Purification and Functional Characterization of SET8, a Nucleosomal Histone H4-Lysine 20-Specific Methyltransferase

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1Department of Biochemistry and Biophysics Among the different covalent modifications identified so far, lysine acetylation is the most intensively studied due to its fundamental role in transcription regulation [1].

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Summary

Background: Covalent modifications of histone N-terminal tails play fundamental roles in regulating chromatin structure and function. Extensive studies have established that acetylation of specific lysine residues in the histone tails plays an important role in transcriptional regulation. Besides acetylation, recent studies have revealed that histone methylation also has significant effects on heterochromatin formation and transcriptional regulation. Histone methylation occurs on specific arginine and lysine residues of histones H3 and H4. Thus far, only 2 residues on histone H4 are known to be methylated. While H4-arginine 3 (H4-R3) methylation is mediated by PRMT1, the enzyme(s) responsible for H4-lysine 20 (H4-K20) methylation is not known.

Results: To gain insight into the function of H4-K20 methylation, we set out to identify the enzyme responsible for this modification. We purified and cloned a novel human SET domain-containing protein, named SET8, which specifically methylates H4 at K20. SET8 is a single subunit enzyme and prefers nucleosomal substrates. We find that H4-K20 methylation occurs in a wide range of higher eukaryotic organisms and that SET8 homologs exist in C. elegans and Drosophila. We demonstrate that the Drosophila SET8 homolog has the same substrate specificity as its human counterpart. Importantly, disruption of SET8 in Drosophila reduces levels of H4-K20 methylation in vivo and results in lethality. Although H4-K20 methylation does not correlate with gene activity, it appears to be regulated during the cell cycle.

Conclusions: We identified and characterized an evolutionarily conserved nucleosomal H4-K20-specific methyltransferase and demonstrated its essential role in Drosophila development.

Introduction

One important breakthrough in our understanding of chromatin is the realization that chromatin structure is not static. Dynamic changes in chromatin structure play important roles in many biological processes, such as DNA replication, repair, recombination, and transcription. One way cells modulate their chromatin structure is through covalent modifications of core histone tails. Among the different covalent modifications identified so far, lysine acetylation is the most intensively studied due to its fundamental role in transcription regulation [1]. Other prominent covalent histone modifications include methylation, phosphorylation, and ubiquitination [2].

Although discovered three decades ago [3], histone methylation remained one of the least-understood forms of posttranslational modification until recently [4, 5]. Methylation of histones occurs on both arginine and lysine residues. While the in vivo evidence for histone arginine methylation and its function in nuclear receptor-mediated transcriptional activation is fairly recent [6–9], methylation on lysines 4, 9, 27, and 36 of H3 and lysine 20 of H4 have been documented for years [2, 10].

Studies on chromatin proteins involved in epigenetic mechanisms, such as position effect variegation (PEV), revealed an evolutionarily conserved sequence motif called the SET domain [11, 12]. Based on the sequence similarity between the SET domain of SUV39H1 and several SET domain-containing methyltransferases from plants [13, 14], Jenuwein and colleagues demonstrated that SUV39H1 and its S. pombe homolog Clr4 contain intrinsic H3-K9-specific methyltransferase activity [15]. Subsequent studies indicate that H3-K9 methylation plays an important role in the recruitment of the heterochromatin protein HP1 and the establishment of heterochromatin [16–18]. More recent studies have suggested that H3-K9 methylation is an early event in X chromosome inactivation, although Suvar39 genes do not seem to be responsible for the H3-K9 methylation in the inactive X chromosome [19–21]. In addition to SUV39H1/Clr4, G9a and ESET/SETDB1 have also been reported to target H3-K9 for methylation [22–24]. Whether either of these two H3-K9-specific HMTases is involved in the methylation of the inactive X chromosomes remains to be determined.

In addition to H3-K9, great progress has also been made in the studies of H3-K4 methylation. Early studies in Tetrahymena suggested that H3-K4 methylation correlates with gene activation because H3-K4 methylation mainly occurs in the transcriptionally active macronuclei where histone acetylation is also enriched [10]. Subsequent chromatin immunoprecipitation (ChIP) analysis of the fission yeast mating-type locus and the chicken β-globin locus revealed that H3-K4 and H3-K9 methylation are enriched in transcriptionally active euchromatin and transcriptionally repressed heterochromatin, respectively [25, 26]. Recent studies have identified SET1 as the enzyme responsible for H3-K4 methylation in budding yeast [27–30]. Interestingly, biochemical purification of HMTase activities from HeLa cells identified SET7 (also called Set9), which has little sequence similarity to yeast SET1 outside the SET domain, as the H3-K4-specific methyltransferase [31, 32]. Whether mammalian
SET7 is the functional homolog of yeast SET1 remains to be determined.

Identification and characterization of the HMTases mentioned above is an important step toward understanding the function of histone methylation. However, since different modifications or the same modification at different lysines have different outcomes [5], it would not be possible to completely dissect the "histone code" [33] without identifying the HMTases that target other histone lysine residues for methylation. Here we report the purification, molecular identification, and functional characterization of a nucleosomal H4-K20-specific HMTase. We found that an evolutionarily conserved SET domain-containing protein, named SET8, is responsible for H4-K20 methylation both in vitro and in vivo. In addition, H4-K20 methylation is widespread in the genome and appears to be regulated during the cell cycle. Finally, disruption of SET8 in Drosophila by a P element insertion results in lethality that correlates with decreased H4-K20 methylation.

