

Corrections

CELL BIOLOGY. For the article “Lack of myostatin results in excessive muscle growth but impaired force generation,” by Helge Amthor, Raymond Macharia, Roberto Navarrete, Markus Schuelke, Susan C. Brown, Anthony Otto, Thomas Voit, Francesco Muntoni, Gerta Vrbóva, Terence Partridge, Peter Zammit, Lutz Bunker, and Ketan Patel, which appeared in issue 6, February 6, 2007, of *Proc Natl Acad Sci USA* (104:1835–1840; first published January 31, 2007; 10.1073/pnas.0604893104), the authors note that the corresponding author’s e-mail address appeared incorrectly. The correct address is helge.amthor@psl.aphp.fr. The online version has been corrected.

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MEDICAL SCIENCES. For the article “NOTCH1 directly regulates *c-MYC* and activates a feed-forward-loop transcriptional network promoting leukemic cell growth,” by Teresa Palomero, Wei Keat Lim, Duncan T. Odom, Maria Luisa Sulis, Pedro J. Real, Adam Margolin, Kelly C. Barnes, Jennifer O’Neil, Donna Neuberg, Andrew P. Weng, Jon C. Aster, Francois Sigaux, Jean Soulier, A. Thomas Look, Richard A. Young, Andrea Califano, and Adolfo A. Ferrando, which appeared in issue 48, November 28, 2006, of *Proc Natl Acad Sci USA* (103:18261–18266; first published November 17, 2006; 10.1073/pnas.0606108103), the authors wish to acknowledge that Andrea Califano is a co-senior author of this work.

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NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth

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The NOTCH1 signaling pathway directly links extracellular signals with transcriptional responses in the cell nucleus and plays a critical role during T cell development and in the pathogenesis over 50% of human T cell lymphoblastic leukemia (T-ALL) cases. However, little is known about the transcriptional programs activated by NOTCH1. Using an integrative systems biology approach we show that NOTCH1 controls a feed-forward-loop transcriptional network that promotes cell growth. Inhibition of NOTCH1 signaling in T-ALL cells led to a reduction in cell size and elicited a gene expression signature dominated by down-regulated biosynthetic pathway genes. By integrating gene expression array and ChIP-on-chip data, we show that NOTCH1 directly activates multiple biosynthetic routes and induces c-MYC gene expression. Reverse engineering of regulatory networks from expression profiles showed that NOTCH1 and c-MYC govern two directly interconnected transcriptional programs containing common target genes that together regulate the growth of primary T-ALL cells. These results identify c-MYC as an essential mediator of NOTCH1 signaling and integrate NOTCH1 activation with oncogenic signaling pathways upstream of c-MYC.

T cell lymphoblastic leukemia

NOTCH receptors play a critical role in lineage specification decisions in development. Physiologic activation of NOTCH receptors (NOTCH1–4) triggered by interaction with a DSL ligand expressed on the surface of a neighboring cell induces two consecutive proteolytic cleavages in the receptor, first by an ADAM metalloprotease and subsequently by the γ -secretase complex, which release the intracellular portion of the NOTCH protein from the membrane (1). This fragment (“intracellular NOTCH” or ICN) rapidly translocates to the nucleus and interacts with the CSL DNA binding protein to activate the expression of target genes (2). Thus, NOTCH receptors operate both as recipients of extracellular signals at the cell surface and as transcription factors regulating gene expression in the nucleus.

NOTCH1 signaling is involved in the maintenance of hematopoietic stem cells and is essential for the development of T cells (3). The key role of NOTCH1 in T cell development underlies the hypothesis that NOTCH1 signaling regulates cell growth, proliferation, differentiation, and apoptosis, processes that when dysregulated are the hallmark of cancer in developing thymocytes. Initially identified because of its involvement in the *t*(7;9)(q34;q34.3) chromosomal translocation in a patient with T cell lymphoblastic leukemia (T-ALL), NOTCH1 is a major factor in the pathogenesis of immature T cell tumors (4, 5). The *t*(7;9) translocation juxtaposes a truncated NOTCH1 gene next to the *TCRB* locus, leading to the aberrant expression of an intracellular constitutively active form of NOTCH1 (4). More frequently, activating mutations in NOTCH1, inducing ligand independent activation of the receptor or increased

stability of ICN1 in the nucleus, are found in over 50% of human T-ALL cases (5).

Importantly, inhibition of NOTCH1 signaling with small molecule inhibitors of the γ -secretase complex (GSI) induces cell cycle arrest and apoptosis in leukemic cells, demonstrating that aberrant NOTCH signaling is necessary for maintenance of the malignant phenotype in T-ALL and making NOTCH1 a major target for the development of molecularly tailored antileukemic drugs (5–7).

Despite the prominent roles of NOTCH1 in T cell development and transformation, only a few NOTCH1 target genes have been identified to date in T cells and the mechanisms controlling cell growth, proliferation, and apoptosis downstream of NOTCH1 remain largely unexplored. To gain insight into the molecular mechanisms that underlie the functions of NOTCH1, we undertook the genome wide identification of direct target genes regulated by NOTCH1 using a combination of gene expression profiling with oligonucleotide microarrays and ChIP-on-chip analysis. Our results identify NOTCH1 as a direct regulator of cell growth; integrate NOTCH1 activation with signaling pathways controlling cell growth upstream of c-MYC, and illustrate the power of genomic approaches to elucidate the structure of key transcriptional networks in human cancer.

