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The Role of Notch Receptors in Transcriptional Regulation

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Notch signaling has pleiotropic context-specific functions that have essential roles in many processes, including embryonic development and maintenance and homeostasis of adult tissues. Aberrant Notch signaling (both hyper- and hypoactive) is implicated in a number of human developmental disorders and many cancers. Notch receptor signaling is mediated by tightly regulated proteolytic cleavages that lead to the assembly of a nuclear Notch transcription complex, which drives the expression of downstream target genes and thereby executes Notch's functions. Thus, understanding regulation of gene expression by Notch is central to deciphering how Notch carries out its many activities. Here, we summarize the recent findings pertaining to the complex interplay between the Notch transcriptional complex and interacting factors involved in transcriptional regulation, including co-activators, cooperating transcription factors, and chromatin regulators, and discuss emerging data pertaining to the role of Notch-regulated noncoding RNAs in transcription.

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The Notch signaling pathway is highly conserved in multicellular animals. Named after the notched wing phenotype of flies with heterozygous loss-of-function mutations in *Drosophila* Notch, Notch participates in a pathway that is normally activated by engagement of Notch receptors by Notch ligands expressed on adjacent cells (Fig. 1; for review, see Kopan and Ilagan, 2009). Mammals have four Notch receptors, Notch1–4, each of which is a single-pass transmembrane protein. Notch ligands in mammals fall into two families of single-pass transmembrane proteins that are homologous to *Drosophila* Delta (DLL1, DLL3, and DLL4) or Serrate (JAG1 and JAG2). Binding of ligand initiates events that produce a conformational change in the Notch juxtamembrane negative regulatory domain (NRR). This alteration renders Notch sensitive to successive cleavages by ADAM metalloproteases and the multiprotein γ-secretase complex. The latter cleavage releases the intracellular domain of Notch (ICN) from the membrane, allowing it to translocate into the nucleus and form a Notch transcription activation complex (NTC) with two other factors, RBPJ (also known as CSL in mammals, Su(H) in flies, and Lag-1 in worms), and co-activators of the Mastermind-like (MAML) family. In the absence of activated Notch, RBPJ interacts with multiple transcriptional repressors. Thus, regulatory elements containing functional RBPJ binding sites can mediate both activation and repression of nearby genes, an arrangement that may serve to tighten the Notch-dependency of Notch target genes.

This seemingly simple signaling pathway is remarkably pleiotropic in its functional outcomes. In humans, this is perhaps most clearly demonstrated by observations showing that Notch1 is a key oncogene in some cancers (e.g., T cell acute lymphoblastic leukemia [T-ALL]) and an important tumor suppressor gene in others (e.g., squamous cell carcinoma of the skin) (South et al., 2012). These diverse outcomes are presumably mediated by the action of cell-context specific Notch target genes and lineage specific cooperating factors. In this review, we discuss recent studies that address the question of how Notch regulates gene expression at the level of transcription.

Before the signal: Transcriptional repression by RBPJ complexes

In the absence of ICN, RBPJ associates with several different corepressors and inhibits gene expression (reviewed in Borggrefe and Oswald, 2009). RBPJ-interacting corepressors include SKIP (Ski-interacting protein), CIR (CBF1 interacting corepressor), KyoT2, *Drosophila* Hairless, and SPEN (also known as SHARP and MINT), as well as the histone demethylase KDM5A (Liefke et al., 2010). SPEN directly interacts with RBPJ and recruits other factors that mediate transcriptional repression, including CtBP, NcoR, CtIP, and histone deacetylases. Loss-of-function mutations in SPEN have recently been described in certain cancers, such as adenoid cystic carcinoma, in which Notch1 gain-of-function mutations are also common (Stephens et al., 2013), suggesting that the selective advantage of SPEN loss-of-function in such tumors is related to increased expression of Notch target genes. KyoT2's LIM domain interacts with the Polycomb group protein RING1 and mediates transcription repression by RBPJ (Qin et al., 2004). The crystal structure of the RBPJ–KyoT2 complex (Collins et al., 2014) shows that like the ICN RAM domain, KyoT2 binds the RBPJ BTD, indicating that (as might perhaps be expected) binding of ICN and KyoT2 to RBPJ are mutually exclusive.

