Post-translational modifications of histone tails directly influence nuclear processes including transcription, DNA repair, and chromatin packaging. Lysine 20 of histone H4 is mono-, di-, or trimethylated in vivo, but the regulation and significance of these methylations are poorly understood. The SET domain proteins PR-Set7 and Suv4-20 have been implicated in mono- and trimethylation, respectively, however, enzymes that dimethylate lysine 20 have not been identified. Here we report that Drosophila Suv4-20 is a mixed product specificity methyltransferase that dimethylates approximately 90% and trimethylates less than 5% of total H4 at lysine 20 in S2 cells. Trimethylation, but not dimethylation, is reduced in Drosophila larvae lacking HP1, suggesting that an interaction with HP1 regulates the product specificity of Suv4-20 and enrichment of trimethylysine 20 within heterochromatin. Similar to the Drosophila enzyme, human Suv4-20h1/h2 enzymes generate di- and trimethylysine 20. PR-SET7 and Suv4-20 are both required for normal levels of methylation, suggesting they have non-redundant functions. Alterations in the level of lysine 20 methylation following knock-down or over-expression of Suv4-20 did not affect lysine 16 acetylation, revealing that these two modifications are not competitive in vivo. Depletion of Suv4-20h1/h2 in HeLa cells impaired the formation of 53BP1 foci, suggesting dimethylysine 20 is required for a proper DNA damage response. Collectively, the data indicate that Suv4-20 generates nearly ubiquitous dimethylation that facilitates the DNA damage response and selective trimethylation that is involved in heterochromatin formation.

Histone posttranslational modifications (PTMs) play key roles in regulating genomic processes including gene transcription, chromatin assembly, DNA replication, recombination, and DNA repair (1-3). Diverse, context-specific regulation of these processes has been proposed to be facilitated by interacting regulatory factors which recognize specific histone PTMs or combinations of PTMs, individually or collectively, and direct distinct regulatory outcomes (4). Lysine residues targeted by methyltransferases (and demethylases) afford the greatest potential combinatorial and functional complexity among histone PTMs since the identity of the site, whether it is unmodified (0m) or mono- (1m), di- (2m) or trimethylated (3m), and the presence of additional PTMs at nearby sites can potentiate the binding of site-specific regulatory factors (5,6).

Only one lysine residue, K20, is known to be methylated in histone H4 (7,8). A single SET domain-containing protein, Set9, forms all 1m, 2m and 3mK20-H4 in S. pombe and the function of
K20 methylation seems to be limited to DNA damage responses in this organism (9). This role appears to be conserved in higher eukaryotes since Crb2 and 53BP1, the homologous DNA damage checkpoint signaling proteins of S. pombe and humans, both recognize K20 dimethylation (10). However, additional functions have been ascribed to K20 methylation in higher eukaryotes and the SET domain-containing proteins PR-Set7, Suv4-20, Ash1 and NSD1 have all been implicated in methylating K20 in such organisms (11-15). Work by several labs has established that PR-Set7 forms 1mK20-H4 exclusively in vitro (13,14,16-18). Analyses with antisera specific for 1m, 2m or 3mK20-H4 suggest that the product specificity of the SET domains of the murine Suv4-20h1 and Suv4-20h2 isoforms in vitro is predominantly K20 trimethylation (11). Systematic characterization of the product specificities of Ash1 and NSD1 has not been reported.

Recent evidence suggests that 1m, 2m and 3mK20-H4 are functionally distinct in higher eukaryotes although several contradictory findings have yet to be resolved. In mammals, immunocytochemical analyses suggest that although 1mK20-H4 is enriched on the inactive X chromosome, it is also distributed throughout euchromatin and heterochromatin, 2mK20-H4 is preferentially localized to inactive euchromatin and 3mK20-H4 is enriched in constitutive heterochromatin (19,20). The phenotype of Drosophila embryos lacking PR-Set7 suggests a role for this enzyme in mitosis (21,22). Similarly, depletion of PR-Set7 in U2OS cells and normal human fibroblasts is associated with defects in DNA replication and repair that lead to aberrant mitosis and growth inhibition, but surprisingly these phenotypes are not apparent in HeLa cells (23,24). It is not clear whether these are due to a requirement for K20 monomethylation or for other aspects of PR-Set7 function. In contrast, ChIP analyses showing preferential association of 1mK20-H4 with transcriptionally active genes imply a role in transcriptional regulation (25-27). Similarly, preferential localization of 3mK20-H4 to constitutive heterochromatin in mammals and dominant suppression of position effect variegation by Suv4-20 in Drosophila suggest that K20 trimethylation is involved in gene silencing (11,28), even though ChIP analyses suggest that 3mK20-H4 is not enriched at the promoters of inactive genes (26,29).