Results

NOTCH1 Regulates Leukemic Cell Growth. To address the leukemogenic regulatory programs activated by NOTCH1, we first analyzed global gene expression profiles after inhibition of NOTCH1 signaling in T-ALL cells. Blocking NOTCH1 activation with Compound E (CompE), a highly active GSI (5, 8), induces a drastic decrease in the levels of ICN1 (Fig. 1a). Duplicate samples of seven T-ALL cell lines harboring activating mutations in NOTCH1 were treated with CompE or with vehicle (DMSO) only. RNA was collected 24 h posttreatment and analyzed with Affymetrix Human Genome U133 Plus 2.0 arrays. Nearest-neighbor analysis was used to look for gene expression changes associated with GSI or vehicle only treatment. This analysis identified 201 genes that were significantly down-regulated ($P < 0.0001$) and 38 that were significantly

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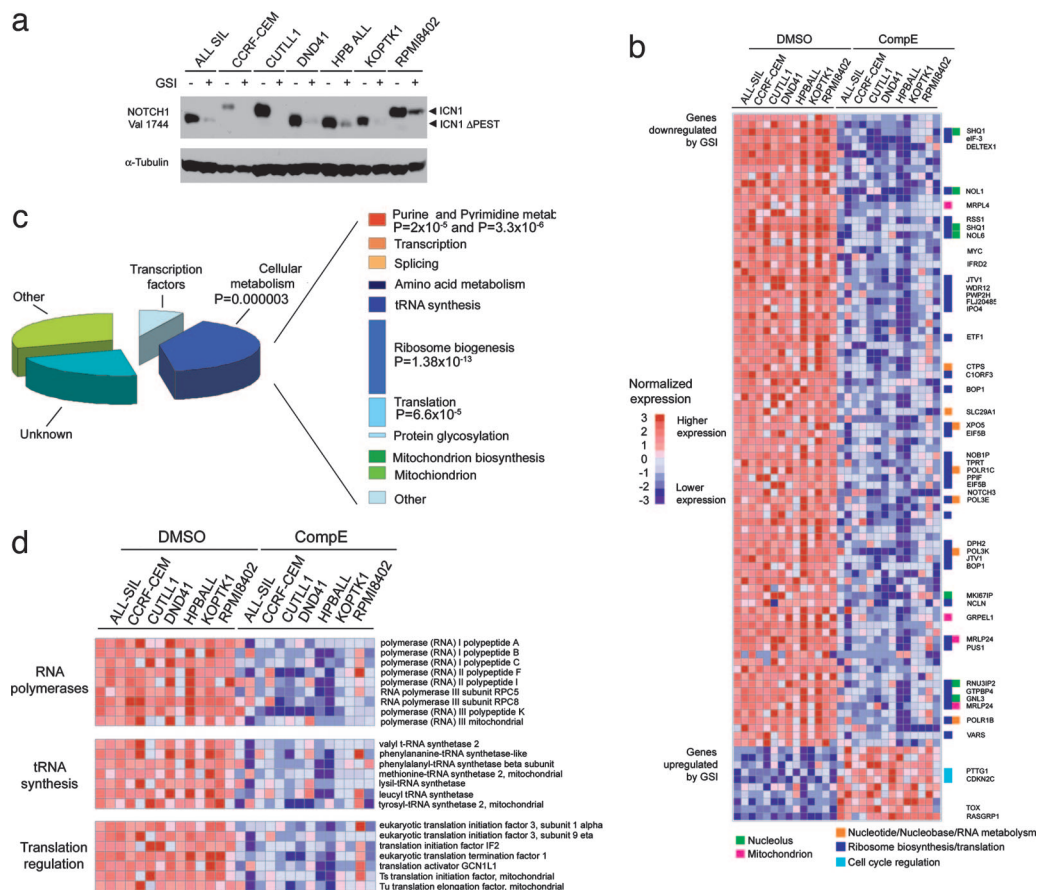
The authors declare no conflict of interest.

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Abbreviations: CompE, Compound E; GSEA, gene set enrichment analysis; GSI, γ -secretase inhibitor; ICN1, intracellular NOTCH1; T-ALL, T cell acute lymphoblastic leukemia.

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up-regulated ($P < 0.0001$) after inhibiting NOTCH1 signaling (Fig. 1b). This collection of regulated genes included known direct transcriptional targets of NOTCH1, such as *DELTEX1* and *HES1*. Importantly, the set of down-regulated genes was highly enriched in functional categories related to cellular metabolism, such as ribosome biosynthesis ($P = 1.38 \times 10^{-13}$), protein translation ($P = 6.6 \times 10^{-5}$) and purine and pyrimidine metabolism ($P = 2 \times 10^{-5}$ and $P = 3.3 \times 10^{-6}$, respectively) (Fig. 1 c and d). Furthermore, analysis of cell size changes following the inhibition of NOTCH1 signaling by GSI treatment showed a marked reduction in cell diameters ($P < 0.001$ by paired *t* test) (Fig. 2a and b). GSI treatment also affects the processing of other γ -secretase substrates expressed in the thymus, including APP and CD44, however, the effect of CompE in gene expression and cell size was specific for NOTCH, as it was rescued by forced expression of ICN1 (Fig. 2c and Fig. 7, which is published as supporting information on the PNAS web site). Similarly, lentiviral expression of a shRNA sequence targeting *NOTCH1* (9) depleted T-ALL cells from ICN1, down-regulated the expression of *DELTEX1* (Fig. 4b), and induced a smaller cell size in DND41 cells compared with cells expressing a control shRNA sequence targeting the luciferase gene (Fig. 2d). Genes up-regulated upon NOTCH1 inactivation included important cell cycle regulators such as *p27/KIP1* and *p18/INK4C*, consistent with the induction of G₁ cell-cycle arrest in T-ALL cells after inhibition of NOTCH1 signaling (Fig. 8, which is published as supporting information on the PNAS web site). These results highlight the role of NOTCH1 as regulator of cell growth and place NOTCH1 signaling upstream of transcriptional programs encompassing essential metabolic and biosynthetic pathways.

