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Cancer Cell. Author manuscript; available in PMC 2015 December 08.

Published in final edited form as:
Cancer Cell. 2014 December 8; 26(6): 896–908. doi:10.1016/j.ccell.2014.10.009.

AF10 Regulates Progressive H3K79 Methylation and *HOX* Gene Expression in Diverse AML Subtypes

Aniruddha J. Deshpande[1,2,7], **Anagha Deshpande**[7], **Amit U. Sinha**[7], **Liying Chen**[1], **Jenny Chang**[7], **Ali Cihan**[6], **Maurizio Fazio**[7], **Chun-wei Chen**[7], **Nan Zhu**[7], **Richard Koche**[7], **Liuda Dzhekieva**[3], **Gloria Ibáñez**[3], **Stuart Dias**[1], **Deepti Banka**[1], **Andrei Krivtsov**[7], **Minkui Luo**[3], **Robert G. Roeder**[6], **James E. Bradner**[4], **Kathrin M. Bernt**[5], and **Scott A. Armstrong**[1,2,7,*]

[1]Division of Hematology/Oncology, Children's Hospital Boston, Boston, MA, 02115

[2]Department of Pediatric Oncology and Harvard Medical School, Boston, MA, 02115, USA

[3]Molecular Pharmacology and Chemistry Program, Memorial Sloan Kettering Cancer Center, New York, NY, 10065

[4]Department of Medicine, Harvard Medical School, Boston

[5]Department of Pediatrics, University of Colorado Anshutz Medical Campus, Aurora, CA

[6]Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, NY 10065, USA

[7]Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, NY, 10065

SUMMARY

Homeotic (*HOX)* genes are dysregulated in multiple malignancies including several AML subtypes. We demonstrate that H3K79 dimethylation (H3K79me2) is converted to mono-methylation (H3K79me1) at *HOX* loci as hematopoietic cells mature thus coinciding with a decrease in *HOX* gene expression. We show that H3K79 methyltransferase activity as well as H3K79me1 to H3K79me2 conversion is regulated by the DOT1L co-factor AF10. AF10 inactivation reverses leukemia-associated epigenetic profiles, precludes abnormal *HOXA* gene expression and impairs the transforming ability of MLL-AF9, MLL-AF6 or NUP98-NSD1 fusions – mechanistically distinct *HOX*-activating oncogenes. Furthermore, NUP98-NSD1 transformed cells are sensitive to small-molecule inhibition of DOT1L. Our findings demonstrate that pharmacological inhibition of the DOT1L/AF10 complex may provide therapeutic benefit in an array of malignancies with abnormal *HOXA* gene expression.

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Correspondence: armstros@mskcc.org.

Supplemental Data

Supplemental data including detailed experimental procedures can be found in the accompanying supplemental section.

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INTRODUCTION

The clustered homeobox (*HOX*) genes are conserved throughout evolution and play important roles in development (Lappin et al., 2006). Multiple human malignancies, including approximately 50% of acute myeloid leukemia (AML), show dysregulated expression of the clustered homeobox genes (Argiropoulos and Humphries, 2007). Specific members of the *HOX* cluster are critical for hematopoietic stem cell (HSC) self-renewal and the expression of a particular sub-set of *HOXA* gene cluster, specifically the *Hoxa7-10* genes, is sharply down-regulated when murine HSCs differentiate to granulocyte macrophage progenitors (GMPs) (Krivtsov et al., 2006). *HOXA* gene repression in myeloid progenitors such as GMPs is critical, since aberrant *HOXA gene* expression is a central component of a leukemogenic gene expression program driven by diverse oncogenes (Argiropoulos and Humphries, 2007).

One of the classic examples of a malignancy with aberrant *HOXA* activation is leukemia with rearrangements of the Mixed Lineage Leukemia gene (*MLL*), an aggressive disease with limited treatment options and poor survival rates. In these leukemias, *MLL* is fused to one of more than 60 different fusion partners leading to the formation of dominantly acting oncogenic fusion proteins (Krivtsov and Armstrong, 2007). Expression of the MLL-fusion oncoprotein perpetuates inappropriate high level *HOXA* gene expression in GMPs and induces leukemias in mice that are arrested at a GMP-like differentiation stage (Krivtsov et al., 2006; Somervaille and Cleary, 2006). Some of the recurrent C-terminal fusion partners of MLL such as AF9, ENL, AF17, and AF10 normally interact with the histone methyl-transferase DOT1L (reviewed in Deshpande et al., 2012). DOT1L is the sole histone methyltransferase that catalyzes histone 3 lysine 79 monomethylation (H3K79me1), dimethylation (H3K79me2) and trimethylation (H3K79me3) (reviewed in Nguyen and Zhang, 2011), chromatin modifications that are widely associated with highly expressed genes in mammalian cells (Steger et al., 2008). MLL-fusions with DOT1L interacting proteins are believed to misdirect DOT1L to the promoters of *HOXA* genes, leading to H3K79 methylation and constitutive activation of these genes in *MLL*-rearranged leukemia. However, recent studies have reported that MLL-fusions with no apparent DOT1L recruiting activity are also dependent on the H3K79 methylating activity of DOT1L for transformation (Chang et al., 2010; Deshpande et al., 2013). These studies offer an alternative, though not mutually exclusive possibility: H3K79 methylation may be broadly important for the epigenetic regulation of specific *HOXA* genes and *HOXA* gene mediated oncogenesis. The most interesting implication of this alternative hypothesis is that the transforming activity of other *HOX*-activating oncogenes may also depend on H3K79 methylation.

There is tremendous interest in DOT1L as a potential therapeutic target in *MLL*-rearranged leukemia and clinical trials with a DOT1L small molecule inhibitor are currently ongoing. However, little is known about potential co-factors that mediate the chromatin modifying activity of DOT1L. DOT1L has been identified as a component of large multi-protein complexes that are associated with transcribed genes (Mohan et al., 2010; Mueller et al., 2009) although critical constituents of the DOT1L complex are not well defined. DOT1L binds to the PHD and leucine zipper containing protein AF10, an interaction that is highly

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conserved throughout evolution. Despite the fact that *AF10* is involved in recurrent chromosomal translocations that fuse it to one of at least five different fusion partners apart from *MLL* in human leukemia (Brandimarte et al., 2013; Dreyling et al., 1996; Soler et al., 2013; Zhang et al., 2012), there have been few studies aimed at understanding the functional and physiological role of AF10 in mammalian cells (Chamorro-Garcia et al., 2012; Linder et al., 2000). *AF10*-rearranged (*AF10*-R) leukemias show two recurrent features: consistent retention of the leucine rich DOT1L-interacting AF10 domain in the fusion and the aberrant upregulation of *HOXA* cluster genes. Strikingly, these two features span all *AF10*-R leukemias regardless of the N-terminal fusion partner. These observations, taken together with the fact that DOT1L as well as AF10 orthologs regulate the expression of the *bithorax* gene cluster in Drosophila (Perrin et al., 2003; Shanower et al., 2005) strongly suggest that DOT1L and AF10 may be generally involved in *HOXA* gene regulation.

RESULTS

Normal and malignant *HOXA* gene expression correlates with higher H3K79 methylation states

We wanted to test a potential role for DOT1L in the regulation of *HOXA* gene expression in murine hematopoietic stem and progenitor cells. We sorted lineage$^-$Sca-1$^+$c-Kit$^+$ (LSK) cells, which are enriched for HSCs, from bone marrow (BM) prepared from *Dot1l*$^{fl/fl}$ mice crossed with interferon inducible Mx1-Cre transgenic mice in which *Dot1l* deletion was induced upon administration of polyinosinic-polycytidylic acid (pIpC) and compared these LSKs to their pIpC injected *Dot1l* wild-type counterparts. Gene expression analysis showed that *Hoxa9* and *Meis1* were significantly down-regulated in LSKs with homozygous *Dot1l* deletion (Figure 1A) demonstrating a role for DOT1L in the normal regulation of these homeobox genes.

Since the state of H3K79 methylation could have different functions, we assessed H3K79me1, H3K79me2 and H3K79me3 in LSKs, which express high levels of *Hoxa7-10* and *Meis1* genes, and compared them to GMPs, which express much reduced levels of these genes (Krivtsov et al., 2006). We performed chromatin immunoprecipitation followed by sequencing (ChIP-seq) using well-characterized antibodies that predominantly recognize H3K79me1, H3K79me2, or H3K79me3 (Figure S1A). Each of the three H3K79 methylation states directly correlated with gene expression levels and showed broadly overlapping patterns across the genome (Figure S1B). H3K79me3 demonstrated a more contracted pattern surrounding the transcription start sites (TSS) (Figure S1B). Interestingly, this analysis showed the sharp decrease in *Hoxa7-10* expression in GMPs compared to LSKs was accompanied by a diminution in H3K79me2 and H3K79me3 at the *HOXA* genes with minimal changes in H3K79me1 (Figure 1B). In fact some genes such as *Hoxa9* showed higher H3K79me1 in GMPs than in LSKs. Furthermore, the LSK-GMP transition was associated with an encroachment of H3K27me3 into the *Hoxa7-10* gene cluster (Figure 1B), consistent with the observed down-regulation of these transcripts. These results demonstrate that *HOXA* gene expression is correlated with higher H3K79 methylation states during hematopoietic differentiation and suggests that H3K79me1 may not be sufficient to maintain high-level *HOXA* gene expression.