To better understand the regulation of H4-K20 methylation, we have used Top Down mass spectrometry (TDMS) for direct identification and relative quantification of singly and multiply modified forms of intact H4. We show here that nearly all H4 in asynchronous Drosophila S2 cells is methylated at K20 with dimethylation present on approx. 90% of the molecules. To identify the enzyme responsible for this abundant dimethylation, we performed an RNAi screen targeting SET domain protein-encoding genes in Drosophila. Depletion of Suv4-20 led to a marked reduction in the level of 2mK20-H4, revealing that Drosophila Suv4-20 is a dual product specificity methyltransferase which forms most, if not all, of the abundant 2mK20-H4 and forms only small amounts of 3mK20-H4 by comparison in vivo. Heterochromatin Protein 1 (HP1) is required for normal levels of 3mK20-H4 in Drosophila larvae, suggesting that the product specificity of Suv4-20 may be modulated by interactions with HP1. Depletion of PR-Set7 led to increased levels of 0mK20-H4, suggesting that PR-Set7 methylates 0mK20-H4 to form 1mK20-H4 in vivo. Comparisons of the results obtained for depletion of PR-Set7 and Suv4-20 individually and in combination suggest that PR-Set7 mediates most K20 monomethylation in vivo and that the majority of 1mK20-H4 formed by PR-Set7 subsequently serves as the physiological substrate.
for Suv4-20. However, 0mK20-H4 can also serve as a substrate for Suv4-20 in the absence of PR-Set7. We show that K20 methylation is regulated similarly in human cells and that K20 dimethylation mediated by Suv4-20h1/h2 is required for efficient 53BP1 foci formation following DNA damage.

**EXPERIMENTAL PROCEDURES**

*Drosophila S2 Cell Culture and RNAi-* S2 cells were grown in Schneider’s medium containing 10% fetal bovine serum (FBS) and penicillin/streptomycin at 26°C. RNAi was performed as described previously (30) with some modifications. Six well plates were seeded with 1 ml of S2 cells per well (2x10^6 cells per ml in media lacking serum and antibiotic) and then 30 μg double stranded RNA (dsRNA) was added to each well. After one hour, 2 ml of complete media containing 15% FBS was added to each well. On day 4, cells were harvested and portions processed for molecular analysis or resuspended as above in media lacking serum and antibiotics and a second dsRNA addition performed for analysis on day 8. Aliquots of cells (approx. 10^6) treated in parallel were harvested at regular intervals to monitor cell cycle progression by flow cytometry as described previously (31).

Templates representing 400-700 bp portions of the 31 known or potential *Drosophila* SET domain-containing genes listed in Supplementary Table 1 were amplified using gene-specific primers fused to T7 promoters. Double stranded RNAs were synthesized from these templates using the T7 MEGAscript Kit (Ambion). Double stranded RNA targeting bases 773-1368 of the coding region of the firefly *luciferase* gene was used as a negative control. The concentration and quality of dsRNA was assessed using absorbance at 260 nm and electrophoresis on 1% agarose gels.

*HeLa S3 Cell Culture and siRNA-* HeLa or HeLa S3 cells were maintained in Dulbecco’s modified Eagle’s medium (D-MEM) containing 10% FBS at 37°C. siRNA was performed using 21 bp siRNA as described previously (32). Briefly, six well plates were seeded with 1x10^5 Hela or 3x 10^5 Hela S3 cells/2 ml D-MEM containing 10% FBS per well. After 24 hours, cells were transfected with 10 μl of 20 μM Suv4-20 siGENOME siRNA (Dharmacon) mixed with 5 μl oligofectamine (Invitrogen). This treatment was repeated after a further 24 hours and subsequently at four day intervals as required. siRNA targeting the sequence 5’-CUUACGCUGAGUACUUCGA-3’ in the firefly *luciferase* gene was used as a negative control. Cell cycle progression of cells treated in parallel was monitored by flow cytometry.

*Generation of larvae lacking HP1-* *Drosophila* HP1 is encoded by the *Su(var)2-5* gene. To examine the levels of K20-H4 methylation in flies lacking HP1, a cross was performed between *y,w*67c23; *Su(var)2-5*04/Cy0, GFP and *y,w*67c23; *Su(var)2-5*05/Cy, GFP flies. The resulting non-GFP progeny are trans-heterozygous for two null mutations in *Su(var)2-5*. Non-GFP third instar larvae were collected, homogenized in SDS sample buffer, heated at 95°C for 5 minutes and the clarified supernatant was used for immunoblot analysis. Similar stage *y,w*67c23 larvae with wild type levels of HP1 were used as a control.

