The histone H3K79 methyltransferase Dot1L is essential for mammalian development and heterochromatin structure

Brendan Jones
Novartis Institutes for Biomedical Research, Cambridge, Massachusetts

Hui Su
Novartis Institutes for Biomedical Research, Cambridge, Massachusetts

Audesh Bhat
Washington University School of Medicine in St. Louis

Hong Lei
Novartis Institutes for Biomedical Research, Cambridge, Massachusetts

Jeffrey Bajko
Novartis Institutes for Biomedical Research, Cambridge, Massachusetts

See next page for additional authors

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Authors
Brendan Jones, Hui Su, Audesh Bhat, Hong Lei, Jeffrey Bajko, Sarah Hevi, Gretchen A. Baltus, Shilpa Kadam, Huili Zhai, Reginald Valdez, Susana Gonzalo, Yi Zhang, En Li, and Taiping Chen
The Histone H3K79 Methyltransferase Dot1L Is Essential for Mammalian Development and Heterochromatin Structure

Brendan Jones1, Hui Su1, Aadesh Bhat2, Hong Lei3, Jeffrey Bajko1, Sarah Hevi1, Gretchen A. Baltus1, Shilpa Kadam1, Huili Zhai4, Reginald Valdez3, Susana Gonzalez2, Yi Zhang5,6, En Li1, Taiping Chen1*

1 Epigenetics Program, Novartis Institutes for Biomedical Research, Cambridge, Massachusetts, United States of America, 2 Department of Radiation Oncology, Radiation and Cancer Biology Division, Washington University School of Medicine, St. Louis, Missouri, United States of America, 3 Developmental and Molecular Pathways, Novartis Institutes for Biomedical Research, Cambridge, Massachusetts, United States of America, 4 Analytical Sciences, Novartis Institutes for Biomedical Research, Cambridge, Massachusetts, United States of America, 5 Howard Hughes Medical Institute, Chevy Chase, Maryland, United States of America, 6 Department of Biochemistry and Biophysics, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, United States of America

Abstract

Dot1 is an evolutionarily conserved histone methyltransferase specific for lysine 79 of histone H3 (H3K79). In Saccharomyces cerevisiae, Dot1-mediated H3K79 methylation is associated with telomere silencing, meiotic checkpoint control, and DNA damage response. The biological function of H3K79 methylation in mammals, however, remains poorly understood. Using gene targeting, we generated mice deficient for Dot1L, the murine Dot1 homologue. Dot1L-deficient embryos show multiple developmental abnormalities, including growth impairment, angiogenesis defects in the yolk sac, and cardiac dilation, and die between 9.5 and 10.5 days post coitum. To gain insights into the cellular function of Dot1L, we derived embryonic stem (ES) cells from Dot1L mutant blastocysts. Dot1L-deficient ES cells show global loss of H3K79 methylation as well as reduced levels of heterochromatic marks (H3K9 di-methylation and H4K20 tri-methylation) at centromeres and telomeres. These changes are accompanied by aneuploidy, telomere elongation, and proliferation defects. Taken together, these results indicate that Dot1L and H3K79 methylation play important roles in heterochromatin formation and in embryonic development.

Introduction

Histones are subject to a variety of post-translational modifications, including acetylation, phosphorylation, ubiquitination, and methylation. These modifications dictate chromatin structure by affecting the recruitment of nonhistone proteins and/or the interactions between nucleosomes [1,2]. Heterochromatin is associated with high levels of methylation at H3K9, H3K27, and H4K20 and low levels of acetylation, whereas actively transcribed euchromatin is typically enriched with acetylation and methylated H3K4, H3K36, and H3K79.

H3K79 modifications occur on residues within the N-terminal tail. In contrast, H3K79 is located in a loop within the globular domain, exposed on the nucleosome surface. The yeast Dot1 and its homologues in other species are the only known H3K79 methyltransferases [3–5]. Unlike other histone lysine methyltransferases, Dot1 family members do not have a SET domain [3–5]. Instead, their catalytic domain contains conserved sequence motifs characteristic of class I methyltransferases such as DNA methyltransferases (DNMTs) and the protein arginine methyltransferase PRMT1 [6].

Dot1 was initially identified as a disruptor of telomeric silencing in Saccharomyces cerevisiae [3]. Subsequent studies showed that both overexpression and inactivation of Dot1 as well as mutations at H3K79 all lead to loss of telomeric silencing [7–9]. Although the mechanisms by which Dot1 affects telomere structure and function are not fully understood, it is believed that H3K79 methylation plays an important role in restricting the Sir proteins at heterochromatic regions [7,8,10]. Dot1-dependent H3K79 methylation has also been shown to be involved in meiotic checkpoint control and in G1 and S phase DNA damage checkpoint functions of Rad9 in yeast [11,12].

