Structural insights into Notch receptor-ligand interactions

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1. Abstract

Pioneering cell aggregation experiments from the Artavanis-Tsakonas group in the late 1980's localized the core ligand recognition sequence in the *Drosophila* Notch receptor to epidermal growth factor-like (EGF) domains 11 and 12. Since then, advances in protein expression, structure determination methods and functional assays have enabled us to define the molecular basis of the core receptor/ligand interaction and given new insights into the architecture of the Notch complex at the cell surface. We now know that Notch EGF11 and 12 interact with the Delta/Serrate/LAG-2 (DSL) and C2 domains of ligand and that membrane-binding, together with additional protein-protein interactions outside the core recognition domains, are likely to fine-tune generation of the Notch signal. Furthermore, structure determination of *O*-glycosylated variants of Notch alone or in complex with receptor fragments, has shown that these sugars contribute directly to the binding interface, as well as to stabilizing intra-molecular domain structure, providing some mechanistic insights into the observed modulatory effects of *O*-glycosylation on Notch activity.

Future challenges lie in determining the complete extracellular architecture of ligand and receptor in order to understand i) how Notch/ligand complexes may form at the cell surface in response to physiological cues, ii) the role of lipid binding in stabilizing the Notch/ligand complex, iii) the impact of *O*-glycosylation on binding and signalling and iv) to dissect the different pathologies that arise as a consequence of mutations that affect proteins involved in the Notch pathway.

2. Notch Receptor Ligand-binding Region (LBR)

The Notch receptor is part of a short-range cell-cell signaling system in metazoans and comprises a large extracellular domain and a short intracellular domain (Fig. 1). Ligand binding to the Notch extracellular domain (Rebay et al., 1991) initiates a process known as intra-membrane regulated proteolysis which releases the Notch intracellular domain (NICD); this then translocates to the nucleus where it assembles into a transcriptional activation complex to initiate expression of target genes (Bray, 2016). The extracellular domain comprises the membrane-proximal negative regulatory region (NRR) and a contiguous set of epidermal growth factor-like (EGF) domains. The number of EGF domains varies from species to species, as does the number of Notch receptors but the molecular architecture (represented in Fig. 1) is similar. Mammals have 4 Notch paralogues (Notch1-4), while *Drosophila melanogaster* has 1 (dNotch) and *C.elegans* 2 (LIN-12, GLP-1). Both dNotch and human Notch1 (hN1) have 36 disulphide-rich EGF domains. These domains are extensively *O*-glycosylated and many of them contain an additional consensus sequence which confers the ability to bind Ca^{2+} (cbEGF).

2.1 Structure of unmodified human Notch1 (hN1) EGF11-13

The solution structure of unmodified (unglycosylated) hN1 EGF11-13, containing the ligand-binding region, was determined using NMR in 2004 (Hambleton et al., 2004). All three EGF domains contain a calcium-binding consensus sequence and binding of this fragment to ligand-expressing cells was shown to be calcium dependent. The high-resolution structure, together with a dynamic analysis, showed that EGF11 and 12 adopted a near linear rod-like orientation, stabilized by calcium binding at the domain-domain interface and a conserved hydrophobic packing interaction between Y444 of EGF11 and I471 of EGF12 (Fig. 2). The tilt angle was similar to that observed for other calcium-binding EGF domain pairs from the extracellular matrix protein fibrillin-1 (reflecting the near-linear structure) but the twist angle was very different (119^{o} compared with $\sim 155^{o}$) (Downing et al., 1996, Smallridge et al., 2003). These features were also observed in a crystal structure (at 2.6Å resolution) of a C-terminal tagged form of unmodified EGF11-13 which, in addition, revealed a very similar pairwise organization of EGF12 and 13 [(Cordle et al., 2008a), Fig. 2]. From these data, it was possible to identify other regions of Notch which were likely to show a similar calcium-stabilized architecture.

2.2 Defining the ligand-binding site in hN1 11-13

Site-directed mutagenesis of each of the calcium-binding sites within EGF11-13 indicated that only loss of calcium binding to EGF12 abrogated binding of this fragment to ligand Delta-like1 (Dll-1) in flow cytometry experiments (Cordle et al., 2008b). This suggested that the Notch EGF11-12 interface and/or Notch EGF12 contained the main ligand-binding site within this fragment. Further amino acid substitutions within EGF12 of hN1 and dN EGF11-13 triple domain fragments were made to probe ligand recognition, whilst retaining calcium binding. Using flow cytometry and cell aggregation assays, L468 and I477 in EGF12 of hN1 and equivalent residues L504 and V513 in dN were identified as being essential for binding (Whiteman et al., 2013). In the crystal structure of EGF11-13, the side chains of these residues are located opposite each other on the central β hairpin of EGF12 and are involved in a hydrophobic interaction (Cordle et al., 2008a, Whiteman et al., 2013). Collectively, these data suggested that these residues, together with contributions from β strand residues in close proximity, provide a stable ligand-binding platform which is conserved from *Drosophila* to human (Fig. 2). Interestingly, this site is adjacent to the amino acid residue T466 which forms part of the *O*-fucosylation consensus (C^2-X_4-(S/T)-C^3) and is subjected to further GlcNAc modification by Fringe enzymes (Rampal et al., 2005, Moloney et al., 2000a, Moloney et al., 2000b, Moloney et al., 1997, Wang et al., 2001).

2.3 *O*-glycosylation of the ligand-binding region and effect on structure

Since *O*-glycosylation of the extracellular domain was known to regulate Notch signaling (Rana and Haltiwanger, 2011) and the ligand-binding site in EGF12 was found to map adjacent to an *O*-fucosylation consensus site, a series of *in vitro* modified forms of hN1 EGF11-13 was prepared and utilized in molecular and cell binding assays (Taylor et al., 2014). Stoichiometric addition of *O*-glucose at S458 (EGF12) and S496 (EGF13) catalyzed by protein O-glucosyltransferase (POGLUT1), and subsequent extension with xylose by glucoside xylosyltransferase 2 (GXYLT2), showed no effect on the binding of EGF11-13 to cells expressing either Notch ligand Jagged-1 or Delta-like 4 (Dll-4), (Taylor et al., 2014). In contrast, *O*-fucose modification of T466 by protein O-fucosyltransferase (POFUT1) within EGF12 and extension with GlcNAc by the enzyme Lunatic Fringe (Lfng) showed effects on cell binding. *O*-fucose monosaccharide addition had a minor effect on the binding of EGF11-13 to Jagged-1 and Delta-like 1 (Dll-1) but the subsequent enzymatic addition of GlcNAc by Fringe to form the disaccharide conferred substantial enhancement of binding to Jagged-1 and Dll-1. Any further enzymatic extension to produce tri- and tetra-saccharide forms failed to show any additional effect. No effect of the mono- or disaccharide was observed on binding to Dll-4 in these flow cytometry assays. The molecular basis of these interactions was further investigated by surface plasmon resonance (SPR) (a biophysical method by which biomolecular interactions can be evaluated in real time) which showed that the disaccharide form of EGF11-13 caused a 9-fold and 18-fold increase in binding to Jagged-1 and Dll-1 respectively compared to the monosaccharide, in agreement with the flow cytometry data. Dll-4 by comparison had a high inherent affinity for unmodified EGF11-13 in the absence of *O*-glycans, explaining why *O*-glycosylation had no effect on binding in flow cytometry experiments and only minor effects when binding was quantified by SPR (Taylor et al., 2014). The high inherent affinity of Dll-4 for Notch was also observed in an independent study (Andrawes et al., 2013).

