

NOTCH1–RBPJ complexes drive target gene expression through dynamic interactions with superenhancers

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The main oncogenic driver in T-lymphoblastic leukemia is NOTCH1, which activates genes by forming chromatin-associated Notch transcription complexes. Gamma-secretase-inhibitor treatment prevents NOTCH1 nuclear localization, but most genes with NOTCH1-binding sites are insensitive to gamma-secretase inhibitors. Here, we demonstrate that fewer than 10% of NOTCH1-binding sites show dynamic changes in NOTCH1 occupancy when T-lymphoblastic leukemia cells are toggled between the Notch-on and -off states with gamma-secretase inhibitors. Dynamic NOTCH1 sites are functional, being highly associated with Notch target genes, are located mainly in distal enhancers, and frequently overlap with RUNX1 binding. In line with the latter association, we show that expression of *IL7R*, a gene with key roles in normal T-cell development and in T-lymphoblastic leukemia, is coordinately regulated by Runx factors and dynamic NOTCH1 binding to distal enhancers. Like *IL7R*, most Notch target genes and associated dynamic NOTCH1-binding sites cooccupy chromatin domains defined by constitutive binding of CCCTC binding factor, which appears to restrict the regulatory potential of dynamic NOTCH1 sites. More remarkably, the majority of dynamic NOTCH1 sites lie in superenhancers, distal elements with exceptionally broad and high levels of H3K27ac. Changes in Notch occupancy produces dynamic alterations in H3K27ac levels across the entire breadth of superenhancers and in the promoters of Notch target genes. These findings link regulation of superenhancer function to NOTCH1, a master regulatory factor and potent oncoprotein in the context of immature T cells, and delineate a generally applicable roadmap for identifying functional Notch sites in cellular genomes.

gene regulation | Notch signaling

Notch signaling has a critical developmental role in metazoan animals, and its dysregulation underlies several human developmental disorders and certain cancers such as T-lymphoblastic leukemia (T-LL), in which *NOTCH1* gain-of-function mutations occur in ~60% of cases (1). Physiological and pathological Notch receptor activities are largely mediated by a canonical signaling pathway, through which Notch directly regulates the expression of downstream target genes (for recent review, see ref. 2). Normally, Notch receptors are activated by binding of ligands of the delta-serrate-lag2 family to the Notch ectodomain, an event that renders the juxtamembrane region of Notch susceptible to successive cleavages by ADAM metalloproteases and γ -secretase. The latter cleavage releases the Notch intracellular domain (NICD) from the membrane, allowing it to translocate to the nucleus and form a Notch transcription complex (NTC) with the DNA-binding protein RBPJ and coactivators of the mastermind-like (MAML) family. MAML interacts with the histone acetyltransferase p300 and is essential for activation of transcription (3, 4), and other work suggests that the NOTCH1 NICD also interacts with the CH3 domain of p300 (5). Recent studies have shown that p300 acetylates H3K18 and H3K27 (6),

and that the H3K27ac mark is characteristic of active enhancers and correlates with transcription activation (6–9).

In cancers such as T-LL, gain-of-function mutations in *NOTCH1* cause excessive Notch activation and exaggerated expression of oncogenic target genes. To further elucidate how NOTCH1 regulates the transcriptomes of T-LL cells, we recently used chromatin immunoprecipitation (ChIP)-Seq to identify RBPJ–NOTCH1-binding sites genomewide in Notch-“addicted” murine and human T-LL cell lines (10). Unexpectedly, we observed that although RBPJ–NOTCH1-binding sites were mainly located within gene promoters (defined as sites <2 kb from a transcriptional start site; TSS), most of these genes did not respond to perturbations of Notch signaling. Conversely, the majority of direct NOTCH1 target genes lacked NOTCH1 binding to their promoters, suggesting that transcriptional response to Notch in T-LL cells is largely mediated through long-range enhancers. Thus, these studies left unresolved both the identity and characteristics of the subset of RBPJ–NOTCH1-binding sites that regulate transcription.

To address this question, we reasoned that functional RBPJ–NOTCH1 genomic-binding sites would be marked by dynamic changes in NOTCH1 occupancy following perturbations of Notch signaling. Here, we use this approach to identify and characterize functional NOTCH1-binding sites genomewide in T-LL cells.

Significance

Studies focused on understanding how transcription factors control gene expression have shown that transcription-factor binding sites generally greatly exceed the number of regulated genes, making it challenging to identify functional binding sites. Using Notch pathway inhibitors, we identified a subset of Notch-binding sites in leukemia cell genomes that are dynamic, changing in occupancy relatively rapidly when Notch signaling is perturbed. Dynamic Notch sites are highly associated with genes that are directly regulated by Notch and mainly lie in large regulatory switches termed superenhancers, which control genes with key roles in development and cancer. This work links Notch signaling to superenhancers and suggests that assessment of transcription factor–genome dynamics can help to identify functionally important regulatory sites.

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Notably, functional sites constitute only a minor subset of NOTCH1-binding sites and are mainly located in distal enhancers, many of which appear to correspond to recently described “superenhancers” (11–14).

Results

Overlap of NOTCH1–RBPJ and ETS1, GABPA, and RUNX1 Binding to the Genomes of Human T-LL Cells. We noted previously that DNA sequences within 500 base pairs of NOTCH1–RBPJ-binding sites in human T-LL cell lines were highly enriched in binding motifs for ETS (E26-transformation specific) factors, RUNX factors, and the zinc finger transcription factor ZNF143 (10). Like NOTCH1, ETS and RUNX factors are required for normal T-cell development (15–18), suggesting that these factors coordinately regulate the transcriptomes of T-cell progenitors. To confirm the associations suggested by motif analysis, we performed steady-state ChIP-Seq for RUNX1 and two ETS family members, ETS1 and GABPA, in the Notch-addicted human T-LL cell line CUTLL1 (19). We analyzed these data as well as prior steady-state ChIP-Seq data for RBPJ, NOTCH1, ZNF143, and the chromatin marks H3K4me1, H3K4me3, and H3K27me3 (summarized in Table S1). As shown in Fig. S1A–C, NOTCH1- and RBPJ-binding sites are highly associated with ZNF143-, ETS1-, GABPA-, and RUNX1-binding sites and are mainly located in promoters. NOTCH1–RBPJ-binding sites are also associated with “activating” chromatin marks (H3K4me1 and H3K4me3) and devoid of repressive H3K27me3 marks (Fig. S1A), consistent with the role of NOTCH1–RBPJ complexes as transcriptional activators. An illustrative example of the overlap among these factors and histone marks can be seen in the chromatin landscapes near *NOTCH3*, a direct NOTCH1 target gene (Fig. S1D).

Dynamic Notch-Binding Sites Are Mainly Located in Distal Enhancers and Are Enriched for RUNX1 Binding. To test the idea that the sensitivity of individual genes to Notch stems from dynamic association of Notch–RBPJ complexes with genomic regulatory elements, we used a method originally described by our group (20) and since exploited by others (21) that relies on reversible inhibition of Notch signaling by gamma-secretase inhibitors (GSI). T-LL cells treated with GSI become depleted of NICD1 and accumulate a pool of membrane-tethered NOTCH1 polypeptides that have undergone ADAM–metalloprotease cleavage. Upon washout of GSI, this pool of partially processed receptors is rapidly cleaved by gamma-secretase, allowing for precisely timed NOTCH1 activation. To study loading of NTCs on regula-

tory elements in T-LL genomes, we performed ChIP-Seq for RBPJ and NOTCH1 (*i*) after treatment with GSI for 72 h and (*ii*) 4 h after GSI washout. Using a linear model to estimate fold-change and a false discovery rate (FDR) of 0.05, we identified 1,012 peaks with significantly increased NOTCH1 occupancy following GSI washout (Fig. 1A and Fig. S2A), hereafter referred to as “dynamic NOTCH1 sites,” which constitute fewer than 10% of NOTCH1 binding sites identified in the “Notch-on” steady-state condition. We used cutoffs based on the bimodal distribution of all 13,986 NOTCH1 peaks (Fig. S2B) to define dynamic and nondynamic sites as being either proximal promoter binding sites (<2 kb from a TSS) or distal enhancer binding sites (>2 kb from a TSS). It is interesting to note that although 57% of the 13,986 NOTCH1 binding sites are located in proximal promoter regions, roughly 90% of the 1,012 dynamic binding sites lie outside of promoters within putative distal enhancers (Fig. 1B and Fig. S2C).

