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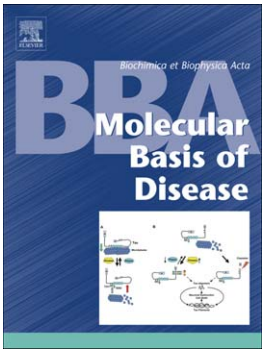

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Mutation of the fucose-specific β1,3 N-acetylglucosaminyltransferase LFNG results in abnormal formation of the spine

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Abstract

Notch signaling is an evolutionarily conserved mechanism that determines cell fate in a variety of contexts during development. This is achieved through different modes of action that are context dependent. One mode involves boundary formation between two groups of cells. With this mode of action, Notch signaling is central to vertebrate evolution as it drives the segmentation of paraxial mesoderm in the formation of somites, which are the precursors of the vertebra. In this case, boundary formation facilitates a mesenchymal to epithelial transition, leading to the creation of a somite. In addition, the boundary establishes a signaling centre that patterns the somite, a feature that directly impacts on vertebral column formation. Studies in *Xenopus*, zebrafish, chicken and mouse have established the importance of Notch signaling in somitogenesis, and indeed in mouse how perturbations in somitogenesis affect vertebral column formation. Spondylocostal dysostosis is a congenital disorder characterized by formation of abnormal vertebrae. Here, mutation in Notch pathway genes demonstrates that Notch signaling is also required for normal somite formation and vertebral column development in humans; of particular interest here is mutation of the *LUNATIC FRINGE* gene, which causes SCD type 3. *LUNATIC FRINGE* encodes for a fucose-specific β1,3-N-acetylglucosaminyltransferase, which modifies Notch receptors and alters Notch signaling activity. This review will focus on Notch glycolsylation, and the role of *LUNATIC FRINGE* in somite formation and vertebral column development in mice and humans.

1. Introduction

Abnormal vertebral segmentation (AVS) is a common congenital abnormality that affects vertebra singly or in multiples. AVS results in a variety of morphologies that affect vertebral size, shape and integrity; these ultimately impact upon the shape and flexure of the spine. AVS may represent an isolated finding or, in the case of multiple AVS is commonly associated with a range of dysmorphic features. In only a small proportion of AVS cases has the genetic component been identified (reviewed in [1]). Spondylocostal dysostosis (SCD) represent one of these cases, with mutation in genes of the Notch signalling pathway, segregating with this disorder with a recessive mode of inheritance. Loss of function mutations in genes of the Notch signalling pathway in mouse have demonstrated the essential role that Notch signalling plays in the formation of the vertebral column and in its precursor, the somites. Somite formation occurs in a reiterative manner during embryonic development, and studies in a number of vertebrate species have lead to the elucidation of numerous components of the Notch signalling pathway that function in somite formation (reviewed in [2]). Notch signalling is instrumental in two aspects of somitogenesis; segmentation of discrete somites from unsegmented mesenchyme and rostrocaudal patterning of the somite. Disruption of either of these processes has a profound impact on the vertebral column (reviewed in [3]).

2. Notch signaling and pathway components

2.1. Mechanisms of action

The Notch signaling pathway instructs developmental processes using different mechanisms of action described as lateral inhibition/specification, inductive signaling and boundary formation. Lateral inhibition/specification occurs between largely “equivalent” groups of cells that express both Notch receptor and ligand. Notch signaling amplifies small differences between these cells until distinct biochemical events occur that dictate cell fate, and results in the generation of cells of different types. Lateral inhibition/specification is instrumental in neurogenesis in vertebrates and invertebrates

(reviewed in [4-6]). Inductive signaling occurs between non-equivalent cells and despite expression of both ligand and receptors in both populations, signaling occurs directionally to alter cell fate. Lymphopoiesis is perhaps the best-characterized example in vertebrates. Here, despite the fact that Notch receptors and ligands are expressed in both bone marrow progenitors and the thymic stroma, Notch1 signaling occurs in bone marrow progenitors and is required for their development into T cells [7, 8] (reviewed in [9]). The role of Notch signaling in boundary formation is the most relevant to this review; here, Notch facilitates the formation of borders between distinct cellular fields. Examples of this exist in disparate developmental contexts; establishment of the dorsoventral wing boundary and the equator in the eye *(Drosophila),* anteroposterior border formation of the boundary between the dorsal and ventral thalamus (chicken), in rhombomeric boundary function of the hindbrain (zebrafish), and in segmentation of developing somites (*Xenopus*, snake, zebrafish, chicken, mouse [10-12] (reviewed in [13, 14]).

Somite formation, like embryonic development in general, is reliant on the activity of a number of key signaling mechanisms to drive fundamental processes such as cell proliferation, apoptosis, fate decisions, differentiation and migration. The complexity of the task is contingent on the integration of these processes and the signaling pathways that drive them. The Notch signaling pathway is known to integrate with fibroblast growth factor (FGF), hedgehog (Hh), janus kinase/signal transducers and activators of transcription (Jak/STAT), receptor tyrosine kinase (RTK), transforming growth factor-β (TGF-β), Wnt, and hypoxia pathways [15] (reviewed in [2, 16]). The degree of Integration between these signaling pathways and Notch has not been fully realized and the subject of future research will be to unravel these interdependencies.

