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An MLL-Dependent Network Sustains Hematopoiesis

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An MLL-dependent network sustains hematopoiesis


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The histone methyltransferase Mixed Lineage Leukemia (MLL) is essential to maintain hematopoietic stem cells and is a leukemia protooncogene. Although clustered homeobox genes are well-characterized targets of MLL and MLL fusion oncoproteins, the range of MLL-regulated genes in normal hematopoietic cells remains unknown. Here, we identify and characterize part of the MLL-dependent transcriptional network in hematopoietic stem cells with an integrated approach by using conditional loss-of-function models, genomewide expression analyses, chromatin immunoprecipitation, and functional rescue assays. The MLL-dependent transcriptional network extends well beyond the previously appreciated Hox targets, is comprised of many characterized regulators of self-renewal, and contains target genes that are both dependent and independent of the MLL cofactor, Menin. Interestingly, PR-domain containing 16 emerged as a target gene that is uniquely effective at partially rescuing MLL-deficient hematopoietic stem and progenitor cells. This work highlights the tissue-specific nature of regulatory networks under the control of MLL/Trithorax family members and provides insight into the distinctions between the participation of MLL in normal hematopoiesis and in leukemia.

Epigenetic regulation is an important mechanism by which gene expression fidelity is maintained during development. The tri-thorax-group (trx-G) and Polycomb-group (Pc-G) genes encode epigenetic factors that act as opposing regulators of clustered homeobox (Hox) gene expression and of axial patterning in most metazoans (1, 2). In addition, numerous studies implicate Pc-G and trx-G homologs in mammals in the maintenance of broader gene expression programs in embryonic and tissue stem cells and in cancer (1, 2). Because of the reversible nature of epigenetic lesions in cancer, targeting oncogenes and tumor suppressors that use epigenetic mechanisms is a promising approach for targeted therapy (3).

The human protooncogene Mixed Lineage Leukemia (MLL) was the first mammalian trx homolog identified because of its characteristic rearrangement in ~70% of infant leukemia. Rearrangement of the human MLL gene by chromosomal translocation also occurs at a lower frequency in childhood acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), and treatment-related and de novo AML in adults (4, 5). Most translocations produce MLL fusion oncoproteins that retain the chromatin-targeting N terminus and acquire a transcriptional effector domain from the C-terminal partner. Partner proteins frequently recruit protein complexes that result in increased histone H3 lysine 79 dimethylation at MLL-fusion targets, overexpression of these target genes, and leukemic transformation (6). Because many of the chromatin-targeting motifs are shared by several protooncogenes MLL fusions and wild-type MLL targeting of MLL-fusion oncoproteins will also require a thorough understanding of normal MLL-dependent regulatory pathways.

Wild-type MLL exists in cells as part of a large multiprotein, chromatin-associated complex that contains chromatin remodeling and histone acetylation/methylation activities (7, 8). MLL itself is thought to regulate genes in part through a highly conserved histone methyltransferase motif, the Su(var)3-9, Enhancer of Zeste, and Trithorax (SET) domain. MLL, like Trithorax, maintains precise domains of Hox gene expression during embryonic development (9, 10).

In addition, MLL has been shown to regulate other tissue-specific processes in immune, hematopoietic, vascular, and neural cell types (11–14). Germ-line disruption of Mll is generally embryonic lethal with multiple developmental defects (9, 15–17); however, conditional deletion of Mll in specific cell types revealed unique functions. For example, hematopoietic-specific deletion of Mll demonstrated that it is essential for maintaining hematopoietic stem and progenitor cells (HSCPs), but dispensable for lineage-committed precursors (13, 18, 19). The breadth of target genes regulated by MLL in specific tissues is largely unknown, although Hox genes are consistently down-regulated in many MLL-deficient cell types (9, 13, 14).

