Conserved crosstalk between histone deacetylation and H3K79 methylation generates DOT1L-dose dependency in HDAC1-deficient thymic lymphoma

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Abstract

DOT1L methylates histone H3K79 and is aberrantly regulated in MLL-rearranged leukemia. Inhibitors have been developed to target DOT1L activity in leukemia, but cellular mechanisms that regulate DOT1L are still poorly understood. We have identified the histone deacetylase Rpd3 as a negative regulator of budding yeast Dot1. At its target genes, the transcriptional repressor Rpd3 restricts H3K79 methylation, explaining the absence of H3K79me3 at a subset of genes in the yeast genome. Similar to the crosstalk in yeast, inactivation of the murine Rpd3 homolog HDAC1 in thymocytes led to an increase in H3K79 methylation. Thymic lymphomas that arise upon genetic deletion of HDAC1 retained the increased H3K79 methylation and were sensitive to reduced DOT1L dosage. Furthermore, cell lines derived from HDac1-/- thymic lymphomas were sensitive to a DOT1L inhibitor, which induced apoptosis. In summary, we identified an evolutionarily conserved crosstalk between HDAC1 and DOT1L with impact in murine thymic lymphoma development.

Keywords Chromatin; H3K79 methylation; histone acetylation; histone ubiquitination; lymphoma

Subject Categories Cancer; Chromatin, Epigenetics, Genomics & Functional Genomics

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Introduction

Aberrant histone modification patterns have been observed in many diseases, and this deregulation of chromatin can play a causative role in disease. Since epigenetic alterations are, in principle, reversible in nature, histone (de)modifiers are attractive therapeutic targets (Brien et al, 2016; Jones et al, 2016; Shortt et al, 2017). Several epigenetic drugs are currently in the clinic or in clinical trials, but for many of the drug targets, we are only beginning to understand their cellular regulation.

The histone H3K79 methyltransferase DOT1L (KMT4; Dot1 in yeast) is an epigenetic enzyme for which inhibitors are in clinical development for the treatment of MLL-rearranged (MLL-r) leukemia (Stein & Tallman, 2016). In MLL-r leukemia, DOT1L recruitment to MLL target genes, such as the HoxA cluster, leads to aberrant H3K79 methylation and increased transcription (reviewed in Vlaming & Van Leeuwen, 2016). Although the DOT1L inhibitor Pinometostat (EPZ-5676) has shown promising results in the laboratory and is currently in clinical development (Bernt et al, 2011; Daigle et al, 2013; Waters et al, 2015; Stein & Tallman, 2016; Stein et al, 2018), the cellular mechanisms and consequences of DOT1L deregulation are only just being uncovered (Vlaming & Van Leeuwen, 2016).

An important mechanism of regulation is the trans-histone crosstalk between monoubiquitination of the C-terminus of histone H2B (H2Bub) at lysine 120 (123 in yeast) and methylation of histone H3K79 (Zhang et al, 2015). The addition of a ubiquitin peptide to the nucleosome at this position occurs in a co-transcriptional manner and promotes the activity of Dot1/DOT1L, possibly by activation of...
DOT1L or coaching it toward H3K79 and thereby increasing the chance of a productive encounter (Vlaming et al., 2014; Zhou et al., 2016). Another mechanism of regulation is mediated by the direct interactions of DOT1L with central transcription elongation proteins (reviewed in Vlaming & Van Leeuwen, 2016). These interactions target DOT1L to transcribed chromatin and provide an explanation for the aberrant recruitment of DOT1L by oncogenic MLL fusion proteins (Deshpande et al., 2014; Li et al., 2014; Chen et al., 2015; Kuntimaddi et al., 2015; Wood et al., 2018). Further characterizing the regulatory network of DOT1L could lead to the identification of alternative drug targets for diseases in which DOT1L is critical and provide alternative strategies in case of resistance to treatment with DOT1L inhibitors (Campbell et al., 2017).

In a previous study, we presented a ChIP-barcode-seq screen (Epi-ID) identifying novel regulators of H3K79 methylation in yeast (Vlaming et al., 2016). The Rpd3-large (Rpd3L) complex was identified as an enriched complex among the candidate negative regulators of H3K79 methylation of a barcoded reporter gene. Rpd3 is a class I histone deacetylase (HDAC) that removes acetyl groups of histones, as well as numerous non-histone proteins, and is generally associated with transcriptional repression (Yang & Seto, 2008). Several inhibitors of mammalian HDACs have been approved for the treatment of cutaneous T-cell lymphoma and other hematologic malignancies, while others are currently being tested in clinical trials (West & Johnston, 2014). HDAC1 and HDAC2, prominent members of the class I HDACs, are found in the repressive Sin3, NuRD, and CoREST complexes (Yang & Seto, 2008). Loss or inhibition of HDAC1/Rpd3 leads to increased histone acetylation, which in turn can lead to increased expression of target genes and cryptic transcripts (Carrozza et al., 2005; Joshi & Struhl, 2005; Li et al., 2007; Rando & Winston, 2012; Brocks et al., 2017; McDaniel & Strahl, 2017).