H3K79 methylation is also a widespread histone modification in mammalian cells [4]. Abnormal H3K79 methylation has been linked to leukemogenesis in humans [13,14]. However, the biological function of H3K79 methylation in mammals remains largely unknown. Here we generated a mouse line containing a null mutation in Dot1L, the murine Dot1 homologue, and investigated the role of Dot1L and H3K79 methylation in embryonic development and cellular function. We provide evidence that Dot1L is required for embryogenesis and for the integrity of constitutive heterochromatin at the cellular level.

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Generation of Dot1L Conditional and Null Alleles in Mice

To target the Dot1L gene, we constructed a targeting vector in which a 2.3-kb genomic region containing exons 3 and 6 and a promoterless β-gal selection cassette were flanked, respectively, by

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Histone methylation plays a critical role in the regulation of gene expression and chromatin structure. Among the sites of histone methylation, lysine 79 of histone H3 (H3K79) is unique in that it is not located within the H3 N-terminal tail but in the globular domain. Our knowledge about H3K79 methylation comes primarily from studies in yeast. This study focuses on the role of H3K79 methylation in mammalian development and cellular function. We show that genetic disruption of Dot1L, the only known H3K79 methyltransferase gene in mouse, results in embryonic lethality. At the cellular level, Dot1L deficiency leads to alterations in constitutive heterochromatin, accompanied by telomere elongation, aneuploidy, and proliferation defects. Our work represents a key step toward understanding the function of H3K79 methylation in mammals.

**Dot1L in Development and Heterochromatin**

Dot1L is essential for embryonic development. We first determined the expression of Dot1L during embryonic development, taking advantage of the fact that cells containing the Dot1Llox allele express lacZ under the control of the endogenous Dot1L promoter. We conducted X-gal staining on Dot1Llox heterozygous embryos and wild-type littermates at different stages of development. Dot1L expression is ubiquitous as early as 7.5-dpc (the earliest time point tested, Figure 2A). At 9.5-dpc, Dot1L expression remains ubiquitous and areas of elevated expression are
apparent. Tissues that demonstrate high levels of lacZ staining include the optic vesicle, the first branchial arch, the limb buds, the heart, the otic pit, and the neural ectoderm (Figure 2B). Dot1L is also expressed at high levels in extra-embryonic tissues, including the visceral endoderm and visceral mesoderm of the yolk sac, and in primitive erythrocytes (Figure 2C). Similar lacZ staining patterns are observed in embryos harvested at 10.5-dpc, 11.5-dpc, and 12.5-dpc (data not shown), suggesting that Dot1L is broadly expressed during embryonic development.

Dot1L1lox/+ mice were grossly normal and fertile. However, intercrosses of Dot1L1lox/+ mice produced no viable Dot1L1lox/lox homozygous offspring, suggesting embryonic lethality (Table 1). Dot1L1lox/lox embryos harvested at 8.5-dpc were indistinguishable from wild-type and Dot1L1lox/+ littermates (data not shown). At 9.5-dpc, Dot1L1lox/lox embryos were undersized, had an enlarged heart (cardiac dilation) and stunted tail (center), while approximately 15% exhibited developmental arrest at E8.5 (right). Dot1L1lox/lox embryos harvested at 10.5-dpc (left) and Dot1L1lox/lox (right) embryos. The vasculature of the Dot1L1lox/lox yolk sac is thinner and less organized than that of the heterozygous littermate. doi:10.1371/journal.pgen.1000190.g002

Table 1. Dot1L deficiency results in embryonic lethality.

<table>
<thead>
<tr>
<th>Litters</th>
<th>8.5-dpc</th>
<th>9.5-dpc</th>
<th>10.5-dpc</th>
<th>11.5-dpc</th>
<th>12.5-dpc Birth</th>
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<td>67</td>
<td>105</td>
<td>37</td>
<td>28</td>
<td>16</td>
</tr>
<tr>
<td>Dot1L+/+</td>
<td>14 (21%)</td>
<td>26 (25%)</td>
<td>11 (30%)</td>
<td>8 (29%)</td>
<td>3 (19%)</td>
</tr>
<tr>
<td>Dot1L1lox/+</td>
<td>34 (51%)</td>
<td>50 (48%)</td>
<td>17 (46%)</td>
<td>13 (46%)</td>
<td>5 (31%)</td>
</tr>
<tr>
<td>Dot1L1lox/lox</td>
<td>14 (21%)</td>
<td>20 (19%)</td>
<td>3 (8%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>resorbed</td>
<td>5 (7%)</td>
<td>9 (8%)</td>
<td>6 (16%)</td>
<td>7 (25%)</td>
<td>8 (50%)</td>
</tr>
</tbody>
</table>

