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Gene expression profiling of precursor T-cell lymphoblastic leukemia/lymphoma identifies oncogenic pathways that are potential therapeutic targets

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Abstract

We compared the gene expression pattern of thymic tumors from precursor T-cell lymphoblastic lymphoma/leukemia (pre-T LBL) that arose in transgenic mice which over-expressed *SCL, LMO1*, or *NUP98-HOXD13 (NHD13)* with that of thymocytes from normal littermates. Only two genes, *Ccl8* and *Mrpl38*, were consistently more than 4-fold over-expressed in pre-T LBL from all three genotypes analyzed, and a single gene, *Prss16* was consistently under-expressed. However, we identified a number of genes, such as *Cfl1, Tcra, Tcrb, Pbx3, Eif4a, Eif4b*, and *Cox8b* that were over or under-expressed in pre-T LBL that arose in specific transgenic lines. Similar to the situation seen with human pre-T LBL, the *SCL/LMO1* leukemias displayed an expression profile consistent with mature, late cortical thymocytes, whereas the *NHD13* leukemias displayed an expression profile more consistent with immature thymocytes. We evaluated two of the most differentially regulated genes as potential therapeutic targets. *Cfl1* was specifically over-expressed in *SCL-LMO1* tumors; inactivation of *Cfl1* using Okadaic acid resulted in suppression of leukemic cell growth. Overexpression of *Ccl8* was a consistent finding in all 3 transgenic lines, and an antagonist for the *Ccl8* receptor induced death of leukemic cell lines, suggesting a novel therapeutic approach.

Keywords

T-cell leukemia; Scl; Nup98; chemokine; cofilin; Pbx3

Introduction

It has been shown that gene expression profiling is a useful technique for classification, subtype discovery, and prognosis in patients with precursor T-cell lymphoblastic leukemia/lymphoma (pre-T LBL) [1, 2]. Many pre-T LBL patients show chromosomal aberrations that result in the generation of fusion genes and/or the aberrant expression of proto-oncogenes. In addition, gene expression profiling and quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) has been shown to detect aberrantly expressed proto-oncogenes in the absence of chromosomal abnormalities. *SCL (TAL1), HOX11, LYL1, LMO1,* and *LMO2* are frequently over-expressed in patients with pre-T LBL [1, 2]. *SCL* was shown to be up-regulated in 49% of the cases with pre-T LBL and considered to be associated with relatively unfavorable prognosis. Additional studies have been employed to identify the important alterations in gene expression, as well as to identify mechanism(s) that lead to altered gene expression [3, 4].

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One advantage of studying genetically engineered mice is that the inciting event is known, which makes classification more straightforward. In order to identify candidate genes that might contribute to T-cell leukemogenesis in the context of aberrant expression of known T-cell oncogenes, we compared the gene expression profile of thymic tumors from pre-T LBL that arose in transgenic mice that over-expressed *SCL, LMO1,* and *NUP98-HOXD13* (*NHD13*) with that of thymocytes from normal littermates. We have proceeded to evaluate several of the candidate genes as potential therapeutic targets for treatment of pre-T LBL.

Materials and Methods

Mouse models for human pre-T LBL

LMO1 transgenic mice that over-express *LMO1* driven by the *lck* promoter were obtained from Dr. Stanley Korsmeyer[5]. *SCL* transgenic mice- that over-expresses *SCL* under control of the *SIL* promoter has been described previously[6]. *SCL-LMO1* double transgenic mice were generated by crossing the *SIL-SCL* mice with lck- *LMO1* mice[6, 7].

NHD13 transgenic mice that expresses a *NUP98-HOXD13* fusion from *vav* regulatory elements have been previously described[8]. Thymic tumors were harvested from clinically ill transgenic mice. Normal thymi were harvested from 40 non-transgenic littermates. Both thymic tumors and normal thymi were immediately frozen on dry ice and transferred to liquid nitrogen. In some cases, single cell suspensions of the thymic tumors were cultured in Iscove's Modified Durbecco's Medium, with 15% FBS[9] in order to establish pre-T LBL cell lines.

Microarray analysis

RNA was isolated from cryopreserved thymic tumors and normal thymocytes with Trizol reagent (Invitrogen, Carlsbad, CA) and purified with RNeasy MiniElute Cleanup kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purified RNA was assessed by electrophoresis through denaturing agarose gels to verify that it was not degraded. The RNA from 40 normal thymi were pooled and used as a reference RNA. First strand cDNA was synthesized and dye-coupled using a FairPlay Microarray labeling kit (Stratagene, La Jolla, CA). The experimental cDNA probe was labeled with Cy3 and the reference cDNA probe was labeled with Cy5. Dye-coupled cDNA was purified with a Qiagen Mini Elute PCR purification kit. The Cy3 labeled experimental probe was combined with the Cy5 labeled reference probe and the mixture was hybridized to an NCI production oligonucleotide DNA microarray containing 22272 long oligonucleotide (70 mer) features (Compugen, San Jose, CA). The microarray was scanned using an Axon GenePix scanner. The fluorescence ratio was quantified for each transcript and reflected the relative abundance of the gene in the experimental mRNA sample compared with the reference mRNA. Statistical analyses, hierarchical clustering, and gene ontology of the differentially-regulated genes were analyzed according to the NCI mAdb web site (http://nciarray.nci.nih.gov/). Briefly, the signal intensity was defined as by the mean pixel intensity minus median background pixel intensity. A log base 2 ratio of tumor to normal signal was calculated by dividing the tumor signal intensity by the normal thymus signal intensity. A raw gene list of those genes either 4 fold up-regulated or 4 fold down-regulated was obtained, and then curated by hand to eliminate duplicate genes. Any multiple occurrences of features were reduced to a single instance by selecting the feature with the strongest signal (Channel A + Channel B). In some cases, the identity of anonymous (such as the Riken collection) genes could be ascertained by comparing to the most recent Genbank relaease.

Okadaic acid and Ccr3 antagonist treatment

Leukemic cell lines established from the thymic tumors of *SCL-LMO1* and *NHD13* transgenic mice were cultured in the presence of the CCR3 antagonist, SB328437 (Sigma-Aldrich, St. Louis, MO), at the concentration of 69 μM for 72 hours, or vehicle (DMSO) alone. In separate

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experiments, *SCL-LMO1* or *NHD13* cell lines were treated with 1 µM okadaic acid (Sigma-Aldrich, St. Louis, MO) or vehicle (dH2O) alone for 4 hours. Viable cells were evaluated by Trypan blue exclusion.

Western blot analysis

Extracts of leukemic cell lines were prepared by lysing cells in HNTG buffer supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO) for one hour. Forty µg of whole cell lysate was separated on an 8% Tris-Glycine Gel (Invitrogen, Carlsbad, CA) at 125V for 90 minutes and then transferred to a nitrocellulose membrane. Membranes were blocked for one hour in SuperBlock Blocking buffer (Pierce, Rockford, IL) and placed in appropriate primary antibody dilution (in TBS, 5% Blotto, 2.5% Tween 20). Primary antibody (anti *Cfl1*, #3311, and anti phospho-*Cfl1*, #3312, Cell Signaling, Danvers, MA) was detected using horseradish peroxidase-linked goat anti-rabbit IgG antibodies and visualized using the chemiluminescent detection system (SuperSignal; Pierce, Rockford, IL).

Transfections of SCL-LMO1 pre-T LBL cell line

We transfected a *NHD13* expression vector into a pre-T LBL cell line (#6812) established from *SCL-LMO1* transgenic-mice using DMRIE-C reagent and the manufacturer's recommended protocol (Invitrogen, Carlsbad, CA). The *NHD13* expression vector used EF1alpha regulatory sequences and the *NHD13* cassette was identical to that previously reported[8].