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MLL-rearranged leukemias show constitutive expression of the *Hoxa7-10* gene cluster and *Meis1*, a process that is dependent on H3K79 methylation. We therefore assessed H3K79 methylation states in LSK-derived MLL-AF9 leukemias by ChIP-seq. Similar to LSKs and GMPs, all three states of H3K79 methylation showed overlapping profiles with H3K79me3 particularly enriched at genes where the MLL-AF9 fusion protein binds (Bernt et al., 2011) (Figure S1C and Table S1). Strikingly, in contrast to the genome wide profiles of H3K79 methylation, promoters of *HOXA* genes were characterized by low levels of H3K79me1 and high levels of H3K79me2/3 (Figure 1C and S1D). The increased conversion of H3K79 methylation to higher methylation states at the *Hoxa7-10* genes was accompanied by the absence of the repressive H3K27me3 mark as expected from the high level expression of these genes in MLL-AF9 leukemia cells (Figure 1C). We then conducted a meta-analysis of H3K79me1/2/3 relationships across MLL-AF9 target genes. Strikingly, MLL-AF9 target genes showed low level H3K79me1 and high level H3K79me2/3 profile observed on the *Hoxa7-10* and *Meis1* genomic loci (Figure 1D). Meta-analysis of the averaged H3K79me1 and H3K79me2 profiles across the body of MLL-AF9 target genes showed that the H3K79me1 dip was especially pronounced near the promoter proximal regions of MLL-AF9 target genes (Figure S1D). In contrast, control sets of non MLL-AF9 target genes showed similar profiles of H3K79me1 and H3K79me2 across the gene body (Figure S1D). These results demonstrate that similar to normal hematopoietic differentiation, higher degrees of H3K79me2/3, rather than H3K79me1, correlate with high *HOXA* gene expression as well as exclusion of the repressive H3K27me3 from the *Hoxa7-10* locus. This suggests that the expression of *HOXA*, and perhaps other genes, is critically dependent on the conversion of H3K79me1 to H3K79me2.

AF10 is critical for higher H3K79 methylated states

Given that H3K79me2, but not H3K79me1, correlated with high level expression of select genes, we wanted to determine how the conversion of H3K79me1 to H3K79me2 is regulated. We found that the expression of *Dot1l* transcripts do not correlate with *Hoxa5-10* gene expression in LSK compared to GMP cells (Figure S2A) suggesting that *Dot1l* transcript levels do not explain the *HOXA* locus associated decrease in H3K79me2 and H3K79me3 in GMPs. There is increasing appreciation of the role of co-factor proteins in the modulation of specific methylation states by chromatin modifying enzymes (Dou et al., 2006; Pasini et al., 2004). We therefore wondered whether a co-factor of DOT1L could be responsible for enhancing the ability of DOT1L to catalyze increased H3K79 di/tri-methylation at the *HOXA* genes. Even though several DOT1L interacting proteins have been reported (Mohan et al., 2010; Park et al., 2010), the composition of the DOT1L complex in human leukemia cells remains undefined. We purified the DOT1L complex from a human *MLL*-rearranged and a *MLL*-germline cell line using previously described protocols (Kim et al., 2009) and identified a number of DOT1L interacting proteins, notably AF10, AF17, ILF2, MEF2C, HDAC1, and CDK9 (Figure S2B and Table S2). Of note, other than AF10 and AF17, most other proteins were either found in low abundance or were occasionally also retrieved from control cells (Table S2). We focused our attention on the PHD and octapeptide-motif leucine zipper containing paralogous proteins AF10 and AF17 for further studies. In order to assess a potential role for AF10 or AF17 in the conversion from H3K79me1 to H3K79me2, we performed shRNA knockdown experiments in human

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leukemia cell lines. We found that *AF10* suppression produced a clear reduction in H3K79me2, but *AF17* suppression had no observable effect on H3K79 dimethylation (Figure S2C). In contrast to *DOT1L* suppression, we observed that *AF10* suppression led to the reduction of H3K79me2 but not H3K79me1 (Figure S2C and S2D). Next we performed proliferation assays with shRNAs targeting *AF10* or *AF17* in human *MLL*-rearranged or *MLL*-germline leukemia cell lines. We found that *AF10* knockdown significantly impaired the proliferation of *MLL*-rearranged cell lines MOLM13, SEMK2 and THP1 but not the *MLL*-germline leukemia cell lines HL60 and Kasumi1 (Figure 2A and Fig. S2E). In contrast, none of the cell lines were sensitive to *AF17* knockdown suggesting that AF10 plays a more prominent role in H3K79 methylation in *MLL*-rearranged leukemia (Figure 2A). Moreover, the levels of *Af10* but not *Af17* correlated with the decrease in *HOXA g*ene expression in murine LSKs compared to GMP cells (Figure S2A) marking AF10 as a prime candidate for further studies.

Given the results above, we interrogated AF10 in more detail. We generated a conditional *Af10* knockout mouse (Figure S2F). We first assessed the effect of AF10 loss on cells insensitive to the loss of H3K79 methylation to avoid the confounding effects of apoptosis or differentiation that might be seen in DOT1L dependent cells. For this, we used bone marrow cells transformed with HOXA9 and MEIS1 or immortalized fibroblast cell lines generated from $Af10^{fl/fl}$ mice. We confirmed the absence of AF10 both at the genomic and the protein level after Cre mediated excision of *Af10* (Figure S2F and Figure S2G). Even though our knockout strategy targeted the 3′ exons of *Af10* encoding the OM-LZ domains, we did not observe a truncated AF10 protein with an anti-AF10 N-terminus antibody suggesting that our model generates a more extensive AF10 loss of function (Figure S2G). We did not observe any changes in the DOT1L or AF17 protein level in AF10 deficient cells, suggesting that these complex members are not adversely affected upon AF10 loss (Figure S2G). Moreover, *Af10* excision had no significant effect on the colony forming ability of HOXA9-MEIS1 transformed cells (Figure S2H) and *Af10* excised single cell clones could be propagated indefinitely (data not shown). Interestingly, AF10 inactivation produced a dramatic decrease in H3K79me2 levels in the HOXA9-MEIS1 transformed cells as well as in the AF10 deficient fibroblasts as assessed by Western blotting, and this loss of H3K79me2 could be rescued by ectopic AF10 expression (Figure 2B and Figure S2I). Notably, we observed that the effect of *Af10* deletion on H3K79me1 was less severe than that of *Dot1l* deletion, which completely eliminated both H3K79me1 and H3K79me2, consistent with DOT1L as the sole H3K79 methyl-transferase (Figure 2B). We next determined H3K79 methylation levels in histones purified from *Af10* deleted HOXA9-MEIS1 transformed single cell clones using Mass Spectrometry (MS) and used *Dot1l* wild-type or deleted clones for comparison. While *Dot1l* deleted clones harbored essentially undetectable levels of H3K79me1 and H3K79me2, *Af10* deleted cells had an average of almost 20 fold reduction in H3K79me2 levels and a much less severe reduction in H3K79me1 levels, recapitulating the results from Western blotting (Figure 2C and Figures S2J and S2K). These results show that loss of AF10 impairs the conversion of H3K79me1 to H3K79me2 in hematopoietic cells.

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Inactivation of AF10 impairs the transforming activity of MLL-AF9 and MLL-AF6

Leukemic transformation driven by different MLL-fusion oncogenes is dependent on DOT1L (Bernt et al., 2011; Chang et al., 2010; Chen et al., 2013; Daigle et al., 2013; Deshpande et al., 2013; Jo et al., 2011; Nguyen et al., 2011). We determined whether the transforming activity of MLL-fusions was dependent on AF10, since it is important for generation of H3K79me2. We used MLL-fusions with fusion partners that interact with DOT1L (MLL-AF9 and MLL-AF10) or an MLL-fusion that is believed to operate through a distinct mechanism (MLL-AF6). We used BM-derived LSKs from wild-type or *Af10*$^{fl/fl}$ mice to establish blast-colony forming cultures expressing the MLL-AF9, MLL-AF6 or MLL-AF10. We then deleted *Af10* using a retrovirus expressing the Cre recombinase and performed colony-forming cell (CFC) assays (Figure 3A). In the first week, *Af10* deletion profoundly impaired blast colony formation from MLL-AF9 transformed LSKs leading to increased myeloid differentiation (Figure 3B–D). Subsequent replatings showed the emergence of blast colonies that had escaped *Af10* excision (data not shown). MLL-AF6 transformed cells showed a similar dependence on AF10 (Figure 3B and Figure 3E). In contrast, the blast colony forming ability of LSKs transformed with MLL-AF10 was unaffected by *Af10* deletion (Figure 3B and Figure S3). These results show that endogenous AF10 is critical for MLL-fusion mediated transformation, and this dependence can be rescued when the AF10$^{OM\text{-}LZ}$ domain is present in the fusion protein as in the case of MLL-AF10.

