Although the tumors rarely present any significant harm to the patient, they are of interest to us because of their causative mutations: PDGFRβ and Notch3 (Lee, 2013; Martignetti et al., 2013). These mutations are predicted to create constitutively active forms of the proteins (Lee, 2013), which fits our current understanding of the relationship between Notch3 and PDGFRβ in promotion of VSMC proliferation (Baeten & Lilly, 2015; Jin et al., 2008).

5.6 Vascular Injury

In situations of vascular injury, many signaling pathways are activated to repair and remodel the vessel. However, these changes can become pathological if not properly regulated and can lead to neointimal hyperplasia, an aggressive migration, and proliferation of VSMCs into the lumen of the vessel (Wu & Zhang, 2009). Expression of members of the Notch signaling pathway, including Notch1, Notch3, HEY1, HEY2, and JAG1, is regulated in response to vascular injury, with an initial downregulation, followed by upregulation after several days (Gridley, 2010; Li, Takeshita, et al., 2009; Lindner et al., 2001). Several of these members have been specifically shown to regulate migration and proliferation of the neointima in injury models, including Notch1, Notch3, and HEY2 (Li, Takeshita, et al., 2009; Sakata et al., 2004; Wang, Campos, et al., 2002). Overall, the general consensus is that the Notch signaling pathway drives neointimal formation after vascular injury and is therefore a potential pharmacological target in situations of restenosis or other vascular injuries.

6. CONCLUSION

Our understanding of the molecular mechanisms of Notch signaling in VSMCs is constantly expanding, and in the last few years we have made great strides in uncovering some of the unique functions of the different Notch family members. There is still much we do not know, however, both in what roles each Notch component has in VSMC development and disease, and the precise mechanisms that allow for functional divergence of the Notch signal. How do the different receptors enact different functional outcomes while utilizing common cofactors? Work from the Gridley lab recently showed that the NICDs of Notch1 and Notch2 are functionally equivalent in development by creating Notch1/2 chimeric proteins (Liu et al., 2015), despite the known differences in Notch1 and Notch2 discussed in this review, especially in regard to VSMC proliferation. Can these differences be explained by different expression profiles and ligand affinities alone? Or are there noncanonical interactions yet to be discovered? The Kitajewski lab has developed decoys that specifically target DLL/Notch or JAG/Notch ligand–receptor interactions, and demonstrated their different functions in tumor angiogenesis (Kangsamaksin et al., 2015). Further use of these tools in VSMCs will go a long way toward understanding the differing functionalities of Notch ligand–receptor pairs. Additionally, the advent of CRISPR technology will greatly expedite our ability to make compound and chimeric Notch receptor mouse models to pinpoint the precise source of the unique functions of the different Notch receptors. These are some of the most pressing questions for the Notch field to answer in the coming years as we strive to uncover the intricacies of Notch signaling in vascular development and disease.

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ABBREVIATIONS

ACTA2	alpha smooth muscle actin
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
ECD	extracellular domain
ECM	extracellular matrix
EGF	epidermal growth factor-like
GOM	granular osmiophilic material
HDACs	histone deacetylases
HES	hairy/enhancer of split
HRT	hairy-related transcription factors
MAML	mastermind-like

MAPK	mitogen-activated protein kinase
NICD	Notch intracellular domain
NRR	negative regulatory region
PAH	pulmonary arterial hypertension
PDA	patent ductus arteriosus
PDGF-B	platelet-derived growth factor B
PEST	domain rich in proline [P]glutamic acid [E], serine [S], and threonine [T]
RBPJ	recombination signal-binding protein for immunoglobulin kappa J region
TGFβ	transforming growth factor β
VSMCs	vascular smooth muscle cells
Wnt	wingless-related integration site

