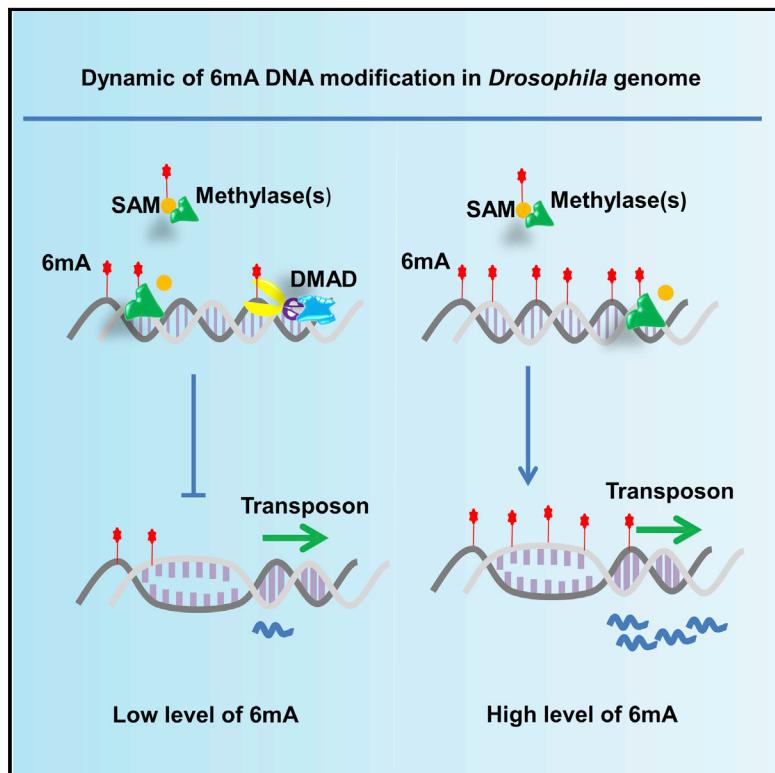


N⁶-Methyladenine DNA Modification in *Drosophila*

Graphical Abstract



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In Brief

DNA N⁶-methyladenine (6mA) modification is present in the *Drosophila* genome. The *Drosophila* Tet-homolog-mediated 6mA demethylation correlates with transposon expression, suggesting a potential role of 6mA in regulating gene expression.

Highlights

- 6mA modification occurs in fly embryo DNA and is regulated by DMAD
- DMAD promotes differentiation of early germ cells in fly ovary
- DMAD catalyzes demethylation of 6mA both in vivo and in vitro
- DMAD-mediated 6mA demethylation is correlated with transposon expression

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N⁶-Methyladenine DNA Modification in *Drosophila*

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SUMMARY

DNA N⁶-methyladenine (6mA) modification is commonly found in microbial genomes and plays important functions in regulating numerous biological processes in bacteria. However, whether 6mA occurs and what its potential roles are in higher-eukaryote cells remain unknown. Here, we show that 6mA is present in *Drosophila* genome and that the 6mA modification is dynamic and is regulated by the *Drosophila* Tet homolog, DNA 6mA demethylase (DMAD), during embryogenesis. Importantly, our biochemical assays demonstrate that DMAD directly catalyzes 6mA demethylation in vitro. Further genetic and sequencing analyses reveal that DMAD is essential for development and that DMAD removes 6mA primarily from transposon regions, which correlates with transposon suppression in *Drosophila* ovary. Collectively, we uncover a DNA modification in *Drosophila* and describe a potential role of the DMAD-6mA regulatory axis in controlling development in higher eukaryotes.

INTRODUCTION

DNA methylation, an epigenetic mechanism, does not change DNA sequence but instead suppresses the transcription factor-DNA association, thereby regulating gene expression and a variety of cellular processes (Feng et al., 2010; Smith and Meissner, 2013). Several methylated bases, including 5-methylcytosine (5mC), N6-methyladenine (6mA), and N4-methylcytosine (4mC), have been found in genomic DNA from diverse species (Cheng, 1995; Ratel et al., 2006; Wion and Casadesús, 2006). These methylated bases have been shown to be products of post-replicative DNA modification generated by specific DNA methylases (Wion and Casadesús, 2006). The prevailing view is that, unlike 5mC, 6mA and 4mC function only in bacteria, protists, and other lower eukaryotes (Cheng, 1995; Wion and Casadesús, 2006). Among these DNA modifications, 6mA plays an important role in controlling a number of biological functions

in bacteria, such as DNA replication and repair, gene expression, and host-pathogen interactions (Reisenauer et al., 1999; Wion and Casadesús, 2006), and is essential for viability of some bacterial strains (Julio et al., 2001; Stephens et al., 1996; Wright et al., 1997). In contrast, 5mC is thought to be the predominant type, if not the only type, of methylated base in mammals (Smith and Meissner, 2013).

Recent studies have suggested that methylation/demethylation at the C-5 position of cytosine in mammals is a dynamic and reversible process controlled by several mechanisms, including passive and active demethylation (Bhutani et al., 2011; Wu and Zhang, 2014). While passive demethylation is attributed to successive cell divisions that cause a progressive loss of 5mC on a genome scale, active demethylation is achieved by ten-eleven translocation (Tet)-mediated oxidation to 5-hydroxymethylcytosine (5hmC) (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009). It has been shown that 5mC can be oxidized by the Tet enzymes in an iterative manner to 5hmC, 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC), and both 5fC and 5caC can be further replaced to unmodified cytosine by excision repair pathway (He et al., 2011; Ito et al., 2011; Maiti and Drohat, 2011).

Given the important roles of 6mA modification in bacteria, we explore whether 6mA plays a role in eukaryotes. However, previous studies have suggested that the 6mA base is present at extremely low levels in genomic DNA of higher eukaryotes (Ratel et al., 2006). We speculate that, if 6mA plays a role, the potential installation of this modification by methyltransferases could be reversed by a demethylase-mediated demethylation process. Since extremely low levels of 6mA are present in higher eukaryotes, we reason that 6mA demethylases might play predominant roles in controlling the dynamics of 6mA DNA modification in higher eukaryotes. Thus, knockout of yet-to-be identified demethylases could lead to accumulation of 6mA and allow its functional investigations. In this study, we identify the *Drosophila* Tet homolog as the DNA demethylase that is responsible for 6mA demethylation in *Drosophila*, and we name it DNA 6mA demethylase (DMAD).

In *Drosophila*, 5mC modification exists at an extremely low level (Lyko and Maleszka, 2011), and the *Drosophila* Dnmt2-dependent methylome lacks defined DNA 5mC patterns (Radatz et al., 2013). Thus, whether the *Drosophila* genome has a

functional DNA modification remains elusive. In this study, we show that DNA modification 6mA is present in the *Drosophila* genome at a considerable level and that the demethylation of 6mA is tightly regulated by DMAD during embryogenesis and tissue homeostasis. We also demonstrate that DMAD is likely a 6mA demethylase since it directly catalyzes 6mA demethylation in vitro. Further genetic and sequencing analyses reveal that DMAD determines 6mA distribution in the *Drosophila* genome and is essential for development.