*Flag-Suv4-20h2 expressing HeLa stable cell line-* HeLa S3 cells were transfected with a pcDNA3 plasmid containing flag-Suv4-20h2 using Lipofectamine 2000 (Invitrogen) and the manufacturer’s protocol. Transfected cells were selected with G418 (1 mg/ml). Clones with high expression were selected and maintained in D-MEM containing 10% FBS and G418 (500 μg/ml).

*Immunofluorescence microscopy-* Flag-Suv4-20h2 expressing cells grown on coverslips were fixed
with 4 % paraformaldehyde in phosphate-buffered saline (PBS) pH 7.5 containing 4 % sucrose for 15 min and then permeabilized with 0.2 % Triton-X100 in tris-buffered saline (TBS) for 15 min. Coverslips were blocked with 2 % bovine serum albumin, 2 % newborn calf serum, 0.1 % Tween-20 in TBS and then incubated with mouse anti-flag M2 antibody (Sigma, 1:5000) or rabbit anti 3mK20-H4 (Abcam, 1:2000) at room temperature for 2 hours. Coverslips were then incubated with fluorescein-conjugated or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch, 1:800). Extensive washes with TBS-0.1 % Tween-20 were performed between incubations. DAPI (0.1 µg/ml final) was included in the last wash solution to stain DNA. Images were captured with Leica DM RXA2 microscope and analyzed using Openlab software. Cells treated with bleocin (1 µg/ml for one hour in normal media) were fixed and permeabilized simultaneously with 4% paraformaldehyde, 0.25% Triton-X100 and 2.5mM MgCl2 in PBS and then stained with mouse anti 53BP1 (Upstate, 1:250) and modified histone antibodies as above. Images were acquired with a Zeiss LSM 510 laser scanning confocal microscope. The average number of 53P1 foci per cell was determined from 6 randomly selected fields each containing 30-100 cells from two separate experiments.

RT-PCR- Total RNA was prepared using Trizol reagent (Invitrogen) following the manufacturer’s protocol. Reverse transcription was performed using a First Strand Synthesis Kit (Invitrogen) and oligo-dT primers. Specific cDNAs were then PCR amplified with gene specific primers (Supplementary Table 2).

Immunoblotting- Cultured cells were washed twice with TBS, lysed in SDS sample buffer and heated at 95°C for 10 minutes. Proteins were separated on SDS-PAGE gels and transferred to PVDF or nitrocellulose membranes. The antiseras to 1mK20-H4 (Abcam), 2mK20-H4 (Upstate) and 3mK20-H4 (Abcam) were diluted 1:3000. Antiseras to individual acetylation sites in H4 (Upstate) were diluted 1:2000. Antiseras to α-tubulin (Sigma) was used at 1:5000. Antiseras to Drosophila PR-Set7 was raised against residues 1-100 expressed in bacteria as a C-terminal His-tag fusion, affinity-purified using antigen coupled to N-hydroxysuccimide-activated agarose beads, and the purified preparation diluted 1:1500. Monoclonal antibody to Drosophila HP1 was obtained from the Developmental Studies Hybridoma Bank (University of Iowa) and used diluted 1:5000.

Histone Purification and Mass Spectrometry- Drosophila S2 cell nuclei were isolated using DNIB buffer (10 mM Tris-HCl, 3 mM MgCl2, 0.25 M sucrose, 0.3 % NP-40, pH 8.0) freshly supplemented with 1 mM DTT, 10 mM Na butyrate, 5 mM microcystin LR, 0.5 mM aminoethyl benzenesulfonyl fluoride (AEBSF). Cells were lysed in cold DNIB buffer for 5 minutes on ice and nuclei collected by low speed centrifugation. Nuclei were washed with DNIB buffer lacking NP-40 and then histones are extracted out with 0.4 N H2SO4. Nuclei were isolated from HeLa S3 cells and histones extracted by a similar procedure using NIB buffer (15 mM Tris-HCl, 60 mM KCl, 15 mM NaCl, 5 mM MgCl2, 1 mM CaCl2, 0.25 M sucrose, 0.3% NP-40, pH 7.5) supplemented as above. Histones were recovered by 20% trichloroacetic acid precipitation followed by washes with acetone/0.1% HCl and acetone. Crude histone was resuspended in H2O and oxidized in 3% H2O2 and 3% formic acid for 4 hours at room temperature prior to reverse phase (RP) HPLC to facilitate analysis of K20 methylation by mass spectrometry (33). Drosophila and human histones were separated on a Vydac C18 column (2.1 mm i.d. x 250 mm, Grace Discovery Sciences)
with a multi-step gradient from buffer A (5 % CH₃CN, 0.1 % TFA) to buffer B (90 % CH₃CN, 0.094 % TFA) and pooled fractions representing the entire single peak obtained for H4 recovered by drying in a Speed Vac. Purified H4 was resuspended in electrospray solvent and mass spectrometry performed as described previously (33).