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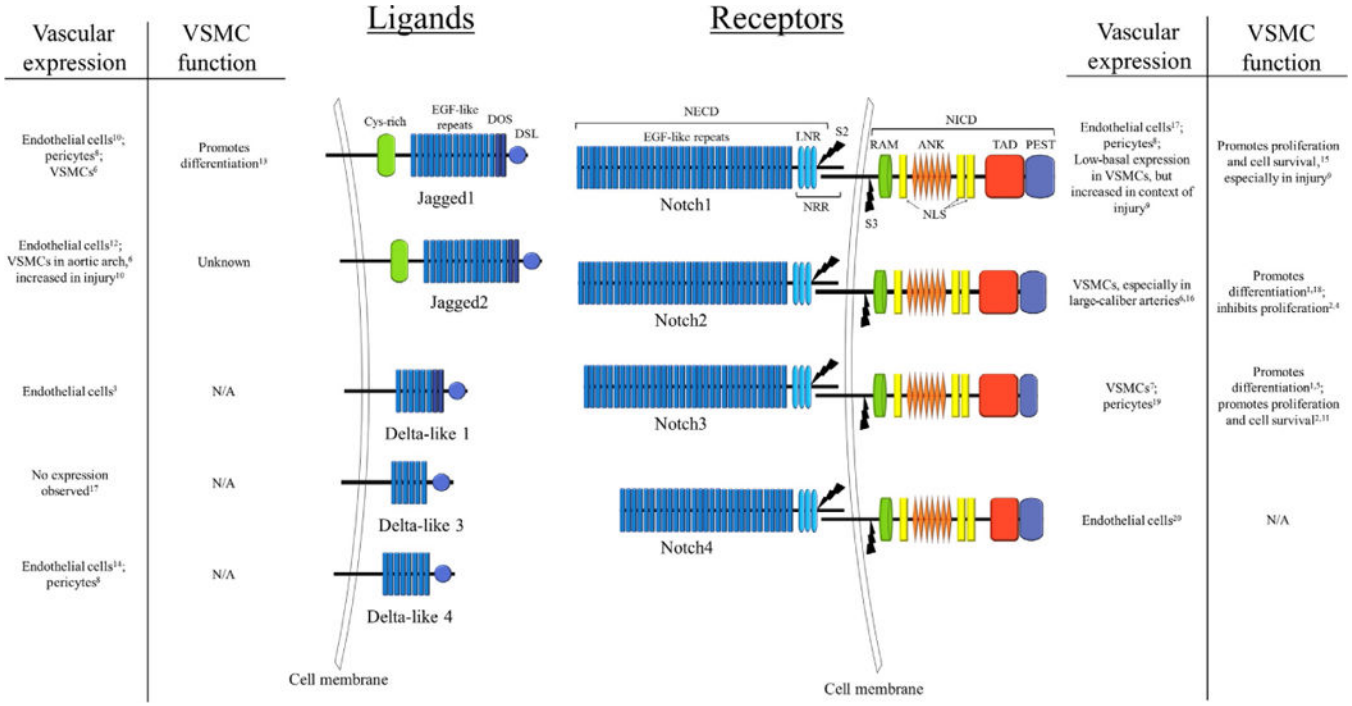

Fig. 1.

Notch family of ligands and receptors. Abbreviations: *ANK*, ankyrin repeats; *Cys-rich*, cysteine-rich region; *DOS*, Delta and OSM11-like proteins motif; *DSL*, Delta/Serrate/Lag2 motif; *LNR*, Lin12-Notch repeats; *NECD*, Notch extracellular domain; *NICD*, Notch intracellular domain; *NLS*, nuclear localization signal; *NRR*, negative regulatory region; *PEST*, proline/glutamic acid/serine/threonine-rich motifs; *RAM*, RBPJassociation module; *S2*, cleavage site 2; *S3*, cleavage site 3; *TAD*, transactivation domain. References: 1, Baeten, Jackson, McHugh, and Lilly (2015); 2, Baeten and Lilly (2015); 3, Beckers, Clark, Wunsch, Hrabe De Angelis, and Gossler (1999); 4, Boucher, Harrington, Rostama, Lindner, and Liaw (2013); 5, Domenga et al. (2004); 6, High et al. (2007); 7, Joutel et al. (2000); 8, Kofler et al. (2015); 9, Li, Takeshita, et al. (2009); 10, Lindner et al. (2001); 11, Liu, Zhang, Kennard, Caldwell, and Lilly (2010); 12, Luo, Aster, Hasserjian, Kuo, and Sklar (1997); 13, Manderfield et al. (2012); 14, Shutter et al. (2000); 15, Sweeney et al. (2004); 16, Varadkar et al. (2008); 17, Villa et al. (2001); 18, Wang, Zhao, Kennard, and Lilly (2012); 19, Wang, Pan, Moens, and Appel (2014); 20, Wu et al. (2005).

