Crumbs top and 'Notch'ed bottom in tumours

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Membrane-bound proteins integrate and coordinate various signalling activities to maintain tissue architecture. In recent years, research updates indicate that these proteins undergo post-translational modification like glycosylation to maintain tissue integrity. Here we discuss research updates from *Drosophila*, about two essential membrane-bound proteins, Notch and Crumbs, that undergo such modifications to interact with each other and their implications in tumorigenesis.

Keywords: Epidermal-growth-factor repeats, glycosyltransferases, membrane-bound proteins, tumorigenesis, xylosylation.

TISSUE architecture of multicellular organisms is maintained by integrating various signalling activities. Interaction among cell surface proteins through post-translational modifications functions as an additional level of regulation to reinforce the maintenance of tissue integrity. Deregulation of these activities leads to various developmental defects and diseases.

As most of these signalling activities and their regulatory mechanisms are well conserved between humans and *Drosophila*, fruit flies serve as a popular model to understand many of these basic molecular and cellular processes. Here we discuss about new updates on an interaction between a cell surface protein Notch and polarity regulator, Crumbs.

While Notch signalling plays a pivotal role in cell proliferation and differentiation[1], the polarity signalling complex plays a crucial role in maintaining cell polarity and cell migration[2]. Perturbations to these activities lead to tumour formation and metastasis. Numerous reports have indicated aberrant Notch signalling activity in several forms of leukaemia and solid tumour development[3–6]. Similarly, deregulated activities of polarity regulators are implicated in tumorigenesis[7–10].

Notch protein may function as an oncogene or as a tumour suppressor depending on its context-specific interaction[11,12]. In humans, besides their role in HER2 signalling pathway[6], Notch1 and ATM are inversely correlated with each other in mediating DNA damage response (DDR) to promote tumour formation[13]. In situations where spurious Notch is activated, DDR-mediated ataxia-telengiectasia mutated (ATM) machinery is deactivated, leading to the accumulation of mutations and progression of carcinogenesis (Figure 1). Notch activity has been shown to be regulated and fine-tuned by another transmembrane protein, Crumbs[14–16], belonging to a polarity signalling complex[17–19]. Crumbs is also shown to be a potential tumour suppressor, implicated in breast cancer development[6,20] and various cancer types[21]. Cell polarity regulators are suggested to serve as a switch between multipotent and differentiated states[22]. However, their interactive mechanism with the Notch signalling pathway, function and significance in maintaining tissue integrity remains elusive.

Unlike other organisms, as the *Drosophila* genome codes for single homologs for both Notch and Crumbs, it facilitates to understand the interactive roles between them. It is well established that Notch activates Crumbs' expression[14], and Crumbs regulates Notch localization on the cell surface[19]. Therefore, tight regulation between these two proteins helps maintain tissue integrity. Due to this essential interaction between them, Crumbs is suggested to be a critical, non-canonical ligand of Notch. Interestingly, Notch receptor[23] and Crumbs[24] are membrane-bound and exhibit structural similarities (Figure 1). Similar to the various glycoproteins that are glycosylated, both Notch and Crumbs are membrane-bound glycoproteins[25]. Based on recent information on various enzymes and post-translation regulatory mechanisms, especially on glycosylation, here we describe the possible interactive modes that exist between them to regulate tissue growth and integrity.

Structural similarities between Notch and Crumbs proteins

Heterodimeric Notch receptor includes (i) the extracellular domain (NECD) containing the epidermal growth factor (EGF) repeats, a small negative regulatory region (NRR) comprising LIN-12-Notch Repeats (LNR) and a heterodimerization domain (HD), and (ii) a transmembrane intracellular domain (NICD) (Figure 1). Notch signalling may be ligand-dependent or ligand-independent depending on the interaction with ligands (Delta/Serrate/LAG-2 (DSL))[1] or other proteins(like Shrub, Crumbs)[14,15,19,26], either in *trans* or *cis* to release NICD. The balance between the *trans* and *cis* interactions is one of the key mechanisms to

