Engineering xxx (xxxx) xxx

Contents lists available at ScienceDirect

Engineering

journal homepage: www.elsevier.com/locate/eng

Research
Natural Medicine—Review

The Notch Signaling Pathway: Mechanistic Insights in Health and Disease

Yao Meng [a,c], Zhihan Bo [b], Xinyi Feng [b], Xinyi Yang [a,c,*], Penny A. Handford [b,*]

[a] Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China
[b] Department of Biochemistry, University of Oxford, Oxford OX1 3QU, UK
[c] State Key Laboratory of Respiratory Health and Multimorbidity, Beijing 100050, China

ARTICLE INFO

Article history:
Received 30 June 2023
Revised 9 October 2023
Accepted 16 November 2023
Available online xxxx

Keywords:
Notch signaling pathway
Structural biology
Glycosylation
Genetic disorders
Cancer
Pharmacological agents

ABSTRACT

The Notch signaling pathway is evolutionarily conserved across metazoan species and plays key roles in many physiological processes. The Notch receptor is activated by two families of canonical ligands (Delta-like and Serrate/Jagged) where both ligands and receptors are single-pass transmembrane proteins usually with large extracellular domains, relative to their intracellular portions. Upon interaction of the core binding regions, presented on opposing cell surfaces, formation of the receptor/ligand complex initiates force-mediated proteolysis, ultimately releasing the transcriptionally-active Notch intracellular domain. This review focuses on structural features of the extracellular receptor/ligand complex, the role of post-translational modifications in tuning this complex, the contribution of the cell membrane to ligand function, and insights from acquired and genetic diseases.

© 2023 THE AUTHORS. Published by Elsevier LTD on behalf of Chinese Academy of Engineering and Higher Education Press Limited Company. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

The Notch pathway, comprising core components—receptor, ligand, transcription factor, and target genes, generates a short-range signal when activated, which is important for many developmental and homeostatic processes [1–6]. These include cell fate determination, cell survival, and stem cell maintenance. Upon binding of specific sites within the extracellular domains (ECDs) of Notch ligand and receptor in *trans* and subsequent application of a pulling force, a proteolytic cleavage event (S2) is triggered in the receptor's membrane-proximal negative regulatory region (NRR) by a disintegrin and metalloprotease (ADAM) family metalloproteases (Fig. 1 [7–11]). This is followed by γ-secretase cleavage of the Notch "stalk" at an intramembrane site (S3). This causes the release of the transcriptionally active Notch intracellular domain (NICD) which then translocates to the nucleus to form a complex with DNA-binding proteins of the recombination signal binding protein for immunoglobulin kappa J region (RBPJ) family (also known as CSL or CBF1/Su(H)/Lag-1) [12,13]. The binding of NICD to RBPJ displaces corepressor proteins and causes the recruitment of co-activators such as Mastermind-like proteins (MAML1–3), resulting in the expression of primary target genes such as the hairy and enhancer of split (*HES*) and HES related family basic helix–loop–helix (bHLH) transcription factor with YRPW motif (*HEY*) (Fig. 1) [14–17]. Unlike many other signaling pathways, there is no amplification of signal, NICD acts as the signal transducer and is responsible for pathway activation [12]. In addition to *trans*-activation, Notch receptors/ligands can form both *cis*-inhibitory and *cis*-activatory complexes when expressed in the same cell. *Cis*-inhibition is important for regulating a number of cell fate decisions such as those that affect tip and stalk cell identity in angiogenesis, wing development, and sensory organ precursor cell selection in *Drosophila* [18–21], whilst *cis*-activation has been shown to occur in a variety of cell types and affect neural stem cell survival *in vitro* [22].

Given this relatively simple circuitry, much of Notch research has focused on understanding how this signaling pathway can dictate so many biological responses. In *Drosophila*, there is one receptor and two different ligands, Delta and Serrate. In mammals, there are four Notch paralogs (NOTCH1–4) and four canonical ligands (JAG1/2 and DLL1/4) expressed on the cell surface and an additional ligand DLL3 which resides in the *trans*-Golgi [23,24]. However, this ligand/receptor repertoire alone, which may show cell type- and developmental stage-specific expression, does not satisfactorily

* Corresponding authors.
E-mail addresses: xinyiyang@imb.cams.cn (X. Yang), penny.handford@bioch.ox.ac.uk (P.A. Handford).

https://doi.org/10.1016/j.eng.2023.11.011
2095-8099/© 2023 THE AUTHORS. Published by Elsevier LTD on behalf of Chinese Academy of Engineering and Higher Education Press Limited Company.
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

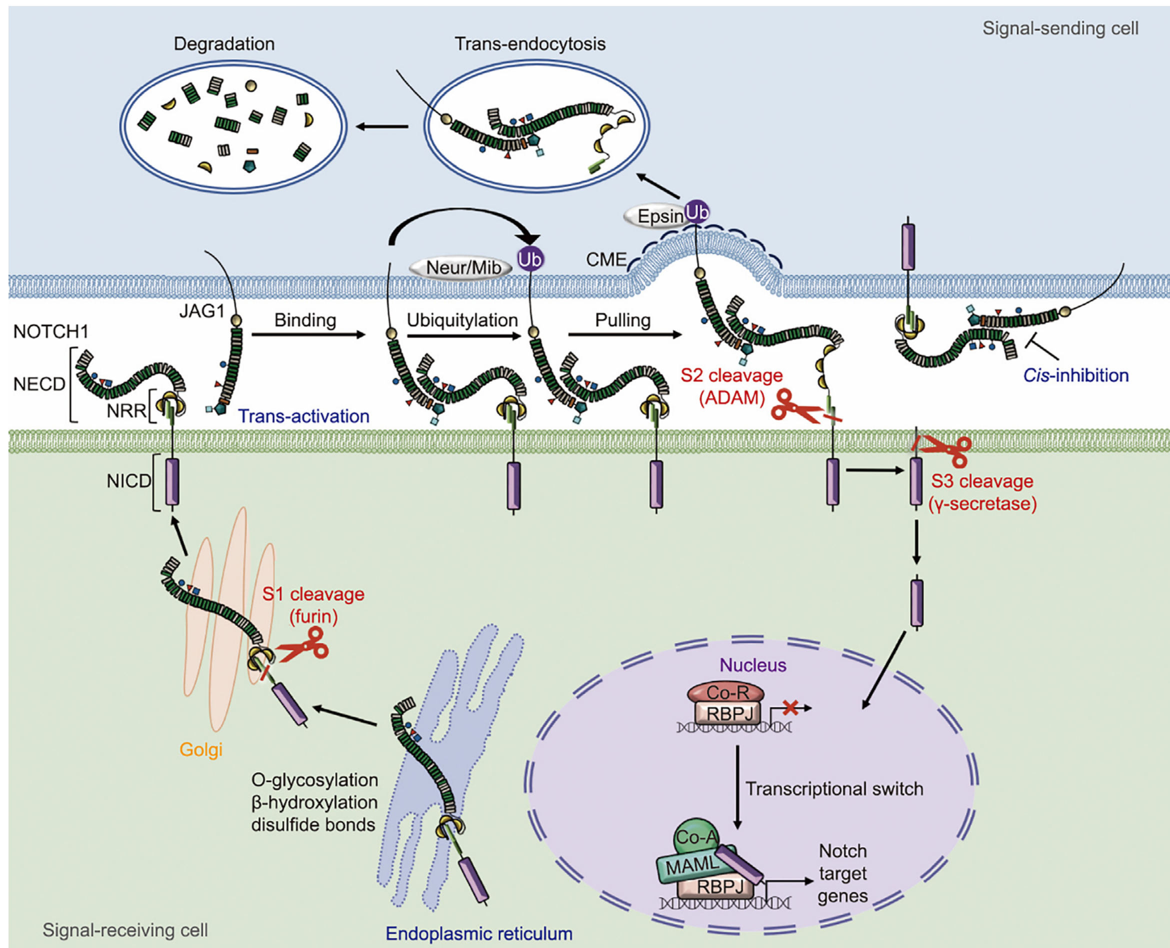

Fig. 1. Overview of the canonical Notch signaling pathway. The newly synthesised Notch receptor (human NOTCH1 shown) undergoes various post-translational modifications (PTMs) to its ECD: a furin-catalysed S1 cleavage yields a heterodimeric form comprising a Notch ECD (NECD) and a Notch transmembrane-intracellular domain (NTM-ICD), O-glycosylation occurs including O-fucose (red triangle), O-glucose (blue circle), and O-linked N-acetylglucosamine (O-GlcNAc; blue square) additions which may be extended further (see sugar extension details in Fig. 5(b)) before NOTCH is translocated to the plasma membrane. At the cell surface, the NOTCH is *trans*-activated in a juxtacrine manner by ligand (which may also undergo PTMs, human Jagged canonical Notch ligand 1 (JAG1) is shown) from the signal-sending cell. The ligand N-terminal C2 domain may bind to the cell membrane aided by a Jagged family specific N-glycan (light blue square) to form a ternary complex required for optimal signalling [7,8,9]. Upon binding, ubiquitylation and endocytosis of JAG1 takes place generating a pulling force which engages a catch bond, acts on the Notch NRR, and exposes the otherwise buried S2 cleavage site to the ADAM metalloprotease (scissors) [10]. The intramembrane S3 cleavage catalysed by γ-secretase (scissors) releases the Notch intracellular domain (NICD), which then translocates to the nucleus, and associates with the transcription factor RBPJ/CSL (wheat). Proteolytic cleavage to release NICD may also occur after endocytosis of the receptor (not shown). NICD binding displaces transcriptional repressors (Co-R; deep salmon) and allows association of Mastermind-like (MAML; blue) and additional coactivators (Co-A; green) to switch on transcription of Notch target genes. The presence of both Notch receptor/ligand on the same cell surface leads to *cis*-inhibition, and *cis*-activation has also been reported to occur. Biophysical data show NOTCH and ligand ECDs are not linear rods, but the structures of the complete ECDs remain undetermined. The antiparallel nature of the core binding regions of ligand and receptor within complexes [10,11] suggests *cis*- and *trans*-interactions may be mediated by one conformation. Ub: ubiquitin; CME: clathrin-mediated endocytosis; Neur/Mib: neuralized and mind bomb.

explain the vastly different physiological responses. Additional mechanisms must operate to regulate/fine tune the signal. In light of recent data, this review focuses on the extracellular receptor/ligand complex, in particular the role of mechanical force, post-translational modifications (PTMs), and membrane interactions in modulating ligand-dependent Notch activity. We draw readers' attention to recent works which cover other aspects of Notch signaling, such as NICD biology and transcriptional output [13,25,26].

2. ECDs of receptors and ligands

The modular architecture of canonical ligands and receptors in most metazoans from *Drosophila* to humans shows they are type I transmembrane proteins which have large ECDs relative to their intracellular portions. The exception to this is *Caenorhabditis elegans* (*C. elegans*) which has shorter receptor/ligand ECDs and a plethora of soluble ligands in addition to transmembrane forms (Fig. 2 [14,27–30]). The Notch ECD is dominated by contiguous epidermal growth factor (EGF)-like domains linked to the membrane-proximal NRR (comprising three LIN-12/Notch repeats (LNRs) and a heterodimerization region). This region, in the absence of applied mechanical force, masks the S2 proteolytic cleavage site which is a substrate for ADAM proteases. The two ligand families share a common N-terminal region which contain core binding sites for Notch. A variable number of EGF domains follow and the presence of a membrane-proximal cysteine-rich domain (CRD) in Jagged/Serrate only, distinguishes the two families from *Drosophila* to humans (Fig. 2).

3. Structural biology of the Notch receptor and its ligands

Historically, both receptor and ligand ECDs were challenging targets for structural biology due primarily to PTMs of their EGF domains such as disulfide bond formation, O-glycosylation, and

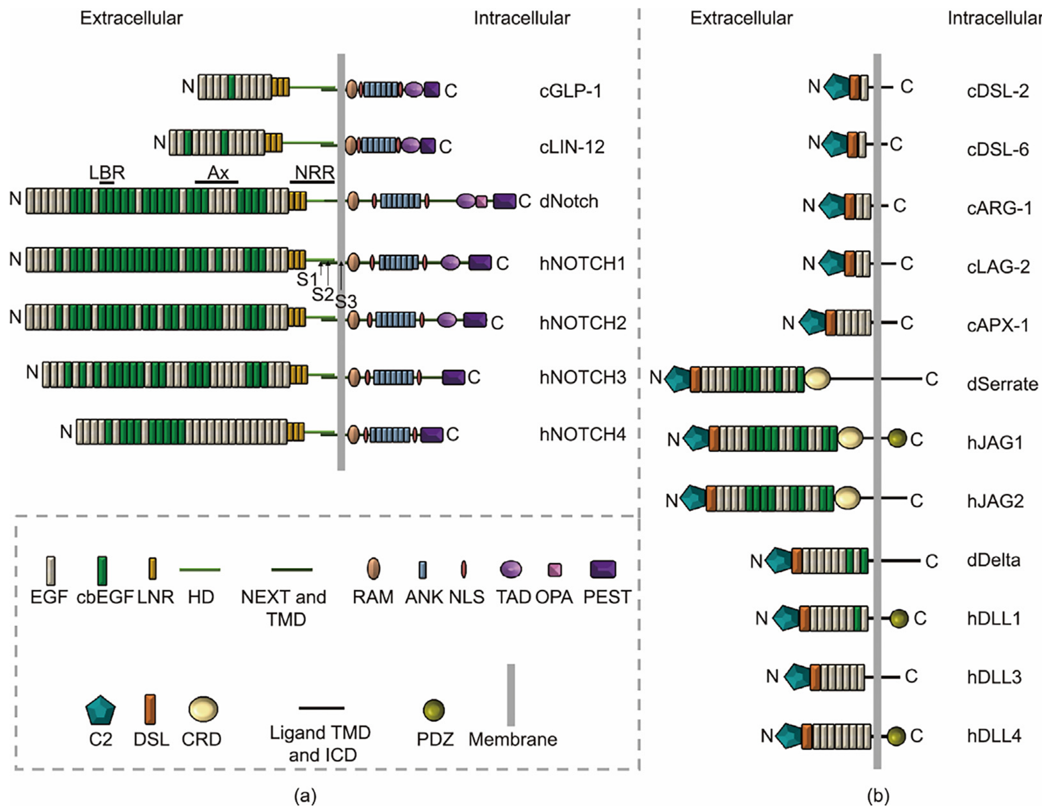

Fig. 2. Domain organisation of Notch receptors and ligands. Notch receptors and ligands are type I transmembrane proteins containing predominantly multiple EGF-like domains, either non-calcium binding (EGF) or calcium binding (cbEGF), in their ECDs. cbEGF repeats are labelled based on a consensus sequence: [D/E/N]–X–[D/N]–[D/E/N/Q]–X*m*–[D/N/Q]*–X*n*–[F/Y] (where *m* and *n* are variables and * indicates possible β-hydroxylation) [27]. (a) *Drosophila* Notch (dNotch) and the four human Notch paralogues (hNOTCH1–4) differ in their number of EGF domains (29–36), whereas the *C. elegans* Notch paralogues (cGLP-1 and cLIN-12) are much shorter. In receptors with 36 EGF domains, EGF11–12 form the core ligand-binding region (LBR) and EGF24–29 form the Abruptex region (Ax), as indicated on dNotch. EGF domains are followed by the NRR, which consists of three cysteine-rich LIN-12/Notch repeats (LNRs) and a heterodimerization domain (HD). Following the Notch transmembrane domain (TMD) is the NICD, which is composed of a RBPJ association module (RAM), nuclear localization sequences (NLSs), ankyrin repeats (ANKs) region, a transactivation domain (TAD), and a conserved proline/glutamic acid/serine/threonine-rich motif (PEST). NOTCH3 or NOTCH4 lacks the TAD [28,29]. dNotch also has a glutamine-rich repeat (OPA) in its TAD [14]. Sites of proteolytic cleavage indicated by S1, S2, and S3. (b) The ECD of a Notch ligand consists of an N-terminal C2 domain and a Delta/Serrate/LAG-2 (DSL) domain, followed by multiple EGF domains. The Jagged/Serrate family contains an additional cysteine-rich domain (CRD) that is not present in the Delta-like family. Some ligands also contain a C-terminal PSD-95/Dlg/ZO-1 (PDZ) motif. There are ten *C. elegans* DSL ligands identified in total, transmembrane ligands (cARG-1, cLAG-2, cAPX-1, cDSL-2/6) are indicated, the soluble ligands (cDSL-1/3/4/5/7) are not shown [30]. NEXT: Notch extracellular truncation.

β-hydroxylation. Many of the early high resolution structures of key domains were obtained from samples which had been *in vitro* refolded and lacked PTMs, with the exception of disulfide bonds [31–34]. The improvement in eukaryotic expression systems using cell lines such as S2, HEK293-T, HEK293-S, and HEK293-F cells, and High Five^{TM}, has facilitated purification of ECD fragments which are natively folded and post-translationally modified. This has led to significant advances in structural knowledge for larger multi-domain fragments and complexes of the core interacting regions of receptor/ligand, thereby giving new insight into possible complexes which may form in *cis* and in *trans* at the cell surface.

3.1. Structure of Notch ECD—Rod-shaped, bent, and flexible

The mature Notch receptors are usually expressed in heterodimeric form on the cell surface, following furin-mediated cleavage at S1 in the secretory pathway, although the *Drosophila* receptor does not require this cleavage for activity [35,36]. The ECDs of mammalian Notch receptors have a variable number of EGF domains linked to the NRR. Human NOTCH1 (hNOTCH1), hNOTCH2 and *Drosophila* Notch (dNotch) are similar, with 36 EGF domains, whilst hNOTCH3 has two less EGF domains and hNOTCH4 has 29 EGF domains. *C. elegans* Notch receptors GLP-1 and LIN-12 are much shorter than mammalian or *Drosophila* counterparts, comprising 10 and 13 EGF domains, respectively (Fig. 2). EGF domains are subjected to a number of PTMs in the secretory pathway. Disulfide bond formation in the oxidizing environment of the endoplasmic reticulum (ER) stabilizes the native EGF fold with a 1–3, 2–4, 5–6 arrangement, β-hydroxylation by the aspartate/asparagine hydroxylase (AspH), ensures correct folding of a subset of EGF domains [37], and O-glycosylation occurs according to distinct consensus sequences with a variety of different effects on function [38,39]. A dissection approach targeting predominantly hNOTCH1 multidomain fragments provided biophysical information. Many of the multiple tandem repeats of the Notch ECD contain the following consensus [D/E/N]-X-[D/N]-[D/E/N/Q]-X$_m$-[D/N/Q]*-X$_n$-[F/Y] (where * indicates possible β-hydroxylation, and *m* and *n* are variables) which is predictive for calcium binding [27]. Pairs of these repeats are expected to adopt near linear and rigid structures in the presence of Ca^{2+} due to the presence of a hydrophobic packing interaction between a conserved aromatic residue in the N-terminal domain and an "XG" dipeptide sequence located on the central β-hairpin in the C-terminal domain [32,33,40]. Where determined, typical Notch domain equilibrium dissociation constant (K_d) values for Ca^{2+} are in the micromolar range (1–200 μmol·L^{-1} at pH 7.5, I = 0.15 (physiological ionic strength equivalent to 150 mmol·L^{-1} NaCl)) [41]. These sites would be expected to be saturated under physiological concentrations (> 1.5 mmol·L^{-1}) of free extracellular Ca^{2+}. As such, bound Ca^{2+} is performing predominantly a structural role. Rarely, K_d values for Ca^{2+} in the millimolar range indicating lower affinity sites have

been observed in other calcium-binding EGF (cbEGF) domain-rich proteins such as the extracellular matrix (ECM) protein, fibrillin-1 [42]. In this case, the cbEGF domain was preceded by a heterologous domain type. Weak binding sites, if present within the receptor, could introduce Ca^{2+} dependent flexibility in the extracellular milieu (Fig. 3(a) [41]).

3.1.1. Ligand-binding region (LBR) is a Ca^{2+} stabilized rod

Notch EGF11–13, comprising three cbEGF domains and encompassing the EGF11–12 LBR first identified in dNotch by cell aggregation experiments, was an early target for structural biology [44]. Initially a nuclear magnetic resonance spectrometry (NMR) solution structure of hNOTCH1 [33] and subsequently X-ray crystal structures of hNOTCH1, hNOTCH2, and dNotch EGF11–13, confirmed its rigid and elongated structure under conditions of Ca^{2+} saturation, as seen in other cbEGF domain fragments (Fig. 3(b) [31,41]) [7,32,45]. Interestingly, most of the Notch cbEGF pairs have two linker residues between the last cysteine of the N-terminal domain and the first calcium-binding residue of the C-terminal domain of a pair, whilst other tandem repeats of cbEGFs in proteins such as fibrillin-1 have one linking residue [33,40]. As a consequence, Notch cbEGF domain pairs have a similar tilt angle (also resulting in an elongated structure) but different twist angles.