**RESULTS**

**RNAi screen for H4-K20 methyltransferases.** Searching the SMART database (34) and BLAST analyses (35) with relaxed restrictions identified 32 candidate SET domain-containing genes in *Drosophila melanogaster* and we performed RNAi for 31 of these candidates in S2 cells (Supplementary Table 1). However, only depletion of PR-Set7 or Suv4-20 affected K20 methylation in immunoblots performed as an initial assay for the effects of RNAi (Supplementary Fig. 1). To confirm and extend these findings, we used TDMS to characterize the modification of H4 recovered from cultures on days 4 and 8 of dsRNA treatment targeting firefly luciferase (control), PR-Set7 alone, Suv4-20 alone, or PR-Set7 and Suv4-20 in combination (Fig. 1).

A major advantage of using TDMS over immunoochemical or other approaches for histone modification analysis is that the relative abundance of differently modified forms can be assessed without bias due to the nature or number of modifications present on individual molecules (36).

Direct TDMS analysis of H4 prepared from luciferase dsRNA treated S2 cells resolved six components differing in molecular mass by multiples of 14 Da due to either methylation or acetylation. Gas phase isolation of individual components followed by fragmentation and tandem MS enabled modifications to be assigned to each form as shown in Fig. 1. All molecules were α-N-acetylated at T1, a co-translational modification, and molecules bearing only this modification are considered to be unmodified. The most abundant form was dimethylated at K20 without additional PTMs (2mK20-H4). The next most abundant form was dimethylated at K20 and acetylated at K16 (aK16,2mK20-H4). Together, these forms accounted for approximately 90 % of all H4 in untreated asynchronous S2 cells and this was not altered by treatment with luciferase dsRNA (Table 1). The remaining four components: 0mK20-H4, 1mK20-H4, aK16,1mK20-H4, and a mixture containing the nearly isobaric 3mK20-H4 and aK16-H4 forms, each accounted for less than 5% percent of the sample in both untreated asynchronous and luciferase dsRNA-treated cells (Table 1). We estimate that 3mK20-H4 represented approximately 1% of total H4 in these control samples, slightly lower than we typically find in mammalian cells (Table 2) (8) and just below our threshold for precise direct quantitation from mixtures (36).

Depletion of PR-Set7 led to a readily apparent increase in the level of 0mK20-H4 (Fig. 1 and Table 1), suggesting that PR-Set7 is responsible for forming at least a portion of 1mK20-H4 in vivo even though the global levels of 1mK20-H4 did not change appreciably (see below). In contrast, depletion of Suv4-20 caused the levels of 1mK20-H4 to increase, and those of 2mK20-H4 to decrease markedly, providing dramatic evidence that Suv4-20 forms most, if not all, 2mK20-H4 in vivo. Immunoblotting with antisera to 3mK20-H4 confirmed that depletion of Suv4-20 also led to decreased levels of 3mK20-H4 (Supplementary Fig. 1). Combined RNAi against mRNAs encoding PR-Set7 and Suv4-20 appeared to prevent the majority of K20 methylation such that 0mK20-H4 ultimately became the predominant form (Fig. 1 and Table 1).
The persistence of 1mK20-H4 following PR-Set7 depletion suggested the possibility that other enzymes might contribute to K20 monomethylation. However, depletion of Ash1 or NSD1 did not affect global levels of 1m, 2m or 3mK20-H4 assessed by immunoblotting (data not shown) or by TDMS (Supplementary Fig. 2). Moreover, combined RNAi targeting mRNAs encoding Ash1 or NSD1 in conjunction with PR-Set7 resulted in H4 modification profiles that were essentially identical to those obtained following RNAi for PR-Set7 alone (compare Fig. 1 and Supplementary Fig. 2), suggesting that neither factor is redundant with PR-Set7 at the global level.

Our finding that 0mK20-H4 predominated when PR-Set7 and Suv4-20 were depleted together, whereas 1mK20-H4 predominated when only Suv4-20 was depleted, clearly suggests that PR-Set7 forms most 1mK20-H4 in vivo. Thus, the apparent perduration of 1mK20-H4 and limited decrease in 2mK20-H4 levels following depletion of PR-Set7 alone indicates that 0mK20-H4 serves as a substrate for Suv4-20 in the absence of PR-Set7, forming detectable amounts of 1mK20-H4 as an intermediate in the formation of 2m/3mK20-H4. This is consistent with the ability of the murine Suv4-20h1/h2 SET domains to methylate bacterially expressed H4 in recombinant nucleosomes in vitro (11).

Taken together, the results in Fig. 1 suggest that PR-Set7 is the major activity responsible for forming 1mK20-H4, most of which is methylated further by Suv4-20 to form predominantly 2mK20-H4 and only a small amount of 3mK20-H4, representing approx. 90 % and 1 % of total H4, respectively, in S2 cells.