2.2. Notch receptor and signal transduction

The Notch signaling pathway, first identified in *Drosophila*, is largely conserved through evolution from invertebrates to vertebrates [17, 18]. Notch is a type I transmembrane receptor that exists on the cell surface as a heterodimer [19, 20]. The receptor is activated upon binding delta or serrate, also type I transmembrane proteins, resident on adjacent cells. This activation of the signaling pathway *in trans* results in the signal-receiving cell often adopting a different fate to that of the signal-sending cell. In mammals, there are four Notch receptors (Notch1-4) (Fig. 1). The extracellular domains of Notch contain between 29 and 36 EGF-like repeats, associated with ligand binding, and three Lin-12/Notch (LIN) repeats that prevent ligand-independent signaling. The intracellular domain contains two protein interaction domains: the RBPj associated molecule (RAM) domain binds RBPj/CSL, and six ankyrin (ANK) repeats moderate protein interactions. Nuclear localization signals (NLS), a transactivation domain (TAD), and a PEST sequence, are also present to various extents.

The Notch signaling pathway is deceptively simple, it lacks second messengers as the intracellular domain (ICD) of the Notch receptor participates directly in target gene transcription (Fig. 2). However it is becoming increasingly clear that signaling specificity brings complexity and this is achieved through the action of Notch signaling modifiers (reviewed in [18]). Notch is synthesized as a single polypeptide. Specific epidermal growth factor (EGF)-like repeats are modified by *O*-fucosylation in the endoplasmic reticulum (ER) by protein O-fucosyltransferase 1 (Pofut1). As Notch traffics through the Golgi, it may be further modified by elongation of the *O*-linked fucose with an N-acetylglucosamine (GlcNAc); this is catalysed by a fucose-specific β1,3-N-acetylglucosaminyltransferase (β1,3-GlcNAc-transferase; fringe) [21, 22]. In the *trans*-Golgi network, Notch is cleaved (S1 cleavage) by a furin-like convertase [19, 20]. The N-terminal extracellular truncation (ECN) and C-terminal transmembrane and intracellular domain (TMIC) fragments form a heterodimer through non-covalent interactions and traffic to the cell surface [23, 24]. Here, the ECN is available to interact with ligand *in trans.* Ligand binding results in proteolytic

cleavage (S2 cleavage) by disintegrin and metalloprotease domain (ADAM) proteases ADAM10 and ADAM17, and in the separation of the Notch heterodimer through *trans*-endocytosis of the ECN into the signal-sending cell [25-30]. This produces a transmembrane bound fragment with the extracellular truncation (EXT), which is subsequently cleaved (S3 cleavage) by the γ-secretase complex [31-33] resulting in the release of the Notch intracellular domain (ICD). Notch ICD translocates to the nucleus where it forms a complex with the DNA-binding protein Rbpj/CSL (CBF1-Suppressor of Hairless-Lag1) [34, 35]. The interaction between ICD and CSL leads to displacement of transcriptional co-repressors previously complexed with CSL [34, 36-39]. Mastermind-like (MAML) is recruited to the Notch ICD-CSL complex ([40, 41], engages proteins required for transcriptional activation such as the histone acetyltransferase p300, and activates Notch target genes [37, 42, 43]. MAML also recruits CycC:CDK8 (cyclinC: cyclin dependent kinase8) to this binary complex, which leads to Notch ICD phosphorylation and degradation ([42, 44]. Known direct targets of Notch include members of the hairy/enhancer-of-split (HES), HES-related (HERP) and MESP families of bHLH transcription factors, Lfng, Cyclin D1, $p21^{WAF1}$, Nodal, PTEN and GFAP [45-54].

2.3. DSL ligands trans-activate and cis-inhibit Notch signalling

Notch receptors bind DSL (Delta/Serrate/Lag2) ligands. In mammals there are five DSL ligands that can be grouped into two classes (Delta-like and Serrate-like) based on homology to *Drosophila* Delta or Serrate. Dll1, Dll3 and Dll4 are Delta-like while Jagged1 and Jagged2 are Serrate-like ligands (Fig 1). The extracellular domain is characterized by a signal peptide, an amino-terminal domain (with limited homology between different DSL ligands), a DSL domain and a series of EGF-like repeats [55]. C-terminal to the transmembrane domain is an intracellular region lacking any recognizable motifs except for a PDZ ligand-binding domain present in Dll1, Dll4 and Jagged1.

The DSL domain is related to an EGF-like repeat and is required for receptor binding *in trans*. Relative affinities of DSL ligands binding *in trans* with Notch receptors have not been exhaustively determined; however, it is known that Dll1 interacts with Notch1 and Notch2, Jagged1 with Notch1-3, and Jagged2 with Notch2 [56-60]. Despite the absence of recognizable motifs in the intracellular domain of DSL ligands, this region is required to activate Notch *in trans.* It facilitates endocytosis of the ligand in association with the ECN part of Notch, into the ligand-expressing cell [27, 28, 61, 62]. This endocytosis requires the intracellular region of the DSL ligand to be ubiquitylated; this is achieved through the action of two E3-ubiquitin ligases, Mindbomb and Neuralized [63-65].

