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The Notch Signaling Pathway: Mechanistic Insights in Health and Disease

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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.

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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

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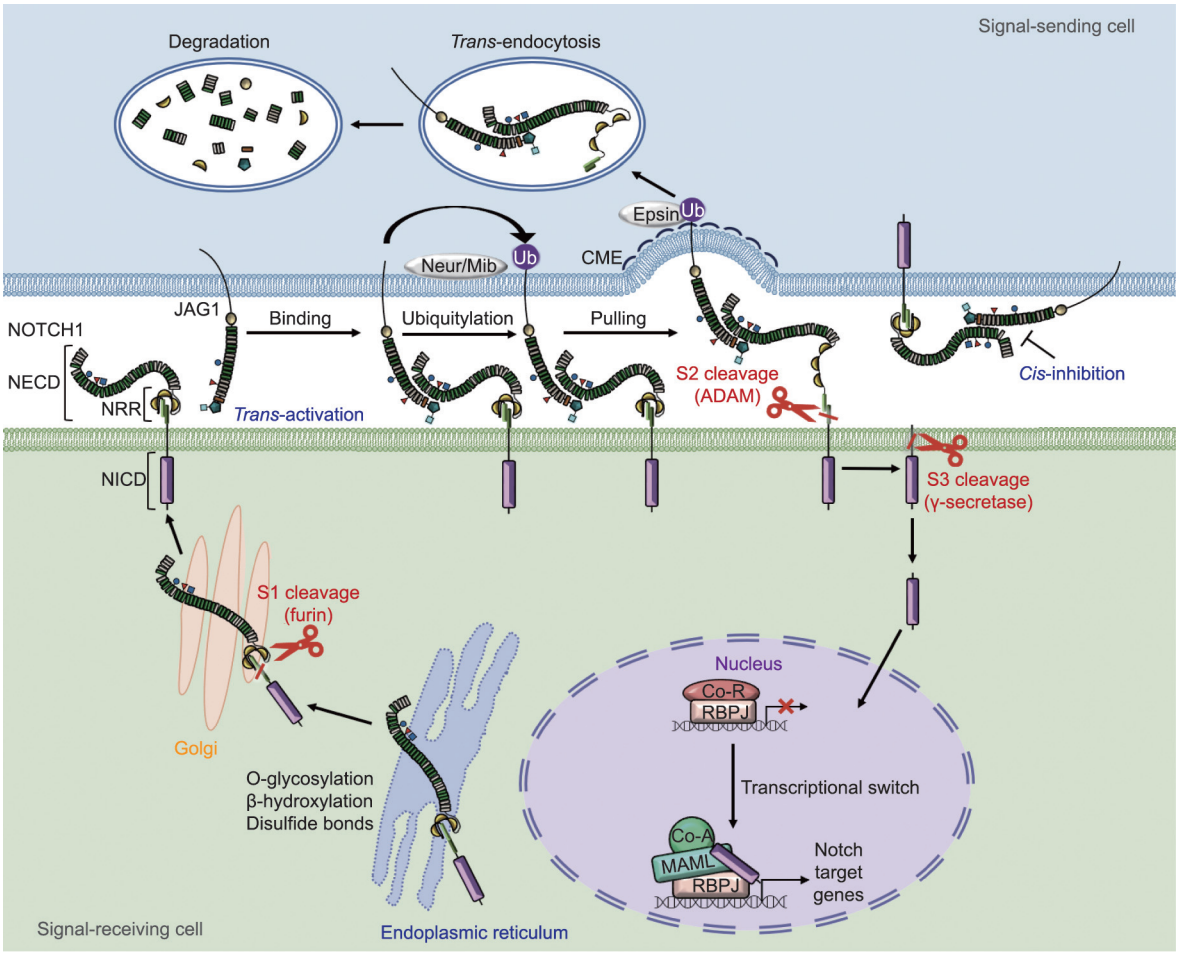

Fig. 1. Overview of the canonical Notch signaling pathway. The newly synthesised Notch receptor (human NOTCH1 shown) undergoes various post-translational modifications (PTMs) to its ECD: a furin-catalysed S1 cleavage yields a heterodimeric form comprising a Notch ECD (NECD) and a Notch transmembrane-intracellular 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

