Published in final edited form as:

Leuk Res. 2011 March ; 35(3): 405–412. doi:10.1016/j.leukres.2010.07.023.

The expansion of T-cells and hematopoietic progenitors as a result of overexpression of the Lymphoblastic leukemia gene, *Lyl1* can support leukemia formation

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Abstract

This study investigates the function of the Lymphoblastic leukemia gene, *Lyl1* in the hematopoietic system and its oncogenic potential in development of leukemia. Overexpression of *Lyl1* in mouse bone marrow cells caused T-cell increase in the peripheral blood and expansion of the hematopoietic progenitors in culture and in the bone marrow. These observations were the result of increased proliferation and suppressed apoptosis of the progenitor cells caused by the *Lyl1*-overexpression. Our studies present substantial evidence supporting the secondary, pro-leukemic effect of *Lyl1* in early hematopoietic progenitors with the potential to cause expansion of malignant cells with a stem/early-progenitor-like phenotype.

Keywords

Lyl1; leukemia; apoptosis; T-cell ALL; AML; T-cells; hematopoietic progenitors

Introduction

The lymphoblastic leukemia 1 (*Lyl1*) gene is a transcription factors containing the basic helix-loop-helix (bHLH) motif [1]. It is related to its highly studied paralog *Tall1/Scl* [2-4].

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Authors' Contributions

G.L.L.: designed and performed experiments, analyzed data and wrote the manuscript; L.R.: designed and performed experiments, analyzed data, wrote segments of the material and methods section and review the manuscript; G.S.: designed and performed experiments; R.M.: performed experiments. M.A.G.: designed experiments, analyzed data, critically review the manuscript and gave the final approval for submission.

Conflict of Interest

The authors declare no financial conflict of interest.

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The bHLH motif facilitates DNA binding via its basic region and protein-protein dimerization via its HLH domain [5,6]. *Lyl1* knock-out mice are viable with reduced populations of hematopoietic stem and progenitor cells, and B-cells [3]. *Lyl1* is not essential for embryonic development, in contrast to *Scl*, which is required for blood formation and blood vessel development during embryogenesis [7,8]. However, in adult mice, *Lyl1* is important in hematopoietic progenitors, and its presence is critical for hematopoietic stem cell (HSC) survival in the absence of *Scl* [9], suggesting functional overlap between the two paralogs. Deletion of both *Lyl1* and *Scl* leads to rapid apoptosis of hematopoietic progenitors.

Lyl1 is expressed broadly in hematopoietic lineages with the exception of T-cells [9-11]. Interestingly, *LYL1* was originally discovered in T-lymphoblasts of patients with T-cell acute lymphoblastic leukemia (T-cell ALL) [1]. The ectopic *LYL1* expression was a result of the chromosomal translocation t(7;19)(q35;p13), juxtaposing it to the T-cell receptor beta chain gene [12]. While *LYL1* genetic alterations were found on average in 2% of all T-cell ALL cases [13], 22% of the children with T-cell ALL in one study had positive expression of *LYL1*, which was not associated with any locus-specific translocations of this gene [14]. In addition to T-cell ALL, *LYL1* translocation and multiple translocation-independent upregulations have also been observed in acute myeloblastic leukemia (AML) [15,16]. Taken together, these reports strongly suggest that *Lyl1* is a proto-oncogene that can be upregulated by multiple mechanisms [17].

The role of *LYL1* in leukemia is further emphasized by the correlation of *Lyl1*-overexpression and a stem-like (CD34⁺) leukemia phenotype with particularly poor prognosis [14,16]. More recent studies have shown that *LYL1* is highly expressed in normal human CD34⁺ bone marrow [16] and umbilical cord blood cells [18]. The involvement of *LYL1* in determining the stem-cell-like leukemia phenotype and in disease prognosis remains unknown.

The oncogenic potential of *Lyl1* has not been extensively studied. Data from transgenic mice which overexpressed *Lyl1* on the elongation factor 1 (EF1) promotor suggested it acts as a weak oncogene, as 30% of the studied *Lyl1* transgenic mice developed T or B-cell malignant lymphomas after an average latent period of 352 days [19]. Remaining unclear is the extent to which the lymphomas were due to *Lyl1*-overexpression specifically in the hematopoietic cells, since *Lyl1* expression was driven by the EF1-promotor, allowing its expression virtually in every cell. The aims of our study were to investigate the effect and the oncogenic potential of *Lyl1*-overexpression specifically in the hematopoietic system and to determine if *Lyl1*-overexpression has the potential to induce a stem- or early progenitor-like leukemia phenotype.