The numbers and percentages of Dot1L+/+, Dot1L1lox/+, Dot1L1lox/lox, and resorbed embryos harvested at the indicated time points are shown. NA: not applicable. doi:10.1371/journal.pgen.1000190.0001
under a dissecting microscope (Figure 2D, center). Approximately 15% of the Dot1L<sup>lox/lox</sup> embryos demonstrated a severe phenotype, exhibiting developmental arrest at E8.5 (Figure 2D, right). Histological examination of 9.5-dpc Dot1L<sup>lox/lox</sup> embryo sections revealed focal areas of extensive apoptosis, but no obvious structural defects (Figure S1). At 10.5-dpc, the percentage of viable Dot1L<sup>lox/lox</sup> embryos was substantially below the expected Mendelian ratio (Table 1), suggesting that many of the Dot1L<sup>lox/lox</sup> embryos die during this time interval. The few that survived to this stage exhibited developmental arrest at E9.5 and severe cardiac dilation (Figure 2E). No Dot1L<sup>lox/lox</sup> embryos survived beyond 10.5-dpc (Table 1).

As stunted growth and enlarged heart are phenotypes that often occur as a result of defects in extraembryonic tissues, we examined the yolk sac and placenta of 9.5-dpc Dot1L<sup>lox/lox</sup> embryos. While the placenta showed no obvious defects, the yolk sac exhibited abnormal vascular morphology. The yolk sac vasculature was present and contained primitive erythrocytes, but was frequently underdeveloped and disorganized when compared to control littermates (Figure 2F). These observations indicate that, in the absence of Dot1L, vasculogenesis took place in the yolk sac but angiogenesis was defective.

**Dot1L Deficiency in ES Cells Results in Growth Defects**

To investigate the cellular function of Dot1L, we derived Dot1L mutant ES cells from blastocysts produced from intercrosses of Dot1L<sup>lox/+</sup> mice. Two Dot1L<sup>lox/lox</sup> and multiple Dot1L<sup>lox/+</sup> and Dot1L<sup>+/+</sup> lines were established. As expected, H3K79 di- and tri-methylation was greatly reduced in Dot1L<sup>lox/lox</sup> cells compared to Dot1L<sup>lox/+</sup> cells (Figure 3A). Dot1L<sup>lox/lox</sup> cells had intermediate levels of H3K979 di- and tri-methylation, indicating haploinsufficiency of Dot1L (Figure 3A). Surprisingly, Western blot analysis using a "mono methyl H3K79" antibody (ab2886, Abcam) detected no change in signal intensity in Dot1L mutant ES cell lines (data not shown). To verify the results, we carried out mass spectrometry. In wild-type ES cells, ~11% of histone H3 showed K79 methylation, among which mono-, di-, and tri-methylation accounted for ~70%, ~30%, and less than 1%, respectively. In Dot1L<sup>lox/lox</sup> ES cells, H3K79 mono- and tri-methylation was absent although trace amount of di-methylation was detected (Figure 3B and Figure S2). We therefore concluded that the Western blot result showing no alteration in H3K79 mono-methylation in the absence of Dot1L was an artifact due to nonspecific recognition of histone H3 by the "mono methyl H3K79" antibody. The low level of H3K79 di-

**Figure 3. Phenotypic analysis of Dot1L mutant ES cells.** (A) Western blot analysis using extracts from ES cell lines of the indicated Dot1L genotypes and antibodies specific for di-, and tri-methylated H3K79. Total histone H3 was used as a loading control. (B) Analysis of H3K79 methylation by mass spectrometry. Quantification of different forms of H3K79 methylation was obtained by comparing the extracted ion chromatogram (EIC) intensity of the ion signals corresponding to the unmodified (Me0), mono-methylated (Me1), di-methylated (Me2), and tri-methylated (Me3) K79-containing peptides. (C) The proliferation of Dot1L<sup>+/+</sup>, Dot1L<sup>lox/lox</sup> and Dot1L<sup>lox/lox</sup> ES cells was determined by doing cell counts every 24 hours for five days. Cells were grown in triplicate, and data shown is representative of three independent experiments. (D) The percentages of apoptotic cells in Dot1L<sup>+/+</sup>, Dot1L<sup>lox/lox</sup> and Dot1L<sup>lox/lox</sup> ES cell cultures. The asterisk indicates P<0.05 (Student t-test). ES cells were stained with propidium iodide (PI) and PE conjugated anti-annexin V antibodies and analyzed by FACS. Apoptotic cells were annexin V positive and PI negative. Cells were grown in triplicate, and data shown are representative of two independent experiments. (E) The percentages of each cell cycle stage in Dot1L<sup>+/+</sup>, Dot1L<sup>lox/lox</sup> and Dot1L<sup>lox/lox</sup> ES cell cultures as determined by PI staining and FACS.

