A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin

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Histone lysine methylation is a central modification to mark functionally distinct chromatin regions. In particular, H3-K9 trimethylation has emerged as a hallmark of pericentric heterochromatin in mammals. Here we show that H4-K20 trimethylation is also focally enriched at pericentric heterochromatin. Intriguingly, H3-K9 trimethylation by the Suv39h HMTases is required for the induction of H4-K20 trimethylation, although the H4 Lys 20 position is not an intrinsic substrate for these enzymes. By using a candidate approach, we identified Suv4-20h1 and Suv4-20h2 as two novel SET domain HMTases that localize to pericentric heterochromatin and specifically act as nucleosomal H4-K20 trimethylating enzymes. Interaction of the Suv4-20h enzymes with HP1 isoforms suggests a sequential mechanism to establish H3-K9 and H4-K20 trimethylation at pericentric heterochromatin. Heterochromatic H4-K20 trimethylation is evolutionarily conserved, and in Drosophila, the Suv4-20 homolog is a novel PEV modifier to regulate position-effect variegation. Together, our data indicate a function for H4-K20 trimethylation in gene silencing and further suggest H3-K9 and H4-K20 trimethylation as important components of a repressive pathway that can index pericentric heterochromatin.

[Keywords: Histone code; histone H4 Lys 20; mono-, di-, trimethylation; Suv4-20h HMTases; heterochromatin; combinatorial histone methyl marks]

Supplemental material is available at http://www.genesdev.org.

Received February 19, 2004; revised version accepted April 5, 2004.

Chromatin is the physiological template of the genetic information and is composed of the DNA polymer, histones, and other chromosomal proteins. The basic repeating unit of chromatin is the nucleosome octamer, which is built by the core histones H2A, H2B, H3, and H4 wrapping 147 bp of DNA [Luger et al. 1997]. Histone N termini [tails] are flexible and protrude from the nucleosome octamer. Histone tails are subject to posttranslational modifications, including acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation [van Holde et al. 1988]. During the recent years, many histone modifying enzymes and several chromatin-associated proteins that specifically bind to these modifications have been identified. Particular histone modification patterns have been associated with distinct chromatin states and are proposed to represent an indexing mechanism that could extend the information potential of the genetic code [Strahl and Allis 2000; Turner 2000; Jenuwein and Allis 2001].

Histone modifications can act as either repressive or active marks. For histone lysine methylation, modification of H3-K4, H3-K36, and of H3-K79 have been correlated with transcriptional activation, whereas methylation of H3-K9, H3-K27, and H4-K20 are marks of repressive chromatin states [for review, see Fischle et al. 2003; Lachner et al. 2003; Vaquero et al. 2003]. Histone lysine residues can be mono-, di-, or trimethylated [Paik and Kim 1971; DeLange et al. 1973], thereby extending the coding potential of a methylatable lysine position. Indeed, the distinctions between di- and trimethylation of H3-K4 [Santos-Rosa et al. 2002] or of H3-K9 residues [Czermin et al. 2002; Kuzmichev et al. 2002; Tamura et al. 2003] have been shown to be relevant for transcriptional regulation or Polycomb-mediated gene silencing. Distinct methylation states or combinations between several methylation marks could further discriminate
different chromatin regions or entire chromosomes. For example, H3-K27 trimethylation in conjunction with H3-K9 dimethylation are considered epigenetic imprints of the inactive X chromosome [Plath et al. 2003; Silva et al. 2003; Okamoto et al. 2004], whereas H3-K9 trimethylation and H3-K27 monomethylation are associated with pericentric heterochromatin [Peters et al. 2003; Rice et al. 2003]. The combinatorial nature of histone lysine modifications requires controlled interplay between different histone lysine methylation systems. Loss of a given HMTase may also affect methylation on lysine residues for which the enzyme has no intrinsic activity. This is exemplified by the conversion of pericentric H3-K27 methylation to H3-K27 trimethylation in the absence of Suv39h enzymes [Peters et al. 2003].

Methylation of H4-K20 has been described as another mark of repressive chromatin domains [Nishioka et al. 2002; Sims et al. 2003]. However, previous studies were focused toward detection of H4-K20 methylation, regardless of the methylation status and characterized PR/SET7 [Nishioka et al. 2002a; Rice et al. 2002] or SET8 [Fang et al. 2002] as the responsible enzyme. Here, we examine all three H4-K20 methylation states in mammalian chromatin with highly selective antibodies that discriminate H4-K20 mono-, di-, and trimethylation. We demonstrate that H4-K20 trimethylation is a novel and evolutionarily conserved mark of pericentric heterochromatin. In addition, we identify two murine SET domain HMTases that localize to pericentric heterochromatin and induce H4-K20 trimethylation in a Suv39h-dependent manner. In Drosophila, mutants of the corresponding gene locus are dominant suppressors of position-effect variegation. These data establish H4-K20 trimethylation as a repressive mark in gene silencing mechanisms and suggest that the sequential induction of H3-K9 and H4-K20 trimethylation by distinct histone lysine methylation systems can index repressive chromatin domains.

Results

H4-K20 trimethylation is a novel mark of pericentric heterochromatin

Pericentric heterochromatin in mammals mainly consists of major satellite repeats and can easily be visualized by the fluorochrome DAPI, which preferentially intercalates with A/T-rich repeat sequences. Differential association of H3-K9 and H3-K27 methylation states in euchromatin versus heterochromatin [Peters et al. 2003] prompted us to analyze other potentially repressive marks, such as histone H4-K20 methylation. Highly specific antibodies that discriminate H4-K20 methylation states were developed by Upstate Biotechnology (UBI) and quality controlled by dot blots presenting a panel of 23 histone tail peptides [Supplementary Fig. S1] and by Western blots of mouse nuclear extracts [Supplementary Fig. S2]. Antibody specificity was further confirmed by peptide competition assays in immunofluorescence analyses [Supplementary Fig. S3]. By using these and our previously characterized H3-K9 methyl antibodies, we performed comparative immunofluorescence in wild type and Suv39h double-null (dn) female mouse embryonic fibroblasts (MEFs). In wild-type MEFs, H4-K20 monomethylation is dispersed in euchromatin but shows focal enrichment at the inactive X chromosome [Fig. 1A, arrow; Kohlmaier et al. 2004]. H4-K20 dimethylation is also broadly distributed over euchromatic regions but shows a more speckled pattern, which resembles H3-K9 dimethylation [Fig. 1A]. In contrast, H4-K20 trimethylation is strongly enriched at DAPI dense regions and reflects the characteristic accumulation of H3-K9 trimethylation at pericentric heterochromatin [Fig. 1A]. We also analyzed H4-K20 methylation states in interphase chromatin of HeLa cells. H4-K20 mono- and dimethylation are uniformly distributed throughout the nuclei [Supplementary Fig. S4]. Notably, H4-K20 monomethylation displays variable signal intensities, suggesting a potential cell-cycle regulation for this mark. In contrast, H4-K20 trimethylation is enriched at several nuclear foci, which represent pericentric heterochromatin as demonstrated by immunofluorescence analysis of metaphase spreads [Supplementary Fig. S4].

