The H3K4 methyltransferase Setd1a is first required at the epiblast stage, whereas Setd1b becomes essential after gastrulation

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ABSTRACT
Histone 3 lysine 4 (H3K4) methylation is a universal epigenetic mark. In mammals, there are six H3K4 methyltransferases related to yeast Set1 and fly Trithorax, including two orthologs of Set1: Setd1a and Setd1b. Here we show that mouse Setd1a is required for gastrulation, whereas Setd1b-deficient embryos survive to E11.5 but are grossly retarded. Setd1a knockout embryos implant but do not proceed past the epiblast. Furthermore, Setd1a is not required until the inner cell mass has formed, at which stage it has replaced Mll2 as the major H3K4 methyltransferase. Setd1a is required for embryonic, epiblast and neural stem cell survival and neural stem cell reprogramming, whereas Setd1b is dispensable. Deletion of Setd1a in embryonic stem cells resulted in rapid losses of bulk H3K4 methylation, pluripotency gene expression and proliferation, with G1 pileup. Setd1b overexpression could not rescue the proliferation defects caused by loss of Setd1a in embryonic stem cells. The precise developmental requirement for Setd1a suggests that gastrulation is regulated by a switch between the major H3K4 methyltransferases.

KEY WORDS: Setd1a, Setd1b, ESC, Reprogramming, H3K4 methylation

INTRODUCTION
Nucleosomes not only serve to package and organize DNA but also as sites for post-translational modifications that contribute to the status of chromatin. These post-translational modifications, notably acetylation and methylation, modify the physical properties of chromatin and are binding sites for factors and complexes that promote the local chromatin status (Bannister and Kouzarides, 2011; Suganuma and Workman, 2011). Methylation on the histone 3 (H3) tail appear to be primary determinants of chromatin status, as exemplified by methylation at H3 lysine 4 (H3K4) or H3K9, which characterize euchromatic or heterochromatic regions, respectively (Noma et al., 2001). Chromatin is further characterized by the degree of methylation, with mono-, di- and trimethylation of lysines indicating a different status. In particular, all expressed euchromatic genes are marked by trimethylated H3K4 (H3K4me3) on promoter nucleosomes, whereas H3K4me2 is found in transcribed regions (Bannister and Kouzarides, 2011; Suganuma and Workman, 2011).

Yeasts have only one H3K4 methyltransferase (Briggs et al., 2001), originally called the Set1 complex (Set1C) (Roguev et al., 2001; Krogan et al., 2002; Noma and Grewal, 2002; Roguev et al., 2003), and the distinction between mono-, di- and trimethylated H3K4 sites is mediated by regulation of its enzymatic SET domain (Kim et al., 2013) or by opposing demethylases (Seward et al., 2007; Kooistra and Helin, 2012). Yeast Set1C contains eight subunits and was the first linkage between H3K4 methylation and Trithorax complex that can both catalyze trimethylation of H3K4 and bind it (Kooistra and Helin, 2012). Yeast Set1C contains eight subunits and was the first linkage between H3K4 methylation and Trithorax group (Trx-G) action (Roguev et al., 2001). This linkage was based on two observations: (1) one of the eight Set1C subunits, Bre2, is the yeast ortholog of the fly Trx-G protein Ash2; (2) among the diversity of SET methyltransferase domains, the SET domain of yeast Set1 is almost identical to that of Drosophila Trx. Because yeast Set1C is an H3K4 methyltransferase, it was likely that Drosophila Trx and the mammalian orthologs Mll1 and Mll2 would also be H3K4 methyltransferases, as was subsequently proven (Milne et al., 2002).

Drosophila has three proteins carrying the Set1/Trithorax-type SET domain, namely dSet1, Trx and Trithorax-related (Trr), whereas mammals have six (Glaser et al., 2006; Ardehali et al., 2011; Mohan et al., 2011; Hallson et al., 2012; Herz et al., 2012). These six are encoded by three pairs of sister genes, including two orthologs of Set1: Setd1a and Setd1b. All six proteins (Mll1-4, Setd1a,b) have been found in complexes based on the same Ash2, Wdr5, Rbbp5 and Dpy30 scaffold as yeast Set1C (yeast Brc2/Ash2, Swd3, Swd1, Sdc1) (Miller et al., 2001; Roguev et al., 2001; Hughes et al., 2004; Yokoyama et al., 2004; Ruthenburg et al., 2007; Ardehali et al., 2011; Mohan et al., 2011; Hallson et al., 2012). Because H3K4 methylation is the most conserved epigenetic modification, the Ash2 scaffold is the most highly conserved core protein complex involved in epigenetic regulation.

Biochemically, the two mammalian Set1 orthologs have been found in virtually identical complexes as yeast Set1C. However, Setd1a and Setd1b do not coexist in the same complex (Lee et al., 2007). All Set1C's contain a PHD finger protein called Cxxc1 or Cfp1 in mammals and Sppl in yeast. This PHD finger binds H3K4me3 (Shi et al., 2007; Eberl et al., 2013). Hence, Set1C is a complex that can both catalyze trimethylation of H3K4 and bind it as well. Potentially, this describes a feed-forward, positively reinforcing loop that could propagate the status of promoter chromatin through cell division and/or add epigenetic momentum to transcriptional decisions.

