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Oncogenic NOTCH1 control of MYC and PI3K: challenges and opportunities for anti-NOTCH1 therapy in T-ALL

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Abstract

The identification of activating mutations in *NOTCH1* in the majority of T-cell acute lymphoblastic leukemias and lymphomas (T-ALL) has brought much interest in inhibiting NOTCH1 signaling as therapeutic target in this disease. Small molecule inhibitors of the γ-secretase complex, which mediates a critical proteolytic cleavage required for NOTCH1 activation, hold the promise of becoming an effective molecular therapy against relapsed and refractory T-ALL. Recent progress in the elucidation of the transcriptional regulatory networks downstream of oncogenic NOTCH1 has uncovered a central role of NOTCH1 signaling in promoting leukemic cell growth and revealed an intricate circuitry that connects NOTCH1 signaling with MYC and the PI3K-AKT signaling pathway. The identification of the downstream effector pathways controlled by NOTCH1 should pave the way for the rational design of anti-NOTCH1 therapies for the treatment of T-ALL.

Background

The NOTCH pathway is an evolutionary-conserved mechanism responsible for the direct transduction of developmental signals at the cell surface into changes in gene expression in the nucleus (1–3). The intrinsic elements of the NOTCH signaling pathway include: (i) the Delta and Serrate family of ligands (Delta-like 1, 3 and 4; and Jagged 1 and 2), (ii) the NOTCH receptors (NOTCH1–4), and (iii) the CSL (CBF1/Su(H)/LAG-1) transcription factor, a DNA binding protein that interacts with the activated forms of NOTCH receptors.

The mature NOTCH1 protein is an heterodimeric transmembrane receptor consisting of an extracellular subunit and a transmembrane and intracellular subunit, which are generated by furin protease cleavage from a precursor polypeptide during its maturation in the trans-Golgi network (4,5). In resting conditions, the two NOTCH1 subunits remain associated forming a heterodimeric complex (2,3). However, upon ligand-receptor interaction, the N-terminus fragment of NOTCH1 is pulled away from the complex. This triggers a double proteolytic processing of the transmembrane and intracellular portion of NOTCH1, first by an ADAM protease (6,7) and subsequently by the γ-secretase complex (8–10). This final proteolytic cleavage releases the intracellular domains of NOTCH1 (ICN1) from the membrane, which then translocates to the nucleus, binds to the CSL DNA-binding protein (11), and recruit the MAML1 transcriptional coactivator to activate the expression of target genes (12). Transcriptional activation terminates NOTCH1 signaling through phosphorylation of the C-terminus PEST domain of ICN by CDK8, which results in FBXW7-CSF mediated degradation

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of the activated receptor in the proteasome (13). NOTCH1 reads and transduces extracellular signals in a quantic way, as one molecule of ligand activates the irreversible proteolytic cleavage and activation of one molecule of receptor, which in turn binds to one promoter to activate gene expression (1–3).

In the hematopoietic system, NOTCH1 plays a critical role in T-cell development and transformation (3,14). During thymocyte development NOTCH1 signals are required for the commitment of multipotent hematopoietic progenitors to the T-cell lineage (15–18) and then along T-cell development for progression through the early DN1, DN2 and DN3 stages of thymocyte maturation (19). Aberrant NOTCH1 signaling was first identified in T-cell acute lymphoblastic leukemias (T-ALLs) harboring the t(7;9)(q34;q34.3), a recurrent chromosomal translocation which truncates the *NOTCH1* gene and misplaces it next to the *TCRB* locus, leading to high levels of expression of a constitutively active intracellular form of NOTCH1 (20,21). However, the most common mechanisms leading to aberrant *NOTCH1* signaling are activating mutations in *NOTCH1*, which are present in over 50% of T-ALL cases (22). Activating *NOTCH1* mutations located in the heterodimerization domain (*HD* alleles) and the juxtamembrane extracellular region (*JME* alleles) induce ligand independent activation of the receptor (23,24), while truncating mutations deleting the *PEST* domain in the C-terminal region of the protein extend NOTCH1 signaling by impairing the proteasomal degradation of ICN1. In addition, mutations in *FBXW7* involving three critical arginine residues that mediate the interaction of this F-box protein with the phosphodegron moiety in the NOTCH1 PEST domain, also extend NOTCH1 signaling by impairing the proteasomal degradation of ICN1 in 15% of TALL cases (25–28). Importantly, about 15–25% of these leukemias harbor two concurrent lesions activating NOTCH1; the first one inducing ligand independent activation of NOTCH1 –an *HD* or *JME* allele –and a second one leading to increased protein stability and extended duration of NOTCH1 signaling –a *PEST* truncation or *FBXW7* mutation –(22,25,27,28).

