

# SETD6 monomethylates H2AZ on lysine 7 and is required for the maintenance of embryonic stem cell self-renewal

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**Abbreviations:** H2AZK7<sup>me1</sup>, histone H2AZ monomethylated on lysine 7; H3K27<sup>me3</sup>, histone H3 tri-methylated on lysine 27; KMT, lysine methyltransferase; SET, Su(var)3-9, enhancer-of-zeste, trithorax; SETD6, SET domain-containing protein 6; mESC, mouse embryonic stem cells; RA, retinoic acid; PRC2, polycomb repressive complex 2; SUZ12, suppressor of zeste 12; EED, embryonic ectoderm development; GST, glutathione S-transferase; ChIP, chromatin immunoprecipitation

The histone H2A variant H2AZ is an essential chromatin signaling factor. Herein, we report that H2AZ is monomethylated at lysine 7 (H2AZK7<sup>me1</sup>) by the lysine methyltransferase SETD6. We observed that methylation of H2AZ increased noticeably upon cellular differentiation of mouse embryonic stem cells (mESCs). H2AZK7<sup>me1</sup> and the repressive H3K27<sup>me3</sup> mark were found near the transcriptional start sites of differentiation marker genes, but were removed upon retinoic acid-induced cellular differentiation. The depletion of Setd6 in mESCs led to cellular differentiation, compromised self-renewal, and poor clonogenicity. These findings demonstrate that mESCs require Setd6 for self-renewal and portray H2AZK7<sup>me1</sup> as a marker of cellular differentiation.

## Introduction

The role of lysine methylation in human pathologies is highlighted by the increasing number of lysine methyltransferases (KMT) involved in the etiology of cancer,<sup>1,2</sup> growth defects<sup>3,4</sup> and neurological disorders.<sup>5</sup> Therefore, the identification and characterization of novel KMT substrates is postulated to be pivotal for the understanding of human pathologies.

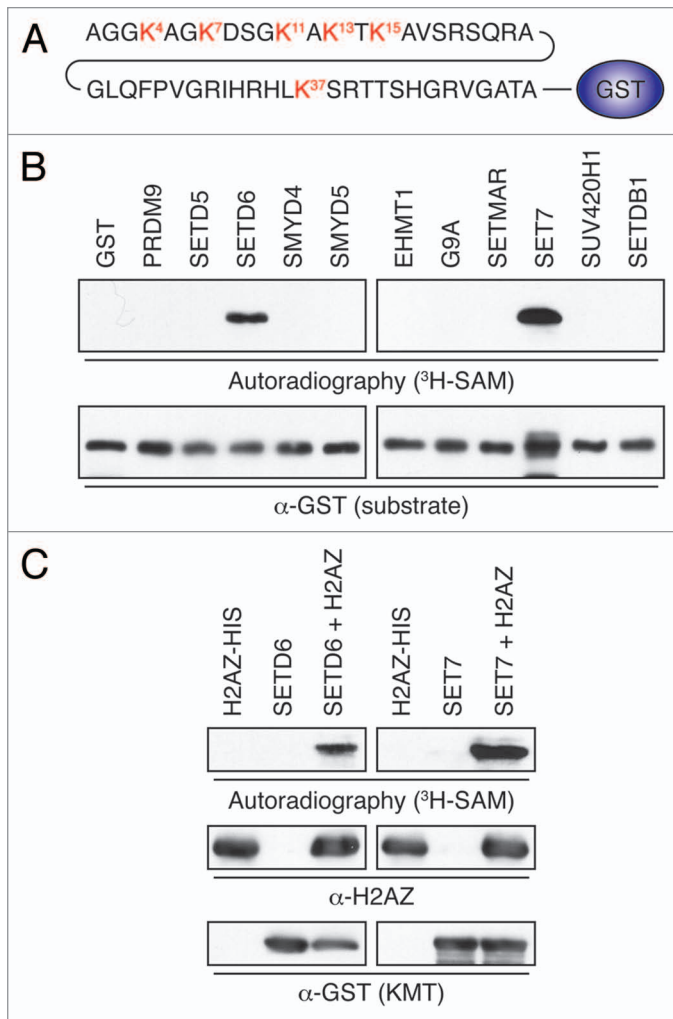
Our current knowledge of how H2AZ functions to regulate DNA-dependent cellular processes is limited. H2AZ is found at actively transcribed promoters in human cells,<sup>6</sup> suggesting a role in favoring open chromatin conformations. Recently, a genome-wide study conducted in human cells by Valdés-Mora et al. showed that the acetylated form of H2AZ was solely found at the transcriptional start site of actively expressed genes, while the deacetylated form of H2AZ localized at silenced promoters.<sup>7</sup> Therefore, posttranslational modifications appear to be a critical component of gene expression regulation by H2AZ.