An interplay between distinct methylation systems at pericentric heterochromatin has been described previously [Peters et al. 2003]. For example, disruption of the Suv39h enzymes results in the loss of H3-K9 trimethylation by also converting H3-K27 monomethylation to H3-K27 trimethylation. Because of the strikingly similar accumulation of H4-K20 trimethylation and H3-K9 trimethylation at pericentric heterochromatin, we analyzed whether H4-K20 methylation states may also depend on the presence of the Suv39h enzymes. In Suv39h dn female MEFs, H4-K20 mono- and dimethylation are not altered, but H4-K20 trimethylation is entirely lost from pericentric heterochromatin [Fig. 1A]. These data were confirmed by analyzing metaphase chromosomes of wild-type and Suv39h dn MEFs. In wild-type mitotic spreads, H4-K20 trimethylation is strongly enriched at pericentric regions and shows a diffuse staining pattern along the chromosomal arms [Fig. 1B]. In contrast, in mitotic spreads from Suv39h dn MEFs, pericentric H4-K20 trimethylation is lost, whereas the chromosomal arms display enriched signals [Fig. 1B].

From these data, we conclude that the presence of the Suv39h enzymes can direct pericentric H4-K20 trimethylation.

Identification of novel heterochromatic SET domain proteins

The requirement of Suv39h enzymes for pericentric H4-K20 trimethylation raised the question whether Suv39h enzymes might contain an intrinsic activity toward the H4-K20 position. However, in previous and extended in vitro HMTase assays [data not shown], the recombinant Suv39h enzymes only target the H3-K9 position, with a weak activity also toward histone H1 [Rea et al. 2000;
Peters et al. 2003). Therefore, other enzymes must exist that can trimethylate the H4-K20 position.

H4-K20 methylation is conserved among eukaryotes, such as *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila*, and mammals, but is not present in *Saccharomyces cerevisiae* [Fang et al. 2002, data not shown]. We therefore chose a candidate approach to identify H4-K20-specific HMTases by comparing all SET domain proteins that are shared among mouse, *Drosophila*, and *S. pombe*. We reasoned that HMTases with similar enzymatic specificities could be classified by their relative homology within the SET domain [Fig. 2]. Within each of the resulting subgroups, the catalytic specificity of known SET domain HMTases is indicated [Fig. 2, gray boxes]. Candidate proteins that are conserved between *Drosophila* and mouse were selected for further analyses and are shown by an asterisk [Fig. 2]. Full-length IMAGE clones [RZPD] were used to express cDNAs as EGFP fusion proteins in MEFs under control of a cytomegalo virus (CMV) promoter. Of the 12 candidate cDNAs tested, Cgi-85 [SuV4-20h1, see below] and Mgc2705 [SuV4-20h2, see below] show a heterochromatic accumulation in wild-type MEFs, which is lost in SuV39h dn cells [Fig. 3B]. In contrast, all other candidate SET domain proteins, including a previously described H4-K20 HMTase [Fang et al. 2002; Nishioka et al. 2002a; Rice et al. 2002], displayed broad nuclear staining patterns [data not shown].

Cgi-85 [876 amino acids] and Mgc2705 [468 amino acids] are two closely related proteins present in mammals, whereas only one corresponding gene product is found in *Drosophila* and *S. pombe* [Supplementary Fig. S2]. As we will show below, the *Drosophila* homolog, CG13363, is a dominant suppressor of position-effect variegation. We therefore classify Cgi-85 and Mgc2705 as novel Su[var] genes and suggest to rename these loci as SuV4-20h1 and SuV4-20h2.

With the exception of the SET domain, which is located close to the N terminus, no other conspicuous domains could be identified in SuV4-20h proteins. However, the SET domain is surrounded by regions that are highly conserved within these orthologs. In addition, there is a small region (~10 amino acids) in the C termini that is shared between the various SuV4-20h proteins [Fig. 3A, Supplementary Fig. S2].

SuV4-20h proteins mediate H4-K20 trimethylation at pericentric heterochromatin

Next, we investigated the function of the putative SuV4-20h enzymes by RNA interference [RNAi] in MEF cells.
MEFs were cotransfected with a pSUPER vector expressing hairpin RNAs and pEGFP-N1 conferring G418 resistance. Transfected cells were cultured for 5 d under selection medium and then analyzed for H4-K20 and H3-K9 methylation patterns by indirect immunofluorescence. As controls, we included RNAi for both Suv39h enzymes.

RNA hairpins directed against both Suv39h1 and Suv39h2 result in the loss of H3-K9 and H4-K20 trimethylation at pericentric heterochromatin (Fig. 3C). Thus, the Suv39h-specific RNAi knock-down accurately reflects the histone methylation defects observed in Suv39h dn cells [Fig. 1]. In contrast, RNAi knock-down of both Suv4-20h1 and Suv4-20h2 selectively impair pericentric accumulation of H4-K20 trimethylation, but do not perturb H3-K9 trimethylation [Fig. 3C].

**Suv4-20h proteins are nucleosomal HMTases with specificity for H4-K20 trimethylation**

Next, we characterized the activity and substrate specificity of Suv4-20h proteins. N-terminal fragments of Suv4-20h1 [amino acids 1–387] and Suv4-20h2 [amino acids 1–280] containing the SET domain were bacterially expressed as GST fusion proteins. Increasing amounts of fusion protein were used in a reaction mix containing [3H]-labeled SAM as methyl donor, and recombinant histone H4, histone octamers, or nucleosomes. Reaction products were separated by SDS-PAGE, and methyl transfer to histones was detected by fluorography. Both Suv4-20h1 and Suv4-20h2 show some HMTase activity toward recombinant octamers but display a significantly higher transfer reaction toward nucleosomes. Peptides and free histones were not accepted as substrates (Fig. 4A; data not shown). The HMTase activity appears to be specific for H4-K20, because no methyl transfer is detected when nucleosomes containing a mutated histone H4 (K20A) were used.