The importance of mutations activating the NOTCH1 pathway is highlighted by the potential role of NOTCH1 as a therapeutic target in T-ALL. Given the strict requirement of γ-secretase cleavage for NOTCH1 activation it was recognized early on that inhibition of this proteolytic step could be exploited to effectively abrogate the function of oncogenic NOTCH1 in T-ALL cells. Importantly, the presenilin γ-secretase complex has been the focus of extensive research over the last decade because of its role in the generation of amyloid deposits in the brains of patients with Alzheimer's disease; and small molecule γ-secretase inhibitors (GSIs), originally designed for the treatment of neurodegenerative disorders, effectively block NOTCH1 signaling. *In vitro* studies showed that GSI treatment of T-ALL cell lines harboring activating mutations in NOTCH1 induced cell cycle arrest in G1 (22,29,30) prompting the investigation of these agents for the treatment of relapsed and refractory T-ALL (31). However, the target genes and effector pathways that mediate the oncogenic activity of NOTCH1 remained largely unknown precluding a fully rational design of anti-NOTCH therapies. This gap has started to be filled as result of microarray gene expression profiling studies of T-ALL cells treated with GSIs. These analyses have demonstrated a major role of NOTCH1 in the regulation of cell growth and metabolism (29,32). Thus, NOTCH1 inhibition in T-ALL cells results in the transcriptional downregulation of numerous genes involved in biosynthesis pathways such as ribosome biosynthesis, protein translation and amino acid and nucleotide biosynthesis (29, 32,33) (Figure 1). Indeed, many of these anabolic genes were shown to be direct targets of NOTCH1 by ChIP-on-chip analysis, thus highlighting the role of NOTCH1 as direct regulator of cell growth (29). Importantly, *MYC* is also a direct trascriptional target downstream of oncogenic NOTCH1 (29,32,33) and MYC regulates the expression of anabolic genes and pathways downstream of NOTCH1 (34). This *NOTCH1-MYC* transcriptional regulatory loop places the control of cell growth anabolic pathways at the core of the mechanisms mediating T-cell transformation by oncogenic NOTCH1 (Figure 1).

A prominent role of NOTCH1 as a regulator of cell growth in immature T-cells was further supported by several lines of evidence indicating that activation of the PI3K-AKT-mTOR pathway is also an important outcome downstream of NOTCH1 signaling. In early work, Sade and coworkers showed that the Src family protein tyrosine kinase $p56^{lck}$ is required for NOTCH1-mediated activation of AKT in T-cells (35). Furthermore phosphoproteomic analysis of T-ALL cells treated with NOTCH1 inhibitors showed a marked decrease in the phosphorylation of mTOR targets (36). In addition, elegant work by Ciofani and coworkers showed that Notch signals regulate the trophic state (cell size, glucose uptake and glycolysis) of T-cell precursors and promote cell survival through maintenance of cellular metabolism (37). Importantly, these critical effects of Notch signaling in the growth, proliferation and survival of T-cell progenitors were mediated by activation of the PI3K-Akt signaling pathway (37).

The PI3K-AKT signal transduction pathway mediates multiple cellular responses triggered by the engagement of growth factor receptors, including increased cell growth, proliferation and survival (38–43). Activation of PI3K by receptor tyrosine kinases and G protein-coupled receptors triggers the phosphorylation of phosphatidylinositol 4,5 biphosphate (PIP2) to generate phosphatidylinositol triphosphate (PIP3). Accumulation of PIP3 at the plasma membrane recruits AKT and induces its phosphorylation by PDK1 and the mTOR-Rictor complex at Thr308 and Ser473, respectively (44,45). In turn, AKT activates the phosphorylation of multiple substrates that promote cell growth, increased glucose uptake and oxidation, cell cycle progression and cell survival by direct and indirect mechanisms (38–41). Signaling by the PI3K-AKT pathway is terminated by the PTEN phosphatase, which dephosphorylates and thereby inactivates PIP3 (38–41).

In this context, activation of the PI3K-AKT pathway downstream of NOTCH1 signaling promotes cell growth at multiple levels and plays an important role in T-cell transformation. However, the specific mechanisms connecting these two pathways remained unclear until a combination of genomic analysis in T-ALL cells and a forward genetic screen in *Drosophila* identified the PTEN downregulation as a major component of the oncogenic program downstream of NOTCH1 (Figure 1) (34). These studies showed that *PTEN* expression in T-ALL is controlled by a dual input transcriptional regulatory circuit operated by HES1, a transcriptional repressor directly controlled by NOTCH1, and MYC, also a direct NOTCH1 target (34). Both HES1 and MYC bind and regulate the *PTEN* promoter, with HES1 working as a strong transcriptional repressor and MYC as a weaker transcriptional activator, so that the overall output downstream of NOTCH1 activation is a controlled downregulation of *PTEN* transcripts (Figure 1) (34).