Embryonic stem cells (ESCs) have the potential to differentiate and give rise to all the cell types constituting multicellular

organisms, an aptitude known as pluripotency. ESCs are also characterized by their ability to self-renew. It has been shown that murine ESCs (mESCs) cellular differentiation is prevented by silencing the expression of H2AZ.<sup>8</sup> In addition, H2AZ colocalizes with the transcriptional silencing H3K27<sup>me3</sup> mark and the PRC2 complex subunits SUZ12 and EED in mESC near the transcription start site of developmental genes.<sup>8,9</sup> However, although H2AZ is important for PRC2 chromatin association,<sup>8</sup> PRC2 subunits are dispensable for H2AZ nucleosome incorporation.<sup>10</sup>

Herein, we demonstrate that SETD6 monomethylates H2AZ on lysine 7 (H2AZK7<sup>me1</sup>) in vitro and that SETD6 contributes to H2AZ methylation in vivo. Mass spectrometric analysis detected lysine methylation of H2AZ on both lysine 4 and lysine 7 on in vitro methylated H2AZ as well as on endogenous H2AZ. A substantial increase of H2AZ lysine methylation upon mESC differentiation led us to investigate the potential role of Setd6 in stem cell biology. Depletion of Setd6 in mESC resulted in cellular differentiation, compromised self-renewal, and reduced clonogenicity. Finally, we observed that H2AZK7<sup>me1</sup> was evicted along with

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**Figure 1.** Identification of H2AZ lysine methyltransferases. **(A)** Representation of H2AZ<sub>WT50</sub>-GST. The first 50 amino acids of H2AZ were fused to the N-terminus of GST. **(B)** Autoradiogram of KMT assays using <sup>3</sup>H-SAM, H2AZ<sub>WT50</sub>-GST as a substrate, and the indicated KMTs. The enzymatic reactions were resolved by SDS-PAGE, transferred to PVDF, and either exposed on film (top) or probed with α-GST to show even H2AZ loading. **(C)** Validation of the potential H2AZ methyltransferases SETD6 and SET7. As in panel B, but full-length recombinant H2AZ-6xHis was used instead of H2AZ<sub>WT50</sub>-GST as the substrate.

the silencing mark H3K27<sup>me3</sup> from the promoter of differentiation markers upon retinoic acid-induced cellular differentiation.

## Results

**Identification of H2AZ lysine methyltransferases.** We hypothesized that H2AZ could be modified and regulated by lysine methylation. We thus fused the first 50 residues of H2AZ to the N-terminus of GST (H2AZ<sub>WT50</sub>-GST; Fig. 1A), which was then affinity purified and used as a substrate in an in vitro screen using a panel of KMTs, described previously.<sup>11</sup> Each recombinant enzyme was incubated with H2AZ<sub>WT50</sub>-GST in the presence of the tritiated form of the methyl donor S-adenosylmethionine (<sup>3</sup>H-SAM). H2AZ was methylated predominantly by SETD6

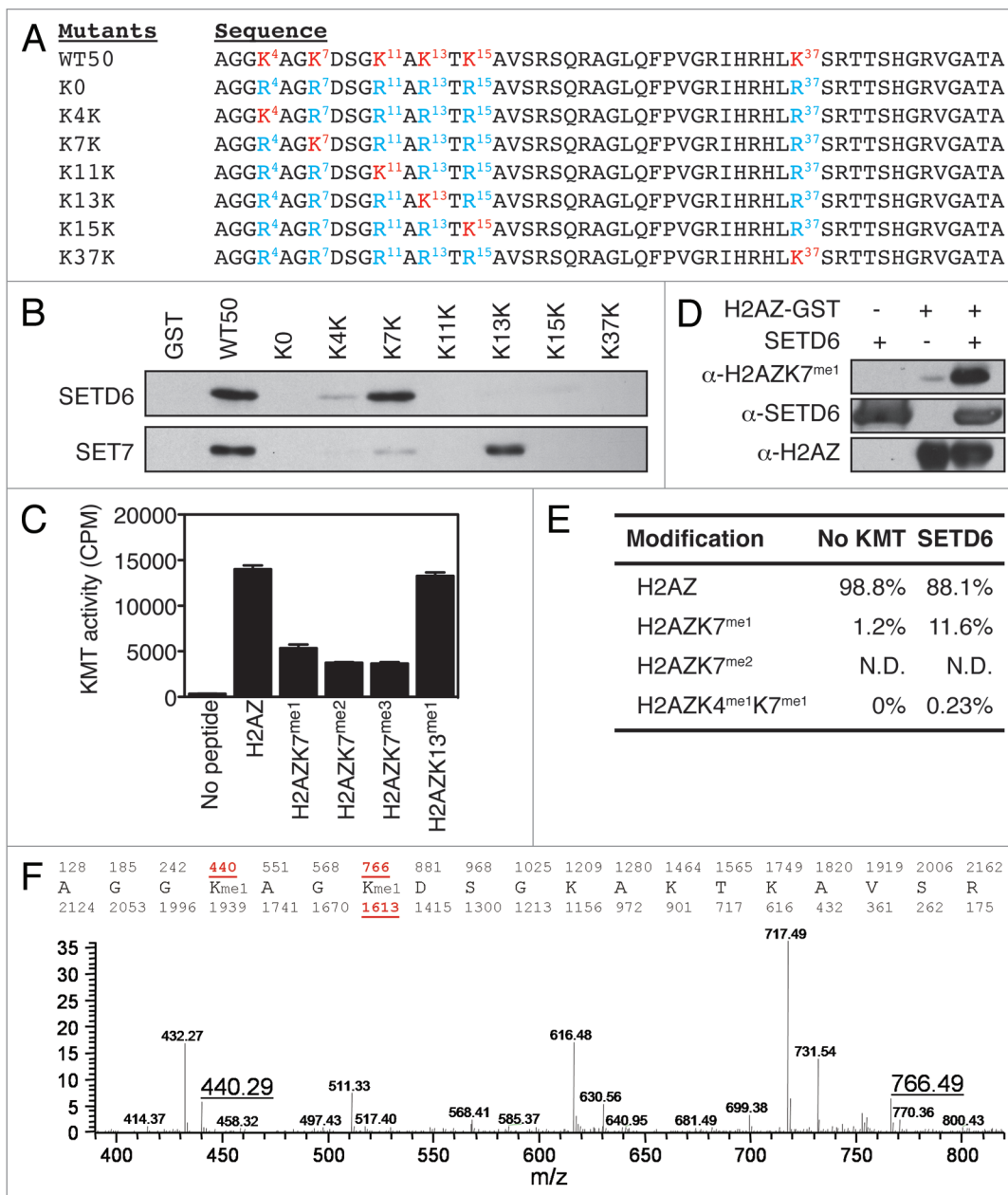
and SET7 (Fig. 1B). These were then validated individually on purified full-length H2AZ containing a C-terminal 6xHis tag and we confirmed that both SETD6 and SET7 methylated H2AZ (Fig. 1C). These findings suggest that H2AZ is a bona fide substrate of SETD6 and SET7.