Results

Purification of an H4-Specific HMTase

By following histone methyltransferase activity, we have previously identified PRMT1 and SET7 as H4-R3- and H3-K4-specific methyltransferases, respectively [6, 31]. However, neither enzyme has significant enzymatic activity toward nucleosomal histones in vitro. To identify HMTase capable of methylating nucleosomal histones, nuclear protein fractions derived from DE52 and P11 columns were analyzed for HMTase activity, using either core histones or oligonucleosomes as substrates. Results shown in Figure 1A indicate that multiple HMTase activities specific for histones H3 and H4 in the form of core histones or oligonucleosomes exist. While multiple fractions contain nucleosomal H3-specific HMTase activity, only one fraction contains nucleosomal H4-specific HMTase activity (Figure 1A, bottom panel). Western blot analysis indicates that this fraction does not contain the previously identified H4-R3-specific methyltransferase PRMT1 (data not shown), suggesting that a protein other than PRMT1 is responsible for the enzymatic activity. Previous studies indicated that lysine methylation in histone H4 is restricted to lysine 20 [10], thus, the fraction likely contains the long sought H4-K20 methyltransferase.

To purify the enzyme, the 1.0 M P11 fraction was fractionated sequentially through four columns (Figure 1A). The enzymatic activity was monitored by HMTase assay using oligonucleosomes as substrates. Analysis of the fractions derived from the last purification step indicated that the activity eluted with a native size of 50–150 kDa between fractions 50 to 56 (Figure 1B, middle panel). Silver staining of an SDS-polyacrylamide gel containing the column fractions revealed multiple proteins present in the enzymatically active fractions (Figure 1B, top panel). The limited amount of the sample prevented us from further purification. Since the activity begins with fraction 50 and ends with fraction 56, the candidate protein bands should be present in fraction 50 but absent in fractions 47 and 59. Using these criteria, we identified six protein bands (Figure 1B, top panel, indicated by dots and star) that are likely responsible for the enzymatic activity.

Identification of a Novel 45 kDa SET Domain-Containing Protein as the H4-Specific HMTase

To determine which of the six candidate proteins is responsible for the detected nucleosomal histone H4-specific methyltransferase activity, each of the six protein bands was excised and subjected to in-gel trypsin digestion. The resulting peptides were analyzed by mass spectrometry [34]. Interestingly, most masses obtained from the 45 kDa protein, marked by a star (Figure 1B, top panel), matched a SET domain-containing protein named PR/SET07 in GenBank (AAF97812). Since all the lysine-specific HMTases identified so far contain a SET domain, the 45 kDa protein is likely responsible for the detected H4-specific HMTase activity. It is important to point out that not all the masses obtained from the 45 kDa protein matched PR/SET07. Either the 45 kDa protein band represents a mixture of PR/SET07 and another protein or the protein is distinct but highly related to PR/SET07.

To differentiate these two possibilities, human EST clones that are similar or identical to that of the PR/SET07 in sequence were obtained and sequenced. Conceptual translation of the cDNA from one of the EST clones (BE867579) generated an ORF (open reading frame) of 352 amino acids. Since all nine peptide sequences obtained from the 45 kDa protein band precisely match sequences within the 352 amino acids ORF (Figure 2A), we conclude that the 45 kDa protein is distinct from but highly similar to PR/SET07 (84% identical). Several lines of evidence indicate that the 352 amino acids encoded by the cDNA represent the full-length protein. First, all the masses obtained from the 45 kDa protein are accounted for in the ORF. Second, the calculated molecular mass 40 kDa is close to the estimated 45 kDa apparent molecular mass. Third, the nucleotide sequence around the first methionine conforms to the Kozak initiator sequence [35]. Fourth, transfection of a mammalian expression vector containing the ORF into 293T cells generated a product that migrates at the same position as that of the native protein (data not shown).

Analysis of the putative nucleosomal H4-specific methyltransferase revealed a SET domain at its C terminus (Figure 2A). Similar to the previously identified H3-K4-specific HMTase SET7 [31], neither a cysteine-rich preSET nor a postSET domain was identified. A search of the GenBank databases with the amino acid sequence revealed apparent homologs in Drosophila (CG3307) and C. elegans (T26A5.7). Alignment of these homologs with their human counterpart revealed that these proteins are highly conserved in the SET domain and its adjacent sequences (Figure 2A). However, the lengths of the N-terminal regions of these proteins are variable in different organisms. Interestingly, no apparent homolog was found in the budding yeast Saccharomyces cerevisiae. Since the SET domain is the only recognizable motif found in this protein, the protein has no homology to any of the six yeast SET domain-containing proteins.
Figure 1. Purification of an H4-Specific Methyltransferase
(A) Schematic representation of the steps used to purify the H4-specific HMTase. Numbers represent the salt concentrations (mM) at which the HMTase activity elutes from the columns. Substrates used in the HMTase assays are indicated.

(B) Silver staining of a polyacrylamide-SDS gel (top panel), HMTase activity assay (middle panel), and Western blot analysis (bottom panel) of the fractions derived from the gel-filtration Superose 200 column. The elution profile of the protein markers is indicated on top of the panel. The protein size marker on SDS-PAGE is indicated to the left of the panel. The polypeptides that coelute with the HMTase activity are indicated by a *, and the enzyme (hSET8) is marked by a †.

outside the SET domain, and the protein is different from the H3-K4-specific HMTase SET7 [31] or the putative protein PR/SET07, we name this protein SET8.

Although all the lysine-specific HMTases identified so far contain SET domains, the presence of a SET domain is not sufficient for HMTase activity [15]. To confirm that SET8 is indeed the nucleosomal H4-specific methyltransferase that we are pursuing, recombinant SET8 was generated and tested for its activity using nucleosomes as substrates. Results shown in Figure 2B demonstrate that SET8 is indeed capable of methylating nucleosomal H4. Moreover, recombinant SET8 and the native SET8 seem to have comparable activity when equal amounts of SET8 were compared (Figure 2B, compare lanes 1 and 3). Thus, we conclude that SET8 is a novel nucleosomal H4-specific methyltransferase.

