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Linking Notch Signaling, Chromatin Remodeling, and T-cell Leukemogenesis

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Abstract Intercellular communication that controls the developmental fate of multipotent cells is commonly mediated by the Notch family of transmembrane receptors. Specific transmembrane ligands activate Notch receptors on neighboring cells inducing the proteolytic liberation and nuclear translocation of the intracellular domain of Notch (N^{IC}). Nuclear N^{IC} associates with a transcriptional repressor known as C-promoter binding factor/RBP-Jκ, suppressor of hairless, or LAG-1, converting it from a repressor into an activator. Through physical interactions with chromatin remodeling enzymes and potentially with components of the transcriptional machinery, N^{IC} activates target genes that mediate cell fate decisions. As *Notch1* is disrupted via a chromosomal translocation in a subset of human T-cell leukemia, leading to a truncated polypeptide resembling N^{IC}, deregulated chromatin remodeling and transcription may fuel uncontrolled cell proliferation in this hematopoietic malignancy. This review summarizes the mechanics of Notch signaling and focuses on prospective molecular mechanisms for how constitutively active Notch might derail nuclear processes as an initiating step in T-cell leukemogenesis. J. Cell. Biochem. Suppl. 35:46–53, 2000.

Key words: notch; signal transduction; chromatin; leukemogenesis

The development of complex multicellular organisms involves numerous decisions that orchestrate the fate of stem/progenitor cells. A common theme of differentiation processes is the requirement for communication between distinct cell types to coordinate cell proliferation and differentiation. An ever-increasing number of cell surface proteins and secreted factors have been implicated as mediators of intercellular communication. Although it is daunting to consider the combinatorial complexity of the signal transduction pathways initiated by these molecules, great progress has been made in defining a paradigm for how the Notch family of receptors controls cell fate decisions from the nematode *C. elegans* to man.

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Molecular Components of the Notch Signaling Pathway: Multiple Ligands, Receptors, and More

It is not the intent of this article to comprehensively review all components in the Notch signaling cascade, but it is important to consider the major mammalian factors. Single-pass transmembrane Notch receptors (Notch 1–4) are activated via direct interaction with transmembrane ligands (Jagged1, Jagged2, Delta-like1, Delta-like3, and Delta-like4) expressed on the surface of neighboring cells (Fig. 1) [Artavanis-Tsakonas et al., 1999]. Certain cells co-express ligand and receptor, raising the possibility that ligand-dependent Notch activation can also occur in a single cell. In response to ligand binding, the disintegrin metalloprotease TNFα-converting enzyme (TACE) cleaves Notch extracellularly [Brou et al., 2000; Mumm et al., 2000], followed by intramembranous γ-secretase-mediated cleavage [Annaert and De Strooper, 1999], liberating the intracellular domain of Notch (N^{IC}). Presumably through usage of two intrinsic nuclear localization signals, N^{IC} translocates into the nucleus and associates with the transcriptional repressor known as C-promoter binding factor/RBP-Jκ (mammalian),

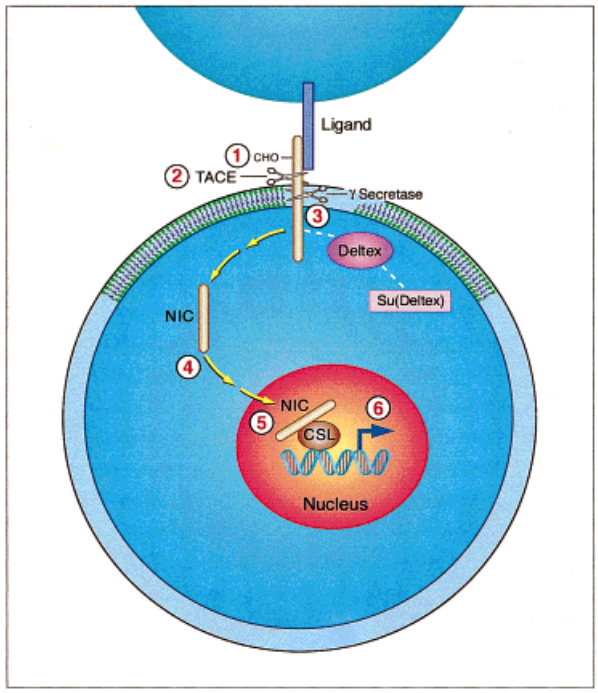

Fig. 1. Signal transduction through the classical Notch pathway. (**1**) Ligand binding stimulates receptor activation and Fringe-mediated glycosylation of Notch modulates Notch signaling. Receptor activation occurs via initiation of a proteolytic cascade involving cleavage of the extracellular domain of Notch by TACE (**2**), secondary cleavage by γ-secretase (**3**), resulting in release of the intracellular domain of Notch (NIC). NIC then translocates into the nucleus (**4**), forms a complex with DNA-bound CSL (**5**), and induces transcriptional activation of Notch target genes (**6**).

