Chapter 4
Out on the Fringe: Modulation of Notch Signaling by Glycosylation

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Abstract Differential glycosylation of Notch, often as part of a feedback loop, represents a powerful mechanism by which signaling is regulated. Together with Dll (Delta) and Jagged (Serrate) ligands, Fringe, Rumi, and other sugar transferase proteins form a remarkably versatile system to coordinate Notch-dependent tissue patterning. When Fringe is induced in the same cell as Dll, it enhances signal reception through Notch, downregulates Dll through cis-inhibition, and helps to make neighboring cells distinct. When induced in a Jagged-expressing cell, it helps to create a hybrid signal sender/receiver identity with low levels of Notch signal reception, accompanied by (Jagged) signal sending activity without cis-inhibition. In this situation, Fringe can help drive neighbors to the same state. Fringe can even work together with Dll3 to inhibit Notch signaling in neighboring cells. A detailed mechanism by which Fringes control development of several tissues has begun to emerge. With time, studies on Notch glycosylation should help define how this system is used to control development in most tissues and how it can be exploited for therapeutic benefit in the fight against cancer and cardiovascular disease.

Keywords Notch · Lunatic Fringe · Manic Fringe · Radical Fringe · Rumi · Delta · Serrate · Dll · Jagged · Somitogenesis · Lymphocyte development · T-cells · Human cancer

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4.1 Introduction

In development, tissue boundaries often form “organizers” or signaling centers that help to pattern new structures, including limbs, organs, and elements of the nervous system. This is the case with a boundary formed between dorsal (D) and ventral (V) cells of the developing fly wing. In 1994, Irvine and Wieschaus described isolation and characterization of a *Drosophila* mutant with altered D/V boundary formation and loss of distal wing tisssue [1]. The mutant in question was in a gene coding for a novel secretory pathway protein, dubbed Fringe. *Fringe* is normally expressed in dorsal but not ventral cells of the developing wing imaginal disc. This gene had the remarkable property whereby a boundary between cells expressing it and those that didn’t, formed a new wing margin, just like the boundary that formed between dorsal and ventral cells in the wild-type imaginal disc. Indeed, ectopic wing margins formed at the boundary between Fringe mutant clones (Fringe$^-$) in the dorsal compartment and surrounding (*Fringe*-expressing) dorsal cells. Similarly, an ectopic margin formed at the boundary between ventral cells programed to express ectopic Fringe (*Fringe*$^+$) and neighboring ventral cells that do not express. Fringe also helped to prevent the mixing of dorsal and ventral cells at the boundary [2]. Next, Irvine and colleagues tested for *Fringe*-mediated regulation of other developmental events. Indeed, they found a critical role for a *Fringe* expression boundary in specifying the D/V midline of the eye, which forms an equatorial organizer. Altered *Fringe* function or expression in this context resulted in small eyes as well as altered chirality of ommatidial units [3]. In the developing fly leg, *Fringe* is expressed in alternate segments, where an expression boundary is required for specification of joints [4]. During oogenesis, *Fringe* is expressed and dynamically regulated in somatic follicle cells, where it’s required for specification of the polar cell fate [5]. Once again, as polar cells show organizer activity, a *Drosophila Fringe* expression boundary helps to control development of a major structure by coordinating formation of an organizer at a tissue boundary [6].

With description of such an unusual boundary-sensing protein, many labs began searching for vertebrate homologues. Indeed, in very short order, similar proteins were identified in frogs [7], chickens [8, 9], and mammals [10, 11]. Vertebrate *Lunatic Fringe (Lfng)*, *Manic Fringe (Mfng)*, and *Radical Fringe (Rfng)* showed remarkable expression patterns, suggestive of a conserved role in development at many tissue boundaries [10–12]. For example, *Lfng* was expressed in a striped pattern within developing somites and plays an important role in this context (see below). *Rfng* was expressed in the apical ectodermal ridge (AER), which promotes limb bud outgrowth. Functional studies in chicken suggested a role for *Rfng* in limb patterning, echoing to some extent the function identified in fruit fly wing development [8, 9] (a role for *Rfng* in AER-dependent limb outgrowth has not been seen in mice [13–15]).

Notch proteins are receptors, activated at the cell surface by Serrate and Delta family ligands. Interestingly, Notch activation at the D/V boundary of the wing imaginal disc is required for margin specification. Indeed, loss-of-function mutations in *Notch* or *Serrate* cause loss of wing tissue at the distal edge. It is this wing phenotype that led to the name, Notch. Similarly, *Serrate* mutants have a serrated

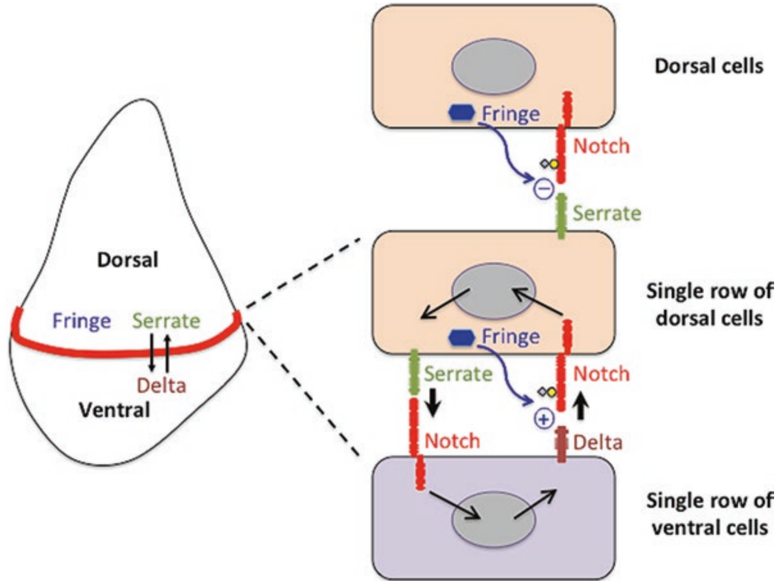

Fig. 4.1 Fringe-modulated Notch signaling in the developing *Drosophila* wing. A wing imaginal disc, which gives rise to the adult wing, is divided into dorsal and ventral compartments. Fringe and Serrate are expressed in dorsal cells, while Delta is expressed in ventral cells. Fringe potentiates Delta signaling to dorsal cells, but inhibits Serrate-mediated signaling. As a result, Notch activation occurs along the interface between both compartments (shown in red)

wing margin. As the Notch ligand/receptor system controls *Drosophila* wing margin specification and *Fringe* expression boundaries can induce ectopic margins, it seemed likely that these two systems interacted in some way. In this regard, *Drosophila* Fringe was found to block activation of Notch by Serrate in the dorsal compartment of the wing disc (but not in the single row of *ventral cells* at the D/V boundary [16, 17]). Also, Fringe promotes Delta-mediated Notch activation in the single row of *dorsal cells* at the D/V boundary and enhances Serrate expression in dorsal boundary cells [18]. Thus, the *Fringe* expression boundary limits Notch activation to a single row of ventral cells and a single row of dorsal cells, each on opposing sides of the D/V compartment boundary [17, 19] (Fig. 4.1).

4.2 Controlling Notch Activation Through Glycosylation

In 1997, Yuan et al. discovered a striking similarity between the sequence of Fringe proteins and glycosyltransferases [20]. This was a major breakthrough. Soon after, Haltiwanger and colleagues reported that Notch is glycosylated on specific serine and threonine residues within EGF-like repeats of the extracellular domain [21]. Some sugar chains were O-linked fucose tetrasaccharides (sialic

acid-α2,6-galactose-β1,4-N-acetylglucosamine-β1,3-fucose-α1-O- (or Sia-α2,6-Gal-β1,4-GlcNAc-β1,3-Fuc-α1-O-)), linked to serine or threonine residues (S/T) preceding the third of six conserved cysteines ($C_2X(4)S/TC_3$) [21–23] within EGF-like repeats. Others were O-linked glucose trisaccharides linked to serine residues between the first and second cysteine ($C_1XSX(A/P)C_2$) of many EGF-like repeats [21, 23, 24]. In this case, the specific modification was Xylose-α1,3-Xylose-α1,3-Glucose-β1-O-Serine (Xyl-α1,3-Xyl-α1,3-Glc-β1-O-Ser) [21] (see below). Remarkably, Fringe was the β1,3-N-acetylglucosaminyltransferase responsible for addition of the second sugar [25, 26] within O-linked fucose moieties. Thus, in cells that don't express Fringe, Notch appears on the surface with O-linked fucose at $C_2X(4)S/TC_3$ sites. Alternatively, in cells expressing Fringe, Notch has a mixture of disaccharide (GlcNAc-β1,3-Fuc-α1-O-Ser/Thr), trisaccharide (Gal-β1,4-GlcNAc-β1,3-Fuc-α1-O-Ser/Thr), and tetrasaccharide (Sia-α2,6-Gal-β1,4-GlcNAc-β1,3-Fuc-α1-O-Ser/Thr) modifications at these sites, depending on the expression of each glycosyltransferase [21, 25, 27] (Note: in flies, the disaccharide is either not extended or extended though addition of β1–4-linked glucuronic acid [27–29]). Fringe-mediated addition of GlcNAc is *the* critical determinant of reduced Serrate binding to Notch together with enhanced binding of Delta [27]. These findings help explain how Fringe can block activation of Notch by Serrate while enhancing activation by Delta [17]. Genetic and biochemical analysis established a critical role for Golgi localization and glycosyltransferase activity of Fringe [25, 26, 30–33]. Vertebrate Lfng, Mfng, and Rfng are also fucose-specific and Notch-directed β1,3-N-acetylglucosaminyltransferases, with Lunatic being the most potent enzyme of the three [25, 31]. Like its *Drosophila* counterpart, Lfng-mediated modification of Notch1 prevents its activation by Jag1 (mammalian Serrate homologue), while enhancing activation by Dll1 (mammalian Delta homologue) [34, 35].

