Unique and Independent Roles for MLL in Adult Hematopoietic Stem Cells and Progenitors

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SUMMARY

The Mixed Lineage Leukemia (MLL) gene is essential for embryonic hematopoietic stem cell (HSC) development, but its role during adult hematopoiesis is unknown. Using an inducible knockout model, we demonstrate that Mll is essential for the maintenance of adult HSCs and progenitors, with fatal bone marrow failure occurring within 3 weeks of Mll deletion. Mll-deficient cells are selectively lost from mixed bone marrow chimeras, demonstrating their failure to self-renew even in an intact bone marrow environment. Surprisingly, HSCs lacking Mll exhibit ectopic cell-cycle entry, resulting in the depletion of quiescent HSCs. In contrast, Mll deletion in myelo-erythroid progenitors results in reduced proliferation and reduced response to cytokine-induced cell-cycle entry. Committed lymphoid and myeloid cells no longer require Mll, defining the early multipotent stages of hematopoiesis as Mll dependent. These studies demonstrate that Mll plays selective and independent roles within the hematopoietic system, maintaining quiescence in HSCs and promoting proliferation in progenitors.

INTRODUCTION

To maintain the adult hematopoietic system, an ongoing and flexible interplay between lineage-instructive transcription factors and epigenetic regulators is likely to play a central role in determining appropriate numbers of each cell type. Several sequence-specific transcriptional regulators have been shown to play important roles in hematopoiesis, but the role of chromatin-modifying epigenetic regulators in this process is less clear. Polycomb (PcG) and trithorax (trxG) family proteins first identified in Drosophila melanogaster influence target gene expression in a manner that is heritable through multiple daughter cell divisions (Cavalli and Paro, 1999), making these proteins uniquely qualified to influence cell identity and plasticity during development or to regenerate tissues such as the hematopoietic system.

The Mixed Lineage Leukemia (MLL) gene was the first trxG gene identified as a proto-oncogene (Djabali et al., 1993; Domer et al., 1993; Gu et al., 1992; Tkachuk et al., 1992). Chromosomal translocations disrupting the MLL gene are primarily associated with acute lymphocytic (ALL) or myelogenous leukemia (AML) of infants, as well as secondary AML, accounting for more than 70% of each of these two groups (reviewed in Biondi et al., 2000; Pui and Relling, 2000). MLL fusion genes produced by chromosomal translocation encode proteins composed of the N-terminal ~1/3 of MLL fused to one of over 50 identified translocation partners. The expression of MLL fusion proteins can influence lineage fidelity within the hematopoietic system (Drynan et al., 2005) and can produce cells with mixed lineage identity in human leukemia (Armstrong et al., 2002).

Retroviral expression of MLL fusion proteins in bone marrow cells is sufficient to produce AML or ALL in the mouse, as is the expression of MLL fusions by knockin or cre-mediated de novo chromosomal translocation (Ayton and Cleary, 2001; Daser and Rabbitts, 2005). The retroviral transduction model enabled structure-function assays that identified motifs within the MLL N terminus and the fusion partner that are essential for transformation. Collectively, such experiments indicate that MLL fusion proteins act via a gain-of-function mechanism through the acquisition of ectopic transactivation or dimerization capacity (reviewed in Ayton and Cleary, 2001). The N-terminal portion of MLL is likely to play a large role in targeting the fusion proteins to many of the same loci regulated by endogenous MLL, as fusion proteins have been shown by chromatin immunoprecipitation (ChIP) to localize to at least some of the same regulatory sequences as full-length wild-type MLL (Milne et al., 2005; Xia et al., 2005) and increase expression of those target genes (Horton et al., 2005).

Recent advances in purifying native MLL and complexes from cell lines have yielded important insights into the mechanism by which MLL acts on its target genes. Such complexes harbor histone methylation, acetylation,
and chromatin remodeling activities (Milne et al., 2002; Nakamura et al., 2002; Wysocka et al., 2005; Yokoyama et al., 2004). The Su(var)3-9/Enhancer of zeste/Trithorax (SET) domain at the extreme C terminus of MLL methylates histone H3 at lysine 4 (H3 K4), a modification associated with active transcription (Santos-Rosa et al., 2002). Consistent components of these complexes include WDR5, ASH2L, and menin. WDR5 and ASH2L interact with the C-terminal SET domain, acting as cofactors for the histone methyltransferase activity (Dou et al., 2006; Steward et al., 2006). Menin interacts with the MLL N terminus and bridges MLL to the basal transcriptional machinery (Yokoyama et al., 2004). The interaction of menin with the N terminus of MLL is important for maintaining expression of an endogenous MLL target gene in HeLa cells and is essential for transformation by MLL fusion proteins (Yokoyama et al., 2004, 2005). Other proteins have been described as part of the MLL complex, or as transient interactors, but scant in vivo data are available to validate the biological importance of these interactions.

To understand the mechanisms by which MLL fusion proteins are leukemogenic, it is critical to determine the normal function of MLL in vivo, particularly in the cell types that may represent the target for transformation by MLL oncogenes. Several groups have disrupted the Mll gene by different strategies, and in most cases, homozygous Mll mutants die during embryogenesis (Ayton et al., 2001; Yagi et al., 1998; Yu et al., 1995). Interestingly, the deletion of the SET domain alone is not lethal, but homozygous mutants exhibit mild loss-of-function phenotypes, including homeotic transformation and reduced expression of several Hox genes (Terrarova et al., 2006). Due to the embryonic lethality of most Mll mutants, studies aimed at identifying the role of Mll in the hematopoietic system have focused on embryonic hematopoiesis. Experiments using Mll-deficient embryonic stem (ES) cells demonstrated that these cells were incapable of differentiating into any hematopoietic cell types in adult animals or in the fetal liver. This multilineage block was due to the lack of HSCs in Mll-deficient embryos as measured functionally (Ernst et al., 2004a). Using an in vitro system, we showed that the block in hematopoietic development was accompanied by global reduction in Hox gene expression and could be rescued by the reintroduction of individual Hox genes (Ernst et al., 2004b). These experiments indicate that Mll plays an essential role in the development of definitive HSCs during embryogenesis and suggest that this is due to global Hox gene misexpression. However, the early and severe nature of the block in hematopoietic development precluded a detailed analysis of the mechanisms underlying the lack of HSCs or the assessment of Mll function in more differentiated hematopoietic lineages. To address these questions, we developed a mouse model in which Mll can be inducibly inactivated in the bone marrow and in specific lineages to provide a model system in which such functions could be studied in more detail.