Given the universal roles played by H3K4 methylation in eukaryotic gene expression, why do yeasts have one, flies three and mammals six Set1/Trithorax-type H3K4 methyltransferases? This question cannot be answered as yet, but recent evidence from flies points to functional specialization as part of the answer. Drosophila Set1 is the major bulk H3K4 di- and trimethyltransferase (Ardehali
et al., 2011; Mohan et al., 2011; Hallson et al., 2012), Trx is restricted to a limited number of regulatory aspects such as homeotic gene regulation (Breen and Harte, 1993; Kuzin et al., 1994), and Trr, which is a co-factor for the Ecdysone receptor (Sedkov et al., 2003), might be a monomethyltransferase with enhancer specificity (Herz et al., 2012). In mice, only the two Trx orthologs Mll1 and Mll2 have been examined sufficiently to compare their roles. Despite ubiquitous expression during development, the two knockouts revealed very different roles. Mll1 is first required at ~E12.5 for definitive hematopoiesis (Yagi et al., 1998; Ernst et al., 2004), whereas Mll2 is widely required shortly after gastrulation from ~E7.5 onwards (Glaser et al., 2006). During development and in the adult neither enzyme is required for bulk H3K4 methylation in any cell type examined. However, Mll2 is the major di- and trimethyltransferase in developing oocytes and during the first cell divisions after fertilization (Andreu-Vieyra et al., 2010).

Although Set1 is the most conserved epigenetic regulator in evolution, the function of neither Setd1a nor Setd1b has been studied in mammalian development before now. Knockout of the Set1C subunit Cxxc1 (also called Cfp1) resulted in peri-implantation lethality in mice (Carlone and Skalnik, 2001). Deletion of Cxxc1 in embryonic stem cells (ESCs) led to a reduction of H3K4me3 levels particularly at promoters of highly transcribed genes (Clouaire et al., 2012). However, ESCs lacking Cxxc1 are viable and express pluripotency markers (Carlone et al., 2005). Here we apply targeted and conditional mutagenesis to examine and compare the functional roles of Setd1a and Setd1b in mouse development, ESCs and somatic cell reprogramming. Both proteins are essential. However, despite their virtually identical proteomic profiles, they convey radically different functions.

**RESULTS**

**Setd1a and Setd1b are highly conserved paralogs**

As evident from their gene structures, Setd1a and Setd1b clearly arose by gene duplication in evolution. At least 10 of 19 (Setd1a) or 17 (Setd1b) exons are bounded by identically placed splice sites, among other conserved features (supplementary material Fig. S1). Both mouse proteins have the same architecture based on an N-terminal RNA recognition motif (RRM) and C-terminal N-SET plus SET/postSET domains, with 72% (49/68), 61% (86/140) and 86% (108/125) amino acid sequence identity, respectively, and high overall identity (~38%) and similarity (~68%).

**Setd1a and Setd1b knockouts die at different embryonic stages**

To explore the function of these highly conserved proteins, we established multipurpose alleles (Testa et al., 2004) for both Setd1a and Setd1b by gene targeting. In our multipurpose allele strategy, the 5′-most frameshifting exon is flanked by loxP sites (exon 4 for Setd1a, Fig. 1A and supplementary material Fig. S2; exon 5 for Setd1b, Fig. 1D and supplementary material Fig. S2) and a stop cassette is inserted in the intron upstream of this exon. The stop cassette is flanked by FRT sites and contains a lacZ reporter. The 5′ splice site of the cassette captures the target gene transcript and the polyadenylation site terminates its transcription, thereby producing a null allele that we term the ‘A’ allele. Staining of heterozygous Setd1a<sup>A+</sup> and Setd1b<sup>B+</sup> embryos for β-galactosidase expression indicated that both genes are expressed ubiquitously in the developing embryo, including the extra-embryonic tissues. Ubiquitous expression was also indicated by qRT-PCR analyses (supplementary material Fig. S2; Fig. 2A).

Removal of the stop cassette by Flp recombination establishes the ‘F’ allele (for Flp recombined) and restores wild-type expression. Cre recombination of the F allele generates the ‘FC’ allele (C for Cre recombined) in which the loxP-flanked exon has been deleted, thereby frameshifting the open reading frame (Fig. 1A). The A and FC alleles are designed to knockout the gene in different ways so that they interrogate the null phenotype twice. If both A/A and FC/FC genotypes provoke the same phenotype, it is likely to be a null. By application of this test to Setd1a and Setd1b, we conclude that both alleles for both genes are nulls (Tables 1, 2). For technical reasons, a rox-flanked cassette was included next to the 3′ loxP site in the Setd1b targeting construct. This cassette was removed by Dre recombination to establish the ‘D’ allele (for Dre recombined), which is operationally the same as an A allele (Fig. 1D). Notably, the Setd1a and Setd1b null phenotypes are dramatically different. No Setd1a<sup>A+/A+</sup> or Setd1a<sup>A+FC/FC</sup> embryos were found after E7.5, whereas Setd1b<sup>DD/DD</sup> or Setd1b<sup>DDDC/DDDC</sup> embryos were found at Mendelian ratios until E10.5.

Although Setd1a<sup>A+/A+</sup> embryos implanted they failed to gastrulate, and antibody staining confirmed the loss of Setd1a protein expression (Fig. 1B,C). The Setd1a<sup>A+/A+</sup> mutant embryos retained mitotic activity, as detected by immunostaining with anti-H3S10P, and expression (Fig. 1B,C). The knockouts displayed apoptosis as determined by TUNEL assay (supplementary material Fig. S4). By contrast, Setd1b<sup>DD/DD</sup> embryos continued to develop until the grossly retarded embryos died at ~E11.5; however, growth retardation was observable from E7.5 (Fig. 1E). The presence of all germ layers in Setd1b knockout embryos was validated by histological analysis (supplementary material Fig. S3). Loss of Setd1b protein was confirmed by western analysis of whole embryos (Fig. 1F).