Transcriptional activation by the NTC—Biochemical insights

Once Notch is activated, biochemical and structural studies are consistent with a model in which the RAM domain of ICN initially binds the RBPJ β–trefoil domain (BTD), an event that

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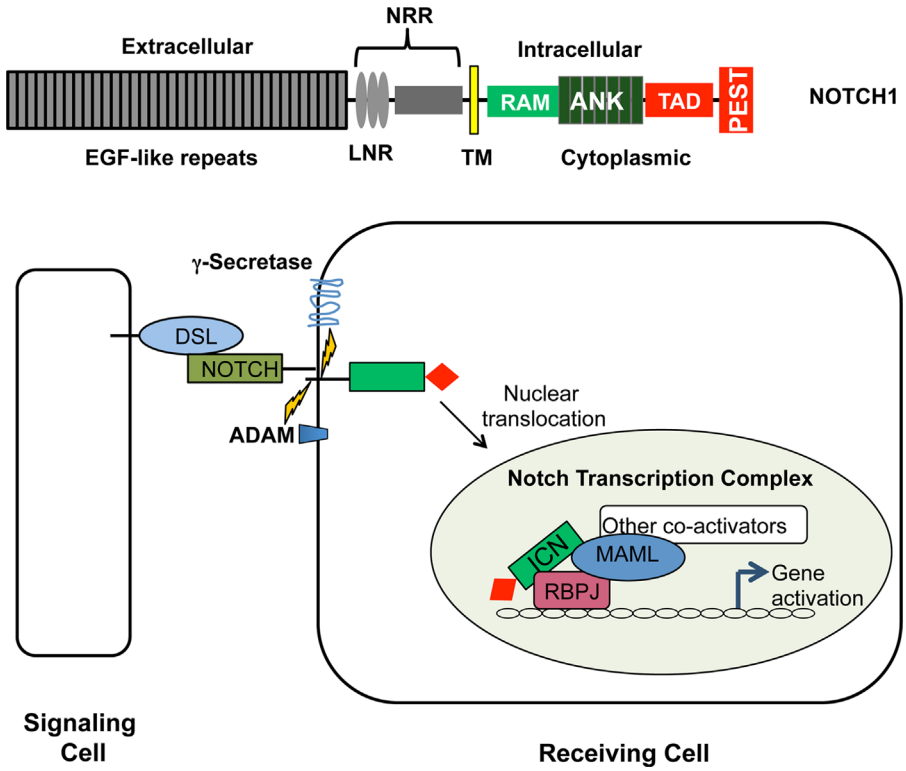

Fig. 1. Overview of Notch signaling. Upper panel: Notch receptor structure. NRR, negative regulatory region; LNR, Lin-12/Notch repeat region; TM, transmembrane domain; ANK, ankyrin repeat domain; TAD, transcriptional activation domain; PEST, PEST degron domain. Lower panel: Notch receptor function. Following binding of ligand to the EGF repeat region, successive cleavages by ADAM10 and gamma-secretase releases intracellular Notch (ICN) from membrane. ICN translocates into the nucleus and forms a ternary Notch transcription complex (NTC) with RBPJ and MAML coactivators on the regulatory elements. The NTC then recruits other coactivators and the basal transcriptional machinery, turning on target gene expression.

fosters docking of a secondary low affinity ankyrin repeat (ANK) binding site in ICN to RBPJ (reviewed by Kopan and Ilagan, 2009). The RBPJ–ANK interface then creates a bipartite binding site for the amino terminal portion of MAML. Notably, MAML cannot bind either RBPJ or ICN alone, and its recruitment is thus strictly restricted to RBPJ/ICN binary complexes.

Further understanding of how Notch functions in the nucleus requires a detailed knowledge of what other factors are recruited upon loading of NTC complexes and how these factors contribute to transcription. Older studies have shown that once assembled the NTC interacts with chromatin modifiers associated with transcriptional activation, such as the histone acetyltransferases p300 and PCAF (reviewed by Kopan and Ilagan, 2009). Several recent studies have markedly expanded the roster of potential interacting factors that contribute to NTC-mediated transcriptional activation. Novel ICN-interactors identified biochemically include the PBAF nucleosome-remodeling complex subunits BRG1 and PB1, the histone demethylases LSD1 and PHF8, and a novel coactivator AF4p12 (Yatim et al., 2012). In T-ALL cells, these factors associate with the RBPJ-ICN1 regulatory elements of several Notch target genes in an ICN1-dependent fashion except for LSD1. Intriguingly, LSD1 (KDM1A) appears to have dual functions as both a repressor and an activator (Wang et al., 2007; Di Stefano et al., 2011; Mulligan et al., 2011; Yatim et al., 2012) of Notch target genes, depending on whether RBPJ is associated with co-repressor complexes or with ICN/MAML; how this functional switch occurs is uncertain. Yatim et al. also noted that RNF40, a subunit of an E3 ubiquitin ligase known as Bre1 that carries out the monoubiquitination of histone H2B, is associated with ICN1, consistent with a previous study showing that Bre1 is required for Notch target gene expression in flies (Bray et al., 2005).