HP1 is required for the normal levels of 3mK20-H4. Polytene chromosome staining with 3mK20-H4 antisera is diminished in HP1 null larvae, consistent with the notion that interactions with HP1 contribute to the preferential localization of Suv4-20 to pericentric heterochromatin (11). Given our finding that Suv4-20 forms 2mK20 on approx 90 % of all H4 in S2 cells, we investigated whether HP1 was required for global levels of either 2m or 3mK20-H4. Remarkably, immunoblot analyses revealed that the level of 3mK20-H4 was significantly reduced in Drosophila larvae lacking HP1, but that the levels of 1m and 2mK20-H4 were unaffected (Figure 2). These data suggest the intriguing possibility that interactions with HP1 regulate both the product specificity of Suv4-20 and the enrichment of 3mK20-H4 in heterochromatin.

Human Suv4-20 proteins mediate K20 dimethylation. High levels of 2mK20-H4 are also present in normal human diploid cells, cancer cell lines and normal animal tissues (8). To determine whether the dual di- and trimethylation product specificity of Drosophila Suv4-20 is conserved in humans, we used a mixture of siRNA sequences to deplete HeLa cells of both the h1 and h2 isoforms of Suv4-20 (Fig. 3A). An increase in the level of 1mK20-H4 and decreased levels of both 2m and 3mK20-H4 were apparent in immunoblots from Suv4-20 siRNA-treated cells (Fig. 3B). The changes in the mass spectra of H4 from Suv4-20 siRNA-treated HeLa cells (Fig. 3C) were similar to those observed for S2 cells following Suv4-20 depletion except that the reduction in 2mK20-H4 on day 8 of treatment was not quite as great. Nonetheless, these results demonstrate that the human Suv4-20 proteins also mediate the formation of 2m and 3mK20-H4.

To further investigate the regulation of K20 di- and trimethylation, we created a HeLa S3 cell line stably expressing flag-Suv4-20h2. The level of 3mK20-H4 in these cells is approximately 4-fold greater compared to parental HeLa S3 cells and this is accompanied by decreases in the relative levels of 1mK20-H4, 2mK20-H4 and...
aK16,2mK20-H4, but not other forms (Figs. 4A and Table 2). Since K20 methylation in HeLa cells is progressive with 1mK20-H4 formed by PR-Set7 serving as the precursor to 2m- and 3mK20-H4 (8), the decreased level of 1mK20-H4 presumably reflects methylation by Suv4-20h2 to form 2m/3mK20-H4 whereas the reduction in 2mK20 forms presumably reflects methylation by Suv4-20h2 to form 3mK20-H4. The finding that the levels of unmethylated H4 (0mK20-H4 and aK16-H4) are unaltered in these Suv4-20h2 overexpressing cells also supports the notion that 2m- and 3mK20-H4 are formed primarily from 1mK20-H4 in vivo. In agreement with previous observations (11), flag-Suv4-20h2 and 3mK20-H4 localized preferentially to intensely DAPI-stained heterochromatin in these cells (Figs. 4B and 4C).

Dimethyllysine 20 formed by Suv4-20 is involved in the DNA damage response. An earlier study suggested that depletion of PR-Set7 impairs recruitment of the 53BP1 DNA damage checkpoint signaling protein into damage-associated foci following exposure of HeLa cells to ionizing radiation (10). Immunoblotting indicated that global levels of both 1m and 2mK20-H4 were reduced in PR-Set7-depleted cells, suggesting that the defect in 53BP1 recruitment could be due to the reduction in either 1mK20-H4 or 2mK20-H4, or possibly both forms, since the estimated affinity of 53BP1 in vitro for 2mK20-H4 is only slightly higher than that for 1mK20-H4 (10). Since our results indicate that depletion of Suv4-20h1/h2 affects the levels of 2mK20-H4 more than depletion of PR-Set7, we determined whether depleting Suv4-20h1/h2 affected 53BP1 foci formation. HeLa cells treated with control or Suv4-20h1/h2 siRNA were exposed briefly to the radiomimetic compound bleocin to induce double strand DNA breaks (37). 53BP1 foci formation and H4-K20 methylation status were analyzed by immunofluorescence microscopy (Figure 5). Consistent with our immunoblot and TDMS analyses (Figs. 3B and 3C), depletion of Suv4-20h1/h2 lead to a marked increase in the level of 1mK20-H4 and significant decreases in the levels of 2m- and 3mK20-H4. Despite this marked increase in the level of 1mK20-H4, significantly fewer 53BP1 foci were formed following bleocin treatment in cells depleted of Suv4-20h1/h2, suggesting that 2mK20-H4 preferentially mediates 53BP1 foci formation in vivo compared to 1mK20-H4.