Suppressor of hairless (*Drosophila*), or LAG-1 (*C. elegans*) (referred to as CSL). N^{IC} binding to CSL masks a repression domain, leading to the activation of target genes that mediate developmental processes [Hsieh and Hayward, 1995]. Besides functioning via derepression, N^{IC} recruits coactivators that may directly mediate activation [Kurooka and Honjo, 2000], and this will be discussed in detail below. It is unclear where N^{IC} meets CSL, but it is likely that this occurs where CSL is bound at regulatory regions of target genes. Prototypical target genes in mice and humans, respectively, are *hairy/enhancer of split-1 (HES-1)* and *hairy (HRY),* encoding broadly expressed basic helix-loop-helix (bHLH) transcription factors [Kageyama and Nakanishi, 1997]. *HES-1* and *HRY* repress transcription in a bimodal fashion—by heterodimerization with other bHLH factors, thereby inhibiting DNA binding, and by occupancy of a variant E box sequence (N box). A classically defined developmental consequence of Notch signaling involves restriction of the developmental fate of multipotent cells [Kimble and Simpson, 1997].

In addition to Notch receptors, ligands, and CSL, other proteins modulate Notch signaling. Genetic studies in *Drosophila* have provided evidence that Deltex is a positive regulator [Xu and Artavanis-Tsakonas, 1990], presumably through physical interactions with N^{IC}. It is unclear whether Deltex binds Notch at the plasma membrane or binds N^{IC} after liberation from the membrane. No biochemical activities have been ascribed to Deltex, but we found that it has a conserved ring finger domain. Since these domains have intrinsic ubiquitin ligase activity [Freemont, 2000], Deltex may ubiquitinate a protein as an important step in Notch signaling. In conjunction with other components of the ubiquitination machinery, ubiquitin ligases catalyze the ubiquitination of proteins, leading to degradation via the proteasome or non-proteolytic influences such as altered protein trafficking [Hershko and Ciechanover, 1998]. Interestingly, a suppressor mutation of *deltex* in *Drosophila* was mapped to a HECT domain ubiquitin ligase gene termed *suppressor of deltex (SuD)* [Cornell et al., 1999], although *SuD* has no known substrates.

As there are greater than twenty highly homologous human HECT domain ubiquitin ligases [Schwarz et al., 1998], it is unclear which one is the mammalian *SuD* ortholog. We cloned mouse and *C. elegans* candidate *suppressor of deltex* orthologs (*Wwp1* and *CeWwp1*, respectively) [Huang et al., 2000] and found that disruption of *CeWwp1* by RNA interference resulted in an early block in embryogenesis at the comma stage and embryonic lethality. Additional analyses are required to assess whether *CeWwp1* mutants are compromised in Notch signaling. Another homolog, the murine HECT domain ubiquitin ligase Itch, can ubiquitinate Notch and therefore also represents a candidate *SuD* ortholog [Qiu et al., 2000].

Besides proteins that regulate N^{IC}, the Fringe family of factors can modulate the ligand-dependent activation of Notch. Fringe is a glycosyltransferase that catalyzes the elongation of O-linked fucose associated with epidermal growth factor (EGF) repeat modules of the Notch extracellular domain. In one report,

glycosylation enhanced the binding of Delta to Notch [Bruckner et al., 2000], whereas another group showed that glycosylation reduced the responsiveness of Notch to Jagged without affecting binding [Moloney et al., 2000]. Thus, Fringe-mediated glycosylation of Notch represents a novel mode of modulating Notch activation.

Control of Hematopoiesis by Notch Signaling: Lineage-Specific Actions?

Considering the numerous developmental processes regulated by Notch, it is not surprising that Notch controls hematopoiesis, the generation of diverse blood cells from stem cells. It has been hypothesized that Notch signaling would stimulate hematopoietic stem/progenitor cell renewal, while inhibiting differentiation. Considerable effort is underway to test this hypothesis and to define whether Notch exerts lineage-specific functions, possibly independent of differentiation. Approaches to analyzing the role of Notch in hematopoiesis have involved ectopic expression of N^{IC} in primary cells and transformed cell lines, targeted expression of N^{IC} in specific hematopoietic compartments in transgenic mice, examination of hematopoietic phenotypes of knock-out mice, and bone marrow reconstitution studies with immunodeficient mice; some of these studies are summarized below. However, this work is in its infancy, and generalities cannot be made with confidence at this time.