The fact that the native enzymatic activity eluted at a slightly larger mass than 45 kDa (Figure 1B) prompted us to ask whether any other factors coexist with SET8 in a protein complex. To this end, a polyclonal rabbit antibody against SET8 was generated. Western blot analysis of partially purified HeLa extracts using affinity-purified SET8 antibody demonstrated that the antibody is highly specific (Figure 2C). To verify that SET8 is indeed present in our last purification step, fractions derived from the gel filtration S200 column were analyzed by Western blot using SET8 antibody. Results shown in Figure 1B indicated that the elution profile of SET8 overlaps the nucleosomal H4-specific HMTase activity. Surprisingly, although fraction 56 contains no detectable SET8, it contains a relatively high level of enzymatic activity. One explanation is that this fraction contains another unidentified H4-specific HMTase (see below).

In order to identify possible SET8 associated protein(s), input for the phenyl sepharose column was used for immunoprecipitation followed by silver staining and Western blot analysis using SET8 antibody. As a control for specificity, a polyclonal antibody against GST was used in parallel experiments. Results shown in Figure 2D demonstrate that no apparent polypeptide coimmunoprecipitates with SET8 (bands around 26 kDa present in both samples are IgG light chain). Since the native size of SET8 is between 66 and 150 kDa (Figure 1B, bottom panel), it is possible that SET8 exists as a homodimer. Consistent with this possibility, recombinant SET8 eluted at exactly the same fractions as that of the native SET8 on the S200 column (compare Figures 2E and 1B, bottom panel). Collectively, the above data support that SET8 is a nucleosomal H4-specific HMTase and that SET8 functions as a homodimer.

SET8 Prefers Nucleosomal Histone H4 as Substrate
Having established that SET8 is able to methylate nucleosomal histone H4, we also assessed whether it can methylate H4 in the form of a mixture of core histones. Results shown in Figure 3A indicate that at a low enzyme concentration (enzyme/octamer molar ratio 1:625 to 1:12) SET8 almost exclusively methylates nucleosomal histone H4 (Figure 3A, lanes 1–8). However, as the enzyme concentration increases (enzyme/octamer molar ratio 1:6 to 1:3), SET8 is able to methylate H4 in both octamer and nucleosome forms, with a preference for the nucleosomal substrate (Figure 3A, lanes 9–12). Given that SET8 has a strong preference toward
nucleosomal histone H4 in a wide range of enzyme concentrations, we believe that nucleosomes are likely to be the physiological substrate of SET8.

To begin to understand why SET8 prefers nucleosomal histone H4 as its substrate, we tested reconstituted nucleosomes prepared from recombinant Xenopus core histones and plasmid DNA by the salt dilution method [36, 37]. The reconstituted nucleosomes were subjected to methylation by SET8 at an enzyme/octetamer molar ratio of 1:30 at which SET8 only methylates nucleosomal histone (Figure 3A). As controls, histone octamers alone and octamer plus unassembled plasmid DNA were also used in the HMTase assay. Results shown in Figure 3B indicate that, while SET8 is not able to methylate histone H4 in octamers, it can efficiently methylate histone H4 in the presence of DNA. However, we cannot rule out the possibility that the plasmid DNA and histones form a nucleosome-like structure during the reaction.

The SET Domain and Its Adjacent Sequences Are Required for SET8 HMTase Activity

Having demonstrated that SET8 is a potent HMTase with preference for nucleosomal histone H4 (Figure 3B), we attempted to determine the minimal amino acid sequences required for its HMTase activity. Previous studies on SUV39H1, SET7, and ESET indicated that the SET domain is required for each of these HMTases’ activity [15, 31, 23]. To determine whether the same is true for SET8, a SET8 mutant protein that contains a single amino acid change (H299A) in the most conserved NHS motif of the SET domain was generated. HMTase assay demonstrated that the point mutation greatly impaired the enzymatic activity toward nucleosomal H4 (Figure 3D, compare lanes 1 and 2). Thus, the SET domain of SET8 is critical for its HMTase activity.

In addition to the SET domain, our previous work on SET7 has demonstrated that the adjacent sequences, whether cysteine rich or not, are also required for HMTase activity [31]. Given that SET8 targets histone H4 instead of H3, we asked whether any sequences in SET8 other than the SET domain are required for its HMTase activity. To this end, a series of N-terminal and C-terminal deletion mutant SET8 proteins were generated and tested for HMTase activity. Similar to the H299A mutation in the SET domain, deletion of the nine amino acids at the extreme C terminus of SET8 dramatically decreased its enzymatic activity toward nucleosomal histone H4 (Figure 3D, compare lanes 1 and 7). In contrast to the stringent requirement for the C terminus, the N-terminal 174 amino acids do not seem to be required for its HMTase activity (Figure 3D, compare lanes 3–5 with lane 1). On the contrary, the N-terminal amino acid sequences seem to inhibit the SET8 HMTase activity, as deletion of the N-terminal 53 amino acids almost doubled the SET8 HMTase activity (Figure 3D, compare lanes 3 and 4 with 1). Further deletion of the N-terminal sequences to the SET domain completely abolished its enzymatic activity (Figure 3D, compare lanes 5 and 6). Similar results were obtained when core histones were used as substrates (data not shown). The above studies indicate that about half of the SET8 protein is dispensable for its HMTase activity. We believe that this is the smallest domain identified so far that possesses nucleosomal HMTase activity. It is interesting to note that the minimum domain of SET8 that possesses HMTase activity correlates with the region that is most conserved among the SET8 homologs from different organisms. Thus, the SET8 homologs from Drosophila and C. elegans are likely to have HMTase activity (see below).