3.1.2. Other Notch EGF domains have more variable interfaces

Properties of Notch fragments containing non-cbEGFs are less easy to predict than those containing calcium binding sites. A study focusing on the N-terminal portion of the hNOTCH1 ECD showed that the presence of these domains can have pleiotropic effects. For example, NMR residual dipolar coupling studies, which define interdomain orientation, showed that the interface between cbEGF9 and non-cbEGF10 was flexible [41]. In contrast, the crystal structure of NOTCH1 EGF4–7, together with residual dipolar coupling studies, showed that the interface between cbEGF5 and non-cbEGF6, was bent and rigid [41]. These studies, together with the available structure for the LBR, allowed a structural model of the EGF4–13 region to be constructed (Fig. 3(b)). It is reasonable to suggest, assuming K_d values for Ca^{2+} are similar to those already measured for other EGF domains, that sections of the ECD such as EGF14–21, EGF23–25, and EGF31–33, which are comprised of contiguous cbEGF domains, are likely to be extended and rigid in conformation. However, additional biophysical studies of non-cbEGF domains which occur at EGF22, EGF26, EGF28–30, and EGF33–36 are required to address the overall architecture and flexibility/-rigidity of the C-terminal ECD. Recent small angle X-ray scattering (SAXS) analysis of a full-length NOTCH1 ECD fragment performed in the presence of physiologically relevant (2 $mmol·L^{-1}$) Ca^{2+} levels, demonstrated flexible properties, supporting the earlier NMR study of EGF9–10 [46]. In addition, the ECD had a maximum

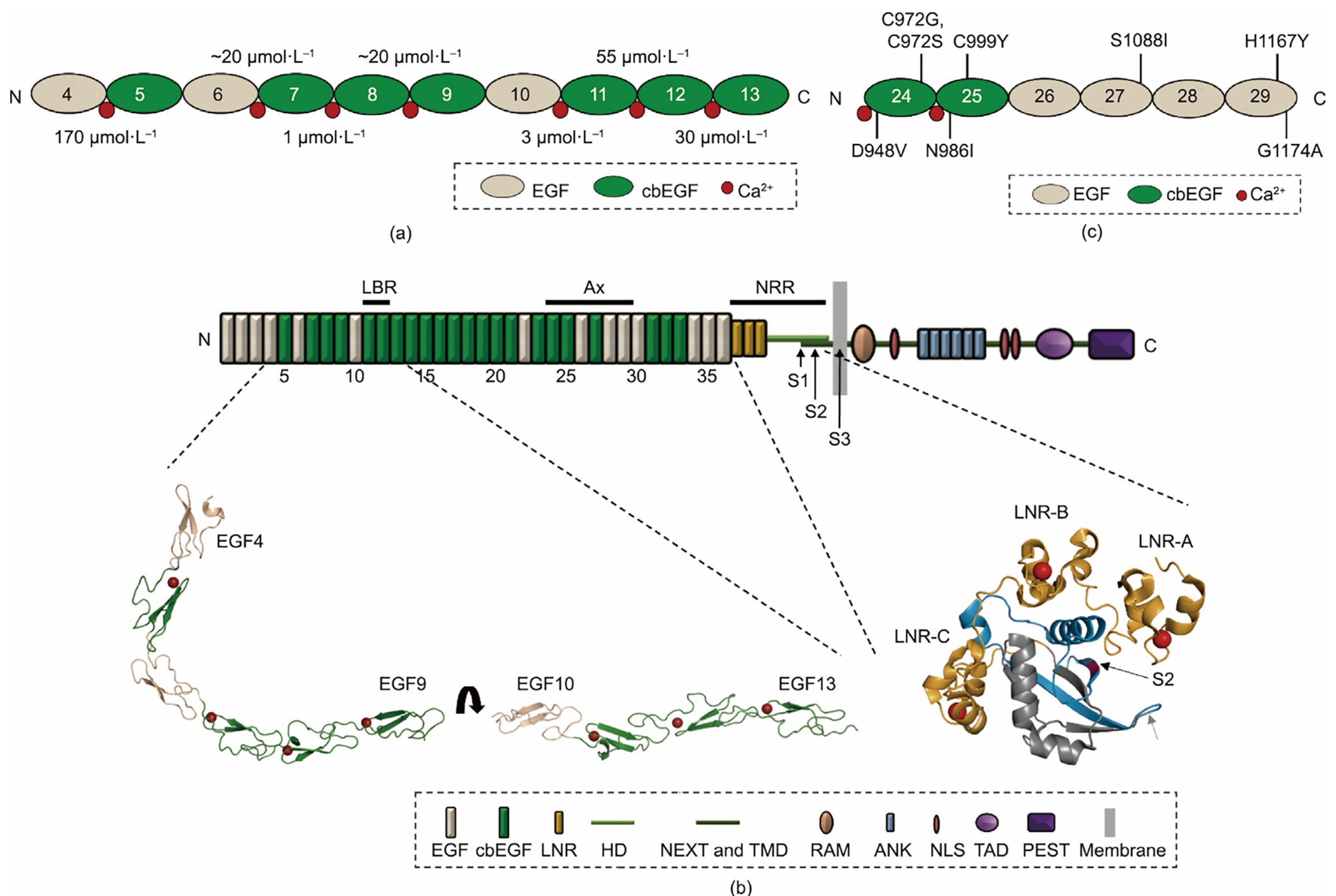

Fig. 3. Notch ECD structural features. (a) The Ca^{2+} dissociation constants at pH 7.5 and I = 0.15 for hNOTCH1 EGF4–13 measured by Weisshuhn et al. [41]. (b) Model of EGF4–13 region of the ECD of hNOTCH1. The models for the EGF4–9 and EGF10–13 regions of NOTCH1 are based on the X-ray structures (Protein Data Bank (PDB): 5FMA, 2VJ3) and residual dipolar coupling (RDC) measurements by Weisshuhn et al. [41]. The structure of furin-cleaved NOTCH1 NRR (PDB: 3ETO) [31] is shown with the LNR modules in brown, the region of the HD domain preceding the S1 cleavage site in grey, and the region after in blue. Ca^{2+} ions shown in red. The S2 cleavage site, between the residues A1721 and V1722 (coloured purple) is indicated by a black arrow. The position of a deleted loop (residues 1623–1669) containing the S1 cleavage site is indicated by a grey arrow. (c) Schematic diagram showing the positions of Ax substitutions identified in dNotch EGF24–29 [43]. EGF domains and Ca^{2+} ions are colored as in (a).

dimension (D_{max}) of ~38 nm. Since each EGF domain is approximately 3 nm in length, these data suggest that the fragment is not simply near linear and rigid, which would impart a length of 108 nm (36 × 3 nm) for EGFs alone, and may incorporate bent and flexible regions as seen in the EGF4–13 region. In the absence of cryo-electron microscopy (cryo-EM) and X-ray crystal structures of the full length transmembrane form, SAXS analysis of shorter multidomain EGF fragments, together with targeted NMR and related biophysical/calcium binding studies, should help to further define ECD shape.

3.1.3. Negative regulatory region

The NRR consists of three LNRs and a membrane-proximal heterodimerization domain (HD), and acts as the mechanosensor in the Notch activation pathway (Fig. 3(b)). It is held in a protease-resistant and inhibited state, until ligand binding to LBR and application of a pulling force releases the autoinhibition, allowing ADAM protease to cleave at S2. High resolution crystal structures revealed the molecular basis of autoinhibition (Fig. 3(b)) [34]. Each LNR donates ligands to a single Ca^{2+}, and the three Ca^{2+} bound domains protect the HD stalk resulting in occlusion of the S2 protease cleavage site. This conformation can be disrupted by mechanical pulling, removal of Ca^{2+} by chelators, and missense mutations which destabilize the autoinhibited state [47,48].

A synthetic Notch system (synNotch) exploiting the NRR mechanosensory mechanism of activation has been designed to sense extracellular and mechanical cues and record cell–cell contact history [49]. Using cells engineered to express synNotch where the receptor/ligand pairing is replaced with an antibody–antigen pairing, and the NICD with a unique transcriptional factor, cells expressing disease-specific antigens can be detected and treated by activated downstream targets [50]. Various synNotch systems have been developed for chimeric antigen receptor T cell (CAR-T) therapies targeting different types of tumor-associated markers, including the apelin receptor, AXL receptor tyrosine kinase, alkaline phosphatase placental-like 2, and EGF receptor splice variant III [51–54]. Whilst a high level of ligand-independent activation was a major limitation of early forms of synNotch, next generation versions have been improved by the addition of a hydrophobic RAM sequence to the base of the transmembrane domain (TMD), or by fusion of a single chain variable fragment derived from an NRR-stabilizing antibody to the NRR, thereby enhancing its autoinhibited conformation [55,56]. Furthermore, protein-engineered substitutions have further tuned NRR's mechanosensitivity making synNotches which activate in response to a wide range of biologically-relevant forces [55]. With its high editability and continuous improvement in specificity, synNotch is a promising therapeutic tool.

Furthermore, a Synthetic Notch Assay for Proteolytic Switches (SNAPS) assay has been developed to study novel putative proteolytic switches, by replacing the NRR with the proteolytically sensitive regions of other receptors sharing structural homology to Notch, but retaining the native Notch ligand-binding interaction with DLL4 as input and the NICD-induced Gal4 transcriptional response as output [57]. The cryptic S2 cleavage site in NRR is housed in a sea urchin enterokinase agrin (SEA)-like domain, where interdomain interactions between the SEA-like and its neighbouring domain prevent protease access [34,58,59]. In the SNAPS assay, several juxtamembrane domains from other surface receptors, which have been identified/predicted to contain a similar SEA-like fold [60], were shown to substitute for NRR's proteolytic switch and induce transcriptional response upon DLL4-induced activation [57]. Despite the similar switch-like behavior observed in these chimeric receptors, structural analysis reveals differential modes of interactions between the different SEA-like domains and the respective neighbouring domains, providing opportunities for engineering new proteolytic switches for synthetic biology [49]. Moreover, SNAPS could detect membrane shedding of diverse receptors without the SEA-like domains, making it a potential tool to study the mechanisms of proteolytic regulation in a wide range of transmembrane proteins. SNAPS can also be exploited to screen for modulators of shedding, such as herceptin and a function-blocking E-cadherin antibody DECMA-1, thus offering potential receptor-specific therapeutic targets in disease where proteolysis is dysregulated [57].

3.1.4. Other regions of interest from functional studies—Abruptex region (Ax)

EGF24–29 of the Notch receptor, known as the Ax (Fig. 2), has also been shown to be important for function [61]. The region was first defined in flies where phenotypes distinct from the *dNotch* null "notched wing" phenotype [61] were found to contain localized missense mutations (Fig. 3(c) [43]). *Ax* alleles can be divided into three classes, known as homozygous lethal, *N* suppressor, and *N* enhancer (Table 1) [43]. Lethal alleles which result in cysteine substitutions in EGF24 and EGF25, are not viable even when expressed in the heterozygous state with *dNotch* null or other *Ax* alleles [62]. Since the cysteine residues affected are involved in native disulfide bonding, these substitutions are likely to generate large structural changes, caused by misfolding, leading to faulty transportation through the biosynthetic pathway. Interestingly, both *N* suppressor variants contain substitution of a residue associated with the EGF domain calcium-binding consensus sequence. By analogy to similar changes observed in other cbEGF domain-rich proteins, these substitutions might be expected to increase K_d values for Ca^{2+} and introduce some flexibility between EGF23–24 and EGF24–25 interfaces [27,63]. Such changes could affect protein–protein interactions, biomechanical properties, a spacer function, or introduce proteolytic susceptibility.

When *Ax* alleles are crossed with a *dNotch* null allele (N^-), *N* suppressor and *N* enhancer mutations suppress or enhance the N^- phenotype, respectively [61,62]. *N* suppressors and *N* enhancers are homozygous viable inducing the same phenotype as their heterozygote form (Ax/N^+) [62,64]. The combination of an *Ax* allele associated with homozygous lethality (*e.g.* *Ax-M1*) and N^- or other class of *Ax* allele is lethal.

3.2. Structure of Notch ligands

Canonical Notch ligands belong to the Delta/Serrate/LAG-2 (DSL) family due to the presence of a novel domain type. Usually DSL ligands are expressed as transmembrane proteins on the cell surface with the exception of DLL3, a negative regulator of Notch which is localized to the *trans*-Golgi [23], and some soluble forms in *C. elegans*. All DSL ligands have an N-terminal C2 domain (previously known as module at the N terminus of Notch (MNNL)) followed by the DSL domain and a variable number of EGF domains. The DSL confers binding to Notch, explaining the

Table 1
Ax alleles and their corresponding amino acid change identified in *Drosophila melanogaster* [43].

Ax alleles	EGF	Amino acid change in dNotch	Effect
Ax9	24	D948V	*N* suppressor
Ax59b	24	C972G	Homozygous lethal
Ax59d	24	C972S	Homozygous lethal
Ax1	25	N986I	*N* suppressor
Ax-M1	25	C999Y	Homozygous lethal
Ax71d	27	S1088I	*N* enhancer
Ax-E2	29	H1167Y	*N* enhancer
Ax16	29	G1174A	*N* enhancer

absolute requirement of this domain for canonical ligand function. Of the two ligand families, Jagged ligands are larger and can be distinguished from Delta by the presence of a membrane proximal CRD (Fig. 2). A number of ligand structures have been determined, identifying common features.

3.2.1. C2 domain and lipid/membrane binding

In 2013, the X-ray structure of JAG1 N-terminal fragment comprising MNNL, DSL, and EGF1–3 (NE3, Fig. 4(a) [1,8,65]) was determined [65] and revealed that the MNNL was a common lipid-binding C2 module, which, in principle, could confer peripheral membrane-binding properties in addition to the ligand's C-terminal transmembrane region. This domain type, which has a hydrophobic core formed from a 2 × 4 β-sheet sandwich, is more usually associated with intracellular proteins involved in vesicle/membrane targeting such as synaptotagmin and phospholipase A2, a notable exception being perforin [66,67]. Additional structures show that the C2 domain (previously referred to as the MNNL domain) is present in both ligand families (Fig. 4(b) [68]), and in all metazoan species studied so far (*Drosophila*, human, and rat) [7,11,69]. All of them have a type II topology that is most similar to the protein kinase C (PKC)-C2 family. Jagged C2 domains bind Ca^{2+} in the apical region (one Ca^{2+} in JAG1 C2 and three Ca^{2+} in JAG2), whilst the Delta-like family does not. In many intracellular C2 domains, the apical loop regions, connecting β-strands 1–2 (loop β1–2) and β-strands 5–6 (loop β5–6), which form the major lipid-binding site, have several hydrophobic residues. However, in Notch ligands the loops are less hydrophobic, suggesting they

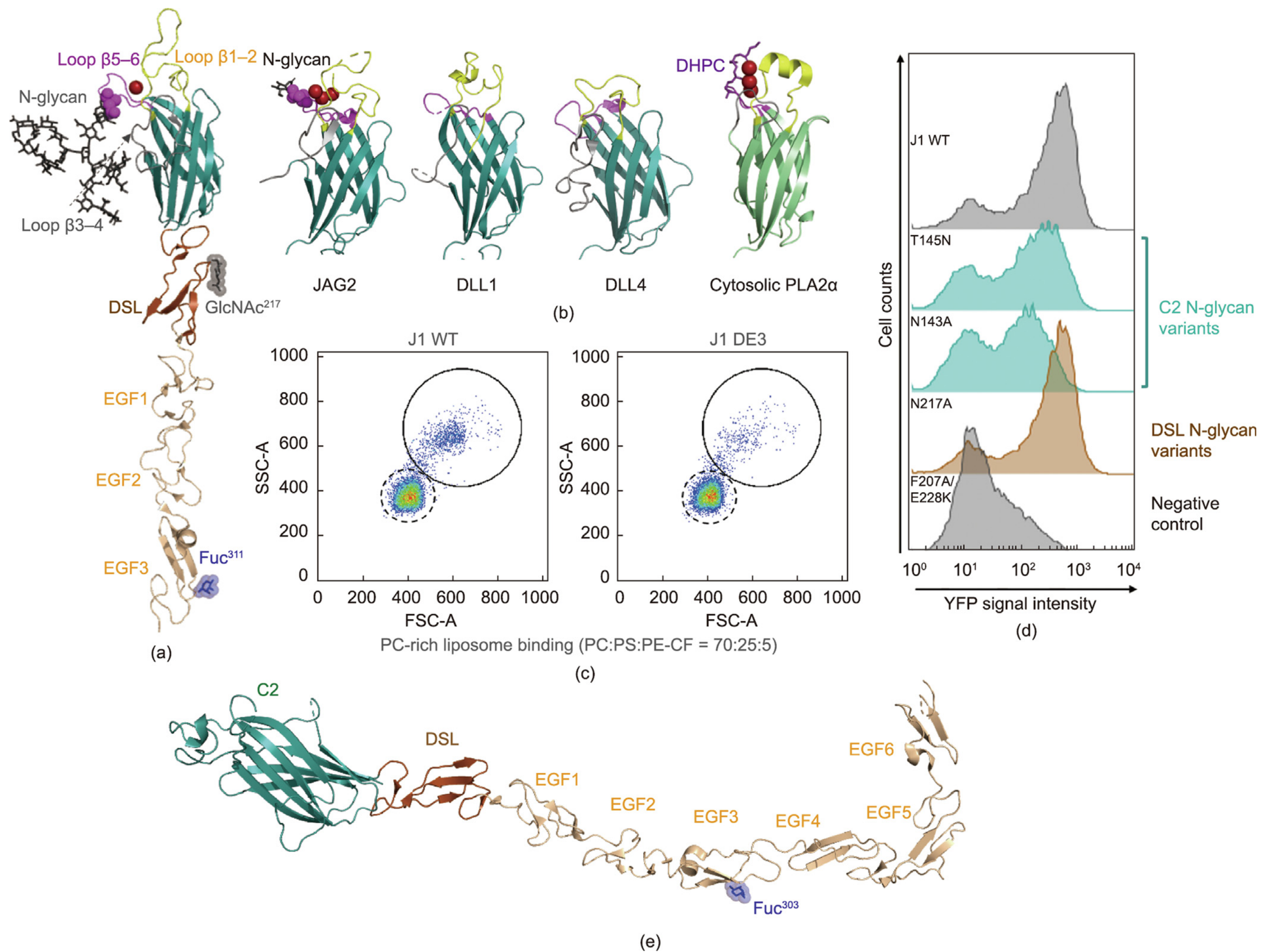

Fig. 4. Ligand structural and functional features. (a) The structure of JAG1 C2-EGF3 (NE3; PDB: 4CBZ) is shown in ribbon representation, and C2 domains are colored in teal, DSL domains in orange, and EGF domains in wheat. The Ca^{2+} ion bound at the apex of the C2 domain is drawn as a red sphere. A tetra-antennary form of a complex N-glycan on the C2 N143 residue (pink spheres) is modelled based on JAG2 (PDB: 5MWF) and *Drosophila* Delta structure (PDB: 7ALK) and functional experiments [1,8,65]. Loop β1–2 is shown in yellow, loop β3–4 is coloured in grey, and loop β5–6 is in magenta. Additional glycans are indicated including an N-glycan on N217 ($GlcNAc^{217}$) in the DSL domain and an O-fucose on T311 (Fuc^{311}) on EGF3. (b) Structures of C2 domains of different Notch ligands are shown alongside cytosolic phospholipase A2-α (cPLA2α)-C2 (PDB: 6IEJ), which is complexed with 1,2-diheptanoyl-sn-glycero-3-phosphocholine (DHPC) lipid (purple) [68]. Note the hydrophobic core in each case. The apical loops β1–2 and β5–6 (same colour scheme as (a)) differ in length and conformation (PDB: 5MW5-JAG2, 4XBM-DLL1, and 5MVX-DLL4, respectively) and may affect lipid-binding preferences. The JAG1 and JAG2 C2 domains bind Ca^{2+} (red), unlike Delta family ligands. An N-glycan on loop β5–6 of JAG2 C2 domain is shown in dark grey. (c) Liposome-binding analysis of bead-immobilised purified JAG1 fragments by flow cytometry [8]. There is a higher aggregate population (black solid circle) in the liposome/JAG1 C2-EGF3 (J1 WT) sample compared to liposome/JAG1 DSL-EGF3 (J1 DE3), which lacks the C2 domain. Dashed black circle indicates single bead population. (d) Flow cytometry-based Notch activation assay by N-glycan variants with J1 WT and a NOTCH-binding negative control (F207A/E228K) using a Chinese hamster ovary (CHO) cell line with a yellow fluorescent protein (YFP) readout [19]. Variants lacking the N-glycan on loop β5–6 of C2 (cyan; T145N and N143A) showed less YFP signal compared to J1 WT and J1 N217A (brown; without the DSL N-glycan) suggesting the C2 N-glycan is important for the ability of JAG1 to activate Notch in this assay [8]. (e) X-ray structure of DLL1 ectodomain (PDB: 4XBM) is shown in the same color scheme as (a). C2-EGF4 and EGF5–6 show a near linear arrangement, respectively, and the junction between EGF4 and EGF5 is approximately 90°. A fucose on T303 (Fuc^{303}) in EGF3 is shown in blue. SSC-A: side-scatter area; FSC-A: forward-scatter area; PC: phosphatidylcholine; PS: phosphatidylserine; PE-CF: phosphatidylethanolamine-carboxyfluorescein.

are not deeply buried in the cell membrane [7]. Data from *in vitro* assays showed that the C2 domains of all canonical Notch ligands can bind lipids (Fig. 4(c) [8]), with a preference shown by JAG1 ligands for sphingomyelin-rich liposomes and DLL4 ligands for ganglioside-rich liposomes [7,8]. Loops at the apex of the C2 domain are highly variable in both length and conformation among Notch ligands (as is a more lateral loop connecting β-strands 3–4) suggesting functional diversity, and consistent with different lipid-binding preferences seen *in vitro* (Fig. 4(b)). The subsequent identification of a Notch-binding surface on the C2 domain (distal from the main lipid-binding region) prompted liposome/ligand binding assays to be performed in the presence of Notch. The inclusion of the LBR Notch fragment was found to enhance recruitment of liposomes to immobilized N-terminal ligand fragments [7]. In addition, variants containing amino acid substitutions (protein-engineered and disease-causing) in the apical loops of the JAG1 C2 domain reduced Notch activation in reporter assays suggesting that the lipid-binding ability of the N-terminal region can play an important role in modulating Notch signaling [7,8]. Collectively these data suggested that a ternary complex of Notch receptor, ligand and lipid (cell membrane) is required for optimal Notch activation, possibly through facilitating the formation of the ligand/receptor complex, prior to catch bond engagement (see Section 5.2 for details).