RESULTS

Characterization of 6mA Modification in *Drosophila* Genomic DNA

Previous studies suggested that 5mC modification in *Drosophila* DNA occurs at very low levels, and the *Drosophila* Dnmt2-dependent methylome lacks defined DNA 5mC methylation patterns (Lyko et al., 2000; Raddatz et al., 2013). To further explore this issue, we employed ultra-high-performance liquid chromatography-triple quadrupole mass spectrometry, coupled with multiple-reaction monitoring (UHPLC-MRM-MS/MS) analysis, an extremely sensitive assay for detecting base modification (Yin et al., 2013), to measure the abundance of oxidized 5mC derivatives, 5hmC, 5fC, and 5caC in multiple tissues. The UHPLC-MRM-MS/MS assays showed that, although 5hmC was detected in *Drosophila* DNA at extremely low levels and fewer than 100 of the cytosine bases per genome were modified to be 5hmC (Figures S1A–S1C), 5fC and 5caC were not detectable in *Drosophila* DNA. These observations prompted us to explore whether DNA methylation could occur at other bases. We turned our attention to explore the possible existence of adenine methylation in *Drosophila* DNA.

We used an antibody that is specifically against the 6mA base in DNA (Figure S1D) and performed dot blot experiments to detect the 6mA signal in *Drosophila* DNA samples isolated from various adult tissues and from embryos at various stages. As shown in Figure 1A, while relatively weak signals of 6mA were detected in DNA from adult tissues and late-stage embryos, a very strong 6mA signal was found to be present in embryos at the very early stage, suggesting the existence of 6mA in *Drosophila* DNA and that the status of 6mA modification might be dynamic during embryogenesis.

We next sought to quantify 6mA in *Drosophila* DNA using the UHPLC-MRM-MS/MS method (Figure S1E) and first focused on measuring the 6mA abundance in DNA at the embryonic stages. As shown in Figures 1B and 1C, abundance of the 6mA base appeared to display a peak (~0.07%, 6mA/dA) at the ~0.75 hr stage but was dramatically reduced to a very low level (~0.001%, 6mA/dA) at the 4–16 hr stages, confirming that 6mA is dynamic in *Drosophila* DNA during embryonic development. Additionally, we also quantified the abundance of 6mA in adult tissues (e.g., brain and ovary) and found that it exhibited similar low levels to those found in the late-stage embryonic genome (Figures 1D and 1E). To confirm that the signal indeed reveals 6mA modification in *Drosophila*, we collected the peak fraction containing 6mA (Figure S1F) and performed a further high-resolution mass spectrometry analysis. As shown in Figures 1F–1H, we observed an accurate mass/charge ratio of

266.1250 au (M+H), which matched the theoretic monoisotopic mass of 6mA (266.1248 au) with a deviation of 1.02 ppm. Notably, the isolated compound displayed the same fragment pattern (20 fragments) as the standard 6mA. Collectively, our findings support that 6mA is present in fly DNA and is highly dynamic during early embryogenesis.

Drosophila Embryos Possess DNA 6mA Demethylation Activity

The observation of a dramatic reduction in 6mA levels in the *Drosophila* genome from the very early to the late stages of embryonic development prompted us to ask the intriguing question of whether active 6mA demethylation occurs during *Drosophila* embryogenesis. To explore this issue, we established an in vitro DNA 6mA demethylation assay. In this assay, we employed the AlkB, a known 6mA demethylase from bacteria (Li et al., 2012), as a positive control enzyme (Figure 2A). As shown in Figure 2B, contrary to the control reaction with adding the GFP protein, the methylated DNA substrates were significantly oxidized in the presence of AlkB in a dose-dependent manner. We then used this established system to determine whether the embryonic nuclear extracts have enzymatic activity for 6mA demethylation. As shown in Figure 2C, addition of serially diluted nuclear extracts in the enzymatic reaction catalyzed 6mA demethylation in a dose-dependent manner. By contrast, no or a low background signal of 6mA demethylation was measured in the control reactions in which GFP was added. Of note, we found that no or only a low level of background demethylation signal was detected when we added the same number of boiled nuclear extracts in a parallel control reaction (data not shown), suggesting that a potential small amount of DNA from nuclear extracts did not interfere with the signal that we collected from in vitro reactions.

Interestingly, we detected an increase in 6mA demethylation activity from nuclear extracts during embryonic development. As shown in enzymatic assays, the 6mA demethylation activity of nuclear extracts was relative low at the very early stage but gradually increased and reached a peak at the 6 hr stage (Figure 2D). This result demonstrated that 6mA demethylation activity and abundance of 6mA in embryonic DNA are mutually complementary with each other during embryonic development (see Figures 1A, 1C, and 2D). Thus, our findings not only support that DNA 6mA modification is a dynamic process during early *Drosophila* embryonic development, but also raise a possibility that 6mA demethylation is regulated by a specific DNA dioxygenase.

DMAD Is Involved in Regulating DNA 6mA Demethylation

We next aimed to search for the specific enzyme responsible for 6mA demethylation. Previous studies have shown that Tet proteins in mammals play important roles in DNA demethylation through converting 5mC to 5hmC (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009). The *Drosophila* genome contains a gene, CG2083, which encodes a putative dioxygenase protein. Sequence alignment and domain structure analysis suggested that this protein contains highly conserved domains, including a CXXC zinc finger (645 aa–684 aa), a Cys-rich domain (1695 aa–1867 aa), and a DSBH domain (1888 aa–2918 aa),