We then used the same strategy to investigate RBPJ loading onto dynamic NOTCH1 sites. We noted a strong association between NOTCH1 and RBPJ binding to dynamic sites (Fig. 1C), an observation consistent with prior data in fly (22) and mammalian cells (10), suggesting that Notch enhances RBPJ binding to DNA. In contrast to steady-state associations, we found that chromatin proximal to dynamic NOTCH1 sites is enriched for strong RUNX1 binding (55% overlap), and for pervasive (56% overlap), but weaker, ETS1 binding, compared with ZNF143 and GABPA binding (Fig. 1C). This association is in line with motif enrichment analysis performed on genomic sequences within 600 base pairs of the summit of dynamic NOTCH1-binding sites, which revealed that the most highly enriched motifs after RBPJ are those for RUNX factors, followed by E-box-binding factors (Fig. S2D). In line with their predominant nonpromoter location, dynamic NOTCH1 sites are also strongly associated with H3K4me1 marks, which have a significantly broader distribution around dynamic NOTCH1 sites than around non-dynamic, nonpromoter NOTCH1 sites (Fig. S2E).

***IL7R* Is Coregulated by NOTCH1 and RUNX1 Through 3' Enhancer Elements.** Enrichment of RUNX1 binding near NOTCH–RBPJ dynamic sites suggested that RUNX1 and NOTCH1 might coregulate the expression of certain genes. We studied the functional importance of RUNX1 in human T-LL cells by knocking down RUNX1 and by expressing a dominant negative Runx factor, RUNT. Like GSI and dominant negative MAML1 (DN-MAML, a specific inhibitor of NTCs), knockdown of *RUNX1* and expression of RUNT both inhibit T-LL cell growth (Fig. S3A–C).

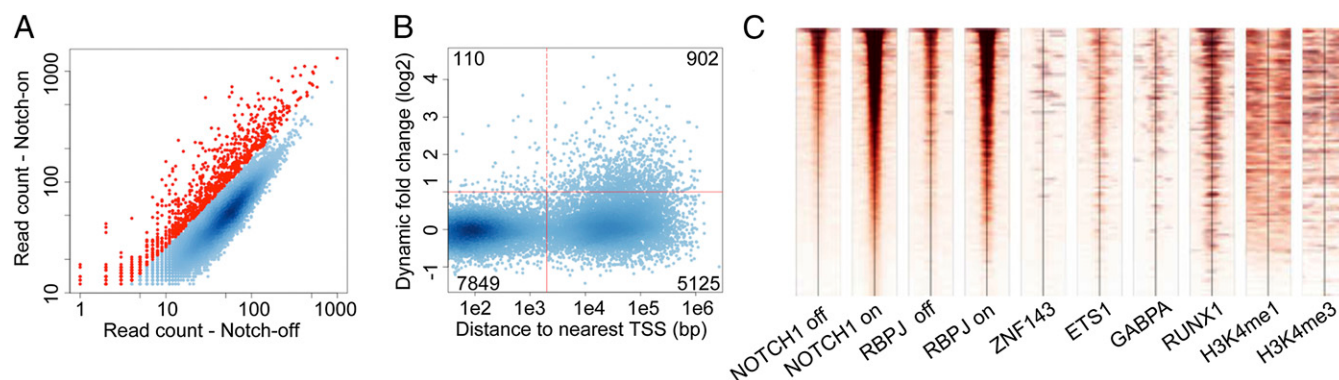


Fig. 1. NOTCH1 activation reveals dynamic NOTCH1–RBPJ-binding sites. (A) Scatterplot of NOTCH1 ChIP-seq read counts. Each dot represents a NOTCH1 peak identified in CUTLL1 T-LL cells following GSI washout. ChIP-seq reads within 600 bp of a peak summit were counted in the Notch-on and Notch-off states. Red dots indicate dynamic sites. (B) Classes of NOTCH1-binding sites defined by genomic location and dynamism. Each dot is a NOTCH1 peak plotted according to its distance to the nearest gene’s transcriptional start site (TSS) and its signal-fold change from the Notch-off to the Notch-on states. The red vertical line separates proximal promoters and distal enhancers, and the red horizontal line corresponds to a signal-fold-change threshold with an FDR < 0.05. *Inset* numbers correspond to peaks found in each quadrant. (C) Heat map of dynamic NOTCH1 sites, ranked by ChIP-Seq signal intensity, and associated transcription factor and histone mark signals across a 1-kb window centered on NOTCH1-binding summits.

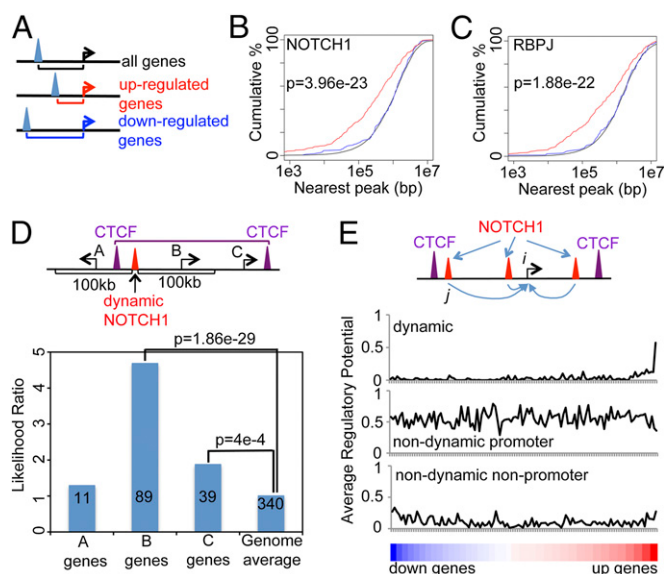


Fig. 3. Target gene activation through dynamic NOTCH1-binding sites. (A–C) Dynamic NOTCH1–RBPJ-binding sites are preferentially located near genes that are up-regulated by Notch. The distance from the nearest binding site to the TSS of each gene was recorded (A) and cumulative distributions of 340 up-regulated genes (red), 187 down-regulated genes (blue), and the genomic background (black) were plotted for dynamic NOTCH1 (B) and dynamic RBPJ sites (C). P values were calculated by the Kolmogorov–Smirnov test. (D) Constitutive CTCF-binding sites define domains that restrict NOTCH1 regulation of nearby genes. Genes near dynamic NOTCH1 sites were classified into three categories: A, within 100 kb but in a different CTCF domain; B, within 100 kb and in the same CTCF domain; C, more than 100 kb away and in the same CTCF domain. The ratio between the likelihood of finding an activated gene in each category and the likelihood of finding an activated gene randomly are shown in the bar plot; numbers in each bar correspond to the activated genes in each category. P values were calculated by Fisher exact test. (E) Relationships between regulatory potentials and Notch-dependent changes in gene expression. The schematic shows how regulatory potential is calculated (see *Materials and Methods* for details). The lower panels show relationships between regulatory potentials of NOTCH1-binding sites and differential expression of genes (see *Supporting Information* for details).

was calculated for dynamic sites, nondynamic promoter sites, and nondynamic nonpromoter sites (Fig. 3E). We found that the genes that are up-regulated upon NOTCH activation are highly enriched for those with high dynamic NOTCH1 regulatory potentials. Of note, although thousands of genes have nondynamic NOTCH1-binding sites in their promoter regions that yield a high average regulatory potential, there is no correlation between the calculated regulatory potential of these genes and actual changes in gene expression following perturbation of Notch signaling.

These results suggest that the calculated dynamic regulatory potential can help to identify direct Notch target genes, particularly those that are under the control of distal enhancer elements. In line with this idea, 87 genes with high dynamic regulatory potential were up-regulated following GSI washout (Table S2). This list includes many previously identified putative direct NOTCH1 target genes, most of which appear to be regulated in part or in whole by distal-enhancer elements.

