

1 **Evolutionarily-conserved chromatin crosstalk generates a DOT1L-dose dependency in thymic**  
2 **lymphoma caused by loss of HDAC1**

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25 **Running title:**

26 Negative regulation of DOT1L by Rpd3/HDAC1

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30 DOT1L, Dot1, HDAC1, H3K79, histone methylation, histone acetylation, histone ubiquitination,  
31 lymphoma.

32 **Abstract**

33 DOT1L methylates histone H3K79 and is aberrantly regulated in MLL-rearranged leukemia. Inhibitors  
34 have been developed to target DOT1L activity in leukemia but the cellular mechanisms that regulate  
35 DOT1L are still poorly understood. Here we identify the budding yeast histone deacetylase Rpd3 as a  
36 negative regulator of Dot1. At its target genes, the transcriptional repressor Rpd3 restricts H3K79  
37 methylation, explaining the absence of H3K79me3 at a subset of genes in the yeast genome. Similar to  
38 the crosstalk in yeast, inactivation of the murine Rpd3 homolog HDAC1 in thymocytes led to an  
39 increase in H3K79 methylation. Thymic lymphomas that arise upon genetic deletion of *Hdac1* retained  
40 the increased H3K79 methylation and were sensitive to reduced DOT1L dosage. Furthermore, cell  
41 lines derived from *Hdac1*<sup>Δ/Δ</sup> thymic lymphomas were sensitive to DOT1L inhibitor, which induced  
42 apoptosis. In summary, we identified an evolutionarily-conserved crosstalk between HDAC1 and  
43 DOT1L with impact in murine thymic lymphoma development.

## 44 **Introduction**

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

50 The histone H3K79 methyltransferase DOT1L (KMT4; Dot1 in yeast) is an epigenetic enzyme for which  
51 inhibitors are in clinical development for the treatment of MLL-rearranged (MLL-r) leukemia (Stein and  
52 Tallman 2016). In MLL-r leukemia, DOT1L recruitment to MLL target genes, such as the HoxA cluster  
53 leads to aberrant H3K79 methylation and increased transcription (reviewed in Vlaming and Van  
54 Leeuwen 2016). Although the DOT1L inhibitor Pinometostat (EPZ-5676) has shown promising results  
55 in the lab and is currently in clinical development (Bernt et al. 2011; Daigle et al. 2013; Waters et al.  
56 2015; Stein and Tallman 2016; Stein et al. 2018), the cellular mechanisms and consequences of DOT1L  
57 deregulation are only just being uncovered (Vlaming and Van Leeuwen 2016).

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

69 and provide alternative strategies in case of resistance to treatment with DOT1L inhibitors (Campbell  
70 et al. 2017).

71 In a previous study, we presented a ChIP-barcode-seq screen (Epi-ID) identifying novel regulators of  
72 H3K79 methylation in yeast (Vlaming et al. 2016). The Rpd3-large (Rpd3L) complex was identified as  
73 an enriched complex among the candidate negative regulators of H3K79 methylation of a barcoded  
74 reporter gene. Rpd3 is a class I histone deacetylase (HDAC) that removes acetyl groups of histones, as  
75 well as numerous non-histone proteins, and is generally associated with transcriptional repression  
76 (Yang and Seto 2008). Several inhibitors of mammalian HDACs have been approved for the treatment  
77 of cutaneous T-cell lymphoma and other hematologic malignancies, while others are currently being  
78 tested in clinical trials (West and Johnstone 2014). HDAC1 and HDAC2, prominent members of the  
79 class I HDACs, are found in the repressive Sin3, NuRD, and CoREST complexes (Yang and Seto 2008).  
80 Loss or inhibition of HDAC1/Rpd3 leads to increased histone acetylation, which in turn can lead to  
81 increased expression of target genes and cryptic transcripts (Carrozza et al. 2005; Joshi and Struhl  
82 2005; Li et al. 2007; Rando and Winston 2012; McDaniel and Strahl 2017; Brocks et al. 2017).

83 Here, we demonstrate that Rpd3 restricts H3K79 methylation at its target genes. Most euchromatic  
84 genes in the yeast genome are marked by high levels of H3K79me<sub>3</sub>. We observed that a subset of the  
85 genes that do not follow this pattern has lower H3K79me<sub>3</sub> levels due to the action of the Rpd3L  
86 complex, which deacetylates its targets and imposes strong transcriptional repression and absence of  
87 H2Bub1. Importantly, the Rpd3-Dot1 crosstalk is conserved in mammals: genetic ablation of *Hdac1* in  
88 murine thymocytes also leads to an increase in H3K79 methylation *in vivo*. High H3K79me is  
89 maintained in the lymphomas these mice develop, and a reduction in DOT1L activity by heterozygous  
90 deletion of *Dot1L* reduces tumor burden, an effect that was not observed upon homozygous deletion  
91 of *Dot1L*. Furthermore, DOT1L inhibitors induce apoptosis in *Hdac1*-deficient but not *Hdac1*-proficient  
92 thymic lymphoma cell lines, suggesting a DOT1L-dose dependence. Taken together, our studies reveal  
93 a new, evolutionarily-conserved mechanism of H3K79me regulation by Rpd3/HDAC1 with relevance  
94 for cancer development.

95 **Results**

96

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

98 We recently reported a systematic screening strategy called Epi-ID to identify regulators of H3K79  
99 methylation (Vlaming et al. 2016). In that screen, relative H3K79 methylation (H3K79me) levels at two  
100 DNA barcodes (UpTag and DownTag) flanking a reporter gene were measured in a genome-wide  
101 library of barcoded deletion mutants, thus testing thousands of genes for H3K79me regulator activity  
102 at these loci (Fig. 1A). Since higher Dot1 activity in yeast leads to a shift from lower (me1) to higher  
103 (me3) methylation states (Frederiks et al. 2008), the H3K79me3 over H3K79me1 ratio was used as a  
104 measure for Dot1 activity. A growth-corrected H3K79me score was calculated to account for the effect  
105 of growth on H3K79 methylation and groups of positive and negative candidate regulators were  
106 identified (Vlaming et al. 2016). Components of the Rpd3L complex were enriched among candidate  
107 negative regulators (10-fold over-representation,  $p=1.2E-4$ ) (Vlaming et al. 2016). The histone  
108 deacetylase Rpd3 is found in two complexes, the large Rpd3L complex and the small Rpd3S complex,  
109 which also share the subunits Sin3 and Ume1 (Carrozza et al. 2005; Keogh et al. 2005). A closer  
110 inspection of the Rpd3 complexes revealed that deletion of Rpd3L subunits resulted in an increase in  
111 H3K79 methylation on both the UpTag and DownTag (promoter and terminator context, respectively;  
112 Fig. 1B), with the exception of two accessory subunits that play peripheral roles (Lenstra et al. 2011).  
113 Deletion of the two Rpd3S-specific subunits did not lead to an increase in H3K79me (Fig. 1B), which is  
114 consistent with Rpd3S binding and acting on coding sequences (Drouin et al. 2010) and thus away  
115 from the intergenic barcodes. To validate the effect on a global scale, we performed targeted mass  
116 spectrometry analysis to determine the relative levels of the different H3K79me states (me0 to me3)  
117 in *rpd3Δ* and *sin3Δ* strains. On bulk histones, these strains showed an increase in H3K79me (increase  
118 in H3K79me3 at the cost of lower methylation states; Fig. 1C). The H3K79me increase was not caused  
119 by an increase in Dot1 protein (Supplemental Fig. 1A) or mRNA expression (Kemmeren et al. 2014).

120 Thus, although these regulators were identified using only two 20-base-pair barcodes to read out  
121 H3K79me levels at a reporter locus, their effects could be validated globally.

122

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

124 We next asked at which regions Rpd3 and Sin3 regulate H3K79 methylation in yeast, other than the  
125 barcoded reporter gene. To address this, we performed ChIP-seq analysis for H3K79me1, H3K79me3,  
126 and H3 in wild-type and *rpd3Δ* strains. In addition, we included ChIP-seq for H2B and H2Bub using a  
127 site-specific antibody that we recently developed (Van Welsem et al. 2018). First, we considered the  
128 patterns in the wild-type strain. Both the coverage at one representative locus and across all genes in  
129 a heatmap showed that H3K79me3 is predominantly present throughout coding sequences of most  
130 genes, where H2Bub is also high, as reported previously (Fig. 1D, 1E, Supplemental Fig. 1B; Schulze et  
131 al. 2009; Weiner et al. 2015; Sadeh et al. 2016; Magraner-Pardo et al. 2014). In contrast, H3K79me1  
132 was found in transcribed as well as intergenic regions (Fig. 1D, Supplemental Fig. 1B). This is consistent  
133 with published ChIP-seq data and our previous ChIP-qPCR results (Weiner et al. 2015; Vlaming et al.  
134 2016). In agreement with the distributive mechanism of methylation of Dot1 (Frederiks et al. 2008; De  
135 Vos et al. 2011), H3K79me1 and H3K79me3 anti-correlated, and H3K79me1 over the gene body was  
136 found on the minority of genes that lacked H3K79me3 and H2Bub (Fig. 1D). Among these low  
137 H3K79me3, high H3K79me1 genes were subtelomeric genes, where the Sir silencing complex  
138 competes with Dot1 for binding to nucleosomes and H2Bub levels are kept low by the  
139 deubiquitinating enzyme Ubp10 (Gardner et al. 2005; Emre et al. 2005; Gartenberg and Smith 2016;  
140 Kueng et al. 2013) (Supplemental Fig. 1C, 1E).

141 We then compared the patterns in wild-type versus *rpd3Δ* mutant strains. In metagene plots, the  
142 mutant showed a decrease in H3K79me1 and an increase in H3K79me3 just after the transcription  
143 start site (TSS; Supplemental Fig. 1B), suggesting that in this region Rpd3 suppresses the transition  
144 from lower to higher H3K79me states. To assess whether the changes observed in the metagene plots  
145 were explained by a modest effect on H3K79me at all genes or a stronger effect at a subset of genes,

146 we determined the H3-normalized H3K79me3 level in the first 500 bp of each gene and ranked the  
147 genes based on the change in H3K79me3 upon loss of Rpd3. A heatmap of H3K79me3 by this ranking  
148 showed that the absence of Rpd3 leads to an increase of H3K79me3 at a subset of genes (Fig. 1F).

149

#### 150 Rpd3 represses H3K79me at its target genes

151 To characterize the genes at which H3K79me is regulated, we calculated the levels of H3K79me1 and  
152 H3K79me3 per gene in the same 500 bp window and plotted values in the rank order of H3K79me3  
153 changes described above, using locally weighed regression (Fig. 2A; corresponding heatmaps can be  
154 found in Supplemental Fig. 2A). Inspection of these plots revealed that the ORFs on which H3K79me3  
155 was increased in the *rpd3Δ* mutant showed a simultaneous decrease in H3K79me1 (groups III-IV; Fig.  
156 2A). Strikingly, these Rpd3-regulated ORFs were on average marked with a relatively high level of  
157 H3K79me1 and low H3K79me3 in the wild-type strains but became more similar to the average yeast  
158 gene upon loss of Rpd3, consistent with the presence of a negative regulator of H3K79me acting on  
159 these ORFs. To test whether H3K79me-regulated genes were direct Rpd3 targets or indirectly  
160 affected, we assessed the relation between H3K79me changes and published data on Rpd3 binding  
161 and H4 acetylation (McKnight et al. 2015). The genes with the strongest increase in H3K79me3 upon  
162 Rpd3 loss had the highest Rpd3 occupancy, both at the promoter and in the 500 bp window  
163 downstream of the TSS (group IV; Fig. 2A). Rpd3 was also found to be active at these genes, since they  
164 were devoid of H4 acetylation in wild-type cells and H4 acetylation was restored in the *rpd3Δ* mutant  
165 (Fig. 2A). Finally, the top-regulated genes were also enriched for meiotic genes, which are known Rpd3  
166 targets, and binding sites of Ume6, the Rpd3L subunit known to recruit Rpd3 to early meiotic genes  
167 (Fig. 2B, 2C) (Rundlett et al. 1998; Kadosh and Struhl 1998; Carrozza et al. 2005; Lardenois et al. 2015).  
168 Together, our results suggest that the genes at which Rpd3 restricts the build-up of H3K79me are  
169 direct targets of Rpd3.

170 Notably, a small subset of genes loses H3K79me3 in the absence of Rpd3 (Fig. 1F, group I in Fig. 2A).

171 This group of genes already has low H3K79me3 and H2Bub levels in wild-type cells and is highly

172 enriched for subtelomeric genes (Fig. 2A, 2D). Loss of Rpd3 is known to enhance Sir-mediated  
173 silencing at subtelomeric regions (Ehrentraut et al. 2010, 2011; Gartenberg and Smith 2016; Thurtle-  
174 Schmidt et al. 2016). Our findings show that the stronger transcriptional silencing occurs with a  
175 concomitant reduction in H3K79me3 and H2Bub in the coding regions of heterochromatic genes.  
176 Whether or not the loss of these modifications contributes to the stronger silencing in *rpd3Δ/sin3Δ*  
177 mutants or is a consequence of it remains to be determined.

178

#### 179 Strong repression of H3K79me by Rpd3 coincides with repression of H2Bub and transcription

180 To understand the mechanistic basis for the crosstalk between Rpd3 and Dot1, we examined the  
181 changes in H2B ubiquitination and transcription. The expression of the H2Bub machinery is not  
182 deregulated in these mutants (Kemmeren et al. 2014) and no upregulation of H2Bub could be  
183 detected by immunoblot (Supplemental Fig. 1A). Because subtle changes can be missed by blot, we  
184 proceeded to generate H2Bub ChIP-seq data in wild-type and mutant strains using an antibody we  
185 recently described (Van Welsem et al. 2018). We found that the strongest H3K79me3 repression by  
186 Rpd3 (group IV) coincided with repression of H2Bub as well as transcription (Fig. 2A; RNA-seq data  
187 from (McKnight et al. 2015)). Moreover, these genes had below-average H2Bub and transcription  
188 levels in wild-type cells (Fig. 2A). H2Bub changes were confirmed by ChIP-qPCR (Supplemental Fig. 2B,  
189 2C).

