A chromatin-wide transition to H4K20 monomethylation impairs genome integrity and programmed DNA rearrangements in the mouse

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H4K20 methylation is a broad chromatin modification that has been linked with diverse epigenetic functions. Several enzymes target H4K20 methylation, consistent with distinct mono-, di-, and trimethylation states controlling different biological outputs. To analyze the roles of H4K20 methylation states, we generated conditional null alleles for the two Suv4-20h histone methyltransferase (HMTase) genes in the mouse. Suv4-20h-double-null (dn) mice are perinatally lethal and have lost nearly all H4K20me3 and H4K20me2 states. The genome-wide transition to an H4K20me1 state results in increased sensitivity to damaging stress, since Suv4-20h-dn chromatin is less efficient for DNA double-strand break (DSB) repair and prone to chromosomal aberrations. Notably, Suv4-20h-dn B cells are defective in immunoglobulin class-switch recombination, and Suv4-20h-dn deficiency impairs the stem cell pool of lymphoid progenitors. Thus, conversion to an H4K20me1 state results in compromised chromatin that is insufficient to protect genome integrity and to process a DNA-rearranging differentiation program in the mouse.

Keywords: H4K20 methylation; Suv4-20h enzymes; DNA repair; genome integrity; B-cell differentiation; class-switch recombination

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Abrogation of H4K20me1 in mitosis results in severe chromosomal segregation defects [Julien and Herr 2004; Houston et al. 2008], and H4K20me1 has been proposed to be an important mark for chromosomal memory [Trojer and Reinberg 2006]. In *S. pombe*, H4K20 methylation by Set9/KMT5 (a PrSet7 ortholog) has been implicated in DNA damage repair [Sanders et al. 2004], although it remained unclear which H4K20 methylation state would operate in this pathway, and H3K79 methylation has also been shown to weaken the DNA damage response [Huyen et al. 2004]. Recently, structural studies confirmed association of S3BP1, a DNA damage response factor, with H4K20me2 and, to a lesser extent, also H4K20me1 [Botuyan et al. 2006]. However, despite several recent studies to interfere with H4K20 methylation states by RNAi knockdown of H4K20 methylating enzymes in a variety of cell lines [Botuyan et al. 2006; Jorgensen et al. 2007; Tardat et al. 2007; Huen et al. 2008; Yang et al. 2008], the true in vivo functions of H4K20me2 in mammals are largely unknown. In contrast, H4K20me3 is implicated in pericentric heterochromatin formation and is induced by Suv4-20h1/KMT5B and Suv4-20h2/KMT5C enzymes in a sequential pathway that depends on pre-existing H3K9me3 marks [Schotta et al. 2004].

While the above examples illustrate the diverse functions of H4K20 methylation at a cellular level, it is not clear whether this modification affects differentiation or, in general, mammalian development. Several HMTases such as Ezh2, G9a, Eset, and Mll2 have been implicated in early mammalian development (O’Carroll et al. 2006; Tachibana et al. 2002; Dodge et al. 2004; Glaser et al. 2006). In addition, Ezh2 and H3K27me3 modulate higher-order chromatin structure and thereby regulate V(D)J rearrangement during early B-cell differentiation [Su et al. 2003]. Similarly, Su39h1 and H3K9me3 function in class-switch recombination (CSR), a process required for antibody isotype diversification [Bradley et al. 2006].

To dissect the biological functions of H4K20 methylation during mammalian development and to reduce the complexities of mono-, di-, and trimethylation, we generated conditional mouse knockouts for both *Suv4-20h1* and *Suv4-20h2* genes. *Suv4-20h*-double-null (dn) mice are perinatally lethal and their chromatin has nearly lost all H4K20me3 and H4K20me2, resulting in a genome-wide transition to H4K20me1. This H4K20 monomethylated chromatin displays increased stress sensitivity and defective DNA damage repair, and reveals an important function for the Suv4-20h enzymes in developmentally programmed pathways for DNA rearrangements and during lineage commitment of lymphoid cells.

**Results**

**Suv4-20h mutant mice display perinatal lethality**

Murine *Suv4-20h* HMTases are encoded by two loci on chromosome 19 (*Suv4-20h1*) and chromosome 7 (*Suv4-20h2*). Whereas *Suv4-20h1* is ubiquitously expressed during embryogenesis and in adult tissues, *Suv4-20h2* mRNAs are much less abundant in the embryo and display restricted expression profile in only some adult tissues [Fig. 1A, Supplemental Fig. S1]. Conditional knockout alleles of *Suv4-20h1* and *Suv4-20h2* were engineered using standard technology [Fig. 1A]. Germline disruptions of floxed *Suv4-20h1* or of *Suv4-20h2* were subsequently generated by crossing to deleter strains [Mox2-Cre] that induce Cre-mediated recombination of loxP sites in the early embryo.

Intercrosses of *Suv4-20h1−/−* mice produced *Suv4-20h1−/−−* pups at sub-Mendelian ratios, indicative of embryonic lethality. Null mutant embryos are born smaller than wild-type littermates and die perinatally a few hours after birth [Fig. 1B], probably due to an alveolar defect in the lungs [data not shown]. In contrast, *Suv4-20h2−/−−* mice have no apparent defects and develop normally. To exclude functional compensation between *Suv4-20h1* and *Suv4-20h2*, we generated *Suv4-20h-dn* mice by intercrossing *Suv4-20h1−/−−* and *Suv4-20h2−/−−* mice. Similar to *Suv4-20h1-null* mice, *Suv4-20h-dn* mice are born at sub-Mendelian ratios, are smaller, and display perinatal lethality [Fig. 1B]. These data indicate an essential function for the *Suv4-20h1* enzyme during embryonic and postnatal development.