Dynamic Notch Binding Activates Target Genes Through Interactions with H3K27ac-Marked Superenhancers. We further investigated how dynamic NOTCH1 sites induce transcriptional activation by studying histone H3K27 acetylation (H3K27ac), an active enhancer mark, under steady-state, Notch-off, and Notch-on conditions using the GSI washout strategy. We noted that the H3K27ac landscapes around dynamic NOTCH1 sites near Notch target genes are remarkably sensitive to alterations in Notch signaling. For ex-

ample, five dynamic NOTCH1 sites located 5' of the Notch-regulated ankyrin repeat protein (*NRARP*) gene body lie within a 20-kb H3K27ac peak that shows striking Notch-dependent changes across its full breadth (Fig. 4A). Similarly, the three dynamic NOTCH1 sites located 5' of the *HES5* gene body are found within a >10-kb H3K27ac peak that also shows large Notch-dependent changes (Fig. S5A) that are accompanied by marked changes in *HES5* expression (Fig. S5B). The wide breadth of H3K27ac peaks associated with these dynamic NOTCH1 sites earmarks these regions as superenhancers, recently described genomic elements linked to

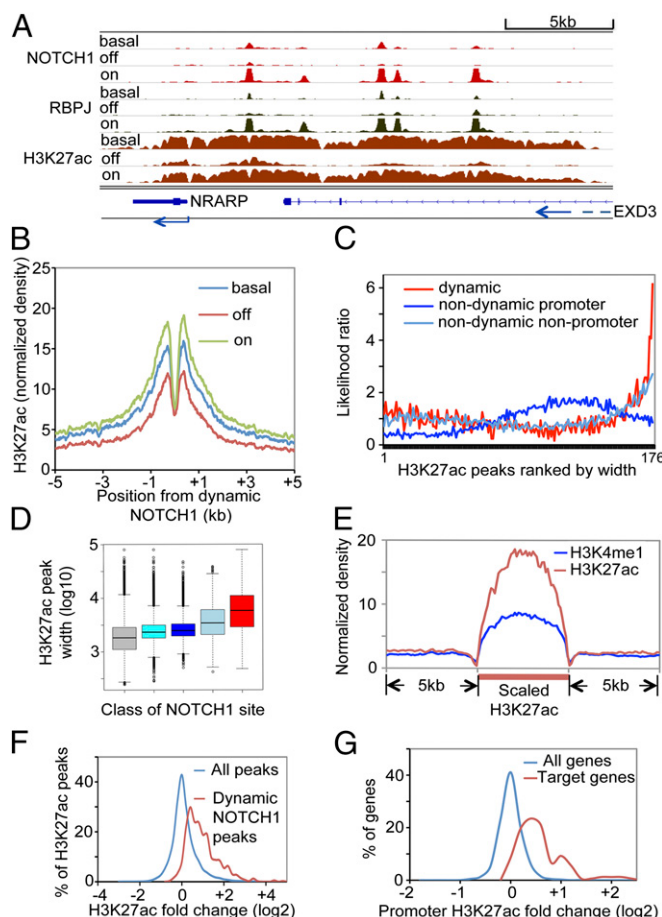


Fig. 4. Dynamic NOTCH1 sites are preferentially located within H3K27ac-marked superenhancers. (A) Chromatin landscapes near the NOTCH1 target gene *NRARP*. The flanking *EXD3* gene is not expressed in T-LL. (B) Composite profiles of H3K27ac flanking dynamic NOTCH1 sites under steady-state (basal), Notch-off, and Notch-on conditions. (C) Enrichment of dynamic NOTCH1 sites in the broadest H3K27ac peaks. Genomic H3K27ac peaks ($n = 35,244$) were ranked by peak width and grouped into 176 bins, each containing 200 peaks. H3K27ac peaks associated with dynamic or nondynamic NOTCH1 sites were counted in each bin, and the ratio of (i) the likelihood of NOTCH1 binding to H3K27ac peaks and (ii) the average likelihood NOTCH1 binding to all genomic H3K27ac peaks is plotted for each class of NOTCH1 sites. (D) Distribution of H3K27ac peak width for all H3K27ac peaks (gray), all promoter H3K27ac peaks (cyan), H3K27ac peaks associated with nondynamic promoter NOTCH1 (dark blue), nondynamic nonpromoter NOTCH1 (light blue), and dynamic NOTCH1 (red) is shown. (E) Composite profiles of H3K27ac and H3K4me1 on dynamic NOTCH1 associated H3K27ac peaks (see *Supporting Information* for details). (F) Fold-change distribution of H3K27ac peaks associated with dynamic NOTCH1 sites versus all H3K27ac peaks. Fold change is the ratio of H3K27ac level in the Notch-on and Notch-off states. (G) Fold-change distribution of promoter H3K27ac levels on NOTCH1 target genes and all genes. H3K27ac level is measured as the normalized read count within 2 kb of transcriptional start sites.

the regulation of genes that are involved in oncogenesis and differentiation (11, 12, 14).

Guided by these local observations, we next assessed the effect of Notch on H3K27ac peaks genomewide. We found that 83% (843 out of 1,012) of dynamic NOTCH1 sites overlap with H3K27ac peaks, and that H3K27ac levels flanking dynamic NOTCH1 sites are reduced by GSI treatment and restored by GSI washout (Fig. 4B and Fig. S5C); by contrast, H3K27ac levels near nondynamic NOTCH1 sites are insensitive to short-term treatment with GSI and subsequent GSI washout (Fig. S5D). Moreover, H3K27ac peaks associated with dynamic NOTCH1 sites are much more likely than nondynamic sites to be located in the widest H3K27ac peaks in the genome (Fig. 4C), and as a result are significantly broader than the genomewide average sizes of all H3K27ac peaks and H3K27ac peaks associated with nondynamic NOTCH1 sites (Fig. 4D). Overall, the median width of H3K27ac peaks associated with dynamic NOTCH1 sites is 5,863 bp, with 56% of peaks being over 5 kb in width, while the median width of all 35,244 H3K27ac peaks in the CUTLL cell genome is 1,830 bp, with only 9% being over 5 kb in width (Fig. S5E). The particularly large size of distal regulatory elements associated with dynamic NOTCH1 sites is also reflected in a broad distribution of the enhancer mark H3K4me1 around this subset of H3K27ac peaks (Fig. 4E), an association that was hinted at in Fig. S2E. Furthermore, changes in H3K27ac levels between Notch-off and Notch-on states are selectively observed near dynamic NOTCH1-binding sites (Fig. 4F), and increased H3K27ac near dynamic sites is accompanied by increased H3K27 acetylation of Notch target gene promoters (Fig. 4G), both of which are consistent with transcriptional regulatory function for the dynamic NOTCH1 sites.

To further characterize Notch-sensitive superenhancers, we performed ChIP-Seq for p300, a factor recruited by Notch transcription complexes (5) that acetylates H3K27 (6), as well as BRD4 and mediator (MED1), factors enriched in superenhancers (11, 12, 14). As anticipated, dynamic NOTCH1 sites were associated with p300 binding (Fig. S5F), and H3K27ac-marked NOTCH1-associated superenhancers were accompanied by broad regions of BRD4 and MED1 binding (Fig. S5G). The Notch-sensitive superenhancers near the Notch target genes *NRARP*, *HES5*, *DTX1*, and *IGF1R* (Fig. S6) illustrate that p300 tends to bind near dynamic NOTCH1-RBPJ sites, and that BRD4 and MED1 bind more broadly across entire superenhancer regions.

To extend the association between dynamic NOTCH1 binding and superenhancer function to other T-LL lines, we studied how changes in NOTCH1 activity affect the *DTX1* superenhancer in the T-LL cell lines KOPT-K1, DND41, and HPBALL, each of which have gain-of-function mutations involving *NOTCH1* (1). Similar to CUTLL cells, we observed that depletion of NOTCH1 with GSI results in loss of H3K27ac across the entire breadth of the *DTX1* superenhancer in all of these lines (Fig. S7). Taken together, these findings suggest that Notch-superenhancer interactions are of general importance in regulation of gene expression in Notch-addicted T-LL cells.

Discussion

ChIP-Seq has revealed in great detail where transcription factors bind in genomes, but factor-binding sites generally greatly outnumber regulated genes, and many functionally important binding sites lie in distal enhancers rather than promoters. These considerations make identification of binding sites that are directly involved in transcriptional regulation challenging. To overcome this limitation, we used small-molecule Notch-pathway inhibitors to toggle between the Notch-on and Notch-off states, and by doing so identified a relatively small subset of dynamic NOTCH1 sites in T-LL cells that are highly associated with Notch target genes. Our results underscore the importance of using transcription-factor perturbations to identify binding sites that govern dynamic changes in gene expression. Factors that de-

termine the dynamism (or lack thereof) of NOTCH1 sites in T-LL genomes remain to be established. It also remains to be determined whether functional roles different from acute regulation of direct Notch target genes exist for nondynamic NOTCH1 sites.