**H2AZ is methylated on lysines K4 and K7 in vitro.** To identify the precise lysine methylation site(s) on H2AZ, all N-terminal lysines were converted to arginines (R) to obtain an unmethylable H2AZ form (K0). Then, each R was individually converted back to K, so that H2AZ contained only one K that could be methylated (Fig. 2A). These mutant forms of H2AZ were then subjected to KMT assays with either SETD6 or SET7. The K7K mutant was methylated by SETD6 to the same extent as the wild-type form of H2AZ (WT50) (Fig. 2B), suggesting that the SETD6-targeted methylation site is lysine 7. In a similar experiment, K13K appeared to be the major site for SET7 (Fig. 2B).

Subsequently, C-terminally biotinylated and mono-, di-, or tri-methylated H2AZK7 peptides were synthesized and subjected to in vitro methylation assays. These assays further demonstrate that H2AZ was effectively methylated by SETD6 (Fig. 2C). In agreement with the results in Figure 2B, methylation of H2AZ by SETD6 was hindered by pre-methylation of K7, further suggesting that SETD6 methylates K7 of H2AZ. Specifically, the monomethyl group on K7 impaired the methylation of the peptide by approximately 60%, while the dimethyl modestly impacted H2AZ methylation by an additional 10% decrease in activity, and the trimethyl had no further effect on SETD6 activity (Fig. 2C). These results suggest that SETD6 is capable of monomethylating H2AZ. The residual KMT activity on the K7<sup>me2</sup> and K7<sup>me3</sup> peptides [about 10 times above the no peptide (-) control background in Fig. 2C] suggests that SETD6 may methylate secondary sites such as K4, as observed by a faint methylation signal on the recombinant K4K H2AZ-GST protein (Fig. 2B). Equivalent amount of biotinylated peptides were used in the KMT assays (Fig. S1A). In agreement with K7 being the major target site of SETD6, single mutation of K7 to an arginine led to a significant decrease in H2AZ methylation (Fig. S1B). A similar experiment was also conducted with SET7 and the panel of K-R mutants. The latter were all methylated similarly to the wild-type H2AZ substrate (Fig. S1B), suggesting that SET7 indiscriminately methylates multiple sites on H2AZ. Henceforth, we focused our efforts on H2AZK7<sup>me1</sup> and SETD6, as little is known about posttranslational modifications of H2AZ, especially lysine methylation, and SETD6 has no reported enzymatic activity on histones.

Recombinant H2AZ was then in vitro methylated by SETD6 and analyzed by immunoblotting using our methyl-specific α-H2AZK7<sup>me1</sup> antibody (Fig. S2). The signal from the H2AZK7<sup>me1</sup> antibody was substantially increased in the presence of H2AZ and SETD6 (Fig. 2D), convincingly indicating that SETD6 monomethylates H2AZ.

