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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.

proliferation | HSC | epigenetics

Epigenetic regulation is an important mechanism by which gene expression fidelity is maintained during development. The *trithorax-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 metazoa (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 supressors that use epigenetic mechanisms is a promising an 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 between oncogenic 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 embryo 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 (HSPCs), 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$^-$ (LSK/CD48^{neg}) HSC-enriched cells from the bone marrow (BM) of polyinosinic:polycytidylic acid (pI:pC)-injected control *Mll*$^{F/F}$ or *Mx1-cre;Mll*$^{F/F}$ animals 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/CD48^{neg} cells revealed 1,935 differentially expressed genes using Significance Analysis of Microarrays (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*)

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Conflict of interest statement: P.E. is a shareholder of Amgen stock.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE47205).

See Commentary on page 11670.

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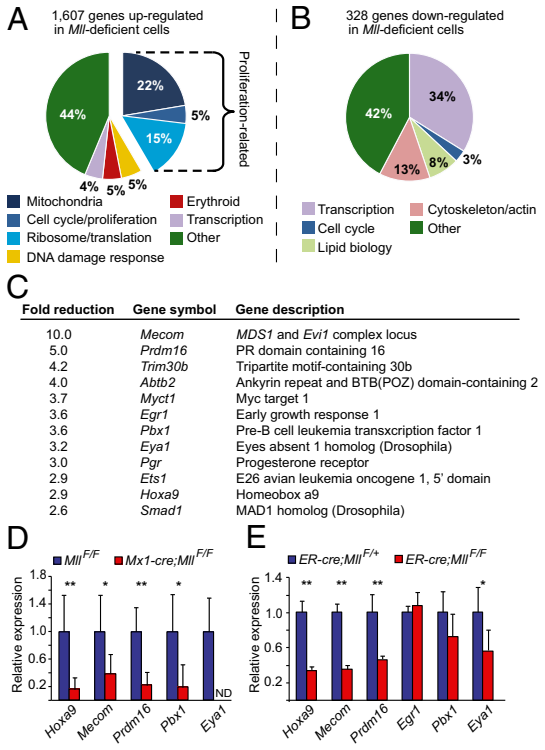

C

Fold reduction	Gene symbol	Gene description
10.0	*Mecom*	*MDS1* and *Evi1* complex locus
5.0	*Prdm16*	PR domain containing 16
4.2	*Trim30b*	Tripartite motif-containing 30b
4.0	*Abtb2*	Ankyrin repeat and BTB(POZ) domain-containing 2
3.7	*Myct1*	Myc target 1
3.6	*Egr1*	Early growth response 1
3.6	*Pbx1*	Pre-B cell leukemia transxcription factor 1
3.2	*Eya1*	Eyes absent 1 homolog (Drosophila)
3.0	*Pgr*	Progesterone receptor
2.9	*Ets1*	E26 avian leukemia oncogene 1, 5' domain
2.9	*Hoxa9*	Homeobox a9
2.6	*Smad1*	MAD1 homolog (Drosophila)

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^{neg} cells compared with controls. Cells were sorted from pI:pC-injected control *Mll*$^{F/F}$ or *Mx1-cre;Mll*$^{F/F}$ 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^{neg} cells listed by fold reduction (see also Dataset S2). (*D*) RT-qPCR validating down-regulated genes in independent control *Mll*$^{F/F}$ (blue) or *Mll*-deficient (red) LSK/CD48^{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*$^{F/+}$ (blue) or *ER-cre;Mll*$^{F/F}$ 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). $*P \leq 0.07$, $**P \leq 0.05$. ER-cre, estrogen receptor^{T2} mutant fused to cre recombinase.

