

RESEARCH ARTICLE

# Setdb1 is implicated in the methylation of pericentric satellite DNA

Sunwha Cho<sup>1,2</sup>, Jung Sun Park<sup>1</sup>, Young Sun Jeong<sup>1</sup>, Yong-Kook Kang<sup>1,2</sup>

<sup>1</sup>Development and Differentiation Research Center, KRIBB, 111 Gwahang-no, Yuseong-gu, Daejeon 305-806, South Korea

<sup>2</sup>Department of Functional Genomics, University of Science and Technology (UST), 113 Gwahangno, Yuseong-gu, 305-333 Daejeon, South Korea

Correspondence: Yong-Kook Kang

E-mail: ykkang@kribb.re.kr

Received: July 30, 2015

Published online: August 13, 2015

**Setdb1/Eset, a histone H3-lysine 9 (H3K9)-specific methyltransferase, interacts with various transcription factors to modify chromatin at target genomic regions. In this study, we provide evidence supporting that Setdb1 also regulates genomic DNA methylation. Whereas 5-methylcytosine (5-mC) signal majorly localizes at pericentric heterochromatin regions in wild type cells, knocking down Setdb1 resulted in diffuse localization of 5-mC signal. Setdb1 knockdown also reduced the expression and subcellular localization of the DNA methyltransferases Dnmt1 and Dnmt3a. Bisulfite mutagenesis studies revealed that Setdb1 knockdown reduced the methylation of satellite DNA sequences (heterochromatin) but not of intracistronic A particle (IAP) sequences (euchromatin). Our results suggest that Setdb1 directly or indirectly regulates the expression of DNA methyltransferases and thus affects DNA methylation and the structural organization of heterochromatin.**

**Keywords:** Setdb1; DNA methylation; Dnmt1; Dnmt3A; satellite sequences; IAP

**To cite this article:** Sunwha Cho, et al. Setdb1 is implicated in the methylation of pericentric satellite DNA. Stem Cell Epigenet 2015; 2: e938. doi: 10.14800/sce.938.

## Introduction

H3K9 trimethylation (H3K9me3) and DNA methylation are enriched in pericentric genomic regions, and thus are considered characteristic hallmarks of heterochromatin. Previous studies in *Neurospora crassa*<sup>[1]</sup> and *Arabidopsis thaliana*<sup>[2]</sup> have shown that mutations in the proteins responsible for H3K9 methylation disrupt methylation of all the tested DNA sequences. These observations suggest a causal relationship between H3K9 methylation and DNA methylation<sup>[3]</sup>.

Setdb1 is a H3K9-specific histone lysine methyltransferase (HKMTase)<sup>[4]</sup> that associates with diverse kinds of transcription factors to regulate the

expression of target genes (for review, see<sup>[5]</sup>). In mammals, there exist redundant HKMTase enzymes, including Suv39h<sup>[6, 7]</sup>, G9a<sup>[8]</sup>, and Glp/Eu-HKMTase<sup>[9]</sup>. Among them, Setdb1 is unique in that, in addition to the distinct feature of a bifurcated SET domain structure, it contains a methyl-CpG binding domain (MBD)<sup>[10, 11]</sup>, the latter being a sequence motif both necessary and sufficient for binding to methylated DNA<sup>[12, 13]</sup>. The presence of an MBD motif suggested that Setdb1 might bridge different epigenetic programs, namely DNA methylation and histone H3K9 methylation. In this study, we characterized Setdb1's potential role in regulating DNA methylation.

## Materials and Methods

## Antibodies and immunostaining

The primary antibodies used were against  $\alpha$ -Setdb1 (07-378; Upstate),  $\alpha$ -DNMT1 (sc-20701; Santa Cruz),  $\alpha$ -DNMT3a (sc-10231; Santa Cruz),  $\alpha$ -Ran-BP (610340; BD Transduction Laboratories),  $\alpha$ -5-mC (BI-MECY-1000; Eurogentec) and  $\alpha$ -H3K9me3 (07-523; Upstate). For immunostaining, cells were fixed in 4% formaldehyde solution at 4°C for 20 min. Fixed samples were permeabilized in phosphate-buffered saline (PBS) containing 0.5% Triton-X 100 for 30 min and blocked for 30 min using PBST (0.05% Tween20 in PBS) supplemented with 2% BSA. For 5-mC staining, the cells were additionally treated with 4 N HCl for 30 min at room temperature (RT) before blocking. After brief washes, the samples were incubated with primary antibody for 2 h at 37°C and then with Alexa-488- or Alexa-594-conjugated secondary antibody (Molecular Probes) for 1 h at RT. After extensive washes, the samples were mounted using Vectashield (Vector) containing DAPI. Images were taken using a Carl Zeiss Axiovert 200M fluorescence microscope equipped with an ApoTome. Images were digitally captured using different filter sets and merged using Axiovision (v4.5 or v4.7) or Adobe Photoshop (v7.0).