4.3 Preparing Notch for Fringe: The Role of Fucosylation

In 2001, a gene coding for the enzyme responsible for O-fucosylation of Notch on EGF-like repeats was identified in multiple species including human, mouse, *Drosophila*, and *C. elegans* (Note: Pofut1 and Ofut1 are the names for this protein in vertebrates and flies, respectively. *Pofut1* and *Ofut1* are the gene names) [36]. Knockdown of *Ofut1* impaired *Fringe*-dependent *and Fringe*-independent Notch activation in flies [37]. This gene was also identified as *Neurotic*, which is required for Delta/Notch signaling [38]. Shi and Stanley reported on altered somitogenesis, as well as cardiac, blood vessel, and neuronal development in *Pofut1* mutant mice, phenotypes almost indistinguishable from those seen in mice with dramatic impairment of Notch signaling (as in *Rbpjk*$^{-/-}$ mutants) [39, 40]. Interestingly, deletion of *Pofut1* within the hematopoietic compartment, or replacement of Notch1 with a mutant that cannot be fucosylated on EGF-like repeat 12 (T466A), impaired Notch1-dependent T-cell development (see below) [41, 42].

A number of studies have begun to address exactly how Pofut1/Ofut1 functions to regulate Notch. Surprisingly, this protein may control Notch through slightly dif-

ferent mechanisms in flies and vertebrates. This could be related to the difference in temperature at which each develops, and potentially to differences in the complex process of Notch extracellular domain folding and trafficking in each case. Alternatively, apparent differences may be a reflection of context-specific functions for Pofut1/Ofut1 and Notch fucosylation, as well as to the very challenging nature of the question being addressed: how to define biological function of a protein in the presence or absence of a modification, in this case Notch fucosylation? Indeed, fucosylation of Notch occurs on the majority of EGF-like repeats within the extracellular domain, and each of these may have different or even opposite functions. Therefore, ultimately, the function of fucosylation will have to be defined for each residue subject to this modification [43]. As a first step, however, loss-of-function mutations in the glycosyltransferase can be used to test for phenotypes caused by loss of the modification on all sites (including those on other EGF-like repeat containing proteins [44]). As noted above, this results in loss of Notch signaling in both flies and mice. Strikingly, a fucosyltransferase-defective mutant (*Ofut1*^{R254A}) could rescue aspects of this phenotype, an effect attributed to the ability of Ofut1 to chaperone Notch from the endoplasmic reticulum to the cell surface [45, 46]. Specifically, *Ofut1*^{R254A} rescued the requirement for Ofut1 during neurogenesis and resulted in phenotypes associated with loss of Fringe. Thus, while Ofut1 protein may function to enhance trafficking of Notch to the cell surface and also to facilitate regulation by Fringe, fucose addition per se is not absolutely required for activation of Notch by either ligand [46]. Based solely on this data, one might consider three scenarios in the fly: (i) Notch expressed in the absence of Ofut1 shows reduced transport or stability on the cell surface but can be activated by either Delta or Serrate once there; (ii) Notch co-expressed with Ofut1 should be efficiently trafficked to the cell surface, but once there, it functions as a Serrate (as opposed to Delta) receptor; and (iii) Notch co-expressed with Ofut1 and Fringe, in which case it will be efficiently trafficked to the cell surface and activated by Delta, but not Serrate.

Interestingly, mouse *Pofut1* is also required for Notch1 trafficking to the cell surface, or perhaps for its stabilization at the membrane of cells within developing presomitic mesoderm [47, 48]. Surprisingly, however, *Pofut1* is not required for cell surface expression of Notch in mouse embryonic stem cells (mES). Also, while ligand binding and Notch signaling is defective in mutant ES cells, both properties can be rescued, at least partially, by *Pofut1*^{R254A} [49]. These findings highlight the importance of Pofut1 chaperone activity, not necessarily for transport to the surface, but for accumulation of a ligand-activatable form of Notch. To complicate things, ectopic expression of an unrelated enzymatically inactive ER-resident glucosidase (α-Gcs1^{S440F}) was also found to rescue ligand binding and Notch signaling. Therefore, induction of the unfolded protein response is likely responsible for rescue, through increased nonspecific chaperone activity within the secretory pathway [49]. Stahl et al. have also looked at ligand binding and Notch signaling in Lec13 Chinese hamster ovary (CHO) cells, which are wild type for *Pofut1*, but have a mutation in the gene coding for GDP-mannose-4,6-dehydratase (*Gmd*). As a result, Lec13 have extremely low levels of GDP-fucose (~3% of the levels seen in parental CHO cells). This deficiency can be corrected simply by growing cells in the presence of fucose [50–53]. Lec13 cells therefore express wild-type levels of ER-resident Pofut1 but insufficient GDP-fucose to modify Notch as it is synthesized. As with

Pofut1$^{-/-}$ mES cells, Notch synthesized in Lec13 cells is expressed on the surface but shows reduced ligand binding and a dramatic loss of ligand-dependent signaling. Importantly, ligand-independent signaling was not effected. In this case, ligand binding and signaling were both rescued through addition of fucose to the media, which restores fucosylation [52]. In sum, fucosylation of Notch *and* chaperone activity of Pofut1/Ofut1 both play an important role for this enzyme in *Fringe*-dependent and *Fringe*-independent Notch-mediated development.

4.4 The Specificity of Ligand-Receptor Interaction and the Role of Specific EGF-Like Repeats

Most EGF-like repeats in Notch, whether *Drosophila* Notch or human Notch (1 through 4), have an O-fucose addition consensus sequence ($C_2X(4)S/TC_3$), and these are efficiently modified [22]. Also, many of the fucose addition sites are conserved: EGF-like repeats 2, 3, 5, 6, 8, 9, 12, 16, 17, 18, 20, 21, 23, 24, 26, 27, 30, 31, 32, 35, and 36 [22]. Notable among these are EGF-like repeat 12 (which is within a critical ligand-binding domain) and EGF-like repeats 24, 26, and 27 (which are within the Abruptex region, which is subject to mutations that impair cell-autonomous, or cis-, inhibition by ligands) [54–56]. EGF-like repeat 12 of Notch represents a particularly critical surface for binding both Delta and Serrate family ligands [57]. Luca et al. recently reported on structural analysis of a Notch1 ligand-binding fragment (EGF [11–13]) in contact with an affinity-evolved mutant fragment from Dll4 ($Dll4_{SLP}$(N-EGF2)). Their data showed binding between EGF-like repeat 11 in Notch1 and the DSL (Delta/Serrate/Lag-2) domain of Dll4, as well as between EGF-like repeat 12 in Notch1 and the Dll4 MNNL (*m*odule at the *N*-terminus of *N*otch *l*igands) domain [58]. Remarkably, addition of O-linked fucose to threonine 466 within EGF-like repeat 12 increased the affinity of interaction with Dll4 by acting as a surrogate amino acid, hydrogen bonding to tyrosine 65 within MNNL [58]. Indeed, fucosylation of T466 also increased the affinity of Notch1 EGF [11–13] for Dll1 and Jagged1, and this effect was even stronger than for Dll4 [59]. This makes sense, as Dll4 shows a higher affinity for Notch1 to start with [60]. Finally, Fringe-mediated addition of GlcNAc to T466-O-fucose further increased affinity of EGF [11–13] for Delta and Serrate family ligands [59]. These data provide a detailed molecular description for how Fringe can enhance ligand-receptor interaction. Despite this, many questions remain. For example, the mechanism by which Fringe impairs Serrate family ligand binding to Notch has yet to be determined, although other EGF-like repeats seem likely to be involved [43, 59]. The importance of further elongation at GlcNAc-β1,3-Fuc-α1-O-Ser/Thr disaccharide moieties has been described but not fully explored [61, 62]. Also, while in vitro and in vivo (see below) studies show that specific Notch receptors can respond differently to the same Fringe [34], and different Fringe proteins seem to effect individual ligands and receptors in unique ways [35, 63–65], the details underlying such complexity have yet to be defined.

4.5 Pofut1, Fringe, and Direct Modification of Ligand EGF-Like Repeats

Just like Notch, its Delta and Serrate family ligands are transmembrane proteins with multiple EGF-like repeats, and many of these have potential O-fucose acceptor sites. Indeed, *Drosophila* and vertebrate ligands are substrates for Ofut1 and for elongation of fucosylated residues by Fringe [23]. Despite this, *Ofut1* mutant clones can induce activation of Notch signaling in adjacent wild-type cells, and therefore Notch ligands maintain signaling function, even in the absence of fucosylation [37]. The importance of Dll1 fucosylation, with subsequent Fringe-mediated extension of fucose moieties through addition of GlcNAc, has been studied in vitro and in vivo. In both cases, Dll1 was found to reach the cell surface in the absence of Pofut1-mediated fucosylation [66]. In contrast, a role for fucosylation and GlcNAc addition does seem to be important for Dll3, an unusual Delta family protein in mammals that functions as a cell-autonomous mediator of Notch cis-inhibition (see below) [67–70].

4.6 Vertebrate Fringe Proteins in Paraxial Mesoderm Segmentation

In 1998, the Gridley and Johnson labs described phenotypic consequences of *Lfng* deletion in the mouse [71, 72]. These mutants were viable but small, with very short tails, as well as vertebral and rib patterning defects. All major abnormalities were traced to impaired anterior-posterior patterning of somites, transient epithelial structures that form from paraxial mesoderm during embryogenesis, which go on to generate vertebrae, ribs, and skeletal muscle. This phenotype was related to but milder than mutants for *Dll1*, *Notch1*, *Rbpjk*, *Psn1*, and *Mesp2* [73–80]. Interestingly, *Lfng* mutant mice were almost indistinguishable from *Dll3* mutants [81]. Also, congenital spondylocostal dysostosis, a vertebral disorder in humans with similarity to the phenotype seen in *Dll3* or *Lfng* mutant mice, is associated with loss-of-function mutations in *DLL3* or *LFNG*, as well as in other Notch pathway genes like *HES7*, *MESP2*, and *RIPPLY2* [82–90]. The similarity between *Lfng* and *Dll3* mutant phenotypes in mice and humans raised the possibility that Lfng and Dll3 function together during somitogenesis [70].