Lymphopoiesis

The development of distinct subtypes of B- and T-cells requires interactions between progenitors and stromal cells in the hematopoietic microenvironment. Multiple lines of evidence support a role for Notch signaling in controlling T-cell development. (i) Notch1 and Jagged2 are expressed in thymocytes and thymic epithelial cells [Felli et al., 1999]. (ii) *Notch1* is disrupted by a chromosomal translocation in acute T-cell lymphoblastic leukemias [Elisen et al., 1991]. (iii) Expression of Notch1 in bone marrow induced T-cell leukemia upon transplantation into recipient mice [Pear et al., 1996]. (iv) Expression of N^{IC} transgenes in mice under the control of the *Lck* promoter disrupted T-cell differentiation. $Notch1^{IC}$ ($N1^{IC}$) increased the generation of single positive $CD8^+$ cells at the expense of $CD4^+$ cells [Robey et al., 1996]. Another group showed that $N1^{IC}$ increased single positive $CD4^+$ and $CD8^+$ cells and decreased $CD4^+/CD8^+$ double positive cells [Deftos et al., 2000]. (v) Disruption of murine *Jagged2* and *Notch1* via homologous recombination confirmed a role for these proteins in T-cell development; other blood cell lineages were not affected [Swiatek et al., 1994; Jiang et al., 1998]. Disruption of *Jagged2* yielded embryonic lethality with multiple phenotypes including impaired differentiation of γδ T-cells, with no effect on other T-cell subtypes. A study involving bone marrow reconstitutions from $Notch1^{+/+}$ and $Notch1^{+/-}$ mice into *rag1* mutant mice observed lower numbers of γδ T-cells from $Notch1^{+/-}$ mice and a block in early B-cell lymphopoiesis [Pui et al., 1999]. The conditional disruption of *Notch1* provided evidence for a cell autonomous requirement of *Notch1* early in T-cell development before expression of T-cell lineage markers [Radtke et al., 1999]. Collectively, this work showed that Notch signaling controls specific aspects of T-cell development.

Myelopoiesis

Overexpression of $N1^{IC}$ and $N2^{IC}$ in 32D myeloid progenitors or treatment with a soluble Jagged1 peptide inhibited granulocytic differentiation [Li et al., 1998], suggesting that Notch signaling inhibits myelopoiesis. Another study showed that a conditionally active estrogen receptor $N1^{IC}$ fusion protein and ligand-dependent Notch1 activation increased granulocytic differentiation of 32D cells [Schroeder and Just, 2000]. Since myelopoiesis is not affected by knockouts of Notch components, a physiological role of Notch signaling in myelopoiesis is uncertain. Certain components may be functionally redundant, or the effects may be unique to 32D cells. As $N1^{IC}$ and $N2^{IC}$ differentially inhibited granulocyte macrophage colony stimulating factor and granulocyte colony stimulating factor induced granulocytic differentiation of 32D cells [Bigas et al., 1998], this raised the intriguing possibility that $N1^{IC}$ and $N2^{IC}$ engage in unique modes of cross-talk with signaling pathways. Since certain cells co-express Notch subtypes, selective interactions between Notch subtypes and signaling molecules would increase the diversity of signals generated by Notch within a single cell.

Erythropoiesis

We identified CSL as a factor that bound to the β-globin locus control region in erythroleu-

kemic cells [Lam and Bresnick, 1996, 1998]. This prompted us to ask whether other Notch components are expressed and whether Notch signaling might control erythroid maturation [Lam et al., 2000]. Indeed, *Notch1* and *Jagged1* were expressed, and expression of $N1^{IC}$ inhibited erythroid maturation without affecting megakaryocytic maturation of human K562 cells. $N1^{IC}$ inhibited induction of diverse genes activated upon erythroid maturation, suggesting that this system has promise for identifying Notch target genes that mediate growth and differentiation. Although our study used transformed cells, we hypothesized that Notch signaling might control erythropoiesis. This is supported by studies with *Notch1*$^{-/-}$ murine embryonic stem (ES) cells, which differentiated more efficiently than wild-type cells into primitive erythroid cells in vitro [Hadland et al., 1999]. Signals generated by Notch1 may therefore negatively regulate erythropoiesis. However, as knockouts of Notch components have not yielded erythroid phenotypes, components may have redundant activities, or a Notch requirement may not be evident in vivo, in which progenitors are bathed in a complex milieu of regulatory factors.