RT-PCR

RNAs were extracted with Trizol reagent, and 1.0 µg of RNA was reverse transcribed using Superscript II reverse transcriptase with an oligo (dT) primer (Invitrogen, Carlsbad, CA). The first strand cDNAs were amplified with mouse *Pbx3F* primers 5'-CAATTATAGAGCAGGCAAGGTTCACCCTG-3' and mouse *Pbx3R* primers 5'-GTTCAGAGGTAGTAAGTGAGCGCTCTA-3' in the volume of 20 µl. After a "hot start" at 94°C, 35 cycles of 94°C for 1 min., 62°C for 1 min., and 72°C for 1 min. was used, followed by a terminal 10 min. extension at 72°C. PCR products were analyzed by agarose gel electrophoresis.

Results

Hierarchical Clustering of tumors obtained from different lines of transgenic mice

We used two-color hybridization of high density oligonucleotide microarrays containing 22272 features to compare the gene expression profiles of individual mouse tumors to a reference of normal thymus RNA. In order to produce a large supply of normal thymus reference RNA, 40 mice aged (3–6 months) were euthanized and the thymi harvested. We assayed 12 *SCL/LMO1*, 10 *NHD13*, and 4 *LMO1* only pre-T LBL samples, as well as 2 normal thymi. Hierarchical clustering of the arrays using a classical Pearson analysis revealed four major groups: *SCL/LMO1* tumors, normal thymus, *NHD13* tumors, and a fourth group consisting primarily of *LMO1* only tumors (Figure 1). All tumors of the same genotype clustered together, with the exception of two *NHD13* tumors (arrays ma18–40C and –43C, mouse # 1149 and 1901).

Differentially expressed genes and gene ontology (Table 1–Table 4)

Table 1 shows the genes that were most highly over- or under-expressed relative to normal thymus. There were only 12 genes whose mean expression levels were more than 4-fold increased when all three groups of tumors (*LMO1, SCL/LMO1,* and *NHD13*) were analyzed, and none of these were more than 7.5-fold increased. These represented a diverse group of genes including notch-signaling pathway (*Dtx1*), immune-system genes (*Iltifb, Igh, Ccl8,*

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Tcrb), polypeptide elongation factors (*Eef2, Eef-tu, and Mrpl38*), *Lmo1*, and *Ftl1*. Genes with the highest fold -decrease included 3 related fatty acid binding proteins (*Fabp4, Fabp9,* and *Fabp3*), apoptosis pathway genes (*Cox8b, Cidea*) and *CD83*. These results suggested that there were relatively few genes consistently over-expressed in tumors arising in all three transgenic lines compared to normal thymus.

Since tumors derived from mice with the same genotypes clustered closely together, we repeated the analysis of the most differentially expressed genes using tumors from each genotype individually. Table 2 shows genes that are more than 4-fold increased in the *SCL-LMO1* tumors compared to normal thymus. The two the most highly over-expressed genes were ferritin light chain (*Ftl1*) and cofilin 1 (*Cfl1*). Additional over-expressed genes included a large number that encoded proteins involved in the immune response (*Tcra, Tcrb, Igh, Iltifb, Ccl8, Tcf7*), cell proliferation and division (*Pin1, Cdk4, Map2k4, Cdc37,* and *Ctnnb1*), protein translation (*Eef1a1, Eef-tu, Eif4a1, Eef2, Eif5a, Tceal8, Eif4b, Mrpl38,* and *L13*), and *Notch* signaling (*Notch1* and *Dtx1*). Genes with the most significantly decreased expression again included apoptotic pathway genes (*Cox8b, Cox7a1, Ucp1,* and *Cidea*), genes encoding fatty acid binding proteins (*Fabp3, 4, 9*), and *CD83*.

Somewhat surprisingly, there was relatively little overlap between the most highly over-expressed genes in the *SCL-LMO1* set and the *LMO1* only set (Table 3). Of the 22 genes that were 4-fold increased in the *LMO1* tumors compared to normal thymus, only 5 (*Igh, Dtx1, Iltifb, Ccl8,* and *Mrpl38*) were also at least 4-fold increased in the *SCL-LMO1* tumors. There also was relatively little overlap between the *SCL-LMO1* group and the *LMO1* only group among the genes with the highest fold decrease compared to normal thymus. *Prss16, Tctex1, Cidea, Dstb,* and *Diras2* were 5 of the only genes that showed decreased expression in both groups. Parodoxically, several genes that were at least 4-fold increased in the *SCL-LMO1* set were at least 4-fold decreased in the *LMO1* set. These genes included *Tcrb* and *Tcra*.

The most highly over-expressed gene in the *NHD13* tumors was *Pbx3*, which was not over-expressed in any of the other groups of tumors. *Pbx3* overexpression was of interest since *Pbx3* is known to bind to *Hox* genes. Additional genes that were at least 4-fold over-expressed included *Ccl8, Tfrc, Mpo, Ctse*, and several genes involved in protein synthesis (*Mrpl38, Eef2*, and *Eif3s9*).

Ccl8 is important for growth of leukemic cells. (Fig. 2)

We focused on *Ccl8* since it was over-expressed in all three series of tumors [*SCL-LMO1* (5.0-fold), *LMO1* (10-fold), and the *NHD13* (5.4-fold) as well as *OLIG2-LMO1* tumors (8.2-fold) [10]], and therefore a candidate for a gene that was generally important for malignant transformation of thymocytes. Since Ccl8 functions through the chemokine receptor (Ccr3), we investigated whether *Ccr3* signaling was important for growth of the Ccl8-expressing cell lines. Murine pre-T LBL cell lines established from *SCL-LMO1* mice, *OLIG2-LMO1* mice, and *NHD13* mice were treated with the Ccr3 antagonist SB328437. As shown in Figure 2, SB328437 inhibits the growth of the *SCL-LMO1, OLIG2-LMO1*, and *NHD13* cell lines, whereas growth of a non-T cell control (murine erythroleukemic) cell line (F4–6) was unaffected.

Cfl1 phosporylation is associated with growth inhibition of SCL-LMO1 tumors. (Fig. 3)

Cfl1 was 10.9-fold up-regulated specifically in the *SCL-LMO1* tumors. Cfl1 has been shown to be phosphorylated and inactivated in peripheral T-lymphocytes. Activation through an accessory receptor leads to dephosphorylation of Cfl1 by PPA2[11]. Dephosphorylated Cfl1 can bind to polymeric F-actin and process it to mono- or oligo-meric G-actin, which can then gain access into the nucleus. In the nucleus, G-actin contributes to transcription via inhibition of

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DNAse I and activation of RNA polymerase II[12, 13]. Okadaic acid (OA) has been shown to specifically inhibit PPA2 so that Cfl1 is phosphorylated and inactivated[14]. In order to investigate whether overexpression of *Cfl1* is important for cell growth in *SCL-LMO1* tumors, we treated leukemic cell lines with OA at the concentration of 1 μM for 4 hours. A murine pre-T LBL cell line (6812), that was established from a *SCL-LMO1* thymic tumor, showed activated (dephosphorylated) Cfl1. OA treatment led to simultaneous suppression of cell growth and dephosphorylation of Cfl1 in the 6812 cell line. By way of comparison, a murine erythroleukemic cell line (F4–6), which expresses less Cfl1 than the 6812 cell line, was unaffected by OA treatment, either in terms of cell viability or Cfl1 dephosphorylation.

