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REVIEW

It's T-*ALL about Notch*

RM Demarest[1], F Ratti[1] and AJ Capobianco

Molecular and Cellular Oncogenesis, The Wistar Institute, Philadelphia, PA, USA

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive subset of ALL with poor clinical outcome compared to B-ALL. Therefore, to improve treatment, it is imperative to delineate the molecular blueprint of this disease. This review describes the central role that the Notch pathway plays in T-ALL development. We also discuss the interactions between Notch and the tumor suppressors Ikaros and p53. Loss of Ikaros, a direct repressor of Notch target genes, and suppression of p53-mediated apoptosis are essential for development of this neoplasm. In addition to the activating mutations of Notch previously described, this review will outline combinations of mutations in pathways that contribute to Notch signaling and appear to drive T-ALL development by 'mimicking' Notch effects on cell cycle and apoptosis.
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Introduction

Acute lymphoblastic leukemia (ALL) is a neoplastic disorder of lymphoblasts that are committed to the B-cell lineage (B-ALL) or the T-cell lineage (T-ALL). Approximately 5200 new cases of leukemia will be classified as ALL in 2008 (http://www.leukemia-lymphoma.org). T-ALL accounts for approximately 10–15% and 25% of ALL cases in children and adults, respectively (Thiel *et al*., 1989; Goldberg *et al*., 2003; Pui *et al*., 2004). Over the past 20 years, mortality rates for leukemia (all subtypes, collectively) have remained relatively the same (http://www.cancer.gov). In contrast, over this same period of time, mortality rates of T-ALL patients have significantly decreased due to advances in the treatment of this aggressive subset of ALL (Grabher *et al*., 2006). Five-year survival rates (FSR) for children and adolescents with this disease are 75–85%, whereas, adult T-ALL patients have a 35–40% FSR. T-ALL patients have essentially the same FSR of patients with B-ALL (Goldberg *et al*., 2003). However, certain aspects about T-ALL make it a more aggressive disease with a poorer clinical outcome than B-ALL. T-ALL patients have a higher percentage of induction failure, and rate of relapse and invasion into the central nervous system (reviewed in Aifantis *et al*., 2008). The challenge to acquiring 100% remission in T-ALL treatment is the subset of patients (20–25%) whose disease is refractory to initial treatments or relapses after a short remission period due to drug resistance. Therefore, it is imperative to delineate the molecular blueprint that collectively accounts for the variety of subtypes in T-ALL. This will allow for the development of targeted therapies that inhibit T-ALL growth by disrupting the critical pathways responsible for the neoplasm. Targeted therapies would not only reduce cytotoxicity associated with the traditional regimen employed to treat T-ALL, but, ideally, also target the subset of tumors that do not respond or develop resistance to treatment. Such an approach would improve the quality of life and ease suffering for T-ALL patients. This review outlines the role of the Notch pathway in T-ALL. We discuss the regulatory networks that are regulated by Notch or that serve to regulate Notch function and how mutations in these circuits drive T-ALL development. We propose that deregulation of the Notch pathway is central to the development of T-ALL and that combinations of mutations in other genes drive T-ALL by 'mimicking' Notch effects on cells.

The Notch signaling pathway

The Notch pathway is an evolutionarily conserved signaling mechanism that regulates numerous cellular programs, including cell fate specification, proliferation and apoptosis. The Notch family is comprised of four paralogues, termed Notch 1–4 that share a high degree of structural similarity. Notch proteins are single-pass transmembrane receptors noncovalently joined as heterodimers through a structural motif termed the heterodimerization domain (HD) (Sanchez-Irizarry *et al*., 2004). All Notch receptors respond to ligands of the Delta-Serrate-Lag2 (DSL) family, located on the surface of neighboring cells (reviewed in D'Souza *et al*., 2008, this issue). Notch proteins overall share a high degree of domain topology. The extracellular domain of the Notch proteins largely consists of a variable number of epidermal growth factor (EGF)-like repeats (between 29 and 36) that mediate interaction with DSL

Correspondence: Dr AJ Capobianco, Molecular and Cellular Oncogenesis, The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA.
E-mail: acapobianco@wistar.org
[1]These authors contributed equally to this work.

ligands (Rebay *et al*., 1991). The LNR/cysteine-rich (CR) domain, located just C terminus to the EGF repeats, is composed of three CR Lin-12 repeats. The LNR/CR region functions by preventing ligand-independent activation of the Notch pathway (Sanchez-Irizarry *et al*., 2004). Both the HD and CR comprise a negative regulatory region (NRR) of which the conformation blocks proteolytic cleavage by a metalloproteinase in the absence of ligand–receptor interaction (Gordon *et al*., 2007). Notch signaling is primarily mediated by the intracellular domain (N^{ICD}), which functions as a transcription factor. The intracellular domain of Notch is composed of several identified domains that have distinct functions such as directing protein–protein interaction and regulating Notch activity. For example, the CDC10/ANK, composed of seven tandem copies of ankyrin-like repeats, in conjunction with the RAM domain, mediates interaction with the DNA-binding protein, CSL (Fortini and Artavanis-Tsakonas, 1994; Tamura *et al*., 1995). The C-terminal portion of the intracellular region contains a PEST domain, which mediates Notch protein turnover (Gupta-Rossi *et al*., 2001; Oberg *et al*., 2001; Thompson *et al*., 2008; Figure 1).