**Setd1a is required shortly after inner cell mass formation**

Both Setd1a and Setd1b are expressed during preimplantation development (Fig. 2A). High expression is detected in the oocyte, which reflects the maternal mRNA stock that declines to a stable level from the 8-cell stage until E8.5 (Fig. 2A). Expression of Setd1a and Setd1b in the blastocyst is mainly nuclear and found in both the inner cell mass (ICM) and the trophectoderm (Fig. 2B). Both Setd1a and Setd1b are expressed during preimplantation development (Fig. 2A). High expression is detected in the oocyte, which reflects the maternal mRNA stock that declines to a stable level from the 8-cell stage until E8.5 (Fig. 2A). Expression of Setd1a and Setd1b in the blastocyst is mainly nuclear and found in both the inner cell mass (ICM) and the trophectoderm (Fig. 2B). Both Setd1a and Setd1b are expressed during preimplantation development (Fig. 2A). High expression is detected in the oocyte, which reflects the maternal mRNA stock that declines to a stable level from the 8-cell stage until E8.5 (Fig. 2A). Expression of Setd1a and Setd1b in the blastocyst is mainly nuclear and found in both the inner cell mass (ICM) and the trophectoderm (Fig. 2B).
Fig. 1. Allele design and embryonic phenotype of Setd1a and Setd1b mutant mouse embryos. (A) The Setd1a knockout first allele (Setd1a\(^A\)). This allele is converted to conditional (Setd1a\(^A\)) upon Flp recombination. Cre recombination leads to excision of the frameshifting exon 4, generating the conditional mutant allele (Setd1a\(^F\)). (B) Embryos from Setd1a\(^A\)\/+ intercrosses at E6.5 and E7.5. At E6.5, Setd1a\(^A\)/A embryos are growth retarded compared with littermates. (C) Immunostaining with a Setd1a-specific antibody on sagittal sections of Setd1a\(^A\)/+ and Setd1a\(^A\)/A embryos at E7.5. Nuclei in the Setd1a\(^A\)/A embryo are devoid of specific staining, confirming loss of protein. The staining is visible in Setd1a\(^A\)/+ embryonic cells and in the deciduum of the Setd1a\(^A\)/A embryo. Scale bars: 150 \(\mu\)m. Red boxes indicate the regions enlarged beneath. (D) The Setd1b knockout first allele (Setd1b\(^A\)). This allele is similar to that resulting after Dre recombination (Setd1b\(^D\)) to remove the PGK-hygromycin selection cassette. The subsequent recombination steps are identical to the Setd1a targeting strategy (A). The frameshifted allele (Setd1b\(^F\)) is based on excision of exon 5. (E) Embryos from Setd1b\(^D\)\/+ intercrosses at E6.5, E7.5, E9.5 and E11.5. Growth retardation is apparent from E7.5 and embryos die before E11.5. (F) Immunoblot on whole cell extracts from Setd1b mutant embryos confirms the loss of Setd1b protein. For abbreviations see supplementary material Fig. S2.
We speculated that the early lethality caused by loss of Setd1a was due to exhaustion of maternally supplied Setd1a protein/mRNA oocyte stockpiles, which might mask an even earlier requirement in development. Therefore, we conditionally mutated Setd1a during oocyte development by crossing the Setd1a conditional line with the Gdf9-Cre line, which excises from early folliculogenesis onwards (Lan et al., 2004).

Setd1aF/F;Gdf9-Cre/+ females [hereafter termed Setd1aGdf9 conditional knockout (cKO)] and heterozygous Setd1aF/+;Gdf9-Cre/+ controls were bred to wild-type males. Setd1a mRNA was almost undetectable in Setd1aGdf9 cKO oocytes (Fig. 3B). Recombination was complete in all pups that carried the conditional allele and both the number of litters born and litter sizes from Setd1aGdf9 cKO females were comparable to those of controls (Fig. 3C). Therefore, unlike the corresponding Mll2Gdf9 cKO (Andreu-Vieyra et al., 2010), loss of Setd1a from developing oocytes did not perturb fertility. Notably, mating of Setd1aGdf9 cKO females to Setd1aFC/+ males recapitulated the null phenotype with no observable increase in severity (Fig. 3D). Furthermore, Setd1aGdf9 cKO; Oct4-GFP embryos had detectable GFP expression in the epiblast (Fig. 3E). This indicates that the null phenotype revealed at E7.5 is not due to the exhaustion of maternal stores. From these experiments we conclude that there is no direct requirement for maternal Setd1a transcripts or protein in the embryo and, moreover, no direct requirement for Setd1a until the ICM has formed.

Setd1a, but not Setd1b, is essential in ESCs

To define the roles of Setd1a and Setd1b more precisely, we derived ESC lines from blastocysts carrying conditional Setd1a or Setd1b alleles combined with the heterozygous Rosa-Cre-ERT2 (RC+/+) allele. To induce the Setd1a or Setd1b frameshift mutations, 4-hydroxytamoxifen (4OHT) was added to the medium for 48 hours, by which time both proteins are almost completely lost (Fig. 4A,B). Conditional deletion of Setd1a severely affected the growth rate of ESCs, which stopped proliferating 72 hours after 4OHT addition (Fig. 4C). The proliferation defect of Setd1aFC/FC;RC/+ ESCs could be rescued by a Setd1a BAC transgene, which restored protein expression (Fig. 4C,D). The rescue was observed with two different clones that carried either one (1c) or two (2c) copies of the BAC transgene. Cell cycle analysis of Setd1aF/F;RC/+ ESCs before and after 4OHT induction revealed a reduction in S phase, an apparent increase in G1 and high levels of disintegrating cells (Fig. 4E). By contrast, conditional deletion of Setd1b did not affect cellular proliferation, morphology (data not shown) or cell cycle distribution (Fig. 4F).

The reduced cell number upon Setd1a deletion in ESCs was accompanied by increased apoptosis, as shown by TUNEL staining (Fig. 4G) and increased annexin V-positive ESCs (Fig. 4H). Notably, the loss of a single copy of Setd1a also promoted higher levels of annexin V-positive cells (Fig. 4H). This indication of apparent Setd1a haploinsufficiency was supported by the observation that the
rescued Setd1a<sup>FC/FC;RC/+</sup> ESC clone containing one copy of the Setd1a BAC transgene (BAC 1c) showed comparable numbers of annexin V-positive cells to the heterozygously deleted Setd1a<sup>FC/+;RC/+</sup> clone, whereas in the rescued mutant with two copies of the BAC transgene (BAC 2c) annexin V-positive cells returned to wild-type levels. However, we have not yet been able to identify a heterozygous Setd1a phenotype in mice.