Genome editing of the C2 domain loop β1–2 in *Drosophila* Delta, resulting in the removal of four residues required for lipid binding, has provided further *in vivo* evidence that this region is required for robust Notch signaling, particularly for developmental decisions that are dependent on lower levels of Notch signal such as microchaete spacing and photoreceptor fate [9]. Further questions remain about the importance of membrane/ligand interactions. Is there ligand-specific selectivity towards lipids in the outer membrane leaflet as suggested by the different preferences shown *in vitro,* and C2 domain structures? Which membrane does the C2 domain bind to—that of the signal-sending or -receiving cell or both?

3.2.2. JAG1 C2 domain and N-glycosylation

Recent data following an analysis of the Catalogue Of Somatic Mutations In Cancer (COSMIC) ligand variants have revealed the importance of an N-glycan, located on the JAG1 C2 domain lipid-binding loop, for Notch activation. An NxS/T glycosylation motif located on the loop β5–6 (Fig. 4(a)) is highly conserved in Jagged/Serrate but not Delta ligands, with the exception of *Drosophila* Delta [8]. JAG1 variants with amino acid substitutions T145N and N143A, which alter the consensus C2 N-glycosylation site, reduced JAG1-mediated Notch activation in cell-based reporter assays (Fig. 4(d) [8,19]) and in a JAG1-dependent vascular smooth muscle cell (VSMC) differentiation assay [8]. This is in contrast to a DSL N-glycan variant N217A, which showed no detrimental effect on activity. The C2 N-glycan variant was also shown to reduce JAG1 binding to liposomes. These data are consistent with a role for this N-glycan in promoting a lipid-binding conformation required for JAG1 function.

3.2.3. Ligand DSL domain/EGF domain/CRD

The X-ray crystal structure of an *in vitro* refolded DSL-EGF3 JAG1 fragment, followed by structure determination of JAG1 C2-EGF3, purified from HEK293S cells, showed that the DSL domain consists of double-stranded anti-parallel β-sheets, reminiscent of the EGF domain fold, prior to a C-terminal disulfide-bonded loop (Fig. 4(a)) [32,65]. However, DSL has a different disulfide pattern (C1–C2, C3–C4, C5–C6), suggesting it may have evolved from the truncation of two tandemly connected short EGF domains [32]. A surface loop of the DSL was shown to contain a highly conserved cluster of charged amino acids, bounded by two aromatic residues, essential for Notch binding [32]. Structure-informed *Drosophila* Serrate functional analyses demonstrated that the Notch-binding loop residues were required for both *trans*-activation and *cis*-inhibition suggesting the same DSL surface is involved in both types of interaction [32]. Both ligand families have variable numbers of EGF domains (Fig. 2). The two non-cbEGF domains, EGF1 and EGF2, adjacent to the DSL domain have a role in facilitating Notch binding [70,71]. Structural data show that these domains exhibit a highly-truncated version of the EGF-fold (referred to as a DOS domain) (Fig. 4(a)) with no canonical secondary structure, and more distant structural homologies to other EGF-like domains. EGF3, in contrast, has a more classic fold with a central β-hairpin. The structure of the complete DLL1 ectodomain was solved in 2015 [69], revealing an extended conformation, and showed electron density for six of the eight EGF domains present (Fig. 4(e)). This comprised a linear arrangement of the N-terminal C2 domain, the ligand-binding DSL domain and the first four EGF-like domains, which are non-calcium binding. The C2-EGF2 regions of DLL1 and JAG1 overlay closely, suggesting these domains adopt a rigid and linear arrangement in solution. EGF3 and EGF4 both have a classical EGF fold and are essentially linear, like EGF12 and EGF13 of NOTCH1, despite lacking calcium-binding sites. The interface between EGF4–5 is bent and mediates a turn of approximately 90° (reminiscent of that seen with EGF5 and EGF6 of NOTCH1) with the following domains EGF5 and EGF6 of DLL1 in a linear arrangement (Fig. 4(e)). EGF7 and EGF8 were not visible. This study once more underscores the difficulty of predicting EGF domain interfaces and their effects on receptor/ligand architecture.

The Jagged/Serrate-specific CRD, located between the EGF domains and the TMD, shares partial homology with the Willebrand factor type C-like domain [72]. No high resolution structure of this region has been determined, but deleting the CRD in *Xenopus* Serrate caused abnormal expression of N-tubulin in primary neurons via the Notch/Su(H) pathway, which suggested this membrane-proximal region is important for activating Serrate-mediated Notch signaling and regulating neurogenesis of *Xenopus* embryos [73].

Whilst advances have been made in determining structures for key functional domains within Notch ligands, information is still lacking. The DLL1 structure provides the most complete picture of ligand architecture that is available, but the absence of data regarding the membrane-proximal two EGF domains limits our knowledge of the ECD of DLL1 at the cell surface. Furthermore, information is required for additional Jagged EGF domains and the membrane-proximal CRD. Overall, knowledge of complete structures for the integral membrane protein forms of ligand and receptor would facilitate our understanding of the molecular basis for *cis*- and *trans*-interactions.

3.3. Notch/ligand complexes

Two seminal studies identifying DLL4 and Jag1 N-terminal fragments in complex with Notch1 LBR fragments (EGF11–13 and EGF8–12, respectively) (Fig. 5(a) [10]) gave new insights into this important interaction and confirmed many experimental observations made previously [10,11]. Crystallization of each complex was made possible using an affinity maturation technique to overcome the observed low affinity interaction between ligand and receptor. *In vitro* evolution of higher affinity forms of each ligand facilitated purification of complexes and co-crystallization. The first complex to be determined was that of rat Notch1 EGF11–13 in complex with DLL4 [11]. Key observations that were made included an antiparallel organization of receptor/ligand fragments within the crystal, which suggested that one complex could underlie both *cis*- and *trans*-interactions. Two core interaction sites along the longitudinal axis were observed, with C2 and DSL domains binding to

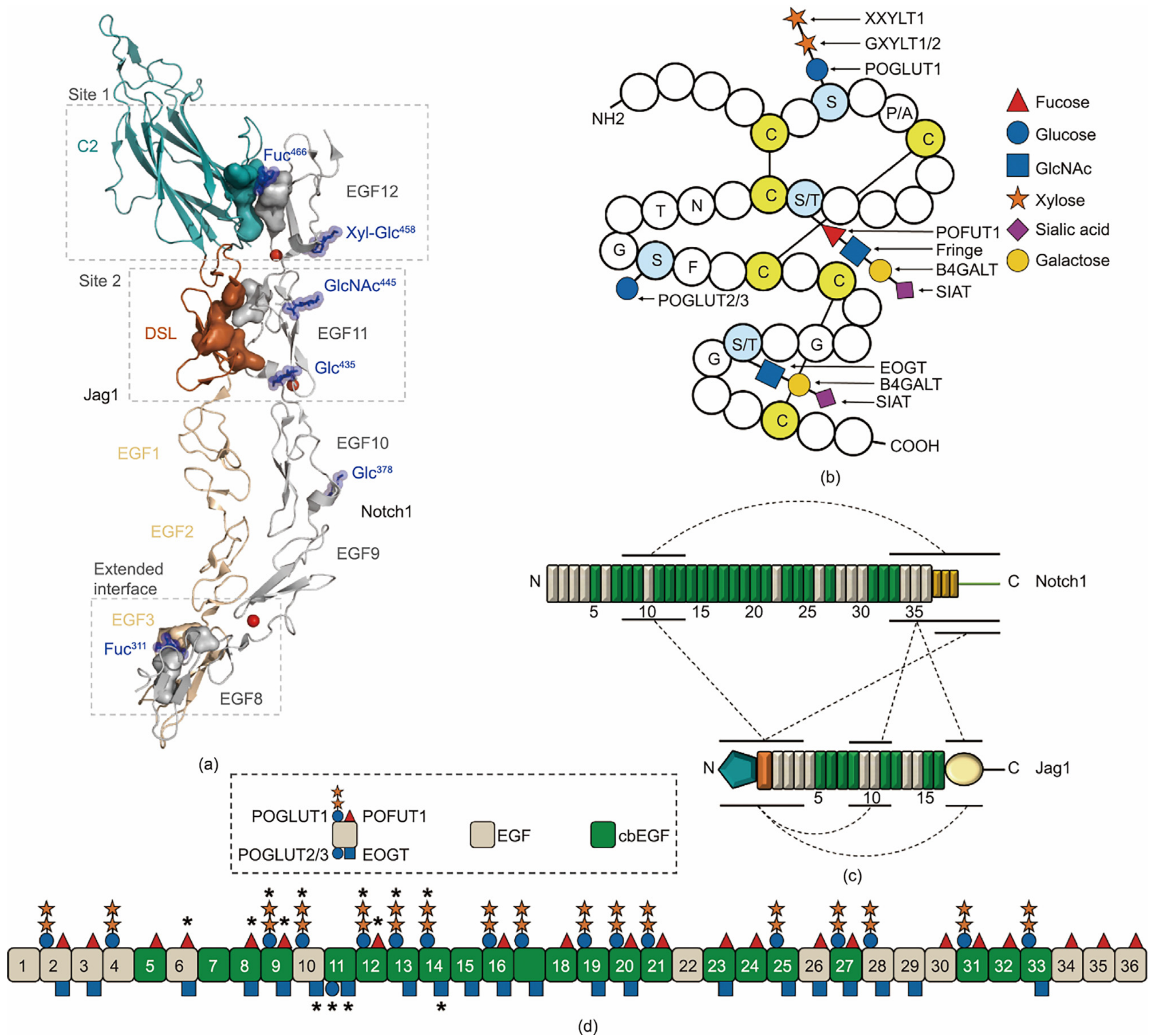

Fig. 5. Structural basis of Notch-ligand interaction and O-glycosylation. (a) X-ray structure of Notch1 EGF8–12/Jag1 C2-EGF3 complex (PDB: 5UK5) [10]. Jag1 C2 domain and DSL domain are shown in teal and orange. The non-cbEGF domains are shown in wheat. Notch1 is shown in grey. The Ca^{2+} ions are shown as red spheres. Key residues involved in the binding of Jag1 C2 to Notch1 EGF12 (site 1), Jag1 DSL to Notch1 EGF11 (site 2), and Jag1 EGF3 to Notch1 EGF8 (extended interface) are shown as surface representations. O-glycans are coloured blue. (b) Schematic diagram of an EGF domain showing the locations and structures of O-glycans and the enzymes catalysing the additions. A typical EGF repeat consists of six conserved cysteine residues linked by three disulfide bonds. Consensus sequences for O-glycan modification are shown. (c) Schematic representation of the reported inter- and intra-molecular interactions in the Jag1/Notch1 full ectodomain complex based on cross-linking mass spectrometry (XL-MS) data and verified by quantitative interaction assays [46]. Direct interactions between different constructs (solid black lines) are shown as dotted lines (inter-molecular: straight dotted lines; intra-molecular: curved dotted lines). (d) Schematic diagram showing the O-glycosylation sites in the EGF repeats of the hNOTCH1 ECD predicted by consensus sequence matching. EGF repeats harbouring the recognition consensus sequences for POGLUT1, POGLUT2/3, protein O-fucosyltransferase 1 (POFUT1), and EGF domain-specific O-linked GlcNAc transferase (EOGT) are shown. O-glycosylation sites that have been confirmed experimentally in hNOTCH1 are indicated by asterisks [45,79–82]. Fuc: fucose; Glc: glucose; Xyl: xylose; XXYLT: xyloside xylosyltransferases; GXYLT: glucoside xylosyltransferases; B4GALT: β-1, 4-galactosyltransferases; SIAT: sialyltransferases.

EGF12 and EGF11 at sites 1 and 2, respectively. Analysis of the receptor/ligand interface verified the role of key residues identified previously from mutagenesis and structural studies, and directly showed the importance of specific O-glycans in interface formation, specifically an O-fucose modification on Thr466 of Notch EGF12. A glucose added by protein O-glucosyltransferase 2 (POGLUT2) and POGLUT3 in the interface was also identified linked to a serine residue within Notch EGF11 but was not associated with a known consensus sequence (see Section 4.2, Fig. 5(b)). The DLL4 study was followed by structure determination of a complex of Jag1 with a longer fragment of Notch EGF8–12 (Fig. 5(a)). This also showed the same antiparallel arrangement, and conservation of sites 1 and 2, but in addition showed a third contact site between Notch EGF8 and EGF3 of Jag1, with a conserved valine buried in the interface. This explained the functional effect of the *Notch^{jigsaw}* mutation V361M, identified in a *Drosophila* screen, which selectively affected Serrate-dependent Notch activation and reduced Notch binding [74]. Crystallization of Jag1 with a longer Notch fragment EGF8–12 further emphasized the direct role of O-fucose in the maintenance of the interface, with contributions made at the third contact site as well as site 1. O-fucose modification of Thr311 of Notch1 EGF8 was shown to hydrogen bond to the side chain of Jag1 EGF3 Asn298. Interestingly, an O-fucose modification of Thr311 of Jag1 EGF3 formed a Van der Waals contact with

His313 of Notch1 EGF8 demonstrating that ligand O-glycosylation also contribute to the receptor/ligand binding interface.

3.3.1. Synthetic biology approach to creating higher affinity forms of DLL4

Whilst an additional binding interface between Jag1 EGF3 and Notch1 EGF8 (site 3) was demonstrated to contribute substantially to Jag1/Notch1 interactions [10], this site has a minimal effect on DLL4/Notch1 interactions [11]. However, by using a site-directed mutant library to select for DLL4 variants that recapitulate the site 3 interactions between Jag1 and Notch1, a higher binding-affinity version of DLL4, referred to as "DLL4.v2" (N-EGF5), was produced which displayed increased signaling capacity [75]. Interestingly, the generation of higher-affinity DLL4 variants also provides insight into the mechanism of affinity enhancement. Structural analysis of the DLL4.v2 substitutions in the context of the Jag1/Notch1 complex suggested that three of them (N257P, T271L, Q305P) enhance affinity by improving hydrophobic packing at the binding interface, whereas the other two (F280Y and S301R) may act by either stabilizing the overall fold of DLL4 (F280Y) or introducing additional contacts between DLL4 and Notch1 (S301R). Moreover, when combining DLL4.v2, which harbors structure-guided site 3 changes, with previously reported affinity-enhancing substitutions [11], a synthetic Delta^{MAX} ligand was engineered with maximized binding affinity that is 500- to 1000-fold higher than wild-type human DLL4 [75]. The affinity-matured Notch ligand, Delta^{MAX}, not only exhibited higher signaling potency that allows it to stimulate increased human CD8^{+} T cell proliferation and expression of effector markers, but also functioned as a Notch-specific inhibitor when administered as a soluble decoy.

3.3.2. New methods helping to define extracellular receptor/ligand interactions

Cross-linking mass spectrometry (XL-MS), utilizing a lysine-targeted PhoX reagent, has recently been used to identify previously undetected intra- and inter-molecular interactions mediated by ECDs of receptor/ligand (Fig. 5(c) [46]). Three regions of Jag1, C2-EGF3, EGF10, and CRD were identified as being in contact with the membrane-proximal Notch1 EGF29-NRR. No interactions were identified between the known core interacting regions of ligand and receptor in this study, but this was attributed to lysine residues being buried in the interface and/or the presence of O-glycans which may prevent the cross-linking reaction. Subsequent studies by surface plasmon resonance (SPR) and microscale thermophoresis (MST) with limited fragments demonstrated a specific interaction of high/moderate affinity (K_d = 0.6 μmol·L^{-1}) between Notch1 NRR and Jag1 C2-EGF3, whilst only low affinity sites for Jag1 EGF8–11 and CRD fragments were observed with a larger EGF33-NRR fragment (Fig. 5(c)). Intra-molecular interactions between Jag1 regions C2-EGF1, EGF5–6, EGF9–12, and CRD were observed by XL-MS, with weak binding between C2-EGF3 and EGF8–11 and CRD confirmed in quantitative interaction assays. Low affinity interactions were also observed for Notch EGF8–13 and EGF33-NRR fragments. Given SAXS data for ECD constructs which suggests flexibility, together with these cross-linking and interaction data, the authors propose that more interactions appear possible than those observed between the core receptor/ligand-binding regions (LBRs). However, these data need to be tested in functional Notch activation assays to assess their physiological importance.

4. O-glycosylation—Sweetening the Notch signal

The discovery of *Drosophila* Fringe (Fng) [76] and its mammalian homologs [77,78] first suggested that glycosyltransferases were an important component of the Notch pathway which had the potential to modulate receptor/ligand interactions. Three major types of O-linked glycans have been identified, specifically O-fucose, O-glucose, and O-linked N-acetylglucosamine (O-GlcNAc). These monosaccharide modifications are added to EGF domains by distinct enzymes in the ER and can be elongated in the Golgi. Mapping of O-glycans throughout the Notch ECD by glycoproteomics has shown that each modifying enzyme is associated with a specific consensus sequence and the O-glycans added have distinct roles (Figs. 5(b) and (d) [45,79–82]) [83,84].

4.1. O-fucosylation

O-fucose is added to the consensus site $C^2–X_{4–5}–(\underline{S/T})–C^3$ within most mouse and dNotch EGF domains by protein O-fucosyltransferase 1 (POFUT1 in mammals and O-fut1 in *Drosophila)* (Figs. 5(b) and (d)) [85–87]. The O-fucose can be extended by Fng, which adds a GlcNAc residue. There is one Fng in *Drosophila* but three homologs in mammals, known as manic (MFNG), radical (RFNG), and lunatic Fng (LFNG) [88,89]. The GlcNAc-fucose-O-disaccharide can be further extended in mammals to tri- and tetra-saccharides by two other distinct enzymes [87]. The functional importance of O-glycan modifications was recognized by early work in wing development in *Drosophila* [76], where Serrate and Delta activity is regulated by Fng modification to Notch to help define boundary cells in the wing margin. Insight into the *cis*-inhibitory effects of Fng has come from both *in vivo* and cell-based experiments which show that reduction in Fng activity increases the *cis*-interaction between Notch and Serrate whilst reducing the *cis*-interaction between Notch and Delta, therefore modulating the *trans*-activity of ligand in each case [90]. Furthermore, loss of Fng modification at Notch EGF8 and EGF12 increases *cis*-inhibition of Serrate [91]. The situation is even more complex in mammals, LFNG and MFNG inhibit NOTCH1 activation by JAG1 acting through sites in EGF6 and EGF36, whilst all three Fngs enhance activation from DLL1 by modification of EGF8 and EGF12 [92]. Insight into the molecular basis for some of these effects has come from analysis of defined, modified fragments. The affinity of *in vitro* O-fucosylated NOTCH1 EGF11–13 for JAG1 and DLL1 N-terminal fragments was shown in cell and molecular assays to increase upon Fng modification, whilst the affinity for DLL4 was already substantially higher before further modification and did not increase further [45]. Subsequent structure determination of Notch/ligand complexes showed that the DLL4/Notch interface at sites 1 and 2, buries a greater surface area than that of Jag1/Notch1, and whilst O-fucose modifications directly contribute to interface formation at site 1 in both ligands, site 3 seen in Jag1/Notch1 also involves O-fucose interactions [10,11]. Interestingly, O-fucose analogues were synthesised and incorporated into Notch EGFs, which inhibited Delta-induced, but not Jagged-induced Notch signaling, thus indicating ligand specific differences [93]. Collectively, these data indicate the advances made but also the challenges associated with understanding *cis*- and *trans*-Notch ligand interactions, and how they may be tuned by O-glycosylation.