The proposed mechanism of Notch signaling occurs as follows. Activation of Notch signaling is initiated through interaction of the Notch extracellular domain with a DSL ligand located on the surface of a neighboring cell. This interaction leads to two successive cleavage events in Notch. Upon ligand binding with the EGF repeats, the NRR region undergoes a conformational change exposing a proteolytic site that allows cleavage by a metalloproteinase of the ADAM family. This cleavage event allows for the immediate cleavage by the Presenilin-dependent γ-secretase complex at a second site, resulting in release of the Notch intracellular domain (N^{ICD}) from the plasma membrane (Schroeter *et al*., 1998; Struhl and Adachi, 1998; Brou *et al*., 2000; Mumm and Kopan, 2000; Mumm *et al*., 2000; Gordon *et al*., 2007). The current model proposes that in the absence of active Notch signaling the transcription factor, CSL, represses target gene transcription (Dou *et al*., 1994; Waltzer *et al*., 1995). Upon release from the membrane and translocation to the nucleus, N^{ICD} associates with CSL, displaces co-repressors and recruits additional co-activators, including Mastermind-like (Maml) and CBP/p300, which results in transcriptional activation of target genes (Hsieh *et al*., 1996; Wu *et al*., 2000; Fryer *et al*., 2002; Jeffries *et al*., 2002; Figure 2). Therefore, the function of Notch appears to direct the conversion of a transcriptional repressor into an activator. Notch transcriptional activation is terminated by phosphorylation of Notch by cyclinC:cdk8, which mediates turnover of the activation complex. Phosphorylation of Notch in the PEST region by cyclinC:cdk8 leads to binding of the F-box protein, Fbw7 (Fryer *et al*., 2004). Ubiquitination of the Notch PEST domain by SCF^{FBW7} increases proteolytic degradation of Notch by the proteosome (Gupta-Rossi *et al*., 2001; Oberg *et al*., 2001; Thompson *et al*., 2008).

All members of the Notch family have been implicated in cancer, including breast, medulloblastoma, colorectal, melanoma, pancreatic and leukemia (reviewed in Koch and Radtke, 2007; Roy *et al*., 2007). Moreover, recent data have indicated that Notch can also function as a tumor suppressor in mouse skin, as well as a growth inhibitor in keratinocytes, hepatocellular carcinoma and small-cell lung cancer (Sriuranpong *et al*., 2001; Nicolas *et al*., 2003; Qi *et al*., 2003; Nguyen *et al*., 2006; reviewed in Dotto, 2008, this issue). The cues that govern whether Notch acts as an oncogene or tumor suppressor are not clearly defined, however, cellular context appears to be involved in determining Notch function.

The *Notch1* gene was originally identified as having a role in human leukemogenesis through identification of the chromosomal translocation t(7;9)(q34;q34.3) in cells derived from a T-ALL patient (Ellisen *et al*., 1991). This

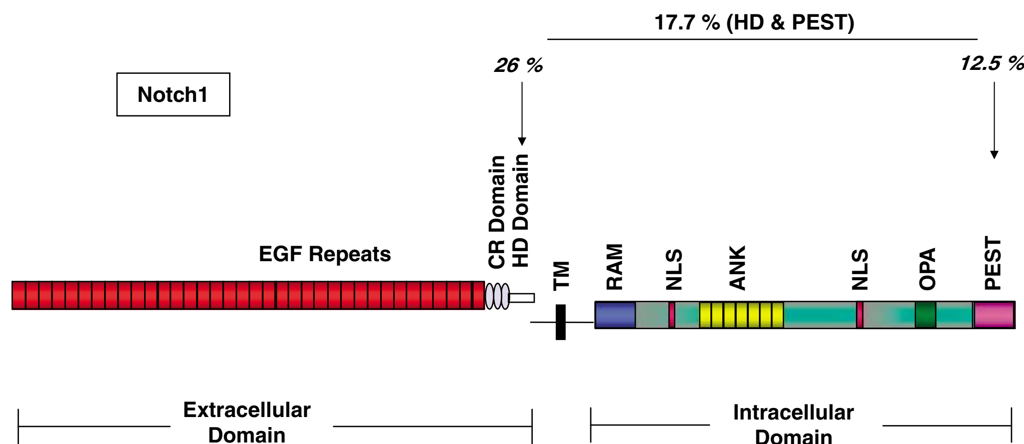

Figure 1 Structure of Notch1. Notch is a single-pass transmembrane receptor. The extracellular domain contains epidermal growth factor (EGF)-like repeats and a cysteine-rich region (CR). The intracellular domain contains the RAM domain, nuclear localization signals (NLS), seven tandem copies of ankyrin-like repeats (ANK), a region rich in glutamine (OPA) and a C-terminal PEST domain. The intracellular and extracellular domains are linked by the heterodimerization domain (HD). A screening of 19 T-ALL cell lines and 96 primary human tumors revealed activating Notch1 mutations in all molecular subtypes. All mutations found were located in the HD and PEST domains, either in *trans* (26% HD and 12.5% PEST) or in *cis* (17.7%).

