RESEARCH COMMUNICATION

Jmjd2b antagonizes H3K9 trimethylation at pericentric heterochromatin in mammalian cells

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Histone lysine trimethyl states represent some of the most robust epigenetic modifications in eukaryotic chromatin. Using a candidate approach, we identified the subgroup of murine Jmjd2 proteins to antagonize H3K9me3 at pericentric heterochromatin. H3K27me3 and H4K20me3 marks are not impaired in inducible Jmjd2b-GFP cell lines, but Jmjd2b also reduces H3K36 methylation. Since recombinant Jmjd2b appears as a very poor enzyme, we applied metabolic labeling with heavy methyl groups to demonstrate Jmjd2b-mediated removal of chromosomal H3K9me3 as an active process that occurs well before replication of chromatin. These data reveal that certain members of the jmjC class of hydroxylases can work in a pathway that actively antagonizes a histone lysine trimethyl state.

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Histone lysine methylation is a central epigenetic modification with both activating and repressive roles in eukaryotic chromatin (Kouzarides 2002; Fischle et al. 2003; Lachner et al. 2004; Reinberg et al. 2004). There are five lysine residues in the histone N termini that are prominently methylated, with H3K4 and H3K36 methylation primarily transducing activating functions, whereas H3K9, H3K27, and H4K20 methylation is mainly associated with repressed chromatin. Histone lysine methylation can be presented as mono-, di-, or trimethyl states, where each distinct methyl state confers different biological read-outs (Santos-Rosa et al. 2002; Lachner et al. 2004). Histone lysine trimethyl states, particularly for the repressive functions, appear relatively robust, since they are stably propagated during several cell divisions (Lachner et al. 2004; Reinberg et al. 2004; Schotta et al.

2004) and resist reprogramming in early mammalian embryos (Santos et al. 2003).

Until recently, no enzymatic mechanism had been described that would directly remove histone lysine methylation, although amine oxidation or radical attack by hydroxylation could destabilize the amino-methyl bond (Kubicek and Jenuwein 2004; Bannister and Kouzarides 2005; Trewick et al. 2005). Indeed, a lysine-specific demethylase 1 (LSD1) has been shown to demethylate H3K4me2 (an active mark) (Shi et al. 2004) and, when associated with a different protein complex, also appears to convert H3K9me2 (a repressive mark) (Metzger et al. 2005). Based on the reaction mechanism, LSD1 cannot act on a lysine trimethyl state, and more potent mechanisms, involving attack by oxygen radicals via hydroxylases/dioxygenases, have been postulated (Clissold and Ponting 2001; Trewick et al. 2005). This could be inferred from the activities of the *Escherichia coli* AlkB enzyme to counteract alkylating damage of DNA or from other protein hydroxylases, such as the jmjC (jumonji) domain containing protein Factor Inhibiting Hypoxia (FIH) (Lando et al. 2002). In *Schizosaccharomyces pombe*, there is one silencing modifier, Epe1, which also carries a jmjC domain, and this factor antagonizes repressive H3K9me2 at centromeric regions (Ayoub et al. 2003). In addition, there are several jmjC proteins that have been shown to be important transcriptional/chromatin regulators (Takeuchi et al. 1995; Gildea et al. 2000; Ahmed et al. 2004; Jin et al. 2004; Katoh and Katoh 2004; Gray et al. 2005; Zhang et al. 2005). Extensive biochemical analyses have recently disclosed the jmjC protein Fbx11 as the first bona fide histone lysine demethylase of the class of hydroxylases, and this enzyme was named JHDM1 (Tsukada et al. 2006). Surprisingly, JHDM1 is selective to remove H3K36me2, but seems again unable to attack a trimethyl state, which may be a consequence of limited substrate recognition or restricted potential of the catalytic pocket to accommodate a histone lysine trimethyl state (Tsukada et al. 2006).