Materials and Methods

Antibodies

All antibodies we purchased from BD Farmingen unless otherwise specified.

Mice

For our studies we used C57B1/6-CD45.1 and CD45.2 isotype mice. The animals were housed, used for experiments and sacrificed in a humane manner following Institutional Animal Care and Use Committee (IACUC) guidelines.

Retroviral transduction of bone marrow progenitor cells, transplantation and blood analysis

The viral MSCV expression constructs were prepared by insertion of the coding sequence of the wild-type (WT) mouse Lyl1 or GFP into the MSCV vector using Gateway recombination methods (Invitrogen). Lyl1 expression was confirmed by western blotting. Consequently the viral plasmids were packed by co-transfection with pCL-Eco in 293T cells [20]. The overexpression of Lyl1 in the mouse hematopoietic system was achieved following procedures described previously [10]. In short, Sca-1⁺ WT hematopoietic progenitor cells (HPCs) from C57B1/6-CD45.2 mice were transduced with MSCV-gene-IRES-GFP and transplanted into lethally irradiated recipient C57B1/6-CD45.1 mice. Peripheral blood (PB) lineage analysis was done as described [10]. In short, after erythrocytolysis, the leucocytes were incubated on ice for 20 min. with the following antibodies: anti-CD45.2-APC, anti-B220-PacBlue, anti-B220-PE-Cy7, anti-CD4-PacBlue, anti-CD8-PacBlue and anti-Mac-1-PE-Cy7. The cells were analyzed on a LSRII flow cytometer (BD) in Hanks' balanced salt solution + 2% FBS (HBSS⁺) supplemented with Propidium Iodide (PI, 1 µg/mL).

In vitro Colony-Forming-Unit (CFU-C) assay

For CFU-C assays, transduced Sca-1⁺ bone marrow cells were cultured in complete StemPro34 media supplemented with thrombopoietin (TPO) and stem cell factor (SCF) (PeproTech) at 37°C, 5% CO₂ for 48 hours. GFP⁺, Sca-1⁺ cells were then sorted (MoFlo flow cytometer) into 96-well plates (1 cell/well), containing M3434 MethoCult medium (Stem Cell Technologies), and incubated at 37°C, 5% CO₂. After 12-14 days of culture, hematopoietic colonies were counted. Colonies larger than 2 mm were collected, washed with HBSS⁺ and stained for 20 minutes on ice with anti-Sca-1-APC and anti-c-Kit-PE antibodies. Cells were analyzed on a LSRII.

Hematopoietic progenitors in cultured bone marrow cells

Sca-1⁺ bone marrow cells were transduced and cultured for 48 hours as described above. The cells were then stained for 20 min. on ice with anti-Sca-1-APC, anti-c-Kit-PE and a cocktail of Pe-Cy5 conjugated anti-lineage antibodies (Mac-1, Gr-1, CD4, CD8, B220, and Ter119). Samples were analyzed on a MoFlo (Beckman Coulter).

Hematopoietic progenitors in mouse bone marrow

The analysis of the KSL (c-Kit⁺, Sca-1⁺, Lineage⁻) and the common lymphoid progenitors (CLPs) was performed as described [21]. Whole bone marrow cells isolated 3 weeks post-transplantation were stained with the following antibodies: biotinylated lineage markers (Mac-1, Gr-1, CD4, CD8, B220, and Ter119), IL7 α -PeCy7 (eBioscience), Sca-1-APC, and c-Kit-PE for 20 min on ice. Cells were then spun down, resuspended, and stained with streptavidin-Pacific Blue for 10 min on ice. Samples were analyzed on a LSRII.