Because histone lysine positions can be mono-, di-, or trimethylated, we next investigated the methylation state of the reaction products. Bacterially expressed N termini of Suv4-20h1 and Suv4-20h2 show some HMTase activity toward recombinant octamers but display a significantly higher transfer reaction toward nucleosomes. Peptides and free histones were not accepted as substrates [Fig. 4A; data not shown]. The HMTase activity appears to be specific for H4-K20, because no methyl transfer is detected when nucleosomes containing a mutated histone H4 (K20A) were used.

Because histone lysine positions can be mono-, di-, or trimethylated, we next investigated the methylation state of the reaction products. Bacterially expressed N termini of Suv4-20h1 and Suv4-20h2 were used in an HMTase assay with nonradioactive SAM and recombinant substrates. Reaction products were separated by SDS-PAGE, and the methylation state of histone H4 was

**Figure 2.** Neighbor-joining tree of mouse SET domain proteins. Sequences of mouse SET domain proteins were identified from public databases. SET domains sequences were aligned, and a neighbor-joining tree showing related SET domain proteins was constructed. Homologous protein sequences in *Drosophila* and *S. pombe* were identified by blast searches. HMTase specificity of subtreens was assigned according to the described HMTase activity of representative enzymes, such as H3-K4 [Ash1 [Beisel et al. 2002], Mll [Milne et al. 2002], Set7/9 [Nishioka et al. 2002b; Wang et al. 2001]], H3-K9 [Suv39h1 [Rea et al. 2000], ESET [Yang et al. 2002], G9a [Tachibana et al. 2001]], H3-K27 [Ezh2 [Kuzmichev et al. 2002, Müller et al. 2002]], H3-K36 [Nsd1 [Rayasam et al. 2003]]; and H4-K20 [Pr-Set7 [Nishioka et al. 2002a; Fang et al. 2002]]. Proteins that were selected for the candidate approach are indicated by an asterisk.
and/or selection medium, cells were stained with Suv39h1/Suv39h2 or Suv4-20h1/Suv4-20h2. After 5 d in
marker and pSUPER vector expressing hairpin oligos directed type MEFs were cotransfected with pEGFP-N1 as transfection
knock-down of Suv39h and Suv4-20h proteins. Female wild-
represent pericentric heterochromatin. (Fig. 5A). To further define the domain re-
quired for heterochromatic targeting, we also analyzed subfragments of the C terminus. One subfragment (S#4: amino acids 280–349) shows aberrant nuclear distribution. Although there are EGFP speckles in the nucleus, these do not correspond to DAPI dense heterochromatin. All other protein fragments display heterochromatic as-
ociations. Thus, the minimal heterochromatic targeting module of Suv4-20h2 is localized within amino acids 348–441.

A possible mechanism for Suv39h-dependent targeting of Suv4-20h2 could be an interaction with other hetero-
chromatic proteins, such as HP1, the localization of which also depends on Suv39h function [Bannister et al. 2001]. Therefore, we investigated whether Suv4-20h2 could directly interact with HP1 iso-
forms. Protein fragments of the above-mentioned local-
ization assay were generated as recombinant GST fusion proteins and bound to glutathione-Sepharose beads. Full-
length myc-tagged HP1α, HP1β, and HP1γ were expressed by in vitro translation and incubated with GST-
Suv4-20h2 protein variants. After extensive washing steps, bound proteins were separated on SDS-PAGE, and Western blots were probed with α-myc antibody for de-
tection of HP1 isoforms. The N terminus of Suv4-20h2 cannot bind HP1 isoforms, but the C terminus interacts with HP1α, HP1β, and HP1γ [Fig. 5B]. Recombinant GST does not interact with HP1, excluding unspecific binding to the GST tag [Fig. 5B]. Subfragments of the C terminus show a differential interaction pattern with HP1 iso-
forms. Suv4-20h2 S5, which was found to be the minimal heterochromatic targeting module [Fig. 5A], is also the minimal subfragment that interacts with HP1α, HP1β, and HP1γ. However, the aberrantly localized sub-
fragment S#4 does not interact with HP1α or HP1β but can still associate with HP1γ. Thus, although there are several distinct HP1 binding sites in amino acid region 280–441 (which are different from the conserved C-terminal domain), it is the interactions with HP1α and HP1β that appear to localize Suv4-20h2 to pericentric heterochromatin (Fig. 5C).

The in vitro binding data together with the hetero-
chromatic localization of Suv4-20h2 suggest a directed targeting of this HMTase by interaction with HP1. If this model is correct, Suv4-20h enzymes and H4-K20 trimethylation should be lost in HP1-deficient cells. This question cannot easily be addressed in mammals, be-
cause of the lack of available HP1 mutants. Moreover, our attempts using RNAi-mediated knock-down of all three HP1 isoforms in MEFs failed to deplete HP1 func-

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**Figure 3.** Identification of Suv4-20h1 and Suv4-20h2 as hetero-
chromatic H4-K20 trimethylating HMTases. (A) Protein structure of Suv4-20h proteins. The SET domain is located in the N terminus and flanked by N- as well as C-terminal extensions of high sequence homology within Suv4-20h orthologous proteins. In addition, there is a conserved region in the C terminus of these proteins. (B) EGFP-tagged Suv39h1, Suv4-20h1, and Suv4-20h2 were expressed in wild-type and Suv39h double-null (dn) female MEFs. After fixation, distribution of EGFP-tagged pro-
teins was analyzed by fluorescence microscopy. DAPI dense foci represent pericentric heterochromatin. (C) RNAi mediated knock-down of Suv39h and Suv4-20h proteins. Female wild-
type MEFs were cotransfected with pEGFP-N1 as transfection marker and pSUPER vector expressing hairpin oligos directed against Suv39h1/Suv39h2 or Suv4-20h1/Suv4-20h2. After 5 d in
selection medium, cells were stained with α-trimethyl H3-K9 and α-trimethyl H4-K20 antibodies. Immunostaining was ex-
amined in EGFP-positive cells.

analyzed by probing Western blots with H4-K20 mono-, di-, and trimethyl-specific antibodies. No H4-K20 mono-
methylated product could be observed [Fig. 4B]. The weak signal visualized by the H4-K20 monomethyl-
ty antibody is a cross-reactivity with unmodified histone H4 (shown by an asterisk). In contrast, reaction products of Suv4-20h enzymes display some H4-K20 dimethylation but are strongly enriched for H4-K20 trimethylation [Fig. 4B]. The absence of H4-K20 monomethylation in reaction products of Suv4-20h2 was further confirmed by a
time-course experiment. Reactions of Suv4-20h2 with recombinal nucleiosomes were stopped after sequential incubation times and analyzed by Western blotting. In the reaction products, H4-K20 di- and trimethylation increased over time, but even at very early time points [2 min], no H4-K20 monomethylation is detected [Supplementary Fig. S3]. The collective interpretation of the results presented above allow us to conclude that Suv4-
20h enzymes are nucleosomal HMTases with a prefer-
atal activity to direct H4-K20 trimethylation.