Here, we demonstrate that Rpd3 restricts H3K79 methylation at its target genes. Most euchromatic genes in the yeast genome are marked by high levels of H3K79me3. We observed that a subset of the genes that do not follow this pattern has lower H3K79me3 levels due to the action of the Rpd3L complex, which deacetylates its targets and imposes strong transcriptional repression and absence of H2B ubiquitination. Importantly, the Rpd3-Dot1 crosstalk is conserved in mammals: Genetic ablation of Hdac1 in murine thymocytes also leads to an increase in H3K79 methylation in vivo. High H3K79me3 is maintained in the lymphomas these mice develop, and no increase in DOT1L activity by heterozygous deletion of Dot1L reduces tumor burden, an effect that was not observed upon homozygous deletion of Dot1L. Furthermore, DOT1L inhibitors induce apoptosis in Hdac1-deficient but not Hdac1-proficient thymic lymphoma cell lines, suggesting a DOT1L-dose dependence. Taken together, our studies reveal a new, evolutionarily conserved mechanism of H3K79me regulation by Rpd3/HDAC1 with relevance for cancer development.

Results

Identification of the Rpd3L complex as a negative regulator of H3K79 methylation

We recently reported a systematic screening strategy called Epi-ID to identify regulators of H3K79 methylation (Vlaming et al., 2016). In that screen, relative H3K79 methylation (H3K79me) levels at two DNA barcodes (UpTag and DownTag) flanking a reporter gene were measured in a genome-wide library of barcoded deletion mutants, thus testing thousands of genes for H3K79me regulator activity at these loci (Fig 1A). Since higher Dot1 activity in yeast leads to a shift from lower (me1) to higher (me3) methylation states (Frederiks et al., 2008), the H3K79me3 over H3K79me1 ratio was used as a measure for Dot1 activity. A growth-corrected H3K79me score was calculated to account for the effect of growth on H3K79 methylation, and groups of positive and negative candidate regulators were identified (Vlaming et al., 2016). Components of the Rpd3L complex were enriched among candidate negative regulators (10-fold over-representation, \( P = 1.2E-4 \); Vlaming et al., 2016). The histone deacetylase Rpd3 is found in two complexes, the large Rpd3L complex and the small Rpd3S complex, which also share the subunits Sin3 and Ume1 (Carrozza et al., 2005; Keogh et al., 2005). A closer inspection of the Rpd3 complexes revealed that deletion of Rpd3L subunits resulted in an increase in H3K79 methylation on both the UpTag and the DownTag (promoter and terminator context, respectively; Fig 1B), with the exception of two accessory subunits that play peripheral roles (Lenstra et al., 2011). Deletion of the two Rpd3S-specific subunits did not lead to an increase in H3K79me (Fig 1B), which is consistent with Rpd3S binding and acting on coding sequences (Drouin et al., 2010) and thus away from the intergenic barcodes. To validate the effect on a global scale, we performed targeted mass spectrometry analysis to determine the relative levels of the different H3K79me states (me0 to me3) in rpd3A and sin3A strains. On bulk histones, these strains showed an increase in H3K79me (increase in H3K79me3 at the cost of lower methylation states; Fig 1C). The H3K79me increase was not caused by an increase in Dot1 protein (Fig EV1A) or mRNA expression (Kemmeren et al., 2014). Thus, although these regulators were identified using only two 20-base-pair barcodes to read out H3K79me levels at a reporter locus, their effects could be validated globally.

Rpd3 represses H3K79 methylation at the 5' ends of a subset of genes

We next asked at which regions Rpd3 and Sin3 regulate H3K79 methylation in yeast, other than the barcoded reporter gene. To address this, we performed ChIP-seq analysis for H3K79me1, H3K79me3, and H3 in wild-type and rpd3A strains. In addition, we included ChIP-seq for H2B and H2Bub using a site-specific antibody that we recently developed (Van Welsem et al., 2018). First, we considered the patterns in the wild-type strain. Both the coverage at one representative locus and across all genes in a heatmap showed that H3K79me3 is predominately present throughout coding sequences of most genes, where H2Bub is also high, as reported previously (Figs 1D and EV1B; Schulze et al., 2009; Magrainer-Pardo et al., 2014; Weiner et al., 2015; Sadeh et al., 2016). In contrast, H3K79me1 was found in transcribed as well as intergenic regions (Figs 1D and EV1B). This is consistent with published ChIP-seq data and our previous ChIP-qPCR results (Weiner et al., 2015; Vlaming et al., 2016). In agreement with the distributive mechanism of methylation of Dot1 (Frederiks et al., 2008; De Vos et al., 2011), H3K79me1 and H3K79me3 anti-correlated, and H3K79me1 over the gene body was found on the minority of genes that lacked H3K79me3 and H2Bub (Fig 1D). Among these low H3K79me3, high H3K79me1 genes were subtelomeric genes, where the SIR silencing
complex competes with Dot1 for binding to nucleosomes and H2Bub levels are kept low by the deubiquitinating enzyme Ubp10 (Gardner et al., 2005; Emre et al., 2005; Gartenberg & Smith, 2016; Kueng et al., 2013; Fig EV1C and E).

We then compared the patterns in wild-type versus rpd3Δ mutant strains. In metagene plots, the mutant showed a decrease in H3K79me1 and an increase in H3K79me3 just after the transcription start site (TSS; Fig EV1B), suggesting that in this region Rpd3 suppresses the transition from lower to higher H3K79me3 states. To assess whether the changes observed in the metagene plots were explained by a modest effect on H3K79me at all genes or a stronger effect at a subset of genes, we determined the H3-normalized H3K79me3 level in the first 500 bp of each gene and ranked the genes based on the change in H3K79me3 upon loss of Rpd3. A heatmap of H3K79me3 changes by this ranking showed that the absence of Rpd3 leads to an increase in H3K79me3 at a subset of genes (Fig 1F).