Real-Time qPCR analysis

Sca-1⁺ bone marrow cells were transduced and maintained at 37°C, 5% CO₂ for 48 hours as described. Subsequently, GFP⁺ cells were sorted directly into lysis buffer and RNA extraction was performed using the RNA Extraction kit from GE Healthcare. cDNA synthesis was then performed using Superscript II (Invitrogen), according to manufacturer instructions. Real-Time qPCR was performed using TaqMan master mix, gene specific TaqMan Expression assays for Lyl1 (Mm00493219_m1), Sca-1 (Mm00726565_s1), c-Kit (Mm00445212_m1) and the 18S rRNA Endogenous Control (VIC/MGB Probe) following company protocol (Applied Biosystems). The samples were amplified for 50 cycles using AbiPrism 7900HT (Applied Biosystems).

AnnexinV staining

Sca-1⁺ bone marrow cells were transduced and cultured for 48 or 96 hours as described. The cells were then washed with PBS and stained for 10 min. on ice with anti-Sca-1-PE. Next, the cells were washed and stained with AnnexinV-APC (Invitrogen) and PI following company protocol. The analyses were performed on a LSRII.

In vivo BrdU labeling of KSL cells

Mice were injected i.p. with BrdU (Sigma-Aldrich) (3.33mg/mouse in 0.5 ml of sterile PBS) 3 hrs prior sacrificing. Next, Sca-1⁺ bone marrow cells were purified and stained with anti-c-Kit-APC-AlexaFluor 750 (eBioscience), PE conjugated anti-lineage antibodies and Streptavidin-PE-Cy7 (to bind anti-Sca-1-biotin used during Sca-1 enrichment). Following the staining, KSL cells were sorted over 500,000 to 1,000,000 PE⁺ carrier B-cells, using a FacsAria flow cytometer (BD). The BrdU content of the KSL cells was analyzed using BD Biosciences BrDU-APC analysis kit and a LSRII, following company protocol.

Results

Lyl1-overexpression causes T-cell expansion in the peripheral blood

Our primary goal is to evaluate the effect and the oncogenic potential of Lyl1-overexpression in the hematopoietic system. We achieved Lyl1-overexpression using retroviral transduction of Sca-1⁺ mouse bone marrow cells followed by syngeneic transplantation. In short, isolated Sca-1⁺ bone marrow cells, representing an enriched heterogeneous population of stem and progenitor cells, from C57B1/6-CD45.2 mice were transduced with mouse stem cell retrovirus (MSCV) expressing either Lyl1 and GFP or only GFP as a control. The treated cells were then transplanted into lethally irradiated C57B1/6-CD45.1 recipient mice. In order to measure the engraftment and the output of white blood cells, we analyzed the GFP expression in the peripheral blood by flow cytometry every 2 or 4 weeks from week 4 to week 30 post-transplantation (Figure 1). We observed that the Lyl1 mice had a higher percentage of GFP⁺ cells, suggesting that the Lyl1 positive bone marrow engrafted better and/or generated higher number of white blood cells (Figure 2A) perhaps as a result of increased proliferation or increased survival due to decreased apoptosis [9].

To investigate the effect of Lyl1-overexpression on the white peripheral blood cells, and particularly on the lymphoid compartment, we measured the fractions of transduced myeloid, B-cell and T-cell compartments. Using flow cytometry, the donor derived GFP⁺ blood cells were analyzed based on the expression of classical lineage markers: Mac1 for the myeloid lineage, B220 for B-cells, and CD4 and CD8 for T-cells (Figure 1). We observed that the T-cells in the Lyl1 mice represented almost half (45%) of the GFP⁺ white blood cells compared to approximately 15% in the control mice, or 3 fold higher (Figure 2B). The T-cells appeared normal, mature, single positive for CD4 or CD8 and in proportions similar to those in the control set (data not shown), suggesting increased but normal T-cell development. The B-cells were lower in the Lyl1 mice (45% compared to 75-80%), suggesting that Lyl1 may drive lymphoid progenitor-development towards T-cell (Figure 2C). There was no significant difference between the myeloid compartments (Figure 2D).

Overexpression of Lyl1 in mouse bone marrow did not result in leukemia

Despite the T-cell increase in the Lyl1-overexpressing mice, during the 30-week study period we did not observe any signs suggesting cancer formation. The mice (GFP control – 2 groups: 5 and 7 mice per group; Lyl1 – 2 groups: 3 and 5 mice per group) appeared in good general condition without any detectable tumors. The parallel T- and GFP⁺-cell decrease with time in the Lyl1 set (Figure 2A, B) underlines the Lyl1 dependency of the T-cell expansion. However, it also suggests that, alone the lymphoproliferative effect of Lyl1-

overexpression cannot drive oncogenicity but, it could be an excellent support in leukemia formation.

