

REVIEW

The developmental biology of genetic Notch disorders

Jan Mašek and Emma R. Andersson*

ABSTRACT

Notch signaling regulates a vast array of crucial developmental processes. It is therefore not surprising that mutations in genes encoding Notch receptors or ligands lead to a variety of congenital disorders in humans. For example, loss of function of Notch results in Adams-Oliver syndrome, Alagille syndrome, spondylocostal dysostosis and congenital heart disorders, while Notch gain of function results in Hajdu-Cheney syndrome, serpentine fibula polycystic kidney syndrome, infantile myofibromatosis and lateral meningocele syndrome. Furthermore, structure-abrogating mutations in *NOTCH3* result in CADASIL. Here, we discuss these human congenital disorders in the context of known roles for Notch signaling during development. Drawing on recent analyses by the exome aggregation consortium (EXAC) and on recent studies of Notch signaling in model organisms, we further highlight additional Notch receptors or ligands that are likely to be involved in human genetic diseases.

KEY WORDS: Adams-Oliver syndrome, Alagille, CADASIL, Notch, Development, Genetics

Introduction

Notch signaling arose in metazoans (Gazave et al., 2009; Richards and Degnan, 2009) and is considered one of the core signaling pathways that controls embryonic development. Indeed, from sponges and roundworms to mice, Notch signaling controls multiple crucial processes during development (Andersson et al., 2011). Importantly, since the discovery of a mutant fly with notched wings, earning the gene the name *Notch* (*N*), over 100 years ago (Dexter, 1914), and the subsequent identification of the genomic region responsible (Morgan, 1917), a wealth of studies – ranging from the elucidation of the Notch pathway (reviewed by Bray, 2016; Kopan and Ilagan, 2009), to the generation of knockouts in model organisms and the discovery of Notch genes mutated in humans (Gridley, 2003) – has confirmed an essential role for Notch signaling in human development.

Different species have distinct repertoires of Notch receptors and ligands (Fig. 1 and Gazave et al., 2009). Humans, for example, express four Notch receptors (NOTCH1-NOTCH4) and five different Notch ligands (JAG1 and JAG2, and DLL1, DLL3 and DLL4) (Fig. 2), whereas fruit flies have one receptor (Notch) and two ligands (Delta and Serrate). Pathway activation typically occurs when a membrane-bound heterodimeric single-pass Notch receptor interacts with a Notch ligand on a contacting cell (Fig. 3). This interaction leads to a series of proteolytic cleavages, mediated by

ADAM secretase and the γ -secretase complex, resulting in release of the Notch intracellular domain (NICD), which translocates to the nucleus where it acts together with RBP β k and MAML to activate transcription. The process is further fine-tuned by numerous post-translational modifications of both receptors and ligands, and via co-activators or inhibitors that function at every level of the, deceptively simple, signaling pathway (Andersson et al., 2011; Bray, 2016).

Mutations in components of the Notch family are generally constrained by the pathway's essential developmental functions, although mutations that confer grave congenital disorders and fitness costs have been identified (Table 1). The discovery of congenital diseases related to defective Notch signaling began in 1996, with the linkage analysis-based discovery of mutations on chromosome 19, more specifically *NOTCH3* mutations, in individuals diagnosed with CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) (Joutel et al., 1996). Soon after, two groups showed that *JAG1* was the gene within chromosome 20p12 that was responsible for Alagille syndrome (Li et al., 1997; Oda et al., 1997). Since then, several inherited disorders caused by mutations in Notch genes have been identified (Table 1). Prior to these discoveries, chromosomal rearrangement of human *NOTCH1* and viral integration into murine *Notch4* had been shown to induce T-ALL and mammary tumors, respectively (Ellisen et al., 1991; Uyttendaele et al., 1996), highlighting a key role for Notch in cancer (reviewed by Nowell and Radtke, 2017).

Many of the congenital diseases linked to the Notch pathway are rare, with prevalences of just a few per 100,000, emphasizing just how crucial Notch signaling components are to human survival, but also presenting serious hurdles to studying the impact of these genes in humans. Fortunately, the generation of knockout mice and the study of other animal models have provided researchers with ample information regarding Notch gene function, allowing the role of specific Notch components in human development and disease to be teased apart. In this Review, we describe Notch-driven human congenital diseases in light of our current knowledge regarding Notch gene function in animal models. The Notch pathway has been implicated in the development of most organs, and a comprehensive review of Notch control of embryonic development is not in the scope of this Review. Rather, we focus on the developmental processes underlying the pathologies manifested in Notch-related congenital disorders, and discuss future routes of research to discover which unknown pathologies may be Notch related. We also discuss recent 'big data' analyses of whole-exome and whole-genome sequencing that have revealed the presence or absence of mutations in Notch components in the human population, confirming that specific Notch components are essential to species fitness (Table 2) and presenting exciting future avenues of research.

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Adams-Oliver syndrome: roles for NOTCH1, DLL4 and RBP β k in human development

Prior to the 1940s, children born with underdeveloped upper or lower extremities were defined as having congenital amputations, a

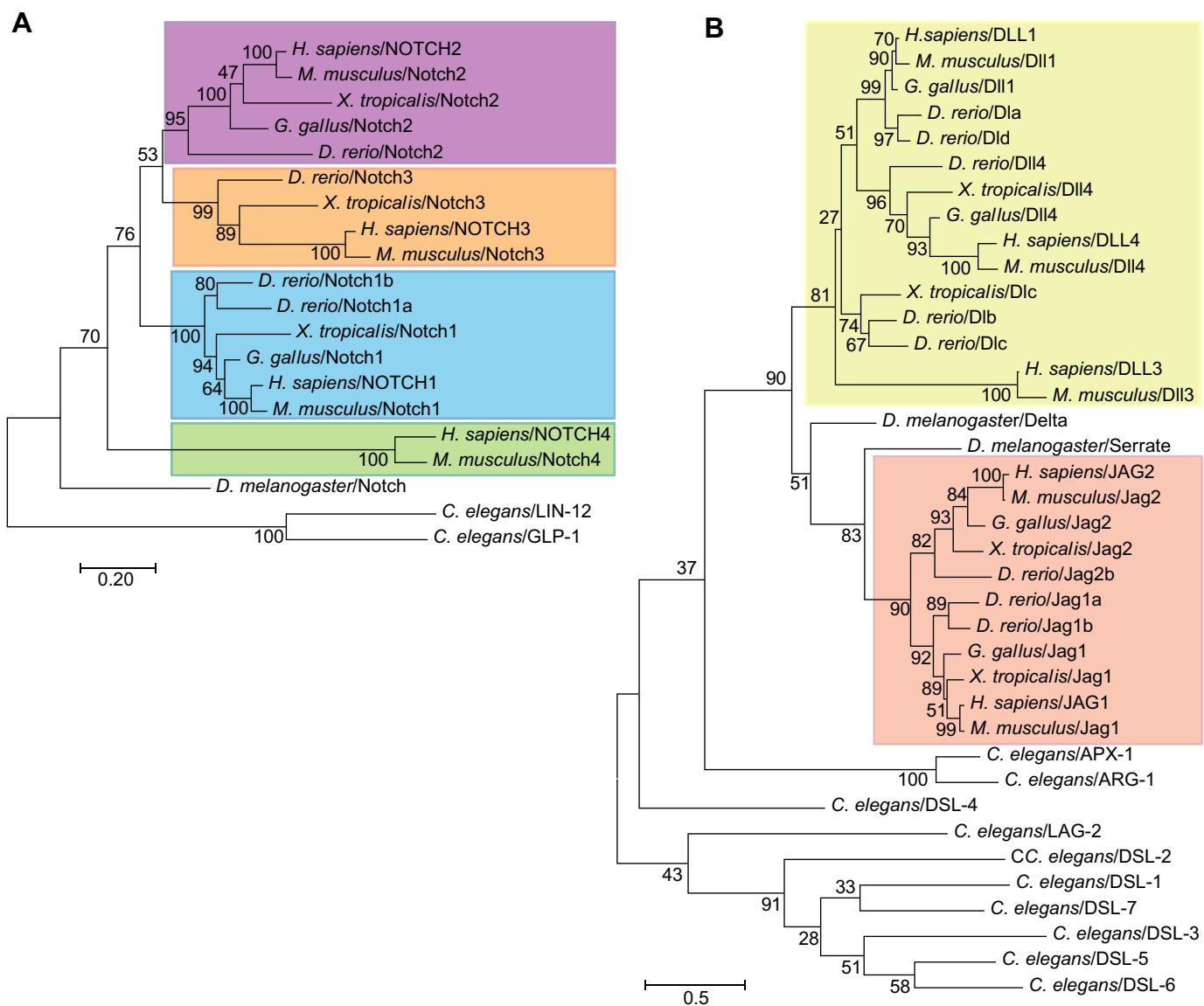


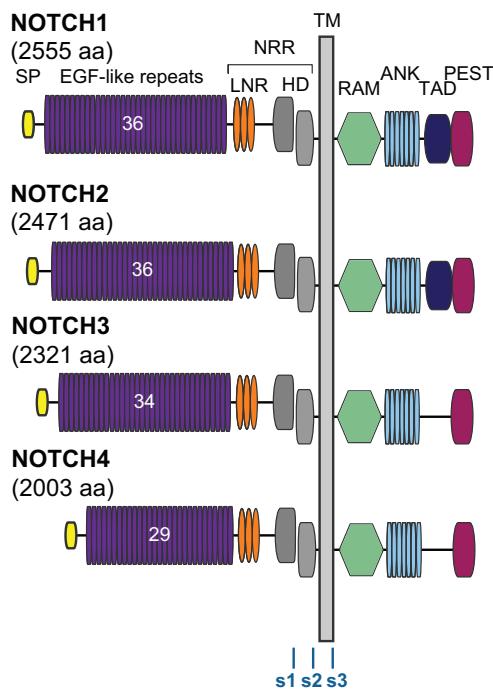
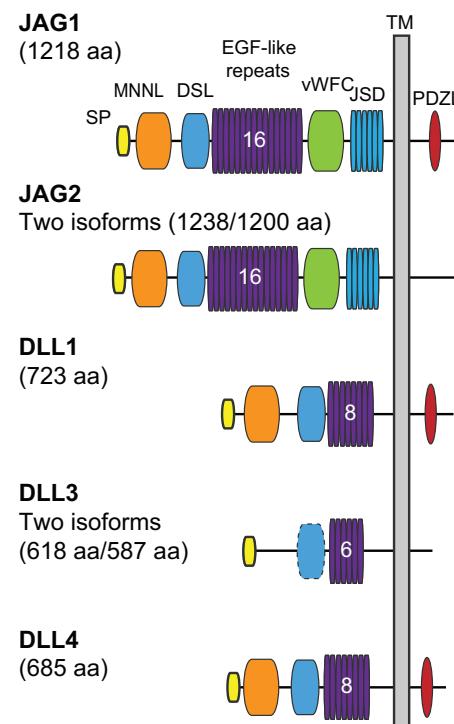
Fig. 1. The evolution of Notch receptors and ligands. Protein sequences of Notch receptors (A) and ligands (B) were aligned using multiple sequence alignment by MAFFT L-INS-I and default parameters (<http://www.genome.jp/tools/mafft/>). The evolutionary history was inferred using the maximum likelihood method based on the JTT matrix-based model (Jones et al., 1992). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 940 positions in the final dataset A and 163 in B. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). Groups of vertebrate receptors and ligands clustering with individual human NOTCH receptors and JAGGED and DLL ligands are enclosed in separate brackets. For accession numbers, please see Table S1.

condition now known as terminal transverse limb deficiencies. These defects were attributed to amniotic band or umbilical cord constriction of the extremities (amniotic band syndrome). In 1945, Clarence Paul Oliver and Forrest H. Adams described a patient with anomalies in the feet and one hand, and also a denuded area of the scalp, with a thinner skull. Most importantly, they showed that multiple family members had similar symptoms, and suggested that the condition was hereditary (Adams and Oliver, 1945). Since then, the diagnosis, genetics and underlying biology of Adams-Oliver syndrome, as it has come to be known, has become more complex.