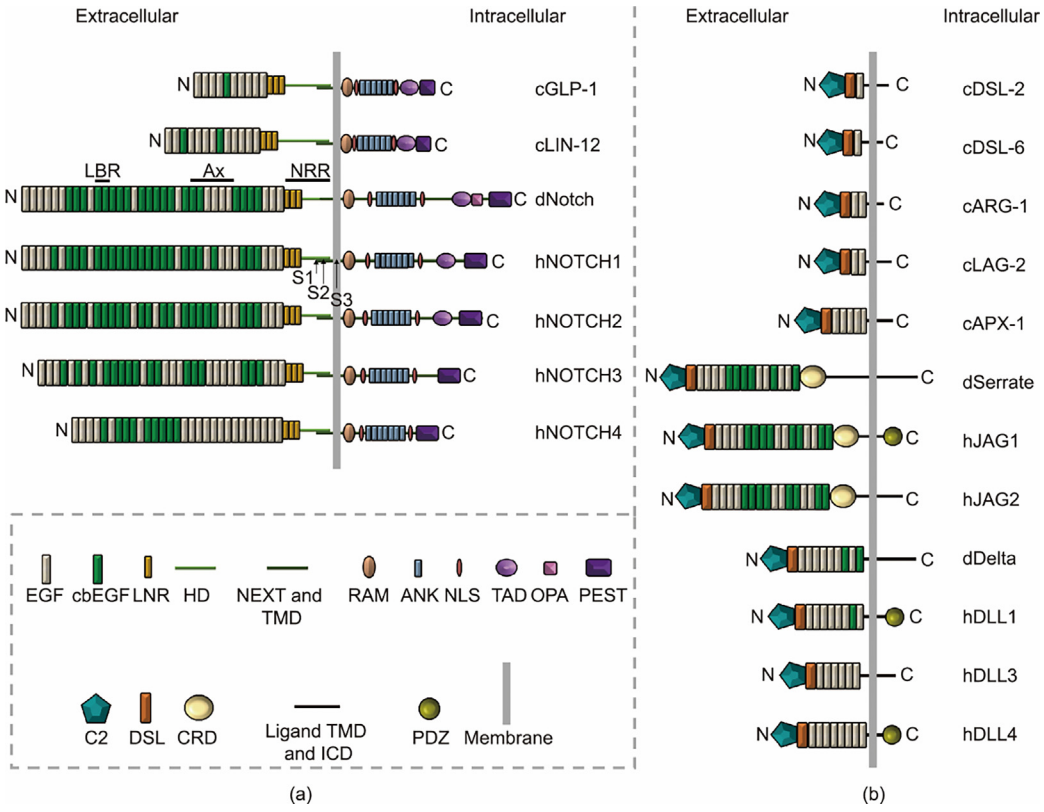

Fig. 2. Domain organisation of Notch receptors and ligands. Notch receptors and ligands are type I transmembrane proteins containing predominantly multiple EGF-like domains, either non-calcium binding (EGF) or calcium binding (cbEGF), in their ECDs. cbEGF repeats are labelled based on a consensus sequence: [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™, 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 $mmol \cdot L^{-1}$) Ca^{2+} levels, demonstrated flexible properties, supporting the earlier NMR study of EGF9–10 [46]. In addition, the ECD had a maximum

