

The Histone Methyltransferase Activity of MLL1 Is Dispensable for Hematopoiesis and Leukemogenesis

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SUMMARY

Despite correlations between histone methyltransferase (HMT) activity and gene regulation, direct evidence that HMT activity is responsible for gene activation is sparse. We address the role of the HMT activity for MLL1, a histone H3 lysine 4 (H3K4) methyltransferase critical for maintaining hematopoietic stem cells (HSCs). Here, we show that the SET domain, and thus HMT activity of MLL1, is dispensable for maintaining HSCs and supporting leukemogenesis driven by the MLL-AF9 fusion oncoprotein. Upon *Mll1* deletion, histone H4 lysine 16 (H4K16) acetylation is selectively depleted at MLL1 target genes in conjunction with reduced transcription. Surprisingly, inhibition of SIRT1 is sufficient to prevent the loss of H4K16 acetylation and the reduction in MLL1 target gene expression. Thus, recruited MOF activity, and not the intrinsic HMT activity of MLL1, is central for the maintenance of HSC target genes. In addition, this work reveals a role for SIRT1 in opposing MLL1 function.

INTRODUCTION

Understanding the contribution of chromatin-modifying complexes to precise and heritable patterns of gene regulation in mammals has been a challenging task due to the complexity, redundancy, and, often, ubiquitous expression of such complexes. Among the first factors shown to influence the heritable transmission of gene expression states were trithorax Group (trxG) and Polycomb group (PcG) proteins (Cavalli and Paro, 1999; Schuettengruber et al., 2011). Following their genetic identification in *Drosophila melanogaster*, a common protein domain

defined by Su(var)3-9, Ezh2, Trithorax (SET) homology was subsequently demonstrated to possess histone methylation activity. More recently, it has become clear that histone methylation enzymes beyond the trxG/PcG homologs function in many aspects of epigenetic gene regulation from yeast to human (Shilatifard, 2012).

The first identified mammalian member of the trxG family, MLL1, was initially noted as a gene frequently rearranged in poor-prognosis leukemia (Grossmann et al., 2012; Krivtsov and Armstrong, 2007). Mammals possess three pairs of related SET-domain containing histone methyltransferase (HMT) proteins, MLL1/2, SET1A/B, MLL3/4, and a divergent MLL5 protein. In addition to *MLL1* translocations in leukemia, mutations in several trxG/MLL family members are associated with developmental disorders and cancer (Jones et al., 2012; Morin et al., 2011; Ng et al., 2010; Parsons et al., 2011; Pasqualucci et al., 2011). How these broadly expressed proteins influence particular physiologic and pathologic processes in specific cell types is largely unknown but, due to their enzymatic activities, represent attractive drug targets.

Tissue-specific loss-of-function models together with biochemical approaches have delineated biologically important target genes and candidate mechanisms by which mammalian trxG/MLL family proteins function. The fact that these large proteins function within multiprotein complexes and frequently lack satisfactory in vivo structure-function assays has limited our understanding of gene regulatory mechanisms used in the tissues in which they function. To determine MLL1 function in vivo, we employed inducible *Mll1* loss-of-function alleles and demonstrated that *Mll1* plays a unique and essential role in HSCs and developing B cells (Artinger et al., 2013; Jude et al., 2007; Li et al., 2013). These studies also revealed MLL1 target genes that were MLL1 dependent only in hematopoietic cells, presumably due to tissue-specific mechanisms of recruitment (Artinger et al., 2013).

The native MLL1 complex purified from cell lines revealed several stoichiometric components, particularly a C-terminal

subcomplex that is critical for HMT activity and specificity (Dou et al., 2005, 2006; Nakamura et al., 2002; Patel et al., 2011; Steward et al., 2006; Yokoyama et al., 2004). Detailed mechanistic studies show that the MLL1 SET domain, in conjunction with the Wdr5/RbBP5/Ash2L/Dumpy-30 (WRAD) subcomplex, acts as a H3K4 mono- and dimethylation, or in some conditions, a trimethylation enzyme (Dou et al., 2006; Patel et al., 2011; Steward et al., 2006). Given the connection between H3K4me1 and active enhancer regions as well as H3K4me3 and active/poised transcriptional start sites (reviewed in Maunakea et al., 2010), investigators have naturally hypothesized that MLL1 family HMTs regulate transcription via promoter or enhancer targeted H3K4 methylation.

To determine the mechanisms by which MLL1 maintains expression of its target genes in hematopoietic cells, we investigated the requirement for HMT activity using domain-specific deletion and conditional knockout mouse models. Surprisingly, we found that the SET domain of MLL1 was not necessary for maintaining the expression of direct target genes in hematopoietic populations, or for facilitating MLL-AF9-mediated leukemogenesis. Through acute deletion of *Mll1*, we identified a histone H4 modification that was rapidly and synchronously reduced as the expression of MLL1 target genes ceased. This modification implicates the MLL1-associated MOF histone acetyltransferase as the dominant activity maintaining transcription of MLL1 target genes. Remarkably, pharmacologic inhibition of H4K16Ac deacetylases was sufficient to restore MLL1-dependent gene expression in *Mll1*^{ΔΔ} cells. Collectively, our data implicate MLL1-recruited H4K16Ac activity, and not HMT activity, as the major mechanism by which gene expression is maintained in hematopoietic stem and progenitor cells (HSPCs). Furthermore, our data implicate Sirtuins as opposing factors in the transcriptional network maintained by MLL1 in HSPCs.