AF10 loss of function impairs the maintenance of diverse MLL-driven leukemias

Next we investigated the effects of *Af10* deletion on leukemia initiation from MLL-AF9 transformed cells. *Af10* deletion significantly delayed leukemia initiation by MLL-AF9 (Figure S4A). We then deleted *Af10* from established MLL-AF9 or MLL-AF6 driven leukemias and injected cells into secondary recipient mice to assess the impact on leukemia maintenance. AF10 inactivation significantly increased the latency of secondary MLL-AF9 leukemias (Figure 4A) and completely abrogated MLL-AF6 leukemia propagation (Figure 4B). Gene expression analysis by quantitative PCR (q-PCR) showed that expression of key *HOXA* cluster genes (*Hoxa5-10*) and *Meis1* was dramatically reduced in AF10 deficient leukemias (Figure S4B). Next we assessed global H3K79 methylation in *Af10* deleted MLL-AF9 leukemia cells. Similar to the HOXA9-MEIS1 transformed cells, we observed that AF10 inactivation drastically reduced H3K79me2, whereas H3K79me1 was affected to a lesser degree (Figure 4C and Figure S4C). We then investigated whether AF10 deficient MLL-AF9 leukemias were still dependent on DOT1L for their continued proliferation. We performed proliferation assays on wild type and AF10 deficient MLL-AF9 leukemias cells using the DOT1L inhibitor EPZ004777 (Daigle et al., 2011). Treatment of the AF10 deficient MLL-AF9 leukemias with EPZ004777 eliminated H3K79me2 and residual H3K79me1 and further reduced the expression of *Hoxa7-10* and *Meis1* genes (Figure 4D and Figure S4D). Strikingly, AF10 deficient MLL-AF9 leukemia cells showed an increased sensitivity to DOT1L inhibition compared to wild-type MLL-AF9 leukemia cells. AF10 deficiency substantially lowered both the time and the dose required to impair proliferation of MLL-AF9 leukemia cells (Figure 4E). These results demonstrate that AF10 may be a

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therapeutic target in *MLL*-rearranged leukemias and that targeted disruption of the DOT1L-AF10 interaction may also sensitize leukemias to small-molecule DOT1L inhibition.

AF10 inactivation reverses MLL-AF9 driven gene expression and H3K79 methylation profiles

Next we performed a comprehensive investigation of histone methylation and gene expression in the *Af10* deleted leukemias compared to wild-type counterparts. We found dramatic locus-specific changes in H3K79 methylation in the AF10 deficient leukemias by ChIP-seq. The most remarkable change was the marked decrease in H3K79me3 in the AF10 deficient cells (Figure S5A and S5B). Specifically at the *HOXA* gene cluster, we observed a progressive reduction in H3K79 methylation with the most profound reduction in H3K79me3, accompanied by a substantial decrease of H3K79me2 (Figure S5C). H3K79me1 however, appeared to be either retained or increased at the *Hoxa7-10* genes (Figure S5C). This discrepancy between H3K79me1 and H3K79me2/3 prompted us to analyze the genome wide relationship between H3K79me1 and H3K79me2 in greater detail. Our analysis showed that thousands of genes, including most of the *HOXA* cluster genes, lost H3K79me2 (Figure 5A lower 2 quadrants) consistent with our previous assessments of global H3K79me2 levels. Interestingly, a few of these H3K79me2 depleted genes appeared to gain H3K79me1 (Figure 5A red box). We found that the genes with H3K79me2 loss accompanied by an apparent H3K79me1 gain were largely those with the highest absolute H3K79me2/3 levels in MLL-AF9 leukemia (Figure 5A, colored from yellow to red), and were highly enriched for MLL-AF9 target genes (Figure 5A, circled dots). We confirmed the decrease in H3K79me2/3 and retention of H3K79me1 at *HOXA/MEIS1* promoters upon *Af10* excision more quantitatively by ChIP-qPCR where the input sample was used to assess relative enrichment (Figure S5D). We then conducted a meta-analysis of all 129 MLL-AF9 target genes to assess changes in the relationships between H3K79me1 and H3K79me2 upon *Af10* deletion across the gene body. Strikingly, we observed that *Af10* deletion altered the relationship between H3K79me1 and H3K79me2 at the majority of MLL-AF9 target genes restoring it to a pattern resembling non MLL-AF9 target genes (Figure 5B and Figure 5C green dots vs. yellow dots). This reversal happened at all but a few MLL-AF9 target genes such as *Eya1*, *Hectd1*, and *Baz1a* (Figure S5E and data not shown). These results demonstrate that AF10 loss of function prevented the conversion of H3K79me1 to H3K79me2/3 at most MLL-AF9 target genes *such as Hoxa7, Hoxa9, Hoxa10, Meis1, Mef2c and Runx2*, resulting in the depletion of H3K79me2/3 and the preservation or accumulation of H3K79me1.

Given that *Af10* excision erased H3K79me2, but not H3K79me1, at critical target genes, we wanted to determine how this influenced the expression of MLL-fusion target genes. Microarray analysis of AF10 deficient leukemias showed significant reduction in the expression of key transcripts associated with *MLL*-rearranged leukemia (Table S3), and gene-set enrichment analysis (GSEA) showed that transcripts down-regulated upon *Af10* deletion were significantly enriched for MLL-AF9 target genes (Figure 5D). Transcripts down-regulated in AF10 deficient leukemias were also significantly enriched for a previously reported gene-set of DOT1L regulated genes in MLL-AF9 leukemia (Figure S5F) (Bernt et al., 2011). Therefore, the lack of conversion of H3K79me1 to H3K79me2

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corresponds with a decrease in gene expression for most of the critical MLL-AF9 target genes. However there are a few MLL-AF9 target genes such as *Eya1*, which show elevated H3K79me2 and increased expression after AF10 inactivation suggesting that these genes may be regulated by alternative DOT1L complexes.

Af10 deletion impairs Dot1L localization and promotes H3K27me3 at *HOXA* genes

Next, we assessed whether chromatin localization of MLL-AF9 or of DOT1L was influenced in AF10 deficient cells. For this, we made use of *in vivo* biotin-tagging to tag either MLL-AF9 or DOT1L and performed ChIP using Streptavidin-conjugated beads followed by qPCR. While there was no significant difference in MLL-AF9 occupancy in $Af10^{-/-}$ compared to $Af10^{fl/fl}$ cells, (Figure S5G) we observed significantly lower DOT1L occupancy on the promoter-proximal regions of the *Hoxa7-10* genes in the $Af10^{-/-}$ cells (Figure 5E). In contrast to the *Hoxa7-10* genes, we observed significantly increased DOT1L localization at the *Eya1* promoter-associated region in $Af10^{-/-}$ compared to $Af10^{fl/fl}$ leukemia cells (Figure S5H), which corresponds with increased *Eya1* promoter associated H3K79me2/3 and mRNA expression upon *Af10* deletion.

We next assessed if there were changes in H3K27 methylation at MLL-AF9 target genes after *Af10* deletion. Since the *Drosophila* AF10 ortholog plays a role in Polycomb repulsion from homeotic loci, we wanted to determine whether AF10 played a similar role in the spread of repressive PRC2 activity on *HOX* genes in MLL-AF9 leukemia. Our ChIP-qPCR analysis of H3K27me3 showed a significant increase in H3K27me3 at the promoters of *Hoxa7-10* genes in $Af10^{-/-}$ leukemias (Figure S5I). We then performed ChIP-seq for H3K27me3 to assess whether H3K79 diminution was generally accompanied by H3K27 increase in other loci. Our analysis showed alterations of H3K27 on various genes in the *Af10* deleted leukemias, but strikingly genes that lost H3K79me3 or H3K79me2 and concomitantly gained high levels of H3K27me3 were almost exclusively comprised of exactly those *HOXA* cluster genes that are highly expressed in LSKs and are associated with stem-cell self renewal (Krivtsov et al., 2006) (Figure S5J and Figure S5K). These results demonstrate that AF10 has specific chromatin modulatory activity at the self-renewal associated *HOXA* cluster genes in MLL-AF9 leukemia cells. Importantly, similar to our observation in normal GMPs, the continued presence of H3K79me1 was insufficient to prevent the increase in H3K27me3 at the *Hoxa7-10* gene promoters in the *Af10* deleted leukemias, thereby disrupting the mutually exclusive nature of H3K79 and H3K27 methylation normally observed across the genome (Figure S5L) (Bernt et al., 2011). These results show that inactivation of AF10 leads to impaired DOT1L association with *HOXA* cluster genes, loss of higher H3K79 methylated states, and the spread of H3K27me3, which coincides with a decrease in gene expression.