Rpd3 represses H3K79me at its target genes

To characterize the genes at which H3K79me is regulated, we calculated the levels of H3K79me1 and H3K79me3 per gene in the same 500-bp window and plotted values in the rank order of H3K79me3 changes described above, using locally weighted regression (Fig 2A; corresponding heatmaps can be found in Fig EV2A). Inspection of these plots revealed that the ORFs on which H3K79me3 was increased in the rpd3Δ mutant showed a simultaneous decrease in H3K79me1 (groups III–IV; Fig 2A). Strikingly, these Rpd3-regulated ORFs were on average marked with a relatively high level of H3K79me1 and low H3K79me3 in the wild-type strains but became more similar to the average yeast gene upon loss of Rpd3, consistent with the presence of a negative regulator of H3K79me3 acting on these ORFs. Next, we compared the genes with H3K79me changes with published data on Rpd3 binding and H4 acetylation (McKnight et al., 2015; Data ref: McKnight et al., 2015). The genes with the strongest increase in H3K79me3 upon Rpd3 loss had the highest Rpd3 occupancy, both at the promoter and in the 500-bp window downstream of the TSS (group IV; Fig 2A). Rpd3 was also found to be active at these genes, since they were devoid of H4 acetylation in wild-type cells and H4 acetylation was restored in the rpd3Δ mutant (Fig 2A). The role of the deacetylase activity of Rpd3 was confirmed by ChIP-qPCR analysis of two previously characterized mutants of Rpd3 that lack catalytic activity (Kadosh & Struhl, 1998; Sun &
While re-expression of wild-type Rpd3 in the rpd3Δ strain restored low H3K79me3 levels at Rpd3 target genes, the Rpd3-H188A and Rpd3-H150A-H151A mutants did not rescue the loss of RPD3 (Fig 2B). Therefore, we conclude that the Rpd3 controls H3K79 methylation via its deacetylase activity. Finally, the top-regulated genes were also enriched for meiotic genes, which are known as Rpd3 targets, and binding sites of Ume6, the Rpd3L subunit known to recruit Rpd3 to early meiotic genes (Fig 2C and D) (Kadosh & Struhl, 1998; Rundlett et al., 1998; Carrozza et al., 2005; Lardenois et al., 2015). Together, our results suggest that the genes at which Rpd3 restricts the buildup of H3K79me are direct targets of Rpd3.

Notably, a small subset of genes loses H3K79me3 in the absence of Rpd3 (Fig 1F, group I in Fig 2A). This group of genes already has targets of Rpd3. The loss of H3K79me3 in the rpd3Δ H188A and Rpd3-H150A-H151A mutants did not rescue the rpd3Δ phenotype. While re-expression of wild-type Rpd3 in the rpd3Δ strain restored low H3K79me3 levels at Rpd3 target genes, the Rpd3-H188A and Rpd3-H150A-H151A mutants did not rescue the loss of RPD3 (Fig 2B). Therefore, we conclude that the Rpd3 controls H3K79 methylation via its deacetylase activity. Finally, the top-regulated genes were also enriched for meiotic genes, which are known as Rpd3 targets, and binding sites of Ume6, the Rpd3L subunit known to recruit Rpd3 to early meiotic genes (Fig 2C and D) (Kadosh & Struhl, 1998; Rundlett et al., 1998; Carrozza et al., 2005; Lardenois et al., 2015). Together, our results suggest that the genes at which Rpd3 restricts the buildup of H3K79me are direct targets of Rpd3.

Notably, a small subset of genes loses H3K79me3 in the absence of Rpd3 (Fig 1F, group I in Fig 2A). This group of genes already has low H3K79me3 levels in wild-type cells and is highly enriched for subtelomeric genes (Fig 2A and E). Loss of Rpd3 is known to enhance Sir-mediated silencing at subtelomeric regions (Ehrentraut et al., 2010, 2011; Gartenberg & Smith, 2016; Thurtle-Schmidt et al., 2016). Our findings show that the stronger transcriptional silencing occurs with a concomitant reduction in H3K79me3 and H2Bub in the coding regions of heterochromatic genes. Whether or not the loss of these modifications contributes to the stronger silencing in rpd3Δ/sin3Δ mutants or is a consequence of it remains to be determined.

**Strong repression of H3K79me by Rpd3 coincides with repression of H2Bub and transcription**

To understand the mechanistic basis for the crosstalk between Rpd3 and Dot1, we looked into other known functions of Rpd3 and other

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**Figure 2.** Rpd3 represses transcription, H2B ubiquitination, and H3K79 methylation at its target sites.

A ChIP-seq and RNA-seq data for genes ranked on H3K79me3/H3 in rpd3Δ/WT, smoothed using locally weighted regression. The gray band around the line shows the 95% confidence interval. Vertical dashed lines separate 4 groups with distinct changes upon RPD3 deletion. ChIP-seq data of H3K79me1, H3K79me3, and H2Bub were generated in this study (plotted is the average coverage in reads per genomic content, RPGC). Rpd3 binding, H4ac, and WT gene expression data were from McKnight et al. (2015), and the relative expression in rpd3Δ/WT was from Kemmeren et al. (2014).

B H3K79me3/H3 ChIP-qPCR efficiencies (relative to a non-transcribed region, which was unaffected by RPD3 deletion) in wild-type and in rpd3Δ cells harboring empty or RPD3-encoding CEN plasmids. The H388A and H150A-H151A mutations have previously been shown to abrogate catalytic activity (Kadosh & Struhl, 1998). Error bars indicate standard deviation of three biological replicates. *P < 0.05, **P < 0.01, and ***P < 0.001 by two-way ANOVA, comparison to wild type.

