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Notch Signaling in Cancer

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Abstract

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Notch signaling was first linked to tumorigenesis through identification of a recurrent t(7;9)(q34;q34.3) chromosomal translocation involving the human *Notch 1* gene that is found in a small subset of human pre-T-cell acute lymphoblastic leukemias (T-ALL).¹ Since this discovery, aberrant Notch signaling has been suggested to be involved in a wide variety of human neoplasms. In this review, we will focus on recent studies linking aberrant Notch signaling with cancer. First, we discuss various mechanisms through which Notch signaling may influence cellular transformation. Then, we critically review literature pertaining to the role of Notch signaling in several cancers, and discuss possible therapeutic targets in the Notch pathway.

Keywords

notch, cancer, transformation, development, oncogene, tumor suppressor

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Review Article

Notch Signaling in Cancer

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ABSTRACT

Notch signaling plays a key role in the normal development of many tissues and cell types, through diverse effects on differentiation, survival, and/or proliferation that are highly dependent on signal strength and cellular context. Because perturbations in the regulation of differentiation, survival, and/or proliferation underlie malignant transformation, pathophysiologic Notch signals potentially contribute to cancer development in several different ways.

Notch signaling was first linked to tumorigenesis through identification of a recurrent t(7;9)(q34;q34.3) chromosomal translocation involving the human *Notch1* gene that is found in a small subset of human pre-T-cell acute lymphoblastic leukemias (T-ALL).¹ Since this discovery, aberrant Notch signaling has been suggested to be involved in a wide variety of human neoplasms. In this review, we will focus on recent studies linking aberrant Notch signaling with cancer. First, we discuss various mechanisms through which Notch signaling may influence cellular transformation. Then, we critically review literature pertaining to the role of Notch signaling in several cancers, and discuss possible therapeutic targets in the Notch pathway.

NOTCH SIGNALING

Notch genes, named after the notched wing phenotype of mutant *Drosophila*,² encode highly conserved cell surface receptors. The Notch signaling pathway, in which almost all elements are conserved from *Drosophila* to humans, consists of Notch receptors, ligands, negative and positive modifiers, and transcription factors. In mammals, these functional classes each have multiple members, and the interplay between these molecules is not yet fully understood. Studies in *Drosophila* suggest, however, that Notch receptors and ligands generally influence lineage specification through two mechanisms: lateral inhibition and lateral induction (for review, see ref. 3). In lateral inhibition, a cluster of equivalent precursor cells evolves into two distinct classes (Notch^{hi} and Notch^{lo}) through an assortment of negative feedback loops that create cell-to-cell variation in Notch signaling tone. A classic example is *Drosophila* sensory organ development, during which equivalent precursor cells expressing both ligand and receptor become either epithelial cells (Notch^{hi}) or sensory organ precursor cells (Notch^{lo}) through amplification of small stochastic differences in the initial levels of Notch signaling. In contrast, inductive signaling involves two non-equivalent cell types that express either the receptor or the ligand. The receptor-expressing cell responds to ligand stimulation, triggering a cell fate decision dependent on access to the appropriate ligand(s). During the development of complex tissues, both mechanisms may be operative.

Notch Structure and Pathway. The four mammalian Notch receptors (Notch1–4) are large, Type I transmembrane proteins comprised of multiple structural motifs (reviewed in ref. 4) (Fig. 1). In route to the cell surface, the Notch receptor is proteolytically cleaved by furin-like convertases in the trans-Golgi network, giving rise to the two subunits of the mature receptor. The extracellular Notch (ECN) subunit consists largely of a ligand-binding domain composed of tandem epidermal growth factor (EGF)-like repeats, and three LIN12/Notch repeats that appear to restrain inappropriate, ligand-independent receptor activation. The transmembrane Notch subunit (NTM) includes a short extracellular domain, a single transmembrane domain, and a large intracellular domain comprised of a RAM sequence, seven iterated cdc10/ankyrin-like repeats, two nuclear localization signals (NLS), and a C-terminal PEST sequence. In addition, mammalian Notch 1, 2 and 3 contain cytokine response (NCR) regions, and Notch 1 and 2 have C-terminal transcriptional activation domains (TAD).