**A genome-wide transition to H4K20 monomethylation in *Suv4-20h-dn* mouse embryonic fibroblasts (MEFs)**

In order to address the effect of *Suv4-20h* abrogation on H4K20 methylation, we first analyzed all three H4K20 methylation states in wild-type and *Suv4-20h-null* primary MEFs (pMEFs) by indirect immunofluorescence (IF). In wild-type cells, H4K20me1 shows a broad nuclear staining with enrichment at the inactive X chromosome [Xi] in female cells [Fig. 2A, arrows]. H4K20me2 also displays a broad, yet more speckled nuclear staining without an apparent subnuclear enrichment; however, H4K20me2 IF signals may be under-represented due to reduced accessibility of the epitope [see the Material and Methods]. As previously reported, H4K20me3 accumulates at pericentric heterochromatin [Fig. 2A; Schotta et al. 2004]. In *Suv4-20h-null* pMEFs, H4K20me2 is reduced, whereas H4K20me3 appears unaltered. In contrast, *Suv4-20h2-null* cells show selective loss of H4K20me3, but maintain H4K20me2. Importantly, in *Suv4-20h-dn* pMEFs both H4K20me2 and H4K20me3 are nearly lost, with a concomitant increase in H4K20me1 that now becomes detectable at pericentric heterochromatin [Fig. 2A].

To quantify the observed changes in H4K20 methylation patterns, we next used mass-spectrometry of bulk histones [see the Material and Methods]. In wild-type pMEFs, H4K20me2 is the most abundant histone lysine methyl mark and is present in around 85% of all histone H4 molecules [Fig. 2B]. H4K20me1 (∼5%) and H4K20me3 (∼10%) are much less frequent. In *Suv4-20h-null* cells, H4K20me2 is reduced to ∼60%, with a concomitant increase in H4K20me1 (∼30%). In agreement with the IF data, *Suv4-20h2-null* cells selectively lose H4K20me3. Notably, in *Suv4-20h-dn* pMEFs, there is a pronounced...
conversion of H4K20 methylation states, such that H4K20me2 and H4K20me3 are almost lost and ∼90% of the H4 molecules now carry the H4K20me1 mark (Fig. 2B). These data suggest that Suv4-20h1 is largely responsible for H4K20me2, whereas Suv4-20h2 primarily regulates H4K20me3. In contrast to isolated cell cultures—such as, e.g., pMEFs—H4K20me3 is not fully abrogated in embryonic tissues of Suv4-20h2-null mice (Supplemental Fig. S2), indicating partial compensation by Suv4-20h1 or by a different HMTase.

We also examined acetylation and methylation states of H3K9, H3K14, H3K23, H3K27, H3K36, and H4 by a similar mass-spec analysis comparing wild-type and Suv4-20h-dn pMEFs. No significant changes in any of these modifications could be detected [Supplemental Fig. S3], indicating partial compensation by Suv4-20h1 or by a different HMTase.

We further examined the biological role of Suv4-20h-dn deficiency, first compared proliferation rates and cell cycle progression of wild-type and Suv4-20h-dn pMEFs. Under normal culture conditions, wild-type cells start entering crisis after more than five passages. Suv4-20h-dn cells show reduced proliferation rates and plateau much earlier (Fig. 3A). FACS analysis for DNA content revealed broader G1 and G2 peaks in Suv4-20h-dn cells at higher passage numbers (Fig. 3B). Suv4-20h-dn cells do not appear to enter aberrant apoptosis, because no significant sub-G1 peak is detected. However, BrdU labeling of wild-type and Suv4-20h-dn pMEFs indicates higher percentages of non-cycling cells in early passage Suv4-20h-dn populations [Supplemental Fig. 4A].

A more detailed analysis of cell cycle stages after BrdU pulse-labeling reveals reduction of S-phase cells with a concomitant increase of G1-phase cells, indicating a partial block in G1–S transition [Fig. 3C]. To address whether G1–S transition is indeed delayed in Suv4-20h-dn cells, we next synchronized early passage pMEFs by serum starvation and monitored S-phase entry after serum addition. There is a significant delay for Suv4-20h-dn cells in S-phase entry as compared with wild-type cells [Fig. 3D]. Together, these data indicate proliferation defects in Suv4-20h-dn cells that would be consistent with impaired cell cycle progression and higher sensitivity to stress-induced damage [Kodama et al. 2001].

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We next asked whether proliferation defects in Suv4-20h-dn pMEFs could be due to defects in DNA damage response. We first tested the G2–M checkpoint that prevents entry into mitosis when DNA damage is not completely repaired (Sancar et al. 2004). DNA double-strand breaks (DSB) were induced with varying doses of ionizing radiation (IR). Both wild-type and Suv4-20h-dn pMEFs show dose-dependent reduction in the number of mitotic cells, indicating that the G2–M checkpoint is functional (Supplemental Fig. S4B). We then analyzed chromosomal abnormalities in mitotic spreads of wild-type and Suv4-20h-dn pMEFs that would be indicative of defective DNA damage repair. Exponentially growing pMEFs were treated with 2 Gy IR and left for 1 h to allow for DSB repair. Following this, cells were arrested in mitosis by nocodazole treatment and metaphase spreads were prepared. Wild-type cells can efficiently repair DNA damage, as only a few chromosomal gaps are observed in the metaphase spreads (Fig. 3E). In contrast, the number of aberrant chromosomes displaying gaps and breaks is modestly increased in Suv4-20h-dn pMEFs (Fig. 3E,F). In a second series of experiments, cell cycle arrest was blocked with the ATM/ATR inhibitor caffeine 1 h post-treatment. These conditions allow cells with DNA damage to proceed to mitosis, resulting in a higher percentage of chromatid breaks [Fig. 3F].