RESULTS

Hematopoiesis in the Absence of the MLL1 SET Domain

All MLL family members harbor a SET domain with specificity for H3K4 methylation, dependent upon associated cofactors or posttranslational modifications (Patel et al., 2014; Schuetten-gruber et al., 2011). Most *Mll1* disruption alleles are embryonic lethal; however, homozygotes for a germline deletion of the SET domain of MLL1 (hereafter, ΔSET) survive into adulthood, providing an opportunity to assess the role of the SET domain, thus HMT activity of MLL1, in adult tissues (Terranova et al., 2006). Homozygous ΔSET mutants were generated, and western blotting of thymocyte extracts confirmed the expression of the predicted size MLL1 C-terminal band (Figure S1A). Mature lymphoid and myeloid populations in the bone marrow and blood of ΔSET animals were present at normal frequencies (P.E., data not shown). Because HSPCs are extremely sensitive to loss of *Mll1* (Gan et al., 2010; Jude et al., 2007; McMahon et al., 2007), we carefully assessed the phenotype and function of HSPCs in ΔSET mutant mice.

Steady-state HSPCs from wild-type (WT), heterozygotes (ΔSET/+), and homozygous ΔSET mice were quantified by determining the total lineage-negative (lin^{neg}), Sca-1-positive, c-Kit-positive (LSK) cells in animals of several age ranges (Figures

1A–1C). Given that there were no significant differences in this population, we examined the more HSC-enriched LSK/CD150⁺/CD48^{neg} population and found the number of these cells was indistinguishable between WT and ΔSET animals (Figures 1D and 1E). For functional quantification of HSCs, we performed competitive repopulation unit (CRU) assays using unfractionated bone marrow cells. Consistent with the phenotypic data, WT and ΔSET bone marrow exhibited overlapping CRU frequencies (Figure 1F). To identify potentially subtle gene expression defects in ΔSET cells, we performed real-time quantitative PCR (qPCR) analyses using sorted LSK cells. We found that the strongly MLL1-dependent target genes *Hoxa9*, *Mecom* (locus encoding *MDS-Evi1* and *Evi1* transcripts), and *Prdm16* (Artinger et al., 2013) were expressed at normal levels in ΔSET cells (Figure 1G). Similarly, H3K4me3 and H3K4me1 enrichment around the transcription start site (TSS) of these loci was also unchanged in ΔSET HSPCs (Figure S1B). These surprising findings suggested that the MLL1 HMT activity is dispensable for regulating target genes and therefore HSPC function. To assess potential compensation by members of the WRAD subcomplex (Cao et al., 2010; Patel et al., 2011) or other HMTs, we knocked down components of HMT complexes in WT and ΔSET in murine embryo fibroblasts (MEFs) and lin^{neg} bone marrow cells. Depletion of Ash2L had no effect on *Hoxa9* expression in either WT or ΔSET MEFs, whereas depletion of Wdr5 reduced expression of *Hoxa9* equivalently in both genotypes (Figures S1C and S1D). Because Wdr5 is also required for SET1A/B HMT complexes (Lee and Skalnik, 2008), we knocked down Wdr82, a subunit uniquely required for SET1A/B complex function (Lee and Skalnik, 2008; Austenaa et al., 2012). Depletion of Wdr82 reduced *Hoxa9* expression slightly in both WT and ΔSET MEFs (Figure S1E). Similar trends were observed with the hematopoietic-specific (Artinger et al., 2013) target gene *Mecom* in lin^{neg} bone marrow cells (Figures S1F and S1G), although less effective knockdown in this cell type reduced the statistical significance. Thus, ΔSET cells do not exhibit an increased dependence on potential compensatory HMTs or C-terminal subcomplex components.

MLL-AF9 Transforms ΔSET Cells

Several lines of evidence suggested that endogenous MLL1 is required for transformation by MLL1 fusion oncoproteins (Milne et al., 2010; Thiel et al., 2010). For example, in *Mll1*^{-/-} MEFs MLL-AF9 cannot localize to the *Hoxa9* gene, but reintroduction of a human MLL1 cDNA restored *Hoxa9* promoter localization (Milne et al., 2010). To test whether histone methylation by endogenous MLL1 is required for MLL-AF9-mediated transformation, we initiated leukemia in both WT and ΔSET HSPCs and followed quantitative and qualitative features of the resulting acute myelogenous leukemia (AML). As shown in Figure 2A, the latency of MLL-AF9 initiated AML in the ΔSET background was indistinguishable from that initiated in WT cells. Leukemia initiated from either WT or ΔSET cells was characterized by similar high white blood cell (WBC) counts, percentages of Gr-1[±]/Mac-1⁺ cells, suppression of erythropoiesis, and splenomegaly (Figures 2B–2E). Qualitatively, animals receiving either WT or ΔSET MLL-AF9-transformed cells exhibited similar histopathology (Figure S2A) and comparable levels of *Hoxa9*