Due to reduced proliferation, deletion of Setd1a led to smaller colonies, which still expressed the classic ESC marker alkaline phosphatase (AP) (Fig. 4I). However, qRT-PCR evaluation of the ESC pluripotency factors Oct4, Nanog and Klf4 revealed strong downregulation that was comparable to, or greater than, the downregulation in the same time frame that accompanies exit from self-renewal promoted by LIF withdrawal (Fig. 4J). Therefore, we looked for upregulation of early lineage markers. Although a 2- to 3-fold increase in Fgf5, brachyury (<i>T</i>) and Gata4 mRNA expression was observed, these responses were not equivalent to those promoted by LIF withdrawal (Fig. 4K). These results show that

### Table 1. Embryonic lethality of Setd1a<sup>AA</sup> and Setd1a<sup>FCFC</sup> embryos

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*The percentage of total is shown in parentheses.
‡The number of litters (n) is shown in parentheses.

### Table 2. Embryonic lethality of Setd1b<sup>DD</sup> and Setd1b<sup>FD/FD</sup> embryos

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<td>84 (48.5)</td>
<td>58 (33.5)</td>
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*The percentage of total is shown in parentheses.
‡The number of litters (n) is shown in parentheses.
Setd1a is essential for proliferation and maintenance of the transcriptional profile of key pluripotent genes in ESCs in culture, whereas Setd1b is dispensable.

**Setd1b overexpression cannot rescue the ESC proliferation defects caused by loss of Setd1a**

Although qRT-PCR results indicated that Setd1a and Setd1b mRNA levels are similar (Fig. 2A), a recent proteomic analysis of ESCs suggests that Setd1a is much more highly expressed than Setd1b (van Nuland et al., 2013). Possibly, therefore, the proliferation defects caused by loss of Setd1a can be compensated by overexpressing Setd1b. To test this proposition we transfected Setd1a conditional ESCs with a Setd1b BAC. Four stable colonies overexpressing Setd1b (2- to 4-fold) were identified and induced for the loss of Setd1a by 4OHT administration. No rescue of the proliferation defect was observed (data not shown). Because 4-fold Setd1b overexpression might not be enough to rescue the proliferation defect, we made a chimeric Setd1 BAC. The exonic coding region of the rescuing Setd1a BAC (Fig. 4C,D) was replaced with the exonic coding Setd1b region (Fig. 5A). After transfection of the chimeric BAC into Setd1a<sup>F/F</sup> ESCs, strong overexpression of Venus-tagged Setd1b from the Setd1a promoter and gene context was achieved (Fig. 5B,C). Despite high levels of Setd1b expression, no rescue of the ESC proliferation defect caused by loss of Setd1a was observed (Fig. 5D,E). This finding implies that Setd1a and Setd1b regulate different targets in ESCs.

**Setd1a is also required for EpiSC and NSC proliferation**

To test whether Setd1a is required in other cell types apart from ESCs, we established epiblast stem cells (EpiSCs) from Setd1a<sup>F/F;RC/+</sup> ESCs and from those carrying the Setd1a-Venus BAC (supplementary material Fig. S5A). As expected, in EpiSCs the expression of Rex1 (Zfp42 – Mouse Genome Informatics) was downregulated and the expression of Oct4 and Fgf5 upregulated (supplementary material Fig. S5B). Loss of Setd1a upon 4OHT administration in EpiSCs resulted in reduced proliferation and downregulation of Oct4 (supplementary material Fig. S5B,C). A similar proliferation defect was observed with neural stem cells.

### Table 3. Genotypes of ESC lines derived from Setd1a<sup>A+</sup> and Setd1b<sup>D+</sup> intercrosses

<table>
<thead>
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<th>Cross</th>
<th>Setd1a&lt;sup&gt;A+&lt;/sup&gt; × Setd1a&lt;sup&gt;A+&lt;/sup&gt;</th>
<th>Setd1b&lt;sup&gt;D+&lt;/sup&gt; × Setd1b&lt;sup&gt;D+&lt;/sup&gt;</th>
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<td>A/A</td>
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*n=73 blastocysts; ‡n=45 blastocysts.
Fig. 4. See next page for legend.
**Fig. 4. Setd1a deletion in ESCs results in a proliferation defect without obvious signs of differentiation.** (A,B) Immunoblot using Setd1a (A) or Setd1b (B) antibodies on whole cell extracts from ESCs carrying Rosa26-Cre-ERT2 (RC/+ ) and conditional homozygous (F/F) or heterozygous (F/+ ) alleles at different time points after 4OHT addition. Neither protein is detectable 96 hours after 4OHT treatment. Tubulin serves as loading control. Setd1a protein levels are unchanged upon Setd1b deletion. (C) Proliferation timecourse after 4OHT administration. The proliferation rate of Setd1a<sup>Cre<sub>RC</sub></sup> ESCs is dramatically decreased after 72 hours. This defect can be rescued by transfecting a Setd1a-containing BAC (BAC 1c has one copy integrated; BAC 2c has two integrated copies). Error bars indicate s.d. of n=3 replicates. (D) Immunoblot showing that integration of Setd1a BAC transgenes restores Setd1a levels in Setd1a<sup>Cre<sub>RC</sub></sup> ESCs treated with 4OHT. (E) Cell cycle profile of Setd1a<sup>FF;RC+</sup> ESCs before and after 4OHT treatment. Note the increase of G1 phase and decrease of S phase. (F) Cell cycle profile is unchanged in induced Setd1b mutant ESCs. (G) Setd1a conditional mutant ESCs show increased apoptosis, as detected by TUNEL staining. Nuclei are stained with DAPI. Scale bar: 25 μm. (H) Quantification (%) of apoptotic annexin V-positive ESCs by flow cytometry at different time points after 4OHT induction. Error bars indicate s.e.m. of n=3 replicates. (I) Alkaline phosphatase (AP) staining of Setd1a conditional mutant and BAC-rescued ESCs at 0 and 120 hours after 4OHT induction. Setd1a<sup>Cre<sub>RC</sub></sup> ESC colonies are smaller but still AP<sup>+</sup> and they do not show any signs of differentiation. Insets illustrate colonies enlarged. (J) qRT-PCR analysis of the pluripotency genes Oct4, Nanog and Klf4. (K) qRT-PCR analysis of the early differentiation genes Fgf5, brachyury (T) and Gata4. Error bars indicate s.e.m. of n=3 replicates.