doi:10.1371/journal.pgen.1000190.g003
methylation detected in 

Dot1Llox/lox samples could be from feeder cells present in the culture or due to incomplete inactivation of Dot1L. Taken together, these results indicated that Dot1L is most likely the sole H3K79 methyltransferase in mice.

Dot1L mutant ES cells maintained an undifferentiated state, as judged by morphology and ES cell markers such as Oct4 and mouse embryonic stem (ES) cell markers. We plated 3\times10^5 Dot1Llox/+ and Dot1Llox/lox ES cells in standard ES cell medium and monitored proliferation. By 24 hours, the number of Dot1Llox/+ ES cells (7.3 x 10^5) was significantly higher than the number of Dot1Llox/+ or Dot1Llox/lox cells (4.2 x 10^5) and 3.6 x 10^5, respectively. P<0.05, Figure 3C). Over the 5 days examined, Dot1Llox/+, Dot1Llox/lox, and Dot1Llox/lox ES cells had average doubling times of 16, 22, and 26 hours, respectively. The fact that both Dot1Llox/+ and Dot1Llox/lox cells exhibited growth defects showed the importance of Dot1L gene dosage in cellular function. The reduction of H3K79 methylation in Dot1Llox/+ ES cells (haploinsufficiency) suggested that Dot1L level is relatively low. Interestingly, Dot1Llox/+ mice were apparently normal despite the defects in Dot1Llox/+ ES cells. It is possible that 50% of Dot1L can barely maintain normal cellular function under ideal conditions (e.g. in vivo) but is not sufficient to do so under suboptimal conditions (e.g. in culture).

We next examined apoptosis and cell cycle status of the Dot1L mutant ES cells. Annexin V staining revealed that 4.0% of the Dot1Llox/lox ES cells and 3.1% of the Dot1Llox/+ ES cells were annexin V positive, while only 1.2% of the Dot1Llox/+ ES cells were annexin V positive (Figure 3D). This indicates that more than twice as many of the Dot1L mutant ES cells were undergoing apoptosis compared to wild-type ES cells. Furthermore, cell cycle analysis by propidium iodide staining revealed an elevated percentage of G2 cells and a reduced percentage of G1 cells among the Dot1L mutant ES cells when compared to the wild-type ES cells (Figure 3E). These results suggest that both elevated apoptosis and G2 cell cycle arrest contribute to the reduced growth rate of Dot1L mutant ES cells.

Dot1L-Deficient ES Cells Show Telomere Elongation and Aneuploidy

Dot1-deficient S. cerevisiae show telomere elongation and defects in telomere silencing [3]. We therefore evaluated the effect of Dot1L inactivation on telomere length. First, we used Southern blot terminal restriction fragment (TRF) analysis to estimate telomere length in two ES cell lines of each Dot1L genotype. Both Dot1Llox/lox lines and one of the Dot1Llox/+ lines showed telomere elongation, as evidenced by the presence of high molecular weight (MW) TRFs and the increase in the lengths of bulk TRFs compared to wild-type controls (Figure 4A). Next, we carried out quantitative fluorescence in situ hybridization (Q-FISH) using a telomere-specific probe to determine the mean telomere length (ml) and the distribution of telomere lengths for each cell line (Figure 4, B and C). Consistent with the TRF results, both Dot1Llox/lox lines had higher ml, greater percentages of elongated (>100 kb) telomeres, and reduced percentages of short (<50 kb) telomeres compared to Dot1Llox/+ lines (Figure 4C). The heterozygous ES cells again showed an intermediate phenotype (Figure 4C).

Examination of the Q-FISH samples revealed frequent aneuploidy in Dot1L-deficient cells (Figure 4B). To further investigate this phenotype, we prepared metaphase chromosome spreads from Dot1Llox/lox and Dot1Llox/+ ES cells and examined them for chromosomal defects. Dot1Llox/+ cells were karyotypically stable, as the vast majority had 40 chromosomes. In contrast, over 40% of metaphase Dot1Llox/lox cells were aneuploid. Most of the aneuploid cells showed gain of chromosomes and some ended up being tetraploid (Figure 4, B and D). Aside from aneuploidy, no obvious chromosomal abnormalities were frequently observed in Dot1L-deficient cells (Figure 4B). These results point to defects in chromosome segregation in the absence of Dot1L.