Characteristics and genetics of Adams-Oliver syndrome

Adams-Oliver syndrome is diagnosed based on terminal transverse limb malformations, an absence of skin (termed aplasia cutis

congenita) and a partial absence of skull bones (Fig. 4A). The skin is most significantly affected in the skull region, though the aplasia cutis congenita may also affect the skin on the abdomen. Typically, by birth, the affected skin region resembles healed but scarred skin, and a skin biopsy reveals absent epidermis, dermal atrophy and a lack of skin structures and elastic fibers. However, symptoms range from a complete absence of skin to patches of skin that lack hair. Similarly, skull symptoms may range from an absence of skull to a near-normal skull (Lehman et al., 1993). In addition, some individuals have vascular anomalies, including dilated surface blood vessels, which give the skin a marbled appearance (cutis marmorata telangiectatica), pulmonary or portal hypertension, and retinal hypervascularization; around 23% have congenital heart defects. It has been suggested that most symptoms of Adams-Oliver

A Notch receptors**B Notch ligands****Fig. 2. The human Notch repertoire.**

Protein domain arrangement of human Notch receptors (A) and ligands (B). Structures are based on InterPro protein domain prediction and other studies (Ehebauer et al., 2005; Lubman et al., 2005). ANK, ankyrin repeats; DLL, Delta-like protein; DSL, Delta/Serrate/LAG-2 domain; EGF, epidermal growth factor; HD, heterodimerization domain; JAG, jagged; JSD, Jagged Serrate domain; LNR, Lin-Notch repeats; MNNL, Notch ligand N-terminal domain; NRR, negative regulatory region; PDZL, PDZ ligand domain [PDZ, post synaptic density protein (PSD95)]; PEST, proline (P), glutamic acid (E), serine (S) and threonine (T) degradation domain; RAM, Rbp-associated molecule domain; s, cleavage site; SP, signal peptide; TAD, transactivation domain; TM, transmembrane domain; vWFC, von Willebrand factor type C domain.

syndrome are due to impaired circulation (Patel et al., 2004; Stitrich et al., 2014; Swartz et al., 1999).

This rare genetic disorder can be autosomal dominant, autosomal recessive or caused by *de novo* mutations. The autosomal recessive forms are caused by mutations in *EOGT*, which encodes a component of the Notch pathway, or in *DOCK6*, which encodes a regulator of Rho GTPase signalling (Lehman et al., 2014; Shaheen et al., 2011, 2013; Sukalo et al., 2015a,b), whereas the dominant forms are caused by mutations in *NOTCH1*, *RBPJ* or *DLL4*, all of which are Notch pathway components, or in *ARHGAP31*, which encodes another Rho GTPase regulator (Hassed et al., 2012; Isrie et al., 2014; Meester et al., 2015; Southgate et al., 2011, 2015; Stitrich et al., 2014). In the case of *DLL4*, it was noted that the disease-associated mutations are distributed throughout the ligand (Meester et al., 2015; Fig. 4B), and so far no distinct pattern of mutation has been identified. However, some genotype-phenotype correlations are beginning to emerge. For example, individuals with *NOTCH1* mutations more often have cardiac defects compared to individuals with *ARHGAP31* or *DOCK6* mutations (Southgate et al., 2015). Together, the mutations discovered thus far account for ~50% of patients, suggesting that more associated genes are likely to be discovered.

It should be noted that *NOTCH1* mutations can also cause an array of isolated cardiovascular defects, including aortic valve defects, hypoplastic left heart syndrome and tetralogy of Fallot (Garg et al., 2005; Kerstjens-Frederikse et al., 2016; McBride et al., 2008; McKellar et al., 2007; Mohamed et al., 2006). Such mutations include missense, nonsense and truncation mutations, implying that heterozygous loss of *NOTCH1* function can lead to either Adams-Oliver syndrome (Fig. 4C) or congenital heart defects. Further supporting the idea that given mutations may cause heart defects or Adams-Oliver syndrome, it has been shown that *ARHGAP31* mutations lead to both Adams-Oliver syndrome (Southgate et al., 2011) and congenital heart defects (Kerstjens-Frederikse et al.,

2016). However, in families with either familial Adams-Oliver syndrome or congenital heart disease, asymptomatic family members bearing the disease-causing mutations have been reported, demonstrating that penetrance is not 100%. This is similar to Alagille syndrome (discussed below), in which family members sharing the same *JAG1* mutations can present with different symptoms, or even be asymptomatic.

Why are *NOTCH1* mutations, and indeed other Notch component mutations, not 100% penetrant? The linear Notch signalling pathway, which does not include signal-amplification steps, is exquisitely dose sensitive. Genetic and environmental factors thus likely shift the intrinsic duration or strength of Notch signalling, altering mutation tolerance in different individuals. Indeed, screens for modifiers of Notch-dependent phenotypes or signalling per se in *Drosophila* (Go and Artavanis-Tsakonas, 1998; Shalaby et al., 2009), mouse (Rubio-Aliaga et al., 2007) and *in vitro* (Mourikis et al., 2010) have revealed many candidate modifiers of Notch-dependent disease. For example, loss of *Itch* (a negative regulator of Notch signalling) in mice interacts with gain of *Notch1* in developing thymocytes to produce autoimmune disease, while loss of one allele of *Dll3* in *Notch1* heterozygous mice results in axial segmentation defects in 30% of double heterozygous mice (Loomes et al., 2007a). This suggests that Notch signalling strength, which can be modified by these interactions, translates into a given output. In support of this, it has been shown that, in the haemogenic endothelium of mice, distinct levels of Notch signal activation in response to Jag1 versus Dll4 create a switch between acquiring a haemogenic versus an arterial endothelial fate (Gama-Norton et al., 2015). It is thus likely that modifications to genes that impact Notch signalling also impact disease presentation.

The biology of Adams-Oliver syndrome: insights from knockout mice
Notch1, *Dll4* and *Rbpj*, or their homologs, are required for the embryonic development of most animal models (Conlon et al.,

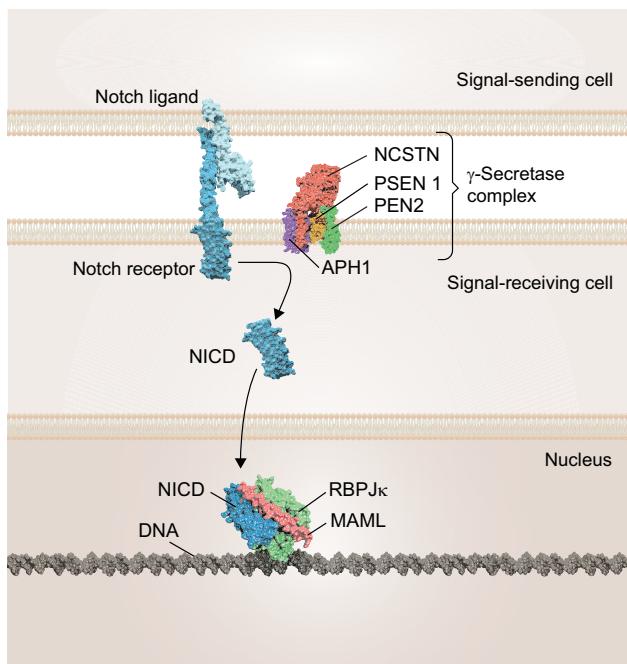


Fig. 3. The core Notch pathway. The canonical Notch signaling pathway is at its core a straightforward signaling mechanism in which a Notch ligand on a signal-sending cell binds to a heterodimeric Notch receptor on a contacting signal-receiving cell. Binding leads to cleavage of the receptor by ADAM secretase (not pictured) and subsequent cleavage by the γ -secretase complex, which is composed of nicastrin (NCSTN), presenillin enhancer 2 (PEN2), presenillin 1 (PSEN1) and anterior pharynx 1 (APH1). Cleavage releases the Notch intracellular domain (NICD), which translocates to the nucleus to activate the transcription of target genes, acting together with recombination signal binding protein for immunoglobulin kappa J region [RBPJ κ , also known as CSL for CBF1/Su(h)/LAG-1] and the co-activator mastermind-like (MAML).

1995; Dexter, 1914; Gale et al., 2004; Oka et al., 1995; Swiatek et al., 1994), and these models have been invaluable for elucidating the mechanisms driving Adams-Oliver syndrome. While *NOTCH1*, *DLL4* and *RBPJ* mutations cause autosomal dominant disease in humans, in mice only *Dll4* mutation results in a severe phenotype in the heterozygous state (Gale et al., 2004), although in this case it is so severe that most heterozygous *Dll4* knockout mice die *in utero*. Similarly, *Dll4* heterozygous-null mice display background-dependent embryonic lethality, with impaired vascular remodeling and embryonic growth retardation (Duarte et al., 2004; Koch et al., 2008; Krebs et al., 2004). *Dll4* knockout mice generated from the few surviving heterozygous mice die by embryonic day (E) 10.5 exhibiting growth delay, smaller hearts and a failure to undergo vascular remodeling (Duarte et al., 2004). These studies, together with various other studies of Notch signaling during vascular development (summarized in Box 1) reveal that dose-sensitive *Dll4*-induced Notch1 signaling is required for arterial development and gross survival.

Notch1 homozygous knockout mice are also embryonic lethal prior to E11 (Conlon et al., 1995; Swiatek et al., 1994), and this is recapitulated in processing-deficient Notch1 embryos (Huppert et al., 2000) and in *Rbpj* $^{-/-}$ embryos (Oka et al., 1995), indicating that canonical Notch1 signaling is required for vascular development and embryonic survival. However, endothelial deletion of *Notch1* using a Tie2-Cre specifically expressed in endothelial cells is far less severe and 50% of these mice survive for at least 8 weeks, albeit with vascular anomalies (Alabi et al., 2016).

This is in stark contrast to an earlier study in which the use of another Tie2-Cre strain (Limbourg et al., 2005) suggested that loss of endothelial *Notch1* was the sole cause of embryonic death seen in null mice; however, it was subsequently noted that the Tie2 promoter used in this strain is also active in the female germ line (de Lange et al., 2008).

Vascular development is clearly abrogated by loss of *Notch1*, and these studies in mice beg the question of whether defective vasculature is the underlying cause of Adams-Oliver syndrome. This hypothesis was suggested before the first causative genes were identified (Swartz et al., 1999), and has more recently been refined to suggest that pericyte dysfunction, in particular, is the main driver of both scalp and skull defects, as well as limb defects in humans (Patel et al., 2004; Stittrich et al., 2014). In further support of this, the transient silencing of Notch signaling (using dominant negative Maml) in vascular smooth muscle cell (vSMC) precursors inhibits their differentiation and leads to hemorrhages in the head and interdigital space (Chang et al., 2012). However, mesenchymal Notch1 and Notch2 (Pan et al., 2005) are activated by Jag2 (Jiang et al., 1998; Sidow et al., 1997) to regulate limb development through Rbpj, indicating that several Notch-regulated processes may act together to control limb development.

Various studies have indicated that Notch1 signaling also has essential roles in skin development (reviewed by Nowell and Radtke, 2013). *Notch1* deletion in murine skin leads to tumors (Demehri et al., 2009; Nicolas et al., 2003) and atopic dermatitis (Dumortier et al., 2010), suggesting that individuals with Adams-Oliver syndrome should be assessed for risk of skin malignancies or skin conditions, as they may be predisposed to developing skin conditions. In addition, considering that neonatal silencing of *Notch1* leads to a reduced thymus and blocked T-cell development (Radtke et al., 1999), it is remarkable that Adams-Oliver patients do not experience thymic insufficiency.

Overall, the phenotypes of mouse models suggest that the various symptoms of Adams-Oliver syndrome reflect the dose-sensitive role of the Notch pathway in the development of the vasculature. Targeting the developing vasculature may prove difficult therapeutically, and current treatment options typically include surgery to close the scalp or skull, or heart surgery. While many current clinical trials focus on inhibition of Notch signaling through small-molecule inhibitors or antibodies (Andersson and Lendahl, 2014), therapeutic activators of Notch signaling have proven more difficult to develop – but may prove beneficial to transiently and precisely boost vascular development.

Alagille and Hajdu-Cheney syndromes: roles for NOTCH2 and JAG1 in development

Alagille syndrome and Hajdu-Cheney syndrome are autosomal dominant, multisystem disorders with an extensive overlap of affected tissues. Alagille syndrome (also known as Alagille-Watson syndrome or arteriohepatic dysplasia) is characterized by defects in the liver, eyes, ears, kidneys, pancreas, heart, vascular system, face and skeleton, as well as by delayed growth (Fig. 5) (Alagille et al., 1975; Watson and Miller, 1973; for a review, see Penton et al., 2012). Individuals with Hajdu-Cheney syndrome, on the other hand, suffer from osteoporosis and progressive focal bone destruction, defective craniofacial development, heart defects, hearing deficits and renal cyst formation (Cheney, 1965; Hajdu and Kauntze, 1948; for a review, see Canalis et al., 2014).