Results and Discussion

Histone lysine demethylation and the family of murine jmjC domain proteins

To address the potential of mammalian jmjC proteins as putative demethylases for a histone lysine trimethyl state, we first compared the amino acid sequences of the conserved jmjC domain of known chromatin modifiers (e.g., Epe1) (Ayoub et al. 2003) or of the protein hydroxylase FIH (Lando et al. 2002) with those of murine jmjC factors, as had been done previously (Trewick et al. 2005). This sequence alignment resulted in the discrimination of ~30 jmjC-containing mouse proteins, 14 of which can be categorized into three classes comprising an Epe1-JHDM1 subgroup, a Jarid subgroup, and a Jmjd2 subgroup (Supplementary Fig. S1). While the jmjC domain is the sole signature motif for the Epe1-JHDM1 subgroup (Fbx110, Fbx111, Phf2, Phf8, and A630082K20RIK), the Jarid subgroup (Rbbp2, Plu1, Smcx, and Smcy) has additional domains, such as BRIGHT/ARID, Zn-fingers, and a jmjN domain of no proposed function (Clissold and Ponting 2001). The Jarid subgroup also contains a *Drosophila* gene, termed *lid* (Gildea et al. 2000), that classi-

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fies as an *E(var)* gene in position effect variegation (PEV) (G. Reuter and H. Baisch, pers. comm.). This genetic characterization would be consistent with the wild-type Lid protein being capable of counteracting a repressive function in heterochromatin or enhancing activating properties of euchromatin, as is the case for Epe1 in *S. pombe* (Ayoub et al. 2003). The Jmjd2 subgroup (Jmjd2a, Jmjd2b, Jmjd2c, Jmjd2d, and XP916094) has jmjN and jmjC domains, and three members also contain additional PHD Zn-fingers and two Tudor domains that would indicate a possible chromatin association (Katoh and Katoh 2004; Gray et al. 2005; Kim et al. 2006).

Jmjd2b abrogates pericentric H3K9me3

GFP fusion constructs of murine jmjC proteins were transiently expressed in female iMEFs, followed by indirect immunofluorescence (IF) with highly selective H3K9, H3K27, and H4K20 methyl-lysine histone antibodies. For each of these repressive marks, we analyzed the described distributions for mono-, di-, and trimethylation profiles (Lachner et al. 2004) and also included stainings for H3K4 methylation. As shown in Figure 1A, overexpression of Jmjd2b results in loss of H3K9me3 at the characteristic pericentric foci in GFP-positive cells (top panel). There was no apparent difference for the dispersed H3K9me2 distribution; however, a significant increase for H3K9me1 was observed. These data suggest conversion from H3K9me3 to H3K9me1 by overexpression of Jmjd2b-GFP. Also accumulation of pericentric HP1 α was lost, but there was no detectable shift for H3K9 acetylation toward pericentric heterochromatin (Fig. 1A, bottom panels). In parallel experiments, we observed no gross changes in the characteristic staining patterns for H3K27me1 (pericentric), H3K27me3 (Xi), or for H4K20me1 (Xi) and H4K20me3 (pericentric) in Jmjd2b-GFP-positive cells (Supplementary Fig. S2). Abrogation of pericentric H3K9me3 also resulted upon overexpression of the other closely related Jmjd2 subgroup members, such as Jmjd2a, Jmjd2c, Jmjd2d, and XP916094 (Supplementary Fig. S3).

Together, our candidate approach identified the Jmjd2 class of predicted hydroxylases to significantly alter pericentric accumulation of H3K9me3. With all the other overexpressed jmjC proteins (see Supplementary Fig. S1), we did not observed gross changes for any of the three methylation states of H3K9, H3K27, H4K20, or H3K4 (data not shown).

Cellular activity of Jmjd2b depends on the jmjN and jmjC domains

We next performed a mutant analysis to identify domains in Jmjd2b (1086 amino acids) that would be responsible for the observed abrogation of pericentric H3K9me3. Deletion of the jmjN (amino acids 14–56) or of the jmjC (amino acids 143–309) domains resulted in a mutant protein that no longer reduced pericentric H3K9me3 (Fig. 1B, top panels). In contrast, removal of the PHD Zn-fingers and Tudor domains generated a truncated protein (amino acids 1–424) that remained functional *in vivo*. Point mutations in this N-terminal version that are predicted to impair coordination of Fe²⁺ (H189A) or association of the cofactor 2-oxoglutarate (F186A) (data not shown) neutralized the activity. Intriguingly, the biologically active 1–424 Jmjd2b-GFP

fragment displays enrichment at pericentric regions (arrows in Fig. 1B) that is not observed for the 1–424 (H189A) mutant nor for the full-length protein, which rather broadly localizes in the nucleus. Moreover, pericentric accumulation for the 1–424 Jmjd2b-GFP variant is lost in *Suv39h* dn iMEFs (Fig. 1B, bottom panel).