Aberrant Telomere Elongation in Dot1L-Deficient Cells Correlates with Activation of the ALT Pathway

Our data suggested a role for Dot1L in the homeostasis of telomere length. Two main mechanisms have been described for the maintenance of mammalian telomeres: the addition of telomeric repeats by telomerase and the so-called alternative lengthening of telomere (ALT) mechanism that relies on homologous recombination between telomeric sequences [15,16]. Dot1L mutant ES cells showed increased telomere heterogeneity (Figure 4), which is a hallmark of ALT cells [17]. To determine whether the ALT pathway is activated in Dot1L-deficient cells, we assessed the presence of ALT-associated PML bodies (APBs, colocalization of PML and telomeres), another hallmark of ALT [17]. Dot1Llox/+, Dot1Llox/lox, as well as Dnm3a+/−/−/−, −/−/−/− (positive control) ES cells were immuno-stained with antibodies against TRF1 (a telomere-binding protein) and PML. In the absence of Dot1L, both the frequency of cells showing APBs and the number of APBs per cell were significantly increased compared to wild-type cells (Figure 5, A−G). χ² tests, P<0.001, suggesting that aberrant elongation of telomeres in Dot1L-deficient cells was due, at least in part, to activation of the ALT pathway.

Dot1L Deficiency Results in Loss of Heterochromatin Marks at Telomeres and Centromeres

Aneuploidy and telomere elongation can result from defects in the chromatin structure at centromeres and telomeres, respectively [18-23]. To evaluate changes in chromatin structure in Dot1L mutant cells, we used chromatin immunoprecipitation (ChIP) to examine histone modifications at major satellite repeats (present at pericentric regions), minor satellite repeats (present at centromeric regions), telomeric repeats, and subtelomeric regions (Figure 6). H3K79 di-methylation was detected in all these heterochromatin regions in Dot1Llox/+ cells (Figure 6, A and B). As expected, this modification was reduced in Dot1Llox/lox cells and almost absent in Dot1Llox/lox cells (Figure 6, A and B), validating our experimental procedures. As further controls, the levels of centromere- and telomere-bound histone H3 were similar in wild-type and Dot1L mutant cells (Figure 6, A and B), and the telomere-binding protein TRF1 associated with telomeric repeats, but not with major satellite repeats (Figure 6B).

In Dot1Llox/lox cells, H4K20 tri-methylation, a hallmark of constitutive heterochromatin, was greatly reduced at minor satellite repeats and sub-telomeric regions, and moderately reduced at major satellite repeats (Figure 6A). Consistent with this observation, immunofluorescence analysis revealed the loss of enrichment of H4K20 tri-methylation at pericentric heterochromatin in the absence of Dot1L (Figure S3). H3K9 di-methylation, but not H3K9 tri-methylation, was reduced in all regions examined in Dot1Llox/lox cells (Figure 6, A and B). Concomitantly, H3K9 mono-methylation showed marked increases at major satellite repeats and minor satellite repeats (Figure 6A), and H3K9 acetylation, a mark of euchromatin, was elevated in all regions examined (Figure 6, A and B). These changes appeared to be heterochromatin-specific, as all histone modifications examined, except H3K9 methylation, showed no global changes in Dot1L mutant cells (Figure S4). Dot1L deficiency did not cause...
alterations in DNA methylation at major satellite repeats and minor satellite repeats as well as other genomic regions such as the intracisternal A-particle (IAP) retroviral elements (Figure S5). Altogether, these results suggest that loss of H3K79 methylation results in a less compacted (or more open) chromatin state at centromeres and telomeres.

**Discussion**

In this report, we provide genetic evidence that Dot1L and, by implication, H3K79 methylation are essential for mammalian development and normal cellular function. We show that loss of Dot1L results in yolk sac angiogenesis defects and embryonic...
lethality. Furthermore, our characterization of Dot1L-deficient ES cells reveals that, like in yeast, H3K79 methylation plays a critical role in heterochromatin structure in mammalian cells. Considering the differences in chromatin structure between yeast and mammals, the phenotypic similarities in mutants of these organisms are both striking and surprising. For example, both mutant organisms exhibit telomere elongation, but mammalian telomeres contain H3K79 methylated histones, while \textit{S. cerevisiae} telomeres contain no histones at all. Furthermore, while \textasciitilde{}7% of the budding yeast genome is packaged as heterochromatin (including rDNA), \textasciitilde{}55% of the mammalian genome is composed of heterochromatin [24].