These two syndromes represent two sides of the same coin. Alagille syndrome is caused by haploinsufficiency for *JAG1* (~94%

Table 1. Genetic disorders associated with mutations in Notch receptors or ligands

Disease	Prevalence	Gene	Inheritance	Frequency	Notch effect	Symptoms
Adams-Oliver syndrome	0.44:100,000 (Martínez-Frías et al., 1996)	<i>NOTCH1</i> (Southgate et al., 2015; Stittrich et al., 2014) (OMIM 616028; AOS5)	AD	23%	Loss of function	Underdeveloped skull and absent or scarred skin (<i>aplasia cutis congenita</i>) in head region, terminal transverse limb defects (Adams and Oliver, 1945). Autosomal dominant forms are also caused by mutation of <i>EOGT</i> (AOS4), a gene encoding an O-GlcNAc transferase that post-translationally modifies Notch receptors. Recessive forms are caused by <i>ARGHAP31</i> and <i>DOCK6</i> mutation (Fig. 3).
		<i>RBPJ</i> (Hassed et al., 2012) (OMIM 614814; AOS3)	AD	<10%		
		<i>DLL4</i> (Meester et al., 2015) (OMIM 616589; AOS6)	AD	10%		
Alagille syndrome	1-3:100,000	<i>JAG1</i> (Li et al., 1997; Oda et al., 1997) (OMIM 118450; ALGS1)	AD	94% (Warthen et al., 2006)	Loss of function	Bile duct paucity, heart malformations, characteristic facial features, butterfly vertebrae and posterior embryotoxon (Alagille et al., 1975) (Fig. 4).
		<i>NOTCH2</i> (McDaniell et al., 2006) (OMIM: ALGS2)	AD	2%		
Aortic valve disease	1-2:100 (twice as common in males compared with females)	<i>NOTCH1</i> (Garg et al., 2005) (OMIM 109730; AOVD1)	AD	8-15%	Loss of function	In AOVD, aortic valves have two instead of three leaflets (bicuspid aortic valves). This can be benign or lead to aortic valve stenosis or insufficiency, and in more severe cases can lead to hypoplastic left heart syndrome (Emanuel et al., 1978).
Cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL)	2-4:100,000 (Bianchi et al., 2015; Kalimo et al., 2002; Razvi et al., 2005)	<i>NOTCH3</i> (Joutel et al., 1996) (OMIM 125310; CADASIL1)	AD	100%	NOTCH3 oligomerization, possible neomorph	Defects in small cerebral arteries lead to subcortical infarcts and white matter damage. Vascular dementia in one-third of patients over the age of 60. Males have increased risk of disease progression. Small arteries in all organs are affected, but symptoms are neurological and limited to the brain.
Early-onset arteriopathy and cavitating leukoencephalopathy	One patient described	<i>NOTCH3</i> (Pippucci et al., 2015)	AR	Unknown	Loss of function	The patient had childhood-onset arteriopathy and severe cavitating leukoencephalopathy. Parents were heterozygous carriers (father had some CADASIL-like symptoms).
Hajdu-Cheney syndrome and serpentine fibula polycystic kidney syndrome	Unknown, few patients described	<i>NOTCH2</i> (Isidor et al., 2011a; Majewski et al., 2011; Simpson et al., 2011) (OMIM: 102500; HJCYS)	AD	92% (Isidor et al., 2011a; Majewski et al., 2011; Simpson et al., 2011)	Gain of function	HJCYS: acro-osteolysis (loss of bone tissue) particularly in hands and feet, osteoporosis, craniofacial dysmorphology, kidney defects and tooth loss (Cheney, 1965; Hajdu and Kauntze, 1948).

Continued

Table 1. Continued

Disease	Prevalence	Gene	Inheritance	Frequency	Notch effect	Symptoms
		<i>NOTCH2</i> (Gray et al., 2012; Isidor et al., 2011b; Majewski et al., 2011; Simpson et al., 2011) (SFPKS)	AD	100% (Isidor et al., 2011b; Narumi et al., 2013; Wang et al., 2011) (5/5, all female)		SFPKS: skeletal dysplasia characterized by elongated serpentine fibulae, with polycystic kidneys and dysmorphic facial features.
Infantile myofibromatosis and lateral meningocele syndrome (also known as Lehman syndrome)	0.25–0.67:100,000	<i>NOTCH3</i> (Martignetti et al., 2013) (OMIM: IMF2)	AD	11% [1/9 (Cheung et al., 2013; Martignetti et al., 2013)]	Gain of function	Mesenchyme proliferation defects leading to benign tumors in skin, muscle and bone. Can also lead to tumors in internal organs, then with poor prognosis (mortality rate >70%). Rarely hereditary, usually spontaneous. When genetic it is, more often associated with a <i>PDGFRB</i> mutation (Martignetti et al., 2013).
	Unknown, few patients described	<i>NOTCH3</i> (Gripp et al., 2015) (OMIM 130720; LMNS)	<i>De novo</i>	100% (6/6, all male)		Distinctive facial features: elongated skull, widely spaced eyes, drooping eyelids, jaw misalignment (micro-retrognathia), high-arched palate, long flat vertical groove between the base of the nose and the edge of the upper lip, and low-set ears. Hyperextensibility, hypotonia and protrusions of the arachnoid and dura through spinal foramina (characteristic lateral meningoceles).
Spondylocostal dysostosis (also known as Jarcho-Levin syndrome, JLS)	Unknown, few patients described	<i>DLL3</i> (Bulman et al., 2000) (OMIM 277300; SCDO1)	AR	70% (Turnpenny et al., 2013)	Loss of function	Axial skeletal disorders, with vertebral segmentation defect, shortened thorax and rib misalignment. Can result in decreased numbers of ribs and variable intercostal fusion (Rimoin et al., 1968). Can also be caused by mutations in <i>HES7</i> (SCDO4), <i>LFNG</i> (SCDO3), <i>MESP2</i> (SCDO2), <i>TBX6</i> (SCDO5) and <i>RIPPLY2</i> (SCDO6). JLS also includes spondylothoracic dyostosis.

AD, autosomal dominant; AR, autosomal recessive; ARHGAP31, Rho GTPase activating protein 31; DLL, Delta-like; DOCK6, dedicator of cytokinesis 6; EOGT, EGF domain-specific O-linked N-acetylglucosamine transferase; HES7, Hes family bHLH transcription factor 7; JAG1, jagged 1; LFNG, lunatic fringe homolog (O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase); MESP2, mesoderm posterior bHLH transcription factor 2; PDGFRB, platelet derived growth factor receptor β ; RBPJ, recombination signal binding protein for immunoglobulin kappa J region; TBX6, T-box 6; RIPPLY2, rippy transcriptional repressor 2.

of cases) (Li et al., 1997; Oda et al., 1997) or by mutations in *NOTCH2* (~2% of patients) (McDaniell et al., 2006), and is considered to be a Notch loss-of-function phenotype (Fig. 6A). By contrast, Hajdu-Cheney syndrome, which is driven by production of a stabilized *NOTCH2* lacking a functional PEST degradation domain, is caused by gain-of-function mutations in *NOTCH2* (Fig. 6B) (Gray et al., 2012; Han et al., 2015; Isidor et al., 2011a,b; Majewski et al., 2011; Simpson et al., 2011). As we highlight below, the vast number of tissues and organs affected in these syndromes

are likely a reflection of the varied and indispensable roles – as revealed by various *in vitro* studies and knockout studies in animal models – of *Jag1* and *Notch2* in developmental processes.

Notch2 and Jag1 function in the liver

Mammalian liver development is a complex process that is regulated by multiple signaling pathways, including the Notch pathway. Notch signaling is, in particular, tightly linked with the development of bile ducts (reviewed by Gordillo et al., 2015). In

Table 2. Components of the Notch pathway are crucial for human development

Gene	Missense constraint (z)	Loss-of-function constraint (pLI)	CNV (z)
NOTCH1	4.48	1.00	-0.79
NOTCH2	3.78	1.00	nan
NOTCH3	4.79	0.21	0.97
NOTCH4	2.45	0.00	-0.28
JAG1	4.05	1.00	0.62
JAG2	2.63	0.99	-1.54
DLL1	2.23	1.00	0.69
DLL3	2.62	0.00	0
DLL4	3.24	0.98	0.93
PSEN1	1.81	1	1.25
PEN2	1.05	0.54	-0.25
APH1A	2.41	0.13	0.24
APH1B	-0.86	0	0.88
NCSTN	1.39	1	1.48
RBPJK	3.73	1	0.64
MAML1	-0.03	1	0.16
MAML2	-1.32	1	0.34
MAML3	0.77	0.33	0.2

The Exome Aggregation Consortium (EXAC) have aggregated and harmonized sequencing data from 60,706 individuals, and established an online resource allowing investigation of how common or rare mutations in specific genes are (<http://exac.broadinstitute.org/>, version 0.3.1). Loss of function was defined as nonsense mutation, splice acceptors and splice donors. For each gene, EXAC predicts how many mutations are expected in a given gene, and compares this with how many were actually observed in the sampled populations. From this, a constraint metric is calculated (loss-of-function constraint, pLI), where <0.9 (no highlight) is tolerant to loss of function, and values >1 (graded from yellow to red) denote extreme intolerance to loss of function (haploinsufficient genes). Missense and copy number variant (CNV) constraint (z) are greater than 0 if fewer mutations were found than expected, and less than 0 if more mutations were discovered than expected. Two of the four Notch receptors, and four of the five ligands in humans, are extremely intolerant to loss of function. It is noteworthy that NOTCH3 is relatively unconstrained when it comes to loss of function mutations, but highly constrained when it comes to missense mutations, in line with our current understanding of CADASIL. Similarly, γ -secretase components and transcription factor complex components are largely intolerant to loss of function. APH1, anterior pharynx 1; DLL, delta-like; JAG, jagged; MAML, mastermind like; nan, not a number (data insufficient); NCSTN, nicastrin; PEN, presenilin enhancer; PSEN, presenilin; RBPJ, recombination signal binding protein for immunoglobulin kappa J region.

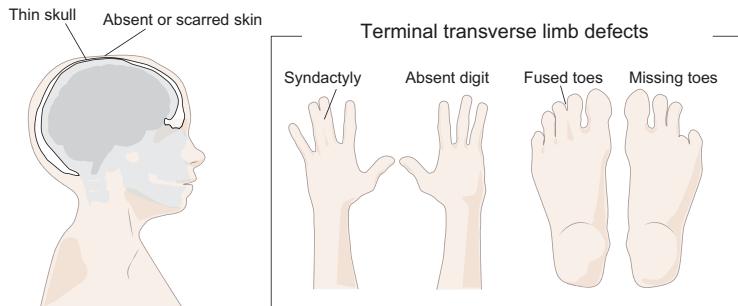
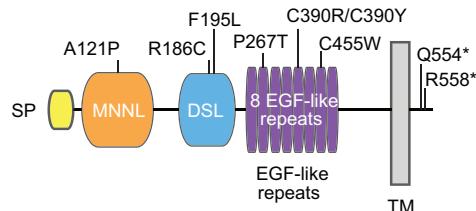
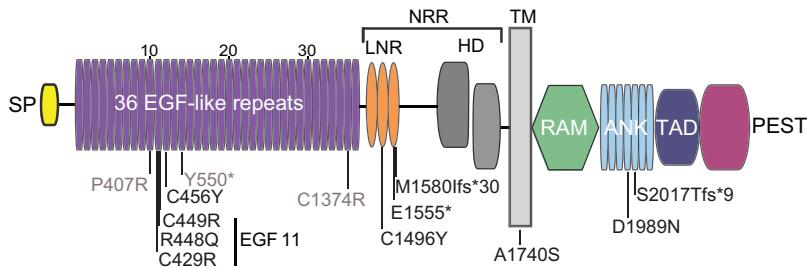
mice, the initiation of Jag1 expression in the portal vein mesenchyme (PVM) marks the onset of bile duct development around E12.5 (Hofmann et al., 2010; Zong et al., 2009). Here, Jag1 interacts with Notch2 in adjacent biliary epithelial cells to induce the expression of Hes1, Hnf1 β and Sox9, which further regulate ductal plate formation and intrahepatic bile duct morphogenesis (Antoniou et al., 2009; Geisler et al., 2008; Kodama et al., 2004; Zong et al., 2009). Jag1 is required specifically in the PVM and not in the portal vein endothelium (Hofmann et al., 2010) nor in biliary epithelial cells (Loomes et al., 2007b), where it is nevertheless also expressed. Compound heterozygous *Jag1* and *Notch2* hypomorphic mice mimic several features of Alagille syndrome, including jaundice, growth retardation, disrupted differentiation of intrahepatic bile ducts, and heart, eye and kidney defects (McCright et al., 2002). Interestingly, similar to other Jag1 phenotypes (Kiernan et al., 2007), the biliary phenotype is highly background dependent, as backcrossing of *Jag1* $^{+/+}$ into a C57BL/6J background results in defects similar to those observed in *Jag1/Notch2* double heterozygotes (Thakurdas et al., 2016). This study also revealed that Jag1 stability is negatively regulated by O-glucosyltransferase 1

(POGLUT1, also known as *Rumi*), and that reduced Rumi levels in *Jag1* $^{+/+}$ /*Rumi* $^{+/+}$ animals rescue the biliary phenotype of *Jag1*-deficient animals. *Notch2* deficiency leads to bile duct agenesis perinatally and secondary bile duct formation after weaning (Felix et al., 2014), a process that appears to be Notch independent (Walter et al., 2014). A similar recovery of the liver phenotype with age has been reported in individuals with Alagille syndrome (Riely et al., 1979), although it is not yet clear which *JAG1* or *NOTCH2* genotypes, if any, are linked to recovery.

