NOTCH AFTER CLEAVAGE

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The discovery that Notch activation involves a proteolytic cleavage to release the intracellular domain (NICD) revolutionized the field of Notch signalling. It resulted in a simple model whereby the cleaved NICD enters the nucleus and activates expression of genes by forming a DNA bound complex with CSL. However, is it really this simple? The realization that the outcome from activating Notch varies greatly from cell to cell raised many questions about what governs the target gene selections in different cell types. Insights have come from recent genome-wide studies, which highlight the importance of tissue-specific transcription factors and epigenetics. Co-factors also have been identified that participate in the regulation of enhancers. Finally, it is generally assumed that once cleaved, NICD goes on to do its job, but with a burgeoning number of post-translations, it may not be that simple.

MAIN SECTIONS:

Background

Notch is the receptor in a highly conserved cell-cell signaling pathway [1] [2]. When Notch engages with its ligands on neighboring cells, they render it susceptible to proteolytic cleavage by metalloproteases of the ADAM family (Figure 1). Elegant structural studies have shown how force from ligand-receptor interactions displaces a protective shield to reveal the cleavage site [3]. Once the extracellular domain is cut away, the residual transmembrane fragment becomes a substrate for a further cleavage by the gamma-secretase complex, containing presenilin. This last cleavage liberates the Notch intracellular domain- NICD [4]. Although there may be some exceptions, most evidence points to the released NICD translocating rapidly into the nucleus, where it cooperates with a DNA binding protein CSL (CBF-1, Suppressor of Hairless, Lag-1) to regulate gene transcription (Figure 1). The complex formed contains a third protein, Mastermind, (Mam; MamL1-3) in addition to CSL and NICD (Figure 1) [5-7] and its primary role appears to be to activate transcription.

While the basic principles are well established, there remain many intriguing questions about how Notch (NICD) performs its role and whether the mechanism is the same in all cells and at all genes that are regulated. It is also currently not known whether different ligands (of which there are 2 in Drosophila and 4 in mammals) produce NICD with different characteristics. Although it is hard to envisage how the type of ligand could release NICD with distinct properties, they may affect how much NICD is produced and how rapidly.

Furthermore, in mammals where there are 4 Notch receptors, there is potential for the individual flavours of NICD to have different features. Similarly, although the three human Mam proteins appear to have similar functions, they have quite diverse structures so may also diverge in their activities [8]. Such differences are outside the scope of this review, which will focus on general properties of NICD after it is released by cleavage.

Characteristics of Notch intracellular domain

The key role of NICD is to assemble a transcription regulatory complex, which it does in a stepwise manner ([5-7]). Two regions, the N-terminal RAM domain and the ANK domain (Figure 1C) are involved in the interaction with its DNA binding partner, CSL. The ANK/CSL interface that is generated forms a groove to accommodate the N-terminal helix of the large Mam co-activator. Assembly of this CSL-NICD-Mam complex is the critical step in activating transcription, and it is likely that Mam has a major role in the recruitment of p300/CBP histone actyl transferases and other factors required for transcription initiation. A transactivation domain (TAD; Figure 1C) has also been defined in Notch1 and Notch2, but as this maps to a region with poor sequence conservation between the Notch paralogues it is unclear whether all NICD share this capability. However, in mice, a deletion of Notch1 TAD resulted in embryonic lethality and in decreased transcription of Notch target genes demonstrating its importance [9]. Finally the C-terminal region of NICD is a so-called PEST domain, (rich in prolines, glutamines serines, and threonines; Figure 1C) containing degrons-- motifs that promote degradation-- which are thought to confer a short half-life.

NICD/CSL complexes can clearly function as individuals, binding to their well-conserved DNA motif (Figure 1D) at target enhancers. However, NICD also has the capability to form dimers through its ANK domain [10]. This permits cooperative binding at positions where there are appropriately spaced head-to-head motifs, so-called SPS. Elegant experiments in which binding was detected by reconstitution of a Dam methylase in the presence and absence of specific mutations that perturb N1ICD demonstrated that binding at certain loci, including Hes1 and NRARP, was indeed dimerization sensitive [11]. Likewise dimerization mutants in N1ICD were compromised in their ability to induce expression of a subset of Notch1 targets, including Myc, with consequences for both T-cell development and transformation [12]. While clearly enriched among responsive enhancers, it remains to be established what distinct effects are conferred by the head-to-head motifs, especially since many enhancers contain both SPS and monomeric sites [13].