In addition to DSL ligands *trans*-activating Notch, they can also inhibit signalling when expressed in the same cell *(in cis)* as Notch [66, 67]. When coexpressed, Notch1 interacts with Dll1 or Jagged1 not on the cell surface but inside the cell [57]. The mechanism through which this occurs is not known, however co-expression of Lfng (β1,3 N-acetlyglucosyltransferase) inhibits this cell autonomous interaction [57]. Recently it has been shown that the DSL ligand Dll3 is distinct; it does not *trans*-activate Notch but rather interacts with Notch only *in cis* to inhibit signalling, and it is predominantly expressed in the *cis*-Golgi [68, 69].

3. Notch and DSL ligands are modified by glycosylation

3.1. O-glycosylation of Notch

The N-terminal region of Notch receptors contain tandem EGF-like repeats that are small protein domains of 40 amino acids with six conserved cysteines that form three conserved disulfide bonds [70]. EGF-like repeats are of two types: human EGF-like (hEGF) and complement Clr-like (cEGF) [71]. The EGF-like repeats of Notch are the hEGF type and

this subtype is present in proteins that are shed from the cell surface and mediate intercellular signaling [71]. Specific EGF-like repeats of Notch.undergo *O*-linked glycosylation with the addition of *O*-glucose and *O*-fucose (Fig. 3). These modifications occur on the hydroxyls of serine or threonine residues in the EGF-like repeats; *O*-glucose sites are located between the first and second conserved cysteine residues (C1XSXPC2) and *O*-fucose sites between the second and third conserved cysteine residues (C2X4-5S/TC3) [72, 73]. Both *O*-glucose and *O*-fucose can be elongated by the sequential action of other glycosyltransferases to yield a trisaccharide or a tetrasaccharide, respectively (Fig. 3). The structure, based on other EGF-associated *O*-glucose trisaccharide, is predicted to be Xylα1,3Xylα1,3Glc (where Xyl is xylose and Glc is glucose, in which glucose is attached to serine [74]. The tetrasaccharide attached to Notch is Sia-α2,3/6-Gal- β1,4-GlcNAc-β1,3-Fuc-α1-*O*-Ser (where Sia is sialic acid, Gal is galactose, GlcNAc is N-acetylglucosamine and Fuc is fucose, in which fucose is attached to serine or threonine [74].

O-glucosyltransferase activity using an EGF-like repeat as the acceptor and UDP-[(3)H]glucose as the donor was identified in mammalian cells and tissues; however, the gene encoding this activity was not idenitifed [75]. Only recently, a genetic screen designed to isolate temperature-sensitive mutants of *Drosophila* that affected Notch signaling identified *rumi*, a mutant that completely lacks *O*-glycosyltransferase activity [76]. The *rumi* gene encodes a CAP10 domain protein *O*-glycosyltransferase that acts in the endoplasminc reticulum (ER) lumen to regulate Notch folding. In the absence of rumi, the ability of Notch to be expressed on the cell surface and to bind Delta is not impaired, however S2 cleavage of Notch does not occur. This suggests that *O*-glucose modification by rumi is required for the ligand-induced conformational change that is required for S2 cleavage of Notch in *Drosophila*.

O-fucosyltransferase catalyses the addition of *O*-fucose to serine and threonine residues of Notch EGF-like repeats (Ofut1 in *Drosophila* and Pofut1 in mammals). In *Drosophila*, loss of Ofut1 phenocopies the loss of fringe and indicates that the absence of *O*-fucose is equivalent to the absence of elongated *O*-fucose. In mouse loss of *Pofut1* confirms that *O*-fucosyltransferase activity essential for Notch function; however, the phenotype is more similar to that generated when Notch signaling is blocked through, for example, the loss of RBPj/CSL [74, 77-80]. While most fucosyltransferases are type II membrane proteins in the *trans*-Golgi, Ofut1/Pofut1 is a soluble protein in ER [81, 82]. Functions for Ofut1, independent of, and dependent on transferase-activity, have been proposed that include folding of Notch in the ER, endocytic trafficking of Notch, and Notch-ligand binding [78, 79, 82-84]. Recent evidence provides compelling support for the non-catalytic activity of Ofut1 as a regulator of Notch trafficking to the cell surface, probably through action as a chaperone in the ER to promote the proper folding of the extracellular domain of Notch [85]. In addition, expression of Ofut1 (lacking catalytic activity) rescued Notch receptor activity and led to phenotypes mimicking loss of fringe activity (see below) [85]. In contrast to *Drosophila* Ofut1, mammalian Pofut1 is required for the generation of optimally functional Notch receptors, but is not an essential chaperone for stable, cell surface expression of Notch receptors [86].