Roles for Notch2 and Jag1 in the development of sensory organs

Individuals with Alagille syndrome exhibit inner ear and eye defects, highlighting roles for Jag1 and Notch2 in the development of sensory organs. One of the most easily observed hallmarks of Alagille syndrome – posterior embryotoxon (an irregularity of Schwalbe's line) – is a benign defect that is relatively common in the general population (Emerick et al., 1999; Ozeki et al., 1997). However, it should be noted that posterior embryotoxon is difficult to study in rodents, which instead manifest eye defects, such as iris abnormalities (Xue et al., 1999). *Jag1* and *Notch2* are expressed in the developing lens and ciliary body (CB), and Notch2 is expressed in the retinal pigmented epithelium (RPE) (Le et al., 2009; Saravanamuthu et al., 2012). During development, the Jag1-expressing inner CB interacts with the Notch2-expressing outer CB (derived from RPE) to regulate proliferation and BMP signaling during CB morphogenesis (Zhou et al., 2013). It has also been shown that the ectoderm-specific deletion (using Ap2a-Cre) of *Jag1* results in arrested separation of the lens vesicle from the surface ectoderm and an arrest in lens development (Le et al., 2012). *Notch2* deletion in the lens (via Lens-Cre) also disrupts lens differentiation (Saravanamuthu et al., 2012), although this phenotype is similar to the phenotype of the heterozygous Lens Cre-expressing mouse strain itself (Dorà et al., 2014).

Jag1 and Notch2 also regulate inner ear development. Deafness and impaired balance have been identified in four ethylnitrosourea (ENU)-induced *Jag1* mutant mouse strains: Slalom (Tsai et al., 2001), Headturner (Kiernan et al., 2001), Ozzy (Vrijens et al., 2006) and Nodder (Hansson et al., 2010). These phenotypes are caused when Jag1-dependent Notch signaling fails to define the presumptive sensory epithelium of the ear and maintain an appropriate ratio of proliferation between populations of hair cells and supporting cells, via Hes1-dependent expression of p27^{kip} (Brooker et al., 2006; Kiernan et al., 2006; Murata et al., 2009; Pan et al., 2010). Conversely, expression of Notch1ICD (Notch1 intracellular domain) in the developing otic vesicle causes ectopic formation of sensory and supportive cells in the cochlea and vestibule (Pan et al., 2010), offering a possible explanation for the hearing deficits found in Hajdu-Cheney patients (Isidor et al., 2011a). Sensory organ development shows similar dose-sensitivity to other Notch-regulated processes, wherein a carefully titrated, moderate reduction of Notch signaling activity mediated by the glycosyltransferases lunatic fringe (Lfng) and manic fringe (Mfng) creates a border between the prosensory primordium of the cochlear domain and the Kölliker's organ. This occurs prior to the fate decision of the first differentiating inner hair cells and their associated supporting cells, affirming the sensitivity of this organ to even very mild changes in Notch signaling intensity (Basch et al., 2016). It is also worth noting that truncated posterior semicircular canals and missing ampullae are observed in *Jag1* $^{\text{del}1/+}$ and *Foxg1* $^{\text{Cre}+/+}$; *Jag1* $^{fl/+}$ heterozygous mice (Kiernan et al., 2006), and that the severity of the vestibular phenotype in *Jag1* $^{\text{del}1/+}$ mice depends on genetic background.

A Adams-Oliver syndrome hallmarks**B DLL4 mutations****C NOTCH1 mutations****Notch2 and Jag1 function during kidney development**

Kidney development is tightly regulated by Notch signaling and, although it is not a diagnostic criteria, many individuals with Alagille syndrome suffer from serious kidney problems (Kamath et al., 2013). During kidney development, Notch2 is first expressed in the branched ureteric bud and the surrounding cap mesenchyme, while Jag1 expression arises, together with Notch2 and Notch1 expression, in epithelial vesicles (the aggregates derived from cap mesenchyme via mesenchymal-to-epithelial transition – MET). These vesicles transform through the stages of comma-shaped bodies and S-shaped bodies into fully developed nephrons in which Jag1 is found in the glomerular endothelium, and both Notch1 and Notch2 are found in glomerular epithelial cells (extensively reviewed by Kamath et al., 2013; Kopan et al., 2014). Consistent with these expression patterns, it was shown that mice haploinsufficient for *Notch2* and lacking one allele of *Jag1* exhibit defective glomerulogenesis (McCright et al., 2001), while the cap mesenchyme-specific depletion of Notch2, but not Notch1, blocks the development of podocytes and proximal tubules prior to S-shaped body formation, resulting in early postnatal lethality (Cheng et al., 2007). Intriguingly, both Notch1 and Notch2 are activated by either Jag1 or Dll1 (Liu et al., 2013), so this unequal requirement for Notch1 and Notch2 signaling during renal development is probably caused by differences in their extracellular domains or interaction with the Lfng, or both. Indeed, Lfng enhances Notch2-mediated signaling to a greater extent than Notch1-mediated signaling, and thus may be a key factor in allowing it to reach the threshold required for induction of proximal structure formation (Liu et al., 2013). However this remains to be tested in genetic

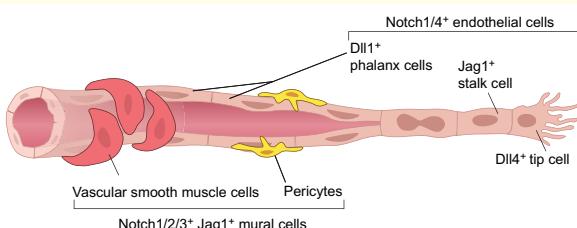
Fig. 4. Adams-Oliver syndrome. (A) The hallmarks of Adams-Oliver syndrome include absent or scarred skin, usually occurring in the scalp region, with an underlying thin skull, and terminal transverse limb defects. Terminal transverse limb defects can resemble amputations, and patients may also have syndactyly. Adams-Oliver syndrome can be caused by mutations in DLL4 (B) or in NOTCH1 (C), as well as in RBPJ, EOGT, ARHGAP31 or DOCK6 (not pictured). DLL4 mutations appear more randomly distributed in the ligand, even including two truncation mutations of the C-terminal domain. NOTCH1 mutations are most often missense mutations in cysteines, especially in EGF repeat 11, in particular in the ligand-binding domain. However, truncation mutations, splice sites and entire deletions are also involved in Adams-Oliver syndrome. Mutations known to have incomplete penetrance are in gray. Asterisks indicate stop; fs is frameshift. NOTCH1 and DLL4 structures are based on InterPro protein domain prediction and other studies (Ehebauer et al., 2005; Lubman et al., 2005). ANK, ankyrin repeats; DSL, Delta/Serrate/LAG-2 domain; EGF, epidermal growth factor; fs, frame shift; HD, heterodimerization domain; LNR, Lin-Notch repeats; MNNL, Notch ligand N-terminal domain; NRR, negative regulatory region; PEST, proline (P), glutamic acid (E), serine (S) and threonine (T) degradation domain; RAM, Rbp-associated molecule domain; SP, signal peptide; TAD, transactivation domain; TM, transmembrane domain.

experiments. It should also be noted that, although MET can occur without Notch signaling (Cheng et al., 2007; Chung et al., 2016), Notch signaling can replace the Wnt/β-catenin pathway during MET (Boyle et al., 2011), and its activity in medial and proximal segments, which is promoted by BMP signaling, is mutually exclusive, with high levels of Wnt/β-catenin signaling (Lindström et al., 2015).

While the studies described above highlight key roles for Notch2 and Jag1 in kidney development, it is not clear how specific human mutations associated with these syndromes lead to kidney defects. For example, no kidney phenotype was described in the Hajdu-Cheney syndrome mouse model harboring the *Notch2*^{Q2319X} mutation (Canalis et al., 2016). Nonetheless, several results illustrate that high levels of Notch signaling negatively impact on kidney development. For example, constitutively active Notch1ICD (Cheng et al., 2007) or Notch2ICD (Fujimura et al., 2010) in the metanephric mesenchyme (Six2-GFP::Cre) drive pathological kidney development. While overexpression of Notch1ICD drives single ureteric bud formation, accompanied by excessive proximal tubule transformation into podocytes and distal tubules (at the expense of mesenchymal progenitor differentiation) (Cheng et al., 2007), an over-abundance of Notch2ICD upregulates *Wnt4* expression at E11.5, causing premature tubule differentiation and depletion of nephron progenitors by E14.5, followed by formation of numerous cysts and general deterioration of the kidney (Fujimura et al., 2010).

Notch2 and Jag1 in the pancreas

Impaired pancreatic function in Alagille syndrome was generally considered common (Rovner et al., 2002), and pancreatitis has also

Box 1. The Notch pathway and vascular development

Notch signaling controls most aspects of vascular development, from vasculogenesis and angiogenesis to arterial identity and mural cell attachment, identity and maintenance. For example, during sprouting angiogenesis, Dll4⁺ endothelial tip cells extend numerous filopodia sprouting forwards while trailing Jag1⁺ stalk cells proliferate to form the vessel trunk. Vascular endothelial growth factor (VEGF) upregulates Dll4 in tip cells, which activate Notch1 on adjacent stalk endothelial cells, downregulating VEGF receptor 2/3 (VEGFR2/3) and suppressing the tip cell phenotype in these trailing cells. Accordingly, Dll4 and Jag1 mutations lead to blood vessel architectural defects, in both mouse models and human disease. Notch signaling is also important in mural cells: Notch3⁺ mural cells, such as vascular smooth muscle cells and pericytes, are recruited to maturing blood vessels in a process that is dependent on endothelial Jag1. In line with this, loss of Jag1 in the endothelium or defective Notch3 in mural cells leads to defective mural cell coverage of arteries and capillaries; in humans this results in, for example, the vascular dementia syndrome CADASIL.

been associated with Alagille syndrome (Devriendt et al., 1996). However, this view was recently revised after a different methodology showed imbalance in pancreatic function in only two out of 42 individuals with Alagille syndrome (Kamath et al., 2012). Nevertheless, it is known that *Notch2* and *Jag1* play essential roles during murine pancreas development, potentially explaining the pancreas defects observed in some patients. Notch signaling controls both the primary (occurring at E8.5–E12.0 of mouse gestation) (Ahnfelt-Rønne et al., 2012; Jensen et al., 2000) and secondary (occurring at E13.0–E16.0) (Murtaugh et al., 2003; Shih et al., 2012) waves of pancreatic progenitor differentiation that give rise to full set of endocrine (α -, β -, δ -, ϵ - and PP-cells), acinar and duct cells (Li et al., 2016; for a review, see Afelik and Jensen, 2013). *Jag1* regulates pancreas development through inhibition of Dll1-Notch signaling during embryonic stages and through activation of Notch signaling during postnatal stages (Golson et al., 2009a). Conditional deletion of pancreatic epithelial *Jag1* (using Pdx1-Cre) leads to defective ductal formation, fibrosis and chronic pancreatitis (Golson et al., 2009b), while conditional compound deletion of *Notch1* and *Notch2* leads to surprisingly mild effects on pancreatic epithelial cell proliferation (Nakhai et al., 2008), although it is possible that the phenotype is rescued by *Notch3* (Apelqvist et al., 1999).

Notch2 and Jag1 function in heart development

Heart development requires concerted induction, proliferation, differentiation, migration and complex morphogenesis events, including tube formation and looping (for a review, see Sedmera, 2011). *Jag1* and *Notch2* are both expressed from early stages of the formation of the heart, and – together with other components of the Notch pathway (summarized in Boxes 2 and 3; for reviews, see D'Amato et al., 2016a; Luxán et al., 2016) – regulate several crucial steps of cardiac development. Although it is still unclear how to link discrete *JAG1* mutations, which have variable effects on *JAG1* trafficking and activity, to the range of cardiac defects

observed in individuals with Alagille syndrome (Bauer et al., 2010), it has been shown that the development of several compartments of the heart is dependent on the balanced activities of *Jag1* and *Notch2*.