In *Drosophila* and related invertebrates, segmentation of the embryo takes place synchronously, along the entire anterior-posterior axis. In contrast, vertebrate segmentation occurs through sequential addition of body segments as the embryo grows at the caudal or posterior end [91]. Many events required to initiate, propagate, and precisely coordinate this process have been uncovered through studies in model systems, including mouse, chicken, zebrafish, and *Xenopus*. The number of somites, and the time taken to add a somite, can vary from one vertebrate species to another. Despite this, a related *clock-and-wavefront* model has been proposed to

explain segmentation in each case [91–93]. While a detailed discussion of sequential somite addition is beyond the scope of this review, in simple terms, it involves coordination and crosstalk between FGF, Wnt, retinoic acid, and Notch signaling pathways [91, 93–98]. At the core of this system is a segmentation clock, by which each somite is generated with passage of a species-specific time window. This window can vary greatly. For example, in zebrafish, somite addition takes ~30 min, whereas in humans the somite addition cycle is 6 h long. The mouse clock takes approximately 90 min to traverse one cycle and therefore to add one somite on either side of the neural tube. Fundamentally, the clock and wavefront are both dependent on induction of delayed negative feedback loops for the above signaling pathways [99, 100]. For example, FGF signaling from caudal mesoderm induces expression of Dusp phosphatases and Sprouty, both of which suppress FGFR/Mapk signaling [101]. FGFR also activates expression of CYP26, which helps to degrade retinoic acid. This limits antagonism of FGF and Wnt signaling pathways by somite and anterior presomitic mesoderm-derived retinoic acid [102–104]. Wnt signaling induces expression of Axin2 and Dickkopf-related protein 1 (Dkk1), the former inhibiting Wnt signaling within cells and the latter inhibiting Wnt-Lrp6 interaction at the cell surface [96, 105]. Finally, Notch signaling induces expression of *Hes7*, *Nrarp*, and *Lfng*, all three of which inhibit Dll1-Notch1 signaling in this context [91, 106–108].

Interestingly, the absolute level of *Lfng* expression is important for precise coordination of somitogenesis, especially in anterior somites [109, 110]. Also, oscillation of Notch activation, rather than a Notch activation boundary per se, is *the* critical determinant of somite border formation and patterning within somites [111]. For Lfng, its fluctuation is controlled by Notch-dependent induction, followed by delays associated mostly with splicing and mRNA export from the nucleus [100, 108]. As for Lfng levels, these are limited through mir-125-5p-mediated *Lfng* mRNA destabilization [112, 113], as well as through proteolytic cleavage-mediated secretion of Lfng protein, which functions to decrease accumulation within the Golgi [114].

4.7 Cell-Autonomous and Non-cell-Autonomous Regulation of Notch and Its Ligands by Fringe Proteins

The function for *Lfng* as an inhibitor of Delta-Notch signaling in somitogenesis is striking and seems to contradict what is known about *Fringe* in *Drosophila*. Recently, an explanation for this discrepancy has begun to emerge. Indeed, recent insights on Fringe function have shown how it can be used to coordinate Notch-dependent processes quite distinct from the type of inductive signaling involved in D/V boundary formation in the fly wing. For example, unlike its positive effect on Delta-mediated Notch activation in the wing, Lfng-mediated inhibition of Notch1 signaling in presomitic mesoderm is *non-cell autonomous* [115]. This so-called transrepression activity is dependent on Dll3 and Dll1 in signaling cells and also correlates with fucosylation of Dll3 EGF-like repeats 2 and 5 at S286 and T403,

respectively [115]. Fucose residues on these sites can be extended through Lfng-mediated GlcNAc addition, at least in vitro [67]. As noted, Dll3 is not a typical Delta-family ligand. Indeed, it's not even expressed on the surface of presomitic mesoderm and cannot activate Notch expressed in neighboring cells [69, 70, 84]. Thus, the most parsimonious model to explain Lfng-mediated transrepression of Notch1 in the segmentation clock involves Pofut1- and Lfng-dependent addition of fucose and GlcNAc on Dll3, which then functions either to change Dll1 into a competitive inhibitor of Notch1 activation in neighboring cells or perhaps simply to decrease the Notch activating properties of Dll1. More studies will be required to precisely determine how Lfng interacts with Dll3 (and Dll1) in somitogenesis.

While the precise mechanism by which Fringes function to coordinate Notch signaling during boundary formation in flies, the segmentation clock in vertebrates, and the myriad other developmental, homeostatic, or pathological states in which it has been implicated remains to be determined at high resolution, sophisticated cell culture techniques and mathematical modeling experiments, together with the structural studies discussed above, are beginning to shed light on cis- and trans-interactions between Notch and its ligands as well as how Fringe can effect both. For example, Sprinzak and colleagues found that mutual interaction between Delta and Notch within the same cell is used to control an ultrasensitive switch between signal sender (Delta high/Notch low) and receiver (Delta low/Notch high) states [116]. The effect of Fringe proteins on cis-interactions is very similar to its effect on trans-interactions, in that Fringe enhances (cis-inhibitory) interactions between Delta-family ligands and Notch, when all three are expressed in the same cell. Consistent with this, Fringe actually cell-autonomously reduces (cis-inhibitory) interactions between Jagged/Serrate family ligands and Notch [117]. Remarkably, the cell-autonomous and non-cell-autonomous activities of Fringe can therefore be used to precisely regulate the specific Delta/Serrate signal sender *and* Delta/Serrate signal receiver functions of neighboring cells. Indeed, Troost and Klein have teased apart two sequential Fringe-dependent events during D/V boundary formation in the wing imaginal disc, the first involving dorsally expressed Serrate activating Notch in ventral cells [19]. This causes upregulation of Delta in the ventral compartment, which subsequently activates Notch in nearby boundary cells on the dorsal side [19].

Mutsado et al. used a "synthetic biology" approach to establish a bistable system in cultured cells, whereby Lfng-mediated positive feedback facilitated propagation of a Delta-Notch signal across the population [118]. In related experiments, they went on to show that Notch-mediated induction of *Lfng* can promote lateral inhibition by partitioning a population of bipotential cells into $Dll1^{+}$ signal sender and $Notch1^{+}$ signal receiver cells [119]. In this context, Notch-induced transcriptional repression of Delta and induction of Lfng, both in receiver cells, acted redundantly to enhance the signal receiving state (at the expense of the signal sender state). This redundancy may well explain why Fringe is not considered essential with respect to lateral inhibition in vivo. Indeed, with multiple feedback systems operational, the Notch activation system can be extremely robust in some biological contexts. For example, while *Lfng* knockout or knockdown cell-autonomously reduced the number of $Hes1^{+}$ signal-receiving cells in the developing mouse brain, this effect did not lead to altered neuronal differentiation or patterning [119, 120].

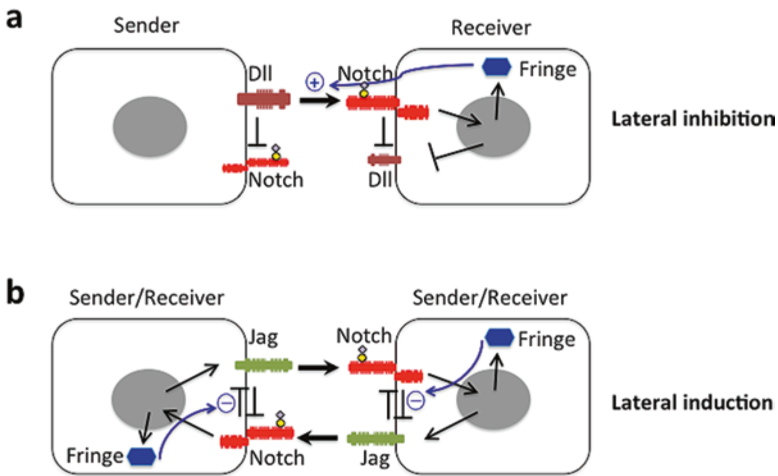

Fig. 4.2 A scheme for roles of Fringe in lateral inhibition and lateral induction. (**a**) Fringe can promote partitioning of bipotential cells into Delta^{Hi}/Notch^{Lo} signal sender and Delta^{Lo}/Notch^{Hi} signal receiver cells (lateral inhibition). In this context, Notch induces transcriptional repression of Delta and induction of Fringe both in receiver cells to enhance the signal receiving state at the expense of the signal sending state. (**b**) Fringe can facilitate formation of the hybrid Sender/Receiver state, leading to a more uniform phenotype among neighbors (lateral induction). In this regard, Notch upregulates both Serrate/Jagged and Fringe, creating a potent positive feedback loop, whereby Fringe prevents cis-inhibition of Notch in these cells

Genes coding for Delta and Jagged/Serrate family ligands are subject to distinct forms of regulation. For example, active Notch^{ICD} directly binds to and transcriptionally activates genes coding for Hes/Hey/E(spl) bHLH proteins. These, in turn, downregulate expression of *Dll1*. In contrast, Notch^{ICD} directly activates expression of *Jagged1* in some cells [121]. As noted above, Delta-Notch signaling facilitates lateral inhibition or partitioning of initially equivalent neighbors to distinct cell states: sender cells (Delta^{Hi}/Notch^{Lo}) and receiver cells (Delta^{Lo}/Notch^{Hi}). With a positive feedback loop between Notch and Jagged/Serrate, activation by ligand promotes formation of a third state: the hybrid sender/receiver state. In this hybrid state, cells can send and receive Notch signals, leading to a more uniform phenotype among neighbors (lateral induction). This idea, based to a large extent on mathematical modeling by Boareto et al., helps to explain how Fringe functions at the D/V boundary of the wing imaginal disc [122] (Fig. 4.2). In this regard, *Fringe* is upregulated by Notch^{ICD}. This creates a potent positive feedback loop, whereby Serrate and Fringe in one cell (e.g., a dorsal compartment cell) can accumulate to very high levels without cis-inhibition of Notch. The Serrate can therefore activate Notch in ventral cells. The dorsal Notch, modified by Fringe, will be very responsive to Delta, which is expressed in ventral neighbors. This model also helps to explain how Jagged and Fringe can facilitate Notch signaling between the epithelium and stroma, as well as between tumor and stromal elements [122].