SET8 Methylates H4 at Lysine 20

Having established that SET8 is an H4-specific HMTase, we attempted to determine the methylation site. Microsequencing of SET8 methylated H4 followed by liquid scintillation counting revealed that lysine 20 is the methylation site (Figure 4A). Since SET8 is capable of methylating recombinant H4 as well as H4 peptide at a higher enzyme concentration (data not shown), we verified the site mapping result using wild-type and mutant H4 tail peptides as substrates. While SET8 can efficiently methylate wild-type H4 tail peptide, it is completely inactive toward the same peptide with a K20L mutation (Figure 4B). Taken together, these results support that SET8 specifically methylates H4 at K20 in vitro.

H4-K20 Methylation Occurs in a Wide Range of Higher Eukaryotic Organisms

To investigate the role for SET8 in H4-K20 methylation in vivo, we generated a polyclonal antibody against a dimethyl-K20 H4 tail peptide. To examine the specificity of the antibody, reconstituted nucleosomes were methylated by SET8 and analyzed by Coomassie staining and Western blots. To demonstrate that the antibody did not simply recognize any methylated residues on H4, recombinant H4 that was methylated by PRMT1 at R3 was included as a control. Results shown in Figure 4C demonstrate that the antibody recognized SET8-methylated histone H4 (lane 4), while it does not recognize nonmethylated (lanes 1 and 3) or R3-methylated H4 (lane 2). Thus, we conclude that the antibody is H4-mK20-specific.

Having demonstrated the specificity of the antibody, we tested for H4-K20 methylation in different organisms. Core histones isolated from chicken, Drosophila, and the budding yeast Saccharomyces cerevisiae were analyzed for the existence of H4-K20 methylation by Western blotting. As negative and positive controls, recombinant H4 and HeLa core histones were included in the assay. Consistent with the existence of a SET8 homolog in Drosophila but not in yeast (see above), H4-mK20-specific antibody reacted with H4 purified from Drosophila but not from yeast (Figure 4D). Given that the minimum sequences on SET8 required for HMTase activity are highly conserved in the putative Drosophila and C. elegans homologs (Figure 2A), it is likely that H4-K20 methylation also occurs in C. elegans. Thus, we believe that H4-K20 methylation is likely to occur in most if not all multicellular organisms.

dSET8 Contributes to H4-K20 Methylation In Vivo

Having established that H4-K20 methylation occurs in Drosophila, we attempted to determine whether the putative Drosophila SET8 homolog encoded by CG3307 also has H4-K20-specific HMTase activity. Toward this
Figure 2. Identification of the H4-Specific HMTase hSET8

(A) Alignment of the amino acid sequences of the novel SET domain-containing H4-specific HMTase from human (AY102937), Drosophila (AAF55047), C. elegans (T34384), and the PR/SET07 (AAF97812). Peptides obtained from mass spectrometric analysis are underlined. Amino acids that are identical to hSET8 are indicated by “-“. Gaps are indicated by “.”. Highlighted sequences represent SET domain. * indicates the amino acid that, when mutated, dramatically reduced the hSET8 HMTase activity.

(B) Comparison of the native and recombinant hSET8 HMTase activity. Fixed amounts of native hSET8 were compared with different amounts of recombinant hSET8 indicated at the top of the panel (top panel) for their HMTase activities (middle panel) using equal amounts of oligonucleosome substrates (bottom panel).
Figure 3. Characterization of the SET8 HMTase Activity

(A) SET8 preferentially methylates nucleosomal H4. Equivalent amounts of octamers or nucleosomes (bottom panel) purified from HeLa cells were methylated using different amounts of recombinant hSET8. The amounts of enzyme used and the corresponding molar ratios of rhSET8 to octamers are indicated on top of the panels.

(B) The HMTase activity of SET8 is stimulated by the presence of DNA. Recombinant octamers in the presence or absence of DNA were compared with equivalent amounts of reconstituted nucleosomes for methylation by 100 ng of SET8 (enzyme/octamer ratio of 1:30).

(C) Schematic representation of the different hSET8 deletion mutants. The SET domain and the point mutation are indicated. The amino acid numbers for each construct are indicated.

(D) Equal amounts of wild-type and mutant GST-hSET8 proteins (top panel) were compared for their HMTase activities (middle panels) using equal amounts of oligonucleosomes as substrates. The relative HMTase activity represents the average of two independent experiments. Variations between the two experiments are indicated by error bars.

end, a CG3307 cDNA clone, LD12042, was obtained from the Drosophila EST project and was sequenced. A GST-fusion protein containing amino acids 25 to 689 of the fly protein was generated and analyzed for HMTase activity using reconstituted nucleosomes as substrates. Results shown in Figure 5A demonstrate that the fly
Figure 4. SET8 Methylates Histone H4-Lysine 20 In Vitro

(A) In vitro hSET8 methylated H4 was blotted onto PVDF membrane and subjected to N-terminal automated sequencing. $^3$H-radioactivity eluted from each cycle was counted. The amino acids identified at each cycle of microsequencing are listed.

(B) HMTase assay using wild-type and mutant (K20L) synthetic H4 tail peptide as substrates.

(C) Characterization of the H4-mK20 antibody. Recombinant histone H4 or equivalent amounts of reconstituted nucleosomes were methylated by PRMT1 or hSET8, respectively. Samples were then analyzed by Western blots using the H4-mR3 and H4-mK20 antibodies.