Ablation of *Jag1* expression in the endocardium leads to outflow tract (OFT) defects, aortic valve hyperplasia, tetralogy of Fallot and valve calcification, recapitulating the spectrum of cardiac pathologies often present in Alagille syndrome (Hofmann et al., 2012; MacGrogan et al., 2016). These phenotypes are, at least partially, linked to cardiac neural crest (CNC) cells, a highly migratory cell population that originates from the neural plate border (Jiang et al., 2000). CNC-specific deletion (using Pax3-Cre) of either *Jag1* (Manderfield et al., 2012) or *Notch2* (Varadkar et al., 2008) revealed that they are not required for CNC migration per se, but that *Jag1* is a key inducer of CNC-derived vSMC differentiation. *Notch2*-mediated signaling, meanwhile, maintains vSMC proliferation around the aortic arch arteries and OFT (Varadkar et al., 2008). Impaired *Jag1* and *Notch2* signaling also results in ventricular septation defects, aortic arch patterning defects and pulmonary artery stenosis, all of which are conditions present in individuals with Alagille syndrome (Manderfield et al., 2012; Varadkar et al., 2008). Another explanation for the congenital heart disease found in Hajdu-Cheney patients (Crifasi et al., 1997) is provided by the role of *Notch2* in the formation of trabecular myocardium: under physiological conditions, *Notch2* activity must be suppressed by Numb and Numbl to balance the formation of compact versus trabecular myocardium, and its overabundance causes hypertrabeculation, non-compaction and septation defects (Yang et al., 2012). Further studies are required to explain how *Notch2* achieves these roles, when the developing myocardium is devoid of *Notch2* mRNA expression (D'Amato et al., 2016b).

Notch2 and Jag1 function during skeletal development

Notch signaling also plays an important role in developing skeleton and, in line with this, skeletal defects are a shared feature of Alagille and Hajdu-Cheney syndromes (reviewed by Zanotti and Canalis, 2016), as well as other congenital Notch disorders (e.g. spondylocostal dysostosis; see below). The systemic deletion of *Jag1* or *Notch2* did not reveal any somite-related phenotype that would suggest their involvement in the early events of bone formation (Hamada et al., 1999; Xue et al., 1999). However, *Jag1* and *Notch2* in the skeletogenic mesenchyme negatively regulate the differentiation of mesenchymal progenitors into osteoblasts, both *in vitro* and in adolescent mice, and their ablation leads to progressive bone loss in adult mice (Hilton et al., 2008; Nobata et al., 2005; Youngstrom et al., 2016). Importantly, *Jag1* deletion in mesenchymal progenitors causes expansion of the cortical bone, while diminishing trabecular bone mass, suggesting opposing effects of *Jag1* signaling on cortical versus trabecular osteoblasts (Youngstrom et al., 2016). This imbalance leads to spine defects and the formation of butterfly vertebrae, a characteristic feature of Alagille syndrome (Emerick et al., 1999; Youngstrom et al., 2016). Furthermore, both clinical and genome-wide association studies indicate a positive correlation between mutations in *JAG1* and decreased bone mineral density and osteoporotic fractures (Bales et al., 2010; Kung et al., 2010). The formation of craniofacial bone, which arises from intramembranous ossification of neural crest (NC)-derived mesenchyme, also requires *Jag1*: its deletion in NC cells disrupts mesenchymal differentiation and leads to abrogated mineralization and deformities of the craniofacial skeleton, another feature shared by individuals with Alagille and Hajdu-Cheney syndromes (Hill et al., 2014; Humphreys et al., 2012).

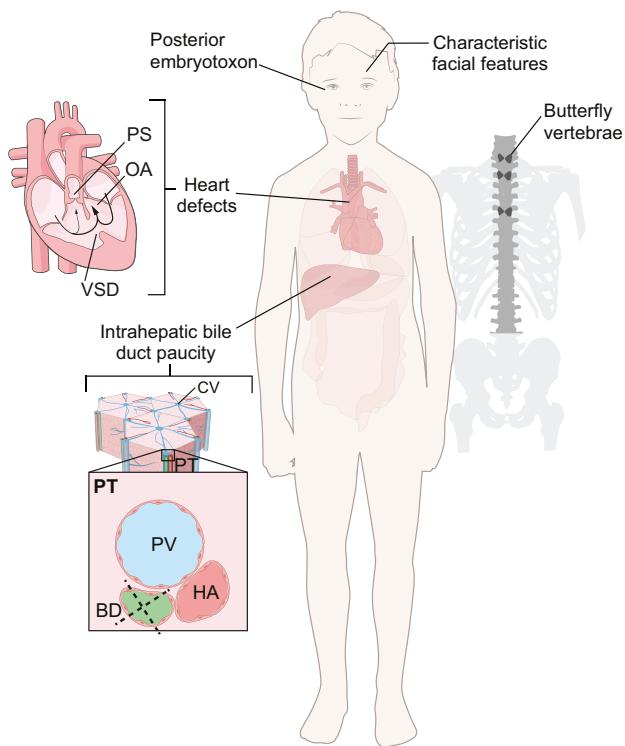
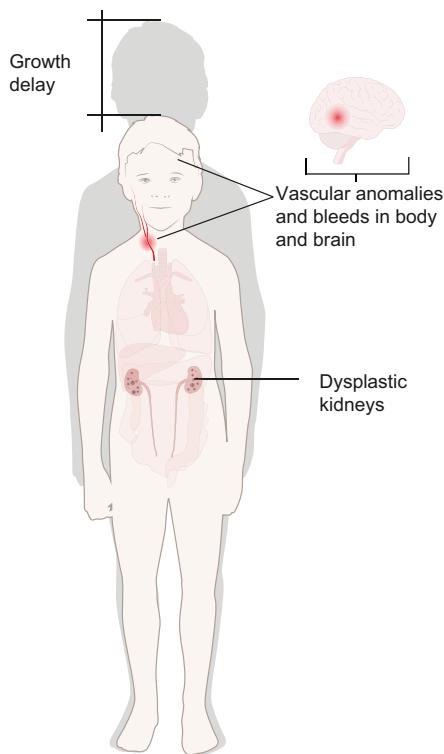
A Alagille syndrome – diagnostic criteria**B Alagille syndrome – associated symptoms**

Fig. 5. Hallmarks of Alagille syndrome. (A) Alagille syndrome is diagnosed based on the presence of five hallmarks of disease: (1) characteristic facial features, including a prominent forehead, pointed chin and deep-set eyes; (2) an eye defect known as posterior embryotoxon; (3) heart defects ranging from pulmonary stenosis to tetralogy of Fallot; (4) vertebral defects, such as butterfly vertebrae; and (5) jaundice/cholestasis due to intrahepatic bile duct paucity. (B) In addition to the diagnostic hallmarks, 50–90% of patients are growth delayed (Alagille et al., 1975; Emerick et al., 1999), 40% of patients experience renal symptoms (Kamath et al., 2013), and 10–25% of patients have vascular structural anomalies and bleeds (Emerick et al., 1999; Kamath et al., 2004). BD, bile duct; CV, central vein; HA, hepatic artery; OA, overriding aorta; PS, pulmonary stenosis; PT, portal triad; PV, portal vein; VSD, ventricular septation defect.

Recently, gain of function *Notch2* mice bearing a Q2319X mutation were shown to exhibit enhanced osteoclastogenesis, resulting in cancellous and cortical bone osteopenia and increased bone resorption (Canalis et al., 2016). This phenotype is strikingly different from the phenotypes observed in odontoblast- and osteocyte-specific Notch1ICD gain-of-function mice (Canalis et al., 2013). This variation might be caused by differences between constitutive and Cre-dependent approaches, different levels of Notch activation, or unknown factors extrinsic to skeletogenic mesenchyme that are responsible for the Hajdu-Cheney syndrome phenotype.

Roles for Notch2 and Jag1 in the vasculature

Components of the Notch pathway regulate several aspects of vascular development, from vascular growth and endothelial tip and stalk cell selection to vSMC development (see Box 1). The systemic knockout of *Jag1* is embryonic lethal in mice at ~E11.5 due to defects in angiogenesis of the embryonic and yolk sac vasculature (Kiernan et al., 2007; Xue et al., 1999). Likewise, homozygous *Notch2* knockout mice die at ~E10.5, displaying widespread apoptosis (Hamada et al., 1999; McCright et al., 2006). The endothelial-specific deletion (via Tie1- or Tie2-Cre) of *Jag1* phenocopies systemic *Jag1* deletion, revealing that a lack of *Jag1* signaling from the vascular endothelium likely causes the differentiation defects, loss of vSMCs and severe disruption of angiogenesis observed in *Jag1* mutants (Benedito et al., 2009; High et al., 2008). A similar loss of vSMCs is observed in embryos with homozygous hypomorphic *Notch2* (McCright et al., 2001; Wang

et al., 2012). More recently, it has been proposed that the perivascular coverage of newly formed vessels by vSMCs and pericytes is facilitated by *Jag1*-induced expression of integrin $\alpha v\beta 3$, which provides binding to a basement membrane-specific von Willebrand factor protein (Schepke et al., 2012). In adulthood, *Jag1* instead acts downstream of *Dll4/Notch1* signaling to promote maturation of vSMCs after injury through *P27kip1*-mediated repression of proliferation (Boucher et al., 2013; Pedrosa et al., 2015).

Jag1 also regulates sprouting during angiogenesis; both gain- and loss-of-function experiments in endothelial cells show that *Jag1* promotes the sprouting of new tip cells during retinal angiogenesis (High et al., 2008; for review, see Benedito and Hellström, 2013). Interestingly, balanced sprouting is achieved by *Dll4*-induced ‘high’ Notch signaling and suppression of sprouting, via inhibition of VEGFR signaling in tip cells, which is antagonized in stalk endothelial cells exhibiting *Jag1*-mediated ‘low’ Notch signaling (Benedito et al., 2009; Pedrosa et al., 2015). Although these various aspects of *Jag1* and *Notch2* signaling have not yet been linked to Alagille or Hajdu-Cheney syndromes, they may contribute to the severity of these conditions, and the likelihood of vascular accidents, including ruptured aneurysms and bleeding (Kamath et al., 2004).

Do primarily vascular defects cause Alagille pathologies?

Several pathologies in Alagille and Hajdu-Cheney syndromes appear to have their roots in defective development of vasculature. As mentioned previously, *Jag1* expressed in the portal vein

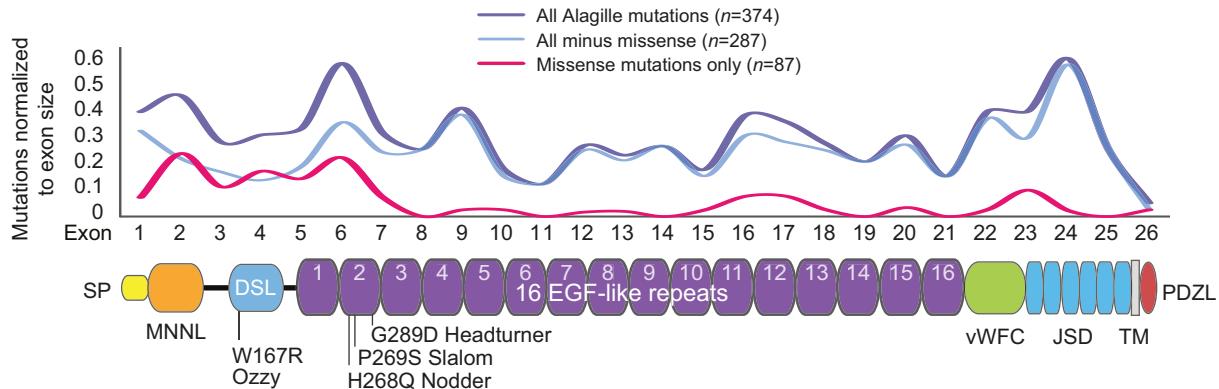
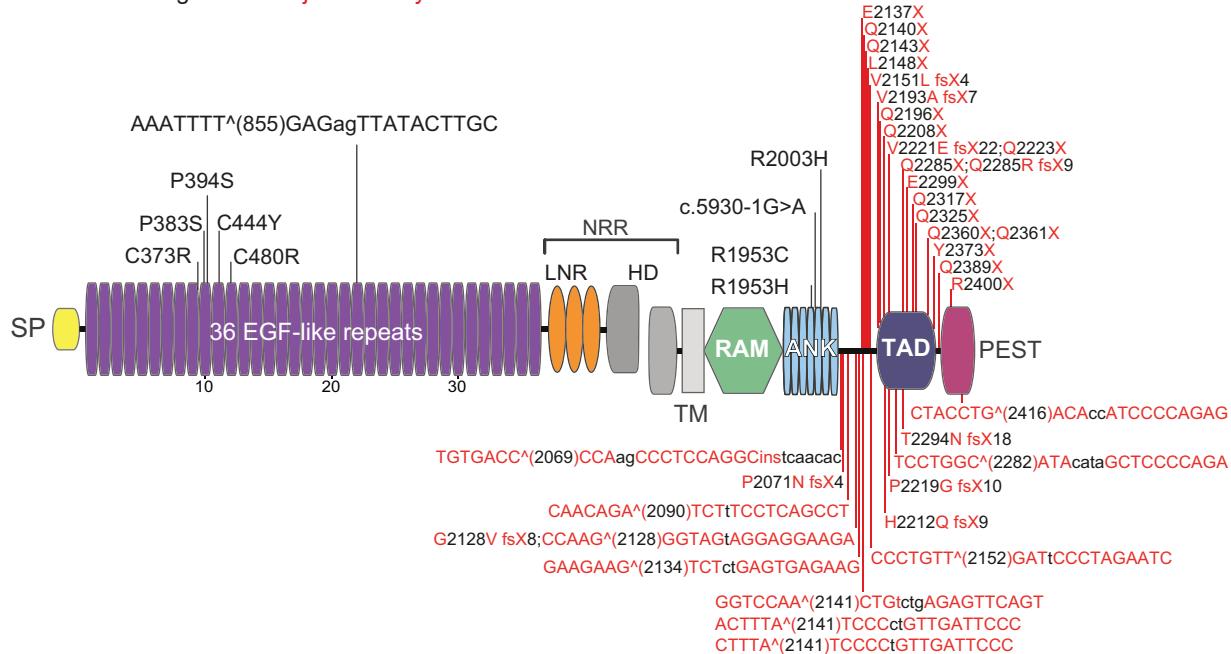
A JAG1 - Alagille mutations**B NOTCH2 - Alagille and Hajdu-Cheney mutations**