In this study, we investigate the molecular circuitry underlying the critical role of Mll in maintaining hematopoiesis as a means to understand trx-G function in normal and pathologic gene regulation. We used inducible loss-of-function models to identify hematopoietic stem cells (HSC)-specific MLL-regulated genes and delineated a network of transcriptional regulators that are direct transcriptional targets of MLL. We then tested reexpression of a subset of these genes in Mll-deficient hematopoietic cells to determine the epistatic relationships among transcriptional targets, to identify cross-regulatory relationships, and assess their individual ability to restore function in Mll-deficient cells. These studies reveal a coherent MLL pathway that coordinates self-renewal, proliferation, and lineage-specific gene expression fidelity in HSCs. Furthermore, this work distinguishes the MLL-dependent transcriptional network from that controlled by MLL fusion oncoproteins in leukemia.

Results

Short-Term Consequences of Mll Deletion in HSCs. To identify Mll-dependent genes involved in maintaining HSCs, we analyzed differentially expressed transcripts after Mll deletion. Lineage-negative, stem cell antigen-1 (Sca-1)+, c-Kit+, CD48−, CD150+ (LSK/CD48neg) HSC-enriched cells from the bone marrow (BM) of polyinosinic:polyribocytidylic acid (pI:pc)-injected control and -deleted mice were purified 6 d after the first pI:pc injection, the optimal timing for Mll deletion, cell yield, and down-regulation of homeobox protein a9 (Hoxa9), a bona fide Mll target gene (13). Assessment of normalized gene expression differences between control and Mll-deficient LSK/CD48neg cells revealed 1,935 differentially expressed genes using Significance Analysis of Microarrays (SAM) analysis (which does not impose a fold cutoff; Fig. 1) (20). Functional classification of genes differentially expressed in Mll-deficient HSCs compared with controls resulted in three global observations: (i)
more genes were up-regulated than down-regulated, (ii) a subset of erythroid-specific genes were up-regulated, and (iii) the largest category of annotated down-regulated genes was comprised of transcriptional regulators.

Among the up-regulated genes, the largest group corresponds to HSC proliferation and ribosome or mitochondrial biogenesis (Fig. L4 and Dataset S1). Up-regulation of genes involved in ribosome biogenesis reflected the greater proportion of cycling Mll-deficient LSK/CD48\textsuperscript{neg} cells (45% G0 in Mll-deleted cells versus 75% G0 in controls; ref. 13). Ten percent in this category and 17% in the mitochondrial group were also identified in proliferating HSCs (21), [Dataset S1]. Thus, many of the up-regulated genes reflect the expected changes based on the proliferation state of Mll-deficient LSK/CD48\textsuperscript{neg} cells. Unexpectedly, 5% of the genes that were up-regulated in Mll-deficient LSK/CD48\textsuperscript{neg} cells encode erythroid-specific proteins, including transcriptional regulators such as GATA binding protein 1 (Gata1) and Kruppel-like factor 1 (Klf1), as well as spectrin, Kell protein (Kell), Erythrophoetin receptor (EpoR), and hemoglobin biosynthesis genes (Dataset S1). Gene set enrichment analysis (GSEA) also identified a GATA1-induced gene signature and a tendency toward erythroid identity (Fig. S1 A and B). The up-regulation of erythroid genes was validated by using an independent in vitro Mll deletion system, illustrating that the scale of gene up-regulation was consistent with derepression rather than full induction of erythroid genes (Fig. S1 C and D). Furthermore, this derepression was not sufficient to impart erythroid fate as demonstrated by colony assay (Fig. S1E).

Depression of erythroid genes likely occurs through an indirect mechanism, thus we focused on the down-regulated genes as potential MLL effectors in the maintenance of HSCs.