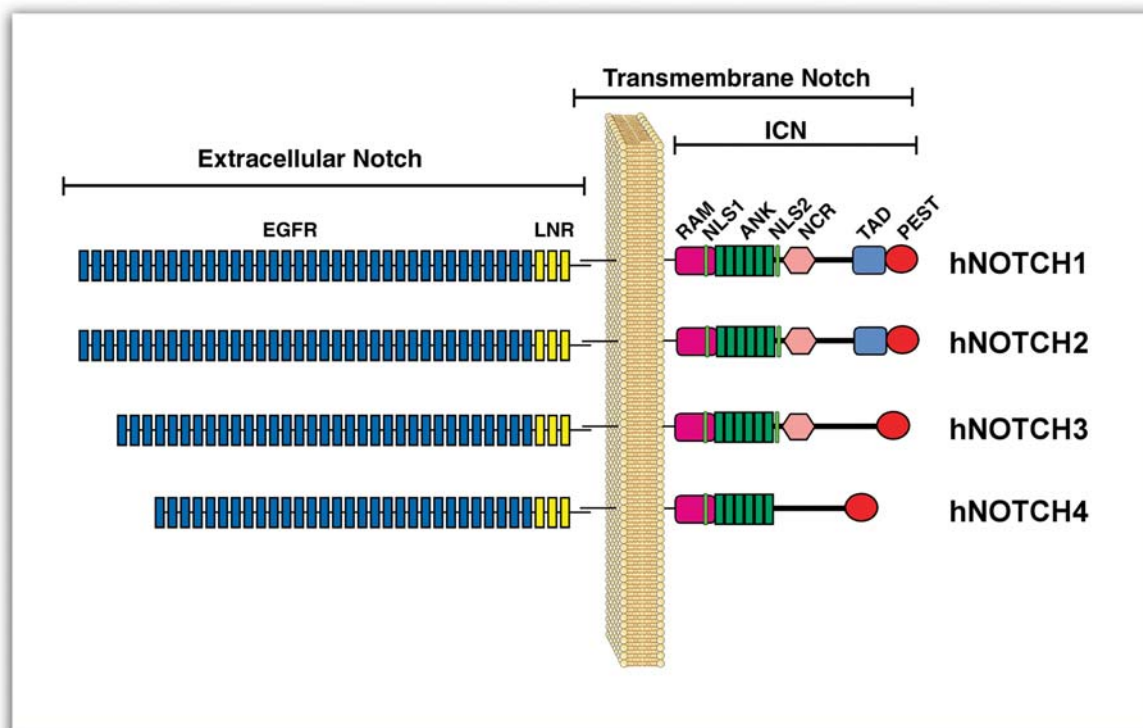


Figure 1. Human Notch receptors. Diagrammatic representations of the 4 known human Notch receptors (hNotch). The full-length proteins are expressed on the cell surface as heterodimers composed of non-covalently associated extracellular and transmembrane Notch subunits. All Notch receptors contain epidermal growth factor like repeats (EGFR), Lin12 Notch repeats (LNR), a RAM23 domain (RAM), Ankyrin repeats (ANK), PEST (P) sequences, and two nuclear localization signals (NLS1, NLS2). The highest degree of homology between Notch receptors is in the ankyrin repeats, whereas the C-terminal sequences show the greatest degree of divergence. Human Notch1-3 contain Cytokine Response (NCR) sequences immediately C-terminal of the ankyrin repeats that regulate functional activity. hNotch1 and hNotch2 also contain strong and weak, respectively, transcriptional activation domains (TAD) in between the NCR and PEST sequences.

Notch signaling begins with ligand binding, which triggers two successive proteolytic cleavages that release the active intracellular domain of Notch (ICN) from the cell membrane (Fig. 2) (for review, see ref. 5). ICN translocates to the nucleus, where it interacts with transcriptional regulatory factors. The only characterized transcription factor that binds ICN is CSL (CBF1/RBP-Jk, Suppressor of Hairless, LAG-1). In the absence of ICN, CSL is as a transcriptional repressor, associated with histone deacetylases and corepressors.⁶ Binding of ICN to CSL displaces corepressor complexes, and recruits coactivators, leading to transcription from promoters containing CSL-binding elements. The Notch transcriptional activation complex or enhanceosome appears to be a large (~1.5Mda) protein complex, of which ICN, CSL, and Mastermind-like (MAML) polypeptides are key components.⁷ In vitro reconstitution of transcription from CSL-dependent promoter elements on chromatin templates requires ICN, CSL and MAML1.⁸ MAMLs are glutamine-rich nuclear proteins that binds to Notch ankyrin repeats, form stable ternary complexes with ICN and CSL on DNA, and recruit CBP/p300, a histone acetylase.⁸ In cells, intact MAML1 amplifies activation of CSL reporter genes dramatically, whereas truncated forms of MAML1 behave as dominant negative inhibitors of ICN-dependent CSL activation.⁹ Based on its size,⁷ the stable enhanceosome complex must contain other unknown components. Another potential component is SKIP, which interacts with the CSL-ICN corepressor complex and assists in its conversion to an activator complex.¹⁰ Lastly, several reports are consistent with the existence of CSL-independent signaling pathways, the details of which are not yet determined.^{11,12}

Assembly of the Notch enhanceosome results in transcription of a variety of downstream targets. In many cells, Notch activates expression of a group of basic-helix-loop-helix-orange (bHLH-O) proteins, including several members of the mammalian Hairy/Enhancer of Split (HES) genes and Hairy-related genes (HRT) (for review, see ref. 13). These proteins repress gene expression through recruitment of transcriptional corepressors of the groucho/TLE family.¹⁴ The bHLH-O proteins also heterodimerize with bHLH activators, which may constitute a second level of bHLH inhibition. Other putative direct targets of Notch transcriptional activation include the *C. elegans* MAPK phosphatase LIP1, which regulates early events in vulval development;¹⁵ pre-T alpha,¹⁶ an important regulator of T cell development; and cell-cycle regulators such as cyclinD1 and p21^{WAF1-CIP1}.¹⁷⁻¹⁹ These diverse targets illustrate that the transcriptional consequences of Notch signaling vary depending on cell type and stage of differentiation, despite the stereotyped nature of the central Notch/CSL signaling axis. Major challenges in Notch biology are to identify the relevant targets of Notch signaling in particular contexts, and to understand the basis for divergent effects in the different contexts.

Notch signaling is subject to regulation at several levels. Notch activation by ligands of the Jagged/Serrate family is inhibited by glycosyl transferases of the Fringe family, which appear to modify the extracellular domains of Notch receptors,²⁰⁻²⁴ whereas activation by Delta ligands is enhanced by Neuralized, an E3 ligase (for review, see ref. 25). Two ligand-dependent cleavages are performed successively by ADAM family metalloproteases, which cleave the extracellular domain of NTM, and the presenilin protease complex, which is

responsible for cleavage within transmembrane domain to create active ICN (for review, see ref. 26). Within the cell, Notch signaling is modified by a number of additional factors, including: Numb, a cytoplasmic negative regulatory protein;²⁷ Deltex, a predominantly cytoplasmic protein that enhances Notch signaling in some contexts^{28,29} and inhibits it in others;^{30,31} SEL-10, an F-box protein that promotes ICN turnover;³² and unidentified kinases that mark ICN for degradation. Precise regulatory mechanisms are still emerging, but the multiplicity of regulators suggests that fine-tuning of Notch signals is of great biological significance.

POTENTIAL MECHANISMS OF NOTCH IN CANCER

Selecting from the growing literature on Notch and cancer, we present here several relatively well-characterized examples of how Notch participates in tumor development. First, we discuss the role of constitutive activation of the Notch pathway in lymphoblastic leukemia/lymphomas, other lymphoproliferative disorders, and mammary gland tumors. We then contrast these findings with results suggesting certain epithelial tumors arise through downregulation of Notch signaling. The ability of Notch to potentially function as an oncoprotein or a tumor suppressor in certain contexts is unusual, but perhaps not surprising given its diverse roles during normal development.

Notch and Lymphoid Neoplasms. In the late 1980s, Jeff Sklar's group identified a recurrent translocation t(7;9) associated with a small subset of T-ALLs.^{1,33} Analysis of these tumors showed that the breakpoints on chromosome 9 fell within the NOTCH1 locus and resulted in the juxtaposition of the T-cell receptor- β promoter/enhancer region with the 3' end of NOTCH1 on the derivative chromosome 9.¹ As TCR β is continuously expressed in T cells, the translocation caused dysregulated expression of a series of tumor-specific 5'-deleted NOTCH1 mRNA transcripts (reviewed in ref. 34) (Fig. 3). All known t(7;9) breakpoints fall within a single intron within the coding sequence EGF repeat 34 of Notch1. However, biochemical studies have shown that the t(7;9)-specific transcripts encode a predominantly nuclear ICN1-like molecule.³⁵ A murine bone marrow transplant model has confirmed the central role of Notch1 in the pathogenesis of this tumor, as mice reconstituted with bone marrow stem cells expressing constitutively active Notch1 rapidly and uniformly develop T-ALL.^{36,37} One primary effect of constitutive Notch1 activation is the maturation arrest of T lymphoblasts at the CD4⁺CD8⁺ double positive (DP) stage, an event that correlates with subsequent development of T-ALL (for a review, see ref. 34).