Pbx3 is a down-stream target of NHD13 (Fig. 4)

Pre-B cell leukemia transcription factors (PBXs) are important co-factors for the transcriptional regulation mediated by a number of Hox proteins[15]. *PBX1* was first identified in chromosomal translocations in B-lineage leukemia and is required for normal hematopoiesis. *PBX2* and *PBX3* were later identified as members of this highly conserved family by their strong homology to *PBX1*. We found that *Pbx3* was 8.3-fold up-regulated exclusively in the *NHD13* tumors. To investigate whether *Pbx3* is a downstream target of *NHD13*, we transfected an *NHD13* expression vector into a murine pre-T LBL cell line, 6812, which was established from an *SCL-LMO1* tumor that did not overexpress *Pbx3*. Two *NHD13* pre-T LBL primary tumors, 2975 and the 2413 expressed *Pbx3* and were used as controls. 6812 cells transfected with the *NHD13* expression vector showed an expression of *Pbx3* comparable to the *NHD13* primary thymic tumors, whereas 6812 cell transfected with the empty vector showed only low level *Pbx3* expression, supporting the hypothesis that expression of *NHD13* leads to up-regulation of *Pbx3*.

Discussion

In order to gain insight into the molecular events that lead to pre-T LBL, we compared the gene expression profile of murine pre-T LBL samples to that of the normal murine thymus. We chose to analyze tumors that were initiated by defined mutations (*SCL* and/or *LMO1* over-expression, or expression of a *NUP98-HOXD13* fusion gene). Our experimental approach was designed to identify two groups of differentially expressed genes. The first group of genes were those that were differentially expressed in all pre-T LBL samples, irrespective of the initiating mutation; these should be genes that are universally important for the malignant transformation of thymocytes. The second group of genes we were interested in identifying were those that were differentially expressed only in a specific subgroup of pre-T LBL (ie, only in *SCL/LMO1* mice, only in *NHD13* mice, etc). Genes identified in this manner should point to genes and pathways that might not be generally important for malignant transformation of thymocytes, but that are important for genetically defined subsets. Hierarchical clustering using a classical Pearson analysis indicated that the 3 groups of pre-T LBL tumors (*SCL/LMO1, LMO1* only, and *NHD13*) could be distinguished from one another, as well as from normal thymus.

We identified 12 genes whose mean expression levels were at least 4-fold over-expressed and 30 genes that were at least 4-fold under-expressed when sample from all of the different genotype groups were analyzed together. The over-expressed genes included several known to be involved in protein synthesis (*Eef2, eEF-Tu, Mrpl38*), Notch signaling (*Dtx1*), and *Ccl8*. Several classes of genes were commonly under-expressed in the pre-T LBL samples, including apoptotic pathway genes (*Cox8b, Cidea*)[16], genes involved in T-cell differentiation (*Prss16, Ccl25, CD83, Spatial, Fkbp6, and Tcrd*) [17–20], a group of fatty acid binding proteins (*Fabp3, Fabp4*, and *Fabp9*)[21], and *Bop1*[22] and *Rb1cc1*[23], two genes whose over-expression is linked to decreased cell proliferation. The Fabp proteins are typically expressed in

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adipocytes[21], and their under-expression in pre-T LBL relative to normal thymus may reflect a decreased proportion of adipocytes in the pre-T LBL tumor samples compared to normal thymus. Given the rapid rate at which the malignant thymocytes proliferate, it is not surprising that several of the over-expressed genes are known to be involved in new protein synthesis. Moreover, several of the genes under-expressed in pre-T LBL with respect to normal thymus were pro-apoptotic or associated with decreased cell proliferation. However, since we used the mean expression level to generate Table 1, it was possible that some of the genes were identified because they were highly up-regulated in one subset only. To investigate this possibility, we analyzed differential gene expression in specific genotypes. When each genotype was analyzed separately, we were surprised to find that only two genes (*Ccl8* and *Mrpl38*) were at least 4-fold over-expressed in all three genotypes, and a single gene (*Prss16*) was at least 4-fold under-expressed in all three genotypes.

We identified a number of genes that were over-expressed in *SCL/LMO1* tumors. Several of these genes were found to be over-expressed only in the *SCL/LMO1* tumors. These included *Ftl1, Cfl1, Tcrb, Tcra, Pin1*, and a large number of genes involved in protein synthesis (*Eef1a1, Eef-tu, Ef2, Eif4a1, Eif5a, Eif4b, Tceal8*, and *Rpl13*). Of note, *TCRA* and *TCRB* were two of the most differentially up-regulated genes in human leukemias that activated *SCL*[1]; these findings likely reflect a differentiation arrest at the late cortical stage of thymic development[1, 24]. Also, the identification of a large number of genes involved in protein synthesis, particularly *Eif4a1* and *Eif4b*, was of interest given recent studies showing activation of the EIF4 complex through *mTOR* signaling[25]. In addition, over-expression of *Eif5a* was of interest, as this is the only eukaryotic protein known to be activated by post-translational hypusination, and hypusination inhibitors have demonstrated an anti-proliferative effect on leukemic cell lines *in vitro*[26]. Several other genes, including *Dtx1, Iltifb, Igh*, were found to be over-expressed in both the *SCL/LMO1* and *LMO1* only tumors. Although we had predicted that the *SCL/LMO1* and *LMO1* only tumors would be fairly similar, the list of commonly over-expressed genes was relatively small. Moreover, *Tcra* and *Tcrb*, among the most highly over-expressed genes in the *SCL/LMO1* tumors were more than 4-fold under-expressed in the *LMO1* only tumors; this difference may reflect an earlier stage of thymocyte differentiation arrest in the *LMO1* tumors, prior to the expression of *Tcra* and *Tcrb*. Of note, this finding is consistent with prior observations that overexpression of a closely related gene (*LMO2*), blocks thymocyte differentiation at the CD4-/CD8- (DN) stage of differentiation[27, 28].

The genes most highly over-expressed in the *NHD13* pre-T LBL had little overlap with those most highly over-expressed in the *SCL/LMO1* and *LMO1* only tumors, except for *Mrpl38* and *Ccl8*, discussed above, and transferrin receptor (*Tfrc*). There were several genes over 4-fold under-expressed in both the *NHD13* and *LMO1*, but no the *SCL/LMO1* tumors. These genes included *Tcra, Tcrb, H2-A, Crip3, Ubd*, and *Diras2*, and, similar to the case with human pre-T LBL that overexpress *HOX11*[1, 3], likely reflect differentiation arrest at an earlier stage of thymocyte differentiation, prior to expression of *Tcra* and *Tcrb*[24]. The overexpression of *Mpo*, a gene typically expressed in myeloid cells, in the *NHD13* tumors may reflect a biphenotypic differentiation potential in *NHD13* pre-T LBL, a possibility consistent with the finding that *NHD13* mice typically develop a MDS, which often progresses to an AML. Alternatively, the *Mpo* expression might be explained by the contamination of small number of myeloid leukemic cells that were seeded from the co-incident MDS present in the *NHD13* mice.

We were intrigued by the observation that *Ccl8* was consistently over-expressed, and hypothesized that *Ccl8* might be an autocrine growth factor for pre-T LBL. Since *Ccl8* exerts its effects through the Ccr3 receptor, we reasoned that inhibition of the Ccr3 receptor might suppress the growth of pre-T LBL cell lines. We verified that the Ccr3 receptor was expressed in pre-T LBL cell lines, and treated four pre-T LBL cell lines, derived from *SCL-LMO1,*

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OLIG2-LMO1, or *NHD13* tumors with a Ccr3 antagonist (SB328437). All four of these pre-T LBL cell lines demonstrated growth inhibition, by as much as 85%, suggesting that treatment with a Ccr3 antagonist might be an effective anti-leukemic therapy.