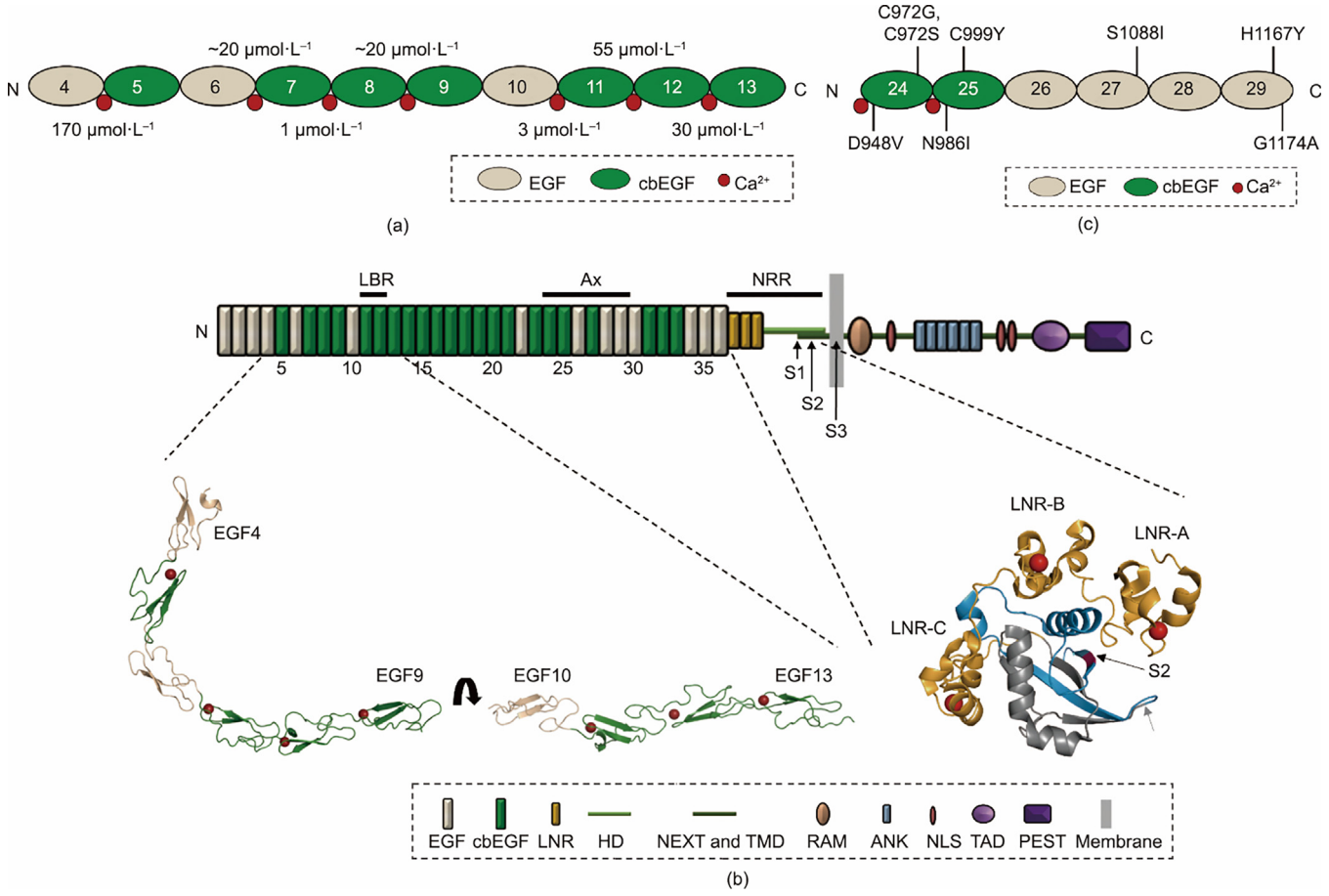

Fig. 3. Notch ECD structural features. (a) The Ca^{2+} dissociation constants at pH 7.5 and I = 0.15 for hNOTCH1 EGF4–13 measured by Weisshuhn et al. [41]. (b) Model of EGF4–13 region of the ECD of hNOTCH1. The models for the EGF4–9 and EGF10–13 regions of NOTCH1 are based on the X-ray structures (Protein 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