Tissue specific gene responses

Genome wide studies indicate that NICD/CSL complexes occupy many sites, binding to the canonical motif recognized by CSL (Figure 1C). However, of the circa 12,000 optimal CSL binding-motifs in the Drosophila genome, only a small proportion (1%) are occupied in any given cell [14]. This emphasizes that other factors must influence their recruitment. Indeed, the bound motifs are located in so-called active chromatin, where there are histone modifications associated with active or poised enhancers and where the DNA is quite accessible [14,15].

There are a handful of genes that respond widely to NICD, which include the HES/Hey gene family and Myc, the latter being particularly critical in several cancers [16-18]. However, the outcome from activating Notch receptors differs extensively according to the cellular context (Figure 1B) because different cohorts of genes are regulated by NICD [19] [14,20,21]. A key question is what enables the different cell-type specific responses to be mounted. How does NICD find the right set of genes in a given cell-type? As yet, there is a paucity of knowledge about this.

In a few cases, tissue specific factors that cooperate with NICD to confer specificity have been identified. These include proteins of the Ets and Runx families. For example, Runx motifs are enriched in the regions bound by NICD in T-ALL cells [11,15] and Drosophila hemocytes [22]. Binding of Runx and other TFs is thus likely to be a critical component to permit cell-type specific enhancers to respond to CSL-NICD. However, it remains unclear whether there is a specific subset of TFs that have the capability to co-operate with CSL-NICD or whether any nearby TF could do so.

Although CSL-NICD bound regions in a given cell type are often enriched for the recognition motifs of specific transcription factors, such as Runx, these enhancers didn't exhibit any stereotyped arrangement of motifs. Instead the binding of Runx, or equivalent transcription factors, in the vicinity of CSL motifs appears sufficient to render an enhancer responsive [15] [22]. One model is that the pre-binding of TFs creates the appropriate chromatin environment and/or exposes CSL motifs, so that the CSL-NICD-Mam complex is able to access them. This model implies that the main contribution from co-operating factors may be to establish a permissive chromatin environment. In support, expression of a Runx factor in *Drosophila* cells was sufficient to alter the local chromatin and to enhance CSL recruitment [14]. In T-ALL cells, many CSL bound regions lie within a larger enhancer domain or super-enhancer [15], arguing that there may be multiple inputs that collaborate to activate the enhancers.

These examples highlight the positive role played by cooperating TFs. However, equally important is the potential of cell-type specific TFs to decommission an enhancer, so that it stops responding. This is illustrated by Drosophila neural progenitors, which change their competence to respond to constitutively active Notch signalling due to the presence of the TF Eyeless/Pax6 [23]. Eyeless/Pax6 appears to directly or indirectly result in the decommissioning of key enhancers associated with progenitor maintenance. Likewise, in the optic lobe neural lineages, Notch activity in neuronal progeny results in two different fates, first inducing cell death and later promoting survival. This is dependant on a temporal switch in the TFs that are present [24]. Altogether these examples highlight the importance cell type-specific transcription factors, some acting positively to make an enhancer available others acting negatively to close enhancers down, so that in each cell context, a specific subset of CSL motifs will be rendered accessible. In this way different Notch responses can be elicited, explaining the diverse outcomes from signaling.

The change in enhancer accessibility is thus likely to require the recruitment of epigenetic regulators that locally modify the chromatin. Indeed, many different histone modifying and remodelling complexes have been found to influence activity of Notch targets [25] [26]. An interplay between Notch activity and epigenetic regulators may also establish conditions that contribute to disease progression. A persistent cell population in glioblastoma are dependent on Notch signalling and have up-regulated histone lysine demethylase (KDM6) activity, which leads to a reactivation of some developmental enhancers [27]. However, the consequences from altered epigenetic regulation are likely to differ depending on the chromatin landscape in a given cell-type, making the design of therapeutic strategies challenging.

The concept that differential recruitment of transcription factors confers tissue/cell specific responses is not unique to Notch. For example, the activation of neural specific Shh and BMP target genes depends on transcription factors of the SoxB family [28]. Indeed, a neural-type response to Shh was generated when SoxB family members were expressed in the limb bud [29]. Possibly because HES genes are responsive in many different contexts, the fact that cells mount different responses to Notch has been much less emphasized than for other signaling pathways, where the concept of specific cellular competence has been well-established. However, the realization that Notch activity can be tumour promoting in some contexts and tumour suppressing in others (Figure 1: [30,31]), is now focusing more attention on the importance of understanding how different responses are programmed.