Bone marrow cells transduced with *Ly11* give rise to progenitor colonies double-positive for Sca-1 and c-Kit

As discussed above, in T-cell ALL and AML cells high LYL1 expression was associated with expression of markers typical for primitive progenitors [14,16,22]. Therefore, we examined the effect of *Ly11*-overexpression on transduced murine hematopoietic progenitors. Initially, we analyzed the ability of *Ly11* to generate hematopoietic colonies with stem/progenitor phenotype based on their expression of c-Kit and Sca-1. Forty-eight hours post-transduction, we sorted single GFP⁺, Sca-1⁺ bone marrow cells into wells of a 96-well plate containing methylcellulose and hematopoietic cytokines (Figure 3A). After 14 days, we found no significant difference in the total number of colonies between the *Ly11*-transduced and the control, GFP-only cells. On average in each set, 60-70 of the 96 sorted single cells gave rise to colonies. All colonies, with a size of 2 mm or larger were subsequently picked and analyzed for c-Kit and Sca-1 expression. Approximately half of the analyzed colonies (15 of 33) in the *Ly11* set consisted primarily (90% or higher) of cells double-positive for c-Kit and Sca-1, compared to only 1 of the 26 analyzed colonies in the GFP set (Figure 3B). In contrast, only 3 colonies (9%) of the *Ly11* set compared to more than half of the colonies in the control set contained 50% or less c-Kit and Sca-1 double positive cells. These observations show that *Ly11*-overexpression can induce dramatic expansion of cultured bone marrow cells double positive for c-Kit and Sca-1, a combination of markers associated with primitive hematopoietic stem and progenitor cells.

***Ly11*-overexpression causes expansion of the progenitor compartment *in vivo* and in culture**

To better understand the effect of *Ly11*-overexpression on the hematopoietic stem and progenitor cells, we analyzed the KSL (c-Kit⁺, Sca-1⁺, Lineage⁻) population, containing the most primitive hematopoietic progenitor and stem cells, in the mouse bone marrow *in vivo* and in culture. First, we measured the KSL percentage in cultured Sca-1⁺ bone marrow cells (Figure 3A). After 48 hrs of culture, we observed that the KSL population in the *Ly11* set was three fold higher than the GFP controls (Figure 3C).

This observation was further supported by our *in vivo* data (Figure 4A). Mice overexpressing *Ly11* have a higher percentage of KSLs in the bone marrow compared to the control set (Figure 4B-C). Furthermore, we observed a similar increase of the common lymphoid progenitors (CLPs) in the *Ly11*-transduced mice compared to the control. Our data suggest that the T-cell lymphoproliferative effect of *Ly11*-overexpression is likely due to the expansion and perhaps increased activity of the lymphoid fraction of the hematopoietic progenitors in the bone marrow.

***Ly11* expands the hematopoietic progenitors by stimulating proliferation and suppressing apoptosis**

There are several possible mechanisms that could lead to expansion of the progenitor population. One possibility is that *Ly11* stimulates Sca-1 and c-Kit transcription. Since *Scl*, the *Ly11* paralog, is required for c-Kit expression [23,24], it is possible that *Ly11* could also regulate the expression of c-Kit and/or Sca-1. Another possibility is that *Ly11* has a proliferative effect, leading to an increased progenitor population. Finally, as shown previously *Ly11* deletion induces apoptosis of Sca-1⁺ cells [9]. Therefore, *Ly11*-overexpression could decrease apoptosis or extend cell survival, which could account for the expansion of the progenitor population.

To investigate if *Lyl1* has a direct effect on c-Kit and Sca-1 transcription, we performed a Real-Time qPCR analysis on cDNA derived from cultured, transduced bone marrow cells (Figure 5A). There was no significant difference in the c-Kit and Sca-1 mRNA levels between the *Lyl1* and the control samples (Figure 5B) suggesting, that the progenitor expansion is not due to a transcriptional activation of Sca-1 and c-Kit.