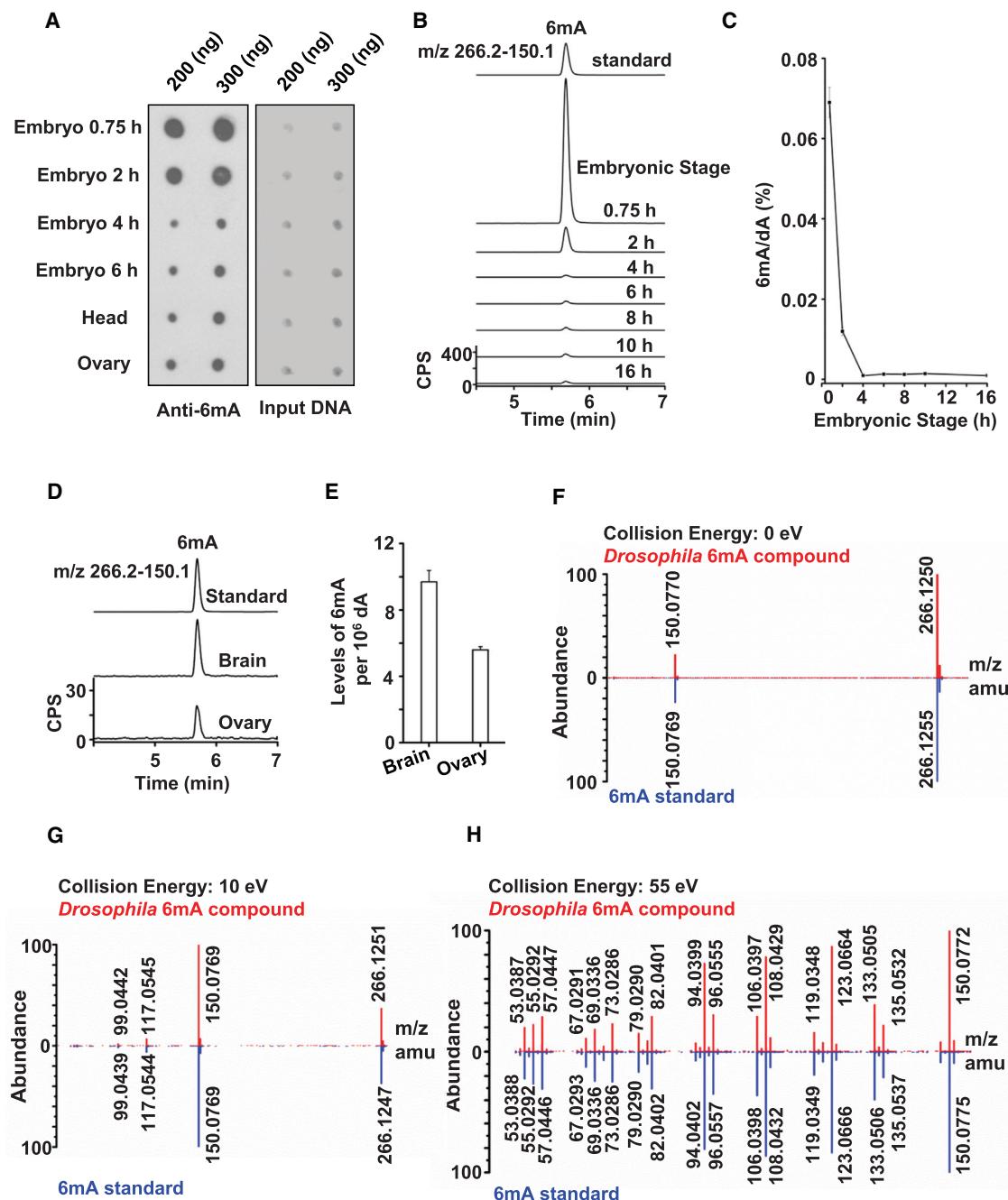


Figure 1. Characterization and Quantification of 6mA in *Drosophila* DNA

(A) Genomic DNAs from embryos at various stages and adult tissues as indicated were subjected to dot blot assays using a specific anti-6mA antibody (left). Methylene blue staining was performed to determine the signal of input DNA (right). (B–E) UHPLC-MRM-MS chromatograms (B and D) and quantification (C and E) of 6mA in genomic DNA of embryos (B and C) and adult tissues (D and E). (F–H) Control compound and the isolated 6mA compound from fly genomic DNA from 0.5–1.5 hr embryos were subjected to further high-resolution mass-spectrometry analysis. The collision energy was set at 0 eV (F), 10 eV (G), and 55 eV (H). The experiments were carried out by triplicates, and the standard deviations were calculated by Excel. See also Figure S1.

which are also present in mammalian Tet proteins (Figure 2E). It is worthwhile to note that the bacterial AlkB protein also contains a DSBH domain (Figure 2E). Based on the biochemical function of the CG2083-encoding protein that we characterized below,

we thereafter called it *Drosophila* DNA 6mA demethylase and abbreviated it as DMAD. Given that DMAD looks more like mammalian Tet proteins, we first performed an *in vitro* enzymatic assay and found that the catalytic domain of DMAD (DMAD-CD),

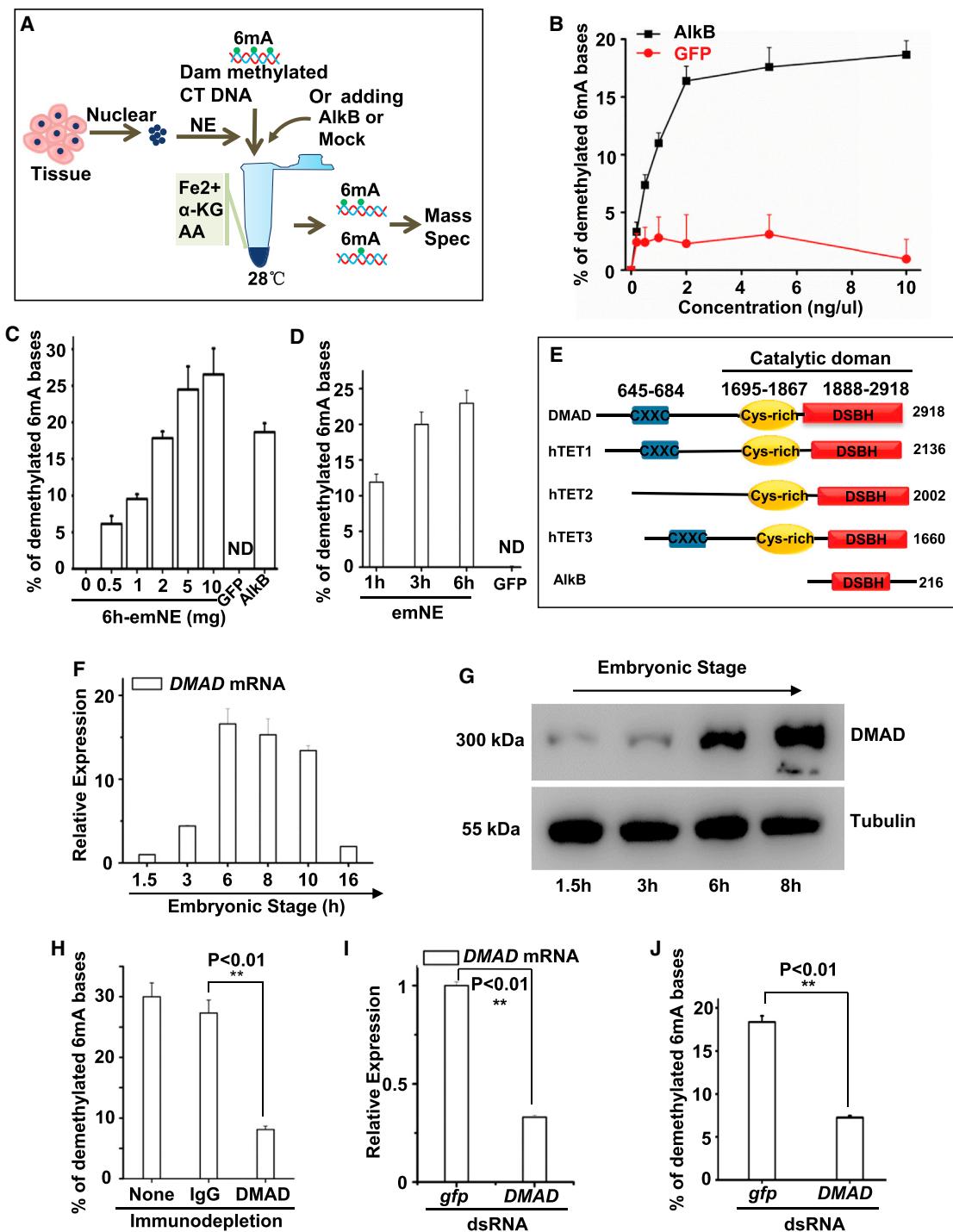


Figure 2. Demethylation of DNA 6mA Modification by *Drosophila* Embryo Nuclear Extracts

(A) Schematic representation of in vitro 6mA demethylation. AlkB- and mock-treated nuclear extracts were used in the enzymatic reaction to catalyze the demethylation of the 6mA in methylated CT DNA, and the products were then subjected to mass spectrometry.

(B) *E. coli* AlkB, but not mock protein (GFP), catalyzes the demethylation of the 6mA in a dose-dependent fashion.

(C) Nuclear extracts from 6 hr embryos (from 0.5, 1, 2, 5, 10 mg embryos as indicated) were tested in the demethylation reaction. In this experiment, AlkB and GFP were used as positive or negative controls, respectively.