AF10 enhances the enzymatic activity of DOT1L

Next we tested whether AF10 can directly affect the histone methyl-transferase (HMTase) activity of DOT1L in cell-free HMTase assays using purified flag-tagged human DOT1L, alone or in a complex with full length human AF10, and reconstituted unmodified mononucleosomes and radioactive ^{3}H-S-adenosyl-methionine (^{3}H-SAM) substrates (Figure 6A and Figure 6B). We found that the DOT1L-AF10 complex had greater HMTase activity

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than DOT1L alone, as assessed by ^{3}H incorporation into histones in filter binding assays (Figure 6C). Next, we extracted histones from the DOT1L or DOT1L-AF10 methylated mononucleosomes and investigated H3K79me0, H3K79me1 and H3K79me2 using MS, which confirmed that the DOT1L-AF10 complex had a significantly higher overall methylating activity as compared to DOT1L alone (Figure 6D and Figure S6A–C). In addition, histones methylated by the DOT1L-AF10 complex had a significantly higher H3K79me2/me1 ratio than those methylated by DOT1L alone (Figure 6E). These results demonstrate that AF10 plays a crucial role in enhancing the HMTase activity of DOT1L, influencing its ability to stimulate higher order H3K79 methylation.

AF10 controls *HOXA* gene expression and proliferation of NUP98-NSD1 transformed cells

Our observations from LSKs, GMPs and different MLL-fusion leukemias suggest that higher degree H3K79 methylation mediated by the DOT1L-AF10 complex is critical for *HOX* gene expression irrespective of the cellular context. *D. melanogaster* studies have shown that both DOT1L and AF10 orthologs are required for normal *HOX* gene expression (Perrin et al., 2003; Shanower et al., 2005), suggesting an evolutionarily conserved function of the DOT1L-AF10 complex in homeotic gene expression. Since aberrant *HOX* gene expression is not limited to *MLL*-rearranged leukemia, we wondered whether the DOT1L-AF10 complex might also regulate *HOX* gene expression and consequently transforming activity of other AML inducing oncogenes that show aberrant *HOX* gene activation. The NUP98-NSD1 fusion resulting from a chromosomal translocation is observed in 16% of cytogenetically normal pediatric AML and in a small subset of adult AML (Fasan et al., 2013; Hollink et al., 2011; Thol et al., 2013). NUP98-NSD1 positive AMLs possess high-level *HOXA* gene expression and have a very poor prognosis (Hollink et al., 2011). We generated a retrovirus that expresses NUP98-NSD1 and transduced LSK cells from either wild-type or homozygous *Af10* floxed mice. NUP98-NSD1 transduced cells proliferate indefinitely in culture and displayed features of differentiation arrest as assessed by immunophenotype and morphology (Figure S7A and S7B). Bi-allelic *Af10* deletion led to a dramatic decrease in H3K79 dimethylation accompanied by a more subtle decrease in H3K79 monomethylation in the NUP98-NSD1 transformed cells (Figure S7C). Furthermore, AF10 inactivation led to a significantly reduced expression of the *Hoxa7-10* cluster (Figure S7D). Importantly, deletion of either *Af10* or *Dot1l* in NUP98-NSD1 transformed bone marrow cells dramatically impaired their proliferation and increased apoptosis (Figure 7A, Figure 7B and S7E) and cells growing after 14 days had incomplete deletion of the *Dot1l* or *Af10* (data not shown). NUP98-NSD1 cells deleted of either *Af10* or *Dot1l* demonstrated a cellular morphology consistent with differentiation (Figure 7C and data not shown). These findings suggest that, similar to *MLL*-rearranged leukemias, oncogenic transformation driven by NUP98-NSD1 is critically dependent on H3K79 methylation mediated by the DOT1L-AF10 complex.

NUP98-NSD1 transformed cells are sensitive to small-molecule DOT1L inhibition

The observations above using *Dot1l* or *Af10* conditional knockout mice indicated that cells transformed with *HOX*-activating oncogenes other than MLL-fusion proteins might show sensitivity to therapeutic targeting of DOT1L activity. Given the clear clinical implication of this hypothesis, we tested the sensitivity of NUP98-NSD1 transformed cells to

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pharmacological DOT1L inhibition. Treatment of the NUP98-NSD1 transformed cells with the DOT1L inhibitor EPZ004777 significantly reduced global and promoter associated H3K79me2, and a significant reduction in *Hoxa7-10* gene expression (Figure S8A, Figure 8A and Figure 8B). Notably, the effect of EPZ00477 exposure on *HOXA* gene expression led to a dramatic dose-dependent decrease in proliferation starting from day 7 after exposure to the inhibitor (Figure 8C) accompanied by increased differentiation and apoptosis (Figure 8D and Figure S8B). The sensitivity of the NUP98-NSD1 cells to EPZ004777 could be completely rescued by retroviral overexpression of HOXA9-MEIS1 (Figure S8C) demonstrating that NUP98-NSD1 mediated transformation was critically dependent on DOT1L-mediated regulation of *HOXA/MEIS1* gene expression. These results provide compelling evidence in support of a broader role for H3K79 methylation, mediated by the DOT1L-AF10 complex, in leukemogenic *HOXA* gene regulation and oncogenesis. These data also indicate that therapeutic targeting of the DOT1L activity may provide benefit in multiple leukemia subtypes showing aberrant *HOXA* gene activation.

DISCUSSION

The biological responses associated with histone methylation are determined both by the amino acid residue and the degree of methylation (Kouzarides, 2007). H3K79 methylation is associated with actively transcribed genes and euchromatin in multiple species. Mono-, di-, and trimethylation of H3K79 are all solely catalyzed by DOT1L, in multiple organisms (reviewed in Nguyen and Zhang, 2011). In *S. cerevisiae*, mutation of H3K79 influences SIR2 binding and heterochromatin formation thus affecting gene activity. Whether H3K79 methylation influences similar mechanisms to control gene expression in mammalian cells is unclear. Our results suggest that different levels of H3K79 methylation serve as a “volume control” mechanism for gene expression during hematopoiesis and leukemia development, with higher degrees of H3K79 methylation correlating with elevated levels of gene expression. Of particular interest, genes decorated with H3K79me1 but not H3K79me2/3 are unable to maintain high-level expression either in developing hematopoietic cells or leukemia. Of note, H3K27me3 invaded *HOX* loci even though H3K79me1 was present. Therefore, the loss of one methyl group on H3K79 appears to have allowed gene repression and H3K27me3 accumulation. This suggests that during hematopoietic development the H3K79 methylation state decreases as myeloid development progresses thus ensuring a finely tuned decrease in gene expression to the point at which repressive mechanisms dominate. Such processes thus allow developing myeloid cells the potential to rapidly and faithfully modulate expression of genes that are highly expressed in less differentiated cells until cells are fully committed to a particular fate. However, this mechanism presents a risk of being coopted by leukemogenic oncoproteins, a mechanism that is taken advantage of by MLL or NUP98-fusions.

There is growing appreciation of aberrant chromatin modifications in cancer cells. One fascinating aspect is the apparent oncogenic impact of abnormal variations in the degree of histone methylation at particular histone residues. In MLL-AF9 leukemia cells we find a decrease in H3K79me1 and concomitant increase in H3K79me2/3 at MLL-AF9 target genes which suggests continuous conversion of H3K79 methylation to higher methylated states. The MLL-AF9 fusion protein prevents a differentiation associated decrease in higher degree

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H3K79 methylation at key MLL-AF9 target genes including the *HOXA* genes, leading to the perpetuation of a constantly self-renewing state. These findings underscore the critical roles that specific methylation states play in oncogenesis. Other examples of specific degrees of histone methylation contributing to oncogenic development are the recently described gain of function mutations in the H3K27 methyl-transferase EZH2 leading to hyper di and trimethylation of H3K27 in B-cell lymphoma (McCabe et al., 2012a; Sneeringer et al., 2010) as well as the accumulation of H3K36me2 in subsets of multiple myeloma and acute lymphoid leukemia (ALL) cells harboring chromosomal translocations or activating mutations of the H3K36 methyl-transferase MMSET/NSD2 (Martinez-Garcia et al., 2011).

The low H3K79me1/high H3K79me2 pattern that we observed on MLL-AF9 target genes could reflect enhanced DOT1L recruitment and/or enzymatic activity at these genes. A recent study demonstrated that inactivation of the *C. elegans* AF10 homolog abrogates chromatin localization of the *C. elegans* homolog of DOT1L (Cecere et al., 2013). In our study, we demonstrate that AF10 depletion reduces DOT1L localization to *HOX* genes indicating that AF10 plays a role in locus-specific DOT1L recruitment. Our observation that some genes, including some MLL-AF9 target genes such as *Eya1*, *Hectd1* and *Baz1a* gain H3K79 methylation upon *Af10* deletion suggests that AF10 is responsible for DOT1L localization on most, but not all, MLL-AF9 target genes, and in the absence of AF10, DOT1L levels and consequently H3K79 methylation may be relocated to other chromatin loci, potentially through other DOT1L co-factors. Although AF10 inactivation dramatically impairs leukemia initiation and maintenance, AF10 deficiency does not completely preclude MLL-AF9 leukemic outgrowth. In the case of MLL-AF6 however, AF10 depletion seems to completely inhibit leukemia development. The difference in the degree of AF10 dependence between these two leukemias may be attributable to the ability of MLL-AF9, but not MLL-AF6, to recruit DOT1L to *HOX* and other loci. It is important to mention that both leukemias show absolute dependence on H3K79 methylation, since DOT1L inhibition in AF10 deficient MLL-AF9 leukemias swiftly and completely eliminates those cells.