Crystal structures of hN1 EGF11-13 modified with either *O*-fucose or GlcNAc-fucose were subsequently determined in the presence of Ca^{2+} and compared to the previously determined structure for the unmodified protein (Taylor et al., 2014). The *O*-fucose sugar added to T466 was found to point away from the central β-sheet region. Both the side chain and sugar modification were well ordered in the structure and were seen to make intramolecular contacts with EGF12 (specifically the C6 methyl group of the *O*-fucose ring was packed between residues I477 and M479). Fringe-catalyzed addition of GlcNAc to the *O*-fucose group was shown to extend the sugar further away from the central β hairpin, thus increasing the potential ligand-binding surface (Fig. 2). Similar to the *O*-fucose sugar, GlcNAc was observed to make intra-molecular contacts with neighboring residues D464 and M479. Although both the monosaccharide and disaccharide made extensive contacts with the protein, no conformational change was observed in EGF11-13 and the backbone structure, tilt and twist angles were unaffected (Fig. 2). Importantly, these data indicate that the increase in binding seen on Fringe modification is most likely due to the increased affinity of hN1 EGF11-13 for Jagged-1 and Dll-1 ligands. Since other EGF domains in the Notch

extracellular domain contain the *O*-fucose consensus and are modified (Shao et al., 2003, Harvey et al., 2016), it is possible that Fringe modification may modulate the receptor/ligand interaction at other sites. Very recently, this has been confirmed using a mass spectrometry approach (Kakuda and Haltiwanger, 2017). Mammalian Lunatic, Manic, and Radical Fringe proteins leave distinctive enzymatic signatures on hN1 (resulting in patterns of disaccharide modifications to Notch EGF domains which are specific to each enzyme) which influence whether or not the different ligands activate or inhibit signaling.

2.4 Structure of Unmodified Human Notch1 (hN1) EGF4-13

Although many of the EGF domains of the EGF5-25 region of the human Notch extracellular domain are of the calcium-binding type and predicted to form rod-like structures similar to that observed for EGF11-13, they are interspersed with non-calcium-binding domains EGF6, EGF10 and EGF22 (Downing et al., 1996, Hambleton et al., 2004, Handford et al., 1991). These have the potential to introduce sites of flexibility and impart dynamic properties to the extracellular domain or allow it to adopt a non-linear structure which could impose a "jack-knife" conformation or stabilise a linear conformation which extends the extracellular domain away from the cell surface. To address this, $\{^{1}H\}$-^{15}N nuclear Overhauser effects, residual dipolar couplings and X-ray crystallography were used to identify a non-linear organization for the EGF4-13 region comprising a rigid bent conformation for EGF4-7, a single flexible linkage between EGF9-10 connected to the calcium-stabilized ligand-binding region EGF11-13 (Weisshuhn et al., 2016). Overall, these data suggested a non-linear but not jack-knifed organization. The near-linear calcium-stabilised section of EGF6-9, N-terminal to the flexible EGF9-10 linkage, suggested that the receptor may align with ligand along its longitudinal axis, with additional weak interactions outside the core recognition region contributing to the overall affinity of receptor for ligand. These data highlight the need for a careful study of pairwise domain interfaces involving a C-terminal non-calcium binding EGF domain, since their properties are not easily predictable from sequence.

2.5 Calcium binding to Notch EGF domains

The free Ca^{2+} concentration in the extracellular milieu is ~1.4 mM (Breitwieser, 2008). Ca^{2+} affinities of EGF domains containing the calcium-binding consensus sequence D/N-x-D/N-E/Q-x_m-D*/N*-x_n-Y/F (where* denotes possible β-hydroxylation) in the EGF4-13 region have been measured to identify which sites would be saturated under physiological conditions thus conferring rigidity to interdomain interfaces (Handford et al., 1990, Handford et al., 1991, Weisshuhn et al., 2016). Two methods have been useful for obtaining these data; chromophoric chelation for high affinity Ca^{2+} binding sites (Linse et al., 1991), NMR titrations for low and medium affinity sites and to assign high affinity sites to specific domains [Fig. 3, (Suk et al., 2004; Whiteman et al., 2014)]. Almost all the calcium-binding domains contain the canonical calcium-binding consensus sequence and the aromatic packing residue in the preceding domain but EGF7 and 9 replace the E/Q consensus residue with a D

residue. All calcium-binding EGF domains in a native context (i.e. with a covalently linked N-terminal domain) have K_d values in the range 1-60 μM, with the exception of EGF5 which has an affinity of 170 μM [Fig. 3, (Weisshuhn et al., 2016)]. Since all measurements were collected at pH 7.5 and at physiological ionic strength (I=0.15), these data suggested that in the extracellular milieu all sites measured so far would be occupied in >90% of molecules, conferring rigidity to domain interfaces (Fig. 3).

3. Ligand Structure

All Notch ligands contain a variable number of EGF domains, a Delta/Serrate/Lag-2 (DSL) domain and an N-terminal domain which until recently was known as the MNNL (Module at the N terminus of Notch Ligand). There are two ligand families which may be distinguished by the presence (Serrate/Jagged) or absence (Delta/Delta-like) of a cysteine–rich region [(Chillakuri et al., 2012), Fig. 1]. A number of mutagenesis and deletion studies previously showed that the DSL domain conferred specificity of binding to Notch and that covalent linkage of EGF1 and 2 to the DSL domain facilitated binding (Shimizu et al., 1999, Henderson et al., 1997).