Roughly 90% of dynamic NOTCH1 sites in T-LL cells lie outside of gene promoters. One factor that is hypothesized to influence pairing of distal regulatory elements with promoters is their spatial relationship to constitutive CTCF-binding sites, which appear to organize the mammalian genome into functional chromatin domains (25–27). Using constitutive CTCF binding data from the ENCODE consortium, we identified a strong bias toward colocalization of dynamic NOTCH1 sites and high-confidence direct Notch target genes within the same CTCF domain. Our data, however, do not exclude the possibility that some genes are regulated by Notch responsive enhancers across CTCF boundaries; indeed, some Notch target genes (such as *MYC*) lack candidate enhancers, and some putative Notch responsive enhancers fall within CTCF domains without evident NOTCH target genes. This is similar to the current view of the CTCF interactome in embryonic stem cells, in which most CTCF-delineated chromatin domains are created by loops formed between loci on the same chromosome, but a significant minority involves pairs of loci on different chromosomes (26, 30). Studies using methods that provide an unbiased view of three-dimensional chromatin organization will likely be needed to elucidate how Notch regulates certain target genes, such as *MYC*.

Transcription is regulated by the interplay of transcription factors, chromatin regulators, and core components of the basal transcriptional machinery. A recent study showed that in T-LL cells the chromatin regulators LSD1, PHF8, AF4p12, and BRG1 associate with NTCs on regulatory elements such as the *DTX1* and *IL7R* enhancers, and are required for expression of at least a subset of NOTCH target genes (31). An additional finding of our work is the strong correlation between the occupancy of dynamic NOTCH1-binding sites and levels of H3K27 acetylation of Notch response elements and associated target gene promoters. Both NICD1 and MAML1 bind p300 (3–5), which carries out H3K27 acetylation (6), and in line with this we observe that p300 is recruited to dynamic NOTCH1 sites genomewide. Runx factors also recruit p300 to chromatin, and it is possible that optimal recruitment of p300 to Notch response elements such as the *IL7R* enhancers requires both Notch and Runx factors. Of note, Yatim et al. identified RUNX1 as a component of the NOTCH1 interactome in T-LL cells (31), raising the possibility that NOTCH1 and RUNX1 might physically contact each other on genomic response elements; however, dynamic NOTCH1 sites and nearby RUNX1 sites do not show any preferred spacing in T-LL genomes, making direct physical interaction unlikely as a general rule. Further work will be needed to define the basis and extent of Notch/Runx transcriptional interplay in T-LL cells and normal hematopoietic progenitors.

Perhaps the most striking observation emerging from this study is the association of functional Notch binding sites with superenhancers. Superenhancers are recently characterized “giant” regulatory switches that appear to have important roles in regulating the expression of genes that control lineage specification during development (14) and of oncogenes in transformed cells (11, 14). Our findings suggest that NOTCH1, an oncoprotein and master regulator of T-lineage specification, functions in large part in T-LL cells through dynamic binding to superenhancers. NOTCH1 unloading and reloading are associated with extensive and dramatic changes in the H3K27ac levels of superenhancers that are spatially associated with robust NOTCH1 target genes. It will be of interest to determine if the interaction of Notch with superenhancers noted in T-LL cells extends to other cellular contexts, and if so, whether superenhancers will prove to have a general role in integrating signaling inputs involving Notch and other pathways.

Materials and Methods

Cell Culture and GSI Washout Studies. Human T-LL CUTLL1, KOPT-K1, DND41, and HPBALL cells were cultured as previously reported (20). T-LL cells were treated with the GSI compound E (1 μ M) for 72 h to establish the Notch-off state, and Notch was then reactivated by washing out GSI, as described (20). Notch-on cells were harvested 4 h after GSI washout.

ChIP and Next-Generation Sequencing. ChIP-seq was performed as described (10). Antibody information is provided in the [SI Materials and Methods](#). ChIP-seq data have been deposited in the Gene Expression Omnibus (accession number GSE51800). Primer sequences for local ChIP are given in the [Supporting Information](#).

ChIP-Seq Data Analysis. Uniquely mapped, nonredundant sequence reads aligned to human genome build hg19 were retained (32). Genomic enrichment was identified using MACS 1.4 under a P value threshold of 10^{-5} (29, 33). Dynamic binding sites were determined using a linear model described in [Supporting Information](#). Heat maps and composite profiles of ChIP-seq enrichment were generated as described (34).

Regulatory Potential Calculation and Data Integration. For each gene i , the regulatory potential, P_i , of associated NOTCH1-binding sites was calculated by $P_i = \sum \exp[-\Delta_{ij}/\lambda]$, where Δ_{ij} is the distance from the TSS of the gene i to the j th binding site located within the CTCF domain (see schematic in Fig. 3E), and λ is a scale factor determined by empirical fitting (29). T-score and FDR of differentially expressed genes were determined using linear models for

microarray data (LIMMA) (35). The rank mean in [Table S2](#) was calculated as the arithmetic mean of the ranks of a gene in the regulatory potential list and in the differential expression t-score list. Additional details are in [Supporting Information](#).

Reporter Constructs. A luciferase reporter plasmid containing a TATA box was assembled by replacing the SV40 promoter region of pGL3 (Promega) with the sequence GATCTCCAGATATATATAGAGGCCGCCAGGGCTCGGATCACACAGA. To create *IL7R* enhancer reporter constructs, 311 (E5) and 200 bp (E3) genomic DNA sequences were amplified by PCR, sequenced, and cloned into the pGL3 TATA box construct individually or in tandem. Mutations in transcription binding site motifs were created by PCR or by QuikChange site-directed mutagenesis kit (Stratagene) according to provided protocols. Primer and genomic DNA sequences of reporter constructs are available on request.

Reporter Gene Assays. CUTLL1 cells were transfected with empty pGL3 or pGL3 *IL7R* enhancer reporter plasmids mixed with an internal control *Renilla* luciferase plasmid, pRL-TK, at a 50:1 ratio using Lipofectamine LTX reagent (Invitrogen). At 48 h posttransfection, cells were lysed and dual luciferase assays were performed as described (36).

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Supporting Information

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SI Materials and Methods

ChIP-Seq Antibodies. Antibodies used for ChIP were as followed: ETS1 (sc-350), GABPA (sc-22810), RUNX1 (Abcam ab23980), H3K27ac (ab4729), p300 (sc-585), BRD4 (Bethyl A301-985A), and MED1 (Bethyl A300-793A).

ChIP-Seq Data Processing. Sequence reads from ChIP-seq experiments were aligned (1) to human genome build hg19 and uniquely mapped, nonredundant reads were retained. After peak calling by model-based analysis for ChIP-Seq (MACS) 1.4, genomic wiggle traces were normalized by the total nonredundant read count of each dataset and displayed using innovative genomics viewer (IGV) Browser (2). Sequence motif analyses were performed using MDSeqPos (3). Heat maps (Figs. 1A and 2C) were generated using the Cistrome analysis pipeline (4).

Quantitative Identification of Dynamic Binding Sites. Binding strength of NOTCH1 peaks was measured by the normalized read count located in a 600-bp region centered on the peak summit. To compare the Notch-on and Notch-off states, a metric to define fold change was calculated as described (5) using a linear model

$$M = kA + C,$$

where $M = \log_2(x_t) - \log_2(x_c)$, and $A = 1/2(\log_2(x_t) + \log_2(x_c))$, with x_t and x_c referring to the normalized read counts in the treated (Notch-on) and control (Notch-off) conditions, respectively. Here, k and C are the parameters of the linear model, and C is used as the metric for dynamism, termed “dynamic fold change” as plotted in Fig. 1B and Fig. S24. Dynamic sites were identified using an empirical false discovery rate (FDR) of 0.05 that was estimated assuming that the background distribution of the adjusted fold change is symmetric to 0 and decreased NOTCH1 binding to sites in the Notch-on state represents a false positive signal (Fig. S24).

Composite Profiles of Chromatin Immunoprecipitation (ChIP)-Seq Signal on a Set of Genomic Loci. ChIP-Seq signals were measured by normalized read density on identified peak regions. For profiles around transcription-factor binding sites (e.g., Fig. 4B and Figs. S2E and S5 D and F), all binding sites were aligned by their summits and nonredundant reads were counted in non-overlapping 100-bp bins from 5 kb upstream to 5 kb downstream flanking the summits. For profiles around a set of broad peak regions (e.g., Fig. 4E and Fig. S5G), nonredundant reads were assigned to nonoverlapping bins equal to 2% of the peak length within the peak region and 100-bp bins outside the peak region from 5 kb upstream to 5 kb downstream. For both cases, the read counts in each bin were then normalized by the total non-redundant read count of the given ChIP-seq dataset and the total base pairs of the bins to obtain the normalized density.