The physical contacts that recruit these cofactors to NTCs are largely unknown, and it is unclear which of the interactions described above are direct. At least some are likely to involve MAML cofactors, which are essential for NTC function. MAMLs are long unstructured molecules containing multiple motifs that participate in protein–protein interactions. N-terminal portions of MAMLs bind RBPJ–ICN binary complexes and p300 (Saint et al., 2007), while more C-terminal regions have been implicated in recruitment of mediator complexes, including the kinase module (Fryer et al., 2004). The E6 proteins of β-human papilloma viruses (β-HPV) inhibit Notch-dependent transcription by binding to a C-terminal motif that is homologous to other E6 targets sequences (Brimer et al., 2012; Tan et al., 2012; Meyers et al., 2013), such as one found in TP53. At present, the normal cellular protein(s) that binds this MAML motif is unknown.

Termination of NTC-dependent transcriptional activation

Cancers in which Notch signaling has an oncogenic role, such as T-ALL, chronic lymphocytic leukemia, and marginal zone B cell lymphoma, often harbor acquired "gain-of-function" frameshift or stop codon mutations that result in loss of a C-terminal PEST

degron domain and stabilization of ICN (South et al., 2012). Two sequences in the C-terminal region of mammalian Notch receptors appear to contribute to ICN turnover in the nucleus: a motif that is recognized by the Fbwx7 E3 ubiquitin ligase complex (for review, see Andersson et al., 2011); and a second WSSSSP motif that lies immediately C-terminal of the Fbwx7 motif (for review, see Aster et al., 2008). Loss-of-function mutations in Fbwx7 homologs such Sel10 produce Notch gain-of-function mutations in invertebrates (Lan et al., 2007) and are common in a number of cancers, including T-ALL (O'Neil et al., 2007), indicating that mechanisms that terminate NTC function by degrading ICN are highly conserved and essential for proper regulation of Notch signaling.

Both the Fbw7 and WSSSSP motifs undergo phosphorylation (Li et al., 2014), which is believed to trigger ICN turnover. One model proposes that MAML binds the cyclin C-CDK8 module of the mediator complex, which phosphorylates the Fbwx7 motif, leading to recruitment of Fbwx7, ubiquitinylation of ICN, and proteasomal degradation. This type of transcription-coupled degradation is appealing, as it would sharply limit the activity of individual NTCs to single regulatory elements, providing for very tight regulation. Consistent with the importance of cyclin C-CDK8 in limiting NTC activity, knockout of cyclin C in mice leads to thymic hyperplasia and enhances the development of Notch-dependent T-ALL (Li et al., 2014). Other kinases, such as SGK1, have also been implicated in Fbwx7-mediated ICN degradation. The kinase(s) responsible for phosphorylation of the WSSSSP motif is unknown, but mutations in this site that leave the Fbwx7 motif intact promote leukemogenesis in mice (reviewed in Aster et al., 2008), implying that the WSSSSP motif has a important independent negative regulatory function.

Beyond the Fbwx7 and WSSSSP motifs, mammalian ICN1 is subject to phosphorylation at several dozen other sites, and is also acetylated on specific lysine residues. Several different acetylases have been implicated in the latter modifications. Deletion of SIRT1 in endothelial cells has been reported to stabilize ICN1 and disrupt angiogenesis (Guarani et al., 2011), which depends on tightly coordinated crosstalk between the Notch and VEGF signaling pathways. SIRT1 has also been reported to interact with LSD1 (Mulligan et al., 2011), which as already mentioned is required for regulation of Notch-dependent transcription. There is also evidence that p300 acetylates MAML and ICN1 (Popko-Scibor et al., 2011), which may be another point of feedback regulation of NTC activity.