These data demonstrate that both the jmjN and jmjC domains are essential for the observed reduction of pericentric H3K9me3, whereas the PHD Zn-fingers and Tudor domains are dispensable, although they may contribute to overall Jmjd2b activity, for example, via chromatin affinity and/or protein complex formation with associated factors.

Jmjd2b reduces H3K9me3 and H3K36me2 in bulk histones

From the above *in vivo* analyses, we reasoned to have identified the major components (enzyme and substrate) for the direct demonstration of biochemical activity of Jmjd2b. However, all our efforts with recombinant full-length Jmjd2b or with the 1–424 Jmjd2b variant, either purified from bacteria or insect cells and even when isolated as a complex from mammalian cells, have been unsuccessful in *in vitro* demethylation assays with H3K9me3 peptides, calf thymus histones, and *in vitro* methylated nucleosomes. These negative results suggest that recombinant Jmjd2b has only very poor enzymatic activity or that Jmjd2b-mediated removal of H3K9me3 may not be a simple two-component system or requires native chromatin substrates.

We therefore focused again on *in vivo* approaches by generating stable mouse cell lines (NIH3T3 Tet-off) that inducibly express GFP-tagged full-length Jmjd2b (clone IB21), the 1–424 truncation variant (clone T2J3), and the 1–424 (H189A) point mutation (clone T2M4). As analyzed by IF, ~35% of the IB21, 92% of T2J3, and none of

Figure 1. IF analysis of female iMEFs transiently transfected with Jmjd2b-GFP constructs. Cells were costained with DAPI and the indicated antibodies, and GFP-positive cells were visualized by excitation under a wavelength of 488 nm. (A) Overexpression of the full-length Jmjd2b-GFP construct. (B) Overexpression of various Jmjd2b-GFP mutants. The 1–424 Jmjd2b-GFP truncation, but not the 1–424 (H189A) point mutant, becomes enriched at pericentric heterochromatin (indicated by arrows). This pericentric localization is lost in *Suv39h* double-null (*Suv39h* dn) iMEFs.

the T2M4 cells lost pericentric H3K9me3 signals after a 2-d induction period (Supplementary Fig. S4). Expression levels for the ectopic proteins in T2J3 and T2M4 cells are comparable, as demonstrated by Western blot with an α -GFP antibody (Supplementary Fig. S5). Data for additional independent clones are shown in Supplementary Figure S6.

We then examined changes in histone lysine methylation states by mass spectrometry of histone H3 that had been isolated from bulk histone preparations in uninduced and induced (2 d) IB21, T2J3, and T2M4 cells, following previously described methods (Peters et al. 2003). Under uninduced conditions, and similar to the parental NIH3T3 Tet-off cells, there are comparable levels for H3K9me1 (15%–20%), H3K9me2 (42%–45%), and H3K9me3 (18%–28%) (Fig. 2A). Upon induction of IB21 and T2J3 cells, there is a selective decrease of H3K9me3 that is accompanied by an increase in the H3K9me1 levels, whereas the H3K9me2 states are largely unaltered.

Figure 2. Mass spectrometry analysis of H3K9 and H3K36 methylation states in bulk histone preparations. Mouse cell lines (NIH3T3 Tet-off) that inducibly express GFP-tagged full-length Jmjd2b (clone IB21), the 1–424 Jmjd2b truncation (clone T2J3), and the 1–424 (H189A) point mutation (clone T2M4) were either uninduced or induced for 2 d, and then histone H3 was isolated from nuclear extracts. The inducibility of the various clones differs such that in 35% of IB21 cells, in 92% of T2J3 cells, and in none of the T2M4 cells, pericentric H3K9me3 is lost (see Supplementary Fig. S4). (A) Mass spectrometry analysis for H3K9 methylation states using a histone H3 peptide that spans amino acid positions 9–17. (B) Mass spectrometry analysis for H3K36 methylation states using a histone H3 peptide that spans amino acid positions 27–40. H3K36me3 is underrepresented (<1%) in the analyzed cell lines (data not shown).

This shift was most pronounced in T2J3 cells, which display a reduction of H3K9me3 from 18% to 4% and an increase in H3K9me1 from 20% to 30%. Although there are minor variations for H3K9 methylation in induced T2M4 cells, no decrease in H3K9me3 was detected. Surprisingly, the mass-spectrometry analyses further revealed that the 1–424 Jmjd2b variant, but not the 1–424 (H189A) mutant, also reduced H3K36 methylation levels, particularly for the H3K36me2 state (Fig. 2B). Since H3K36me3 is generally very low in these cells (data not shown), we could not determine whether biologically active Jmjd2b would also attack H3K36me3.