Identification of CTCF Domains in the Human Genome. ChIP-Seq data for CTCF in 69 human cell lines were downloaded from the Encyclopedia of DNA elements (ENCODE) consortium. CTCF-binding sites in each cell line were identified using MACS1.4 with default parameters. Constitutive CTCF-binding sites were

defined as sites present in >90% of cell lines and that lie outside of gene promoters. The entire human genome was then partitioned into nonoverlapping CTCF domains using these 11,326 constitutive CTCF-binding sites.

Calculation of Average Regulatory Potential in Bins of Genes. The regulatory potential of each differentially expressed gene between Notch-off and Notch-on states was calculated as described in the *Materials and Methods*. All differentially expressed genes (340 up and 187 down, FDR < 0.05) were then ranked according to their differential expression t score and binned by every 20 genes. The color bar in Fig. 3E is a schematic of the differential expression of genes in all bins. Within each bin ($n = 20$), the average regulatory potential was calculated by

$$P = \frac{1}{n} \sum_{i=1}^n P_i$$

and plotted against the rank of bins.

Retroviral Expression of cDNAs. A MigR1 retrovirus vector encoding the dominant negative RUNT domain of RUNX1 was the kind gift of Dr. Andrew Weng (BC Cancer Agency, Vancouver, Canada). MigR1 and MigR1-DN-MAML retroviral constructs been described (6, 7). Pseudotyped retrovirus was produced by transfection of 293T cells as described (8). GFP+ cells transduced by MigR1 and MigR1-RUNT were isolated by cell-sorting.

Real-Time RT-PCR. Total RNA was prepared with RNeasy Mini kit (Qiagen) and cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed in triplicates using the SYBRgreen supermix and primers for *IL7R*, *GAPDH*, and 18s rRNA with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Data were analyzed with CFX manager software (BioRad).

Primer Sequences Used for DTX1 Superenhancer ChIP and IL7R RT-PCR. *DTX1* Pair1: forward 5'-CGGGAGGGTTGAAGAGTG-AC-3', reverse 5'-CCCCTCTGTGAAATGGGACC-3'; *DTX1* Pair2: forward 5'-CCAGCTGGGTGACCTTAGTG-3', reverse: 5'-GCCTTACCCTAAGCCAAGA-3'; *DTX1* Pair3 (dynamic Notch site): forward 5'-AGGGGTGAAGGCTGGCTCCC-3', reverse 5'-GCAGGCCTCCTGCGAGAACC-3'; *DTX1* Pair4: forward 5'-ATCCCTGCGCCAAATGATGA-3', reverse 5'-AATGAACACAGCTCCCAGAG-3'; *DTX1* Pair5: forward 5'-CTTTCTGGTGCCAACTGTGC-3', reverse 5'-GACAGCTGG-AGAAGGAGCTG-3'; DNA negative control region: forward 5'-AATGCTGGGCTTCCAAGGA-3', reverse 5'-GACCTTGGT-GACTGTTGAGGAAAC-3'; *IL7R* mRNA: forward 5'-TGT-CGTCTATCGGGAAGGAG-3', reverse 5'-CGGTAAGCTAC-ATCGTGCATTA-3'; 18s rRNA: forward 5'-TAGAGTGTTT-AAAGCAGGCCC-3', reverse 5'-CCAACAAAATAGAACCG-CGGT-3'; *GAPDH* mRNA: forward 5'-GAAGGTGAAGG-TCGGAGTCAAC-3', reverse 5'-TGGAAGATGGTGATGG-GATTTC-3'.

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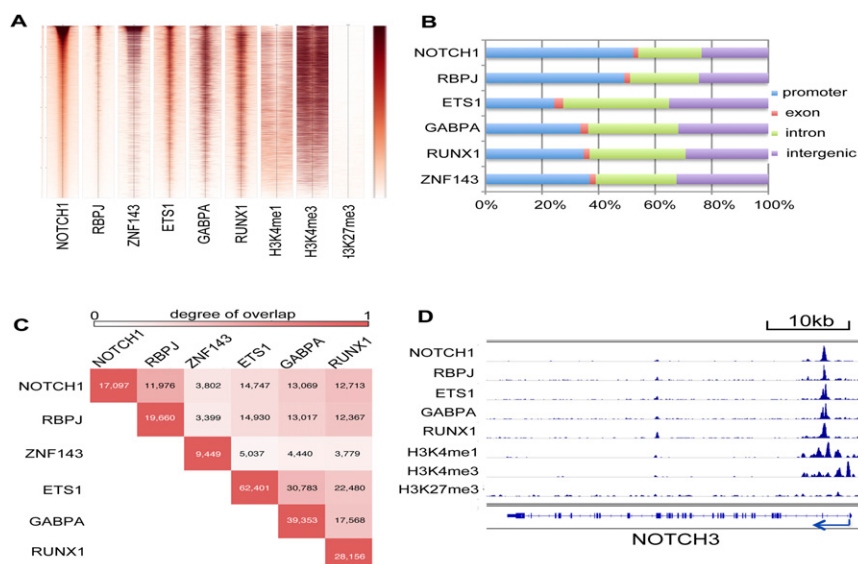


Fig. S1. Overlap of NOTCH1–RBPJ steady-state genomic binding sites with ETS1, GABPA, and RUNX1 sites in T-LL cells. (A) Heat map of genomic NOTCH1 genomic-binding sites, ranked by ChIP-Seq signal intensity, and associated signals of the indicated transcription factors and histone marks across a 1-kb window centered on NOTCH1-binding peak summits. (B) Distribution of genomic-binding sites of NOTCH1, RBPJ, and other transcription factors. Promoter binding is defined as a site <2 kb from a mapped transcriptional start site (TSS). (C) Pairwise comparison of transcription-factor binding sites. The central diagonal in the grid shows the total number of binding sites for each factor, and the pairwise comparisons indicate the number of overlapping sites for each pair of factors. The intensity of the red coloring of each factor pair corresponds to the significance of the overlap, as measured by Jaccard index (shown above). (D) Transcription-factor-binding sites and associated chromatin marks in the *NOTCH3* locus. An arrow indicates the direction of transcription.