Notch Signaling Gone Awry: An Etiological Role in Acute T-cell Lymphoblastic Leukemia?

As with any multi-step signaling mechanism, there are many nodal points in the Notch mechanism at which signal transmission may go awry. In this regard, a t(7;9)(q34;q34.3) chromosomal translocation involving the translocation-associated Notch homolog *TAN1* (identical to *Notch1*) and *T-cell receptor* β is associated with a subset of human acute T-cell lymphoblastic leukemias [Ellisen et al., 1991]. This translocation truncates *Notch1,* giving rise to a constitutively active protein resembling $N1^{IC}$. It is easy to envision how bypassing the requirement for ligand-mediated activation might perturb differentiation, leading to uncontrolled proliferation associated with this T-cell malignancy. Despite this, it has not been proven that the translocation initiates leukemogenesis, although a causative role is suggested by the transgenic mouse studies described above. It is of great interest to define the molecular mechanisms that endow N^{IC} with a leukemogenic property in mice, and this is the subject of the following discussion.

Molecular Mechanisms Underlying N^{IC}-Induced Leukemogenesis: Is the Physiological Notch Pathway Even Relevant?

A central question is whether the leukemogenic activity of overexpressed N^{IC} results from the uncontrolled stimulation of the Notch pathway (hyperactivation mechanism), or from perturbing cellular processes not normally regulated by Notch (Notch-independent mechanism). It is likely that overexpressed N^{IC} would ectopically induce Notch target genes such as *HRY*. By heterodimerizing with compatible bHLH factors, HRY would deregulate genes controlled by these factors. Since N^{IC} activates transcription of *deltex* [Deftos et al., 2000], a facilitator of Notch signaling, increased Deltex in response to overexpressed N^{IC} may disrupt autoregulation and thus amplify an already constitutive Notch signal. As the activities of proteins encoded by physiological Notch target genes would mediate leukemogenesis in the hyperactivation mechanism, identifying these genes and defining their spatiotemporal expression patterns are critical for testing the relevance of this mechanism.

By contrast to hyperactivation, N^{IC} overexpression may deregulate proteins not in the Notch pathway. N^{IC} has multiple interaction domains, and overexpressed N^{IC} could engage in interactions that would not be of sufficient affinity to occur at normal N^{IC} concentrations. Such interactions may occur in the cytoplasm or nucleus and may stimulate or inhibit the activity of the interactor. In this scenario, elucidating the normal Notch mechanism would not reveal the basis of leukemogenesis.

With regard to a Notch-independent mechanism, the ankyrin repeats of N^{IC} can interact with the NF-κB transcription factor, resembling binding by the inhibitor IκB [Guan et al., 1996]. Overexpression of $N1^{IC}$ in transient assays in Jurkat cells inhibited NF-κB-mediated transactivation. Thus, the ankyrin repeats may engage in interactions detrimental to cell growth controls. By contrast, we see no effect of $N1^{IC}$ expression on NF-κB-mediated transactivation in K562 cells, under conditions in which N^{IC} strongly inhibits transactivation mediated by the protein kinase C-responsive transcription factor activator protein 1 (AP1) [Chu and Bresnick, 2000]. Since AP1 regulates multiple

genes involved in growth and differentiation, this represents an intriguing mechanism for how overexpressed N^{IC} might deregulate hematopoiesis.

N^{IC} can induce neoplastic transformation of baby rat kidney (RKE) in vitro [Capobianco et al., 1997], and it is instructive to consider these studies vis-à-vis the leukemogenic activity of N^{IC}. Nuclear-localized $N1^{IC}$ cooperated with Adenovirus E1A to transform RKE cells [Jeffries and Capobianco, 2000]. The minimal transforming domain included the ankyrin repeats and 107 carboxy-terminal amino acids, but lacked the RAM (RBP-Jκ associated molecule) domain. The RAM domain has been implicated in CSL binding, and RAM domain mutants have been used to argue that N^{IC} can function in a CSL-independent mode. However, the ankyrin repeats can physically and functionally interact with CSL [Kato et al., 1997], complicating the interpretation of experiments with RAM mutants. It will be important to assess whether similar mechanisms underlie N^{IC}-mediated neoplastic transformation in RKE and hematopoietic systems.