## Knockdown experiments

siRNA oligonucleotides for Setdb1 were purchased from Open Biosystems. To label NIH 3T3 cells knocked down for Setdb1, we simultaneously transfected a GFP expression vector and siRNA oligonucleotides at 1:8 - 1:10 mass ratios. Cells expressing GFP were also likely knocked down for the protein targeted by siRNAs. Thirty six to forty hours after transfection, cells were fixed for immunostaining. For lentiviral shRNA-mediated knockdown, recombinant lentiviruses were produced by co-transfecting 293T cells with lentiviral shRNA vectors (Open Biosystems, pCMV-VSVG-RSV-Rev, and pMDL g/p RRE vectors). Culture supernatants were collected 72 h after transfection and filtered through a 0.45- $\mu$ m filter. For knockdown, the NIH 3T3 cells were incubated with virus-containing supernatants, supplemented with 10  $\mu$ g/ml polybrene (Sigma Aldrich), for 72 h.

## RT-PCR and western blotting

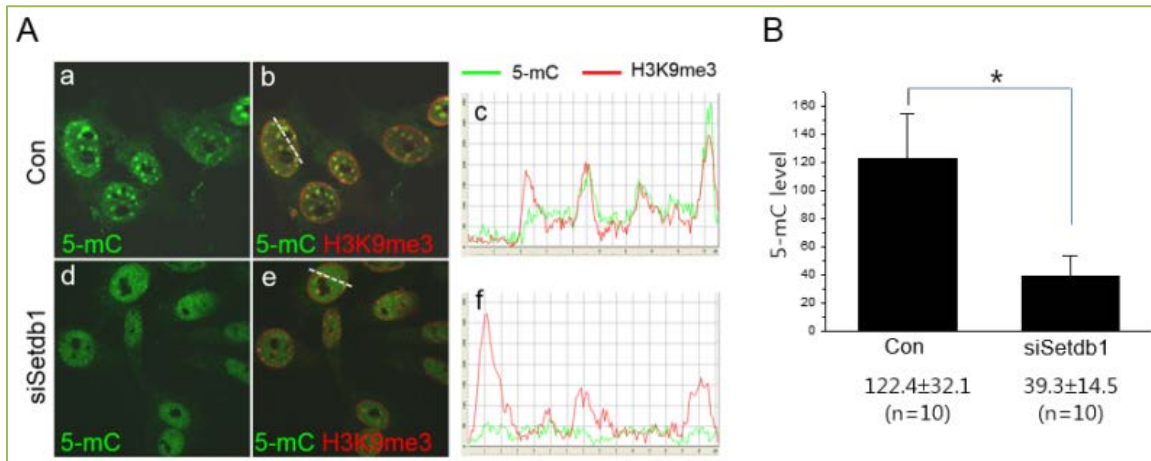
For RT-PCR, total RNA was extracted from NIH 3T3 cells using an RNeasy Mini kit (Qiagen). Complementary DNA was synthesized using 2  $\mu$ g of

total RNA with oligo-dT primers and SuperScript II RNase H-Reverse Transcriptase (Invitrogen), following the manufacturer's instruction. For western blot analysis, cells were harvested and treated with protein lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 $\times$  protease inhibitor cocktail) for 1 h at 4°C. Cell lysates were sonicated and centrifuged at 13,000 rpm for 20 min. Equal amounts of total protein were loaded onto a SDS-PAGE gel, electrophoresed, and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 1.5% (w/v) BSA in TBST (0.05% Tween-20 in TBS) for 1 h at RT and probed with antibodies against  $\alpha$ -Setdb1 (07-378; Upstate),  $\alpha$ -DNMT1 (sc-20701; Santa Cruz),  $\alpha$ -DNMT3b (sc-20704; Santa Cruz),  $\alpha$ -DNMT3a (sc-10231; Santa Cruz),  $\alpha$ -H3K9me2 (ab1220; Abcam),  $\alpha$ -H3K9me3 (07-523; Upstate),  $\alpha$ -H3 (sc-10809; Santa Cruz), and  $\alpha$ - $\alpha$ -Tubulin (T-5168; Sigma). Appropriate HRP-conjugated secondary antibodies were used, and antibody binding was visualized using a chemiluminescent substrate (BM chemiluminescent blotting substrate, Roche).