Fig. 6. Mutations associated with Alagille syndrome or Hajdu-Cheney syndrome. (A) Mutations in *JAG1* cause Alagille syndrome. Mutations can be deletions, truncations, splice site, nonsense or missense. It was previously thought that mutations could occur anywhere in the CDS for *JAG1*, but analysis of 87 missense mutations reveals that the Alagille-causing deleterious mutations predominantly cluster in the N-terminal region of *JAG1*, with two other smaller sub-clusters: one in EGF repeats 11–12 and one in the von Willebrand Factor type C/Jagged Serrate domain (also known as the cysteine-rich domain). *Jag1* mouse mutants generated in ENU mutagenesis screens, such as Ozzy (Vrijens et al., 2006), Headturner (Kiernan et al., 2001), Slalom (Tsai et al., 2001) and Nodder (Hansson et al., 2010), harbor mutations that cluster in the N-terminal missense-mutation hotspot. (B) Mutations in *NOTCH2* lead to Alagille syndrome (black) or Hajdu-Cheney syndrome (red). Alagille *NOTCH2* mutations generally abrogate cysteines in the ligand-binding EGF repeats, or arginines in the ankyrin repeats, while Hajdu-Cheney *NOTCH2* mutations are generally frameshift or nonsense mutations that lead to absence of the PEST domain and thus gain of function of *NOTCH2* activity (Descartes et al., 2014; Gray et al., 2012; Han et al., 2015; Isidor et al., 2011a,b; Majewski et al., 2011; Narumi et al., 2013; Simpson et al., 2011; Zhao et al., 2013). ANK, ankyrin repeats; DSL, Delta/Serrate/LAG-2 domain; EGF, epidermal growth factor; HD, heterodimerization domain; JSD, Jagged Serrate domain; LNR, Lin-Notch repeats; MNNL, Notch ligand N-terminal domain; NRR, negative regulatory region; PDZL, PDZ ligand domain [PDZ, post synaptic density protein (PSD95)]; PEST, proline (P), glutamic acid (E), serine (S) and threonine (T) degradation domain; RAM, Rbp-associated molecule domain; SP, signal peptide; TAD, transactivation domain; TM, transmembrane domain; vWFC, von Willebrand factor type C domain.

mesenchyme is required for liver bile duct development (Hofmann et al., 2010). Similarly, disruption of Notch signaling in endothelial cells impairs bone vessel formation and the Notch-dependent release of noggin [a bone morphogenetic protein (BMP) antagonist] from endothelial cells, leading to reduced osteogenesis, shortening of long bones, chondrocyte defects, loss of trabeculae and decreased bone mass (Ramasamy et al., 2014). This is especially interesting in light of the capillary defects observed in digits of individuals with Hajdu-Cheney syndrome with acroosteolysis (Damian et al., 2016).

Finally, maxillae and palate sections from mutants with neural-crest specific *Jag1* deletion show decreased PECAM (platelet endothelial cell adhesion molecule) and SMA (smooth muscle actin) staining, again suggesting a contribution of disrupted vasculature to the phenotypes seen in other organ systems (Hill et al., 2014; Humphreys et al., 2012). In summary, *Jag1* and *Notch2* regulate the development of several different organ systems, and their abrogation leads to wide-ranging defects and symptoms in Alagille syndrome and Hajdu-Cheney syndrome.

Box 2. Notch function during heart development

Notch signaling regulates multiple aspects of mammalian heart development, being expressed and acting in various tissue types and compartments (also see Box 3). Loss-of-function mutations in *NOTCH1* (Garg et al., 2005; Theodoris et al., 2015) and the E3 ubiquitin ligase mind bomb1 (*MIB1*) (Luxán et al., 2013) have been implicated in calcific aortic valve disease (CAVD), and left ventricular noncompaction (LVNC) congenital cardiovascular diseases, respectively. Dll4 is a key Notch1 inducer that regulates endothelial-to-mesenchymal transformation (EndoMT) (MacGrogan et al., 2016). As Dll4 expression in the endocardium diminishes with the progression of endocardial cushion formation around E10, Jag1/Notch1 signaling-induced expression of heparin-binding EGF-like growth factor (Hbegf) becomes crucial to limit the BMP-driven proliferation of cardiac valve mesenchyme (MacGrogan et al., 2016). Jag1, together with Jag2, also regulates the maturation and compaction of the ventricular chamber myocardium. At first, Jag1/Jag2-mediated activation of Notch1 is suppressed by Dll4 and Mfng in the endocardium, but later, after E11, Dll4 and Mfng diminish and Jag1/2 can activate Notch1 signaling, inducing proliferation and compaction of the chamber myocardium. Notch signaling also functions in the epicardium, which is a crucial source of cells for coronary vessel formation (for a review, see Perez-Pomares and de la Pompa, 2011). Balanced Notch signaling in/from the epicardium is indispensable for correct heart development (Grieskamp et al., 2011). Accordingly, the ablation of Notch1 negatively affects the formation of coronary vasculature in the compact myocardium, while Notch1ICD overexpression abrogates subepicardial ECM, decreases thickness of compact myocardium, and disrupts the integrity of the epicardium (Del Monte et al., 2011). The exact roles of Jag1 and Notch2, which are also expressed in the epicardium, and Notch3 and Dll4, which are expressed in epicardium-derived vSMCs, remain to be elucidated.

NOTCH3 in development: a cornucopia of congenital disorders

Both autosomal dominant and recessive *NOTCH3* mutations have been described and associated with at least four different disorders: cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), early-onset arteriopathy with cavitating leukodystrophy, lateral meningocele syndrome, and infantile myofibromatosis (Table 1). Among these are mutations affecting the form, but not function, of NOTCH3, nonsense mutations resulting in loss of canonical function, and nonsense mutations resulting in prolonged canonical signaling and gain of function due to loss of the degradation domain. These observations, together with studies of animal models, paint a vivid picture of Notch3 function in development.

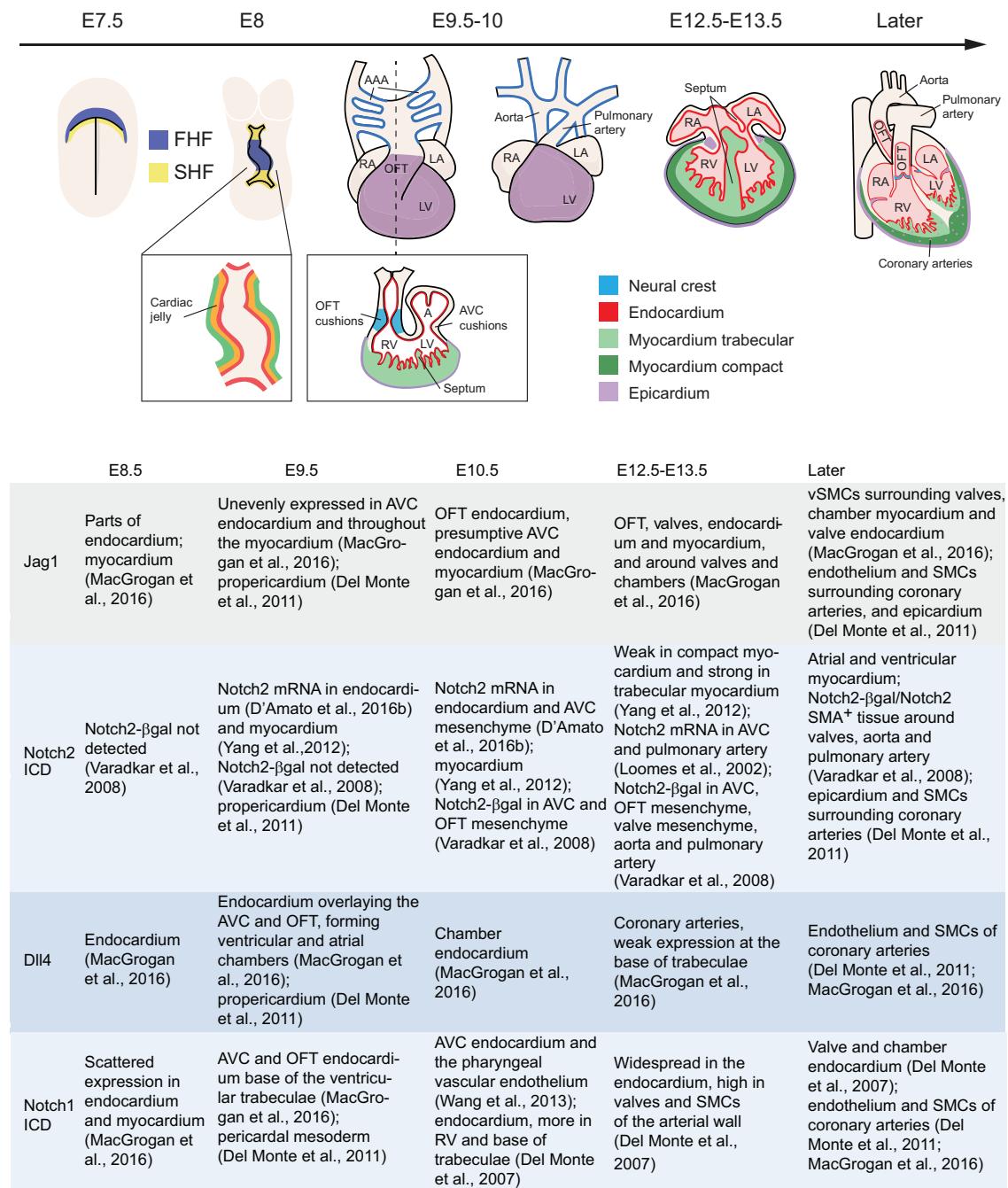
CADASIL, which is caused by heterozygous mutations in *NOTCH3*, is an autosomal dominant hereditary stroke disorder resulting in vascular dementia (Fig. 7A,B; Joutel et al., 1996). Patients experience multiple ischemic strokes and magnetic resonance imaging (MRI) reveals white matter lesions. This arteriopathy is due to breakdown of vSMCs – a cell population in which NOTCH3 is highly expressed (Joutel et al., 2000). Although Notch3 regulates vSMC proliferation, maturation and survival (Domenga et al., 2004; Wang et al., 2012), most CADASIL-related NOTCH3 mutations do not abrogate the capacity of the receptor to mediate signaling. Instead, most, if not all, CADASIL-causing NOTCH3 mutations lead to addition or deletion of a cysteine residue in the extracellular EGF repeats (Fig. 7A), resulting in aggregation of the NOTCH3 extracellular domain (ECD) into extracellular deposits of granular osmophilic material (GOM). This mode of pathogenesis suggests that CADASIL-causing NOTCH3 mutations are not loss of function; rather they are likely neomorphic or toxic.

This is further supported by the observation that patients harboring homozygous CADASIL mutations experience similar or only slightly more severe symptoms (Abou Al-Shaar et al., 2016; Liem et al., 2008; Pippucci et al., 2015; Ragno et al., 2013; Soong et al., 2013; Tuominen et al., 2001; Vinciguerra et al., 2014); if the mutations were loss of function, homozygous patients would be expected to suffer more severe consequences. Interestingly, previous reports have described a clustering of CADASIL-related *NOTCH3* mutations in exon 4 (Fig. 7B; Joutel et al., 1997), or in exon 11 (Dotti et al., 2005), but the mapping of missense mutations, normalized to exon size (this Review), reveals four ‘hotspots’ for CADASIL mutations, rather than one or two (Fig. 7A).