**Targeting of Suv4-20h2 by HP1 interactions**

To identify the targeting mecha

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H3-K9 and H4-K20 trimethylation at heterochromatin
We therefore extended our studies to *Drosophila*, in which H4-K20 trimethylation is present at pericentric heterochromatin and in a high number of bands (as judged by comparison with propidium iodide labeling of DNA) on salivary gland chromosomes (Fig. 6). In *Drosophila*, pericentric H4-K20 trimethylation is severely reduced in *Su(var)3-9* mutants (Fig. 6A), indicating that cross-talk between H3-K9 and H4-K20 trimethylation systems is evolutionarily conserved. In *Su(var)2-5* (HP1) mutants, H3-K9 methylation and pericentric accumulation...
tion of SU(VAR)3-9 remains unaltered (Schotta et al. 2002). Intriguingly, H4-K20 trimethylation is nearly lost from polytene chromatin in these mutants (Fig. 6A). This result indicates that the induction of heterochromatic histone lysine methylation marks occurs in a sequential pathway. At pericentric heterochromatin Su-(var)3-9-mediated H3-K9 methylation would precede H4-K20 trimethylation by the Suv4-20 enzymes. Other H3-K9-specific HMTases could direct HP1 and subsequently H4-K20 trimethylation to euchromatic bands.

Drosophila Suv4-20 is a dominant suppressor of position-effect variegation

Our data suggest H4-K20 trimethylation to be a mark of silenced chromatin domains. We therefore investigated whether this modification would indeed be important for gene silencing in well-described PEV models in Drosophila [Reuter and Spierer 1992]. A single, homozygous viable P-element insertion (P[GT1]BG00814) into the third exon of Suv4-20 has been identified in the course of the Drosophila gene disruption project (Flybase). H4-K20 trimethylation at polytene chromatin is nearly lost in homozygous mutant larvae (Fig. 6A), demonstrating that the P-element insertion (Suv4-20 BG00814) represents a strong hypomorphic allele of Suv4-20. Because the Suv4-20 locus maps on the X chromosome, the classical PEV rearrangement In(1)wmt [Reuter and Spierer 1992] cannot be used to analyze a potential modifier effect of Suv4-20. Therefore, we analyzed another PEV rearrangement that translocates a different marker, Stubble (Sb), close to pericentric heterochromatin [T(2;3)SbV]; Sinclair et al. 1983). The dominant mutation Stubble induces short bristles, but heterochromatin-induced silencing of SbV results in long bristles [Fig. 6B]. Homozygous Suv4-20[BG00814] as well as control wild-type females were crossed to T(2;3)SbV males. In the progeny, the extent of SbV reactivation was determined as the ratio of short bristles [active SbV] to long bristles [inactive SbV] in Suv4-20[BG00814] flies, because now ~25% of the bristles are short [Fig. 6B]. This result classifies Suv4-20 as a dominant PEV modifier and further indicates a functional role for Suv4-20-dependent H4-K20 trimethylation in gene silencing.

Discussion

A combinatorial histone methyl code for constitutive heterochromatin

One of the predictions of the “histone code” hypothesis suggests that distinct combinations of histone modifications participate in the formation of different chromatin domains (Strahl and Allis 2000; Turner 2000; Jenuwein and Allis 2001). Of the five lysine positions in the histone N termini that display prominent methylation (Peters et al. 2003), H3-K9, H3-K27, and H4-K20 are supposed to have repressive functions [Fischle et al. 2003; Lachner et al. 2003; Vaquero et al. 2003]. A combination of H3-K27 trimethylation together with H3-K9 dimethylation and H4-K20 monomethylation has been described to index the inactive X chromosome [Silva et al. 2002].
prominent marks of constitutive heterochromatin are H3-K9 trimethylation and H3-K27 monomethylation [Peters et al. 2003; Kohlmaier et al. 2004]. However, the contribution of the three distinct H4-K20 methylation states in the formation of chromosomal subdomains has not been analyzed. Previous studies indicated H4-K20 methylation is broadly enriched at mitotic chromosomes [Fang et al. 2002, Nishioka et al. 2002a, Rice et al. 2002]. By using highly selective antibodies, developed by UBI, which discriminate H4-K20 mono-, di-, and trimethylation, we now describe H4-K20 trimethylation as a third component of repressive histone lysine methyl marks in pericentric heterochromatin. Based on our novel data, we propose the following model for sequential induction of H3-K9 and H4-K20 trimethylation at constitutive heterochromatin in mammals [Fig. 7]. In a first step, Suvs39h enzymes would be targeted to repeat-rich sequences at pericentric heterochromatin, presumably by interacting with protein complexes containing components of the RNAi machinery [Hall et al. 2002; Jenuwein 2002; Volpe et al. 2002; Schramke and Allshire 2003; Pal-Bhadra et al. 2004; Verdel et al. 2004]. Because pericentric heterochromatin is also enriched for H3-K27 monomethylation, and the Suvs39h enzymes prefer an H3-K9 monomethylated substrate [Peters et al. 2003], this first step most likely also requires the activity of currently unknown H3-K27 and H3-K9 monomethylases. Following stabilized binding of HP1α and HP1β to H3-K9 trimethylated nucleosomes [Bannister et al. 2001, Lachner et al. 2001], HP1 molecules, probably in conjunction with nucleosomal surfaces, would then recruit the Suvs4-20h enzymes, which in turn trimethylate H4-K20. This tentative model would involve collaboration of at least four distinct HMTases to induce the observed combination of H3-K9 trimethyl, H4-K20 trimethyl, and H3-K27 monomethyl marks at pericentric heterochromatin. Future studies are aimed to identify whether HP1 binding is stabilized by a combination of H3-K9 and H4-K20 trimethylation or if other heterochromatin-specific components may exist that could selectively be recruited by this combinatorial histone lysine methylation pattern.