**DISCUSSION**

Despite highly homologous protein sequences and identical protein complexes, Setd1a and Setd1b play very different essential roles in mouse development. Setd1a is required immediately before gastrulation, shortly after implantation. At this stage and in ESCs, it is the major H3K4 methyltransferase. It is required for the maintenance of pluripotency factors in ESCs as well as for ESC, iPSC, EpiSC and NSC proliferation. By contrast, none of these functions applies to Setd1b, which is required after gastrulation during organogenesis.

Because their protein complexes, Setd1aC and Setd1bC, are otherwise identical (Lee and Skalnik, 2005; Lee et al., 2007), the different functions of Setd1a and Setd1b must be conveyed by differences in their (1) expression patterns, (2) transient protein-protein interactions or (3) epigenetic interactions. First, all evidence so far indicates that both mRNAs are ubiquitously expressed (Fig. 2; supplementary material Fig. S2). Whether the two proteins are expressed in different patterns, or at very different levels as has been recently suggested (van Nuland et al., 2013), remains to be determined. At least in ESCs, overexpression of Setd1b cannot rescue the proliferation defect caused by loss of Setd1a. Second, three different protein-protein interactions have been described so far. Setd1a, but not Setd1b, interacts with host cell factor 1 (Hcf1, or Hcf1c), whereas Setd1b, but not Setd1a, interacts with Rbm15 (Lee and Skalnik, 2008; Lee and Skalnik, 2012). Both Hcf1 and Rbm15 are expressed in ESCs (data not shown). It has also been reported that Setd1a interacts with β-catenin and binds to the Tert promoter. Although β-catenin (Ctnnb1<sup>−/−</sup>) ESCs are viable, the Tert promoter loses H3K4me3 and telomerase activity is downregulated (Hoffmeyer et al., 2012). However, we could not detect Tert downregulation in our Setd1a conditional mutant ESCs (data not shown). Current information does not permit an explanation of the differences between Setd1a and Setd1b knockout phenotypes based on these different protein-protein interactions. Third, Setd1a and Setd1b might differentially interact with the epigenetic circuitry. A role for the N-SET domain in the regulation of epigenetic writing was recently assigned to yeast Set1 (Kim et al., 2013). Also, RNA binding by the RRM domain, which conceptually presents great potential for regulatory specificity, has been described for yeast Set1 (Trésaugues et al., 2006). Differences between the functions of the N-SET or RRM domains in Setd1a and Setd1b might be mediated by the divergent sections of these very similar proteins, are likely to be the source of their different roles in mouse development.

**Setd1a in mouse development**

We found that Setd1a is required for ESC, iPSC, EpiSC and NSC proliferation. Hence, we considered whether it is required for proliferation of all cell types and, if so, then the very early embryonic lethality of the Setd1a knockout could report the moment reduction in all three H3K4 methylated species (Fig. 7A,B). This observation was supported by western blots using the Setd1a conditional ESCs after addition of 4OHT (Fig. 7C). Whereas total H3, H2B and H3K27me3 levels remained constant, all three methylation marks at lysine 4 showed a significant reduction, which was restored in BAC-rescued ESC lines (BAC 1c and BAC 2c). However, H3K4me3 levels were not restored in Setd1a conditional ESCs overexpressing Setd1b from the chimeric Setd1 BAC (data not shown). In contrast to Setd1a, deletion of Setd1b in ESCs did not affect global H3K4 mono-, di- and trimethylation levels (Fig. 7D). Likewise, we were unable to detect any changes in global H3K4 methylation in E10.5 Setd1b<sup>Δβ</sup> embryos (Fig. 7E).

**Setd1a is essential for the derivation of induced pluripotent stem cells**

We tested the role of Setd1a and Setd1b in the reprogramming of a somatic state to induced pluripotent stem cells (iPSCs). NSCs were isolated from E15.5 <i>Rosa-Cre-ERT2</i> (RC<sup>−/−</sup>) telencephali (supplementary material Fig. S6A,B).
when the oocyte stores of maternal Setd1a are exhausted. However, the removal of Setd1a from oocytes by Gdf9-Cre disproved this proposition and showed that Setd1a is not required for early proliferation of the embryo. Deletion of Setd1a expression during oogenesis had no impact on fertility or the early embryonic lethal phenotype. By contrast, deletion of Mll2 using the same Gdf9-Cre driver caused complete infertility through loss of ovulation and oocyte degeneration (Andreu-Vieyra et al., 2010). Because the kinetics of recombination-promoted protein loss for these two genes (and also Setd1b) in ESCs are the same (Glaser et al., 2009) (Fig. 4), we conclude that Setd1a is not required for oogenesis or cell division in the embryo before ICM formation.

Furthermore, use of an Oct4-GFP reporter and attempts to establish Setd1a-deficient ESCs precisely defined the point in development at which Setd1a becomes essential. Our analyses showed that the ICM is established in the absence of Setd1a but not sustained after implantation. Setd1a knockout embryos develop primitive endoderm but do not gastrulate. Hatching from cultured blastocysts occurred but further proliferation was impaired and no ESC lines could be established. Hence, Setd1a is required at, or immediately after, formation of the epiblast. Setd1a is also essential in ESCs for proliferation and maintenance of the self-renewal circuitry. However, loss of this circuitry in Setd1a knockout ESCs did not provoke default differentiation, suggesting that Setd1a might be required for ESC transcriptional activity in general, which is a proposition supported by the fact that it is the major ESC H3K4 methyltransferase. These observations are reinforced by our reprogramming experiments from NSCs. Setd1a is required for NSC proliferation and its removal at any stage during a reprogramming timecourse impeded the acquisition of iPSC lines.

