The Notch driven long non-coding RNA repertoire in T-cell acute lymphoblastic leukemia

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ABSTRACT

Genetic studies in T-cell acute lymphoblastic leukemia have uncovered a remarkable complexity of oncogenic and loss-of-function mutations. Amongst this plethora of genetic changes, *NOTCH1* activating mutations stand out as the most frequently occurring genetic defect, identified in more than 50% of T-cell acute lymphoblastic leukemias, supporting a role as an essential driver for this gene in T-cell acute lymphoblastic leukemia oncogenesis. In this study, we aimed to establish a comprehensive compendium of the long non-coding RNA transcriptome under control of Notch signaling. For this purpose, we measured the transcriptional response of all protein coding genes and long non-coding RNAs upon pharmacological Notch inhibition in the human T-cell acute lymphoblastic leukemia cell line CUTLL1 using RNA-sequencing. Similar Notch dependent profiles were established for normal human CD34$^+$ thymic T-cell progenitors exposed to Notch signaling activity *in vivo*. In addition, we generated long non-coding RNA expression profiles (array data) from *ex vivo* isolated Notch active CD34$^+$ and Notch inactive CD4$^+$CD8$^+$ thymocytes and from a primary cohort of 15 T-cell acute lymphoblastic leukemia patients with known *NOTCH1* mutation status. Integration of these expression datasets with publicly available Notch1 ChIP-sequencing data resulted in the identification of long non-coding RNAs directly regulated by Notch activity in normal and malignant T cells. Given the central role of Notch in T-cell acute lymphoblastic leukemia oncogenesis, these data pave the way for the development of novel therapeutic strategies that target hyperactive Notch signaling in human T-cell acute lymphoblastic leukemia.

Introduction

The Notch pathway comprises a highly conserved signaling pathway that regulates various cellular processes in all metazoans, including stem cell maintenance, regulation of cell fate decisions, cellular proliferation, differentiation, cell death and adult tissue homeostasis.[1] As such, Notch signaling is critically involved in many different tissues including epithelial, neuronal, blood, bone, muscle and endothelial cells.[2] Precise regulation and duration of Notch signaling activity is of critical importance to ensure appropriate execution of the various developmental cues and cellular processes. Consequently, constitutive or acquired perturbation of Notch signaling frequently leads to human disease and cancer.[1-4]

Notch signaling plays multiple roles in hematopoiesis and is essential for the establishment of definitive hematopoiesis through the generation of hematopoietic stem cells,[5] as well as for their subsequent differentiation in an expanding number of blood cell types.[6-9] The role of Notch signaling has been particularly well documented in T-cell development where Notch1/Dll4 interactions are crucial to induce T-lineage differentiation at the expense of other hematopoietic lineages.[10-14] Subsequently, Notch signaling is implemented in TCR- rearrangements,[15,16] modulation of TCR-αβ *versus* -γδ development,[17-21] and in the support of proliferation during β-selection.[22-24] Sustained activation of Notch1 signaling beyond this developmental checkpoint has been shown to cause T-cell acute lymphoblastic leukemia (T-ALL) and *NOTCH1* activating mutations are amongst the most frequently observed genetic alterations in T-ALL.[25,26] Importantly, γ-secretase inhibitors (GSIs) that block S3 cleavage of the Notch1 receptor and subsequent release of the intracellular signaling domain (ICN) are the subject of intensive investigation as novel drugs to combat T-ALL. However, single compound therapies almost invariably lead to resistance. Therefore, a deeper understanding of Notch signaling in normal thymocyte maturation[27] and in Notch1 activated T-ALLs could yield novel insights that could make treatment more effective.

Activation of Notch1 converts the intracellular domain (ICN1) of the Notch1 receptor into a transcriptional activator and ICN1 subsequently acts as a direct regulator of multiple target genes.[28] However, despite intensive investigation, the nature of these genes, as well as their context-dependent acti-

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vation, remains largely elusive. In general, oncogenic Notch signaling promotes leukemic T-cell growth through direct transcriptional upregulation of multiple anabolic genes involved in ribosome biosynthesis, protein translation, and nucleotide and amino acid metabolism. Furthermore, Notch1 positively regulates G1/S cell cycle progression in T-ALL[29-31] and up-regulates several cyclins and CDKs,[30] in addition to the recurrent oncogene MYC. Furthermore, Notch signaling regulates cell size, glucose uptake and PI3K-AKT activated glycolysis through HES1-mediated *PTEN* repression. Besides direct regulation of *HES1*, Notch1 is also implicated in the control of essential early T-cell genes such as pre-TCRα (*PTCRA*) and *IL7R*.[32-34] Taken together, these genes and pathways, as well as a further expanding list controlled by Notch1 in T-ALL and normal T-cell development, illustrate the complexity and vastness of the Notch1 controlled regulatory program.

Recent transcriptome-wide profiling efforts have uncovered an unanticipated pervasiveness of transcription of the human genome, most of which is not translated into protein.[35-38] Evidence is now emerging that more than 60% of the entire genome is transcribed.[39] In addition to previously well-characterized untranslated RNA molecules such as tRNAs, snoRNAs and microRNAs (miRNAs), thousands of so-called long non-coding RNAs (lncRNAs) have been annotated to the human genome.[40,41] Although functional studies still need to be carried out on the vast majority of these lncRNA sequences, important cellular functions are rapidly being attributed to some of them, including roles in disease processes such as cancer.[42] In contrast to microRNAs, a picture is emerging in which lncRNAs can exhibit a myriad of different functions. These include various regulatory mechanisms of gene transcription, splicing, post-transcriptional control, protein activity and nuclear architecture.[43-45] Despite this initial progress, mechanisms of upstream regulation of lncRNAs have so far remained largely unexplored.

In this study, we investigate the role of Notch in the control of lncRNA transcription in the context of normal T-cell development and T-ALL. To this end, lncRNA expression was measured following modulation of Notch signaling in the T-ALL cell line CUTLL1 as well as in normal human thymocytes, and the recently published data on genome-wide Notch1 binding sites was used to reveal the potential for direct regulation.[34] Using this approach, we identified a total of 40 Notch-driven lncRNAs, thereby revealing a novel layer in the molecular machinery that mediates Notch signaling.

Methods

GSI treatment of T-ALL cell lines

HPB-ALL, TALL-1, ALL-SIL and CUTLL1 cells (see also *Online Supplementary Methods*) were seeded at a density of $1x10^6$ cells/mL and treated with either DMSO or 1 μM of Compound E (Enzo Life Sciences). Cells were harvested 12 and 48 h after treatment.