Identification of NOTCH1 Direct Target Genes. To test the hypothesis that NOTCH1 directly controls genes regulating cell growth, we

Fig. 1. Oncogenic NOTCH1 signaling activates transcriptional programs regulating leukemic cell growth. (a) Activated intracellular NOTCH1 levels after γ -secretase cleavage were detected by Western blot analysis using the Val-1744 antibody that specifically recognizes ICN1, in cell lysates from T-ALL cell lines treated with GSI (CompE 500 nM) for 24 h or mock-treated (DMSO) controls. Smaller molecular weight bands (ICN1- Δ PEST) are observed in cell lines with truncating mutations in the PEST domain of NOTCH1. α -Tubulin levels are shown as loading control. (b) Gene expression profiling was performed in duplicate samples from 7 T-ALL cell lines treated with GSI (CompE) or vehicle (DMSO) for 24 h. Heat map represents color coded expression levels for each sample with respect to mock treatment controls. Top significant genes ($P < 0.00001$) ranked by t test are shown. (c) Functional annotation of top genes ($P < 0.0001$) down-regulated after NOTCH inhibition by GSI treatment using the DAVID tool. (d) Detailed heat maps representing expression changes induced by GSI in selected functional categories.

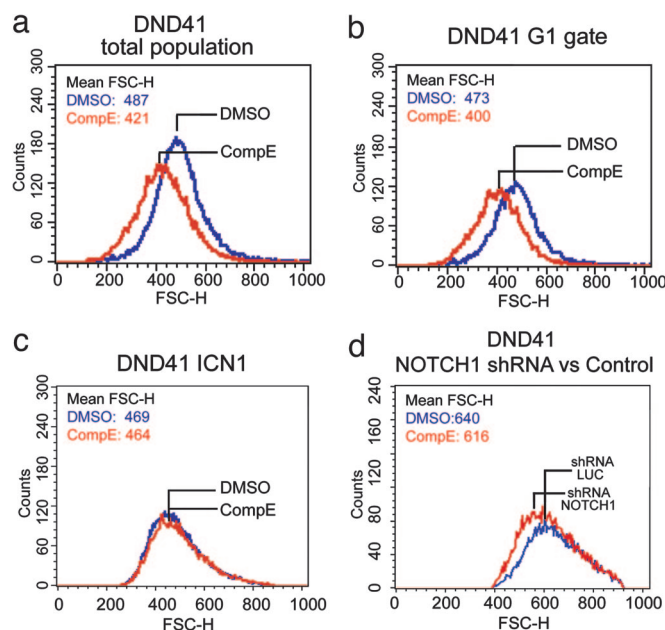


Fig. 2. Inhibition of NOTCH1 signaling impairs cell growth in T-ALL cells. (a) DND41 T-ALL cells treated with GSI for 6 days showed a reduction in cell diameters compared with vehicle (DMSO) treatment. Representative histograms of triplicate experiments are shown. Similar results were obtained in HPB-ALL, CUTLL1, and KOPTK1 cell lines. (b) GSI-induced changes in cell size are independent of cell cycle as demonstrated by analysis of G₁ (2N DNA content) gated cells. (c) Forced expression of ICN1 rescues the effects of GSI in cell size. (d) Inhibition of NOTCH1 signaling via lentiviral expression of shRNAs targeting *NOTCH1* (pGK GFP shRNA NOTCH1) or the luciferase gene (pGK GFP shRNA LUC) used as control.