At some point in early mouse development, there is a shift of the major regulator of H3K4me2/3 from Mll2 to Setd1a. Mll2 plays this role during oogenesis and the early cleavage stages after fertilization. Thereafter, knockout of Mll2 had no effect on bulk H3K4 methylation (Lubitz et al., 2007; Glaser et al., 2009) indicating that (an)other H3K4 methyltransferase(s) acquired this role at some point after compaction. Our findings implicate Setd1a, and not Setd1b, as the major H3K4 di- and trimethyltransferase in question and suggest that Setd1a acquires this role at, or immediately before, the epiblast stage when its function becomes required. Whether Setd1a remains the major H3K4 methyltransferase in adults remains to be determined.

**H3K4 methyltransferases in mouse development**

The embryonic phenotype of the Setd1b knockout bears a striking resemblance to that of the Mll2 knockout. In both cases, the
embryos appear normal until E7.5 but further development is retarded. However, all organs and structures appear to arise albeit in an increasingly retarded context and death occurs before E11.5. Neither protein is required for ESC self-renewal. Despite extensive examination we do not know how loss of Mll2 promotes this pleiotropic dysfunction. The fact that Setd1b appears to promote a similar phenotype might help solve the puzzle. Of the three other Set1/Trithorax-type H3K4 methyltransferases, namely Mll1, Mll3 and Mll4, only the knockout phenotype of Mll1 is known with certainty. It is first required in development, and continues to be required in adults, for definitive hematopoiesis (Yu et al., 1995; Yagi et al., 1998; Ernst et al., 2004; Jude et al., 2007; McMahon et al., 2007; Milne et al., 2010). Hence, Mll1 appears to have a very specialized function, as opposed to the apparently broader requirements for Setd1a, Setd1b and Mll2.

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**Fig. 6. Setd1a knockout NSCs cannot generate iPSCs.** (A) The NSC to iPSC reprogramming timecourse. NSCs were treated with 4OHT either before (P-1), simultaneously (d0-3), 5 days after (d5-8) or 10 days after transfection of the reprogramming factors. iPSC colonies were stained for AP on day 14 after transfection and classified into weak or strong staining. From each experiment, iPSC colonies were picked, expanded and immunostained for Nanog and SSEA1 between days 28 and 35 after transfection. (B) Reprogramming efficiency shown as a percentage of iPSC colonies derived at different time points of 4OHT induction relative to uninduced cells from the four different conditional lines. (C,D) Micrographs of AP-stained iPSCs from Setd1a<sup>F/F</sup>;RC/+ and Setd1b<sup>F/F,D/D</sup>;RC/+ lines. (E,F) iPSCs from Setd1a<sup>F/F</sup>;RC/+ and Setd1b<sup>F/D,D/D</sup>;RC/+ generated without or after 4OHT treatment and immunostained for Nanog and SSEA1. Note that Setd1a<sup>F/C,F/C</sup> iPSC colonies could not be sustained and hence are not shown. Scale bars: 1 mm in C,D; 100 μm in E,F.
Several subunits of Set1C have been knocked down or out. In the Ash2 scaffold, knockdown of Wdr5 in ESCs resulted in reduced self-renewal, increased differentiation and global loss of H3K4me3 (Ang et al., 2011). However, knockdown of Dpy30 and Rbbp5 had no effect on self-renewal, but decreased differentiation into the neural lineage and a global reduction of H3K4me3 levels (but not H3K4me1 and H3K4me2) were observed (Jiang et al., 2011). These Dpy30 and Rbbp5 knockdown phenotypes are similar to the Mll2 knockout in ESCs, which showed delayed exit from self-renewal and impaired neuronal differentiation (but no change in H3K4 methylation) (Lubitz et al., 2007). Possibly, the discrepancy with the Wdr5 knockdown phenotype is due to the presence of Wdr5 in several other protein complexes [e.g. ATAC (Nagy and Tora, 2007; van Nuland et al., 2013)]. Embryos homozygous for an Ash2l hypomorphic allele showed a more severe phenotype than that of the Setd1a knockout, consistent with the inclusion of Ash2l in other H3K4 methyltransferase complexes. No Ash2l hypomorphic embryos implanted, and attempts to derive ESCs from blastocysts failed (Stoller et al., 2010). By contrast, knockdown of Ash2l induced ESC differentiation (Wan et al., 2013).

Knockout of the Setd1C-specific subunit Cxxc1 caused an early embryonic lethality apparently similar to that of the Setd1a knockout (Carlone and Skalnik, 2001). However, Cxxc1 knockout ESCs are viable but cannot undergo differentiation and there is no global change in H3K4me3 levels (Carlone et al., 2005; Lee and Skalnik, 2005; Tate et al., 2009). But there was a repositioning of H3K4me3 away from active promoters to new ectopic sites indicating that Cxxc1 restricts Setd1Cs to active promoters (Tate et al., 2009; Tate et al., 2010; Clouaire et al., 2012). Because Setd1a is responsible for bulk H3K4 methylation in ESCs, we predict that it is the enzyme that is being mispositioned. If so, it will be interesting to examine whether these ectopic sites are subject to other aspects specific to Setd1a, such as Hcf1 activity (Lee and Skalnik, 2008).