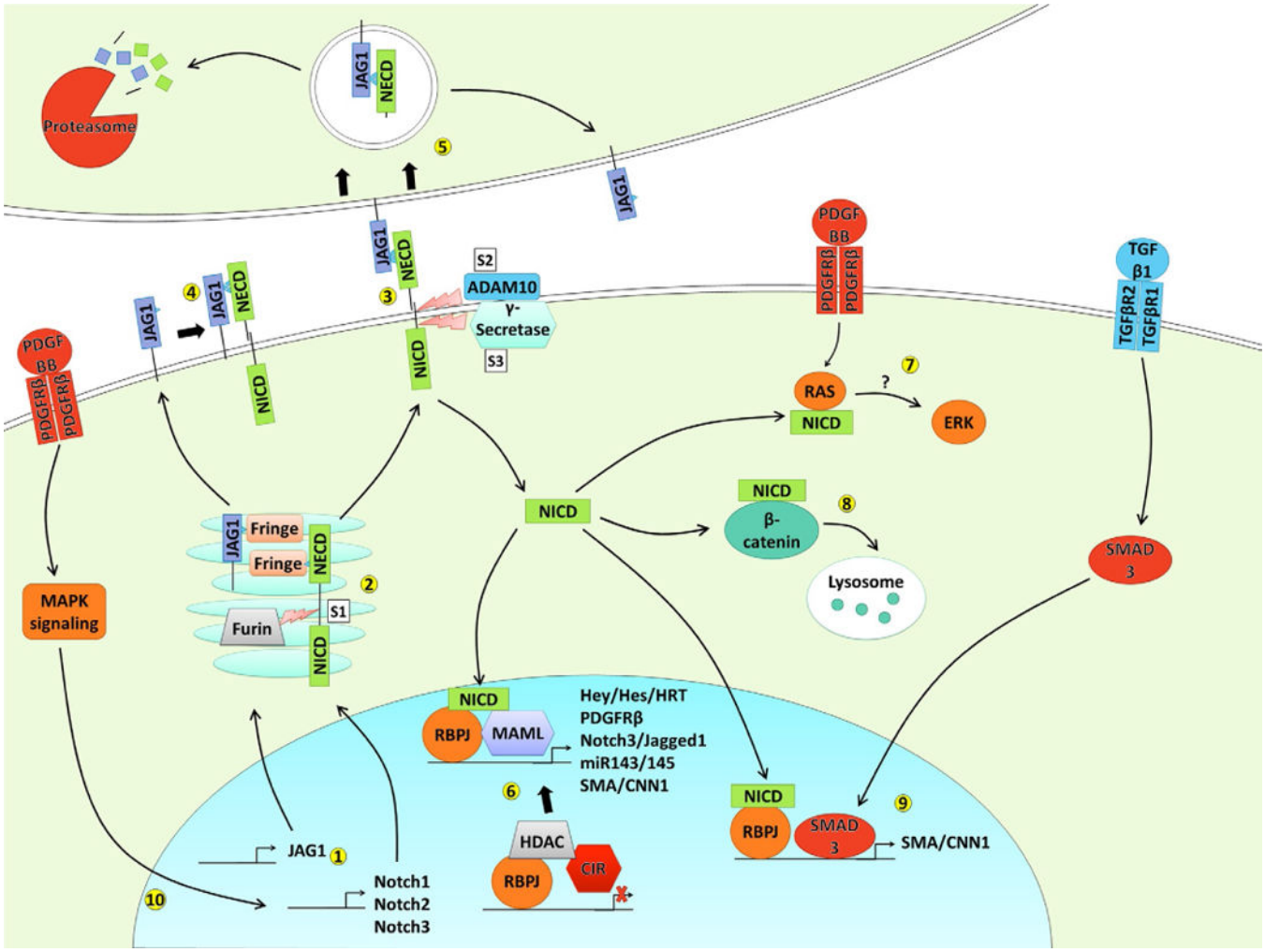

Fig. 2.

Notch signaling pathway. (1) Notch receptor and ligand genes are transcribed and translated into protein. (2) During posttranslational processing in the Golgi, Notch receptors are cleaved by furin to create heterodimers; both ligands and receptors are glycosylated on their EGF-like repeats by Fringe family enzymes. (3) *trans* activation of Notch receptor by Notch ligand from a neighboring cell, triggering cleavage by ADAM10 at S2 and γ-secretase at S3, releasing the NICD. (4) *cis* inhibition of Notch receptor by Notch ligand on the same cell, preventing activation. (5) Endocytosis of Notch ligand with associated NECD can be degraded by the proteasome or recycled back to the cell surface. (6) NICD is shuttled to the nucleus where it binds to RBPJ, displacing HDACs and corepressors such as CIR and recruiting MAML to activate transcription of Notch target genes. (7) NICD can both directly interact with Ras and promote ERK phosphorylation, though the mechanism is not yet known. (8) NICD can bind to the Wnt signaling mediator β-catenin and target it for degradation in the lysosome. (9) Transcription of some genes, including smooth muscle contractile genes, is coregulated by NICD/RBPJ and the TGFβ transcription factor SMAD3.

(10) Many signaling pathways can regulate expression of Notch components, including PDGFRβ signaling via the MAPK pathway.

Table 1

Vascular Diseases Associated with Mutations of Notch Pathway Components