3.1 Structure of hJagged-1 DSL-EGF3

Based on these data a DSL-EGF3 four-domain fragment from human Jagged-1 was expressed in bacteria, *in vitro* refolded to form the native disulphide-stabilised fold and purified to homogeneity for structure determination (Cordle et al., 2008a). The X-ray structure of hJagged-1 DSL-EGF3 was determined at a resolution of 2.5Å and showed an extended linear arrangement of domains (Cordle et al., 2008a). All EGF domains in this fragment were of the non-calcium-binding type but EGF1 and 2 had much shorter loops between the cysteine residues involved in disulphide bonding, whilst EGF3 adopted a more classical fold. The DSL domain was shown to have a distinct fold but with some similarity to EGF domain structure, suggesting it may have evolved from a pair of EGF domains. Sequence alignments of DSL domains from both Jagged/Serrate and Delta ligand families identified a series of highly conserved residues which were mapped onto the Jagged-1 structure. A subset of these clearly performed a structural role but others (F199, R201, R203, D205, F207) were surface exposed on one face of the DSL domain suggesting these may form a Notch-binding site (Fig. 4A). To confirm these observations, a series of alanine substitutions was generated at equivalent residues within Serrate, the Jagged homologue in *Drosophila*, for testing in an *in vivo* wing imaginal disc model of Notch activity. Each construct was ectopically expressed along the anterior/posterior compartment boundary and *wingless* expression measured as a downstream marker of Notch activity. All Serrate variants gave functional effects on Notch signaling, consistent with a role in Notch binding. F207 was found to have a particularly crucial role, confirmed by alanine substitution of this residue in

either *Drosophila* Serrate (F257A) or hJagged-1 (F207A) which abrogated Notch interaction in binding assays. It was also possible to show that the same face of the DSL domain was involved in mediating both Notch trans-activation and cis-inhibition, since both induction of an ectopic wing margin (trans) and suppression of endogenous Notch activity at the dorsal-ventral boundary (cis) could be observed in this system. Subsequent to this, it was demonstrated that a region containing the ligand binding portion of the receptor (dNotch EGF10-12) was required for cis-inhibition mediated by Serrate (Becam et al., 2010), suggesting that interactions between DSL and EGF11-12 underlie both cis- and trans-Notch complexes.

3.2 Structure of N-terminus-EGF3 (N-EGF3) of human Jagged-1

The importance of the region N-terminal to the DSL domain of Notch ligands was suggested by an early experiment performed by Henderson and colleagues (Henderson et al., 1997), since its deletion in the *C.elegans* ligand LAG-2 abolished function. Expression of an N-terminal fragment of human Jagged-1, comprising the complete N-terminus, DSL domain and three contiguous EGF domains (N-EGF3), in HEK cells enabled purification of sufficient material for structure determination (Chillakuri et al., 2013). The N terminal region was found to adopt the fold of a common phospholipid-recognition C2 domain which packed on top of the DSL domain, thereby extending the linear organization of the ligand identified in the DSL-EGF3 structure (Fig. 4A). The Jagged-1 C2 domain had strong structural homology to the calcium-binding C2B domain of Munc13 (an intracellular protein involved in priming synaptic vesicles) with a typical β sandwich fold comprising two four-stranded β sheets at its centre (Cho and Stahelin, 2006, Shin et al., 2010). Although crystallization conditions for Jagged-1 N-EGF3 did not originally contain Ca^{2+}, the strong homology to Munc13 prompted us to assess the Jagged C2 domain for its calcium-binding properties using limited proteolysis performed in the presence and absence of Ca^{2+}. Addition of Ca^{2+}, but not Mg^{2+}, conferred protection against proteolysis, suggesting the presence of Ca^{2+} binding sites within the protein. Further crystallization trials were performed in the presence of Ca^{2+} and the structure of the holo form was solved. Comparison of both apo- and holo- crystal forms showed that Ca^{2+} occupancy increased the degree of structure in the loop regions responsible for co-ordinating Ca^{2+}, regions that are located at the apex of the C2 domain.

In parallel with our structural data, the biological significance of the C2 domain was investigated (Chillakuri et al., 2013). Liposome binding assays confirmed that the C2 domain conferred a phospholipid-binding capability to Jagged-1 N-EGF3 and other Notch ligands which was abrogated by a C2 domain deletion (Fig. 4B). In a quantitative split luciferase Notch activation assay, a Jagged-1 variant containing a double D140A/D144A substitution, designed to prevent Ca^{2+} coordination, was found to substantially reduce activation compared to its wild type (WT) counterpart, despite still being able to bind to the Notch receptor (Chillakuri et al., 2013). Collectively, these data suggested that phospholipid binding, in addition to the core receptor/Jagged-1 interaction, facilitates generation of the Notch signal.

3.3 Additional Ligand Structures

Other crystal structures of the N-terminal region of Notch ligands have been determined since the Jagged-1 structure was published. An eight-domain fragment of hDll-1 showed an extended, near-linear conformation for C2, DSL and EGF1-4 (Kershaw et al., 2015), confirming the presence of a C2 domain and a similar arrangement of domains in both ligand families. Interestingly, a 90^{o} bend was then observed between EGF4 and 5 of Dll-1, with EGF5 and 6 forming a near linear arrangement. Since the EGF4-5 interface was the site of a crystal contact, it is unclear whether or not the bent conformation observed is present in solution or is more dynamic. However, this structure, together with the identification of EGF domain interfaces with very different properties (bent, flexible, rigid) in the Notch EGF4-13 region, underscores the importance of a detailed study of these regions when elucidating the architecture of both ligand and receptor. With the availability of crystal structures for a member of each ligand family, the authors further identified by comparative sequence analysis a conserved patch in EGF2 which may represent an additional receptor-ligand interaction site. More recently, the structures of the N-terminal regions of hJagged-2 and hDll-4 have been determined (Suckling et al., 2017). The C2 domains of Jagged-1 and -2 bind calcium ions while the Delta family do not and, in each case, the loop regions of the C2 domains are highly variable suggesting different lipid preferences (Fig. 4C).

4. Structures of Notch/Ligand complexes

4.1 Structure of Notch/Dll-4

A milestone in Notch structural biology was reached in 2015 when the crystal structure of a Notch1/Dll-4 complex, comprising the core recognition regions, was solved at 2.3Å [(Luca et al., 2015), Fig. 5)]. The technical challenge of crystallising the relatively low affinity Notch complex was circumvented by targeting the rat Notch1/Dll-4 interaction for affinity maturation. The N-terminal region-EGF5 region from Dll-4 was expressed as a fusion protein on the surface of yeast and higher affinity variants (generated using error-prone PCR) were identified using Notch1 EGF1-14, immobilized to magnetic beads, as bait. Any ligand construct selected was subjected to a second round of mutagenesis/selection to further enhance affinity. Subsequent DNA sequencing revealed a series of missense mutations in the C2 and DSL domains of the ligand construct. Affinity-matured variants were subsequently expressed in insect cells. The N-EGF2 form of Dll-4$_{SLP}$ (containing G28S, F107L and L206P substitutions) showed a >70-fold enhancement in affinity relative to the WT construct, attributed to the slower dissociation rate of the complex. In activity assays using a luciferase reporter construct, N-EGF2 Dll-4$_{SLP}$ showed no reduction in activity compared to the WT construct and the dose response curve was shifted to the left consistent with the increased affinity measured. Crystal forms of glycan-trimmed Dll-4$_{SLP}$ (N-EGF2 or N-EGF1) bound to

EGF11-13, also purified from insect cells, were obtained and their structures solved by molecular replacement.