## Bisulfite sequencing

Genomic DNA was isolated using a DNeasy tissue kit (Qiagen) and subjected to bisulfite mutagenesis using an EpiTect kit (Qiagen), following the manufacturer's protocol. Bisulfite-treated DNA was used as templates to PCR amplify IAP (GenBank #D63767.1) and satellite sequences (GenBank #AJ296864.1). The PCR conditions were as follows: 30 cycles of 94°C for 30 s, 45°C for 40 s, and 72°C for 30 s in the first PCR and then another 30 cycles of 94°C for 30 s, 50°C for 40 s, and 72°C for 30 s for hemi-nested PCR. The list of primers we used were: 5'-ACCTCTATCTCCACTCTCATTA-3' and 5'-TTTTGATTGGTTGTAGTTTATGGT-3' for the first PCR of IAP sequences, and 5'-ATTAATATAAATAACCTATTTACTCTTA-3' and 5'-TTTTGATTGGTTGTAGTTTATGGT-3' for hemi-nested PCR of IAP; 5'-TACACACTAAAAAACTAAATTATAA-3' and 5'-TTYGTTATATTTTAGGTTTTTTAGAGA-3' for the first PCR of satellite repeats, and 5'-ATACACACTTTAAAACATAAAAATATAA-3' and 5'-TTYGTTATATTTTAGGTTTTTTAGAGA-3' for hemi-nested PCR of satellite repeats. The PCR products were purified, cloned into pGEM-T Easy vector (Promega), and sequenced.

## Results



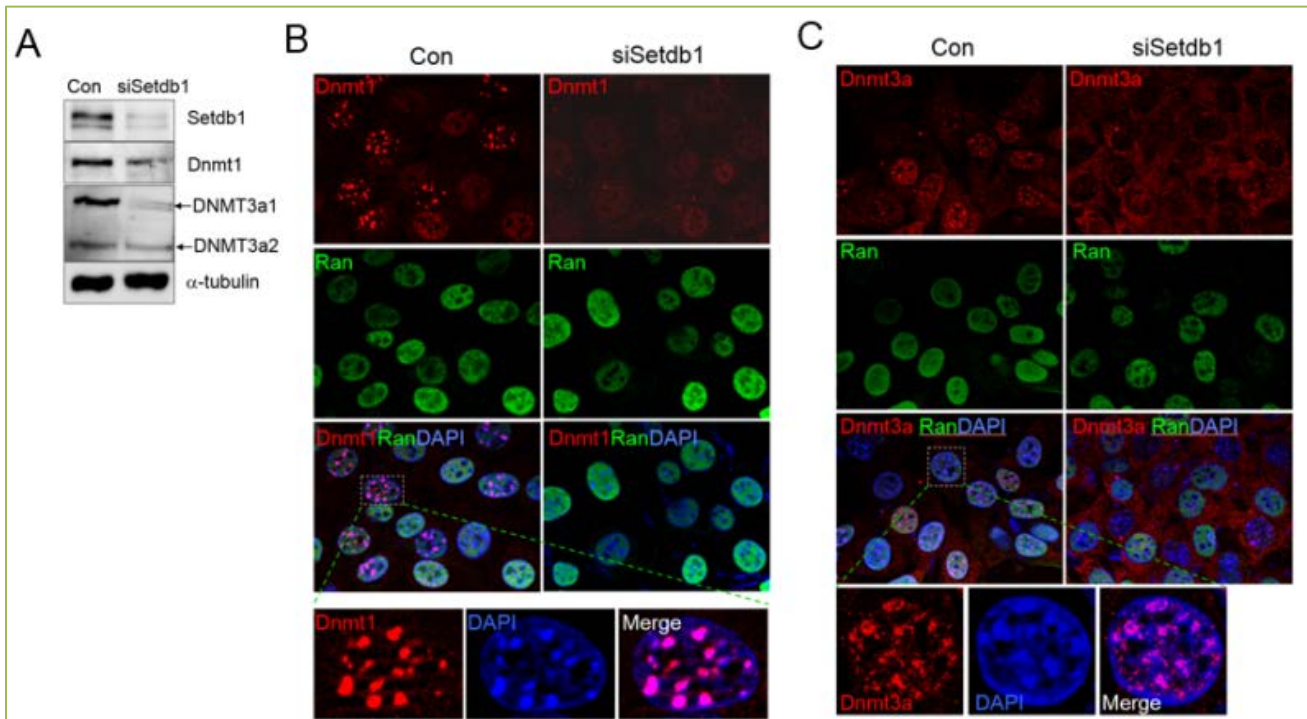
**Fig 1. Changes in DNA methylation in Setdb1 knockdown cells.** **A**, Immunostaining result. NIH 3T3 cells were infected with lentiviruses expressing Setdb1 shRNAs (siSetdb1) and immunostained for 5-methylcytosine (5-mC, green) and H3K9me3 (red). Linescan profiles of fluorescence intensity over the indicated regions (dashed lines) in b and e are shown in c and f, respectively. **B**, 5-mC level. 5-mC fluorescence signal intensity was measured at randomly chosen linear positions that pass through DAPI-dense regions using Axiovision software (v4.7). Statistics (mean  $\pm$  standard deviation) are shown below the graph. Asterisk indicates a significant difference between the groups (*t*-test,  $p < 0.001$ ). Numbers in parentheses indicate the number of cells counted.