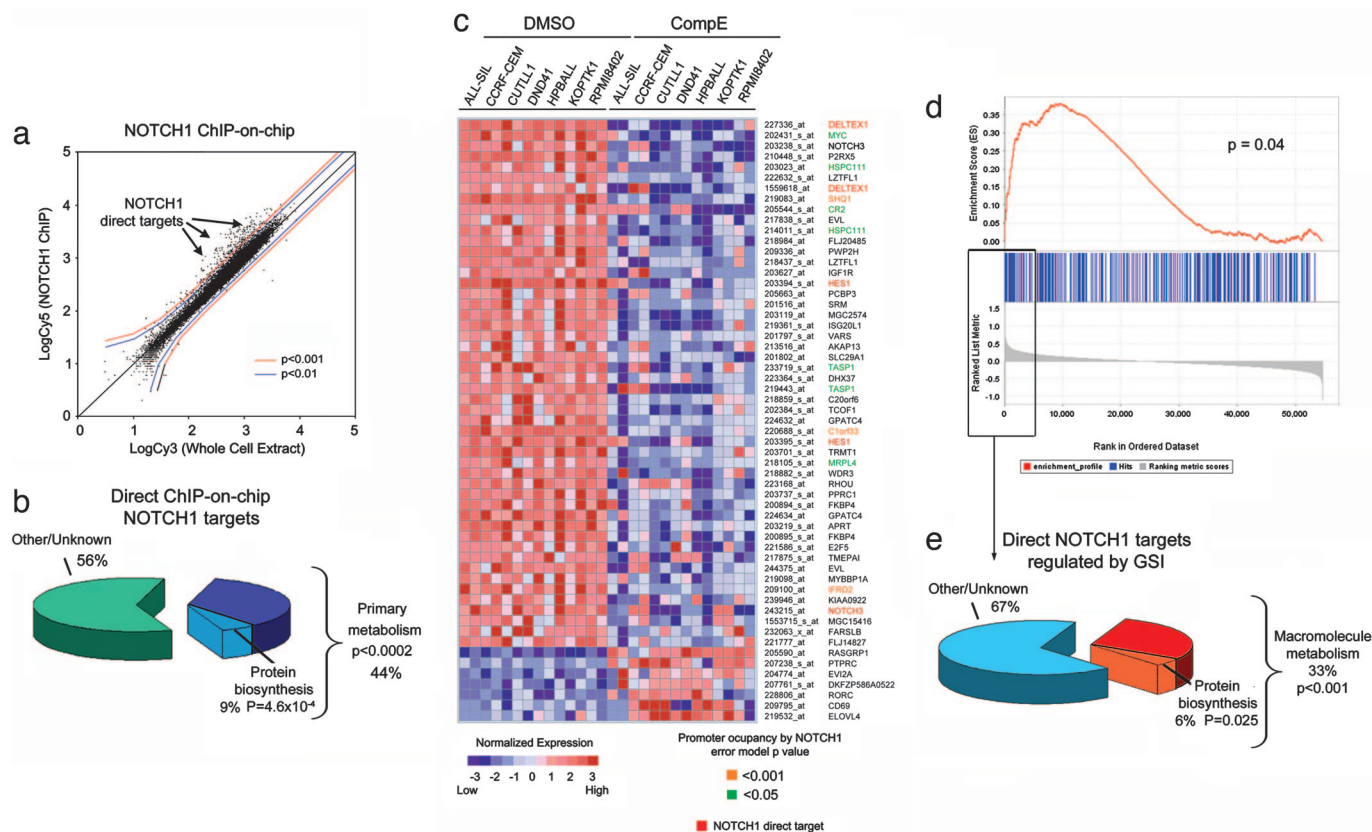


Fig. 3. NOTCH1 is a direct regulator of genes involved in cell growth and metabolism. (a) Scatter plot showing the combined results of three independent ChIP-on-chip experiments with the N1-TAD antibody recognizing the transactivation domain of NOTCH1 in HPB-ALL cells. The log fluorescence values for control DNA labeled with Cy3 are plotted on the x axis, and those for the NOTCH1 IP DNA labeled with Cy5 are plotted on the y axis. Promoter sequences bound by NOTCH1 (arrowheads) are shifted to the upper left side from the diagonal. (b) Functional classification of NOTCH1 direct targets identified by ChIP-on-chip ($P < 0.0001$). (c) Top gene expression changes induced by GSI in T-ALL cell lines ranked by signal-to-noise ratio. Heat map represents color-coded expression levels for each sample with respect to mock treatment controls. Previously known NOTCH1 direct target genes and genes identified on ChIP-on-chip analysis are highlighted. (d) Graphic representation of the GSEA enrichment score and distribution of NOTCH1 occupied promoter genes (binding P value < 0.0001) along the rank of GSI-regulated transcripts. (e) Annotation of NOTCH1 direct target genes along the top GSI-regulated transcripts ranked by signal-to-noise ratio.

undertook the identification of NOTCH1 direct target genes by ChIP-on-chip analysis using a spotted promoter array platform. In this experiment, triplicate chromatin immunoprecipitations using an antibody against the transactivating domain of NOTCH1 (N1-TAD) were performed in the HPB-ALL T-ALL cell line and hybridized to HU19K promoter arrays. The HU19K genomic array platform contains 13,000 human proximal promoter sequences, typically located between -700 to $+200$ bp relative to the transcription initiation site, plus sequences encompassing the promoters of all human transcription factor genes up to -3 kb from the transcription initiation site (10, 11). This analysis identified highly significant NOTCH1 binding ($P < 0.0001$) to the promoter regions of 134 genes (Fig. 3a) (Table 1, which is published as supporting information on the PNAS web site). Importantly, quantitative ChIP analysis of a set of 12 randomly selected genes among these putative NOTCH1 targets, confirmed NOTCH1 binding in 10 of them (83%) (Table 2, which is published as supporting information on the PNAS web site). In agreement with these results, analysis of the proximal promoters of genes identified as direct NOTCH1 targets by ChIP-on-chip demonstrated a highly significant overrepresentation of CSL DNA binding sequences (TGGGAA) compared with randomly selected nonoccupied promoter sequences ($P = 1.10 \times 10^{-13}$). Functional annotation of NOTCH1-bound promoters revealed that genes involved in primary cellular metabolism were markedly overrepresented (44%, $P < 0.002$) (Fig. 3b), further suggesting that NOTCH1 signaling directly activates downstream transcriptional programs that regulate cell growth. How-

ever, promoter occupancy by transcription factors is variably associated with regulation of gene expression.