Dot1L recruitment is coupled with gene transcription [25] and H3K79 methylation is enriched in euchromatin, which seem to be counterintuitive to the heterochromatin phenotype of Dot1L-deficient cells. One possible explanation is that Dot1L inactivation alters the expression of specific factors involved in heterochromatin assembly. Alternatively, global loss of H3K79 methylation may result in redistribution of heterochromatin factors, thereby reducing their relative abundance at constitutive heterochromatin. Indeed, loss of Dot1 in yeast leads to mislocalization of the Sir proteins, which promote heterochromatin formation and telomere silencing [7,8].

H3K9 tri-methylation and H4K20 tri-methylation are hallmarks of constitutive heterochromatin, such as that at centromeres and...
telomeres [20,21,23,26,27]. Based on the observation that H3K9 trimethylation by the Suv39h methyltransferases is required for the induction of H4K20 tri-methylation by the Suv4-20h methyltransferases at pericentric heterochromatin, a sequential model of chromatin assembly at constitutive heterochromatin has been proposed in which Suv4-20h enzymes act downstream of the Suv39h enzymes [26]. Dot1L-deficient cells show loss of H4K20 trimethylation at telomeres and centromeres, suggesting that Dot1L functions upstream of the Suv20h enzymes. Given that H3K9 trimethylation shows no obvious alterations in Dot1L-deficient cells, it is possible that Dot1L acts in parallel or downstream of the Suv39h enzymes. Interestingly, despite the relatively normal levels of H3K9 tri-methylation, H3K9 di-methylation is severely reduced at constitutive heterochromatin in the absence of Dot1L. Because the
total levels of H3K9 di-methylation and of several H3K9 methyltransferases (Suv39h1, ESET, and G9a) are not altered in Dot1L-deficient cells (Figure S4), we speculate that Dot1L deficiency may affect the targeting of one or more H3K9 methyltransferases or demethylases to constitutive heterochromatin. Further studies will be required to elucidate the mechanisms by which Dot1L and H3K979 methylation regulate heterochromatin.

Perturbation of epigenetic marks at constitutive heterochromatin has been shown to cause chromosome instability and telomere elongation [20–23]. Therefore, aberrant changes in chromatin at centromeres and telomeres most likely underlie the aneuploidy and telomere elongation observed in Dot1L-deficient ES cells. How the observed alterations in chromatin structure and cellular function contribute to the developmental abnormalities in Dot1L-deficient embryos is less clear. The requirement of Dot1L for normal cellular function does not appear to be ES cell-specific, as RNAi-mediated Dot1L knock-down in somatic cell lines also leads to growth arrest and cell death [13]. It is thus probable that intrinsic defects in cellular proliferation and viability, which themselves are likely the result of heterochromatin alterations, contribute to the growth defects and apoptosis observed in Dot1L mutant embryos. However, we believe that yolk sac defects are a major cause of embryonic lethality. In the absence of Dot1L, yolk sac angiogenesis is severely impaired. As both the endoderm and mesoderm cell layers of the visceral yolk sac are critical for blood vessel development [28,29] and both express Dot1L, we speculate that, in the absence of Dot1L, aberrant changes in gene expression and chromatin structure in one or both cell layers may underlie the yolk sac vascular defects. Some embryonic abnormalities, such as cardiac dilation, could be secondary to yolk sac vascular defects. Although Dot1L is also expressed in primitive erythrocytes, loss of Dot1L does not appear to have an obvious impact on erythropoiesis. It remains to be determined, however, whether the erythrocyte function is impaired.

Materials and Methods

The Dot1L conditional targeting vector, in which a 2.3-kb genomic region containing exons 5 and 6 was flanked by loxP sites, was constructed by sequentially subcloning Dot1L genomic fragments and a floxed βGeo cassette into pBluescript SK (Stratagene). The Dot1L genomic fragments were generated by PCR using mouse genomic DNA as the template. The primer pairs used were: 5′-TTC ACT AGT GCC CAC CTG TGG ATT G-3′ and 5′-GGC ACT AGT GTC ACA CAC CTG TA-3′ for the 5′ arm, 5′-CAT GTG GAC ACC GTG TAG TGG TGG GA-3′ and 5′-CTG GGC GGG CCT TGC TGG TGG CAGC-3′ for the 3′ arm, and 5′-GAG ACC GGC TCT GCC GAA CCT TT TGG G-3′ and 5′-CTG GGG GCC GCA CCA GCA ACA AAC TACG-3′ for the floxed region (underlined are the restriction sites used for cloning). The identity of the vector was verified by DNA sequencing.