Genes whose expression levels were altered specifically in *SCL-LMO1* tumors represent candidates for genes important in malignant transformation of these cells. Of those candidates, *Cfl1* was 10.9-fold up-regulated. Since Cfl1 has been shown to be an inhibitor of glucocorticoid receptor that is consistent with the relative resistance of pre-T LBL overexpressing *SCL* compared to pre-T LBL with other genetic alterations[1, 29, 30], we investigated whether the up-regulation of the *Cfl11* is important for proliferation of leukemic cell lines derived from *SCL-LMO1* mice. It has been shown that both Ras and a costimulation of TCR/CD3 and CD28 activate MAPK/ERK kinase and PI3K, which induces the dephosphorylation of cofilin1. An activation of PI3K by stimulation through CD28 also down-regulates a cyclin-dependent-kinase inhibitor $p27^{kip1}$[31]. Those cascades lead to the production of cytokines such as IL-2 and subsequent proliferation of T-lymphocytes[31, 32]. OA, an inhibitor for the serine/threonine phosphatase type2A that phosphorylates cofilin1, treatment demonstrated inhibition of leukemic cell growth, suggesting that activation of Cfl1 is important for growth of leukemic cell lines that overexpress *SCL* and *LMO1*. Similarly, *Pbx3* was specifically up-regulated in *NHD13* pre-T LBL; transfection of T-cell lines with an *NHD13* expression vector led to upregulation of *Pbx3*, suggesting that *Pbx3* may be a direct downstream target of *NHD13*.

We have used gene expression profiling to identify genes and pathways that may be important for malignant transformation of T-cells in general, as well as those genes and pathways that may be important for transformation of T-cells that express known oncoproteins. We found relatively few genes consistently over or under-expressed in pre-T LBL compared to normal thymus, and a larger number of genes that may be important for transformation of thymocytes that express known oncoproteins. Similar to findings with human pre-T LBL, we suspect that the differences in gene expression profile seen in different genetically defined subsets of pre-T LBL may reflect different stages of thymocyte maturation arrest. We tested and confirmed several of the genes that were candidates for general or specific involvement in the malignant transformation of thymocytes, and have identified Ccr3, the Ccl8 receptor, as a novel potential target for treatment of pre-T LBL.

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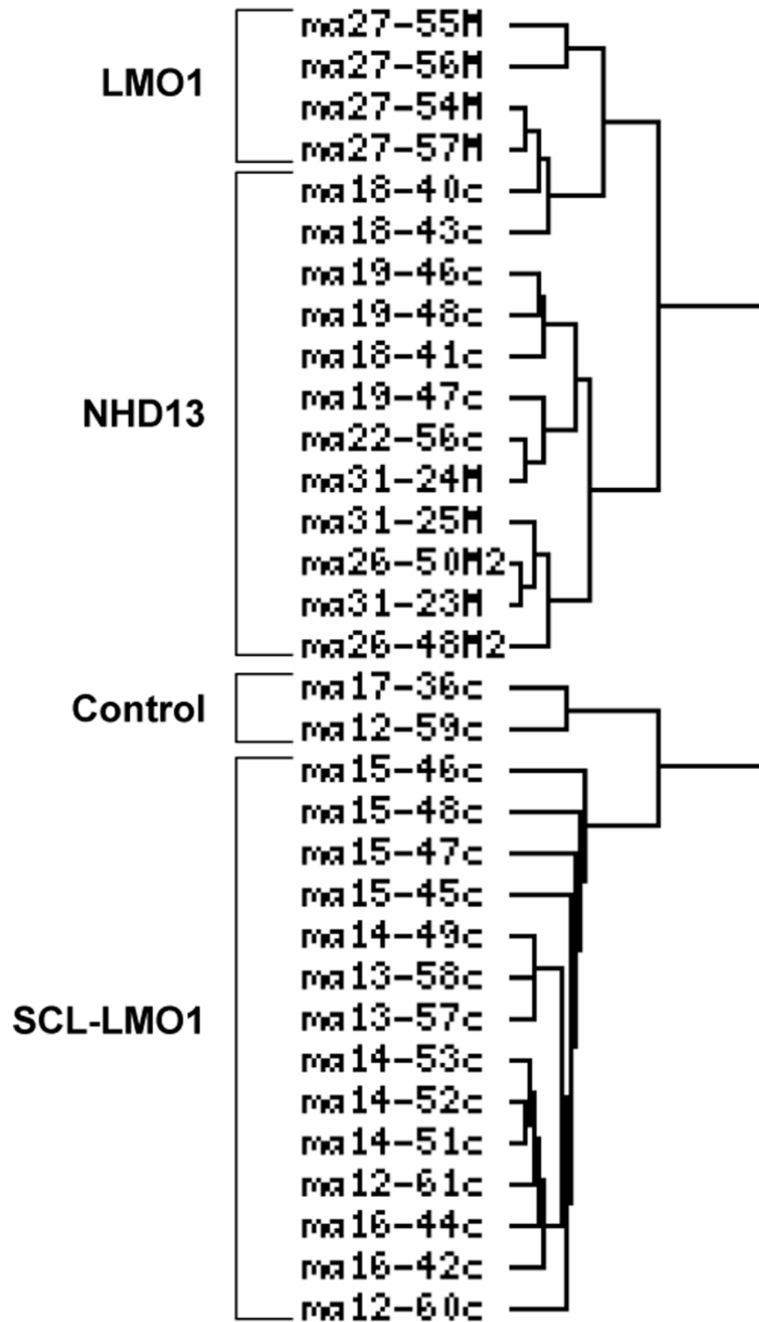

Figure 1. Hierarchical clustering of murine pre-T LBL gene expression profiles

Hierarchical clustering was determined using the classical Pearson method. The first hierarchical break distinguished a group of the *LMO1* and *NHD13* tumors from the group of the *SCL-LMO1* tumors and normal thymus.