RESULTS

Excision of Mll Exons 3 and 4 Generates a Loss-of-Function Allele

To create an inducible loss-of-function model for Mll, we designed an in-frame deletion to remove the AT hooks, nuclear targeting sequences (NTS), and subnuclear localization (SNL) sequences by flanking exons 3 and 4 with loxP sites (“floxed,” MllF). Thus, cre recombinase-mediated excision produces an intragenic deletion (MllΔN), resulting in an MLL mutant protein lacking several NTS and other interaction motifs (Figure 1A). We recently introduced this allele into ES cells in combination with a strong loss-of-function allele (Mll-lacZ) and found that these cells exhibited a block in hematopoietic development (Ernst et al., 2004a) and a block in embryoid body hematopoiesis (Ernst et al., 2004b). Embryos homozygous for the MllΔN allele died in utero at embryonic day 12.5, one day later in embryonic development than MllΔN/Mll-lacZ and two days later than Mll-lacZ homozygotes (Figure S1 in the Supplemental Data available with this article online). MllΔN/+ animals exhibit normal viability and fertility and can be backcrossed to the C57Bl/6 strain without loss of heterozygote viability, in contrast to the exon 3 insertion Mll-lacZ strain (Yu et al., 1995). Thus, both the age of embryonic lethality and the hematopoietic phenotypes indicate that the MllΔN allele is a strong hypomorphic allele of Mll, slightly less severe than the Mll-lacZ allele.

To confirm the predicted subcellular distribution of the MllΔN protein, we expressed an N- and C-terminally tagged version of the predicted protein encoded by MllΔN in 293T cells. MLL is processed by proteolytic cleavage to produce N- and C-terminal polypeptides that associate with each other within a large protein complex (Hsieh et al., 2003; Yokoyama et al., 2002). To determine the localization of both protein fragments, cells transfected with tagged wild-type and MllΔN constructs were visualized by fluorescence microscopy using antibodies to the N- and C-terminal tags, as well as an internal MLL antibody that recognizes an epitope in the N-terminal fragment. MllΔN was clearly excluded from the nucleus as determined by the N-terminal anti-Flag or anti-MLL antibodies, as well as the C-terminal anti-HA antibody (Figure 1B). In contrast, the full-length protein was exclusively nuclear as expected (Figures 1B and 1C). This subcellular localization was also observed with antibodies that detect the endogenous MllΔN protein produced by cre-mediated Mll excision in MllΔN/F bone marrow-derived macrophages (Figure 1C). Western blot analysis demonstrated similar levels of the MllΔN protein compared to wild-type (data not shown).

To independently assess the function of the MllΔN protein, we examined the expression of Hoxa7, Hoxa9, and Hoxa10, well-characterized MLL target genes, in MllΔN/ΔN hematopoietic cells. As shown in Figure 1D, we observed a progressive reduction (2- to ~50-fold) in Hoxa7, Hoxa9, and Hoxa10 expression between days 6 and 11 after cre-mediated excision of Mll. Other Hox genes, such as Hoxb3, were not affected by Mll loss.
Furthermore, highly purified c-Kit+/lineageneg/low/Sca-1+ (KLS) cells exhibited a similar fold reduction in Hoxa gene expression (Figure S3). Thus, excision of Mll exons 3 and 4 results in the production of a stable protein that fails to become imported into the nucleus and, based on the genetic, biochemical, and gene expression data above, results in a nonfunctional protein and a loss-of-function phenotype.

Figure 1. The Mll<sup>ΔN</sup> Allele Encodes a Cytoplasmic Protein with Reduced Activity

(A) Diagram of wild-type MLL with the deletion of sequences encoded by exons 3 and 4 indicated by dashed lines. Homology motifs are shown as colored bars with the following indicated: AT hooks, minor groove DNA binding motif; SNL1 and 2, subnuclear targeting motif; NTS1 and 3, nuclear targeting signals; MT, DNA methyltransferase homology and CpG binding motif; PHD, plant homeodomain homology regions; T, taspase cleavage sites; AD, activation domain; and SET, Su(var)3-9/Enhancer of zeste/Trithorax homology region.

(B) Localization of tagged transfected wild-type MLL versus MLL<sup>ΔN</sup>. 293T cells were transfected with the construct labeled on the right of the panels and stained with antibodies indicated at the left. Examples shown are representative of three independent experiments.

(C) Subcellular localization of endogenous MLL in wild-type (upper panels) and Mll<sup>ΔNΔN</sup> macrophages (lower panels) derived in vitro from bone marrow cells.

(D) Relative Hox gene expression levels were determined by quantitative PCR using lin<sup>neg/low</sup> bone marrow cells isolated the days indicated after Mll deletion. Each bar represents the average expression level from at least three animals. Error bars represent the standard deviation of the average relative expression among animals. The asterisk denotes an undetectable Ct.
Acute \( \text{Mll} \) Loss Results in Rapid Bone Marrow Failure

To determine the role of \( \text{Mll} \) during adult steady-state hematopoiesis, we employed an inducible transgenic cre mouse strain (\( \text{Mx1-cre} \); Kuhn et al., 1995). Control and \( \text{Mx1-cre;Mll}^{F/F} \) animals were subjected to a brief series of polyninosinic:polycytidylic acid (pI:pC) injections to induce \( \text{cre} \)-mediated excision of \( \text{Mll} \) in the bone marrow. Prior to pI:pC injection, bone marrow populations did not differ between control and \( \text{Mx1-cre;Mll}^{F/F} \) animals (Figure S4). As early as 4 days after the first pI:pC injection, nearly all bone marrow cells had undergone \( \text{cre} \)-mediated excision (Figure S5). Approximately 19 days after inducing \( \text{cre} \) expression, animals in which 80% or more of the bone marrow expressed the \( \text{Mll}^{DN} \) allele exhibited bone marrow cytopenia and died or had to be sacrificed (Figure 2). The bone marrow of the few \( \text{Mx1-cre;Mll}^{F/F} \) animals surviving the pI:pC injections was invariably composed of cells that had not undergone \( \text{cre} \)-mediated \( \text{Mll} \) excision (data not shown). \( \text{Mx1-cre;Mll}^{F/F} \) pI:pC-injected animals that were also injected with wild-type bone marrow cells were healthy beyond 6 months (data not shown), demonstrating that the cause of death was from bone marrow failure.