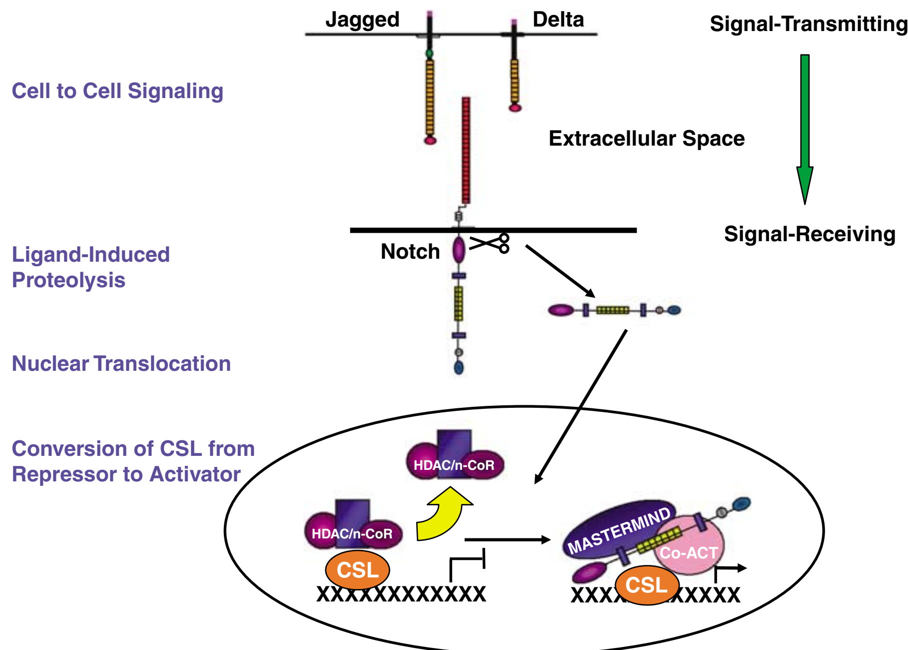

Figure 2 Schematic of Notch signaling. The interaction with DSL ligands (Jagged and Delta) causes a series of proteolytic cleavages of the Notch receptor, which releases the intracellular domain (N^{ICD}) from the plasma membrane. N^{ICD} translocates to the nucleus, binding to CSL, displacing co-repressors and recruiting other co-activators, such as Maml, to cause the transcription of target genes.

translocation results in juxtaposition of the 3′ region of *Notch* into the *TCR-β* locus, resulting in constitutive expression of active N^{ICD}. Although this translocation is rare in T-ALL patients (<1%), 56% of T-ALL cases examined contained activating *Notch* mutations (Weng *et al*., 2004). Sequencing of T-ALL cell lines and patient samples revealed that the majority of mutations found in the *Notch1* locus are located in two regions, the HD and PEST domains. Mutations found in the HD region result in ligand-independent proteolytic cleavage of Notch, leading to constitutive activation of the Notch signaling pathway (Weng *et al*., 2004; Malecki *et al*., 2006). Whereas, mutations in the PEST domain appear to increase the half-life of the intracellular domain by preventing Fbw7 interaction and, thereby, targeting of Notch to the proteosome (Thompson *et al*., 2008). The HD and PEST domain mutations were found in *trans* in 26% and 12.5%, respectively, and in *cis* in 17.7% of cases examined (Weng *et al*., 2004; Figure 1). These mutant forms of Notch have been demonstrated to increase Notch transcriptional activity *in vitro*, however, the ability of these forms to induce T-ALL in a mouse model remains to be resolved.

Ikaros: a transcriptional repressor and inhibitor of Notch-induced T-ALL

Ikaros (Ik) is a transcriptional regulator expressed exclusively in the lymphoid system that is required for the development of all lymphoid lineages (Georgopoulos *et al*., 1992). The *Ik* gene encodes multiple isoforms that are generated by alternative splicing (Hahm *et al*., 1994; Molnar and Georgopoulos, 1994). Ik isoforms are classified into two categories, DNA binding and dominant inhibitory (DI-Ik). The primary distinction between these two categories is the presence of a functional DNA-binding zinc-finger domain. All isoforms contain two C-terminal zinc-finger protein dimerization domains, which enable hetero- and homodimerization of Ik proteins, however, not all isoforms contain the N-terminal zinc-finger domains required for DNA binding (Hahm *et al*., 1994; Molnar and Georgopoulos, 1994; Sun *et al*., 1996). Ik binds DNA as an obligate dimer and mediates transcriptional repression of target genes. DI-Iks are dominant inhibitors of Ik function. DI-Ik heterodimerizes with DNA-binding isoforms and prevents DNA binding, thereby relieving repression of target genes (Sun *et al*., 1996).