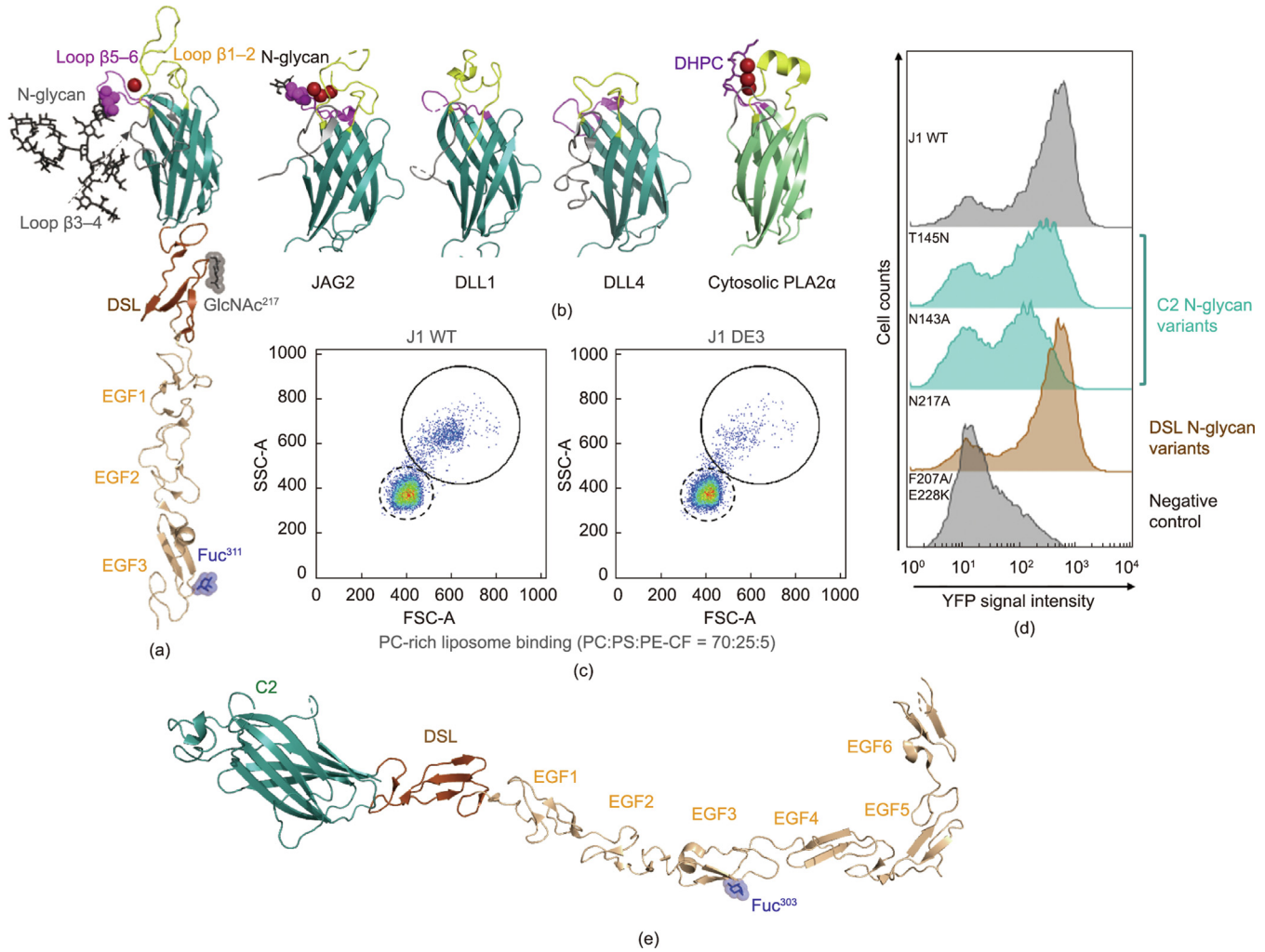

Fig. 4. Ligand structural and functional features. (a) The structure of JAG1 C2-EGF3 (NE3; PDB: 4CBZ) is shown in ribbon representation, and C2 domains are colored in teal, DSL domains in orange, and EGF domains in wheat. The Ca^{2+} ion bound at the apex of the C2 domain is drawn as a red sphere. A 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