The Maintenance of Adult HSCs Requires Continuous MLL Function

We previously demonstrated that MLL was essential for the development of HSCs in the embryo, so we first determined whether acute loss of \( \text{Mll} \) affected the HSC-enriched KSL population in the bone marrow. Phenotypic and functional analyses were performed from day 4 after...
cre-mediated Mll deletion until the point at which Mx1-cre;Mll+/+ or Mll+/F red animals died. Notably, the number of KSL cells dropped precipitously and failed to recover (Figure 3A).

To determine whether the reduction in KSL cells reflected a cell-intrinsic defect, we performed competitive transplantation experiments with sorted pooled cells from the day 8 time point. We injected up to 10,000 viable KSL cells purified by fluorescence-activated cell sorting (FACS) from pI:pC-treated Mx1-cre;Mll+/+; or control animals into lethally irradiated recipients. Animals injected with wild-type KSL cells all exhibited donor-derived cells in the peripheral blood; however, MllD/D KSL cells failed to yield any significant donor contribution as early as 3 weeks after transplantation (Figure 3A, inset) or as late as 20 weeks (data not shown).

To determine whether HSC activity was present independent of the cell-surface phenotype, unfractionated MllD/D bone marrow cells were tested in competitive transplantation experiments. We tested either equal numbers (1:1) or a 10-fold excess (10:1) of MllD/D bone marrow mixed with wild-type bone marrow cells (Figures 3B and 3C). Remarkably, even a 10-fold excess of MllD/D cells yielded recipients that were engrafted exclusively by wild-type cells (Figure 3C). Thus, Mll deletion results in the rapid decline in a phenotypically-defined HSC-enriched population and loss of HSC activity from this population and, in fact, from the bone marrow in general.

Consequences of Mll Deletion on HSC Viability and Proliferation

One explanation for the inability of MllD/D KSL to compete with wild-type cells in transplantation experiments, as well as their disappearance from conditional knockout bone marrow, is that cells undergo programmed cell death in response to Mll deletion. In fact, an Mx1-cre-induced knockout of the antiapoptotic Mcl-1 gene exhibits very similar kinetics of bone marrow cytopenia and HSC loss (Opferman et al., 2005). To determine whether cell death occurs in response to Mll loss, we assessed Annexin V and propidium iodide (PI) staining of KSL cells throughout the time course after Mll deletion. As shown in Figure 4A, we found no significant difference in the proportion of Annexin V+ KSL cells at any time point. Annexin V+ cells were detectable in other populations in the bone marrow, but there were no significant differences between the two genotypes in any population tested, nor was there a difference in the overall viability (PI+) at early time points (day 0–11, data not shown).

Because programmed cell death did not account for the engraftment and homeostasis defects identified in Mll-deficient KSL cells, we considered growth arrest as an alternative hypothesis. For these experiments, we assessed the proportion of KSL cells in cycle by using 12 hr bromodeoxyuridine (BrdU) pulses (Figures 4B and 4C). Cell-surface staining was performed to identify KSL cells followed by intracellular staining for BrdU detection...
identified cells in S phase during the prior 12 hr). Surprisingly, we found a significant increase in BrdU+ KSL cells in MllΔN/ΔN bone marrow starting at day 8 after Mll deletion, becoming more severe by day 13 (Figure 4B). Conversely, there were almost no BrdU-negative cells in the MllΔN/ΔN KSL population from day 11 (Figures 4C and 4D). We

Figure 4. Cell Death and Proliferation within the MllΔN/ΔN KSL Population

(A) Control (blue) or MllΔN/ΔN (red) cells were stained with lineage, c-Kit, and Sca-1 antibodies and Annexin V to assess cell death within the KSL population over time. The percentage of Annexin V+ cells within the KSL gated population is shown from individual animals with the average shown as a bar. For gating examples, see Figure S6B and S6C. Control (blue) or MllΔN/ΔN (red) cells were stained as above, fixed, and then stained with anti-BrdU and 7-AAD. Data in (B) indicate the percentage of BrdU-positive KSL cells from at least three individual animals per time point with the error bars representing 95% confidence intervals. Data in (C) represent the percentage of BrdU-negative KSL cells (G0/G1 gate, [D]).

(E) Representative Hoechst/Pyrion staining of sorted KSL/CD48− cells at day 4 post cre induction, and (F) compiled Hoechst/Pyrion results from four individual animals per genotype based on the gates shown in (E). Similar results were obtained in at least three independent experiments.

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further enriched HSCs from this population to examine the proportion of quiescent (G0) cells by sorting CD48neg/KSL cells (Kiel et al., 2005). Four days after cre induction (prior to our increase in S phase cells), we found that MllA/NANANAN cells consistently exhibited a shift from G0 to G1 relative to control cells (Figures 4E and 4F). Therefore the first defect observed in MllA/NANANAN HSCs is the failure to maintain quiescence, followed shortly thereafter by an increased number of cycling KSL cells. These data suggest that the depletion of HSCs upon Mll loss is due to their failure to self-renew and maintain a noncycling population of HSCs.