To investigate whether Setdb1 regulates genomic DNA methylation, we knocked down Setdb1 in NIH 3T3 cells and characterized changes in the DNA methylation state. Immunostaining for 5-methylcytosine (5-mC) in wild type cells showed that 5-mC localized to numerous dense foci corresponding to pericentric heterochromatic regions (Fig. 1Aa). Knocking down Setdb1 disrupted the normal localization of 5-mC; 5-mC was localized diffusely throughout the nucleus, and few to no foci were observed (Fig. 1Ad). Linescan profiles of fluorescence signal intensity (FSI) demonstrated the co-localization of 5-mC with H3K9me3 in wild type cells (Fig. 1Ac), which was disrupted in Setdb1 knockdown cells (Fig. 1Af). In fact, 5-mC appeared to be not correlated or anti-correlated with H3K9me3 in Setdb1 knockdown cells. In addition, we estimated global 5-mC levels by measuring the FSI of 5-mC at random DAPI-dense positions within the nucleus. As shown in Fig. 1B, Setdb1 knockdown reduced the global levels of 5-mC to about one-third of the 5-mC levels observed in wild type cells, indicating that Setdb1 regulates DNA methylation globally.

Our immunostaining results showed that Setdb1 knockdown changes both the degree and pattern of genomic DNA methylation. We hypothesized that Setdb1 could indirectly affect DNA methylation by regulating proteins that directly supervise DNA methylation. Setdb1 knockdown did not significantly affect the protein expression of Dnmt1, a maintenance

DNA methyltransferase (Fig. 2A). However, immunostaining for Dnmt1 in Setdb1 knockdown cells revealed that Dnmt1 was markedly depleted from DAPI-dense heterochromatin regions, where Dnmt1 is normally localized in a good proportion of wild type cells (Fig. 2B). We next examined Dnmt3a, a de novo DNA methyltransferase. Western blots of the two variants, Dnmt3a1 and Dnmt3a2, showed that protein expression only of the former was decreased by knocking down Setdb1 (Fig. 2A). In addition, Dnmt3a, which is normally localized to heterochromatin foci in wild type cells, was nearly undetectable in the nucleus of Setdb1 knockdown cells (Fig. 2C). In agreement with our result, an interaction between Setdb1 and Dnmt3a was reported previously [14]. Collectively, our results indicate that Setdb1 knockdown downregulates or displaces DNA methyltransferases, such as Dnmt1 and Dnmt3a, from heterochromatin regions.