Insights into possible mechanisms relevant to the pathogenesis of Notch1-related T-ALL have come from studies looking at the role of Notch signaling in normal T cell development. Both Notch1 expression and activity appear to be triphasic during normal thymocyte development (reviewed in ref. 4). Levels are highest at the earliest stages of T cell development (CD4⁺CD8⁻ (DN) cells), low in CD4⁺CD8⁺ DP cells, and intermediate in both the CD4⁺ and CD8⁺ single positive (SP) cells.^{38,39} A variety of gain and loss of function studies have shown that Notch1 signaling through CSL is required for T cell commitment from a common lymphoid progenitor.⁴⁰⁻⁴⁴ Although constitutive Notch1 activity drives common lymphoid progenitors to the T lineage, failure to downregulate Notch signaling at the double positive (DP) stage prevents further maturation.⁴⁵ Thus, one potential Notch1 transforming activity is to allow survival of DP cells otherwise destined for apoptosis, and/or to cause maturation arrest at the DP stage.

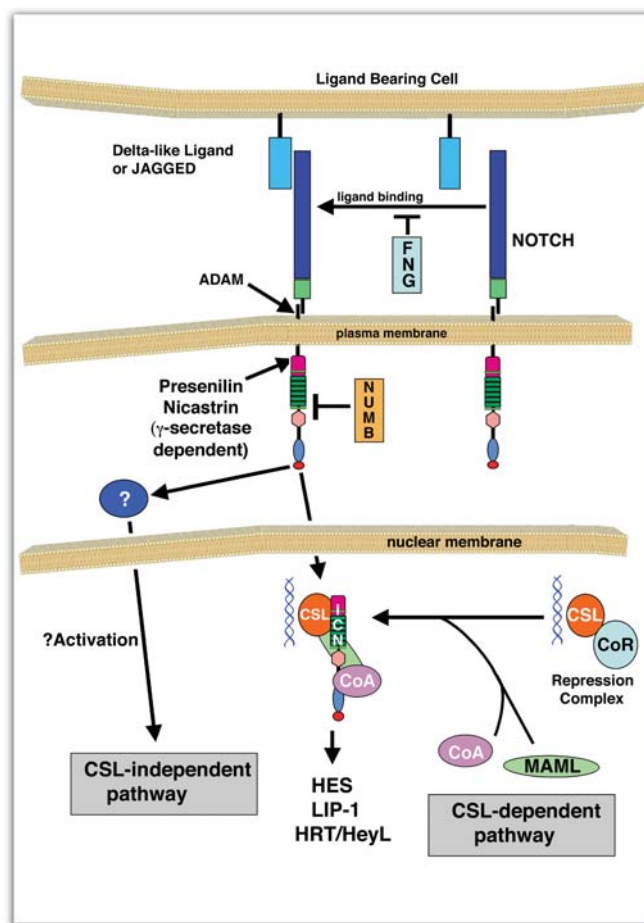


Figure 2. Ligand-induced Notch signaling pathways. Binding of Notch receptors to ligands of the Jagged and Delta-like ligand (Dll) families result in successive cleavages, first in the extracellular domain by ADAM-type proteases, and then in the transmembrane domain by presenilin-dependent proteases, which release ICN and permit its translocation to the nucleus. The ability of Jagged/Serrate ligands to activate Notch is antagonized by Fringe (FNG) glycosylases, which modify Notch extracellular domains. In the nucleus, ICN activates target gene expression by binding the transcription factor CSL, displacing corepressors (CoR), and recruiting coactivators (CoA), including Mastermind-like polypeptides (MAML). Poorly characterized CSL-independent pathways also exist. Notch signals are negatively regulated by the cytoplasmic protein, Numb. See the text for additional details.

The first T cells to appear in the bone marrow of mice reconstituted with ICN1-expressing stem cells resemble small, non-cycling DP thymocytes. This indicates that outgrowth of highly proliferative ICN-associated T-ALL requires secondary events, which have yet to be identified in sporadic human T-ALLs and corresponding murine models. Within the hematopoietic compartment, ICN1 has a striking oncogenicity for T cell progenitors. Stem cells bearing deficiencies of recombination activating genes (RAG), which prevent the development of normal CD4⁺CD8⁺ T cells, fail to form lymphoid malignancies in reconstituted mice even if followed for more than a year.⁴² The malignant phenotype is recreated in the RAG^{-/-} background by the addition of a TCR β transgene, which permits maturation to the CD4⁺CD8⁺ stage.⁴² Whether a functional TCR β merely allows the development of cells (the DP T cell) susceptible to ICN1 transformation, or actively participates in propagation of transforming signals (such as through activation of MAPK) (for review, see refs. 46, 47) remains to be ascertained.