To further confirm the site and state of methylation on H2AZ by SETD6, we conducted mass spectrometric (MS) analyses of KMT assays on biotinylated H2AZ peptides. These analyses showed that in vitro SETD6 catalyzes the monomethylation of

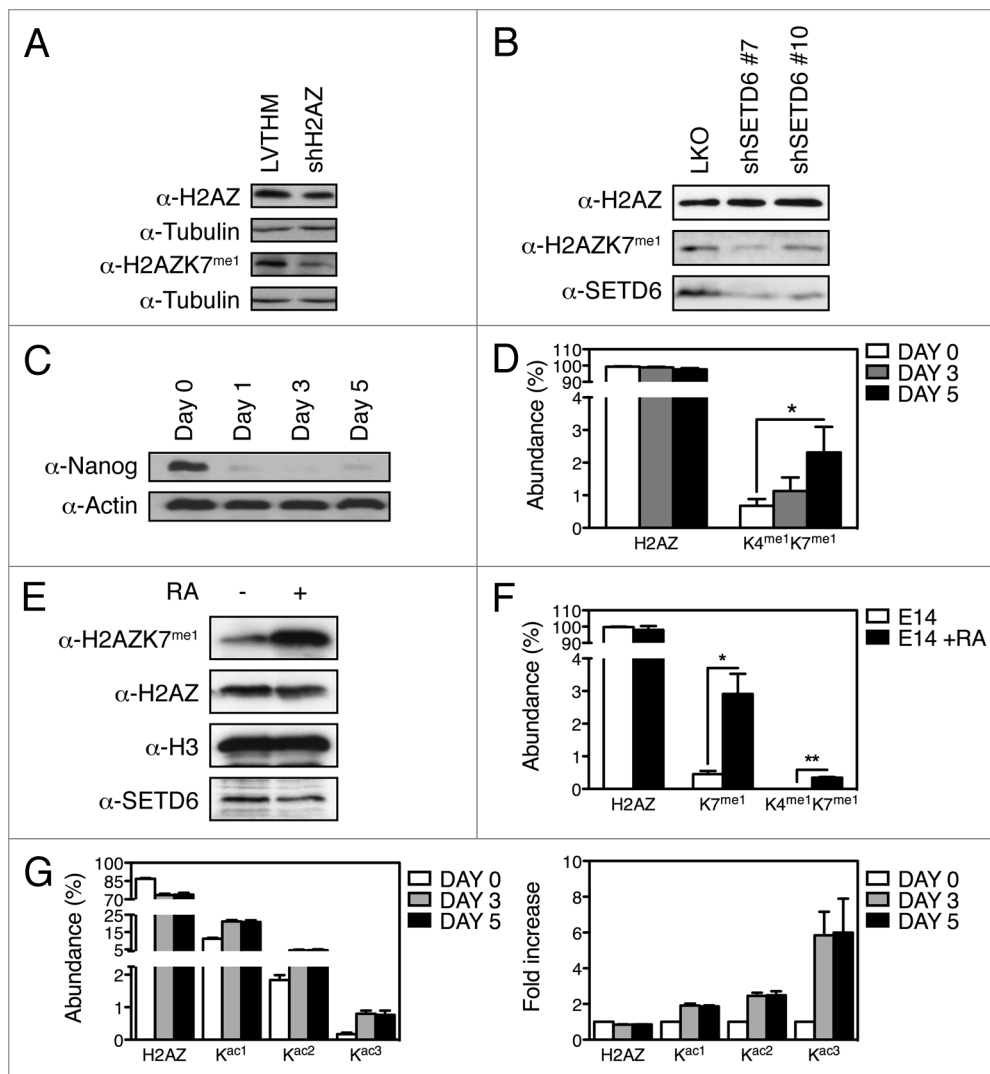


**Figure 2.** H2AZ is methylated on lysines K4 and K7 in vitro. (A) Representation of the H2AZ mutants. Lysines are highlighted in red. (B) Autoradiogram of the KMT assays using SETD6 (top) or SET7 (bottom) and H2AZ mutants shown in panel A. (C) Flashplate KMT assays using SETD6 and indicated H2AZ-biotin peptides as substrates. Each condition was performed in quadruplicate and the error bars represent the standard error of the mean. The KMT activity is expressed as <sup>3</sup>H counts per minute. (D) KMT assays on H2AZ<sub>WT50</sub>-GST were analyzed by immunoblotting using the indicated antibodies. (E) Table summarizing the quantitative MS analysis of SETD6-methylated H2AZ-biotin peptide. (F) Chromatogram of the quantitative MS analysis performed to generate the table in panel E. The molecular weights of mono-methylated K4 and K7 peptides are highlighted (440.29 and 766.49, respectively).

K7 (H2AZK7<sup>me1</sup>) while di-methylation on K7 (H2AZK7<sup>me2</sup>) could not be detected on H2AZ peptides (Fig. 2E). Also, low levels of H2AZ methylation were detected simultaneously on both K4 and K7. A detailed MS2 chromatogram highlights the monomethylation of H2AZ at both K4 (molecular mass 440.29) and K7 (molecular mass 766.49) (Fig. 2F; Fig. S3). Importantly, monomethylation on K7 was detected on two different peptides (molecular masses 766.49 and 1613.85 in Fig. S3). Altogether,

these results show that SETD6 preferentially monomethylates H2AZK7 in vitro, but also methylates secondary sites, such as K4, as detected by mass spectrometric analyses.