4.2. O-glucosylation

O-glucose is added to the EGF domain consensus motif $C^1–X–\underline{S}–X–(P/A)–C^2$ by POGLUT1 in mammals and Rumi in *Drosophila* (Figs. 5(b) and (d)) [94,95]. Unlike POFUT1, which can target both serine and threonine, POGLUT1 can only add O-glucose to serine [96–99]. In addition to POGLUT1, there are two mammalian O-glucosyltransferase homologs, POGLUT2 and POGLUT3 (formerly known as KDELC1 and KDELC2), which have their own distinct consensus motifs and have only been reported to glucosylate Notch1

EGF11 and Notch3 EGF10 [79,100]. O-glucose monosaccharides on Notch EGF can be extended by glucoside xylosyltransferases (GXYLT1/2 in mammals, Shams in *Drosophila*) and xyloside xylosyltransferases (XXYLT1 in mammals, Xxylt in *Drosophila*). However, whilst the majority of Poglut1 target sites in mouse Notch1 are elongated to a trisaccharide form [98], xylosylation only occurs to a subset of glucosylated dNotch EGFs [101,102]. Instead of directly modulating receptor/ligand interactions, like O-fucosylation and Fng extension, O-glucosylation has been suggested to act downstream of ligand-binding, and upstream or at the level of S2 cleavage [94,103,104]. *Drosophila* studies have shown that deficiency of Notch O-glucosylation results in a temperature-sensitive loss of Notch signaling, without affecting the Notch surface expression level [94,104,105] or its ligand-binding capacity [94,106]. On the other hand, *Drosophila* S2 cells with either *Rumi* or *Kuzbanian*/*ADAM10*-knockouts displayed the same abnormal Notch cleavage pattern [94,107], suggesting that O-glucose modification of Notch plays a key role in the modulation of S2 cleavage, by helping to maintain the autoinhibited Notch ECD (NECD) conformation prior to ligand binding and the application of mechanical force [104,108]. Similar observations were also made in *Poglut1*$^{-/-}$ mouse models [103,106,109–111], as well as in respective mouse and human cell lines [103,112,113]. In the structures for receptor/ligand complexes, it was striking that consensus O-glucose modifications of Notch did not contribute directly to the interface, but were more peripherally located. There was one exception: the non-consensus glucose modification of Ser435 of Notch EGF11, identified in the DLL4/Notch interface, which had the potential to modulate binding [11,80]. The authors suggested that O-glucose modifications may prevent hydrophobic sites within Notch from aggregating, possibly upon receptor clustering, therefore facilitating protease cleavage at the membrane. This may explain defective trafficking of Notch1 observed in *Poglut1* null HEK293T cells [39].

4.3. O-GlcNAcylation

O-GlcNAc is added to the consensus sequence C^5–X–X–G–X–(S/T)–G–X–X–C^6 by EGF domain-specific O-linked GlcNAc transferase (EOGT) (Figs. 5(b) and (d)) [114,115]. Mass spectrophotometric data have shown that only five out of eighteen O-GlcNAc consensus sites are efficiently modified in *Drosophila* [101], whereas the majority of the seventeen consensus sites present in mouse Notch1 are modified, with a subset of sites extended [116–118]. Wing-specific knockdown of *Eogt* may result in enhanced Notch signaling in *Drosophila* [119], whereas *Eogt* null mice do not show gross morphological abnormalities, with mild defects similar to the phenotypes of decreased Notch signaling [120,121]. Furthermore, *EOGT* loss-of-function mutations have only been implicated in reducing Notch1–DLL1/DLL4 binding and signaling [121]. Direct modification of Notch EGF11 with GlcNAc was identified in the Jag1/Notch1 structure, but like most other O-glucose modifications did not contribute to the binding interface [10]. These data suggest a more nuanced role for this modification.

4.4. Notch ligand O-glycosylation

Compared to Notch receptors, glycosylation of Notch ligands has been less well studied. O-glycans on Notch ligands have been identified through structural and mass spectrometric analyses and a number of ligand EGF domains contain consensus sequences for modifications [10,11,122]. The Jag1/Notch1 co-crystal structure showed the importance of a ligand O-fucose modification in the Notch1 EGF8–Jag1 EGF3 interface [10]. Both *Drosophila* Serrate and mammalian Jag1 O-fucose modifications can be extended by Fng proteins [122], and Serrate is a substrate for O-GlcNAc modification by Eogt [119]. Jag1 also has four O-glucosylation sites on its ECD, all of which are efficiently modified by Poglut1 in the C57BL/6 mouse model [123]. However, unlike Notch receptors, studies showed that O-fucose analogs incorporated into ligands did not affect Notch activity in cell-based reporter assays [93]. Interestingly, in a mouse model heterozygous for Jag1, removal of a single copy of *Rumi* suppressed the defect seen in bile duct development, suggesting a reduction in O-glucosylation compensates for the reduced level of Jag1 [123].

5. Mechanical force

Many insightful experiments performed in *Drosophila* suggested that Notch activation was dependent on mechanical forces. Soluble ligand ECDs or those lacking tails were signaling inhibitors [124,125], whilst loss of function phenotypes of components known to be involved in endocytosis resembled Notch signaling-deficient phenotypes [126,127]. With the advent of structure(s) for the NRR, observations of ligand ECDs within the signal-sending cell, combined with earlier genetic experiments, collectively suggested that a mechanosensory mechanism was operating, with a pulling force generated by endocytosis of ligand.

5.1. Ligand endocytosis

Ligand endocytosis is initiated by ubiquitylation of the intracellular tails—performed by E3 ubiquitin ligases such as Neuralized (Neur) and Mind bomb (Mib) identified in model organisms *Drosophila* and *Xenopus* (Fig. 1) [128–130] The mammalian homolog of Mib, MIB1, acts on all ligands and appears to be the major player, although homologs of Neur do exist. Once ubiquitylation of DSL ligands has occurred the modified proteins are recognized by Epsin [121–133], which interacts with clathrin to create the clathrin-coated pit. Dynamin is likely to be employed for the scission of the invagination to form the endocytic vesicle as studies have confirmed that dynamin plays a role in Delta endocytosis, and Serrate-dependent Notch *trans*-endocytosis is reduced in *Drosophila* wing disc cells carrying a mutation in *shibire*, the *Drosophila* dynamin [127,134–138].

Whilst many studies have identified ligand endocytosis as the source of the pulling force for receptor activation, additional research also suggests the signal-receiving cell may play an important role, with Notch ubiquitinylation by Deltex E3 ubiquitin ligase 4 (DTX-4) and bilateral endocytosis proposed to occur prior to S2 cleavage by ADAM proteases [139,140]. Further work is required to understand the relative contribution of these different elements and the tissue/cell types in which they might occur.

5.2. Catch bond formation

A tension gauge tether assay was used to demonstrate catch bond behavior for Jag1 and DLL4/receptor complexes, and authors suggested that this was mediated through changes in domain interfaces on application of a mechanical force [10]. High resolution structural data for the Jag1/Notch complex identified the C2-DSL intra-molecular interface as a potential inflexion point, and different domain arrangements within isolated structures of ligands were observed [7]. These data help to rationalize how a relatively weak interaction between ligand and receptor, observed in many studies, might ultimately result in NRR cleavage on application of a pulling force. Factors such as O-glycosylation, lipid-binding, and clustering which may favor initial formation/affinity of the receptor/ligand complex, prior to engagement of the catch bond, are presumably required to facilitate generation of the Notch signal under many different physiological conditions in time and space.

6. Insights from genetic disorders

Like many core metazoan pathways, a significant burden of genetic disease is associated with the Notch pathway (Table 2 and Fig. 6 [141–165]). From a biochemical perspective, analysis of genetic disease has helped to rationalize the roles of receptor/ligand paralogs which often show tissue-specific and developmental stage-specific expression. Furthermore, particular receptor/ligand pairings may be identified within the mutational spectrum of a specific disease (see Alagille syndrome (ALGS) and Adams-Oliver syndrome (AOS) below). Missense mutations associated with gain- or loss-of-function may highlight regions of functional importance, whilst mutations resulting in null alleles or nonsense-mediated decay inform on the importance of receptor/ligand quantity.

6.1. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL)

Autosomal dominant mutations in *NOTCH3* are associated with CADASIL (Table 2 and Fig. 6) [157,158]. This is one of the most common inherited small artery diseases of the brain and is characterized by multiple strokes without vascular risk factors, migraine headaches, and vascular dementia in middle-aged adults. Most of the pathogenic mutations are associated with changes in the number of cysteines that stabilize the EGF fold, either by substitution or creation of an additional cysteine, although some atypical variants are described (Fig. 6) [157,166]. This is postulated to lead to receptor misfolding and aggregation, with granular osmiophilic material (GOM), consisting of NOTCH3 ECD, visible in the extracellular space, and located close to the cell surface in smooth muscle cells [166]. This insoluble material is thought to contribute to the vessel wall thickening and decreased blood flow in brain arteries [166]. Widespread white-matter abnormalities can be observed in patients using neuroimaging. Although, a detailed pathological mechanism is still unclear, cellular and transgenic mouse experiments have further suggested that CADASIL mutations result in increased accumulation of NOTCH3 [167–169]. Recently, in both diagnosed European and Japanese patients, it has been found that pathogenic variants affecting N-terminal Notch domains EGF1–6 were significantly correlated with a more severe CADASIL phenotype than those in EGF7–34 [159,170]. It is known that missense mutations affecting EGF-like and related disulfide-rich domains can have surprisingly different outcomes for passage through the secretory pathway suggesting the individual properties of the domains as well as the type of mutation introduced affect their fate [171].

6.2. Bicuspid aortic valve (BAV) disease

Affected individuals with BAV disease have an abnormal aorta in strength and size, which contributes to high risk of developing thoracic aortic aneurysms (TAAs) and acute aortic dissection. Nonsense, missense, and frameshift mutations in *NOTCH1* have been identified in both familial and sporadic cases (Table 2 and Fig. 6) [141,172]. These mutations which cause an early developmental defect in the aortic valve and later calcium deposition, show autosomal-dominant transmission usually via a haploinsufficiency mechanism [172,173]. In BAV patients, messenger RNA (mRNA) levels of Notch signalling components including Notch receptors and downstream transcriptional regulators are found to decrease [174]. *NOTCH1* mutation within neural crest VSMCs of the BAV is suggested to drive VSMC apoptosis leading to disruption of ECM and aortic wall weakness at the same time promoting the contractile phenotype of VSMC, indicative of differentiation, which is unable to upregulate ECM gene expression [173]. While *NOTCH1* mutations have been linked to non-syndromic TAA caused by BAV disease, a recent study identified two *NOTCH1* mutations in

Table 2
Summary of inherited diseases caused by gene mutations affecting Notch ligands and receptors and glycosyltransferases.

Disease	Related genes	Mutations	Phenotypes
ALGS	*JAG1, NOTCH2*	Missense/frameshift/splice site leading to decay/retention of *JAG1*	Hepatic, skeletal, cardiac, renal developmental disorders
EHBA	*JAG1*	Missense site leading to reduced ability of activating Notch	Partial or total absence of bile duct
ToF	*JAG1*	Missense/frameshift mutation, most reported missense mutations cluster at the extreme N-terminus	Hole in septum of heart
CMD	*JAG2, POGLUT1*	Missense, frameshift, nonsense, in-frame deletion, and a larger deletion encompassing *JAG2*	Progressive muscle weakening
CMT2	*JAG1*	Missense mutations in *JAG1*	Partial paralysis of vocal fold and peripheral neuropathy
CADASIL	*NOTCH3*	Predominantly cysteine-related missense mutations in EGF domains	Impaired differentiation and maturation of VSMCs, accumulation of proteins in matrix around VSMCs
AOS	*NOTCH1, DLL4, EOGT*	Addition/deletion of a cysteine residue in EGF domains	Terminal limb-reduction defects, skin/skull absence, and cardiovascular anomalies
BAV disease	*NOTCH1*	Missense/frameshift mutation	Abnormal aortic valve leaflets and aorta
DDD	*POFUT1, POGLUT1*	Non-sense, frameshift and missense mutations contributing to haploinsufficiency of *POFUT1* and *POGLUT1*	Lacy/net-like pattern of abnormally darkened skin
SD	*LFNG, DLL3*	Missense mutation, in-frame deletion of *LFNG* leading to loss of the enzyme activity; in-frame deletion/insertion of *DLL3* leading to truncations of ECD; or missense mutations disrupt EGF repeats of *DLL3*	Fused/deleted/uneven/severe curvature spine, truncal shortening

ALGS: Alagille syndrome; EHBA: extrahepatic biliary atresia; ToF: tetralogy of Fallot; CMD: congenital muscular dystrophy; CMT2: Charcot-Marie-Tooth disease type 2; CADASIL: cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; AOS: Adams-Oliver syndrome; BAV: bicuspid aortic valve; DDD: Dowling-Degos disease; SD: spondylocostal dysostosis.

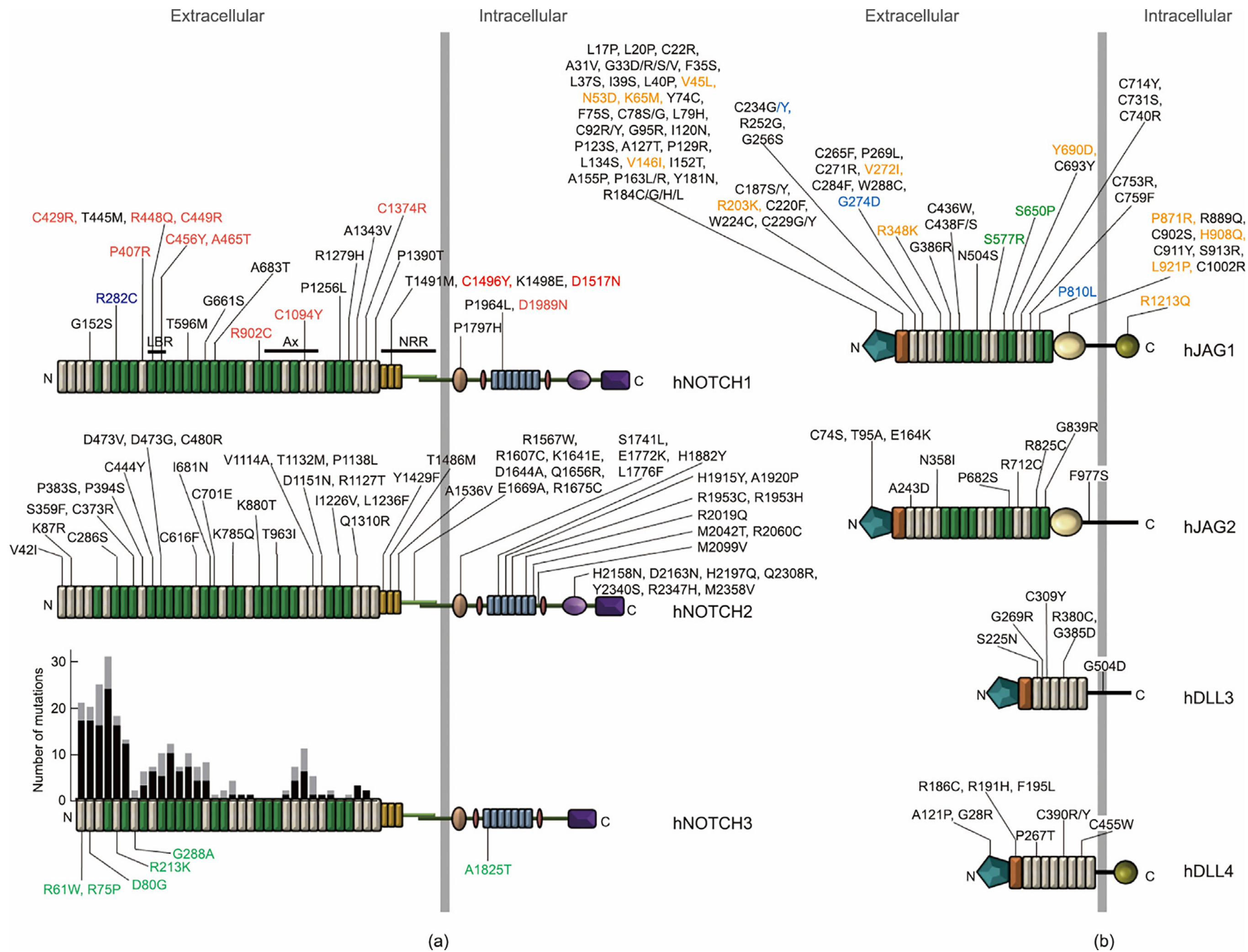

Fig. 6. Map of Notch ligand and receptor missense mutations associated with genetic disease. Reported amino acid substitutions in Notch ligands and receptors are mapped onto schematic diagrams with the same color scheme as Fig. 2. (a) In hNOTCH1, missense mutations leading to BAV disease are labelled in black [141–146], thoracic aortic aneurysm (TAA)/tricuspid aortic valve (TAV) in blue [147], and AOS in red [148–150]. ALGS-associated substitutions are mapped in hNOTCH2 [151–156]. Over 200 CADASIL missense mutations in hNOTCH3 have been reported, and substitutions affecting each EGF domain were counted and labelled (grey: gain-of-cysteine, black: loss-of-cysteine, green: cysteine-sparing) [157–162]. Genome-wide association studies (GWASs) suggested hNOTCH4 as a susceptibility locus for schizophrenia [163] and systemic sclerosis [164]. (b) ALGS (black), ToF (blue), CMT2 (green), and EHBA (orange) substitutions are mapped in hJAG1. CMD, SD, and AOS-associated substitutions are labelled in hJAG2, hDLL3, and hDLL4, respectively. Haploinsufficiency of hDLL1 has been identified as the cause of neurodevelopmental disorder with nonspecific brain abnormalities and with or without seizures (NEDBAS) [165].

TAA in patients with tricuspid aortic valve (TAV), suggesting haploinsufficiency of NOTCH1 might be a pathogenic factor for TAA in the absence of BAV disease (Fig. 6) [147].

6.3. Alagille syndrome (ALGS)/extrahepatic biliary atresia (EHBA)/ tetralogy of Fallot (ToF)/Charcot-Marie-Tooth disease type 2 (CMT2)

ALGS is an autosomal dominant disease, and 94% of identified cases are caused by mutations in *JAG1* [151,175–178] with a small number affecting *NOTCH2* (Table 2 and Fig. 6) [151,152]. Patients with ALGS usually have a variety of developmental disorders such as bile duct paucity, heart and vascular defects, skeletal abnormalities, and liver disease [179,180]. Frameshift, nonsense, and splice site mutations have all been observed, demonstrating haploinsufficiency is the major mechanism underlying dominance. Missense mutations and gene deletions have also been found in patients, but at a lower frequency. Many missense mutations disrupt the JAG1 C2 domain by altering residues in the hydrophobic core of the domain which leads to domain misfolding and a functional haploinsufficiency of JAG1 on the cell surface [65]. By studying ALGS, it has been found that Notch signaling not only participates in liver development and repair, biliary and bile duct development, but is also involved in vascular, cardiac, pulmonary, and kidney development [181–183].

A small number of *JAG1* missense mutations were found to cause an isolated disorder, EHBA (Table 2 and Fig. 6). EHBA is a neonatal liver disease with partial or total absence of bile duct between porta hepatis and the duodenum [184]. Two of these variants affect the loop β1–2 structure of the JAG1 C2 domain associated with lipid-binding and result in the production of folded JAG1, but have a reduced ability to activate Notch in a split luciferase reporter cell line [7]. *In vitro* assays indicate that these variants have a reduced capacity for lipid-binding, with Notch-binding unaffected, suggesting that extrahepatic bile duct development is particularly sensitive to the membrane interaction with JAG1 [7]. In addition, a unique missense mutation in EGF2 of *JAG1*,

p.Gly274Asp, was found to correlate with a ToF phenotype which is the most common form of complex congenital heart malformation involving ventricular septal defect, aortic dextroposition, and right ventricular hypertrophy disorders. *In vitro* experiments suggest this missense mutation affects native folding of EGF2 and leads to partial retention of JAG1 [185–187]. The relatively mild quantitative defect, rather than that associated with haploinsufficiency, may explain why this variant presents as an isolated disorder rather than the more complex phenotype associated with ALGS.

More recently, mutations affecting *JAG1* EGF domains have been identified in patients with autosomal dominant peripheral neuropathy, specifically CMT2 (Table 2 and Fig. 6) [188]. Two serine substitutions in *JAG1*, p.Ser577Arg and p.Ser650Pro, appear to affect its cell surface expression and showed ER retention. Mouse models of these variants were created using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) gene editing and heterozygotes displayed mild peripheral neuropathy, whilst homozygotes were embryonic lethal by mid-gestation. However, the exact cause of defective trafficking of these variants is not established and it is unclear why these missense mutations result in neuropathy. Ser650Pro affects a conserved residue whose backbone carbonyl group may provide a ligand for Ca^{2+} in some cbEGF domains [189].

6.4. Congenital muscular dystrophy (CMD) and JAG2

Mutations in *JAG2*, have recently been shown to be associated with rare forms of CMD, a genetic disorder leading to progressive skeletal muscle weakening (Table 2 and Fig. 6) [190]. The original study covers an international cohort of 23 individuals from 13 families, wherein 15 different *JAG2* mutations were identified through whole exome sequencing, including ten missense, two frameshifts, one nonsense, one in-frame deletion, and one larger deletion encompassing *JAG2* [190]. Through *in silico* analysis of the *JAG2* structure, it is suggested that many *JAG2* missense mutations, which affect C2, DSL domains, and EGF domains, could result in structural changes and protein misfolding [190]. Unusually, compared to other genetic disorders affecting cell surface expressed Notch ligands, the disease shows recessive inheritance, suggesting *JAG2* is less sensitive to gene dosage. The reason for this is unknown, but is also observed for *DLL3*, where mutations lead to spondylocostal dysostosis (SD) [191–193].