MATERIALS AND METHODS

Targeting constructs and BAC transgenes

The targeting constructs for Setd1a and Setd1b were generated using recombineering (Fu et al., 2010). For Setd1a, the engrailed-intron-splice-acceptor-IRES-lacZ-neomycin-polyA cassette flanked by FRT sites for Flp recombination was inserted in intron 3 of the gene. The critical exon 4, which upon deletion results in a frameshift and a premature stop codon, was flanked by loxP sites. For targeting the second allele of Setd1a we generated a similar targeting construct by replacing the neomycin resistance gene with blasticidin resistance. For Setd1b, the engrailed-intron-splice-acceptor-2A-lacZ-neomycin-polyA cassette flanked by FRT sites was inserted in intron 4 and the critical exon 5 was flanked by loxP sites. The 3′ loxP was accompanied by a PGK-hygromycin-polyA cassette flanked by rox sites for Dre recombination. The homology arms were 5 kb 5′ and 4.9 kb 3′ for Setd1a and 2.3 kb 5′ and 6.4 kb 3′ for Setd1b. Bacterial artificial chromosomes (BACs) containing Setd1a (clone name: RP23-188K7) and Setd1b (clone name: bMQ-33K9) genes were modified by recombineering to introduce an N-terminal Venus tag cassette directly after the second codon (Ciotta et al., 2011). The Setd1a/Setd1b chimeric BAC was made by

**Fig. 7. Global reduction of H3K4 methylation levels in Setd1a but not Setd1b knockout ESCs and embryos.** (A) Immunohistochemistry on sections of Setd1aA/+ and Setd1aA/A embryos with H3, H3K4me1, H3K4me2 and H3K4me3 antibodies. (B) Quantification of the methylation levels by measuring the signal intensity of the nuclei in the embryo relative to the intensity in the neighboring deciduum. Error bars indicate s.d. of 20 nuclei per region from n=6 different embryo sections of each genotype. (C) Immunoblot on whole cell extracts from Setd1a conditional mutant and BAC-rescued ESCs using antibodies against H3K4me3, H3K4me2, H3K4me1, H3, H3K27me3 and H2B. Note the decrease in bulk H3K4 methylation in Setd1aCPC ESCs. (D) Immunoblot on whole cell extracts from Setd1bCPC and Setd1bCPC ESCs using antibodies against antibodies against H3K4me3, H3K4me2, H3K4me1, H3 and H3K27me3. (E) Immunoblot on whole cell extracts from Setd1bA/+ and Setd1bA/A embryos at E10.5. Scale bars: 100 μm.
swapping the Setd1a gene body with that of Setd1b in the Setd1a BAC. All three BACs contained a ubiquitin C-fragment-polyA (UbC-BSD-pA) selection cassette inserted close to the bacterial backbone.

Gene targeting and generation of conditional knockout mice
R1 ESCs were cultured with fetal calf serum (FCS)-based medium [DMEM + GlutamaxTM (Invitrogen), 15% FCS (PAA), 2 mM L-glutamine (Invitrogen), 1x non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.1 mM β-mercaptoethanol, in the presence of 1000 units LIF (Chemicon) per ml] on mitomycin-C inactivated mouse embryonic fibroblasts. Cells (1×10^6) were electroporated with 40 μg linearized targeting construct using standard conditions (250 V, 500 μF) and selected with 0.2 mg/ml G418. Colonies were screened for correct targeted events by Southern blot hybridization using an internal probe and external probes. For removing the selection cassettes, correctly targeted Setd1a clones were electroporated with a CAG-Flpo-IRES-puro expression vector (Kranz et al., 2010). For both Setd1a and Setd1b, two correctly targeted ESC clones were injected into blastocysts and the resulting chimeras were subsequently bred to C57BL/6 mice. Successfully bred mice were genotyped by PCR using primers specific for the targeting cassette inserted and the wild-type allele.

Rolf Jessberger. Primers for genotyping are provided in supplementary material Table S1.

Differentiation of ESCs to EpiSCs
ESCs were passaged using accutase (PAA), washed twice in DMEM/F12 (Gibco) and 1×10^6 cells per cm^2 were seeded on fibronectin-coated (10 ng/ml in PBS, Millipore) 6-well plates and glass coverslips in EpiSC medium [50% DMEM/F12, 50% Neurobasal medium (Gibco), 0.5× self-made N2 supplement, 2 mM glutamate, 100 μM β-mercaptoethanol, 12 ng/ml recombinant human FGF2 (Protein Facility, MPI-CBG), 20 ng/ml recombinant human activin A (MPL-CBG)], Stable EpiSC lines were established after three to eight passages.

Reverse transcription and quantitative PCR (qRT-PCR) analysis
Total RNA from oocytes, embryos or cells was isolated using Trizol reagent (Ambion) and reverse transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen). Real-time quantitative PCR was performed with Go Taq qPCR Master Mix (Promega) on a Mx3000P multiplex PCR instrument (Agilent). Ct values were normalized against Gapdh or Plik1. Primer sequences and the lengths of the amplified products are given in supplementary material Table S1. Fold differences in expression levels were calculated according to the 2^(-ΔΔCt) method (Livak and Schmittgen, 2001).

Western blot analysis
ESCs were homogenized in buffer E (20 mM HEPES pH 8.0, 350 mM NaCl, 10% glycerol, 0.1% Tween 20, 1 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF) and protein extracts were obtained after three cycles of freezing and thawing. Whole cell extracts were separated by SDS-PAGE (3-8% Tris-acetate gel for Setd1 proteins, 12% Tris-glycine gel for histone proteins), transferred to PVDF membranes and probed with primary antibodies: Setd1a (1:50; A300-289, Bethyl), anti-Setd1b (1:50; A302-281A, Bethyl)] for 1 hour in blocking buffer followed by washing with 10 mM phosphate buffer (pH 6.0) for 45 minutes. Sections were incubated in blocking serum (5% goat serum) for 1 hour at room temperature. Incubation with the primary antibody [anti-phospho-HIS10 (1:200; #06-570, Upstate), anti-Setd1a (1:50; A300-289, Bethyl), anti-Setd1b (1:50; A302-281A, Bethyl)] was performed in 10 mM citrate buffer (pH 6.0) for 45 minutes. Sections were then washed in 100 mM Tris-maleate pH 9.0, 8 mM EDTA, 1% (v/v) Fast Red TR (Sigma). To detect apoptotic cells, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining was performed with the In Situ Cell Death Detection Kit (Roche) and ESCs were counterstained with DAPI. TUNEL staining on whole-mount embryos was performed accordingly. To determine the levels of apoptosis, ESCs were stained with APC-labeled annexin V according to the manufacturer’s instructions (BD Biosciences). Cells were subjected to flow cytometry and analyzed by FACSDiva software (BD Biosciences).