4.8 Hematopoiesis and Lymphocyte Development: The Role of Lfng and Mfng

Notch signaling controls development and homeostasis within the hematopoietic system. Indeed, altered *Notch* gene function has been linked to a number of hematopoietic malignancies [123]. Once again, development of this system is complex, and roles for Notch signaling in this context are varied. We therefore refer readers to other reviews for a more exhaustive discussion [124–130]. Here we highlight a few key areas that are likely to involve Fringe or for which Fringe protein function has already been studied in detail.

Hematopoietic stem cells (HSC) derive from cells within the aorta-gonad-mesonephros (AGM) region of the embryo [131]. It is within the AGM that cells are partitioned between endothelial and hematopoietic fates on the basis of Notch1 signaling [132]. Cells exposed to a high level of Dll4 ligand are fated to become endothelium, whereas low-level Notch1 activation in response to Jagged1 induces HSC/ $CD45^{+}$ specification [130, 133–136]. The dosage-dependent effects documented in venous vs. arterial vs. hematopoietic cell fate specification within the AGM may be paradigmatic for many Notch-dependent developmental and homeostatic events. Interestingly, Fringe genes are downregulated in AGM endothelium after exposure to Jagged1 [130]. This downregulation may well increase sensitivity to Jagged1, while reducing sensitivity to Dll4, thereby reinforcing commitment to the hematopoietic stem cell fate. Once generated, HSC migrate to the fetal liver and ultimately to the bone marrow.

The generation and diversification of T-lymphocytes proceeds through multiple Notch1-dependent steps [137]. Firstly, Dll4 expressed by $Osteocalcin^{+}$ cells in the bone marrow stimulates development of thymus-seeding progenitors (TSP) [138]. Upon arriving in the thymus, TSP encounters Dll4 expressed by stromal cells. Dll4-mediated Notch1 activation then stimulates differentiation of TSPs into early T-cell precursors (ETPs). This step is associated with rapid loss of B-lymphocyte developmental potential [139–141]. ETPs then respond to Dll4 on thymic stroma by differentiating into $CD4^{-}CD8^{-}$*d*ouble *n*egative 2 (DN2) thymocytes, which lose the ability to generate myeloid cell types [142]. DN2 cells then differentiate into DN3a cells and then into one of two alternative cell types: $TCR\gamma\delta^{+}$ or $TCR\beta^{+}$ (DN3b). The $TCR\beta^{+}$ cell requires Notch1 as it proliferates and differentiates into a $CD4^{+}/CD8^{+}$ double-positive (DP) cell. Notch1 expression is significantly reduced as cells differentiate into DP. Also, Dll3 is expressed in DP cells, where it functions cell-autonomously to inhibit Notch-mediated induction of *Hes5*. Biologically, inhibition of Notch by Dll3 in this context is important for positive selection of αβ T-cells [143].

Lfng is highly expressed in ETP, DN2, and DN3, but not DP thymocytes. In competition experiments, *Lfng* is cell-autonomously required for Notch1-dependent T-cell development, as well as for suppression of the B-cell fate [144]. Interestingly, transgenic overexpression of *Lfng* in DN3 or DP causes them to bind so well to Dll4, that they non-cell-autonomously block wild-type TSP and ETP

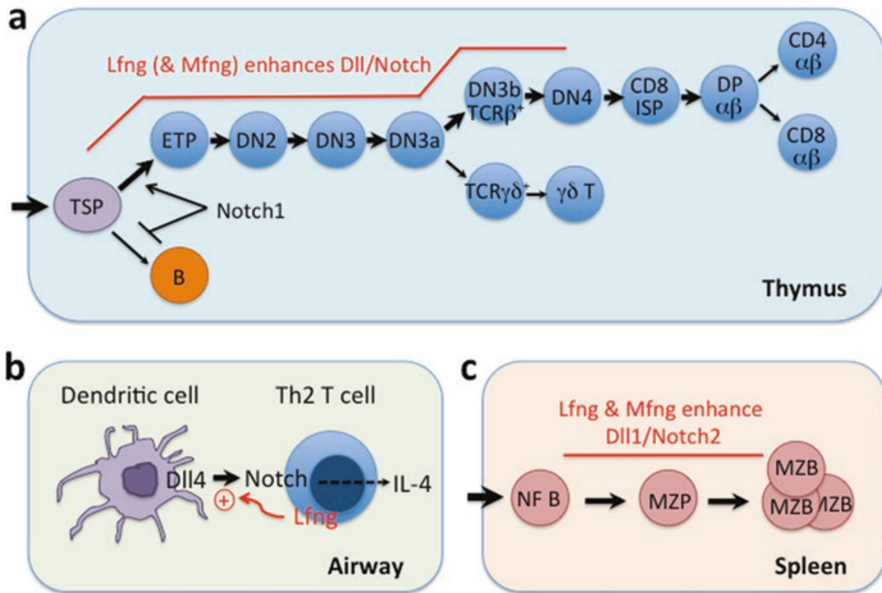

Fig. 4.3 Roles of Fringe-modulated Notch signaling in the immune system. (**a**) Lfng (and Mfng) promotes T-cell development while suppressing the B-cell fate through enhancement of Notch1 signaling. (**b**) Lfng contributes to the augmented Th2 response during viral exacerbation of existing airway allergy by enhancing Dll4-Notch activation. (**c**) Lfng and Mfng cooperatively enhance the Dll1-Notch2 interaction to promote marginal zone (MZ) B cell development in the spleen

from efficiently binding to the thymic niche, as required for Dll4-Notch1 signaling [145, 146]. As a result, transgenic cells impair T-cell specification and differentiation of immature transgenic thymocytes that have yet to activate the transgene [147] as well as wild-type immature cells that are present in chimeric mice [145]. Notch signaling also plays a role in mature effector T-cell differentiation, and this process is responsive to signaling from Jagged/Serrate *and* from Delta family ligands [148–150]; a role for Fringe proteins is to be anticipated. Indeed, *Lfng* helps control Notch-dependent T-cell differentiation in response to immunological challenge [151] (Fig. 4.3a,b).

In the thymus, Notch1 signaling promotes T-cell development at the expense of B-cells as noted above; however, Notch2 controls innate B-cell development in the spleen [152]. Dll1 expressed by fibroblasts within the splenic marginal zone (MZ) red pulp activates Notch2 in newly formed (NF) B-cells from the bone marrow [153]. Interestingly, this interaction is relatively weak [154], and hemizygous mutations in *Dll1* or *Notch2* impair innate B-cell generation [155, 156]. Indeed, this interaction is so weak that it requires Lfng- or Mfng-mediated glycosylation of Notch2 in NF B-cells [63] (Fig. 4.3c).

4.9 Role of Lfng and Mfng in Mammary Gland Development and Breast Cancer

Breast cancer is a heterogeneous disease. Most tumors express estrogen and progesterone receptors. A distinct group expresses elevated levels of the HER2/ErbB2 receptor tyrosine kinase. Another, very diverse group of tumors is defined on the basis of not expressing any of these receptors and is therefore described as "triple negative breast cancer" (TNBC) [157]. Transcriptional profiling has also been used to classify breast tumors into at least six molecular subtypes: basal-like, claudin-low, luminal A, luminal B, HER2-enriched, and normal-like. Among these, basal-like and claudin-low subtypes account for the majority of TNBCs [158–160]. Basal-like tumors expresses markers of myoepithelium/basal cells, share features with bipotent progenitors, and are thought to have originated from bipotent/luminal progenitor cells [159, 161, 162]. Claudin-low breast cancers (CLBCs) on the other hand share features with mammary stem cells (MaSCs) and cells that have undergone epithelial-to-mesenchymal transition (EMT)[158]. This has led to the suggestion that MaSCs could be the cell-of-origin for CLBC [158]. BLBC and CLBC are notoriously aggressive and prone to recurrence and metastasis. At present, no effective treatment exists for either. Understanding the unique biology of both subtypes should help provide insight into recurrence and metastasis and may identify specific targets for treatment.

It is widely believed that cancer stem cells (CSC) play an important role in tumor maintenance, tumor relapse, and metastasis. Also, EMT is known to promote metastasis in many contexts, and EMT by itself may generate cells with stem cell properties [163–166]. Notch signaling controls stem cell self-renewal, cell fate specification, and differentiation in the mammary gland [167–169]. Notch activation has also been shown to regulate EMT in developmental and pathological conditions [170]. Thus, it's not surprising that Notch plays an important role in breast cancer, especially in aggressive subtypes with features of stem cells and EMT. Indeed, high-level expression of *Jagged1* as well as *Notch1* and/or *Notch3* is associated with poor overall survival [171]. Also, functionally significant mutations, including *Notch1* and *Notch2* rearrangements, PEST domain mutations in *Notch1*, *Notch2*, and *Notch3*, and focal amplifications of *Notch2* and *Notch3*, have been linked to TNBC formation [172–174]. Recent studies have suggested many functions for Notch signaling in mammary development and cancer. For instance, Notch1 hyperactivation causes cyclin D1-dependent BLBC formation, and Notch1 signaling controls expansion of the MaSC compartment [167, 175, 176]; Notch2 genetic fate mapping reveals two previously unrecognized mammary epithelial lineages involved in branching morphogenesis and may have important implications vis-à-vis cell-of-origin for some breast tumors [177]; Notch3 marks clonogenic luminal progenitor cells, is required for luminal filling, and may induce cyclin D1-dependent luminal inflammatory breast tumors [178–180]; and Notch4 is implicated in self-renewal of breast cancer stem cells, EMT, and endocrine therapy resistance [181–183]. As a result, Notch has emerged as a potential drug target for poor prognosis breast cancer

[184–186]. However, Notch receptors, ligands, and modulators can exert complex effects on the pathogenesis of breast cancer, depending on tumor subtype, cellular context, and stage of disease progression. Therefore, it's critical to define roles for receptors, ligands, modulator, and downstream target in *each* subtype.