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. 1*A* and Dataset S1). Up-regulation of genes involved in ribosome biogenesis reflected the greater proportion of cycling *Mll*-deficient LSK/CD48^{neg} cells (45% G_0 in *Mll*-deleted cells versus 75% G_0 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^{neg} cells. Unexpectedly, 5% of the genes that were up-regulated in *Mll*-deficient LSK/CD48^{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 (*Kel*), Erythropoietin 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. S1*E*). Derepression 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^{neg} cells (Fig. 1*B* 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. S1*F*). *Mll* itself (Fig. S1*G*) 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. 1*C*). 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 *Mx1-cre;Mll*$^{F/F}$ animals (Fig. 1*D*), as well as cells in which *Mll* was deleted in vitro by using 4-hydroxytamoxifen (4-OHT; Fig. 1*E*). 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 *Mx1-cre* and *ER-cre* systems. Of the annotated transcription factors down-regulated >2.5-fold (Fig. 1*C*), MDS and Evi1 complex locus (*Mecom*), *Prdm16*, Pre-B cell leukemia homeobox protein 1 (*Pbx1*), Eyes absent homolog 1 (*Eya1*) and *Hoxa9* were consistently *Mll*-dependent (Fig. 1*E*). 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, Prdm16*, 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 *Prdm16* 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 5×10^4 BM cells (31). Based on previous results demonstrating MLL binding near transcription start

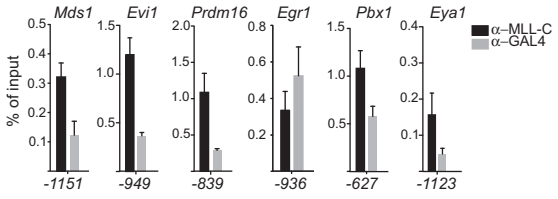

Fig. 2. MLL binds directly to the promoter regions of a subset of genes identified by expression array. ChIP results demonstrating specific enrichment at the *Mecom* locus (*Mds1* and *Evi1* start sites) and the *Prdm16, Pbx1*, and *Eya1* promoter regions. Anti-MLL C-terminal (black) or control (anti-GAL4, 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^{neg}) population and LSK cells (Fig. S3*A*). Control ChIP experiments demonstrated MLL binding to the *Hoxa9* but not *Gapdh* TSS regions (Fig. S3*B*). Using lin^{neg} 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. S3 *C–G*). Interestingly, we did not observe MLL binding to the Early growth response 1 (*Egr1*) promoter (Fig. 2*B* and Fig. S3*H*), consistent with the observation that this gene was not *Mll*-dependent in both model systems (Fig. 1*E*). Therefore, we conclude that like *Hoxa9*, the expression of *Mecom*, *Prdm16*, *Pbx1*, and *Eya1* is maintained directly by MLL in normal lin^{neg} 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/lens 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. 3*A*). Despite efficient excision of *Men1* (Fig. 3*B*), *Prdm16* and *Pbx1* levels were not affected (Fig. 3*A*), 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 *Evi1* 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-Evi1* and *Evi1*) 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*, *Evi1* 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 observed in 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. *Evi1* expression increased *Prdm16* and *Hoxa9* transcripts in *Mll*-deficient LSK cells back to the wild-type levels (Fig. 4 *A* and *B*). *Mds1-Evi1* 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-Evi1* and *Evi1* 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 *Evi1*, 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.

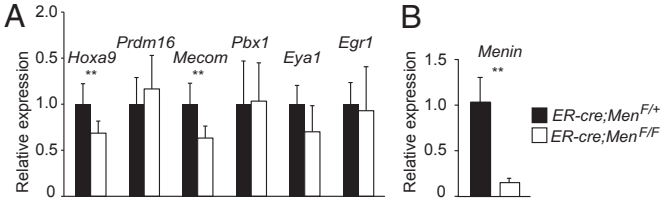

Fig. 3. Menin loss affects some but not all MLL targets in LSK cells. (*A*) RT-qPCR of *Mll*-regulated genes in LSK cells sorted from control *ER-cre;Men1*$^{F/+}$ (black) or *ER-cre;Men1*$^{F/F}$ cells (white) cultured for 72 h after initiating *Menin* deletion. Expression levels were normalized to rRNA. (*B*) Menin transcript levels in LSK cells treated as in *A*. Error bars represent 95% CI; $n = 4$–8 animals per genotype. $^{**}P \leq 0.05$.