C–E Gene set enrichment analysis on genes ranked on H3K79me3/H3 in rpd3Δ/WT; all genes have been ranked, and the ranks of the genes in the subsets are indicated by vertical lines. Meiotic (C) and Ume6-bound (D) genes are enriched among the genes at which Rpd3 represses H3K79 methylation, and subtelomeric genes (<30 kb of telomere) (E) are enriched among genes at which H3K79 methylation is decreased in rpd3Δ cells.

Source data are available online for this figure.
known regulators of H3K79me. Given the role of Rpd3 in repressing antisense transcription (Venkatesh et al., 2013; Castelnuovo et al., 2014; Murray et al., 2015), we compared our H3K79me data with data on antisense transcription in wild-type cells (as calculated by Brown et al. (2018) using data from Churchman and Weissman (2011)). This analysis showed that Rpd3 does not specifically affect H3K79 methylation at genes with high or low antisense transcription, which agrees with the notion that Rpd3 represses antisense transcription via the Rpd3S complex (Venkatesh et al., 2013; Castelnuovo et al., 2014; Murray et al., 2015) while it regulates H3K79me via the Rpd3L complex (Fig 1B). We also compared the changes in H3K79 methylation in mutants lacking Rpd3 with changes in H3K79 methylation in mutants lacking INO80 (Data ref: Xue et al., 2015). This chromatin remodeler has been shown to keep transcription and H3K79me3 at intergenic regions at bay (Xue et al., 2015, 2017). Our analysis shows that inactivation of the INO80 complex by deletion of ARP5 does not specifically affect H3K79 methylation at the promoters of genes that are also regulated by Rpd3, although INO80 might affect Rpd3 target genes somewhat more than non-target genes (Fig EV2F). This finding is in agreement with the observation that INO80 affects the majority of genes in the yeast genomes (Xue et al., 2015, 2017) whereas Rpd3 regulates H3K79 methylation at a smaller subset of genes. Finally, we examined the role of H2B ubiquitination in mediating the effect of Rpd3. The expression of the H2Bub machinery is known to be deregulated in Rpd3L mutants (Kemmeren et al., 2014), and no upregulation of H2Bub could be detected by immunoblot (Fig EV1A). Because subtle changes can be missed by blot, we proceeded to generate H2Bub ChIP-seq data in wild-type and mutant strains using an antibody we recently described (Van Welsem et al., 2018). We found that the strongest H3K79me3 repression by Rpd3 (group IV) coincided with repression of H2Bub as well as transcription (Fig 2A; RNA-seq data from McKnight et al. (2015); Data ref: McKnight et al., 2015). Moreover, these genes had below-average H2Bub and transcription levels in wild-type cells (Fig 2A). H2Bub changes were confirmed by ChIP-qPCR (Fig EV2B and C).

The H2B ubiquitination machinery is known to be recruited cotranscriptionally and promote H3K79 methylation, so transcriptional repression provides a likely explanation for the restriction of H3K79 methylation at these genes. However, despite these established causal links, there is no simple linear relation between transcription level and H3K79me3 level, while H2Bub correlates with transcription perfectly (Fig EV2D; Schulze et al., 2009, 2011; Weiner et al., 2015). It appears that other processes counteract H3K79 methylation (see Discussion), especially at highly transcribed genes, but that these processes do not affect the Rpd3-regulated genes as much, since they form a subset of genes at which transcription and H3K79me3, and their changes upon RPD3 deletion, are correlated.

In addition to genes where Rpd3 has a strong effect on H3K79me, we also observed genes at which H3K79 methylation was more modestly affected by the deletion of RPD3 (group III; Fig 2A). Rpd3 is found at the promoters of these genes, but H4 acetylation, transcription, and H2B ubiquitination are not affected (Fig 2A). Although some of the differences may be caused by differences in antibody strength, the results suggest that at these loci, another, still unknown additional mechanism could be at play.

Taken together, we identified Rpd3 as a bona fide negative regulator of H3K79 methylation in yeast that restricts H3K79me3 at its euchromatic targets, probably mostly by repressing target gene transcription and H2Bub, but other mechanisms seem to be involved as well.

**HDAC1 loss increases H3K79me in murine thymocytes**

Having uncovered a role for Rpd3 in restricting H3K79me at its targets and finding that this can explain a significant part of the H3K79 methylation variance between genes in yeast, we next wanted to investigate the biological relevance of this regulation in mammals. Histone deacetylases are conserved between species and can be divided into four classes (Yang & Seto, 2008). Rpd3 is a founding member of the class I HDACs, which in mammals includes HDAC1, HDAC2, HDAC3, and HDAC8. Of these, HDAC1 and HDAC2 are found in Sin3 complexes, like yeast Rpd3 (Yang & Seto, 2008). Given that both HDACs and DOT1L play critical roles in T-cell malignancies, we employed conditional early thymocyte-specific Hdac1 deletion (Lck-Cre;Hdac1<sup>fl/fl</sup>), resulting in Hdac1<sup>−/−</sup> (thymocytes) in the mouse to investigate whether the regulation that we observed in yeast also exists in T cells. We focused on HDAC1 because it is more active in mouse thymocytes than HDAC2 (Dovey et al., 2013; Heideman et al., 2013). First, we measured the relative abundance of H3K79 methylation states on bulk histones by mass spectrometry in wild-type and Hdac1-deleted thymocytes of 3-week-old mice (Fig 3A). In general, the overall levels of H3K79 methylation were much lower than in yeast and H3K79me1 was the most abundant methylation state, followed by H3K79me2, consistent with previous reports in mouse and human cells (Jones et al., 2008; Leroy et al., 2013). As seen in Fig 3A, Hdac1-deleted thymocytes had more H3K79me2 and H3K79me1 and less H3K79me0. Considering the distributive activity of Dot1 enzymes (Fig EV3A), this suggests that Rpd3/HDAC1 is a conserved negative regulator of H3K79 methylation.