**Identifying an Mll-Dependent Transcriptional Network**. Transcriptional regulators comprised the largest single annotated category of down-regulated genes in Mll-deleted LSK/CD48\textsuperscript{neg} cells (Fig. 1B and Dataset S2). Because many of these regulators are highly expressed in HSCs relative to more differentiated cell types (22), we asked whether Mll-deficient HSCs exhibit a global shift in cell fate by assessing the relatedness of our gene expression data to other hematopoietic populations (23, 24). This analysis showed an enrichment of erythroid identity as described earlier, but did not suggest that HSCs were generally differentiated, because HSC and multipotent progenitor signatures were equivalently enriched by GSEA (Fig. S1F). Mll itself (Fig. S1G) and well-characterized MLL targets such as Hoxa9 were down-regulated although the majority of the genes in this category were not previously known to be Mll targets (Fig. 1C). We confirmed the Mll dependence for all annotated transcription factors >2.5-fold down-regulated by quantitative RT-PCR (RT-qPCR) using independently sorted samples from Mxl-cre/Mll\textsuperscript{fl} animals (Fig. 1D), as well as cells in which Mll was deleted in vitro by using 4-hydroxytamoxifen (4-OHT; Fig. 1E). Each inducible knockout model has its characteristic limitations, so to discover genes that were truly Mll-dependent, we only pursued genes down-regulated in both Mxl-cre and ER-cre systems. Of the annotated transcription factors down-regulated >2.5-fold (Fig. 1C), MDS and Evi1 complex locus (Mecom), Pdm16, Pre-B cell leukemia homeobox protein 1 (Pbx1), Eyes absent homolog 1 (Eya1) and Hoxa9 were consistently Mll-dependent (Fig. 1E). Tripartate motif-containing 30B (Trim30B) is not characterized, so we focused on the other five genes for the following studies.

Several of the transcriptional regulators identified above individually play critical roles in HSC homeostasis. For example, the proteins encoded by the Pbx1, Pdm16, and Mecom genes act to restrain HSC proliferation and/or promote self-renewal (25–29), as has been demonstrated for Mll (13, 18). Interestingly, Mecom and Pdm16 were not Mll-dependent in fibroblasts or in Mll knockout embryos overall, despite coexpression of Mll and these genes (Fig. S2).

**Mll Binds Directly to the Promoter Regions of a Subset of Mll-Dependent Genes. Mll** and its homolog Trithorax typically act to maintain expression of their direct target genes (30), thus we evaluated the down-regulated transcription factors as potential direct MLL targets. To assess whether MLL acts directly to promote expression of the identified transcriptional regulators, we used a mini-ChIP procedure optimized for 5 × 10\(^6\) BM cells (31). Based on previous results demonstrating MLL binding near transcription start

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**Fig. 1.** Identification of Mll-regulated genes in HSCs. General overview of genes up-regulated (A) or down-regulated (B) in Mll-deficient LSK/CD48\textsuperscript{neg} cells compared with controls. Cells were sorted from mI:pC-injected control Mll\textsuperscript{fl} or Mxl-cre/Mll\textsuperscript{fl} animals at day six. Gene Ontology assignments were based on the criteria in Datasets S1 and S2. (C) The top down-regulated transcription factors in Mll-deficient LSK/CD48\textsuperscript{neg} cells listed by fold reduction (see also Dataset S2). (D) RT-qPCR validating down-regulated genes in independent control Mll\textsuperscript{fl} (blue) or Mll-deficient (red) LSK/CD48\textsuperscript{neg} cells, n = 8 animals per genotype; ND, not detected. (E) RT-qPCR validation of transcripts in LSK cells sorted from control ER-cre/Mll\textsuperscript{fl} (blue) or ER-cre/Mll\textsuperscript{fl} animals (red) cultured for 72 h after initiating Mll deletion. Relative expression levels were determined by normalizing to Gapdh, n = 4 animals per genotype. Error bars represent 95% confidence interval (CI). *P ≤ 0.07, **P ≤ 0.05. ER-cre, estrogen receptor\textsuperscript{22b} mutant fused to cre recombinase.