Insights from genome-wide studies of notch response elements

Early work to identify Notch regulatory elements in genomes were largely restricted to study of candidate RBPJ binding sites in the promoters of known Notch target genes. The advent of unbiased genome-wide studies using chromatin immunoprecipitation coupled to next generation sequencing (ChIP-Seq) or whole genome tiled arrays has demonstrated the limitations of these previous studies, while also raising new questions. Our group conducted the first ChIP-Seq studies of RBPJ and ICN1, using T-ALL cells with activating mutations in Notch1 as a model system (Wang et al., 2011). As anticipated, these studies revealed a high degree of overlap between RBPJ and ICN1 ChIP-Seq binding sites, which were mainly associated with gene promoters and with "activated" chromatin marks such as H3K4me3 and H3K4me1. There was also a strong enrichment for DNA binding motifs of members of the Runx and Ets family in DNA immediately adjacent to the RBPJ/ICN1 sites, suggesting that these factors might interact with Notch to regulate expression of target genes. However, the number of RBPJ/ICN1 binding sites greatly outnumbered high confidence direct Notch target genes, and thus the relationship between Notch binding and regulation of target gene expression was uncertain.

Subsequently, our group and that of Castel et al. have performed similar studies in which changes in RBPJ/ICN1 occupancy were determined genome-wide following acute activation of Notch1 by γ-secretase inhibitor (GSI) washout in T-ALL cells (Wang et al., 2014) or by ligand treatment of myogenic murine C2C12 cells (Castel et al., 2013). In T-ALL cells, roughly 90% of sites characterized by rapid loading of RBPJ/ICN1 complexes are located in distal enhancers ($>$ 2Kb from any TSS). These dynamic binding sites are highly associated with Notch1 target genes and are mostly found within broad regions defined by binding of Brd4, Med1, and p300 and high levels of H3K27 acetylation. These features are consistent with so-called super-enhancers, large regulatory switches that have been implicated in controlling the expression of lineage-specific "master regulatory factors" during development and oncogenes in cancer cells (Hnisz et al., 2013; Loven et al., 2013; Parker et al., 2013). In total, at least 1,000 dynamic and presumably functional RBPJ/Notch1 binding sites were identified in T-ALL cells. Castel et al. identified a more limited number of dynamic RBPJ/Notch1 binding sites in C2C12 cells (roughly 200–250), which are also mainly located in enhancer elements. Like the sites identified by Wang et al., these dynamic sites are also associated with Notch target genes, H3K27 acetylation, and p300 binding.

Certain differences between these studies also emerged. Wang et al. identified many more high confidence RBPJ/ICN1 binding sites, 90% of which were "stable," not showing dynamic changes in occupancy as cells were toggled between the Notch-on and -off states over a period of hours to several days. Whether these sites are of any functional importance or merely "noise" related to the high level of constitutively active Notch1 that is present in Notch-mutated T-ALL cells is uncertain. Castel et al., by contrast, identified a small number of RBPJ sites that did not load ICN1 and whose occupancy was unaffected by the Notch activation status of the cells. Of note, prior work has shown that ICN-independent association of RBPJ with the bHLH factor PTF1a is required for normal development of the pancreas and the central nervous system (Hori et al., 2008). By analogy, the RBPJ sites identified by Castel et al. may point to the existence of other Notch-independent RBPJ functions.

Another common finding in these studies and prior work from Bray's group in *Drosophila* cells (Krejci and Bray, 2007) is that loading of ICN onto Notch response elements appeared to result in increased RBPJ occupancy. One possible explanation for this observation would be for loading of ICN and MAML to increase the affinity of RBPJ for DNA, but in purified systems the affinity of RBPJ and RBPJ/ICN1/MAML complexes for DNA are similar. Further work directed at determining the dwell time of different RBPJ complexes on genomic response elements will be needed to sort out the basis for this phenomenon, which is at least superficially at odds with the previously discussed model in which NTCs are subject to transcription-coupled degradation.