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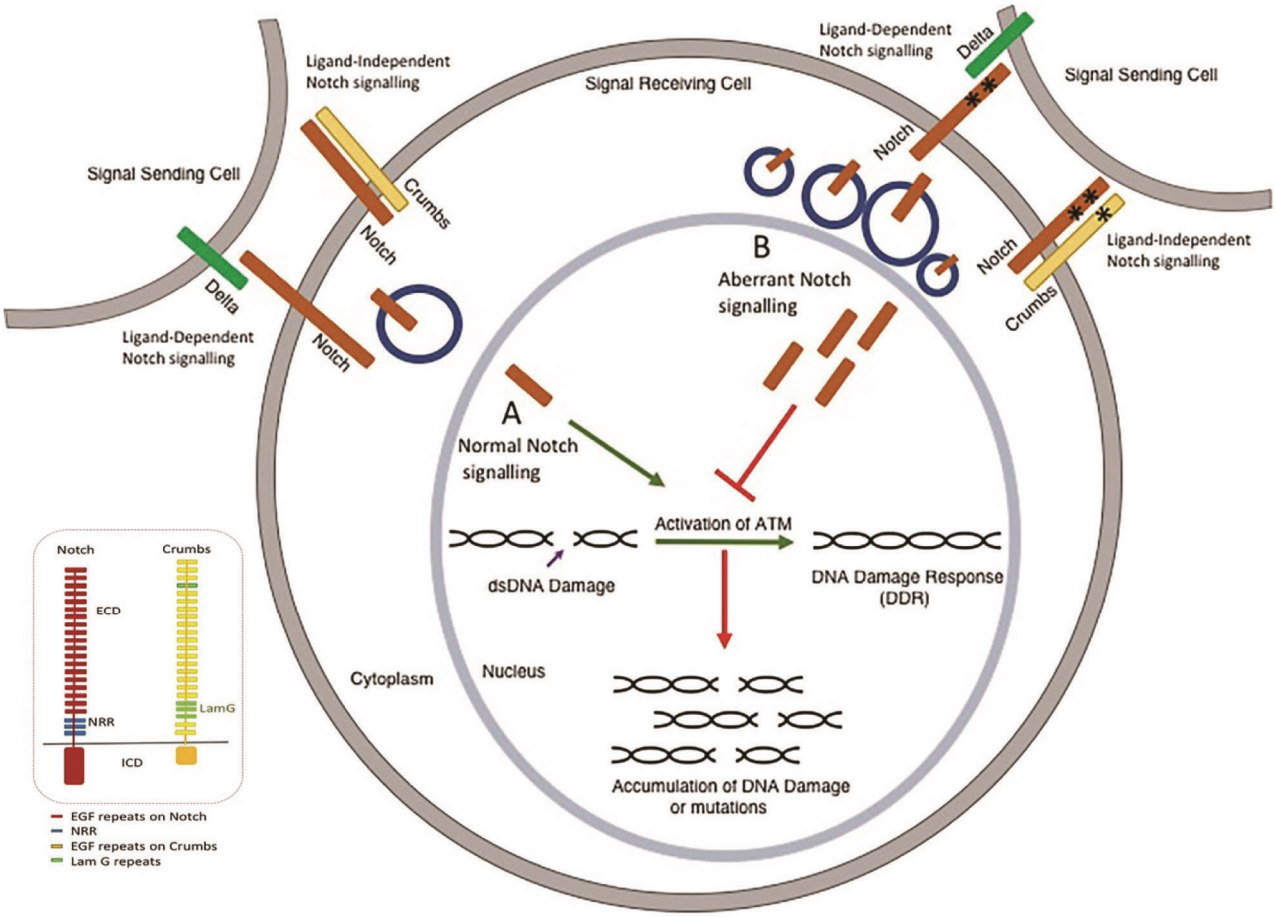

Figure 1. Aberrant Notch signalling leads to tumorigenesis. Notch and Crumbs are represented on the cell surface. Notch receptor comprises epidermal growth factor (EGF) repeats containing extracellular domain (NECD) and a transmembrane intracellular domain (NICD). On NECD, in addition to EGF repeats, a small negative regulatory region (NRR) comprising LIN-12-Notch Repeats (LNR) and a hetero-dimerization domain (HD) is sandwiched between EGF repeats and the trans-membrane (TM) region. Crumbs is a large, evolutionarily conserved transmembrane protein with an array of EGF-like repeats and laminin A–G-like repeats on the extracellular domain (ECD) and a short, well-conserved cytoplasmic tail containing 4.1/ezrin/radixin/moesin (FERM) domain and PSD-95/Dlg/ZO-1 (PDZ) domain. A, Normal Notch signalling – Notch protein interacts with ligands (delta) or other proteins (Crumbs) on the cell surface leading to ligand-dependent or ligand-independent signalling pathways respectively. When dsDNA damage takes place during normal Notch signalling, the ATM complex is recruited as a DNA damage response (DDR) to rectify it. B, Aberrant Notch signalling – Due to deregulated glycosylation (shown as ** on Notch and * on Crumbs proteins), ligand-dependent or ligand-independent signalling pathways lead to spurious Notch activity. During aberrant Notch signalling, activated Notch inactivates the ATM machinery, leading to accumulation of DNA mutations, thereby promoting tumorigenesis.

fine-tune Notch activity[16,27]. Irrespective of the mode of signalling, NECD transduces the cue to cleave NICD, leading to Notch activation. It is suggested that the interaction of NECD with LNR which permits cleavage of NICD is critical for Notch activity[28]. Therefore, interacting components that promote NICD cleavage must overcome the resistance imposed by LNR. Aberrant Notch signalling is also due to deregulation of factors that interact with the Notch protein. Among the various post-translational modifications (PTMs) that occur on Notch protein[29], evidence indicates that glycosylation (a process of adding sugar moieties to the protein), which brings about changes in the conformation of EGF domains to alter binding affinities between the residues, is pivotal for promoting *trans* or *cis* interaction[6] or ligand-dependent/ligand-independent interactions[12,30,31].