To address the role of Ik in hematopoiesis mice lacking three of the DNA-binding zinc-fingers of Ik (Ik-null) were generated (Georgopoulos *et al*., 1994). Ik-null homozygous mice are smaller than wild type at birth and die postnatally between 1 and 3 weeks of age (Georgopoulos *et al*., 1994). These mice do not produce natural killer cells, dendritic cells and T and B lymphocytes, implicating Ik function as essential in the development of all lymphoid-derived cells (Georgopoulos *et al*., 1994). Ik-null heterozygous mice have essentially normal lymphoid systems at 1 month of age

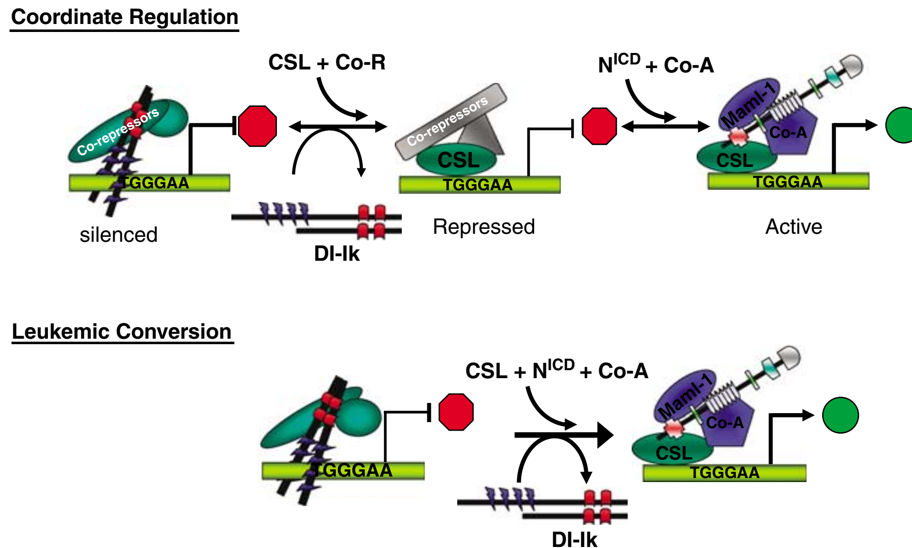

Figure 3 Model of differential gene expression by Notch and Ikaros. In a model of coordinate regulation, such as T-cell differentiation, the gene is repressed by Ik and its co-repressors. Expression of DI-Ik isoforms results in removal of Ik, allowing CSL binding to the core consensus DNA-binding region. This event maintains the gene in a repressed state. Following activation of the Notch receptor, N^{ICD} translocates to the nucleus and recruits Maml and co-activators displacing the co-repressor proteins associated with CSL to activate transcription. In the context of leukemic conversion, the *Ik* locus is preferentially expressed as DI-Ik, resulting in unfettered access of CSL to the core consensus sequence. In conjunction with constitutive Notch signaling this event leads to aberrant expression of the target genes.

(Winandy *et al*., 1995). However, mice eventually developed lymphoproliferative disease that progressed to B- or T-cell lymphoma (100% by 3 months). In addition, disease progression in these mice was concomitant with loss of the Ik wild-type allele (Winandy *et al*., 1995; Sun *et al*., 1996). These data demonstrate that Ik functions as a tumor suppressor in the lymphoid system. In support of this model, loss of Ik activity has been associated with human leukemia. Loss of DNA-binding Ik isoforms and/or overexpression of DI-Ik was observed in nearly 100% of childhood and adolescent T-ALL cases examined (Sun *et al*., 1999a, b, c). No mutations in the *Ik* locus have been described to account for the shift in isoform expression. The current model suggests that the increase in expression of DI-Ik isoforms is a result of alternative splicing, and not as a result of genomic alterations.

A series of experiments have demonstrated cooperation between Notch and Ik in leukemogenesis. Transgenic mice that constitutively express N^{ICD} under the control of a T-cell-specific promoter develop T-ALL. MuLV insertional mutagenesis was performed on these mice to identify genes that are important in the development of Notch-induced T-ALL. Analysis of these tumors revealed that 40% of the samples examined harbor integrations in the *Ik* locus, resulting in loss of Ik expression or production of DI-Ik isoforms (Beverly and Capobianco, 2003). Consistent with these results, the Piqueras group reported that 87.5% of lymphomas generated by thymic irradiation of mice exhibited an increase in Notch1 expression (Lopez-Nieva *et al*., 2004). Increase in the expression of DI-Ik isoforms and c-myc was observed in 92.8% of these Notch-expressing samples (Lopez-Nieva *et al*., 2004). These data indicate that loss of Ik tumor suppressor function is a cooperative event with Notch activation in leukemogenesis. Furthermore, a recent report from the Berns group confirms that mutation in Notch and Ik is concomitant in a high percentage of MuLV-derived T-cell tumors in mice (Uren *et al*., 2008).