Clinical-translational advances

The induction of cell cycle arrest and a decrease in cell size in T-ALL cell lines treated with GSIs is in agreement with the role of NOTCH1 as a direct regulator of cell growth. However, the most striking direct implication of the elucidation of the transcriptional circuitry controlling cell growth downstream of NOTCH1 is that it sets the conceptual framework to understand the mechanisms that mediate primary resistance to GSI therapy in T-ALL. It was recognized early on that only a fraction of T-ALL cell lines with activating mutations in *NOTCH1* show a decreased cell size and cell cycle arrest response when treated with GSIs (22). However, analysis of GSI sensitive and GSI resistant T-ALL cell lines showed no differences in the effect of GSI treatment on inhibition of NOTCH1 processing, the rate and kinetics of ICN1 clearance or the transcriptional response triggered by NOTCH1 inhibition in these cells (34). These results demonstrated that GSI resistance was not the result of increased drug metabolism, increased drug export or decreased ability of this GSI to interact with and inhibit the γ-secretase complex; and supported that activation of an alternative oncogenic pathway was taking over

the role of NOTCH1 as central regulator of growth and metabolism in GSI resistant T-ALL cells. This hypothesis was confirmed upon discovering that mutational loss of *PTEN* and consequent constitutive activation of the PI3K-AKT pathway was present in GSI resistant T-ALL lines, but not in GSI-sensitive leukemias (34). Further analysis established a mechanistic link between loss of PTEN, activation of AKT and increased metabolism as a mechanism of resistance to NOTCH1 inhibition in T-ALL cell lines (34). Importantly, loss of PTEN was also found in 17% of primary T-ALL samples, suggesting that primary resistance to GSI therapy may be readily present in a significant fraction of T-ALL patients (34).

Treatments that target oncogenic signaling pathways controlling the growth and survival of malignant tumor cells are based on the concept of oncogene addiction, which proposes that adaptation to oncogenic signals require an irreversible rewiring of the cellular machinery which renders tumor cells dependent on continuous oncogene activity for proliferation and survival (46). Following on this premise we predicted that constitutive AKT activation could circumvent the requirement of NOTCH1 signaling for cell growth and proliferation, at the expense of inducing an oncogene addiction switch that would render PTEN null/GSI-resistant cells addicted to AKT signaling. Treatment of PTEN-positive/GSI-sensitive and PTEN-null/GSI-resistant TALL cells with an AKT inhibitor confirmed this prediction showing a 10 fold higher sensitivity to this drug in cells resistant to NOTCH-inhibition (34). Thus, small molecule inhibitors of PI3K-AKT signaling currently under clinical development (47) may constitute an effective treatment in T-ALL tumors with mutational loss of *PTEN*.

Future developments

Despite strong rationale and much enthusiasm towards GSIs as a targeted therapy against T-ALL tumors harboring activating mutations in *NOTCH1*, the results of the first clinical trial testing the MK-0752 GSI, in relapsed and refractory T-ALL have shown no significant clinical responses and a high incidence of dose limiting gastrointestinal toxicity (31). Thus, new approaches aiming to increase the therapeutic window of GSIs are required for the successful implementation of anti-NOTCH based therapies in T-ALL. Combination therapies exploiting the interaction of NOTCH inhibitors with drugs targeting the PI3K-AK-mTOR pathway such as rapamycin and perifosin may increase the intrinsic activity of GSIs and facilitate dose schedules that avoid the development of gastrointestinal toxicity.

Recent studies have shown that, in addition to its direct and indirect effects on cell growth and metabolism, NOTCH1 signaling may regulate the activity of p53 (48) and the NFKB pathway (49). Although the specific mechanisms mediating these interactions have not been elucidated yet, these results suggest that combinations of GSIs with DNA damaging agents inducing p53 activation, or with drugs interfering with the activity of the NFKB pathway such as bortezomib, may have a synergistic effect in the treatment of T-ALL.