To determine whether MllA/NANANAN HSCs and their progeny are ultimately depleted in vivo due to a failure to self-renew, we generated chimeric animals in which cotransplanted wild-type bone marrow supports long-term animal viability. Bone marrow chimeras were generated with Ly5.2+ wild-type cells mixed with either Mx1-cre;Mll+/+ or Mx1-cre;MllA/F/F transgene-negative MllA/F/F cells. After pi:pC treatment, the Mx1-cre;MllA/F/F (now MllA/NANANAN) cells were selectively lost from chimeras (Figure 5). This loss occurred first in the bone marrow, with the linneglow/c-Kit+ pool depleted of MllA/NANANAN cells by 6 weeks (Figures 5A and 5B), followed shortly after by a decline in MllA/NANANAN neutrophils, B-, and T cells in the peripheral blood (Figures 5C–5E). Because whole bone marrow was transferred, the fact that peripheral blood T- and B cells were not completely depleted (Figures 5C and 5D) likely reflects the long half-life of these cells (>12 weeks; Forster and Rajewsky, 1990; Kondo et al., 1997), whereas the more rapid and complete loss of MllA/NANANAN neutrophils (half-life of 1 to 2 days) reflects the complete exhaustion of MllA/NANANAN HSCs. These data demonstrate that Mll-deficient HSCs are completely depleted, even in a bone marrow environment that includes wild-type neighboring hematopoietic cells and does not become cytopenic. However, these data do not account for the rapid cell loss in pi:pC-injected Mx1-cre;MllA/F/F animals (Figure 2A). To investigate this further, we focused on the consequences of Mll deletion on progenitor populations.

**Mll Plays an Independent Role in Maintaining Progenitor Pools**

To determine whether lineage-committed progenitors were also affected by the loss of MLL function, we analyzed representative myeloid and lymphoid progenitor populations by cell-surface phenotype. We found that the absolute number of common lymphoid progenitors (CLP), common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP), and megakaryocyte-erythrocyte progenitors (MEP) was reduced 1.4- to 4-fold by day 11 after Mll deletion (Figure 6A). This was
predominantly due to an overall reduction in \textit{c}-Kit$^+$/lineage$^{neg/low}$/Sca-1$^+$, KLS; common lymphocyte progenitors, CLP; common myeloid progenitors, CMP; granulocyte-megakaryocyte progenitors, GMP; and megakaryocyte-erythroid progenitors, MEP. Averages are from three to four animals, and error bars represent 95% confidence intervals.

To examine progenitor frequencies prior to their phenotypic reduction, we subjected bone marrow from the day 6 time point to in vivo (colony-forming units-spleen day 8 [CFU-S 8]) and in vitro (colony-forming unit-culture [CFU-C]) clonal progenitor assays. Reduced myeloid, erythroid, and lymphoid colony frequencies were observed with \textit{Mll}\textsuperscript{DN/DN} bone marrow samples (2- to 5-fold, Figures 6B–6F). The reduction in all colony types suggests a uniform requirement for \textit{Mll} in the primitive hematopoietic compartment.

Figure 6. Reduction in Lymphoid and Myeloid Progenitors upon \textit{Mll} Deletion

(A) Average cell number per two hindlimbs at day 11 post cre induction for the indicated cell types: \textit{c}-Kit$^+$/lineage$^{neg/low}$/Sca-1$^+$, KLS; common lymphocyte progenitors, CLP; common myeloid progenitors, CMP; granulocyte-megakaryocyte progenitors, GMP; and megakaryocyte-erythroid progenitors, MEP. Averages are from three to four animals, and error bars represent 95% confidence intervals.

(B) Colonies per spleen (CFU-S\textsubscript{8}) produced from pI:pC-injected control (\textit{Mll}\textsuperscript{FF}, blue) or \textit{Mx1-cre};\textit{Mll}\textsuperscript{FF} donors (red). Donor cells (1 \times 10^5) were harvested from animals 4 days after cre induction. At least four donor animals and 16 irradiated recipients were used per genotype. Error bars represent 95% confidence intervals.

(C and D) Representative spleens for \textit{Mll}\textsuperscript{+/+} (C) or \textit{Mll}\textsuperscript{DN/DN} (D) donor cells.

(E) CFU-preB frequency in line$^{neg/low}$ bone marrow. Control (\textit{Mll}\textsuperscript{FF}, blue) or \textit{Mll}\textsuperscript{DN/DN} cells (red) were generated as in (B) but were enriched in line$^{neg/low}$ cells, plated, and scored (see Experimental Procedures). Error bars represent 95% confidence intervals.

(F) CFU-C assay using bone marrow cells prepared as in (E) but anti-IL7R\textalpha and -Sca-1 were included in the lineage mix. Cells from at least three donors per genotype were plated in triplicate, and colonies were scored 7 days later. Represented are the averages from all replicates with error bars indicating the 95% confidence interval of the averages. Megakaryocyte/erythroid, Meg/E; burst formation unit-erythroid, BFU-E; granulocyte, G; macrophage, M; granulocyte/macrophage, GM; and granulocyte, erythroid, macrophage, megakaryocyte colony, GEMM.

(G) CFU-C assay using in vitro-produced \textit{Mll}\textsuperscript{DN/DN} bone marrow cells. Lin$^{neg/low}$ cells from \textit{Mll}\textsuperscript{+/+} or \textit{Mll}\textsuperscript{FF} bone marrow were infected with a cre-ires-GFP retrovirus, and 5000 GFP$^+$ cells were plated in methylcellulose in triplicate as described in (E). Error bars represent 95% confidence intervals.

(H) Lin$^{neg/low}$/IL7R\textalpha ;/Sca-1$^+$ cells were enriched from control (\textit{Mll}\textsuperscript{FF}, blue) or \textit{Mll}\textsuperscript{DN/DN} (red) samples prepared as in (F) from animals 11 days after cre induction. Samples were cultured in liquid medium containing SCF, IL-3, and IL-6. Cells were pulsed for 1 hr with BrdU, harvested, and analyzed by flow cytometry at the indicated time points, reflecting days of in vitro culture. Error bars represent 95% confidence intervals.

(I) Cells as described above were incubated with low serum and cytokine-free medium overnight, followed by readdition of serum and cytokines for 1 hr followed by BrdU pulses as in (H). Error bars represent 95% confidence intervals.