**H2AZ is methylated on lysines K4 and K7 in vivo.** To confirm the biological existence of methyl-H2AZ, in vivo methylation of H2AZ was assessed by immunoblotting total protein lysates from control cells (LVTHM; parental shRNA control vector) and H2AZ depleted cells (shH2AZ). The unmodified-H2AZ



**Figure 3.** H2AZ is methylated on lysines K4 and K7 in vivo. (A) Protein extracts from U2OS control (LVTHM) or H2AZ knockdown (shH2AZ) cells were analyzed using the indicated methyl-specific antibody. (B) Lentiviruses were used to transduce U2OS cells to express the indicated human SETD6-targeting shRNAs and protein extracts were analyzed by immunoblotting using the indicated antibodies. (C) Immunoblot analysis of protein extracts from shNanog mESC shows loss of Nanog throughout the differentiation time course. (D) Quantitative MS analyses of the H2AZ peptides derived from differentiating mESC induced by Nanog knockdown at the indicated times. \*p value of 0.05. (E) Immunoblot analysis of H2AZK7me1 levels in untreated and RA-treated E14 cells. (F) Quantification by MS of total H2AZ, H2AZK7me1, and H2AZK4me1K7me1 from E14 mESC differentiated with RA. \* and \*\* denote p values of < 0.05 and < 0.02, respectively. (G) Quantification by MS of H2AZ acetylation in mESC during shNanog-induced differentiation shown as abundance (left) and fold increase (right).

and H2AZK7<sup>me1</sup> antibodies detected a decrease of H2AZ in the knockdown cell extract (Fig. 3A). Importantly, cells depleted of SETD6 by two independent shRNAs (shSETD6 #7 and shSETD6 #10) had reduced levels of H2AZK7<sup>me1</sup> (Fig. 3B), suggesting that SETD6 monomethylates H2AZ on lysine 7 in vivo.

To detect dynamic changes in H2AZ lysine methylation in an in vivo biological system that requires H2AZ, we used an shRNA-mediated method<sup>12</sup> (Fig. S4A) for the depletion of Nanog (Fig. 3C) and induction of mESC differentiation (Figs. S4B and C). Subsequently, histones from mESCs were analyzed by MS to validate the existence of methyl-H2AZ in vivo. Interestingly,

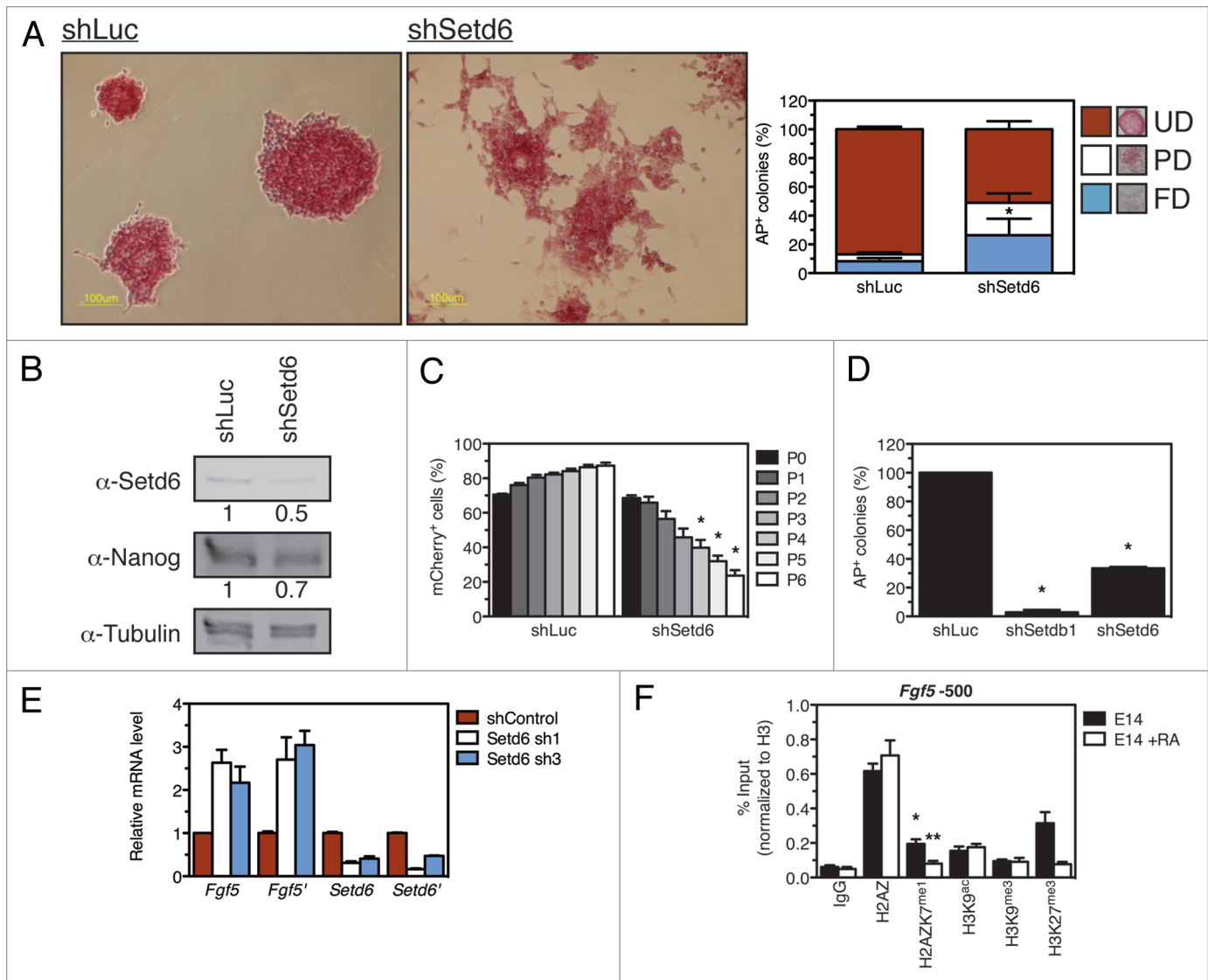
H2AZK7<sup>me1</sup> was detected solely in conjunction with H2AZK4<sup>me1</sup> (Fig. 3D). But, most importantly, methylation of H2AZ at K4 and K7 was increased by over 3-fold following mESC differentiation (Fig. 3D; Fig. S4D). As observed with the shNanog-induced cellular differentiation system, induction of cellular differentiation by retinoic acid (RA) led to a noticeable increase in H2AZK7<sup>me1</sup> and in H2AZK4<sup>me1</sup>K7<sup>me1</sup> detected by immunoblotting of H2AZK7<sup>me1</sup> (Fig. 3E) and by mass spectrometry (Fig. 3F), while the global levels of H2AZ and SETD6 appeared to diminish (Fig. 3E). Together, these data demonstrate that H2AZ is monomethylated in vivo on lysines K4 and K7. To conclude, the levels of H2AZK4<sup>me1</sup>K7<sup>me1</sup> increased noticeably in response to cellular differentiation induced by either RA or the silencing of Nanog expression.