Human thymocytes and OP9-DLL1 co-cultures

Pediatric thymus samples were obtained and used according to the guidelines of the Medical Ethical Commission of the Ghent University Hospital, Belgium. $CD34^+$ thymocytes were purified using magnetic activated cell sorting (MACS, Miltenyi Biotec) to a purity of more than 98% and seeded onto confluent OP9-GFP or OP9-DLL1 plates for 48 h in α-MEM media supplemented with 20% heat-inactivated FCS plus 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and the T-lineage supporting cytokines SCF, Flt3-L and IL-7 at 5 ng/mL each.[20] Following 48 h of OP9 co-culture, cells were harvested by forceful pipetting and stained with CD45-PE (Miltenyi) to purify $CD45^+$ human thymocytes through sorting to remove contaminating OP9 stromal cells. For validation of selected lncRNAs, CD34 MACS purified thymocytes were labeled with CD34, CD1 and CD4 to sort $CD34^+CD1^-CD4^-$ uncommitted and $CD34^+CD1^+CD4^-$ committed early thymocytes, while $CD4^+CD8^+CD3^-$ and $CD4^+CD8^+CD3^+$ double positive thymocytes were sorted following CD4, CD8 and CD3 labeling of a total thymus suspension.[20] Sorted cells were lysed in 700μl QIAzol (Qiagen) and stored at -70°C prior to RNA isolation.

Clinical samples

Diagnostic blood samples of 15 individuals with T-ALL were acquired after informed consent from the Department of Pediatric Hemato-Oncology at Ghent University Hospital, Belgium. This cohort includes 8 wild-type *NOTCH1* cases and 7 mutant *NOTCH1* cases (all *FBXW7* wild type). Sequencing was performed as described by Mavrakis *et al.*[46]

Correlation analysis was performed on bone marrow lymphoblast samples from 64 T-ALL patients (unknown *NOTCH1* mutation status), which were collected after informed consent according to the Declaration of Helsinki from Saint-Louis Hospital, Paris, France. The study was approved by the Institut Universitaire d'Hématologie Institutional Review Board. This primary T-ALL cohort had been previously investigated[47] and the high-quality RNA samples from this cohort were used for lncRNA micro-array based expression profiling.

RNA sequencing

RNA samples from the CUTLL1 cells treated with GSI and thymocytes cultured on OP9-GFP/DLL1 were prepared (see also *Online Supplementary Methods*). RNA-seq was performed after unstranded poly-A library prep with an average coverage of $130x10^6$ paired-end reads. Reads were mapped to the hg19 reference genome using Tophat and transcript assembly was performed with Cufflinks. Differential expression analysis was carried out with DESeq2 in R. The design formula was adjusted to take into account the paired nature of the data.

Micro-array based gene expression profiling

RNA samples (see also *Online Supplementary Methods*) were profiled on a custom designed Agilent micro-array covering all protein coding genes and 12,000 lncRNAs (23,042 unique lncRNA probes) as described by Volders *et al.*[48] The data-analysis workflow can be found in the *Online Supplementary Methods*.

The data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus[49] and are accessible through GEO Series accession number GSE62006.

Complete details of study methods can be found in the *Online Supplementary Appendix*.

Results

Pharmacological Notch inhibition followed by RNA-sequencing reveals a set of Notch regulated lncRNAs in T-ALL

To identify lncRNAs that are regulated through Notch signaling activity in the context of T-ALL, we used the γ-secretase (GSI) inhibitor responsive T-ALL cell line CUTLL1 as a model system, since genome-wide informa-

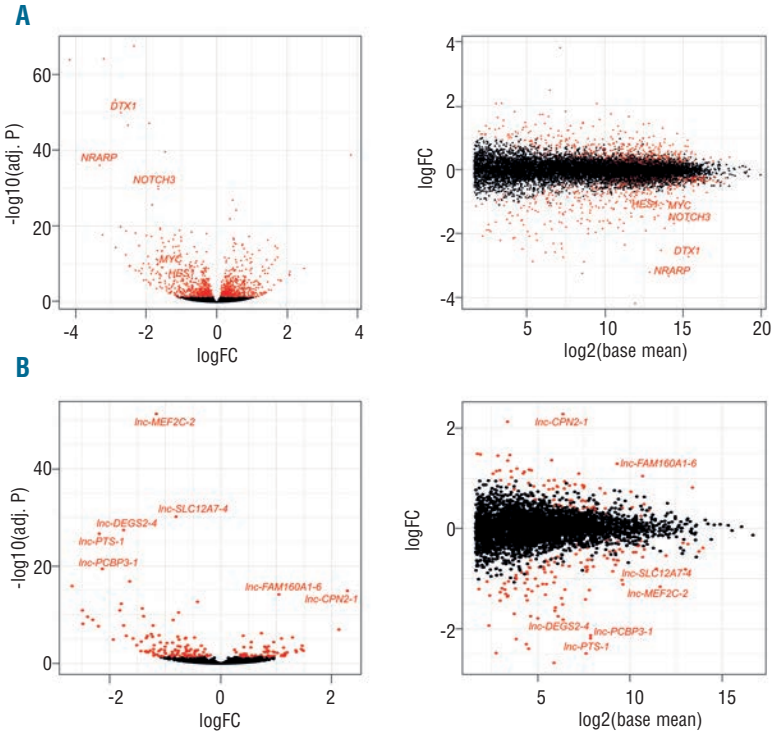

Figure 1. Pharmacological Notch inhibition followed by RNA-sequencing identifies a set of Notch regulated lncRNAs in T-ALL. (A) Volcano (left) and MA plot (right) representation of the differential expression of protein coding genes in CUTLL1 cells upon GSI treatment. Red dots represent the significant differentially expressed genes (adjusted *P*-value <0.05). (B) Volcano (left) and MA plot (right) representation of the differential expression of previously annotated lncRNAs in CUTLL1 cells upon GSI treatment. Red dots represent the significant differentially expressed genes (adjusted *P*-value <0.05; n=2). LncRNAs names depicted in the plots are the top differentially regulated lncRNAs.

tion on this cell line is available with respect to Notch1 binding[34] and the Notch dependent expression of coding genes.[50] CUTLL1 T-ALL cells were treated with GSI for 12 h and 48 h in triplicate. Genome-wide transcriptional changes determined by performing differential expression analysis on the RNA-seq data (see alignment summary in *Online Supplementary Table S1*) with DESeq2 using Ensembl (release 75) as a reference, showed robust down-regulation of several of the canonical Notch1 protein coding target genes (e.g. *DTX1, NRARP, NOTCH3*) upon GSI treatment (Figure 1A). A decrease in ICN1 protein levels was shown by Western blot analysis and downregulation of the canonical Notch1 target gene *DTX1* upon GSI treatment was further validated by RT-qPCR (*Online Supplementary Figure S1*). Amongst previously annotated lncRNAs[48] we could detect significant differential expression (adjusted *P*-value <0.05) for 83 lncRNAs, using a base mean cut off of 100 (Figure 1B). In total, 50 out of the 83 differentially expressed lncRNAs were down-regulated after GSI treatment.

Besides previously annotated lncRNAs, we also detected differential expression of non-coding transcripts that had not been previously annotated in other databases (Gencode, lncRNAdb, Broad Institute and Ensembl release 64).[48] Differentially expressed lncRNA loci with a base mean higher than 100 and identified as "unknown, intergenic transcript" or "transfrag falling entirely within a reference intron" by Cuffcompare were retained for further analysis. This led to a selection of 134 lncRNA loci of which 74 were down-regulated upon GSI treatment.