**Reduced DOT1L dosage increases the latency of Hdac1-deficient thymic lymphomas**

Conditional Lck-Cre;Hdac1<sup>fl/fl</sup> knock-out mice die of thymic lymphomas characterized by loss of p53 activity and Myc amplification, with a 75% incidence and a 23-week mean latency (Heideman et al., 2013). Oncogenic transformation has not occurred yet in 3-week-old mice (Heideman et al., 2013), the age at which H3K79me levels were determined above. Since Hdac1 deletion in thymocytes resulted in an increase in H3K79 methylation, as well as thymic lymphoma formation, we asked whether increased H3K79 methylation was important for tumor development in this mouse model. To address this question, a conditional Dot1L (Dot1L<sup>−/−</sup>) allele was crossed into the Lck-Cre;Hdac1<sup>fl/fl</sup> line such that deletion of Hdac1 was combined with deletion of zero, one or two Dot1L alleles. Immunohistochemistry confirmed the loss of HDAC1 at the protein level, and mass spectrometry confirmed the loss of DOT1L protein activity for the expected genotypes (Figs 3A and B, and EV3B). H3K79me2 was used as an indicator for DOT1L presence, since none of the DOT1L antibodies tested worked for IHC (antibody difficulties have also been described by Sabra et al., 2013).

Mice with conditional Hdac1 alleles but wild type for Dot1L (Lck-Cre;Hdac1<sup>fl/fl</sup>) developed thymic lymphomas for which they had to be sacrificed, with a median latency of 21 weeks and an incidence of 86% during the 40-week length of the experiment, comparable to...
what was observed before (Fig 3C and D) (Heideman et al., 2013). As expected, Lck-Cre-negative control mice rarely developed thymic lymphomas (1 out of 112). Also, Dot1L deletion alone (Lck-Cre; Dot1L$^{ff}$) rarely led to thymic lymphomas, with a 15% incidence in this background (Fig 3C and D), and no cases of thymic lymphoma in another background (data not shown). We then assessed the effect of Dot1L deletion in the Lck-Cre;Hdac1$^{ff}$ model. Loss of one copy of Dot1L increased survival rate and tumor latency (48% incidence, comparison to Hdac1$^{ff}$ alone $P = 0.002$; Fig 3C and D). This effect suggests that there is a causal link between the increase in H3K79 methylation and the development or maintenance of thymic lymphomas upon Hdac1 deletion. Interestingly, homozygous Dot1L deletion, leading to a complete loss of H3K79me, did not extend the latency of thymic lymphomas (81% incidence, 15.7-week median

Figure 3. Hdac1 deletion increases H3K79 methylation in thymocytes in vivo, and simultaneous heterozygous Dot1L deletion prolongs tumor-free survival.

A Mass spectrometry analysis of H3K79 methylation in thymuses from 3-week-old mice, either wild-type (Cre-) or with deleted Hdac1 or Dot1L alleles, as indicated. Mean and individual data points of biological replicates; H3K79me0 is the predominant state, and the y-axis is truncated at 70% for readability. The remaining H3K79 methylation after homozygous Dot1L deletion is probably due to the presence of some cells in which Cre is not expressed (yet). **$P < 0.01$ and ***$P < 0.001$ by two-way ANOVA, comparison to wild type.

B Representative H&E and immunohistochemical staining on sequential sections of thymic lymphomas of the indicated genotypes. A picture with lower magnification of independent samples is included in Fig EV3B.

C Kaplan–Meier curves of mice harboring thymocytes with indicated genotypes. An event was defined as death or sacrifice of a mouse caused by a thymic lymphoma. Mice that died due to other causes or were still alive and event-free at the end of the experiment were censored. Mice for which the cause of death could not be determined were removed from the data. Wild-type mice were the Cre- littermates of the mice that were used for the other curves.

D Summary of the data presented in panel C. A median latency could only be calculated when the tumor incidence was $> 50\%$. The $P$ value was determined by comparing to the Lck-Cre;Hdac1$^{ff}$ curve with a Peto test, but a logrank test yielded the same conclusions.

Source data are available online for this figure.
latency, comparison to Hdac1 deletion alone \( P = 0.463; \) Fig 3C and D). A possible explanation is that the simultaneous deletion of Dot1L and Hdac1 results in the generation of a different class of tumor that does not depend on H3K79 methylation but has acquired other, possibly epigenetic, events that allow oncogenic transformation. A similar model has been proposed for the loss of Hdac2 in the Lck-Cre:Hdac1\(^{-/-}\) model (Heideman et al., 2013). For the heterozygous Dot1L effect, we consider two possible explanations: The oncogenic transformation occurred later because an additional event was required to overcome the lack of high H3K79me, or tumors grew slower due to lower H3K79me, because of either decreased proliferation or increased apoptosis.