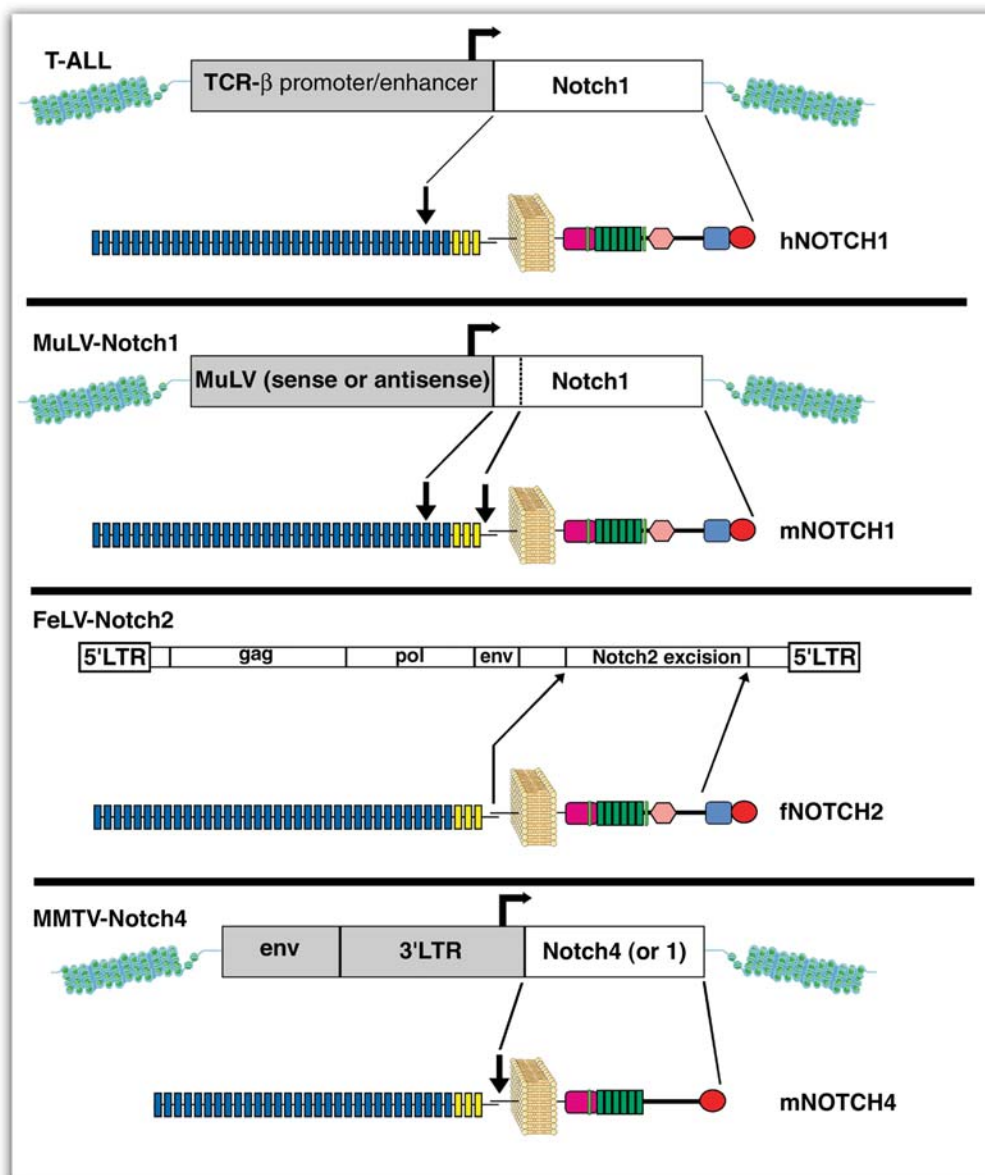


Figure 3. Schematic representation of translocations and proviral insertions involving Notch receptor genes. T-ALL. The t(7;9) translocation involving the human Notch1 gene and the TCR β promoter/enhancer region is shown. The translocation results in a series of tumor-specific 5'-deleted Notch1 mRNA transcripts. All known breakpoints fall within a single intron in the coding sequence for the EGF repeat 34 of Notch1. MuLV-Notch1. Proviral insertions occur just 5' of the Notch1 transmembrane region coding sequence and result in similar 5'-deleted transcripts as produced by the t(7;9). See text for the description of a less well characterized second type of insertion, which is found clustered at the 3' end of the Notch1 gene just 5' of the PEST coding sequence. FeLV-Notch2. A FeLV excision of a 3' portion of the Notch2 gene is depicted. The transduced portion of Notch2 spans the coding sequences from the transmembrane domain to just C-terminal of the ankyrin repeat region. MMTV-Notch4. Proviral insertion occurs within a short stretch of DNA containing exons 21 and 22 of Notch4, just downstream of the three LIN12 repeats and upstream of the transmembrane sequence.

From a therapeutic vantage, it is important to consider whether signaling is required for tumor maintenance once full-blown Notch1-related T-ALL has been established. Recent results from the Aster lab show that inhibition of Notch signaling in several Notch-transformed T cell lines results in a G₁/G₀ cell cycle arrest followed by apoptosis (Weng A et al., submitted). These data provide support for therapeutic targeting of the Notch pathway in T-ALL. The phenotype associated with withdrawal of signals also suggests that Notch contributes to T cell transformation by influencing proliferation and survival, rather than merely blocking differentiation.

Genetic alterations of Notch have also been identified in T cell tumors in other species. Proviral insertions of Moloney Murine

Leukemia Virus (MuLV) and Feline Leukemia Virus (FeLV) causing gain of function mutations of Notch1 and Notch2, respectively, have been found in T-ALL.^{48,49} In both instances, a subset of the insertions cause Notch rearrangements similar to those observed in human T-ALLs associated with the t(7;9) (Fig. 3). A high prevalence of Notch1 insertional mutagenesis was detected in early onset T-ALLs arising after MuLV infection of c-myc or E2A-PBX transgenic mice.^{50,51} Interestingly, two types of proviral insertions were observed. The first occurred just 5' of the Notch1 transmembrane region coding sequence, presumably giving rise to a gain-of-function truncation mutant similar to that produced by the t(7;9). The second type was found clustered at the 3' end of the Notch1 gene

just 5' of the PEST coding sequence. The mechanism of transformation in this second type of proviral insertion has not yet been well characterized, but similar mutations in flies produce gain-of-function phenotypes. In the case of FeLV, a FeLV strain that produced T-ALLs after a shorter latency period than wild type FeLV was found to have recombined with a 3' portion of the *Notch2* gene.⁴⁹ The transduced portion of Notch2 spanned the coding sequences from the transmembrane domain to just C-terminal of the ankyrin repeat region (Fig. 3). The retroviral LTR drives expression of a truncated constitutively nuclear form of Notch2 in transfected cells, suggesting it acts as a gain-of-function mutant. Transgenes encoding activated forms of Notch3 also cause T-ALL in mice.⁵² Similar to Notch1, these tumors are dependent on expression of a functional pre-T cell receptor.⁵³

To date, translocations or mutations involving Notch2 or Notch3 have not been identified in human T-ALL. It could be argued that this is due to differences in the intrinsic transforming potential of ICN1-3, as ICN1 is a more potent activator of CSL-dependent promoter elements than ICN2 or ICN3,⁵⁴ but the ability of ICN2 and ICN3 to cause T-ALL in other mammals makes this doubtful. Of note, many Notch gain-of-function alleles identified in fly and worm genetic screens have proved to be due to point mutations in Notch receptors. Until thorough searches have been conducted for analogous mutations in Notch1-3 in sporadic human T-ALLs, the prevalence of Notch gain-of-function mutations will remain unknown. It is also notable that enforced expression of the Notch ligand Dll4 in hematopoietic stem cells induces T-ALL.⁵⁵ Thus, dysregulation of Notch pathway components other than the receptors could contribute to leukemogenesis in man.