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**Fig. 2.** Mll binds directly to the promoter regions of a subset of genes identified by expression array. ChiP results demonstrating specific enrichment at the Mecom locus (Mds1 and Evi1 start sites) and the Prdm16, Pbx1, and Eya1 promoter regions. Anti-Mll C-terminal (black) or control (anti-GAL4, gray) antibodies were used for ChiP, and enrichment was determined by using quantitative PCR assays. Amplicon position is indicated relative to the TSS for each gene. Results using additional primers surrounding the TSS are shown in Fig. S4. Data represents averages ± SEM for two to four PCR replicates and are representative of at least four independent experiments.
sites (TSS) in cell lines (32, 33), we assessed MLL binding within 2 kb of the TSS by using 3–5 amplicons per gene. MLL-dependence was similarly observed in the BM lineage-negative (lin<sup>−</sup>) population and LSK cells (Fig. S3A). Control ChIP experiments demonstrated MLL binding to the Hoxa9 but not Gapdh TSS regions (Fig. S3B). Using lin<sup>−</sup> BM cells, we observed specific MLL binding around each TSS of the Mecom locus [both Myelodysplastic syndrome 1 (Mds1) and Ecotropic virus integration site 1 (Evi1) promoter regions], as well as the Prdm16, Pbx1, and Eya1 genes (Fig. 2 and Fig. S1 C–G). Interestingly, we did not observe MLL binding to the Early growth response 1 (Egr1) promoter (Fig. 2B and Fig. S3H), consistent with the observation that this gene was not MLL-dependent in both model systems (Fig. 1E). Therefore, we conclude that like Hoxa9, the expression of Mecom, Prdm16, Pbx1, and Eya1 is maintained directly by MLL in normal lin<sup>−</sup> BM cells.

**Only a Subset of MLL-Dependent Genes Are Affected by Men1 Deletion.** MLL itself does not harbor sequence-specific DNA binding motifs. One important chromatin-targeting mechanism occurs through an N-terminal interaction with Menin and p75/leukemia epithelium-derived growth factor (LEDGF), thought to be essential for targeting wild-type MLL to promoter regions based on studies using MLL fusion oncoproteins (34). To understand how the MLL complex localizes to its targets in HSCs, we assessed the Menin dependence of Egr1, Hoxa9, Prdm16, Mecom, Pbx1, and Eya1. Consistent with a previous study (35), we found that Hoxa9 expression was reduced in Menin (Men) 1-deficient LSK cells. Interestingly, Mecom and Eya1 were slightly reduced, but the latter was not statistically significant (Fig. 3A). Despite efficient excision of Men1 (Fig. 3B), Prdm16 and Pbx1 levels were not affected (Fig. 3C), suggesting that a subset of HSC-specific MLL-dependent genes do not require Menin. These data demonstrate that the MLL complex differentially requires the Menin chromatin-targeting cofactor to regulate distinct classes of target genes.

**Structure of the MLL-Dependent Transcriptional Network.** We considered that some of the MLL-dependent transcriptional regulators act in interconnected pathways to modulate HSC function. For example, it has been reported that overexpression of Evil up-regulates Pbx1 in c-kit–enriched BM cells (36). To identify potential expression interrelationships and determine whether the identified genes represent a linear or branched pathway downstream of MLL, we overexpressed Hoxa9, Prdm16, Eya1, Pbx1, or Mecom isoforms (Mds1-Evii and Evil) in wild-type or MLL-deficient LSK cells and assessed the effect on other genes in this network 48 h later. Focusing first on the effects of overexpression in wild-type cells, we found that Hoxa9 could increase levels of Prdm16, Evil could increase both Prdm16 and Hoxa9, and Prdm16 could increase Hoxa9 levels. For MLL-deficient LSK cells infected with the empty retrovirus, we observed reduced expression of Hoxa9, Prdm16, Mecom, Pbx1, and Eya1 (Fig. 4, empty) as compared with unmanipulated MLL-deficient LSK cells (Fig. 1). However, reexpression of Hoxa9, Prdm16, Eya1, or Pbx1 did not restore expression of the other tested genes to wild-type levels in MLL-deficient LSK cells (Fig. 4). In contrast, expression of either of the Mecom isoforms altered the expression of other genes in this network in MLL-deficient LSK cells. Evil expression increased Prdm16 and Hoxa9 transcripts in MLL-deficient LSK cells back to the wild-type levels (Fig. 4 A and B). Mds1-Evii suppressed Prdm16, Hoxa9, Pbx1, and Eya1 expression in wild-type cells to the low levels observed in MLL-deficient LSK cells (Fig. 4, A, B, D, and E), consistent with previous observations that Mds1-Evii and Evil have opposing activities on hematopoietic differentiation and cytokine-stimulated growth (37, 38). These data illustrate that overexpression of individual transcription factors can influence the expression levels of other regulators in this network primarily in wild-type LSK cells, yet in most cases cannot restore normal levels of any of the network genes in MLL-deficient cells. The exception is Evil, which is capable of restoring the expression of two of the five genes in this network in MLL-deficient LSK cells. Taken together, these data exclude that these transcriptional regulators are organized in a linear pathway downstream of MLL and, instead, suggest that they each perform independent functions as downstream effectors of MLL.