A single patient presenting with early-onset arteriopathy and *NOTCH3* loss of function has also been described. This patient was originally diagnosed with Sneddon syndrome at age 11 (Parmeggiani et al., 2000), but a worsening of neurological symptoms, with cavitating leukoencephalopathy, multiple lacunar infarctions and disseminated microbleeds, prompted re-examination of the diagnosis and revealed a homozygous nonsense mutation in EGF25 of *NOTCH3* (Fig. 7C; Pippucci et al., 2015). Both parents were asymptomatic, but had small vessel ischemic changes as revealed by brain MRI. Importantly, the patient did not harbor GOM. Thus, although most CADASIL-associated *NOTCH3* mutations do not abrogate Notch3 signaling (Joutel, 2011), it is possible that a spectrum of vascular diseases are caused by *NOTCH3* mutations with variable penetrance, depending on whether the mutation induces GOM and/or negatively affects Notch3 signaling.

By contrast, *NOTCH3* gain of function – mediated via loss of the PEST degradation domain or destabilization of the heterodimerization domain – is associated with lateral meningocele syndrome (Ejaz et al., 2016; Gripp et al., 2015) and infantile myofibromatosis (Lee, 2013), respectively (Fig. 7D,E). Lateral meningocele syndrome is a rare skeletal disorder, also known as Lehman syndrome, characterized by protrusions of the arachnoid and dura through the spinal foramina, characteristic facial features, hypotonia, and skeletal and urogenital anomalies (Lehman et al., 1977). Patients may also have bicuspid aortic valves and ventricular septation defects. Intriguingly, the skeletal defects are somewhat similar to those seen in Hajdu-Cheney syndrome, serpentine fibula polycystic kidney syndrome and spondylocostal dysostosis with scoliosis, vertebral fusion or scalloping, and wormian bones, suggesting that NOTCH2 and NOTCH3 gain of function result in similar defects. Infantile myofibromatosis is a very different disorder, characterized by the growth of benign tumors in skin, bone, muscle and soft tissue (Purdy Stout, 1954). Sometimes, but more rarely, internal organs are also affected. Only one patient with infantile myofibromatosis and a *NOTCH3* mutation has been described thus far (Lee, 2013). Although both loss of the PEST degradation domain and destabilization of the heterodimerization domain are thought to lead to NOTCH3 gain of function, the presentation of these two diseases is dramatically different, suggesting signaling from these two mutation variants are not equivalent.

A number of studies in mice have attempted to further tease apart the mechanisms underlying the above NOTCH3-related pathologies. For example, it has been shown that *Notch3*-knockout mice are viable and fertile (Krebs et al., 2003) but exhibit defective patterning of the circle of Willis, arterial differentiation defects and vSMC loss and vascular leakage (Domenga et al., 2004; Fouillade et al., 2012; Henshall et al., 2015; for a review, see Joutel, 2015). Importantly, cerebral blood flow regulation is compromised in *Notch3* loss of function mice, resulting in challenge-induced ischemic stroke (Belin De Chantemèle et al., 2008; Domenga et al., 2004). *Notch3* function

Box 3. The expression of Notch pathway components during heart development

Several key Notch pathway components, including Jag1, Dll4, Notch1 and Notch2, are expressed during heart development, as summarized. AAA, aortic arch arteries; A, atrium; AVC, atrioventricular canal; β -gal, β -galactosidase; FHF, first heart field; ICD, intracellular domain; LA, left atrium; LV, left ventricle; OFT, outflow tract; SHF, second heart field; SMA, smooth muscle actin; RA, right atrium; RV, right ventricle; vSMC, vascular smooth muscle cell.

is thus required for arterial and vSMC development. Furthermore, it should be noted that although most CADASIL-related *NOTCH3* mutations do not negatively affect signaling (Joutel, 2011), and some can even rescue the Notch3 loss of function phenotype (Monet et al., 2007), some do abrogate signaling capacity (Peters et al., 2004). Further complicating matters, the archetypal R169C mutation was recently shown to increase Notch3 signaling (Baron-Menguy et al.,

2017). Thus, it would appear that several mechanisms contribute to vascular damage in CADASIL. The NOTCH3ECD cascade hypothesis (Monet-Leprêtre et al., 2013) suggests that NOTCH3-induced GOM scavenges extracellular matrix proteins, contributing to toxic effects. This hypothesis is also supported by animal models in which the strongest CADASIL phenotype is seen in mice expressing the highest levels of mutated Notch3 (Rutten et al., 2015).

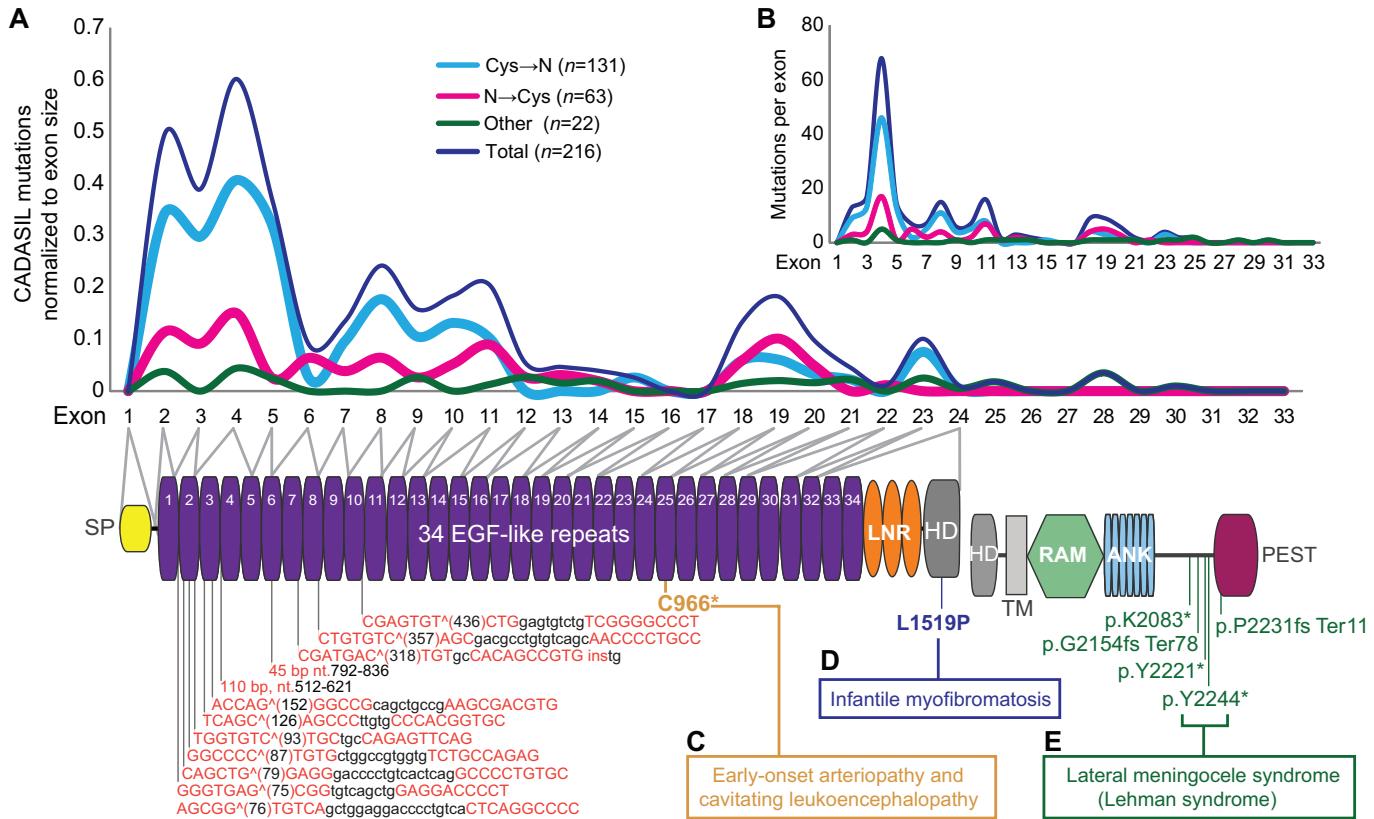


Fig. 7. NOTCH3 mutations are associated with four genetic disorders. (A,B) The most common CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and lesions)-causing mutations in NOTCH3 lead to missense mutations of cysteines, followed by missense of mutations of amino acids into cysteines, resulting in an uneven and unpaired number of extracellular cysteines. (B) When missense mutations per exon are normalized to exon size, it is clear that missense mutations are enriched in EGF repeats 1–6, but also somewhat in EGF repeats 9–15. Calculating mutations per exon without taking exon size into account shows that most mutations (in absolute numbers) occur in exon 4, which encodes EGF repeats 3–5. (C) One patient with early onset arteriopathy and cavitating leukoencephalopathy has been described, who had a homozygous C966* truncation mutation in NOTCH3. (D) One patient with infantile myofibromatosis was found to be heterozygous for an L1519P mutation in the HD domain-encoding region of NOTCH3, which was predicted to result in NOTCH3 gain of function. (E) Six patients with lateral meningocele syndrome were found to have NOTCH3 truncating mutations, resulting in deletion of the PEST domain and stabilization of the NOTCH3 intracellular domain. ANK, ankyrin repeats; EGF, epidermal growth factor; HD, heterodimerization domain; LNR, Lin-Notch repeats; PEST, proline (P), glutamic acid (E), serine (S) and threonine (T) degradation domain; RAM, Rbp-associated molecule domain; SP, signal peptide; TM, transmembrane domain.

The pathology observed in the individual with cavitating leukodystrophy does not appear to be modeled by any of the *Notch3* loss-of-function mice generated thus far, and further studies are clearly needed to understand how *Notch3* loss of function might lead to such early-onset neurological symptoms. Finally, while lateral meningocele syndrome and infantile myofibromatosis have not yet been modeled in mice, a recent ENU screen generated a *Notch3* mutant mouse nicknamed Humpback, which presents with muscle phenotypes and a severely curved spine (<http://www.informatics.jax.org/reference/J:157222>). These mice, in which a *Notch3* splice-donor site in intron 31 is abrogated (Fairfield et al., 2011), may thus be of interest for studying *Notch3* gain-of-function developmental disorders.

Spondylocostal dysostosis: DLL3 in development and disease

Spondylocostal dysostosis is a skeletal disorder characterized by misshapen and fused vertebrae and ribs, a short trunk and scoliosis (Rimoin et al., 1968). Owing to the reduced size of the thorax, breathing capacity may be compromised. Several mutated genes have been identified in patients, but the most frequently found is *DLL3* (Bulman et al., 2000), which encodes an atypical Notch ligand thought to be a negative regulator of Notch signaling (Ladi et al., 2005).

During development, the vertebrae and ribs arise from the central and medioventral part of somites – transient tissue structures generated in a paired and sequential manner, tightly regulated by Wnt, Notch and Fgf signaling (Pourquié, 2011). In line with this, ‘Pudgy’ mice, which harbor a *Dll3* mutation resulting in truncation, display defects in somite formation during embryogenesis and recapitulate spondylocostal dysostosis (Kusumi et al., 1998). It was further shown that the loss of *Dll3* in mice, or deltaD in zebrafish, differentially affects the cycling and stage-specific expression of genes during somite formation (Kusumi et al., 2004; Lewis et al., 2000). Importantly, Notch gene mutations with low penetrance interact with embryonic hypoxia, resulting in more severe phenotypes and high penetrance, suggesting a gene-environment interaction in this disorder (Sparrow et al., 2012). Together, data from the zebrafish, the Pudgy mouse and traditional gene targeting approaches have shown that the function of *Dll3* is to regulate the oscillatory clock during somite segmentation (Dunwoodie et al., 2002; Sewell et al., 2009), thereby explaining the defects seen in spondylocostal dysostosis.

Insights from ‘big data’ analyses

With the advent of whole-genome sequencing, and as this technology becomes more affordable, sequencing efforts across the globe are yielding crucial information regarding the role of genetics in human

diseases, ranging from autism spectrum disorders and schizophrenia to heart disease (McPherson and Tybjaerg-Hansen, 2016; Mitchell, 2011). Recently, the Exome Aggregation Consortium (EXAC) has pooled data and resources to generate the largest database of human sequencing data, including 60,706 individuals (Lek et al., 2016). Importantly, this large dataset has allowed in-depth analyses of which genes or gene sets are mutated in the human population at expected rates, and which are constrained by their essential functions such that they are rarely found mutated in the population. As an example, the most highly constrained genes encode factors involved in core biological processes, including components of the spliceosome, ribosome and proteasome (Lek et al., 2016).