3.2. Fringe elongates O-fucose of Notch EGF-like repeats

Fringe was identified in *Drosophila* as a gene that affected formation of the dorsoventral boundary during wing formation [87]. Genetic analysis revealed that fringe potentates delta-dependent Notch signaling and inhibits serrate-dependent Notch signaling [21, 22, 88, 89]. Fringe encodes a fucose-specific β1,3-N-acetylglucosaminyltransferase (β1,3-GlcNAc-transferase). Fringe activity requires UDP-GlcNAc as a donor and *O*-linked fucose in the alpha conformation as an acceptor [89]. In mammals, three genes encode for fringe activity; *lunatic fringe (Lfng), manic fringe (Mfng)* and *radical fringe (Rfng)* each of which is

capable of complementing mutations in *Drosophila fringe (Dfng)*[90, 91]. Fringe shows considerable preference for a properly folded EGF-like repeat as a substrate, and can glycosylate EGF-like repeats on Notch receptors and DSL ligands [74, 75, 88, 92].

3.3. Mammalian Fringe proteins

Catalytic activity

The catalytic activity of fringe proteins has been compared, and despite the use of different acceptor substrates, Lfng has a significantly higher catalytic efficiency than Mfng, Rfng or DLfng [89, 93]. Given that the substrate greatly affects the measured catalytic activity of the fringe enzymes, the current picture of relative activity may not be representative of specific fringe-notch interactions. Indeed, not all *O*-fucosylated EGF-like repeats are modified by Fringe, suggesting that Fringe may recognize amino acid sequences in individual EGF-like repeats [73]. Of those *O*-fucose EGF-like repeats that are modified by fringe activity, the three mammalian fringes display similar specificity with regard to the EGF-like repeats that are modified [93]; distinctions have been reported, with Mfng showing the least ability to modify *O*-fucose EGF-like repeats, but it is currently unclear if these are due to differences in catalytic activity of the fringes, or substrate recognition [88, 90].

Impact on Notch signaling

The three mammalian fringes have the capacity to modify four receptors; therefore, the effects of fringe activity on Notch signaling in mammals are more complex and not yet fully understood. Generally speaking, as in *Drosophila*, fringe activity enhances delta-mediated Notch signaling and inhibits jagged-mediated Notch activity [56, 94]. Current exceptions are: Rfng potentiates Jagged1-mediated Notch1 signaling [94]. Mfng does not enhance Notch2 signaling [95], and Lfng potentiates Jagged1-mediated Notch2 signaling [56]. These data are incomplete and further uncertainty ensues when gain- and loss-of-function studies with Lfng in chick and mouse embryos conclude that Lfng inhibits Notch1 signaling [96-98].

Fringe activity alters the efficacy of Notch-ligand binding *in trans* to affect Notch signaling [56, 88, 94]. Fringe activity increases the capacity of Notch1 to bind to Dll1 but does not alter Notch1-Jagged1 binding [94]. These binding assays relied on *in vivo* glycosylation so it was unclear if further modification of Notch, subsequent to the addition of GlcNAc by fringe, affected Notch-ligand binding. *In vitro* reconstitution assays demonstrated that addition of GlcNAc by fringe is sufficient to alter the interaction of Notch with its ligands, and that further elongation of the GlcNAc-1,3-Fuc disaccharide by Gal did not alter Notch-ligand binding *in vitro* [99]. In contrast, Chen et al [100] using showed that Fringe modulation of Jagged1-induced Notch signaling requires the addition of Gal to form the trisaccharide O-Fuc- GlcNAc-Gal on Notch. These disparate findings may reflect the fact that binding of *Drosophila* ligands with Notch was assayed in one study *[99],* and Notch signaling in rodent cells was examined in the other [100].

using Chinese hamster ovary (CHO) glycosylation mutants in a co-culture assay, showed that Fringe modulation of Jagged1-induced Notch signaling requires the addition of Gal to form the trisaccharide O-Fuc- GlcNAc-Gal on Notch (Chen et al., 2001). The galactosyl-transferase b4GalT-1 was shown to be necessary to obtain the
Fringe-mediated effects on Notch signaling in this assay (Chen
et al., 2001).J. Chen et al. / Gene Expression Patterns 6 (2006) 376–382 377
[100, 101]

As previously discussed, Notch receptors and DSL ligands also interact *in cis* leading to inhibition of signalling [57, 68, 69]. Expression of Lfng inhibits the intracellular interaction of Notch1 with Dll1 or Jagged1 [57] but not with Dll3 [69]. The mechanism of

ligand-dependent *cis*-inhibition of Notch signaling, and the role of fringe activity in this inhibition, is unknown.

4. Spondylocostal dysostosis (SCD) is caused by mutation of the fucose-specific β1,3 N-acetylglucosaminyltransferase LFNG