**Prdm16 Exhibits a Unique Capacity to Partially Rescue MLL-Deficient Cells.** One to two weeks after inducing cre, the attrition of BM cells in Mlx1-cre;Mll<sup>−/−</sup> animals results in animal death accompanied by multiple defects in HSPCs (13). To evaluate the relative functional importance of the identified Mll targets, we assessed whether reexpression of individual genes could rescue Mll-deficient cell attrition from BM chimeras. To this end, the Mll target genes identified above were overexpressed individually in sorted LSK cells from uninduced control Mll<sup>−/−</sup> or Mlx1-cre;Mll<sup>−/−</sup> mice, then engrafted into lethally irradiated recipients together with uninfected wild-type BM cells. After stable engraftment, Mll excision was induced by pI-pC injection and the persistence of Mll-deficient BM cells expressing the reintroduced gene was determined 2 wk later (Fig. S4A). Thus, in this assay, “rescue” is defined as the selective persistence of retrovirus-infected cells within the population of Mll-deleted cells (Fig. S4A). The use of Mll itself as a positive control was precluded by the large size of the Mll transcript (>11 kb), because it could not be packaged into a retrovirus.

Upon Mll deletion, uninfected or empty retrovirus-expressing donor cells were lost rapidly from chimeric animals (Figs. S4 B and C). Hoxa9 overexpression resulted in the expansion of donor-derived cells in chimeras (Hoxa9 versus empty) but also Hoxa9 expressing Mll-deficient cells were protected from attrition, as evidenced by their overrepresentation in the Mll-deficient population (Fig. 5B, red versus blue). Surprisingly, Prdm16 reexpression resulted in the most significant rescue of Mll-deficient cells. Despite its greater ability to influence other network genes, reexpression of Evil only marginally protected Mll-deficient cells from attrition, and Mds1-Evii, Pbx1, and Eya1 had no specific activity in this assay (Fig. 5B). Because of the low contribution of Evil-expressing cells in chimeras, we considered in this case that overexpression may suppress hematopoiesis overall, but we found that a retrovirus producing ~10-fold less Evil produced similar results (Fig. S4 E–F). Complete Mll deletion in the persisting cells of chimeras was confirmed by a quantitative genomic PCR assay (Fig. S4D). We found that retroviral overexpression of the individual genes resulted in a similar contribution to lymphoid and myeloid lineages, with the exception being the suppression of B-lymphopoesis by Prdm16 (Fig. S4D) as has been noted (26). Taken together, these data suggest that in addition to Hoxa9, Prdm16 is an important direct target of MLL in HSCs and is capable of partially rescuing Mll-deficient hematopoietic cells from attrition in BM chimeras without restoring the entire transcriptional network.

**Prdm16 Can Correct the Intrinsic Proliferation Defect of MLL-Deficient HSCs.** To determine the mechanism by which Prdm16 partially rescued Mll-deficient BM cells, we examined the consequences of
Prdm16 reexpression on LSK cell proliferation. We demonstrated that more Mll-deleted LSK cells are in S phase compared with wild-type, and that the CD45^S^ subset of these cells were largely in G1/S rather than G0 (13). Thus, we first assessed whether we could recapitulate any aspects of the hyperproliferative phenotype in vitro, then assessed the impact of Prdm16 in this setting.