Generation of Dot1L Mutant Mice

The Dot1L conditional targeting vector was transfected into ES cells via electroporation, and transfected cells were selected with G418. Clones with homologous recombination (Dot1Llox/+ ) were identified using Southern blot. genomic DNA was digested with EcoRI and hybridized with a 5′ external probe (The probe was generated by PCR using the following primers: 5′-CTG TGG TAC CTT TGT TGT TAT ACA G-3′ and 5′-CTG TCA AGT CGA CTA GAT GAA G-3′). Multiple Dot1Llox/+ clones were used to generate chimeric mice and F1 heterozygotes.

Deletion of exons 5 and 6 as well as the βGeo cassette was achieved by crossing Dot1Llox/+ mice with Zp3-Cre transgenic mice, which express the Cre recombinase in the germline. Mutant mice were maintained on a C57BL/6 inbred or a C57BL/6J-129Sv hybrid background. Primers used for PCR genotyping were: F1: 5′-GGG ACT TCT GAA CCA GGT AGC-3′, DR1: 5′-CAC TCC CCT GGT CTA ACA ACA ACG-3′, and DR2: 5′-CTG TCC CCT GAG GCA GC-3′ (Figure 1).

Embryo Collection, X-Gal Staining, and Histology

Female mice in Dot1Llox/+ intercrosses were examined for plug formation to establish the timing of copulation. Deedics were isolated from euthanized females at various time points following copulation, and embryos were examined under a dissecting microscope. DNA from the yolk sac was used for genotyping by PCR using the primers described above. X-gal staining was performed on 7.5- to 12.5-dpc Dot1Llox/+ embryos and littersmates as previously described [30]. Embryo, yolk sac, and placental tissue specimens, which were harvested at 9.5-dpc, were fixed in Bouin’s solution, washed extensively in 70% ethanol, processed routinely for paraffin embedding, sectioned at 5 μm, stained with hematoxylin and eosin, and then evaluated by bright field microscopy.

ES Cell Derivation and Culture

Dot1L mutant ES cells were derived from blastocysts produced from intercrosses of Dot1Llox/+ mice, as previously described [31]. Established ES cells were maintained in ES cell medium [32]. Apoptosis was analyzed using an Annexin V-PE apoptosis detection kit (BD Pharmingen). Cell cycle analysis was done using a PI/RNase Staining Buffer (BD Pharmingen).

Immunofluorescence and Immunoblot Analyses

Immunoblot and indirect immunofluorescence analyses were carried out using standard procedures. The following antibodies were used: anti-H3K79Me1 (Abcam), anti-H3K79Me2 (Abcam), anti-H3K79Me3 (Abcam), anti-H3 (Millipore), anti-H3K4Me2 (Millipore), anti-H3K4Me3 (Millipore), anti-H3K9Me1 (Millipore), anti-H3K9Me2 (Millipore), anti-H3K9Me3 (Millipore), anti-H3K27Me1 (Millipore), anti-H3K27Me3 (Millipore), anti-H3K9Ac (Millipore), anti-H4K20Me3 (Millipore), anti-H4Ac (Millipore), anti-Suv39h1 (Upstate), anti-ESET (Upstate), anti-G9a (Cell Signaling), anti-TRF1 (Abcam), anti-PML (Chemicon), Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes), Alexa 555-conjugated goat anti-mouse IgG (Molecular Probes), and peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG (Jackson ImmunoResearch Laboratories).

Mass Spectrometry Analysis

Histone H3 purified from ES cells was digested with trypsin, and the resulting peptides were analyzed using a LTQ-FT mass spectrometer (Thermo Fisher Scientific Inc.) hyphenated with an Agilent 1200 HPLC system (Agilent). Identification of the peptides was performed by searching the MS/MS fragmentation data against the histone H3 sequence using MASCOT search software (Matrix Science, version 2.1). All identifications were manually inspected for correctness. The abundance of each identified and validated peptide was calculated from its peak intensity using extracted ion chromatogram (XIC) of LC/MS spectra. Relative quantification of different forms of H3K79 methylation was performed by comparing the signal intensities of the tryptic peptide EIAQDFK (m/z 668.35), EIAQDFK (m/z 675.36), EIAQDKF (m/z 682.35), and EIAQDKF (m/z 689.35) in Dot1L mutant and wild-type ES cells.
Telomere Length Analysis
To analyze telomere length, we performed Q-FISH and TRF analyses according to procedures described previously [22].