The importance of Ik tumor suppressor function loss in T-ALL development lies in its ability to coordinately regulate gene expression with the Notch activation complex. It was observed that Ik and CSL bind the same core DNA consensus sequence and that Ik-1 was able to inhibit Notch-mediated gene transactivation *in vitro* (Beverly and Capobianco, 2003). These data indicate that CSL and Ik bind in a mutually exclusive manner to target gene promoters. On the basis of this work, investigators proposed a model of differential gene regulation by Notch and Ik (Figure 3). Under normal physiological conditions, Ik and Notch tightly regulate gene expression. Ik binds the core consensus sequence in target genes preventing CSL access to the promoter. An increase in DI-Ik isoforms, such as during T-cell development or in T-ALL, leads to inhibition of Ik transcriptional repressor function. This event allows CSL to bind promoter regions of genes that the Notch activation and Ik inhibitory complexes differentially regulate. In T-cell development, replacement of Ik at gene promoter regions by CSL coupled with Notch activation leads to expression of Notch target genes. In the case of leukemia, DI-Ik isoforms are prevalent and Ik cannot bind target gene promoters, resulting in unfettered access of CSL to gene regulatory regions. This event coupled with the ligand-independent Notch activation observed in T-ALL would lead to constitutive target gene expression (Beverly and Capobianco, 2004).

Additional data lend support to this model of differential regulation of target genes by Ik and Notch. Thymocytes from Ik-null mice are blocked at the DN3 stage of T-cell development ($CD4^-$, $CD8^-$, TCR^-, $CD25^+$, $CD44^-$). Ectopic expression of Ik1 in these cells results in a decrease in expression of Hes1, a Notch target gene, which is accompanied by expression of genes that indicate T-cell differentiation (Kathrein *et al*., 2008).

Several target genes have been identified that are differentially regulated by Notch and Ik, including Hes1 and Deltex. Interestingly, these two proteins have been shown to be upregulated not only in thymic lymphomas, but also in premalignant thymocytes of mice expressing low levels of Ik (Dumortier *et al*., 2006). Increase in Hes1 and Deltex was accompanied by truncating mutations in the PEST domain of Notch in a large percentage of tumors.

Although the current model suggests that the switch in Ik isoform expression in T-ALL is due to alternative splicing, the precise mechanism that regulates this switch is unclear. A clue to the mechanism was given by a recent report by the Screpanti group that suggests splicing of Ik mRNA is regulated, at least in part, by Notch3 induction of HuD (Bellavia *et al*., 2007b; reviewed in Bellavia *et al*., 2008, this issue). They found that in T-ALL tumors from Notch3 transgenic mice the *Ik* locus was expressed predominantly as DI-Ik isoforms. Furthermore, they propose a model that suggests Notch3 induces HuD expression that then regulates Ik isoform selection. However, it is not clear whether Notch1 regulates HuD expression or alternative splicing of Ik. It is intriguing to speculate that perhaps Notch1 can induce Notch3 to regulate Ik alternative splicing through HuD in T-ALL.

Notch suppresses p53 in T-ALL

The ARF–mdm2–p53 pathway is a central tumor surveillance mechanism that is a major checkpoint of genome integrity. This pathway is disrupted in the majority of cancer types. Inactivating mutations in the *p53* locus are present in more than 50% of all human malignancies (reviewed in Sherr, 1998). Those tumors that express wild-type *p53* often exhibit mutations in other pathway effectors, such as *mdm2* amplifications or loss of *ARF* (reviewed in Sherr, 2006). p53 is a transcription factor that functions by inhibiting cell-cycle progression or inducing apoptosis in response to cellular stress or DNA damage to maintain genome integrity (reviewed in Levine *et al*., 2006). Proteolytic degradation is the primary mechanism by which p53 expression is regulated in the cell. Mdm2 is an E3 ubiquitin ligase that binds to and ubiquitinates p53, targeting it for degradation by the proteasome. ARF binds to mdm2, disrupting the mdm2–p53 interaction thereby preventing ubiquitination of p53. This leads to accumulation of p53 in the cell and expression of downstream target genes, such as *p21*, *PUMA*, *NOXA*, *14-3-3-σ* and *GADD-45*, resulting in cell-cycle arrest or apoptosis. Numerous post-translational modifications of both p53 and mdm2 have been determined that serve to regulate the response to cellular stress signals. Most of which modulate the p53–mdm2 association, either disrupting or enhancing this interaction (Meek and Knippschild, 2003; Brooks and Gu, 2006).