190 The H2B ubiquitination machinery is known to be recruited co-transcriptionally and promote H3K79  
191 methylation, so transcriptional repression provides a likely explanation for the restriction of H3K79  
192 methylation at these genes. However, despite these established causal links, there is no simple linear  
193 relation between transcription level and H3K79me3 level, while H2Bub correlates with transcription  
194 perfectly (Supplemental Fig. 2D; Schulze et al. 2009, 2011; Weiner et al. 2015). It appears that other  
195 processes counteract H3K79 methylation (see discussion), especially at highly transcribed genes, but  
196 that these processes do not affect the Rpd3-regulated genes as much, since they form a subset of  
197 genes at which transcription and H3K79me3, and their changes upon *RPD3* deletion, are correlated.



198 In addition to genes where Rpd3 has a strong effect on H3K79me, we also observed genes at which  
199 H3K79 methylation was more modestly affected by the deletion of *RPD3* (group III; Fig. 2A). Rpd3 is  
200 found at the promoters of these genes, but H4 acetylation, transcription, and H2B ubiquitination are  
201 not affected. Therefore, at these loci another, still unknown additional mechanism could be at play.

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

205

#### 206 HDAC1 loss increases H3K79me in murine thymocytes

207 Having uncovered a role for Rpd3 in restricting H3K79me at its targets and finding that this can explain  
208 a significant part of the H3K79 methylation variance between genes in yeast, we next wanted to  
209 investigate the biological relevance of this regulation in mammals. Histone deacetylases are conserved  
210 between species and can be divided into four classes (Yang and Seto 2008). Rpd3 is a founding  
211 member of the class I HDACs, which in mammals includes HDAC1, -2, -3 and -8. Of these, HDAC1 and -  
212 2 are found in Sin3 complexes, like yeast Rpd3 (Yang and Seto 2008). Given that both HDACs and  
213 DOT1L play critical roles in T-cell malignancies, we employed conditional early thymocyte-specific  
214 *Hdac1* deletion (*Lck-Cre;Hdac1<sup>f/f</sup>*, resulting in *Hdac1<sup>Δ/Δ</sup>* thymocytes) in the mouse to investigate  
215 whether the regulation that we observed in yeast also exists in T cells. We focused on HDAC1 because  
216 it is more active in mouse thymocytes than HDAC2 (Heideman et al. 2013; Dovey et al. 2013). First, we  
217 measured the relative abundance of H3K79 methylation states on bulk histones by mass spectrometry  
218 in wild-type and *Hdac1*-deleted thymocytes of 3-week-old mice (Fig. 3A). In general, the overall levels  
219 of H3K79 methylation were much lower than in yeast and H3K79me1 was the most abundant  
220 methylation state, followed by H3K79me2, consistent with previous reports in mouse and human cells  
221 (Jones et al. 2008; Leroy et al. 2013). As seen in Figure 3A, *Hdac1*-deleted thymocytes had more  
222 H3K79me2 and H3K79me1 and less H3K79me0. Considering the distributive activity of Dot1 enzymes

223 (Supplemental Fig. 3A), this suggests that Rpd3/HDAC1 is a conserved negative regulator of H3K79  
224 methylation.

225

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

227 Conditional *Lck-Cre;Hdac1<sup>ff</sup>* knock-out mice die of thymic lymphomas characterized by loss of p53  
228 activity and *Myc* amplification, with a 75% incidence and a 23-week mean latency (Heideman et al.  
229 2013). Oncogenic transformation has not occurred yet in 3-week-old mice (Heideman et al. 2013), the  
230 age at which H3K79me levels were determined above. Since *Hdac1* deletion in thymocytes resulted in  
231 an increase in H3K79 methylation, as well as thymic lymphoma formation, we asked whether  
232 increased H3K79 methylation was important for tumor development in this mouse model. To address  
233 this question, a conditional *Dot1L* (*Dot1L<sup>ff</sup>*) allele was crossed into the *Lck-Cre;Hdac1<sup>ff</sup>* line such that  
234 deletion of *Hdac1* was combined with deletion of zero, one or two *Dot1L* alleles.  
235 Immunohistochemistry confirmed the loss of HDAC1 at the protein level and mass spectrometry  
236 confirmed the loss of DOT1L protein activity for the expected genotypes (Fig. 3A, B, Supplemental Fig.  
237 3B). H3K79me<sub>2</sub> was used as an indicator for DOT1L presence, since none of the DOT1L antibodies we  
238 tested worked for IHC (antibody difficulties have also been described by Sabra et al. 2013).

239 Mice with conditional *Hdac1* alleles but wild-type for *Dot1L* (*Lck-Cre;Hdac1<sup>ff</sup>*) developed thymic  
240 lymphomas for which they had to be sacrificed, with a median latency of 21 weeks and an incidence of  
241 86% during the 40-week length of the experiment, comparable to what was observed before (Fig. 3C,  
242 D) (Heideman et al. 2013). As expected, *Lck-Cre*-negative control mice rarely developed thymic  
243 lymphomas (1 out of 112). Also, *Dot1L* deletion alone (*Lck-Cre;Dot1L<sup>ff</sup>*) rarely led to thymic  
244 lymphomas, with a 15% incidence in this background (Fig. 3C, 3D), and no cases of thymic lymphoma  
245 in another background (data not shown). We then assessed the effect of *Dot1L* deletion in the *Lck-*  
246 *Cre;Hdac1<sup>ff</sup>* model. Loss of one copy of *Dot1L* increased survival rate and tumor latency (48%  
247 incidence, comparison to *Hdac1<sup>ff</sup>* alone p=0.002; Fig. 3C, D). This effect suggests that there is a causal  
248 link between the increase in H3K79 methylation and the development or maintenance of thymic

249 lymphomas upon *Hdac1* deletion. Interestingly, homozygous *Dot1L* deletion, leading to a complete  
250 loss of H3K79me, did not extend the latency of thymic lymphomas (81% incidence, 15.7 weeks median  
251 latency, comparison to *Hdac1* deletion alone  $p=0.463$ ; Fig. 3C, D). A possible explanation is that the  
252 simultaneous deletion of *Dot1L* and *Hdac1* results in the generation of a different class of tumor that  
253 does not depend on H3K79 methylation but has acquired other, possibly epigenetic, events that allow  
254 oncogenic transformation. A similar model has been proposed for the loss of *Hdac2* in the *Lck-*  
255 *Cre;Hdac1<sup>f/f</sup>* model (Heideman et al. 2013). For the heterozygous *Dot1L* effect, we consider two  
256 possible explanations: the oncogenic transformation occurred later because an additional event was  
257 required to overcome the lack of high H3K79me, or tumors grew slower due to lower H3K79me,  
258 either because of decreased proliferation or increased apoptosis.

259

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

261 To further study the *Dot1L* dependence of *Hdac1*-deficient thymic lymphomas in a more controlled  
262 environment, we turned to *ex vivo* experiments. Cell lines were derived from *Hdac1*-deficient thymic  
263 lymphomas (Heideman et al. 2013). Since these cell lines had an inactivating mutation in p53, cell lines  
264 derived from p53-null thymic lymphomas were used as *Hdac1*-proficient control lines (Heideman et al.  
265 2013). First, we examined whether *Hdac1*-deficient tumor cells retained the increased H3K79  
266 methylation levels seen prior to the oncogenic transformation. Both by immunoblot and by targeted  
267 mass spectrometry on independent samples (Fig. 4A, 4B), *Hdac1*-deficient tumor cell lines had more  
268 H3K79 methylation than their *Hdac1*-proficient counterparts. Thus, the effect of HDAC1 on H3K79  
269 methylation observed *in vivo* in 3-week old pre-malignant thymuses was maintained in the thymic  
270 lymphoma cell lines. Importantly, *Hdac1*-deficient cell lines also possessed high levels of ubiquitinated  
271 H2B compared to *Hdac1*-proficient controls (Fig. 4C). High H2Bub is consistent with the increase in  
272 H2Bub seen at Rpd3 targets in yeast. Therefore, a plausible model is that at least part of the observed  
273 increase in H3K79me upon loss of HDAC1 activity is mediated via H2Bub, consistent with what we  
274 observed in yeast. However, contributions from other regulatory mechanisms cannot be excluded (see

275 discussion). To test the DOT1L dependence of the cell lines, DOT1L was depleted using shRNAs in  
276 *Hdac1*-proficient and -deficient cell lines. As can be seen in Figure 4D, shRNAs that reduce Dot1L  
277 expression (Supplemental Fig. 4A) affected proliferation of the *Hdac1*-deficient cell lines. Compared to  
278 the control lines, the *Hdac1*-deficient cell lines were also more sensitive to two different DOT1L  
279 inhibitors (Fig. 4E). Both inhibitors, EPZ-5676 (Pinometostat) and SGC-0946, effectively lowered H3K79  
280 methylation (Supplemental Figure 4B). Thus, shRNA-mediated DOT1L knockdown and chemical DOT1L  
281 inhibition showed that the *Hdac1*-deficient thymic lymphoma cell lines depended on DOT1L activity.  
282 The reduced growth upon inactivation of DOT1L could be explained by a block in proliferation or an  
283 increase in cell death. To measure apoptosis induction, the levels of AnnexinV and DAPI staining of  
284 non-permeabilized cells were determined by flow cytometry. In the DMSO-treated condition most  
285 cells were alive, although *Hdac1*-deficient lines had a slightly higher basal apoptosis level (Fig. 5A,B).  
286 This combination of proliferation and apoptosis has also been observed in *Hdac1*<sup>Δ/Δ</sup> teratomas (Lagger  
287 et al. 2010). However, DOT1L inhibition by 5μM of SGC-0946 dramatically induced apoptosis in *Hdac1*-  
288 deficient cells, whereas no effect on apoptosis was observed in the control cell lines (Fig. 5A,B). Thus,  
289 DOT1L inhibition induced apoptosis specifically in *Hdac1*-deficient thymic lymphoma cell lines.

290 **Discussion**

291

292 Here we describe that the yeast HDAC, Rpd3, is a negative regulator of H3K79 methylation that  
293 restricts methylation at the 5' ends of its target genes. Similar to what we observe for Rpd3 in yeast,  
294 deleting *Hdac1* in murine thymocytes leads to an increase in H3K79 methylation. This regulation is  
295 relevant in a cancer context, since heterozygous deletion of *Dot1L* prolongs the survival of mice that  
296 develop *Hdac1*-deficient thymic lymphomas. Cell lines derived from *Hdac1*-deficient thymic  
297 lymphomas undergo apoptosis upon DOT1L inhibition or depletion, which indicates a form of non-  
298 oncogene addiction to DOT1L.

299

300 Rpd3 target genes

301 In the yeast genome, most euchromatic genes are marked by H2Bub and H3K79me3 in their  
302 transcribed region. While the levels of H2Bub correlate well with transcription levels, it is evident that  
303 H2Bub is not the only determinant of H3K79me3 in yeast because the relation between transcription  
304 and H3K79me3 is more complex (Fig. 2A). While genes silenced by the SIR complex have low  
305 H3K79me3 levels due to active repression mechanisms (Gartenberg and Smith 2016), the majority of  
306 euchromatic genes contain H3K79me3 irrespective of their expression level (Supplemental Fig. 2D;  
307 Schulze et al. 2009, 2011; Weiner et al. 2015). Some genes contain lower H3K79me3 and higher  
308 H3K79me1 levels than the average gene, however. A subset of these deviants has been identified as  
309 genes undergoing antisense transcription (Murray et al. 2015; Brown et al. 2018), possibly resulting in  
310 nucleosome instability and increased histone turnover, which counteracts the buildup of higher  
311 H3K79me states but does not affect the more dynamic H2Bub modification (Weiner et al. 2015). Here  
312 we provide insight into the low H3K79me3/high H3K79me1 levels of another subset of yeast genes.  
313 H3K79me CHIP-seq in yeast revealed that Rpd3 restricts H3K79me3 at its direct target genes. This  
314 subset of genes showed great overlap with the minority of euchromatic genes that is marked with  
315 H3K79me1 instead of H3K79me3. Thus, the regulation by Rpd3 provides an explanation for the

316 variation in H3K79 methylation between genes and thereby seems to be an important determinant of  
317 the H3K79 methylation pattern. The H3K79me effect of Rpd3 was most notable at the 5' end of genes,  
318 which is in agreement with previous studies on Rpd3 activity. The deacetylation activity of  
319 Rpd3/Rpd3L is reported to be strongest in coding sequences, particularly at the 5' ends (Weinberger  
320 et al. 2012). At which genes HDAC1 regulates H3K79 methylation in murine thymocytes is an  
321 interesting question, but addressing it is not straight-forward. Unlike in yeast, in mammals H3K79  
322 methylation is tightly linked to the transcriptional activity at genes. Processes through which  
323 transcription promotes H3K79 methylation are known, but in turn, H3K79 methylation may affect  
324 transcription as well (reviewed in Vlaming and Van Leeuwen 2016). Therefore, while assessing  
325 H3K79me changes in *Hdac1*-deficient cells, it will be challenging to separate direct effects from  
326 indirect effects on DOT1L activity through transcriptional changes.

327

### 328 Mechanism of regulation

329 What could be the mechanism of H3K79me regulation by Rpd3/HDAC1? Until now, the H2B  
330 ubiquitination machinery was the only described H3K79me regulator conserved from yeast to  
331 mammals (Weake and Workman 2008). Here we describe another conserved regulator, Rpd3/HDAC1,  
332 and our results indicate that it acts in part by restricting H2Bub. Although transcription and H3K79  
333 methylation are not clearly positively correlated in wild-type yeast cells (Supplemental Fig. 2D; Weiner  
334 et al. 2015; Magraner-Pardo et al. 2014; Schulze et al. 2011), we observed that the H3K79 methylation  
335 changes in *rpd3Δ* correlate very well with transcriptional changes. These data, together with the well-  
336 established causal relationships between transcription and H2B ubiquitination on the one hand and  
337 H2B ubiquitination and H3K79 methylation on the other hand, suggest that there is indeed a causal  
338 link between (sense) transcription and the placement of H3K79 methylation at a subset of the yeast  
339 genome. At higher transcription levels however, this relationship can be obscured by other processes,  
340 most likely histone turnover, counteracting the high Dot1 activity (Radman-Livaja et al. 2011; Murray  
341 et al. 2015). We have recently identified the conserved histone acetyltransferase Gcn5 as a negative

342 regulator of H3K79me and H2Bub (Vlaming et al. 2016). At first glance, it seems counterintuitive that a  
343 HAT and an HDAC have overlapping effects. However, acetylation at non-overlapping histone or non-  
344 histone lysines may explain this discrepancy. For example, Gcn5 most likely negatively regulates  
345 H2Bub and H3K79me by affecting the deubiquitinating module of the SAGA co-activator complex in  
346 which Gcn5 also resides (Vlaming et al. 2016). H2B ubiquitination by human RNF20/40 has also been  
347 shown to be regulated by histone acetylation (Garrido Castro et al. 2018). Treatment of acute  
348 lymphoblastic leukemia cell lines with the non-selective HDAC inhibitor Panobinostat showed changes  
349 in H2Bub, with decreased H2Bub in MLL-r leukemia lines and increased H2Bub in non MLLr-leukemia  
350 lines, suggesting context-dependent mechanisms (Garrido Castro et al. 2018).