(D) Nuclear extracts (from 2 mg embryos) from various embryonic stages possess enzyme activity for 6mA demethylation.

(E) Schematic diagram showing that DMAD contains three conserved domains—the CXXC zinc finger, the Cys-rich domain, and the DSBH domain—that are also present in mammalian Tet proteins. The bacterium AlkB contains DSBH, which possesses enzyme activity for 6mA demethylation.

(legend continued on next page)

but not DMAD-CD^{mut}, could convert 5mC to 5hmC in vitro (Figures S2A–S2D). However, the in vitro activity of DMAD-CD that mediates oxidation of 5mC was about 30-fold lower than that of mouse Tet1-CD (data not shown). Nevertheless, only a few cytosine bases (less than 100) as mentioned above could be detected as the hydroxymethylated form, 5hmC, per genome (Figures S1B and S1C). We speculated that DMAD might play a role in catalyzing other forms of DNA modification in fly, for example, 6mA.

We found that the DMAD is weakly expressed during early embryonic stages but is highly expressed during the later embryonic stages (Figures 2F and 2G). Thus, the DMAD expression has a complementary expression pattern as 6mA during embryogenesis. Because nuclear extracts from the late-stage embryos exhibited considerable 6mA demethylation activity and high levels of DMAD expression, we asked whether DMAD is involved in regulating the 6mA demethylation. To do this, we used a specific anti-DMAD antibody and then performed antibody-depletion experiments. Nuclear extracts with depleted DMAD were then used in in vitro 6mA DNA demethylation assays. As shown in Figure 2H, depletion by the anti-DMAD antibody, but not IgG, significantly blocked the demethylation activity of the nuclear extracts from the late-stage embryos, arguing that the DMAD is involved in regulating 6mA demethylation.

To obtain further evidence to support our argument, we next employed the double-strand RNA (dsRNA) knockdown method and further evaluated the specificity of DMAD's role in regulating 6mA demethylation. As shown in Figure 2I, injection of dsRNA against the *DMAD* mRNA in embryos significantly reduced the *DMAD* expression. As shown in Figure 2J, nuclear extracts from embryos treated with *DMAD* dsRNA exhibited much less 6mA demethylation activity than extracts from control embryos, further confirming the important role of DMAD in demethylating 6mA. In line with this, we observed that knockdown of DMAD increased the levels of 6mA in late-stage (15 hr) embryos (Figure S2E). In addition, we found that injection of *DMAD* dsRNA at different developmental time points caused significant lethality at the late embryonic stage when compared with control dsRNA injection (Figure S2F), suggesting that DMAD possibly contributes to embryonic development.

DMAD Is Required for *Drosophila* Development

To investigate the biological role of DMAD and its relevance to fly DNA 6mA demethylation in vivo, we sought to generate the *DMAD* mutant flies by employing the CRISPR/Cas system. According to the method described previously (Cong et al., 2013; Mali et al., 2013), we designed two sgRNAs containing non-overlapping sequences targeting the *DMAD* gene and generated two alleles, *DMAD*¹ and *DMAD*², with an independent genetic background (Figure 3A and Extended Experimental Procedures). As shown in a western blot assay, DMAD expression

was completely abolished in the *DMAD*¹ and *DMAD*² mutant allelic combination (Figure 3B), revealing that these two *DMAD* mutants are null alleles. To determine the biological role of DMAD, we performed a genetic complementation test and found that, while most of *trans*-heterozygous mutant animals were lethal at the pupa stage, a small population of mutant animals were able to pass through the pupa stage but died within 3 days post-eclosion (Figure 3C).

We next determined the role of DMAD in demethylating 6mA in vivo. We prepared genomic DNA from both wild-type and *DMAD*^{1/2} mutant flies and measured the abundance of the 6mA base. As shown in Figure 3D, loss of *DMAD* led to a significant increase in the overall 6mA abundance in genomic DNA. Of note, we found no difference in the abundance of 5mC and 5hmC between the wild-type and *DMAD* mutant flies (Figures 3E, S3A, and S3B), strongly arguing that the *Drosophila* DMAD has no apparent in vivo role in regulating the conversion of 5mC to 5hmC. Moreover, we found that, while N³-methylcytosine (3mC) and O⁶-methylguanine (m6G) were not detectable, N¹-methyladenine (1mA) (below 0.6 adduct per million dA) and N³-methyladenosine (3mA) (about 2 adducts per million dA) were present at low levels in both wild-type and *DMAD* mutant flies, and no difference in relative abundance of 1mA and 3mA bases was detected between wild-type and *DMAD* mutant flies (Figure S3C; see Discussion). Additionally, we failed to detect any apparent difference in levels of m6A abundance in RNA between wild-type and *DMAD* mutant flies (Figure S3D). These results together suggest that the DMAD specifically suppresses the in vivo modification of 6mA, rather than 5mC and other methylated DNA bases tested in this study, and 6mA in RNA.

We then sought to determine the functional requirements of the conserved domains (Figure S3F) in the DMAD protein by generating specific domain-deletion alleles. To do so, we designed two additional sgRNAs and attempted to use the Cas9/sgRNA technique to locally produce truncated proteins of the DMAD (Figure 3F). According to the experimental design, we successfully obtained two new *DMAD* alleles, *DMAD*^{del-CXXC} and *DMAD*^{del-CD}. These two alleles encode putative truncated proteins, in which the CXXC domain and the catalytic domain were deleted in DMAD, respectively (Figure 3F and Figure S3E).

Our genetic experiments showed that *DMAD*^{del-CXXC} homozygous mutant animals are viable and fertile and that the *DMAD*^{del-CXXC} allele is able to complement both *DMAD*¹ and *DMAD*² alleles (Figure 3C). In addition, UHPLC-MRM-MS/MS assays showed that the CXXC domain deletion did not cause significant change in 6mA abundance in DNA between *DMAD*^{del-CXXC} homozygous and wild-type flies (Figure 3G). Of note, in our western blot assays, we found that wild-type flies also expressed a similar size protein as present in *DMAD*^{del-CXXC} homogote (Figure S3E). Taken together, these results suggested that the CXXC domain is dispensable for the

(F) Expression levels of *DMAD* at different embryonic stages as measured by qRT-PCR.

(G) Expression levels of DMAD protein at different embryonic stages measured by western blot assays.

(H) Nuclear extracts treated with anti-DMAD or IgG or without treatment were used in in vitro 6mA demethylation assays.

(I) Relative expression levels of *DMAD* in embryos treated with dsRNA against *DMAD* or *gfp* were measured by qRT-PCR.

(J) Nuclear extracts from embryos treated with dsRNA against *DMAD* or *gfp* were used in in vitro 6mA DNA demethylation assays.

The experiments were carried out by triplicates, and the standard deviations were calculated by Excel. See also Figure S2.