Revisiting the NICD "switch"

A model, that NICD displaces co-repressors such as SMRT-HDAC from CSL to convert it from a repressor to an activator [32] is widely accepted and has many attractive features. It implies, firstly, that target loci are repressed by CSL in the absence of Notch activity. There is good evidence that CSL complexed with co-repressors is important to prevent or dampen transcription in the absence of NICD at least at some loci (e.g. [33]. The effects are often quite subtle— de-repression leads to lower levels of expression that activation, emphasizing the important contribution from NICD beyond alleviating repression.

The second characteristic of the original model, was that CSL remained resident on the DNA while NICD displaced the co-repressors. How NICD achieves this has not been solved and recent data may be best explained by a revised model, where exchange occurs between different pre-formed CSL complexes. For example, an exchange model can accommodate the similar measured affinity of CSL for NICD and for co-repressors [34-36] and the observed increase in levels of CSL bound at target loci in the presence of NICD [15,21,37,38]. Further studies examining the real-time dynamics of CSL interactions with DNA and with its partners will be needed to resolve which of these models is correct.

It is also possible that there are different modes of operation depending on the target enhancer. ChIP studies in mammalian cells found that some regions had enhanced CSL binding in the presence of NICD while others did not [15,21]. Binding of NICD itself was correlated with those positions where CSL binding was enhanced and was associated with genes that had clear-cut changes in expression. However, in both T-ALL and C2C12 cells, only a fraction of CSL bound enhancers behaved in this manner [15,21]. Therefore it remains to be established firstly whether enhanced CSL binding is a hallmark for all Notch regulated genes and secondly what occurs at the other CSL bound regions. If they are not Notch responsive, is CSL complexed with different co-repressors that make those positions "blind" to NICD or are there other factors present that mitigate any impact from changes in NICD? Deciphering the characteristics of different types of enhancer we will be important for answering these questions.

Several lines of evidence indicate that target genes are sensitive to the levels of NICD. One scenario is that genes undergo linear concentration dependant responses to NICD, which appears the case with some isolated enhancers in transfection assays. Results from precise analysis of nascent transcripts from two Notch (Glp1) regulated genes in *C.elegans* gonad also revealed a graded response. Nuclei had a probability of transcriptional firing that was

sensitive to the levels of NICD, a mutant with reduced levels of activity had fewer sites of active transcription and fewer nascent transcripts [39]. Another possibility is that target enhancers respond when NICD reaches a certain threshold, and then they do so in an all or none manner so that Notch exerts an on off switch rather than a graded response. One example of a threshold effect is Bcl11b in T-cell lineages, where increasing ligand concentrations enhanced the frequency of Bcl11b expressing cells but not the magnitude of Bcl11b expression in individual cells [40]. These studies also revealed that Bcl11b activation required a lower Notch signal compared to other processes analyzed. Interestingly, Bcl11b expression was sustained if Notch activity was removed once the gene had been activated. This suggests that NICD triggers BCl11b expression but is not required to maintain it, in contrast to many of the well-studied enhancers whose expression is lost when signaling is switched off.

Modulating NICD stability

The C-terminal PEST domain of NICD contains several motifs that are recognized by E3-ubiquitin ligases when phosphorylated, i.e. that function as phosphodegrons. The discovery that many cancer-associated mutations in Notch1 truncate the so-called PEST domain at its C-terminus [41] (Figure 2) has emphasized the importance of this region [42] [43]. These mutations lead to Notch hyperactivity due to increased stability of NICD. Truncating mutations affecting Notch2 PEST have also been linked to Hajdu-Cheney syndrome (a rare skeletal disorder] [44]), although it has not yet been shown whether these also increase the stability of the ICD.

Phosphodegrons in N1-ICD include Cdc4-type motifs, which are recognized by Fbw7, the substrate recognition subunit of an E-3 ubiquitin ligase complex [45]. Specific serine residues within this motif can be phosphorylated by CDK8 (Figure 2), a kinase associated with the Mediator complex and also with Cyclin C [46]. Its association with Mediator has led to the model that CDK8 recruitment to active promoters could limit N1-ICD activity by promoting degradation [47]. Cdc4-phosphodegron motifs are commonly phosphorylated by glycogen synthase kinase 3 (GSK3), which has been shown to down-regulate N2ICD [48]. Currently we have a paucity of knowledge about the relative contributions of different kinases to NICD stability and about whether this differs according to the context.