351 Besides H2Bub, other mechanisms are likely to contribute to the observed H3K79me increase in the  
352 absence of Rpd3/HDAC1 as well. Histone acetylation is increased in the absence of the deacetylase  
353 HDAC1, and histone acetylation has been previously linked to DOT1L recruitment through the YEATS  
354 domain transcription elongation proteins AF9 and ENL (Li et al. 2014; Kuntimaddi et al. 2015; Erb et al.  
355 2017; Wan et al. 2017). Very recently, preferential Dot1 binding to acetylated H4K16 has been shown,  
356 and the histone acetyltransferase Sas2 was found to be a positive regulator of H3K79 methylation in  
357 yeast, probably via acetylation of H4K16 (Lee et al. 2018). Our identification of Rpd3/HDAC1 as a  
358 regulator of DOT1L underscores the intimate relationship between histone acetylation and H2Bub and  
359 H3K79me and provides evidence for a specific HDAC involved in the crosstalk: HDAC1.

360

### 361 DOT1L in tumor maintenance

362 Loss of HDAC1 leads to oncogenic transformation and higher H3K79me in murine thymocytes.  
363 Heterozygous *Dot1L* deletion prolonged the survival of mice with thymocyte-specific *Hdac1* deletion  
364 due to a lower incidence and increased latency of thymic lymphomas. Our analysis of *Hdac1*-deficient  
365 thymic lymphoma cells *ex vivo* provided more insight into the possible mechanisms for the reduced  
366 tumor burden. Using DOT1L inhibitors and a knock-down approach, we established that DOT1L was  
367 required for survival of the tumor cells by preventing the induction of apoptosis, suggesting that

368 DOT1L is required for tumor maintenance. The DOT1L dependency of the thymic lymphomas  
369 resembles that of MLL-rearranged leukemia (Wang et al. 2016) as well as breast and lung cancer cell  
370 lines (Kim et al. 2012; Zhang et al. 2014). The full genetic deletion of *Dot1L* did not reduce tumor  
371 burden. The reasons for this are currently unknown and require further study. One possible reason is  
372 that some remaining DOT1L activity and H3K79 methylation might be required to induce apoptosis in  
373 the tumor cells. We note that in the *ex vivo* experiments where *Dot1L* knock down and DOT1L  
374 inhibitors were found to lead to induction of apoptosis, some residual H3K79me was indeed still  
375 present. Another possibility is that the simultaneous loss of *Hdac1* and *Dot1L* imposes oncogenic  
376 transformation through alternative, epigenetic mechanisms that bypass the apoptotic-prone state.  
377 This would be in agreement with the known role of DOT1L in the maintenance of cellular epigenetic  
378 states (Onder et al. 2012; Soria-Valles et al. 2015; Breindel et al. 2017). Regardless of possible  
379 mechanisms, the finding that HDAC1 affects DOT1L activity in yeast as well as mouse T cells, warrants  
380 further investigation. For example, it will be interesting to determine whether and under which  
381 conditions HDAC1 activity influences DOT1L activity in human MLL-r leukemia and whether the  
382 crosstalk is involved in the response of CTLC to HDAC inhibitors in the clinic. Our findings in murine  
383 lymphoma add to a growing list of cancers that rely on DOT1L activity, and therefore underline the  
384 importance of understanding the regulation of DOT1L.



## 385 **Materials and methods**

386

### 387 Yeast strains

388 Yeast strains used in this article are listed in Supplemental Table 1. Yeast media were described  
389 previously (Van Leeuwen and Gottschling 2002). The generation of the barcoded deletion library used  
390 for the Epi-ID experiment was described previously (Vlaming et al. 2016). Yeast *RPD3Δ* and *SIN3Δ*  
391 strains were taken from this library and independent clones were generated by deleting these genes  
392 in the barcoded wild-type strain NKI4657, using the NatMX selection marker from pFvL99 (Stulemeijer  
393 et al. 2011). Gene deletions were confirmed by PCR.

394

### 395 Cell culture

396 Thymic lymphoma cell lines (Heideman et al. 2013) were cultured under standard conditions in RPMI  
397 1640 (Gibco) supplemented with 10% FBS (Sigma-Aldrich), antibiotics and L-Glutamine. HEK 293T cells  
398 were cultured in DMEM (Gibco) supplemented with 10% FBS (Sigma-Aldrich) and L-Glutamine.

399

### 400 Mouse survival analysis

401 The generation and crosses of the conditional knock-out mice is described in the Supplemental  
402 Methods. Mice were monitored over time, up to 40 weeks of age. A power analysis performed  
403 beforehand determined group sizes of 20-25 mice per genotype. Mice were sacrificed before 40  
404 weeks when they displayed breathing issues caused by the thymic lymphoma, or serious discomfort  
405 unrelated to tumor formation. After death or sacrifice, mice were checked for the presence of a  
406 thymic lymphoma. Mice that died without a lymphoma were censored in the survival curves. Mice of  
407 which the cause of death could not be determined were left out of the survival curve, together with  
408 their littermates. All experiments were approved by a local ethical committee and performed  
409 according to national guidelines. Mice were housed under standard conditions of feeding, light and  
410 temperature with free access to food and water.

411 Protein lysates, immunoblots and mass spectrometry

412 Yeast whole-cell extracts were made as described previously (Vlaming et al. 2014). Nuclear extracts  
413 were prepared of murine cells, see supplemental methods for details. The immunoblotting procedure  
414 was as described in (Vlaming et al. 2014). All antibodies used in this study are listed in the  
415 supplemental methods. Mass spectrometry measurements on yeast strains and thymic lymphoma cell  
416 lines were as described in (Vlaming et al. 2014). Measurements on thymus tissue were performed  
417 using the method described in (Vlaming et al. 2016).

418

419 ChIP-sequencing and ChIP-qPCR

420 ChIP and ChIP-qPCR experiments were performed as described before (Vlaming et al. 2016). Primers  
421 for qPCR are shown in Supplemental Table 2. Details on the ChIP-seq library preparation and the first  
422 analysis steps are provided in the Supplemental Methods. In short, preparation and sequencing were  
423 performed by the NKI Genomics Core Facility. Reads were mapped to the *Saccharomyces cerevisiae*  
424 reference genome R64-2-1 and extended to 150 bp. Samples were depth-normalized, and when data  
425 from biological duplicates was found to be similar, data sets were merged for further analyses.  
426 Metagene plots and heatmaps were generated with custom scripts in R/Bioconductor (Cherry et al.  
427 2012; Huber et al. 2015). Reads were aligned in a window of -500 to 1 kb around the TSS of each  
428 verified ORF recorded in SGD. Genes that contained a coverage of 0 or an average coverage in the first  
429 500 bp below 0.5 were filtered out (leaving 5006 out of 5134 genes). For heatmaps, the coverage was  
430 grouped in bins of 10 bp. The plots in Figure 2A and Supplemental Fig. 2D were created with custom  
431 scripts in R by using locally weighed regression (LOESS). Transcription in WT cells was obtained from  
432 (McKnight et al. 2015), and transcription in *rpd3Δ*/WT from (Kemmeren et al. 2014). Gene set  
433 enrichment plots were created with the barcodeplot function from the limma package (Ritchie et al.  
434 2015). Ume6 targets were obtained from the Yeabstract database (Teixeira et al. 2014) and filtered for  
435 simultaneous DNA binding and expression evidence. The list of meiosis factors was generated by  
436 searching for genes with a GO term containing "meio" or children thereof. The distance of each gene

437 from the telomere on the same chromosome arm was calculated manually by using genome feature  
438 information from SGD.

439

#### 440 Histology/immunohistochemistry

441 Tissues were fixed in EAF (ethanol, acetic acid, formol saline) for 24 hours and subsequently  
442 embedded in paraffin. Slides were stained with hematoxylin and eosin (H&E), or  
443 immunohistochemistry was performed as described (Heideman et al. 2013).

444

#### 445 Knockdown/viability assays and Dot1L mRNA analysis

446 To produce lentiviral particles, HEK 293T cells were co-transfected with shRNA-containing pLKO.1 and  
447 three packaging plasmids containing Gag and Pol, Rev and VSV-G, respectively, using PEI. The media  
448 was replaced after 16 hours and virus-containing medium was harvested 72 hours after transfection.  
449 Virus particles were 10x concentrated from filtered medium using AmiCon 100 kDa spin columns. For  
450 lentiviral infections, 100,000 cells were seeded in 96-well tissue culture plates and infected using 7.5  
451  $\mu$ L concentrated virus, in the presence of 8  $\mu$ g/ml Polybrene. The medium of infected cells was  
452 replaced with puromycin-containing medium 48 hours after infection and refreshed again 72 hours  
453 after infection, after which a cell viability assay was performed every 24 hours. Cell viability was  
454 determined by a CellTiter-Blue (Promega) assay, measuring conversion to resorufin after three hours  
455 with the EnVision Multilabel Reader (PerkinElmer). All cells treated with a particular shRNA were  
456 pooled for RNA isolation using the RNeasy Mini kit (Qiagen). DNaseI (New England Biolabs) digestion  
457 was performed and RNA was reverse transcribed into cDNA using SuperScript II Reverse Transcriptase  
458 (Invitrogen) according to the manufacturer's protocol. *Dot1L* transcript abundance was measured by  
459 qPCR using SYBRgreen master mix (Roche) and the LightCycler 480 II (Roche). qPCR primers can be  
460 found in Supplemental Table 2.

461

462

463 Inhibitor treatment

464 100,000 cells were seeded in 96-well tissue culture plates, in 200  $\mu$ L culture medium containing the  
465 indicated concentration of inhibitor. Two inhibitors were used: SGC-0946 (Structural Genomics  
466 Consortium) and EPZ-5676 (Pinometostat; Selleck Chemicals). Cell viability was determined after three  
467 days, as described above. Data was normalized, with the maximum of each cell line to 100% and  
468 background fluorescence set to 0%. Graphpad Prism was used to fit log(inhibitor) vs normalized  
469 response curves with a variable slope.

470

471 Apoptosis FACS

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

475

476 Statistics

477 Survival curves were plotted in Graphpad Prism, and Peto Mortality-Prevalence tests were performed  
478 in SAS to compare the curve of *Lck-Cre;Hdac1<sup>ff</sup>* mice with the *Lck-Cre;Hdac1<sup>ff</sup>;Dot1L<sup>f/WT</sup>* and *Lck-*  
479 *Cre;Hdac1<sup>ff</sup>;Dot1L<sup>ff</sup>* curves. The same conclusions could be drawn based on the standard logrank test  
480 in Graphpad Prism. Mass spectrometry data were compared using two-way ANOVA, comparing  
481 samples of all genotypes to the wild-type sample and using the Dunnett correction for multiple  
482 comparisons. The significance thresholds used were  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*). The  
483 asterisks indicate significant differences compared to wild type.