Within the developing mammary gland, *Lfng* is targeted to the stem cell-enriched cap cell layer of terminal end buds [187]. Consistent with this finding, human MaSC and/or bipotent progenitors express 20-fold higher levels of *Lfng* in comparison to luminally restricted progenitors [169]. Tissue-specific deletion of *Lfng* in mouse mammary epithelium (*Lfng$^{flox/flox}$;MMTV-Cre*) induced ectopic proliferation, expansion of the basal compartment, and ultimately, development of triple negative tumors. Gene expression profiling revealed that about two-thirds of these were basal-like and one-third claudin-low. Histological and immunohistochemical analyses confirmed features of both subtypes, with type I (basal-like) showing broad co-expression of markers for luminal (K8) and myoepithelial cells (K14) and type II (claudin-low) containing mostly spindle-shaped cells that were positive for EMT markers including vimentin and twist. Of note, the vast majority of human basal-like and a subset of claudin-low breast cancers show lower *Lfng* expression as compared with other subtypes or normal mammary tissue [187]. Thus *Lfng* deficiency represents a hallmark for basal-like mammary tumors in both mouse and human. Interestingly, most mammary tumors in *Lfng$^{flox/flox}$;MMTV-Cre* mice harbor Met/Caveolin gene amplification, causing elevated Met accumulation and activation. Met, a tyrosine kinase receptor, is frequently expressed at high levels in aggressive human breast cancer with EMT features, and expression of oncogenic Met (together with p53 loss) induced basal-like as well as claudin-low mammary tumors in transgenic mice [188–191]. Taken together, *Lfng* deficiency cooperates with Met/Caveolin amplification to induce BLBC (and less frequently, CLBC). Indeed, combination targeting of Met and Notch may prove beneficial for TNBC patients with Met overexpression and Notch hyperactivation [192].

It's noteworthy that very low levels of *Lfng* expression are seen in basal-like tumors, but only a small fraction of these harbor Notch-activating mutations [172–174]. Therefore downregulation of *Lfng* is more prevalent than functional mutations in Notch. The *Lfng* deletion model for TNBC will complement models that overexpress Notch intracellular domain fragments. In addition, the latter models are not good for testing most Notch-targeting agents (such as γ-secretase inhibitors), which block cleavage of the intact receptor.

While *Lfng* deficiency is a hallmark of BLBC, *Mfng* is highly expressed in CLBC, functioning as an oncogene in this context [193]. *Mfng* regulates Notch activation in human and mouse CLBC cell lines, as well as in the mouse mammary gland at puberty and during involution [193]. Knockdown of *Mfng* in CLBC cell lines reduced cell migration and tumorsphere formation associated with diminution of MaSC and/or bipotent progenitor cell populations, as well as reduced tumorigenicity. In addition, deletion of *Mfng* in the *Lfng$^{flox/flox}$;MMTV-Cre* mouse caused a tumor subtype shift away from CLBC. In this study, *Pik3cg*, which encodes PI3K catalytic subunit γ, was identified as a direct transcriptional target of *Mfng*-facilitated RBPJk-dependent Notch signaling. This finding may well shed light on why Notch pathway activation can confer resistance to PI3K inhibitors [194]. *Pik3cg* is aber-

rantly expressed in many invasive breast tumors, and its expression level correlates with metastatic potential of breast cancer cell lines [195]. Indeed, pharmacologic inhibition of PI3Kγ blocked migration and tumorsphere formation by CLBC cell lines [193]. Fringe-regulated Notch signaling within myeloid cells could also control response to tumor-derived chemoattractants that stimulate GPCRs coupled to PI3Kγ. In vivo studies will be required to determine precise roles for this Mfng-Notch-Pik3cg axis in CLBC pathogenesis, in particular, in the enrichment of cancer stem cells, induction of EMT, and recruitment of myeloid cells. Nonetheless, identification of Pik3cg as a Notch target prompts a new targeting strategy for treating CLBC and perhaps other poor prognosis breast cancers. As discussed above, Met was amplified in basal-like *and* claudin-low tumors from *Lfng$^{flox/flox}$;MMTV-Cre* mice, and Met can synergize with mutant *Trp53* to induce claudin-low like mammary tumors in the mouse [190]. Therefore, combination therapy against Met and PI3Kγ could represent an effective strategy for treatment of CLBC. *MFNG* expression in human breast cancer is highly correlated with expression of *NOTCH4*, but not with other Notch receptors. In addition, *Mfng* silencing in CLBC cell lines consistently decreased Notch4 activation/cleavage as did *Mfng* deletion in the mouse mammary gland [193]. Therefore, Mfng appears to control Notch4-mediated signaling in mammary epithelium. Given that Notch4 is enriched in MaSC, a putative cell-of-origin for CLBC, the Mfng-Notch4-Pik3cg signaling cascade may drive pathogenesis for this subtype.

The phenotypic response to Notch is often determined by the level of pathway activation, and this affects the balance between growth-stimulating and growth-suppressing effects [196]. In this context, Fringe may be used to precisely modulate signaling in distinct cell types of the mammary epithelial hierarchy. Recent studies in mice and in humans support a model whereby *Lfng* inhibits Notch activation within luminal progenitor cells to prevent BLBC, while Mfng enhances Notch4 signaling in mammary stem cells to promote CLBC initiation and progression. Of note, deletion of *Lfng and Mfng* dramatically decreased activation of multiple Notch receptors and induced adenosquamous carcinoma [193], suggesting a redundant role for these genes in MaSCs and/or multipotent progenitors of the mammary gland.

4.10 Roles of Lfng and Mfng in Lung Development and Lung Cancer

The lung is a highly specialized organ that facilitates rapid and efficient oxygenation of blood. This is achieved at the interface of alveoli and microcapillaries, each organized in a complex labyrinth of interconnected thin-walled sacs and tubules. During alveogenesis, the final stage of lung development, a multilayered structure is transformed into a thin epithelial/capillary wall composed of type II and type I alveolar cells, microcapillaries, fibroblasts, and elastic extracellular matrix. This event must occur in a highly coordinated manner under conditions where the lung is subjected to elastic forces associated with breathing. *Lfng* and other Notch pathway

components are highly expressed in the developing mouse lung. At 16.5–18.5 dpc, *Lfng* is expressed in saccular cells and in cells of the distal lung mesenchyme. The highest levels of *Lfng* expression are seen in pulmonary neuroendocrine cells (PNECs), which are organized in clusters known as neuroepithelial bodies (NEBs) or as solitary cells. At P5, *Lfng* is still expressed in NEBs but now also seen in endothelial cells of large pulmonary veins. Notch1 is expressed in bronchiolar cells at 16.5 dpc, whereas *Notch2* and *3* are highly expressed in mesenchyme and airway epithelium at 17.5 dpc and beyond. *Dll1* is also expressed in PNEC throughout lung development and in vascular endothelial cells after P5. Expression of *Dll4* is localized to endothelial cells scattered throughout the mesenchyme and in PNEC at 17.5 dpc. Finally, Jagged1 is expressed in pulmonary veins and arteries throughout development and in bronchiolar epithelial cells starting at 16.5 dpc [197].

Lfng null mice exhibit a dramatic defect in lung structure, with altered patterning and reduced vascular branching. Histological analysis of lung development in these mice revealed a delay in saccule expansion starting at 16.5 dpc, followed by defective alveolar septation, which persisted in older animals. *Lfng* mutant lungs show delayed distal epithelial cell differentiation, as manifested by lower expression of integrin β6 in saccular epithelial cells and decreased level of aquaporin 5 in type I alveolar cells. Expression of the type II alveolar cell marker SP-C, the Clara cell marker CC10, and PNEC markers, Ascl1 and CGRP, are largely normal in mutant lungs. The most striking defect is that smooth muscle actin α (sma), a myofibroblast differentiation marker, is expressed in myofibroblasts of wild-type but not in *Lfng* mutant mesenchyme at 17.5 dpc. During postnatal alveolar development, sma is localized in myofibroblast cells that have migrated to branch or septation points in the developing alveoli of wild-type lungs. In contrast, sma is expressed at a low level in a small number of *Lfng* mutant mesenchymal cells, most of which are trapped within multilayered walls separating large alveoli. Myofibroblast progenitor cells in the developing distal lung require PDGFRα [198]. This receptor is expressed similarly in wild-type and mutant lungs during late embryogenesis, suggesting that differentiation but not specification or spreading of myofibroblast progenitors is affected. Myofibroblasts express and deposit elastin, which is critical for alveogenesis and lung function. Indeed, *Lfng* mutant lungs exhibit aberrant deposition of elastin, with few elastic fibers evident at P14. Despite this, elastin accumulates and is trapped within multilayered walls at 6 wk. of age. Taken together, alveolar development is impaired in Lfng mutant mice, starting in the saccule phase of fetal development, with impaired differentiation of myofibroblasts, accompanied by a modest delay of type I alveolar epithelial cell differentiation [197].

Lfng could either be enhancing or suppressing Notch activation during distal lung development.

Interestingly, *Notch2* and *3* are both highly expressed during saccular development in the distal lung, and myofibroblast differentiation is impaired in *Notch2*$^{+/-}$*Notch3*$^{-/-}$ compound mutant embryos but not in single mutants, suggesting that these receptors function redundantly to induce myofibroblast differentiation. Moreover, conditional deletion of RBPJκ caused a similar impairment of myofibroblast differentiation. A simple model to explain these findings involves a requirement for *Lfng*-mediated facilitation of Dll-induced Notch2/3 activation in

myofibroblast progenitor cells. In this case, Dll4 expressed in endothelial cells of the distal lung would be the likely ligand.