To examine progenitor frequencies prior to their phenotypic reduction, we subjected bone marrow from the day 6 time point to in vivo (colony-forming units-spleen day 8 [CFU-S\textsubscript{8}]) and in vitro (colony-forming unit-culture [CFU-C]) clonal progenitor assays. Reduced myeloid, erythroid, and lymphoid colony frequencies were observed with \textit{Mll}\textsuperscript{DN/DN} bone marrow samples (2- to 5-fold, Figures 6B–6F). The reduction in all colony types suggests a uniform requirement for \textit{Mll} in the primitive hematopoietic compartment.

The maintenance of progenitors in vivo may reflect an intrinsic requirement for \textit{Mll} within the progenitor pool or...
Figure 7. *Mll* Is Dispensable for Homeostasis of Lineage-Committed Cells and Is Not Compensated by Close Homologs in the Primitive Hematopoietic Compartment

(A) Analysis of bone marrow (top four panels) and peritoneal cells (bottom four panels) in *lysozyme M-cre;Mll^F/+* (left column) and *lysozyme M-cre;Mll^F/F* animals (right column). Inset in the lower four panels indicates F/480 expression on Mac-1/Gr-1 gated cells.

(B) Analysis of bone marrow cells of *CD19-cre;Mll^F/+* (left column) and *CD19-cre;Mll^F/F* (right column) animals.

(C) Analysis of thymocytes in *lck-cre;Mll^F/+* (left column) and *lck-cre;Mll^F/F* (right column) animals. The lower four panels represent CD4^+/CD8^+/ gated cells stained with the indicated antibodies. Two representative animals are shown for each strain.

(D–G) The expression of *Mll* homologs within purified hematopoietic populations. Quantitative real-time PCR results are shown for *Mll* (Mll-1) (D), Mll-2 (E), Mll-3 (F), and Mll-4 (G). Populations are as described in Figure 6, with KSL cells further fractionated to enrich HSCs with short- and long-term engrafting potential (Flt3^+^ and Flt3^+-, respectively). Also tested are the populations predicted to be affected in the lineage-specific cre strains:
a prior effect in generating progenitors from HSCs. To distinguish between these possibilities, we excised Mll in myelo-erythroid progenitors (lin<sup>neg/low</sup>/Sca-1<sup>-</sup> /IL-7R<sup>α</sup>-cells) purified from Mll<sup>F/F</sup> or Mll<sup>-/-</sup> bone marrow and determined CFU-C frequencies with Mll<sup>AN/AN</sup> cells generated in vitro through retroviral introduction of cre recombinase. This approach also revealed ~2-fold reduced CFU-C from Mll<sup>AN/AN</sup> myelo-erythroid progenitors, with all colony types similarly reduced (Figure 6G). Together, these data indicate that Mll is required for the maintenance of myelo-erythroid progenitors through a cell-intrinsic mechanism.

**Proliferation Defects in Mll<sup>AN/AN</sup> Myeloid-Erythroid Progenitors**

To determine the basis for the functional and phenotypic reduction in Mll<sup>AN/AN</sup> progenitors, we assessed cell death and proliferation. We found no significant difference between the viability of Mll<sup>AN/AN</sup> cells in vivo or using the in vitro cre excision strategy described above (data not shown). We assessed proliferation in vitro by using a pool of myeloid-erythroid progenitors (lin<sup>neg/low</sup>/IL7R<sup>α</sup>-/ c-Kit<sup>+/</sup>Sca-1<sup>-</sup> cells encompassing the CMP, GMP, and MEP populations). Using a time course of in vitro BrdU pulses, we found that Mll<sup>AN/AN</sup> progenitors exhibited reduced proliferation relative to controls (Figure 6H). In addition, serum/cytokine starvation experiments demonstrated that Mll<sup>AN/AN</sup> progenitors exhibited a 2.6-fold reduction in cells that could re-enter the cell cycle upon serum and cytokine restoration (Figure 6I). Importantly, the composition of the cell populations before or after in vitro culture was not significantly different between wild-type and Mll<sup>AN/AN</sup> populations (Figure S7 and data not shown). Based on these results, we conclude that Mll is required to sustain cytokine-driven proliferation of myeloid-erythroid progenitors. Thus, the combination of ectopic, nonrenewing HSC proliferation in conjunction with the reduced proliferation within the larger progenitor pool (lin<sup>neg/low</sup>/c-Kit<sup>+</sup>) is likely to play a major role in the rapid attrition of cells in the pl:pC-injected Mx1-cre;Mll<sup>F/F</sup> animals.

**Differentiating T-, B-, and Myeloid Cells No Longer Require Mll for Homeostasis**

To determine whether Mll is continuously required throughout the differentiation of myeloid and lymphoid cells, we used lineage-specific cre transgenic or knockin strains to delete Mll in cells subsequent to lineage commitment. In contrast to the results with more primitive progenitors, we found no difference in the steady-state number or function of peripheral B cells, T cells, or macrophages and neutrophils in CD19-cre, lck-cre, or lysozyme-M cre;Mll<sup>F/F</sup> animals, respectively (data not shown). We carefully examined the populations representing the beginning of maximal cre expression in each strain and found that there were no differences in cell number or phenotype despite efficient gene deletion within the appropriate population (Figures 7A–7C and data not shown). Thus, these data delineate an early window of hematopoietic development as Mll dependent, encompassing the stem and progenitor pools. These data also demonstrate that our failure to detect mature Mll<sup>AN/AN</sup> leukocytes in transplanted animals (Figures 3 and 5) was not due to a late block in T cell, B cell, or granulocyte differentiation, and therefore accurately reflect a defect in stem/progenitor function.

Due to the contrast of these results with the severe, multilineage phenotype observed in early progenitors, we considered compensation by Mll homologs as an explanation for the differential effect of Mll deletion on primitive versus more mature, lineage-committed cell types. The Mll homologs Mll-2 through -4 exhibit similar domain organization and encode SET domains predicted to methylate histone substrates with the same specificity (Fitzgerald and Diaz, 1999; Prasad et al., 1997; Ruault et al., 2002). Mll-2, the most closely related homolog, is expressed in a similar manner to Mll in the hematopoietic system (see below). Mll-2 has been isolated in similar protein complexes as Mll and has also been localized to the same target gene by ChiP (Dou et al., 2005; Hughes et al., 2004; Nakamura et al., 2002; Yokoyama et al., 2004).