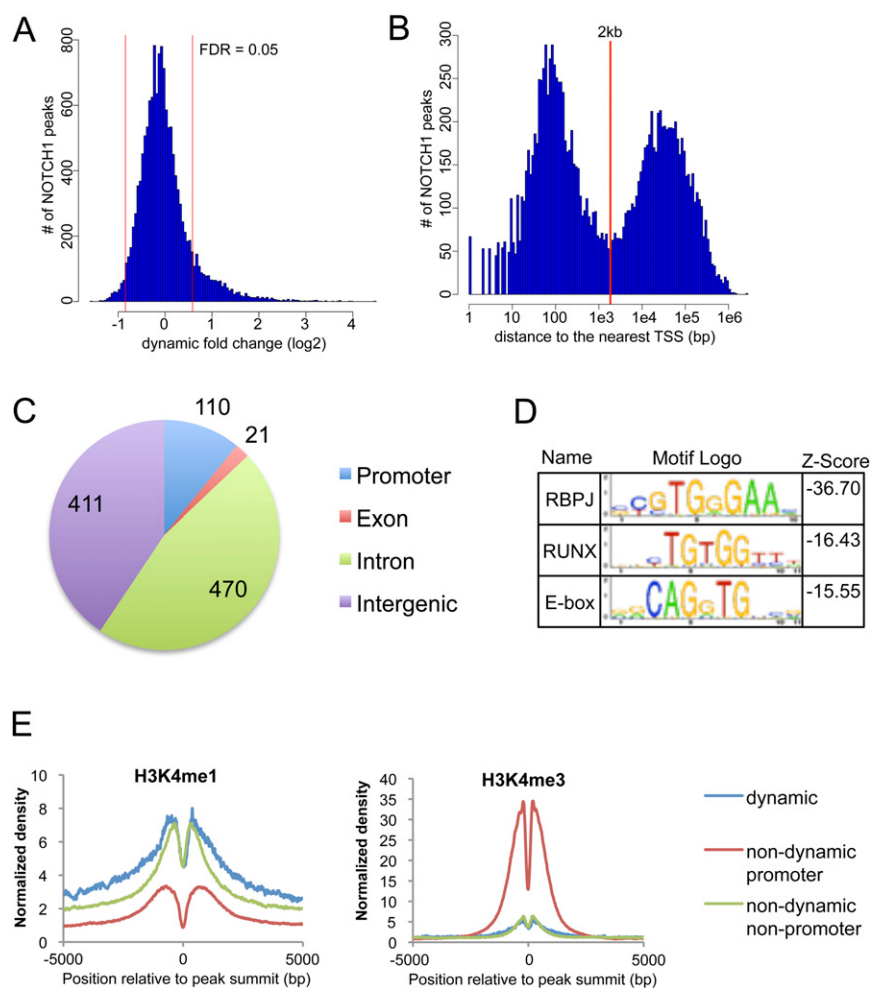


Fig. S2. Identification and characteristics of dynamic and nondynamic NOTCH1-binding sites. (A) Distribution of dynamic fold change from Notch-off to Notch-on for all NOTCH1-binding sites. Red lines indicate the cutoffs with FDR = 0.05. (B) Distribution of distances separating NOTCH1-binding sites and the nearest TSSs. (C) Distribution of dynamic NOTCH1 sites in the genome. (D) DNA sequence motifs enriched near dynamic NOTCH1-binding sites. MDSeqPos Z score indicates the significance of motif enrichment. (E) Composite H3K4me1 and H3K4me3 ChIP-seq signals aligned around the summits of dynamic and nondynamic NOTCH1-binding sites.

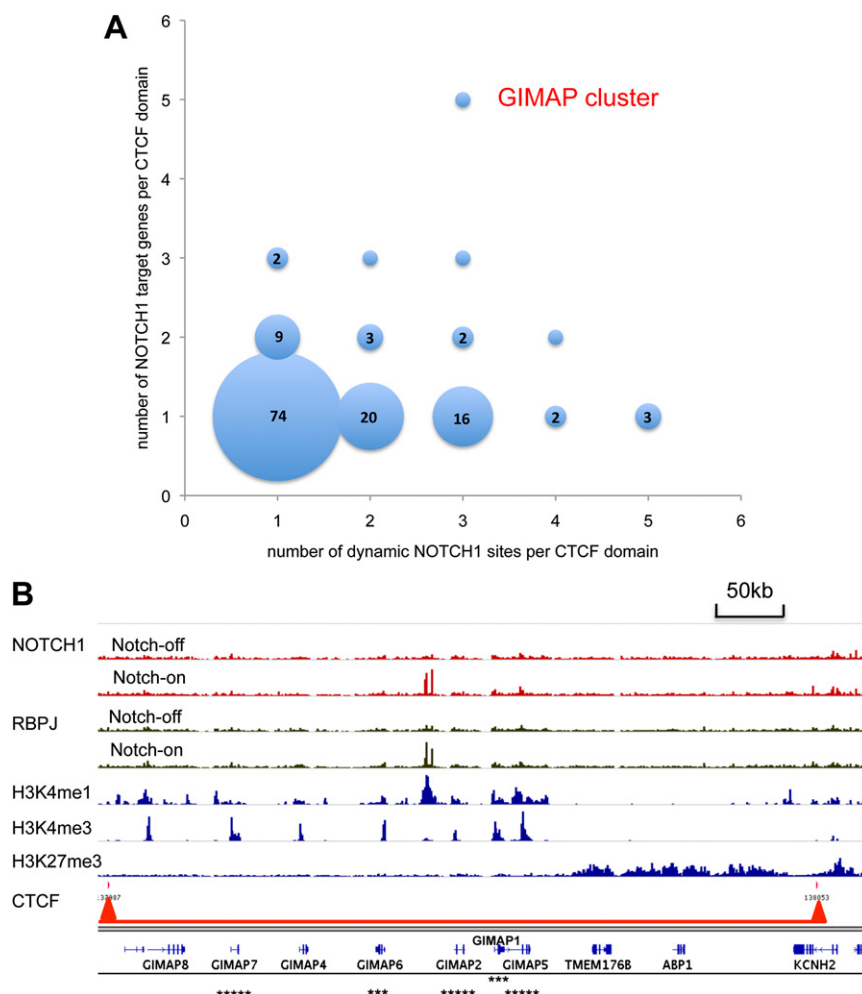


Fig. 54. Numbers of dynamic NOTCH1 sites and NOTCH1 target genes in CTCF domains with at least one dynamic NOTCH1 site and at least one NOTCH1 target gene. (A) Genomewide summary. The size of the circles is proportional to the number of constitutive CTCF domains in the genome that contain the indicated combinations of dynamic NOTCH1 sites and NOTCH1 target genes. The smallest circles correspond to combinations that occur only once in the genome, such as the CTCF domain harboring the *GIMAP* gene cluster, which contains three dynamic NOTCH1 sites and five NOTCH1 target genes. The number of CTCF domains containing other combinations of target genes and dynamic binding sites is given. (B) Chromatin landscapes around the *GIMAP* gene cluster. Three dynamic NOTCH1–RBPJ sites lie close together within a central element with high levels of the H3K4me1-enhancer mark. *GIMAP1*, *GIMAP2*, *GIMAP5*–*7* are all up-regulated when NOTCH1 is activated by GSI washout. ***** $P < 10^{-5}$; *** $P < 10^{-3}$.