Since the majority of functional RBPJ/ICN binding sites within genomes are located within distal enhancers, how looping interactions of these distal sites with their target genes is regulated is an issue of central importance. We recently found that the regulatory activity of dynamic functional RBPJ/ICN sites is largely restricted to chromatin domains bounded by constitutive CCCTC-binding factor (CTCF) sites (Wang et al., 2014), defined as CTCF binding sites that are retained in most cellular lineages (Cuddapah et al., 2009; Handoko et al., 2011; Hawkins et al., 2011). Genes located in the same CTCF domain as one or more dynamic RBPJ/ICN sites are significantly more likely to be activated by Notch than genes located in a flanking CTCF domain, even if the latter genes are closer to the site of

RBPJ/Notch1 binding. The CTCF sites at domain boundaries are co-occupied by cohesin and form stable long-range interactions, which may serve to segregate enhancers and gene bodies into functional domains (Kagey et al., 2010; Dowen et al., 2014). It is apparent that higher order chromatin structures such as looping interactions must have a major role in the transcriptional activation of target genes by NTC complexes bound to enhancers (Fig. 2). Several core components of cohesin complexes involved in chromatin looping (including SMC3, SMAC1A, PDS5A, and MAU2) have been identified in the ICN interactome (Yatim et al., 2012), raising the question of whether NTC loading directly or indirectly affects higher order chromatin conformation. Lake et al. (2014) have reported that RBPJ sites in the genomes of F9 embryonal carcinoma cells are enriched for CTCF-binding motifs and have speculated that RBPJ

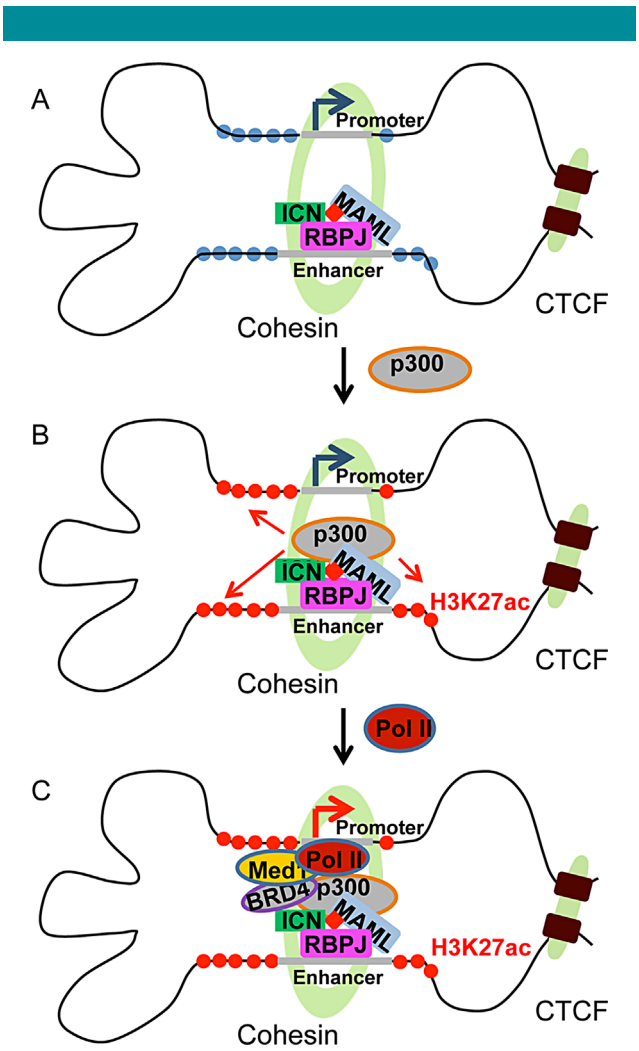

Fig. 2. Model of Notch-dependent activation of target genes through interaction with long-range enhancers. (A) Within a CTCF-organized regulatory domain, following Notch activation, the NTC binds to accessible enhancer elements. In this scenario, as appears to be the case with Notch-dependent regulation of Myc in T-ALL cells, preexisting chromatin loops established by cohesin complexes have brought the enhancer into close proximity with the proximal target gene promoter. (B) NTC recruits p300, which acetylates H3K27 throughout the enhancer and the target gene promoter. (C) Acetylation histones serve as binding sites for BRD4, and BRD4, together with other recruited factors, brings mediator and RNA pol II to the proximal promoter, turning on target gene transcription.

may participate in establishing chromatin domains, an idea that requires further investigation.