To ascertain the connection between *Lyl1*-overexpression and apoptosis, we stained cultured, transduced Sca-1⁺ bone marrow cells with AnnexinV (Figure 5A). Initially, 48 hrs of culture did not lead to a difference in the percentage of apoptotic progenitors between *Lyl1*-overexpressing and control cells. However, 96 hrs of culture resulted in 50% more apoptotic cells in the control compared to the *Lyl1* set (Figure 5C). This observation confirmed our expectations that *Lyl1*-overexpression has a counter-apoptotic or pro-survival effect, which could contribute to the expansion of the KSL and CLP progenitors.

Next, we investigated the proliferative effect of *Lyl1*-overexpression in the progenitor cells. Therefore, we performed an *in vivo* BrdU labeling assay in order to determine if *Lyl1*-overexpression can stimulate proliferation of the KSL cells. We used mice transplanted with Sca-1⁺ bone marrow cells, transduced with *Lyl1* and *GFP* or only with *GFP*. Eight weeks post-transplantation, mice were injected with BrdU, and three hours later bone marrow was harvested. KSL cells were purified using flow cytometry, fixed, labeled with anti-BrdU antibody and analyzed for BrdU content (Figure 5A). It appears that the *Lyl1* mice have higher percentage of BrdU-positive KSLs compared to the controls (Figure 5D), suggesting that these early progenitors are more proliferative when *Lyl1* is overexpressed.

Together, the evidence presented shows that *Lyl1*-overexpression can cause expansion of the hematopoietic progenitors by stimulating cell proliferation and by suppressing apoptosis. Therefore, the hypothesis that *Lyl1*-overexpression could have a secondary oncogenic effect by stimulating the expansion of leukemic cells generated in the early stages of hematopoiesis, with stem- or early progenitor-like phenotype seems plausible.

Deletion of *Lyl1* delays development of NICD driven T-cell leukemia in mice by promoting apoptosis

Previously published work has shown that overexpression of the intracellular domain of *NOTCH1* (*NICD*) results in development of severe T-cell (CD4⁺ and CD8⁺) leukemia, causing all animals to die by 15 weeks post-transplantation [25,26]. We used the powerful oncogenic drive of *NICD* to study leukemia development in the absence of *Lyl1*. We transplanted wild-type and *Lyl1* knock-out (*Lyl1*^{-/-}) Sca-1⁺ bone marrow cells, transduced with bicistronic MSCV virus expressing *NICD* and *GFP* or *GFP*-only, into wild-type mice (Figure 6A). Four weeks post-transplantation, the peripheral blood output of the *NICD*-transduced wild-type bone marrow was predominantly composed of leukemic T-cells (88%). Normally, as observed in the wild-type control or *GFP*-only set, the fraction of T-cells is about 2% (Figure 6B). The transduced *Lyl1*^{-/-} bone marrow gave rise to 44% leukemic cells, or half of the amount produced by the *NICD*-transduced wild-type bone marrow. This delay of leukemic cell production was only temporary; by the 6th week, the leukemic cell output was the same between the wild-type and the *Lyl1*^{-/-} mice.

Simultaneously with our mouse study we performed an AnnexinV staining of cultured Sca-1⁺ bone marrow cells. We found that *NICD*-transduced, *Lyl1*^{-/-} progenitors were significantly more apoptotic than the wild-type (Figure 6C). Our observations show that the absence of *Lyl1* temporarily delays the development of the *NICD* leukemia, most likely due to increased apoptosis of the progenitors giving rise to the peripheral blood leukemic cells.

Discussion

In this study we examined the potential role of *Lyl1* in development of leukemia using the murine hematopoietic system as a model. *Lyl1*-overexpression caused significant increase of peripheral blood T-cells. The T-cell lymphoproliferative effect strongly supports the connection in humans between *Lyl1*-overexpression and T-cell malignancies. However, by itself it was unable to induce leukemia or lymphoma during the 7-month study period in a cohort of 20 mice. This was somewhat surprising, because ~30% of the *Lyl1* transgenic mice developed malignant lymphomas, although a year was required for their development [19]. This long time-lag suggests that *Lyl1*-overexpression predisposes the mice toward lymphoma but, additional secondary mutations must accumulate prior to development of cancer [27,28]. In addition, the oncogenicity of *Lyl1* in the transgenic mice might have been enhanced by its overexpression in non-hematopoietic cells, since it was an EF1 promotor-driven.