Crumbs is a large, evolutionarily conserved transmembrane protein with an array of EGF-like repeats[24] and laminin A–G-like repeats on the extra cellular domain (ECD) and a short cytoplasmic tail (Figure 1). Interestingly, EGF-like repeats and laminin repeats of Crumbs ECD are suggested to interact with other cell-surface proteins[32]. Crumbs homologs like Crb (in *Drosophila*), CRB1 and CRB2 (in humans) contain both ECD and cytoplasmic domain, while CRB3 has only the cytoplasmic domain. Crumbs being a tumour suppressor, its expression is negatively associated with tumour progression. In a recent study, CRB3 was found to be weakly expressed in breast epithelial cell lines[22,33], and shown to promote tumorigenesis by activating the Hippo signalling pathway. It has been clearly demonstrated that in *Drosophila* homolog Crb, Notch–Crumbs interaction takes place only

by ECD and not by the cytoplasmic intra cellular domain (ICD)[19], suggesting that ECD of Crumbs is required for interacting with Notch signalling pathway and ICD is needed for activating other signalling pathways.

Relation between Notch and Crumbs in tumorigenesis

Unlike Notch proteins which show ubiquitous expression in most of the tissues[1], Crumbs displays tissue-specific and differential expression[10]. According to the human protein atlas (www.proteinatlas.org), in normal tissues CRB1 is expressed only in eyes while CRB2 is highly expressed both in eyes and brain. In glioma patients, both CRB1 and CRB2 are not expressed; however, 11 out of 12 patients show high/medium levels of Notch protein indicating an oncogenic role of Notch1 (www.proteinatlas.org). In contrast, the profile of 4 out of 12 affected breast cancer patients showed low levels of CRB1 protein expression. Apparently spurious CRB1 expression may be the driving force to promote tumorigenesis. In human retinal epithelial tissues, CRB1 and CRB2 are shown to be downregulated[34] and Notch is upregulated. It has been shown that Crumbs and Notch stabilize each other at the cell surface[14]. However, during tumour malignancy, whether loss of Crumbs destabilizes Notch, or deregulation of factors that stabilize the Notch–Crumbs interaction leads to aberrant Notch activity is not known yet and requires further studies.

Role of glycosylation on Notch and Crumbs proteins

Earlier in *Drosophila*, Crumbs was shown to interact with Notch mediating ligand-dependent pathways[14,35]. In these studies, absence of Crumbs is suggested to enhance γ-secretase-mediated proteolytic processing of NICD. In contrast, studies from vertebrates suggested that ECD of Crumbs binds to Notch and inhibits its activity with its ligands in *cis*[36]. In support of this, a recent study in *Drosophila* suggests Crumbs to regulate Notch by promoting endocytosis and activate Notch in a ligand-independent manner[15,19]. Taken together, all these studies suggest that in situations or certain conditions where Notch activity is spurious, Crumbs tightens the interaction with Notch through its EGF repeat associations, thereby reducing NICD release. Although context- and tissue-specificity have been the basis for regulatory differences among these studies, other regulatory processes like glycosylation might have a role in Notch–Crumbs interaction.

Interestingly, EGF repeats on the extracellular domains of both Notch[12] and Crumbs[21,32] undergo rigorous glycosylation. Both Notch and Crumbs EGF repeats undergo *O*-glycosylation and *N*-glycosylation in Golgi bodies[37,38]. Following these modifications, mature glycosylated Crumbs and Notch proteins might undergo a homophilic interaction at the EGF domain, which might facilitate distribution and interaction on the cell surface. However, compared to Notch–ligands interactions, Notch–Crumbs interactions at EGF repeats of ECD are not well established.