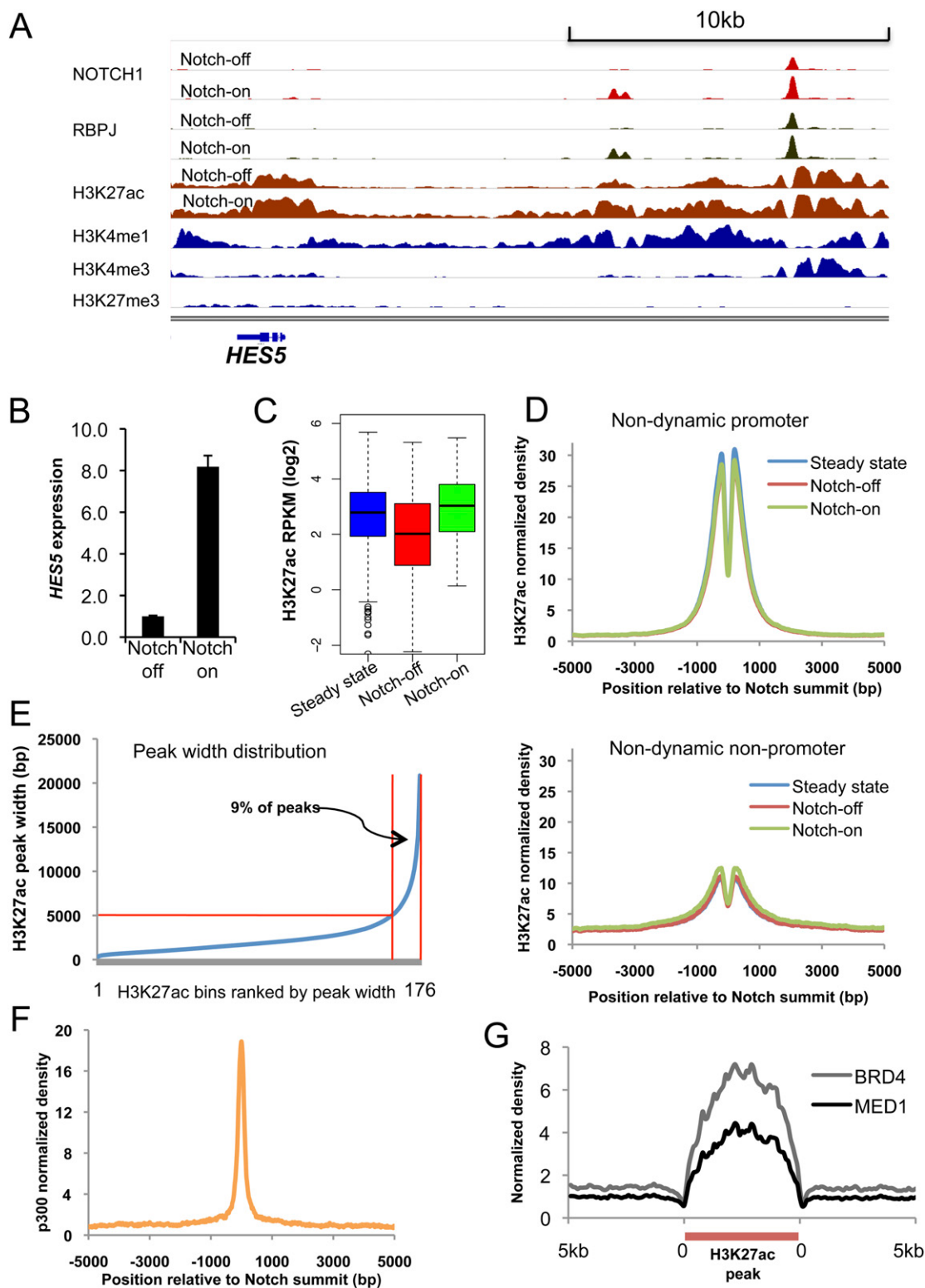
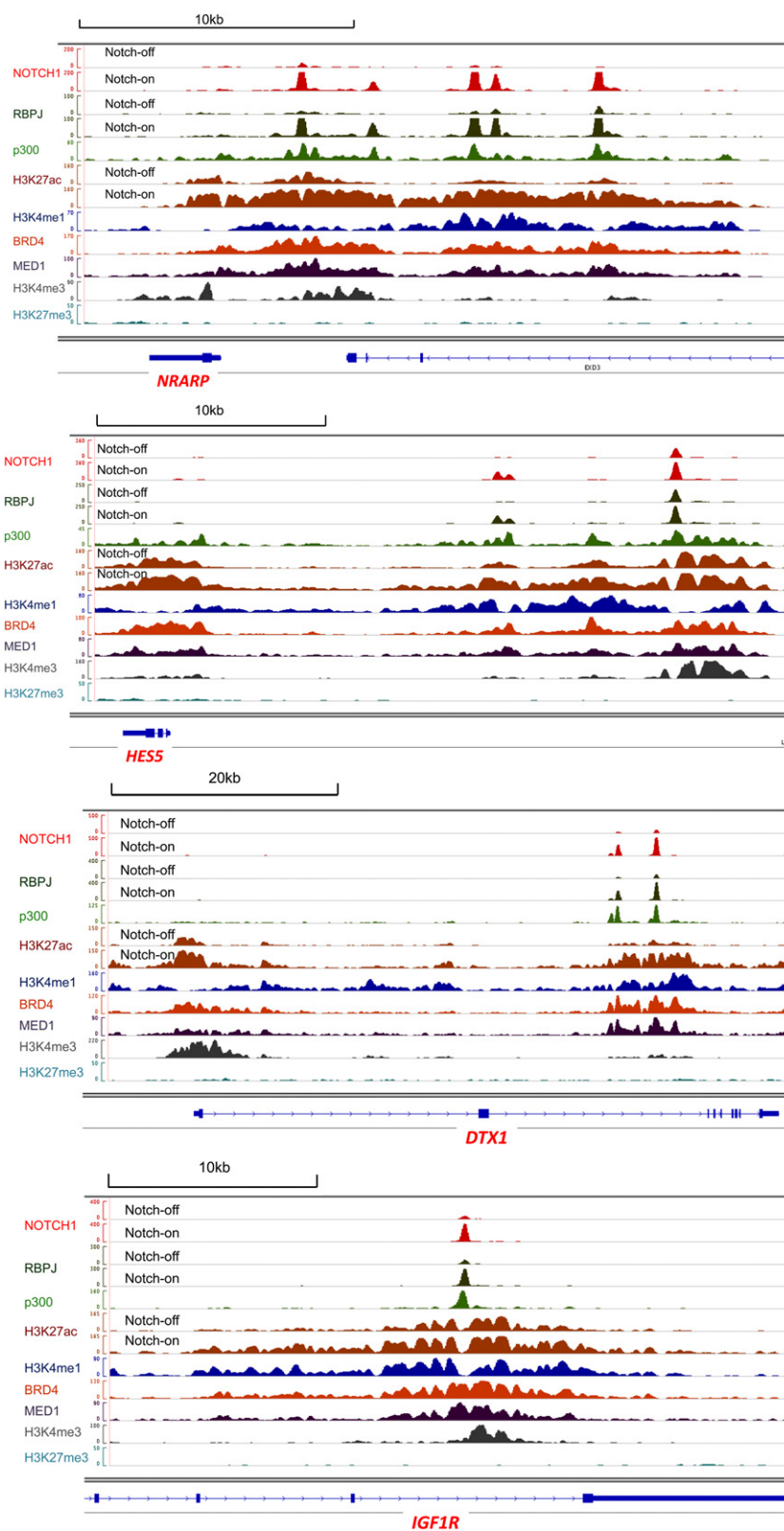


Fig. 55. Effect of Notch activation status on chromatin landscapes around dynamic and nondynamic NOTCH1 sites. (A) NOTCH1 reactivation and loading of NOTCH1–RBPJ complexes on a superenhancer located 5' of *HES5* increases H3K27ac levels across a region of >10 kb in width. (B) Changes in *HES5* expression associated with NOTCH1 reactivation as assessed by qRT-PCR. All data points were obtained in triplicate. (C) H3K27ac levels of H3K27ac peaks associated with dynamic NOTCH1 sites in steady-state, Notch-off, and Notch-on states. (D) Composite profiles of H3K27ac aligned around the summits of nondynamic promoter NOTCH1 sites (Upper) and nondynamic nonpromoter sites (Lower) in the steady-state, Notch-off, and Notch-on states. (E) Genomewide width distribution of H3K27ac peaks. All 35,244 H3K27ac peaks were ranked by peak width and binned as described in Fig. 4C. Median width of the peaks in each bin was plotted on the y axis. Only 9% of H3K27ac peaks are >5 kb in width. (F) Composite profiles of P300 signal aligned around the summits of dynamic NOTCH1-binding sites. (G) Composite profiles of BRD4 and MED1 aligned on dynamic NOTCH1-associated H3K27ac peaks. H3K27ac peaks were scaled to the same width. BRD4 and MED1 signals associated with scaled H3K27ac peaks were plotted across the H3K27ac peaks as well as flanking 5-kb regions.