**Hdac1-deficient thymic lymphoma lines depend on DOT1L activity**

To further study the Dot1L dependence of Hdac1-deficient thymic lymphomas in a more controlled environment, we turned to *ex vivo* experiments. Cell lines were derived from Hdac1-deficient thymic lymphomas (Heideman et al., 2013). Since these cell lines had an inactivating mutation in p53, cell lines derived from p53-null thymic lymphomas were used as Hdac1-proficient control lines (Heideman et al., 2013). First, we examined whether Hdac1-deficient tumor cells retained the increased H3K79 methylation levels seen prior to the oncogenic transformation. Both by immunoblot and by targeted mass spectrometry on independent samples (Fig 4A and B), Hdac1-deficient tumor cell lines had more H3K79 methylation than their Hdac1-proficient counterparts. Thus, the effect of HDAC1 on H3K79 methylation observed in *vivo* in 3-week-old pre-malignant thymuses was maintained in the thymic lymphoma cell lines. Importantly, Hdac1-deficient cell lines also possessed high levels of ubiquitinated H2B compared to Hdac1-proficient controls (Fig 4C). High H2Bub is consistent with the increase in H2Bub seen at Rpd3 targets in yeast. Therefore, a plausible model is that at least part of the observed increase in H3K79me upon loss of HDAC1 activity is mediated via Rpd3 targets. To test the DOT1L dependence of the cell lines, DOT1L was depleted using shRNAs in Hdac1-proficient and Hdac1-deficient cell lines. As can be seen in Fig 4D, shRNAs that reduce Dot1L expression (Fig EV4A) affected proliferation of the Hdac1-deficient cell lines. Compared to the control lines, the Hdac1-deficient cell lines were also more sensitive to two different DOT1L inhibitors (Fig 4E). Both inhibitors, EPZ-5676 (Pinometostat) and SGC-0946, effectively lowered H3K79 methylation (Fig EV4B). Thus, shRNA-mediated DOT1L knockdown and chemical DOT1L inhibition showed that the Hdac1-deficient thymic lymphoma cell lines depended on DOT1L activity.

The reduced growth upon inactivation of DOT1L could be explained by a block in proliferation or an increase in cell death. To measure apoptosis induction, the levels of Annexin V and DAPI staining of non-permeabilized cells were determined by flow cytometry. In the DMSO-treated condition, most cells were alive, although Hdac1-deficient lines had a slightly higher basal apoptosis level (Fig 5A and B). This combination of proliferation and apoptosis has also been observed in Hdac1\(^{+/+}\) teratomas (Lagger et al., 2010). However, DOT1L inhibition by 5\(\mu\)M of SGC-0946 dramatically induced apoptosis in Hdac1-deficient cells, whereas no effect on apoptosis was observed in the control cell lines (Fig 5A and B). Thus, DOT1L inhibition induced apoptosis specifically in Hdac1-deficient thymic lymphoma cell lines.
Here, we describe that the yeast HDAC, Rpd3, is a negative regulator of H3K79 methylation that restricts methylation at the 5’ ends of its target genes. Similar to what we observe for Rpd3 in yeast, deleting Hdac1 in murine thymocytes leads to an increase in H3K79 methylation. This regulation is relevant in a cancer context, since heterozygous deletion of Dot1L prolongs the survival of mice that develop Hdac1-deficient thymic lymphomas. Cell lines derived from Hdac1-deficient thymic lymphomas undergo apoptosis upon DOT1L inhibition or depletion, which indicates a form of non-oncogene addiction to DOT1L.

Rpd3 target genes

In the yeast genome, most euchromatic genes are marked by H2Bub and H3K79me3 in their transcribed region. While the levels of H2Bub correlate well with transcription levels, it is evident that H2Bub is not the only determinant of H3K79me3 in yeast because the relation between transcription and H3K79me3 is more complex (Fig 2A). While genes silenced by the SIR complex have low H3K79me3 levels due to active repression mechanisms (Gartenberg & Smith, 2016), the majority of euchromatic genes contain H3K79me3 irrespective of their expression level (Fig EV2D; Schulze et al., 2009, 2011; Weiner et al., 2015). Some genes contain lower H3K79me3 and higher H3K79me1 levels than the average gene, however. A subset of these deviants has been identified as genes undergoing antisense transcription (Murray et al., 2015; Brown et al., 2018), possibly resulting in nucleosome instability and increased histone turnover, which counteracts the build-up of higher H3K79me states but does not affect the more dynamic H2Bub modification (Weiner et al., 2015). Here, we provide insight into the low H3K79me3/high H3K79me1 levels of another subset of yeast genes. H3K79me ChIP-seq in yeast revealed that Rpd3 restricts H3K79me3 at its direct target genes. This subset of genes showed great overlap with the minority of euchromatic genes that is marked with H3K79me1 instead of H3K79me3. Thus, the regulation by Rpd3 provides an explanation for the variation in H3K79 methylation between genes and thereby seems to be an important determinant of the H3K79 methylation pattern. The H3K79me effect of Rpd3 was most notable at the 5’ end of genes, which is in agreement with previous studies on Rpd3 activity. The deacetylation activity of Rpd3/Rpd3L is reported to be strongest in coding sequences, particularly at the 5’ ends (Weinberger et al., 2012). At which genes HDAC1 regulates H3K79 methylation in murine thymocytes is an interesting question, but addressing it is not straightforward. Unlike in yeast, in mammals H3K79 methylation is tightly linked to the transcriptional activity at genes. Processes through which transcription promotes H3K79 methylation are known, but in turn, H3K79 methylation may affect transcription as well (reviewed in Vlaming & Van Leeuwen, 2016). Therefore, when assessing H3K79me changes in Hdac1-deficient cells, it will be challenging to separate direct effects from indirect effects on DOT1L activity through transcriptional changes.