The structures of the individual components of the complex confirmed the elongated structure of ligand and receptor identified previously as well as the lack of calcium binding to the C2 domain of Delta ligands first observed by Kershaw and colleagues (Kershaw et al., 2015). However, striking new observations included an antiparallel arrangement of receptor relative to ligand in the complex and two sites of interaction observed between C2 and EGF12 (Site 1) and DSL and EGF11 [Site 2, (Fig. 5)]. The antiparallel arrangement, coupled with the observed sites of flexible/bent EGF interfaces in hNotch1 (Weisshuhn et al., 2016), may suggest that a single receptor/ligand complex underlies trans-activation and cis-inhibition modes of Notch activity, since flexible regions either side of the core recognition element Notch EGF11-12 would allow a single complex to form in cis or trans.

The direct binding role of the *O*-glycan modifications on T466 in EGF12, proposed by Taylor and colleagues (Taylor et al., 2014), was confirmed in this structure with the *O*-fucose making a network of glycan-amino acid contacts within the C2 domain. Modelling of the Fringe extension based on this complex suggested that, as in the crystal structure of Fringe-modified hN1 EGF11-13 (Fig. 2), the disaccharide extends away from the *O*-fucose and makes further contacts with ligand and receptor. *O*-glucose modifications present within EGF11-13 in the complex were not involved in direct contacts with ligand, in agreement with the lack of effect on ligand binding observed by Taylor and colleagues (Taylor et al., 2014). In contrast, DSL-EGF11 contacts at Site 2 were mainly mediated by protein-protein interactions but an *O*-glycan at S435 in EGF11 was observed to interact directly with DSL residues. The functional significance of this is yet to be elucidated but Ser 435 is not absolutely conserved across Notch paralogues suggesting it plays a lesser regulatory role in Notch activity than the Fringe modification. On the basis of sequence comparisons, the authors suggested that Site 2 (EGF11-DSL) is the main common determinant of ligand binding, while Site 1 (EGF12-C2) is less specific, allowing different receptor/ligand combinations to form. It should be noted, however, that other regions of contact may exist away from the core recognition surfaces that also modulate different receptor ligand pairings. A model for hN1 EGF10-13 (Weisshuhn et al., 2016) has been superimposed on EGF11-13 of the complex; small rearrangements were required in order to accommodate the longer receptor fragment and suggested that additional interactions may occur between EGF10 of Notch and EGF1 of ligand. Since possible interface residues are not conserved between ligand families, this and other similar sites such as in EGF 2 of Dll-1 which are outside the core recognition sites may contribute to ligand-specific differences in binding. A comparison of new ligand structures from both Jagged and Delta families showed that two different conformations existed in the crystals. The straighter conformation is compatible with the binding of longer fragments of Notch [Fig. 5, (Suckling et al., 2017)].

4.2 Structure of Notch/Jagged-1

Very recently a 2.5Å crystal structure of a Notch/Jagged-1 complex has been obtained using affinity maturation to select for high affinity ligand variants (Luca et al., 2017). It confirms the Site 1 and Site 2 core interaction sites, as observed in the Notch/Dll-4 complex, but utilizing longer five-domain constructs it shows an extensive interface that forms along the whole length of the longitudinal axis. Specifically, Notch EGF12, EGF11, EGF10, EGF9 and EGF8 interact with Jagged-1 C2, DSL, EGF1, EGF2 and EGF3. This complex thus corroborates the observation made by Weisshuhn and colleagues, that domain rearrangements were necessary to accommodate longer Notch constructs in complex with ligand (Weisshuhn et al., 2016). The Notch EGF8/Jagged-1 EGF3 interaction provides a structural explanation for the *jigsaw* mutation identified in a *Drosophila* screen which mapped to V324 in dNotch EGF8 (Yamamoto et al., 2012). Interestingly, the Notch/Jagged-1 complex showed that *O*-fucose modification of T311 in Notch EGF8 plays a direct role in the interaction with Jagged-1 EGF3, highlighting the importance of *O*-glycans in Notch domains other than EGF12 for selective ligand binding. It was also observed in this study that Jagged-1 altered its conformation on Notch binding and exhibited catch bond behavior (where the lifetime of the receptor/ligand interaction is increased on application of a tensile mechanical force), providing an explanation of how relatively weak interactions between receptor and ligand are able to survive cellular forces and result in NRR cleavage.

5. Implications for disease

These structural data for the extracellular portions of the receptor and ligands have provided insight into molecular mechanisms underlying genetic disease associated with the Notch pathway (Table 1). Many missense mutations affecting Jagged-1 have been identified which cause Alagille syndrome, a disease affecting liver, heart and kidney development (Penton et al., 2012) and related disorders such as extrahepatic biliary atresia and Tetralogy of Fallot. Prior to structural work identifying the C2 domain, a number of *JAG1* mutations associated with nonsense-mediated decay of RNA were known to cause Jagged-1 haploinsufficiency. Mapping Alagille missense mutations onto the structure of Jagged-1 C2 suggested they destabilized the hydrophobic core and prevented native folding (Chillakuri et al., 2013, Luca et al., 2015). This was corroborated by expression of Jagged-1 N-EGF3 disease-causing variants, which mainly resulted in little or no protein secretion, thus underscoring haploinsufficiency as the main dominant mechanism underlying Alagille syndrome (Chillakuri et al., 2013). However, a few missense mutations were observed which might act through disruption of ligand domain interfaces, a direct effect on Notch binding (Luca et al., 2015) or an effect on lipid binding (Suckling et al., 2017).

Adams Oliver syndrome, a developmental disorder affecting the scalp and cranium, and limb development is associated with a subset of mutations in the *NOTCH1* and *DLL4* genes. The missense mutations identified affected C2, DSL, EGF6, 8, 9 domains of Dll-4

(Meester et al., 2015). Many are implicated in domain misfolding by altering the highly conserved cysteine residues which stabilize the fold, suggesting that the main autosomal dominant mechanism underlying *DLL4* mutations is haploinsufficiency. Missense mutations causing Adams Oliver disease have also been identified in *NOTCH1* (Stittrich et al., 2014)*;* a C429R change in EGF11 is also likely to cause domain misfolding of which one can speculate may lead to cellular retention of this variant.