6.5. AOS and NOTCH1 and DLL4

Autosomal dominant mutations in *NOTCH1* and *DLL4* genes are associated with AOS, a rare genetic condition associated with abnormal skin development and limb defects which are present from birth (Table 2 and Fig. 6) [194,195]. Diagnosis is based on the presence of terminal limb-reduction defects, an absence or scarred skin, a partial absence or thin skull, and cardiovascular anomalies. Other Notch pathway-related genes may also cause AOS including notably *RBPJ* (dominant) and *EOGT* (recessive). Types of *DLL4* mutations seen include nonsense, frameshift, and missense with no mutational hotspot (Table 2 and Fig. 6) [148,195–197]. The molecular basis of a number of *DLL4* missense mutations has been described which supports a loss of function mechanism, these include p.Gly28Arg (ligand/receptor interface) [197], p.Ala121Pro (β5 strand of hydrophobic core) in the C2 domain, and DSL residue p.Arg186Cys (ligand/receptor interface) [195]. Due to the nature of the substitution, these latter two variants may result in misfolding and a quantitative defect. *NOTCH1* mutations that cause AOS include nonsense, frameshift, and missense [148,149], and it is of interest that some of these are associated with symptoms of BAV disease. Since there is no obvious difference in the nature of the *NOTCH1* mutation which leads to BAV disease or AOS, this suggests that additional genetic modifiers and/or environmental conditions are required for these phenotypes to diverge [150]. AOS missense mutations that are of particular note, following structure determination of Notch/ligand complexes are p.Pro407Arg, p.Arg448Gln, p.Cys449Arg, and p.Cys456Tyr which are in the LBR, although substitution of proline and cysteine residues are likely to lead to EGF domain misfolding with possible cellular retention [149]. Irrespective of the type of dominant mechanism, these mutations are loss-of-function. The phenotypic symptoms of AOS reinforce important roles of NOTCH1 and DLL4 in vasculature and skeletal formation.

6.6. SD and DLL3

SD is an abnormal vertebral segmentation syndrome characterized by rib fusions and deletions, hemivertebrae and loss of vertebrae, which leads to truncal shortening. Most cases are caused by mutations in *DLL3* with an autosomal recessive mode of inheritance, while some cases result from mutation of other Notch signaling-related genes such as lunatic Fng (*LFNG*), *HES7*, and mesodermal posterior 2 (*MESP2*) (Table 2 and Fig. 6) [198]. SD missense mutations in *DLL3* distribute across C2, DSL, and EGF domains with no special hotspots [193,198]. In addition to vertebral and rib malformations, SD patients associated with *DLL3* mutations also exhibit a respiratory infection clinical phenotype [198,199]. Molecular and cellular analysis of X-ray-induced mouse mutation pudgy (*pu*) showed that mutations in *DLL3* alter expression of genes in the segmentation clock, including *LFNG*, *HES1*, and *HES5* [199]. By studying mouse embryo rostral presomitic mesoderm and human cell lines, it has been indicated that DLL3 exerts its function by co-localizing with NOTCH1 in late endosomes and degradative lysosomes, which alters Notch1 trafficking during somitogenesis [23].

6.7. Insights from Notch glycosylation-related disease

Genetic mutations affecting the O-glycosylation machinery have given insights into the importance of such modifications for Notch signaling (Table 2). Despite other EGF domain-containing proteins also acting as substrates for glycosyltransferases, in many cases prominent effects on cell types strongly dependent on Notch are seen [200].

Nonsense, frameshift, and missense mutations affecting *POFUT1* and *POGLUT1* have been reported in the Dowling-Degos disease (DDD), an autosomal dominant pigmentation disorder of relatively late onset, suggesting a particular requirement of melanosome transfer and melanocyte and keratinocyte differentiation for these modifications [201]. In DDD studies, all reported missense mutations are in proximity to the enzyme active site, and *in vitro* assays confirmed that these missense mutations reduced Notch activation [202–204]. A different missense mutation in *POGLUT1* was found to cause a rare autosomal recessive form of limb girdle muscular dystrophy which led to Notch-dependent loss of muscle-specific adult stem cells (satellite cells) [38,110]. Mutations in the *LFNG* gene cause an autosomal recessive form of SD, characterized by abnormal vertebrae and rib development. Using a co-culture system, the authors showed that the LFNG disease-causing variants failed to modulate Notch activation by DLL1, whereas wild type (WT) LFNG potentiated activation. These data confirmed the vital role of LFNG-tuned Notch signaling in skeleton patterning [191,192]. In recent years, an autosomal recessive form of *EOGT* deficiency has also been found to cause AOS (more commonly caused by dominant mutations *NOTCH1* or *DLL4* genes) [205]. These genetic diseases emphasize the functional importance of glycan modifications for Notch signaling with some cell types more sensitive than others to deficiency.

6.8. Mechanistic insights in cancer

6.8.1. Notch as an oncogene

As a key regulator of growth and homeostasis, aberrant Notch signaling has been shown to contribute to the development of various types of cancer (Table 3 [206–235]) [236]. NOTCH plays an oncogenic role in T-cell acute lymphoblastic leukemia (T-ALL), colorectal cancer, breast cancer, ovarian cancer, prostate cancer, splenic marginal zone lymphoma, lung adenocarcinoma, hepatocellular carcinoma, adenoid cystic carcinoma, and glioma, where abnormal NOTCH activation is often linked to poor prognosis, lower overall and relapse-free survival (Table 3) [206,224,225,237–253]. While upregulation of NOTCH activation can be caused by constitutive receptor/ligand expression, activating *NOTCH* mutations detected in multiple types of cancers have provided insight into the oncogenic mechanism [236]. The t(7;9)(q34;q34.3) chromosomal translocation identified in a small number of cases of T-ALL creates a truncated NOTCH1 lacking the NRR which is subjected to rapidly proteolysis to release the NICD, thereby upregulating Notch signaling [254].

Missense mutations which directly affect the NRR of NOTCH1 (Fig. 3) are most commonly associated with T-ALL. Their gain-of-function mechanism can be explained following structure determination of this region. Missense mutations may either enhance dissociation of the two polypeptide chains within the NOTCH HD revealing the S2 site, or destabilize the autoinhibited state of the NRR, causing exposure of the S2 cleavage site without changing the stability of the heterodimer [255]. In addition to T-ALL and related hematological cancers, mutations disrupting the NRR in triple-negative breast cancers, and adenoid cystic carcinoma, have also been found, leading to constitutive NOTCH expression [206,222,223,237,256]. Although not a focus of this review, oncogenic NOTCH mutations which disrupt the intracellular proline/glutamic acid/serine/threonine-rich motif (PEST) domain are also associated with various cancers. These may prevent the normal recognition of phosphorylated PEST domain and subsequent NICD degradation mediated by E3-ubiquitin ligases such as FBXW7, thus enhancing the half-life and accumulation of NICD [206,217,219,222,223,237,257].

6.8.2. Notch as a tumor suppressor

A number of studies of different squamous cell carcinomas (SCC) including head and neck and esophageal SCC have identified Notch as a tumor suppressor (Table 3) [227,258–262]. Mutational analysis has identified loss of function mutations predominantly in *NOTCH1*, but also *NOTCH2* and *NOTCH3*. These may be nonsense, frameshift, or missense mutations affecting regions known to be functionally important including the ligand binding region [227,228,259,263]. Intriguingly, sequencing of aging normal human esophagus has identified the presence of *NOTCH1* loss-of-function mutations (Table 3) [264]. Analysis of normal mouse esophagus recapitulated these data showing that the presence of heterozygous or biallelic *Notch1* mutations in cells conferred a competitive advantage compared to clones expressing WT Notch. The authors further show using a carcinogenesis model that *Notch1* mutations were less common in tumor epithelium compared to normal tissue and *Notch1* deletion resulted in the same outcome, inhibition of tumor growth, as anti-Notch1 treatment. Thus, paradoxically in this system, loss of Notch was protective and use of NOTCH1 inhibitors was proposed as a potential treatment for prevention of esophageal SCC [265]. Possible explanations given to explain the previous association of Notch mutations with esophageal SCC were either that the mutations identified were not in fact tumorigenic but came from normal tissue, or that multiple genomic changes, including *NOTCH1* mutations, collectively caused transformation. At least in the case of esophageal SCC analysis the role of NOTCH1 as a tumor suppressor warrants further analysis.

6.8.3. Cancer-associated mutations

Data bases such as COSMIC and National Cancer Institute (NCI)'s Genomic Data Commons (GDC) identify sequence variants found in a variety of cancer types. JAG1 variants affecting C2 domain residues were shown to exhibit predominantly loss-of-function

Table 3
Summary of *NOTCH*-associated mutations in cancer.

Cancer	Related genes	Mutations	References
Gain-of-function (oncogene)			
T-cell acute lymphoblastic leukemia	*NOTCH1*, *NOTCH3* (rare)	Missense in the NRR and late truncation leading to PEST mutations, frequent frameshift, or nonsense mutations	[206,207]
Early T cell progenitor acute lymphoblastic leukemia	*NOTCH1*	Missense in the NRR and late truncation leading to PEST mutations	[208–210]
Adult T-cell leukemia/lymphoma	*NOTCH1*	Missense, nonsense mutations, and deletions affecting the PEST domain	[211,212]
B-cell chronic lymphocytic leukemia	*NOTCH1*	Late truncation leading to PEST mutations in *NOTCH1*	[213–216]
Mantle cell lymphoma	*NOTCH1*, *NOTCH2*	Late truncation leading to PEST mutations	[217,218]
Marginal zone B cell lymphoma	*NOTCH2*	Missense and late truncation leading to PEST mutations	[219–221]
Breast carcinoma/triple negative breast cancer	*NOTCH1*, *NOTCH2*	Missense mutations, deletions, and gene rearrangement affecting NRR or PEST domains	[222,223]
Adenoid cystic carcinoma	*NOTCH1*	Missense, frameshift mutations in *NOTCH1*, truncating mutations in *NOTCH2*	[224,225]
Glomus tumor	*NOTCH1* (rare), *NOTCH2*, *NOTCH3*	Gene rearrangement leading to significant expression of the NICD	[226]
Loss-of-function (tumor suppressor)			
Squamous cell carcinoma	*NOTCH1*, *NOTCH2*, *NOTCH3*	Missense, in-frame deletions in *NOTCH1/3*, and early truncation resulting from nonsense or frameshift mutations before or within the ANK domain	[227–230]
Bladder transitional cell carcinoma/urothelial carcinoma	*NOTCH1*, *NOTCH2*, *NOTCH3*	Missense, in-frame deletions in *NOTCH1/2*, and early truncation resulting from nonsense or frameshift mutations before or within the ANK domain	[231]
Small-cell lung carcinoma	*NOTCH1*, *NOTCH2*	Missense and early truncation resulting from nonsense or frameshift mutations before or within the ANK domain	[232]
Esophageal carcinoma	*NOTCH1*, *NOTCH2*, *NOTCH3*	Missense, frameshift, and splice site mutations in *NOTCH1*	[233]
Lower-grade glioma	*NOTCH1*	Missense, in-frame deletions, and early truncation resulting from nonsense or frameshift mutations before or within the ANK domain	[234]
Chronic myelomonocytic leukemia	*NOTCH2*	Missense	[235]

molecular phenotypes in reporter cell assays, and a functionally important N-glycan on the loop β5–6 was identified which was highly conserved in the Jagged/Serrate family [14]. A study of cancer-associated O-fucosylation NOTCH1 variants revealed both loss- and gain-of-function phenotypes [81]. NOTCH1 mutations, G310R and T311P in EGF8, and G347S and T349P in EGF9, found in cancers where Notch plays a tumor-suppressive role, reduced O-fucosylation, Notch expression at the cell surface, and ligand-induced Notch activation, suggesting glycosylation of Notch may facilitate Notch trafficking to the membrane. In contrast, G309R, which reduces O-fucosylation of EGF8, had no effect on cell surface Notch levels and hyperactivated ligand-induced Notch activation by an unclear mechanism. On the other hand, N386T in EGF10 and D464N in EGF12 both increased O-fucosylation in the (extended) LBR but had opposing effects on Notch signaling. These studies facilitate our understanding of the mechanistic impact of individual NOTCH/ligand mutations accumulating in cells. A key future challenge is to understand the consequences of such changes for cell lineages and transformation. In addition to specific NOTCH missense mutations altering O-glycosylated residues, changes in expression patterns of the enzymes responsible for O-glycosylation are also associated with cancers including gliomas, T-cell lymphoma, breast cancers, T-ALL, acute myeloid leukemia, pancreatic ductal adenocarcinoma, and chronic lymphocytic leukemia [266–274].

7. Pharmacological agents and natural products targeting the Notch signaling pathway

As abnormal Notch signaling contributes to a broad spectrum of human diseases, pharmacological agents targeting this pathway have attracted a widespread attention. These reagents including γ-secretase inhibitors (GSIs), monoclonal antibodies, antibody-drug conjugates (ADCs), and therapeutic microRNAs, and the majority of these studies have focused on tumor treatments [275,276].

GSIs are the most well-studied small chemical compounds of pan-Notch inhibitors and were initially developed for decreasing amyloid-β peptide production in Alzheimer's disease. Clinical development was aborted due to severe adverse events in patients when used as a life-long treatment. Since more short-term treatment regimes may be appropriate for cancer, GSIs are now widely being studied in Notch-related tumors [275,276], and have been tested in clinical trials for treating advanced solid tumors, desmoid tumors, and triple-negative breast cancer. As GSIs also block the processing of more than 90 other substrates, to improve the specificity of Notch pathway-targeted pharmacological agents, monoclonal antibodies (mAbs) have been developed. For Notch receptor blocking mAbs, two regions known to be functionally important have been targeted, the NRR and the LBR [275]. Meanwhile, mAbs targeting specific Notch ligands have also been developed. For example, DLL4 mAbs were examined for their potential to control tumor angiogenesis [277,278], whilst JAG1-neutralizing mAbs were developed for breast cancer treatment [279]. In recent years, novel ADC agents have been studied to improve the efficiency of delivery of cytotoxic chemicals, such as anti-NOTCH3 ADC PF-06650808, was proved to inhibit tumor growth safely in a phase I clinical trial [280]. Recently, a novel orally active small molecule inhibitor of Notch signaling, CB-103, has been discovered to disrupt RBPJ-NICD transcription complex, which provides a new pharmacological strategy for targeting the Notch signaling pathway [281]. Patients with advanced tumors tolerated CB-103 well in the phase I clinical study, and it is currently in a phase II trial [282].

Natural products and their derivatives are also rich sources of drug discovery, especially for cancer and infectious diseases, and several have been reported to modulate the Notch signaling pathway (Table 4) [283]. Natural products affecting the pathway are mainly grouped in three types: GSIs, receptor modulators, and ligand modulators. These Notch-regulating natural components have been found in either plants or animals. For example, in preclinical studies, Cinobufagin extracted from skin secretions of the Chinese giant toad showed an anti-tumor activity both *in vitro* and *in vivo* through inhibiting NOTCH1 expression [284]. In addition to preclinical studies, several natural products under clinical trials showed Notch-modulating activity (Table 4). For instance, ginsenoside RG3, a bioactive ginseng compound, was found to modulate γ-secretase activity in lipid rafts by increasing levels of phosphoinositide [285]. It has been tested, in combination with transarterial chemoembolization (TACE), on hepatocellular carcinoma patients exhibiting high expression of NOTCH1 [286]. Data showed that the drug combination improved the overall survival time of carcinoma patients compared with TACE alone [286]. Natural products remain an untapped source of potential drugs alongside more conventional drug development pipelines. Meanwhile, recent development of technological methods, including analytical tools improvement and genome editing and microbial culturing advances, empower natural product-based Notch-targeting drug discovery [283].

Table 4
Selected active and recently completed clinical studies of natural products which target the Notch pathway.

Compound	Resources	Target	Clinical trial identifier	Phase	Status	Tumor type/condition	Sponsor and country
Epigallocatechin gallate	Green tea, black tea, etc.	GSI	NCT01183767	II/III	Completed	Duchenne muscular dystrophy	Charite University, Germany
			NCT02891538	I	Recruiting	Colorectal cancer	The University of Texas Health Science Center at San Antonio, USA
Curcumin	Turmeric	GSI	NCT03072992	II	Completed	Advanced breast cancer	National Center of Oncology, USA
			NCT04294836	II	Withdrawn	Advanced cervical cancer	Instituto Nacional de Cancerologia, Colombia
Ginsenoside RG3	Panax ginseng	Lipid/presenilin interaction	NCT02724358	I	Completed	Hepatocellular carcinoma with high NOTCH1 expression	Eastern Hepatobiliary Surgery Hospital, China
Honokiol	Magnolia species	JAG1, γ-secretase expression level	CTR20170822	I	Ongoing	Non-small cell lung cancer	Chengdu Jinrui Foundation Biology Science and Technology Co., Ltd., China
Resveratrol	Grape skins, peanuts, etc.	GSI	NCT01476592	Not provided	Completed	Low grade gastrointestinal tumors	University of Wisconsin, USA

8. Summary

Rapid progress has been made on understanding the mechanism of Notch activation and inhibition in the extracellular space, with genetic and acquired disease associated with core components and their modifiers underscoring the contribution of different elements to this signal transduction pathway. Advances in the toolboxes for structural, cell, and molecular biology are starting to identify features of ligand/receptor complexes which may underlie *cis*- and *trans*-interactions, and how the Notch signals initiated by different Notch ligand combinations are interpreted by cells. We still need to refine our understanding of how Notch signaling output is modulated by various types of glycosylation in ligands and receptors. Fundamental research of the pathway has facilitated the decoding of mutational data revealed by exome sequencing of normal and tumor cell lines, giving insight into the different effects of the Notch signal on the cell. Collectively, these data will ultimately help drive drug discovery and targeted treatments for disease. As Notch regulates a wide variety of physiological processes, targeting Notch signaling pathway safely, precisely, and effectively is challenging. Over the past two decades, a number of novel approaches targeting the Notch pathway have been investigated, which allows the development of more combinatorial treatments. In recent years, more natural products have been reported to modulate Notch signaling, although precise mechanisms of their molecular targets still need to be identified. It would be also useful to explore whether these natural products could be engineered to act more locally and effectively. As discussed, since aberrant Notch signaling leads to a wide variety of diseases, development of Notch-related therapies is urgently required.

Acknowledgments

Penny A. Handford is supported by the Medical Research Council (MRC) Grant (MR/V008935/1). Yao Meng is supported by the National Natural Science Foundation of China (82304596), the Fundamental Research Funds for the Central Universities (3332022057), and the CAMS Innovation Fund for Medical Sciences (2022-I2M-1-016). Xinyi Yang is supported by National Natural Science Foundation of China (81973383).

We apologize in advance to researchers in the field for any of their references which have been omitted due to space limitations.

Compliance with ethics guidelines

Yao Meng, Zhihan Bo, Xinyi Feng, Xinyi Yang, and Penny A. Handford declare that they have no conflicts of interest or financial conflicts to disclose.