**Suvs4-20h enzymes are nucleosomal-specific HMTases**

Recombinant Suvs4-20h HMTases possess selective activity toward the H4-K20 position in a nucleosomal context. Recombinant histone H4 is not used as a substrate, and only poor activity is detected with histone octamers [Fig. 4]. In contrast, Suvs39h enzymes preferably methylate free histone H3 and recombinant octamers, whereas nucleosomes are only very poor in vitro substrates [data not shown]. Thus, selective generation of H3-K9 and H4-K20 methylation marks could occur in a stepwise mechanism [Vermaak et al. 2003], in which histone H3-H4 dimers [Tagami et al. 2004] or tetramers might be methylated by Suvs39h and, after nucleosome assembly, Suvs4-20h enzymes would trimethylate the H4-K20 position.

In vitro, Suvs4-20h enzymes can directly trimethylate unmodified H4-K20 positions [Fig. 4], without requirement for a monomethyl substrate. This is in contrast to Suvs39h function, which prefers an H3-K9 monomethylated position [Peters et al. 2003]. In Suvs39h dn cells, H3-K9 monomethylation becomes enriched at pericentric heterochromatin [Peters et al. 2003]; however, we did not detect an increase for H4-K20 monomethylation in Suvs4-20h RNAi knockdown experiments [data not shown]. In addition, H3-K9 trimethylation is not required for Suvs4-20h activity in vitro [Fig. 4], although cross-tail interactions between nucleosomal H3-K9 and H4-K20 positions may exist in vivo that could affect enzymatic activity of Suvs4-20h HMTases.

PR/SET7 is another HMTase with selective activity toward nucleosomal H4-K20 [Nishioka et al. 2002a, Fang et al. 2002], but in contrast to Suvs4-20h enzymes, it appears to be an exclusively monomethylating enzyme [K. Sarma, D. Reinberg, and S. Gamblin, unpubl.]. Furthermore, PR/SET7 fails to localize to pericentric heterochromatin when expressed as an EGFP fusion protein [data not shown]. N- and C-terminal extensions of the SET domain, such as pre- and post-SET domains of Suvs39h enzymes, are involved in substrate binding and in conferring enzyme selectivity toward mono-, di-, and trimethylation [Xiao et al. 2003]. Suvs4-20h enzymes contain extensions to the SET domain that are distinct from pre- and post-SET domains, and that are only conserved
within their subgroup of orthologous proteins. In addition, there is no significant homology to PR/SET7 within these conserved regions of Suv4-20h enzymes.

Suv4-20h HMTases and PR/SET7 possibly recognize the H4-K20 position in a different nucleosomal context. Indeed, the two H4 tails of one nucleosome are presented in different molecular projections, such that one H4 tail is directed toward the DNA backbone, whereas amino acids 16–25 of the second histone H4 molecule contact the H2A/H2B dimer of the adjacent nucleosome (Luger et al. 1997). It is according to these structural definitions that the H4 tail may be particularly suited for directing transitions between 11- and 30-nm chromatin fibers, and H4-K20 trimethylation appears as a good candidate mark to contribute in facilitating these higher order nucleosomal organizations.

**H4-K20 trimethylation is evolutionarily conserved**

In mammals, ~50 SET domain genes have been identified (Lachner and Jenuwein 2002, Kouzarides 2002), of which a subset is evolutionarily conserved in *Drosophila* and *S. pombe*. Intriguingly, nearly all putative SET domain HMTases that target repressive lysine positions (H3-K9, H3-K27, H4-K20) are absent in *S. cerevisiae*. In agreement, H3-K9, H3-K27, and H4-K20 methylation cannot be detected in *S. cerevisiae* (R. Sengupta and T. Jenuwein, unpubl.), indicating that this organism largely lacks extensive heterochromatic domains and uses other repressive systems (e.g., SIR proteins) to silence gene activity (Kurdistani and Grunstein 2003).

H3-K9 methylation has emerged as an important and evolutionarily conserved mark to silence gene activity at repressive chromatin domains. The concerted function of Suv39h and Suv4-20h enzymes described here raises the question as to the functional role of H4-K20 methylation in well-described epigenetic paradigms. Homologs of the Suv39h/Suv4-20h methylation systems are also conserved in *Drosophila* [Su(var)3-9 and Suv4-20] and *S. pombe* (Clr4 and SET9). Surprisingly, H3-K9 methylation appears severely underrepresented (and can hardly be detected) in bulk histone preparations from *S. pombe* extracts, which instead display robust signals for all three H4-K20 methylation states (R. Sengupta and T. Jenuwein, unpubl.). Because HP1 is also conserved in *S. pombe* [as the Swi6 protein], a functional synergy between H3-K9 and H4-K20 methylation systems might exist in *S. pombe* that could be operative to safeguard gene silencing and/or to index distinct chromosomal regions. Together, our definition of novel H4-K20 trimethylating HMTases and the presence of H4-K20 trimethylation from *S. pombe* to mammals provides evidence for an evolutionary conservation of the proposed silencing pathway between H3-K9 and H4-K20 methylation.

**Suv4-20h enzymes are novel Su(var) genes**

H4-K20 trimethylation is a novel hallmark of pericentric heterochromatin in mammals, but the current lack of mutants for Suv4-20h enzymes prevents the analysis of its regulatory function during mouse development. However, the identification of a P-element insertion in *Drosophila* Suv4-20, which results in nearly complete loss of H4-K20 trimethylation [Fig. 6A], allowed a first functional analysis. In *Drosophila*, there are ≥50 Su(var) loci (Reuter and Spierer 1992, Schotta et al. 2003), which encode products involved in heterochromatin-induced gene silencing. Only ~15 of these Su(var) genes have been identified to date, the most revealing of which include Su(var)2-5 [HP1], Eisenberg et al. 1990, Su(var)3-7 (Cleard et al. 1997), and Su(var)3-9 (Tschiersch et al. 1994). Here we show that *Drosophila* Suv4-20 is a novel, dominant suppressor of position-effect variegation [Fig. 6B]. The Su(var) effect of Suv4-20 is about as strong as that of Su(var)3-9 [data not shown], suggesting that H3-K9 and H4-K20 trimethylation may have a similar potential to establish heterochromatin-induced gene silencing. In addition, both the hypomorphic Suv4-20 and the Su(var)3-9 null mutants are homozygous viable. Based on the observed synergy, it is conceivable that the combined disruption of Suv4-20 and Su(var)3-9 genes might have a much more pronounced phenotype that could compromise establishment and stability of heterochromatin.