DISCUSSION

Recent data implicates 1m, 2m and 3mK20-H4 in regulating transcription, DNA damage responses and heterochromatin function, respectively, suggesting that different states of K20 methylation are functionally distinct (10,11,26). The discovery that 2mK20-H4 is far more abundant than 1m and 3mK20-H4 in both Drosophila and human cells (Figs. 1 and 3) compelled us to investigate which enzyme(s) mediate formation of 2mK20-H4. Combining RNAi depletion of SET domain proteins in Drosophila S2 cells with immunoblot and TDMS analyses of H4 modification, we established that Suv4-20 mediates the formation of most, if not all 2mK20-H4 in vivo (Fig. 1, Table 1 and Supplementary Fig. 1). PR-Set7 appears to be the major source of 1mK20-H4 which serves as the primary substrate for Suv4-20. Additional proteins may possibly contribute to low levels of K20 methylation but our results suggest that although Ash1 and NSD1 have been implicated in K20 methylation previously (12,15), they do not mediate K20 methylation detectable at the global level (Supplementary Fig. 2). These proteins may mediate levels of K20 methylation that are too small to be detected using the approach employed here. Alternatively, their physiological substrates
may not include H4 (38,39).

The murine Suv4-20h1 and h2 isoforms both localize preferentially to pericentric heterochromatin (11). However, our finding that Suv4-20 proteins direct K20 dimethylation that affects approximately 80 and 90 % of total H4 in HeLa and S2 cells, respectively, implies that Drosophila Suv4-20 and human Suv4-20h1/h2 act widely throughout chromatin. Among the possible explanations for this discrepancy, we favor the hypothesis that Suv4-20 proteins associate with chromatin in a dynamic fashion, giving rise to 2mK20-H4 at most loci, but that they interact differently with sites in constitutive heterochromatin and others scattered sparsely throughout the genome to give rise to 3mK20-H4. Our finding that HP1 is required for normal levels of 3mK20-H4 (Fig. 2) suggests that it, and possibly other heterochromatin-associated proteins, may interact with Suv4-20 proteins to favor formation of 3mK20-H4 at heterochromatic loci. This view is supported by the ability of murine Suv4-20h2 to interact directly with HP1 in vitro and the observation that 3mK20-H4 staining of the chromocenter is diminished in polytene chromosomes from HP1 null flies (11). Moreover, members of the retinoblastoma (RB) protein family interact with Suv4-20h1/h2 in vivo. Global levels of 3mK20-H4 are reduced in RB1/RBL1/RBL2 triple knock out MEFs (40,41) and mutations in the LXCXE motif of the RB1 "binding pocket" diminish the pericentric heterochromatin localization of 3mK20-H4 without disrupting the interaction ability of RB1 with Suv4-20 proteins (41). Together, these observations suggest that Suv4-20 is regulated by multiple factors.

Based on evidence that H4 acetylation at K16 and methylation at K20 are competitive in enzyme assays in vitro, are localized differently in vivo, and that K20 methylation activity is greatest during mitosis (13,42), K20 methylation has been proposed to function as an epigenetic mark which enables the transcriptionally silent state of genes to be transmitted to daughter cells through mitosis (43). Our finding that the most abundant form of H4 that is acetylated at K16 in control cultures of S2 cells and HeLa cells is also dimethylated at K20 clearly argues against this (Figs. 1 and 3, Tables 1 and 2). Moreover, we did not detect any changes in acetylation at lysines 5, 8, 12 and 16 in S2 cells by immunoblotting despite the significant decreases in the levels of 2m and 3mK20-H4 that followed depletion of Suv4-20 alone or in combination with PR-Set7 (Supplementary Fig. 1). TDMS analyses revealed that depletion of Suv4-20 in S2 cells led to decreases in the level of aK16,2mK20-H4 and corresponding increases in the level of aK16,1mK20-H4 relative to the luciferase RNAi controls (Fig. 1 and Table 1). This is most consistent with the notion that the increased abundance of aK16,1mK20-H4 is due to the persistence of K20 monomethylation during Suv4-20 depletion on molecules which would otherwise become aK16,2mK20-H4 rather than a global enhancement in H4 acetylation. We found that global H4-K16 acetylation, approximated by the sum of the abundances of aK16,1mK20-H4, aK16,2mK20-H4 and the mixture containing 3mK20-H4+aK16-H4 (Table 1), differed little between control and Suv4-20 depleted S2 cells. The degree of K20 methylation does not appear to be a significant factor in this regard since global acetylation was also not enhanced following RNAi for PR-Set7 (Fig. 1 and Table 1). Similarly, depletion of Suv4-20h1/h2 (Fig. 3) or PR-Set7 (data not shown), did not enhance global H4 acetylation in HeLa cells. Overexpression of flag-Suv4-20h2 in HeLa cells led to a dramatic increase in 3mK20-H4 and concomitant decreases in the level of 2mK20-H4 and aK16,2mK20-H4 (Fig. 4 and Table 2). However, we believe that the
decreased abundance of aK16,2mK20-H4 in this case, like that of 2mK20-H4, is a consequence of the high level of 3mK20-H4 attained, rather than antagonism between Suv4-20h2 and K16 acetyltransferases, since all methylation states compete for the same K20 residue. Taken together, our data support the conclusion that H4 acetylation and K20 methylation are not competitive with each other at the global level in vivo.