Suppression of the p53-mediated apoptotic response was demonstrated to be critical in Notch-induced leukemogenesis. In a mouse model of Notch-induced T-ALL, tumors express low levels of p53 protein without a reduction in mRNA levels (Beverly *et al*., 2005). This led investigators to hypothesize that Notch may regulate p53 by increasing its proteolytic degradation (Beverly *et al*., 2005). Disruption of mdm2–p53 association by nutlin or γ-irradiation resulted in an increase in p53 protein levels, demonstrating that the mdm2–p53 mechanism is functional in Notch-induced T-ALL tumors. However, levels of ARF protein are undetectable and examination of the *ARF* locus revealed that it is intact, indicating that Notch regulates ARF (Beverly *et al*., 2005). This suggests the possibility that Notch suppresses p53 by inhibiting ARF leading to continuous mdm2-induced degradation of p53 by the proteosome. Inhibition of the Notch transgene results in 100% tumor regression with a concomitant increase in p53 expression, suggesting that p53 reactivation mediates tumor regression (Beverly *et al*., 2005). In support of this model, Notch-induced tumors in T-ALL mice that were heterozygous for p53 exhibited delays in tumor regression and apoptosis (Beverly *et al*., 2005). As Notch suppresses p53, this would indicate that activating mutations in the Notch pathway and loss of p53 activity would be mutually exclusive events. Indeed, in a comparison of lymphomas generated by MuLV insertional mutagenesis of p53 wild type and $p53^{-/-}$ mice, Notch insertions were primarily found in p53 wild-type compared to $p53^{-/-}$ tumors (Uren *et al*., 2008).

Examination of human T-ALL cell lines lends further support to the importance of suppressing the p53 apoptotic response in lymphomagenesis. The Ferrando group examined T-ALL cell lines that express activated Notch and are resistant to GSI. Interestingly, upon treatment, activated Notch was inhibited in these samples. This led investigators to hypothesize that the GSI resistance observed might be due to molecular abnormalities in signaling pathways that promote cell growth downstream of Notch1. Examination of these T-ALL cell lines revealed that they all contained inactivating phosphatase and tensin homologue deleted on chromosome 10 (PTEN) mutations (Palomero *et al*., 2007). The investigators propose that Akt may be a downstream Notch target and that GSI resistance is due to aberrant Akt signaling as a consequence of PTEN loss. Given the evidence that Notch suppresses p53 in T-ALL, the function of PTEN in suppression of Akt activity may provide an explanation for the resistance to GSI in these samples. Akt is a serine-threonine kinase that phosphorylates mdm2 enhancing its activity and nuclear localization, resulting in increased p53 degradation (Zhou *et al*., 2001; Mayo *et al*., 2002). Therefore,

upon inhibition of Notch by GSI in these T-ALL cell lines, the p53 apoptotic response will not be induced as a result of increased Akt activity due to loss of PTEN.

How do the observations in T-ALL cell lines translate to the human disease? Screening of primary human T-ALL samples revealed that PTEN was inactivated in 17% of cases examined. In these tumors that were PTEN null, Notch is activated in only a minor subset (11.4%; Palomero *et al.*, 2007). Therefore, the majority of the cases examined contained wild-type Notch, suggesting that in T-ALL cases where loss of PTEN occurs there is no preference to acquire activating Notch mutations. This can be explained by the ability of PTEN, like Notch, to regulate both cell-cycle progression and apoptosis. Therefore, is loss of PTEN sufficient to mimic Notch activation in T-ALL?

Experiments in mouse models of T-ALL provide insight into this issue. $PTEN^{-/-}$ mice are embryonic lethal, however, $PTEN^{+/-}$ mice develop a variety of cancers accompanied by loss of heterozygosity. Of the cancers that developed in $PTEN^{+/-}$ mice, 88% were classified as T-ALL (Suzuki *et al.*, 1998). Furthermore, mice reconstituted with $PTEN^{-/-}$ hematopoietic stem cells (HSCs) developed T-ALL that harbored translocations resulting in aberrant expression of c-myc (Guo *et al.*, 2008). Interestingly, no activating Notch mutations were found in these tumors, however, the phenotype was the same as those tumors induced by N^{ICD} ($CD4^+CD8^+$), suggesting that PTEN inactivation can compensate for some Notch-mediated processes in T-ALL, namely, suppression of p53-mediated apoptosis (Figure 4). However, other mutations would be required to drive cell-cycle progression, such as mutations in c-myc.

The ability of PTEN to suppress p53-mediated apoptosis may be a hurdle to GSI-based treatment response in T-ALL. Notch inhibition by GSI would provide a strong selective pressure to acquire mutations leading to inhibition of the p53 pathway, such as loss of p53 or PTEN. In support of this hypothesis human T-ALL relapses present a higher percentage of p53 mutations compared to primary T-ALL tumors (28 vs 1%) (http://p53.free.fr/p53_info/p53_cancer.html).

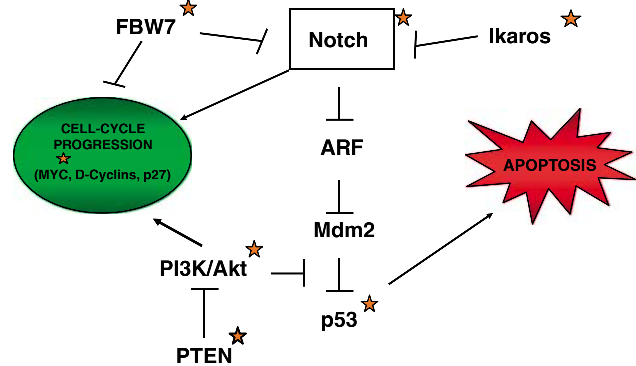

Figure 4 The Notch signaling pathway. The Notch signaling pathway governs cell-cycle progression and apoptosis. ‘Stars’ indicate pathway regulators and effectors demonstrated to be mutated in T-ALL. Combinations of these mutations may be able to ‘mimic’ activated Notch signaling.