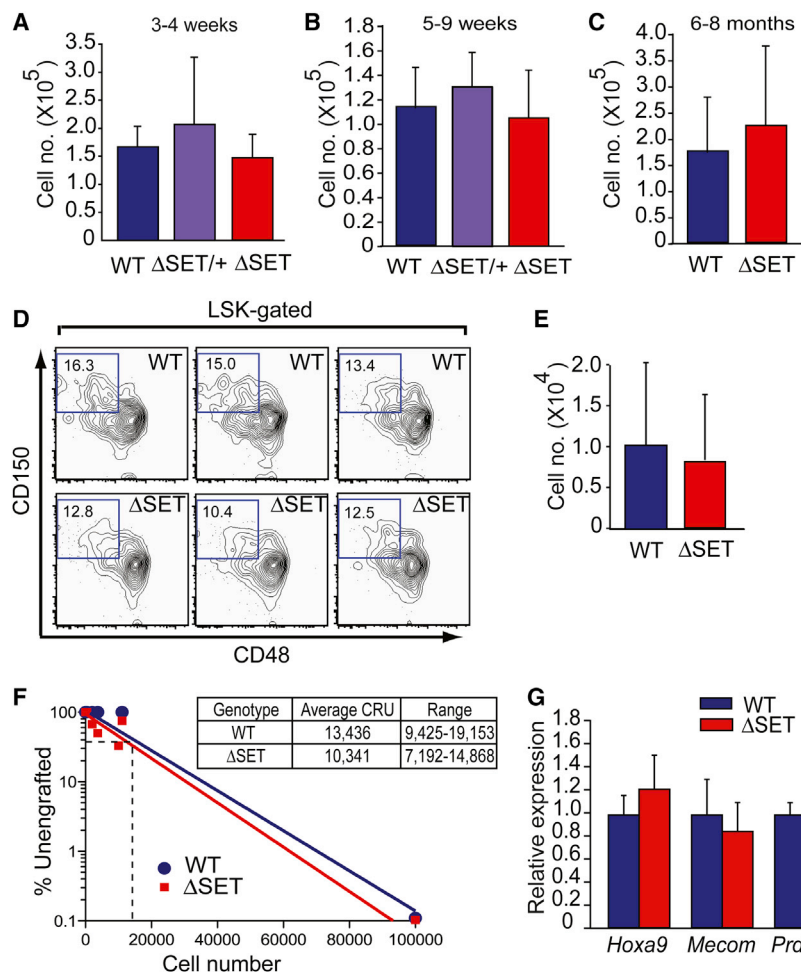


Figure 1. Normal HSC Number, Function, and Gene Expression in Δ SET Animals

(A–C) LSK cells were quantified from littermates of 3–4 weeks (A), 5–9 weeks (B), or 6–8 months old (C) ($n = 3–7$ animals for each genotype). The cell number on the y axis reflects total number per two hindlimbs.

(D) Representative fluorescence-activated cell sorting plots of LSK-gated cells.

(E) Total LSK/CD150⁺/CD48^{neg} cells per two hindlimbs, $n = 5–6$ animals per genotype at 4–6 weeks of age.

(F) CRU quantification for bone marrow from WT or Δ SET animals, $n = 3–7$ recipients and three donors per cell dose. Inset table shows mean CRU \pm range, determined using L-Calcul software.

(G) Expression of MLL1 target genes in WT or Δ SET LSK cells. LSK cells were sorted from the bone marrow of 6- to 12-week-old animals, $n = 5–6$ per genotype. Error bars reflect mean gene expression level per donor animal $\pm 95\%$ confidence interval.

expression and histone modifications at the *Hoxa9* promoter, including the H3K79Me2 modification recruited by the AF9 fusion partner (Figures S2B–S2E). Furthermore, secondary transplantation of leukemia cells (Figure 2F) and serial replating assays (Figure 2G) demonstrated that leukemia-initiating capacity and self-renewal are not affected by the loss of endogenous MLL1 HMT activity. In summary, none of the known hematopoietic functions of MLL1 appear to require the HMT activity or presence of the SET domain generally.

Acute Deletion of MLL1 Has No Impact on H3K4 Methylation in HSPCs

To identify changes in chromatin that are directly influenced by the MLL1 complex, we established a system to temporally resolve gene downregulation and chromatin modifications in primitive hematopoietic cells. Using 4-hydroxytamoxifen (4-OHT) inducible cre recombinase (*ER^{T2}-cre*) animals in which ex vivo deletion of *Mll1* resulted in rapid target gene downregulation (Artinger et al., 2013), we determined transcript levels and chromatin modifications over a period of 3 days, during which lin^{neg} bone marrow cells remain phenotypically undifferentiated (B.P.M., data not shown). *ER^{T2}-cre;Mll1^{F/+}* cells were used as controls because target gene expression is indistinguishable

despite dramatic changes in gene expression, acute MLL1 loss does not result in H3K4 methylation differences at its target gene promoters.

To test whether these observations were generalizable in a more HSC-enriched population, we performed ChIP-seq with sorted LSK cells from control *ER^{T2}-cre;Mll1^{F/+}* or *ER^{T2}-cre;Mll1^{F/F}* animals. In this system, 48 hr was sufficient to reduce MLL1 target gene expression (Figures S4A and S4B). We examined *Hoxa9*, *Evi1*, and *Prdm16* TSS regions and found a remarkable consistency in H3K4me3 and H3K4me1 enrichment comparing control and *Mll1^{Δ/Δ}* samples (Figure 4A), which was true genome-wide at all enriched loci (Figures 4B and 4C). Despite the downregulation of over 300 genes (Artinger et al., 2013), the changes in H3K4me enrichment vary only within the range observed between replicate samples (Figures S4C and S4D). These data demonstrate that the HMT activity of MLL1 is not responsible for the maintenance of target gene expression in HSPCs.

H4K16 Acetylation at MLL1 Targets Is Lost in Parallel with Transcription

To identify chromatin changes that occur upon *Mll1* deletion, we surveyed histone modifications at MLL1 target gene promoters