Other modifications such as acetylation and methylation can also influence NICD stability, although the effects on activity are not straightforward. For example, different outcomes from acetylation/deacteylation on NICD stability have been reported. In endothelial cells,

inhibitors of the deacetylase SIRT1 elicited an increase in N1-ICD levels and enhanced endothelial Notch responses, implying that acetylated NICD is more stable [49]. p300 mediated acetylation of N3-ICD in T-ALL cells had the converse effect, acetylation was found to prime ubiquitinylation and proteasomal-mediated degradation [50]. Whether these differences are paralogue specific or context specific remains to be established. Notably, Mam and CSL may also become acetylated and/or ubiquitinylated, which could further confound the outcomes [51,52]. The consequences from NICD methylation also demonstrate that stabilization of NICD may not always be indicative of hyperactivity. The arginine methylase CARM1 was found to methylate the TAD of N1-ICD at five conserved arginine residues (Figure 2). When methylation was prevented N1-ICD was more stable but, surprisingly, this led to decreased rather than increased activity [53]. Altogether it is evident that the outcome of signalling will be profoundly influenced by the type, level and combination of post-translational modifications to NICD, especially given their impact on its stability.

Role of other co-factors

The presence or absence of different co-factors also has the potential to modulate activity of NICD. Two categories of co-factors that interact with NICD-CSL complexes can be envisaged. The first category encompasses factors that are required for assembling a functional initiation complex, to drive transcription of target genes. These include the p300/CBP histone acetyl-transferases, which interact with Mam and are recruited to NICD bound genomic regions where they are necessary for enhancer activation. Mediator complex and other components of the preinitiation complex must also be recruited, although whether this involves direct or indirect interactions remains to be established. Two other positively acting factors, the RNA helicase Ddx5 [54,55] and the Parafibromin component of the CAF complex [56] are reported to stimulate NICD induced transcription. Likewise, interactions with Super elongation complex have also been detected in neural stem cells, and are proposed to augment the transcription at target loci [57]. Further investigations will be needed to discover how widespread the role of these different factors and whether the constellation of co-factors varies according to the specific gene context. Its also important to consider the possibility that, besides regulating stability, post-translational modifications of NICD also have the potential to alter interactions with CSL and other key factors associated with transcriptional activity, as suggested for nemo-like kinase (NLK) in zebrafish [58] DYRK1A in neuroblastoma cells [59] and Casein kinase 2 (CK2) in human embryonic kidney cells [60] (Figure 2).

A second category of co-factors would include those that modulate the activity of the CSL-NICD complex in response to specific conditions. For example, a proteomics analysis identified over 100 N1ICD interacting proteins that include proteins implicated in signaling pathway crosstalk and other protein modifiers [61].   In more directed studies, interactions of NICD with β-catenin and SMADs have been detected that result in a cross-talk between the different signaling pathways [62]. However, the consequences are not clear-cut, and it remains to be established how frequently these interactions occur. In other cases, signaling pathways may converge onto a common co-regulator, as proposed for Parafibromin, whose phosphorylation state may be a mechanism that integrates the information from different signals [56]. Several of the other NICD interacting proteins that have emerged from specific interrogations contribute to repression rather than activation, including the de-methylases Lsd1 and Kdm5a/lid, and may be involved in mechanisms that shut down transcription [61,63,64]. A final point for consideration is that these interactions may be dynamic, to enable fine-tuning of the response.

Conclusions

The structure of the NICD-CSL-Mam complex cemented the fundamental role of NICD as a transcriptional activator. The challenge in recent years has been to understand how this complex is deployed and regulated. Significant insights have come from genome wide studies, that have demonstrated the diversity of target genes bound and have shed light on the epigenetic mechanisms that contribute to the cell-type specificity. However, there are still many unanswered questions about what delimits the responsive enhancers in a given context and whether these exhibit different modes of regulation, as suggested by studies of Bcl11b [40]. Might some enhancers be sensitive to levels of NICD while others exhibit an all-or-none concentration independent response, as recently found for the Bicoid morphogen? [65] Indeed, we still know little about the amounts of NICD needed and the extent that post-translational modifications control this. A better understanding of the latter is of particular relevance for diseases like some cancers in which mutations affecting NICD stability are implicated. Finally, we know that the actions of NICD in the nucleus occur remarkably rapidly after receptor activation but the voyage of NICD from the membrane to the nucleus, and the extent that this is regulated, is still uncharted territory.