(D) H4-K20 methylation occurs in a wide range of multicellular organisms. Equivalent amounts of histones from different organisms indicated on top of the panel were analyzed by Western blot using the H4-mK20-specific antibody.

The fact that the mutant retains substantial levels of H4-K20 methylation suggests that additional H4-K20-specific HMTases exist in Drosophila. Consistent with this possibility, we observed additional H4-K20 methyltransferase activity in HeLa cells which does not correlate with hSET8 in chromatography (data not shown and also see Figure 1B, compare middle and bottom panels). Identification of the additional H4-K20-specific HMTase will be required to fully understand the function of H4-K20 methylation in vivo.

To further investigate the timing and possible causes
Figure 5. Disruption of dSET8 Results in Decreased H4-K20 Methylation In Vivo
(A) Recombinant Drosophila SET8 (dSET8) is capable of methylating nucleosomal H4-K20 in vitro. Equivalent amounts of reconstituted nucleosomes (bottom panel) were methylated by recombinant human or Drosophila SET8. The HMTase activity (top panel) and H4-K20 specificity (middle panel) are shown.
(B) A P element insertion greatly reduces dSET8 expression. Cell lysates from wild-type and homozygous mutant larvae were analyzed by Western blot for dSET8. Equal loading was verified by Western blot for tubulin (bottom panel).
(C) dSET8 is partially responsible for H4-K20 methylation in vivo. Equivalent amounts of histones (top panel) extracted from wild-type and homozygous mutant Drosophila larvae were analyzed for H4-K20 methylation level by Western blot (bottom panel).
(D) Distribution of K20-methylated H4 in polytene chromosomes derived from a wild-type larval salivary gland. Arrow indicates the chromocenter, and asterisks indicate chromosome puffs.
(E) Distribution of K20-methylated H4 in polytene chromosomes derived from a dSET8 mutant of genotype l(3)neo41/Df(3R)red31. Arrow indicates chromocenter.

of lethality in l(3)neo41 mutants, we analyzed l(3)neo41/Df(3R)red31 animals, where Df(3R)red31 is a third chromosome deficiency that uncovers lethality of the l(3)neo41 insertion ([38, 40] and Experimental Procedures). This combination appears to be semilethal, as six adult survivors were recovered from a cross yielding 754 total progeny. These rare escapers displayed multiple phenotypes, including rough eyes, notched wings, and patches of disorganized thoracic and abdominal bristles. The lethality prior to adulthood appeared to be polyphasic, with death occurring at both pupal and prepupal stages. Taken together, these results show
K20 staining is generally reduced, although not elimi-

nated by p300 [6]. Since histone acetylation has been linked to gene activation [1], we thought that understanding the effect of H4-K20 methylation on subsequent histone acetylation might help us to understand the potential role of H4-K20 methylation on transcription. To this end, recombinant histone H4 was incubated with SET8 with or without the presence of the methyl donor S-adenosyl-L-methionine (SAM). After confirmation of the H4-K20 methylation status (Figure 6B, top panel), the reaction mixtures were subjected to acetylation by p300 in the presence of [3H]-acetyl-CoA. Results shown in Figure 6B (middle panel) indicate that no significant differences in the acetylation efficiency of the nonmethylated and methylated H4 were observed. Thus, we conclude that methylation on H4-K20 does not significantly affect subsequent H4 acetylation by p300 under our assay conditions.

We next sought to determine the effect of histone acetylation on subsequent histone methylation. We first addressed this question in vivo by purifying both hyper-acetylated and hypoacetylated core histones from HeLa cells and comparing their H4-K20 methylation status. The acetylation status of the core histones were revealed by Coomassie staining of a Triton-Acetic Acid-Urea (TAU) gel (Figure 6C, lanes 1 and 2). Western blot analysis of parallel samples using H4-mK20-specific antibodies revealed that the H4-K20 methylation occurs on all the different acetylated H4 isoforms and appears to be slightly enriched in the nonacetylated and mono-acetylated isoforms (Figure 6C, compare lanes 1 and 3). Thus, H4-K20 methylation level does not seem to be significantly affected by histone acetylation level in vivo. To compare the efficiency of different acetylated H4 isoforms to serve as substrates for SET8, both hyper-acetylated and hypoacetylated core histones (Figure 6C, lanes 1 and 2) were methylated by SET8 in the presence of [3H]-SAM and analyzed by TAU gel. Results shown in Figure 6C (lanes 5 and 6) indicate that, while SET8 is able to methylate all the different H4 isoforms, it preferentially methylates hyperacetylated H4 isoforms (lane 5) in vitro. A simple explanation for this preferential methylation is that the hyperacetylated forms were at a lower methylation level in vivo before being subjected to in vitro methylation. Collectively, our data do not support significant interdependence between H4-K20 methylation and H4 acetylation.

H4-K20 Methylation Is Cell Cycle Regulated

Previous studies indicated that histone methylation is regulated during the cell cycle [41], although H4-K20 modification was not specifically assessed. To analyze the H4-K20 methylation status during the cell cycle, we arrested HeLa cells at the G1/S border, using a double thymidine block. After releasing the arrested cells from the thymidine block, cells were collected every 2 hr for flow cytometry as well as for the preparation of protein
Figure 6. Distribution of H4-K20 Methylation in NIH3T3 Cell Nuclei, Mitotic Chromosomes, HeLa Cell Cycle, and Tests for Interplay between H4-K20 Methylation and H4 Acetylation

(A) NIH3T3 cell nuclei and mitotic chromosomes were stained with antibodies against H4-mK20 and counterstained with DAPI. Note mK20 staining does not correlate with heavily DAPI stained heterochromatin in interphase cells and is excluded from centromeres in mitotic chromosomes.