Increased DNA damage sensitivity of H4K20me1 chromatin

To address how the genome-wide removal of H4K20me2 and H4K20me3 would impair proliferation, genomic in-
Integrity, and DNA damage repair, we analyzed downstream read-outs. Earlier work in *S. pombe* (Sanders et al. 2004; Du et al. 2006) and mammalian cells (Botuyan et al. 2006) suggested that 53BP1, a protein involved in DNA damage repair, binds to H4K20 methylation. Also, Dot1-mediated methylation of H3K79 has been shown to weaken the DNA damage response and could facilitate 53BP1 recruitment (Huyen et al. 2004; Wysocki et al. 2005). Although instructive, these studies did not address 53BP1 binding to a physiological template. A nucleosomal context for 53BP1 interaction is particularly important for the H4K20 position, since Suv4-20h HMTases specifically methylate nucleosomes, but show only little activity toward histone peptides (Schotta et al. 2004). Therefore, we used Suv4-20h enzymes to generate in vitro methylated nucleosomes, which serve as a strong binding substrate for 53BP1 (Supplemental Fig. S5).

The conserved checkpoint protein 53BP1 localizes to DSBs within minutes post-damage and participates in DNA damage repair (Schultz et al. 2000; Ward et al. 2003). To examine whether H4K20me1 chromatin may be compromised in 53BP1-mediated DNA damage response, we exposed wild-type and *Suv4-20h-dn* pMEFs to 2 Gy of IR and then monitored formation of γH2A.X and 53BP1 foci by IF. Nontreated cells are largely negative for γH2A.X and show dispersed 53BP1 staining. However, as early as 1–2 min post-damage, most wild-type cells have formed small 53BP1 foci that overlap with γH2A.X, whereas *Suv4-20h-dn* cells only display reduced focal enrichment of 53BP1 [Fig. 4A]. At later time points (10 min), nearly all wild-type and *Suv4-20h-dn* cells show comparable γH2A.X and 53BP1 foci [Fig. 4A]. We quantified this differential 53BP1 recruitment, which indicates that only at very early time points after damage (<5 min), there are reduced numbers of 53BP1-positive *Suv4-20h-dn* cells [Fig. 4B].

We next examined DNA damage sensitivity by colony formation assays (see the Materials and Methods) and by comparing *Suv4-20h-dn* with 53BP1-null MEFs [Ward et al. 2003]. 53BP1-null MEFs show pronounced sensitivity toward IR-induced DNA damage, which is not reflected in *Suv4-20h-dn* cells [Fig. 4C, top left panel]. This result is in agreement with data from *S. pombe*, where cells remain insensitive to IR despite impaired recruitment of Crb2 [a 53BP1 ortholog] to DSBs, due to loss of H4K20 methylation or mutations in the Crb2 tudor domain [Du et al. 2006], suggesting that binding of 53BP1 to

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**Figure 4.** Increased DNA damage sensitivity in H4K20me1 chromatin. (A) Wild-type and *Suv4-20h-dn* pMEFs were treated with 2 Gy IR, fixed after different time-points (1–10 min), and stained with antibodies against γH2A.X and 53BP1. (B) Numbers of 53BP1-positive cells (more than one focus per cell) were counted at different time-points post-treatment with 2 Gy IR. At early time-points (2 min), *Suv4-20h-dn* cells display reduced focal enrichment of 53BP1. At later time points (10 min), wild-type and *Suv4-20h-dn* cells show comparable 53BP1 foci. (C) Colony formation assays to assess sensitivity of *Suv4-20h-dn* and 53BP1-null cells to various types of DNA damage. Wild-type, 53BP1-null, and *Suv4-20h-dn* MEFs were plated at low density and treated with indicated doses of IR, etoposide, hydroxyurea, and UV. The percentage of surviving colonies relative to the nontreated samples is plotted.
H4K20me2 is not critical for cell survival after IR damage. We then extended our analysis to other DNA-damaging agents, such as etoposide, a specific inhibitor of topoisomerase II, and to the replication inhibitor hydroxyurea. Under these stress conditions, Suv4-20h-dn cells display a very similar increase in damage sensitivity as compared with 53BP1-null MEFs [Fig. 4C]. In contrast, upon UV-induced damage, Suv4-20h-dn cells are more sensitive than 53BP1-null MEFs [Fig. 4C, bottom right panel]. Together, these data indicate partially overlapping functions for the Suv4-20h enzymes and 53BP1 in certain DNA damage response pathways, but also reveal selective roles for H4K20 methylation that appear to be independent of 53BP1.

Conditional deletion of the Suv4-20h genes in the hematopoietic system

The impairment of the DNA damage response suggests that Suv4-20h deficiency may also affect lineage programs in the mouse, where DSB-mediated DNA rearrangements dictate transitions between progenitor and fully differentiated cells. Paradigms for these processes are B and T cells of the hematopoietic system that rearrange variable regions of antigen receptor loci [V(D)J recombination] and constant regions of immunoglobulin genes [CSR], thereby allowing antibody diversity (Dudley et al. 2005).