Metaphase Spread Analysis
To prepare metaphase spreads, cells were incubated with 0.1 μg/ml of colcemid for 4 hours and then harvested and resuspended in 200 μl PBS. 10 ml of 75 mM KCl solution was added dropwise with constant gentle agitation. Cells were fixed by slow addition of 3:1 methanol/acetic acid solution, and then dropped onto a microscope slide. Slides were washed in 70% acetic acid, stained with DAPI and mounted. Chromosome spreads were observed using a Zeiss fluorescence microscope.

Chromatin Immunoprecipitation Assays
ChIP was performed using 20 × 10^6 ES cells as described in the online protocol provided by Upstate. Antibody sources are described above. Purified DNA was either analyzed with quantitative real-time PCR (qPCR) using Applied Biosystems SYBR PCR mastermix or analyzed with a dot blot assay as described [22]. qPCR primers used were specific for major satellite repeats, minor satellite repeats [33], and subtelomeric regions of chromosome 1 (forward: 5'-TTA GGA CTT CTC GCT GCT G-3', reverse: 5'-AGC TGT GGC AGG CAT CTT GGC GGC-3') and chromosome 2 (forward: 5'-GAA TCG TCC CTG TAG CAG GAG-3', reverse: 5'-GAT AAG CGA TCC AGG TGT G-3'). Enrichment was calculated as 2^(-ΔΔCt) (control CHIP) - 2^(-ΔΔCt) (experimental CHIP), where Ct is equal to the Ct (immunoprecipitated sample) - Ct (input) and normalized so that the wild-type value was 1, with the exception of H3K9Me1 at major satellite repeats where the Dot1L^lox/lox^/^lox^ value was 1. Each sample used in the dot blot contained DNA precipitated from 2.5 × 10^6 cells. Probes used were 32P-labelled oligonucleotides specific for telomeric repeats (TTAGGG)n and major satellite repeats (5'-TAT GGC GAG GAA TGA TTA ATT TAG AAA TGT CCA CTG GAC GAT GGA TAT GGC AAG-3'), respectively.

DNA Methylation Assay
Genomic DNA isolated from ES cells was digested with methylation-sensitive restriction enzymes and analyzed by Southern hybridization using probes specific for the major satellite repeats, the minor satellite repeats, and the intracisternal A particle retrovirus [32,34].

Supporting Information
Figure S1 Elevated apoptosis in Dot1L^lox/lox^ embryos. Representative hematoxylin and eosin-stained sections from 9.5-dpc Dot1L^+/^+/^ (left) and Dot1L^lox/lox^ (right) embryos illustrating focal areas of extensive apoptosis in the Dot1L^lox/lox^ embryo (asterisk). Scale bars = 100 μm.

Figure S2 Identification of H3K79 methylation by Nano-ESI MS/MS. Tryptic digest mixtures were analyzed by ESI MS using an LTQ-FT instrument. The precursor ion (m/z = 668.35), corresponding to the doubly charged (z = 2) version of peptide ion was selected for collision-induced dissociation (CID)-based MS/MS analysis. The fragment ion spectrum was inspected for y ions and the deduced sequence is indicated. The double methylation on K79 was identified from the spectrum. Unmodified, mono-methylated, and tri-methylated peptides were identified in the same way (data not shown).

Figure S3 Loss of H4K20Me3 enrichment at pericentric heterochromatin in Dot1L^lox/lox^ cells. Dot1L^+/^+/^, Dot1L^lox/+^, and Dot1L^lox/lox^ ES cells were immunostained with antibodies specific for the indicated histone modifications and examined using a fluorescent microscope. Dot1L^lox/lox^ cells showed no obvious alterations in the level and localization pattern of all modifications tested, with the exception of H4K20 tri-methylation, which displayed a more diffused nuclear pattern compared to Dot1L^+/^+/^ and Dot1L^lox/+^ cells.

Figure S4 No global changes in histone modifications besides H3K79 methylation in Dot1L^lox/lox^ cells. Lysates from Dot1L^+/^+/^, Dot1L^lox/+^, and Dot1L^lox/lox^ ES cells were analyzed with immunoblotting using antibodies specific for the indicated histone modifications (A) or H3K9 methyltransferases (B).

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Author Contributions
Conceived and designed the experiments: BJ YZ EL TC. Performed the experiments: BJ HS AB HL JB SH GAB SK HZ RV TC. Analyzed the data: BJ SK SG TC. Wrote the paper: BJ TC.

References