Recently described long-range Notch-dependent enhancers are providing an increasingly rich set of experimental substrates with which to address the possible role of Notch in regulating higher-order chromatin structures. For example, in developing T cells and in transformed T-ALL cells, ICN1 regulation of Myc expression is mediated through a distal enhancer located more than 1 Mb 3′ of the Myc gene body (Herranz et al., 2014; Ohtani et al., 2014). Depletion of ICN1 from T-ALL cells over several days does not disrupt looping interactions between this Notch-dependent enhancer element and the Myc proximal promoter (Ohtani et al., 2014), consistent with a model in which NTC loading upregulates Myc expression by acting on a chromatin structure that does not require activated Notch for its maintenance. Other recent studies have focused on IGFR1, which is regulated by a 3′ intronic Notch-sensitive enhancer (Medyouf et al., 2011). Loading of NTCs onto this site upregulates expression of a long non-coding RNA (LncRNA) encoded by an element dubbed LUNAR1 (Trimarchi et al., 2014) that is located immediately 3′ of the IGFR1 gene body. LUNAR1 is a structural RNA that appears to stabilize or promote the interaction of the LUNAR1 locus and the Notch-dependent enhancer with the IGFR1 proximal promoter. In the process of doing so, it also appears to enhance recruitment of RNA polymerase II. Many other LncRNAs are regulated by NTCs in T-ALL cells (Durinck et al., 2014; Trimarchi et al., 2014), suggesting that NTC-regulated LncRNAs have a widespread role in regulating Notch target expression, particularly those that are controlled by Notch-sensitive long-range enhancers.

Another recent insight from ChIP-Seq analyses comes from studies focused on the interplay between the NTC and Ikaros, a master regulator of lymphoid development that suppresses expression of many Notch target genes in developing thymocytes. In thymocytes and in T-ALL cells, Ikaros appears to physically interact with ICN and preferentially binds DNA sequences that are immediately adjacent to NTC binding sites (Geimer et al., 2014). Ikaros loss-of-function mutations contribute to T-ALL development in an RBPJ/Notch1-dependent fashion in mouse models (Jeannet et al., 2010), suggesting that Ikaros function is an important restraint on Notch signaling in this cellular context.

Remaining questions

It is apparent from the discussion above that many fundamental issues pertaining to Notch regulation of transcription remain to be worked out. A functionally diverse set of factors is implicated in RBPJ/Notch-mediated transcriptional processes (Table 1), and how the activities of these factors are coordinated to control transcriptional outputs is largely unknown. Among the remaining areas of uncertainty are three that we have yet to touch on: the assembly of activator and repressor complexes and the dynamics of their exchange; the regulation of context-specific Notch responses; and the role of NTC dimerization in Notch target gene expression.

The simplest way for RBPJ to function would be for it to remain stably associated with DNA and exchange co-repressor complexes for co-activator complexes. However, definitive evidence for this model is lacking, and it remains possible that complexes are exchanged and loaded after assembly off of DNA. Of interest in this regard, Lake et al. (2014) recently described data suggesting that RBPJ remains associated with chromosomes at most genomic binding sites during mitosis. If confirmed, this would indicate that under some instances RBPJ interaction with chromosomal binding sites is quite stable, enabling individual RBPJ molecules to not only act as exchange stations for corepressor complexes and NTCs, but to also