To address a possible role for the Suv4-20h enzymes in these processes, we inactivated Suv4-20h function by crossing compound Suv4-20h1f/f, Suv4-20h2−/− mice to a deleter strain (vav Cre) that expresses the Cre recombinase in hematopoietic stem cells (HSCs) and should, therefore, delete Suv4-20h enzymes in all cells of the hematopoietic system. Suv4-20h-dn vav mice are viable and do not show any gross abnormalities. RT–PCR demonstrates efficient deletion of Suv-20h1f/f, since no Suv4-20h1 transcripts can be detected in Suv4-20h-dn vav CD19+ B cells [Supplemental Fig. S6A]. In addition, we analyzed H4K20 methylation states in wild-type and Suv4-20h-dn vav CD19+ B cells by mass-spectrometry of bulk histones. Deletion of both Suv4-20h enzymes in B cells also leads to a genome-wide conversion to H4K20me1 chromatin [Supplemental Fig. S6B].

V(D)J recombination assembles the variable regions of immunoglobulin (Ig) genes from discontinuous variable (V), diversity (D), and joining (J) gene segments in pro-B cells by a sequence-specific Rag1 and Rag2-mediated recombination mechanism (Dudley et al. 2005). We isolated genomic DNA from sorted wild-type, Suv4-20h-dn vav, and Pax5−/− pro-B cells and quantified successful V(D)J rearrangements by Vκ segment-specific PCR. In contrast to the Pax5 mutant control, which has impaired joining of the more distal Vκ segments [Fuxa et al. 2004], Suv4-20h-dn vav pro-B cells do not show apparent defects in V(D)J recombination [Supplemental Fig. S7]. We also examined V(D)J recombination of the TCRβ locus in wild-type and Suv4-20h-dn vav pro-T cells and could not detect any gross abnormalities [Supplemental Fig. S8]. We conclude that Suv4-20h enzymes have no primary function in V(D)J recombination of both pro-B and pro-T cells.

Suv4-20h-dn B cells are defective in CSR

CSR in the immunoglobulin heavy chain (IgH) locus is a process that changes the antibody effector function by...
replacing the default constant region (Cµ) of the antibody gene with a different constant region. This process is not sequence specific and involves the generation of DSBs in highly repetitive switch (S) regions, which precede every individual constant region (see Fig. 5, bottom diagram). These DSBs are processed by the DNA damage machinery (Dudley et al. 2005). Mature B cells primarily generate the IgM isotype [from the V(D)J most-proximal Cµ region] and, upon specific antigen stimulation with LPS/IL-4, predominantly switch to the IgG3 isotype.

To examine whether CSR is impaired in Suv4-20h-dn^vav mice, we stimulated B220^+ splenic B cells with LPS/IL-4 to induce switching from Cµ to Cγ1. Comparable proliferation rates over a 3-d period between wild-type and mutant samples were confirmed by CFSE fluorescent labeling of B220^+sorted B cells [Fig. 5A]. Whereas 35% of wild-type B cells showed expression of IgG1, only 16% of Suv4-20h-dn^vav B cells were IgG1-positive [Fig. 5B]. The experiment was repeated with B-cell preparations from eight independent Suv4-20h-dn^vav mice, consistently demonstrating reduced efficiency in CSR to IgG1 [Fig. 5C]. We next examined whether CSR to other isotypes may also be impaired by stimulating B220^+ B cells with LPS only to induce switching to IgG1. In wild-type B cells we observed ~6% IgG1-positive cells, whereas in Suv4-20h-dn^vav B cells, this number is decreased to 2%–3% [Fig. 5C].

Efficient CSR correlates with the expression of so-called germline transcripts (GLTs) initiated at the promoters upstream of the switch regions [see bottom diagram in Fig. 5], which are proposed to make the chromatin domain accessible for the induction of DSBs [Dudley et al. 2005]. We therefore measured the abundance of Iµ and Iγ1 GLTs in wild-type versus Suv4-20h-dn^vav B cells by real-time RT–PCR. As shown in Figure 5D, Iµ and Iγ1 GLTs are expressed at similar levels. A byproduct of productive CSR is the generation of a looped-out circular DNA fragment that contains joined segments of the Sµ and S γ1 switch regions that are preceded by an intronic promoter. Joined SµS γ1 transcripts from this circular DNA only occur after productive CSR, and their abundance is directly proportional to the frequency of productive switching events (Dudley et al. 2005). We quantified these SµS γ1 circle transcripts by real-time RT–PCR, which are significantly reduced in Suv4-20h-dn^vav B cells [Fig. 5D].

Altered chromatin structure and increased chromosomal aberrations in Suv4-20h-dn^vav B cells

Next, we addressed whether transcriptional changes in Suv4-20h-dn^vav B cells could be responsible for defective CSR. The activation-induced deaminase (Aid), which targets switch regions and generates a DNA damage signal by deamination of cytidine nucleotides is such an essential factor, since CSR is abrogated in Aid-null mice (Muramatsu et al. 2000). Real-time RT–PCR analysis revealed no significant alteration in Aid expression levels in activated B cells [see Fig. 5D]. We then performed global expression profiling using Affymetrix tiling arrays to analyze possible changes in gene expression between wild-type versus Suv4-20h-dn^vav B cells before and after antigen stimulation. These, and similar analyses in embryonic stem [ES] cells, revealed that out of ~35,000 ESTs, <150 genes were either slightly up- or down-regulated in Suv4-20h-dn^vav B cells [Supplemental Fig. S9]. None of the affected genes is reported to be involved in CSR or DNA damage control [Supplemental Tables S1–S3]. From these data we conclude that Suv4-20h deficiency has only a minor impact on general transcriptional regulation. By comparison, abrogation of the transcription factor Pax5 in B cells results in ~300 genes that are aberrantly regulated with much more significant differences in their expression levels [Delogu et al. 2006; Schebesta et al. 2007].