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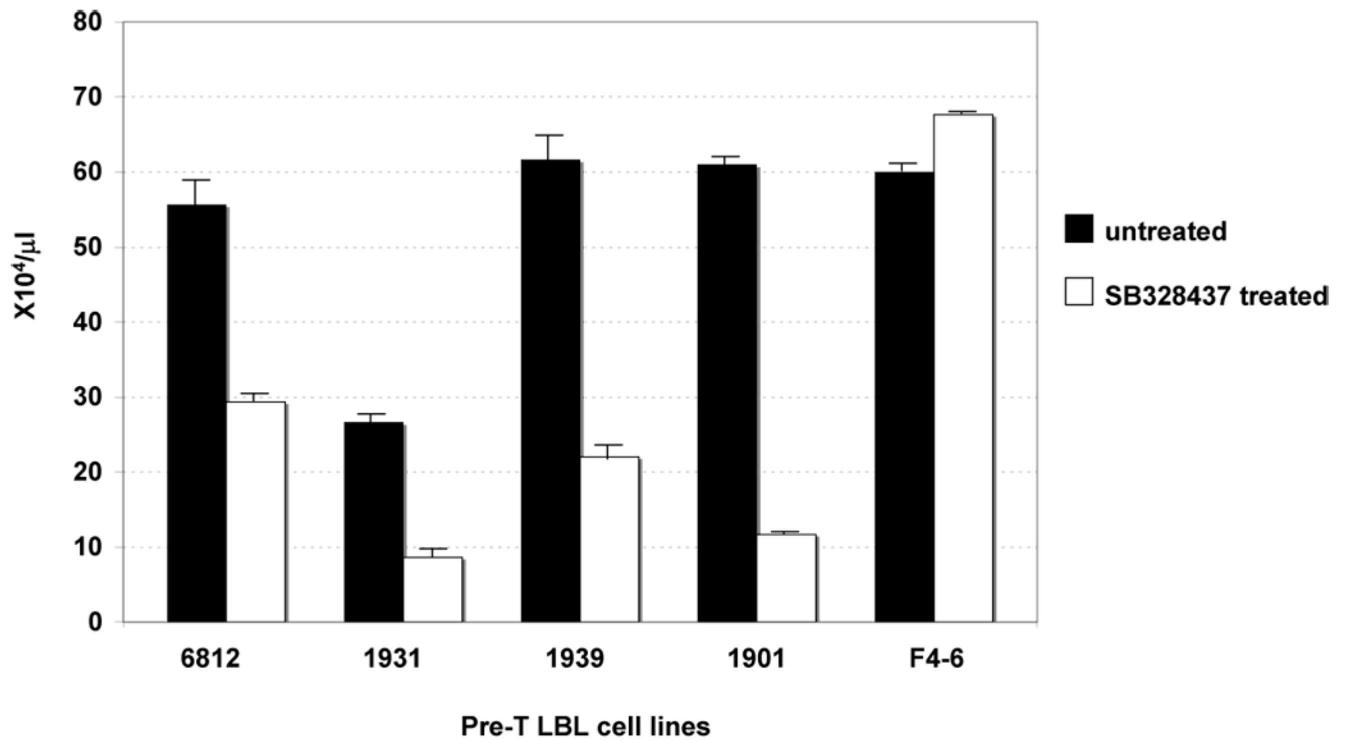

Figure 2. Chemokine ligand 8 is important for leukemic cell survival

Murine pre-T LBL cell lines were treated with the Ccr3 antagonist, SB328437, at the concentration of 69 μM for 72 hours. #6812 and 1901 are pre-T LBL cell lines established from *SCL-LMO1* and *NHD13* mice, respectively. #1931 and 1939 are pre-T LBL cell lines established from *OLIG2-LMO1* mice. F4–6 is a Friend virus-induced murine erythroleukemia cell line.

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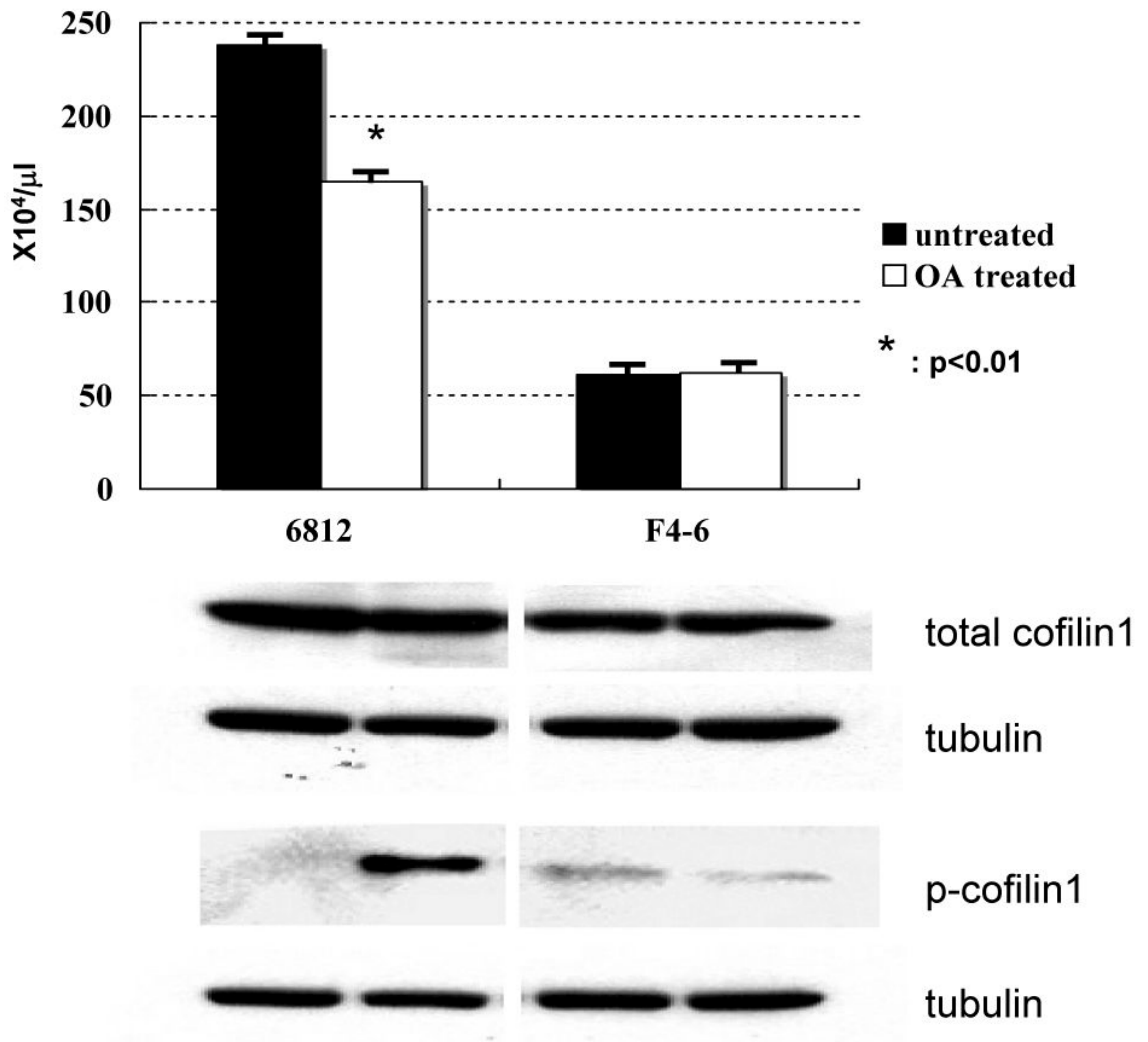

Figure 3. Cofilin1 is important for growth of *SCL-LMO1* pre-T LBL

Treatment of the *SCL-LMO1* pre-T LBL cell line #6812 with Okadaic acid resulted in the suppression of cell growth and an increased proportion of inactivate phospho-cofilin1.