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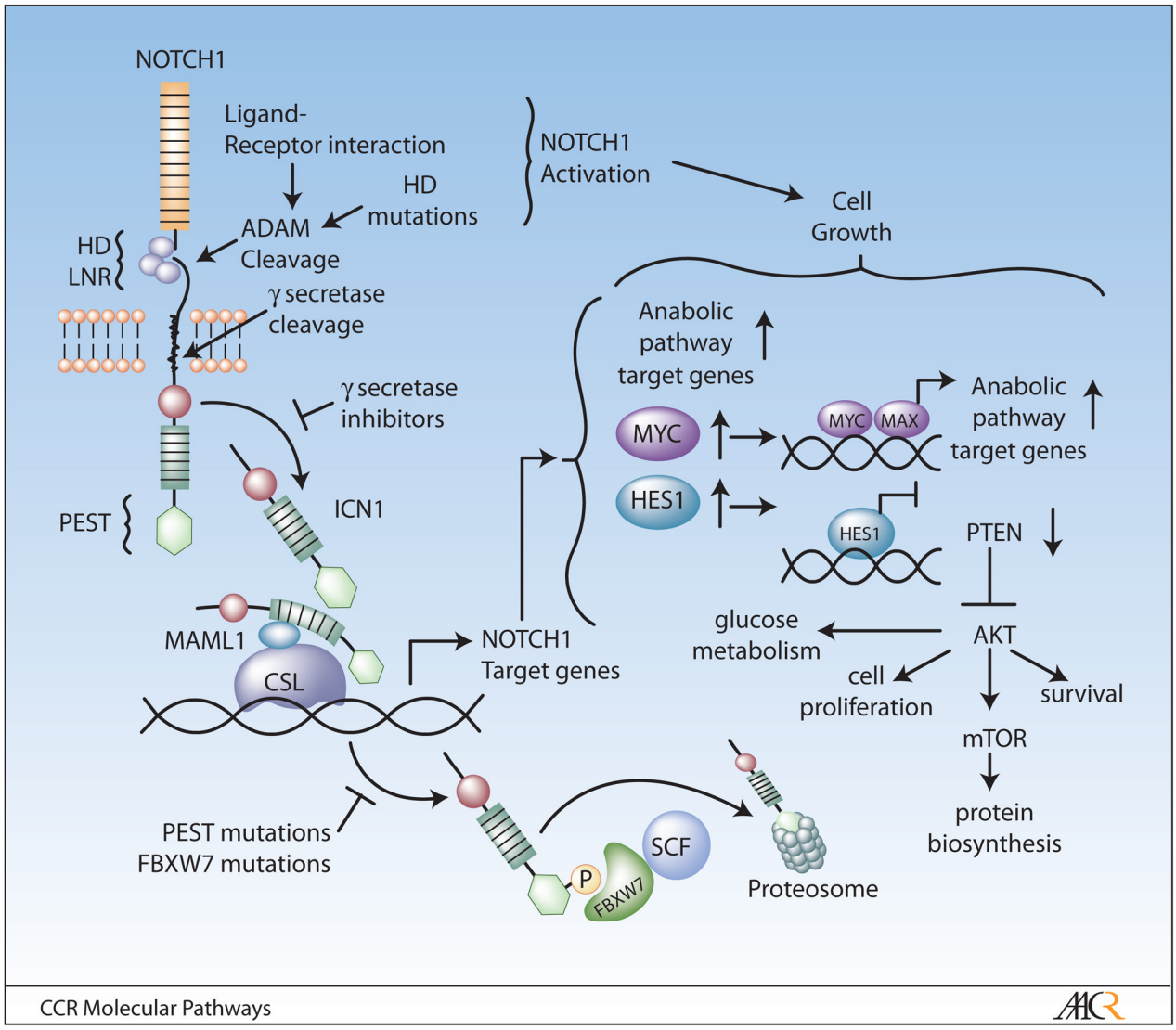

Figure 1. Schematic representation of the NOTCH1 signaling pathway and transcriptional networks promoting leukemic cell growth downstream of oncogenic NOTCH1

The two subunits of the mature NOTCH1 receptor are generated by cleavage by a furin protease in the trans-Golgi network soon after translation of NOTCH1 precursor protein. The extracellular subunit responsible for ligand-receptor interaction and the transmembrane subunit responsible for triggering transcription activation interact by their respective heterodimerization (HD) domains. Upon binding to its ligands –Delta-like 1, 3 and 4; Jagged 1 and 2– the transmembrane portion of NOTCH1 is sequentially cleaved by ADAM proteases and then by the γ-secretase complex. This final proteolytic cleavage liberates the active intracellular fragment of NOTCH1 (ICN1), which translocates to the nucleus and activates the expression of target genes by forming a ternary complex with the CSL DNA binding protein and the MAML1 transcriptional coactivator. Small molecule inhibitors of the γ-secretase block NOTCH1 activation by retaining the receptor at the membrane. NOTCH1 promotes cell growth by transcriptional upregulation of genes involved in anabolic pathways and also by transcriptional upregulation of *MYC*. In addition NOTCH1 induces the expression of *HES1*, a transcriptional repressor that promotes the upregulation of the PI3K-AKT signaling pathway by transcriptional downregulation of *PTEN*. Termination of NOTCH1 signaling is mediated

by phosphorylated-coupled degradation of the activated receptor in the proteasome via FBXW7-SCF.