Transcriptional regulation of Notch-regulated lncRNAs in immature normal human thymocytes

Physiological levels of Notch signaling are essential during the earliest stages of T-cell development, but no information is available on the Notch dependent expression of lncRNAs in these cells.[27] Therefore, and in order to have an independent screening method in addition to the CUTLL1 cell line to identify Notch dependent lncRNAs, we used the *in vitro* OP9-DLL1 co-culture system (Figure 2A). Here, *ex vivo* purified CD34$^+$ thymocytes from healthy human donors (n=2) were cultured on a feeder layer of stromal OP9 cells either expressing GFP (as a negative control) or the Notch1 ligand DLL1 to trigger Notch signaling. CD34$^+$ progenitor cells were collected after 48 h of co-culture and deep RNA-sequencing was performed (see alignment summary in *Online Supplementary Table S2*). The set of differentially expressed protein coding genes and lncRNAs was defined as above for the CUTLL1 cells. Detection of differentially expressed protein coding genes known to be regulated by Notch signaling in early human thymocytes,[20,27] also validated our approach in this model system (Figure 2B). Differential expression analysis for previously annotated lncRNAs revealed 131 significantly up-regulated lncRNAs as a consequence of Notch activation (Figure 2C). From these 131 lncRNAs, 27 overlapped with the set of down-regulated lncRNAs upon GSI treatment of the CUTLL1 cell line (Figure 2D and *Online Supplementary Table S3*). Furthermore, we identified 156 unannotated lncRNA loci (base mean >100; adjusted *P*-value <0.05) in CD34$^+$ thymocytes that were up-regulated by the Notch ligand DLL1. In total, 13 unique unannotated lncRNA loci were identified to be positively regulated by Notch in both normal and malignant T-cell development (Figure 2E and *Online Supplementary Table S4*). Amongst the set of 13 overlapping Notch lncRNA loci, the recently described *LUNAR1*[51] was present, thus supporting the validity of our approach. In addition, we also identified 33 annotated lncRNAs to be up-regulated upon GSI treatment of CUTLL1 cells by RNA-seq, 18 of them overlapping with

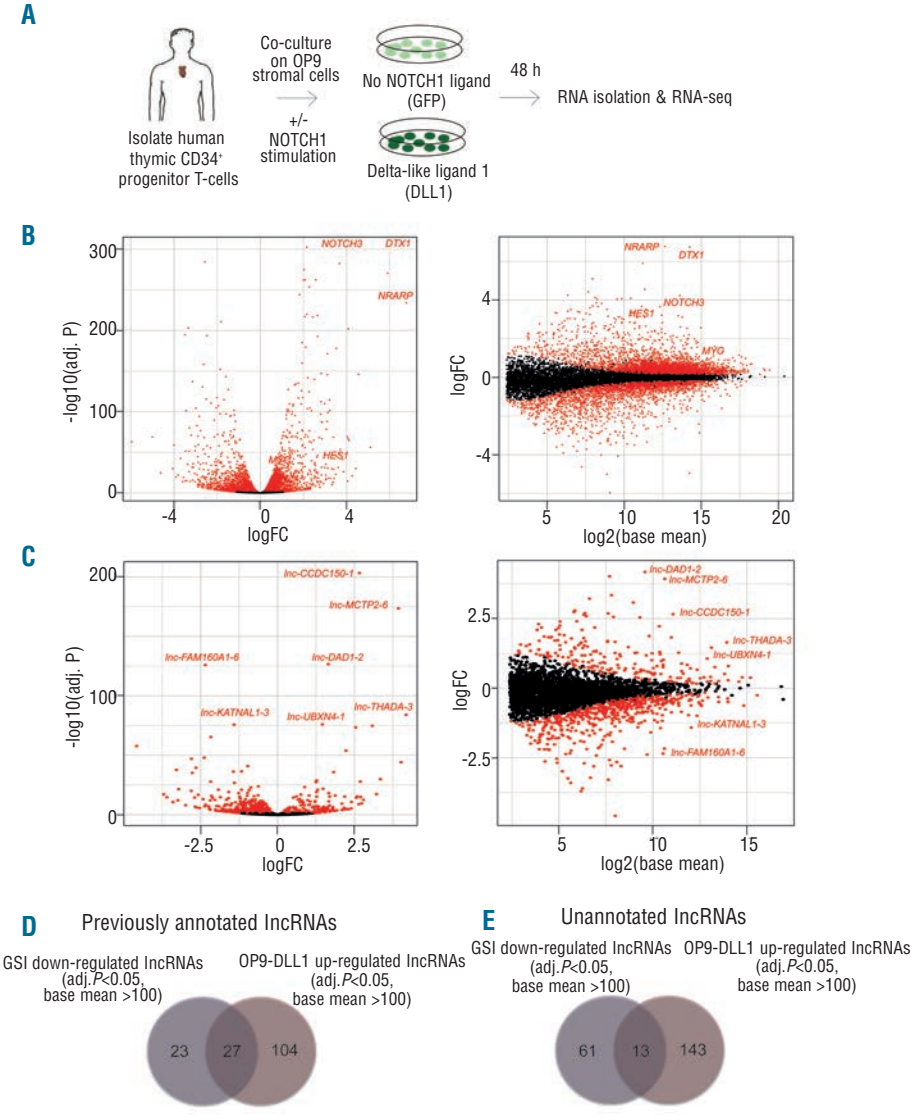

Figure 2. Transcriptional regulation of Notch regulated lncRNAs in immature normal human thymocytes. (**A**) Schematic overview of the OP9-control and -DLL1 co-culture system used to manipulate Notch signaling in healthy human immature CD34$^+$ thymocytes. (**B**) Volcano (left) and MA plot (right) representation of the differential expression of protein coding genes in CD34$^+$ cells upon Notch signaling induction by an OP9-DLL1 feeder layer. Red dots represent the significant differentially expressed genes (adjusted *P*-value <0.05; n=2). (**C**) Volcano (left) and MA plot (right) representation of the differential expression of previously annotated lncRNAs in CD34$^+$ cells upon Notch activation by an OP9-DLL1 feeder layer. LncRNA names depicted in the figure are the top differentially regulated lncRNAs. (**D**) Venn diagram depicting the overlap between previously annotated lncRNAs that are down-regulated upon GSI treatment of the CUTLL1 cell line and up-regulated upon co-culturing of CD34$^+$ thymocytes on the OP9-DLL1 feeder layer. (**E**) Venn diagram depicting the overlap between previously unannotated lncRNAs that are down-regulated upon GSI treatment of the CUTLL1 cell line and up-regulated upon co-culturing of CD34$^+$ thymocytes on OP9-DLL1 stromal cells.

the set of lncRNAs down-regulated in CD34$^+$ T-cell progenitors upon DLL1 exposure in the OP9 *in vitro* culture system (366 in total) (*Online Supplementary Table S5 and Figure S2A*). In a similar manner, 7 of 57 previously unannotated lncRNAs up-regulated upon GSI treatment of CUTLL1 cells overlapped with the set of 320 unannotated lncRNAs down-regulated in CD34$^+$ thymocytes with DLL1 exposure (*Online Supplementary Table S6 and Figure S2B*). Furthermore, we hypothesize that the Notch dependent lncRNAs (both annotated and unannotated) that are not shared between CUTLL1 T-ALL cells and normal human thymocytes can be assumed to have very context-specific functions and should be regarded as potentially interesting for further exploration in future studies. For example, lncRNAs expressed exclusively in T-ALL cells could be restrictively connected to a malignant context. To evaluate the putative protein coding potential of all unannotated lncRNA loci identified by RNA-seq in CUTLL1 T-ALL cells and CD34$^+$ T-cell progenitors cultured on OP9 stromal cells, Phylogenetic Codon Substitution Frequency (PhyloCSF) scores for all loci were calculated and we could confirm that more than 90% of all unannotated lncRNA loci determined are truly 'non-coding' (*Online Supplementary Figure S3*). Putative unannotated lncRNA loci with a PhyloCSF score higher than the determined threshold score are listed and thus predicted to be 'coding' (*Online Supplementary Tables S7 and S8*) (see also *Online Supplementary Methods*).