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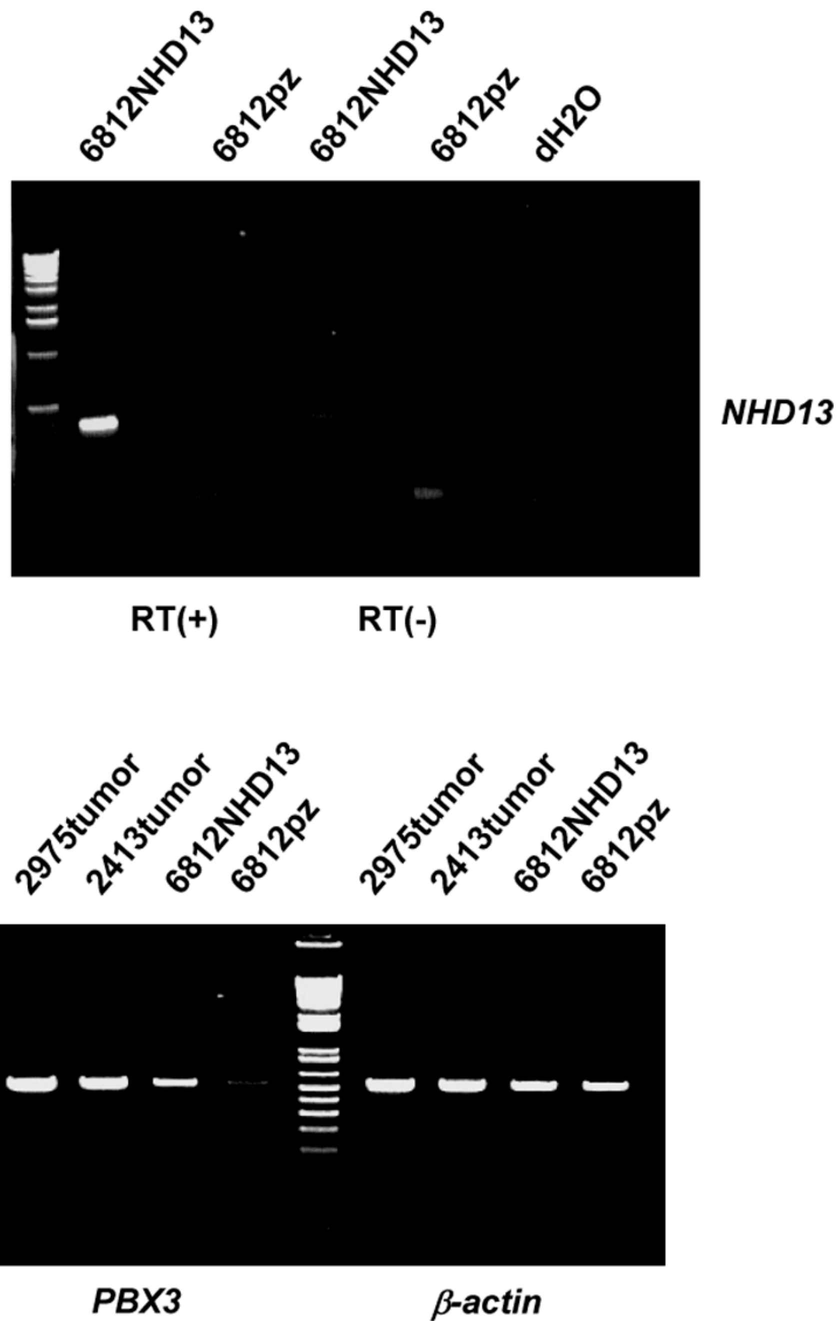

Figure 4. Pbx3 is a downstream target of *NHD13*

Upper panel. A pre-T LBL cell line established from an *SCL-LMO1* mouse (#6812) was transfected with an *NHD13* expression vector or empty vector (lanes 6812*NHD13* and 6812pz respectively). Expression of *NHD13* was detected by RT-PCR; RT(+) and RT (−) indicate presence or absence or reverse transcriptase respectively. **Lower panel.** *PBX3* is up-regulated in the sample transfected with the NHD13 expression vector. 2975 and 2413 are two primary *NHD13* tumors used as positive controls for *PBX3* expression. β-*actin* amplification is used as an RNA quality control.

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Table 1.

Genes most differentially expressed in T-cell tumors compared to normal thymus

Fold increase	Gene	Description
7.5	*Iltifb*	interleukin 10-related T cell-derived inducible factor beta (Iltifb),
7.2	*Dtx1*	deltex 1 homolog (Drosophila) (Dtx1), mRNA.
6.7	*Igh-6*	Immunoglobulin heavy chain 6 (heavy chain of IgM)
5.8	*Ccl8*	chemokine (C-C motif) ligand 8 (Ccl8), mRNA.
5.5	*Tcrb*	T-cell receptor beta-1 chain C region (LOC669847), mRNA.
5.3	*Mrpl38*	mitochondrial ribosomal protein L38 (Mrpl38), mRNA.
4.3	*Gapdh*	glyceraldehyde-3-phosphate dehydrogenase (LOC14433),.
4.3	*Eef2*	eukaryotic translation elongation factor 2 (Eef2), mRNA.
4.3	*Lmo1*	LIM domain only 1 (Lmo1), mRNA.
4.3	*Tubb5*	tubulin, beta 5 (Tubb5), mRNA.
4.2	*eEF-Tu*	Mouse eEF-Tu gene encoding elongation factor Tu, 5′ end.
4.2	*Ftl1*	ferritin light chain 1 (Ftl1), mRNA.

Fold decrease	Gene	Description
4.0	*Arpp21*	cyclic AMP-regulated phosphoprotein, 21 (Arpp21)
4.1	*Ly49n*	Mus musculus natural killer cell receptor (Ly49n) gene
4.2	*Cbr2*	carbonyl reductase 2 (Cbr2), mRNA.
4.2	*Bop1*	block of proliferation 1 (Bop1), mRNA.
4.3	*Tcrd*	T-cell receptor delta
4.3	*Fabp3*	fatty acid binding protein 3, muscle and heart (Fabp3), mRNA.
4.3	*Lcn5*	lipocalin 5 (Lcn5), mRNA.
4.4	*1700084E18Rik*	RIKEN cDNA 1700084E18 gene (1700084E18Rik)
4.4	*Rb1cc1*	RB1-inducible coiled-coil 1 (Rb1cc1), mRNA.
4.5	*Scnn1a*	sodium channel, nonvoltage-gated, type I, alpha (Scnn1a)
4.6	*Fkbp6*	FK506 binding protein 6 (Fkbp6), mRNA.
4.7	*Hba-a1*	hemoglobin alpha, adult chain 1 (Hba-a1), mRNA.
4.7	*Ctsl*	cathepsin L (Ctsl), mRNA.
4.8	*Cstb*	cystatin B (Cstb), mRNA.
4.8	*Slc18a3*	solute carrier family 18, member 3 (Slc18a3)
4.9	*LOC674927*	PREDICTED: similar to melanoma antigen (LOC674927)
5.4	*Erf1*	Mus musculus ETS-related transcription factor ERF (Erf1)
5.4	*Cdo1*	cysteine dioxygenase 1, cytosolic (Cdo1), mRNA.
5.7	*Diras2*	DIRAS family, GTP-binding RAS-like 2 (Diras2), mRNA.
5.7	*Tctex1d1*	Tctex1 domain containing 1 (Tctex1d1), mRNA.
5.8	*Spatial*	Mus musculus fetal thymus spatial protein mRNA
6.1	*Dgat2*	diacylglycerol O-acyltransferase 2 (Dgat2), mRNA.
6.4	*Cidea*	cell death-inducing DNA fragmentation factor, alpha subunit
6.5	*Fabp9*	Fatty acid binding protein 9, testis
6.6	*Cd83*	CD83 antigen (Cd83), mRNA.
7.0	*Krt2-8*	keratin complex 2, basic, gene 8 (Krt2-8), mRNA.
7.3	*Fabp4*	Fatty acid binding protein 4, adipocyte
7.3	*Cox8b*	cytochrome c oxidase, subunit VIIIb (Cox8b), mRNA.
14.5	*Ccl25*	Chemokine (C-C motif) ligand 25
22.4	*Prss16*	protease, serine, 16 (thymus) (Prss16), mRNA.