In summary, there have been substantial exciting advances made recently in structure determination of the extracellular domains of Notch and its ligands. The future challenge will be to determine the remaining architecture to build plausible models of the receptor/ligand complex at the cell surface and combine these data with cell biology experiments. Currently, we have been unable to harness the advances made in electron microscopy (EM) due to the narrow dimensions of both receptor and ligand. However, improvements in protein expression systems will allow us to use small angle X-ray scattering (SAXS) to complement the high-resolution structure data and we may be able to apply EM methods in future when we will have a better understanding of individual structures and their interacting regions. Structure-informed mutagenesis, combined with model organism studies, will be required to determine the physiological role of lipid binding by the C2 domain. These multidisciplinary data will then help to optimise the design of novel therapeutic agents aimed at modulating the Notch signal.

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Figure legends

Fig. 1. Modular organisation of the extracellular domains of human Notch1 (hN1) receptor and the Jagged1 (hJ-1) and Delta-like4 (hDll4) ligands. The negative regulatory region (NRR) of Notch1 and the transmembrane domain (TM) of Notch1, hJ-1 and hDll-4 are indicated, as is the complete extracellular domain (NECD). Individual domains belonging to the Notch intracellular domain (NICD) are not indicated separately. Ca^{2+}-binding and non-Ca^{2+}-binding EGF domains and the cysteine-rich domain are indicated as grey, white and black rectangles, respectively. The C2 and DSL domains are indicated as white octagons and hexagons, respectively. The ligand-binding region (LBR, corresponding to EGF11-13) of hN1 is shown.

Fig. 2. Structure of ligand-binding hN1 EGF11-13. Key features are highlighted including calcium ions, hydrophobic packing interaction of EGF11-12 between Y444 and I471, key ligand-binding residues in EGF12, L468 and I477, and the GlcNAc fucose disaccharide added by POFUT1 and Fringe, respectively onto T466. Note the overall rod-shape of the triple domain fragment stabilized by calcium binding to each EGF domain and hydrophobic packing interactions and the proximity of the disaccharide to the ligand-binding site.

Fig. 3. Measurement of calcium affinities of Notch EGF domains**.** (A) High-affinity Ca^{2+} binding can be determined using the chromophoric chelator 5,5'-Br_2BAPTA. The open circles show the decrease in absorbance at 263 nm when Ca^{2+} is added to a 30 μM solution of 5,5'-Br_2BAPTA alone. This response is altered (filled circles) when Ca^{2+} is added to a mixture of 30 μM 5,5'-Br_2BAPTA and 30 μM hN1 EGF9-11, due to the competition of the high-affinity EGF11 site with the chelator for Ca^{2+}. A K_d value of 3 μM can be determined for EGF11 in the EGF9-11 construct. (B) Lower-affinity Ca^{2+} binding can be determined using NMR. The fractional chemical shift change for aromatic residues in EGF11 (open circles), EGF12 (filled circles) and EGF13 (open triangles) in the EGF11-13 construct are plotted as a function of the free Ca^{2+} concentration. Overlaid ^{1}H-^{1}H NOESY spectra collected with 0, 0.4, 1.2 and 15 mM Ca^{2+} are shown in the inset. K_d values of 310, 55 and 30 μM are fitted for EGF11, EGF12 and EGF13; weak binding is observed for EGF11 because this domain is not in a native context in the EGF11-13 construct. (C) The measured Ca^{2+} dissociation constants (K_d) at pH 7.5 and I = 0.15 for the EGF4-13 region of human Notch-1 receptor. K_d values in the 1-20 μM range were determined by chromophoric chelation. K_d values in the 20 μM to mM range were determined by NMR. Ca^{2+}-binding and non-Ca^{2+}-binding EGF domains are indicated in grey and white, respectively. Ca^{2+} is indicated by a black sphere at the N-terminus of each Ca^{2+}-binding EGF domain.

Fig. 4. Notch ligand structure and liposome binding. (A) Structure of hJagged-1 N-EGF3 fragment showing the C2 domain at the N-terminus, the DSL domain (with the Notch binding residue F207 in the Notch-binding loop is indicated) and three contiguous EGF domains of the non-calcium binding type. (B) The presence of the C2 domain confers liposome binding properties to three diverse Notch ligands (J-1, Dll-1, Serrate), Conversely, each ligand with the C2 domain deleted (ΔC2) shows greatly reduced binding to phosphatidylcholine/phosphatidylserine/phosphatidylethanolamine (PC/PS/PE) liposomes. (C) Comparison of C2 domains of different human Notch ligands Delta-like-4, Jagged-2,

Jagged-1 and Delta-like-1 showing that the Jagged family bind calcium ions but the Delta family does not. Note the diversity in loop structures at the apex of each domain suggesting that each ligand may show different lipid preferences.

Fig. 5. The Delta-like-4/Notch1 complex. Key structural features are indicated including the putative lipid-binding site of the C2 domain (Chillakuri et al., 2013) and receptor/ligand interaction Site 1 (C2-EGF12) and Site 2 (DSL-EGF11) (Luca et al., 2015). Shown, adjacent in cartoon form, are two different ligand conformations seen in crystal structures of various ligands (Suckling et al., 2017) which suggest that i) the straighter form is required to accommodate longer constructs of Notch and ii) that additional contacts may occur along the longitudinal axis (Weisshuhn et al., 2016). C2 domain loops 1-2, 3-4, 5-6 are indicated.