Jmjd2b diminishes chromosomal H3K9me3 at Suv39h target regions

To examine the effects of Jmjd2b overexpression on the conversion of H3K9me3 at higher resolution, we next performed chromatin immunoprecipitation (ChIP) analyses on known H3K9me3 target regions. For example, Suv39h-dependent H3K9 trimethylation accumulates at the major satellite repeats (i.e., pericentric heterochromatin), and is also frequently associated with many other repetitive elements in the mouse genome, such as LINEs and IAPLTRs (Martens et al. 2005). In chromatin from the parental and uninduced IB21 and T2J3 cells, there are highly enriched signals for H3K9me3 at the major satellites, LINE L1 elements, and IAPLTR1 (Fig. 3), at levels that are comparable or even higher than those observed in mouse ES cells (Martens et al. 2005). Following a 2-d induction, H3K9me3 signals in chromatin from IB21 cells are significantly reduced (between threefold and sevenfold) at major satellites, LINE L1, and IAPLTR1, and this decrease is even more pronounced with induced T2J3 cells (>20-fold reduction). H3K9me3 levels persist nearly unaltered in chromatin from T2M4 cells that express the inactive 1–424 (H189A) mutant (Fig. 3). Importantly, the Jmjd2b-mediated loss in chromatin-associated marks is selective for the H3K9me3 state, does not significantly alter H3K9me2 signals, and, at all three target regions, increases H3K9me1. The selectivity of Jmjd2b is further confirmed by comparative ChIP analyses for H4K20me3 and H3K4me3, which, within the experimental variability, do not display significant changes upon forced expression of biologically active Jmjd2b. We have not yet analyzed alterations for chromosomal H3K36 methylation marks upon overexpression of Jmjd2b.

Unaltered histone turnover in Jmjd2b-expressing cells

In the absence of direct biochemical activity of recombinant Jmjd2b, it remained unclear whether the mechanism underlying Jmjd2b-mediated reduction of chromosomal H3K9me3 would reflect active demethylation of H3K9me3 marks or, rather, inhibit their establishment. To discriminate between these possibilities, we designed metabolic labeling experiments that allow to measure both histone turnover and the stability of histone lysine methyl groups. For this, we used a labeled methionine, in which the methyl group is composed of ^{13}C and deuterium isotopes (both nonradioactive). This “heavy” methyl $^{-13}\text{CD}_3$ group (19 Da) can easily be distinguished by mass spectrometry from the normal $-\text{CH}_3$ group (15 Da). Moreover, metabolic labeling of cells with this “heavy” methyl methionine (Ong et al. 2004) will result

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Figure 3. ChIP analysis of known chromosomal Suv39h targets (major satellites, Line L1, and IAPLTR1) in NIH3T3 Tet off (parental cells) and in the IB21, T2J3, and T2M4 cell lines (see Fig. 2). ChIP was performed with the indicated methyl-lysine histone antibodies and with a GFP antibody. Precipitation of enriched chromatin fragments relative to input material was quantified by real-time PCR. In chromatin from induced T2J3 cells, H3K9me3 signals are nearly abrogated (asterisk). Although there is no alteration for H3K9me3 levels in chromatin from uninduced versus induced T2M4 cells, there are differences in ChIP efficiency across the IAPLTR1 element, as compared with parental, IB21, and T2J3 cells.

in the replacement of regular methionine in the amino acid backbone of proteins and also allow for the transfer of “heavy” methyl groups in SAM-dependent HMTase reactions. In histone H3, there is one conserved methionine residue (M120). T2J3 cells were either uninduced or induced for 8 h, then the medium was changed and we determined the relative conversion of M120 “light” histone H3 (reflecting the pre-existing H3 population) toward the “heavy” and newly synthesized H3 pool at different time points (8 h, 16 h, 24 h) by using mass spectrometry on the H3 peptide fragment (amino acids 117–128) comprising the M120 position. The data indicate that histone H3 turnover is not affected by overexpression of the 1–424 Jmjd2b-GFP construct, and, by 24 h, ~50% of the pre-existing H3 had been passively exchanged with newly synthesized H3 (Fig. 4A), consistent with the ~20-h doubling time of the NIH3T3 cell lines.