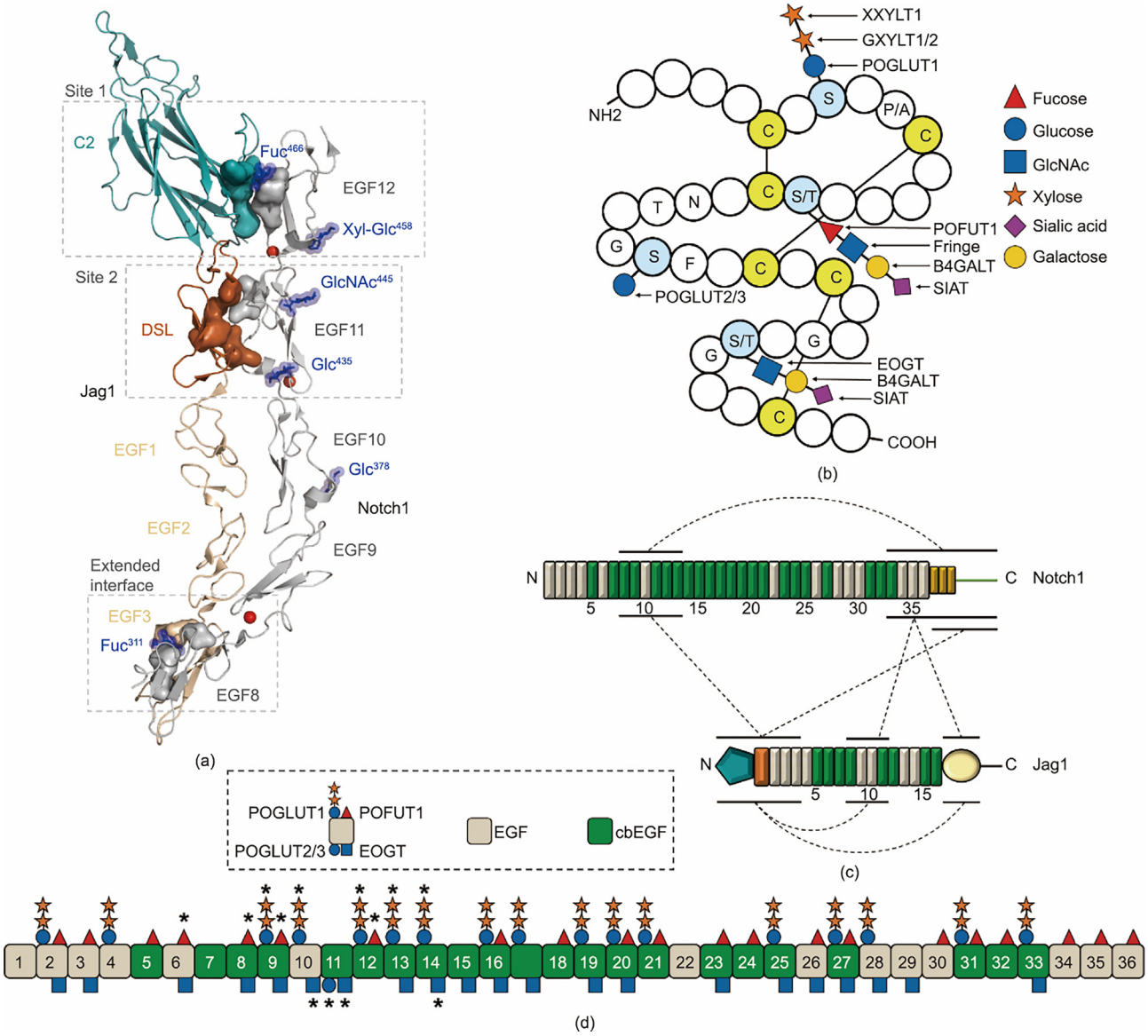

Fig. 5. Structural basis of Notch-ligand interaction and O-glycosylation. (a) X-ray structure of Notch1 EGF8–12/Jag1 C2-EGF3 complex (PDB: 5UK5) [10]. Jag1 C2 domain and DSL domain are shown in teal and orange. The non-cbEGF domains are shown in wheat. Notch1 is shown in grey. The Ca^{2+} ions are shown 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$_{4–5}$–(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–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.