Table 1

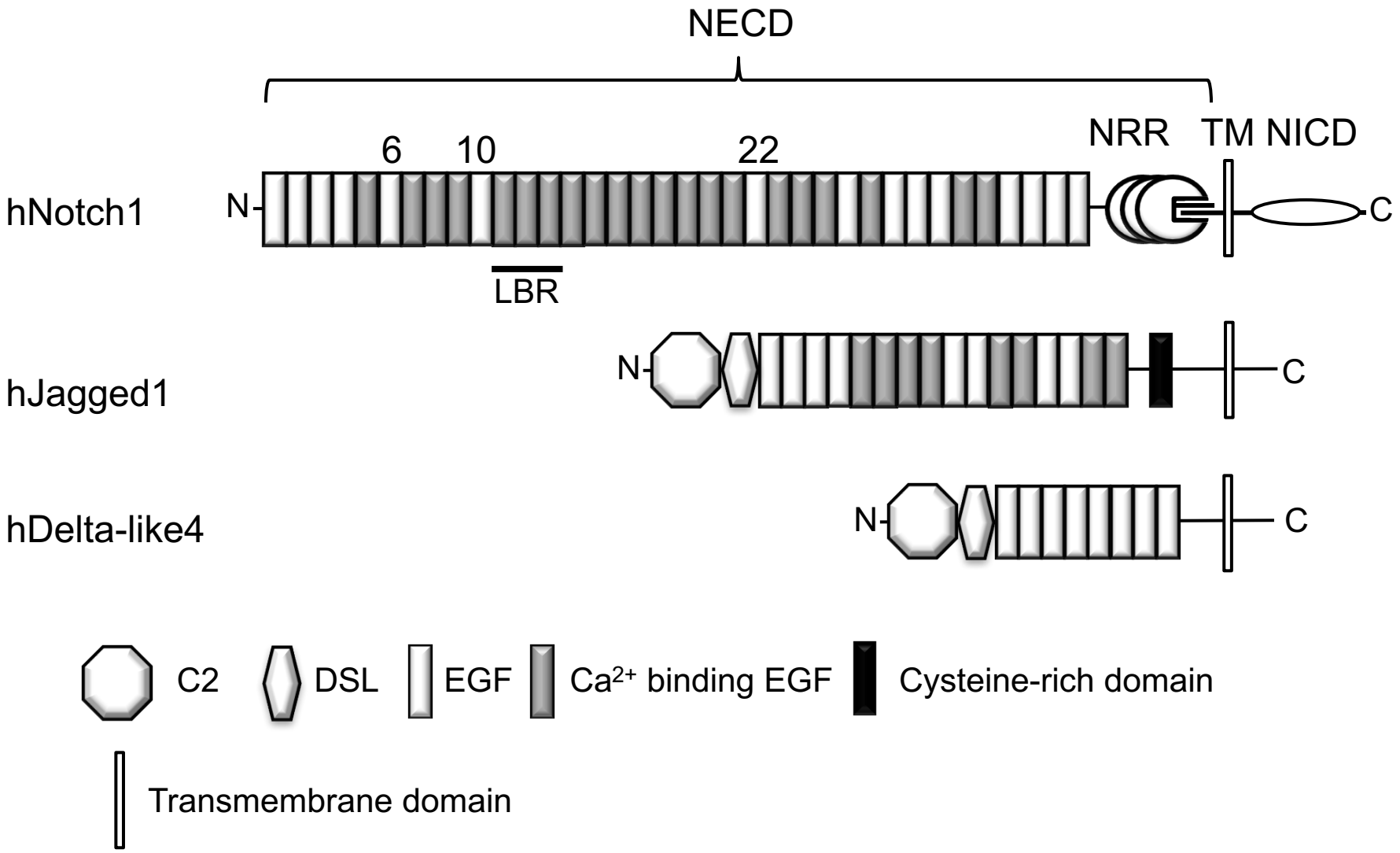
Molecular consequences of disease-causing missense mutations based on structural information for extracellular domains of ligand and receptor discussed in this review.

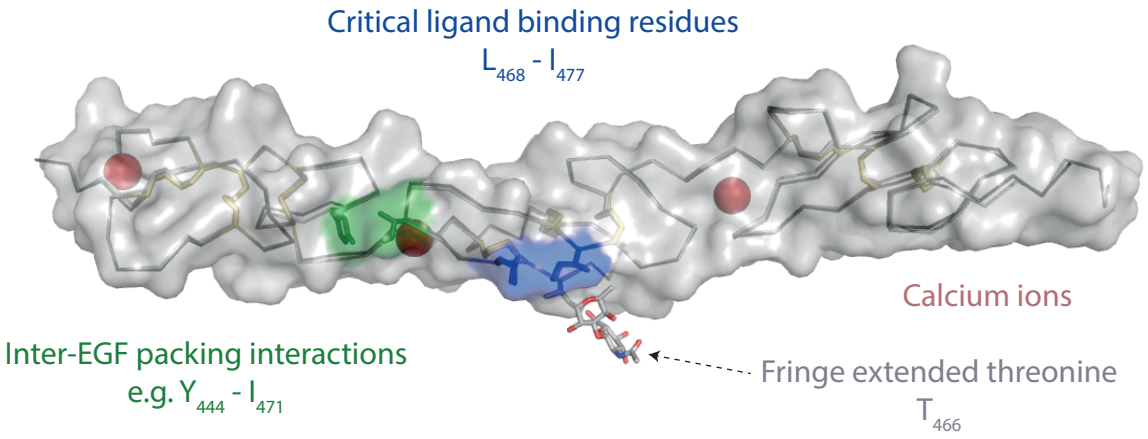
AS (Alagille syndrome), AOS (Adams- Oliver syndrome), EHBA (Biliary atresia, extrahepatic), TOF (Tetralogy of Fallot). Mutation data obtained from the Human Gene Mutation Database (Stenson et al., 2014).

Protein affected	Domain affected	Missense mutation	Disease	Predicted effect of mutation
J1	C2	L20P	AS	defective secretion
J1	C2	C22R	AS	defective secretion
J1	C2	A31V	AS	disruption of signal peptide cleavage
J1	C2	G33D/S/V	AS	stability/ folding
J1	C2	L37S	AS	stability/folding
J1	C2	I39S	AS	stability/folding
J1	C2	L40P	AS	stability/folding
J1	C2	V45L	EHBA	lipid binding affected
J1	C2	N53D	EHBA	lipid binding affected
J1	C2	K65M	EHBA	lipid binding affected
J1	C2	D69G	AS	lipid binding affected
J1	C2	F75S/L	AS	stability/folding
J1	C2	C78R/G/Y/S	AS	Notch binding site1 C2-EGF12 perturbed
J1	C2	L79H/F	AS	stability/folding
J1	C2	K80E	AS	Notch binding site 1 C2-EGF12 perturbed
J1	C2	C92R/Y	AS	Notch binding site 1 C2-EGF12 perturbed
J1	C2	I120N	AS	stability/folding
J1	C2	L122P	AS	stability/folding
J1	C2	P123S	AS	exposed residue (effect unclear)
J1	C2	A127T	AS	Notch binding site 1 C2-EGF12 perturbed
J1	C2	P129R	AS	Notch binding site 1 C2-EGF12 perturbed
J1	C2	L134F	AS	stability/folding
J1	C2	V136G	AS	stability/folding
J1 J1	C2	I152T	AS	stability/folding
J1	C2	A155P	AS	stability/folding
J1 J1	C2	P163L/R	AS	misfolded protein
J1	C2	F179S	AS	stability/folding
J1	C2	Y181N	AS	stability/folding
J1	C2	R184H/G/L/C	AS	stability/folding