484

485 Accession numbers

486 The accession number for the ChIP-seq results reported in this study is GSE107331.

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## 512 References

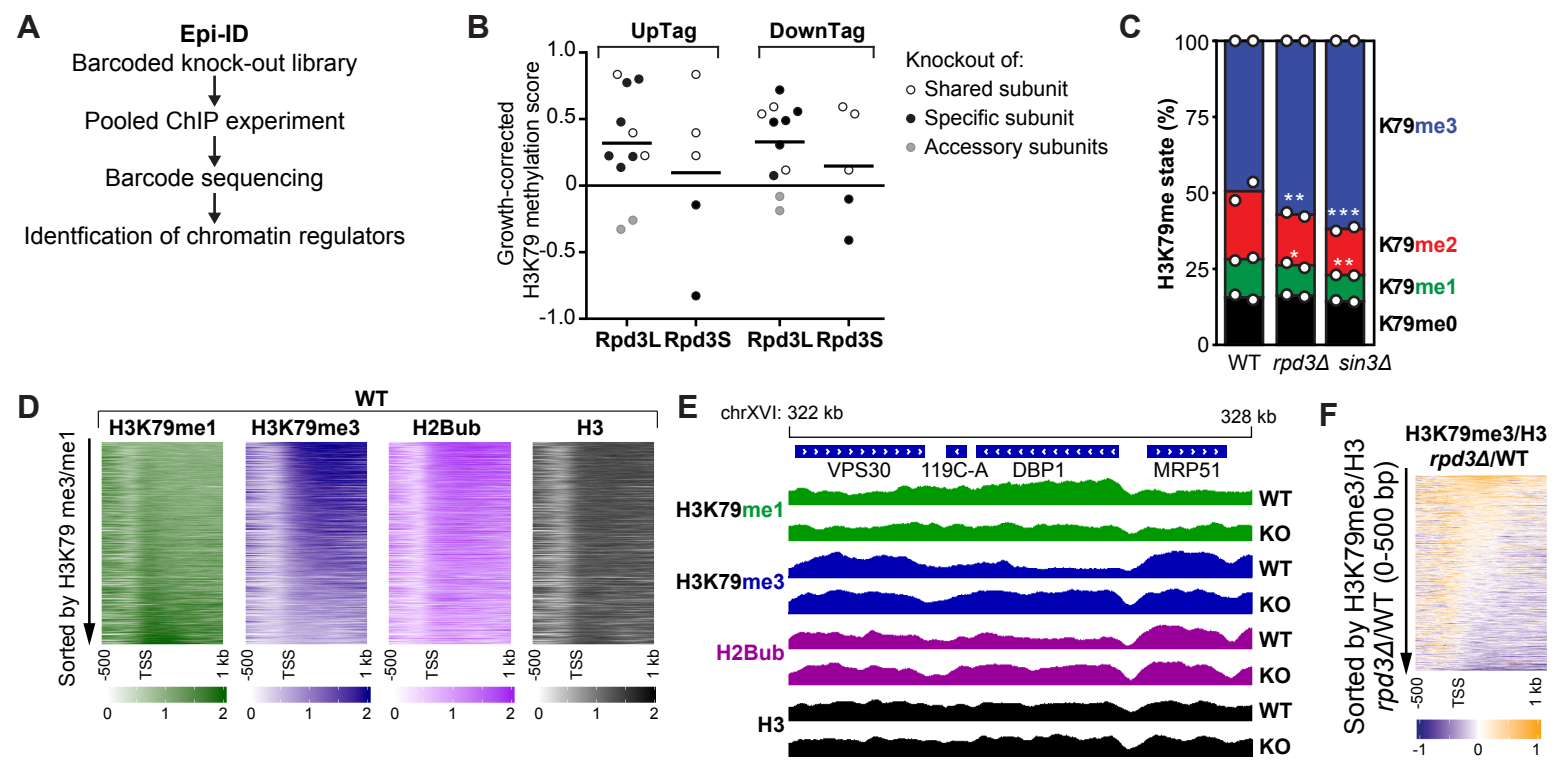
- 513 Bernt KM, Zhu N, Sinha AU, Vempati S, Faber J, Krivtsov A V, Feng Z, Punt N, Daigle A, Bullinger L, et al.  
514 2011. MLL-Rearranged Leukemia Is Dependent on Aberrant H3K79 Methylation by DOT1L.  
515 *Cancer Cell* **20**: 66–78.
- 516 Breindel JL, Skibinski A, Sedic M, Wronski-Campos A, Zhou W, Keller PJ, Mills J, Bradner J, Onder T,  
517 Kuperwasser C. 2017. Epigenetic Reprogramming of Lineage-Committed Human Mammary  
518 Epithelial Cells Requires DNMT3A and Loss of DOT1L. *Stem Cell Reports* **9**: 943–955.
- 519 Brien GL, Valerio DG, Armstrong SA. 2016. Exploiting the Epigenome to Control Cancer-Promoting  
520 Gene-Expression Programs. *Cancer Cell* **29**: 464–476.
- 521 Brocks D, Schmidt CR, Daskalakis M, Jang HS, Shah NM, Li D, Li J, Zhang B, Hou Y, Laudato S, et al.  
522 2017. DNMT and HDAC inhibitors induce cryptic transcription start sites encoded in long terminal  
523 repeats. *Nat Genet* **49**: 1052–1060.
- 524 Brown T, Howe FS, Murray SC, Wouters M, Lorenz P, Seward E, Rata S, Angel A, Mellor J. 2018.  
525 Antisense transcription-dependent chromatin signature modulates sense transcript dynamics.  
526 *Mol Syst Biol* **14**: e8007.
- 527 Campbell CT, Haladyna JN, Drubin DA, Thomson TM, Maria MJ, Yamauchi T, Waters NJ, Olhava EJ,  
528 Pollock RM, Smith JJ, et al. 2017. Mechanisms of Pinometostat (EPZ-5676) Treatment–Emergent  
529 Resistance in MLL -Rearranged Leukemia. *Mol Cancer Ther* **16**: 1669–1679.
- 530 Carrozza MJ, Li B, Florens L, Suganuma T, Swanson SK, Lee KK, Shia W-J, Anderson S, Yates J,  
531 Washburn MP, et al. 2005. Histone H3 Methylation by Set2 Directs Deacetylation of Coding  
532 Regions by Rpd3S to Suppress Spurious Intragenic Transcription. *Cell* **123**: 581–592.
- 533 Chen S, Yang Z, Wilkinson AW, Deshpande AJ, Sidoli S, Krajewski K, Strahl BD, Garcia BA, Armstrong SA,  
534 Patel DJ, et al. 2015. The PZP Domain of AF10 Senses Unmodified H3K27 to Regulate DOT1L-  
535 Mediated Methylation of H3K79. *Mol Cell* **60**: 319–327.
- 536 Cherry JM, Hong EL, Amundsen C, Balakrishnan R, Binkley G, Chan ET, Christie KR, Costanzo MC,  
537 Dwight SS, Engel SR, et al. 2012. Saccharomyces Genome Database: the genomics resource of  
538 budding yeast. *Nucleic Acids Res* **40**: D700–D705.
- 539 Daigle SR, Olhava EJ, Therkelsen C a, Basavapathruni A, Jin L, Boriack-Sjodin PA, Allain CJ, Klaus CR,  
540 Raimondi A, Scott MP, et al. 2013. Potent inhibition of DOT1L as treatment of MLL-fusion  
541 leukemia. *Blood* **122**: 1017–1025.
- 542 De Vos D, Frederiks F, Terweij M, van Welsem T, Verzijlbergen KF, Iachina E, de Graaf EL, Altelaar AFM,  
543 Oudgenoeg G, Heck AJR, et al. 2011. Progressive methylation of ageing histones by Dot1  
544 functions as a timer. *EMBO Rep* **12**: 956–62.
- 545 Deshpande AJ, Deshpande A, Sinha AU, Chen L, Chang J, Cihan A, Fazio M, Chen C, Zhu N, Koche R, et  
546 al. 2014. AF10 Regulates Progressive H3K79 Methylation and HOX Gene Expression in Diverse  
547 AML Subtypes. *Cancer Cell* **26**: 896–908.
- 548 Dovey OM, Foster CT, Conte N, Edwards SA, Edwards JM, Singh R, Vassiliou G, Bradley A, Cowley SM.  
549 2013. Histone deacetylase 1 and 2 are essential for normal T-cell development and genomic  
550 stability in mice. *Blood* **121**: 1335–1344.
- 551 Drouin S, Laramée L, Jacques P-É, Forest A, Bergeron M, Robert F. 2010. DSIF and RNA polymerase II  
552 CTD phosphorylation coordinate the recruitment of Rpd3S to actively transcribed genes. ed. J.D.  
553 Lieb. *PLoS Genet* **6**: e1001173.
- 554 Ehrentraut S, Hassler M, Oppikofer M, Kueng S, Weber JM, Mueller JW, Gasser SM, Ladurner AG,  
555 Ehrenhofer-Murray AE. 2011. Structural basis for the role of the Sir3 AAA+ domain in silencing:  
556 interaction with Sir4 and unmethylated histone H3K79. *Genes Dev* **25**: 1835–46.
- 557 Ehrentraut S, Weber JM, Dybowski JN, Hoffmann D, Ehrenhofer-Murray AE. 2010. Rpd3-dependent  
558 boundary formation at telomeres by removal of Sir2 substrate. *Proc Natl Acad Sci U S A* **107**:  
559 5522–7.
- 560 Emre NCT, Ingvarsdottir K, Wyce A, Wood A, Krogan NJ, Henry KW, Li K, Marmorstein R, Greenblatt JF,  
561 Shilatifard A, et al. 2005. Maintenance of Low Histone Ubiquitylation by Ubp10 Correlates with

- 562 Telomere-Proximal Sir2 Association and Gene Silencing. *Mol Cell* **17**: 585–594.
- 563 Erb MA, Scott TG, Li BE, Xie H, Paulk J, Seo H-S, Souza A, Roberts JM, Dastjerdi S, Buckley DL, et al.
- 564 2017. Transcription control by the ENL YEATS domain in acute leukaemia. *Nature* **543**: 270–274.
- 565 Frederiks F, Tzouros M, Oudgenoeg G, van Welsem T, Fornerod M, Krijgsveld J, van Leeuwen F. 2008.
- 566 Nonprocessive methylation by Dot1 leads to functional redundancy of histone H3K79
- 567 methylation states. *Nat Struct Mol Biol* **15**: 550–557.
- 568 Gardner RG, Nelson ZW, Gottschling DE. 2005. Ubp10/Dot4p regulates the persistence of
- 569 ubiquitinated histone H2B: distinct roles in telomeric silencing and general chromatin. *Mol Cell*
- 570 *Biol* **25**: 6123–39.
- 571 Garrido Castro P, van Roon EHJ, Pinhanços SS, Trentin L, Schneider P, Kerstjens M, te Kronnie G,
- 572 Heidenreich O, Pieters R, Stam RW. 2018. The HDAC inhibitor panobinostat (LBH589) exerts in
- 573 vivo anti-leukaemic activity against MLL-rearranged acute lymphoblastic leukaemia and involves
- 574 the RNF20/RNF40/WAC-H2B ubiquitination axis. *Leukemia* **32**: 323–331.
- 575 Gartenberg MR, Smith JS. 2016. The Nuts and Bolts of Transcriptionally Silent Chromatin in
- 576 *Saccharomyces cerevisiae*. *Genetics* **203**: 1563–1599.
- 577 Heideman MR, Wilting RH, Yanover E, Velds A, de Jong J, Kerkhoven RM, Jacobs H, Wessels LF,
- 578 Dannenberg J-H. 2013. Dosage-dependent tumor suppression by histone deacetylases 1 and 2
- 579 through regulation of c-Myc collaborating genes and p53 function. *Blood* **121**: 2038–2050.
- 580 Huber W, Carey VJ, Gentleman R, Anders S, Carlson M, Carvalho BS, Bravo HC, Davis S, Gatto L, Girke
- 581 T, et al. 2015. Orchestrating high-throughput genomic analysis with Bioconductor. *Nat Methods*
- 582 **12**: 115–121.
- 583 Jones B, Su H, Bhat A, Lei H, Bajko J, Hevi S, Baltus GA, Kadam S, Zhai H, Valdez R, et al. 2008. The
- 584 histone H3K79 methyltransferase Dot1L is essential for mammalian development and
- 585 heterochromatin structure. *PLoS Genet* **4**: e1000190.
- 586 Jones PA, Issa J-PJ, Baylin S. 2016. Targeting the cancer epigenome for therapy. *Nat Rev Genet* **17**:
- 587 630–641.
- 588 Joshi AA, Struhl K. 2005. Eaf3 Chromodomain Interaction with Methylated H3-K36 Links Histone
- 589 Deacetylation to Pol II Elongation. *Mol Cell* **20**: 971–978.
- 590 Kadosh D, Struhl K. 1998. Histone deacetylase activity of Rpd3 is important for transcriptional
- 591 repression in vivo. *Genes Dev* **12**: 797–805.
- 592 Kemmeren P, Sameith K, van de Pasch LAL, Benschop JJ, Lenstra TL, Margaritis T, O’Duibhir E, Apweiler
- 593 E, van Wageningen S, Ko CW, et al. 2014. Large-Scale Genetic Perturbations Reveal Regulatory
- 594 Networks and an Abundance of Gene-Specific Repressors. *Cell* **157**: 740–752.
- 595 Keogh M-C, Kurdistani SK, Morris SA, Ahn SH, Podolny V, Collins SR, Schuldiner M, Chin K, Punna T,
- 596 Thompson NJ, et al. 2005. Cotranscriptional set2 methylation of histone H3 lysine 36 recruits a
- 597 repressive Rpd3 complex. *Cell* **123**: 593–605.
- 598 Kim W, Kim R, Park G, Park J-W, Kim J-E. 2012. Deficiency of H3K79 histone methyltransferase Dot1-
- 599 like protein (DOT1L) inhibits cell proliferation. *J Biol Chem* **287**: 5588–99.
- 600 Kueng S, Oppikofer M, Gasser SM. 2013. SIR Proteins and the Assembly of Silent Chromatin in Budding
- 601 Yeast. *Annu Rev Genet* **47**: 275–306.
- 602 Kuntimaddi A, Achille NJ, Thorpe J, Lokken AA, Singh R, Hemenway CS, Adli M, Zeleznik-Le NJ,
- 603 Bushweller JH. 2015. Degree of Recruitment of DOT1L to MLL-AF9 Defines Level of H3K79 Di- and
- 604 Tri-methylation on Target Genes and Transformation Potential. *Cell Rep* **11**: 808–820.
- 605 Lagger S, Meunier D, Mikula M, Brunmeir R, Schleder M, Artaker M, Pusch O, Egger G, Hagelkruys A,
- 606 Mikulits W, et al. 2010. Crucial function of histone deacetylase 1 for differentiation of teratomas
- 607 in mice and humans. *EMBO J* **29**: 3992–4007.
- 608 Lardenois A, Stuparevic I, Liu Y, Law MJ, Becker E, Smagulova F, Waern K, Guilleux M-H, Horecka J, Chu
- 609 A, et al. 2015. The conserved histone deacetylase Rpd3 and its DNA binding subunit Ume6
- 610 control dynamic transcript architecture during mitotic growth and meiotic development. *Nucleic*
- 611 *Acids Res* **43**: 115–128.
- 612 Lee S, Oh S, Jeong K, Jo H, Choi Y, Seo HD, Kim M, Choe J, Kwon CS, Lee D. 2018. Dot1 regulates