Mechanism of regulation

What could be the mechanism of H3K79me regulation by Rpd3/HDAC1? Until now, the H2B ubiquitination machinery was the only described H3K79me regulator conserved from yeast to mammals (Weake & Workman, 2008). Here, we describe another conserved regulator, Rpd3/HDAC1, and our results indicate that it acts in part
by restricting H2Bub. Although transcription and H3K79 methylation are not clearly positively correlated in wild-type yeast cells (Fig EV2D; Schulze et al., 2011; Magrana-Pardo et al., 2014; Weiner et al., 2015), we observed that the H3K79 methylation changes in rpd3Δ correlate very well with transcriptional changes. These data, together with the well-established causal relationships between transcription and H2B ubiquitination on the one hand and H2B ubiquitination and H3K79 methylation on the other hand, suggest that there is indeed a causal link between (sense) transcription and the placement of H3K79 methylation at a subset of the yeast genome. At higher transcription levels however, this relationship can be obscured by other processes, most likely histone turnover, counteracting the high Dot1 activity (Radman-Livaja et al., 2011; Murray et al., 2015). We have recently identified the conserved histone acetyltransferase Gcn5 as a negative regulator of H3K79me and H2Bub (Vlaming et al., 2016). At first glance, it seems counterintuitive that a HAT and an HDAC have overlapping effects. However, acetylation at non-overlapping histone or non-histone lysines may explain this discrepancy. For example, Gcn5 most likely negatively regulates H2Bub and H3K79me by affecting the deubiquitinating module of the SAGA co-activator complex in which Gcn5 also resides (Vlaming et al., 2016). H2B ubiquitination by human RNF20/40 has also been shown to be regulated by histone acetylation (Garrido Castro et al., 2018). Treatment of acute lymphoblastic leukemia cell lines with the non-selective HDAC inhibitor Panobinostat showed changes in H2Bub, with decreased H2Bub in MLL-r leukemia lines and increased H2Bub in non-MLL-r leukemia lines, suggesting context-dependent mechanisms (Garrido Castro et al., 2018).

Besides H2Bub, other mechanisms are likely to contribute to the observed H3K79me increase in the absence of Rpd3/HDAC1 as well. Histone acetylation is increased in the absence of the deacetylase HDAC1, and histone acetylation has been previously linked to DOT1L recruitment through the YEATS domain transcription elongation proteins AF9 and ENL (Li et al., 2014; Kuntimaddi et al., 2015; Erb et al., 2017; Wan et al., 2017). Very recently, preferential Dot1 binding to acetylated H4K16 has been shown, and the histone acetyltransferase Sas2 was found to be a positive regulator of H3K79 methylation in yeast, probably via acetylation of H4K16 (Lee et al., 2018). Our identification of Rpd3/HDAC1 as a regulator of DOT1L underscores the intimate relationship between histone acetylation and H2Bub and H3K79me and provides evidence for a specific HDAC involved in the crosstalk: HDAC1.

**DOT1L in tumor maintenance**

Loss of HDAC1 leads to oncogenic transformation and higher H3K79me in murine thymocytes. Heterozygous Dot1L deletion prolonged the survival of mice with thymocyte-specific Hdac1 deletion due to a lower incidence and increased latency of thymic lymphomas. Our analysis of Hdac1-deficient thymic lymphoma cells ex vivo provided more insight into the possible mechanisms for the reduced tumor burden. Using DOT1L inhibitors and a knockdown approach, we established that DOT1L was required for survival of the tumor cells by preventing the induction of apoptosis, suggesting that DOT1L is required for tumor maintenance. The DOT1L dependency of the thymic lymphomas resembles that of MLL-rearranged leukemia (Wang et al., 2016) as well as breast and lung cancer cell lines (Kim et al., 2012; Zhang et al., 2014). The full genetic deletion of Dot1l did not reduce tumor burden. The reasons for this are currently unknown and require further study. One possible reason is that some remaining DOT1L activity and H3K79 methylation might be required to induce apoptosis in the tumor cells. We note that in the ex vivo experiments where Dot1l knockdown and DOT1L inhibitors were found to lead to induction of apoptosis; some residual H3K79me was indeed still present. Another possibility is that the simultaneous loss of Hdac1 and Dot1L imposes oncogenic transformation through alternative, epigenetic mechanisms that bypass the apoptotic-prone state. This would be in agreement with the known role of DOT1L in the maintenance of cellular epigenetic states (Onder et al., 2012; Soria-Valles et al., 2015; Breindel et al., 2017). Regardless of possible mechanisms, the finding that HDAC1 affects DOT1L activity in yeast as well as mouse T cells warrants further investigation. For example, it will be interesting to determine whether and under which conditions HDAC1 activity influences DOT1L activity in human MLL-r leukemia and whether the crosstalk is involved in the response of CTLC to HDAC inhibitors in the clinic. Our findings in murine lymphoma add to a growing list of cancers that rely on DOT1L activity, and therefore underline the importance of understanding the regulation of DOT1L.

**Materials and Methods**

**Yeast strains and plasmids**

Yeast strains and plasmids used in this article are listed in Appendix Table S1. Yeast media were described previously (Van Leeuwen & Gottschling, 2002). The generation of the barcoded deletion library used for the Epi-ID experiment was described previously (Vlaming et al., 2016). Yeast rpd3Δ and sin3Δ strains were taken from this library and independent clones were generated by deleting these genes in the barcoded wild-type strain NKI4657, using the NatMX selection marker from pFvL99 (Stulemeijer et al., 2011). Gene deletions were confirmed by PCR. RPD3 expression vectors were derived from YCplac22-RPD3, YCplac22-rpd3_H188A, and YCplac112-rpd3_H150A_H151A (Kadosh & Struhl, 1998). The inserts were released by digestion with SacI and SalI and cloned into the same sites of the single-copy LEU2 vector pRS315 (Brachmann et al., 1998). The mutations were verified by DNA sequencing. Strains harboring pRS315-derived plasmids were grown in synthetic media lacking leucine.