We next analyzed how knocking down Setdb1 affects genomic methylation at the DNA sequence level. Centromeric satellites and IAP sequences, genomic repeats that represent heterochromatic and euchromatic regions, respectively, were studied using bisulfite mutagenesis [15]. We extracted genomic DNA from Setdb1 knockdown cells that we previously showed stained diffusely for 5-mC, as in Fig. 1Ad. We found that centromeric satellites from wild type cells were 70.4% methylated (56 of the 80 CpG sites examined were methylated), but only 45.4% (69/152) were methylated when Setdb1 was knocked down. This



**Fig 2. Downregulation of DNA methyltransferases in Setdb1 knockdown cells.** A, Western blotting. B-C, Immunostaining for Dnmt1 (B) and Dnmt3a (C). NIH 3T3 cells were transfected with Setdb1 knockdown constructs (siSetdb1) and were immunostained for Dnmt1 (red, B)/Ran (green) or Dnmt3a (red, C)/Ran (green). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). Boxed images are enlarged in separate panels below.

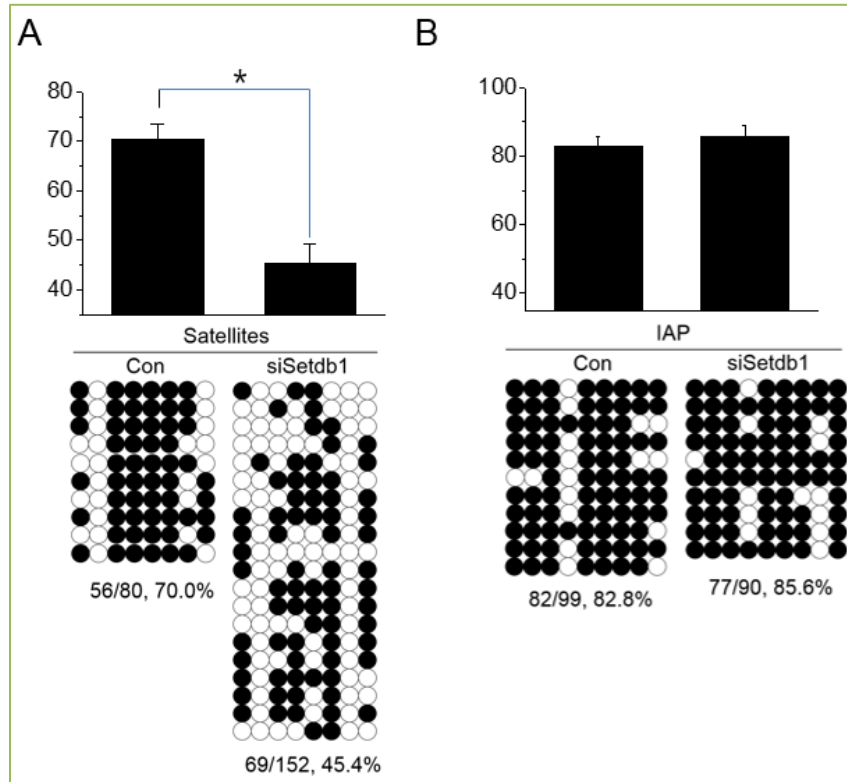
result confirms that Setdb1 promotes DNA methylation at centromeric satellite regions (**Fig. 3A**). In contrast, IAP sequences from Setdb1 knockdown cells and wild type control cells showed no noticeable difference in their methylation level (85.6% and 82.8%, respectively). This result agrees with a recent report that indicated that the effects of Setdb1 are sequence- and cell-type specific<sup>[5, 16]</sup>. Our bisulfite mutagenesis results showing reduced methylation of satellite sequences due to Setdb1 knockdown also agree with our immunofluorescence results showing reduced 5-mC immunostaining at heterochromatic regions (**Fig. 1A**). Taken together, our results suggest that Setdb1 regulates DNA methylation at centromeric satellite regions and, by extension, maintains the structural integrity of heterochromatin.

## Discussion

We provide evidence supporting that Setdb1 is involved in the maintenance of DNA methylation and heterochromatin organization at pericentric DNA regions, possibly by regulating the expression and/or subcellular localization of DNA methyltransferases, as shown in Fig. 2. However, Setdb1 itself is a repressive effector in transcriptional regulation, acting in a way of recruiting various repressive chromatin remodelers such as SIN3-HDAC complex to

target gene promoters and decorating chromatin with repressive epigenetic mark as H3K9me3<sup>[17]</sup>. Furthermore, Setdb1 localizes to euchromatin in general (see below for exceptions). For these reasons, it is unlikely that Setdb1 participates directly in the formation of pericentric heterochromatin. Rather, Setdb1 likely regulates the expression of Dnmt1 and Dnmt3a indirectly.