Validation of Notch regulated lncRNAs in an extended panel of T-ALL cell lines, normal T-cell subsets and primary T-ALLs

To further validate our data, we used a custom designed Agilent micro-array[48] developed in house that contains probes for 15 of the 27 previously annotated lncRNAs and the recently identified *LUNAR1* lncRNA that were shown to be regulated by Notch in the above described RNA-seq data from the T-ALL and normal thymocyte models. First, we treated the T-ALL cell lines ALL-SIL, TALL-1, HPB-ALL and DND-41 with GSI (*Online Supplementary Figure S4A and B*) and carried out gene expression profiling after 12 h and 48 h. Inclusion of the GSI-treated CUTLL1 cell-line samples and the samples of 4 donors of CD34$^+$ thymocytes cultured on OP9 stromal cells, revealed that there was a significant overlap between the RNA-sequencing data and the micro-array data as validated by overlapping

the protein-coding signatures derived from both datasets by Gene Set Enrichment Analysis (GSEA) (*Online Supplementary Figure S4C and D*). Nevertheless, few lncRNAs were significantly Notch dependent over all samples of the extended panel of T-ALL cell lines (ALL-SIL, HPB-ALL, DND-41 and TALL-1), probably related to the difference in the T-ALL genetic subgroup and concomitant differences in maturation arrest of the different cell lines evaluated (Figure 3A). From our selection, only *lnc-PLEKHB2-1* and *lnc-UBXN4-1* were differentially

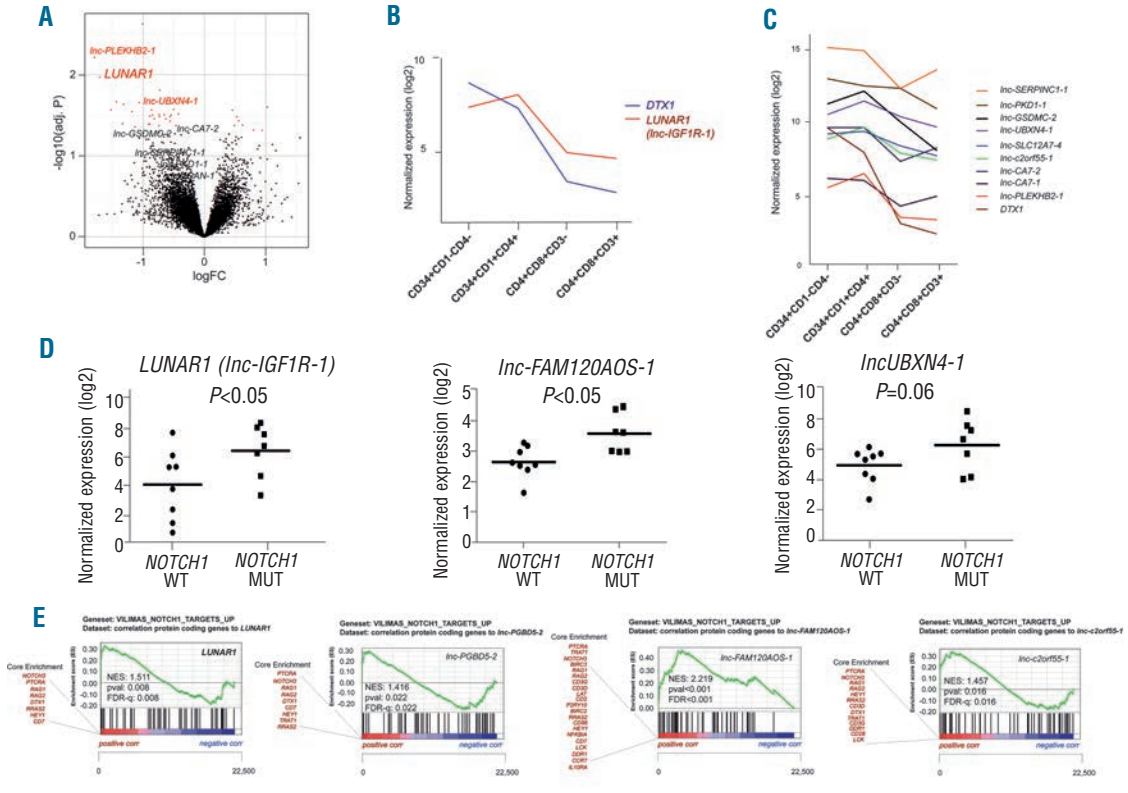

Figure 3. Screening expression of Notch regulated lncRNAs in an extended panel of T-ALL cell lines, normal T-cells subsets and primary T-ALLs. (**A**) Volcano plot representation of the differential expression of lncRNAs upon GSI treatment of ALL-SIL, TALL-1, HPB-ALL and DND-41 cells. Red dots represent the significant differentially expressed genes (adjusted *P*-value <0.05). *LUNAR1* was amongst the top-differentially expressed lncRNAs across the panel of GSI-treated T-ALL cell lines. The other lncRNA names depicted in the figure are some of the selected lncRNAs from the CUTLL1 GSI treatment and the OP9-DLL1 co-culture system. (**B**) Plot representing the expression of lncRNAs in selected Notch-dependent and -independent stages of normal T-cell development for one healthy donor. *LUNAR1* expression is significantly correlated with the expression of *DTX1* (see *also Online Supplementary Table S6*) and the data are representative for 4 independent donors. (**C**) Similar analysis as in (**B**) for the other lncRNAs that are significantly correlated with the expression of *DTX1* (see also *Online Supplementary Table S6*); data are representative for 4 independent donors. (**D**) Expression of *LUNAR1*, *lnc-FAM120AOS-1* and *lnc-UBXN4-1* in *NOTCH1* wild-type (WT) *versus NOTCH1* mutant (MUT) primary T-ALL samples. (**E**) Gene set enrichment analysis (GSEA) using the public gene set 'VILIMAS_NOTCH1_TARGETS_UP'[52] and the Spearman correlations between all protein coding genes and the set of 15 selected annotated candidate Notch lncRNAs was performed. This NOTCH1 signature was significantly enriched within the set of protein coding genes positively correlated to the expression of *lnc-PGBD5-2*, *lnc-FAM120AOS-1* and *lnc-c2orf55-1*. This enrichment was also found for lncRNA *LUNAR1*.