Besides defective myofibroblast differentiation, *Lfng* mutant lungs exhibit a modest delay in saccule epithelial cell development that appears to correct itself by birth. This delay may be related to low-level expression of *Lfng* in saccular cells, which could affect Notch signaling in developing type II or type I cells. Previous studies suggest that Notch signaling is not required for specification of alveolar epithelial cells [199]. *Lfng* could indeed function in saccular cells to prevent Notch activation by Jagged ligands. In this case, ectopic Jagged-Notch activation would likely occur in *Lfng* mutant saccular cells and thereby block or delay alveolar epithelial differentiation [200, 201]. Alternatively, *Lfng* may not function within saccular or alveolar epithelial progenitor cells, and delayed differentiation of these cells may be secondary to impaired differentiation of myofibroblasts or other mesenchymal cell types [202].

A unique Notch expression boundary is noted at the NEB, where *Dll1*, *Dll4*, *Notch4*, and *Lfng* are expressed in PNEC cells, *Notch1*, *2*, and *3* are expressed in SSEA-1^{+}, CC10^{-} cells surrounding the NEB, and Jagged1 is expressed in neighboring epithelial cells [197, 203, 204] (Zhang et al., unpublished data). The NEB and related bronchoalveolar junctions are thought to represent a stem cell niche [205] as well as a signaling center that controls distal lung development [206]. *Lfng* may affect stem cell differentiation kinetics in this context. Interestingly, Branchfield et al. found that PNECs are important regulators of postnatal lung function [207]. In this study, inactivation of *Robo* in the mouse lung resulted in an inability of PNECs to cluster into NEBs. This triggered increased neuropeptide production upon exposure to air, leading to an increase in immune infiltrates, which in turn remodeled the matrix and irreversibly simplified alveoli [207]. Coincidentally, *Lfng* null mice show ectopic CGRP-positive cells in the distal lung, accompanied by accumulation of macrophages and severely disrupted alveoli in postnatal life [197] (Xu and Egan, unpublished data). Thus, expression of *Lfng* in NEBs appears to prevent solitary PNECs from entering the alveolar compartment, which may impact distal lung structure and function. In future, PNEC-specific deletion of *Lfng* will be needed to probe the functional significance of these findings. Finally, PNEC is the cell-of-origin for small cell lung cancer (SCLC) [208]. A recent study identified inactivating mutations in NOTCH family genes in 25% of human SCLC [209]. Activation of Notch signaling in a preclinical SCLC mouse model strikingly reduced the number of tumors and extended the survival of mutant mice, and neuroendocrine gene expression was abrogated by Notch activity in SCLC cells [209]. In this case, although deletion of *Lfng* did not affect specification of PNEC as judged by expression of Ascl1 and CGRP, it enhanced Notch activation in these cells (Xu and Egan, unpublished data), which could influence SCLC development.

As with Notch, differential expression of Fringes suggests non-redundancy and context-dependent function. Unlike *Lfng* mutants, the *Mfng* mutant lung appeared normal (Xu et al., unpublished data) [210]. Despite this, human MFNG is located on chromosome 22q13.1, a region of homozygous deletion in many non-small cell lung cancer (NSCLC) [211, 212]. Indeed, *MFNG* expression is consistently reduced in lung cancer as compared to normal lung tissue [213]. To the contrary, *MFNG* is

one of the most upregulated genes in response to antitumor B (ATB), a Chinese herbal mixture with chemopreventive activity in mouse models for lung cancer [214]. Indeed, reexpression of *MFNG* decreased expression of *HES1* and *HEYL* Notch target genes and reduced transformation-associated phenotype in vitro as well as tumorigenicity in vivo [213]. Surprisingly, these effects appear to be related to modulation of Notch3 protein stability. Indeed, Mfng enhanced degradation of Notch3, and proteasome inhibition reversed this effect [213]. A number of studies have suggested oncogenic roles of Notch3 in lung cancer. Interestingly, a recent study identified a rare population of $CD24^{+}ITGB4^{+}Notch^{hi}$ cells that drive tumor propagation in NSCLC and that require Notch3 for self-renewal [215]. This population is enriched after chemotherapy, and its gene signature correlates with poor prognosis. In agreement with this result, an investigation into inefficacy of erlotinib in advanced lung cancer found that EGFR blockade enriches for lung cancer stem-like cells through Notch3-dependent signaling [216]. Thus, Notch3 may represent an important therapeutic target for NSCLC. In this context, *Mfng*-mediated proteasome-dependent degradation of Notch3 could prove beneficial.

4.11 Tumor-Suppressive Role of Lfng in Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most lethal malignancies owing to its diagnosis at an advanced stage and resistance to most therapies. Extensive pathological studies have established a model for initiation and progression of PDAC. The most common precursor of this disease is a microscopic pancreatic lesion associated with ducts, referred to as pancreatic intraepithelial neoplasia (PanIN). PanINs progress through defined histological and molecular stages, ultimately advancing to invasive PDAC and metastasis. Genomic analyses have identified accumulating genetic alterations associated with disease progression. In this regard, activating mutations in *KRAS* have been detected in more than 90% of the PanIN lesions, whereas mutations in tumor suppressor genes such as *TP53*, *CDKN2A*, and *SMAD4* are associated with more advanced disease [217]. Thus, *KRAS* mutations may contribute to PDAC initiation, and subsequent mutations may promote tumor progression and metastasis.

In recent years, mouse models have been designed to recapitulate pathologic changes associated with human PDAC, including activation of oncogenic *Kras* and/or inactivation of *p53*, *Ink4a/Arf*, and *Smad4*. Additional genes and signals implicated in PDAC have been identified in these models and verified in humans. Indeed, Notch pathway activation has been linked to initiation and progression [218–220]. For example, *Notch2* appears to be required for progression from PanIN to PDAC [221]. Interestingly, loss- *and* gain-of-function mutations in *Notch1* rendered acinar cells more susceptible to *Kras*-induced PanIN formation and progression [218, 222, 223]. On the other hand, deficiency of isoprenylcysteine carboxyl methyltransferase, which methylates Ras and is considered a target for cancer therapy, actually exacerbated Kras-driven PDAC via Notch suppression [224]. Thus, the importance and

complexity of Notch signaling in pancreatic tumorigenesis has become increasingly evident. A dormant progenitor cell population in the adult pancreas is capable of initiating PDAC under conditions of oncogenic stimulation [225]. Acinar cells, not ductal cells, are competent to form *Kras*-induced PanIN, and active Notch signaling synergizes with *Kras* in PanIN initiation and progression [218, 226, 227]. In these studies, although nearly every pancreatic acinar cell expressed activated *Kras*, only a minority gave rise to PanIN. How the transforming activity of *Kras* is constrained, or which subset of acinar cells is preferentially targeted by *Kras*, is unknown. Notch controls cell fate specification and differentiation during pancreatic development as well as in the adult exocrine pancreas [228–231]. In this context, activation of Notch generally maintains the undifferentiated state. Thus, inappropriate Notch activation in the acinar compartment of the adult pancreas may cause accumulation of undifferentiated stem/progenitor cells, which may serve as preferred target for *Kras*-induced tumor initiation.

As noted above, *Lfng* functions to suppress tumor formation in the mammary gland, in part by preventing aberrant accumulation of stem/progenitor cells [187]. In the embryonic pancreas, *Lfng* is expressed in the same cells as Ptf1a, an exocrine cell marker [232]. In adults, *Lfng* expression is restricted to a small subset of acinar cells [233]. Given that Notch inhibits Ptf1 function, and acinar cell differentiation in the developing pancreas [234], *Lfng* could well be controlling Notch signaling to facilitate regulation of acinar cell differentiation during development and homeostasis. In the *Kras*$^{LSL\text{-}G12D/+}$;*Pdx1-Cre* mouse model of PDAC, deletion of *Lfng* in *Kras*-expressing cells caused increased activation of *Notch3* during tumor initiation and progression, as well as activation of Notch1 after disease onset, associated with upregulation of the Notch target gene *Hes1*. Interestingly, deletion of *Lfng* caused accumulation of Aldh1-positive stem-like cells. More importantly, loss of *Lfng* significantly accelerated PDAC development and shortened survival of these mice. Of note, *Lfng*-deficient tumors were typically poorly differentiated, with features of epithelial-to-mesenchymal transition[233]. Deletion of *Lfng* also caused Notch-mediated transcriptional repression of *Tgfb1*, *Tgfb2*, and *Tgfbr2* in otherwise wild-type mice and in the *Kras*$^{LSL\text{-}G12D/+}$*;Pdx1-Cre* pancreas after PDAC onset. In this regard, TGF-β might promote initiation of acinar-ductal metaplasia (ADM) and PanIN. Indeed, a recent report showed that spontaneous transition of human acinar cells toward a ductal and mesenchymal phenotype was decreased through inhibition of TGF-β signaling [235]. On the other hand, decreased TGF-β expression/signaling in animals with accelerated PDAC development suggests that TGF-β may prevent progression from PanIN to PDAC.