Our findings that Suv4-20 mediates ubiquitous K20 dimethylation in Drosophila and human cells and that 53BP1 appears to preferentially recognize 2mK20-H4 compared to 1mK20-H4 following DNA damage suggest a role for Suv4-20 in genome maintenance that has only partially been recognized. Suv4-20 deficiency in mice results in abnormal telomere elongation and recombination that may be linked to oncogenesis or cancer progression (33,44), and decreased expression of Suv4-20h2 has been suggested to occur in human breast cancer cells (45). However, it is not clear if these links reflect the importance of K20 di/trimethylation or other aspects of Suv4-20 function. Evidence suggesting that marked reductions in the levels of 3mK20-H4 and aK16-H4 are common in human cancers has been described (46), but the levels of 3mK20-H4 assessed in both normal and cancer samples by these authors far exceed those we have found in diverse cell types using TDMS (8,33). Further analyses are required to fully address this issue. However, since nearly all K20 dimethylation occurs preferentially on newly synthesized H4 in G1 phase of the cell cycle in the absence of DNA damage (8), we suggest that other aspects of Suv4-20 function are also likely to be significant to the role of Suv4-20 in genome maintenance. Recent evidence that p53 activity is negatively regulated by PR-Set7-mediated monomethylation suggests that non-histone substrates may also be important in Suv4-20 function (47). The data presented here suggesting that the mixed methylation product specificity of Suv4-20 is regulated by interactions with HP1 in Drosophila should serve as the basis for further investigations of the regulation of K20 methylation in chromatin function and the pathogenesis of cancer or other diseases.

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FOOTNOTES

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The abbreviations used are: 0m, unmethylated; 1m, monomethylated; 2m, dimethylated; 3m,
trimethylated; DAPI, 4',6-diamidino-2-phenylindole; K20, lysine 20; FTMS, Fourier transform mass
spectrometry; PTMs, posttranslational modifications; TDMS, Top Down mass spectrometry

FIGURE LEGENDS

Fig. 1. Fourier transform mass spectrometry of histone H4 from Drosophila S2 cells depleted of
Suv4-20 confirms that Suv4-20 mediates the formation of abundant 2mK20-H4. Typical results for days
4 and 8 of RNAi treatment to deplete firefly luciferase (negative control), PR-Set7, Suv4-20 or both PR-Set7
and Suv4-20 are shown. The posttranslational modifications determined to be associated with each
species by tandem mass spectrometry are indicated at the top of the figure. Slightly different amounts of
sample were analysed in each case so the spectra are normalized according to the height of the tallest peak.
The relative abundances of the forms in each sample are listed in Table 1.

**Fig. 2.** HP1 is required for normal levels of 3mK20-H4 in *Drosophila*. Whole cell extracts from wild type and *Su(var)2-5* mutant larvae lacking HP1 were analyzed on immunoblots using H4-K20 methylation state-specific antisera. Extracts were also analyzed with antisera to HP1 to confirm the absence of HP1 in the mutant larvae and antisera to α-tubulin to ensure equivalent loading.

**Fig. 3.** Human Suv4-20 proteins also mediate formation of 2mK20-H4 *in vivo*. Typical results are shown for days 4 and 8 of treatment of HeLa cells with siRNA targeting firefly luciferase (negative control) or pooled siRNA targeting both Suv4-20h1 and Suv4-20h2. *A.* Relative levels of mRNA for Suv4-20h1 and Suv4-20h2 detected by RT-PCR. The levels of actin mRNA were used to ensure equivalent loading. *B.* Relative levels of 1m, 2m and 3mK20-H4 detected by immunoblotting with H4 K20 methylation state-specific antisera. The levels of α-tubulin were used to ensure equivalent loading. *C.* Fourier transform mass spectra of H4 from siRNA-treated cells. Slightly different amounts of sample were analysed in each case so the spectra are normalized according to the height of the tallest peak.