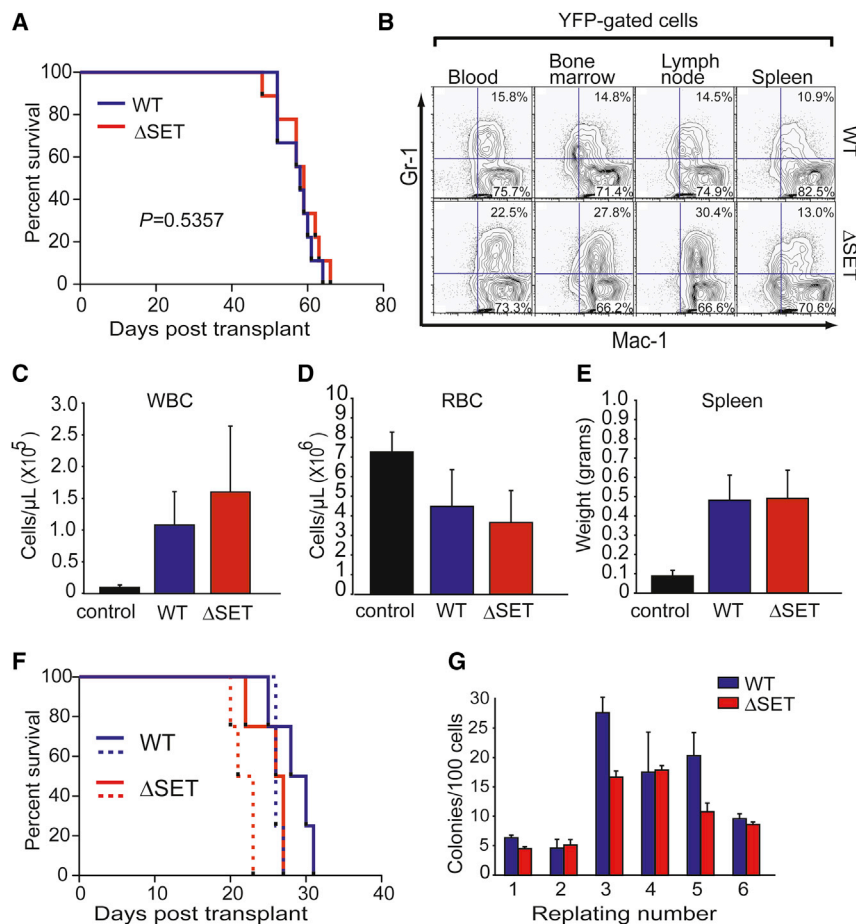


Figure 2. MLL-AF9 AML Is Initiated and Propagated Normally in Δ SET Hematopoietic Cells

(A) Kaplan-Meier plot of primary leukemia in recipients transplanted with MLL-AF9-transduced WT or Δ SET bone marrow cells, $n = 9$ per genotype. (B) Phenotypic analysis of leukemia cells from tissues of moribund animals. Cells from the indicated organs were stained with Mac-1 and Gr-1 antibodies and YFP⁺-gated cells shown. (C and D) Automated differential counts were determined using peripheral blood cells from leukemic animals ($n = 9$ per genotype) and age-matched controls ($n = 2$). White blood cells (WBCs, C) and red blood cells (RBC, D) are shown from the 18 animals represented in (A). (E) Spleen weight of leukemic animals when moribund and control animals as in (C) and (D). (F) Secondary transplantation results using 100 (solid lines) or 1,000 (dashed lines) YFP⁺ leukemia cells, $n = 4$ recipients per genotype. (G) MLL-AF9-transformed cells exhibit similar serial replating activity in vitro. Leukemia cells from primary recipients were plated in methylcellulose medium and colonies quantified and replated every week, corresponding to the replating number below the pairs of bars. Data represent means of triplicate reactions \pm SD.

throughout the time course shown in Figure 3. MLL1 interacts physically and/or genetically with p300/CBP, MOF, and MOZ histone acetyltransferases (Dou et al., 2005; Ernst et al., 2001; Paggetti et al., 2010). Although the CBP/p300-mediated H3K27Ac and MOZ-mediated H3K9Ac modifications were stable throughout the time course (Figures S4E, S5A, and S5B), H4K16Ac was rapidly reduced around the TSS of MLL1 target genes in *Mll1* Δ/Δ cells (Figure 5A) as was MOF itself (Figure 5B). Polycomb-complex-mediated H3K27me3 modifications increased, but late in the time course, suggesting they occur as a secondary consequence of cessation of transcription (Figures S5E and S5F). Interestingly, Δ SET mutant *lin*^{ne9} cells exhibited unchanged H4K16Ac enrichment, whether gene-specific or global levels were assessed (Figures S5G and S5H).

Because MOF-mediated acetylation creates a binding site for Brd4, which recruits the PTEFb transcriptional elongation complex (Yang et al., 2005; Zippo et al., 2009), we assessed promoter Brd4 and Cdk9 levels (the latter representative of the PTEFb complex). As shown in Figures 5C and 5D, Brd4 and Cdk9 were also reduced at MLL1 target genes. Consistent with the reduced Cdk9, the actively elongating form of RNA polymerase II (serine 2 phosphorylated) was also decreased (Figure 5E). These data suggest that MLL1 loss results in the rapid loss of promoter H4K16Ac, and, as a consequence, reduced Brd4

and PTEFb recruitment. In addition, both serine 5 phosphorylated, paused RNA polymerase II (ser5 Pol II, Figure 5F) and nascent *Hoxa9*, *Evi1* (specific isoform from the *Mecom* locus), and *Prdm16* transcripts were reduced upon *Mll1* deletion, demonstrating a role for MLL1 in enhancing transcriptional initiation (Figures S5C and S5D). These data show that the MLL1 complex maintains target gene expression through both transcriptional initiation and elongation, in part, through maintaining H4K16Ac levels by recruitment of MOF.