Removal of H3K9me3 by Jmjd2b is an active process

We next examined the relative stability of H3K9 methylation states in the same H3 preparations as used above.

For this, we applied mass spectrometry on another H3 peptide fragment (amino acids 9–17), where the “heavy” methyl signal can only be derived from SAM-dependent methylation at the H3K9 residue. Since H3K9 methylation can pre-exist in a mono-, di-, or trimethylated form, metabolic labeling results in various combinations of “light” versus “heavy” methyl groups (e.g., for a trimethyl state 3/0, 2/1, 1/2, 0/3), all of which can be discriminated by mass spectrometry. Under uninduced conditions, the data reveal different resident times on histone H3 for the three possible H3K9 methylation states, such that there is significant alteration of H3K9me1, intermediate stability of H3K9me2, but relatively high persistence of H3K9me3 (Fig. 4B). For example, for H3K9me3, less than half of “heavy” methyl groups were incorporated 24 h after medium change.

In contrast, following induction of the 1–424 Jmjd2b-GFP construct, there was a selective and pronounced removal (approximately fivefold reduction) of the “light” H3K9me3 groups (black bars), already by 8 h after me-

Figure 4. Turnover of histone H3 and stability of H3K9 methyl groups in T2J3 cells analyzed by metabolic labeling with “heavy” methionine. (A) Histone H3 turnover was analyzed by mass spectrometry of a histone H3 peptide that spans amino acid positions 117–128. (B) Stability of H3K9 methyl groups was analyzed by mass spectrometry of a different histone H3 peptide that spans amino acid positions 9–17. Metabolic labeling results in various combinations of “light” methyl (pre-existing) and “heavy” methyl (newly synthesized) groups. The black bars indicate the percentage of H3 peptide fragments that carry three “light” methyl groups. The yellow bars show the percentage of H3 peptide fragments that carry three “heavy” methyl groups. Intermediate combinations, such as one “light” methyl and one “heavy” methyl group (1/1; white bars) or (2/1; gray bars) or (1/2; light yellow bars), are also indicated.

dium change that is not compensated by incorporation of “heavy” methyl groups (yellow bars) (Fig. 4B, bottom panel). Even after 24 h, only ~50% of H3K9me3 levels are restored. Interestingly, the newly generated H3K9me3 groups do not appear to be affected by the presence of 1–424 Jmjd2b-GFP. Together, this detailed mass spectrometry analysis strongly suggests that Jmjd2b-mediated elimination of H3K9me3 marks is regulated by an active process and most likely the consequence of direct demethylation rather than passive histone H3 turnover or some other indirect mechanism.

A distinct pathway for H3K9me3 demethylation by Jmjd2b

We provide evidence that the subgroup of murine Jmjd2 proteins, primarily Jmjd2b, can antagonize pericentric H3K9me3. This is one of the first reports to indicate that histone lysine trimethyl marks can be destabilized by the activity of hydroxylases/demethylases. Our findings significantly extend previous observations on jmjC domain genes, such as *Epe1* in *S. pombe*, that suggested the offset of repressive H3K9me2, probably in collaboration with HDACs (Ayoub et al. 2003). There is only sparse H3K9me3 in *S. pombe* (S. Grewal, pers. comm.). Similarly, in *Drosophila*, another jmjC-containing factor, *lid*, classifies as a PEV modifier that enhances activating functions in chromatin (G. Reuter, pers. comm.). H3K9me3 is underrepresented in *Drosophila*, where instead H3K9me2 accumulates at pericentric heterochromatin (Ebert et al. 2004). In contrast, histone lysine trimethyl states are prominent marks for epigenetic control in mammalian chromatin (Lachner et al. 2004). Several members of mammalian jmjC proteins have been linked to transcriptional repression, either via interaction with other transcription factors or by recruiting chromatin-modifying activities that involve histone deacetylation (Gray et al. 2005; Zhang et al. 2005), probably also ubiquitination (Jin et al. 2004) or direct demethylation of H3K9me2 (Yamane et al. 2006). In our studies presented here, we show that the N-terminal half of Jmjd2b is sufficient for abrogating chromosomal H3K9me3 and that this fragment becomes enriched at pericentric heterochromatin in a *Suv39h*-dependent manner (see Fig. 1B). Thus, in line with the jmjN and jmjC domains of certain Jmjd2 proteins to transduce enzymatic activity (Whetstine et al. 2006), these modules may direct substrate binding and thereby impart affinity toward H3K9me3 chromatin, whereas the full-length Jmjd2b protein, with the other PHD and Tudor domains (Kim et al. 2006), has a much broader chromosomal association (see Fig. 1B).