**Cell culture**

Thymic lymphoma cell lines (NKI8996, NKI9002) were derived from Hdac1-deficient thymic lymphomas (Heideman et al., 2013). Since these cell lines had an inactivating mutation in p53, cell lines derived from p53-null thymic lymphomas (NKI8995, NKI8999) were used as Hdac1-proficient control lines (Heideman et al., 2013). Thymic lymphoma cell lines were cultured under standard conditions in RPMI 1640 (Gibco) supplemented with 10% FBS (Sigma–Aldrich), antibiotics, and L-glutamine. The HDAC1 status was confirmed by immunoblot analyses (Fig 4A). HEK 293T cells were cultured in DMEM (Gibco) supplemented with 10% FBS.
the NKI Genomics Core Facility. Reads were mapped to the
first analysis steps are provided in the Appendix Supplementary
Appendix Table S2. Details on the ChIP-seq library preparation and
merged for further analyses. Metagene plots and heatmaps were
biological duplicates were found to be similar, data sets were
100 bp. Samples were depth-normalized, and when data from
reference genome R64-2-1 and extended to
et al
2012; Huber
WT cells was obtained from McKnight
in R by using locally weighted regression (LOESS). Transcription in
lymphoma cell lines were as described in Vlaming
ods. Mass spectrometry measurements on yeast strains and thymic
Measurements on thymus tissue were performed using the method
described in Vlaming et al (2014). All antibodies
viability assay was performed every 24 h. Cell viability was deter-
mixed by a CellTiter-Blue (Promega) assay, measuring conversion
to resorufin after 3 h with the EnVision Multilabel Reader (Perki-
nElmer). All cells treated with a particular shRNA were pooled for
RNA isolation using the RNeasy Mini Kit (Qiagen). DNase I (New
England Biolabs) digestion was performed, and RNA was reverse-
transcribed into cDNA using SuperScript II Reverse Transcriptase
medium was harvested 72 h after transfection. Virus particles were
10^5 concentrated from filtered medium using Amicon 100 kDa spin
Columns. For lentiviral infections, 100,000 cells were seeded in 96-
well tissue culture plates and infected using 7.5 µl concentrated
virus, in the presence of 8 µg/ml polybrene. The medium of infected
cells was replaced with puromycin-containing medium 48 h after
infection and refreshed again 72 h after infection, after which a cell
viability assay was performed every 24 h. Cell viability was deter-
mixed by filter-sterilized (0.22 µm) medium containing the indicated concentration of
inhibitor. Two inhibitors were used: SGC-0946 (Structural Genomics
Consortium) and EPZ-5676 (Pinometostat; Selleck Chemicals). Cell
viability was determined after three days, as described above. Data
were normalized, with the maximum of each cell line to 100% and
background fluorescence set to 0%. GraphPad Prism was used to fit
log(inhibitor) vs normalized response curves with a variable slope.

**Inhibitor treatment**
A total of 100,000 cells were seeded in 96-well tissue culture plates,
in 200 µl culture medium containing the indicated concentration of
inhibitor. Two inhibitors were used: SGC-0946 (Structural Genomics
Consortium) and EPZ-5676 (Pinometostat; Selleck Chemicals). Cell
viability was determined after three days, as described above. Data
were normalized, with the maximum of each cell line to 100% and
background fluorescence set to 0%. GraphPad Prism was used to fit
log(inhibitor) vs normalized response curves with a variable slope.

**Apoptosis FACS**
Cells treated with 0.1% DMSO or 5 µM SGC-0946 were stained with
Annexin V-FITC and DAPI following the protocol of the Annexin V-
FITC Apoptosis Detection Kit (Abcam). Fluorescence was detected
by FACS using the CyAn ADP Analyzer (Beckman Coulter), and data were analyzed using FlowJo software.

**Statistics**

Survival curves were plotted in GraphPad Prism, and Peto mortality-prevalence tests were performed in SAS to compare the curve of Lck-Cre;Hdac1f/f mice with the Lck-Cre;Hdac1f/f;Dot1LWT and Lck-Cre;Hdac1f/f;Dot1L curves. The same conclusions could be drawn based on the standard logrank test in GraphPad Prism. Mass spectrometry and ChIP-qPCR data were compared using two-way ANOVA, comparing samples of all genotypes to the wild-type sample and using the Dunnett’s correction for multiple comparisons, using GraphPad Prism.

**Data availability**

The ChIP-seq data from this publication have been deposited to the GEO database (www.ncbi.nlm.nih.gov/geo/) and assigned the identifier accession number GSE107331.

**Expanded View for this article is available online.**

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**Author contributions**

HV, CMM, and FvL designed the studies and the cell line and mouse studies together with HJ and J-HD. Yeast experiments were performed by HV, TMM, and TtvW; mouse experiments by CMM, HV, SH, EMK-M, MFA, and CL; and cell line experiments by CMM, SP, and HV. TK performed ChIP-seq and genomics analyses, SK made IHC pictures and gave histology advice, and LH and TTS performed mass spectrometry measurements and were advised by AFMA. HV and FvL wrote the manuscript, with help from CMM, HJ, and J-HD.

**Conflict of interest**

The Netherlands Cancer Institute and FvL are entitled to royalties that may result from licensing the yeast H2BK123ub-specific monoclonal antibody according to IP policies of the Netherlands Cancer Institute. The other authors declare that they have no conflict of interest.

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