Role of Sirtuin Family Deacetylases in Opposing MLL1-Dependent Gene Expression

If MLL1-associated MOF is required for maintaining MLL1 target genes in HSPCs, we predicted that blocking H4K16 deacetylation might enhance the expression of MLL1 target genes. Both SIRT1 and SIRT2 deacetylate H4K16Ac, the former acting constitutively in the nucleus and the latter cytoplasmic enzyme acting mainly on chromatin at the G₂/M transition (Martinez-Redondo and Vaquero, 2013). To test the role of these enzymes in MLL1-dependent gene expression, we used the class III histone deacetylase inhibitor nicotinamide or the SIRT1-specific inhibitor Ex-527 (Napper et al., 2005) in conjunction with *Mll1* deletion. Remarkably, either inhibitor could restore MLL1 target gene expression in *Mll1* Δ/Δ cells (Figure 6). Interestingly, treatment with Ex-527 had a negligible effect on MLL1 target gene expression in WT cells, although induction of *Cdkn1* was observed (Figures S6A and S6B). Induction *Cdkn1* occurs through deacetylation and thereby activation of the nonhistone SIRT1 target

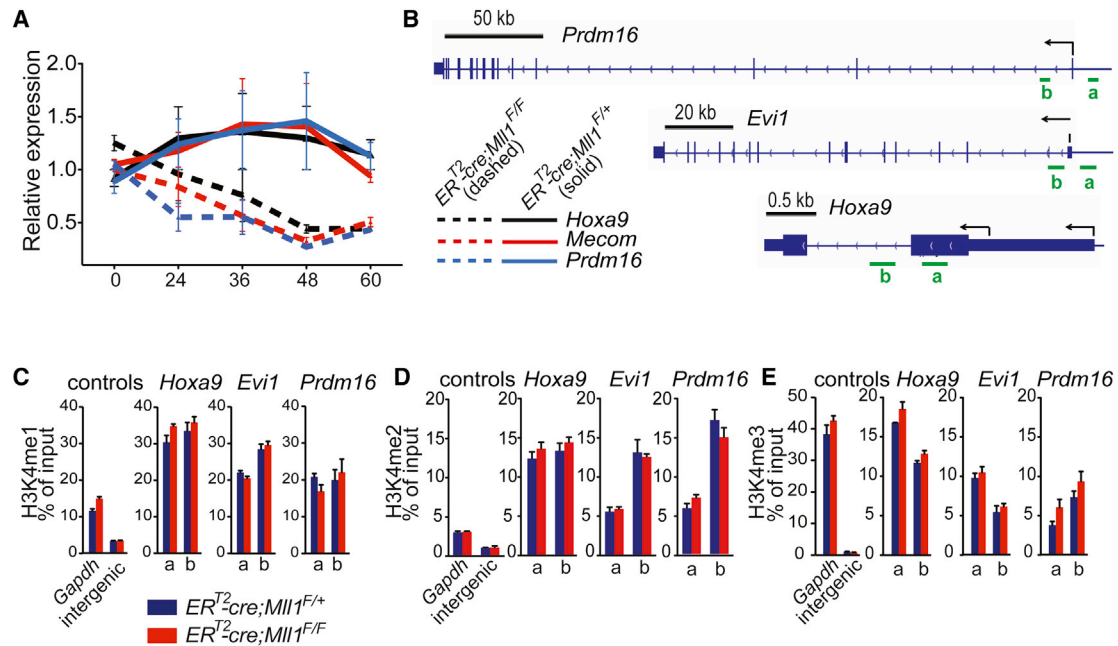


Figure 3. Loss of MLL1 in Hematopoietic Cells Does Not Affect H3K4me Enrichment at Target Genes

(A) Transcripts corresponding to three representative MLL1 target genes decrease rapidly upon 4-OHT-induced excision of *Mll1* in vitro. Lin^{neg} cells were purified from control ER^{T2}cre;Mll1^{F/+} or ER^{T2}cre;Mll1^{F/F} bone marrow and were cultured in vitro in medium containing 400 nM 4-OHT. Triplicate samples were harvested every 12 hr for real-time qPCR (A) and ChIP (C–E) assays.

(B) Diagram of target gene loci showing ChIP amplicon location, approximately 1 kb to either side of the TSS (see Table S1).

(C–E) Anti-H3K4me1, -H3K4me2, and -H3K4me3 ChIP assays were performed using samples from the 48 hr time point. Enrichment of the indicated histone modification is shown for each amplicon as the mean of four PCRs ±SEM. ChIP results are representative of at least three independent experiments.

p53 (Yuan et al., 2013). Ex-527 treatment did not affect the efficiency of *Mll1* deletion (Figure S6D) but did restore H4K16Ac levels of MLL1 target genes without having an impact on H3K4me3 levels (Figures S6E and S6F). These data demonstrate that the role of MLL1 in maintaining hematopoietic-specific target genes occurs largely through associated MOF acetylation activity, which continuously counters the potential repressive actions of SIRT1.

DISCUSSION

We show that MLL1 maintains the expression of its hematopoietic target genes using mechanisms that do not depend on its HMT activity or other molecules that interact with the SET domain. Despite the fact that Δ SET homozygous animals exhibit transient *Hox* gene misexpression and altered H3K4me enrichment during embryogenesis (Terranova et al., 2006), these perturbations are apparently not sufficient to cause embryonic lethality or overt phenotypes in adult animals. Careful analysis of the hematopoietic system in Δ SET animals revealed normal cellular homeostasis, engraftment, support of MLL-AF9 leukemogenesis, gene expression, and target gene histone modifications in HSPCs. In contrast, acute deletion of *Mll1* in HSPCs had a dramatic impact on gene expression but was also not accompanied by reduced H3K4me at MLL1 target genes. Rather, the coordinate reduction in MOF-mediated H4K16Ac and MLL1 target gene expression suggested a major

role for this associated acetyltransferase in maintaining MLL1 target genes in HSPCs. The correlation between MLL1 function and H3K4me3 promoter modification may be the indirect result of the gene maintenance activity, such that active transcription results in H3K4me3 modifications through the action of the more broadly active SET1A/B complexes. This hypothesis is supported by our finding that Δ SET and WT cells are equivalently sensitive to depletion of *Wdr5*, *Ash2L*, and *Wdr82*, all required for SET1A/B activity (Figures S1C–S1G). In the case of acute deletion of *Mll1*, we propose that failure to recruit MOF to MLL1 target genes results in the rapid loss of H4K16Ac enrichment through opposing SIRT1 action, making the genes susceptible to Polycomb-mediated repression and ultimately the reduction in H3K4me3 levels. Our observations do not rule out that H3K4me3 modification at MLL1 target genes is involved in the heritability of expression state but illustrate particularly in the case of the Δ SET mutant that these modifications are likely performed by a distinct enzyme and not MLL1 itself.