We demonstrate that AF10 augments the HMTase activity of DOT1L. AF10 is therefore important for both recruitment and enzymatic activity of DOT1L, analogous to co-factors of other chromatin modifying complexes (Dou et al., 2006; Wysocka et al., 2005). There is increasing interest in the development of small molecule inhibitors against chromatin modulators. Studies have shown that inhibition of chromatin modifying enzymes can have therapeutic benefits in pre-clinical models (Chen et al., 2013; Daigle et al., 2011; Deshpande et al., 2013; McCabe et al., 2012b). A small molecule DOT1L inhibitor is currently in phase I clinical trials for the therapy of *MLL*-rearranged leukemia (Daigle et al., 2013). It is worth noting that most drug-screening efforts aimed at therapeutically targeting the HMTase activity of DOT1L use the catalytically active DOT1L N-terminal 1-416 amino acid fragment. In our study, we have demonstrated that full-length DOT1L in complex with AF10 not only has a significantly enhanced HMTase activity but also is better able to produce H3K79 dimethylation than DOT1L alone. Our data not only provides an important starting point for further detailed analyses into the enzymology of H3K79 methylation, but also indicate that the DOT1L-AF10 complex might be a more physiologically relevant target for future drug development efforts.

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Efforts to target chromatin-modifying proteins are increasingly gaining traction. One prime example of this is small molecule inhibition of the PRC2 complex in lymphoma cells for which clinical trials are ongoing (Knutson et al., 2012; McCabe et al., 2012b; Qi et al., 2012). These rapid advances predict an emerging wave of anti-cancer therapeutics based on the inhibition of chromatin regulatory factors. Our results demonstrate that in addition to histone methyltransferases themselves, the identification of oncogenic dependencies on chromatin co-factors may present additional avenues for therapeutic intervention in malignancies with aberrant changes in the epigenome. Targeting the DOT1L-AF10 interaction may synergize with and thus improve the efficacy of agents that inhibit DOT1L. These studies present a rationale for therapeutic targeting of the DOT1L-AF10 interaction and suggest that dimethylation and trimethylation of H3K79 via the DOT1L complex is a potential therapeutic target in an array of different leukemias, and perhaps other tumor types associated with elevated *HOX* gene expression.

EXPERIMENTAL PROCEDURES

ChIP-qPCR and ChIP-Seq

ChIP was performed as described previously (Bernt et al., 2011). Briefly, crosslinking was performed with 1% formalin, and the cells were lysed in SDS buffer. Sonication was used to fragment DNA. ChIP for H3K79me1, H3K79me2, H3K79me3, and H3K27me3 was performed using the antibodies ab2886, ab3534, ab2621 (Abcam) and 07-449 (Millipore) specific to the respective modifications. Eluted DNA fragments were analyzed by qPCR, or subjected to sequencing using next-generation Ilumina sequencing. More details can be obtained in the supplemental methods section.

Mice

A pFlexible-based targeting vector containing a ~2.7 kb genomic locus region that included exons 17 and 18 of *Af10* (reference transcript: REFSEQ NM_010804) flanked by loxp sites were used to target CJ9 ES cells (129 background). Conditional mice were maintained on a B6/129 background (Taconic). The generation of *Dot1l* knockout mice was previously described (Bernt et al., 2011). *Dot1l*$^{fl/fl}$ mice were crossed to Mx1-Cre mice (Kuhn et al., 1995) (Jackson Labs, Bar Harbor, ME), and the *Mx1-Cre* allele was maintained as a heterozygous allele on a mixed B6/129 background. Genotyping strategies, primers and conditions are described in the supplemental experimental procedures. All animal experiments described in this study including the experiments with conditional knockout mice as well as mouse BM transplantation models were approved by, and adhered to guidelines of, the Memorial Sloan-Kettering Cancer Center and the Children's Hospital Boston institutional animal care and use (IACUC) committees.

Small molecule inhibitor

EPZ004777 was synthesized by the laboratory of Jay Bradner (Dana Farber Cancer Institute, Boston, MA). 50 mM stock solutions were prepared in DMSO and stored at −20° C. Serial dilutions of stock solutions were carried out just prior to use in each experiment and final DMSO concentrations were kept at, or below 0.02%.

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Accession information

ChIP and Microarray data is deposited at the NCBI gene expression omnibus (GEO) - accession number: GSE54500.

Data Analysis and Statistical Methods

Significance for all individual experiments described in the manuscript was calculated using a 2-tailed unpaired Student's T-test in Microsoft Excel or GraphPad Prism. For mouse survival curves the Log-Rank test was used to calculate statistical significance using GraphPad Prism. Raw expression data was normalized using Genepattern software by RMA algorithm with quantile normalization and background correction. Probesets that had 50% or more present calls (using MAS5 algorithm) were marked as expressed and the remainder as not expressed. The expressed probe-sets were further divided into 3 groups of high, medium and low expression based on raw expression values. Gene set enrichment analysis of microarray was performed with GSEA (Subramanian et al., 2005), and relative changes in methylation values were plotted using Icanplot software (www.icanplot.org) (Sinha and Armstrong, 2012).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors acknowledge Ronald Hendrickson, Yuko Fujiwara and Stuart Orkin for valuable assistance with MS analysis and knockout mouse generation. This work was supported by the American Cancer Society, the Leukemia and Lymphoma Society (LLS), Gabrielle's Angel Foundation and the NCI (CA105423, CA140575, CA176745) to S.A.A. and the NCI K99 Award to A.J.D.. S.A.A. is a consultant for Epizyme Inc.. The protein complex purification work and the MS studies were supported by the LLS SCOR grant 7132-08 to R.G.R. and an NCI Cancer Center support grant P30 CA08748 to MSKCC.

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SIGNIFICANCE

A broad array of human malignancies shows aberrant expression of the clustered homeobox genes, particularly the genes of the *HOXA* gene cluster. Our study demonstrates that the DOT1L interacting protein AF10 regulates the HMTase activity and H3K79me1 to H3K79me2 conversion by DOT1L. AF10 inactivation causes widespread changes to the leukemia epigenome, suppressing the ability of multiple *HOX*-activating oncogenes to induce leukemic transformation. Our results provide evidence that transformation driven by MLL-fusions as well as the recurrent AML-associated NUP98-NSD1 fusion oncogene is critically dependent on the ability of AF10 to stimulate DOT1L activity. These findings have profound therapeutic implications as they provide a strong rationale for therapeutically targeting the DOT1L-AF10 complex in multiple types of refractory leukemias.

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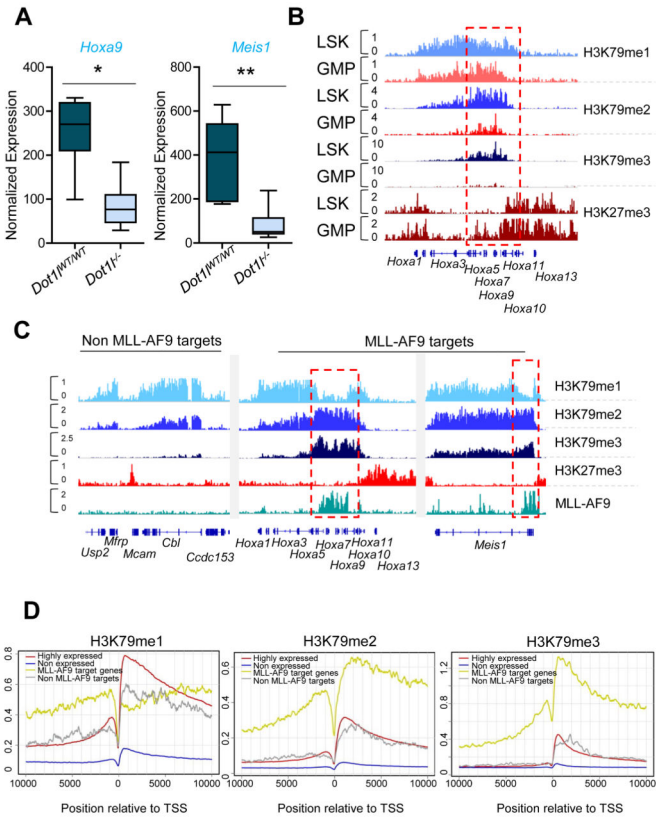

Figure 1. Histone methylation profiles in normal cells and MLL-AF9 leukemia

(A) Expression of *Hoxa9* and *Meis1* in LSKs sorted from *Dot1l*$^{fl/fl}$ or *Dot1l*$^{WT/WT}$ mice crossed with Mx1-Cre mice treated with pIpC 10 days before. The box plot shows normalized expression values from 6 independent replicates. Whiskers represent the upper and lower limits of the range. Boxes represent the first and third quartile, and the line represents the median. (B) Representative profiles for ChIP-seq using anti-H3K79me1, H3K79me2, H3K79me3 and H3K27me3 antibodies in LSK and GMP cells at the *HOXA* cluster. (C) Representative profiles for ChIP-seq using anti-H3K79me1, H3K79me2, H3K79me3 and H3K27me3 antibodies at various genomic loci in MLL-AF9 leukemia cells. Binding of MLL-AF9 fusion gene is shown at the bottom in green. In both B and C, the y-axis scales represent read density per million sequenced reads and the *Hoxa5-10* genomic region is marked by a dotted red rectangle. (D) Meta-analysis of averaged ChIP-seq signal at four sets of genes across a +10 kb to −10 kb genomic region around the transcriptional start site. See also Figure S1 and Table S1.