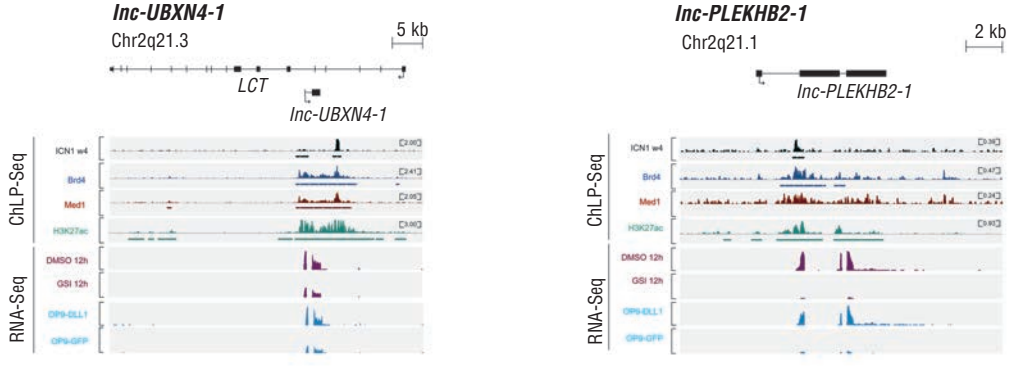

Figure 4. Notch1 ChIP-seq reveals direct binding of Notch1 to a subset of regulated lncRNAs. Representation of ChIP-sequencing tracks[34] for Notch1, Brd4, Med1 and H3K27ac and representative RNA-sequencing tracks for CUTLL1 DMSO/GSI treatment and OP9-GFP/DLL1 of *lnc-UBXN4-1* and *lnc-PLEKHB2-1*.

expressed at a significant level, while *lnc-GSDMC-2* and *lnc-CA7-2* narrowly failed to reach significance. As we were able to detect the previously unannotated and recently described lncRNA *LUNAR1*[51] on our custom designed micro-array platform, this lncRNA was one of the strongest overlapping and significantly differentially expressed lncRNAs amongst the four GSI-treated T-ALL cell lines screened. Secondly, we validated the Notch dependency of selected lncRNAs in normal thymocytes by analyzing their expression in the most immature Notch dependent CD34$^+$ stages in comparison to the Notch independent CD4$^+$CD8$^+$ double positive stages of human T-cell development. As is evident from the profiles of the Notch target gene *DTX1*, we could show that in these two T-cell subpopulations *LUNAR1* follows the expression pattern of this canonical Notch target (Figure 3B). Remarkably, 9 out of 15 lncRNAs (and *LUNAR1*) from this selection significantly correlated with *DTX1* expression (Spearman rho correlation), supporting their regulation by Notch during early stages of normal T-cell development (Figure 3C and *Online Supplementary Table S9*).

Moreover, we also had access to 15 primary T-ALL samples of which 7 harbored activating *NOTCH1* mutations while 8 were wild type (all cases are *FBXW7* wild type). There was a significant difference in expression of *LUNAR1* and *lnc-FAM120AOS-1* between *NOTCH1* wild type and mutant cases (Figure 3D and *Online Supplementary Tables S10 and S11*). By implying an additional dataset of 64 primary T-ALL patient samples, we could correlate the expression of lncRNAs *lnc-PGBD5-2*, *lnc-FAM120AOS-1*, *lnc-c2orf55-1* and *LUNAR1* with the Notch1 positively regulated gene set Vilimas_NOTCH1_targets_up[52] by GSEA (Figure 3E).

Overall, these independent experiments confirm the Notch dependent regulation of the selected lncRNAs, thereby validating the RNA-seq data from the GSI treated CUTLL1 T-ALL cell line and the Notch perturbed normal human thymocytes.

Genome-wide analysis reveals direct Notch1 binding to selected lncRNAs

To further validate the direct regulation of selected lncRNAs by Notch, publicly available ChIP sequencing (ChIP-seq) data from the CUTLL1 cell line were analyzed for Notch1 binding at specific loci.[34] From the Notch-driven annotated lncRNAs that overlapped between normal and malignant thymocytes (Figure 2D), 13 out of the 27 lncRNAs were bound by ICN1 (*Online Supplementary Table S12*) as illustrated for *lnc-UBXN4-1* and *lnc-PLEKHB2-1* (Figure 4). Remarkably, 12 out of the 13 lncRNAs with a Notch1 binding peak also show Brd4 and Med1 binding. Notably, from the putative Notch regulated lncRNAs that showed correlated expression with *DTX1* in CD34$^+$ and CD4$^+$CD8$^+$ normal thymocytes, 6 out of 9 (Figure 4 and *Online Supplementary Figure S5*) showed binding of ICN1, suggesting that the majority are a direct Notch target.

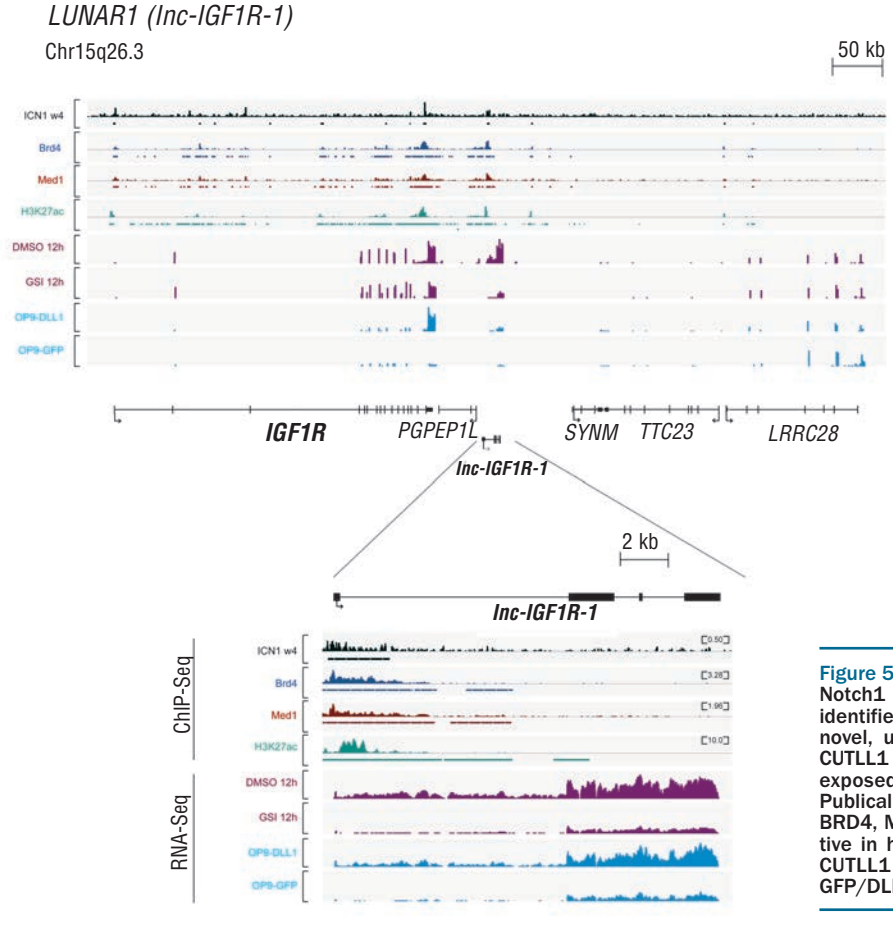

Figure 5. Notch1 ChIP-seq reveals direct binding of Notch1 to *LUNAR1*. *LUNAR1*[51] (*lnc-IGF1R-1*) was identified amongst the top differentially expressed novel, unannotated lncRNAs in both GSI-treated CUTLL1 cells and CD34$^+$ thymic progenitor cells exposed to DLL1-triggered Notch signaling. Publically available ChIP-seq tracks[34] for ICN1, BRD4, MED1 and H3K27ac as well as representative in house generated RNA-seq data tracks for CUTLL1 DMSO/GSI treated cells and OP9-GFP/DLL1 are shown at the *LUNAR1* locus.