Mutation or chromosomal rearrangements of Notch receptor genes have not yet been identified outside of T-ALL, but evidence in some other lymphoid tumors points to the possibility of dysregulated Notch signaling. The expression of Notch1 was recently studied by immunohistochemistry in various subsets of human lymphomas.⁵⁶ Strong staining for Notch1 was consistently found in Hodgkin's lymphoma (HL), a tumor derived from germinal center B cells, and anaplastic large cell lymphoma (ALCL), a tumor of cytotoxic T cells. Staining was weak in Burkitt's lymphoma, diffuse large B cell lymphoma, and weak or absent in other entities. High expression of Notch1 and Notch2 was also observed in HL and ALCL cell lines. When cocultured with feeder cell lines bearing the ligand Jagged1, both tumor cell lines upregulated the Notch target gene HES-1 and exhibited an increased growth fraction and decreased apoptosis. Immuno-histochemistry showed Jagged1 was present in primary tumors, both on HL and ALCL tumor cells and surrounding reactive cell types, suggesting that Notch signaling could be triggered in vivo through cell-cell interactions. Much additional work is needed to determine whether Notch signaling is necessary for HL and ALCL growth and survival in vitro and in vivo. For example, mouse models of ALK-associated lymphoma would be helpful in further evaluating the role of Notch signaling in ALCL.

Circumstantial evidence also suggests a role for Notch2 in B-cell chronic lymphocytic leukemia (CLL).⁵⁷ High surface expression of the transmembrane glycoprotein CD23 is characteristic of CLL cells. While investigating CD23 expression, Hubmann et al. identified putative CSL-binding sites within the CD23 promoter, and showed Notch2 to be present in a multiprotein nuclear complex that binds the CD23 promoter. An anti-apoptotic role for the Notch pathway in CLL cells has been proposed,⁵⁷ but additional experimentation is needed to test this rigorously.

Recent evidence suggests Notch signaling within the murine naïve B cell compartment is necessary for specification of splenic marginal zone fate, but not splenic germinal center B cell fate.⁵⁸ Of interest, based on expression profiling studies, human B-cell CLL cells most closely resemble normal marginal zone B cells,⁵⁹ offering a possible link between this form of B cell neoplasia and a physiologic role for Notch signals in the peripheral B cell compartment. Conversely, Notch signaling promotes growth arrest and/or apoptosis of some avian and murine B cell lines^{60,61} (He Y, Pear WS, in preparation). Thus, Notch signaling might also inhibit the growth and survival of certain lymphoid malignancies.

Notch and Mammary Tumors. An important role for Notch signaling in murine mammary cancer has been well documented. Mouse Mammary Tumor Virus (MMTV) is a retrovirus that causes mammary tumors through insertional mutagenesis of the mouse genome.⁶² One such recurrent integration site, *int-3*, lies within the *Notch4* gene.⁶³⁻⁶⁵ Proviral insertion commonly occurs within a short stretch of DNA containing exons 21 and 22 of Notch4, just downstream of the three LIN12 repeats and upstream of the transmembrane sequence (Fig. 3). The 3' LTR drives the expression of a truncated 2.3 kb mRNA that includes the coding sequences for the transmembrane domain and the intracellular portion of Notch4. Removal of extracellular domain regulatory sequences, such as the LIN12 domain, is predicted to give rise to active forms of Notch4. It is not clear whether such polypeptides associate transiently with membranes (and thus require presenilin for activation), or are constitutively nuclear.

The *int-3/Notch4* oncoprotein transforms epithelial cells in a variety of murine assays. Enforced expression of *int-3/Notch4* in cultured mammary epithelial cells induces anchorage-independent growth, matrix invasion and loss of contact inhibition.^{63,66} In vivo, an *MMTV-int-3/Notch4* transgene causes abnormal proliferation and partial maturation arrest of mammary epithelium, followed by development of frank adenocarcinoma.⁶⁷ Abnormal growth of epithelia also occurs in other tissues where the MMTV promoter is active, such as salivary glands and epididymis, indicating that effects of *int-3/Notch4* are not strictly restricted to the mammary gland.⁶⁷ The potential of *int-3/Notch4* to induce malignant transformation in other cellular compartments has not been reported, and downstream mechanisms of transformation are largely unknown.

The *Notch1* gene has also been identified as a site of MMTV proviral insertion in murine mammary carcinomas. MMTV infection was used to screen for insertion sites that accelerated tumorigenesis caused by the *Erb2* transgene.⁶⁸ Of 24 tumors analyzed, two had proviral insertions within the *Notch1* gene that are predicted to give rise to aberrant transcripts encoding constitutively active forms of Notch1.

In contrast to the animal models, information on Notch in human breast cancer is scarce and indirect.^{69,70} Notch4 mRNA is expressed in selected human breast cancer cell lines.⁶⁹ Interestingly, Northern blot analyses have detected the presence of a truncated 1.8 kb Notch4 transcript in two breast cancer, one colon carcinoma, and two lung carcinoma cell lines. This transcript contains only 3' coding sequences and is predicted to encode a truncated intracellular polypeptide consisting of much of the intracellular portion of Notch4. The relevance of these findings remains to be studied. The expression of other Notch family members in breast cancer has not been documented.

NOTCH AND LUNG CANCER

Notch has also been linked to the pathogenesis of small-cell lung cancer (SCLC), a tumor with neuroendocrine (NE) differentiation. In contrast to the previous examples of T cell leukemia and MMTV-induced breast cancer, there is good evidence development of SCLC involves the up-regulation of a specific pathway normally antagonized by Notch signaling.^{71,72}

Similar to T cell development and leukemia, Notch signaling is involved in both normal pulmonary development and tumorigenesis. During human fetal development, pulmonary neuroendocrine cells (PNEC) selectively express a highly conserved bHLH protein, Achaete-scute homologue-1 (ASH-1), which is required for the NE features of these cells.^{71,73,74} During this process, Notch signaling controls pulmonary epithelial cell fate by activating HES, which in turn suppresses genes required for NE cell differentiation, such as ASH1.⁷⁴ In experimental models of the developing lung, ASH1 is expressed in PNEC, while Notch1 and HES1 are strongly expressed in non-PNEC.^{71,74} ASH1 forms a heterodimer with other ubiquitously expressed bHLH factors, and drives the expression of downstream genes needed for neuronal or NE differentiation.^{73,74} Both HES1 and HES3 bind the hASH1 promoter and repress hASH1 transcription,^{75,76} providing one mechanism for downregulation of ASH1 by Notch. ICN1 may also induce hASH1 degradation through TAD-dependent polyubiquitination of the hASH1 protein.⁷⁷