Since H2AZ is acetylated on lysines K4, K7, K11, K13 and K15,<sup>7,13</sup> we also investigated lysine acetylation during mESC differentiation. Interestingly, we observed that H2AZ acetylation at lysines K4, K7, and K11 increased substantially following Nanog depletion. Specifically, the mono- and di-acetylated forms increased by about 2-fold, from roughly 10% to 20% abundance and from approximately 2% to 4% abundance, respectively. However, the triacetylated (H2AZK4acK7acK11ac) form increased close to 6-fold from 0.15% to 0.8% abundance

(Fig. 3G). Since the MS analysis did not detect the presence of lysine acetylation and methylation simultaneously on the same peptide, our data indicate that the two modifications on H2AZ are mutually exclusive.

**SETD6 is required to maintain self-renewal of mESCs.** Because of the compelling data showing an increase of H2AZK4<sup>me1</sup>K7<sup>me1</sup> upon mESC differentiation, we further investigated the potential role of Setd6 and methyl-H2AZ in biological mESC properties. E14 mESC were transduced with shRNA-expressing lentiviral particles to target *Setd6*. While control cells retained mESC morphology, *Setd6*-depleted cells





**Figure 4.** Setd6 maintains mESC self-renewal. **(A)** E14 mESC were transduced with either shLuc- or shSetd6-expressing lentiviral particles, selected with puromycin, and stained with alkaline phosphatase (AP). AP-stained colonies were scored as fully differentiated (FD), partially differentiated (PD), or undifferentiated (UD) according to the legend. \*Denotes a p value of 0.008. **(B)** Setd6, Nanog, and Tubulin immunoblots from control and shSetd6 mESC. **(C)** Competition assay in which mCherry-expressing cells (shLuc or shSetd6) were mixed with control cells at a 4:1 ratio, maintained over the course of 6 passages, and monitored for mCherry expression by FACS. \*Denotes a p value of < 0.004. **(D)** Clonogenicity assays in which shLuc, shSetdb1, and shSetd6 cells were plated at clonal density and stained for AP after 10 d. \*Denotes a p value of < 0.0002. **(E)** qPCR validation of *Setd6* knockdown and analysis of *Fgf5* expression. **(F)** ChIP analysis of chromatin approximately 500 base pairs upstream of *Fgf5* transcriptional start site (TSS) in undifferentiated mESC (E14) or retinoic acid-induced differentiated mESC (E14 + RA). The error bars represent the standard error of the mean calculated from a technical replicate triplicate from a representative biological replicate. \*Denotes a p value of < 0.02 between the IgG signal and H2AZK7me1 signal. \*\*Denotes a p value of < 0.05 between the E14 untreated control and the E14 + RA samples.

appeared differentiated (Fig. 4A). Specifically, *Setd6* depletion induced a significant increase (p value 0.008) in partially and fully differentiated colonies accompanied by a drastic decrease (p value 0.01) in the undifferentiated colonies (Fig. 4A). *Setd6* depletion was validated by immunoblotting (Fig. 4B) and differentiation of shSetd6 mESC confirmed by decreased Nanog level (Fig. 4B).