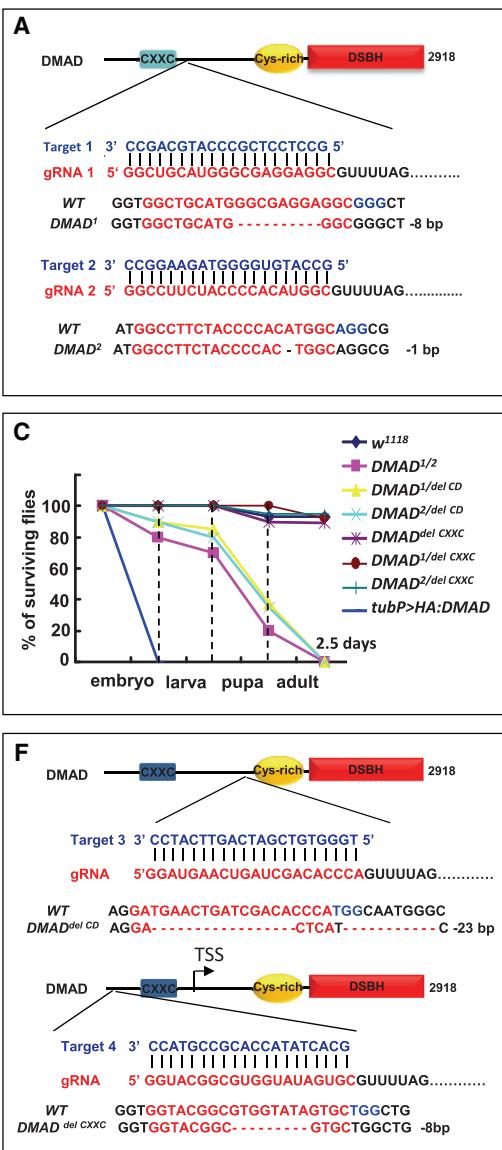


Figure 3. DMAD Is Required for *Drosophila* Development

(A) Schematic representation of DMAD mutant allele generation using the CRISPR/Cas system. The primer sequences of sgRNAs and information for DMAD¹ and DMAD² are indicated.

(B) Western blot experiments showed that the DMAD protein was completely abolished in the DMAD¹ and DMAD² allelic backgrounds.

(C) Survival rates of wild-type, different DMAD mutant, or overexpression flies at different developmental stages indicated were measured.

(D and E) Abundance of 6mA (D) and 5mC (E) in DNA from wild-type and DMAD^{1/2} mutant flies was measured by mass spectrometry.

(F) Schematic representation for the generation of mutant alleles of DMAD using the CRISPR/Cas system. The primer sequences of sgRNAs and information for DMAD^{del-CXXC} and DMAD^{del-CD} are provided.

(G and H) Abundance of 6mA in DNA from wild-type, DMAD^{del-CXXC} (G) and DMAD^{2/del-CD} (H) mutant flies was measured by mass spectrometry. The experiments were carried out by triplicates, and the standard deviations were calculated by Excel. See also Figure S3.

driver, *tub-gal4*, at 29°C caused lethality at the late embryonic stage, since embryos ($n = 735$) expressing DMAD completely failed to develop to the larva stage. However, relative low levels of DMAD expression by the Gal4/Gal80ts system (data not shown) permitted ~28% ($n = 810$) of the DMAD expression embryos to develop to the larva stage. Interestingly, when we induced the expression of the DMAD at the 10 hr embryonic stage by taking advantage of the temperature-dependent activity of Gal80ts (see Extended Experimental Procedures), we found that ~49% of DMAD-expressing embryos ($n = 530$)

could develop to the larva stage. Thus, our findings suggested that the DMAD expression must be under tight control during embryonic development.

DMAD Promotes Differentiation of Early Germ Cells in *Drosophila*

We next explored the potential roles of the DMAD in tissue homeostasis. The *Drosophila* ovary offers an excellent model system to study a number of important biological processes, such as germline stem cell (GSC) regulation, oocyte determination, and epigenetic control (Lin, 2002; Ohlstein et al., 2004; Spradling et al., 2001). A wild-type female contains a pair of ovaries, each of which is composed of 16–20 ovarioles that consist of an anterior functional unit (called “germarium”) and a linear string of differentiated egg chambers (Figures S4A and S4B). In the tip of germarium, GSCs divide asymmetrically to

role of DMAD in development and in suppressing 6mA modification. By contrast, the DMAD^{del-CD} completely failed to complement either the DMAD¹ or DMAD² allele. The trans-heterozygous mutant DMAD^{del-CD}/DMAD² and DMAD^{del-CD}/DMAD¹ displayed strong developmental defects (Figure 3C). Interestingly, we found that the levels of 6mA modification were also increased in the trans-heterozygous mutant background that carried DMAD^{del-CD}, compared to wild-type (Figure 3H). These results together suggested that the catalytic domain is essential for the role of DMAD in development and in suppressing 6mA modification in vivo.

Additionally, we also examined the phenotypes in animals with ectopic expression of the DMAD by generating the transgenic flies, P{UASp-HA:DMAD}, in which the HA-tagged full-length DMAD was placed under the control of the UASp promoter. As shown in Figure 3C, overexpression of DMAD by the ubiquitous

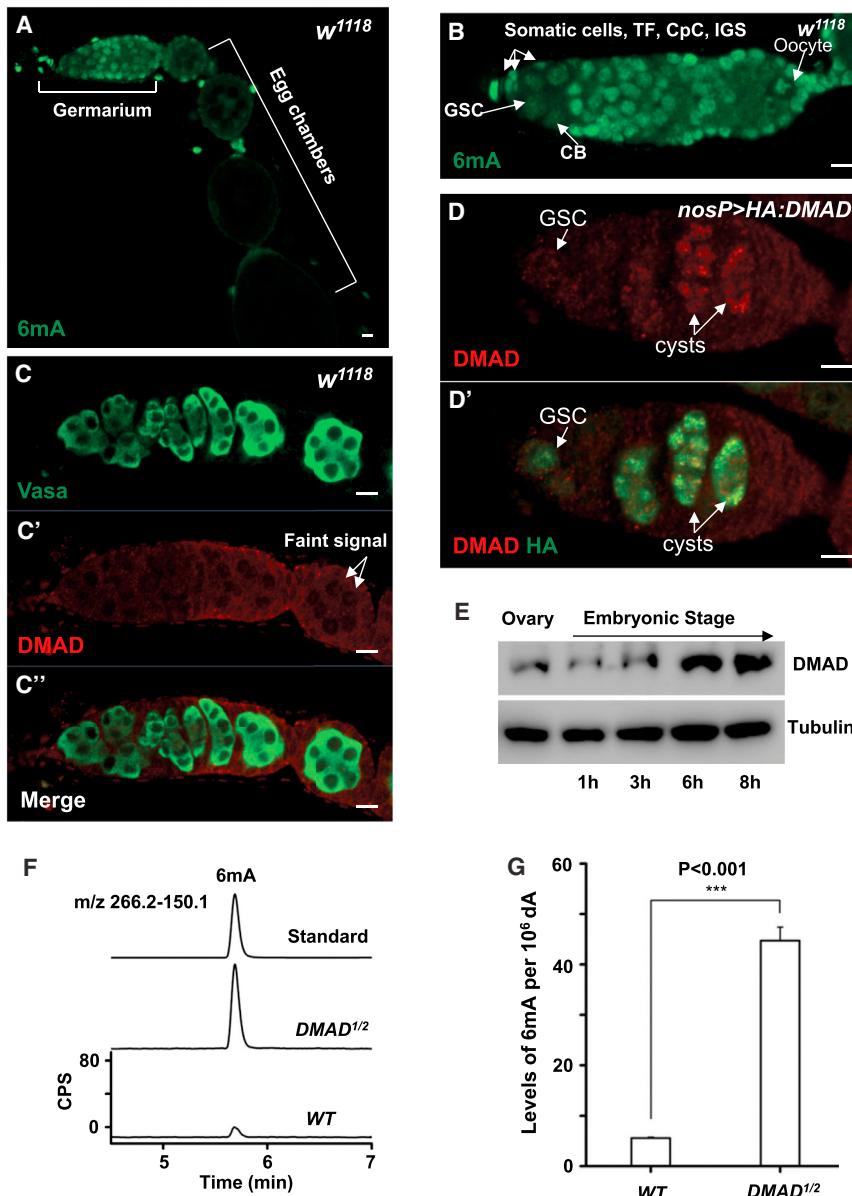


Figure 4. DMAD and 6mA Patterns in the *Drosophila* Ovary

(A and B) Ovaries from wild-type flies were stained with anti-6mA antibody. (A) shows that 6mA signal is highly expressed in the germarium region and becomes gradually reduced and ultimately disappears in germ cells of late egg chambers. (B) indicates that 6mA marks both germ cells and somatic cells. Scale bar, 10 µm.