To demonstrate that promoter occupancy by NOTCH1 drives transcription of the affected genes, we combined our ChIP-on-chip results with gene expression profiling data on T-ALL cell lines treated with GSI. This analysis revealed a significant overlap between expression data and ChIP-on-chip targets (47% of ChIP-on-chip targets with binding P value < 0.001 are regulated with expression P values < 0.0001 , Fisher's exact test $P = 9 \times 10^{-11}$) (Fig. 3c and Figs. 9 and 10, which are published as supporting information on the PNAS web site). Conversely, we observed significant enrichment of genes directly targeted by NOTCH1 in the top rank of sequences down-regulated by γ -secretase inhibition ($P = 0.04$) (Fig. 3d). The high frequency of NOTCH1 direct targets among the top-ranking genes regulated by GSI (Fig. 3d) demonstrates a close relationship between NOTCH1 signaling inhibition and the transcriptional changes induced by GSI treatment. In addition, functional annotation of these top-ranked direct targets of NOTCH1 regulated by GSI revealed a marked overrepresentation of genes involved in macromolecule metabolism (33%, $P = 0.0009$) and protein biosynthesis ($P = 5.5 \times 10^{-6}$) (Fig. 3e) in agreement with an important role of NOTCH1 in the control of cell growth.

NOTCH1 Directly Regulates c-MYC Expression. Remarkably, the c-MYC oncogene, whose product is a master regulator of cell growth, showed significant binding by NOTCH1 in our ChIP-on-chip analysis ($P = 0.037$) and was ranked among the top tier of genes

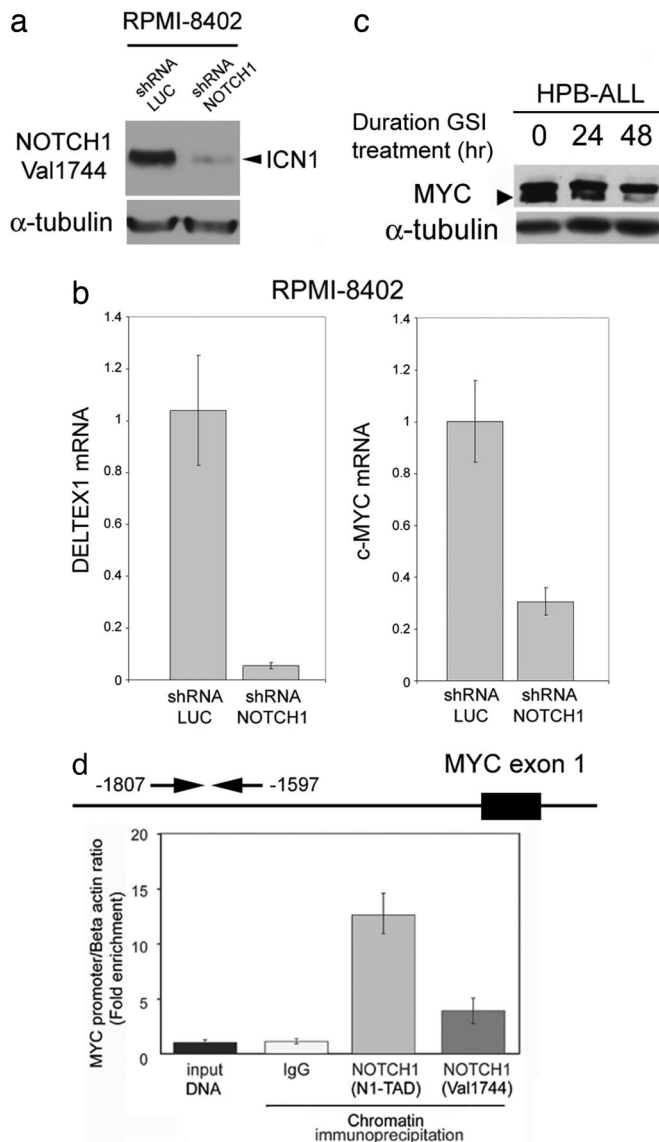


Fig. 4. NOTCH1 binds to *c-MYC* proximal promoter sequences and regulates *c-MYC* mRNA and protein expression in T-ALL. (a) Western blot analysis of ICN1 levels in RPMI 8402 T-ALL cells infected with lentiviruses (pLKO puro) driving expression of shRNAs targeting *NOTCH1* (shRNA NOTCH1) or control shRNAs targeting the luciferase gene (shRNA LUC). (b) Quantitative RT-PCR analysis of *DELTEX1* and *c-MYC* transcript levels in RPMI 8402 T-ALL cells upon NOTCH1 shRNA knockdown and in control shRNA-treated cells. (c) Western blot analysis of *c-MYC* in HPB-ALL T-ALL cells showing down-regulation of *c-MYC* expression upon NOTCH1 inhibition with GSI (ComP 500 nM). The arrowhead indicates the *c-MYC*-specific band. (d) ChIP analysis of NOTCH1 binding to *c-MYC* promoter sequences. Quantitative PCR analysis of *c-MYC* promoter sequences normalized to β -actin levels in control DNA and chromatin immunoprecipitates performed with two NOTCH1 antibodies (N1-TAD and Val 1744) and IgG as control in HPB-ALL cells.