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Table 2

Genes most differentially expressed in SCL-LMO1 T-cell tumors

Fold increase	Gene	Description
15.6	*Ftl1*	ferritin light chain 1 (Ftl1), mRNA.
15.0	*Cfl1*	cofilin 1, non-muscle (Cfl1), mRNA.
11.5	*Tcrb*	T cell receptor beta chain mRNA, partial cds.
11.0	*Hspa8*	heat shock protein 8 (Hspa8), mRNA.
11.0	*Lmo1*	LIM domain only 1 (Lmo1), mRNA.
10.9	*Eef1a1*	Eukaryotic translation elongation factor 1 alpha 1
10.7	*Tuba7*	tubulin, alpha 7 (Tuba7), mRNA.
10.4	*Eef-tu*	Mouse eEF-Tu gene encoding elongation factor Tu
9.4	*Ef2*	Elongation factor 2 (EF-2) (LOC673429), mRNA.
9.2	*Igh-6*	Immunoglobulin heavy chain 6 (heavy chain of IgM)
8.9	*Gapdh*	glyceraldehyde-3-phosphate dehydrogenase
8.4	*Dtx1*	deltex 1 homolog (Drosophila) (Dtx1), mRNA.
8.4	*Cnbp2*	cellular nucleic acid binding protein 2 (Cnbp2), mRNA.
8.2	*Tubb5*	tubulin, beta 5 (Tubb5), mRNA.
8.0	*Zmym1*	zinc finger, MYM domain containing 1 (Zmym1), mRNA.
7.9	*Siglece*	sialic acid binding Ig-like lectin E (Siglece), mRNA.
7.8	*Eif4a1*	eukaryotic translation initiation factor 4A1 (Eif4a1), mRNA.
7.6	*Bzw2*	basic leucine zipper and W2 domains 2 (Bzw2), mRNA.
7.5	*Eef2*	eukaryotic translation elongation factor 2 (Eef2), mRNA.
7.2	*Eif5a*	eukaryotic translation initiation factor 5A (Eif5a), mRNA.
6.8	*Tcf7*	transcription factor 7, T-cell specific (Tcf7), mRNA.
6.3	*Aes*	amino-terminal enhancer of split (Aes), mRNA.
6.3	*Iltifb*	interleukin 10-related T cell-derived inducible factor beta (Iltifb)
6.3	*Tcra*	T-cell receptor alpha chain (TCRA)
6.0	*Coro1a*	coronin, actin binding protein 1A (Coro1a), mRNA.
5.5	*Pin1*	protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1 (Pin1), mRNA.
5.4	*Cdk4*	cyclin-dependent kinase 4 (Cdk4), mRNA.
5.4	*Tceal8*	Transcription elongation factor A (SII)-like 8
5.3	*Map2k4*	mitogen activated protein kinase kinase 4 (Map2k4), mRNA.
5.1	*Eif4b*	eukaryotic translation initiation factor 4B (Eif4b), mRNA.
5.0	*Ccl8*	chemokine (C-C motif) ligand 8 (Ccl8), mRNA.
4.8	*Dap*	death-associated protein (Dap), mRNA.
4.7	*Dennd2d*	DENN/MADD domain containing 2D (Dennd2d), mRNA.
4.5	*Mrpl38*	mitochondrial ribosomal protein L38 (Mrpl38), mRNA.
4.3	*Rpl13*	Ribosomal protein L13
4.3	*Hnrpu*	heterogeneous nuclear ribonucleoprotein U (Hnrpu), mRNA.
4.3	*Notch1*	Notch gene homolog 1 (Drosophila)
4.2	*Hnrpl*	heterogeneous nuclear ribonucleoprotein L (Hnrpl), mRNA.
Fold decrease	**Gene**	**Description**
6.1	*Cd83*	CD83 antigen (Cd83), mRNA.
6.1	*Prr6*	Proline-rich polypeptide 6
6.4	*Erf1*	Mus musculus ETS-related transcription factor ERF (Erf1) mRNA
6.5	*Krt2-8*	keratin complex 2, basic, gene 8 (Krt2-8), mRNA.
6.6	*Mgst1*	microsomal glutathione S-transferase 1 (Mgst1), mRNA.
6.6	*Ucp1*	uncoupling protein 1 (mitochondrial, proton carrier) (Ucp1), mRNA.
6.8	*Fabp3*	fatty acid binding protein 3, muscle and heart (Fabp3), mRNA.
6.9	*Diras2*	DIRAS family, GTP-binding RAS-like 2 (Diras2), mRNA.
7.0	*Cox7a1*	cytochrome c oxidase, subunit VIIa 1 (Cox7a1), mRNA.
8.5	*Slc18a3*	solute carrier family 18 (vesicular monoamine),
10.7	*Fabp4*	Fatty acid binding protein 4, adipocyte
11.3	*Cdo1*	cysteine dioxygenase 1, cytosolic (Cdo1), mRNA.
11.3	*Cox8b*	cytochrome c oxidase, subunit VIIIb (Cox8b), mRNA.
11.6	*Fabp9*	Fatty acid binding protein 9, testis
12.1	*Prss16*	protease, serine, 16 (thymus) (Prss16), mRNA.
12.3	*Cidea*	cell death-inducing DNA fragmentation factor, alpha subunit
14.3	*Dgat2*	diacylglycerol O-acyltransferase 2 (Dgat2), mRNA.
15.7	*Ccl25*	Chemokine (C-C motif) ligand 25
20.3	*Snap25*	Synaptosomal-associated protein 25

* some genes between 4 and 6-fold upregulated not listed due to space limitations

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Table 3.