- 613 nucleosome dynamics by its inherent histone chaperone activity in yeast. *Nat Commun* **9**: 240.  
614 Lenstra TL, Benschop JJ, Kim T, Schulze JM, Brabers NACH, Margaritis T, van de Pasch LAL, van Heesch  
615 SAAC, Brok MO, Groot Koerkamp MJA, et al. 2011. The Specificity and Topology of Chromatin  
616 Interaction Pathways in Yeast. *Mol Cell* **42**: 536–549.
- 617 Leroy G, Dimaggio PA, Chan EY, Zee BM, Blanco MA, Bryant B, Flaniken IZ, Liu S, Kang Y, Trojer P, et al.  
618 2013. A quantitative atlas of histone modification signatures from human cancer cells.  
619 *Epigenetics Chromatin* **6**: 20.
- 620 Li B, Carey M, Workman JL. 2007. The role of chromatin during transcription. *Cell* **128**: 707–19.  
621 Li Y, Wen H, Xi Y, Tanaka K, Wang H, Peng D, Ren Y, Jin Q, Dent SYR, Li W, et al. 2014. AF9 YEATS  
622 Domain Links Histone Acetylation to DOT1L-Mediated H3K79 Methylation. *Cell* **159**: 558–571.
- 623 Magraner-Pardo L, Pelechano V, Coloma MD, Tordera V. 2014. Dynamic remodeling of histone  
624 modifications in response to osmotic stress in *Saccharomyces cerevisiae*. *BMC Genomics* **15**: 247.
- 625 McDaniel SL, Strahl BD. 2017. Shaping the cellular landscape with Set2/SETD2 methylation. *Cell Mol*  
626 *Life Sci* **74**: 3317–3334.
- 627 McKnight JN, Boerma JW, Breeden LL, Tsukiyama T. 2015. Global Promoter Targeting of a Conserved  
628 Lysine Deacetylase for Transcriptional Shutoff during Quiescence Entry. *Mol Cell* **59**: 732–743.
- 629 Murray SC, Haenni S, Howe FS, Fischl H, Chocian K, Nair A, Mellor J. 2015. Sense and antisense  
630 transcription are associated with distinct chromatin architectures across genes. *Nucleic Acids Res*  
631 **43**: 7823–7837.
- 632 Onder TT, Kara N, Cherry A, Sinha AU, Zhu N, Bernt KM, Cahan P, Marcarci BO, Unternaehrer J, Gupta  
633 PB, et al. 2012. Chromatin-modifying enzymes as modulators of reprogramming. *Nature* **483**:  
634 598–602.
- 635 Radman-Livaja M, Verzijlbergen KF, Weiner A, van Welsem T, Friedman N, Rando OJ, van Leeuwen F.  
636 2011. Patterns and mechanisms of ancestral histone protein inheritance in budding yeast. ed.  
637 P.B. Becker. *PLoS Biol* **9**: e1001075.
- 638 Rando OJ, Winston F. 2012. Chromatin and Transcription in Yeast. *Genetics* **190**: 351–387.
- 639 Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. 2015. limma powers differential  
640 expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* **43**: e47-.
- 641 Rundlett SE, Carmen AA, Suka N, Turner BM, Grunstein M. 1998. Transcriptional repression by UME6  
642 involves deacetylation of lysine 5 of histone H4 by RPD3. *Nature* **392**: 831–835.
- 643 Sabra M, Texier P, El Maalouf J, Lomonte P. 2013. The Tudor protein survival motor neuron (SMN) is a  
644 chromatin-binding protein that interacts with methylated lysine 79 of histone H3. *J Cell Sci* **126**:  
645 3664–77.
- 646 Sadeh R, Launer-Wachs R, Wandel H, Rahat A, Friedman N. 2016. Elucidating Combinatorial Chromatin  
647 States at Single-Nucleosome Resolution. *Mol Cell* **63**: 1080–8.
- 648 Schulze JM, Hentrich T, Nakanishi S, Gupta A, Emberly E, Shilatifard A, Kobor MS. 2011. Splitting the  
649 task: Ubp8 and Ubp10 deubiquitinate different cellular pools of H2BK123. *Genes Dev* **25**: 2242–  
650 2247.
- 651 Schulze JM, Jackson J, Nakanishi S, Gardner JM, Hentrich T, Haug J, Johnston M, Jaspersen SL, Kobor  
652 MS, Shilatifard A. 2009. Linking cell cycle to histone modifications: SBF and H2B  
653 monoubiquitination machinery and cell-cycle regulation of H3K79 dimethylation. *Mol Cell* **35**:  
654 626–41.
- 655 Shortt J, Ott CJ, Johnstone RW, Bradner JE. 2017. A chemical probe toolbox for dissecting the cancer  
656 epigenome. *Nat Rev Cancer* **17**: 160–183.
- 657 Soria-Valles C, Osorio FG, Gutiérrez-Fernández A, De Los Angeles A, Bueno C, Menéndez P, Martín-  
658 Subero JI, Daley GQ, Freije JMP, López-Otín C. 2015. NF-κB activation impairs somatic cell  
659 reprogramming in ageing. *Nat Cell Biol* **17**: 1004–13.
- 660 Stein EM, Garcia-Manero G, Rizzieri DA, Tibes R, Berdeja JG, Savona MR, Jongen-Lavrenic M, Altman  
661 JK, Thomson B, Blakemore SJ, et al. 2018. The DOT1L inhibitor pinometostat reduces H3K79  
662 methylation and has modest clinical activity in adult acute leukemia. *Blood* **131**: 2661–2669.
- 663 Stein EM, Tallman MS. 2016. Emerging therapeutic drugs for AML. *Blood* **127**: 71–78.

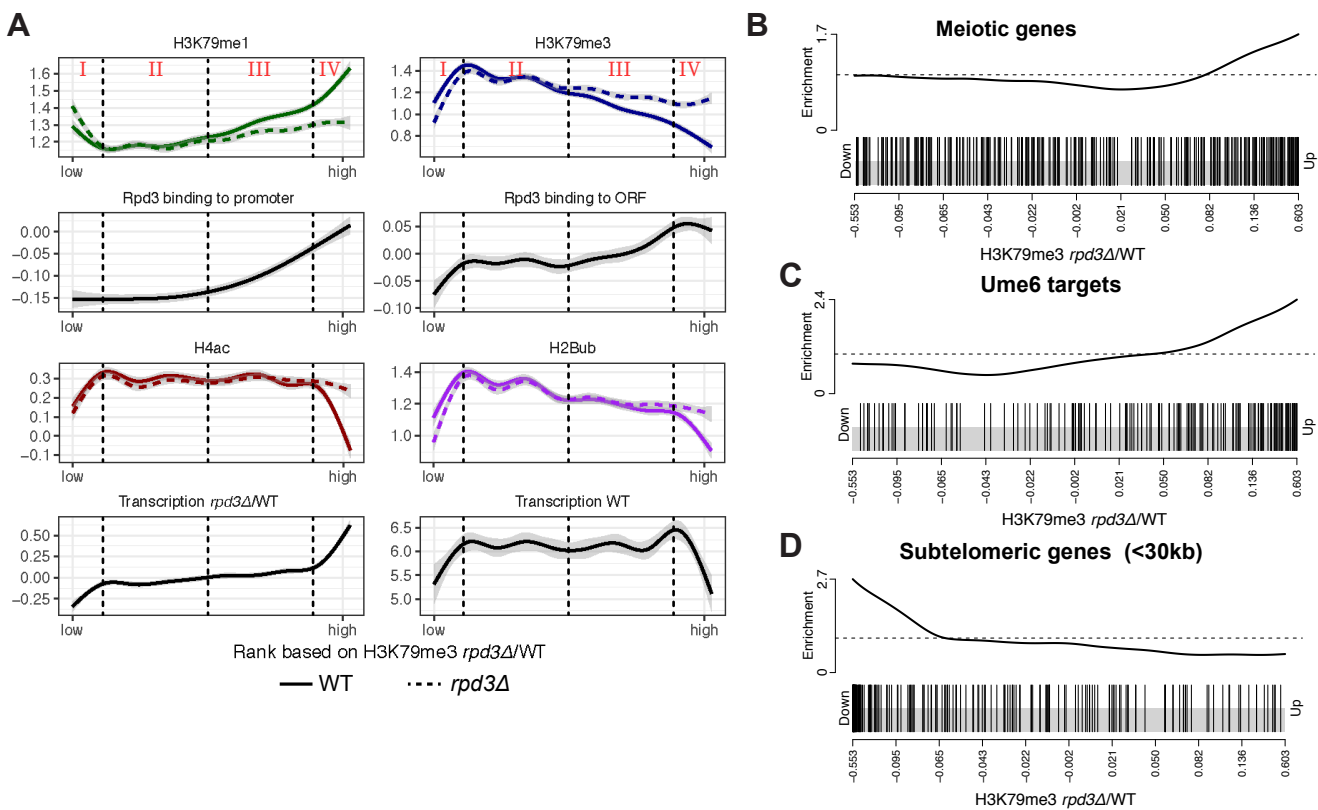


- 664 Stulemeijer IJ, Pike BL, Faber AW, Verzijlbergen KF, van Welsem T, Frederiks F, Lenstra TL, Holstege FC,  
665 Gasser SM, van Leeuwen F. 2011. Dot1 binding induces chromatin rearrangements by histone  
666 methylation-dependent and -independent mechanisms. *Epigenetics Chromatin* **4**: 2.
- 667 Teixeira MC, Monteiro PT, Guerreiro JF, Gonçalves JP, Mira NP, dos Santos SC, Cabrito TR, Palma M,  
668 Costa C, Francisco AP, et al. 2014. The YEASTRACT database: an upgraded information system for  
669 the analysis of gene and genomic transcription regulation in *Saccharomyces cerevisiae*. *Nucleic  
670 Acids Res* **42**: D161–D166.
- 671 Thurtle-Schmidt DM, Dodson AE, Rine J. 2016. Histone Deacetylases with Antagonistic Roles in  
672 *Saccharomyces cerevisiae* Heterochromatin Formation. *Genetics* **204**: 177–190.
- 673 Van Leeuwen F, Gottschling DE. 2002. Assays for gene silencing in yeast. In *Methods in enzymology*,  
674 Vol. 350 of, pp. 165–186.
- 675 Van Welsem T, Korthout T, Ekkebus R, Morais D, Molenaar TM, van Harten K, Poramba-Liyanage DW,  
676 Sun SM, Lenstra TL, Srivas R, et al. 2018. Dot1 promotes H2B ubiquitination by a  
677 methyltransferase-independent mechanism. *Nucleic Acids Res* **46**: 11251–11261.
- 678 Vlaming H, Molenaar TM, van Welsem T, Poramba-Liyanage DW, Smith DE, Velds A, Hoekman L,  
679 Korthout T, Hendriks S, Altelaar AM, et al. 2016. Direct screening for chromatin status on DNA  
680 barcodes in yeast delineates the regulome of H3K79 methylation by Dot1. *Elife* **5**: e18919.
- 681 Vlaming H, Van Leeuwen F. 2016. The upstreams and downstreams of H3K79 methylation by DOT1L.  
682 *Chromosoma* **125**: 593–605.
- 683 Vlaming H, van Welsem T, de Graaf EL, Ontoso D, Altelaar AM, San-Segundo PA, Heck AJ, Van Leeuwen  
684 F. 2014. Flexibility in crosstalk between H2B ubiquitination and H3 methylation in vivo. *EMBO  
685 Rep* **15**: 1077–1084.
- 686 Wan L, Wen H, Li Y, Lyu J, Xi Y, Hoshii T, Joseph JK, Wang X, Loh Y-HE, Erb MA, et al. 2017. ENL links  
687 histone acetylation to oncogenic gene expression in acute myeloid leukaemia. *Nature* **543**: 265–  
688 269.
- 689 Wang X, Chen C-W, Armstrong SA. 2016. The role of DOT1L in the maintenance of leukemia gene  
690 expression. *Curr Opin Genet Dev* **36**: 68–72.
- 691 Waters NJ, Daigle SR, Rehlaender BN, Basavapathruni A, Campbell CT, Jensen TB, Truitt BF, Olhava EJ,  
692 Pollock RM, Stickland KA, et al. 2015. Exploring drug delivery for the DOT1L inhibitor  
693 pinometostat (EPZ-5676): Subcutaneous administration as an alternative to continuous IV  
694 infusion, in the pursuit of an epigenetic target. *J Control Release* **220**: 758–765.
- 695 Weake VM, Workman JL. 2008. Histone Ubiquitination: Triggering Gene Activity. *Mol Cell* **29**: 653–663.
- 696 Weinberger L, Voichek Y, Tirosh I, Hornung G, Amit I, Barkai N. 2012. Expression Noise and Acetylation  
697 Profiles Distinguish HDAC Functions. *Mol Cell* **47**: 193–202.
- 698 Weiner A, Hsieh T-HSS, Appleboim A, Chen HV V., Rahat A, Amit I, Rando OJJ, Friedman N. 2015. High-  
699 Resolution Chromatin Dynamics during a Yeast Stress Response. *Mol Cell* **58**: 371–386.
- 700 West AC, Johnstone RW. 2014. New and emerging HDAC inhibitors for cancer treatment. *J Clin Invest*  
701 **124**: 30–39.
- 702 Wood K, Tellier M, Murphy S. 2018. DOT1L and H3K79 Methylation in Transcription and Genomic  
703 Stability. *Biomolecules* **8**: 11.
- 704 Yang X-J, Seto E. 2008. The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice  
705 and men. *Nat Rev Mol Cell Biol* **9**: 206–18.
- 706 Zhang L, Deng L, Chen F, Yao Y, Wu B, Wei L, Mo Q, Song Y. 2014. Inhibition of histone H3K79  
707 methylation selectively inhibits proliferation, self-renewal and metastatic potential of breast  
708 cancer. *Oncotarget* **5**: 10665–10677.
- 709 Zhang T, Cooper S, Brockdorff N. 2015. The interplay of histone modifications - writers that read.  
710 *EMBO Rep* **16**: 1467–1481.
- 711 Zhou L, Holt MT, Ohashi N, Zhao A, Müller MM, Wang B, Muir TW. 2016. Evidence that ubiquitylated  
712 H2B corrals hDot1L on the nucleosomal surface to induce H3K79 methylation. *Nat Commun* **7**:  
713 10589.
- 714