Prdm16 Exhibits a Unique Capacity to Partially Rescue *Mll*-Deficient Cells. One to two weeks after inducing cre, the attrition of BM cells in *Mx1-cre;Mll*$^{F/F}$ 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*$^{F/F}$ or *Mx1-cre;Mll*$^{F/F}$ 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. 5*A*). Thus, in this assay, "rescue" is defined as the selective persistence of retrovirus-infected cells within the population of *Mll*-deleted cells (Fig. S4*A*). 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 as expected (Fig. 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. 5*B*, 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 *Evi1* only marginally protected *Mll*-deficient cells from attrition, and *Mds1-Evi1*, *Pbx1*, and *Eya1* had no specific activity in this assay (Fig. 5*B*). Because of the low contribution of *Evi1*-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 Evi1 produced similar results (Fig. S4 *E–H*). Complete *Mll* deletion in the persisting cells of chimeras was confirmed by a quantitative genomic PCR assay (Fig. S4*D*). 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-lymphopoiesis by *Prdm16* (Fig. S4*I*) 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*-deleted BM cells, we examined the consequences of

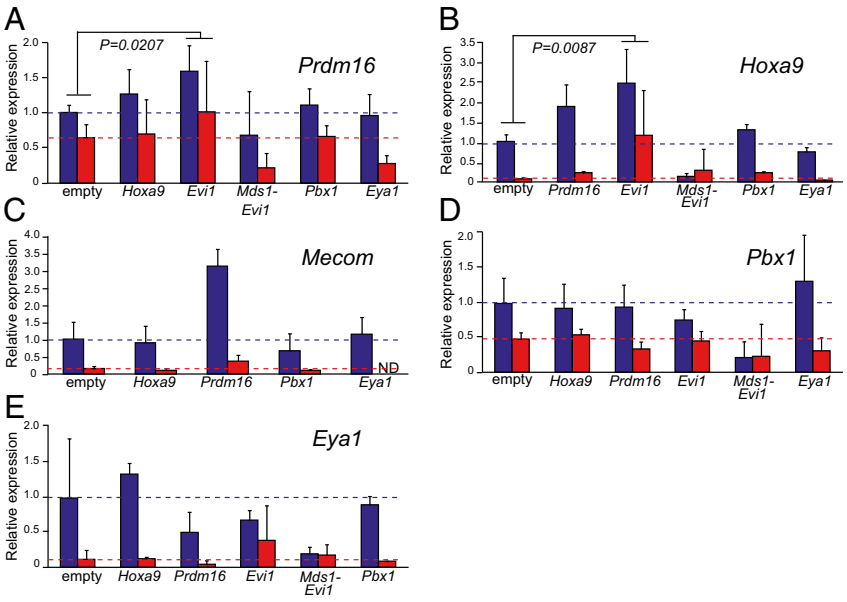

Fig. 4. Effect of reexpression of individual *Mll* targets on others in the network. RT-qPCR of genes in LSK cells reexpressing the cDNA indicated below each set of bars. Cells were produced in vivo by pI:pC injection, sorted 6 d later, then infected with a retrovirus without an added cDNA (empty) or cDNA as indicated. Two days later, retrovirally infected cells were sorted and RT-qPCR assays were performed. (*A*) *Prdm16* expression levels in control $Mll^{F/F}$ (blue) or *Mll*-deficient (red) LSK cells infected with the retrovirus indicated below each set of bars. Expression levels were normalized to the average expression level empty retrovirus-infected $Mll^{F/F}$ cells and to *Gapdh* in each sample. Expression of *Hoxa9* (*B*), *Mecom* transcripts (*C*), *Pbx1* (*D*), and *Eya1* (*E*) were analyzed and normalized as in *A*. Dashed lines indicate the average expression level in wild-type or *Mll*-deficient, empty retrovirus infected cells; four to five animals per genotype were used for each experiment, and error bars represent 95% CI. *P* values are shown for the comparison between pairs of empty vector and *Evi1*-expressing cells, calculated with the paired Student *t* test. ND, not detected.

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 $CD48^{neg}$ subset of these cells were largely in G_1/S rather than G_0 (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;*$Mll^{F/F}$) LSK/$CD48^{neg}$ cells were sorted from pI: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. 6*A*). Importantly, the percentage of surviving clones was similar between wild-type and *Mll*-deleted cells (Fig. S5*A*), 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. 6*E*). 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. 6*C*), and by 72 h, the mode was one full cell division ahead (Fig. 6*D*). To address the possibility that *Mll*-deficient LSK/$CD48^{neg}$ cells exhibit earlier cell division because more are initially in G_1/S compared with wild type, we performed higher resolution studies examining the initial three cell divisions (Fig. 6*E*). 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. S5*B*). 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 G_0 (13).