Evidently, *Lyl1* has an important role in early hematopoietic progenitors. We show that *Lyl1*-overexpression causes progenitor expansion by promoting proliferation and suppressing apoptosis, and not by induction of the progenitor phenotype via stimulation of Sca-1 and c-Kit transcription. In considering further the possible mechanisms that may explain these effects, we examined the chromatin-IP-followed by sequencing (ChIP-Seq) analysis performed recently to identify Scl targets [29]. Because Scl and *Lyl1* have overlapping functions in the adult HSCs and progenitors, and almost identical DNA-binding and HLH domains, we anticipate that the targets of Scl and *Lyl1* may be at least partially overlapping. Göttgens' group showed that Scl targets were highly enriched for hematopoietic transcription factors and for members of the mitogen-activated protein kinase (MAPK) and the focal adhesion kinase pathways, which facilitate a wide range of proliferative signals. It is conceivable that *Lyl1* also targets some of the same genes responsible for stimulating stem and progenitor cell proliferation. The increased survival of the *Lyl1*-overexpressing progenitors correlates well with the increased apoptosis when *Lyl1* is deleted [9]. Thus, it is not surprising that there was a delay in the development of the NICD-induced leukemia in the absence of *Lyl1*.

Increased proliferation and decreased apoptosis are complementary mechanisms through which the pro-oncogenic effects of *Lyl1*-overexpression may be manifest. These properties, in addition to the T-cell lymphoproliferative effect fortify the role of *Lyl1* in T-cell leukemia/lymphoma.

The hypothesis that *Lyl1*-overexpression can induce expansion of leukemic cells with a stem/progenitor-like phenotype is reinforced by our results showing *Lyl1* functioning within and expanding the early progenitor populations. Previous studies have also shown that cancer cells with stem/progenitor-like phenotype have decreased drug sensitivity due to increased capacity for drug efflux [30-32]. Therefore, it is not surprising that the *LYL1*⁺ T-cell ALL subtype also has poor response to therapy [14]. In addition, the U937 lymphoma cells become less sensitive to the cytotoxic drug cytarabine when *LYL1* is overexpressed [16]. It appears that *LYL1* could be a good candidate as a marker of therapy-resistant leukemia. Further exploration in this direction could lead to identifying targetable differences between leukemia subtypes offering a real possibility for development of more specific and effective medicinal agents. Undoubtedly, *Lyl1* has the potential to act as an oncogene and as a prognostic factor. It might not have a strong cancer initiation effect, but it has the potential to support and influence leukemia formation, development and outcome.

Acknowledgments

We would like to extend our appreciation to Fabian Zohren and the rest of our colleagues from the Goodell lab for the helpful discussions and support throughout this study. We also like to thank Dr. Brendan Lee from Baylor College of Medicine for donating a plasmid copy of NOTCH1. The financial support for this work was provided by NIH grants DK58192, HL081007, and Ellison Foundation grant number AGSS1787.

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Figure 1. Lyl1-overexpression outline

Schematic representation of the MSCV retroviral constructs and the overexpression experimental design. Peripheral blood, pre-cleared from red blood cells, was treated with a cocktail of antibodies and then analyzed by flow cytometry. The donor-derived white blood cells (CD45.2 positive) were separated based on their GFP positivity and then further fractionated as myeloid, B- and T-cells.

Figure 2. Effect of Lyl1-overexpression on the peripheral blood

Peripheral blood lineage analyses of mice transplanted with control (*GFP*-transduced) or *Lyl1*-transduced (*Lyl1* + *GFP*) Sca-1⁺ bone marrow cells. The blood was analyzed by flow cytometry every 2 or 4 weeks starting 4 weeks (for total GFP expression) or 12 weeks (for peripheral blood lineage compartments) until 30 weeks post-transplantation. The graphs represent the analysis of the GFP⁺ donor-derived white blood cells: A) GFP⁺ donor-derived white blood cells, represented as a percent of the total white blood cells; B-D) lineage distribution within the GFP⁺ donor-derived cells: T-cells (B), B-cells (C) and Myeloid (D). The error bars represent the average deviation of 3 to 5 mice. The p value for the GFP

expression profile was calculated using Graph Pad Prism Version 5 software and a third order polynomial curve fit. * $p < 0.05$; ** $p < 0.06$, determined by unpaired, two tailed t test.