In addition, we evaluated the presence of H3K27 acetylation (H3K27ac) ChIP-seq signal at these lncRNA loci, a histone mark indicative for putative enhancer regions. For 18 out of the 27 selected lncRNAs, H3K27ac ChIP-seq signal was present in close proximity of the promoter region (*Online Supplementary Table S12*), suggesting the presence of enhancer sequences. Moreover, this public ChIP-seq data also showed *LUNAR1* to be directly bound by ICN1, Brd4, Med1 and H3K27ac (Figure 5). We also evaluated ICN1 binding at annotated lncRNA loci up-regulated upon GSI treatment of CUTLL1 T-ALL cells and down-regulated in $CD34^+$ T-cell progenitors upon DLL1 exposure in the OP9 *in vitro* co-culture system. Only 4 out of the 18 overlapping annotated lncRNAs that are negatively regulated by Notch (*Online Supplementary Figure S2A*) showed direct binding by ICN1. The same analysis was performed on the set of 7 unannotated lncRNAs repressed by Notch1 signaling (*Online Supplementary Figure S2B*). Only 3 out of these 7 lncRNA loci showed ICN1 binding in the proximity of its promoter region. Given the established predominant role of Notch1 as a transcriptional activator, lncRNAs that are negatively affected by Notch1 signaling may actually be indirect targets.

Attributing functional annotation to Notch-regulated lncRNAs through guilt-by-association analysis

As described above, we defined a core set of 27 Notch-driven and previously annotated lncRNAs by considering only those differentially expressed and positively regulated by Notch signaling in the GSI perturbation model in CUTLL1 cells and the *in vitro* OP9-DLL1 co-culture system. As a next step, we aimed to assign potential functionalities to each of these candidates. To this end, we used the so-called 'guilt-by-association' approach (see also *Online Supplementary Methods*). As previously mentioned, 15 out of these 27 lncRNAs (together with *LUNAR1*) were detectable by a probe on our custom designed micro-array platform. In a first step, we calculated the Spearman correlation coefficients between the lncRNAs-of-interest and all protein coding genes using the expression data of a primary T-ALL cohort of 64 patients from which we profiled all samples on the custom designed Agilent array.[48] These correlations were subsequently used as an input for a GSEA pre-ranked analysis. Next, the output of this GSEA analysis was further refined into functional clusters of enriched gene sets using the Cytoscape plug-in enrichment mapping. This analysis yielded markedly different functional clustering patterns for each of the 16 lncRNAs analyzed (including *LUNAR1*). Important putative functionalities were represented in each of the networks as exemplified by TCR-signaling and phospholipid metabolism for *lnc-PLEKHB2-1*, DNA replication and DNA repair for *lnc-UBXN4-1* and splicing and cell cycle regulation for *LUNAR1* (Figure 6A-C and *Online Supplementary Figure S6A-M*).

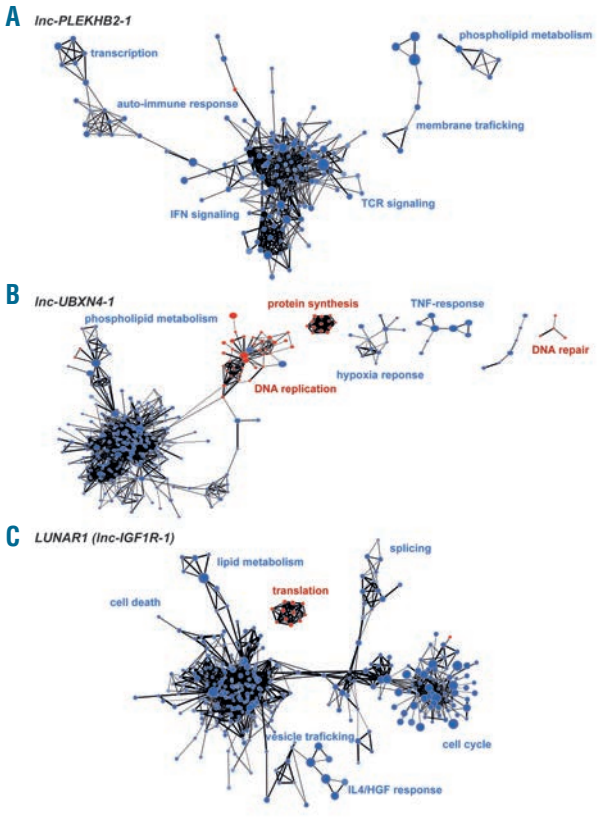

Figure 6. Attributing functional annotation to selected, annotated lncRNAs through guilt-by-association analysis. Enrichment maps of gene sets correlated with the expression of (A) *lnc-PLEKHB2-1*, (B) *lnc-UBXN4-1* and (C) *LUNAR1*. Red nodes represent the positively correlated gene sets to the lncRNA of interest, blue nodes the negatively correlated gene sets. The size of the nodes depicts the size of the gene sets. Nodes that are clustered represent gene sets with the same or similar functional indication.

Discussion

Non-coding RNAs are emerging as important players in normal development and disease, including cancer. In previous studies, we investigated the role of miRNAs in T-cell acute lymphoblastic leukemia (T-ALL), thereby identifying a small set of miRNAs that is responsible for the co-operative suppression of several tumor suppressor genes.[46] These miRNAs produced overlapping and co-operative effects with several *bona fide* T-ALL tumor suppressor genes including *IKZF1, PTEN, BIM, PHF6, NF1* and *FBXW7*, and more recently this network was expanded further with *PHF6*.[53] In order to provide some insight into the genetic components driving long non-coding RNAs in T-ALL formation, we performed an integrated analysis of lncRNA profiling data sets from GSI inhibited Notch-driven T-ALL cell lines and Notch-stimulated immature normal human thymocytes using the OP9 co-culture system, together with publicly available genome-wide data on Notch1 binding and specific chromatin marks. In addition, we correlated the expression of Notch-dependent lncRNAs with the Notch-dependent stages of normal thymocytes during T-cell differentiation. Overall, our work establishes a novel lncRNA network that acts downstream of Notch during normal and malignant thymocyte development.