Although expression of *Lfng* is confined to a small subset of acinar cells, its deletion profoundly altered Notch signaling and dramatically accelerated pancreatic cancer development in the *Kras* model. Thus *Lfng*-expressing acinar cells, or their neighbors, may represent a preferential target for *Kras*-induced pancreatic cancer formation. Alternatively, *Lfng*-expressing cells may be resistant to dedifferentiation and tumor formation as compared to *Lfng*-negative cells. Since *Lfng*-expressing cells account for a small minority (~5%) of acinar cells, deletion of *Lfng* would not have such a dramatic effect on PDAC development in the latter case. However, the

pancreas in this study has already lost *Lfng* expression at the embryonic stage, which may affect differentiation of most cells in the acinar compartment. Therefore, to determine whether *Lfng*-expressing cells were really targeted by *Kras* to form PanIN and PDAC, lineage tracing of *Lfng*-expressing acinar cells will be required. Interestingly, the pancreas in *Lfng*$^{lacZ/lacZ}$ mice contains a dramatically increased number of X-Gal-positive cells as compared with the pancreas in *Lfng*$^{lacZ/+}$ mice [233], suggesting that *Lfng* may negatively regulate propagation of cells in which it is expressed. Notch signaling is required for maintenance of the cancer stem cell population in pancreatic cancer [236]. Indeed, DCLK1-positive preinvasive pancreatic cancer cells have tumor-initiating properties. These cells express high levels of HES1 and HEY1, and pharmacological inhibition of γ-secretase activity reduced accumulation of these cells in murine PanIN [237]. It would be interesting to determine whether these cells derive from *Lfng*-positive cells.

Individual Notch receptors have been found to play distinct, sometimes even opposing, roles in lung and pancreatic cancers [221, 238]. As noted above, Fringe proteins can regulate activation of individual Notch receptors in unique ways, depending on the ligands presented on the surface of neighboring cells. In the adult pancreas, individual Notch receptors are expressed in distinct cell types, and all four receptors are upregulated in precancerous lesions. *Lfng* appears to differentially regulate individual Notch receptors during initiation and progression of PDAC. Indeed, deletion of *Lfng* caused sustained Notch3 activation as early as 2 months of age, long before frank neoplasia. The activation of Notch2 was largely unaffected, whereas Notch1 activation was increased only after 4 months, when PanIN are progressing toward PDAC. *LFNG* knockdown in Miapaca2 human pancreatic cancer cells also caused increased activation of Notch, mostly Notch3 [233]. These data suggest that Notch3 is a major target for *Lfng*-mediated regulation in Kras-induced PDAC. Interestingly, accumulation of nuclear Notch3^{ICD} is associated with adverse clinical features in pancreatic adenocarcinoma [239, 240]. Also, expression of *Notch3* and *Jagged1* are correlated in human PDAC [241]. It therefore seems likely that *Lfng* functions to inhibit Jagged1-Notch3 signaling in the context of *Kras*-induced PDAC. Of note, antibody-mediated inhibition of Notch2/Notch3 (by tarextumab) inhibits tumor growth and decreases tumor-initiating cell frequency [186].

In summary, *Lfng* exerts a potent tumor suppressive role in *Kras*-induced PDAC. While Notch1 and Notch2 are thought to exert opposing effects on PDAC initiation and progression, studies in *Lfng* mutant mice suggests that Notch3 is a major player in pancreatic cancer. Future studies using Notch3 knockout mice could help define roles for Notch3 in pathogenesis of pancreatic cancer. Finally, *Lfng*-expressing acinar cells in the mature pancreas are likely to represent a stem-like population that is uniquely sensitive to oncogenic *Kras*-induced transformation, and self-renewal of these cells are limited by *Lfng* itself. In the future, a *Lfng*-Cre knock-in could be used to test whether *Lfng*-expressing acinar cells represent the cell-of-origin for PDAC and also whether these cells are required not only for initiation but for tumor maintenance. Ultimately, knowledge of these cells may help identify biomarkers for early detection and targeted therapies for this devastating disease.

4.12 Tumor-Suppressive Role of Lfng in Prostate Cancer Initiation

Prostate cancer is the most common malignancy in males. Despite advances in prostate cancer detection and therapy, much about this common malignancy remains unknown or controversial. Defining the cell-of-origin for prostate cancer should provide new insights into mechanism of tumor initiation, which may lead to improved prognosis and therapeutic options. Unfortunately, different approaches to this question have yielded different answers: tissue recombination assays support basal epithelial cells as the cell-of-origin, whereas genetically engineered mice coupled with lineage-tracing suggest luminal cells as the preferred target for transformation [242]. Meanwhile, it remains unclear whether tumors originating from different cell types within the prostate lead to distinct molecular subtypes, each with a distinct clinical course or outcome. In addition, how the normal prostatic epithelial hierarchy is established and maintained, and how it is subverted during oncogenic transformation, needs to be elucidated. From a clinical point of view, two important challenges are (1) the need to find new methods for distinguishing aggressive from indolent prostate cancers and (2) the need to identify effective therapeutic targets for the treatment of advanced castration-resistant prostate cancer.

Notch is a critical regulator of cell differentiation and proliferation in the prostate. Disruption of canonical Notch signaling starting from the earliest stages of prostate development through deletion of *RBPJκ* in *Nkx3.1*$^+$ cells resulted in decreased cell proliferation and loss of epithelial and smooth muscle progenitors. Conversely, expression of activated *Notch1*^{ICD} in *Nkx3.1*$^+$ cells increased cell proliferation and the number of p63$^+$ progenitors in basal epithelium [243]. Interestingly, deletion of *RBPJκ* in both compartments of the adult prostate with ARR2PB-Cre caused ectopic cell proliferation in the basal compartment during regeneration [244]. When Notch is activated exclusively in K8$^+$ luminal cells of the prostate, it stimulated proliferation and resistance to anoikis [245]. Thus, while Notch signaling plays complex roles during development, homeostasis, and regeneration of the prostate gland, it consistently appears to promote luminal specification and expansion of the luminal compartment.

Notch1 expression is elevated in malignant prostatic epithelial cells of primary and metastatic tumors from the TRAMP model of prostate cancer (the TRAMP mouse is a transgenic line with SV40 large T antigen expressed from the rat probasin promoter) [246]. Also, in humans, NOTCH1 and JAGGED1 are overexpressed in high-grade and metastatic prostate cancer as compared to localized prostate cancer or benign prostatic tissue [247, 248]. Interestingly, Notch2 is implicated in acquired docetaxel resistance in castration-resistant tumors [249], whereas Notch3 is activated by chronic hypoxia and contributes to progression [250]. A recent study on Notch signaling in the adult prostate and in prostatic tumor development revealed upregulation of pathway components, in particular, *Jagged1* and *2*, *Notch3*, and *Hey1* [251]. Deregulated Notch signaling in this context induces increased proliferation and expansion of the stem/progenitor compartment, thereby contributing to prostate tumorigenesis.

All three Fringe genes are expressed in the mouse prostate. Interestingly, while *Lfng* is expressed at lower level than either *Mfng* or *Rfng*, its expression is much more restricted to basal layer epithelial cells [244]. Basal cells undergo symmetric and asymmetric divisions leading to distinct cell fates. In contrast, luminal cells only divide symmetrically [252]. In fact, postnatal development of the prostate is mediated by multipotent stem cells in the basal layer that differentiate into basal, luminal, and neuroendocrine cells, as well as by unipotent basal and luminal progenitors [253]. A gene signature specific for basal cells has been identified that is enriched for expression of genes associated with late-stage metastatic prostate cancer, suggesting that aggressive tumors share a conserved transcriptional program with normal adult prostate basal stem cells [254]. Given that Notch signaling regulates basal cell proliferation and differentiation, basally expressed *Lfng* likely regulates activation. Indeed, deregulation of *Lfng*-dependent Notch signaling may promote initiation and/or progression of prostate cancer. This is somewhat reminiscent of the Lfng-modulated Notch signaling that has been shown to control basal stem/progenitor cell self-renewal and differentiation in mammary gland, where *Lfng* deficiency induces basal-like breast cancer [187].

In an attempt to define the function of *Lfng* in the prostate, Zhang et al. studied activation of different Notch receptors in the *Lfng* null prostate as well as epithelial development in this context. Deletion of *Lfng* in mice caused altered Notch activation in the prostate, associated with elevated accumulation of Notch1, Notch2, and Notch4 intracellular domains, decreased levels of the putative Notch3 intracellular fragment, as well as increased expression of Hes1, Hes5, and Hey2. Loss of *Lfng* resulted in expansion of the basal layer, increased proliferation of both luminal and basal cells, and ultimately, prostatic intraepithelial neoplasia. The *Lfng* null prostate showed downregulation of prostatic tumor suppressor gene *Nkx3.1* and increased androgen receptor expression. Interestingly, expression of LFNG and NKX3.1 were positively correlated in publically available human prostate cancer datasets. Knockdown of LFNG in DU-145 prostate cancer cells led to expansion of $CD44^{+}CD24^{-}$ and $CD49f^{+}CD24^{-}$ stem/progenitor-like cell population associated with enhanced prostatosphere-forming capacity. Taken together, these data reveal a tumor-suppressive role for *Lfng* in the prostate through differential regulation of Notch signaling [255]. *Lfng* inhibits activation of Notch1 and Notch4 in basal cells of the prostate gland, and deletion of *Lfng* resulted in accumulation of stem-like cells in the prostate basal compartment. Thus *Lfng* gene deficiency or silencing may contribute to prostate cancer initiation through Notch-mediated expansion of basal multipotent stem cells, one of the cellular origins of prostate cancer. Further, *Lfng*-modulated Notch signaling may be particularly important in the pathogenesis of aggressive prostate cancer, as these cancer cells share a molecular signature with normal adult prostate basal stem cells [254]. Finally, deletion of *Lfng* caused elevated TGF-β signaling [255], which may inhibit proliferation in early-stage lesions. However, this may promote tumor cell invasion and metastasis in advanced stages. Of note, we observed development of sarcomatoid carcinoma, a rare malignant tumor of the prostate having an aggressive clinical course and dismal prognosis, in the occasional *Lfng* null mice (Xu and Egan, unpublished data).

Future comprehensive studies using inducible deletion of *Lfng* in basal or luminal epithelial cells may help clarify the role of specific Notch receptor(s)/ligand(s) during pathogenesis of prostate cancer originated from different cell lineages, which will provide a solid basis for determining whether and how Notch should be employed as a therapeutic target for prostate cancer. Furthermore, Lfng/Notch downstream target genes in prostate cancer initiation, progression, and metastasis may well be biomarkers for the screening of aggressive prostate cancer out of many indolent ones, as well as candidate targets for effective treatment of advanced castration-resistant disease.