4.1 Spondylocostal dysostoses are a group of vertebral malsegmentation disorders

The vertebral column is characterized by the linear arrangement of regularly shaped vertebrae, bound together and supported by tendons and muscles. The vertebrae, tendons and muscles of the axial skeleton all differentiate from somites, which are derived from mesoderm. Somites condense into epithelial spheres on either side of the embryonic midline in a reiterative manner from caudally located presomitic mesoderm (PSM) during embryonic development. The ventral half of the somite (sclerotome) reverts to mesenchyme, which migrates around the neural tube and notochord and becomes partitioned into rostral and caudal halves; the caudal half of one somite combines with the rostral half of its neighboring somite to form an individual vertebra. These mesenchymal cells differentiate into cartilage, which later ossifies forming definitive vertebrae. These aspects of somite patterning and vertebral column formation have been well reviewed [102-104]. Abnormal vertebral segmentation (AVS) is a common congenital abnormality with an estimated prevalence of 0.5-1/1000 [105-108]. Many manifestations of AVS exist resulting in uneven or fused vertebrae that can be present once in the vertebral column or in multiples; AVS follows Mendelian inheritance (autosomal dominant and recessive) and also can occur sporadically [1]. The term spondylocostal dysostosis (SCD) is applied to a wide variety of radiological features that include multiple AVS. These include contiguous involvement of greater than ten vertebrae, often affecting all spinal regions. Despite asymmetric misalignment of some ribs and the presence of rib fusions, there is a basic overall symmetry to the shape of the thorax. Individuals with SCD have reduced stature and nonprogressive kyphosis [1]. Genetic mapping and candidate gene sequencing approaches have identified causative mutations in four genes. SCDO1 (OMIM 277300) [109] represents around 20% of all cases, and is due to mutation of the *DLL3* gene, with 25 distinct causative mutations identified to date [109-112] and unpublished). Mutation in *MESP2* (SCDO2; OMIM 608681 [113]), *LFNG* (SCDO3; OMIM 609813 [114]) and HES7 (SCDO4; [115]) appear to occur less frequently as they are currently represented by single cases. All four genes are part of the Notch signaling pathway; *DLL3* encodes an inhibitory ligand of Notch and *MESP2, LFNG* and *HES7* are Notch target genes expressed in the PSM.

4.2 Mutation of LFNG cause SCDO3

The proband presented with extensive congenital vertebral anomalies (Fig 4), long, slender fingers, and camptodactyly of the left index finger. Multiple vertebral ossification centers in the thoracic spine, with fitted angular shapes were apparent. The severe foreshortening of the spine was emphasized by the comparison of the patient's arm span (186.5 cm) with adult height (155 cm) [114]. Sequence analysis did not identify mutations in *DLL3* or *MESP2*; therefore a candidate gene approach was used to identify the genetic cause of SCD in this patient. A search for mouse mutants that recapitulated the SCD phenotype suggested that *LFNG* was indeed a good candidate for causing SCD. The phenotypes of embryos lacking *Dll3* and *Lfng* are virtually identical [116], indicating that murine somitogenesis is similarly dependent on each gene. Moreover, *Lfng* gene expression is severely disrupted in *Dll3* null embryos, suggesting that *Lfng* expression is dependent on Dll3 function [117, 118]. A homozygous missense mutation (c.564C>A) in exon 3 was detected, resulting in the substitution of leucine for phenylalanine (F188L)

[114]. The proband's consanguineous parents had normal spinal anatomy, and were both heterozygous for the mutant allele. The substituted phenylalanine residue is absolutely conserved in all known fringe proteins from *Drosophila melanogaster* to humans [119] and is close to the Mn^{2+}-binding active site of the enzyme [100] which is DDD 200-202 in human LFNG. Prediction of the structural consequence of the F188L substitution, based on the highly homologous Manic Fringe [120] suggests that the conserved phenylalanine residue (F188) is not directly involved in uridine diphosphate (UDP)–GlcNAc (donor) or protein binding. Rather, it is likely to reside in a helix that packs against the strand containing the Mn^{2+}-ligating residues D200 and the nearby D202. F188 is predicted to form an aromatic cluster with residues F196 and H198, and thus the F188L mutation may either cause steric perturbation of the Mn^{2+}-binding site by altered packing of the smaller mutant (leucine) residue or cause electronic disruption of the enzymatic reaction by removal of required π–π interactions associated with the aromatic ring [114].

Functional analysis of the LFNG F188L variant demonstrated that it did not function like wildtype LFNG and thus it is considered that c.564C>A in exon 3 represent a null mutation in *LFNG* [114]. Firstly localization of wildtype and F188L LFNG, was compared by expressing wildtype mouse LFNG or the F187L, which is the mouse version of F188L in C2C12 myoblasts cells. LFNG is normally localized to the Golgi where it adds β-1,3-N-acetylglucosamine to *O*-fucosylated EGF-like repeats of Notch [56]. This was confirmed with wildtype LFNG, it accumulated in a Golgi-like pattern in the cell and colocalised with GM130, which marks the *cis*-Golgi compartment. In contrast F187L was neither enriched in a Golgi-like pattern nor was there colocalization with GM130. Next, the ability of LFNG to modify Notch1 signaling was determined using a coculture assay [121]. Notch1 signaling is enhanced by LFNG when *trans*-activated by the Dll1 ligand and conversely attenuated when activated by the Jagged1 ligand [56, 94]. It was demonstrated that LFNG F187L did not enhance Dll1-Notch1 signaling and did not reduce Jagged1-Notch1 signaling like wildtype LFNG. Finally the GlcNAc-transferase activity of F187L was examined *in vitro* using UDP-[3H]GlcNAc as the donor and pNp-fucose as the acceptor for the reaction; F187L lacked GlcNAc-transferase activity [114].