(C–C') Ovaries from wild-type flies were stained with anti-DMAD and anti-Vasa antibodies. Weak signal of anti-DMAD was indicated in the nucleus of egg chamber nurse cells. Scale bar, 10 µm.

(D and D') Ovaries from *P{nosP-ga4vp16}/P{UASp-HA:DMAD}* flies were stained with anti-DMAD and anti-HA antibodies. Overlapping signal of DMAD and HA was detected in germ cell nuclei in germaria. Scale bar, 10 µm.

(E) Western blot assays show the levels of DMAD protein expression during different stages of embryonic development and in ovary.

(F and G) UHPLC-MRM-MS/MS chromatograms (F) and quantification (G) showing 6mA abundance in genomic DNA from wild-type and *DMAD^{1/2}* mutant ovaries.

The experiments were carried out by triplicates, and the standard deviations were calculated by Excel. See also Figure S4.

produce two daughters. The anterior daughter cell retains contact with the cap cells as a new stem cell, whereas the posterior differentiating daughter cell becomes a cystoblast (CB) (Figures S4C and S4D). The CB further divides four times with incomplete cytokinesis, resulting in a cyst that sustains oogenesis (Figure S4D). To address whether DMAD has a role in germline, we performed immunostaining experiments to investigate the patterns of 6mA in the ovary. As shown in Figures 4A and 4B, a striking 6mA staining signal was detected in the nucleus of gerarium cells, including germ cells and somatic cells (Figure 4B). In contrast, the 6mA signal was gradually reduced with development and ultimately disappeared in germ cells of mature differentiated egg chambers (Figure 4A), suggesting that 6mA modification occurs in the germ cell in a developmentally regulated fashion. We then determined DMAD expression

in the gerarium using the anti-DMAD antibody. As shown in Figures 4C–4C'', we detected no nuclear staining of the DMAD in gerarium germ cells, but a faint signal was present in the nucleus in egg chambers. To test whether the faint signal from the anti-DMAD antibody was specific, we performed further immunostaining in the gerarium for ectopic expression of the HA-tagged DMAD. As shown in Figures 4D–4D'' and S4E–S4E'', overlapping staining signals of HA with DMAD in the nucleus of germ cells in both gerarium and egg chambers were readily detected. These findings together

suggest that DMAD expression occurs at a low level in the ovary. In support of this, our western blot assays showed that the DMAD protein expression was maintained at low levels in the ovary as compared with in the embryo (Figure 4E). We then tested whether DMAD has a role in regulating 6mA modification in ovaries and found that loss of *DMAD* resulted in an ~10-fold increase in levels of 6mA in ovaries (Figures 4F and 4G).

To test whether DMAD-mediated 6mA modification has a role in regulating early germ cell development, we examined the germ cell behavior in *DMAD* mutant ovaries by performing immunostaining assays using anti-Vasa and anti-Hts antibodies, which were used to visualize germ cells and fusomes, respectively. As shown in Figures 5A–5E, a newly enclosed (1-day-old) wild-type gerarium normally contained 3–4 GSCs/CBs,

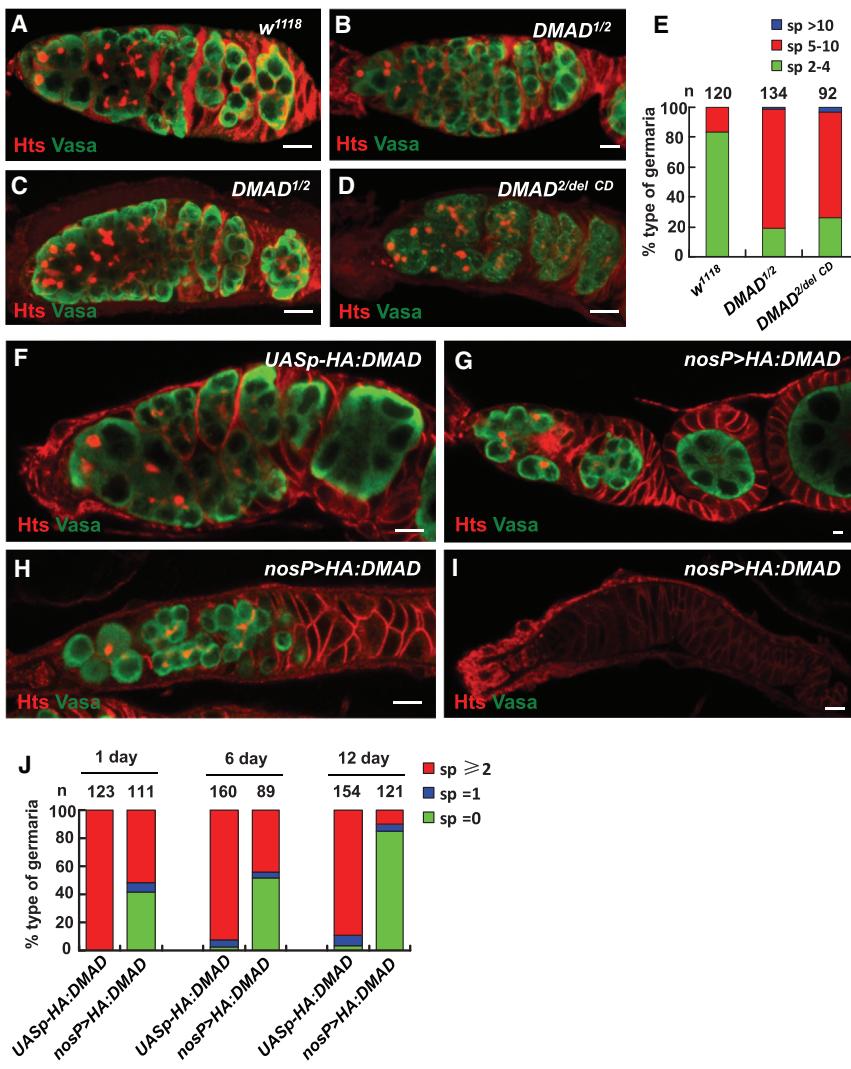


Figure 5. DMAD Promotes Early Germ Cell Differentiation

(A–D) Ovaries from wild-type (A) and different *DMAD* mutant flies as indicated were stained with anti-Hts (Red) and anti-Vasa (Green) antibodies. Scale bar, 10 μ m.

(E) Quantification assay showing percentage of type of germaria in wild-type and different *DMAD* mutant ovaries corresponding to (A–D). The types of germaria were classified according to the number of spectrosome (Sp)-containing germ cells in each germarium.