To further investigate the role of *Setd6* expression in mESC biology, we conducted a competition assay, as described previously,<sup>12,14,15</sup> in which mCherry-expressing (mCherry<sup>+</sup>) shLuc

control or *Setd6*-depleted mCherry<sup>+</sup> populations were each mixed with non-fluorescent mESC (Fig. S5) and maintained over the course of 6 passages. The mixed populations were analyzed for mCherry signal by fluorescence-activated cell sorting (FACS) at each passage. This experiment demonstrates that self-renewal is compromised in the *Setd6*-depleted population (Fig. 4C). Specifically, at passage 0 the population was composed of about 70% *Setd6*-depleted cells, but these contributed to less than 25% of the population by passage 6 (Fig. 4C; Fig. S5). Clonogenicity assays in which depletion of *Setdb1* was

used as a positive control,<sup>16</sup> confirmed that depletion of Setd6 led to impaired mESC self-renewal (Fig. 4D). To refute the possibility that the impaired self-renewal of Setd6-depleted cells could be due to cell death, shSetd6 cells were stained for Annexin V (Fig. S6). Also, the Setd6-depleted population had a normal cell cycle profile, which was measured by FACS (Fig. S7), and unaltered proliferation capacity, which was assessed by Ki67 staining (Fig. S8). These results reiterate<sup>16</sup> and validate that Setd6 is required for mESC self-renewal.

In response to Setd6 depletion, the expression of differentiation and pluripotency markers was analyzed and we found that *Fgf5* was consistently and reproducibly upregulated (Fig. 4E). The efficiency of *Setd6* knockdown was also confirmed by qPCR (Fig. 4E). These results suggest that Setd6 functions to maintain mESC self-renewal, presumably by repressing the expression of differentiation genes.

To address the possibility that H2AZ and H2AZK7<sup>me1</sup> could be involved in the regulation of *Fgf5* expression, we induced E14 differentiation using retinoic acid (RA). We then investigated the incorporation of H2AZ forms at chromatin at the *Fgf5* locus and found by chromatin immunoprecipitation assays that H2AZ was integrated within nucleosomes about 500 base pairs upstream of the *Fgf5* transcriptional start site (TSS; Fig. 4F) as well as at the TSS itself (Fig. S9). However, H2AZK7<sup>me1</sup> was enriched by about 3-fold at the -500 site and essentially absent from the TSS (Fig. 4F; Fig. S9). Interestingly, upon RA-mediated differentiation, the levels of H2AZK7<sup>me1</sup> dropped substantially in correlation with the loss of the epigenetic silencing mark H3K27<sup>me3</sup> (Fig. 4F). In addition, H2AZK7<sup>me1</sup> was enriched at the TSS of *FoxA2* (4-fold) and *Hand2* (3-fold), but depleted along with H3K27<sup>me3</sup> upon RA-mediated differentiation (Fig. S9). The RA treatment also induced increased acetylation of the active transcription H3K9<sup>ac</sup> mark at the TSS of *FoxA2* and *Hand2* (Fig. S9).

## Discussion

Herein, we have identified two potent lysine methyltransferases, SETD6 and SET7, that modify the histone variant H2AZ. Although H2AZ is known to be polyacetylated within the N-terminal tail (at residues K4, K7, K11, K13 and K15)<sup>7,13</sup> as well as monoubiquitylated at the carboxy-terminus (at residues K120 and K121),<sup>17,18</sup> H2AZ had never been reported to be methylated. Interestingly, our methyl-specific H2AZK7<sup>me1</sup> antibody detects a slow-migrating band (not shown), which, based on the observed molecular weight, is likely an ubiquitylated form of H2AZ. These observations suggest that lysine methylation and ubiquitylation may occur together and that there could be some form of cross-talk between the two modifications.

Regarding H2AZ acetylation, we anticipate that methylation of H2AZ at lysines 4 and 7 could prevent subsequent acetylation at these sites, thereby impairing the reported transcriptional functions associated with the acetylation of H2AZ.<sup>7</sup> Indeed, mass spectrometry analysis did not detect any acetylation on the endogenous H2AZ methylated forms.

Mass spectrometry data from shNanog and RA-treated cells, in addition to the data using our methyl-specific H2AZK7<sup>me1</sup>

antibody, highlight the authentic existence of a novel posttranslational modification on H2AZ in human and murine cells. Interestingly, upon RA-induced differentiation, H2AZK7<sup>me1</sup> levels increased drastically although SETD6 decreased slightly at the protein level (Fig. 3E) and mRNA level (not shown). It will be interesting to investigate if SETD6 enzymatic activity is increased upon RA treatment or whether other KMTs are induced by RA. Indeed, we observed increased expression of several KMT in response to RA (not shown). Notably, *SET7* mRNA levels increased by about 2-fold in response to RA (not shown) and, as a H2AZ methyltransferase, may thus contribute to increased H2AZK7<sup>me1</sup> levels. In future experiments, we will explore the contribution of these RA-induced KMTs to H2AZ methylation.