The defects in CSR could be caused by alterations in the chromatin structure at the IgH locus. Therefore, we analyzed major histone lysine methylation marks at the Iγ1 and Iγ1 promoter and at their associated switch regions [Fig. 6A, top diagram] in wild-type versus Suv4-20h-dn^vav B cells by directed chromatin immunoprecipitation [ChIP]. In resting wild-type B cells, the Iγ1 promoter is active, whereas the Iγ1 region is silent [Dudley et al. 2005]. In agreement, we detect significant enrichment for H3K4me3 across the IgH, but not the Iγ1 region, which instead is decorated by the repressive H3K27me3 mark [Fig. 6A, bottom left panels]. For H3K9me3 and H4K20me3, there are only low-level enrichments with both the IgH and Iγ1 probes. Importantly, H4K20me1 is high across IgH, but only with residual signals across Iγ1 [Fig. 6A, top left panel]. We could not examine H4K20me2, since several attempts with distinct H4K20me2 antibodies failed to indicate reliable enrichment at the IgH locus and also at other chromosomal regions [data not shown].

Upon activation of wild-type B cells, this chromatin profile is altered by three major changes, such that H3K4me3 is now present across Iγ1 [with a concomitant decrease for H3K27me3], H4K20me1 signals at IgH are reduced and H4K20me3 is elevated with three out of five Iγ1 probes [Fig. 6A, right panels]. Importantly, in both resting and activated Suv4-20h-dn^vav B cells, the IgH and Iγ1 chromatin structure is locked with high H4K20me1 marks, thereby impairing the downshift for H4K20me1 at IgH and reducing H3K4me3 accumulation at Iγ1 [Fig. 6A, both panels].

The aberrantly high H4K20me1 levels across the Iγ1 region in both resting and activated Suv4-20h-dn^vav B cells could affect processing of Aid-induced DSBs also beyond impaired CSR. Therefore, we examined IgH-associated chromosomal abnormalities in activated wild-type and Suv4-20h-dn^vav B cells by chromosome painting and IgH-telomere FISH analysis [Callen et al. 2007]. Isolated splenic B cells were stimulated with LPS/IL-4 for 3 d, and mitotic chromosome spreads were then prepared. As shown in Figure 6B, left panel, the IgH locus is located close to the telomeric region of chromosome 12. Notably, we detect IgH-associated translocations [T12;12] and deletions [D12;12] in mitotic spreads from six independently isolated Suv4-20h-dn^vav B-cell samples [Fig. 6B]. To determine whether IgH-specific chromosomal instabili-
ties are dependent on impaired CSR, we stimulated B cells from wild-type and Suv4-20h-dn mouse with anti-RP105, which induces proliferation, but not class switching (Callen et al. 2007). In neither wild-type (n = 80) nor Suv4-20h-dn mouse (n = 80) mitotic spreads could we identify IgH-associated chromosomal aberrations (Supplemental Fig. S10).

Figure 6. Altered chromatin structure and increased chromosomal aberrations in Suv4-20h-dn mouse B cells. (A) Histone modifications at the Igµ and Igα promoters and their associated switch regions of the IgH locus were determined by directed ChIP in resting and activated B cells. The top diagram indicates the approximate positions of the primer pairs. Histone modifications for major satellite repeats and tubulin served as controls. (B) IgH-associated chromosomal abnormalities were analyzed in metaphase spreads of activated wild-type and Suv4-20h-dn mouse B cells using chromosome painting in combination with IgH-FISH. In wild-type B cells, the IgH locus (green dots) is located on chromosome 12 (blue), proximal to the telomeres (red dots). Elevated levels of IgH-associated translocations (T) or Deletions (Del) were observed in Suv4-20h-dn mouse B cells. The box plot shows percentages of IgH-associated abnormalities in six independent wild-type (362 spreads) and Suv4-20h-dn mouse (379 spreads) B-cell preparations.

Suv4-20h-dn deficiency impairs the stem cell potential for lymphoid cells

The Suv4-20h enzymes are expressed throughout distinct stages of B-cell development and down-regulated upon B-cell activation. This expression profile is similar to the B-cell-specific transcription factors NFκB
Pax5, but different from Aid, which only becomes transcribed after B-cell activation [Supplemental Fig. S11]. To examine whether B-cell development is impaired in Suv4-20h-dn^{avw} mice, we performed FACS analyses of bone marrow, spleen, and thymus using markers that identify progenitor, immature, and mature B cells, and also granulocytes, macrophages, and CD4^+ , CD8^+ double-positive (DP) T cells. In both bone marrow and spleen of Suv4-20h-dn^{avw} mice, there are fewer numbers of CD19^+ B cells as compared with wild-type controls. In particular, we observed pronounced reductions for recirculating B cells and mature B cells, but not for pro-B and pre-B cells [Fig. 7A]. In addition, numbers of DP T cells in the thymus are reduced. This developmental defect appears specific to the lymphoid lineage, since numbers of myeloid cells [granulocytes and macrophages] are unaltered [Fig. 7A].