To investigate whether *Prdm16* reexpression influenced the proliferation phenotype observed in *Mll*-deficient cells, we sorted LSK cells from control *ER-cre;*$Mll^{F/+}$and *ER-cre;*$Mll^{F/F}$ mice, retrovirally introduced *Prdm16*, and concurrently incubated with 4-OHT to induce *Mll* deletion (Fig. 6*F*). *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. 6*G*). Together, these data suggest that the mechanism by which *Prdm16* can correct *Mll* deficiency is, in part, by restraining proliferation within HSPCs.

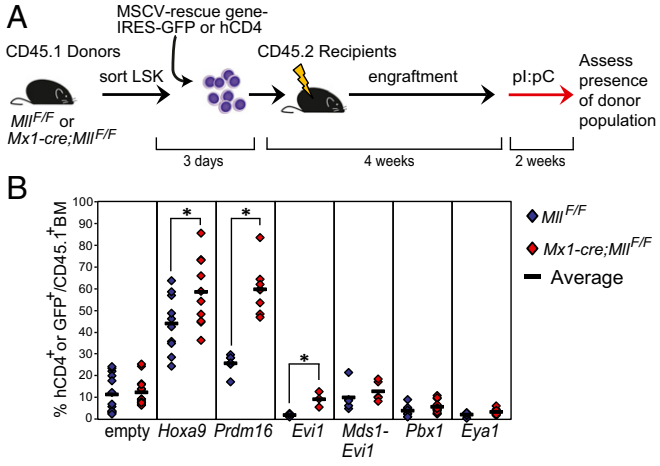

Fig. 5. Reexpression of *Prdm16* partially rescues *Mll* deficiency. (*A*) Experimental scheme to determine effects of reexpression of *Mll*-dependent genes. LSK cells were sorted from control $Mll^{F/F}$ or *Mx1-cre;*$Mll^{F/F}$ donor animals then infected with the indicated retrovirus. The entire pool of infected and uninfected cells was transplanted into irradiated recipients, which were analyzed 6 wk later. (*B*) Results of reexpression of each individual gene in control $Mll^{F/F}$ (blue) or *Mll*-deficient LSK cells (red); each point represents an individual recipient animal, $n = 3$–10 recipients per condition. The percentage of donor-type ($CD45.1^+$) BM cells that are GFP^+ or $hCD4^+$ 2 wk after *Mll* deletion is shown. Data are representative of three independent experiments. $*P \leq 0.05$ was calculated by using the Wilcoxon rank-sum test.