Acknowledgements

Figure Legends

Figure 1: Simplified overview of Notch signaling.

(A) Diagram illustrating Notch pathway activation and release of NICD. Ligands (green) on surface of signal sending cell interact with Notch receptors (purple) on neighbouring cells making it susceptible to cleavage by Adam 10 (light brown) and then γ-secretase (dark brown) to release NICD. NICD forms a complex with CSL (green) and Mastermind (orange) to activate target genes.

(B) Yin-Yang of Notch illustrates that very different outcomes are programmed depending on which target genes are activated.

(C) Diagram of NICD domain architecture

(D) Logo for CSL recognition motif, height of letters indicates frequency of nucleotide at each position.

Figure 2: Mutations and modifications in NICD

Upper: Positions in NICD that are frequently mutated in cancers, LoF, loss of function; GoF, gain of function; T-All, T-cell acute lymphoblastic leukemia; CLL, Chronic lymphocytic leukemia; MCL, Mantle cell lymphoma; HDSCC, head and neck squamous cell carcinoma.

Lower: Sites of post-translational modifications, examples where the modifications have been mapped. P, phosphorylation; Me, methylation; Ac, Acetylation; DYRK1A, Dual-specificity tyrosine phosphorylation-regulated kinase 1A; NLK, nemo-like kinase; CDK8, cyclin dependant kinase 8; GSKβ, Glycogen synthase kinase β; CARM1, Coactivator associated arginine methyltransferase 1;

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Example showing importance of novel post-translational modifications on NICD stability

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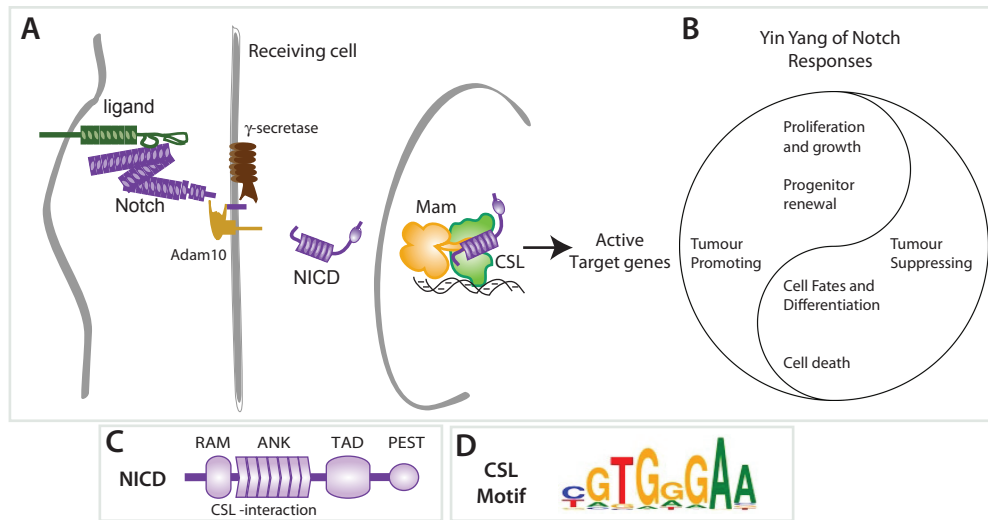
Figure 1

A Receiving cell

ligand

Notch

γ-secretase

Adam10

NICD

Mam

CSL

Active Target genes

B Yin Yang of Notch Responses

Proliferation and growth

Progenitor renewal

Tumour Promoting

Tumour Suppressing

Cell Fates and Differentiation

Cell death

C NICD

RAM ANK TAD PEST

CSL -interaction

D CSL Motif

GTGGGAA

Figure 2

LoF Truncating or nonsense mutations HNSCC

GoF Truncating or nonsense mutations T-ALL, CLL, MCL

Ac

P

P Ac Me

P

p300

DYRK1A CK2

NLK CARM1

p300

CDK8 GSK3B