References

[1] Gozlan O, Sprinzak D. Notch signaling in development and homeostasis. Development 2023;150(4):dev201138.
[2] Duvall K, Crist L, Perl AJ, Pode Shakked N, Chaturvedi P, Kopan R. Revisiting the role of Notch in nephron segmentation confirms a role for proximal fate selection during mouse and human nephrogenesis. Development 2022;149(10):dev200446.
[3] Seymour PA, Collin CA, Egeskov-Madsen AR, Jørgensen MC, Shimojo H, Imayoshi I, et al. Jag1 modulates an oscillatory Dll1–Notch–Hes1 signaling module to coordinate growth and fate of pancreatic progenitors. Dev Cell 2020;52(6):731–47.e8.
[4] Porcheri C, Golan O, Calero-Nieto FJ, Thambyrajah R, Ruiz-Herguido C, Wang X, et al. Notch ligand Dll4 impairs cell recruitment to aortic clusters and limits blood stem cell generation. EMBO J 2020;39(8):e104270.
[5] Kobia FM, Preusse K, Dai Q, Weaver N, Hass MR, Chaturvedi P, et al. Notch dimerization and gene dosage are important for normal heart development, intestinal stem cell maintenance, and splenic marginal zone B-cell homeostasis during mite infestation. PLoS Biol 2020;18(10):e3000850.
[6] Logeay R, Géminard C, Lassus P, Rodríguez-Vázquez M, Kantar D, Heron-Milhavet L, et al. Mechanisms underlying the cooperation between loss of epithelial polarity and Notch signaling during neoplastic growth in *Drosophila*. Development 2022;149(3):dev200110.
[7] Suckling RJ, Korona B, Whiteman P, Chillakuri C, Holt L, Handford PA, et al. Structural and functional dissection of the interplay between lipid and Notch binding by human Notch ligands. EMBO J 2017;36(15):2204–15.
[8] Meng Y, Sanlidag S, Jensen SA, Burnap SA, Struwe WB, Larsen AH, et al. An N-glycan on the C2 domain of JAGGED1 is important for Notch activation. Sci Signal 2022;15(755):eabo3507.
[9] Martins T, Meng Y, Korona B, Suckling R, Johnson S, Handford PA, et al. The conserved C2 phospholipid-binding domain in Delta contributes to robust Notch signalling. EMBO Rep 2021;22(10):e52729.
[10] Luca VC, Kim BC, Ge C, Kakuda S, Wu D, Roein-Peikar M, et al. Notch-Jagged complex structure implicates a catch bond in tuning ligand sensitivity. Science 2017;355(6331):1320–4.
[11] Luca VC, Jude KM, Pierce NW, Nachury MV, Fischer S, Garcia KC. Structural basis for Notch1 engagement of Delta-like 4. Science 2015;347(6224):847–53.
[12] Bray SJ, Gomez-Lamarca M. Notch after cleavage. Curr Opin Cell Biol 2018;51:103–9.
[13] Falo-Sanjuan J, Lammers NC, Garcia HG, Bray SJ. Enhancer priming enables fast and sustained transcriptional responses to Notch signaling. Dev Cell 2019;50(4):411–25.e8.
[14] Kopan R, Ilagan MXG. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 2009;137(2):216–33.
[15] Oswald F, Kovall RA. CSL-associated corepressor and coactivator complexes. Adv Exp Med Biol 2018;1066:279–95.
[16] Henrique D, Schweisguth F. Mechanisms of Notch signaling: a simple logic deployed in time and space. Development 2019;146(3):dev172148.
[17] Sprinzak D, Blacklow SC. Biophysics of Notch signaling. Annu Rev Biophys 2021;50(1):157–89.
[18] De Celis JF, Bray S. Feed-back mechanisms affecting Notch activation at the dorsoventral boundary in the *Drosophila* wing. Development 1997;124(17):3241–51.
[19] Sprinzak D, Lakhanpal A, Lebon L, Santat LA, Fontes ME, Anderson GA, et al. *Cis*-interactions between Notch and Delta generate mutually exclusive signalling states. Nature 2010;465(7294):86–90.
[20] Del Gaudio F, Liu D, Lendahl U. Notch signalling in healthy and diseased vasculature. Open Biol 2022;12(4):220004.
[21] Troost T, Binshtok U, Sprinzak D, Klein T. *Cis*-inhibition suppresses basal Notch signaling during sensory organ precursor selection. Proc Natl Acad Sci USA 2023;120(23):e2214535120.
[22] Nandagopal N, Santat LA, Elowitz MB. *Cis*-activation in the Notch signaling pathway. Elife 2019;8:e37880.
[23] Chapman G, Sparrow DB, Kremmer E, Dunwoodie SL. Notch inhibition by the ligand Delta-like 3 defines the mechanism of abnormal vertebral segmentation in spondylocostal dysostosis. Hum Mol Genet 2011;20(5):905–16.
[24] Serth K, Schuster-Gossler K, Kremmer E, Hansen B, Marohn-Köhn B, Gossler A. O-fucosylation of DLL3 is required for its function during somitogenesis. PLoS One 2015;10(4):e0123776.
[25] Carrieri FA, Murray PJ, Ditsova D, Ferris MA, Davies P, Dale JK. CDK1 and CDK2 regulate NICD1 turnover and the periodicity of the segmentation clock. EMBO Rep 2019;20(7):e46436.
[26] Giaimo BD, Gagliani EK, Kovall RA, Borggrefe T. Transcription factor RBPJ as a molecular switch in regulating the Notch response. Adv Exp Med Biol 2021;1287:9–30.
[27] Handford PA, Mayhew M, Baron M, Winship PR, Campbell ID, Brownlee GG. Key residues involved in calcium-binding motifs in EGF-like domains. Nature 1991;351(6322):164–7.
[28] Bellavia D, Checquolo S, Campese AF, Felli MP, Gulino A, Screpanti I. Notch3: from subtle structural differences to functional diversity. Oncogene 2008;27(38):5092–8.
[29] James AC, Szot JO, Iyer K, Major JA, Pursglove SE, Chapman G, et al. Notch4 reveals a novel mechanism regulating Notch signal transduction. Biochim Biophys Acta 2014;1843(7):1272–84.
[30] Komatsu H, Chao MY, Larkins-Ford J, Corkins ME, Somers GA, Tucey T, et al. OSM-11 facilitates LIN-12 Notch signaling during *Caenorhabditis elegans* vulval development. PLoS Biol 2008;6(8):e196.
[31] Gordon WR, Roy M, Vardar-Ulu D, Garfinkel M, Mansour MR, Aster JC, et al. Structure of the Notch1-negative regulatory region: implications for normal activation and pathogenic signaling in T-ALL. Blood 2009;113(18):4381–90.
[32] Cordle J, Johnson S, Zi J, Tay Y, Roversi P, Wilkin M, et al. A conserved face of the Jagged/Serrate DSL domain is involved in Notch *trans*-activation and *cis*-inhibition. Nat Struct Mol Biol 2008;15(8):849–57.
[33] Hambleton S, Valeyev NV, Muranyi A, Knott V, Werner JM, McMichael AJ, et al. Structural and functional properties of the human Notch-1 ligand binding region. Structure 2004;12(12):2173–83.
[34] Gordon WR, Vardar-Ulu D, Histen G, Sanchez-Irizarry C, Aster JC, Blacklow SC. Structural basis for autoinhibition of Notch. Nat Struct Mol Biol 2007;14(4):295–300.
[35] Kidd S, Lieber T. Furin cleavage is not a requirement for *Drosophila* Notch function. Mech Dev 2002;115(1–2):41–51.
[36] Logeat F, Bessia C, Brou C, LeBail O, Jarriault S, Seidah NG, et al. The Notch1 receptor is cleaved constitutively by a furin-like convertase. Proc Natl Acad Sci USA 1998;95(14):8108–12.
[37] Pfeffer I, Brewitz L, Krojer T, Jensen SA, Kochan GT, Kershaw NJ, et al. Aspartate/asparagine-β-hydroxylase crystal structures reveal an unexpected

epidermal growth factor-like domain substrate disulfide pattern. Nat Commun 2019;10(1):4910.
[38] Matsumoto K, Luther KB, Haltiwanger RS. Diseases related to Notch glycosylation. Mol Aspects Med 2021;79:100938.
[39] Takeuchi H, Yu H, Hao H, Takeuchi M, Ito A, Li H, et al. O-glycosylation modulates the stability of epidermal growth factor-like repeats and thereby regulates Notch trafficking. J Biol Chem 2017;292(38):15964–73.
[40] Downing AK, Knott V, Werner JM, Cardy CM, Campbell ID, Handford PA. Solution structure of a pair of calcium-binding epidermal growth factor-like domains: implications for the Marfan syndrome and other genetic disorders. Cell 1996;85(4):597–605.
[41] Weisshuhn PC, Sheppard D, Taylor P, Whiteman P, Lea SM, Handford PA, et al. Non-linear and flexible regions of the human Notch1 extracellular domain revealed by high-resolution structural studies. Structure 2016;24(4):555–66.
[42] Kettle S, Yuan X, Grundy G, Knott V, Downing AK, Handford PA. Defective calcium binding to fibrillin-1: consequence of an N2144S change for fibrillin-1 structure and function. J Mol Biol 1999;285(3):1277–87.
[43] De Celis JF, Garcia-Bellido A. Modifications of the Notch function by Abruptex mutations in *Drosophila melanogaster*. Genetics 1994;136(1):183–94.
[44] Rebay I, Fleming RJ, Fehon RG, Cherbas L, Cherbas P, Artavanis-Tsakonas S. Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for notch as a multifunctional receptor. Cell 1991;67(4):687–99.
[45] Taylor P, Takeuchi H, Sheppard D, Chillakuri C, Lea SM, Haltiwanger RS, et al. Fringe-mediated extension of O-linked fucose in the ligand-binding region of Notch1 increases binding to mammalian Notch ligands. Proc Natl Acad Sci USA 2014;111(20):7290–5.
[46] Zeronian MR, Klykov O, Portell i de Montserrat J, Konijnenberg MJ, Gaur A, Scheltema RA, et al. Notch–Jagged signaling complex defined by an interaction mosaic. Proc Natl Acad Sci USA 2021;118(30):e2102502118.
[47] Tiyanont K, Wales TE, Aste-Amezaga M, Aster JC, Engen JR, Blacklow SC. Evidence for increased exposure of the Notch1 metalloprotease cleavage site upon conversion to an activated conformation. Structure 2011;19(4):546–54.
[48] Rand MD, Grimm LM, Artavanis-Tsakonas S, Patriub V, Blacklow SC, Sklar J, et al. Calcium depletion dissociates and activates heterodimeric notch receptors. Mol Cell Biol 2000;20(5):1825–35.
[49] Morsut L, Roybal KT, Xiong X, Gordley RM, Coyle SM, Thomson M, et al. Engineering customized cell sensing and response behaviors using synthetic Notch receptors. Cell 2016;164(4):780–91.
[50] Roybal KT, Rupp LJ, Morsut L, Walker WJ, McNally KA, Park JS, et al. Precision tumor recognition by T cells with combinatorial antigen sensing circuits. Cell 2016;164(4):770–9.
[51] Cho JH, Okuma A, Al-Rubaye D, Intisar E, Junghans RP, Wong WW. Engineering Axl specific CAR and SynNotch receptor for cancer therapy. Sci Rep 2018;8(1):3846.
[52] Wang Z, Wang F, Zhong J, Zhu T, Zheng Y, Zhao T, et al. Using apelin-based synthetic Notch receptors to detect angiogenesis and treat solid tumors. Nat Commun 2020;11(1):2163.
[53] Hyrenius-Wittsten A, Su Y, Park M, Garcia JM, Alavi J, Perry N, et al. SynNotch CAR circuits enhance solid tumor recognition and promote persistent anti-tumor activity in mouse models. Sci Transl Med 2021;13(591):591eabd8836.
[54] Choe JH, Watchmaker PB, Simic MS, Gilbert RD, Li AW, Krasnow NA, et al. SynNotch-CAR T cells overcome challenges of specificity, heterogeneity, and persistence in treating glioblastoma. Sci Transl Med 2021;13(591):eabe7378.
[55] Sloas DC, Tran JC, Marzilli AM, Ngo JT. Tension-tuned receptors for synthetic mechanotransduction and intercellular force detection. Nat Biotechnol 2023;2023(9):1–9.
[56] Yang ZJ, Yu ZY, Cai YM, Du RR, Cai L. Engineering of an enhanced synthetic Notch receptor by reducing ligand-independent activation. Commun Bio 2020;3(1):116.
[57] Hayward AN, Aird EJ, Gordon WR. A toolkit for studying cell surface shedding of diverse transmembrane receptors. eLife 2019;8:e46983.
[58] Gordon WR, Zimmerman B, He L, Miles LJ, Huang J, Tiyanont K, et al. Mechanical allostery: evidence for a force requirement in the proteolytic activation of Notch. Dev Cell 2015;33(6):729–36.
[59] Parks AL, Klueg KM, Stout JR, Muskavitch MA. Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. Development 2000;127(7):1373–85.
[60] Pei J, Grishin NV. Expansion of divergent SEA domains in cell surface proteins and nucleoporin 54. Protein Sci 2017;26(3):617–30.
[61] Kelley MR, Kidd S, Deutsch WA, Young MW. Mutations altering the structure of epidermal growth factor-like coding sequences at the *Drosophila* Notch locus. Cell 1987;51(4):539–48.
[62] Portin P. Allelic negative complementation at the Abruptex locus of *Drosophila melanogaster*. Genetics 1975;81(1):121–33.
[63] Ohlin AK, Landes G, Bourdon P, Oppenheimer C, Wydro R, Stenflo J. Beta-hydroxyaspartic acid in the first epidermal growth factor-like domain of protein C. Its role in Ca^{2+} binding and biological activity. J Biol Chem 1988;263(35):19240–8.
[64] Foster GG. Negative complementation at the notch locus of *Drosophila melanogaster*. Genetics 1975;81(1):99–120.
[65] Chillakuri CR, Sheppard D, Ilagan MX, Holt LR, Abbott F, Liang S, et al. Structural analysis uncovers lipid-binding properties of Notch ligands. Cell Rep 2013;5(4):861–7.
[66] Law RHP, Lukoyanova N, Voskoboinik I, Caradoc-Davies TT, Baran K, Dunstone MA, et al. The structural basis for membrane binding and pore formation by lymphocyte perforin. Nature 2010;468(7322):447–51.
[67] Corbalan-Garcia S, Gómez-Fernández JC. Signaling through C2 domains: more than one lipid target. Biochim Biophys Acta 2014;1838(6):1536–47.
[68] Hirano Y, Gao YG, Stephenson DJ, Vu NT, Malinina L, Simanshu DK, et al. Structural basis of phosphatidylcholine recognition by the C2-domain of cytosolic phospholipase A2α. eLife 2019;8:e44760.
[69] Kershaw NJ, Church NL, Griffin MDW, Luo CS, Adams TE, Burgess AW. Notch ligand delta-like1: X-ray crystal structure and binding affinity. Biochem J 2015;468(1):159–66.
[70] Shimizu K, Chiba S, Kumano K, Hosoya N, Takahashi T, Kanda Y, et al. Mouse Jagged1 physically interacts with Notch2 and other Notch receptors. Assessment by quantitative methods. J Biol Chem 1999;274(46):32961–9.
[71] Fleming RJ. Ligand-induced *cis*-inhibition of Notch signaling: the role of an extracellular region of Serrate. Adv Exp Med Biol 2020;1227:29–49.
[72] D'souza B, Miyamoto A, Weinmaster G. The many facets of Notch ligands. Oncogene 2008;27(38):5148–67.
[73] Kiyota T, Kinoshita T. Cysteine-rich region of X-Serrate-1 is required for activation of Notch signaling in *Xenopus* primary neurogenesis. Int J Dev Biol 2002;46:1057–60.
[74] Yamamoto S, Charng WL, Rana NA, Kakuda S, Jaiswal M, Bayat V, et al. A mutation in EGF repeat-8 of notch discriminates between Serrate/Jagged and delta family ligands. Science 2012;338(6111):1229–32.
[75] Gonzalez-Perez D, Das S, Antfolk D, Ahsan HS, Medina E, Dundes CE, et al. Affinity-matured DLL4 ligands as broad-spectrum modulators of Notch signaling. Nat Chem Biol 2023;19(1):9–17.
[76] Irvine KD, Wieschaus E. Fringe, a boundary-specific signaling molecule, mediates interactions between dorsal and ventral cells during *Drosophila* wing development. Cell 1994;79(4):595–606.
[77] Brückner K, Perez L, Clausen H, Cohen S. Glycosyltransferase activity of fringe modulates Notch–Delta interactions. Nature 2000;406(6794):411–5.
[78] Moloney DJ, Panin VM, Johnston SH, Chen J, Shao L, Wilson R, et al. Fringe is a glycosyltransferase that modifies Notch. Nature 2000;406(6794):369–75.
[79] Takeuchi H, Schneider M, Williamson DB, Ito A, Takeuchi M, Handford PA, et al. Two novel protein O-glucosyltransferases that modify sites distinct from POGLUT1 and affect Notch trafficking and signaling. Proc Natl Acad Sci USA 2018;115(36):E8395–402.
[80] Andrawes MB, Xu X, Liu H, Ficarro SB, Marto JA, Aster JC, et al. Intrinsic selectivity of Notch 1 for Delta-like 4 over Delta-like 1. J Biol Chem 2013;288(35):25477–89.
[81] Pennarubia F, Ito A, Takeuchi M, Haltiwanger RS. Cancer-associated Notch receptor variants lead to O-fucosylation defects that deregulate Notch signaling. J Biol Chem 2022;298(12):102616.
[82] Yokoi Y, Nishimura SI. Effect of site-specific O-glycosylation on the structural behavior of NOTCH1 receptor extracellular EGF-like domains 11 and 10. Chemistry 2020;26(54):12363–72.
[83] Saiki W, Ma C, Okajima T, Takeuchi H. Current views on the roles of O-glycosylation in controlling notch-ligand interactions. Biomolecules 2021;11(2):309.
[84] Takeuchi H, Haltiwanger RS. Role of glycosylation of Notch in development. Semin Cell Dev Biol 2010;21(6):638–45.
[85] Wang Y, Lee GF, Kelley RF, Spellman MW. Identification of a GDP-L-fucose: polypeptide fucosyltransferase and enzymatic addition of O-linked fucose to EGF domains. Glycobiology 1996;6(8):837–42.
[86] Wang Y, Shao L, Shi S, Harris RJ, Spellman MW, Stanley P, et al. Modification of epidermal growth factor-like repeats with O-fucose. Molecular cloning and expression of a novel GDP-fucose protein O-fucosyltransferase. J Biol Chem 2001;276:40338–45.
[87] Holdener BC, Haltiwanger RS. Protein O-fucosylation: structure and function. Curr Opin Struct Biol 2019;56:78–86.
[88] Johnston SH, Rauskolb C, Wilson R, Prabhakaran B, Irvine KD, Vogt TF. A family of mammalian fringe genes implicated in boundary determination and the Notch pathway. Development 1997;124(11):2245–54.
[89] Cohen B, Bashirullah A, Dagnino L, Campbell C, Fisher WW, Leow CC, et al. Fringe boundaries coincide with Notch-dependent patterning centres in mammals and alter Notch-dependent development in *Drosophila*. Nat Genet 1997;16(3):283–8.
[90] LeBon L, Lee TV, Sprinzak D, Jafar-Nejad H, Elowitz MB. Fringe proteins modulate Notch-ligand cis and trans interactions to specify signaling states. eLife 2014;3:e02950.
[91] Pandey A, Harvey BM, Lopez MF, Ito A, Haltiwanger RS, Jafar-Nejad H. Glycosylation of specific Notch EGF repeats by O-Fut1 and fringe regulates Notch signaling in *Drosophila*. Cell Rep 2019;29(7):2054–66.e6.
[92] Kakuda S, Haltiwanger RS. Deciphering the fringe-mediated Notch code: identification of activating and inhibiting sites allowing discrimination between ligands. Dev Cell 2017;40(2):193–201.
[93] Schneider M, Kumar V, Nordstrøm LU, Feng L, Takeuchi H, Hao H, et al. Inhibition of Delta-induced Notch signaling using fucose analogs. Nat Chem Biol 2018;14(1):65–71.
[94] Acar M, Jafar-Nejad H, Takeuchi H, Rajan A, Ibrani D, Rana NA, et al. Rumi is a CAP10 domain glycosyltransferase that modifies Notch and is required for Notch signaling. Cell 2008;132(2):247–58.
[95] Takeuchi H, Fernández-Valdivia RC, Caswell DS, Nita-Lazar A, Rana NA, Garner TP, et al. Rumi functions as both a protein O-glucosyltransferase and a protein O-xylosyltransferase. Proc Natl Acad Sci USA 2011;108(40):16600–5.
[96] Moloney DJ, Shair LH, Lu FM, Xia J, Locke R, Matta KL, et al. Mammalian Notch1 is modified with two unusual forms of O-linked glycosylation found on epidermal growth factor-like modules*. J Biol Chem 2000;275(13):9604–11.