Mice lacking ASH1 have no detectable PNEC, while forced expression of ASH1 results in lung hyperplasia and metaplasia, though these cells displayed no detectable NE markers.^{71,74,78} This suggests that while necessary, ASH1 is not sufficient for pulmonary NE differentiation. In contrast, enforced expression of both ASH1 and SV40 Large T Antigen results in aggressive lung adenocarcinomas with NE features, a phenotype previously found only in spontaneous murine tumors.⁷⁸ Of interest, the targets of Large T antigen, p53 and Rb, are frequently inactivated in human lung cancers.⁷⁹

Human ASH1 is highly expressed in many SCLC lines,⁸⁰ but it is not detectable in non-SCLC (NSCLC) cell lines.^{80,81} hASH1 transcripts are also highly over-expressed (1000-fold) in primary SCLC tumors, as compared to non-SCLC tumors and normal bronchial biopsies.⁸² Experimentally, over-expression of ICN1 or ICN2 in two human SCLC cell lines caused marked growth suppression stemming from a G₁ cell cycle arrest⁷² accompanied by up-regulation of the cyclin-dependent kinase inhibitors (CDKI) p21Cip1 and p27Kip1. Overexpression of HES1 only partially arrests cell growth, suggesting other Notch1 targets are involved as well.

NOTCH AND NEUROBLASTOMAS

In addition to NE cell differentiation, ASH1 is also crucial for neuronal tissue determination.⁸³⁻⁸⁵ As in NE cells, Notch antagonizes ASH1 in the developing sympathetic nervous system. In experimental models, ICN1 over-expression inhibits the development of various neuronal-derived cells in wildtype, HES1^{-/-}, and HES5^{-/-} cells, but not in the HES1/HES5-double null background, suggesting both HES proteins can suppress neuronal determination.⁸⁶ In addition, HES1 deficient mice exhibit premature neuronal differentiation and increased ASH1 expression.⁸⁷ The involvement of ASH1 in the development of the nervous system and NE cells, as well as in SCLC, led to an examination of its expression in neuroblastoma (NB), a highly aggressive childhood tumor. Similar to SCLC, NB cells exhibit high expression of ASH1 and low to absent expression of HES

family proteins.^{81,85,88} However, the direct link between ASH1 and the tumor phenotype is less clear than in SCLC.

Current clinical trials are based upon the efficacy of agents such as retinoic acid (RA) or phorbol esters in the growth arrest and differentiation of NB cell lines *in vitro*.⁸⁹ Using the same cell culture systems, several groups found transduction of NB cell lines by either ICN1 or HES1 blocks drug-induced differentiation. This effect contrasts with the ICN1-induced growth arrest seen in SCLC cells.⁸⁸ However, one caveat concerns the complexity of effects mediated by RA treatment. Numerous RA-responsive genes are members of the DEC/STRA/SHARP (DSS) family of bHLH family of proteins.^{13,90} DSS proteins closely resemble HES proteins, but lack the C-terminal WRPW motif that recruits members of the Groucho/TLE family of transcriptional corepressors. It is therefore possible HES proteins antagonize the function of DSS proteins, thereby negating the RA signal to differentiate. Additional work is required to understand the effect of Notch in both normal neural development and transformation.

Another tumor of likely neuroectodermal origin is Ewing's sarcoma, which usually presents in childhood. Most Ewing's sarcomas have translocations involving members of the EWS gene family, which undoubtedly play a central role in the pathogenesis of this neoplasm. In addition, manic fringe (MFNG) is reportedly expressed at high levels in Ewing's sarcoma cells.⁹¹ In an experimental model, overexpression of MNFG renders fibroblasts tumorigenic in SCID mice. The relevance of this finding to the pathogenesis of Ewing's sarcoma awaits further studies.

NOTCH AND SKIN CANCER

Notch receptors are reported to be downregulated in basal cell carcinoma.⁹² In addition, mounting experimental data supports a role for Notch in regulating cell differentiation and growth arrest at the boundary between the basal layer cluster of progenitor cells and the adjacent cells.^{18,93} Whether Notch down-regulation has a role in the pathogenesis of certain types of human skin cancer remains speculative.

The human skin contains clusters of highly proliferative undifferentiated cells in the basal cell layer (for review, see ref. 94) capable of self-renewal as well as differentiation.^{95,96} In model mammal organisms, a "bulge" stem cell with capacity to differentiate into hair follicles and epidermis has been identified, but whether the human basal stem cell clusters retain the ability to form hair follicles remains unknown (for review, see ref. 97).^{98,99} The basal layer of the cutaneous interfollicular epithelium rests on the basement membrane and gives rise to stratified suprabasal layers of increasingly differentiated cells: the relatively undifferentiated stratum spinosum; the stratum granulosum layer, defined by the presence of keratin granules; and a stratum corneum layer comprised of flattened, fused cell remnants consisting mostly of keratin.¹⁰⁰ All four Notch receptors are expressed in regions of the basal and suprabasal layers, but in different patterns, suggesting distinct functional roles. In human adult and fetal skin samples, Notch1 protein and mRNA are expressed in all layers of the epidermis, but at the highest levels in the stratum spinosum.^{93,101} Notch2 protein is distributed only in the basal cell layer, while expression at the basement membrane is limited to Notch3. Both Notch3 and Notch4 protein are coexpressed in the suprabasal layers.¹⁰¹

The expression patterns of Notch ligands and Fringe family modulators add additional layers to this complexity. Transcripts for Delta-like 1 (Dll-1), Jagged 1, and all three known mammalian Fringes are found in the basal cell layer of the interfollicular epider-

mis.^{92,93,101} Interestingly, one group found clustered expression of Dll-1 in the basal cell layer near the dermal papillae, the regions of the dermis that project upward and interdigitate with the epidermal (rete) ridges,⁹³ and reported that this group of cells is resistant to ICN1-induced differentiation.¹⁰⁰ It seems likely that the complex expression patterns of various Notch receptors, ligands and Fringe modulators create other “microniches” within the epidermis that differ with respect to Notch signaling tone, and more generally reflect a requirement for very tight spatiotemporal control of Notch signaling in the skin.