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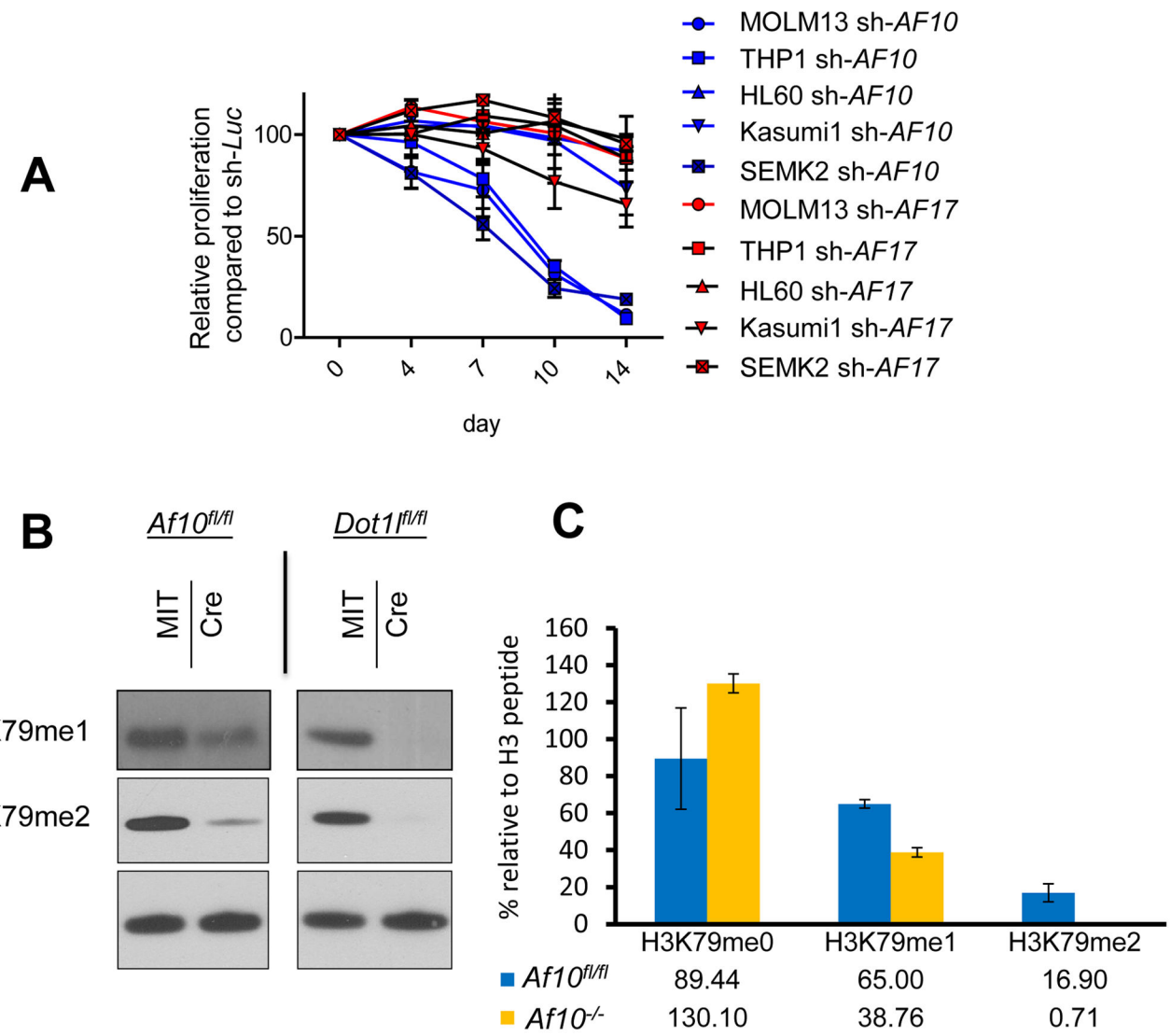

Figure 2. The impact of genomic *Af10* deletion on H3K79 methylation

(A) Growth of human leukemia cell lines MOLM13 (MLL-AF9), THP1 (MLL-AF9), SEMK2 (MLL-AF4), HL60 (non-MLL-rearranged), and Kasumi1 (non-MLL-rearranged) after transduction with anti-*AF10* and anti-*AF17* shRNAs. Viable cells were counted every 3 to 4 days, and cell numbers after transduction with an anti-luciferase shRNA (sh-*Luc*) were set as 100%. (B) Western blots showing H3K79 methylation in HOXA9-MEIS1 transformed clones with indicated floxed allele following empty vector (MIT) or Cre expression. (C) Quantification of integrated signals for area under the curve values of indicated clones. Error bars represent SD. See also Figure S2 and Table S2.

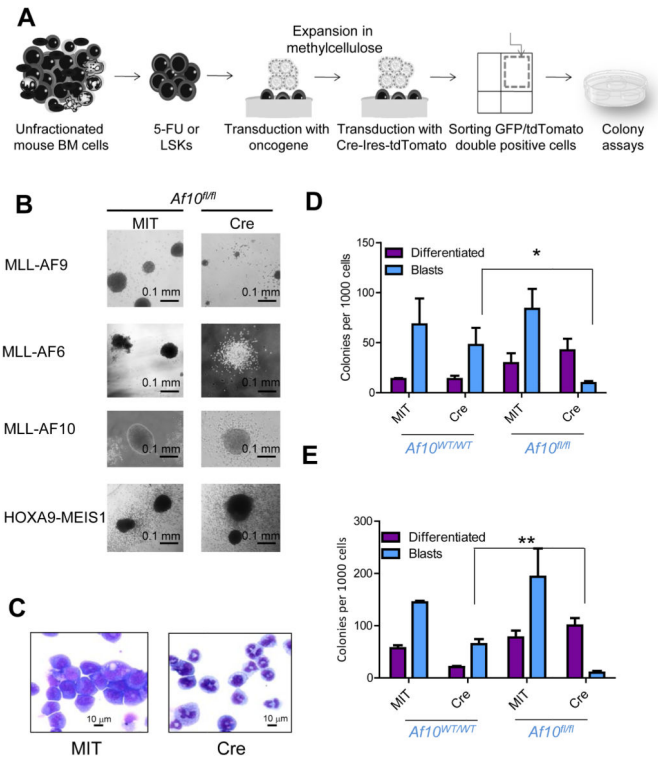

Figure 3. The DOT1L-AF10 interaction is critical for MLL-transformation

(A) Schematic representation of colony assays. (B) Pictures of representative colonies from cells transformed with different oncogenes 7 days after *Af10* excision (Cre) compared to *Af10* floxed colonies (MIT). (C) Wright-Giemsa stained cytospins of MLL-AF9 transformed cells 7 days after expression of the Cre recombinase (Cre) or the control vector (MIT). (D, E) Colony numbers obtained 7 days after expression of the Cre recombinase in wildtype or *Af10* floxed MLL-AF9 (D) or MLL-AF6 (E) transformed cells. *p=0.013, **p=0.04. (D) and (E) represent mean +/− SEM values from 3 independent experiments. p values were calculated between the mean numbers of blast colonies in Cre expressing wildtype cells compared to Cre expressing *Af10* floxed counterparts to account for Cre-mediated toxicity. See also Figure S3.