To examine whether Suv4-20h-dn deficiency would impair the stem cell pool of lymphoid progenitors, we conducted competitive [1:1] transplantation experiments. Equal numbers of wild-type [discriminated by the cell surface marker CD45.1] and Suv4-20h-dn^{avw} [different CD45.2] bone marrow cells were used to repopulate the hematopoietic system of lethally irradiated wild-type mice. The well-defined fraction of multipotent progenitor cells [lin^- , sca-1^+ , c-kit^+ ], which contains short- and long-term HSCs is slightly increased in Suv4-20h-dn^{avw} bone marrow as compared with the wild-type control [Supplemental Fig. S12] and could therefore give rise to even higher repopulation activity. We analyzed reconstituted mice 7 wk [Supplemental Fig. S13] and 11 mo [Fig. 7] after transfer for long-term reconstitution. Intriguingly, numbers of Suv4-20h-dn^{avw} B and T cells are severely underrepresented and amount to <1% of re-

**Figure 7.** Suv4-20h deficiency impairs the stem cell potential for lymphoid cells. (A) Bone marrow, spleen, and thymus of eight independent 2-mo-old wild-type and Suv4-20h-dn^{avw} mice were analyzed by FACS for different B-cell populations, myeloid lineages [macrophages, granulocytes], and DP T cells. Asterisks indicate statistically significant differences (t-test, P < 0.05). (B) Competitive reconstitution experiments [1:1] were performed with equal numbers of wild-type (CD45.1) and Suv4-20h-dn^{avw} (CD45.2) bone marrow cells, which were injected into lethally irradiated mice. The ratio between the CD45.1 and CD45.2 markers was analyzed 11 mo post-transplantation in the different hematopoietic lineages. Whereas myeloid cells show significant [20%–30%] contribution of Suv4-20h-dn^{avw} cells, <1% of the lymphoid lineages (B and T cells) are derived from Suv4-20h-dn^{avw}.
constituted B and T cells (Fig. 7, bottom panel). In contrast, myeloid cells, such as granulocytes and macrophages, still comprised significant numbers (20%–30%) of CD45.2-positive (−Suv4-20h-dn) cells. This is an important control and largely excludes the possibility that Suv4-20h-dn HSCs would fail to repopulate the hematopoietic system because of an attenuated proliferative potential. Together, these in vivo data demonstrate an important function for the Suv4-20h enzymes to ensure the lineage program of DNA-rearranging lymphoid cells.

Discussion

The control of H4K20 methylation states in mammalian chromatin

In this study, we generated Suv4-20h-dn mice, which lack nearly all H4K20me2 and H4K20me3 in mammalian chromatin. Suv4-20h1 primarily induces H4K20me2, whereas Suv4-20h2 is largely responsible for H4K20me3. Surprisingly, H4K20me3 is not fully abrogated in embryonic tissues of Suv4-20h2−/− mice and readily detectable in several adult tissues (Supplemental Fig. S2). These data suggest compensatory functions of Suv4-20h1 or other HMTases. Therefore, selective loss of either H4K20me2 or H4K20me3 during mouse development cannot be uncoupled or independently analyzed. In Suv4-20h-dn mice, there is a conversion to H4K20me1 chromatin, but not to the fully unmodified state. The data demonstrate that Suv4-20h HMRTases are the predominant enzymes for H4K20me2 and H4K20me3 and that H4K20me1 may be their default substrate.

The major HMTase for H4K20me1 is PrSet7, which functions in gene silencing and during mitotic chromosome condensation (Fang et al. 2002; Nishioka et al. 2002). Recent data indicate that PrSet7 generates most H4K20me1 during S phase as it localizes to replication foci and directly interacts with PCNA (Jorgensen et al. 2007; Tardat et al. 2007; Huen et al. 2008). In Drosophila, H4K20me1 seems to be a prerequisite to establish H4K20me2 and H4K20me3, as disruption of PrSet7 results in the removal of all three H4K20 methylation states (Karachentsev et al. 2005). These data suggest that murine PrSet7 may also dictate H4K20 methylation in the mouse. However, probing mammalian PrSet7 functions has been problematic, since abrogation of PrSet7 by RNAi knockdown leads to immediate S-phase defects and a severely reduced proliferation potential (Jorgensen et al. 2007; Tardat et al. 2007). In addition, Prset7 deficiency results in early embryonic lethality (Huen et al. 2008) and PrSet7-null blastocysts fail to progress beyond the four- to eight-cell stage (H. Oda and D. Reinberg, pers. comm.). Together, these observations predict that PrSet7 function cannot be rescued by Suv4-20h enzymes. In contrast, Suv4-20h-dn chromatin is characterized by broad H4K20me1, which compromises, but not fully abrogates some of the biological roles of the other H4K20 methylation states. In this respect, Suv4-20h-dn deficiency reflects a hypomorphic mutation for H4K20 methylation.