An alternative hypothesis might explain how Dnmt1 and Dnmt3a are lost from pericentric regions in Setdb1 knockdown cells. Setdb1 may still directly influence their localization by recruiting DNA methyltransferases to pericentric heterochromatin, which would require Setdb1 to be localized to pericentric regions as well. In support of this hypothetical model, dSETDB1 was shown to localize to heterochromatin in the germline stem cells of *Drosophila*<sup>[18]</sup>. In another study, dSETDB1 was also shown to recruit Dnmt2<sup>[19]</sup>, promoting DNA methylation and silencing of target genes<sup>[20]</sup>. In mammalian cells, Dnmt3a has been shown to directly associate with Setdb1<sup>[14]</sup>. These previous observations suggest that Setdb1 may directly recruit Dnmt3a proteins to pericentric heterochromatin in our studies. However, in mammalian cells, there is no evidence (assayed immunocytochemically or through immunoprecipitations) that Setdb1 localizes to pericentric heterochromatin or that it recruits other partner proteins. Immunostaining for Setdb1 in the nucleus shows that it is



**Fig 3. Decreased DNA methylation at satellite DNA sequences in Setdb1 knockdown cells.** CpG methylation of centromeric satellite repeats (A) and intracistronic A particle (IAP, B) sequences in Setdb1 knockdown cells (siSetdb1). Genomic DNA was extracted from the wild type control and Setdb1 knockdown cell samples and treated with bisulfite. Asterisk indicates a significant difference between groups (*t*-test,  $p < 0.001$ ).

either localized diffusely<sup>[21]</sup> or co-localized in a punctuate form with promyelocytic leukemia (PML) nuclear bodies, completely escaping from DAPI-dense heterochromatin<sup>[22-24]</sup>.

DNA methylation was significantly reduced at satellite sequences but remained unchanged at intergenic IAP sequences in Setdb1 knockdown cells (Fig. 3). These results agree well with earlier studies reporting that satellite regions are partly maintained by Setdb1<sup>[25-27]</sup>, and that IAP sequences are transcriptionally repressed by Setdb1 but their DNA methylation remained unaffected in Setdb1-deleted cells<sup>[16, 28]</sup>. Our results together with these earlier results establish that Setdb1 contributes to the maintenance of regional DNA methylation.

### Acknowledgements

This work was supported by grants from National Research Foundation of Korea (2006-2004082 and 2015R1A2A2A01003117), and NRF-SRC program (2011-0030049).

### Conflict of interest

The authors declare that they have no conflicting interests.

### Abbreviations

IAP: intracistronic A particle; 5-mC: 5-methylcytosine; HKMTase: H3K9-specific histone lysine methyltransferase; H3K9me3: H3K9 trimethylation; MBD: methyl-CpG binding domain; FSI: fluorescence signal intensity.

### References

1. Tamaru H, Selker EU. A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*. *Nature* 2001; 414:277-283.
2. Jackson JP, Lindroth AM, Cao X, Jacobsen SE. Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. *Nature* 2002; 416:556-560.
3. Bird A. Molecular biology. Methylation talk between histones and DNA. *Science* 2001; 294:2113-2115.
4. Yang L, Xia L, Wu DY, Wang H, Chansky HA, Schubach WH, et al. Molecular cloning of ESET, a novel histone H3-specific methyltransferase that interacts with ERG transcription factor. *Oncogene* 2002; 21:148-152.