Altered Notch expression patterns have been reported in basal cell carcinoma (BCC), as well as cutaneous disorders characterized by hyperproliferation, such as psoriasis and wound healing.⁹² Though controversial, most data suggest BCC originate from the basal layer of the outer root sheath of the hair follicle,^{71,102-104} which closely resembles the interfollicular basal layer of the epidermis in terms of surface molecule expression patterns, including Notch.^{101,105} In comparison to normal basal cells, BCCs show lower levels of Notch1, Notch2, Dll-1, Jagged1 and all three Fringe mRNA transcripts.⁹² To date, no other skin malignancies have been reported with abnormal Notch expression patterns.

Experimental mouse models also suggest that Notch drives differentiation and growth arrest in keratinocytes.¹⁸ Conditional ablation of Notch1 in the basal cell layer under the control of the keratin-5 promoter results in epidermal hyperplasia and loss of differentiation. In addition, presenilin1 (PS1) deficient mice develop spontaneous skin cancers.¹⁰⁶ Unlike Notch1 knockout mice, many of the signaling abnormalities in these mice stem from Notch-independent increases in β -catenin stabilization, through loss of negative inhibitory effects of PS1 on Wnt signaling. β -catenin stabilization in turn up-regulates cyclin D1 induction through the transcription factor LEF,¹⁰⁶ eventually leading to accelerated G₁ progression. Possibly, decreased Notch signaling and hyperactive Wnt signaling may synergize to drive skin tumor development in this model system. Of interest, complex interactions between the Notch and Wnt pathways play crucial roles in normal *Drosophila* wing development and segmentation (reviewed in refs. 107, 108). Other signaling pathways, like the Ras pathway, have also been postulated to interact with Notch signals in human fibroblast and epithelial transformed cell lines, however, these data remain speculative and need further analysis.¹⁰⁹

NOTCH IN CERVICAL CANCER

The Notch pathway has also been implicated in cervical cancer. Multiple reports show elevated Notch expression in cervical carcinoma cells.¹¹⁰⁻¹¹³ However, recent data suggests that Notch1 is down-regulated in late-stage HPV-infected tumors, and that Notch signals counteract the HPV-induced transformation,¹¹³ indicating the relationship of Notch signaling to cervical carcinogenesis is complex.

Normally, a squamous epithelium covers the ectocervix and a columnar epithelium covers the endocervical canal. The basal portion of the endocervical epithelium also contains a reserve cell precursor population that can undergo either squamous or columnar cell differentiation.^{114,115} In normal squamous ectocervix, only the stratum spinosum layer has detectable Notch receptors, whereas in normal columnar endocervix Notch1 and Notch2 are present on reserve cells.^{110,111} Human Jagged1 and Dll-1 have an identical pattern of expression as Notch receptors in both endocervix and ectocervix, whereas Jagged2 has not been detected.

The definitive stem cell precursor(s) for each endocervical and ectocervical layer remains to be identified. However, basal epithelial cells at the endocervical-ectocervical junction are prone to metaplastic transformation, and stain strongly with Notch1, Notch2, Jagged1 and Delta1 antibodies.^{110,111} While this suggests that Notch may function to regulate cellular fate decisions at the cervical squamocolumnar junction, the responsible signals and receptors, as well as the identity of the critical target cells, are unclear. Notch signaling may provide a permissive environment for development of early pre-cancerous lesions. Notch 1 and Notch 2 expression levels are high in cervical squamous carcinoma in situ and early dysplastic HPV-induced lesions (CIN I-III).¹¹⁰⁻¹¹² Jagged1, Jagged2 and Delta1 display similar expression patterns in metaplastic epithelia. Progression from CIN III to carcinoma was reported by one group to be associated with decreased surface and cytoplasmic Notch1 staining, and increased nuclear staining.²⁵ However, other data point in the opposite direction. Most notably, Dotto's group recently grouped cervical tumors according to the presence of episomal HPV DNA and the histology of the lesion of origin. Notch1 proteins were readily detected in lysates from HPV-negative cervical carcinoma cells and HPV-positive cell lines derived from low-grade cervical lesions, but HPV-positive high-grade cervical lesions had sharply reduced Notch1 protein levels.¹¹³ Interestingly, Notch2 protein expression did not vary, while Notch3 and Notch4 proteins were undetectable. Normal cervical tissue surrounding the lesions displayed Notch1 and Notch2 protein expression, in agreement with previously published data.

The net effect of Notch1 signaling may be suppression of HPV-dependent activities. ICN1, but not ICN2, antagonizes the HPV-URR promoter, which regulates E6 and E7 activity, in a CSL-independent manner.¹¹³ This occurs through down-regulation of *c-Fos* and upregulation of *Fra1*, two AP-1 family members, the net effect of which is to favor the formation of inhibitory AP-1 complexes composed of Jun-Fra heterodimers.¹¹⁶ The resulting restricted expression of oncoproteins E6 and E7 is hypothesized to antagonize late stages of tumor progression. An additional provocative finding is that E6 binds human MAML1.⁹ Perhaps this serves to inhibit MAML1 activity, thus limiting Notch signaling and promoting progression of late stage cervical neoplasms. Less certain is an explanation for how Notch signaling might promote the development of early stage cervical neoplasms.

NOTCH IN OTHER EPITHELIAL TUMORS

Notch receptor expression has been characterized in experimental models of prostate tumors, though the number of studies is limited. In addition, the Notch pathway is clearly involved in the organogenesis of other tissues like the pancreas, but currently there are no reports linking Notch signals or expression to the etiology of other carcinomas.^{117,118}

NOTCH AND PROSTATE CANCER

The role of Notch in human prostate cancer has not been examined directly. However, a murine model of prostate tumor formation suggests that Notch signals can inhibit prostate cancer progression. Notch1 expression during murine prostate development was examined using a Notch1 promoter transgene driving GFP expression as a surrogate marker. Expression is localized to the basal epithelial layer of the prostate during fetal development, but is down-regulated in

the adult.¹¹⁹ In contrast, expression is elevated in prostate tumors induced by SV40 large T antigen,¹²⁰ but whether this correlates with increased Notch1 signaling is unknown. In contrast, enforced expression of ICN1 inhibits the growth of various prostate cancer cell lines¹¹⁹; however, the relevance of phenotypes produced by high levels of ICN1 is unclear. Although these experiments suggest Notch may regulate growth and development of normal and malignant prostate cells, additional work is required to determine if these findings are relevant to primary human prostate cancer.