TABLE 1. Factors implicated in regulation of transcription by RBPJ and Notch

Protein	Primary function	Proposed link to Notch regulation of gene expression	References
Transcription factors			
BCL11B	Zn finger protein, component of Swi/Snf chromatin remodeling complexes	ICN-associated protein	Yatim et al., 2012
CTCF	CCCTC-binding factor, implicated in organization of chromatin into functional domains by promoting the formation of chromatin loops	Marks the boundaries of chromatin domains that define and limit the ability of Notch enhancer sites to regulate nearby genes. Binding motif enriched near RBPJ binding sites in embryonal carcinoma cell genomes.	Cuddapah et al., 2009; Lake et al., 2014; Wang et al., 2014; Dowen et al., 2014;
ETS1	Ets family protein, regulates T cell development and proliferation	Ets binding motif and ETS1 occupancy enriched near NTC binding sites in T-ALL genomes	Wang et al., 2011
GABPA	Ets family protein, involved in regulation of T cell differentiation	GABPA binding motif and GABPA occupancy enriched near NTC binding sites in T-ALL genomes	Wang et al., 2011
HEB	E-protein, T cell development regulator	ICN1-associated protein	Yatim et al., 2012
IKAROS	Lymphoid-specific transcription factor, regulates lymphoid development	Antagonizes NTC function, binds adjacent to a subset of NTC sites in the genomes of T-ALL cells	Jeannet et al., 2010; Geimer et al., 2014
RUNX1	Critical for adult hematopoiesis and T cell development	Binds adjacent to a subset of NTC sites in T-ALL genomes, co-regulates IL7R expression with Notch1 via a distal enhancer, required with Notch1 for specification of hematopoietic stem cell fate	Terriente-Felix et al., 2013; Wang et al., 2014
ZNF143	Zinc-finger protein, required for normal development, implicated in bidirectional transcription	ZNF143 binding associated with nondynamic Notch binding sites of uncertain function	Wang et al., 2011
Transcription co-activators			
AF4p12	Novel transcriptional regulator	ICN1-associated protein, required for expression of some Notch target genes in T-ALL cells	Yatim et al., 2012
BRD4	Bromodomain-containing protein, chromatin reader, binds acetylated histones and proteins	Likely contributes to transcriptional activation of Notch target genes that are regulated through super-enhancers	Hnisz et al., 2013; Loven et al., 2013; Wang et al., 2014
MAML1, 2, 3	Core components of Notch signaling pathway	Binds ICN and RBPJ, recruits p300 and components of the mediator kinase module, may contribute to transcription coupled degradation of ICN	Saint et al., 2007; Fryer et al., 2004
Mediator1 (MED1)	Mediator complex component, promotes assembly of RNAPII and general transcription factors	Along with BRD4, marker of super-enhancers, recruited to Notch-dependent enhancers	Hnisz et al., 2013; Loven et al., 2013; Wang et al., 2014
Transcription co-repressors			
CIR (CBF1 interacting corepressor)	Transcriptional co-repressor	Links RBPJ to the histone deacetylase complex	Borggrefe and Oswald, 2009
KyoT2	LIM domain protein, transcriptional repressor	Forms repressive complex with RBPJ, inhibits gene expression	Qin et al., 2004; Collins et al., 2014
SPEN (SHARP, MINT)	Transcriptional co-repressor	Directly binds RBPJ, recruits other transcriptional repressors	Borggrefe and Oswald, 2009; Stephens et al., 2013
Chromatin modifiers			
BRG1	Components of the PBAF chromatin remodeling complex	ICN1-associated protein, co-activator, regulates expression of several Notch target genes	Yatim et al., 2012
KDM5A	Histone demethylase	Associated with RBPJ co-repressor complex, demethylates H3K4me3, represses Notch target expression	Liefke et al., 2010
LSD1 (KDM1A)	Histone demethylase	Dual function as co-activator and co-repressor, depending Notch activation status of the cell	Wang et al., 2007; Di Stefano et al., 2011; Mulligan et al., 2011; Yatim et al., 2012
p300	Histone acetyltransferase	Recruited by NTC, increases Notch target gene expression by acetylating H3K27	Popko-Scibor et al., 2011; Wang et al., 2014
PB1	Components of the PBAF chromatin remodeling complex	ICN1-associated protein, functions as co-activator, regulates expression of several Notch target genes	Yatim et al., 2012
PHF8	Histone demethylase	ICN1-associated protein, may enhance Notch target gene expression by demethylating H3K27me2 and H3K9me1/me2.	Yatim et al., 2012
RNF40	Histone H2B ubiquitin ligase	ICN1-associated protein, subunit of Bre1, contributes to Notch target gene expression	Bray et al., 2005; Yatim et al., 2012
SIRT1	NAD-dependent protein deacetylase	Deacetylates H4K16ac, interacts with LSD1, demethylates H3K4me1/2, represses Notch target gene expression	Guarani et al., 2011; Mulligan et al., 2011
Factors involved in protein degradation			
E6 protein	Papillomavirus oncoprotein	Binds MAML, interferes with NTC function	Brimer et al., 2012; Tan et al., 2012; Meyers et al., 2013
Fbwx7	E3 ubiquitin ligase	Binds ICN PEST domain, promotes ICN ubiquitinylation and proteasomal degradation	Lan et al., 2007; O'Neil et al., 2007; Andersson et al., 2011
Chromatin looping factors			
Cohesin	Multiprotein complex that promotes looping enhancer-promoter interactions	Cohesin complex components SMC3, SMAC1A, PDS5A and MAU2 associate with ICN1; CTCF associated with cohesion complex at chromatin domain boundaries may associate with RBPJ in some contexts	Kagey et al., 2010; Yatim et al., 2012; Lake et al., 2014
Long non-coding RNAs			
LUNAR1	Leukemia-specific long non-coding RNA	Notch-dependent long non-coding RNA in T-ALL cells, binds IGF1R enhancer, recruits mediator and RNAPII to the IGF1R promoter	Trimarchi et al., 2014

function as mitotic bookmarks, factors that help to preserve chromatin states in daughter cells. This proposed function is at least superficially at odds with the ability of Notch to induce different cellular outcomes during individual cell divisions or successive divisions of cells within the same lineage, as well as the context-dependent nature of its target genes (described below), and further work is needed to determine whether bookmarking is indeed a general function of RBPJ.