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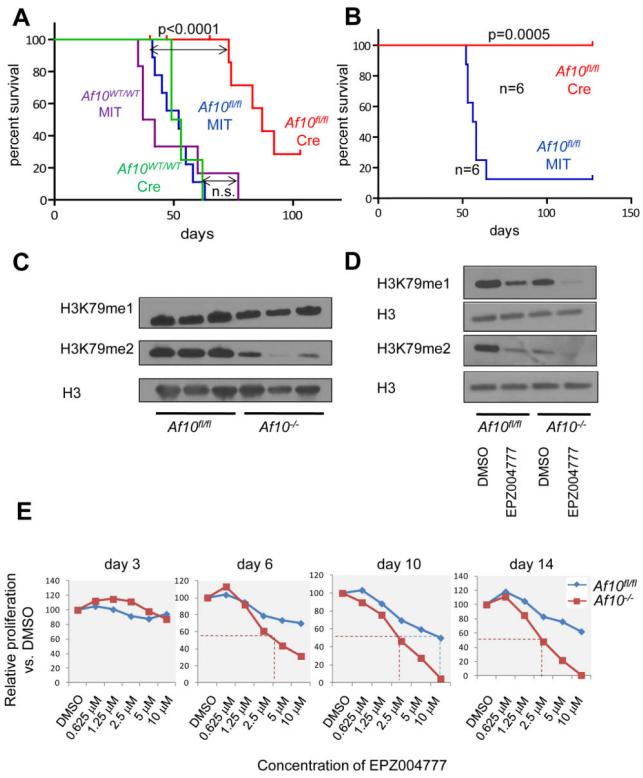

Figure 4. Impact of AF10 inactivation on leukemia maintenance

(A)Survival curves for secondary leukemia from MIT or Cre expressing primary MLL-AF9 leukemias in the *Af10*$^{fl/fl}$ background compared to MLL-AF9 leukemias from the *Af10* wildtype background. (n=6–10 mice for each cohort). (B) Percent survival of mice injected with primary MLL-AF6 leukemia cells in the *Af10*$^{fl/fl}$ background after Cre or MIT expression (n=6 for each cohort). (C) Western blots showing H3K79me1 and H3K79me2 in the BM cells of 3 independent *Af10*$^{fl/fl}$ or *Af10*$^{-/-}$ MLL-AF9 leukemias. Total H3 is used as the loading control. (D) Western blots showing H3K79me1 and H3K79me2 in a representative *Af10*$^{fl/fl}$ or *Af10*$^{-/-}$ MLL-AF9 leukemia treated with 10 μM of EPZ004777 or the DMSO vehicle control. (E) Representative experiment showing relative proliferation as assessed by Trypan blue exclusion for *Af10* floxed (MIT) or deleted (Cre) MLL-AF9 leukemia cells plotted as a percent of vehicle control (DMSO) in various concentrations of EPZ004777 at different time-points (Representative of 3 independent experiments). See also Figure S4.

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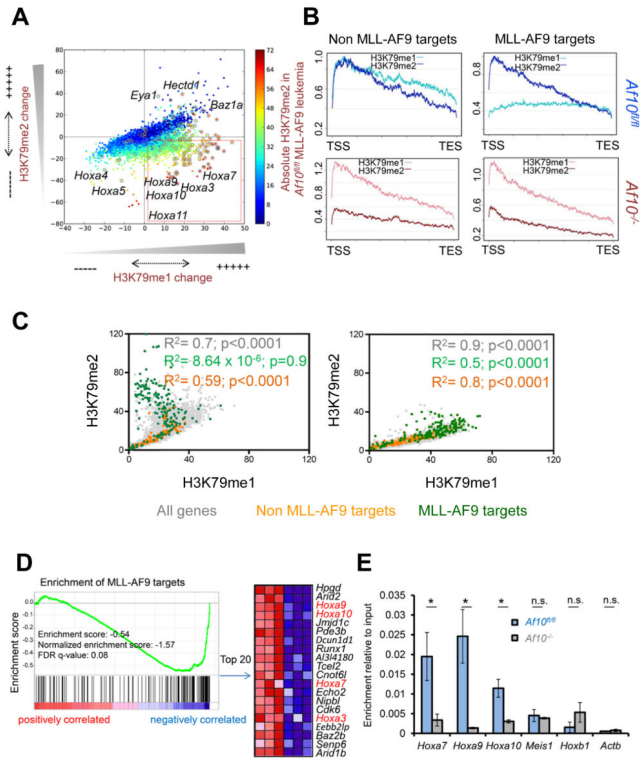

Figure 5. Genomic and epigenomic changes upon *Af10* deletion

(A) Scatter plot showing genome-wide changes in the values of promoter-proximal H3K79me1 (x-axis) or H3K79me2 (y-axis) in $Af10^{fl/fl}$ vs. $Af10^{-/-}$ leukemias. Each dot represents the difference in averaged methylation values around the promoter proximal regions (−2 kb to +2 kb around the TSS); positive and negative values reflect increases and decreases respectively in $Af10^{-/-}$ compared to $Af10^{fl/fl}$ leukemias. Methylation values are colored according to absolute H3K79me2 values in wild type MLL-AF9 leukemia (blue-least methylation, red-highest methylation). (B) A representative meta-analysis plot showing averaged profile across the gene body from the TSS to the transcription end site (TES) of MLL-AF9 target genes compared to control genes. Profiles of $Af10^{fl/fl}$ MLL-AF9 leukemias in (blue) compared to their $Af10^{-/-}$ counterparts (in red) are presented. Light colors represent H3K79me1 and dark colors represent H3K79me2. MLL-AF9 targets are genes bound by MLL-AF9 and non MLL-AF9 target genes are a size and expression matched set of genes that are not bound by MLL-AF9. (C) Scatter plot showing the genome-wide relative relationship between H3K79me1 and H3K79me2 values around promoter-proximal regions. Values are representative of 5 independent replicates for each group. (D) Enrichment of MLL-AF9 target genes in the $Af10^{-/-}$ compared to $Af10^{fl/fl}$ leukemias shown by GSEA. The top 20 MLL-AF9 targets downregulated in $Af10^{-/-}$ leukemia are shown on the right. (E) ChIP for DOT1L protein at the promoter proximal regions of indicated genes in MLL-AF9 leukemias. n=2, * p<0.05, n.s.= not significant.

Error bars represent SD. See also Figure S5 and Table S3.

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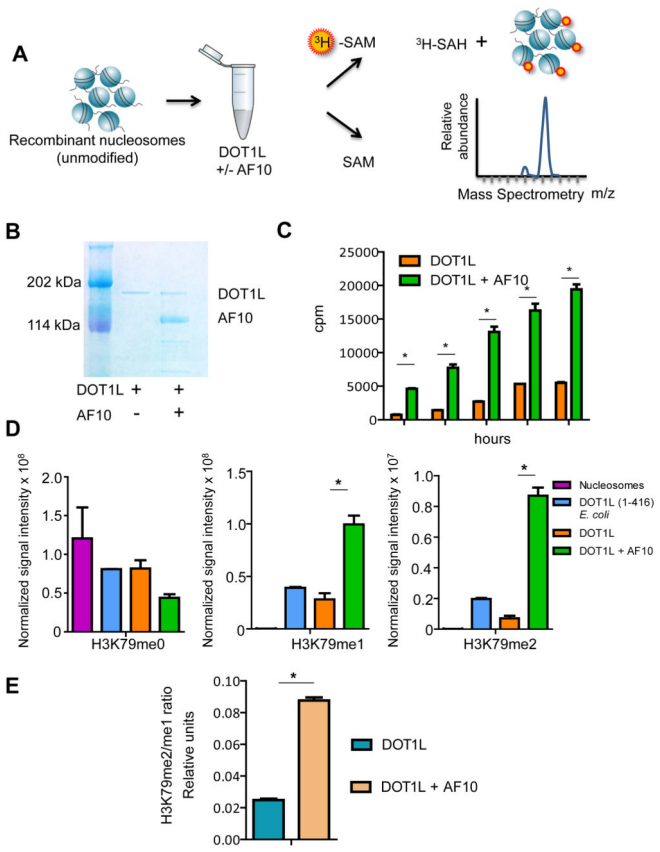

Figure 6. Effect of AF10 on the HMTase activity of DOT1L

(A) Schematic depiction of the experimental design. SAM: S-adenosylmethionine, SAH: S-Adenosylhomocysteine. (B) Flag-tagged DOT1L overexpressed either alone or in combination with untagged AF10 in insect cells and purified using Flag-affinity purification is shown by Coomassie staining. (C) Bar graph showing quantification of methylation as performed by filter binding followed by liquid scintillation counting at various time-points denoted on the x-axis. cpm: counts per million. n=3, *p<0.01 in each case. (D) Comparative levels of H3K79me0, H3K79me1 and H3K79me2 by MS in nucleosomes incubated with a human DOT1L fragment expressed in *E. coli* (positive control), full-length human DOT1L or DOT1L+AF10. (n=2; *p<0.05). Ion intensities of methylated peptides (residues 74–84) are scaled relative to an internal standard. (E) Ratio of H3K79me2/me1 normalized to internal standard in the different conditions is depicted. *p<0.05.

Error bars represent SD. See also Figure S6.