Fbw7 regulates Notch stability and is mutated in T-ALL

Fbw7 is the F-box component of an SCF-E3 ubiquitin ligase complex (SCF^{FBW7}). F-box proteins recognize substrates that are phosphorylated at sequences termed degrons and provide substrate recognition for the SCF complex. Recognition of a specific substrate by SCF^{FBW7} results in ubiquitination of the substrate by the E2 ubiquitin-conjugating enzyme (reviewed in Welcker and Clurman, 2008). Several proto-oncogenes have been identified as substrates of SCF^{FBW7}, such as Myc, Jun and Notch. SCF^{FBW7}-mediated degradation of Notch requires phosphorylation of multiple residues. The first phosphorylation event at S2514 mediates Fbw7 binding, and the second at T2512 is required for polyubiquitination of the protein by SCF^{FBW7} that results in rapid turnover of N^{ICD} by the proteasome (O’Neil *et al.*, 2007; Thompson *et al.*, 2008). Phosphorylation of Notch at S2514 is mediated by CyclinC:cdk8 after recruitment to the Notch activation complex by Maml (Fryer *et al.*, 2004; O’Neil *et al.*, 2007). However, the kinase that phosphorylates N^{ICD} at the T2512 residue has not been definitively determined (Thompson *et al.*, 2008).

Evidence from mouse models indicates that Fbw7 may be involved in T-ALL. Mice reconstituted with HSC lacking both p53 and Fbw7 develop $CD4^+CD8^+$ T-ALL tumors (Matsuoka *et al.*, 2008). This combination of mutations would play the same role as an activating Notch mutation, regulating both cell-cycle progression and apoptosis (Figure 4). Although the status of the Notch locus was not examined in tumors from these mice, evidence from human T-ALL samples gives insight into the Fbw7–Notch dynamic in leukemogenesis. Inactivating mutations of Fbw7 were found in 30% of human T-ALL samples examined, and analysis of these tumors revealed that only wild-type or HD mutant Notch forms were expressed (O’Neil *et al.*, 2007; Matsuoka *et al.*, 2008). Whereas, Notch PEST domain mutations were only found in samples expressing wild-type Fbw7, these data imply that in T-ALL, an inactivating Fbw7 mutation would have the same effect as N^{ICD} PEST mutations, increasing Notch activity by decreasing Notch degradation by the proteosome. Indeed, activating PEST domain mutations identified in primary, human T-ALL cases either result in insertions of translational termination codons or mutation of the Fbw7 degron region (Thompson *et al.*, 2008).

How does the role of Fbw7 in the Notch pathway explain what is observed in human T-ALL samples? In T-ALL tumors harboring HD domain mutations in Notch, Fbw7 loss would inhibit Notch degradation by the proteosome, essentially mimicking HD/PEST mutations in *trans*. In those tumors where wild-type Notch is expressed, loss of Fbw7 would decrease degradation of its substrates by the proteosome. This would result in

increased expression of the proto-oncogenes that Fbw7 regulates contributing to cell-cycle progression. Oncogenic levels of these genes, such as c-myc, would lead to activation of the p53-mediated apoptotic response, requiring inactivation of the ARF–mdm2–p53 tumor surveillance mechanism for leukemogenesis to occur.

Notch regulates cell-cycle progression

Cell-cycle progression is a tightly controlled cellular process that is regulated by several checkpoints that monitor completion of cell-cycle processes and genomic integrity. Many factors that regulate cell-cycle progression are mutated in cancer resulting in deregulated cellular growth. It has been demonstrated that Notch is involved in driving cell-cycle progression by regulating genes involved in the G_1 to S-phase transition (Ronchini and Capobianco, 2000; Satoh *et al*., 2004; Murata *et al*., 2005; Sharma *et al*., 2006; Weng *et al*., 2006). Notch directly regulates D-type cyclins, which, in conjunction with cyclin-dependent kinase (cdk) 4 and cdk6 facilitate progression through the G_1 phase (Ronchini and Capobianco, 2000). In fact, *in vitro* and *in vivo* experiments indicate that upregulation of D-type cyclins is required for Notch-mediated transformation (Sicinska *et al*., 2003; Stahl *et al*., 2006).