(B) Methylation of H4-K20 does not significantly affect subsequent acetylation by p300. Mock- and SET8-methylated recombinant H4 were subjected to p300 acetylation in the presence of [3 H]acetyl-CoA. Samples were analyzed by Western blot, fluorography, and Coomassie staining.

(C) Both hypoacetylated and hyperacetylated H4 can be methylated by SET8. Hyper- (Ac) and hypoacetylated (Non-Ac) core histones were resolved by TAU gel electrophoresis and analyzed by Coomassie staining (left panel) and Western blot (middle panel). These samples were also subjected to SET8 methylation in the presence of [3 H]-SAM and visualized by fluorography (right panel).

(D) Cell extracts and histones derived from different cell cycle stages were analyzed by Western blotting. SLBP and cyclin A were used as cell cycle markers; tubulin and total H4 were used as loading controls. The H4-K20 methylation level was analyzed by probing with the mK20-specific antibodies. The cell cycle stage of each sample is indicated on top of the panels.

effects and histones. Flow cytometry analysis indicated that more than 95% of the cells progress through S phase and enter G2 synchronously (data not shown). The cells were successfully arrested at the G1/S border before release, as evidenced by the accumulation of the histone mRNA stem-loop-binding-protein (SLBP) (Figure 6D). As demonstrated previously [42], SLBP levels stayed high throughout S phase, and as cells exited S phase, SLBP levels dropped rapidly (Figure 6D). The cells complete mitosis about 12 hr after release, as evidenced by the degradation of cyclin A as cells enter anaphase (Figure 6D). To determine whether H4-K20 methylation levels change during the cell cycle, histones isolated from corresponding cells were subjected to Western blot analysis using the H4-mK20-specific antibody. Results shown in Figure 6D demonstrate that H4-K20 methylation peaks during S phase and drops during G2/M phase.

Using a similar approach, we also analyzed the H4-K20 methylation status in G1 phase by releasing cells arrested at M phase by nocodazole block. This study confirmed that H4-K20 methylation level is the lowest during mitosis, maintains an intermediate level during G1, and reaches the highest in S phase (data not shown).
Thus, experiments using two different cell synchronization methods indicate that H4-K20 methylation level is regulated during the cell cycle.

Discussion

SET8 Is a Nucleosomal Histone H4-K20-Specific Methyltransferase

Using biochemical approaches, we purified a nucleosomal histone H4-specific HMTase activity (Figure 1) and found that a single SET domain-containing polypeptide, named SET8, is responsible for the enzymatic activity (Figure 2). This single subunit HMTase is highly specific for nucleosomal H4 at low enzyme concentrations. However, at a higher enzyme concentration, it is capable of methylating H4 present in mixtures of core histones (Figure 3A). To our knowledge, this is the first HMTase identified with this substrate preference, suggesting that it may exclusively target nucleosomal histones for methylation in vivo.

Mutation and deletion studies indicated that the SET domain and its adjacent regions are required for SET8 HMTase activity (Figure 3D). Similarly, we have demonstrated previously that both the SET domain and its adjacent sequence in SET7 are required for its HMTase activity, although neither SET7 nor SET8 contains the cysteine-rich preSET or postSET domains present in SUV39H1 [15, 31]. One possibility is that the sequences adjacent to the SET domain help with the folding of the SET domain to accommodate the histone tails. SET8 deletion studies also revealed that removing the N-terminal 53 amino acids of SET8 almost doubled its enzymatic activity toward nucleosomal histone substrate when compared with the full-length protein. Importantly, we found that the C-terminal 178 amino acids of SET8 are sufficient for full HMTase activity. Further studies are needed to define the domains involved in catalysis and substrate recognition.

Direct sequencing of SET8 methylated H4 and HMTase assays using a K20L mutant peptide strongly argue that SET8 exclusively methylates H4-K20 (Figure 4). Using an H4-mK20-specific antibody, we found that H4-K20 methylation occurs in all tested multicellular organisms from Drosophila to human (Figure 4D). Consistent with our finding that no apparent homolog of SET8 exists in Saccharomyces cerevisiae, H4-K20 methylation does not occur in this organism (Figure 4D). However, we were able to identify a SET8 homolog in Drosophila and demonstrate that this protein is at least partially responsible for H4-K20 methylation in vivo (Figure 5). Our studies, both in mammalian cells and Drosophila, suggest that SET8 is not the only H4-K20-specific methyltransferase. Because different H4-K20 methyltransferases are likely to have functional redundancy, complete understanding of the function of H4-K20 methylation requires the identification of other H4-K20 methyltransferases.

Relationship between H4-K20 Methylation, Transcription, and Cell Cycle Regulation