H4K20 monomethylated chromatin

H4K20 methylation and DNA damage response

The position of Lys 20 at the boundary between the globular and flexible histone H4 domains is proposed to dominate structural transitions of chromatin (Dorigo et al. 2003; Shogren-Knaak et al. 2006). Currently, it is not known whether more rigid or more relaxed chromatin configurations could be induced by binding of proteins to the different H4K20 methylation states, although interaction of L3MBT with H4K20me1 methylated nucleosomes has been shown recently to induce nucleosome compaction in vitro (Trojer et al. 2007). We show that DNA damage repair is only partially defective in Suv4-20h-dn cells (Fig. 4A,B), consistent with a recent report where RNAi knockdown of Suv4-20h in mouse cells modestly reduced 53BP1 foci formation after treatment with the radio-mimetic bleomycin (Yang et al. 2008). This and earlier work (Huyen et al. 2004) suggest that 53BP1 can probably localize to chromatin at DSBs via multiple mechanisms. Although in S. pombe H4K20 methylation is crucial for Crb2 [a 53BP1 ortholog] localization to DSBs (Sanders et al. 2004; Du et al. 2006), Dot1-mediated H3K79 methylation has also been shown to aid 53BP1 recruitment (Huyen et al. 2004) and to contribute to DNA damage checkpoint control in Saccharomyces cerevisiae (Wysocki et al. 2005). Structural definition of 53BP1 binding revealed preferred interaction to H4K20me2 and, at threefold reduced affinity, also to H4K20me1 (Botuyan et al. 2006). Whereas our in vivo analysis of Suv4-20h-dn chromatin substantiates a role for H4K20me2 in facilitating 53BP1 recruitment, it does not exclude other targeting modes such as, e.g., H3K79 methylation.

53BP1 contains distinct domains that mediate interaction with other upstream [e.g., γH2AX] or downstream [e.g., Chk2] factors of the repair machinery and that could further stabilize 53BP1 chromatin interaction in the absence of H4K20me2. The DNA damage response has also revealed chromatin remodeling activities that precede H2A.X phosphorylation (Polo et al. 2006). No apparent defect in γH2AX foci formation was observed in Suv4-20h-dn cells, either after IR damage [Fig. 4A] or after laser microirradiation of euchromatic or heterochromatic subnuclear domains [data not shown]. This probably excludes that H4K20me1 chromatin impairs nucleosome remodeling, although it could favor nucleosome compaction or potential cross-talk to histone H1 (Trojer et al. 2007). Interestingly, mouse cells that are triple-deficient for histone H1 isoforms (Fan et al. 2005) display more relaxed nucleosomal arrays and are more protected against DNA damage (Murga et al. 2007). Further, whereas Suv4-20h deficiency weakens 53BP1 association at sites of DNA damage and reflects a similar sensitivity following etoposide and hydroxyurea treatment [Fig. 4C], there are distinct stress responses between Suv4-20h-dn and 53BP1-null MEFs after IR or UV exposure [Fig. 4C]. These data would be consistent with Suv4-20h enzymes to participate in 53BP1-dependent and 53BP1-independent pathways, where also substrates other than histone H4 may be targeted by Suv4-20h function.
Control of CSR by H4K20 methylation

Several components of the DSB repair machinery are also involved in programmed DNA rearrangements during B- and T-cell maturation. For example, 53BP1 mutant mice are severely defective in CSR, however, they do not show impaired V(D)J recombination (Manis et al. 2004; Ward et al. 2004). Conversely, several HMTases affect processing of chromatin-mediated transitions during B- and T-cell differentiation. The polycomb enzyme Ezh2 coregulates V(D)J rearrangement (Su et al. 2003), and the Suv39h1 HMTase partially controls CSR at the Cε constant region (Bradley et al. 2006). Here, we show that differentiation of B and T cells is significantly perturbed upon conditional deletion of both Suv4-20h enzymes in HSCs [Fig. 7]. In addition, CSR at the Cγ1 and Cγ3 regions is decreased in Suv4-20h-dn B cells [Fig. 5], but there is no apparent defect in V(D)J rearrangement of the IgH region in pro-B cells (Supplemental Fig. S7) or of the TCRβ locus in pro-T cells (Supplemental Fig. S8).

These results could, in principle, be explained by weakened association of 53BP1 to H4K20me1 IgH chromatin, similar to the delayed recruitment at DSBs in Suv4-20h-dn MEFs. While this may seem plausible, we observe significantly elevated H4K20me1 in Suv4-20h-dn mutant mice. In addition, the recent observation that Suv4-20h-dn MEFs display aberrant telomere length (Benetti et al. 2007). In addition, and in contrast to 53BP1-null mice (Ward et al. 2003, Suv4-20h-dn mutant mice are perinatally lethal (Fig. 1B).

Further, H4K20me1 has been associated with transcriptional repression (Nishioka et al. 2002, Karachentsev et al. 2005) or activity (Papp and Muller 2006, Vakoc et al. 2006), which would predict altered gene regulation in chromatin that is severely overrepresented for H4K20me1. However, global expression profiling in mouse ES cells, and in resting and activated B cells (Supplemental Fig. S9), has indicated only very minor changes between wild-type and Suv4-20h-dn cells. In addition, transcriptional regulation of 53BP1 or Aid is not altered in the absence of Suv4-20h enzymes during distinct stages of B-cell maturation (Supplemental Fig. S11).

In summary, the cumulative data suggest a model in which Suv4-20h deficiency induces compromised H4K20me1 chromatin that is sensitive to DNA damage or comparable stress signals, particularly at chromosomal regions largely lacking gene activity (e.g., telomeres and centromeres) or otherwise being exposed for sequence nonspecific DNA rearrangements (e.g., CSR).