INTEGRATED VIRAL PROTEIN PATHWAYS AND NOTCH

Additional insight into the molecular mechanisms through which Notch contributes to transformation came from the study of oncogenic viruses. A diverse group of viral oncoproteins target the Notch pathway, emphasizing its importance in normal cellular regulation of growth and/or differentiation. We have previously discussed the complex interplay between HPV and Notch in the pathogenesis of cervical cancer. We now turn our attention to two oncogenic herpes viruses.

The two predominant types of human herpesviruses associated with human malignancies are Epstein Barr virus (EBV or HHV4) and Kaposi's sarcoma-associated herpesvirus (KSHV or HHV8). Most humans are latently infected with the EBV. Except for cases of infectious mononucleosis, the acute infection is usually asymptomatic, but the virus persists in latent form. If such individuals suffer from acquired deficits of T cell immunity, this small pool of cells can undergo rapid expansion, giving rise to EBV-associated B cell lymphoproliferative disorders.¹²¹ The other herpesvirus, KSHV, also causes several tumors that occur predominantly in immunosuppressed individuals: Kaposi's sarcoma, a tumor of endothelial origin^{122,123}; and an unusual B cell lymphoma that arises in effusions.¹²⁴

The main reservoir for latent EBV and KSHV infection is the B lymphocyte.^{121,125-127} Expression of KSHV RTA is necessary for the switch from viral latency to lytic reactivation, while EBV EBNA-2 helps maintain latency.¹²⁸⁻¹³⁰ Interestingly, multiple proteins necessary for cellular transformation by EBV and KSHV, including EBNA2 and RTA, bind CSL.¹³⁰⁻¹³³ RTA binding results in Notch-independent transcriptional activation of CSL targets, which promotes G₁ arrest and eventually triggers lytic reactivation.¹³⁰ EBNA-2 is involved in the up-regulation of proteins that contribute to latency (for review, see ref. 134). EBNA-3a and EBNA-3c, in contrast, inhibit CSL-dependent transcription, suggesting that EBV latency (and probably B cell transformation) requires tight regulation of CSL activity.¹³⁵ The region of CSL that binds RTA and EBNA-2 is identical^{130,132,136} which may have complex consequences in B cell lymphomas that are co-infected with EBV and KSHV.

The human population is also widely infected with adenovirus type 5. One of its early genes, *E1A*, has been widely used as a model oncogene in rodent cell lines.¹³⁷ Despite this, adenoviruses have not been linked to human tumors, and E1A induces human tumor cell lines to adopt a more mature epithelial phenotype.¹³⁸⁻¹⁴⁰ The activity of E1A as a tumor suppressor or differentiation agent has not yet been mapped. Intriguingly, the 13S-E1A adenoviral oncoprotein binds and activates CSL, similar to EBNA-2 and RTA.¹⁴¹ Like EBNA-3, another adenoviral protein, 12S-E1A, represses CSL-dependent transcription.¹⁴² Understanding the physiological effects of these proteins will require additional studies in naturally infected host cells.

DISCUSSION

The three general mechanisms of tumorigenesis that involve the Notch pathway are summarized in Table 1. Various translocations and proviral insertions in Notch receptor genes lead to ligand-independent production of ICN-like polypeptides. Such events underlie Notch-associated T-ALLs in humans, felines, and mice, and murine mammary carcinomas. In developing T cells, enforced expression of ICN1-3 results in a maturation arrest at the CD4⁺CD8⁺ DP stage that likely contributes to oncogenesis. The second mechanism involves ligand-mediated Notch receptor activation. This mechanism has been demonstrated in experimental models, but its importance in sporadic cancers is unclear. Finally, a diverse group of tumors seem to be associated with dampening of Notch signals through generally uncertain mechanisms. The clearest evidence supporting a role for Notch as a tumor suppressor comes from HPV-driven cervical carcinomas, in which Notch1 downregulation appears to be important during late stages of tumor progression.

The complex and occasionally paradoxical effects of Notch signals on cellular transformation are reflections of the protean effects of this signaling pathway on normally developing tissues and cells. The Notch pathway controls numerous developmental cell fate decisions through the regulation of genes involved in differentiation and proliferation. Notch receptors, ligands, and other signaling components have dynamic overlapping patterns of expression, suggesting that this pathway is subject to extensive fine-tuning to ensure that the timing and strength of Notch signals is appropriate for context. Cancers commonly resemble cells corresponding to normal stages of development, and it is thus not surprising that Notch signaling molecules are widely expressed in diverse neoplasms. A major challenge for the field is to distinguish stage-appropriate expression of Notch signaling components from aberrant expression that is pathophysiologic. This is not trivial, since tumors may recapitulate stages of normal tissue development that occur early in development, or have as their origin rare cells that are difficult to study. The frequent targeting of Notch signaling components by viral oncogenes provides strong presumptive evidence of a general role in cellular transformation. However, clear evidence of a broader

Table 1 **POTENTIAL ROLES OF NOTCH SIGNALING IN HUMAN CANCERS**

Mechanisms of Tumor Propagation	Potential Tumor Examples	Potential Therapies
Gain of function mutations	T-ALL, mouse mammary carcinomas	Intracellular inhibitors of the NOTCH pathway (disrupt ICN nuclear complex, activate Notch inhibitors)
Ligand-mediated activation of the Notch pathway	Lymphoproliferative disorders (CLL, Hodgkin's lymphoma)	Intracellular or extracellular inhibitors of the Notch pathway (block ligand-Notch binding or same targets as above).
Downregulation of the Notch pathway	SCLC, prostate adenocarcinomas, cervical carcinomas, basal cell cancer, neuroblastomas	Activate the Notch pathway (soluble ligands, antibody activation of Notch signaling).

role in sporadic human tumors awaits detection of additional acquired mutations or epigenetic modifications in Notch signaling components that alter function. Development of specific modifiers of Notch signals will also improve our ability to assess their role in particular tumor types.

The current trend in cancer therapy is to replace systemic chemotherapy with target-specific biologicals/chemicals, such as all-trans retinoic acid and imatinib mesylate (STI571). Because Notch is widely expressed and affects many differentiation processes, toxicities associated with targeting of this pathway may be unacceptable, although this remains to be seen. If true, it may be possible to focus on the downstream mediators of Notch signaling, rather than components of the central signaling axis. Ideas for potential targets are presented in Table 1.

In summary, Notch signaling is likely involved in the pathogenesis of a variety of human tumors. As in differentiation, its effect is probably context-specific, inhibiting transformation in some tissues and promoting malignancy in others. An improved understanding of Notch signaling in normal development and malignant transformation may lead to novel cancer therapeutics.

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