What role can glycosylation have in mediating Notch–Crumbs interaction? To understand this, we considered the updates available on the glycosylation process in mediating Notch–Delta interaction. The interaction of Notch with its canonical DSL ligands has been demonstrated to take place at specific EGF repeats[16]. It has been established that interaction between the same EGF repeat (EGF3) and DSL domain of ligands is important for both *trans* activation and *cis* inhibition. It is suggested that on the same surface of both ligand and receptor, depending either on structurally distinct complexes formed[39], or based on additional glycosylations like xylosylation, *trans* activation or *cis* inhibition will be promoted[30]. Supporting this view, studies indicate that steric clashes between the interacting residues can reduce binding affinities. However, glycosylation of these interacting residues can reduce steric hindrance thereby enhancing the binding affinities[40], or it might provide the mechanical force required for cleaving NICD[16].

Extensive evidence demonstrates the indispensable role of glycosylation on Notch interactions with its ligands[12]. Updates on specific EGF repeats and identification of enzymes involved in the glycosylation process underscore the importance of understanding the underlying mechanisms of glycosylation. While glucosylation and fucosylation are positive regulators, xylosylation is a negative regulator of the Notch signalling pathway[41]. On specific folded EGF repeats of Notch, depending on the different surfaces recognized, glycosyltransferases like POFUT1 and POGLUT1/Rumi (in *Drosophila*) utilize similar mechanisms to add fucose and glucose moieties to the specific folded EGF repeats respectively[42]. In addition, POGLUT1 has been shown to possess dual donor substrate specificity, capable of utilizing both glucose and xylose to promote *O*-glucosylation or xylosylation. Also, xylosylation may not only prevent ligand–receptor interaction, but also inhibit interaction of EGF repeats with LNR on NECD, thereby activating ligand-independent Notch activation. In support of this view, mutations in the NRR region that possibly alter the EGF repeats have been shown to increase Notch activity through ligand-independent mode[41].

In addition, like Shams that adds xylose residues to glucose on specific EGF repeats of NECD, Fringe elongates by adding GlcNAc to fucose residues on specific EGF repeats of Notch. It has been demonstrated that specific EGF motifs are marked and modified by Fringe to mediate differential ligand binding[31]. Although it is well established that Fringe is a positive regulator of Notch activity that promotes receptor–ligand interactions by

specific residues, the combined function of various sugar modifications mediated by these enzymes and the regulatory mechanisms are not well understood. Possible interactive roles of various glycosyltransferases with LNR and other structural changes that resulted subsequently are not known yet. The role of Fringe or Shams on Notch–Crumbs interactions needs further research.

Similarly, xylosylation on Notch receptor may be required for Notch–Crumbs interactions. Interestingly, POGLUT1/hCLP46/Rumi is found to be over expressed in several human leukaemia, breast cancer and endometrial cancer cell lines[20] and has been shown to glycosylate Crumbs as well[21]. However, the role of glycosylation in mediating interactions between these cell surface proteins and their implications in tumour malignancy are not well understood. A possible interaction between *O*-glycosylated sugar residues on Crumbs and Notch may be required for Notch–Crumbs stabilization on the cell surface. Shams-mediated xylosylation that functions as a negative regulator of the Notch receptor may corroborate interaction of EGF repeats or sugar residues on Crumbs to reinforce negative regulation on Notch activation[12]. Loss of Shams might impair the interaction between Notch and Crumbs, thereby destabilizing Crumbs and promoting Notch ICD cleavage. Hence loss of Shams and xylosylation might not only enhance ligand–receptor interaction, but might also promote interaction of EGF repeats with LNR on NECD leading to NICD cleavage.

Conclusion

In summary, since Notch is a sensitive signalling pathway, its activation is subjected to additional regulatory mechanisms than predicted before. It is intriguing to understand several regulatory mechanisms operating from various levels to curtail spurious Notch activity. Glycosylated residues are upcoming promising candidates that could indicate the status of Notch activation. Mapping glycosylated residues, identifying the glycosylating complexes and understanding glycosylation-mediated regulatory mechanisms will be highly useful for overcoming tumour progression and treatment. Here we suggest that xylosylation functions not only to balance *trans* activation/*cis* inhibition of ligands with the Notch receptor, but it also stabilizes the Notch levels activated by ligand-dependent and ligand-independent pathways. Future research is necessary to understand the inhibitory or promoting roles of xylosylation on EGF repeats and LNR repeats of NECD. It is noteworthy to mention here that most NOTCH1 point mutations and insertions found in T-cell acute lymphocytic leukaemia (T-ALL) patients are mapped to NRR[43]. Therefore NRR has been identified as a mechanism-based therapeutic target. In addition, both inhibitory and activating antibodies against NRR and human NOTCH3 are available to modulate Notch activity[44]. As the Notch protein is a promising therapeutic target for cancer treatment[4], it would be interesting to understand the possible role of glycosylation processes on Notch–Crumbs interactions, which might facilitate the identification of more mechanism-based therapeutic targets and regulatory glycoproteins for treating human carcinoma.

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