Chromatin Remodeling as an Essential Step in Notch Signaling

An important component of the transactivation property of N^{IC} appears to be its ability to associate with histone acetyltransferases (HATs) [Kurooka and Honjo, 2000] (Fig. 2). The implication of HATs in Notch signaling is exciting but not unexpected, given the broad role for histone acetylation in the control of gene expression [Strahl and Allis, 2000]. HAT-mediated acetylation of lysines on the amino-terminal tails of core histones increases accessibility of nucleosomal DNA to sequence-specific DNA binding proteins. Transactivators present in the nucleus in an active form are commonly excluded from cognate binding sites until a signal induces HAT recruitment to the template and concomitant acetylation-induced chromatin remodeling. HATs are attracted to specific chromosomal sites via physical interactions with DNA-bound transcription factors [Brownell et al., 1996]. Thus, at the earliest step of transcriptional activation, certain factors must bind. their sites within hypoacetylated chromatin. In this regard, the glucocorticoid receptor binds with a similarly high affinity to recognition sites on nucleosomal DNA and naked DNA [Hager et al., 1993]. The instigating factor

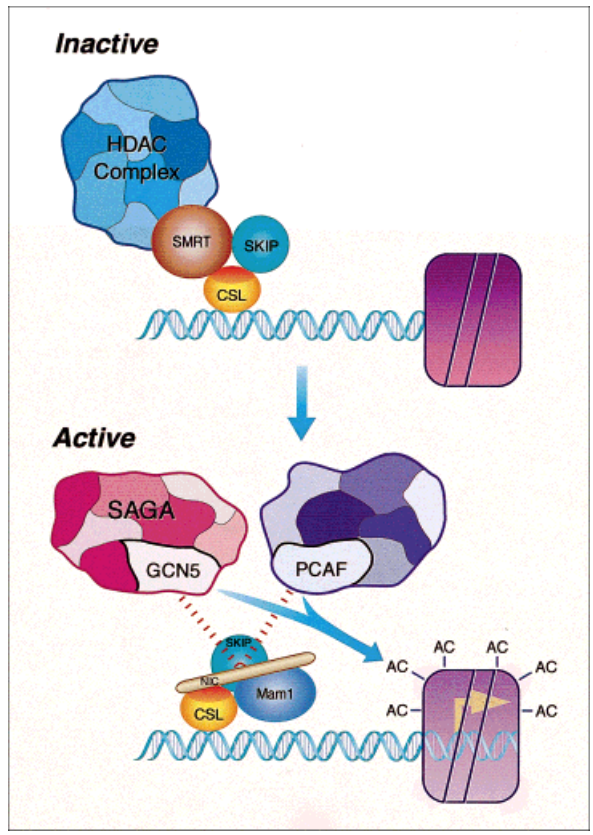

Fig. 2. Model for Notch-mediated transcriptional activation. In the transcriptionally inactive state, DNA-bound CSL associates with a SMRT-containing HDAC complex and SKIP (Ski interacting protein), which also binds SMRT directly. HDAC-mediated histone deacetylation would decrease DNA accessibility and thus repress the target gene. In the active state, NIC binds the repression domain of CSL. Mastermind (Mam1) associates with NIC and CSL simultaneously. SKIP associates with both CSL and the ankyrin repeats of NIC. The ankyrin repeats of NIC also mediate binding to the HATs GCN5 and PCAF. Upon recruitment to the chromatin, these HATs would acetylate histones locally, increasing accessibility of the DNA to the basal transcription machinery and induce transcriptional activation.

would then recruit HATs, leading to local (and potentially broad) histone hyperacetylation, increased DNA accessibility, binding of excluded factors, and assembly of functional nucleoprotein complexes. In addition to facilitation of protein–DNA interactions at the nucleosomal level, acetylation may perturb higher order folding of chromatin into 30 nm fibers and thus increase chromatin accessibility [Tse et al., 1998].

The ankyrin repeats and a carboxy-terminal transactivation domain of N^{IC} mediate binding to the HATs Gcn5 and PCAF in vitro and in a mammalian two-hybrid assay [Kurooka and

Honjo, 2000]. Adenovirus E1A and twist, inhibitors of PCAF and the HAT CBP/p300, inhibited N^{IC} mediated transactivation, supporting a coactivator function for these HATs in the Notch pathway. Gcn5 [Grant et al., 1997] and PCAF [Ogryzko et al., 1998] are broadly expressed and exist in large multi-protein complexes. Microarray analysis in yeast provided evidence for a role of the Gcn5 complex, SAGA (Spt-Ada-Gcn5-acetylase) in the control of many genes [Lee et al., 2000], while the number of PCAF target genes is unclear. Overexpression of a protein such as N^{IC}, capable of interacting with these HATs could disrupt the expression of genes dependent on these factors if they are limiting. This would depend on the concentrations and affinities of the interactors, which may vary during hematopoiesis and therefore may only be limiting in specific cell types at specific stages of hematopoiesis. This could provide a "window of opportunity" for N^{IC} to deregulate genes in a cell- and differentiation stage-specific manner.