**DISCUSSION**

The central finding of this study is that adult steady-state hematopoiesis depends on Mll, based on essential myelomonocytic precursors (M, Mac-1<sup>-</sup>/Gr-1<sup>-</sup>), pro/pre-B cells (B, B220<sup>+</sup>/CD19<sup>+</sup>), and double-positive T cells (T, CD4<sup>+</sup>/CD8<sup>+</sup>). Results represent the mean of triplicate reactions with all expression levels normalized to Gapdh. Error bars indicate standard deviation, and asterisks indicate an undetectable Ct. Similar results were obtained in three independent experiments. 

(H) Model illustrating the essential, nonredundant role of MLL in maintaining stem and progenitor cells. The green box encompasses cells affected by MLL loss, with those outside the box unaffected at the level of steady-state cell numbers. Red boxes indicate point at which lineage-specific cre expression occurs using the strains shown.
functions performed within hematopoietic stem and progenitor cells. In the absence of Mll, HSCs exit from a quiescent state and precociously undergo cell-cycle entry. Ectopic cycling of HSCs occurs without an increase in this population and coincident with functional deficits, suggesting that cell division in this compartment is accompanied by differentiation and not self-renewal. In contrast, the role of Mll within primitive myelo-erythroid progenitors appears to be quite distinct, in that progenitor frequency is reduced upon Mll deletion through a mechanism involving reduced proliferation. In conjunction with germline loss-of-function analyses (Ernst et al., 2004a), the studies presented here demonstrate that Mll is necessary for both the development and maintenance of HSCs. Further characterization will reveal whether all of these MLL-dependent processes utilize common or distinct molecular pathways.

Our data generally support the concept that MLL acts on a selective set of target genes in different cell types. Based on a genome-wide ChIP study in the U937 cell line, it has been proposed that MLL functions as a transcription start site–specific global transcriptional regulator (Guenther et al., 2005). This conclusion is partly based on the large overlap in Pol II and MLL localization at active promoters. If this colocalization indicated a biologically important association, one would expect a severe consequence for all cells in which MLL activity is removed. This prediction is difficult to reconcile with the selective phenotypes observed in vivo upon Mll loss. For example, ES cells and many differentiating tissues in chimeric animals are unaffected by Mll deficiency (Ernst et al., 2004a; Ernst et al., 2004b). These observations, along with the results presented in this study, suggest that selective and distinct target genes are regulated by MLL and its homologs in different tissues.

The reduced proliferation of myelo-erythroid progenitors shown in this study is consistent with previous observations using embryoid body–derived hematopoietic progenitors. In these studies, the reduced expression of multiple Hox genes appeared to be the primary cause for the reduced colony frequency in Mll mutant embryoid bodies (Ernst et al., 2004b). Thus, we predict that the reduced Hox gene expression in progenitors is similarly likely to play a major role in reduced progenitor frequency and proliferation upon Mll deletion. Conversely, Hoxa7 and Hoxa9 are consistently overexpressed in MLL fusion-initiated leukemia in humans and in mouse models, and are rate limiting for leukemogenesis or influential in determining leukemic cell phenotype (Armstrong et al., 2002; Ayton and Cleary, 2003; Kumar et al., 2004; So et al., 2004). Based on the results of the current study and results of Hox expression analyses in Mll loss- and gain-of-function experiments (Ernst et al., 2004b; Horton et al., 2005), we propose that altered Hox gene expression may be responsible for the proliferation component of the leukemogenic program induced by MLL fusion proteins.

The regulation of quiescence in HSCs is less likely to be explained by Hox gene maintenance alone, based on analyses of individual and compound Hox gene knockouts in which such a phenotype has not been reported (Bijl et al., 2006; Brun et al., 2004; Lawrence et al., 2005). This suggests that there are other HSC-specific Mll target genes involved in this process. Such HSC-specific target genes may remain activated by MLL fusion oncoproteins as HSCs differentiate, resulting in the maintenance of a self-renewal program coincident with a proliferation-promoting program mediated by Hox gene overexpression. Candidate genes for this self-renewal program include those reported as reactivated in an MLL-AF9 leukemia model (Krivtsov et al., 2006). Leukemia can be initiated by introducing MLL fusion oncogenes into committed progenitors (Cozzo et al., 2003; Krivtsov et al., 2006; Somervaille and Cleary, 2006), suggesting that the reactivation of such a self-renewal program by MLL fusion oncogenes can occur in progenitors. However, the introduction of MLL fusion oncogenes into HSCs rather than progenitors may facilitate fusion protein access to a quiescence-promoting gene expression program. One prediction of this hypothesis is that the introduction of Mll fusions into HSCs (rather than progenitors) would result in a more persistent, chemotherapy-resistant pool of leukemic cells, a concept that has not been tested in mouse models.

Importantly, our studies suggest that targeting MLL fusion oncogenes through mechanisms that generally interfere with MLL function may cause bone marrow failure. Thus it will be essential to identify strategies that selectively target MLL fusion oncoproteins without compromising wild-type MLL function. This effort will be aided by a comprehensive assessment of target genes affected by MLL fusions versus those affected by wild-type MLL.

**EXPERIMENTAL PROCEDURES**

**Mice, Competitive Transplantation, and CFU-S Assays**

Animals were maintained in accord with the Dartmouth Animal Resources Center and I.A.C.U.C. policies. Mll floxed alleles and ES targeting have been described (Ernst et al., 2004a). To generate Mll^AN animals, Mll^F/F animals were crossed to the Ella-cre strain (Jackson Labs). Mll^AN embryos were generated by crossing heterozygotes. Eight a.m. of the day of vaginal plug observation was considered E 0.5. The Mll^AN strain was backcrossed to C57Bl/6 for more than nine generations then backcrossed to B6.SJL (Jackson Labs) for five generations. Animals were crossed to the Mx1-cre, lysozyme M-cre, CD19-cre (obtained from Drs. Rajewski and Orkin, Harvard Medical School) and lck-cre strains (obtained from Dr. Wilson, University of Washington) to produce homozygous Mll floxed (Mll^AN), cre-expressing strains. Mx1-cre/Mll^AN, Mx1-cre/Mll^AN^+^−^, or Mll^AN^ mice were treated with two or four injections of plpC (GE Healthcare) every other day. Two, three, or four injections were performed for the day 4, 6, or 8–19 time points, respectively. Increasing the number of injections resulted in a higher penetrance of the phenotypes described, without altering the kinetics of their appearance. All animals were 6–12 weeks old for analyses in this study. The following formula was used for the plpC dose: (10 × animal mass [g] + 50) µg plpC.