J1	DSL	C187Y/S	AS	misfolded protein
J1	DSL	R203K	EHBA	Notch binding site 2 DSL-EGF11 perturbed
J1	DSL	C220F	AS	misfolded protein
J1	DSL	W224C	AS	stability/folding
J1	DSL	C229G/Y	AS	misfolded protein
J1	EGF1	R252K/G	AS	exposed residue (effect unclear)
J1	EGF1	G256S/C	AS	disrupt loop conformation
J1	EGF1	G259V	AS	disrupt loop conformation
J1	EGF2	C265F	AS	misfolded protein
J1	EGF2	P269L	AS	disrupt loop conformation
J1	EGF2	C271R	AS	misfolded protein
J1	EGF2	V272F	TOF	exposed residue (effect unclear)
J1	EGF2	G274D	TOF	stability/folding
J1	EGF2	E278D	TOF	exposed residue (effect unclear)
N2	EGF9	C373R	AS	misfolded protein
N2	EGF10	P383S	AS	disrupt loop conformation (based on homology to EGF11)
N2	EGF10	P394S	AS	disrupt loop conformation (based on homology to EGF11)
N2	EGF11	C444Y	AS	misfolded protein
N2	EGF12	C480R	AS	misfolded protein
Dll4	C2	A121P	AOS	stability/folding
Dll4	DSL	R186C	AOS	Notch binding site 2 DSL-EGF11 perturbed
Dll4	DSL	F195L	AOS	Notch binding site 2 DSL-EGF11 perturbed (confirmed experimentally)
Dll4	EGF2	P267T	AOS	disrupt loop conformation
Dll4	EGF5	C390R/Y	AOS	no structure available, misfolded protein predicted
Dll4	EGF7	C455W	AOS	no structure available, misfolded protein predicted
N1	EGF10	P407R	AOS	disrupt loop conformation (based on homology to EGF11)
N1	EGF11	C429R	AOS	misfolded protein
N1	EGF11	R488Q	AOS	Notch binding site 2 DSL-EGF11 perturbed (confirmed experimentally)
N1	EGF11	C449R	AOS	misfolded protein
N1	EGF12	C456Y	AOS	misfolded protein
N1	EGF12	A465T	AOS	stability/folding
N1	EGF35	C1374R	AOS	no structure available, misfolded protein predicted