down-regulated by GSI treatment in T-ALL, next to the NOTCH1 direct target gene *DELTEX1* (Fig. 3c). Furthermore, shRNA knockdown of *NOTCH1* induced down-regulation of *c-MYC* expression (Fig. 4a) and chromatin immunoprecipitation with two independent antibodies against NOTCH1 demonstrated direct NOTCH1 binding to the *c-MYC* promoter (Fig. 4b). These results are in agreement with those of Satoh *et al.* (12), who demonstrated the presence of regulatory elements controlled by *Notch1* in the mouse *c-Myc* promoter, and with recent results by Klinakis *et al.* (13) and Weng *et al.* (14) showing an interaction between *Notch1*

and *c-Myc* in mouse breast tumors and leukemia, respectively. Moreover, Western blot analysis showed a gradual reduction of *c-MYC* protein levels in T-ALL cells after γ -secretase inhibition (Fig. 4c). These findings suggest that NOTCH1 signaling drives a *c-MYC* mediated transcriptional feed-forward loop promoting cellular growth and metabolism (Fig. 5a). However, the slow kinetics of *c-MYC* down-regulation upon GSI treatment suggests that NOTCH1 accounts only for a fraction of *c-MYC* expression in T-ALL cells and are consistent with the delayed (3–6 days) occurrence of reduced cell size and cell cycle arrest in T-ALL cells treated with GSIs (Figs. 2 and 8) (5, 14, 15).

To test this hypothesis we analyzed the overlap between the transcriptional effects of NOTCH1 and a signature of *c-MYC*-regulated genes generated by microarray analysis of T cell lymphoblasts expressing this transcription factor oncogene (16). Gene set enrichment analysis (GSEA) can be used to test the enrichment of a set of features (*c-MYC*-regulated genes) in a list of genes ranked based on the closeness of the association of their expression changes with a particular group of samples (GSI-treated samples). This analysis demonstrated that genes regulated by forced expression of *c-MYC* in T cell lymphoblasts, including multiple biochemically validated *c-MYC* targets, were highly enriched among the top ranking genes regulated by GSI treatment ($P = 0.0001$) (Fig. 5b); underscoring the close functional relationship between NOTCH1 signaling and *c-MYC* regulation.

Based on these results we postulated that forced expression of *c-MYC* in T-ALL cells treated with GSI should restore, at least in part, the cell growth transcriptional program activated by oncogenic NOTCH1. To test this hypothesis we analyzed the effects of GSI treatment in DND41 cells infected with a *c-MYC* expressing retrovirus or GFP controls. Our results demonstrate that forced expression of *c-MYC* can effectively abrogate the reduction in cell size (Fig. 5c) typically induced by NOTCH1 inactivation in this T-ALL line (Fig. 2).

Reverse Engineering of Transcriptional Networks in T-ALL. To further interrogate the transcriptional effects of NOTCH1 and *c-MYC* in T-ALL, we performed reverse engineering of microarray expression data to generate an ARACNe (Algorithm for the Reconstruction of Accurate Cellular Networks) global transcriptional network using the mutual information (MI) algorithm. This analysis establishes the relationship between different genes based on their pattern of expression across multiple experimental conditions and has previously been shown to accurately reproduce the complex transcription interactions regulating cell homeostasis in mammalian cells (17). Analysis of the *c-MYC* hub demonstrated that numerous *c-MYC* first neighbor genes in our T-ALL network were identical to previously reported *c-MYC*-regulated genes, consistent with previous results from ARACNe analysis in B-cell lymphomas (17) (Table 3, which is published as supporting information on the PNAS web site). Because reconstruction of NOTCH1-controlled transcriptional circuits depends on the levels of activated NOTCH1 protein rather than *NOTCH1* transcript levels, we measured activated NOTCH1 levels by Western blot analysis in T-ALL cell lines. This information was used to build a pattern of gene expression or metagene from microarray expression data to serve as a surrogate for activated NOTCH1 protein levels in the analysis of global transcriptional networks. Integration of this NOTCH1 metagene into an ARACNe transcriptional network, constructed with data from 103 microarray measurements (99 primary T-ALLs, 3 T-ALL cell lines, 1 normal thymus) identified 58 genes that were directly connected to this surrogate signature of activated NOTCH1 signaling (i.e., inferred first-neighbors of NOTCH1) (Fig. 6a). Strikingly, NOTCH1 first-neighbor genes were significantly enriched in the rank of γ -secretase-regulated genes by GSEA ($P < 0.004$) and included 11 NOTCH1 direct target genes identified by ChIP-on-chip ($P = 0.0059$) (Fig. 6b and c). Functional annotation of these NOTCH1-associated genes revealed a marked overrepresentation