Major functions of heterochromatin include to safeguard a specialized chromatin structure around centromeres (Karpen and Allshire 1997; Bernard and Allshire 2002) and to protect genome stability by silencing of transposable elements (Birchler et al. 2000; Pal-Bhadra et al. 2004). The pericentric accumulation and the silencing defect observed in Suv4-20 mutants predict H4-K20 trimethylation to participate in these mechanisms, but H4-K20 trimethylation may also be involved in gene repression at euchromatic targets or other functions of chromatin control, in which it could even be independent of Suv39h activity. As histone lysine methylation is implicated in almost all epigenetic paradigms, and H4-K20 trimethylation has been shown to be enriched in aged organs (Sarg et al. 2002), future analyses into the physiological roles of H4-K20 methylation promise not only to provide a better understanding of the interplay between distinct histone lysine methylation systems but may even offer novel insights into fundamental biological processes, such as normal and perturbed development, aging, and regeneration.

**Materials and methods**

**Plasmid construction**

Full-length open reading frames of all candidate SET domain proteins as well as protein truncations of Suv4-20h1 and Suv4-20h2 were PCR amplified from IMAGE cDNA clones (purchased from Deutsches Ressourcenzentrum für Genomforschung GmbH [RZPD] and cloned into the GATEWAY entry vector pDONR207 [Invitrogen]). A list of clones used as well as primer sequences is available on request. The reference sequences of Suv4-20h1 and Suv4-20h2 have been deposited at GenBank [AY555192, AY555193].

**H3-K9 and H4-K20 trimethylation at heterochromatin**
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The two GATEWAY compatible target vectors for expression of EGFP-tagged proteins in mammalian cells [pEGFP-GW] and bacterial expression of GST-tagged proteins [pGEX-GW] were constructed by inserting GATEWAY cassettes into Smal sites of pEGFP-N1 (Clontech) and pGEX-6P1 (Amersham), respectively. Entry clones were recombined into respective target vectors by GATEWAY LR Reaction.

Transient transfection and immunofluorescence on interphase cells

Candidate SET domain proteins were expressed in female wild-type and Suv39h, Suv4-20h2 (1–280) containing the SET domain and N- as well as HMTase assays fluorescence were assumed to also contain pSUPER vector. underrepresented in the transfection mix, cells showing EGFP as described (Peters et al. 2003). Because pEGFP-N1 vector was chamber slides and analyzed by indirect immunofluorescence cells. Five days after transfection, cells were transferred on selection, medium containing 600 µg/mL G418 was applied to the using Lipofectamine (Invitrogen). One day after transfection selection, cells were fixed in 2% PFA/PBS, extracted with 0.1% sodium citrate/0.1% Triton X-100, washed with PBS, and mounted with Vectashield [Vector Laboratories]. EGFP fluorescence was analyzed by fluorescence microscopy. Indirect immunofluorescence was performed according to the method of Peters et al. (2003), using antibodies against H4-K20 mono-, di-, and trimethyl H3-K9, H3-K4 mono-, di-, and trimethylation [Peters et al. 2003].

RNAi knock-down

DNA oligos containing the 19mer targeting sequence, a loop region, and HindIII/BglII restriction sites were cloned into pSUPER [Brummelkamp et al. 2002]. For each target gene, two different 19mer sequences were used. Fifty thousand wild-type MEFs were plated on a 10-cm dish 1 d before transfection. Cells were transfected with a mixture of 2 µg pSUPER plasmids containing the 19mer targeting sequences and 0.2 µg pEGFP-N1 using Lipofectamine [Invitrogen]. One day after transfection selection, medium containing 600 µg/mL G418 was applied to the cells. Five days after transfection, cells were transferred on chamber slides and analyzed by indirect immunofluorescence as described [Peters et al. 2003]. Because pEGFP-N1 vector was underrepresented in the transfection mix, cells showing EGFP fluorescence were assumed to also contain pSUPER vector.

HMTase assays

GST fusion proteins of Suv4-20h1 [amino acids 1–387] and Suv4-20h2 [1–280] containing the SET domain and N- as well as C-terminal associated regions [Fig. 3A] were expressed in Escherichia coli. HMTase assays were performed as described [Nishikoa et al. 2002b]. In particular, samples of the Suv4-20h enzymes were incubate for 1 h at 30°C in reaction buffer (50 mM Tris-HCl at pH 8.5, 5 mM MgCl2, 4 mM DTT) with 1 µM 3H-labeled SAM [Amersham Pharmacia Biotech]. Fifty nanograms of recombinant histone H4 and 2 µg of recombinant octamers/nucleosomes were used as substrates.

In vitro binding assays

GST fusion proteins of Suv4-20h2 were expressed in E. coli and purified on glutathione-S-Sepharose [Pharmacia]. Equal amounts of GST fusion proteins were incubated with in vitro translated myc-tagged HPI isoforms [HPIα, amino acids 5–191; HPIβ, amino acids 5–185; HPIγ, amino acids 5–173; Lachner et al. 2001] in IP buffer [50 mM Tris-HCl at pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10% glycerol, proteinase inhibitor cocktail]. Incubation was carried out overnight at 4°C with gentle agitation. The beads were washed six times with IP buffer and finally resuspended in 50 µL loading buffer. Bound proteins were separated on SDS-polyacrylamid gels and detected by immunoblotting using α-myc antibody [Upstate Biotech, 05-419].

Immunostaining of polytene chromosomes

Preparation of polytene chromosomes was performed as described [Schotta et. al. 2002] with the following modifications: salivary glands of third instar larve were dissected in 0.7% NaCl, fixed in 4% formaldehyde and squashed in 45% acetic acid/2% formaldehyde. Chromosomes were incubated with α-trimethyl H3-K9 [Peters et al. 2003] as well as α-trimethyl H4-K20 antibodies overnight at 4°C, followed by incubation with secondary alexA 488-conjugated antibodies [Molecular Probes] for 2 h at 37°C. DNA was stained with propidium iodide [Molecular Probes]. Preparations were mounted in Vectashield medium and examined with a confocal laser scanning microscope [LSM510, Zeiss].

Phenotypic characterization of Stubble variegation

The P-element P[GT1]BG00814 is inserted into the third exon of Suv4-20 [CG13363] and results in a strong hypomorphic allele (Suv4-20BG00814). The PEV modifier effect of Suv4-20BG00814 was evaluated by using T(2;3)Shv [Sinclair et al. 1983]. This translocation brings the dominant mutation Shv [short-bristles phenotype] near to chromosome 2R heterochromatin. Because Shv is dominant, Suv[vari]s increase the stubble phenotype. Suv4-20BG00814/T(2;3)Shv as well as control wild-type females were crossed to T(2;3)Shv, In[3R]Mo, Shv, st/TM3, males, and 14 defined bristles were scored as being wild type or Sh. The ratio between the total numbers of Stubble to wild-type bristles is a mark for the extent of Shb inactivation and has been evaluated for >25 flies of each genotype.