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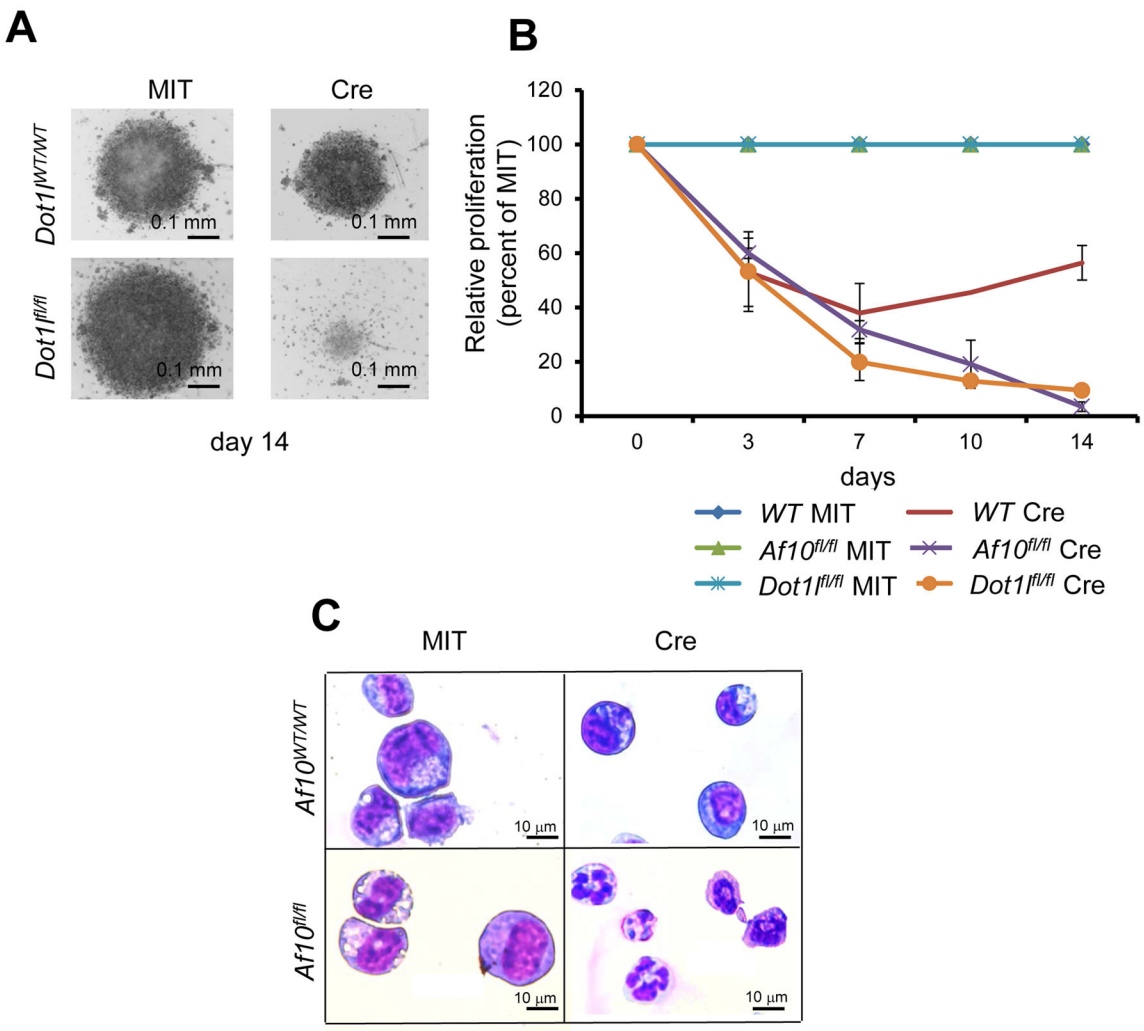

Figure 7. Role of the DOT1L-AF10 complex beyond MLL-rearranged leukemia

(A) Representative picture of a well with MIT or Cre expressing NUP98-NSD1 transformed cells in the *Dot1l*$^{WT/WT}$ and *Dot1l*$^{fl/fl}$ backgrounds at day 14 after culture. (B) Relative proliferation of NUP98-NSD1 cells after *Af10* or *Dot1l* deletion (Cre) compared to wildtype controls are presented as a percentage of their *Af10* or *Dot1l* non deleted (MIT) counterparts. n=3, * p< 0.05. Error bars represent SD. (C) Wright-Giemsa stained cytospins of NUP98-NSD1 transformed cells 7 days after MIT or *Cre* expression in the *Af10*$^{WT/WT}$ or *Af10*$^{fl/fl}$ backgrounds. See also Figure S7.

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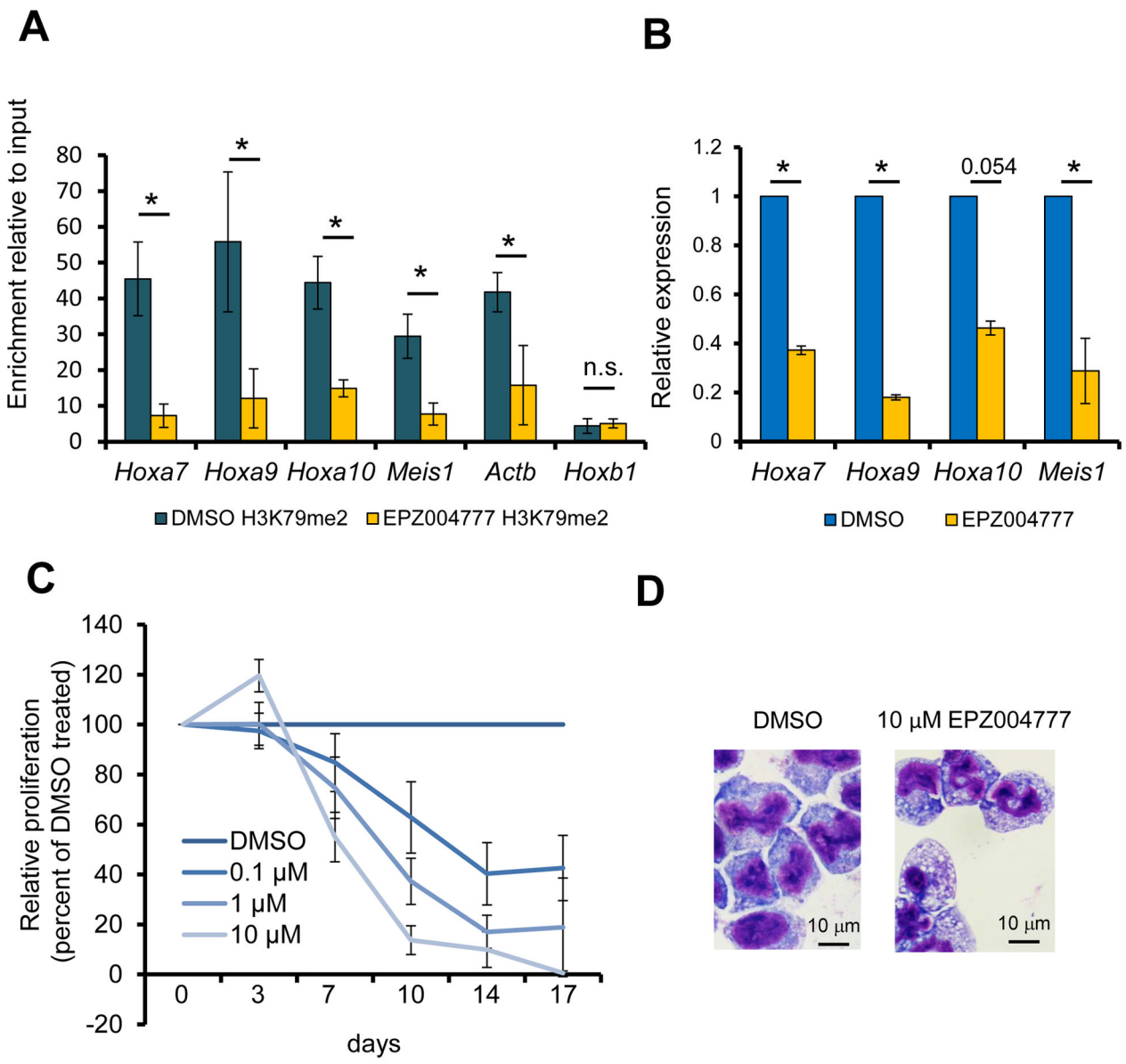

Figure 8. Effect of pharmacological DOT1L inhibition on NUP98-NSD1 transformed cells

(A) The H3K79me2 level at the promoters of the *Hoxa7-10* and *Meis1* genes upon DOT1L inhibition with 10 μM EPZ004777 for 7 days is assessed by ChIP with an H3K79me2 specific antibody followed by qPCR. *p< 0.05, n.s.= not significant. (B) Expression of *HOXA/MEIS1* genes 7 days after treatment of NUP98-NSD1 transformed BM cells with 10 μM EPZ004777 is shown relative to DMSO vehicle carrier treated cells. *p< 0.05. (C) Relative proliferation of NUP98-NSD1 transformed cells during 17 days of incubation with varying concentrations of EPZ004777 is plotted as a percentage of the vehicle control treated cells. (D) Wright-Giemsa stained cytospins of NUP98-NSD1 transformed cells 10 days after treatment with DMSO or the EPZ004777 inhibitor. Error bars represent SD. See also Figure S8.