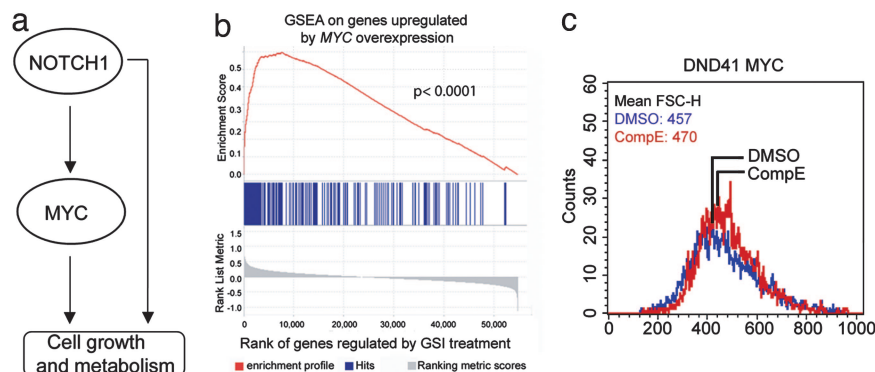


Fig. 5. NOTCH and *c-MYC* constitute a feed-forward regulatory network motif controlling cell growth. (a) Structure of the NOTCH1 feed-forward-loop regulatory motif controlling *c-MYC* and cell growth genes. (b) NOTCH1 inhibition with GSI in T-ALL cells switches off genes up-regulated by forced expression of *c-MYC* in T cell lymphoblasts. Graphic representation of the GSEA enrichment score and distribution of *c-MYC*-regulated genes along the rank of GSI-regulated transcripts. (c) Retroviral expression of *c-MYC* in DND41 rescues cell growth after GSI treatment. Cell size was estimated by flow cytometry in DND41 cells infected with pBMM *c-MYC* IRES GFP retrovirus growing in the presence of CompE or vehicle (DMSO) for 4 days.

of genes involved in cellular and protein biosynthesis (48%, $P = 8.56 \times 10^{-18}$ and 38%, $P = 8.13 \times 10^{-17}$, respectively). Additionally, ARACNe analysis showed a close relationship between the NOTCH1 metagene and *c-MYC* ($MI = 0.2692$, $P < 2 \times 10^{-7}$). Importantly, the direct association between the NOTCH1 metagene and *c-MYC* is filtered in the graphic representation of the NOTCH1 and *c-MYC* transcriptional hubs, because 12 of the transcripts directly connected with both the NOTCH1 metagene and *c-MYC* (Fig. 6d). Indeed, 9 of the 12 genes comprising this common interface encoded components of primary metabolic pathways involved in cell growth ($P = 0.009$), such as ribosomal proteins and chaperones. The strong connectivity between the NOTCH1 metagene and *c-MYC*, as well as the enrichment for common neighbors of NOTCH1 and *c-MYC* in our ARACNe network ($P = 2.35 \times 10^{-52}$), is in agreement with a feed-forward-loop transcriptional network motif and further supports the highly synergistic role of these two transcription factor oncogenes in the regulation of thymocyte cell growth.

Discussion

Oncogenic transcription factors promote tumor formation by activating transcriptional networks that link coregulated genes

important in cell homeostasis. Thus, aberrant cell proliferation, increased survival and increased cell mass, induced by the dysregulation of metabolic pathways during malignant transformation, constitute mainstay characteristics of tumor cells (18).

The canonical NOTCH1 signaling pathway is thought to operate as a signal transduction pipeline that transmits information from developmentally important extracellular signals from the cell surface directly to the nucleus. NOTCH1 signaling plays essential roles in the thymus as regulator of T cell lineage commitment and thymocyte development. Furthermore, constitutive activation of NOTCH1 due to chromosomal translocations or mutations is strongly oncogenic (4, 5). However, the transcriptional programs through which NOTCH1 exerts its potent effects on thymocyte homeostasis and T cell transformation have not been established.

Using a combination of gene expression profiling and ChIP-on-chip analysis we have demonstrated that NOTCH1 is a direct regulator of biosynthesis pathways controlling cell growth and metabolism. Furthermore, pharmacologic inhibition of NOTCH1 signaling at the membrane with GSIs or by shRNA knockdown impairs the growth of T cell lymphoblastic leukemia cells. Importantly, this analysis also identified *c-MYC*, a master regulator of