Although we provide evidence for the abrogation of chromosomal H3K9me3 by Jmjd2b, the enzymatic activity of Jmjd2b has been hard to address. From our studies, we can conclude that Jmjd2b converts an H3K9me3 state to H3K9me1 (see Figs. 1A, 2A). The Jmjd2 subgroup contains five members (see Supplementary Fig. S1), and all five (including Jmjd2a, Jmjd2c, Jmjd2d, and XP916094) are active in cellular assays to decrease chromosomal H3K9me3 (Supplementary Fig. S3). It is possible that these Jmjd2 members differ in their *in vitro* potential to function as demethylases (Whetstine et al. 2006), and that recombinant Jmjd2b may be particularly inefficient. Furthermore, the various Jmjd2 enzymes could display distinct reaction profiles to convert a H3K9me3 state,

similar to diverse HMTases that can either act in a distributive or processive manner (Zhang et al. 2003). In addition, our detailed mass spectrometry analysis with heavy isotope labeling of H3K9 methyl groups (see Fig. 4) has revealed that pre-existing H3K9me3 is rapidly removed by biologically active Jmjd2b, whereas newly synthesized H3K9me3 appears unaffected (Fig. 4B, bottom panel). Thus, the relevant substrate may need to be presented in a native chromosomal context that would require additional components. Since the cellular activity of Jmjd2b is enriched at pericentric heterochromatin, these extra components could involve HP1, other combinatorial histone modifications, DNA methylation, and even noncoding RNAs.

Overexpression of Jmjd2b strikingly phenocopies the conversion of pericentric H3K9me3 to H3K9me1 that also results upon disruption of both *Suv39h* enzymes (Peters et al. 2003). Surprisingly, Jmjd2b not only abrogates pericentric H3K9me3 marks, but our mass spectrometry analyses also indicate a significant reduction in H3K36 methylation, where H3K36me2 is converted to H3K36me1 and unmodified lysine (Fig. 2B; data not shown). Although H3K36 methylation has primarily been associated as an active mark facilitating transcriptional elongation, there is other evidence for its involvement in repressive functions (Strahl et al. 2002). A possible synergy between demethylation of H3K36me2 and H3K9me2 has also been suggested from the studies on JHDM1 (Tsukada et al. 2006). Since *Suv39h* HMTases are involved in processing of repeat-associated noncoding RNAs (Martens et al. 2005), and H3K36 methylation can inhibit spurious intragenic transcripts (Carrozza et al. 2005; Keogh et al. 2005), an RNA moiety may provide the discriminating signal whether *Suv39h* or Jmjd2b activities will prevail to induce or remove histone lysine methylation marks at pericentric heterochromatin. It will be very interesting to test this model by analyzing the occurrence and persistence of H3K9me3 and H3K36me2 at known *Suv39h* targets and in wild-type and mutant backgrounds.

Materials and methods

Cell culture

Cell culture, transfection, stable cell lines, and conditions for induction are described in the Supplemental Material. Metabolic labeling was performed as described (Ong et al. 2004), with the modification that L-methionine- $^{13}\text{C}_5$, D_3 (methyl- $^{13}\text{CD}_3$) (Sigma Isotec) was the only labeled amino acid derivative.

Bioinformatics methods

The jmjC-domain proteins in mouse, *S. pombe*, and *Drosophila* were identified by using a hidden Markov model (HMM) profile for the jmjC domain (PF02373). Details on database searches and sequence alignments are provided as Supplemental Material.

IF staining

IF procedures were described previously (Peters et al. 2003).

Analysis of histone modifications

Mass spectrometry analyses of histone modifications and ChIP were described previously (Peters et al. 2003; Ong et al. 2004; Martens et al. 2005).

Antibodies

H3K9, H4K20, and H3K27 methyl-lysine antibodies were described previously (Peters et al. 2003; Schotta et al. 2004; Martens et al. 2005). Other antibodies are detailed in the Supplemental Material.

DNA constructs

Detailed description of DNA constructs is provided in the Supplemental Material.

Preparation of nuclear extracts and Western blot

Nuclear extracts were prepared as previously described (Peters et al. 2003). Western blotting was performed according to standard protocols.

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