5. Kang YK. SETDB1 in Early Embryos and Embryonic Stem Cells. *Current issues in molecular biology* 2014; 17:1-10.
6. Nakayama J, Rice JC, Strahl BD, Allis CD, Grewal SI. Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science* 2001; 292:110-113.
7. Rea S, Eisenhaber F, O'Carroll D, Strahl BD, Sun ZW, Schmid M, *et al.* Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* 2000; 406:593-599.
8. Tachibana M, Sugimoto K, Nozaki M, Ueda J, Ohta T, Ohki M, *et al.* G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. *Genes Dev* 2002; 16:1779-1791.
9. Ogawa H, Ishiguro K, Gaubatz S, Livingston DM, Nakatani Y. A complex with chromatin modifiers that occupies E2F- and Myc-responsive genes in G0 cells. *Science* 2002; 296:1132-1136.
10. Hendrich B, Bird A. Mammalian methyltransferases and methyl-CpG-binding domains: proteins involved in DNA methylation. *Curr Top Microbiol Immunol* 2000; 249:55-74.
11. Wade PA. Methyl CpG binding proteins: coupling chromatin architecture to gene regulation. *Oncogene* 2001; 20:3166-3173.
12. Meehan RR, Lewis JD, McKay S, Kleiner EL, Bird AP. Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. *Cell* 1989; 58:499-507.
13. Nan X, Meehan RR, Bird A. Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2. *Nucleic Acids Res* 1993; 21:4886-4892.
14. Li H, Rauch T, Chen ZX, Szabo PE, Riggs AD, Pfeifer GP. The histone methyltransferase SETDB1 and the DNA methyltransferase DNMT3A interact directly and localize to promoters silenced in cancer cells. *J Biol Chem* 2006; 281:19489-19500.
15. Kim SH, Kang YK, Koo DB, Kang MJ, Moon SJ, Lee KK, *et al.* Differential DNA methylation reprogramming of various repetitive sequences in mouse preimplantation embryos. *Biochemical and biophysical research communications* 2004; 324:58-63.
16. Matsui T, Leung D, Miyashita H, Maksakova IA, Miyachi H, Kimura H, *et al.* Proviral silencing in embryonic stem cells requires the histone methyltransferase ESET. *Nature* 2010; 464:927-931.
17. Cho S, Park JS, Kang YK. AGO2 and SETDB1 cooperate in promoter-targeted transcriptional silencing of the androgen receptor gene. *Nucleic Acids Res* 2014; 42:13545-13556.
18. Yoon J, Lee KS, Park JS, Yu K, Paik SG, Kang YK. dSETDB1 and SU(VAR)3-9 sequentially function during germline-stem cell differentiation in *Drosophila melanogaster*. *PLoS ONE* 2008; 3:e2234.
19. Kunert N, Marhold J, Stanke J, Stach D, Lyko F. A Dnmt2-like protein mediates DNA methylation in *Drosophila*. *Development* 2003; 130:5083-5090.
20. Gou D, Rubalcava M, Sauer S, Mora-Bermudez F, Erdjument-Bromage H, Tempst P, *et al.* SETDB1 is involved in postembryonic DNA methylation and gene silencing in *Drosophila*. *PLoS ONE* 2010; 5:e10581.
21. Schultz DC, Ayyanathan K, Negorev D, Maul GG, Rauscher FJ, 3rd. SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. *Genes Dev* 2002; 16:919-932.
22. Cho S, Park JS, Kang YK. Dual Functions of Histone-Lysine N-Methyltransferase Setdb1 Protein at Promyelocytic Leukemia-Nuclear Body (PML-NB): MAINTAINING PML-NB STRUCTURE AND REGULATING THE EXPRESSION OF ITS ASSOCIATED GENES. *J Biol Chem* 2011; 286:41115-41124.
23. Cho S, Park JS, Kang YK. Regulated nuclear entry of over-expressed Setdb1. *Genes Cells* 2013; 18:694-703.
24. Yeap LS, Hayashi K, Surani MA. ERG-associated protein with SET domain (ESET)-Oct4 interaction regulates pluripotency and represses the trophectoderm lineage. *Epigenetics Chromatin* 2009; 2:12.
25. Fritsch L, Robin P, Mathieu JR, Souidi M, Hinaux H, Rougeulle C, *et al.* A subset of the histone H3 lysine 9 methyltransferases Suv39h1, G9a, GLP, and SETDB1 participate in a multimeric complex. *Mol Cell* 2010; 37:46-56.
26. Lee J, Hagerty S, Cormier KA, Kim J, Kung AL, Ferrante RJ, *et al.* Monoallele deletion of CBP leads to pericentromeric heterochromatin condensation through ESET expression and histone H3 (K9) methylation. *Human molecular genetics* 2008; 17:1774-1782.
27. Loyola A, Tagami H, Bonaldi T, Roche D, Quivy JP, Imhof A, *et al.* The HP1alpha-CAF1-SetDB1-containing complex provides H3K9me1 for Suv39-mediated K9me3 in pericentric heterochromatin. *EMBO reports* 2009; 10:769-775.
28. Dodge JE, Kang YK, Beppu H, Lei H, Li E. Histone H3-K9 methyltransferase ESET is essential for early development. *Mol Cell Biol* 2004; 24:2478-2486.