4.13 Notch and Rumi

As noted above, Notch is modified through addition of O-linked glucose trisaccharides (Xyl-α1,3-Xyl-α1,3-Glc-β1-O-Ser) [21] attached to serine residues between the first and second cysteine ($C_1XSX(A/P)C_2$) of many EGF-like repeats [21, 23, 24]. The glucose residue is added directly to Notch by a CAP10 domain containing glycosyltransferase, known as Rumi in *Drosophila* and Poglut1 in mammals [256, 257]. Also, in cases where two serines are present within the consensus target site ($C_1XSS(A/P)C_2$), then Rumi/Poglut1 can add glucose to the first serine or *xylose* to the second [258, 259]. Glucose addition by Rumi/Poglut1, as well as elongation of the glucose residue through xylose addition, occurs at many sites within Notch [24]. Genetic analysis in *Drosophila* reveals a temperature dependent loss of Notch signaling in *Rumi* homozygous mutant animals [256]. Similarly, deletion of *Poglut1* in mice causes a wide spectrum of Notch mutant phenotypes, not unlike deletion of *Pofut1* or *RBPJk* [257]. Interestingly, *Poglut1* mutant mice have phenotypes beyond what is expected of animals with defective Notch signaling. Indeed, other targets for Poglut1-mediated glycosylation, including Eyes Shut (Eys) and Crumbs (Crb)/Crumbs2 have been identified [260, 261].

The enzymes responsible for elongation of glucose have been identified. The first xylose is added by a glucoside xylotransferase (either *GXYLT1* or *GXYLT2*) [262]. The second xylose is added by xyloside xylosyltransferase 1 (*XXYLT1*) [263, 264]. Genetic analysis of *Drosophila* glucoside xylotransferase, known as *Shams*, strongly suggests that while glucose addition enhances Notch signaling, xylose elongation of glucose, which occurs primarily within the EGF-like repeat 14–20 region, inhibits Notch signaling by limiting cell surface expression [265]. Not surprisingly, Rumi/Poglut1 is also capable of adding glucose to $C_1XSX(A/P)C_2$ target sequences in Notch ligands. In the case of Jagged1, Rumi/Poglut1 functions to decrease accumulation of ligand in vascular smooth muscle cells (VSMC), and hemizygous deletion of *Poglut1* in VSMC suppresses Alagille syndrome like phenotypes in the liver of *Jagged1*$^{+/-}$ (C57BL/6) mice [266, 267]. Thus, at least in some contexts, *Poglut1* functions to inhibit Notch signaling when expressed in the signal-sending cell and to enhance signaling when expressed in the receiver cell.

4.14 New Frontiers

Fringes and other Notch targeted sugar transferases function to coordinate development of neighboring cells. In many cases they operate together with other Notch regulators as well as feedback loops. Therefore, any effort to define their role will require a detailed picture of which Notch ligands and receptors are expressed in which neighboring cells, which cells express fringe and/or other sugar transferases targeted to the Notch system, what biological states are being regulated by Notch, and how Notch signaling changes with time in the system. Thus far, we have a fairly good description of how fringe proteins control organizer formation at tissue boundaries in flies, as well as how Fringe proteins control somitogenesis and lymphocyte development. Below, we highlight a number of new and exciting research directions on glycosylation of Notch and its importance for development and disease.

(i) *Regulation of Notch by N-linked glycosylation and O-linked GlcNAc*: *Pofut1*-, *Fringe*-, and *Rumi*-mediated Notch modifications represent powerful mechanisms by which development/homeostasis is regulated. However, Notch and its ligands are subject to other sugar-based modifications. For example, N-linked glycosylation occurs and is likely to control folding, transport, signaling, and internalization of these proteins [22, 268–270]. Notch is also modified through direct addition of O-linked GlcNAc [29, 271, 272]. Unfortunately, the function of N-linked glycosylation or O-GlcNacylation on Notch will be very difficult to determine, unless the enzymes responsible are almost exclusively dedicated to regulation of Notch signaling. In other words, such modifications may be very important for regulating Notch, but in as much as they regulate other pathways, their function with respect to Notch will be difficult to pin down.

(ii) *Atypical Fringe proteins*: *Chondroitin sulfate synthase 1* (*CHSY1*), and related *CHSY3*, codes for type II transmembrane proteins that accumulate in the extracellular space. Both contain N-terminal fringe-related and C-terminal type-A glycosyltransferase domains. Humans with recessive mutations in *CHSY1* have preaxial brachydactyly with partial duplication of proximal phalanges [273]. In addition, patients with *CHSY1* mutations exhibit macrophthalmia. Interestingly, *Chsy1* knockdown zebrafish show many of the same phenotypes as humans with *CHSY1* mutations and show dramatically upregulated Jagged expression together with elevated Notch signaling [273]. Suppression of Morpholino-induced phenotypes and Notch hyperactivation was achieved through ectopic expression of a Morpholino-resistant wild-type *Chsy1* cDNA, but not by a cDNA with a mutation in the fringe domain [273]. Thus, Chsy1 and related chondroitin sulfate synthase genes are widely expressed Notch inhibitors, functioning either transiently in the secretory pathway or perhaps in the extracellular space where they accumulate. It remains to be determined how they control Notch activation/signaling.

(iii) *Fringe in development of the nervous system*: While neural development is grossly normal in single, double, and triple fringe mutant mice [210], a role for fringe in fine patterning or differentiation of the nervous system has yet to be

explored. Indeed, Fringe expression boundaries in the developing nervous system are suggestive of a role in compartmentalization and organizer specification [274]. For example, fringe expression boundaries are found between rhombomeres of the developing hindbrain [274–276]. In the developing forebrain, a *Lfng* expression boundary corresponds to the zona limitans intrathalamica (ZFI) organizer [274, 277]. Also, *Lfng* is expressed in the developing inner ear, and a *Lfng* mutation suppresses the effect of *Jagged2* deletion in this tissue [278]. Interestingly, *Dll3* is also expressed in the developing inner ear, as is Dll1 [279]. In the neural tube, as in the inner ear, Notch-dependent lateral inhibition ensures specification and differentiation of the correct cell types in a timely manner. In both cases, *Lfng* is induced downstream of Notch signaling [280]. As multiple feedback loops are at play in lateral inhibition, the role of Fringe can be difficult to detect unless a sensitized system is established and studied. Finally, Fringe has even been implicated in control of neuron-to-glial cell signaling through Notch [281].

(iv) *Fringe-dependent regulation of the cardiovascular system*: The Notch system is a potent regulator of vascular development and homeostasis. Indeed, as discussed above, dose-dependent Notch signaling is used to differentiate hemangioblasts into venous, arterial, and hematopoietic derivative fates, as well as to pattern the vascular network [130, 282]. In this latter context, Dll4 and Jagged1 play opposing and complementary roles [283]. Vegf induces Dll4 expression in endothelial tip cells, which lead to migration and growth of vascular tubes toward the Vegf source. Dll4 then activates Notch1 in neighboring endothelial stalk cells [284]. In these cells, Notch1^{ICD} induces Hey bHLH transcriptional repressors which downregulate Vegfr2 expression, preventing them from becoming tip cells [285, 286]. Jagged1 is also induced in stalk cells. In this context, Jagged1 appears to stabilize tip cell fate in the neighbor, as well as to activate Notch4 within the endothelium, thereby promoting vascular maturation [287]. Finally, Jagged1 is also thought to recruit mural cells to the growing endothelial network [284, 287]. In this system then, Dll4/Jagged1 interaction is thought to create and reinforce a boundary between tip cells and their neighboring stalk cells, which have a hybrid signal sender/receiver state [122, 288]. Fringes can stabilize the hybrid state, and all three fringes are expressed in developing vessels. Thus, in this context, fringes are thought to reinforce the tip/stalk cell-state balance, as well as to establish a dynamic system facilitating rapid cell-state transitions required for patterning [283, 287]. Very recently, *Mfng* has been implicated in coordination of Notch signaling by Dll4, Jagged1, and Jagged2 in the developing heart. Specifically, Mfng is thought to enhance Dll4-Notch1 signaling in the endocardium to promote trabeculation in the developing ventricle. Mfng is then downregulated to facilitate myocardial Jagged signaling [289]. While differential expression of fringes can help explain how tip/stalk cell balance is induced and sustained, much work remains to prove these models and to define detailed mechanisms by which fringes control Dll4 and Jagged signaling in the developing heart and vasculature.

Vascular patterning and homeostasis plays an important role in normal development and in major pathological conditions including cancer and cardiovascular disease. Indeed, much energy has been focused on developing Notch ligand- or receptor-based therapeutics [284, 290–296]. Once again, it remains to be determined what role fringes (and *Rumi/Poglut1*) play in tumor angiogenesis and cardiovascular disease, although they might well represent excellent targets for therapy directed against a limited and pathological set of Notch-controlled events, while sparing healthy vessels and other tissues.

(v) *Novel cancer therapy*: As discussed above, fringe can function as a tumor suppressor in a number of tissues. This finding is based primarily on studying fringe mutant mice, as well as on data from fringe gene expression in human tumors. However, fringe mutations are not common in the cancer genome. Also, surprisingly, fringe can actually induce transformation when overexpressed in cultured cells [297]. This complexity is almost certainly related to the tissue- and context-specific function for Notch itself, which is both an oncogene and a tumor suppressor gene. As fringe proteins are enzymes, and as they are critical regulator of Notch activation in many if not most tissues, they could represent excellent targets for cancer therapy. Before targeting fringe proteins in any pathological setting, however, it will be important to determine how Jagged and Dll ligands are functioning to activate one or other Notch in the normal and diseased tissue. In this regard, single-cell assays and mathematical modeling can help define the role of Pofut1-, Fringe-, and Rumi/Poglut1-mediated differential glycosylation in any Notch-dependent biological process.

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