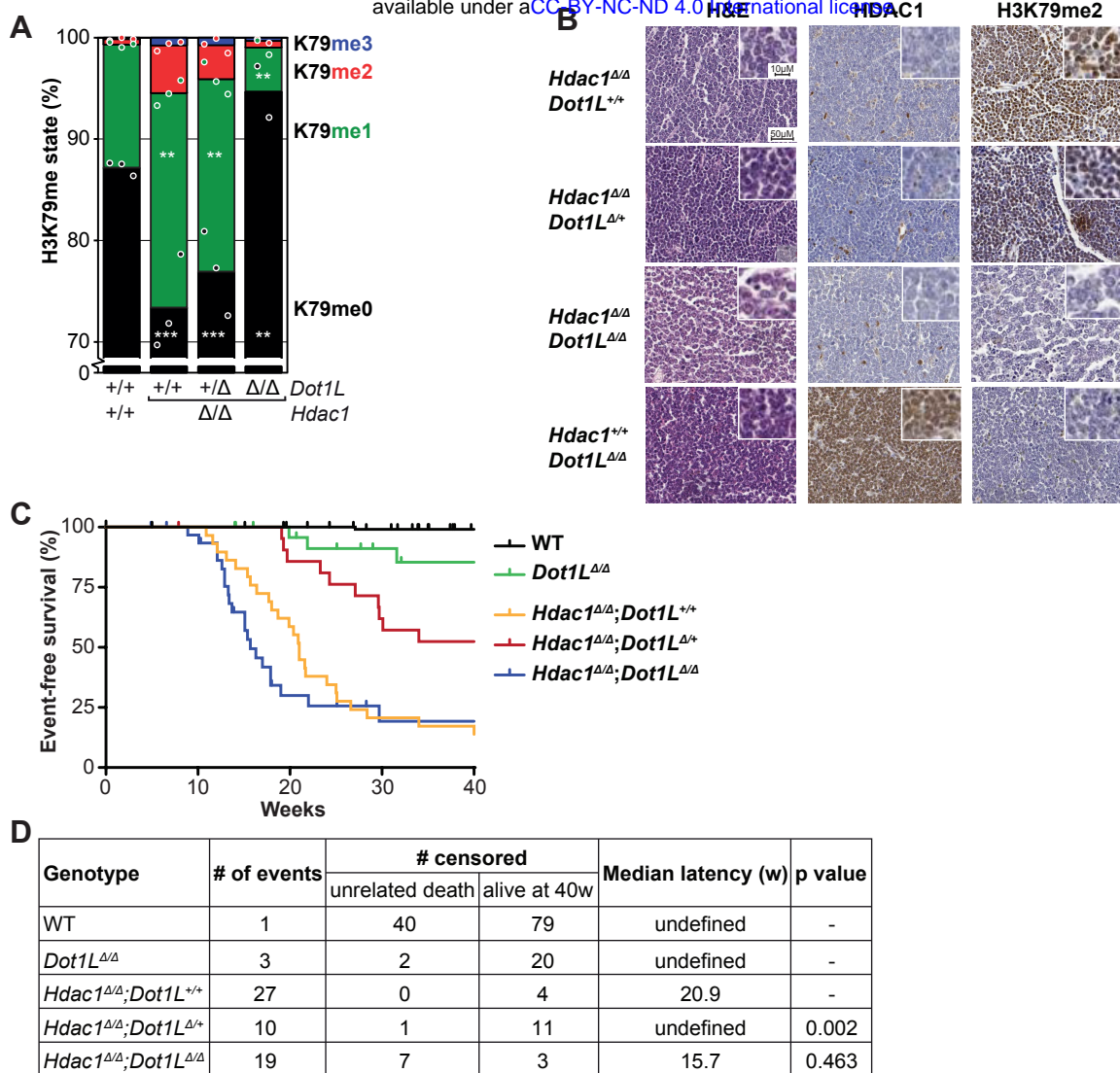
**Figure 1 Rpd3 and other members of the Rpd3L complex negatively regulate H3K79 methylation.**

A) Schematic overview of the Epi-ID strategy. B) Epi-ID H3K79 methylation scores of the deletion mutants of members of the Rpd3L and Rpd3S complexes, calculated as described in the Supplemental Methods, where 0 means a wild-type H3K79me level (log2 scale). The gray dots indicate accessory subunits. UpTag and DownTag are barcode reporters in a promoter and terminator context, respectively. Data was obtained on all Rpd3L/Rpd3S subunits except Sds3. C) Mass spectrometry analysis of H3K79 methylation in wild-type and mutant strains. Mean and individual data points of two biological replicates. D) Heatmaps of H3K79me1, H3K79me3, H2Bub and H3 in wild-type cells, aligned on the TSS. Genes were sorted based on the average H3K79me3/H3K79me1 ratio in the first 500 bp. E) Snapshot of depth-normalized ChIP-seq data tracks from wild-type and *rdp3Δ* strains showing 6 kb surrounding the *DBP1* ORF, which is the top gene in the heatmap in panel F. All tracks have the same y axis (0-2). A snapshot of another top-regulated gene is shown in Supplemental Fig. 1D. F) Heatmap of the H3K79me3/H3 change in *rdp3Δ* versus wild-type cells, aligned on the TSS. Genes were sorted based on the average ratio in the first 500 bp.



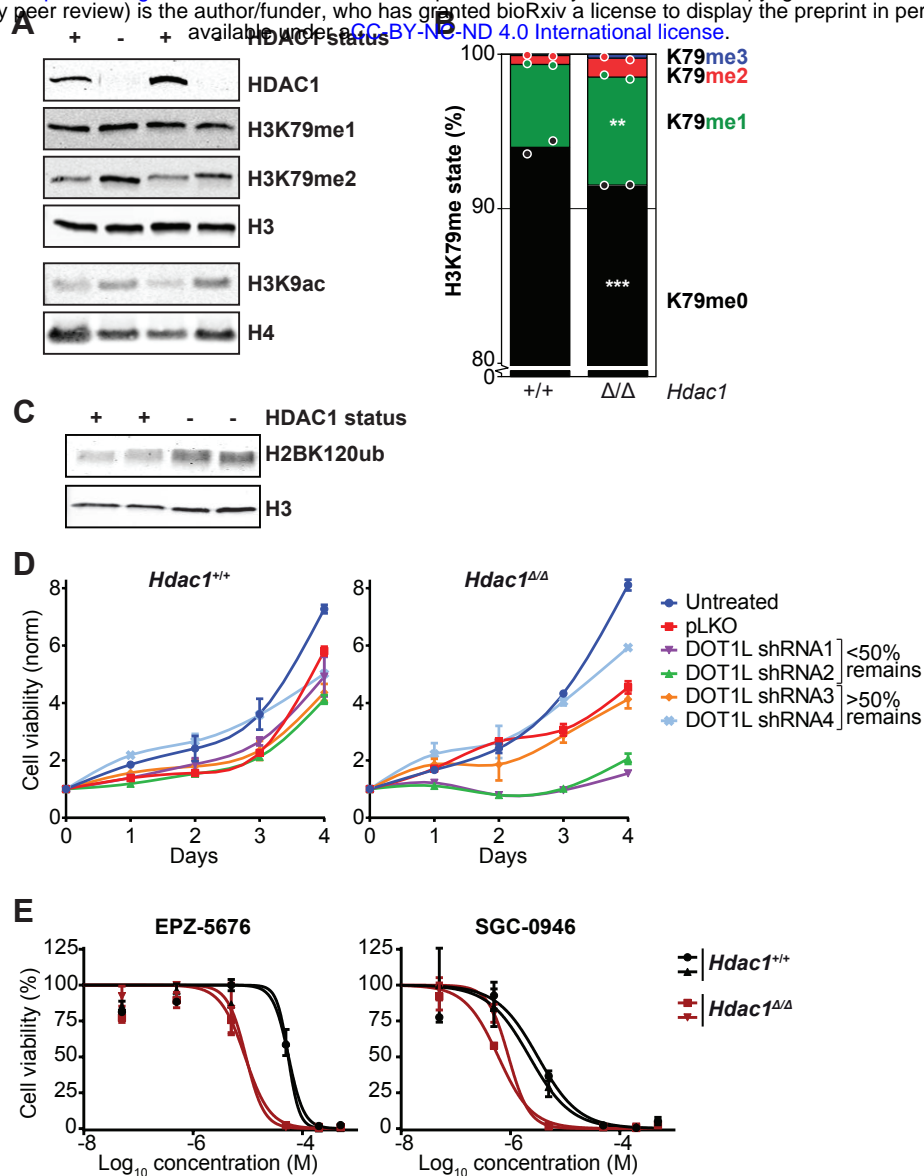
**Figure 2 Rpd3 represses transcription, H2B ubiquitination and H3K79 methylation at its target sites.**

A) ChIP-seq and RNA-seq data for genes ranked on H3K79me3/H3 in *rpd3Δ/WT*, smoothed using locally weighed regression. The gray band around the line shows the 95% confidence interval. Vertical dashed lines separate 4 groups with distinct changes upon RPD3 deletion. For H3K79me1, H3K79me3, H2Bub, H4ac and Rpd3 binding to ORF, the average coverage in the first 500 bp was used. Rpd3 binding to promoter was the average coverage in the 400 bp upstream of the TSS. Transcription in wild-type cells was obtained from (McKnight et al. 2015), and the transcription in *rpd3Δ/WT* from (Kemmeren et al. 2014). B-D) Gene set enrichment analysis on genes ranked on H3K79me3/H3 in *rpd3Δ/WT* shows that meiotic (B) and Ume6-bound (C) genes are enriched among the genes at which Rpd3 represses H3K79 methylation, and subtelomeric genes (<30 kb of telomere) (D) are enriched among genes at which H3K79 methylation is decreased in *rpd3Δ* cells.



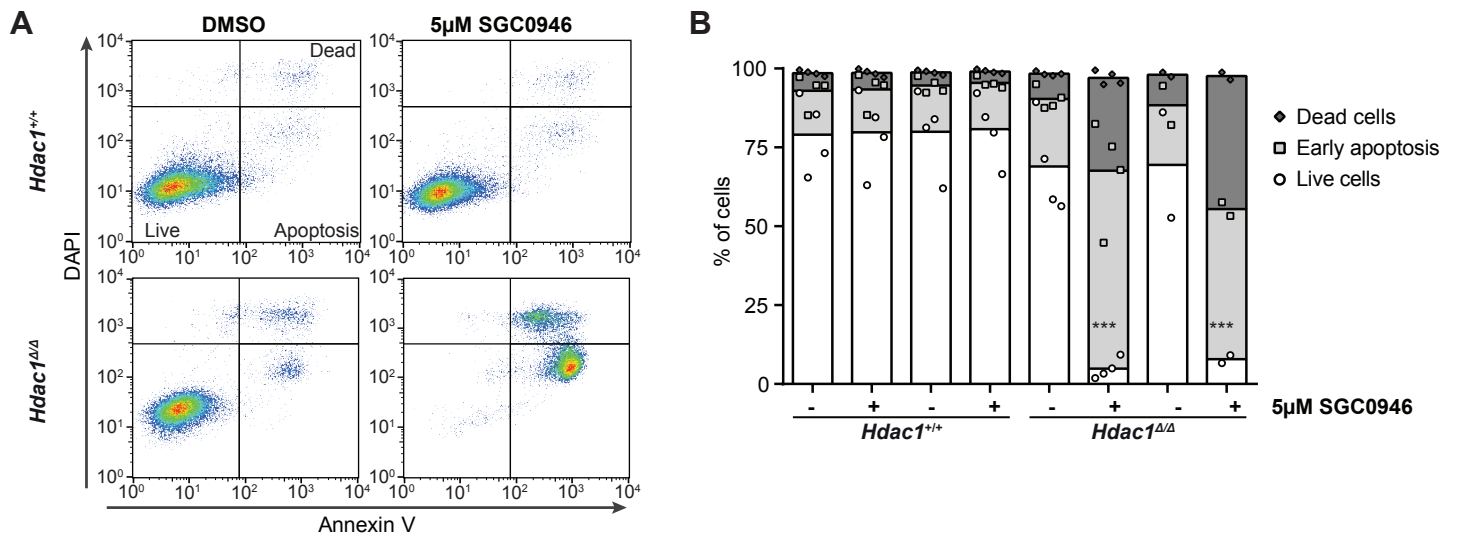
**Figure 3 *Hdac1* deletion increases H3K79 methylation in thymocytes *in vivo*, and simultaneous heterozygous *Dot1L* deletion prolongs tumor-free survival.**

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, 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). B) Representative H&E and immunohistochemical stainings of thymic lymphomas of the indicated genotypes. A picture with lower magnification is included in Supplemental Fig. 3B. 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*<sup>fl/fl</sup> curve with a Peto test, but a logrank test yielded the same conclusions.



### Figure 4 *Hdac1*-deleted thymic lymphoma cell lines depend on DOT1L activity.

A) Immunoblots showing HDAC1 status and H3K79me/H3K9ac levels in nuclear lysates of *Hdac1*-proficient (p53-null) and *Hdac1*-deficient thymic lymphoma cell lines. The top four and bottom two panels are from separate lysates of the same cell lines. B) Mass spectrometry analysis of H3K79 methylation in the cell lines from panel A. Mean and individual data points of two independent cell lines; H3K79me0 is the predominant state, the y axis is truncated at 80% for readability. C) Immunoblot showing H2BK120 ubiquitination levels in *Hdac1*-proficient and -deficient cell lines (two independent lines each). D) Growth curves of *Hdac1*-proficient and -deficient cell lines that were left untreated or were infected with empty virus (pLKO) or shRNAs against *Dot1L* and selected with puromycin. Growth curves were determined by a series of resazurin assays, which measure metabolic activity, starting four days post-infection. Error bars indicate the range of two replicates from independent cell lines. E) Inhibitor dose-response curves of the two DOT1L inhibitors EPZ-5676 (Pinometostat) and SGC-0946 in *Hdac1*-proficient and -deficient cell lines. Cell viability was measured by a resazurin assay after three days of treatment and measurements were normalized to DMSO-treated cells. Two independent cell lines are plotted separately; error bars represent the range of two biological replicates.



**Figure 5 *Hdac1*-deficient thymic lymphoma lines require DOT1L activity for survival.**

A) Representative apoptosis FACS plots of cell lines treated with DMSO or the DOT1L inhibitor SGC-0946 for two days. Annexin V staining and DAPI staining were performed on unpermeabilized cells to distinguish live (Annexin V low; DAPI low), apoptotic (Annexin V high; DAPI low) and dead (Annexin V high; DAPI high) cells. B) Quantification of several independent FACS experiments, including the experiment shown in panel A. Mean (bars) with individual data points of 2-4 replicates each of two independent lines per genotype.