To directly assess proliferation kinetics in vitro, wild-type (Mll^F/F^) or Mll-deleted (Mx1-cre;Mll^f^) LSK/CD48^neg^ cells were sorted from pl:pC-injected animals, deposited into wells as single cells and cultured in serum-free medium containing cytokines to maintain HSC identity and function (39) (Fig. 6A). Importantly, the percentage of surviving clones was similar between wild-type and Mll-deleted cells (Fig. S5A), confirming previous observations that apoptosis is not induced in Mll-deleted HSPCs (13). Integrating individual observations for 158 wild-type and 240 Mll-deleted LSK/CD48^neg^ cells, we found that the proliferation kinetics of the latter were consistently more advanced than wild type (Fig. 6E). After 48 h, the mode (greatest number of cells) of Mll-deleted LSK/CD48^neg^ clones had progressed approximately one-half a division further than the wild-type clones (Fig. 6C), and by 72 h, the mode was one full cell division ahead (Fig. 6D). To address the possibility that Mll-deficient LSK/CD48^neg^ cells exhibit earlier cell division because more are initially in G1/S compared with wild type, we performed higher resolution studies examining the initial three cell divisions (Fig. 6E). We found that Mll-deficient LSK/CD48^neg^ cells enter the cell cycle earlier at all cell divisions; in fact, Mll-deficient cells had a shorter cell cycle (~1 h) than wild-type cells (Fig. S5B). Therefore, Mll-deficiency results in a cell-intrinsic increase in proliferation that is recapitulated in vitro in conditions that maintain HSC identity. This system likely models the increased proportion of LSK cells in S phase we observed in vivo but does not represent the defect in maintaining G0 (13).

To investigate whether Prdm16 reexpression influenced the proliferation phenotype observed in Mll-deficient cells, we sorted LSK cells from control ER-cre;Mll^F/F^ and ER-cre;Mll^f^ mice, retrovirally introduced Prdm16, and concurrently incubated with 4-OHT to induce Mll deletion (Fig. 6F). ER-cre;Mll^F/F^ cells infected with an empty control retrovirus displayed greater cell accumulation than the ER-cre;Mll^f^ control cells, consistent with the single-cell observations. However, Prdm16 reexpression restored the growth of Mll-deficient LSK cells to within the normal range of the control LSK cells (Fig. 6G). Together, these data suggest that the mechanism by which Prdm16 can correct Mll deficiency is, in part, by restraining proliferation within HSPCs.

**Discussion**

Using two complementary conditional knockout models (Mx1-cre and ER-cre), we have identified genes that are consistently Mll dependent in HSC-enriched cell populations. The acute nature of Mll deletion and the use of highly purified cells resulted in the identification of a succinct list of transcriptional regulators with a high level of reproducibility and enrichment for genes that control self-renewal and proliferation specifically in HSCs. Thus, we refer to this set of genes as core components of the MLL HSC-specific transcriptional network. Among the down-regulated genes, Prdm16, Mecom, Pbx1, Eya1, and Hoxa9 emerged as a series of
interconnected Mll-regulated transcriptional nodes, with Pdrm16 exhibiting the greatest activity to replace Mll function in HSCs. We tested these genes individually by overexpression to uncover dominant nodes downstream of Mll, but our data are consistent with the concept that this network functions coordinately to sustain HSC homeostasis through diverse functions, hence the inability of any individual gene to completely replace Mll in the gene expression or functional assays. In fact, each of these genes has distinct targets and loss-of-function phenotypes (25, 27, 29, 41). Ultimately, identification of the minimal network of genes sufficient to replace Mll function will require simultaneous expression of physiologic levels of multiple genes.

Given the mechanisms by which MLL family members regulate gene expression, one surprising finding was the large number of up-regulated genes in Mll-deficient HSCs. However, the majority of these genes reflect the enhanced proliferation that we observe in Mll-deficient HSC-enriched populations in vivo, a finding that we also observe at single-cell resolution in the current study. The direct connection between Mll and enhanced proliferation in HSCs could be explained by three mechanistically distinct hypotheses. First, Pbx1, Mecom, and Pdrm16 have all been suggested to suppress HSC proliferation, based on the analysis of hematopoietic populations in the corresponding knockout animals (25, 27, 29, 40). Thus, the reduction in these three factors would be predicted to result in unrestrained proliferation, specifically in HSCs. Interestingly, responsiveness to TGFβ signaling is attenuated in hematopoietic cells from each of these knockouts (25, 29, 41), suggesting that the overall effect may have a significant impact on TGFβ signaling (Fig. S5 C and D). Alternatively, a distinct mechanism has been proposed to link Mll to proliferation in the setting of DNA damage. In this case, DNA damage-induced delay in origin of replication activation is enforced by wild-type MLL (42). In our conditional knockout system, it is possible that the loss of MLL (even in the absence of overt DNA damage) also results in unrestrained origin activation, a more rapid S phase, and shorter overall cell cycle duration. Finally, a recent demonstration that Mib1-Evi1 and Pdrm16 are H3K9 monomethylases (43) suggests that global derepression of heterochromatinized genes could potentially have a broad impact on the suppression of proliferation or erythropoiesis in Mll-deficient HSCs.