(F–I) Ovaries from wild-type and P{*nosP-gal4*:*vp16*}/P{*UASp-HA:DMAD*} transgenic flies were stained with anti-Hts (Red) and anti-Vasa (Green) antibodies. Scale bar, 10 μ m.

(J) Quantification assay showing percentage of type of germaria in wild-type and P{*nosP-gal4*:*vp16*}/P{*UASp-HA:DMAD*} ovaries corresponding to (F–I).

See also Figure S5.

whereas the 1-day-old *DMAD* mutant had an average of >8 spectrosome-containing germ cells (GSC-like cells) (Figures 5C and 5D), suggesting that *DMAD* plays a role in promoting early germ cell differentiation. We next overexpressed *DMAD* to examine the phenotype in germ cells by generating flies carrying a transgene combination, P{*UASp-HA:DMAD*} and P{*nosP-gal4*:*vp16*}, in which *nosP-gal4*:*vp16* is a germ-cell-specific driver. As shown in Figures 5F–5J, S5A, and S5B, overexpression of *DMAD* led to a significant loss of germ cells, including GSCs, supporting that *DMAD* plays a role in promoting GSC differentiation.

DMAD Directly Catalyzes Demethylation of 6mA

To elucidate the biochemical properties of *DMAD*, we asked whether *DMAD* directly catalyzes 6mA demethylation by performing in vitro demethylation activity assays using the ovarian nuclear extracts from wild-type and *DMAD* mutants. As shown in Figure 6B, while wild-type ovarian nuclear extract has considerable enzymatic activity for 6mA demethylation, *DMAD* mutant nuclear extracts almost completely failed to

support the in vitro 6mA demethylation reaction. In contrast, nuclear extracts from *DMAD* mutant ovaries with addition of the purified *DMAD*-CD protein, but not *DMAD*-CD^{mut} protein, resulted in striking enzymatic activity for 6mA demethylation (Figures 6A and 6B). As shown in Figure 6B, ~46% of 6mA bases in the substrates were demethylated, compared with only ~20% having demethylated 6mA in the control reaction with the addition of AlkB or with nuclear extracts from wild-type ovaries. Thus, our findings support that *DMAD* is essential for 6mA demethylation in *Drosophila*.

We next tested whether *DMAD* has a similar role in other tissues. The fly brain represents another interesting and complementary model to study the *DMAD*-mediated 6mA modification due to two reasons. First, *DMAD* is expressed at a much higher level in the fly brains than in ovaries (Figures 6C and 6D). Second, levels of 6mA are also relatively low in the brain when compared with very early-stage embryos (Figures 1C and 1E). We measured abundance of 6mA in brain genome from wild-type and *DMAD* mutant brain tissue, respectively. Strikingly, we found that loss of *DMAD* resulted in up to a 100-fold increase in 6mA levels in brain (Figures 6E and 6F). Additionally, similar to ovary, nuclear extracts from *DMAD* mutant brain with addition of the purified *DMAD*-CD protein exhibited a considerable 6mA demethylation activity (Figure S6A). Collectively, our findings reveal that *DMAD* plays a critical role in the regulation of 6mA demethylation in *Drosophila*.

Up to 100-fold increases of 6mA abundance in *DMAD* mutant tissues raised a possibility that potential 6mA methylases catalyze 6mA methylation in fly DNA. To explore this issue, we employed nuclear extracts from wild-type and

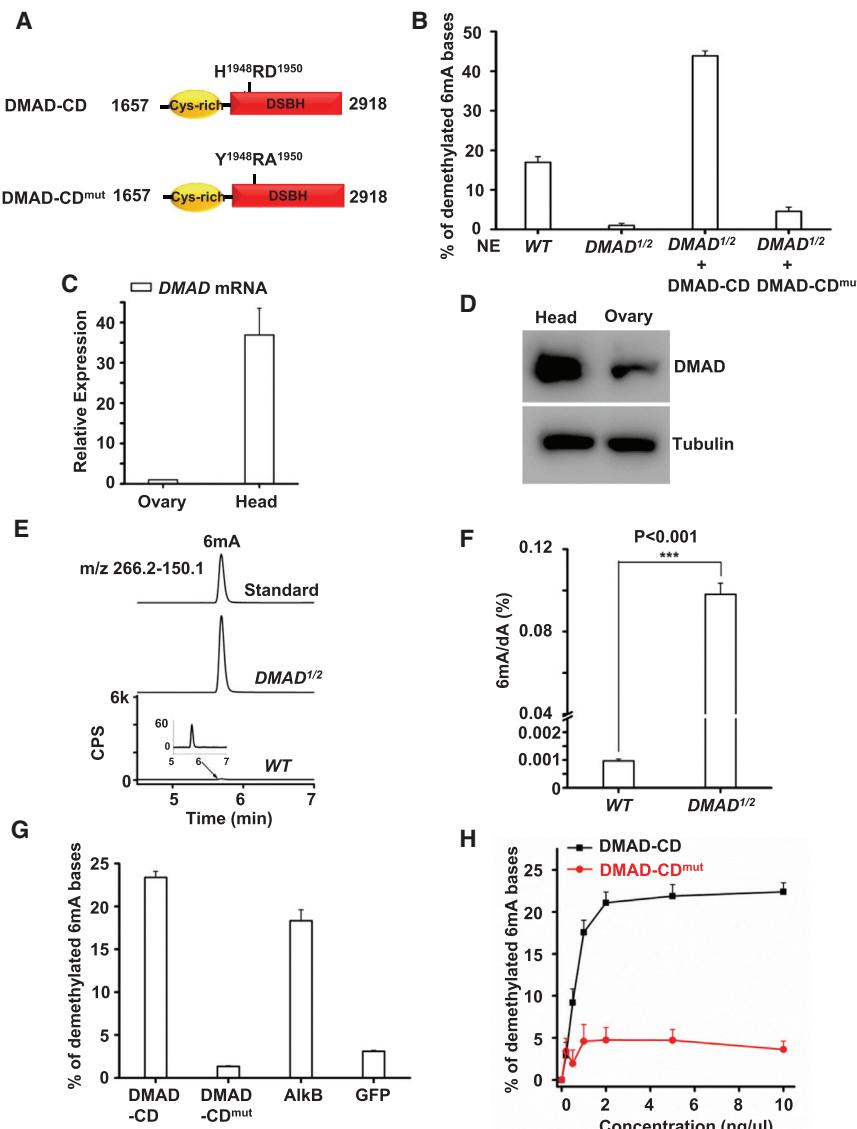


Figure 6. DMAD Directly Catalyzes Demethylation of 6mA

(A) Schematic diagram of the DMAD catalytic domain (DMAD-CD) fragment (aa. 1657–2918) and its mutant (DMAD-CD^{mut}), in which two residues, H1948 and D1950, were mutated to Y and A, respectively. These two Fe(II)-binding sites are located in the highly conserved “H-R/K/Q-D” motif, which has been shown to be important for the catalytic activity in the family of AlkB-like Fe(II)/ α -ketoglutarate-dependent dioxygenases.

(B) The in vitro 6mA demethylation assays were performed to test enzymatic activity in wild-type ovary nuclear extracts, DMAD mutant ovary nuclear extracts without or with addition of the DMAD-CD, or DMAD-CD^{mut} protein as indicated. (C and D) Levels of DMAD mRNA (C) and protein expression (D) in ovary and brain were measured by qRT-PCR and western blot assays, respectively.