What signals oppose N^{IC} induced chromatin remodeling? In the absence of effective concentrations of N^{IC}, repression appears to involve the interaction of DNA-bound CSL with HDACs [Hsieh at al., 1999]. N^{IC} binding to CSL would reconfigure the repressive nucleoprotein complex, displacing HDACs and allowing Gcn5 and/or PCAF binding (Fig. 2). This might occur via competition for HDAC binding, but this seems unlikely based on the low concentration of N^{IC} needed to maximally transactivate, which is likely to be considerably less than the concentrations of HDACs. Other nuclear factors such as LAG-3 (in *C. elegans*)/mastermind (Mam1) (in mice) and ski interacting protein (SKIP) function in a complex with CSL and are important for N^{IC}-mediated transactivation. Cooperative interactions between these factors to form a higher-order nucleoprotein complex may suffice to displace HDACs. Thus, a complex interplay between HATs and HDACs serves as an important determinant of Notch signaling, analogous to other nuclear signaling systems, most notably the nuclear hormone receptor superfamily.

Given the integral role of chromatin remodeling in DNA replication, transcription, recombination, and repair, one might expect the remodeling machinery to be highly regulated by signaling mechanisms. Phosphorylation of transactivators can facilitate HAT recruitment [Goldman et al., 1997], and HATs can be regulated via direct phosphorylation [Lu et al., 1998]. In addition to non-histone protein phosphorylation, Rsk2-mediated phosphorylation of histone H3 facilitates H3 acetylation [Lo et al., 2000], showing that distinct signals can cooperatively regulate remodeling. As histone acetylation can be modulated by the small GTPase Cdc42 [Alberts et al., 1998], Rsk2- and Cdc42-dependent pathways might interact to control remodeling. Histones can also be ubiquitinated [Nickel et al., 1989], but nothing is known about the control of this modification. Lastly, the ATP-dependent chromatin remodeling enzyme Swi/Snf is regulated by mitogenic signaling [Kingston and Narlikar, 1999]. We predict that a major mode of cross-talk between Notch and other pathways involves the integration of signals in the nucleus that control chromatin remodeling.

Future Directions

A number of questions regarding links between Notch signaling, chromatin remodeling, and leukemogenesis arise from the work reviewed in this article. First, it will be critical to define how frequent Notch deregulation occurs in human T-cell leukemias, whether this correlates with aggressiveness or responsiveness to chemotherapy, and whether Notch perturbations accompany other forms of leukemia. Second, is T-cell differentiation the only aspect of hematopoiesis regulated by Notch signaling in vivo? In vitro studies certainly suggest broader roles. The answer to this question should result from the analysis of hematopoietic-specific and compound knockouts of Notch receptors and ligands. Furthermore, conditional knock-outs that bypass early embryonic lethality may reveal unique requirements of Notch signaling in controlling primitive erythropoiesis. Third, does the leukemogenic activity of overexpressed N^{IC} involve hyperactivation or a Notch-independent mechanism? Identifying the N^{IC} sequences required for leukemogenesis and the relevant interactions will allow one to distinguish between these mechanisms. Fourth, if the evidence supports hyperactivation, it becomes critical to define physiological target genes for Notch and N^{IC} induced factors such as HRY. Given the progress in using microarrays to identify differentially expressed genes, this approach is likely to be productive. If the evidence supports a Notch-independent mechanism, the identity of nonphysiological N^{IC}

interactors will be important. Quantitative biochemical studies to define the cellular concentrations of N^{IC} and interactors and the affinity of the interactions will allow one to assess whether N^{IC} sequesters a significant amount of the interactor, thus derailing the corresponding cellular pathway. Both nonphysiological and physiological interactors may be sequestered by overexpressed N^{IC}. Interactions between N^{IC} and chromatin remodeling factors such as GCN5 and PCAF might be of particular relevance. Clearly, more work is required to solidify links between Notch signaling, chromatin remodeling, and leukemogenesis.

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