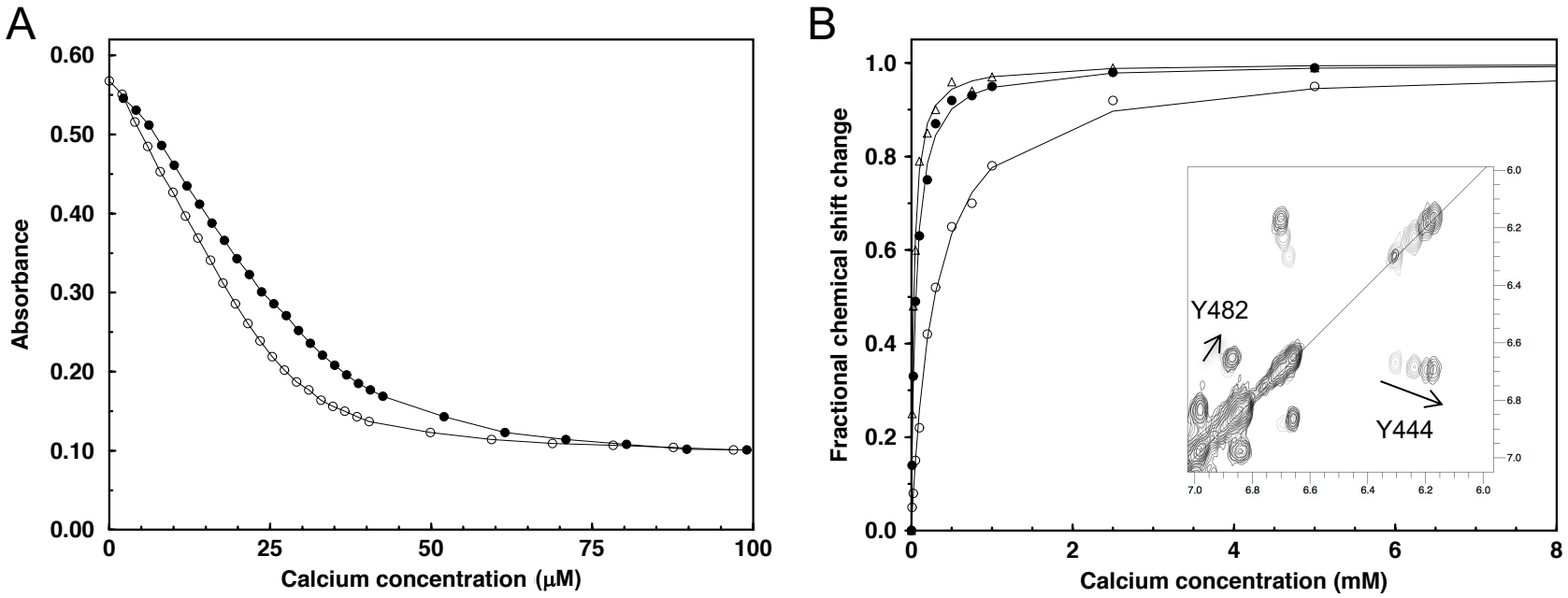
Handford- Fig 2.1

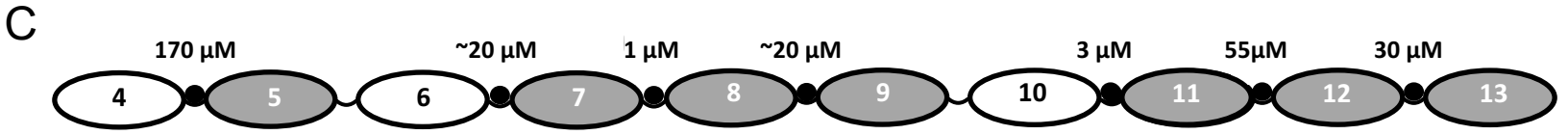




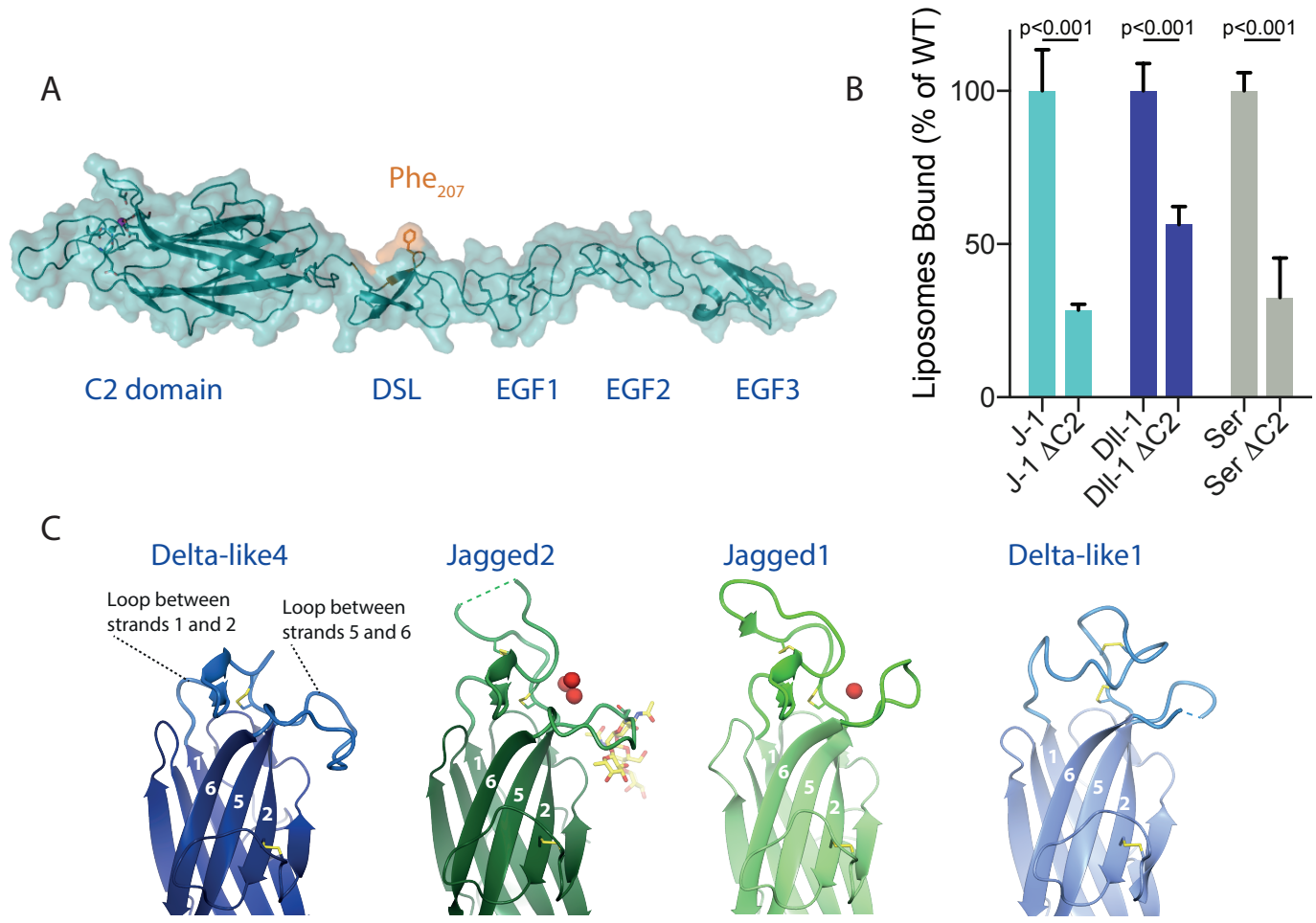
Handford-Fig2.2

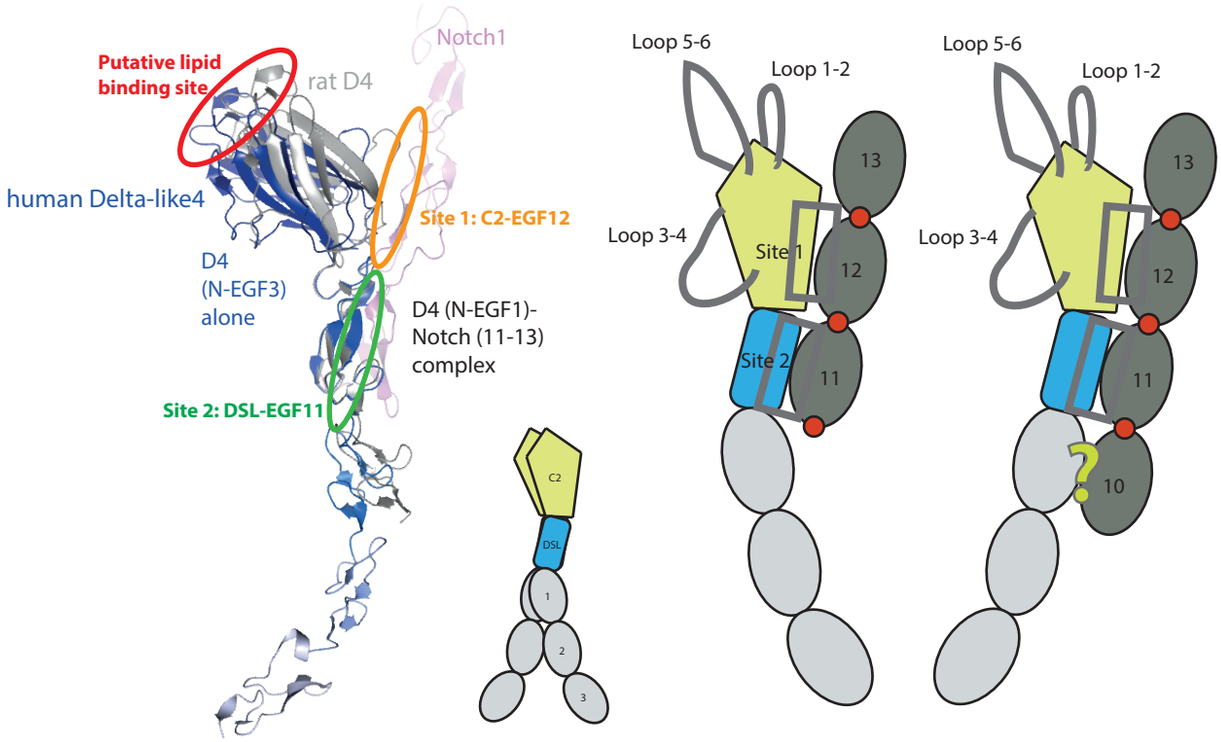
Handford-Fig2.3





Handford-Fig2.4





Handford-Fig2.5