For competitive transplantation experiments, C57BL/6J recipient mice were irradiated with a split dose of 1100 Rads 3 hr apart. Bone marrow cells were prepared by crushing with a mortar and pestle in ice-cold Hank’s buffered saline solution (HBSS) supplemented with 2% fetal bovine serum (FBS). Red cells were removed with ACK lysis buffer (0.13 M NH4Cl, 10 mM KHCO3, 0.1 mM EDTA [pH 7.2]). Bone marrow chimeras were generated by injecting a mixture of 1.25 × 10^6 donor cells (Mx1-cre/Mll^AN^, or Mx1-cre/Mill^AN^, Ly5.1) plus 2.5 × 10^6
carrier cells (wild-type Ly5.2 or Ly5.1/5.2) and waiting >6 weeks for stable engraftment. Animals were maintained on sterile food and water (containing 50 μg/mL Baytril) for 3 weeks after irradiation. For the CFU-S assay, 1 × 106 bone marrow cells from animals receiving 2 pI:pC doses were injected into lethally irradiated recipients. Eight days later, spleens were transferred to Tellesniczyk’s fixative. Colonies were counted and photographed with a Leica MZ7.5 dissecting microscope fitted with a Cannon Powershot S3 IS digital camera.

Flow Cytometry, Annexin V Binding Assays, and G0/G1 Analysis
Lineage staining was accomplished with unconjugated rat anti-CD4, -CD8, -Gr-1, -B220, -CD19, -Ter119, and -Mac-1 and for some experiments anti-Sca-1 and -IL-7Rα (all antibodies were from BD Biosciences except the IL-7Rα [eBiosciences]). Lineage antibodies were detected with goat anti-rat Fab (Fab′)2 tricolor (CalTag), blocked with rat serum (Sigma), and stained with anti-Sca-1 and -c-Kit antibodies (BD Biosciences). Stained cells were incubated with Annexin V FITC as described by the manufacturer and analyzed immediately with a FACSCalibur (BD Biosciences) and FlowJo software (Treestar, Inc). We first determined that Mxl-cre;MllΔ1–22M mice exhibited indistinguishable levels of Annexin V and PI staining after pI:pC injection (data not shown) then used either genotype as controls for the following experiments. Annexin V-positive controls were generated for each analysis by incubating cells with 1 mM H2O2 at 37°C for 5 min. G0/G1 analysis was performed by first FACS purifying KLS/CD48neg cells then incubating 10,000 sorted cells in 10 μg/mL Hoechst 33342 and 100 μM verapamil (both from Molecular Probes) for 45 min at 37°C. Samples were then supplemented to 2 μg/mL Pyronin Y (Sigma), incubated for an additional 15 min, washed, and analyzed immediately on a FACSAnia (BD Biosciences). Anti-CD48 was from BD Biosciences. The unpaired t test was used to evaluate the statistical significance of differences in G0 populations.

Ex Vivo Induction of Cre Recombinase and In Vitro Progenitor Assays
Bone marrow cells were prepared from 6- to 8-week-old MllΔ1–22M- and MllF/F mice. Cells were stained with unlabeled antibodies as described above and depleted with sheep anti-rat magnetic beads (Dynab). Lin-creflow cells were then resuspended in growth medium (IMDM containing 50 ng/mL SCF, 10 ng/mL IL-3, and 10 ng/mL IL-6. Cells were incubated with BrdU at 10 μM for 1 hr. For the starvation experiment, cells were grown for 2 days, washed and incubated in cytokine-free, 0.5% FBS growth medium overnight, shifted to complete medium, and then pulsed with BrdU as described above. For the pre-B colony assay, 10,000 lin-crelow cells were plated in Methocult M3630 (StemCell Technologies) supplemented with 100 ng/mL SCF, 20 ng/mL murine Flt3L, and 10 ng/mL IL-7. Pre-B colonies were scored after 7 days. Cytokines were from R&D Systems.

Real-Time, Quantitative PCR
Total RNA from the sorted populations indicated in Figure 7 was purified with the RNeasy Mini kit (QIAGEN). After extraction, 1 μg MS2 bacteriophage RNA (Roche) was added as a carrier, and mRNA was amplified with the RiboAmp RNA Amplification Kit (Arcturus) per the manufacturer’s recommendations for one round of amplification. Hox gene detection was performed as described (Ernst et al., 2004b), and Mil family primer and probes are shown in Figure S5. Relative expression levels were determined by using the ΔΔCt method (Livak and Schmittgen, 2001) with data from triplicate multiplexed reactions normalized to Gapdh. Quantitative real-time PCR was performed on a 7900HT machine using the 9600 emulation setting (Applied Biosystems).

Histopathology
For bone marrow sections, the humerus was removed, cleaned, and transferred to Bouin’s fixative overnight at 4°C. Fixed samples were sectioned and stained by the Harvard Histopathology Core Facility. Samples were imaged on a Nikon Optiphot-2 microscope with 10x and 40x objectives.

Plasmids
The region spanning the exon 3 to 4 deletion (ΔN) was amplified by PCR from MllΔ324/Δ324 fibroblast RNA. This PCR product was inserted into the Fasel and PvuI sites of the human MLL cDNA. The resulting MllΔ1–22M cDNA was modified to encode the Flag epitope at the 5′ end and the HA epitope at the 3′ end by inserting annealed oligonucleotides encoding the epitope sequence. The tagged cDNAs (wild-type and MLLΔ1–22M) were then subcloned into pCDA3.1(-) (Innotriv) at the Xhol and EcoRV sites.