Our study provides a number of fundamental new insights into Notch-dependent regulation of lncRNAs in T-ALL and normal developing thymocytes. First, we unambiguously demonstrate that a significant number of lncRNAs are directly regulated by Notch signaling activity. Through RNA-sequencing, we identified 40 lncRNAs that are positively regulated by Notch in both normal and malignant T lymphocytes (annotated as well as previously unannotated lncRNAs), supporting an important role for

these lncRNAs in Notch-regulated T-cell biology. This could be related to various functions of Notch signaling, including T-cell lineage specification and commitment, proliferation and differentiation. Importantly, the recently identified lncRNA *LUNAR1*[51] was present amongst the most robustly Notch regulated long non-coding RNAs in our data sets. *LUNAR1* was shown to be required for efficient T-ALL growth as a consequence of its role in enhancing *IGF1R* mRNA expression to sustain IGF1 signaling.[51] As a prelude to assigning functional annotation to the newly assigned Notch-regulated lncRNAs in this study, we applied the so-called 'guilt-by-association' approach in which functions are predicted based upon correlation with known protein coding genes and subsequent gene set enrichment analysis. For the selected lncRNAs, various functions were predicted, several of which are linked to T-cell biology or processes that are perturbed in cancer. This marks these lncRNAs as prime targets for further functional studies in order to unravel their mode of action and assess to what extent they might serve as future therapeutic targets for treatment of T-ALL.

Not all of the 40 overlapping lncRNAs displayed ICN1 binding, as is evident from the publicly available ChIP-seq data.[34] This may relate to the complexity of the chromosomal 3D-structures that are generated when lncRNAs act as cis-regulatory elements, as well as to the sensitivity of the Notch1 ChIP procedure. However, for the previously annotated lncRNAs directly bound by ICN1, all but one displayed Brd4 and Med1 binding. Those lncRNAs that are characterized by Brd4 and Med1 ChIP-seq signal are also characterized by the presence of H3K27ac ChIP-seq signal, which could be indicative of an enhancer activity of these loci.

A second aspect of our study involved the identification of novel, previously unannotated lncRNAs. Indeed, previous studies have shown that lncRNAs are often shown to be very restricted, but with biologically high relevant expression.[40-45] This includes expression during very specific time points during development and/or differentiation, as well as restriction to very specific cell subsets. Typically, these lncRNAs are expressed at significant levels in these cells whereas in other cell types their expression is very low or absent. Here, we identified novel lncRNAs in the CUTLL1 cell line and in the OP9-DLL1 co-culture system. Interestingly, 61 of these lncRNAs were present in T-ALL cells only, suggesting that their ectopic expression could be restricted to the malignant context. Likewise, lncRNAs only present in thymocytes may be implicated in differentiation of normal T cells which is disrupted in T-ALL cells, or may reflect differential Notch3 activity as the DLL1 ligand, to which $CD34^+$ progenitor T cells are exposed in the OP9-DLL1 co-culture system, can activate both Notch1 and Notch3 (*Waegemans E, Van de Walle I and Taghon T, unpublished data on preferential Notch receptor-ligand interactions in human, 2011*) while both receptors are implicated in modulating human T-cell development.[21] Both subsets of lncRNAs may, therefore, serve as novel therapeutic targets for T-ALL treatment.

It is evident that our work, as well as the recent paper by Aifantis and colleagues,[51] strongly favors an important role for lncRNAs in normal T-cell development and T-ALL oncogenesis. Moreover, we show that the Notch1 transcription factor directly controls the transcription of many of these long non-coding RNAs. Therefore, one can predict that other oncogenic transcription factors and drivers in T-ALL, such as TAL1, TLX1/TLX3, LMO1/2 and HOXA genes, as well as other transcriptional regulators of normal T-cell development, will also perform similar transcriptional control. Therefore, specific T-ALL subgroups and more distinct subsets of normal immature developing T cells need to be analyzed in human for detection of all lncRNAs. We predict that this will further dramatically expand the lncRNA landscape for T-ALL and thymocyte maturation, and thus provide an important regulatory framework for understanding some of the unique features that control human T-cell biology.[27] Finally, given the central role of oncogenic Notch1 activation in most if not all T-ALLs, and the current limitations of targeted therapy, further exploration of the new therapeutic opportunities offered by these lncRNAs in the context of their specific functionality is strongly recommended.

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References

1. Hori K, Sen A, Artavanis-Tsakonas S. Notch signaling at a glance. J Cell Sci. 2013;126(Pt 10):2135-40.
2. Aster JC. In brief: Notch signalling in health and disease. J Pathol. 2014;232(1):1-3.
3. Koch U, Radtke F. Notch signaling in solid tumors. Curr Top Dev Biol. 2010;92:411-55.
4. Ntziachristos P, Lim JS, Sage J, Aifantis I. From fly wings to targeted cancer therapies: a centennial for notch signaling. Cancer Cell. 2014;25(3):318-34.
5. Kumano K, Chiba S, Kunisato A, Sata M, Saito T, Nakagami-Yamaguchi E, et al. Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. Immunity. 2003;18(5):699-711.
6. Oh P, Lobry C, Gao J, Tikhonova A, Loizou E, Manent J, et al. In vivo mapping of notch pathway activity in normal and stress hematopoiesis. Cell Stem Cell. 2013;13(2):190-204.
7. Radtke F, MacDonald HR, Tacchini-Cottier F. Regulation of innate and adaptive immunity by Notch. Nat Rev Immunol. 2013;13(6):427-37.
8. Sandy AR, Maillard I. Notch signaling in the hematopoietic system. Expert Opin Biol Ther. 2009;9(11):1383-98.
9. Yuan JS, Kousis PC, Suliman S, Visan I, Guidos CJ. Functions of notch signaling in