## Supplemental Material

<b>Supplemental Table 1</b>	Yeast strains used in this study
<b>Supplemental Table 2</b>	Primers used for qPCR
<b>Supplemental Figure 1</b>	Rpd3 and Sin3 negatively regulate H3K79 methylation
<b>Supplemental Figure 2</b>	ChIP-seq and ChIP-qPCR data from WT vs <i>rpd3Δ</i> cells and the relation between transcription and histone modifications
<b>Supplemental Figure 3</b>	The distributive nature of Dot1, and thymus immunohistochemistry
<b>Supplemental Figure 4</b>	Control experiments belonging to Figure 4
<b>Supplemental Methods</b>	
<b>Supplemental References</b>	

**Supplemental Table 1** Yeast strains used in this study

Strain name	Genotype	Reference	Figures
NatMX-KO Barcoders library	MATa can1Δ::STE2pr-Sphis5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 hoΔ::barcodedKanMX GOI::NatMX	(Vlaming et al. 2016)	1B
NKI4560	MATa can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 hoΔ::barcode(0001)KanMX	(Vlaming et al. 2016)	1C, S1A
NKI4557	MATa can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 hoΔ::barcode(sir2)KanMX dot1Δ::NatMX	(Vlaming et al. 2016)	S1A
NKI4558	MATa can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 hoΔ::barcode(sir3)KanMX bre1Δ::NatMX	(Vlaming et al. 2016)	S1A
NKI4643	MATa can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 hoΔ::barcode(0791)KanMX rpd3Δ::NatMX	From library	1C, S1A
NKI4644	MATa can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 hoΔ::barcode(1002)KanMX sin3Δ::NatMX	From library	1C, S1A
NKI4657	MATa his3Δ200 leu2Δ0 trp1Δ63 ura3Δ0 met15Δ0 hoΔ:: barcode(sir4)KanMX	(Vlaming et al. 2016)	All ChIPs
NKI4713	MATa his3Δ200 leu2Δ0 trp1Δ63 ura3Δ0 met15Δ0 hoΔ:: barcode(sir4)KanMX rpd3Δ::NatMX	This study	All ChIPs

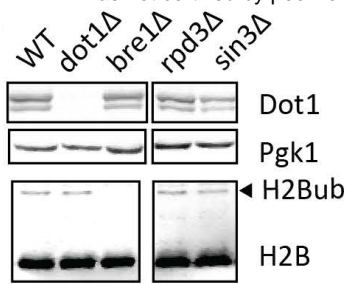
**Supplemental Table 2** Primers used for qPCR

Primer	Species	Position*	Sequence
Gapdh qPCR - F	mouse		CATCTTCTTGTGCAGTGCCAG
Gapdh_qPCR_R	mouse		GTGAGTGGAGTCACTACTGGAACA
Dot1L Exons5-7 qPCR Fw	mouse		CAGAGGATGACCTGTTTGTCTG
Dot1L Exons5-7 qPCR Rv	mouse		CATCCACTTCTGAACTCTCG
SPA2_High_Qfor	budding yeast	379	ATCAAGAGAAGAGGGTTCGACAAG
SPA2_High_Qrev	budding yeast	379	CATCGGCTGCGGTAATGG
PCH2_ORF_Qfor	budding yeast	176	CTGACTCGAAACAAAACAGCA
PCH2_ORF_Qrev	budding yeast	176	CTTCCTTGCCCCCTCTCTCAT
SIP4_ORF_Qfor	budding yeast	268	CTCTGTGAGAAAGGCGCATG
SIP4_ORF_Qrev	budding yeast	268	CGCTGGAAGTTCGATTCATA
DBP1_ORF_Qfor	budding yeast	254	CTGGAAGGCAAACTGGGAAC
DBP1_ORF_Qrev	budding yeast	254	TAGGCCCGGTATATGCTTG
ZIP1_ORF_Qfor	budding yeast	254	ACCCACAAAACCTTCTACCGA
ZIP1_ORF_Qrev	budding yeast	254	TTTCAATTGCGGCAACATCA

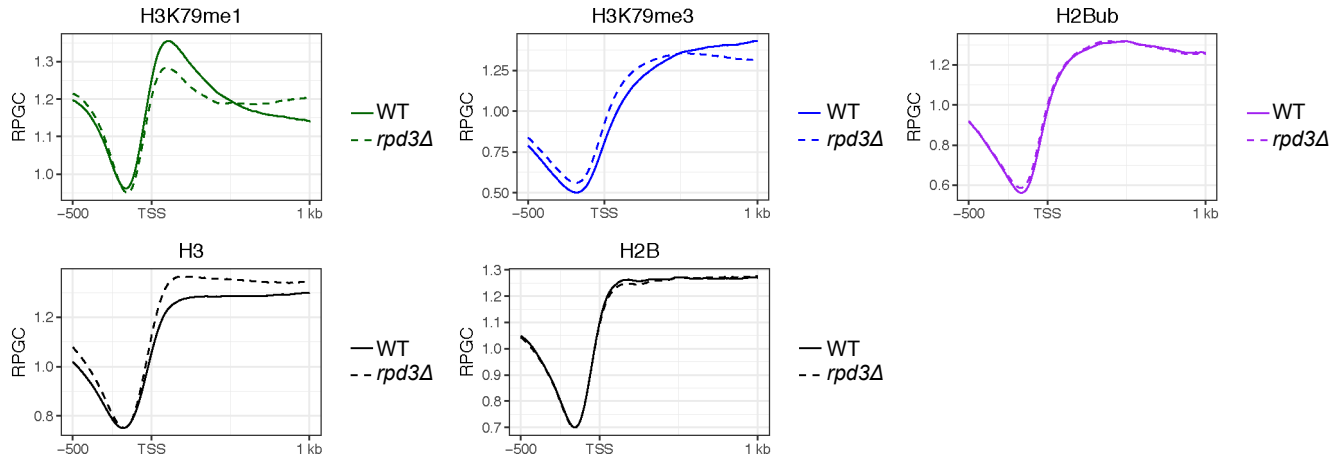
\* distance PCR product from start of coding sequence



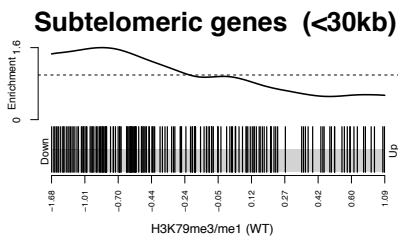
**A**



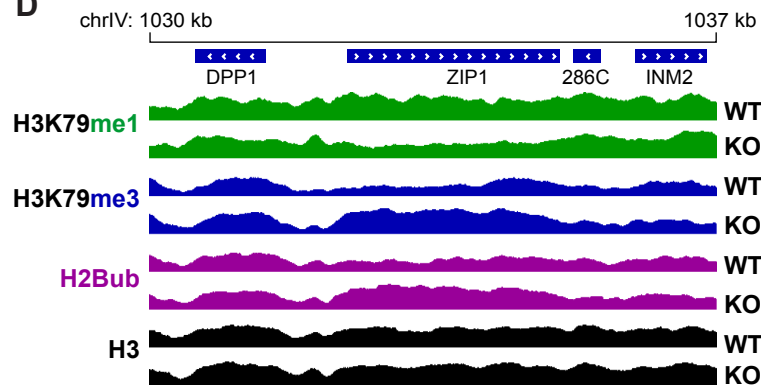
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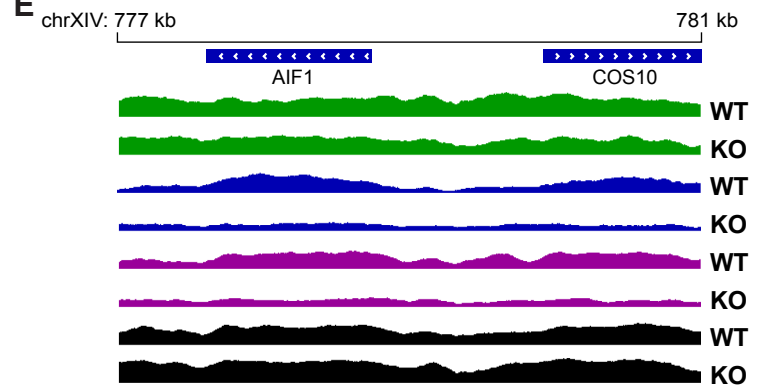
**C**



**D**



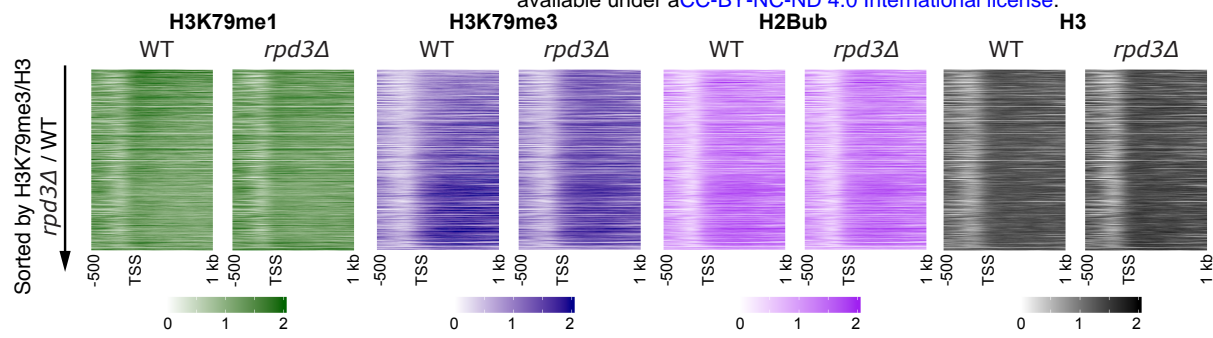
**E**



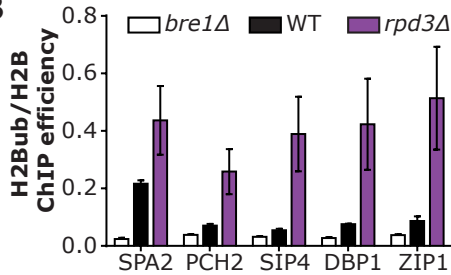
### Supplemental Figure 1 Rpd3 and Sin3 negatively regulate H3K79 methylation.

A) Immunoblots show that deletion of *RPD3* or *SIN3* does not lead to a detectable increase in global H2Bub or Dot1 protein levels. B) Metagene plots of H3K79me1, H3K79me3, H2Bub, H3, and H2B in *rp3Δ* and wild-type strains. C) Gene set enrichment analysis shows that subtelomeric genes (<30 kb of telomeres) are enriched among genes with low H3K79 methylation (measured by the average H3K79me3/H3K79me1 ratio in the first 500 bp of the ORF). D-E) Snapshots of depth-normalized ChIP-seq data tracks from wild-type and *rp3Δ* strains showing 7 kb surrounding the meiotic gene *ZIP1* (D), and telomeric genes *AIF1* and *COS10* (E). All tracks have the same y axis (0-2), which, for comparison, is also the same scale as in Fig. 1E.

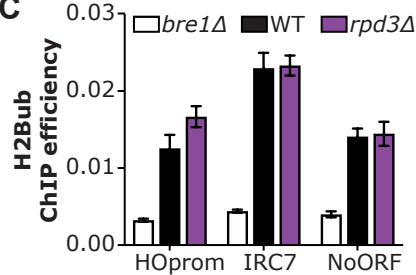
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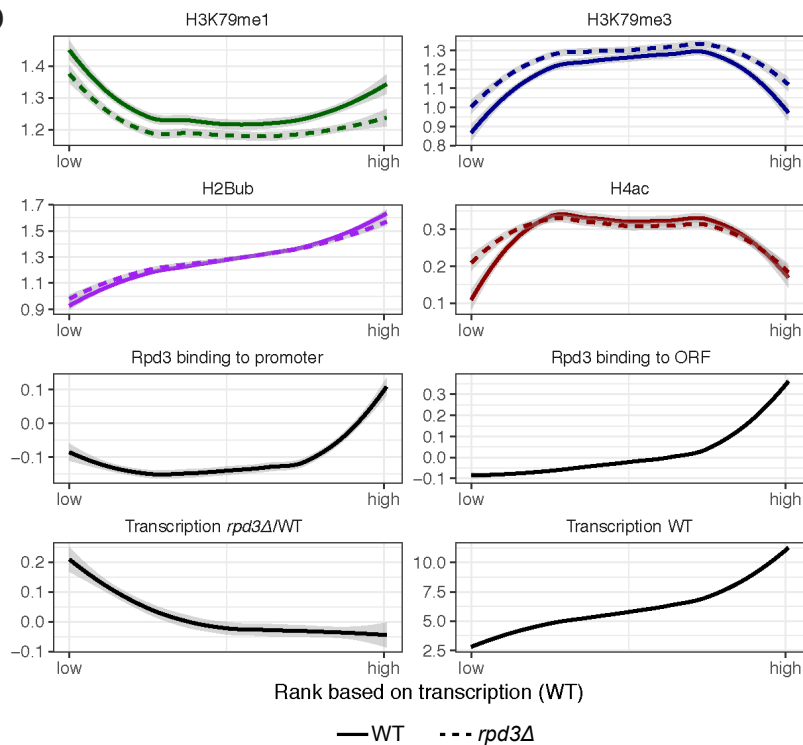
B



C

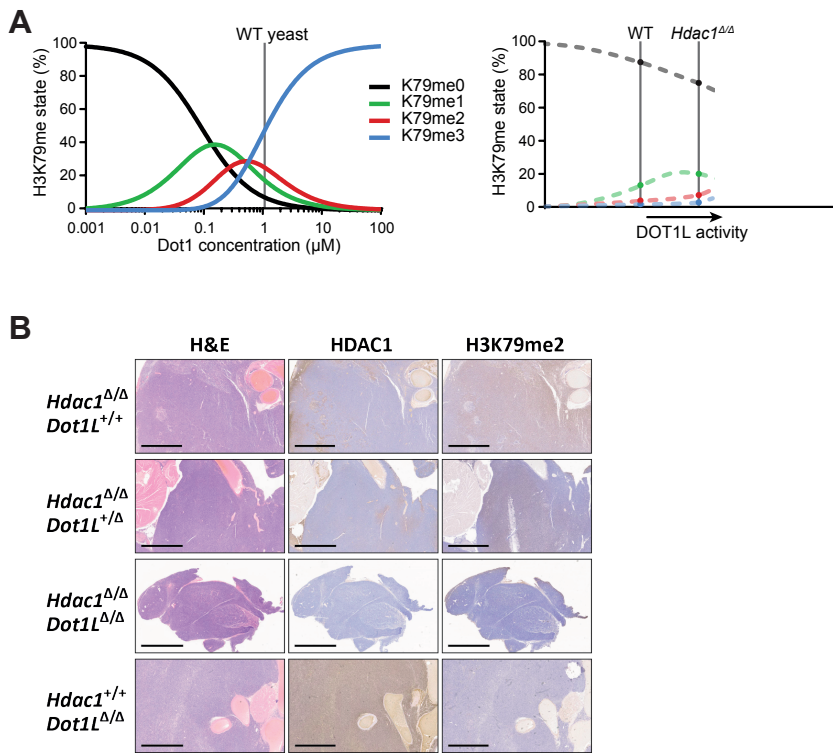


D



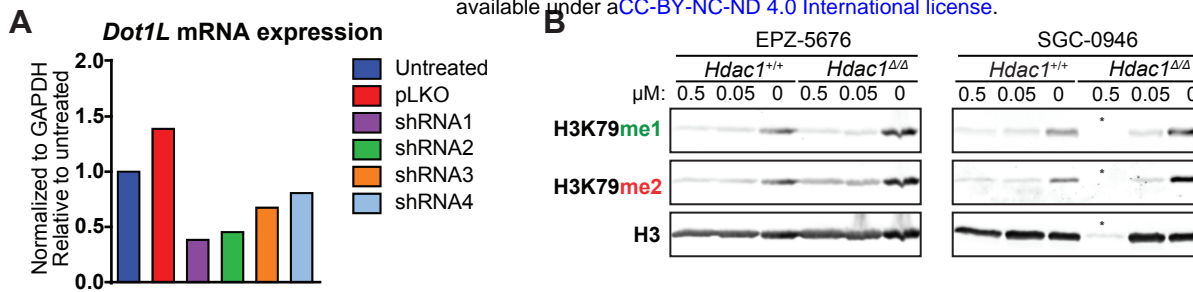
## Supplemental Figure 2 ChIP-seq and ChIP-qPCR data from WT vs *rpd3Δ* cells and the relation between transcription and histone modifications.