Histone tail modification, particularly acetylation, plays an important role in transcription regulation [1]. Recent studies have revealed that histone methylation is also involved in transcription regulation [5]. For example, methylation of H4-R3 and H3-R17 by PRMT1 and CARM1, respectively, play important roles in nuclear hormone receptor-mediated transcriptional activation [6–9]. In contrast, methylation of H3-K9 by SUV39/Clr4 and subsequent recruitment of HP1/Swi6 generally correlates with transcriptional repression [16–18]. To investigate the potential role of H4-K20 methylation in transcription regulation, we used several approaches. Multiple lines of evidence suggest that H4-K20 methylation is not likely to play a major role in regulating transcription activity. First, immunostaining of fly polypytene chromosomes revealed that although H4-K20 methylation is widespread on the euchromatic arms (Figure 5D), staining is low or absent in puffs and interbands, which correspond to the most actively transcribed regions. H4-K20 methylation was also seen at the chromocenter. More work is needed to determine if there is a link between this modification and gene repression or structural organization of chromatin in this region. However, immunostaining of mammalian cells with the same antibody indicated that H4-K20 methylation pattern does not correlate with the transcriptionally repressed heterochromatin (Figure 6A). Although we cannot rule out the possibility that H4-K20 methylation regulates transcription in a species-specific manner, we believe that this possibility is less likely. The second line of evidence that argues against a major role for H4-K20 methylation in transcription comes from the lack of significant interplay between H4-K20 methylation and histone acetylation (Figure 6). It appears that H4-K20 methylation does not significantly affect the subsequent acetylation of H4 by p300 (Figure 6B). However, we note that the interpretation of this result might be complicated by the fact that we do not know what percentage of H4-K20 was methylated in our acetylation assay. Although the use of synthetic peptides might overcome this problem, it is possible that the potential effect of the histone globular domain on the reaction would be lost. In a reciprocal experiment, we also did not detect significant changes in H4-K20 methylation in vivo in response to histone hyperacetylation (Figure 6C). The final evidence that argues against a significant role for H4-K20 methylation in transcription comes from direct tests of SET8 fusion proteins. When both wild-type and HMTase defective SET8 mutant proteins were targeted to reporters through the Gal4-DNA binding domain in either mammalian cells or Xenopus oocytes, no significant transcriptional effect was detected (data not shown). While the negative result from the studies performed in mammalian cells might be attributed to an incompletely chromatinized reporter, the studies performed in Xenopus oocytes used a successfully assembled nucleosome template. Taken together, we believe H4-K20 methylation is unlikely to play a major role in transcription regulation comparable to that of H3-K9 or H3-K4 methylation.

In order to understand the role of H4-K20 methylation in processes other than transcription, we analyzed the methylation status of H4-K20 during the cell cycle. By using two different methods for cell synchronization, we observed that the H4-K20 methylation level changes during the cell cycle, with the highest level at S phase,
changes in H4-K20 methylation during the cell cycle. However, we can think of questions. For example, what is the cause of this change? Does this change bear any relationship to the turnover of methylated histones? Is the change a consequence of cell cycle progression, or does it reflect a role of H4-K20 methylation in controlling the cell cycle? Does the high level of H4-K20 methylation at S phase have any connection to DNA synthesis? Furthermore, we will be required to provide satisfactory answers to these questions. However, we can think of at least three potential explanations for the observed changes in H4-K20 methylation during the cell cycle. First, it is possible that the level or activity of the H4-K20-specific methyltransferases, such as SET8, are themselves regulated during the cell cycle. Due to the low abundance of SET8, our attempts to directly monitor its levels during the cell cycle by Western blot have so far proven unsuccessful. Further studies using more sensitive methods may address this possibility. The second explanation is that K20-methylated H4 might be selectively degraded at a particular cell cycle stage, such as M phase. We note that the H4-mK20-specific antibody stained the mammalian mitotic chromosomes much more weakly than the *Drosophila* polytene chromosomes, which result from multiple rounds of replication without division. The third possibility is that cell cycle-regulated histone demethylation. More studies are needed to determine which of these possibilities underlies the observed change in H4-K20 methylation during the cell cycle.

**Function of dSET8 in Fly Development**

We have demonstrated in *Drosophila* that H4-K20 is an in vivo target of SET8 (Figure 5). However, in mammalian cells as well as in flies, SET8 does not seem to be the only enzyme that can target H4-K20 for methylation. Thus, a complete understanding of the function of H4-K20 methylation requires the identification of all the responsible enzymes. Our preliminary studies using a P element insertion allele indicate that dSET8 plays an important role in normal fly development. The survival of hemizygotes bearing this mutation to late developmental stages, together with the decreased but not complete disappearance of H4-K20 methylation in the mutant (Figure 5), are consistent with at least partial strains used for HMTase assays included oligonucleosomes, mononucleosomes, and core histones purified from HeLa cells [43], synthetic H4-tail peptides, recombinant *Xenopus* H4 and octamers purified as described [36], and reconstituted nucleosomes assembled as described [37]. Hyperacetylated histone purification and TAU gel assays were performed as described [44]. Quantitations were performed using the NIH Image 1.62 software after scanning the original data.

**Plasmids and Antibodies**

A plasmid encoding GST-hSET8 was constructed by digestion of the hSET8 cDNA with NcoI and HindIII, which was then ligated into the Ncol/HindIII sites of the pGEX-KG vector. All the GST-hSET8 deletion constructs were generated by PCR and cloned into the EcoRI/XhoI sites of the pGEX-KG vector. The H299A mutant was generated by overlap PCR. Constructs generated using PCR were verified by DNA sequencing. A plasmid encoding GST-dSET8 was constructed by digestion of the *Drosophila* SET8 cDNA (LD12042) with EcoRI and SacI and insertion into the EcoRI/SacI sites of the pGEX-KG vector. Antibody against hSET8 was generated in rabbit

lacks both this modification and a recognizable SET8 homolog. We demonstrated that a *Drosophila* SET8 homolog has the same substrate specificity as human SET8 in vitro and is at least partially responsible for H4-K20 methylation in vivo. Disruption of dSET8 by a P element insertion produces lethality, indicating that dSET8 function is required for normal fly development. Although the chromosomal distribution of H4-K20 methylation is widespread, our data do not support a primary role in gene activity. H4-K20 methylation levels are regulated during the cell cycle, with the highest levels detected in S phase.