Notch Component	Mutation Site	Notch Signaling Effect	Disease Outcome
Jagged[1]	Identified in every exon and several splice sites	Loss of function	Alagille syndrome[1]
	EGF-like repeat 2	Not known	Tetralogy of Fallot[2]
DLL4	DSL domain, N-terminal domain, and EGF-like repeats	Predicted loss of function	Adams–Oliver syndrome[3]
Notch1	Haploinsufficiency, EGF-like repeat 11, LNR 2, ANK domain 3	Predicted loss of function	Adams–Oliver syndrome[4]
	Haploinsufficiency, EGF-like repeat 29	Predicted loss of function	Bicuspid aortic valve[5]
Notch2	Truncated NICD	Predicted loss of function	Alagille syndrome[6]
	PEST domain	Predicted gain of function	Hajdu–Cheney[7]
Notch3	EGF-like repeats 1–32	Likely loss of function	CADASIL[8–11]
	PEST domain	Predicted gain of function	Lehman[12]
	Heterodimerization domain	Predicted gain of function	Infantile myofibromatosis[13]
	EGF-like repeats 21 and 23	Not known	Childhood PAH[14]
RBPJ	DNA-binding domain	Loss of Function	Adams–Oliver syndrome[15]
EOGT	Domains not defined	Predicted loss of function	Adams–Oliver syndrome[16]

References:

[1] Spinner et al. (2001);

[2] Eldadah et al. (2001);

[3] Meester et al. (2015);

[4] Stittrich et al. (2014);

[5] Garg et al. (2005);

[6] McDaniell et al. (2006);

[7] Simpson et al. (2011);

[8] Chabriat, Joutel, Dichgans, Tournier-Lasserve, and Bousser (2009);

[9] Joutel et al. (2000);

[10] Joutel et al. (1996);

[11] Joutel et al. (1997);

[12] Gripp et al. (2015);

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