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

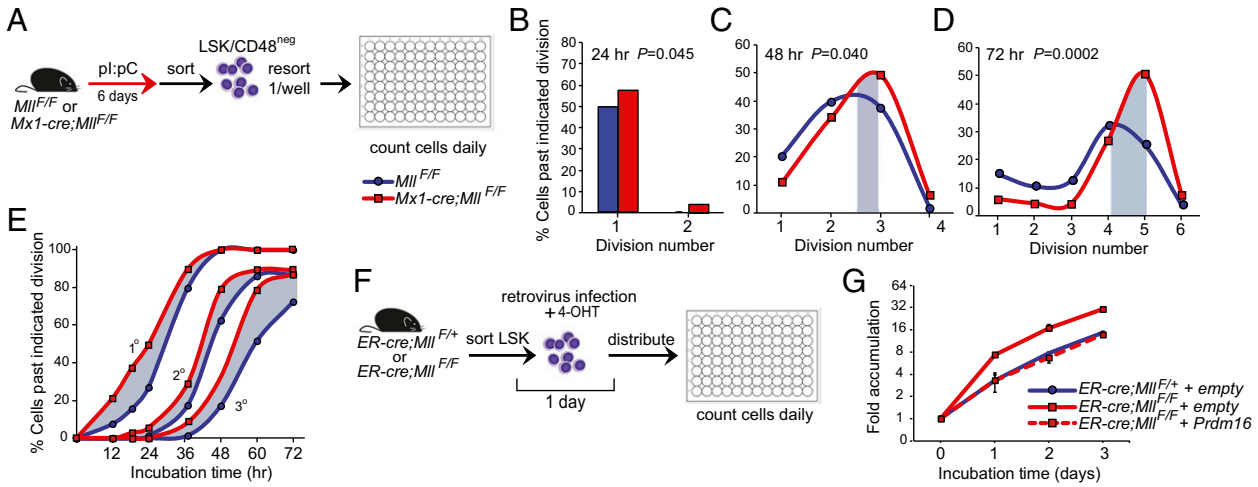

Fig. 6. The intrinsic proliferation defect of *Mll*-deficient HSCs is corrected by reexpression of *Prdm16*. (*A*) Scheme to determine proliferation kinetics of individual LSK/CD48^{neg} cells. *Mll* deletion was performed in vivo, and double-sorted LSK/CD48^{neg} cells were deposited at 1 cell per well. Cell divisions were scored every 24 h. (*B–D*) Cumulative proliferation data from individual control *Mll*$^{F/F}$ (blue) or *Mll*-deficient LSK/CD48^{neg} cells (red). Data represent 158 control *Mll*$^{F/F}$ and 240 *Mll*-deficient cells; $n = 3$–5 animals per genotype. The difference between modes of each line is indicated by gray fill. The Pearson's $\chi 2$ test was performed to determine statistical significance, shown on *B–D*. (*E*) Higher-resolution proliferation kinetics of control *Mll*$^{F/F}$ (blue) or *Mll*-deficient LSK/CD48^{neg} cells (red). Cells were prepared as in *A*, $n = 2$–3 animals per genotype, 93 control *Mll*$^{F/F}$ and 38 *Mll*-deficient cells. The percentage of cells past the first, second, and third divisions are graphed separately (1°, 2°, 3°). (*F*) Scheme to determine the impact of *Prdm16* reexpression in *Mll*-deficient LSK cells. (*G*) Accumulation of LSK cells expressing an empty (solid) or hCD4-*Prdm16* retrovirus (dashed). LSK cells were sorted from control *ER-cre;Mll*$^{F/+}$ (blue) or *ER-cre;Mll*$^{F/F}$ animals (red), cultured in 4-OHT during the retroviral infection to induce *Mll* deletion then enumerated every 24 h for 3 d. Data represent averages ± 95% CI, $n = 4$ animals per genotype, 3 replicates per time point.

interconnected *Mll*-regulated transcriptional nodes, with *Prdm16* 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, 40). 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 *Prdm16* have all been suggested to suppress HSC proliferation, based on the analysis of hematopoietic populations in the corresponding knockout animals (25, 27, 29). 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 *Mds1-Evi1* and *Prdm16* 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, Prdm16*), 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. *Prdm16* 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). Our work illustrates that MLL family members control exquisitely tissue-specific gene programs despite their ubiquitous expression

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*$^{F/F}$ animals and *cre* induction have been described (13). *Men1*$^{F/F}$ 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 cDNAs obtained or cloned as described in *SI Materials and Methods*. Viral supernatants were prepared by cotransfection, 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/CD48^{neg} cells were cultured in HSC expansion medium [StemSpan Serum Free Expansion Medium (SFEM); 300 ng/mL recombinant murine (rm) SCF, 20 ng/mL rmIL-11, and 4 ng/mL rmFlt3L; StemCell Techologies and R&D Systems]. To induce deletion using the *ER-cre* strain, HSC expansion medium was supplemented with 300–400 nM 4-OHT (Sigma).

ChIP. Rabbit polyclonal anti-MLL C terminus (50) or anti-Gal4 (Santa Cruz; SC-577) antibodies were used for ChIP by using lin^{neg} or LSK cells (31) with refinements as indicated in *SI Materials and Methods*. Primer sequences and genomic positions are described in Dataset S3.

Microarray Sample Preparation and Data Analyses. Affymetrix microarray analyses were performed by using sorted LSK/CD48^{neg} cells from five *Mll*$^{F/F}$ or *Mx1-cre*; *Mll*$^{F/F}$ mice 6 d after cre induction. Detailed methods and bioinformatic analyses are found in *SI Materials and Methods* and Dataset S4.

Statistical Analyses. Unless indicated otherwise, the unpaired Student *t* test was used to determine significance, and error bars represent 95% CI. Statistical analyses were performed by using Excel (Microsoft) or Prism (GraphPad) Software.

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