A) Heatmaps of H3K79me1, H3K79me3, H2Bub and H3 sorted on H3K79me3/H3 *rpd3Δ*/WT. B) H2B-normalized H2Bub ChIP-qPCR IP efficiencies at several pre-selected loci in wild-type and *rpd3Δ* cells, with *bre1Δ* cells serving as a negative control. C) H2Bub ChIP-qPCR IP efficiencies at several pre-selected loci in wild-type and *rpd3Δ* cells, with *bre1Δ* cells serving as a negative control. D) ChIP-seq and RNA-seq data for genes ranked on transcription level in wild-type cells, smoothed using locally weighed regression. The shaded band around the line shows the 95% confidence interval. For H3K79me1, H3K79me3, H2Bub, H4ac and Rpd3 binding to ORF, the average coverage in the first 500 bp was used. Rpd3 binding to promoter was the average coverage in the 400 bp upstream of the TSS. Transcription in wild-type cells



**Supplemental Figure 3 The distributive nature of Dot1, and thymus immunohistochemistry.**

A) Yeast Dot1 is a distributive enzyme and shows waves of the different methylation states over a range of Dot1 concentrations in yeast (De Vos et al. 2011), figure from De Vos et al. (2017). The catalytic nature of mammalian DOT1L enzymes is not known, but the observation that the abundance of each methylation state increases upon *Hdac1* deletion does not conflict with a distributive nature. Since H3K79 methylation levels are low in mammalian cells, it is possible we are looking at the start of the methylation waves, as indicated by the dotted lines. B) Representative H&E and immunohistochemical stainings of thymic lymphomas of the indicated genotypes. The scale bar represents 2 mm.



### Supplemental Figure 4 Control experiments belonging to Figure 4.

A) Knockdown efficiency of the shRNAs used in Figure 4D. The shRNAs 1 and 2 give a knockdown of more than 50%. B) Immunoblot analysis of H3K79 methylation levels in the two cell lines with and without inhibitors. Both the effect of the inhibitors and the difference between the cell lines can be observed. \* indicates a lane that was underloaded because most cells had died.

## Supplemental Methods

### Mouse generation and crosses

The *Dot1L*<sup>tm1a(KOMP)Wtsi</sup> mouse was generated by the Wellcome Trust Sanger Institute (WTSI) and obtained from the KOMP Repository ([www.komp.org](http://www.komp.org)) (Skarnes et al. 2011). Since this mouse had a knock-out first allele, FLPe in a C57BL/6 background was crossed in to remove the FRT-flanked cassette (B6.Cg-Tg(ACTFLPe)9205Dym/A, MGI:2448985; Rodríguez et al. 2000). FLPe was crossed out to obtain C57BL/6 *Dot1L*<sup>ff</sup> mice, with LoxP sites flanking exon 2 of *Dot1L*. No thymic lymphomas were observed in C57BL/6 *Lck-Cre;Dot1L*<sup>ff</sup> mice. For this study, mice with the conditional *Dot1L* allele were crossed with mice bearing *Lck-Cre* and conditional *Hdac1* alleles, which were described before (Heideman et al. 2013). All mice analyzed in this study were progeny of this cross and were in a mixed FVB/n, C57BL/6, and 129/Sv background. Wild-type control mice were the *Lck-Cre*-negative littermates of the other mice used in this study.

### Nuclear extract preparation and immunoblotting

Thymic lymphoma cell lines were collected and washed by PBS. Single-cell suspensions of thymuses were obtained by passing the tissues through a 70 µm cell strainer, and cells were pelleted and washed with PBS. Samples were kept cold at all times and all buffers were supplemented with Complete protease inhibitors (Roche), Trichostatin A and nicotinamide. To make nuclear extracts, cells were first incubated in hypotonic lysis buffer (10mM Tris (pH 7.8), 5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.1 mM EDTA, 300 mM sucrose, 5 mM B-glycerol) for ten minutes. Nonidet P-40 was added to an end concentration of 0.12% to rupture the cells. Nuclei were collected by centrifugation and lysed in RIPA buffer (20mM Tris (pH 7.5), 150mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1mM EDTA, 0.1% SDS) for 30 minutes. All buffers were supplemented with Complete protease inhibitors (Roche), Trichostatin A and nicotinamide. Samples were sonicated for 2.5 minutes (10 second pulses) using the Diagenode Biorupter to solubilize chromatin. After this step, debris was pelleted and the supernatant was collected. Protein concentration was determined using the DC protein assay (Bio-Rad). The immunoblotting procedure was as described in (Vlaming et al. 2014). Yeast extracts were loaded on 16% polyacrylamide gels; murine extracts were loaded on gradient gels (4-12% Bis-Tris NuPAGE mini gels).

### Antibodies

Blots with yeast samples were probed with antibodies against Dot1 (RRID: AB\_2631109; Van Leeuwen et al. 2002), Pgc1 (A-6457, Invitrogen) and H2B (39238, Active Motif). Blots with mouse samples were probed with antibodies against HDAC1 (NB100-56340, Novus Biologicals), H3K79me1 and H3K79me2 (RRID:AB\_2631105 and AB\_2631106; Frederiks et al. 2008), H2BK120ub (#5546, Cell Signaling Technology), H3K9ac (ab4441, Abcam), total H3 (ab1791, Abcam), and a newly generated H4 antibody. Rabbit polyclonal antibodies against histone H4 were generated by immunizing with the peptide (C)VYALKRQGRPLYGFG of the C terminus of histone H4 of *S. cerevisiae*. The polyclonal serum recognizes human and mouse histone H4. ChIP experiments were performed using antibodies against H3K79me1, H3K79me3 and total H3 (RRID:AB\_2631105, AB\_2631107, AB\_2631108; Frederiks et al. 2008) and antibodies against H2B (39238, Active Motif) and a new site-specific antibody against yeast H2Bub that we recently developed (Vlaming et al. 2016). For immunohistochemistry, antibodies against HDAC1 (ab31263; Abcam) and H3K79me2 (RRID:AB\_2631106; Frederiks et al. 2008) were used.

### ChIP-seq library preparation and data analysis

Library preparation and sequencing were performed by the NKI Genomics Core Facility, in two batches. Libraries from the ChIP samples from WT #1 were prepared using the TruSeq<sup>®</sup> DNA LT Sample Preparation kit (Illumina, cat no. FC-121-2001), using ten times less adapter in the adapter ligation step. After fifteen PCR cycles, a size selection cleanup was performed using 0.5X Agencourt AMPure

XP PCR Purification beads (Beckman Coulter, cat no A63881) to get rid of large fragments due to crosslinking DNA. The supernatant of the 0.5X cleanup was used to catch the smaller fragments; this supernatant was cleaned up 2 times with 1X beads to remove primers present in the libraries. Samples were pooled equimolarly and subjected to sequencing on an Illumina HiSeq2000 machine in a single-read 50bp run. Libraries from the ChIP samples from WT #2 and two *rpm3Δ* replicates were prepared using the KAPA HTP Library Preparation Kit, Illumina® platforms (KAPA Biosystems KK8234), using Illumina-provided adapters at 200nM. After eleven PCR cycles, cleanup and pooling was as described above. Samples were sequenced in a single-read 65bp run on an Illumina HiSeq2500 machine. ChIP libraries from an *sin3Δ* strain prepared in the first batch (with WT #1) gave results comparable to the two *rpm3Δ* replicates from batch two. Reads were mapped to the *Saccharomyces cerevisiae* reference genome R64-2-1 with BWA version 0.6.1 and filtered for mapping quality 37 (Engel et al. 2014; Li and Durbin 2009). Each read was extended to 150 bp. Each sample was normalized for the sequencing depth by converting to Reads per Genomic Content (RPGC) with DeepTools (Ramírez et al. 2016). This was done by dividing the coverage by the sequencing depth, calculated as (total number of mapped reads \* fragment length) / effective genome size ( $12.1 \times 10^6$  bp). Data from the biological duplicates was found to be similar and the data sets were merged for further analyses.

#### Epi-ID analysis

Data from the Epi-ID H3K79me regulator screen described in Vlaming et al. (2016) were used. As described, a growth-corrected methylation score was calculated by first calculating the H3K79me3/H3K79me1 ratio and then subtracting the value expected based on the growth rate of the strain (Vlaming et al. 2016). The CLIK tool (Dittmar et al. 2013) was used to define groups of candidate regulators, and to determine the enrichment of the Rpd3L complex. Data on all components of Rpd3L and Rpd3S was obtained in the screen. Deletions were checked by PCR and barcodes were checked by Sanger sequencing. All deletions could be confirmed, with the exception of *sds3Δ*, which was eliminated from the plot in Figure 1B.

## Supplemental references

De Vos D, Frederiks F, Terweij M, van Welsem T, Verzijlbergen KF, Iachina E, de Graaf EL, Altelaar AFM, Oudgenoeg G, Heck AJR, et al. 2011. Progressive methylation of ageing histones by Dot1 functions as a timer. *EMBO Rep* **12**: 956–62.

De Vos D, Vlaming H, Bakker BM, Van Leeuwen F. 2017. Modeling Distributive Histone Modification by Dot1 Methyltransferases. In *Epigenetics and Systems Biology*, pp. 117–141, Elsevier.

Dittmar JC, Pierce S, Rothstein R, Reid RJD. 2013. Physical and genetic-interaction density reveals functional organization and informs significance cutoffs in genome-wide screens. *Proc Natl Acad Sci* **110**: 7389–7394.

Engel SR, Dietrich FS, Fisk DG, Binkley G, Balakrishnan R, Costanzo MC, Dwight SS, Hitz BC, Karra K, Nash RS, et al. 2014. The Reference Genome Sequence of *Saccharomyces cerevisiae*: Then and Now. *G3* **4**: 389–398.

Frederiks F, Tzouros M, Oudgenoeg G, van Welsem T, Fornerod M, Krijgsveld J, van Leeuwen F. 2008. Nonprocessive methylation by Dot1 leads to functional redundancy of histone H3K79 methylation states. *Nat Struct Mol Biol* **15**: 550–557.

Heideman MR, Wilting RH, Yanover E, Velds A, de Jong J, Kerkhoven RM, Jacobs H, Wessels LF, Dannenberg J-H. 2013. Dosage-dependent tumor suppression by histone deacetylases 1 and 2 through regulation of c-Myc collaborating genes and p53 function. *Blood* **121**: 2038–2050.

Kemmeren P, Sameith K, van de Pasch LAL, Benschop JJ, Lenstra TL, Margaritis T, O’Duibhir E, Apweiler E, van Wageningen S, Ko CW, et al. 2014. Large-Scale Genetic Perturbations Reveal Regulatory Networks and an Abundance of Gene-Specific Repressors. *Cell* **157**: 740–752.

Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**: 1754–1760.

McKnight JN, Boerma JW, Breeden LL, Tsukiyama T. 2015. Global Promoter Targeting of a Conserved Lysine Deacetylase for Transcriptional Shutoff during Quiescence Entry. *Mol Cell* **59**: 732–743.

Ramírez F, Ryan DP, Grüning B, Bhardwaj V, Kilpert F, Richter AS, Heyne S, Dündar F, Manke T. 2016. deepTools2: a next generation web server for deep-sequencing data analysis. *Nucleic Acids Res* **44**: W160–W165.

Rodríguez CI, Buchholz F, Galloway J, Sequerra R, Kasper J, Ayala R, Stewart AF, Dymecki SM. 2000. High-efficiency deleter mice show that FLP is an alternative to Cre-loxP. *Nat Genet* **25**: 139–40.

Skarnes WC, Rosen B, West AP, Koutsourakis M, Bushell W, Iyer V, Mujica AO, Thomas M, Harrow J, Cox T, et al. 2011. A conditional knockout resource for the genome-wide study of mouse gene function. *Nature* **474**: 337–342.

Van Leeuwen F, Gafken PR, Gottschling DE. 2002. Dot1p Modulates Silencing in Yeast by Methylation of the Nucleosome Core. *Cell* **109**: 745–756.

Vlaming H, Molenaar TM, van Welsem T, Poramba-Liyanage DW, Smith DE, Velds A, Hoekman L, Korthout T, Hendriks S, Altelaar AM, et al. 2016. Direct screening for chromatin status on DNA barcodes in yeast delineates the regulome of H3K79 methylation by Dot1. *Elife* **5**: e18919.

Vlaming H, van Welsem T, de Graaf EL, Ontoso D, Altelaar AM, San-Segundo PA, Heck AJ, Van Leeuwen F. 2014. Flexibility in crosstalk between H2B ubiquitination and H3 methylation in vivo. *EMBO Rep* **15**: 1077–1084.