[97] Shao L, Luo Y, Moloney DJ, Haltiwanger RS. O-glycosylation of EGF repeats: identification and initial characterization of a UDP-glucose: protein O-glucosyltransferase. Glycobiology 2002;12(11):763–70.
[98] Rana NA, Nita-Lazar A, Takeuchi H, Kakuda S, Luther KB, Haltiwanger RS. O-glucose trisaccharide is present at high but variable stoichiometry at multiple sites on mouse Notch1. J Biol Chem 2011;286(36):31623–37.
[99] Li Z, Fischer M, Satkunarajah M, Zhou D, Withers SG, Rini JM. Structural basis of Notch O-glucosylation and O-xylosylation by mammalian protein-O-glucosyltransferase 1 (POGLUT1). Nat Commun 2017;8(1):185.
[100] Williamson DB, Haltiwanger RS. Identification, function, and biological relevance of POGLUT2 and POGLUT3. Biochem Soc Trans 2022;50(2):1003–12.
[101] Harvey BM, Rana NA, Moss H, Leonardi J, Jafar-Nejad H, Haltiwanger RS. Mapping sites of O-glycosylation and fringe elongation on *Drosophila* Notch. J Biol Chem 2016;291(31):16348–60.
[102] Lee TV, Sethi MK, Leonardi J, Rana NA, Buettner FFR, Haltiwanger RS, et al. Negative regulation of Notch signaling by Xylose. PLoS Genet 2013;9(6):e1003547.
[103] Fernandez-Valdivia R, Takeuchi H, Samarghandi A, Lopez M, Leonardi J, Haltiwanger RS, et al. Regulation of mammalian Notch signaling and embryonic development by the protein O-glucosyltransferase Rumi. Development 2011;138(10):1925–34.
[104] Leonardi J, Fernandez-Valdivia R, Li YD, Simcox AA, Jafar-Nejad H. Multiple O-glucosylation sites on Notch function as a buffer against temperature-dependent loss of signaling. Development 2011;138(16):3569–78.
[105] Perdigoto CN, Schweisguth F, Bardin AJ. Distinct levels of Notch activity for commitment and terminal differentiation of stem cells in the adult fly intestine. Development 2011;138(21):4585–95.
[106] Pandey A, Niknejad N, Jafar-Nejad H. Multifaceted regulation of Notch signaling by glycosylation. Glycobiology 2021;31:8–28.
[107] Lieber T, Kidd S, Young MW. Kuzbanian-mediated cleavage of *Drosophila* Notch. Genes Dev 2002;16(2):209–21.
[108] Jafar-Nejad H, Leonardi J, Fernandez-Valdivia R. Role of glycans and glycosyltransferases in the regulation of Notch signaling. Glycobiology 2010;20(8):931–49.
[109] Ramkumar N, Harvey BM, Lee JD, Alcorn HL, Silva-Gagliardi NF, McGlade CJ, et al. Protein O-glucosyltransferase 1 (POGLUT1) promotes mouse gastrulation through modification of the apical polarity protein CRUMBS2. PLoS Genet 2015;11(10):e1005551.
[110] Servián-Morilla E, Takeuchi H, Lee TV, Clarimon J, Mavillard F, Area-Gómez E, et al. A POGLUT1 mutation causes a muscular dystrophy with reduced Notch signaling and satellite cell loss. EMBO Mol Med 2016;8(11):1289–309.
[111] Servián-Morilla E, Cabrera-Serrano M, Johnson K, Pandey A, Ito A, Rivas E, et al. POGLUT1 biallelic mutations cause myopathy with reduced satellite cells, α-dystroglycan hypoglycosylation and a distinctive radiological pattern. Acta Neuropathol 2020;139(3):565–82.
[112] Ma W, Du J, Chu Q, Wang Y, Liu L, Song M, et al. hCLP46 regulates U937 cell proliferation via Notch signaling pathway. Biochem Biophys Res Commun 2011;408(1):84–8.
[113] Chu Q, Liu L, Wang W. Overexpression of hCLP46 enhances Notch activation and regulates cell proliferation in a cell type-dependent manner. Cell Prolif 2013;46(3):254–62.
[114] Matsuura A, Ito M, Sakaidani Y, Kondo T, Murakami K, Furukawa K, et al. O-linked N-acetylglucosamine is present on the extracellular domain of notch receptors. J Biol Chem 2008;283(51):35486–95.
[115] Sakaidani Y, Nomura T, Matsuura A, Ito M, Suzuki E, Murakami K, et al. O-linked-N-acetylglucosamine on extracellular protein domains mediates epithelial cell–matrix interactions. Nat Commun 2011;2(1):583.
[116] Ogawa M, Senoo Y, Ikeda K, Takeuchi H, Okajima T. Structural divergence in O-GlcNAc glycans displayed on epidermal growth factor-like repeats of mammalian Notch1. Molecules 2018;23(7):1745.
[117] Ogawa M, Okajima T. Structure and function of extracellular O-GlcNAc. Curr Opin Struct Biol 2019;56:72–7.
[118] Sakaidani Y, Ichiyanagi N, Saito C, Nomura T, Ito M, Nishio Y, et al. O-linked-N-acetylglucosamine modification of mammalian Notch receptors by an atypical O-GlcNAc transferase Eogt1. Biochem Biophys Res Commun 2012;419(1):14–9.
[119] Müller R, Jenny A, Stanley P. The EGF repeat-specific O-GlcNAc-transferase Eogt interacts with notch signaling and pyrimidine metabolism pathways in *Drosophila*. PLoS One 2013;8(5):e62835.
[120] Varshney S, Stanley P. Multiple roles for O-glycans in Notch signalling. FEBS Lett 2018;592(23):3819–34.
[121] Sawaguchi S, Varshney S, Ogawa M, Sakaidani Y, Yagi H, Takeshita K, et al. O-GlcNAc on NOTCH1 EGF repeats regulates ligand-induced Notch signaling and vascular development in mammals. eLife 2017;6:e24419.
[122] Panin VM, Shao L, Lei L, Moloney DJ, Irvine KD, Haltiwanger RS. Notch ligands are substrates for protein O-Fucosyltransferase-1 and Fringe. J Biol Chem 2002;277(33):29945–52.
[123] Thakurdas SM, Lopez MF, Kakuda S, Fernandez-Valdivia R, Zarrin-Khameh N, Haltiwanger RS, et al. Jagged1 heterozygosity in mice results in a congenital cholangiopathy which is reversed by concomitant deletion of one copy of Poglut1 (Rumi). Hepatology 2016;63(2):550–65.
[124] Sun X, Artavanis-Tsakonas S. The intracellular deletions of Delta and Serrate define dominant negative forms of the *Drosophila* Notch ligands. Development 1996;122(8):2465–74.
[125] Sun X, Artavanis-Tsakonas S. Secreted forms of DELTA and SERRATE define antagonists of Notch signaling in *Drosophila*. Development 1997;124(17):3439–48.
[126] Poodry CA. Shibire, a neurogenic mutant of *Drosophila*. Dev Biol 1990;138(2):464–72.
[127] Meloty-Kapella L, Shergill B, Kuon J, Botvinick E, Weinmaster G. Notch ligand endocytosis generates mechanical pulling force dependent on dynamin, epsins, and actin. Dev Cell 2012;22(6):1299–312.
[128] Le Borgne R, Bardin A, Schweisguth F. The roles of receptor and ligand endocytosis in regulating Notch signaling. Development 2005;132(8):1751–62.
[129] Deblandre GA, Lai EC, Kintner C. Xenopus neuralized is a ubiquitin ligase that interacts with XDelta1 and regulates Notch signaling. Dev Cell 2001;1(6):795–806.
[130] Itoh M, Kim CH, Palardy G, Oda T, Jiang YJ, Maust D, et al. Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. Dev Cell 2003;4(1):67–82.
[131] Xie X, Cho B, Fischer JA. *Drosophila* Epsin's role in Notch ligand cells requires three Epsin protein functions: the lipid binding function of the ENTH domain, a single ubiquitin interaction motif, and a subset of the C-terminal protein binding modules. Dev Biol 2012;363(2):399–412.
[132] Wang W, Struhl G. *Drosophila* Epsin mediates a select endocytic pathway that DSL ligands must enter to activate Notch. Development 2004;131(21):5367–80.
[133] Wang W, Struhl G. Distinct roles for mind bomb, neuralized and spsin in mediating DSL endocytosis and signaling in *Drosophila*. Development 2005;132(12):2883–94.
[134] Okano M, Matsuo H, Nishimura Y, Hozumi K, Yoshioka S, Tonoki A, et al. Mib1 modulates dynamin 2 recruitment via Snx18 to promote Dll1 endocytosis for efficient Notch signaling. Genes Cells 2016;21(5):425–41.
[135] Seugnet L, Simpson P, Haenlin M. Requirement for dynamin during notch signaling in *Drosophila* neurogenesis. Dev Biol 1997;192(2):585–98.
[136] Windler SL, Bilder D. Endocytic internalization routes required for Delta/Notch signaling. Curr Biol 2010;20(6):538–43.
[137] Couso JP, Knust E, Martinez AA. Serrate and wingless cooperate to induce vestigial gene expression and wing formation in *Drosophila*. Curr Biol 1995;5(12):1437–48.
[138] Glittenberg M, Pitsouli C, Garvey C, Delidakis C, Bray S. Role of conserved intracellular motifs in Serrate signalling, *cis*-inhibition and endocytosis. EMBO J 2006;25(20):4697–706.
[139] Chapman G, Major JA, Iyer K, James AC, Pursglove SE, Moreau JLM, et al. Notch1 endocytosis is induced by ligand and is required for signal transduction. Biochim Biophys Acta 2016;1863(1):166–77.
[140] Chastagner P, Rubinstein E, Brou C. Ligand-activated Notch undergoes DTX4-mediated ubiquitylation and bilateral endocytosis before ADAM10 processing. Sci Signal 2017;10(483):eaag2989.
[141] Mohamed SA, Aherrahrou Z, Liptau H, Erasmi AW, Hagemann C, Wrobel S, et al. Novel missense mutations (p. T596M and p.P1797H) in *NOTCH1* in patients with bicuspid aortic valve. Biochem Biophys Res Commun 2006;345(4):1460–5.
[142] McKellar SH, Tester DJ, Yagubyan M, Majumdar R, Ackerman MJ, Sundt III TM. Novel *NOTCH1* mutations in patients with bicuspid aortic valve disease and thoracic aortic aneurysms. J Thorac Cardiovasc Surg 2007;134(2):290–6.
[143] McBride KL, Riley MF, Zender GA, Fitzgerald-Butt SM, Towbin JA, Belmont JW, et al. *NOTCH1* mutations in individuals with left ventricular outflow tract malformations reduce ligand-induced signaling. Hum Mol Genet 2008;17(18):2886–93.
[144] Theis JL, Hrstka SCL, Evans JM, O'Byrne MM, de Andrade M, O'Leary PW, et al. Compound heterozygous *NOTCH1* mutations underlie impaired cardiogenesis in a patient with hypoplastic left heart syndrome. Hum Genet 2015;134(9):1003–11.
[145] Dargis N, Lamontagne M, Gaudreault N, Sbarra L, Henry C, Pibarot P, et al. Identification of gender-specific genetic variants in patients with bicuspid aortic valve. Am J Cardiol 2016;117(3):420–6.
[146] Girdauskas E, Geist L, Disha K, Kazakbaev I, Groß T, Schulz S, et al. Genetic abnormalities in bicuspid aortic valve root phenotype: preliminary results. Eur J Cardiothorac Surg 2017;52(1):156–62.
[147] Torres-Juan L, Rico Y, Fortuny E, Pons J, Ramos R, Santos-Simarro F, et al. *NOTCH1* gene as a novel cause of thoracic aortic aneurysm in patients with tricuspid aortic valve: two cases reported. Int J Mol Sci 2023;24(10):8644.
[148] Stittrich AB, Lehman A, Bodian DL, Ashworth J, Zong Z, Li H, et al. Mutations in *NOTCH1* cause Adams-Oliver syndrome. Am J Hum Genet 2014;95(3):275–84.
[149] Southgate L, Sukalo M, Karountzos ASV, Taylor EJ, Collinson CS, Ruddy D, et al. Haploinsufficiency of the NOTCH1 receptor as a cause of Adams-Oliver syndrome with variable cardiac anomalies. Circ Cardiovasc Genet 2015;8(4):572–81.
[150] Meester JAN, Sukalo M, Schröder KC, Schanze D, Baynam G, Borck G, et al. Elucidating the genetic architecture of Adams-Oliver syndrome in a large European cohort. Hum Mutat 2018;39(9):1246–61.
[151] Gilbert MA, Bauer RC, Rajagopalan R, Grochowski CM, Chao G, McEldrew D, et al. Alagille syndrome mutation update: comprehensive overview of *JAG1* and *NOTCH2* mutation frequencies and insight into missense variant classification. Hum Mutat 2019;40(12):2197–220.
[152] Kamath BM, Bauer RC, Loomes KM, Chao G, Gerfen J, Hutchinson A, et al. *NOTCH2* mutations in Alagille syndrome. J Med Genet 2012;49:138–44.

[153] ShenTu Y, Mi X, Tang D, Jiang Y, Gao L, Ma X, et al. Alagille syndrome caused by *NOTCH2* mutation presented atypical pathological changes. Clin Chim Acta 2021;521:258–63.
[154] Uddin MS, Fulayyih SA, Denaini FFA, Hatlani MMA. Pathogenic novel heterozygous variant c.1076c>T p. (Ser359Phe) chr1: 120512166 in *NOTCH2* gene, type 2 alagille syndrome causing neonatal cholestasis: a case report. Am J Case Rep 2022;23:e935840.
[155] Li ZD, Abuduxikuer K, Wang L, Hao CZ, Zhang J, Wang MX, et al. Defining pathogenicity of *NOTCH2* variants for diagnosis of Alagille syndrome type 2 using a large cohort of patients. Liver Int 2022;42(8):1836–48.
[156] Li J, Wu H, Chen S, Pang J, Wang H, Li X, et al. Clinical and genetic characteristics of Alagille syndrome in adults. J Clin Transl Hepatol 2023;11:156–62.
[157] Joutel A, Corpechot C, Ducros A, Vahedi K, Chabriat H, Mouton P, et al. *Notch3* mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. Nature 1996;383(6602):707–10.
[158] Coupland K, Lendahl U, Karlström H. Role of *NOTCH3* mutations in the cerebral small vessel disease cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. Stroke 2018;49(11):2793–800.
[159] Mukai M, Mizuta I, Watanabe-Hosomi A, Koizumi T, Matsuura J, Hamano A, et al. Genotype–phenotype correlations and effect of mutation location in Japanese CADASIL patients. J Hum Genet 2020;65(8):637–46.
[160] Mizuno T, Mizuta I, Watanabe-Hosomi A, Mukai M, Koizumi T. Clinical and genetic aspects of CADASIL. Front Aging Neurosci 2020;12:91.
[161] Rodriguez CA, Fustes OJH, Arteaga CBT. A novel Notch 3 mutation (pathogenic variant c.1565G>C) in CADASIL. Neurologia 2022;37(3):235–6.
[162] Ni W, Zhang Y, Zhang L, Xie JJ, Li HF, Wu ZY. Genetic spectrum of NOTCH3 and clinical phenotype of CADASIL patients in different populations. CNS Neurosci Ther 2022;28(11):1779–89.
[163] Wei J, Hemmings GP. The *NOTCH4* locus is associated with susceptibility to schizophrenia. Nat Genet 2000;25(4):376–7.
[164] Cardinale CJ, Li D, Tian L, Connolly JJ, March ME, Hou C, et al. Association of a rare *NOTCH4* coding variant with systemic sclerosis: a family-based whole exome sequencing study. BMC Musculoskelet Disord 2016;17(1):462.
[165] Fischer-Zirnsak B, Segebrecht L, Schubach M, Charles P, Alderman E, Brown K, et al. Haploinsufficiency of the Notch ligand DLL1 causes variable neurodevelopmental disorders. Am J Hum Genet 2019;105(3):631–9.
[166] Chabriat H, Joutel A, Dichgans M, Tournier-Lasserve E, Bousser MG. Cadasil. Lancet Neurol 2009;8(7):643–53.
[167] Karlström H, Beatus P, Dannaeus K, Chapman G, Lendahl U, Lundkvist J. A CADASIL-mutated Notch 3 receptor exhibits impaired intracellular trafficking and maturation but normal ligand-induced signaling. Proc Natl Acad Sci USA 2002;99(26):17119–24.
[168] Pippucci T, Maresca A, Magini P, Cenacchi G, Donadio V, Palombo F, et al. Homozygous *NOTCH3* null mutation and impaired NOTCH3 signaling in recessive early-onset arteriopathy and cavitating leukoencephalopathy. EMBO Mol Med 2015;7(6):848–58.
[169] Klein C, Schreyer S, Kohrs FE, Elhamoury P, Pfeffer A, Munder T, et al. Stimulation of adult hippocampal neurogenesis by physical exercise and enriched environment is disturbed in a CADASIL mouse model. Sci Rep 2017;7(1):45372.
[170] Rutten JW, Van Eijsden BJ, Duering M, Jouvent E, Opherk C, Pantoni L, et al. The effect of *NOTCH3* pathogenic variant position on CADASIL disease severity: *NOTCH3 EGFR 1–6* pathogenic variant are associated with a more severe phenotype and lower survival compared with *EGFR 7–34* pathogenic variant. Genet Med 2019;21(3):676–82.
[171] Jensen SA, Iqbal S, Bulsiewicz A, Handford PA. A microfibril assembly assay identifies different mechanisms of dominance underlying Marfan syndrome, stiff skin syndrome and acromelic dysplasias. Hum Mol Genet 2015;24(15):4454–63.
[172] Garg V, Muth AN, Ransom JF, Schluterman MK, Barnes R, King IN, et al. Mutations in *NOTCH1* cause aortic valve disease. Nature 2005;437(7056):270–4.
[173] Harrison OJ, Torrens C, Salhiyyah K, Modi A, Moorjani N, Townsend PA, et al. Defective NOTCH signalling drives smooth muscle cell death and differentiation in bicuspid aortic valve aortopathy. Eur J Cardiothorac Surg 2019;56(1):117–25.
[174] Sciacca S, Pilato M, Mazzoccoli G, Pazienza V, Vinciguerra M. Anti-correlation between longevity gene *SirT1* and Notch signaling in ascending aorta biopsies from patients with bicuspid aortic valve disease. Heart Vessels 2013;28(2):268–75.
[175] Li L, Krantz ID, Deng Y, Genin A, Banta AB, Collins CC, et al. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nat Genet 1997;16(3):243–51.
[176] Kamath BM, Spinner NB, Rosenblum ND. Renal involvement and the role of Notch signalling in Alagille syndrome. Nat Rev Nephrol 2013;9(7):409–18.
[177] Birtel J, Eisenberger T, Gliem M, Müller PL, Herrmann P, Betz C, et al. Clinical and genetic characteristics of 251 consecutive patients with macular and cone/cone-rod dystrophy. Sci Rep 2018;8(1):4824.
[178] Fischetto R, Palmieri VV, Tripaldi ME, Gaeta A, Michelucci A, Delvecchio M, et al. Alagille syndrome: a novel mutation in *JAG1* gene. Front Pediatr 2019;7:199.
[179] Fabris L, Fiorotto R, Spirli C, Cadamuro M, Mariotti V, Perugorria MJ, et al. Pathobiology of inherited biliary diseases: a roadmap to understand acquired liver diseases. Nat Rev Gastroenterol Hepatol 2019;16(8):497–511.
[180] Hankeova S, Van Hul N, Laznovsky J, Verboven E, Mangold K, Hensens N, et al. Sex differences and risk factors for bleeding in Alagille syndrome. EMBO Mol Med 2022;14(12):e15809.
[181] Hankeova S, Salplachta J, Zikmund T, Kavkova M, Van Hul N, Brinek A, et al. DUCT reveals architectural mechanisms contributing to bile duct recovery in a mouse model for Alagille syndrome. eLife 2021;10:e60916.
[182] Meester JAN, Verstraeten A, Alaerts M, Schepers D, Van Laer L, Loeys BL. Overlapping but distinct roles for NOTCH receptors in human cardiovascular disease. Clin Genet 2019;95(1):85–94.
[183] Fabris L, Cadamuro M, Guido M, Spirli C, Fiorotto R, Colledan M, et al. Analysis of liver repair mechanisms in Alagille syndrome and biliary atresia reveals a role for Notch signaling. Am J Pathol 2007;171(2):641–53.
[184] Kohsaka T, Yuan Z, Guo S, Tagawa M, Nakamura A, Nakano M, et al. The significance of human Jagged 1 mutations detected in severe cases of extrahepatic biliary atresia. Hepatology 2002;36(4):904–12.
[185] Guarnaccia C, Dhir S, Pintar A, Pongor S. The tetralogy of Fallot-associated G274D mutation impairs folding of the second epidermal growth factor repeat in Jagged-1. FEBS J 2009;276(21):6247–57.
[186] Eldadah ZA, Hamosh A, Biery NJ, Montgomery RA, Duke M, Elkins R, et al. Familial tetralogy of Fallot caused by mutation in the Jagged1 gene. Hum Mol Genet 2001;10(2):163–9.
[187] Bauer RC, Laney AO, Smith R, Gerfen J, Morrissette JJD, Woyciechowski S, et al. Jagged1 (*JAG1*) mutations in patients with tetralogy of Fallot or pulmonic stenosis. Hum Mutat 2010;31(5):594–601.
[188] Sullivan JM, Motley WW, Johnson JO, Aisenberg WH, Marshall KL, Barwick KE, et al. Dominant mutations of the Notch ligand Jagged1 cause peripheral neuropathy. J Clin Invest 2020;130(3):1506–12.
[189] Lee SSJ, Knott V, Jovanović J, Harlos K, Grimes JM, Choulier L, et al. Structure of the integrin binding fragment from fibrillin-1 gives new insights into microfibril organization. Structure 2004;12(4):717–29.
[190] Coppens S, Barnard AM, Puusepp S, Pajusalu S, Õunap K, Vargas-Franco D, et al. A form of muscular dystrophy associated with pathogenic variants in *JAG2*. Am J Hum Genet 2021;108(5):840–56.
[191] Sparrow DB, Chapman G, Wouters MA, Whittock NV, Ellard S, Fatkin D, et al. Mutation of the *LUNATIC FRINGE* gene in humans causes spondylocostal dysostosis with a severe vertebral phenotype. Am J Hum Genet 2006;78(1):28–37.
[192] Otomo N, Mizumoto S, Lu HF, Takeda K, Campos-Xavier B, Mittaz-Crettol L, et al. Identification of novel *LFNG* mutations in spondylocostal dysostosis. J Hum Genet 2019;64(3):261–4.
[193] Bulman MP, Kusumi K, Frayling TM, McKeown C, Garrett C, Lander ES, et al. Mutations in the human Delta homologue, DLL3, cause axial skeletal defects in spondylocostal dysostosis. Nat Genet 2000;24(4):438–41.
[194] Adams FH, Oliver CP. Hereditary deformities in man. J Hered 1945;36(1):3–7.
[195] Meester JAN, Southgate L, Stittrich AB, Venselaar H, Beekmans SJA, Den Hollander N, et al. Heterozygous loss-of-function mutations in DLL4 cause Adams-Oliver syndrome. Am J Hum Genet 2015;97(3):475–82.
[196] Nagasaka M, Taniguchi-Ikeda M, Inagaki H, Ouchi Y, Kurokawa D, Yamana K, et al. Novel missense mutation in *DLL4* in a Japanese sporadic case of Adams-Oliver syndrome. J Hum Genet 2017;62(9):851–5.
[197] Rojnueangnit K, Phawan T, Khetkham T, Techasatid W, Sirichongkolthong B. A novel *DLL4* mutation in Adams-Oliver syndrome with absence of the right pulmonary artery in newborn. Am J Med Genet A 2022;188(2):658–64.
[198] Umair M, Younus M, Shafiq S, Nayab A, Alfadhel M. Clinical genetics of spondylocostal dysostosis: a mini review. Front Genet 2022;13:996364.
[199] Kusumi K, Mimoto MS, Covello KL, Beddington RSP, Krumlauf R, Dunwoodie SL. Dll3 pudgy mutation differentially disrupts dynamic expression of somite genes. Genesis 2004;39(2):115–21.
[200] Mehboob MZ, Lang M. Structure, function, and pathology of protein O-glucosyltransferases. Cell Death Dis 2021;12(1):71.
[201] Stephan C, Kurban M, Abbas O. Dowling-Degos disease: a review. Int J Dermatol 2021;60(8):944–50.
[202] Buket Basmanav F, Oprisoreanu AM, Pasternack SM, Thiele H, Fritz G, Wenzel J, et al. Mutations in *POGLUT1*, encoding protein O-glucosyltransferase 1, cause autosomal-dominant Dowling-Degos disease. Am J Hum Genet 2014;91(1):135–43.
[203] McMillan BJ, Zimmerman B, Egan ED, Lofgren M, Xu X, Hesser A, et al. Structure of human POFUT1, its requirement in ligand-independent oncogenic Notch signaling, and functional effects of Dowling-Degos mutations. Glycobiology 2017;27(8):777–86.
[204] Li M, Cheng R, Liang J, Yan H, Zhang H, Yang L, et al. Mutations in *POFUT1*, encoding protein O-fucosyltransferase 1, cause generalized Dowling-Degos disease. Am J Hum Genet 2013;92(6):895–903.
[205] Shaheen R, Aglan M, Keppler-Noreuil K, Faqeih E, Ansari S, Horton K, et al. Mutations in *EOGT* confirm the genetic heterogeneity of autosomal-recessive Adams-Oliver syndrome. Am J Hum Genet 2013;92(4):598–604.
[206] Weng AP, Ferrando AA, Lee W, Morris IV JP, Silverman LB, Sanchez-Irizarry C, et al. Activating mutations of *NOTCH1* in human T cell acute lymphoblastic leukemia. Science 2004;306(5694):269–71.
[207] Xu X, Choi SH, Hu T, Tiyanont K, Habets R, Groot AJ, et al. Insights into autoregulation of Notch3 from structural and functional studies of its negative regulatory region. Structure 2015;23(7):1227–35.
[208] Van Vlierberghe P, Ambesi-Impiombato A, Perez-Garcia A, Haydu JE, Rigo I, Hadler M, et al. *ETV6* mutations in early immature human T cell leukemias. J Exp Med 2011;208(13):2571–9.