In summary these studies demonstrate that F1887L did not accumulate in the Golgi, did not modify Notch1 signaling, and lacked GlcNAc-transferase activity. LFNG D202A was used as a control in these assays as it lacks transferase activity [89, 100]. F187L was functionally equivalent to D202A except that D202A localized normally to the Golgi [114]. These findings strongly support the hypothesis that F188L is non-functional and causes SCD in this patient.

4.3 Lfng-null mice model SCDO3 and define the developmental origins of the vertebral anomalies

Lfng expression in mouse

In mouse during gastrulation, *Lfng* transcripts are localized to posterior nascent mesoderm with expression becoming restricted to stripes either side of the node [122]. This expression appears coincident with *Mesp2*, implicating *Lfng* in head-trunk boundary formation and demonstrating the existence of molecular prepatterning in nascent mesoderm [118]. Later in the PSM, *Lfng* transcripts are present in two domains; a broad caudal domain characterized by oscillatory expression, and a rostral domain that narrows in synchrony with somite formation (Fig. 5A). These two domains of *Lfng* expression are driven by distinct regulatory elements and the identification of functional CSL binding sites demonstrates that *Lfng* is a direct target of Notch signaling [45, 49]. Like *Lfng*, other targets of Notch signaling are expressed in an oscillatory pattern in the PSM such as *Hes1, Hes5, Hes7, Hey1* [117, 123-126]; they oscillate in caudal PSM in phase with the detection of Notch1 ICD which is the cleaved, activated form of Notch1 [97, 127].

Somitogenesis relies on the oscillatory progression rostrally of activated Notch1 in

the PSM. A key element of this oscillatory Notch1 activation is the cyclical expression of the transcriptional repressor Hes7 (reviewed in [128]. *Hes7*, like *Lfng*, is a target gene of Notch1 signaling; Hes7 protein inhibits its own transcription as well as that of *Lfng*. These interdependent negative regulatory mechanisms by Hes7 and Lfng are important to maintain the oscillatory activation of Notch1 in the PSM. Recently Shifley and Cole have demonstrated that processing of the mouse Lfng preprotein reduces the half-life of the enzyme, thus facilitating the rapid oscillations of Lfng activity and the segmentation clock that drives somitogenesis [129].

In the rostral PSM *Lfng* expression is also activated by Mesp2, Lfng then inhibits Notch1 signaling in this region creating an interface between cells undergoing Notch1 signaling and cells which are not; this interface marks the site where the next somite boundary will form (Fig. 5C) [97]. In addition, the rostral PSM cells that express *Lfng* adopt a rostral fate due to co-expression with Mesp2; Mesp2 acts as a rostral determinant as it restricts the expression of the caudal determinant, Dll1 to the caudal half-somite [97, 130, 131] (Fig. 5B). The expression of *Lfng* is altered in the PSM of Notch pathway mutants; further supporting the finding that *Lfng* is a target of Notch signaling [117, 118, 132, 133].

In addition to its expression in the PSM, *Lfng* is expressed in a number of other locations in the embryo and postnatal mouse, these include: the segmenting hindbrain, ventricular zone of the developing cortex neural crest cells, olfactory placode, inner ear, lung, kidney, urogenital epithelium and prostate, ovary, thymus and developing T cells, small intestine skin, hair follicle and teeth [90, 91, 133-149].

Somitic defects in mouse

Lfng is the only mammalian fringe protein required for normal somitogenesis [116, 150, 151]. *Lfng*-null mice have a reduced viability at birth and before weaning; their shortened tail consists of few vertebrae and their body is truncated (Fig. 6). The axial skeleton of these mice is severely disrupted along its length consisting of hemi-vertebra, and fusion of vertebrae, neural arches and ribs. These skeletal defects are presaged by anomalies in their precursor tissues, the somites. Somites are divided and patterned in order to impart flexibility and regularity on the components of the vertebral column, in addition they instruct the position of the dorsal route ganglia and the trajectories of the spinal nerves. Cells of the sclerotome condense in the caudal half of each somite and in doing so restrict the passage of neural crest cells and spinal nerve axons through the rostral part of the somite (reviewed [152]). In the absence of *Lfng*, the normal alternating density of sclerotome and the regular spacing of dorsal route ganglia and spinal nerve axons are disrupted. These defects are highly reminiscent of those generated when other components of the Notch signaling pathway, such as *Dll3*, *Mesp2*, *Hes7* and *Rippley2*, are absent during mouse development [117, 123, 153, 154]. *Lfng* is a direct target of Notch, and already noted Lfng inhibits Notch signaling in the PSM of mouse and chick embryos [96-98]. It is therefore consistent with this that Notch1 signaling is disrupted in the PSM of *Lfng*-null embryos and accordingly so is the expression of other known targets of Notch *(Mesp2, Hes5)* [97, 150]. More recently in an effort to dissect further the roles of Lfng in somitogenesis Shifley et al [98] deleted the oscillatory expression of *Lfng*, leaving the gene regulatory elements that control expression in the rostral PSM. In the absence of oscillatory *Lfng* expression in the caudal PSM, Notch1 activation does not cycle and instead is widespread. In addition, the mice developed AVS in the thoracic and lumbar spine, while the sacral and tail vertebrae are only minimally affected. This indicates that oscillatory *Lfng* expression and activation of Notch1 are necessary for normal segmentation and patterning of somites in the thoracic and lumbar regions, and largely dispensable during formation of the more caudal somites.