The pleiotropic nature of cellular responses to Notch signaling implies the existence of lineage-specific Notch response elements. In line with this idea, we recently noted that even within three different cellular contexts in which Notch1 has an oncogenic role (mantle cell lymphoma, T-ALL, and breast carcinoma), only six shared Notch1 target genes were identified (Hey1, Hes4, Myc, Notch3, NRARP, and TFRC) (Stoeck et al., 2014). The roster of Notch target genes in particular cellular contexts is likely to be determined by the action of upstream pioneer factors, master regulators that are capable of binding to DNA sites that are wrapped by nucleosomes. Subsequent nucleosome remodeling can then convert such sites to "open" chromatin that is competent for binding other classes of DNA binding factors. It may be that Notch largely acts on multipotent chromatin states that have been set by upstream pioneer factors, that is, that Notch is an arbitrator between possibilities laid out by pioneer factors. A limitation of most studies to date is that they have used transformed cell lines that are arrested at particular stages of cellular differentiation. An important next step for the field will be to extend these studies to normal developing cells, which should provide insight into the upstream factors and mechanisms that regulate the formation and maintenance of lineage-specific Notch-dependent enhancers.

Pioneer factors that likely contribute to Notch regulation of gene expression in blood cell lineages are transcription factors of the Runx family, one of which, Runx1, was identified in ICN1 pulldowns prepared from T-ALL cells by Yatim et al. (2012) Lozenge, the *Drosophila* homolog of Runx factors, acts upstream of Notch in development of the equivalent of blood cells in the fly, and Runx1 is needed for Notch1-dependent specification of hematopoietic stem cells in developing mammals. A recent study in *Drosophila* showed that Lozenge binds enhancer elements prior to Notch activation and increases recruitment of Su(H) (the fly homolog of RBPJ), probably by increasing chromatin accessibility (Terriente-Felix et al., 2013). Wang et al. (2014) noted that Runx motifs were highly enriched near functional NTC enhancer binding sites, and subsequently showed using ChIP-Seq that these sites bind Runx1. Further work showed that full expression of some key genes, such as the interleukin-7 receptor (IL7R) gene, required both Runx1 and ICN1, implying that Runx1 may have a continuing role in maintaining the expression of certain genes even after an open chromatin state is established. These results suggest that many examples of context- and gene-specific interplay between Notch and various lineage-specific pioneer factors await discovery.

A third area of uncertainty arises from the observation that the genomes of organisms as diverse as humans and flies contain two different types of Notch response elements, sites that bind monomeric NTCs and sites that bind dimeric NTCs. Dimeric sites, termed sequence-paired sites (SPS), are defined by the presence of two RBPJ binding sites in a head-to-head orientation separated by a 15–17 bp spacer. NTC dimerization on SPS sites can be disrupted by mutation of amino acid contacts located in the ICN ANK domain (Arnett et al., 2010). Remarkably, these mutations abolish activation of transcription from genes and reporters that contain SPS sites, while having no effect on the transcriptional responses of elements containing monomeric sites.

The importance of NTC dimerization for expression of certain target genes is highlighted by Notch regulation of Myc in T-ALL cells. Forms of ICN1 that cannot dimerize do not support Myc expression in murine T-ALL cells, and also fail to cause T-ALL when expressed in murine hematopoietic progenitors (Liu et al., 2010). This appears to be due to the presence of a critical SPS site in the murine Myc Notch-regulated long-range enhancer (Ohtani et al., 2014). By contrast, the human Myc gene contains one or more monomeric NTC binding sites that drive Myc expression even when loaded with ICN1 mutants that cannot support NTC dimerization. At present it is unknown why some Notch responses require NTC dimerization, while others do not. Understanding the basis for the divergence in NTC dimer-dependency of the murine and human Myc genes offers a way forward towards resolution of this unsettled issue.

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