Hints of Setd6 potential role in mESC self-renewal surfaced in a genetic screen of chromatin factors.<sup>16</sup> Our exhaustive investigations confirm, through AP staining (Fig. 4A), immunoblotting of Nanog (Fig. 4B), competition (Fig. 4C) and clonogenic assays (Fig. 4D), as well as cell death assays (Fig. S6) and cell cycle profiling (Figs. S7 and S8), that Setd6 is required for mESC self-renewal. Since Setd6-depleted cells appear to differentiate, it will be interesting to investigate how Setd6 contributes to cell lineages and identify the types of differentiated cells that emerge from decreased Setd6 expression. Together with the ChIP analyses of H2AZ and H2AZK7<sup>me1</sup> at *Fgf5*, *FoxA2* and *Hand2* promoters, these results suggest that Setd6 participates in the maintenance of mESC self-renewal by regulating the expression of differentiation markers such as *Fgf5*. In addition, the loss of both H2AZK7<sup>me1</sup> and H3K27<sup>me3</sup> from *Fgf5* upon cellular differentiation implies that H2AZK7<sup>me1</sup> could be involved with H3K27<sup>me3</sup> in an epigenetic program that regulates the expression of lineage commitment genes.

Quite interestingly, although the global level of H2AZK7<sup>me1</sup> increased upon RA-induced mESC differentiation, the level of H2AZK7<sup>me1</sup> assessed at the promoters of *Fgf5*, *FoxA2* and *Hand2* decreased along with the H3K27<sup>me3</sup> mark. Whether H2AZK7<sup>me1</sup> is demethylated by a demethylase or evicted by a histone exchange complex remains to be uncovered. However, it is clear that H2AZK7<sup>me1</sup> levels decrease at some promoters upon RA-induced differentiation. Genome-wide studies, such as H2AZ and H2AZK7<sup>me1</sup> ChIPseq, may reveal more precisely their biological functions. In addition, the eventual identification of H2AZ- or H2AZK7<sup>me1</sup>-specific readers will undoubtedly help concretize the molecular mechanism of chromatin signaling by H2AZK7<sup>me1</sup>.

To conclude, our results demonstrate that SETD6 mono-methylates H2AZ on lysine 7 and that H2AZ is methylated in vivo at lysines 4 and 7. Notably, the H2AZK4<sup>me1</sup>K7<sup>me1</sup> mark is induced upon mESC cellular differentiation. Finally, we show that Setd6 is required for the maintenance of mESC self-renewal. Although H2AZK7<sup>me1</sup> and H2AZK4<sup>me1</sup>K7<sup>me1</sup> increased in differentiated mESC, the self-renewal defects we observed upon silencing of Setd6 are likely due to a combination of lysine methylation signaling events, including H2AZK7<sup>me1</sup> and H2AZK4<sup>me1</sup>K7<sup>me1</sup> as well as other SETD6 substrates, such as the NF- $\kappa$ B subunit, RelA.<sup>11</sup> Future experiments should investigate the mechanism of

H2AZK<sup>me1</sup> and H3K27<sup>me3</sup> marks concomitant loss from promoters and how these histone marks may cooperate to regulate the expression of differentiation genes and maintain mESC self-renewal potential.

## Materials and Methods

**Plasmids, cDNA and peptides.** H2AZ cDNA was cloned from HeLa cells by PCR on reverse transcribed total RNA using *Pfu* SuperMix and SuperScript II Reverse Transcriptase, respectively (Invitrogen; cat. no. 12532-016 and 18064-022, respectively). The indicated H2AZ cDNAs were inserted in either pGEX6-P-1 (GE Healthcare; cat. no. 12532-016) or pET28 (Novagen; cat. no. 69866-3) for bacterial expression. The H2AZ AGGKAGK<sup>me0/me1/me2/me3</sup>DSGKAKTKAVSRS-biotin and AGGKAGK<sup>me0/me1</sup>DSGKAKC peptides were synthesized at Yale University's W.M. Keck Foundation Biotechnology Resource Laboratory.

**Cell culture.** U2OS and HEK293T cell lines were maintained in D-MEM (HyClone). The mouse ES NanogR rescue cell line was maintained as previously described.<sup>12</sup> E14 mESCs were maintained on 0.1% gelatin-coated tissue culture plates without feeder cells for all experiments. To induce differentiation with retinoic acid (RA), LIF (Chemicon; cat. no. ESG1107) was removed and RA (Sigma-Aldrich; cat. no. R2625) was added at a concentration of 5  $\mu$ M. Alkaline phosphatase activity was measured using a commercial detection kit (Sigma-Aldrich).

**Antibodies.** The AGGKAGK<sup>me0/me1</sup>DSGKAKC peptides were conjugated to KLH (Sigma) according to the manufacturer's

suggested procedure, and used to immunize rabbits (Pocono Rabbit Farm and Laboratory). An exhaustive list of the antibodies used can be found in the supplementary information. Full methods and associated references are available in the supplementary methods online.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

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## Supplemental Materials

Supplemental materials may be found here:

[www.landesbioscience.com/journals/epigenetics/article/23416](http://www.landesbioscience.com/journals/epigenetics/article/23416)

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