Whilst defects in somitogenesis and vertebral column formation are the most striking in mice lacking *Lfng*, functions for *Lfng* have also been uncovered in the inner ear and ovary. *Lfng* is expressed in non-sensory supporting cells in the mouse cochlea [140] and whilst deletion of *Lfng* has no effect on hair cell number or pattering, loss of *Lfng* does suppress the production of supernumerary hair cells that arise in the absence of the Notch

ligand Jagged2 [149]. In addition, *Lfng* is expressed in the granulosa cells of the developing ovarian follicles and in its absence folliculogenesis is abnormal with the progression of oocytes through meiosis slow and incomplete [136]. Infertility may be variable in its penetrance as some *Lfng*-null females are reportedly fertile [155].

5. Conclusions and perspectives

There has been incredible progress made as many of the genetic effectors of somitogenesis have been elucidated. Prominent in this increased understanding is the Notch signaling pathway, and the uncovering of the role that it plays in somite formation and patterning. The fucose-specific β1,3-N-acetylglucosaminyltransferase Lfng emerges as a prominent component of Notch1 signaling in the PSM and there is now an understanding of interdependency between target genes of Notch signaling, such as *Hes7* and *Lfng*, and the oscillatory activity of the Notch1 receptor in the PSM. In addition in the rostral PSM there is an understanding of how the transcription factor Mesp2 controls *Lfng* expression, and in doing so narrows the region of Notch1 signaling in the rostral PSM; this is providing great insights into the processes of somite segmentation and pattering. It is still not clear how *Lfng* expression leads to inhibition of Notch1 in the PSM, as *in vitro* studies show that Lfng potentiates Notch1 signalling when the receptor is *trans*-activated by the Dll1 ligand. Since Dll1 is the activating ligand of Notch1 in the PSM one might expect Notch1 activity to be potentiated by Lfng. It is clear that an understanding of Lfng modification of Notch1 ligands, and its potential role in ligand-dependent *cis*-inhibition of Notch1 will need to be achieved in order to address this conundrum.

Interaction between Notch signalling components and FGF and Wnt signalling are clearly important for somiogenesis [156-158] (reviewed in [2]). Effort is being made to establish a hierarchy between FGF, Notch and Wnt signalling in the PSM; this may be difficult to fully appreciate given the degree of negative feedback mechanisms that bind these pathways together. It is indeed this interdependency that likely underpins the robustness of somitogenesis.

The mouse has been at the forefront of research into somitogenesis as genetic modification has enabled definitive roles for many genes to be established (reviewed in [2, 159]). Clinical research into the genetic causes of AVS has clearly benefited from these developmental genetic studies in mice. For example, the discoveries showing that mutation of *DLL3*, *MESP2*, *LFNG* and very recently HES7, cause the congenital AVS disorder SCD were underpinned by the findings that these genes are each required for somite formation in mouse. Genetic analysis in mouse has shown that Notch, FGF and Wnt signalling are central to somitogenesis, therefore components of these pathways represent candidate genes for causing SCD (independent of *DLL3*, *MESP2*, *LFNG* or *HES7*) as well as other AVS disorders.

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Figure legends
Figure 1 Mammalian Notch receptors and DSL ligands.
The 4 Notch receptors (Notch1-4) and 5 DSL ligands (Dll1, Dll3, Dll4, Jagged1 and Jagged2) are type I transmembrane proteins with their carboxy terminus in the cytosol. Notch Receptors: N-terminal to the transmembrane domain, Notch receptors have 29 to

36 epidermal growth factor (EGF)-like repeats that are required for ligand binding, three LIN12/Notch repeats (LNR) that prevent ligand-independent signaling, and a heterodimerisation domain (overlapping parallel lines) which contains the S2 cleavage site. C-terminal to the transmembrane domain the receptors have an RBPj associated molecule (RAM) domain that binds RBPj/CSL, and six ankyrin (ANK) repeats which generally moderate protein interactions and are hydroxylated by factor inhibiting HIF (FIH) in Notch1-3. There is a nuclear localisation sequence (NLS) in the RAM domain and Notch1-3 have a second NLS. There is a transcription activation domain (TAD), and a C-terminal polypeptide enriched in proline, glutamine, serine and threonine residues (PEST) that is associated with protein degradation. A TAD has not been identified in Notch 3 or Notch4. Notch 4 also lacks a second NLS and PEST sequence. DSL Ligands: N-terminal to the transmembrane domain, the DLS ligands (excluding Dll3) have a Delta/Serrate/Lag (DLS) domain and EGF-like repeats that interacts with the EGF-like repeats of Notch. Jagged1-2 have a von Willebrand factor type C domain (VWC) which is generally involved in multiprotein complexes. C-terminal to the transmembrane domain the proteins are unstructured and, excluding Dll3 and Jagged2, there is a PDZ ligand-binding domain (PDZLDB) at the C-terminus. This figure was assembled using the following: ExPASy http://www.expasy.org/sprot/, SMART http://smart.embl-heidelberg.de/, PESTfind Analysis Webtool https://emb1.bcc.univie.ac.at/toolbox/pestfind/pestfind-analysis-webtool.htm, [160-167].