Functions of Suv4-20h enzymes in protecting normal development

We have discussed H4K20me1 and H4K20me2 and how these states are altered in Suv4-20h-dn mice. By comparison, removal of H4K20me3 correlates with rather mild phenotypes. For H4K20 methylation, it appears that a trimethyl state is nonessential for viability, absence of a dimethyl state still allows for full fetal development (Fig. 1B), and lack of a monomethyl state is embryonic lethal [Huen et al. 2008; H. Oda and D. Reinberg, pers. comm.]. H4K20me3 at pericentric heterochromatin is induced by Suv4-20h enzymes in a pathway that is dependent on preceding H3K9me3 by the Suv39h HMTases (Schotta et al. 2004). In agreement, H3K9me3 and HP1 association is maintained at pericentric heterochromatin in the absence of the Suv4-20h HMTases (data not shown). Therefore, Suv4-20h2−/− mice show less severe defects in overall chromatin structure, genome integrity, chromosome segregation (aneuploides), or cancer development as described for Suv39h-dn mice (Peters et al. 2001). However, a strong reduction of H4K20me3 has been correlated with cancer progression (Fraga et al. 2005, H2A.X- or 53BP1-null mice eventually succumb to cancer development in a p53-dependent manner (Bassing et al. 2003, Celeste et al. 2003, Ward et al. 2005, Morales et al. 2006). Although we have not (yet) observed lymphomas in Suv4-20h-dn mice, it will be interesting to analyze these mutants in a p53-deficient background or in other accelerated tumor models.

A aberrantly high levels of H4K20me3 progressively accumulate in senescent cells or in premature aging syndromes (Shumaker et al. 2006). Furthermore, H4K20me3 levels were shown to increase with aging in mammalian tissues (Sarg et al. 2002). Future work is required to address the possible functions of the Suv4-20h enzymes in aging-associated processes and during cancer development using appropriate mouse systems. In summary, the conditional Suv4-20h-dn mice represent a valuable model to analyze functions of H4K20 methylation during mammalian development and clearly demonstrate the divergent biological relevance of the three distinct histone lysine methylation states.

Materials and methods

Generation of conditional Suv4-20h knockout mice

Targeting of both Suv4-20h genes was performed using standard technologies with details provided in the Supplemental Material.

Northern blots

To analyze expression of Suv4-20h enzymes in embryonic development and in different tissues of adult mice, 1.8-kb PCR DNA fragment of the Suv4-20h1 cDNA and a 1.2-kb PCR DNA fragment of the Suv4-20h2 cDNA was hybridized to multiple Northern blots (IBOT2 and BLOT3, Sigma).

Immunofluorescence and mass-spec

Immunofluorescence analyses were performed as described (Peters et al. 2003) using the following antibodies: H4K20me1, H4K20me2, H4K20me3 (Peters et al. 2003), 53BP1 (1:5000, Novus Biologicals), γ-H2A.X [1:2000, UBI]. Five independent H4K20me2 antibodies (Peters et al. 2003; Abcam ab9052, UBI #05-672, UBI #07-747) show only low signal intensities, suggesting reduced accessibility of the H4K20me2 epitope.
For mass-spect, bulk histones from pMEFs and B cells were isolated by acid extraction and analyzed as described [Peters et al. 2003]. For quantification, we used controls to ensure complete digest of the propionylated samples and synthetic peptides to correct for precolumn enrichment, HPLC separation, ionization, and mass-spectrometric detection of cleaved peptide fragments (see the Supplemental Material).

**Proliferation, cell cycle, and DNA damage assays**

These assays were performed using standard procedures as detailed in the Supplemental Material.

**CSR**

CSR assays with splenic B220+ B cells from Suv4-20h-dn^v^ and age-matched wild-type mice were performed according to standard procedures as outlined in the Supplemental Material. To induce proliferation without activation of AID, B cells were stimulated with RP105 (2.5 μg/ml, BD Pharmingen). To measure GLTs and Aid transcripts, RNA from stimulated B cells (LPS/IL-4) was analyzed by quantitative RT–PCR.

**ChIP**

Chromatin from resting and activated B cells from three independent wild-type and Suv4-20h-dn^v^ mice was prepared using standard procedures and precipitated with H4K20me1, H4K20me3, H3K9me3, H3K27me3 (Peters et al. 2003), and H3K4me3 (Abcam, ab8580) antibodies. Immunoprecipitated DNA was analyzed by quantitative PCR using primers specific for Igµ and IgH transcripts, RNA from stimulated B cells (LPS/IL-4) was analyzed by quantitative RT–PCR.

Chromatin from resting and activated B cells from three independent wild-type and Suv4-20h-dn^v^ mice was prepared using standard procedures and precipitated with H4K20me1, H4K20me3, H3K9me3, H3K27me3 (Peters et al. 2003), and H3K4me3 (Abcam, ab8580) antibodies. Immunoprecipitated DNA was analyzed by quantitative PCR using primers specific for Igµ and IgH transcripts, RNA from stimulated B cells (LPS/IL-4) was analyzed by quantitative RT–PCR.

**Competitive bone marrow reconstitution**

Lethally irradiated B6. SJL recipient mice (CD45.1) were injected with 5 x 10^5 bone marrow cells [1:1] derived from wild-type (CD45.1) and Suv4-20h-dn^v^ (CD45.2) mice. Hematopoietic lineages were analyzed by FACS as outlined in the Supplemental Material.

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A chromatin-wide transition to H4K20 monomethylation impairs genome integrity and programmed DNA rearrangements in the mouse

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