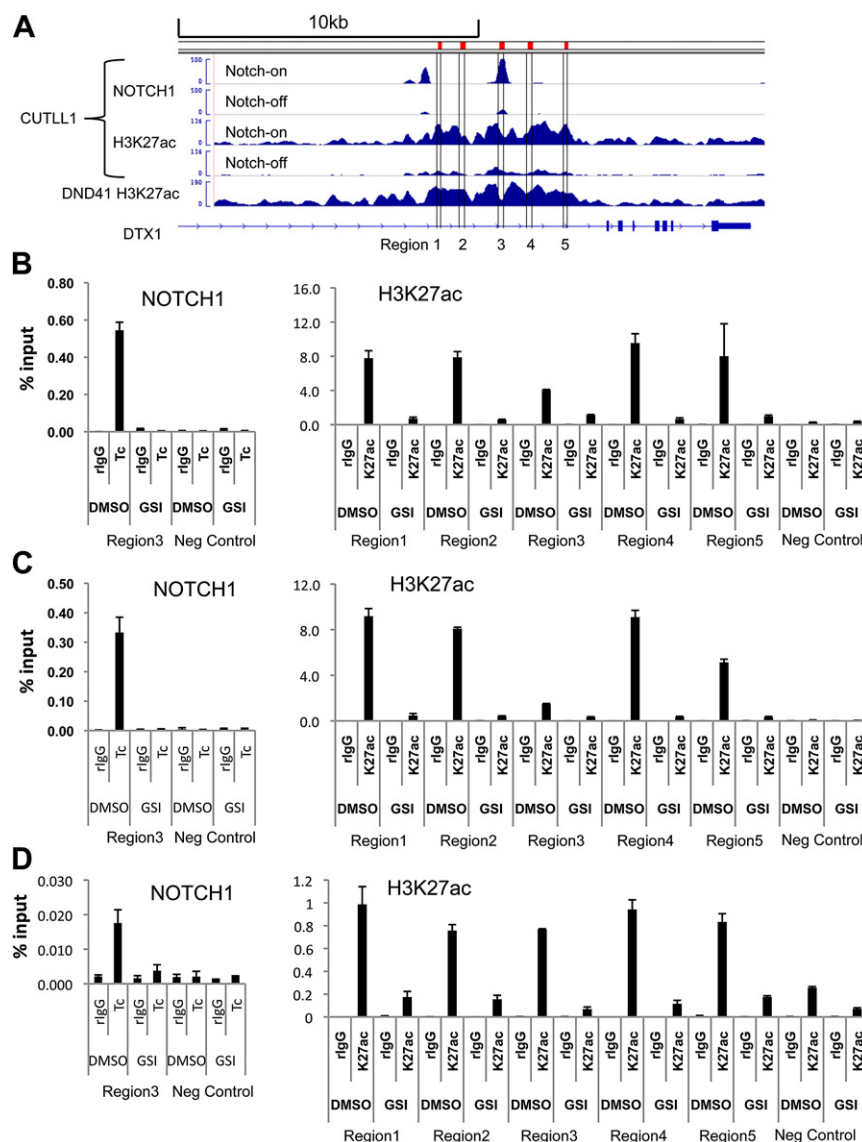


Fig. S7. *DTX1* superenhancer H3K27 acetylation is Notch-dependent in multiple T-LL cell lines. (A) Chromatin landscapes of the *DTX1* superenhancer in CUTLL1 and DND41 T-LL cell lines. CUTLL1 data are from this report, and DND41 data are from the resource paper of Hnisz et al. (9). The vertical lines denote five regions analyzed by local ChIP. (B–D) Results of local ChIP for NOTCH1 (region 3, antibody designated Tc) and H3K27ac (regions 1–5) in KOPT-K1, DND41, and HPB-ALL T-LL cells, respectively. Controls for local ChIP were nonimmune rabbit Ig (rlgG) and a region in the *MYC* gene desert that lacks NOTCH1 binding (negative control). Individual data points were obtained in triplicate. Error bars represent 1 sd from the mean.

Factor/histone mark	Uniquely mapped reads	Nonredundant tags	Peaks/binding sites
H3K4me1	29,904,653	29,585,328	71,325
H3K4me3	19,084,592	17,021,424	27,910
H3K27me3	26,401,660	26,320,910	76,313
H3K27ac	44,883,204	30,921,421	42,843
ZNF143	22,946,868	19,226,402	9,449
ETS1	98,844,473	95,541,903	62,401
GABPA	130,011,244	101,428,669	39,353
RUNX1	30,624,572	30,422,702	28,156
NOTCH1 GSI	24,144,774	23,147,328	8,918
NOTCH1 w4h	18,373,896	18,110,018	13,986
RBPJ GSI	26,012,893	25,262,387	6,600
RBPJ w4h	22,889,385	22,544,520	13,429
H3K27ac GSI	42,059,683	31,297,366	31,847
H3K27ac w4h	44,677,046	31,307,801	33,162
BRD4	37,393,634	29,015,587	28,289
MED1	31,491,572	26,107,721	24,076
p300	25,488,514	20,099,007	25,843

GSI, γ -secretase inhibitor treated cells; w4h, cells harvested 4 h after GSI washout.

Table S2. Genes with high dynamic regulatory potential that are also up-regulated by GSI washout

RefSeq ID	Gene symbol	Regulatory potential	t score	FDR	Rank mean*
NM_001025197	<i>CHI3L2</i>	1.433	19.733	4.50E-12	2
NM_001004354	<i>NRARP</i>	1.303	40.777	1.00E-18	2.5
NM_005524	<i>HES1</i>	1.182	16.148	1.50E-10	6.5
NM_001010926	<i>HES5</i>	1.318	13.621	3.30E-09	7
NM_001040708	<i>HEY1</i>	0.914	21.453	1.30E-12	9
NM_001163446	<i>CPA4</i>	0.882	17.203	4.70E-11	11.5
NM_007261	<i>CD300A</i>	1.256	11.775	4.10E-08	11.5
NM_001017971	<i>ATP6AP1L</i>	1	11.047	1.20E-07	16
NM_020311	<i>CXCR7</i>	0.74	8.619	6.70E-06	31.5
NM_018130	<i>SHQ1</i>	0.595	14.001	2.20E-09	39.5
NM_001142467	<i>HES4</i>	0.606	7.785	2.80E-05	46
NM_000435	<i>NOTCH3</i>	1.025	6.225	5.50E-04	49.5
NM_002334	<i>LRP4</i>	0.629	6.464	3.60E-04	57.5
NM_001003674	<i>C18orf1</i>	0.698	6.15	6.40E-04	59.5
NM_001002926	<i>TWISTNB</i>	0.606	5.964	8.90E-04	70
NM_015660	<i>GIMAP2</i>	0.56	8.427	9.20E-06	71.5
NM_024901	<i>DENND2D</i>	0.807	5.492	2.30E-03	72.5
NM_020909	<i>EPB41L5</i>	0.59	6.773	1.90E-04	77.5
NM_001006658	<i>CR2</i>	0.497	8.258	1.20E-05	89.5
NM_015895	<i>GMNN</i>	0.502	8.151	1.50E-05	90
NM_014746	<i>RNF144A</i>	1.056	4.759	9.20E-03	96
NM_020742	<i>NLGN4X</i>	0.573	5.871	1.10E-03	101
NM_002185	<i>IL7R</i>	0.411	15.679	2.60E-10	103.5
NM_000695	<i>ALDH3B2</i>	0.393	10.401	3.30E-07	117.5
NM_001078174	<i>SLC29A1</i>	0.599	4.712	1.00E-02	126
NM_198841	<i>FAM120AOS</i>	0.403	7.446	5.40E-05	130
NM_001077365	<i>POMT1</i>	0.55	5.05	5.30E-03	131
NM_006352	<i>ZNF238</i>	0.494	5.42	2.60E-03	133
NM_000589	<i>IL4</i>	0.343	12.974	7.40E-09	135.5
NM_001146029	<i>SEMA7A</i>	0.413	6.469	3.60E-04	138.5
NM_001164211	<i>LRCH1</i>	0.54	4.839	8.00E-03	143.5
NM_024980	<i>GPR157</i>	0.525	4.837	8.00E-03	146.5
NM_015382	<i>HECTD1</i>	0.587	4.585	1.20E-02	148.5
NM_006320	<i>PGRMC2</i>	0.459	5.307	3.30E-03	150.5
NM_001037172	<i>ACPL2</i>	0.39	5.995	8.40E-04	155.5
NM_020639	<i>RIPK4</i>	0.313	8.992	3.40E-06	159
NM_001018053	<i>PFKFB2</i>	0.292	8.064	1.80E-05	177
NM_018566	<i>YOD1</i>	0.267	9.944	6.70E-07	182
NM_002976	<i>SCN7A</i>	0.502	4.414	1.70E-02	182.5
NM_024711	<i>GIMAP6</i>	0.317	6.301	4.80E-04	182.5
NM_032735	<i>BEST3</i>	0.253	11.231	9.50E-08	183
NM_003325	<i>HIRA</i>	0.378	5.132	4.50E-03	184
NM_004694	<i>SLC16A6</i>	0.358	5.059	5.20E-03	199.5
NM_020979	<i>SH2B2</i>	0.251	6.812	1.80E-04	210.5
NM_001166663	<i>CD244</i>	0.234	6.878	1.60E-04	221
NM_012388	<i>BLOC1S6</i>	0.258	5.717	1.40E-03	226.5
NM_014612	<i>FAM120A</i>	0.376	4.292	2.10E-02	232.5
NM_001029884	<i>PLEKHG1</i>	0.237	5.516	2.10E-03	246
NM_002165	<i>ID1</i>	0.303	4.633	1.20E-02	248.5
NM_006577	<i>B3GNT2</i>	0.301	4.644	1.10E-02	249
NM_022720	<i>DGCR8</i>	0.441	3.903	4.30E-02	251
NM_004416	<i>DTX1</i>	0.17	12.415	1.60E-08	251.5
NM_032373	<i>PCGF5</i>	0.158	17.429	4.10E-11	253
NM_000299	<i>PKP1</i>	0.197	6.004	8.40E-04	261
NM_001098614	<i>PUS7L</i>	0.196	5.565	2.00E-03	274
NM_024093	<i>C2orf49</i>	0.235	4.672	1.10E-02	283
NM_080605	<i>B3GALT6</i>	0.288	4.013	3.50E-02	303
NM_153000	<i>APCDD1</i>	0.111	13.829	2.50E-09	303.5
NM_130759	<i>GIMAP1</i>	0.163	5.689	1.50E-03	305
NM_001031680	<i>RUNX3</i>	0.161	5.716	1.40E-03	305.5
NR_002779	<i>NUDT9P1</i>	0.156	5.748	1.40E-03	308
NM_018092	<i>NETO2</i>	0.163	5.354	3.00E-03	312.5