Cell proliferation and cell cycle analysis
The proliferation count assay is described elsewhere (Lubitz et al., 2007). For cell cycle analysis, asynchronously growing ESCs were fixed with ethanol and stained with 1 μg/ml propidium iodide containing 0.1 mg/ml RNase A (both from Sigma-Aldrich). Cells were analyzed by flow cytometry, and G1, S, and G2/M cell cycle distribution was determined using ModFit LT software (Verity Software House, Topsham, ME, USA).

Alkaline phosphatase staining and apoptosis assays
ESCs were fixed in 4% formaldehyde, permeabilized with 0.5% Triton X-100/PBS, and stained with 25 mM Tris-maleate pH 9.0 (Sigma), 8 mM MgCl2, 0.4% (w/v) 1-naphthyl phosphate (Sigma) and 1% (w/v) Fast Red TR (Sigma). To detect apoptotic cells, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining was performed with the In Situ Cell Death Detection Kit (Roche) and ESCs were counterstained with DAPI. TUNEL staining on whole-mount embryos was performed accordingly. To determine the levels of apoptosis, ESCs were stained with APC-labeled annexin V according to the manufacturer’s instructions (BD Biosciences). Cells were subjected to flow cytometry and analyzed by FACSDiva software (BD Biosciences).

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ESCs were passaged using accutase (PAA), washed twice in DMEM/F12 (Gibco) and 1×10^6 cells per cm^2 were seeded on fibronectin-coated (10 ng/ml in PBS, Millipore) 6-well plates and glass coverslips in EpiSC medium [50% DMEM/F12, 50% Neurobasal medium (Gibco), 0.5× B27 supplement (Gibco), 0.5× self-made N2 supplement, 2 mM glutamate, 100 μM β-mercaptoethanol, 12 ng/ml recombinant human FGF2 (Protein Facility, MPI-CBG), 20 ng/ml recombinant human activin A (MPL-CBG)]. Stable EpiSC lines were established after three to eight passages.

Differentiation and derivation of NSCs to neurons and astrocytes
Mouse NSCs were isolated from E15.5 Setd1a and Setd1b (F/F;RC+/+) forebrains and cultured in NSC medium [EUromed-N (Biozol), 1× N2 (self-made), 2 mM l-glutamine, 10 ng/ml recombinant FGF2 (MIP-CBG), 10 ng/ml EGF (Peprotech), supplemented with 0.5× B27]. NSCs were passaged using accutase, washed in DMEM/F12 and 1×10^5 cells per cm^2 were seeded on laminin-coated (2 μg/ml, Roche) glass coverslips for subsequent fixation and permeabilization for immunofluorescence using mouse anti-nestin [1:20, Rat-401, Developmental Studies Hybridoma Bank (DSHB)] or mouse anti-Pax6 (1:20, DSHB) antibodies.

Reverse transcription and quantitative PCR (qRT-PCR) analysis
Total RNA from oocytes, embryos or cells was isolated using Trizol reagent (Ambion) and reverse transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen). Real-time quantitative PCR was performed with Go Taq qPCR Master Mix (Promega) on a Mx3000P multiplex PCR instrument (Agilent). Ct values were normalized against Gapdh or Plik1. Primer sequences and the lengths of the amplified products are given in supplementary material Table S1. Fold differences in expression levels were calculated according to the 2^(-ΔΔCt) method (Livak and Schmittgen, 2001).

Western blot analysis
ESCs were homogenized in buffer E (20 mM HEPES pH 8.0, 350 mM NaCl, 10% glycerol, 0.1% Tween 20, 1 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF) and protein extracts were obtained after three cycles of freezing and thawing. Whole cell extracts were separated by SDS-PAGE (3-8% Tris-acetate gel for Setd1 proteins, 12% Tris-glycine gel for histone proteins), transferred to PVDF membranes and probed with primary antibodies: Setd1a (1:500; A300-289, Bethyl), Setd1b (1:500; A302-281A, Bethyl), alpha tubulin (1:10,000; 1876-1, Epitomics), H3 (1:3500; ab1791, Abcam), H3K4me1 (1:2500; ab8889, Abcam), H3K4me2 (1:2500; ab7766, Abcam), H3K4me3 (1:1000; 07-473, Millipore), H3K27me3 (1:2500; 07-449, Millipore) and ubiquitylated H2B (1:2500; ab1790, Abcam).
NSCs and reprogramming

Mouse NSCs were cultured as described above. Cre-mediated recombination was induced with 0.1 μM 4OHT. For reprogramming, 8–10th NSCs were nucleofected with circular PiggyBac-OSKM and HA-mPB transposase plasmids (Yusa et al., 2009) using the Mouse Neural Stem Cell Nucleofector Kit (Lonza). From day 2 onwards, medium was changed to FCS-based ESC medium. Fourteen days after nucleofection, induced pluripotent stem cell colonies from each well were picked on mitomycin-C-treated mouse embryonic fibroblasts and expanded for verification of complete recombination by genotyping. Subsequent analysis comprised immunofluorescence staining for Nanog (1:200, sc1000, Calbiochem) and SSEA1 (1:10, MC-480, DSHB). Residual colonies on reprogramming plates were stained for alkaline phosphatase expression.

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References