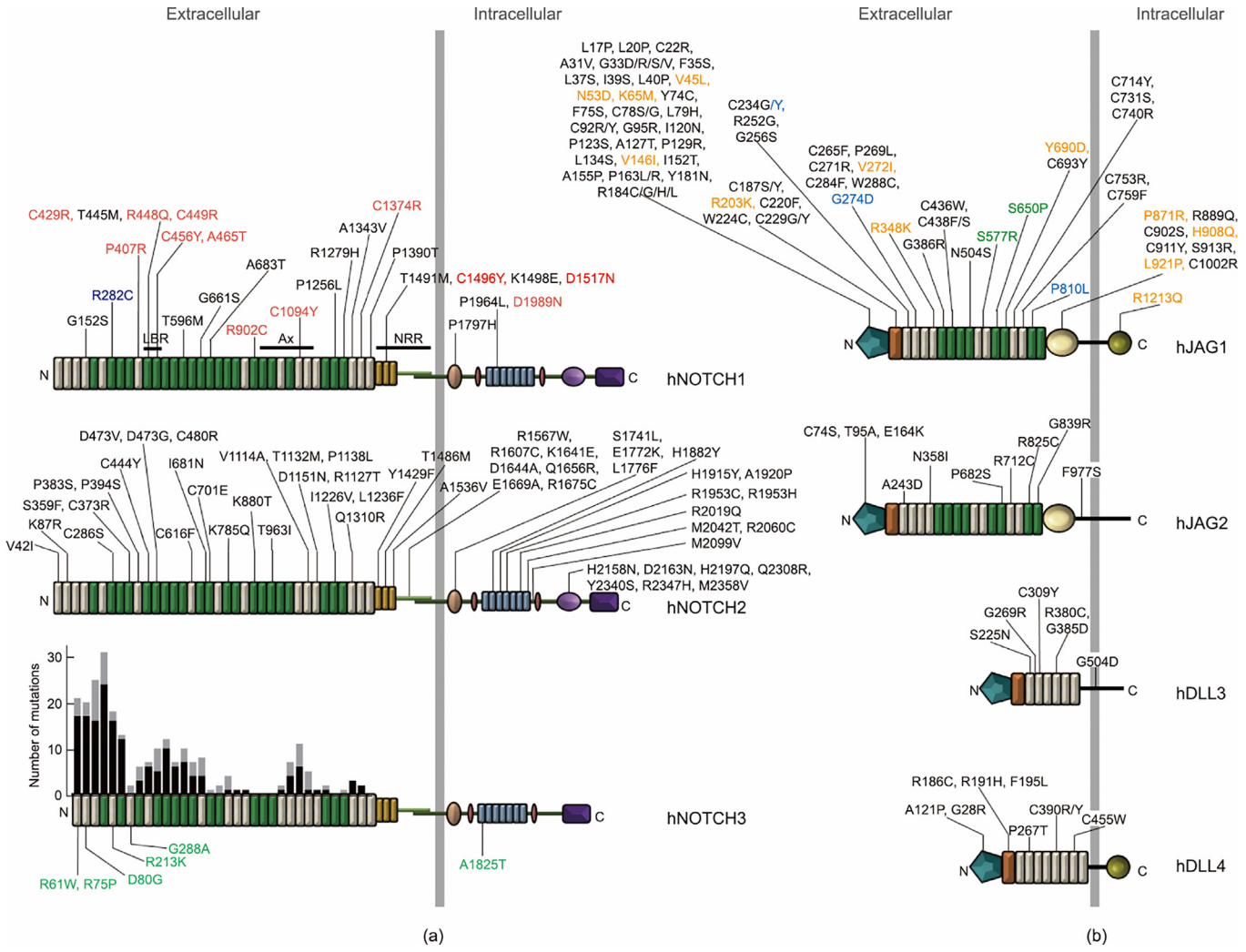

Fig. 6. Map of Notch ligand and receptor missense mutations associated with genetic disease. Reported amino acid substitutions in Notch ligands and receptors are mapped onto schematic diagrams with the same color scheme as Fig. 2. (a) In hNOTCH1, missense mutations leading to BAV disease are labelled 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.

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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.

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