[209] Zhang J, Ding L, Holmfeldt L, Wu G, Heatley SL, Payne-Turner D, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature 2012;481(7380):157–63.
[210] Neumann M, Heesch S, Schlee C, Schwartz S, Gökbuget N, Hoelzer D, et al. Whole-exome sequencing in adult ETP—all reveals a high rate of *DNMT3A* mutations. Blood 2013;121(23):4749–52.
[211] Shimizu D, Taki T, Utsunomiya A, Nakagawa H, Nomura K, Matsumoto Y, et al. Detection of *NOTCH1* mutations in adult T-cell leukemia/lymphoma and peripheral T-cell lymphoma. Int J Hematol 2007;85(3):212–8.
[212] Pancewicz J, Taylor JM, Datta A, Baydoun HH, Waldmann TA, Hermine O, et al. Notch signaling contributes to proliferation and tumor formation of human T-cell leukemia virus type 1-associated adult T-cell leukemia. Proc Natl Acad Sci USA 2010;107(38):16619–24.
[213] Sportoletti P, Baldoni S, Cavalli L, Del Papa B, Bonifacio E, Ciurnelli R, et al. *NOTCH1* PEST domain mutation is an adverse prognostic factor in B-CLL. Br J Haematol 2010;151(4):404–6.
[214] Di Ianni M, Baldoni S, Rosati E, Ciurnelli R, Cavalli L, Martelli MF, et al. A new genetic lesion in B-CLL: a *NOTCH1* PEST domain mutation. Br J Haematol 2009;146(6):689–91.
[215] Puente XS, Beà S, Valdés-Mas R, Villamor N, Gutiérrez-Abril J, Martín-Subero JI, et al. Non-coding recurrent mutations in chronic lymphocytic leukaemia. Nature 2015;526(7574):519–24.
[216] Puente XS, Pinyol M, Quesada V, Conde L, Ordóñez GR, Villamor N, et al. Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. Nature 2011;475(7354):101–5.
[217] Kridel R, Meissner B, Rogic S, Boyle M, Telenius A, Woolcock B, et al. Whole transcriptome sequencing reveals recurrent *NOTCH1* mutations in mantle cell lymphoma. Blood 2012;119(9):1963–71.
[218] Beà S, Valdés-Mas R, Navarro A, Salaverria I, Martín-Garcia D, Jares P, et al. Landscape of somatic mutations and clonal evolution in mantle cell lymphoma. Proc Natl Acad Sci USA 2013;110(45):18250–5.
[219] Kiel MJ, Velusamy T, Betz BL, Zhao L, Weigelin HG, Chiang MY, et al. Whole-genome sequencing identifies recurrent somatic *NOTCH2* mutations in splenic marginal zone lymphoma. J Exp Med 2012;209(9):1553–65.
[220] Rossi D, Trifonov V, Fangazio M, Bruscaggin A, Rasi S, Spina V, et al. The coding genome of splenic marginal zone lymphoma: activation of NOTCH2 and other pathways regulating marginal zone development. J Exp Med 2012;209(9):1537–51.
[221] Trøen G, Wlodarska I, Warsame A, Hernández Llodrà S, De Wolf-Peeters C, Delabie J. *NOTCH2* mutations in marginal zone lymphoma. Haematologica 2008;93(7):1107–9.
[222] Robinson DR, Kalyana-Sundaram S, Wu YM, Shankar S, Cao X, Ateeq B, et al. Functionally recurrent rearrangements of the MAST kinase and *Notch* gene families in breast cancer. Nat Med 2011;17(12):1646–51.
[223] Stoeck A, Lejnine S, Truong A, Pan L, Wang H, Zang C, et al. Discovery of biomarkers predictive of GSI response in triple-negative breast cancer and adenoid cystic carcinoma. Cancer Discov 2014;4(10):1154–67.
[224] Ho AS, Kannan K, Roy DM, Morris LGT, Ganly I, Katabi N, et al. The mutational landscape of adenoid cystic carcinoma. Nat Genet 2013;45(7):791–8.
[225] Stephens PJ, Davies HR, Mitani Y, Van Loo P, Shlien A, Tarpey PS, et al. Whole exome sequencing of adenoid cystic carcinoma. J Clin Invest 2013;123 (7):2965–8.
[226] Mosquera JM, Sboner A, Zhang L, Chen CL, Sung YS, Chen HW, et al. Novel MIR143–NOTCH fusions in benign and malignant glomus tumors. Genes Chromosomes Cancer 2013;52(11):1075–87.
[227] Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K, Li RJ, et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in *NOTCH1*. Science 2011;333(6046):1154–7.
[228] Wang NJ, Sanborn Z, Arnett KL, Bayston LJ, Liao W, Proby CM, et al. Loss-of-function mutations in Notch receptors in cutaneous and lung squamous cell carcinoma. Proc Natl Acad Sci USA 2011;108(43):17761–6.
[229] Durinck S, Ho C, Wang NJ, Liao W, Jakkula LR, Collisson EA, et al. Temporal dissection of tumorigenesis in primary cancers. Cancer Discov 2011;1 (2):137–43.
[230] Cancer Genome Atlas Research Network. Comprehensive genomic characterization of squamous cell lung cancers. Nature 2012;489(7417):519–25.
[231] Rampias T, Vgenopoulou P, Avgeris M, Polyzos A, Stravodimos K, Valavanis C, et al. A new tumor suppressor role for the Notch pathway in bladder cancer. Nat Med 2014;20(10):1199–205.
[232] George J, Lim JS, Jang SJ, Cun Y, Ozretić L, Kong G, et al. Comprehensive genomic profiles of small cell lung cancer. Nature 2015;524(7563):47–53.
[233] Song Y, Li L, Ou Y, Gao Z, Li E, Li X, et al. Identification of genomic alterations in oesophageal squamous cell cancer. Nature 2014;509(7498):91–5.
[234] Brat DJ, Verhaak RGW, Aldape KD, Yung WKA, Salama SR, Cooper LAD, et al. Comprehensive, integrative genomic analysis of diffuse lower-grade gliomas. N Engl J Med 2015;372(26):2481–98.
[235] Klinakis A, Lobry C, Abdel-Wahab O, Oh P, Haeno H, Buonamici S, et al. A novel tumour-suppressor function for the Notch pathway in myeloid leukaemia. Nature 2011;473(7346):230–3.
[236] Aster JC, Pear WS, Blacklow SC. The varied roles of Notch in cancer. Annu Rev Pathol 2017;12(1):245–75.
[237] Bernasconi-Elias P, Hu T, Jenkins D, Firestone B, Gans S, Kurth E, et al. Characterization of activating mutations of *NOTCH3* in T cell acute lymphoblastic leukemia and anti-leukemic activity of NOTCH3 inhibitory antibodies. Oncogene 2016;35(47):6077–86.
[238] Bonfiglio F, Bruscaggin A, Guidetti F, Terzi di Bergamo L, Faderl M, Spina V, et al. Genetic and phenotypic attributes of splenic marginal zone lymphoma. Blood 2022;139(5):732–47.
[239] Choi JH, Park JT, Davidson B, Morin PJ, Shih IM, Wang TL. Jagged-1 and Notch3 Juxtacrine loop regulates ovarian tumor growth and adhesion. Cancer Res 2008;68(14):5716–23.
[240] Gao J, Liu J, Fan D, Xu H, Xiong Y, Wang Y, et al. Up-regulated expression of Notch1 and Jagged1 in human colon adenocarcinoma. Pathol Biol 2011;59 (6):298–302.
[241] Guo D, Ye J, Li L, Dai J, Ma D, Ji C. Down-regulation of Notch-1 increases co-cultured Jurkat cell sensitivity to chemotherapy. Leuk Lymphoma 2009;50 (2):270–8.
[242] Jubb AM, Browning L, Campo L, Turley H, Steers G, Thurston G, et al. Expression of vascular Notch ligands Delta-like 4 and Jagged-1 in glioblastoma. Histopathology 2012;60(5):740–7.
[243] Kim EJ, Kim SO, Jin X, Ham SW, Kim J, Park JB, et al. Epidermal growth factor receptor variant III renders glioma cancer cells less differentiated by JAGGED1. Tumour Biol 2015;36(4):2921–8.
[244] Reedijk M, Odorcic S, Chang L, Zhang H, Miller N, McCready DR, et al. High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. Cancer Res 2005;65 (18):8530–7.
[245] Santagata S, Demichelis F, Riva A, Varambally S, Hofer MD, Kutok JL, et al. JAGGED1 expression is associated with prostate cancer metastasis and recurrence. Cancer Res 2004;64(19):6854–7.
[246] Strati TM, Kotoula V, Kostopoulos I, Manousou K, Papadimitriou C, Lazaridis G, et al. Prognostic subcellular Notch2, Notch3 and Jagged1 localization patterns in early triple-negative breast cancer. Anticancer Res 2017;37(5):2323–34.
[247] Sugiyama M, Oki E, Nakaji Y, Tsutsumi S, Ono N, Nakanishi R, et al. High expression of the Notch ligand Jagged-1 is associated with poor prognosis after surgery for colorectal cancer. Cancer Sci 2016;107(11):1705–16.
[248] Vaish V, Kim J, Shim M. Jagged-2 (JAG2) enhances tumorigenicity and chemoresistance of colorectal cancer cells. Oncotarget 2017;8(32):53262–75.
[249] Westhoff B, Colaluca IN, D'Ario G, Donzelli M, Tosoni D, Volorio S, et al. Alterations of the Notch pathway in lung cancer. Proc Natl Acad Sci USA 2009;106(52):22293–8.
[250] Yuan X, Wu H, Xu H, Han N, Chu Q, Yu S, et al. Meta-analysis reveals the correlation of Notch signaling with non-small cell lung cancer progression and prognosis. Sci Rep 2015;5(1):10338.
[251] Zheng CG, Chen R, Xie JB, Liu CB, Jin Z, Jin C. Immunohistochemical expression of Notch1, Jagged1, NF-κB and MMP-9 in colorectal cancer patients and the relationship to clinicopathological parameters. Cancer Biomark 2015;15 (6):889–97.
[252] Zhu C, Ho YJ, Salomao MA, Dapito DH, Bartolome A, Schwabe RF, et al. Notch activity characterizes a common hepatocellular carcinoma subtype with unique molecular and clinicopathologic features. J Hepatol 2021;74 (3):613–26.
[253] Lim JS, Ibaseta A, Fischer MM, Cancilla B, O'Young G, Cristea S, et al. Intratumoural heterogeneity generated by Notch signalling promotes small-cell lung cancer. Nature 2017;545(7654):360–4.
[254] Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD, et al. *TAN-1*, the human homolog of the *Drosophila* Notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell 1991;66(4):649–61.
[255] Malecki MJ, Sanchez-Irizarry C, Mitchell JL, Histen G, Xu ML, Aster JC, et al. Leukemia-associated mutations within the NOTCH1 heterodimerization domain fall into at least two distinct mechanistic classes. Mol Cell Biol 2006;26(12):4642–51.
[256] Ferrarotto R, Mitani Y, Diao L, Guijarro I, Wang J, Zweidler-McKay P, et al. Activating *NOTCH1* mutations define a distinct subgroup of patients with adenoid cystic carcinoma who have poor prognosis, propensity to bone and liver metastasis, and potential responsiveness to Notch1 inhibitors. J Clin Oncol 2017;35(3):352–60.
[257] Ianni MD, Baldoni S, Rosati E, Ciurnelli R, Cavalli L, Martelli MF, et al. A new genetic lesion in B-CLL: a *NOTCH1* PEST domain mutation. Br J Haematol 2009;146(6):689–91.
[258] Hammerman PS, Voet D, Lawrence MS, Voet D, Jing R, Cibulskis K, et al. Comprehensive genomic characterization of squamous cell lung cancers. Nature 2012;489(7417):519–25.
[259] Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, et al. The mutational landscape of head and neck squamous cell carcinoma. Science 2011;333(6046):1157–60.
[260] Alcolea MP, Greulich P, Wabik A, Frede J, Simons BD, Jones PH. Differentiation imbalance in single oesophageal progenitor cells causes clonal immortalization and field change. Nat Cell Biol 2014;16(6):615.
[261] Sawangarun W, Mandasari M, Aida J, Morita K, Kayamori K, Ikeda T, et al. Loss of Notch1 predisposes oro-esophageal epithelium to tumorigenesis. Exp Cell Res 2018;372(2):129–40.
[262] Boukhatmi H, Martins T, Pillidge Z, Kamenova T, Bray S. Notch mediates inter-tissue communication to promote tumorigenesis. Curr Biol 2020;30 (10):1809–20.e4.
[263] Nowell CS, Radtke F. Notch as a tumour suppressor. Nat Rev Cancer 2017;17 (3):145–59.
[264] Martincorena I, Fowler JC, Wabik A, Lawson ARJ, Abascal F, Hall MWJ, et al. Somatic mutant clones colonize the human esophagus with age. Science 2018;362(6417):911–7.

[265] Abby E, Dentro SC, Hall MWJ, Fowler JC, Ong SH, Sood R, et al. *Notch1* mutations drive clonal expansion in normal esophageal epithelium but impair tumor growth. Nat Gene 2023;55(2):232–45.
[266] Zhang S, Chung WC, Wu G, Egan SE, Miele L, Xu K. Manic fringe promotes a claudin-low breast cancer phenotype through Notch-mediated PIK3CG induction. Cancer Res 2015;75(10):1936–43.
[267] Wang S, Itoh M, Shiratori E, Ohtaka M, Tohda S. *NOTCH* activation promotes glycosyltransferase expression in human myeloid leukemia cells. Hematol Rep 2018;10(3):7576.
[268] Yang C, Hu JF, Zhan Q, Wang ZW, Li G, Pan JJ, et al. SHCBP1 interacting with EOGT enhances O-GlcNAcylation of NOTCH1 and promotes the development of pancreatic cancer. Genomics 2021;113(2):827–42.
[269] Libisch MG, Casás M, Chiribao ML, Moreno P, Cayota A, Osinaga E, et al. GALNT11 as a new molecular marker in chronic lymphocytic leukemia. Gene 2014;533(1):270–9.
[270] Barua R, Mizuno K, Tashima Y, Ogawa M, Takeuchi H, Taguchi A, et al. Bioinformatics and functional analyses implicate potential roles for EOGT and L-fringe in pancreatic cancers. Molecules 2021;26(4):882.
[271] Wang Y, Chang N, Zhang T, Liu H, Ma W, Chu Q, et al. Overexpression of human CAP10-like protein 46KD in T-acute lymphoblastic leukemia and acute myelogenous leukemia. Genet Test Mol Biomarkers 2010;14(1):127–33.
[272] Xu K, Usary J, Kousis PC, Prat A, Wang DY, Adams JR, et al. Lunatic fringe deficiency cooperates with the Met/Caveolin gene amplicon to induce basal-like breast cancer. Cancer Cell 2012;21(5):626–41.
[273] Kroes RA, Dawson G, Moskal JR. Focused microarray analysis of glyco-gene expression in human glioblastomas. J Neurochem 2007;103(Suppl 1):14–24.
[274] Larose H, Prokoph N, Matthews JD, Schlederer M, Högler S, Alsulami AF, et al. Whole exome sequencing reveals *NOTCH1* mutations in anaplastic large cell lymphoma and points to Notch both as a key pathway and a potential therapeutic target. Haematologica 2021;106(6):1693–704.
[275] Majumder S, Crabtree JS, Golde TE, Minter LM, Osborne BA, Miele L. Targeting Notch in oncology: the path forward. Nat Rev Drug Discov 2021;20(2):125–44.
[276] Andersson ER, Lendahl U. Therapeutic modulation of Notch signalling—are we there yet? Nat Rev Drug Discov 2014;13(5):357–78.
[277] Ridgway J, Zhang G, Wu Y, Stawicki S, Liang WC, Chanthery Y, et al. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. Nature 2006;444(7122):1083–7.
[278] Noguera-Troise I, Daly C, Papadopoulos NJ, Coetzee S, Boland P, Gale NW, et al. Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. Nature 2006;444(7122):1032–7.
[279] Masiero M, Li D, Whiteman P, Bentley C, Greig J, Hassanali T, et al. Development of therapeutic anti-JAGGED1 antibodies for cancer therapy. Mol Cancer Ther 2019;18(11):2030–42.
[280] Rosen LS, Wesolowski R, Baffa R, Liao KH, Hua SY, Gibson BL, et al. A phase I, dose-escalation study of PF-06650808, an anti-Notch3 antibody–drug conjugate, in patients with breast cancer and other advanced solid tumors. Invest New Drugs 2020;38(1):120–30.
[281] Lehal R, Zaric J, Vigolo M, Urech C, Frismantas V, Zangger N, et al. Pharmacological disruption of the Notch transcription factor complex. Proc Natl Acad Sci USA 2020;117(28):16292–301.
[282] Lopez Miranda E, Stathis A, Hess D, Racca F, Quon D, Rodon J, et al. Phase 1 study of CB-103, a novel first-in-class inhibitor of the *CSL-NICD* gene transcription factor complex in human cancers. J Clin Oncol 2021;39 (15_suppl):3020.
[283] Atanasov AG, Zotchev SB, Dirsch VM, Supuran CT. Natural products in drug discovery: advances and opportunities. Nat Rev Drug Discov 2021;20 (3):200–16.
[284] Cao Y, Yu L, Dai G, Zhang S, Zhang Z, Gao T, et al. Cinobufagin induces apoptosis of osteosarcoma cells through inactivation of Notch signaling. Eur J Pharmacol 2017;794:77–84.
[285] Kang MS, Baek SH, Chun YS, Moore AZ, Landman N, Berman D, et al. Modulation of lipid kinase PI4KIIα activity and lipid raft association of presenilin 1 underlies γ-secretase inhibition by ginsenoside (20S)-Rg3. J Biol Chem 2013;288(29):20868–82.
[286] Zhou B, Yan Z, Liu R, Shi P, Qian S, Qu X, et al. Prospective study of transcatheter arterial chemoembolization (TACE) with ginsenoside Rg3 versus TACE alone for the treatment of patients with advanced hepatocellular carcinoma. Radiology 2016;280(2):630–9.