Although the SET domain/enzymatic activity of several MLL-related proteins have been shown to be critical for gene regulation (Ding et al., 2012; Lee et al., 2006; Tie et al., 2014), recent evidence suggests that MLL1 and MLL2 exhibit very specialized H3K4me activities that are restricted to particular cell types and target genes (Andreu-Vieyra et al., 2010; Austenaa et al., 2012; Glaser et al., 2009; Ladopoulos et al., 2013). Nonetheless, both MLL1 and MLL2 regulate many unique gene expression

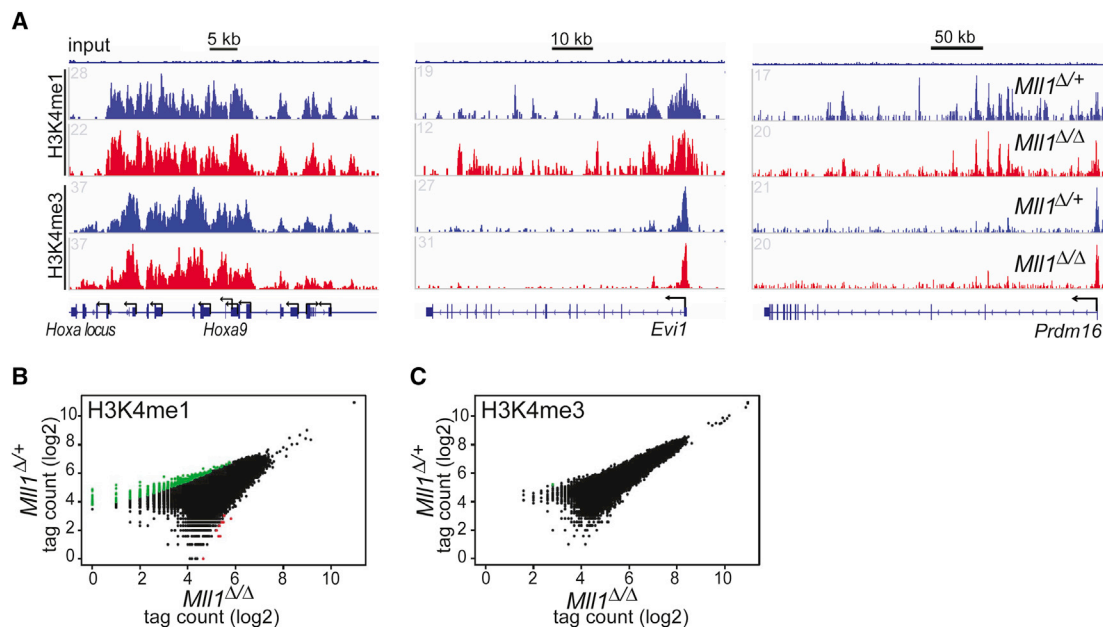


Figure 4. Genome-wide Comparison of H3K4me1 and H3K4me3 Enrichment in Purified *Mll1*^{ΔΔ} HSPCs

(A) Representative ChIP-seq tracks showing reads per million (y axis). LSK cells were sorted from control *ER*^{T2-cre};*Mll1*^{F/+} or *ER*^{T2-cre};*Mll1*^{F/F} animals and cultured in vitro in HSC medium 48 hr (300 nM 4-OHT for only the first 24 hr). Complete deletion of *Mll1* was confirmed (Figure S4B). The locus is diagrammed below the tracks.

(B and C) Scatterplots showing the concordance between enriched regions in a representative pair of control versus *Mll1*^{ΔΔ} samples. Each dot represents enrichment of the indicated histone modification within a bin of 1 kb. Differentially enriched bins in control *Mll1*^{Δ+/+} LSK cells are shown in green and those enriched in *Mll1*^{ΔΔ} LSK cells are shown in red. Pairwise comparisons between different replicate pairs yielded similar results and concordance between replicates is shown in Figures S4C–S4E.

programs above and beyond genes that are directly H3K4-methylated. Interestingly, an HMT-inactive mutant of the *Arabidopsis* *Trx* ortholog, *Atx1*, retained its ability to recruit preinitiation components to the promoters of its target genes, but exhibited reduced RNA Pol II elongation (Ding et al., 2012), demonstrating that non-HMT functions of *Atx1* can play significant and separable roles in transcriptional maintenance. Our data reinforce the concept that HMTs, particularly MLL family members, affect target gene expression through mechanisms other than their HMT activity.

MOF interacts with the C-terminal portion of MLL1 (Dou et al., 2005). Because this region is not retained in most MLL1 fusion oncoproteins, this interaction is not predicted to play a role in leukemogenesis, where the de novo recruitment of PTEFb or DOT1 complexes by the fusion partner may override the need for MOF activity. Given these qualitative differences in how fusion proteins and endogenous MLL1 regulate genes, selective targeting of the oncoprotein may be more feasible than originally imagined.