the immune system: consensus and controversies. Annu Rev Immunol. 2010;28:343-65.
10. De Obaldia ME, Bell JJ, Wang X, Harly C, Yashiro-Ohtani Y, DeLong JH, et al. T cell development requires constraint of the myeloid regulator C/EBP-alpha by the Notch target and transcriptional repressor Hes1. Nat Immunol. 2013;14(12):1277-84.
11. Hozumi K, Mailhos C, Negishi N, Hirano K, Yahata T, Ando K, et al. Delta-like 4 is indispensable in thymic environment specific for T cell development. J Exp Med. 2008;205(11):2507-13.
12. Koch U, Fiorini E, Benedito R, Besseyrias V, Schuster-Gossler K, Pierres M, et al. Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. J Exp Med. 2008;205(11):2515-23.
13. Radtke F, Wilson A, Stark G, Bauer M, van Meerwijk J, MacDonald HR, et al. Deficient T cell fate specification in mice with an induced inactivation of Notch1. Immunity. 1999;10(5):547-58.
14. Wilson A, MacDonald HR, Radtke F. Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. J Exp Med. 2001;194(7):1003-12.
15. De Smedt M, Hoebeke I, Reynvoet K, Leclercq G, Plum J. Different thresholds of Notch signaling bias human precursor cells toward B-, NK-, monocytic/dendritic-, or T-cell lineage in thymus microenvironment. Blood. 2005;106(10):3498-506.
16. Wolfer A, Wilson A, Nemir M, MacDonald HR, Radtke F. Inactivation of Notch1 impairs VDJbeta rearrangement and allows pre-TCR-independent survival of early alpha beta Lineage Thymocytes. Immunity. 2002;16(6):869-79.
17. Ciofani M, Knowles GC, Wiest DL, von Boehmer H, Zuniga-Pflucker JC. Stage-specific and differential notch dependency at the alphabeta and gammadelta T lineage bifurcation. Immunity. 2006;25(1):105-16.
18. Garbe AI, von Boehmer H. TCR and Notch synergize in alphabeta versus gammadelta lineage choice. Trends Immunol. 2007;28(3):124-31.
19. Taghon T, Yui MA, Pant R, Diamond RA, Rothenberg EV. Developmental and molecular characterization of emerging beta- and gammadelta-selected pre-T cells in the adult mouse thymus. Immunity. 2006;24(1):53-64.
20. Van de Walle I, De Smet G, De Smedt M, Vandekerckhove B, Leclercq G, Plum J, et al. An early decrease in Notch activation is required for human TCR-alphabeta lineage differentiation at the expense of TCR-gammadelta T cells. Blood. 2009;113(13):2988-98.
21. Van de Walle I, Waegemans E, De Medts J, De Smet G, De Smedt M, Snauwaert S, et al. Specific Notch receptor-ligand interactions control human TCR-alphabeta/gammadelta development by inducing differential Notch signal strength. J Exp Med. 2013;210(4):683-97.
22. Ciofani M, Zuniga-Pflucker JC. Notch promotes survival of pre-T cells at the beta-selection checkpoint by regulating cellular metabolism. Nat Immunol. 2005;6(9):881-8.
23. Maillard I, Tu L, Sambandam A, Yashiro-Ohtani Y, Millholland J, Keeshan K, et al. The requirement for Notch signaling at the beta-selection checkpoint in vivo is absolute and independent of the pre-T cell receptor. J Exp Med. 2006;203(10):2239-45.
24. Taghon T, Van de Walle I, De Smet G, De Smedt M, Leclercq G, Vandekerckhove B, et al. Notch signaling is required for proliferation but not for differentiation at a well-defined beta-selection checkpoint during human T-cell development. Blood. 2009;113(14):3254-63.
25. Van Vlierberghe P, Ferrando A. The molecular basis of T cell acute lymphoblastic leukemia. J Clin Invest. 2012;122(10):3398-406.
26. Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science. 2004;306(5694):269-71.
27. Taghon T, Waegemans E, Van de Walle I. Notch signaling during human T cell development. Curr Top Microbiol Immunol. 2012;360:75-97.
28. Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell. 2009;137(2):216-33.
29. Dohda T, Maljukova A, Liu L, Heyman M, Grander D, Brodin D, et al. Notch signaling induces SKP2 expression and promotes reduction of p27Kip1 in T-cell acute lymphoblastic leukemia cell lines. Exp Cell Res. 2007;313(14):3141-52.
30. Joshi I, Minter LM, Telfer J, Demarest RM, Capobianco AJ, Aster JC, et al. Notch signaling mediates G1/S cell-cycle progression in T cells via cyclin D3 and its dependent kinases. Blood. 2009;113(8):1689-98.
31. Rao SS, O'Neil J, Liberator CD, Hardwick JS, Dai X, Zhang T, et al. Inhibition of NOTCH signaling by gamma secretase inhibitor engages the RB pathway and elicits cell cycle exit in T-cell acute lymphoblastic leukemia cells. Cancer Res. 2009;69(7):3060-8.
32. Gonzalez-Garcia S, Garcia-Peydro M, Martin-Gayo E, Ballestar E, Esteller M, Bornstein R, et al. CSL-MAML-dependent Notch1 signaling controls T lineage-specific IL-7R{alpha} gene expression in early human thymopoiesis and leukemia. J Exp Med. 2009;206(4):779-91.
33. Reizis B, Leder P. Direct induction of T lymphocyte-specific gene expression by the mammalian Notch signaling pathway. Genes Dev. 2002;16(3):295-300.
34. Wang H, Zang C, Taing L, Arnett KL, Wong YJ, Pear WS, et al. NOTCH1-RBPJ complexes drive target gene expression through dynamic interactions with superenhancers. Proc Natl Acad Sci USA. 2014;111(2):705-10.
35. Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, Maeda N, et al. The transcriptional landscape of the mammalian genome. Science. 2005;309(5740):1559-63.
36. Consortium EP, Birney E, Stamatoyannopoulos JA, Dutta A, Guigo R, Gingeras TR, et al. Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature. 2007;447(7146):799-816.
37. Djebali S, Davis CA, Merkel A, Dobin A, Lassmann T, Mortazavi A, et al. Landscape of transcription in human cells. Nature. 2012;489(7414):101-8.
38. Katayama S, Tomaru Y, Kasukawa T, Waki K, Nakanishi M, Nakamura M, et al. Antisense transcription in the mammalian transcriptome. Science. 2005;309(5740):1564-6.
39. Okazaki Y, Furuno M, Kasukawa T, Adachi J, Bono H, Kondo S, et al. Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature. 2002;420(6915):563-73.
40. Cabili MN, Trapnell C, Goff L, Koziol M, Tazon-Vega B, Regev A, et al. Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. Genes Dev. 2011;25(18):1915-27.
41. Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, et al. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature. 2009;458(7235):223-7.
42. Gutschner T, Diederichs S. The hallmarks of cancer: a long non-coding RNA point of view. RNA Biol. 2012;9(6):703-19.
43. Geisler S, Coller J. RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts. Nat Rev Mol Cell Biol. 2013;14(11):699-712.
44. Kung JT, Colognori D, Lee JT. Long noncoding RNAs: past, present, and future. Genetics. 2013;193(3):651-69.
45. Ulitsky I, Bartel DP. lincRNAs: genomics, evolution, and mechanisms. Cell. 2013;154(1):26-46.
46. Mavrakis KJ, Van Der Meulen J, Wolfe AL, Liu X, Mets E, Taghon T, et al. A cooperative microRNA-tumor suppressor gene network in acute T-cell lymphoblastic leukemia (T-ALL). Nat Genet. 2011;43(7):673-8.
47. Clappier E, Gerby B, Sigaux F, Delord M, Touzri F, Hernandez L, et al. Clonal selection in xenografted human T cell acute lymphoblastic leukemia recapitulates gain of malignancy at relapse. J Exp Med. 2011;208(4):653-61.
48. Volders PJ, Helsens K, Wang X, Menten B, Martens L, Gevaert K, et al. LNCipedia: a database for annotated human lncRNA transcript sequences and structures. Nucleic Acids Res. 2013;41(Database issue):D246-51.
49. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 2002;30(1):207-10.
50. Palomero T, Lim WK, Odom DT, Sulis ML, Real PJ, Margolin A, et al. NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. Proc Natl Acad Sci USA. 2006;103(48):18261-6.
51. Trimarchi T, Bilal E, Ntziachristos P, Fabbri G, Dalla-Favera R, Tsirigos A, et al. Genome-wide Mapping and Characterization of Notch-Regulated Long Noncoding RNAs in Acute Leukemia. Cell. 2014;158(3):593-606.
52. Vilimas T, Mascarenhas J, Palomero T, Mandal M, Buonamici S, Meng F, et al. Targeting the NF-kappaB signaling pathway in Notch1-induced T-cell leukemia. Nat Med. 2007;13(1):70-7.
53. Mets E, Van Peer G, Van der Meulen J, Boice M, Taghon T, Goossens S, et al. MicroRNA-128-3p is a novel oncomiR targeting PHF6 in T-cell acute lymphoblastic leukemia. Haematologica. 2014;99(8):1326-33.