Figure 2 Notch1 signalling in mammalian somitogenesis.
Notch1 is synthesised as a single polypeptide. (A) Specific EGF-like repeats are modified by *O*-fucosylation in the endoplasmic reticulum (ER) by protein O-fucosyltransferase 1 (Pofut1). As the Notch1 polypeptide passes through the Golgi, these *O*-fucosylated EGF-like repeats may undergo elongation with the addition of GlcNAc by lunatic fringe (Lfng). (B) S1 cleavage by a furin-like convertase occurs in the *trans*-Golgi network (TGN). Unlike Dll1, the divergent ligand Dll3 is located in the *cis*-Golgi and not at the cell surface. (C) The Notch1 heterodimer consisting of the N-terminal extracellular truncation (ECN) and C-terminal transmembrane and intracellular domain (TMIC) traffics to the cell surface. (D) The DSL ligand Dll1 binds Notch1 ECN *in trans* and activates S2 cleavage by the disintegrin and metallopeptidase domain (ADAM) proteases ADAM10 or ADAM17. (E) This releases Notch1 ECN, which bound to Dll1, is *trans*-endocytosed into the signal-sending cell. (F) The membrane anchored Notch1 extracellular truncation (EXT) may undergo ubiquitylation (ub) facilitating endocytosis. Notch1 EXT undergoes S3 cleavage in the transmembrane domain, this is mediated by γ-secretase and releases Notch1 ICD. (G) Notch1 ICD binds the DNA-binding protein CSL, this triggers the release of corepressor (CoR) proteins and histone deacetylases (HDACs), and facilities the binding of coactivators (CoA) which results in transcription of target genes such as *Mesp2, Lfng* and *Hes7*. [18, 19, 168, 169].

Figure 3 *O*-linked glycosylation sites of mouse Notch1 EGF-like repeats.
EGF-like repeats that contain consensus sites for *O*-linked fucose (blue), *O*-linked glucose (yellow), or both (green) are indicated. Identified *O*-fucose glycans include sialic acidα(2,3)Galβ(1,4)GlcNAcβ(1,3)Fuc-*O* and *O*-glucose glycans include Xylα(1,3)Xylα(1,3)O-Glc-*O*. The N-terminus of Notch1 is at the top. Standardised symbols and colors were used from the Consortium for Functional Glycomics to represent glycans. [73, 74, 170].

Figure 4 Vertebral anomalies caused by mutation of *LFNG*.
Radiograph (A) and T2-weighted coronal MRI images (B, C) in the vertebral plane. (A) Severe vertebral segmentation anomalies throughout the vertebral column. (B) Thoracic spine showing vertebral centres with a fitted angular shape. (C) Cervical and lumbar spine

showing similar segmental anomalies. Reprinted from Sparrow et al [114], © 2005 by The American Society of Human Genetics.

Figure 5 Cycling expression of *Lfng*.
Dorsal views of whole-mount RNA *in situ* hybridization preparations of mouse embryo presomitic mesoderm showing *Lfng* expression (purple), rostral at the top. (A) Cycling *Lfng* expression is represented in three phases at E9.5. Embryos have been arranged to display selected stages in the temporal sequence of expression. (B) *Lfng* expression in embryos, bisected into axial halves. The left half was fixed, while the right half was cultured for 45 min. Rostral bands of *Lfng* expression display an anteriograde shift, and caudal regions of expression also change. Arrowheads indicate rostral *Lfng* bands of expression. Asterisks show caudal expression. (C) A schematic depiction of the genetic activities involved in Mesp2 activation and the subsequent events regulated by Mesp2. *Mesp2* is expressed in the anterior presomitic mesoderm by the cooperative function of *Tbx6* and Notch signaling (NICD dotted line). The Notch signal oscillates in the presomitic mesoderm, thus the dotted line moves anteriorly (white arrow). Once Mesp2 protein is generated, NICD is suppressed and several genes are activated that establish the segmental border and rostral–caudal polarity within the somites. S1, formed somite; S0, forming somite; S-1, S-2, somite primordia. Asterisks show caudal presomitic mesodermal expression. Figures 5A,B is reproduced from Kusumi et al [118], and 5C from Saga et al [130].

Figure 6 Skeletal phenotype of *Lfng*-null mice.
a, Photograph of 8-week-old wild-type and mutant littermates. Mutant animals have a very small and bent tail and their body axis is shortened from the neck to the rump. b–e, Alcian blue/Alizarin red skeletal preparations of newborn wild-type and mutant mice. b, Side view with limbs removed (WT, wild type; -/-, homozygous mutant). c, Dorsal and ventral views of the cervical, thoracic and lumbar regions, with the ventral ribs and sternum removed. c, Dissected ventral ribs and sternum. Reprinted by permission from Macmillan Publishers Ltd: Nature, Evrard et al [150], copyright (1998).

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