The involvement of Sirtuins in opposing trxG function and working in parallel with PcG proteins has been previously noted. In *Drosophila*, Sir2 homozygous mutations enhance E(Z) and other homeotic PcG phenotypes, although Sir2 mutations alone do not cause homeotic phenotypes. These authors also showed a physical association between SIR2 and E(Z) in larval extracts and on chromatin (Furuyama et al., 2004). The observation that genes with trithorax binding sites are among those selectively

activated by the MOF/NSL complex further suggests that a trx-MOF-Sir2-PcG pathway may be broadly used in development (Feller et al., 2012) such that the sequential actions of H4K16 deacetylation then PcG repression terminate transient developmental gene expression. The biochemical activity of trx required to confer heritability of expression state to target genes remains unclear, but interestingly, early studies using the Fab-7 trx/PcG response element implicated histone H4 acetylation as the epigenetic mark leading to the heritable maintenance of Fab-7 linked reporter genes (Cavalli and Paro, 1999). Thus, the MOF-Sirtuin balance may play a role in such epigenetic decisions.

Recent work (Cao et al., 2014) concluded that the HMT activity of MLL1 is required to support MLL-AF9-mediated leukemogenesis. The authors demonstrated that the MLL1 HMT activity depends on WDR5 interaction and that a novel pharmacologic inhibitor of this interaction both reduces MLL-AF9 colony growth and affects gene expression in a manner similar to the conditional deletion of endogenous *Mll1* in the MLL-AF9-transformed cells. Significant differences in approaches used between our two studies may underlie the distinct conclusions. For example, in vivo effects of the pharmacologic inhibitor may result from mechanisms other than the MLL1 HMT inhibition demonstrated in vitro. In addition, the distinct hypomorphic *Mll1* conditional allele used exhibits milder phenotypes than that used in our study. Future studies using HMT inhibitors and ΔSET/MLL-AF9 cells may clarify this discrepancy.

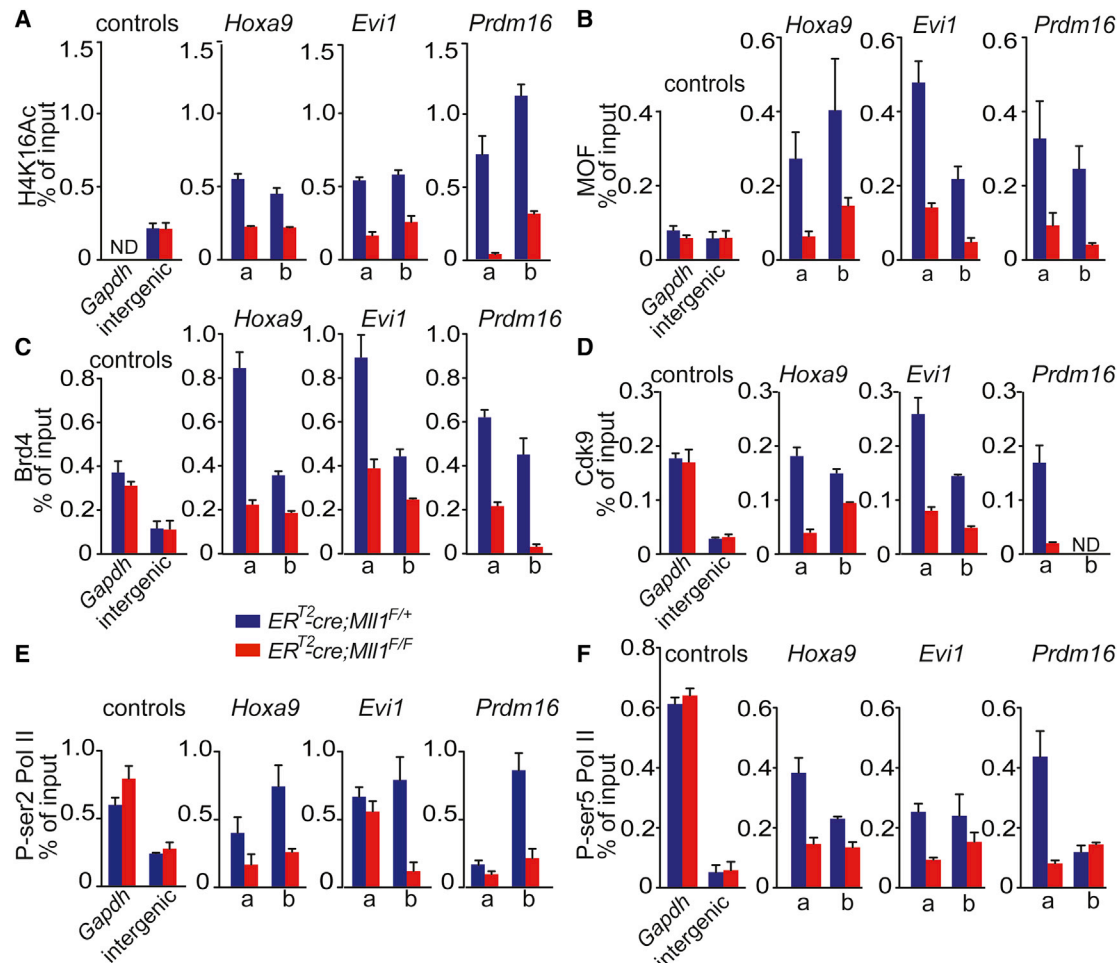


Figure 5. MOF-Mediated H4K16 Acetylation Parallels MLL1 Target Gene Expression

ChIP assays performed 48 hr after initiating *Mll1* deletion as in Figure 3A. Nuclei from *lin^{neg}* cells were fixed and ChIP performed using anti-H4K16Ac (A), -MOF (B), -Brd4 (C), -Cdk9 (D), -phospho-ser2 Pol II (E), and phospho-ser5 Pol II (F). Amplicons are as diagrammed in Figure 3B. Data represent mean of two to four replicates \pm SEM and are representative of at least three experiments. ND, not determined.