Bioinformatic methods

SET domain proteins in mouse were identified by using a hidden Markov model (HMM) profile for the SET domain [Pfam, PF00856]. IPI database [http://www.ebi.ac.uk/IPI] was searched by using HMMPER software [http://hmmer.wustl.edu]. An alignment of all SET domains and the neighbor-joining tree was created by using the t-coffee alignment program [Notredame et al. 2000]. Orthologs of mouse SET domain proteins in S. pombe and Drosophila were identified by using protein blast searches [http://www.ncbi.nlm.nih.gov/blast].

Acknowledgments

We are indebted to UBI for exchange of H4-K20 antibodies and thank Jim Bone, Mary-Ann Jelinek, and Judy Nisson for helpful advice. We also acknowledge Susanne Opravil and Monika Kauer for detailed characterization of the H4-K20 antibodies. We are further grateful to Alexander Schleiffer and Anton Beyer for their expert help in sequence comparison and homology searches of mouse SET domain genes. This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) to G.R. Studies conducted in the laboratory of D.R. were supported by a grant from the National Institutes of Health (GM37120) and the Howard Hughes Medical Institute. Research in the laboratory of T.J. is supported by the IMP through Boehringer Ingelheim, and by grants from the Vienna Economy Promotion Fund (WWFF), the European Union (EU-network HPRN-CT 2000-
References

Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C., and Kouzarides T. 2001. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature 410: 120–124.

Beisel, C., Imhof, A., Greene, J., Kremmer, E., and Sauer, F. 2002. Histone methylation by the Drosophila epigenetic transcriptional regulator Ash1. Nature 419: 857–862.

Bernard, P. and Allshire, R. 2002. Centromeres become unstuck without heterochromatin. Trends Cell. Biol. 12: 419–424.

Birchler, J.A., Bhadra, M.P., and Bhadra, U. 2000. Making noise about silence: Repression of repeated genes in animals. Curr. Opin. Genet. Dev. 10: 211–216.

Brummelkamp, T.R., Bernards, R., and Agami, R. 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science 296: 550–553.

Cleard, F., Delattre, M., and Spierer, P. 1997. SU(VAR)3-7, a Drosophila heterochromatin-associated protein and companion of HP1 in the genomic silencing of position-effect variegation. EMBO J. 16: 5280–5288.

Czermin, B., Melfi, R., McCabe, D., Seitz, Y., Imhof, A., and Pirotta, V. 2002. Drosophila Enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal polycomb sites. Cell 111: 185–196.

De Lange, R.J., Hooper, J.A., and Smith, E.L. 1973. Histone H3.3: Sequence studies on the cyanogen bromide peptides; comparison with calf thymus histone 3. J. Biol. Chem. 248: 3261–3274.

Eisenberg, J.C., James, T.C., Foster-Hartnett, D.M., Hartnett, T., Ngan, V.K.W., and Elgin, S.C. 1990. Mutation in a heterochromatin-associated protein and companion of HP1 in the genomic silencing of position-effect variegation. Nature. 347: 389–392.

Fang, J., Feng, Q., Ketel, C.S., Wang, H., Cao, R., Xia, L., Erdjument-Bromage, H., Tempst, P., Steward, R., et al. 2002a. PR-Set7 is a nucleosome-specific methyltransferase that modifies lysine 20 of histone H4 and is associated with silent chromatin. Mol. Cell 20: 1201–1213.

Fischle, W., Yang, W., and Allis, C.D. 2003. Extending the histone code: Modification cassettes and switches. Nature 425: 475–479.

Hall, I.M., Shankaranarayana, G.D., Noma, K., Ayoub, N., Cohen, A., and Grewal, S.I. 2002. Establishment and maintenance of a heterochromatin domain. Science 297: 2232–2237.

Jenuwein, T. 2002. Molecular biology: An RNA-guided pathway for the epigenome. Science 297: 2215–2218.

Jenuwein, T. and Allis, C.D. 2001. Translating the histone code. Science 293: 1074–1080.

Karpen, G.H. and Allshire, R.C. 1997. The case for epigenetic effects on centromere identity and function. Trends Genet. 13: 489–496.

Kohlmayer, A., Savarese, F., Lachner, M., Martens, J., Jenuwein, T., and Wutz, A. 2004. Kinetics of histone methylation triggered by Xist reveals an epigenetic memory independent of silencing. PLOS (in press).

Kouzarides, T. 2002. Histone methylation in transcriptional control. Curr. Opin. Genet. Dev. 12: 198–209.

Kurdistani, S.K. and Grunstein M 2003. Histone acetylation and deacetylation in yeast. Nat. Rev. Mol. Cell. Biol. 4: 276–284.

Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. 2002. Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes & Dev. 16: 2893–2905.

Lachner, M. and Jenuwein, T. 2002. The many faces of histone lysine methylation. Curr. Opin. Cell. Biol. 14: 286–298.

Lachner, M., O’Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. 2001. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature 410: 116–120.

Lachner, M., O’Sullivan, R.J., and Jenuwein, T. 2003. An epigenetic road map for histone lysine methylation. J. Cell. Sci. 116: 2117–2124.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. 1997. Crystal structure of the nucleosome core particle at 2.8 Å resolution. Nature 389: 251–260.

Milne, T.A., Briggs, S.D., Brock, H.W., Martin, M.E., Gibbs, D., Allis, C.D., and Hess, J.L. 2002. MLL Targets SET domain methyltransferase activity to Hox gene promoters. Mol. Cell 10: 1107–1117.

Müller, J., Hart, C.M., Francis, N.J., Vargas, M.L., Sengupta, A., Wild, B., Miller, E.L., O’Connor, M.B., Kingston, R.E., and Simon, J.A. 2002. Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell 111: 197–208.

Nishioka, K., Rice, J.C., Sarma, K., Erdjument-Bromage, H., Werner, J., Wang, Y., Chuiikov, S., Valenzuela, P., Tempst, P., Steward, R., et al. 2002b. PR-Set8 is a nucleosome-specific methyltransferase that modifies lysine 20 of histone H4 and Is associated with silent chromatin. Mol. Cell 9: 1201–1213.

Notredame, C., Higgins, D.G., and Heringa, J. 2000. T-Coffee: A novel method for fast and accurate multiple sequence alignment. J. Mol. Biol. 302: 205–217.

Okamoto, I., Otte, A.P., Allis, C.D., Reinberg, D., and Heard, E. 2004. Epigenetic dynamics of imprinted x inactivation during early mouse development. Science 303: 644–649.

Paik, W.K. and Kim, S. 1971. Protein methylation. J. Biol. Chem. 246: 6816–6823.