Cell Culture, Transfection, and Immunofluorescence
293T cells were maintained in DMEM supplemented with 10% FBS, 200 mM L-gln, P/S, and 100 mM NEAA and transfected with FuGENE 6 (Roche). Thirty-six hours posttransfection, cells were plated on coverslips and grown overnight before fixing in 4% paraformaldehyde in PBS for 5 min. Cells were then blocked with PBS containing 2% goat serum and 0.2% Triton X-100 overnight. Anti-Flag M2 (1:100, Sigma), anti-MLL1 (BL1290), and anti-HA (both 1:100, Bethyl Labs) were incubated with coverslips for 30 min at room temperature, then washed three times in PBS. Detection was performed with Alexa Fluor-568 goat anti-mouse or Alexa Fluor-488 goat anti-rabbit (both at 1:1000, Molecular Probes). Stained cells were then washed three times in PBS, with the final wash containing 1μg/mL 4′,6-diamidino-2-phenylindole (DAPI). Coverslips were mounted onto slides with ProLong Gold antifade medium (Molecular Probes). Images were captured with a Zeiss AxioVisionZ1 microscope and analyzed in Adobe Photoshop. Macrophages were cyto spun onto slides and stained as described above.

Supplemental Data
Supplemental Data include eight figures and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/1/3/324/DC1/.

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de novo translocations can induce lineage reassignment in tumorigenesis. EMBO J. 24, 3136–3146.


of the Hox a9 locus, promoting transcription and multiple histone modifications. Cancer Res. 65, 11367–11374.


Supplemental Data

Unique and Independent Roles for MLL in Adult Hematopoietic Stem Cells and Progenitors

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Figure S1. Age of lethality for homozygous Mll<sup>ΔΝ/ΔΝ</sup> embryos. Mll<sup>ΔΝ/+</sup> animals were intercrossed and embryo age was defined considering the day of plug observation (8 a.m.) as day 0.5 post-coitum (dpc). Pregnant females were sacrificed at the embryonic day indicated and viable embryos were scored based on cardiac contractions. All embryos were genotyped by Southern blot or the 3 primer assay presented in Fig. S5 using yolk-sac or limb bud genomic DNA.
Figure S2. Expression of *Hoxb3* and *Mll-2* in lin$^\text{neg/low}$ bone marrow cells 11 days after cre induction as determined by quantitative real-time PCR. Error bars represent 95% confidence intervals.
Figure S3. Expression of Hox genes in sorted KLS cells six days after cre induction. A) Gating used to sort KLS cells from bone marrow of Mll\(^{+/+}\) and Mll\(^{\Delta N/\Delta N}\) mice. Samples in which one antibody is withheld to demonstrate controls for positioning the gates are shown in the top row. Examples of Mll\(^{+/+}\) and Mll\(^{\Delta N/\Delta N}\) cells are shown in the bottom two rows. B) Results of quantitative real-time PCR assays using amplified RNA from sorted
KLS cells. Asterisk denotes undetectable expression. C) Expression of *Hoxb3* and *Mll-2* in sorted KLS cells as determined by quantitative real-time PCR. Error bars represent 95% confidence intervals. D) Table of raw *Gapdh* Ct values in the sorted KLS samples.
Figure S4. The FACS plots demonstrate the KSL and CLP gating for Mx1-cre;Mll^{F/F}(top row) and Mll^{+/+} samples (bottom row).
Figure S5. Efficiency of cre-mediated Mll deletion in bone marrow cells. A) Low-cycle PCR products using a $^{32}$P-labeled forward primer were fractionated on an 8% acrylamide gel and visualized by autoradiography. The black arrow indicates the $Mll^{\Delta N}$ product and the open arrowhead indicates the $Mll^{F}$ product. B) Quantification of Mll deletion efficiency in a subset of the individual mice used for Figure 3. PCR products were quantified by exposing dried gels to phosphorimager analysis to determine the relative ratio of $Mll^{\Delta N}$ versus $Mll^{F}$ alleles. A standard curve was run for each experiment to ensure measurements in the linear range of the PCR reaction.
Figure S6. Representative examples of staining of $Mll^{+/+}$ and $Mll^{ΔN/ΔN}$ bone marrow samples used for the data shown in Fig 3A and 4A. Untreated control and H$_2$O$_2$-treated (annexin V positive control) analyses are shown in the final column.
Figure S7 Composition of the cell populations before and after in vitro culture. The average percentage of A) cells depleted from crude bone marrow, and B) lin$^{neg/low}$ bone marrow cells after depletion of lineage-positive bone marrow cells. Percentages are shown for both genotypes. After depletion, cells were re-stained with a cocktail of lineage-specific antibodies (see Experimental Procedures) and detected with an anti-rat
fluorochrome conjugate. **C-D)** Gating used to identify myeloid progenitor cells. **C)** Unstained control. **D)** Lineage staining of crude bone marrow cells. Representative examples of progenitor cells enrichment of **E)** Mll+/+, and **F)** MllΔN/ΔN cells after lineage depletion. **G)** Composition of the *in vitro* progenitor cultures after 5 days in SCF, IL-3 and IL-6 as described in the Experimental Procedures. Cells cultured in parallel with those used for the proliferation experiments were stained with the indicated antibodies and analyzed by flow cytometry. Total percentages of the indicated cells are shown for *Mll*+/+ (blue bars) or *Mll*ΔN/ΔN cells (red bars).
**Figure S8.** Design and characteristics of MLL homolog PCR primers and probes.  

**A)** Amplicon characteristics for the genes indicated. Gene nomenclature corresponds to our use in the text with alternate names shown. 

**B)** Primer and probe sequences. Probes were synthesized with 5’-FAM and 3’-TAMRA modifications. The default ABI cycling conditions were used for all reactions. 

**C)** Expression of MLL and homologs in testis. 

*Mll-1, Mll-2, Mll-3 and Mll-4* gene expression levels in testis were determined as described in the Experimental Procedures and Fig. 7 legends. Results represent the mean expression level relative to *Gapdh* and displayed in log scale. A value of 1 is equal to the
level of *Mll-4* expression in the myelomonocytic population. Error bars represent standard deviation. Experiments were repeated at least three times with similar results.