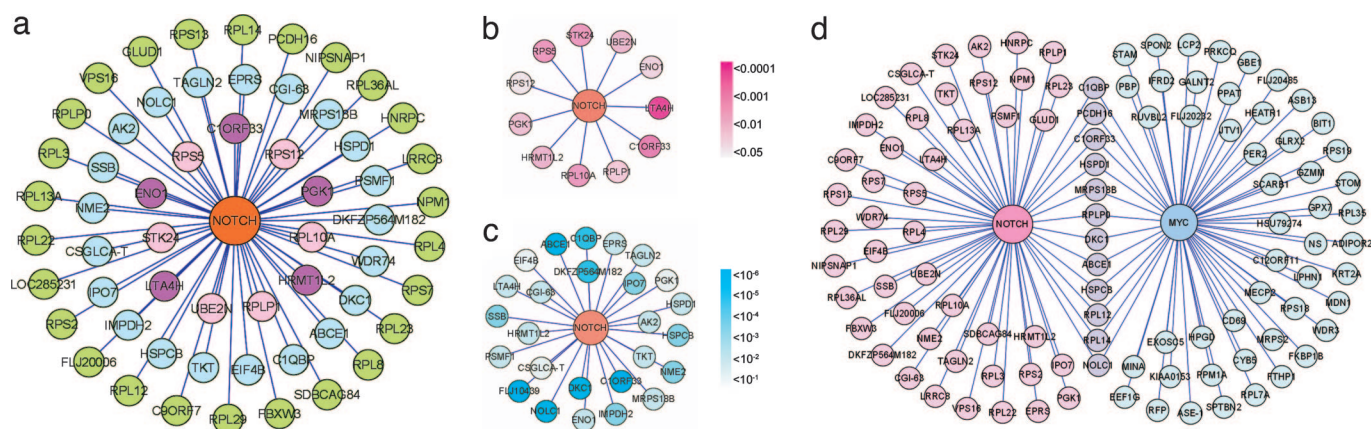


Fig. 6. Activated NOTCH1 and *c-MYC* regulatory network in T-ALL. (a) A metagene based on the gene expression signature associated with levels of activated NOTCH1 protein in T-ALL cell lines was integrated in an ARACNe global regulatory network constructed with microarray expression data from T-ALL samples. Neighbors of the NOTCH1 metagene identified as NOTCH1 direct target genes by ChIP-on-chip are shaded in pink, neighbors regulated in T-ALL cells treated with a GSI are shown shaded in blue, and neighbor genes showing both significant promoter occupancy by ChIP-on-chip and regulation upon GSI treatment are shaded in purple. (b) Detailed representation of overlap between NOTCH1 neighbor genes and ChIP-on-chip data. The intensity of each neighboring node represents the significance level of promoter occupancy by NOTCH1. (c) Detailed representation of NOTCH1 neighbor genes regulated in T-ALL cells treated with a GSI. The intensity of each neighboring node, corresponding to the scale panel on the right, represents the significance level of gene regulation by GSI treatment. (d) Representation of direct neighbor genes associated with the NOTCH1 metagene and with *c-MYC*.

multiple biosynthesis and metabolic pathways (19), as a direct target gene regulated by NOTCH1. Reverse engineering of transcriptional networks from microarray expression data demonstrated that NOTCH1 signaling and *c-MYC* are closely related and share numerous target genes. These results demonstrate that the interaction between NOTCH1 and *c-MYC* composes a feed-forward-loop transcriptional regulatory motif regulating leukemic cell growth (Fig. 11, which is published as supporting information on the PNAS web site). In agreement with this model, NOTCH and *c-MYC*-regulated genes were broadly overlapping and forced expression of *c-MYC* was able to rescue the deleterious effects of NOTCH1 inhibition by GSI treatment on the growth of T-ALL cells.

The identification of NOTCH1 as direct regulator of cell growth genes highlights the role of this transcription factor in T cell transformation and provides a mechanism that couples cell metabolism and cell cycle progression with developmental decisions in lymphocyte development. Overall, our identification of *c-MYC* as a direct target gene regulated by NOTCH1 in T-ALL highlights the importance of this interaction in the pathogenesis of human cancer. We predict that the intensity of input signals that enter the NOTCH1-MYC feed-forward regulatory circuitry upstream of *c-MYC* may tune the cell growth response to NOTCH1 activation and may potentially enhance or attenuate the antitumor effect of drugs targeting NOTCH1 signaling. Finally, the expression data and ChIP-on-chip analysis reported here show that NOTCH1 regulates multiple genes in important developmental signaling pathways and transcriptional networks. Further studies are warranted to test the significance of these interactions in T cell development and transformation.

Materials and Methods

DNA Microarray Analysis. Samples for microarray analysis were prepared according to the manufacturer's instructions and as described (17).

Significant changes in gene expression associated with GSI treatment were estimated by using one-sample *t* test with 13 degrees of freedom after normalization of expression levels with respect to mock-treated controls.

Functional annotation based on the Gene Ontology classification was performed with the DAVID tool (20).

GSEA was performed on normalized expression data as described (21).

An ARACNe mutual information network (17) was built based on a large panel of data from primary human T-ALL samples ($n = 99$), cell lines ($n = 3$), and normal thymus ($n = 1$) using Human U133A GeneChip data (22) after GC-RMA array normalization. A

metagene entry proportional to the concentration of activated NOTCH1 protein in the nucleus was constructed by using stepwise regression from quantitative measurements of ICN1 protein level in T-ALL cell lines, was integrated into the microarray gene expression profile measurements, and was used to build an ARACNe network for the NOTCH1 signaling hub (23).

ChIP-on-Chip Analysis. Complete protocols are available for download in pdf format at <http://web.wi.mit.edu/young/NOTCH1>. Typically, 5×10^7 to 1×10^8 HPB-ALL were used per chromatin immunoprecipitation.

Quantitative ChIP Analysis. Relative quantitation by real-time PCR of promoter sequences were normalized to β -actin levels in chromatin immunoprecipitates performed with antibodies against NOTCH1 or IgG.

Western Blot Analysis. Antibodies against activated NOTCH1 (Val 1744, Cell Signaling) *c-MYC* (N-262, Santa Cruz Biotechnology) and α -tubulin (TU-02, Santa Cruz Biotechnology) were used according to standard procedures. Activated NOTCH1 protein levels relative to tubulin expression were calculated by using infrared detection with the Odyssey detector (LI-COR Biosciences).

Cell Size Assays. Changes in cell size induced by GSI treatment were monitored by flow cytometry in asynchronous cell populations and in cells in G_1 phase of the cell cycle (2N DNA content by Draq5 staining).

shRNA Knockdown. Oligo sequences for shRNAs targeting NOTCH1 (9) or the luciferase gene were cloned in the pLKO-puro and pGK-GFP lentiviral vectors (gift from William Hahn of the Dana-Farber Cancer Institute). Lentivirus production and infections were performed as described (24).

A detailed description of ChIP-on-chip, expression and network analysis is provided in *Supporting Methods*, which is published as supporting information on the PNAS web site.

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