Genes most differentially regulated in LMO1 tumors

Fold increase	Gene	Description
107.8	*Iltifb*	interleukin 10-related T cell-derived inducible factor beta (Iltifb), mRNA.
24.7	*Igh-6*	Immunoglobulin heavy chain 6 (heavy chain of IgM)
17.4	*Bst1*	bone marrow stromal cell antigen 1 (Bst1), mRNA.
9.9	*Ccl8*	chemokine (C-C motif) ligand 8 (Ccl8), mRNA.
8.0	*Gzma*	granzyme A (Gzma), mRNA.
7.5	*Mrpl38*	mitochondrial ribosomal protein L38 (Mrpl38), mRNA.
6.8	*Dennd2d*	DENN/MADD domain containing 2D (Dennd2d), mRNA.
6.7	*Il9*	interleukin 9 (Il9), mRNA.
5.4	*Ccdc18*	coiled-coil domain containing 18 (Ccdc18), mRNA.
5.0	*Dtx1*	deltex 1 homolog (Drosophila) (Dtx1), mRNA.
4.7	*Adam19*	a disintegrin and metallopeptidase domain 19 (meltrin beta) (Adam19)
4.6	*Il12rb2*	interleukin 12 receptor, beta 2 (Il12rb2), mRNA.
4.4	*Hdgfrp3*	hepatoma-derived growth factor, related protein 3 (Hdgfrp3), mRNA.
4.2	*Aldh1b1*	aldehyde dehydrogenase 1 family, member B1 (Aldh1b1), mRNA.
4.2	*Pabpc1*	poly A binding protein, cytoplasmic 1 (Pabpc1), mRNA.
4.2	*Ck2*	Mus musculus casein kinase 2 beta subunit (gMCK2) gene
4.2	*Dpp4*	dipeptidylpeptidase 4 (Dpp4), mRNA.
4.1	*Flnc*	PREDICTED: filamin C, gamma (actin binding protein 280), transcript variant 3 (Flnc), mRNA.
4.1	*Igk-V23*	Immunoglobulin kappa chain variable 23 (V23)
4.1	*Ifi205*	interferon activated gene 205 (Ifi205), mRNA.
4.0	*Hes1*	hairy and enhancer of split 1 (Drosophila) (Hes1), mRNA.
4.0	*Tfrc*	transferrin receptor (Tfrc), mRNA.
Fold decrease	**Gene**	**Description**
4.0	*Anxa2*	annexin A2 (Anxa2), mRNA.
4.0	*Laptm4b*	lysosomal-associated protein transmembrane 4B (Laptm4b), mRNA.
4.0	*Nrbp*	nuclear receptor binding protein (Nrbp), mRNA.
4.1	*Cstb*	cystatin B (Cstb), mRNA.
4.1	*Igfbpl1*	Insulin-like growth factor binding protein-like 1
4.1	*Ctdsp2*	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 2
4.2	*Krt2-6a*	Keratin complex 2, basic, gene 6a
4.2	*Gtf2h4*	general transcription factor II H, polypeptide 4 (Gtf2h4), mRNA.
4.3	*Rb1cc1*	RB1-inducible coiled-coil 1 (Rb1cc1), mRNA.
4.4	*Plxdc2*	plexin domain containing 2 (Plxdc2), mRNA.
4.5	*Cidea*	cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (Cidea), mRNA.
4.9	*Arhgef1*	Rho guanine nucleotide exchange factor (GEF) 1 (Arhgef1), mRNA.
4.9	*Cxcl11*	chemokine (C-X-C motif) ligand 11 (Cxcl11), mRNA.
4.9	*Diras2*	DIRAS family, GTP-binding RAS-like 2 (Diras2), mRNA.
5.0	*H2-Ab1*	histocompatibility 2, class II antigen A, beta 1 (H2-Ab1), mRNA.
5.2	*Zfp236*	Zinc finger protein 236
5.2	*S100a16*	S100 calcium binding protein A16 (S100a16), mRNA.
5.4	*Plxnd1*	Plexin D1
5.5	*LOC674927*	PREDICTED: similar to melanoma antigen (LOC674927), mRNA.
5.5	*Ubd*	ubiquitin D (Ubd), mRNA.
5.8	*Serinc3*	serine incorporator 3 (Serinc3), mRNA.
6.0	*Tcrd*	T-cell receptor delta
6.2	*5830406J20Rik*	RIKEN cDNA 5830406J20 gene (5830406J20Rik), mRNA.
6.2	*Cox6a2*	cytochrome c oxidase, subunit VI a, polypeptide 2 (Cox6a2), mRNA.
6.7	*A430035B10Rik*	RIKEN cDNA A430035B10 gene
7.0	*Coro1a*	coronin, actin binding protein 1A (Coro1a), mRNA.
7.1	*Cbr2*	carbonyl reductase 2 (Cbr2), mRNA.
7.2	*Itk*	IL2-inducible T-cell kinase
7.3	*Tcra*	T-cell receptor alpha chain V region CTL-F3 precursor
7.6	*Tceal8*	Transcription elongation factor A (SII)-like 8
7.9	*LOC14433*	similar to glyceraldehyde-3-phosphate dehydrogenase (LOC14433), mRNA.
10.4	*Tcrb*	T-cell receptor beta, variable 13
10.7	*Crip3*	cysteine-rich protein 3 (Crip3), transcript variant TLP-B, mRNA.
10.9	*Tctex1d1*	Tctex1 domain containing 1 (Tctex1d1), mRNA.
55.2	*Prss16*	protease, serine, 16 (thymus) (Prss16), mRNA.

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Table 4

Genes most differentially regulated in NHD13 tumors

Fold increase	Gene	Description
12.9	*Pbx3*	pre B-cell leukemia transcription factor 3 (Pbx3), mRNA.
6.4	*Cox6a2*	cytochrome c oxidase, subunit VI a, polypeptide 2 (Cox6a2), mRNA.
5.6	*Mrpl38*	mitochondrial ribosomal protein L38 (Mrpl38), mRNA.
5.6	*Eef2*	eukaryotic translation elongation factor 2 (Eef2), mRNA.
5.4	*Ccl8*	chemokine (C-C motif) ligand 8 (Ccl8), mRNA.
5.3	*Gapdh*	glyceraldehyde-3-phosphate dehydrogenase (LOC14433), mRNA.
5.2	*Ctse*	cathepsin E (Ctse), mRNA.
4.8	*Tfrc*	transferrin receptor (Tfrc), mRNA.
4.4	*Eif3s9*	eukaryotic translation initiation factor 3, subunit 9 (eta) (Eif3s9), mRNA.
4.2	*Mpo*	myeloperoxidase (Mpo), mRNA.
4.1	*H2-D1*	Histocompatibility 2, D region locus 1
4.0	*Farsla*	phenylalanine-tRNA synthetase-like, alpha subunit (Farsla), mRNA.
Fold decrease	**Gene**	**Description**
4.0	*Spo11*	sporulation protein, meiosis-specific, SPO11 homolog (S. cerevisiae)
4.1	*Ubd*	ubiquitin D (Ubd), mRNA.
4.1	*Tcra*	T-cell receptor alpha.
4.2	*Gtf2h4*	general transcription factor II H, polypeptide 4 (Gtf2h4), mRNA.
4.5	*Cfd*	complement factor D (adipsin) (Cfd), mRNA.
4.5	*1700084E18Rik*	PREDICTED: RIKEN cDNA 1700084E18 gene (1700084E18Rik), mRNA.
4.6	*Fabp9*	Fatty acid binding protein 9, testis
4.6	*Ly49n*	Mus musculus natural killer cell receptor (Ly49n) gene
4.7	*Vamp1*	vesicle-associated membrane protein 1 (Vamp1), mRNA.
4.7	*Diras2*	DIRAS family, GTP-binding RAS-like 2 (Diras2), mRNA.
4.9	*Ccni*	Cyclin I
4.9	*9130430L19Rik*	RIKEN cDNA 9130430L19 gene
5.8	*H2-Aa*	histocompatibility 2, class II antigen A, alpha (H2-Aa), mRNA.
6.0	*Erf1*	Mus musculus ETS-related transcription factor ERF (Erf1) mRNA.
6.3	*Fabp4*	Fatty acid binding protein 4, adipocyte
6.5	*5830406J20Rik*	RIKEN cDNA 5830406J20 gene (5830406J20Rik), mRNA.
6.6	*Cd8a*	CD8 antigen, alpha chain, transcript variant 1 (Cd8a), mRNA.
6.8	*Arpp21*	cyclic AMP-regulated phosphoprotein, 21 (Arpp21)
6.9	*Crip3*	cysteine-rich protein 3 (Crip3), transcript variant TLP-B, mRNA.
7.2	*Cd74*	CD74 antigen
7.4	*Dntt*	deoxynucleotidyltransferase, terminal (Dntt), mRNA.
8.8	*Igh-6*	Immunoglobulin heavy chain 6 (heavy chain of IgM)
8.9	*Cd83*	CD83 antigen (Cd83), mRNA.
9.5	*Tctex1d1*	Tctex1 domain containing 1 (Tctex1d1), mRNA.
11.4	*Tcrb-V13*	T-cell receptor beta, variable 13
11.7	*H2-Eb1*	histocompatibility 2, class II antigen E beta (H2-Eb1), mRNA.
12.2	*LOC545854*	Immunoglobulin kappa chain
16.3	*LOC674927*	PREDICTED: similar to melanoma antigen (LOC674927), mRNA.
17.6	*Ccl25*	Chemokine (C-C motif) ligand 25
32.8	*Prss16*	protease, serine, 16 (thymus) (Prss16), mRNA.