p27 is a cdk inhibitor that sequesters cyclin E to prevent cdk2 activation and S-phase progression (reviewed in Sherr and Roberts, 1999). p27 is also required for assembly of the cyclin D/cdk4/6 complexes in dividing cells (Blain *et al*., 1997). Therefore, as cells enter the cell cycle, levels of p27 expression decrease and the remaining p27 is required for the formation of cyclinD/cdk4/6 complexes to progress through the G_1 phase. The cyclinE/cdk2 complex is then activated allowing progression through S phase (Sheaff *et al*., 1997). It has been demonstrated that downregulation of p27 is required for T-cell development and proliferation, and its upregulation causes apoptosis in T-ALL cells (Barata *et al*., 2001; Tsukiyama *et al*., 2001). Notch decreases p27 protein levels in T-ALL cell lines by driving the expression of SKP2, an E3 ubiquitin ligase that targets p27 for degradation. Treatment of these T-ALL cell lines with GSI to prevent Notch cleavage results in a G_0/G_1 arrest. This arrest is marked by an increase in p27 protein levels with a concomitant decrease in SKP2 (Dohda *et al*., 2007). Furthermore, it has been demonstrated that ectopic expression of Notch1 results in an increase in cdk2 activity, during Notch-mediated transformation of epithelial cells (Ronchini and Capobianco, 2000). This evidence suggests that an increase in Cdk2 activity by virtue of p27 downregulation might be a required event for Notch-mediated leukemogenesis.

Notch induces cell-cycle progression also through direct transactivation of c-myc (Satoh *et al*., 2004; Sharma *et al*., 2006; Weng *et al*., 2006). Normally, c-myc is only expressed in dividing cells and induces cell-cycle progression through induction of D-type cyclins, cyclin E, cdk4 and cdc25A, as well as repression of p27 expression (reviewed in Amati *et al*., 1998). Myc is frequently overexpressed in human neoplasia and exogenous expression of c-myc can induce tumorigenesis in multiple cell types, including hematopoietic cells (Marcu *et al*., 1992; Dang *et al*., 1999). Oncogenic levels of c-myc activate p53-mediated apoptosis, therefore, mutations that inhibit the ARF–mdm2–p53 pathway are commonly found in myc-induced tumorigenesis (Hermeking and Eick, 1994). Screening of a panel of tumors generated in $MMTV^{D}$/myc transgenic mice infected with MuLV revealed that 52% had proviral insertions into the *Notch* locus (Girard *et al*., 1996). Interestingly, no insertions were found in the *myc* locus in MuLV-infected N^{ICD} transgenic mice (Beverly and Capobianco, 2003). Why do c-myc tumors contain proviral insertions in the *Notch* locus, whereas Notch tumors do not acquire proviral insertions in the *c-myc* locus? What accounts for this lack of reciprocity? The need for c-myc to inhibit the p53-mediated apoptotic response during lymphomagenesis has been demonstrated, as 80% of tumors examined contained mutations in the ARF–mdm2–p53 pathway (Eischen *et al*., 1999). Therefore, Notch activation would provide an advantage to c-myc-induced tumors by virtue of its ability to suppress p53 and, therefore, apoptosis.

Concluding remarks

Notch signaling is critical for T-cell development and is important in T-ALL. The Notch pathway regulates two critical processes that govern cellular transformation, cell-cycle progression and apoptosis. This is in contrast to other proto-oncogenes, such as c-myc, which only regulate proliferation, and in fact, induces apoptosis if proliferation is not kept in check (Figure 4). In addition to Notch, other oncogenes have been shown experimentally to induce T-ALL in mice. Expression of LMO1/2, LYL1, TAL1/2, HOX11, HOX11L2 or MYC in mouse bone marrow leads to development of a heterogeneous population of T-ALL subtypes. Interestingly, activating Notch1 mutations have been found in all subtypes of T-ALL, suggesting that Notch activation is a dominant event necessary for leukemogenesis even in the presence of other T-ALL-associated genes (Weng *et al*., 2004). Expression of certain genes, *Lyl*, *Hox11*, *Hox11L2*, *Tal1*, *Calm-10* and *MLL*, is used to classify the subtype of T-ALL, as these are correlated with tumor phenotype and clinical outcome (Ferrando *et al*., 2002). These genes may also greatly contribute to treatment response based on the stage of T-cell development at which the tumors are blocked, as suggested for HOX11. $HOX11^{+}$ tumors are in the early cortical stage and are therefore more sensitive to apoptotic signals (Ferrando *et al*., 2002). Therefore, in developing T-ALL treatments that target the Notch pathway, the tumor classification and how cellular context influences treatment response should be taken into account. Activating Notch mutations in cells of the T lineage induce both cell-cycle

progression and apoptotic inhibition. Whereas other acquired genetic mutations observed in T-ALL (loss of p53/Fbw7/PTEN or activation of c-myc) only result in regulation of one of these two processes. Recent reports have demonstrated that combinations of mutations in pathways that contribute to Notch signaling are modulated in mouse models of T-ALL, such as p53 and Fbw7 loss or loss of PTEN and activation of c-myc (Figure 4). Inhibition of Notch could, in fact, provide a strong selective pressure that might lead to mutations in these pathways. For example, in the TOP-Notch model of T-ALL, inhibition of Notch induces p53 and tumor regression (Beverly *et al*., 2005). However, 100% of these tumors relapse. This may also occur in human T-ALL. For example, upon treatment with GSI a subset of T-ALL tumors may acquire loss of PTEN with concomitant activation of c-myc leading to chemoresistance and relapse. These combinations of mutations may contribute to primary T-ALL in which activating point mutations in *Notch* are not observed, reinforcing the model that the Notch pathway is central in T-ALL development.

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