EXPERIMENTAL PROCEDURES

Flow Cytometry and Cell Sorting

Lin^{neg} bone marrow cells were prepared using a custom rat antibody mix and Dynal goat anti-rat magnetic beads, routinely yielding cells that are ~80% lineage negative/low; flow cytometry and cell sorting were performed as described (Artinger et al., 2013).

Animals, Cell Culture, and In Vitro Induction of cre Recombinase

Animal studies were performed in accordance with the Dartmouth Institutional Animal Care and Use Committee. Δ SET animals (Terranova et al., 2006) and *Mll1^{F/F}* animals (Jude et al., 2007) were backcrossed to the B6.SJL strain using the Dartmouth speed congenic facility. MEFs were prepared using standard methods from day 14 embryos. For ChIP-qPCR and ChIP-seq studies, *Mll1* was deleted in vitro from either lineage-depleted bone marrow or sorted LSK cells from two to six pooled *ER^{T2}-cre;Mll1^{F/+}* or *ER^{T2}-cre;Mll1^{F/F}* animals as detailed in the Supplemental Information.

RNA Preparation and Real-Time qPCR

Total RNA was extracted with Trizol (Life Technologies) and purified with RNeasy kits (QIAGEN). For real-time qPCRs, 0.5 μ g RNA was used to synthesize cDNA using Superscript III (Life Technologies), and analyses were per-

formed on a 7500 fast real-time PCR system (Applied Biosystems) using primers or TaqMan assays in the Supplemental Information; relative expression was normalized to *Gapdh*.

Retroviruses and Leukemia Analyses

The MLL-AF9-IRES-YFP MSCV plasmid was from Dr. Armstrong (MSKCC). Retroviral supernatants were prepared by cotransfection as described (Artinger et al., 2013). MLL-AF9-transduced cells were mixed with 4×10^5 C57Bl/6 bone marrow cells and injected into lethally irradiated recipients, and animals were sacrificed when moribund. YFP⁺ splenocytes from moribund animals were injected into lethally irradiated secondary recipients. Peripheral blood and organ analyses are detailed in the Supplemental Information. Small hairpin RNA (shRNA) plasmids were from Drs. Vakoc (Cold Spring Harbor Laboratories) or Natoli (IFOM-IEO, Milan). Replating experiments were performed by culturing fresh leukemia cells in methylcellulose plus cytokines (M3434, STEMCELL Technologies), counting colonies, and replating every 6–7 days.

ChIP-qPCR and ChIP-Seq

A miniChIP protocol (Attema et al., 2007) was used with antibodies detailed in the Supplemental Information. ChIP-seq libraries were prepared following the manufacturer's suggestions (Ovation Ultralow Library Systems, NuGEN).

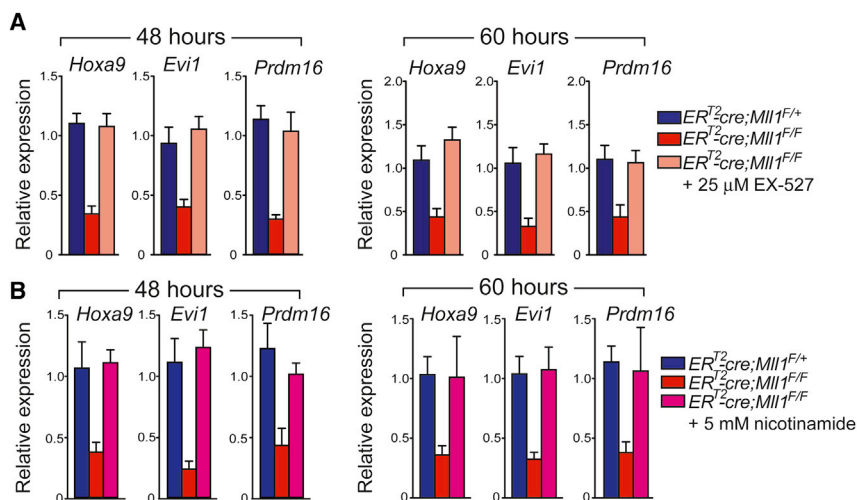


Figure 6. Restoration of MLL1 Target Expression and H4K16 Acetylation by SIRT1 Inhibition

ER^{T2}-cre;Mll1^{F/+} or ER^{T2}-cre;Mll1^{F/F} cells were cultured as in Figure 3A to initiate Mll1 deletion. Duplicate ER^{T2}-cre;Mll1^{F/F} samples were incubated with both 4-OHT and either 25 μM Ex-527 (A) or 5 mM nicotinamide (B). After 48 or 60 hr, Mll1 target gene expression was determined by real-time qPCR. Means of four PCRs ±SEM are shown. Data are representative of two to three independent experiments.

Multiplexed sequencing was performed on a HiSeq2000 instrument (Illumina). Details are provided in the [Supplemental Information](#).

SIRT1 Inhibitor Studies

Lin^{neg} cells from ER^{T2}-cre;Mll1^{F/+} or ER^{T2}-cre;Mll1^{F/F} animals were cultured in medium supplemented with 400 nM 4-OHT dissolved in ethanol and Ex-527 (Sigma) or Nicotinamide (Sigma) were dissolved in DMSO. One million cells were collected in Trizol or fixed as described above and stored at -80°C until use.

Statistics

CRU data were calculated with L-Calc (STEMCELL Technologies). Statistics and graphing were performed with Excel (Microsoft) or Prism (GraphPad). Error bars reflect mean ± SEM unless otherwise indicated in the legends. Significance for Kaplan-Meier curves was calculated using the log-rank test.

ACCESSION NUMBERS

The GEO accession number for the ChIP-seq data reported in this paper is GSE56456.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.04.015>.

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