**Experimental Procedures**

**Purification of the H4-K20 HMTase**

HeLa nuclear proteins were separated into nuclear extract and nuclear pellet fractions as previously described [6]. After solubilization, nuclear pellet proteins (4 grams) were resuspended in buffer D (50 mM Tris-HCl [pH 7.9], 0.1 mM EDTA, 2 mM DTT, 0.2 mM PMSF, and 25% glycerol) containing 20 mM ammonium sulfate (BD20) and loaded onto a 500 ml DEAE52 column equilibrated with BD20. Proteins that bound to the DEAE52 column were step eluted with BD350 and BD500. The BD350 fraction was dialyzed into buffer C (20 mM Tris-HCl [pH 7.9], 0.2 mM EDTA, 1 mM DTT, 0.2 mM PMSF, and 20% glycerol) containing 100 mM KCl (BC100) and loaded onto a 400 ml phosphocellulose P11 column. Bound proteins were step eluted with BC300, BC500, and BC1000. The BC1000 fraction was then dialyzed to BD20 and loaded onto a 45 ml HPLC-DEAE-SPW column (TosoHaas). The bound proteins were eluted with a 12 column-volume (cv) linear gradient from BD20 to BD600. The fractions containing the HMTase activity were combined and dialyzed into buffer P (5 mM HEPES-KOH [pH 7.5], 40 mM KCl, 0.01% Triton X-100, 0.01 mM CaCl2, 0.5 mM PMSF, 1 mM DTT, 10% glycerol containing 10 mM potassium phosphate (BP10) and loaded onto a 5 ml hydroxyapatite column. The bound proteins were eluted with a 12 cv linear gradient from BP10 to BP600. The fractions containing the HMTase activity were pooled and adjusted to BD1000 using saturated ammonium sulfate and loaded onto a 1 ml FPLC Phenyl Sepharose column (Pharmacia). The bound proteins were eluted with 0.20 cv linear gradient from BD1000 to BD500. The peak fraction containing the HMTase activity were pooled and dialyzed into BC50, concentrated on a 0.2 ml P11 column, and then fractionated on a Sepharose 200 gel-filtration column (Pharmacia). The HMTase activity elutes with a relative molecular mass between 150 and 66 KDa.

**Protein Identification, Methylation Site Determination, HMTase, and HAT Assays**

Protein identification and methylation site determination were performed exactly as previously described [31]. HMTase and HAT assays were also performed essentially as described [31]. Substrates used for HMTase assays included oligonucleosomes, mononucleosomes, and core histones purified from HeLa cells [43], synthetic H4-tail peptides, recombinant *Xenopus* H4 and octamers purified as described [36], and reconstituted nucleosomes assembled as described [37]. Hyperacetylated histone purification and TAU gel assays were performed as described [44]. Quantitations were performed using the NIH Image 1.62 software after scanning the original data.
using GST-hSET8(54-352) as an antigen. Methyl-K20-specific antibody was generated by injecting rabbits with a KLH conjugated synthetic H4 peptide (AKHRKmKVLRDN). H3-mR and SLBP antibodies have been previously described [31, 42]. All the antibodies used in this study were affinity purified.

Cell and Mitotic Chromosome Staining, Cell Synchronization, and Flow Cytometry

For nuclei and mitotic chromosomes staining, NIH 3T3 cells were treated with 0.4 µg/ml nocodazole (Sigma) for 16 hr before harvesting. The cells were then incubated with 75 mM KCl for 10 min at 37°C. The swollen cells were spun down onto polylysined coverslips at 1800 rpm for 5 min. Following cyt centrifugation the coverslips were immersed immediately in KCM buffer (120 mM KCl, 20 mM NaCl, 10 mM Tris-Cl [pH 7.5], 0.5 mM EDTA, and 0.1% Triton X-100) for 10 min. The cells were then incubated with antibodies against H4-mK20 (1:250 dilution in 1% BSA/KCM) for 1 hr at room temperature. After washing with KCM twice, the cells were incubated with FITC-conjugated goat anti-rabbit IgG for 1 hr. The cells were then washed twice with KCM, stained with DAPI for 10 s, and fixed with 4% formaldehyde in KCM for 10 min. Coverslips were mounted and viewed using a Zeiss microscope. Cell synchronization and flow cytometry were performed exactly as described [45].

**Drosophila** Genetics, Polytenic Chromosome Staining, and Histone Isolation from Larvae

The is(3)neo41 chromosome has genotype mwh red is(3)neo41 e and was reassembled over TM6B, Tb e, is(3)neo41 homozygotes were collected from grape agar plates as roving, second instar larvae that were red (red Malpighian tubules) and non-Tb (non-Tubby). The mutant extracts and histone preparations used in Figures 6B and 6C were prepared from these hand-sorted homozygotes. Wild-type extracts were prepared from bulk-collected larvae of genotype y Df(1)w67c2/Df(3R)red31. Drosophila embryos were used as a control for the effects of chromosome position-effect variegation suppressor gene Su(var)3-9 complex. EMBO J. 9, 3822–3831. Preimmune serum tested in parallel at 1:100 did not produce chromosome staining. Isolation of histones from wild-type and mutant Drosophila larva was performed using a modification of a published procedure [47].

**Acknowledgments**

We thank William Marzluff and Yue Xiong for antibodies; Huck-Hui Ng for chicken and yeast histones; Lori Wallrath, Guillermo Marques, Tom Hays, and Tom Neufeld for discussions and technical advice; and Erin Henry for critical reading of the manuscript. Y.Z. is a Kimmel Scholar and is supported by the National Institutes of Health (GM63067-01) and the American Cancer Society (RSG-00-351-01-GMC). The work was also supported by NIH grant GM49850 to J.A.S.; National Cancer Institute grant P01CA08748 to P.T., and funding from NIH training grant HD07480 to C.S.K.

**References**

An H4-K20-Specific Methyltransferase

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Accession Numbers

The GenBank accession number for the hSET8 sequence reported in this paper is AY102937.