Figure 3. Analyses of cultured bone marrow cells transduced with *Lyl1*

A) Sca-1⁺ bone marrow cells, transduced with *GFP* or *Lyl1-GFP* were cultured as described. After 48 hrs, the KSL population was measured and single Sca-1⁺, GFP⁺ cells were sorted into each well of a 96-well plate containing M3434 MethoCult medium. B) After 14 days of incubation, each colony larger than 2 mm was analyzed for Sca-1 and c-Kit expression using flow cytometry. Cells, double positive for c-Kit and Sca-1 were designated as KS. Total of 26 (GFP) and 33 (Lyl1) colonies, from 2 separate experiments were analyzed. Each data entry represents the percentage of KS cells for each colony. The calculated medians are 42.9 (GFP) and 88.4 (Lyl1). C) Graphical representation of the KSL compartment as a percentage of the GFP⁺ cells from three independent experiments and total of 5 (GFP) and 6 (Lyl1) biological replicates. The error bars are the corresponding calculated SEM. The p values were derived using two tailed, unpaired t test.

Figure 4. Effect of Lyl1-overexpression on the KSL and CLP progenitors in the mouse bone marrow

A) Bone marrow derived from mice transplanted with Sca-1⁺ bone marrow cells transduced with GFP- or Lyl1-expressing MSCV virus was isolated and the KSL and CLP progenitors analyzed as follow: B) the KSL population was derived from the GFP⁺, Lineage⁻ cells which were further separated as double positive for Sca-1 and c-Kit; the CLP population, also double positive for Sca-1 and c-Kit was derived from the IL7Rα⁺, Lineage⁻, GFP⁺ cells. C) The bar graphs represent the KSL or the CLP populations as a percentage of the total GFP⁺ bone marrow cells. The error bars represent the SEM derived from three to five biological replicates.

Figure 5. Role of Lyl1 in cell proliferation, apoptosis, and Sca-1 and c-Kit expression

A) Sca-1⁺ mouse bone marrow cells, transduced with *GFP*-only or *Lyl1* and *GFP* were transplanted into recipient mice or cultured for 48 or 96 hrs as described. The cultured cells were used in the following two ways: B) GFP⁺ cells were sorted into lysis buffer for RNA isolation which consequently was used for cDNA synthesis. The cDNA was then used as a template in Real-Time qPCR expression analyses of *Lyl1*, *Sca-1*, *c-Kit* and 18S as a control. Cycle thresholds (Ct) specific for each gene were normalized to the 18S Ct by subtraction (Δ Ct). Next the Δ Cts from the *Lyl1* set were subtracted from the Δ Cts of the *GFP* set, thus obtaining the $\Delta\Delta$ Ct. The fold change was calculated from the formula $2^{-(\Delta\Delta Ct)}$. The bar graph represents the average values obtained from triplicates of three biological replicates.

For each gene the GFP value has been set to 1. The error bars are the corresponding SEM. C) The cultured cells were first incubated with anti-Sca-1-PE conjugated antibody and then with AnnexinV-APC conjugate following company protocol. The AnnexinV⁺ cells from three independent experiments were measured and the average plotted as a percent of the GFP⁺, Sca-1⁺ cells. The error bars represent the SEM. The p value was derived using two tailed, unpaired t test. D) The average BrdU⁺, donor KSLs were presented graphically as a percent of the total donor KSLs. The averages were obtained from 4 (GFP) and 6 (Lyl1) biological replicates and two independent experiments. The error bars represent the SEM.

Figure 6. Development of NICD driven leukemia in the absence of Lyl1

A) Wild-type and Ly11^{-/-} Sca-1⁺ mouse bone marrow cells, transduced with *GFP*-only or *NICD* and *GFP* were transplanted into recipient mice or cultured for 96 hrs as described. B) The percent of CD4 and CD8 positive cells in the transduced donor derived peripheral blood was analyzed using flow cytometry. The averages from 3 to 7 specimens with the corresponding SEM were graphed. C) The number of the apoptotic cultured progenitors was measured using AnnexinV staining as shown previously. The AnnexinV⁺ cells were presented as a percent of the transduced Sca-1⁺ cells. The p values were derived using two tailed, unpaired t test.