Pal-Bhadra, M., Leibovitch, B.A., Gandhi, S.G., Rao, M., Bhadra, U., Birchler, J.A., and Elgin, S.C. 2004. Histone H3 lysine methylation: a novel histone methyltransferase that facilitates transcription by precluding histone tail modifications required for heterochromatin formation. Genes & Dev. 16: 479–489.

Plath, K., Fang, J., Mlynarczyk-Evans, S.K., Cao, R., Worringer, K.A., Wang, H., de la Cruz, C.C., Otte, A.P., Panning, B., and Zhang, Y. 2003. Role of histone H3 lysine 27 methylation in X inactivation. Science 300: 131–135.

Rayasam, G.V., Wendling, O., Angraud, P.O., Mark, M., Niederrreither, K., Song, L., Lerouge, T., Hager, G.L., Chambon, P., and Losson, R. 2003. NSD1 is essential for early post-implantation development and has a catalytically active SET domain. EMBO J. 22: 3153–3163.

Rea, S., Eisenhaber, F., O’Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C.P., Allis,
Schotta et al.

C.D., et al. 2000. Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 406: 593–599.

Reuter, G. and Spierrer, P. 1992. Position effect variegation and chromatin proteins. Bioessays 14: 605–612.

Rice, J.C., Nishioika, K., Sarma, K., Steward, R., Reinberg, D., and Allis, C.D. 2002. Mitotic-specific methylation of histone H4 Lys 20 follows increased PR-Set7 expression and its localization to mitotic chromosomes. Genes & Dev. 16: 2225–2230.

Rice, J.C., Briggs, S.D., Uckerheide, B., Barber, C.M., Shabano-witz, J., Hunt, D.F., Shinkai, Y., and Allis, C.D. 2003. Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. Mol. Cell 12: 1591–1598.

Santos-Rosa, H., Schneider, R., Bannister, A.J., Schreiber, S.L., Mellor, J., and Kouzarides, T. 2002. Active genes are tri-methylated at K4 of histone H3. Nature 419: 407–411.

Sarg, B., Kourtzamani, E., Helliger, W., Rundquist, I., and Lindner, H.H. 2002. Postsynthetic trimethylation of histone H4 at lysine 20 in mammalian tissues is associated with aging. J. Biol. Chem. 277: 39195–39201.

Schotta, G., Ebert, A., Krauss, V., Fischer, A., Hofmann, J., Rea, S., Jennewein, T., Dorn, R., and Reuter, G. 2002. Central role of Drosophila SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing. EMBO J. 1: 1121–1131.

Schotta, G., Ebert, A., Dorn, R., and Reuter, G. 2003. Position-effect variegation and the genetic dissection of chromatin regulation in Drosophila. Semin. Cell. Dev. Biol. 14: 67–75.

Schramke, V. and Allshire, R. 2003. Hairpin RNAs and retrotransposon LTRs effect RNAi and chromatin-based gene silencing. Science 301: 1069–1074.

Silva, J., Mak, W., Zvejkova, I., Appanah, R., Nesterova, T.B., Webster, Z., Peters, A.H., Jennewein, T., Otte, A.P., and Brockdorff, N. 2003. Establishment of histone H3 methylation on the inactive X chromosome requires transient recruitment of eed–enx1 polycomb group complexes. Dev. Cell. 4: 481–495.

Sims, R.J., Nishioika, K., and Reinberg, D. 2003. Histone lysine methylation: A signature for chromatin function. Trends Genet. 19: 629–639.

Sinclair, D.A.R., Mottus, R.C., and Grigliatti, T.A. 1983. Genes which suppress position effect variegation in Drosophila melanogaster are clustered. Mol. Gen. Genet. 191: 326–333.

Strahl, B.D. and Allis, C.D. 2000. The language of covalent histone modifications. Nature 403: 41–45.

Tachibana, M., Sugimoto, K., Fukushima, T., and Shinkai, Y. 2001. Set domain-containing protein, G9a, is a novel lysine-prefering mammalian histone methyltransferase with hyperactivity and specific selectivity to lysines 9 and 27 of histone H3. J. Biol. Chem. 276: 25309–25317.

Tagami, H., Ray-Gallet, D., Almouzni, G., and Nakatani, Y. 2004. Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. Cell 116: 51–61.

Tamura, H., Zhang, X., McMillen, D., Singh, P.B., Nakayama, J., Grewal, S.I., Allis, C.D., Cheng, X., and Selker, E.U. 2003. Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in Neurospora crassa. Nat. Genet. 34: 75–79.

Tschiersch, B., Hofmann, A., Krauss, V., Dorn, R., Korge, G., and Reuter, G. 1994. The protein encoded by the Drosophila position-effect variegation suppressor gene Su(var)3-9 combines domains of antagonistic regulators of homeotic gene complexes. EMBO J. 13: 3822–3831.

Turner, B.M. 2000. Histone acetylation and an epigenetic code.

Bioessays 22: 836–845.

van Holde, K.E. 1988. Chromatin. Springer, New York.

Vaqero, A., Loyola, A., and Reinberg, D. 2003 The constantly changing face of chromatin. Sci. Aging Knowl. Environ. 14: RE4. http://sageke.sciencemag.org/cgi/content/full/sageke/2003/14/re4.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S.I., and Moazed, D. 2004. RNAi-mediated targeting of heterochromatin by the RITS complex. Science 303: 672–676.

Vermaak, D., Ahmad, K., and Henikoff, S. 2003. Maintenance of chromatin states: An open-and-shut case. Curr. Opin. Cell. Biol. 15: 266–274.

Volpe, T.A., Kidner, C., Hall, I.M., Teng, C., Grewal, S.I., and Martienssen, R.A. 2002. Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science 297: 1833–1837.

Wang, H., Cao, R., Xia, L., Erdjument-Bromage, H., Borchers, C., Tempst, P., and Zhang, Y. 2001. Purification and functional characterization of a histone H3-lysine 4-specific methyltransferase. Mol. Cell 8: 1207–1217.

Xiao, B., Wilson, J.R., and Gamblin, S.J. 2003. SET domains and histone methylation. Curr. Opin. Struct. Biol. 13: 699–705.

Yang, L., Xia, L., Wu, D.Y., Wang, H., Chansky, H.A., Schubach, W.H., Hickstein, D.D., and Zhang, Y. 2002. Molecular cloning of ESET, a novel histone H3-specific methyltransferase that interacts with ERG transcription factor. Oncogene 21: 148–152.
A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin

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*Genes Dev.* 2004, 18: Access the most recent version at doi:10.1101/gad.300704

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