As we briefly highlight below, this valuable resource (<http://exac.broadinstitute.org/>) now allows researchers to query which genes are mutated in the general population and which are constrained by their essential function (Table 2), and, based on studies of model organisms, to predict which other congenital disorders might be linked to mutations in Notch pathway components.

Identifying highly constrained genes and predicting Notch-associated diseases

As expected, genes known to be the cause of severe disorders, such as *JAG1* for Alagille syndrome or *DLL4* for Adams-Oliver syndrome, are highly constrained, and loss-of-function mutations in these genes are not found in the general population. However, *NOTCH3* mutations that cause adult-onset dementia in the heterozygous state, or *DLL3* mutations that cause spondylocostal dysostosis, are not so deleterious at the population level and appear to have minor or no impact on reproductive capacity.

Intriguingly, *JAG2* and *DLL1* are not yet linked to any specific disorder in the online Mendelian inheritance in man (OMIM) database, yet EXAC data reveal these genes to be highly constrained: among more than 60,000 sequenced individuals, only five individuals carry loss-of-function alleles of *JAG2* (compared with the expected ~36 individuals), whereas no individuals carry *DLL1* loss of function mutations (compared with ~20 expected), revealing that these genes are highly constrained (Table 2). This is in line with the severe homozygous lethal phenotypes seen for both genes in mice (de Angelis et al., 1997; Jiang et al., 1998; Sidow et al., 1997), although it is unclear why one defective allele is sufficient to confer grave developmental defects in humans, while mice heterozygous for these and other Notch components are relatively normal. A notable exception is the lethality of *Dll4* heterozygous mice (Gale et al., 2004; Krebs et al., 2004), and more recently it has become clear that *Jag1* heterozygous mice model Alagille syndrome to some extent (Thakurdas et al., 2016). It is thus interesting to speculate whether congenital disorders caused by mutations of *JAG2* or *DLL1* are likely to exist in the human population, or whether haploinsufficiency in humans is incompatible with life. Below, based on the phenotypes observed in knockout mice, we discuss this and highlight which symptoms or pathologies could be expected in *JAG2*- or *DLL1*-related human disorders.

Jag2 mutated mice were first observed as spontaneously occurring mutants that were nicknamed *syndactylism* (*sm*) based on soft tissue or bone fusions in the digits on their fore- and hindfeet (Grüneberg, 1956). The *Jag2sm* phenotype is recessive and homozygous lethal in several pups after birth. Interestingly, Hans Grüneberg observed that several phenotypes are dependent on genetic background, which, as mentioned previously, is also the case for *Jag1* (Kiernan et al., 2007). Furthermore, *sm/sm* mice often have twisted or kinked tails, which can be indicative of neural tube

defects, although in this case appears to be related to epidermal hyperplasia. It was also noted that *sm/sm* embryos undergo premature skin keratinization, resulting in wart-like structures, although these features are not detected after birth. The *sm* mutation is a G-to-A missense mutation resulting in a glycine-to-serine substitution (G267S) in the first EGF repeat (Sidow et al., 1997), and appears to be a hypomorphic allele when compared with *Jag2* mutants in which the DSL domain has been knocked out (*Jag2^{ΔDSL}*). *Jag2^{ΔDSL/ΔDSL}* mice are homozygous lethal at birth, owing to severe cleft palate leading to breathing difficulties (Jiang et al., 1998), which is also the likely explanation for the deaths seen in a proportion of *sm/sm* mice. *Jag2^{ΔDSL/ΔDSL}* mice also display the same syndactyly as *sm/sm* mice, as well as thymic defects.

Based on these phenotypes seen in *Jag2* mutant mice, it is tempting to speculate that human disorders involving syndactyly or cleft palate may involve *JAG2* mutations. In fact, mutations in *JAG2* have been associated with cleft palate in a number of studies (De Araujo et al., 2016; Ghazali et al., 2015; Neiswanger et al., 2006; Paranaíba et al., 2013; Scapoli et al., 2008; Vieira et al., 2005), although they account for only around 2% of cases. Indeed, single-nucleotide polymorphisms (SNPs) have been identified in intronic regions of *JAG2* but also in its EGF repeats 8, 9, 10 and 11, where they lead to missense mutations. This localization is somewhat surprising as this region is not considered to have key functions, unlike the DSL domain and the first two EGF repeats, in which the *sm* mutation is localized. A dose-sensitive role for Notch signaling in palate formation is also highlighted by the fact that individuals with lateral meningocele and *NOTCH3* gain-of-function mutations sometimes also exhibit cleft palate (Gripp et al., 2015) or a high arched palate (Ejaz et al., 2016).

In contrast, *Dll1* is expressed and implicated in the development of multiple organs in mice. It is highly expressed in the presomitic mesoderm, condensed somites and myotome (Bettenhausen et al., 1995), where it regulates rostrocaudal somite segmentation (de Angelis et al., 1997). Later in development, *Dll1* is expressed in a range of organs, including the developing kidneys and pancreas, skeletal muscle and smooth muscle of the gut, the brain, vascular endothelial cells, and sensory organs (Beckers et al., 1999). It controls cell fate and epithelial branching in the pancreas (Apelqvist et al., 1999), craniofacial and trunk muscle development (Czajkowski et al., 2014), and marginal zone B-cell development but not T-cell development (Hozumi et al., 2004). *Dll1* is also required for arterial development (Limbourg et al., 2007; Sorensen et al., 2009) and regulates hair cell development in the inner ear (Kiernan et al., 2005). Furthermore, *Dll1* haploinsufficiency leads to defects in metabolism, the immune system and the skeletal system (Rubio-Aliaga et al., 2009). In addition, mice bearing a missense mutation in *Dll1* (*Dll1^{E26G}*) sometimes display an ectopic neural tube, and, like *Dll1* knockout mice (Przemek et al., 2003), display randomized heart looping (Wansleeben et al., 2011). Thus, *Dll1* is crucially required for a great number of developmental processes, suggesting that humans bearing mutations in *DLL1* are likely to suffer grave consequences. Perhaps this is why no loss-of-function mutations are found in the EXAC database; *DLL1* mutation in humans may in fact be incompatible with viability.

Conclusions

In summary, the Notch signaling pathway regulates a vast array of developmental processes that are essential to life. Mutations in genes encoding components of the Notch pathway therefore have detrimental effects, leading to an array of congenital disorders in humans. Considering the fine-tuning of Notch signaling by

auxiliary mechanisms, such as glycosylation, fucosylation and phosphorylation, to name but a few (Andersson et al., 2011; Bray, 2016), it is likely that a great many genetic modifiers influence the risk for, and dictate the severity of, various disorders. This may also partially explain why both Alagille syndrome and Hajdu-Cheney syndrome present with variable penetrance and an alternating spectrum of symptoms. Furthermore, investigation into the role of Notch in the vasculature, as a driver of several of the pathologies, may open up new avenues for therapeutic options, while ongoing efforts to develop more specific Notch agonists and antagonists offer exciting possibilities for personalized medicine.

Ongoing efforts to map human genetic variation will be essential for an improved understanding of which combinations of Notch components and modifiers regulate human development. It is interesting to note that many Notch mutations in congenital disorders are *de novo* mutations occurring in germ line cells of a parent, meaning that a large proportion of patients will have a genetic disorder that is not manifested in either parent. Such diseases are more challenging to diagnose, and their genetic components are only now, thanks to whole-genome sequencing, being elucidated. Further efforts into understanding the transcriptional control of Notch genes, and the transcriptional machinery of the Notch signaling pathway itself, will be essential for deciphering how non-protein-coding mutations affect gene regulation and impact on disease. Mutations in DNA encoding non-coding RNAs (Makrythanasis and Antonarakis, 2013) and in regulatory elements, including enhancers and insulators, are gaining recognition as causes of Mendelian disorders (Chong et al., 2015; Lowe and Reddy, 2015). Identifying all Notch-associated genetic conditions will require continued improvements in whole-genome sequencing and analysis, standardization of patient phenotyping, and global sharing of genomic and phenotypic data.

Acknowledgements

We thank Mattias Karlen for help with Figs 2A, 3A and 4C,D, and the figures in Boxes 1 and 2. Mutations across some Notch genes were mapped using HGMD (Stenson et al., 2014). The authors also thank the Exome Aggregation Consortium and the groups that provided exome variant data for comparison. A full list of contributing groups can be found at <http://exac.broadinstitute.org/about>. We also acknowledge and thank Chrysoula Pantzartzzi for help with phylogenetic trees.

Competing interests

The authors declare no competing or financial interests.

Funding

Work in the E.R.A.'s lab is supported by the Center for Innovative Medicine (CIMED), by the Karolinska Institutet, by Vetenskapsrådet and by Stockholms Läns Landsting. J.M. is supported by a Wenner-Gren Foundation postdoctoral fellowship.

Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.148007.supplemental>

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Table S1. Accession numbers for sequences used to construct PhylogenogramsNotch Receptors

H.Sapiens/NOTCH1/NP_060087.3
 H.Sapiens/NOTCH2/NP_077719.2
 H.Sapiens/NOTCH3/NP_000426.2
 H.Sapiens/NOTCH4/NP_004548.3
 M.Musculus/Notch1/NP_032740.3
 M.Musculus/Notch2/NP_035058.2
 M.Musculus/Notch3/NP_032742.1
 M.Musculus/Notch4/NP_035059.2
 C.Elegans/LIN-12/NP_499007.1
 C.Elegans/GLP-1/NP_499014.1
 D.Melanogaster/Notch/NP_476859.2
 G.Gallus/Notch1/NP_001025466.1
 G.Gallus/Notch2/NP_001238962.1
 X.Tropicalis/Notch1 ENSXETP00000001676/no accession number for protein (see gene instead)
 X.Tropicalis/Notch2/F7E314_XENTR (Uniprot)
 X.Tropicalis/Notch3/F6XA19_XENTR (Uniprot)
 D.Rerio/Notch1a/A0A0R4IT11_DANRE (uniprot)
 D.Rerio/Notch1b/A0A0R4IMV9_DANRE (Uniprot)
 D.Rerio/Notch2/F1RCH4_DANRE (Uniprot)
 D.Rerio/Notch3/F1QZF2_DANRE (Uniprot)

Notch ligands

H.Sapiens/JAG1/NP_000205.1
 H.Sapiens/JAG2/NP_002217.3
 H.Sapiens/Dll1/NP_005609.3
 H.Sapiens/Dll3/NP_058637.1
 H.Sapiens/Dll4/NP_061947.1
 M.Musculus/Jag1/NP_038850.1
 M.Musculus/Jag2/NP_034718.2
 M.Musculus/Dll1/NP_031891.2
 M.Musculus/Dll3/NP_031892.2
 M.Musculus/Dll4/NP_062327.2
 G.Gallus/Jag1/XP_415035.4
 G.Gallus/Jag2/XP_001235689.2
 G.Gallus/Dll1_NP_990304.1
 G.Gallus/Dll4_XP_421132.2
 D.Melanogaster/Serrate/NP_524527.3
 D.Melanogaster/Delta/NP_477264.1
 C.Elegans/LAG-2/NP_503877.1
 C.Elegans/APX-1/NP_503882.2
 C.Elegans/DSL-1/NP_500054.1
 C.Elegans/ARG-1(encodes Delta-like protein 1)/NP_001024615.1
 D.Rerio/Jag1a/XP_005168261.1
 D.Rerio/Jag1b/NP_571938.2
 D.Rerio/Jag2b/Q5TZK8_DANRE(Uniprot)
 D.Rerio/Dll4/Q5SPB5_DANRE(Uniprot)
 D.Rerio/Dla/AAC41249.1
 D.Rerio/Dld/NP_571030.2

D.Rerio/Dlb/NP_571033.1
D.Rerio/Dlc/NP_571019.1
X.Tropicalis/Jag1/XP_002931784.1
X.Tropicalis/Jag2_ENSXETT00000060633.1/F6R4F7_XENTR (Uniprot)
X.Tropicalis/Dlc_ENSXETG00000002875/F7CZA9_XENTR (Uniprot)
X.Tropicalis/Dll-4_ENSXETT00000046677.2/F6XIJ0_XENTR (Uniprot)
C.Elegans/DSL-2/NP_500052.1
C.Elegans/DSL-3/NP_500108.1
C.Elegans/DSL-4/NP_510346.2
C.Elegans/DSL-5/NP_502191.1
C.Elegans/DSL-6/NP_502148.2
C.Elegans/DSL-7/NP_001023803.1

Sequences are from NCBI unless otherwise specified.