By identifying this transcriptional network, we discovered three important features of this HSC-specific Mll pathway. First, some (e.g., Hoxa9, Mecom), but not all (e.g., Pbx1, Pdrm16), of the direct Mll target genes also require the cofactor Menin. This finding illustrates that MLL uses distinct chromatin-targeting motifs for distinct categories of its direct target genes. Second, the genes identified here as Mll dependent in HSCs are not universally regulated by Mll in other tissues, with the exception of Hoxa9. This observation suggests that tissue-specific targeting and restriction mechanisms are behind the tissue-specific activity of MLL family members. Third, we note that not all of the HSC-specific, Mll target genes are up-regulated in leukemia, possibly reflecting the distinction between the chromatin targeting/activation mechanisms used by fusion oncoproteins in contrast to those used by wild-type MLL. For example, it is clear that Hoxa9 is consistently overexpressed in MLL translocation leukemia, whether T-cell ALL (T-ALL), B-cell ALL (B-ALL), or AML (44–46). Evi1 and Eya1 have recently been implicated as targets of MLL fusion oncoproteins in some leukemia subsets (33, 47), but they are not consistently up-regulated in either ALL or AML harboring an MLL rearrangement. Pdrm16 is not up-regulated in MLL-translocation leukemia yet can be activated by retroviral insertion in leukemia by translocation in other contexts, therefore has leukemogenic potential (48). Thus, our data begin to delineate a normal and reversible HSC-specific maintenance pathway, of which a selective portion is subverted to result in leukemia. Interestingly, Hoxa9, Mecom, and possibly Eya1 are the Mll-dependent genes we found to be affected by Menin loss, providing an intriguing connection between chromatin-targeting mechanism and leukemogenic versus normal HSC regulatory networks. The selective dependence on particular protein–protein interactions may render leukemia-specific gene programs driven by Mll-fusion oncogenes more sensitive to inhibitors than normal HSCs, as suggested by the study of compounds that disrupt the Menin–MLL interaction (49).
patterns, underscoring the complexity of mechanisms that must be used to regulate diverse gene expression programs in vivo.

Materials and Methods

Mice and in Vivo Induction of cre Recombinase. Mx1-cre;Mll-Tf animals and Mll-Tf mice have been described (13). Mx1-cre;Mll-Tf mice (kind gift of Matthew L. Meyerson, Harvard Medical School, Boston, MA) were back-crossed by using the DartMouse speed congenic facility then crossed to the ER-cre strain.

Flow Cytometry, Cell Sorting, and Culture. Flow cytometry and cell sorting were performed on a FACSCalibur and FACSAria, respectively (BD Biosciences). Data were analyzed by using FlowJo software (Tree Star). Fluorochrome-labeled antibodies and procedures are detailed in SI Materials and Methods.

Plasmids, Retroviral Infection, Cell Culture, and Transplantation. Murine stem cell virus (MSCV)-based retroviral expression plasmids were constructed by using cDNA obtained or cloned as described in SI Materials and Methods. Viral supernatants were prepared by cotationransfection, and sorted LSK cells were infected by using retronectin (Takara). Retrovirally infected cells were cotransplanted into lethally irradiated (950 Rads, split dose) C57BL/6J female mice. For proliferation assays, LSK and LSK/CD48cre F/F mice were cultured in HSC expansion medium [StemSpan Serum Free Expansion Medium (SFEM); 300 ng/mL recombinant murine (rm) SCF, 20 ng/mL rmIL-7, and 4 ng/mL rmFlt3L; StemCell Technologies and R&D Systems]. To induce deletion using the ER-cre strain, HSC expansion medium was supplemented with 300–400 nM 4-OHT (Sigma).

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