**Fig. 4.** Constitutive expression of Suv4-20h2 preferentially increases 3mK20-H4. *A.* Fourier transform mass spectra of H4 from asynchronous control HeLa S3 cells (upper panel) and cells stably expressing flag-Suv40-20h2 (lower panel). The spectra are normalized according to the height of the tallest peak. *B.* Immunofluorescence microscopy reveals that most cells in this line express flag-Suv4-20h2 and that it localizes to nuclei. The upper panel shows staining with DAPI to reveal nuclei; the bottom panel shows anti-flag staining. *C.* Double staining with antisera to 3mK20-H4 and anti-flag reveals that flag-Suv4-20h2 and 3mK20-H4 are largely co-localized in heterochromatin.

**Fig. 5.** 53BP1 recruitment to DNA damage foci is impaired in Suv4-20 siRNA cells. HeLa cells transfected with luciferase siRNA or Suv4-20 siRNA targeting both Suv4-20h1 and Suv4-20h2 for 8 days were treated with 1µg/ml bleocin for 1hr to induce DNA damage prior to staining with the antisera to K20 methylated H4 or 53BP1 as shown. The histogram displays the average number of 53BP1 foci observed per cell in 6 randomly selected fields (each containing 30 - 100 cells) from each group in two different experiments. Error bars represent the standard error of the mean. The T-test with unequal variance shows that the change is significant with p < 0.05.
Table 1

*Global Changes in H4 Modification Following Depletion of PR-Set7 or Suv4-20 in Drosophila S2 Cells*

The relative abundances, in percentage of the total of H4 characterized by Fourier transform mass spectrometry in each sample, were determined from the recorded spectra. The data represent the average of 4 independent experiments ± standard error of mean. PTMs: post-translational modifications. Asynch S2: asynchronous growing cells.

<table>
<thead>
<tr>
<th>PTMs</th>
<th>Asynch S2</th>
<th>Luciferase</th>
<th>PR-Set7</th>
<th>Suv4-20</th>
<th>PR-Set7 + Suv4-20</th>
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<tbody>
<tr>
<td></td>
<td>Day 4</td>
<td>Day 8</td>
<td>Day 4</td>
<td>Day 8</td>
<td>Day 4</td>
</tr>
<tr>
<td>0mK20</td>
<td>1.3±0.2</td>
<td>1.9±0.5</td>
<td>1.3±0.4</td>
<td>15.4±1.5</td>
<td>16.5±1.2</td>
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<tr>
<td>1mK20</td>
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<td>2.4±0.4</td>
<td>2.0±0.2</td>
<td>2.0±0.3</td>
<td>2.1±0.4</td>
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<tr>
<td>2mK20</td>
<td>75.8±2.8</td>
<td>76±4.2</td>
<td>79.1±2.0</td>
<td>62.1±1.6</td>
<td>63.8±2.0</td>
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<tr>
<td>3mK20</td>
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<td>4.6±3.3</td>
<td>1.6±0.5</td>
<td>7.7±1.2</td>
<td>6.2±1.2</td>
</tr>
<tr>
<td>aK16 &amp; 1mK20</td>
<td>1.5±0.3</td>
<td>1.4±1.2</td>
<td>0.6±0.3</td>
<td>0.6±0.3</td>
<td>0.6±0.3</td>
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<tr>
<td>aK16 &amp; 2mK20</td>
<td>15.6±2.0</td>
<td>13.8±1.4</td>
<td>15.5±1.6</td>
<td>12.2±1.1</td>
<td>10.8±0.4</td>
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</tbody>
</table>

Table 2

*Global Changes in H4 Modification in HeLa S3 Cells Constitutively Expressing Flag-Suv4-20h2*

The relative abundances, in percentage of the total of H4 characterized by Fourier transform mass spectrometry in each sample, were determined from the recorded spectra. Data representative of two independent analyses are reported. PTMs: post-translational modifications. Asynch. HeLa S3: asynchronous growing non-transfected cells. Asynch. Flag-Suv4-20h2 HeLa S3: asynchronous growing clonal cell line stably expressing flag-tagged Suv4-20h2.

<table>
<thead>
<tr>
<th>PTMs</th>
<th>Asynch. HeLa S3</th>
<th>Asynch. Flag-Suv4-20h2 HeLa S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0mK20</td>
<td>4.4</td>
<td>4.8</td>
</tr>
<tr>
<td>1mK20</td>
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<tr>
<td>aK16</td>
<td>4.0*</td>
<td>4.4*</td>
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<tr>
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<td>1.1</td>
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<tr>
<td>2mK20 &amp; aK16</td>
<td>25.7</td>
<td>18.7</td>
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</tbody>
</table>

* Monoacetylation at K16 and K12 are both detected at a ratio of approx. 4:1 for aK16:aK12.
Figure 5

1mK20-H4  53BP1
Luciferase siRNA

Suv4-20 siRNA

3mK20-H4  53BP1
Luciferase siRNA

Suv4-20 siRNA

53BP1 foci per cell
siRNA: Luciferase  Suv4-20