(E and F) UHPLC-MRM-MS/MS chromatograms (E) and quantification (F) showing 6mA abundance in genomic DNA from wild-type and DMAD^{1/2} mutant brains.

(G) Comparison for the enzymatic activity of DMAD with its CD mutant form, AlkB, in the in vitro 6mA demethylation assays.

(H) An in vitro enzymatic assay showing that the DMAD protein directly catalyzes the 6mA demethylation in a concentration-dependent manner. The experiments were carried out by triplicates, and the standard deviations were calculated by Excel. See also Figure S6.

DMAD-Mediated 6mA Demethylation Is Correlated with Transposon Expression

We next sought to test whether DMAD influences 6mA modification of the *Drosophila* genome. We collected genomic DNA from 1-day-old wild-type and DMAD mutant ovaries and performed DNA immunoprecipitation (DNA-IP) experiments using anti-6mA antibody

and then generated DNA libraries, which were subjected to a high-throughput sequencing analysis. In this assay, the IgG-immunoprecipitated DNA from an equivalent amount of wild-type ovaries was used as the control, and 4.2–5.5 million reads were obtained through high-throughput sequencing. We then used MACS software (2.0 version, Zhang et al., 2008) to identify the 6mA-enriched regions. In sum, we identified 161 and 491 peaks from wild-type and DMAD mutant samples, respectively (Figure 7A). 88% of peaks identified in wild-type are also identified in DMAD mutant sample, while 73% of peaks in DMAD mutant sample were unique (Figure 7A). As shown in Figures 7B and 7C, signal of 6mA was stronger in DMAD mutant sample than wild-type sample with respect to both common peaks and DMAD mutant unique peaks, providing further evidence that 6mA demethylation is regulated by DMAD.

DMAD mutant flies to perform in vitro 6mA methylation assays. As shown in Figures S6B and S6C, wild-type nuclear extracts showed a weak enzymatic activity for 6mA methylation, and DMAD mutant nuclear extracts exhibited relatively high 6mA methylation activities. These findings suggest that potential 6mA methylases and DMAD constitute an antagonistic loop to control 6mA base modification. Nevertheless, our findings suggest that demethylation activity of DMAD plays a predominant role in maintaining low levels of 6mA in genome. To determine whether DMAD directly participates in 6mA demethylation, we performed in vitro DNA demethylation assays. As shown in Figures 6G, 6H, and S6D, the purified catalytic domain of DMAD (DMAD-CD), but not its dead form of DMAD (DMAD-CD^{mut}), is sufficient to promote 6mA demethylation, suggesting that DMAD is the *Drosophila* 6mA demethylase.

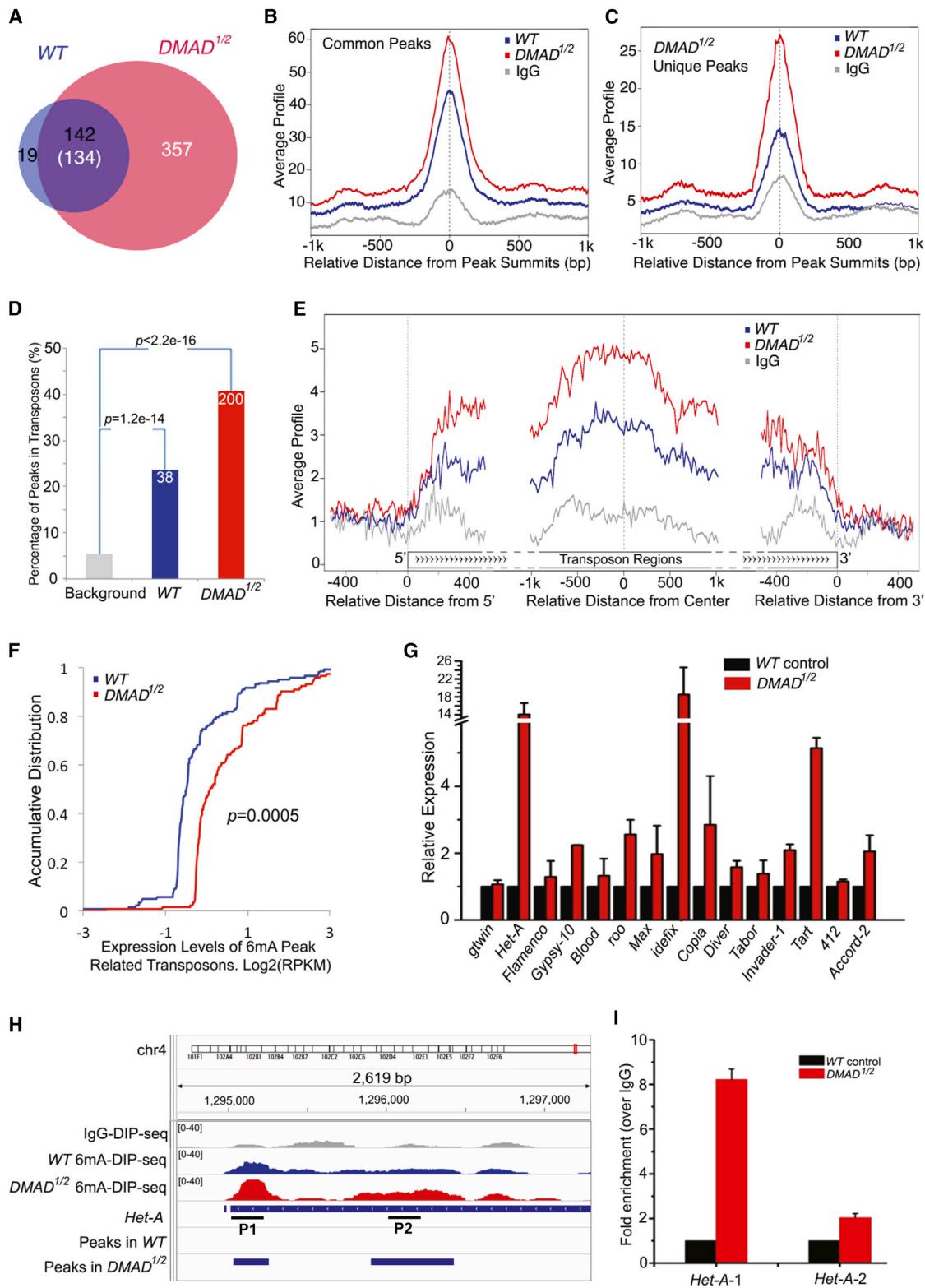


Figure 7. DMAD Controls 6mA Modification on Genome and Transposon Silencing

(A) 6mA enrichment peaks identified from wild-type and *DMAD* mutant ovary samples. A significant portion of peaks are shared by wild-type and *DMAD* mutant ovary samples.

(legend continued on next page)

Of note, our immunostaining assays revealed an evident expansion of γ -H2Av expression domain in late meiosis in *DMAD* mutant germlaria, compared with the wild-type control (Figures S5C–S5D'). This phenomenon is also present in *ago3* mutant germlaria (Huang et al., 2014). We thus reasoned that *DMAD* might be involved in transposon silencing by regulating 6mA modification. Indeed, we found that 24% and 41% of peaks from wild-type and *DMAD* mutants are located in the transposon regions, respectively (Figure 7D), indicating that the transposon sequence is the important 6mA-modified target by *DMAD* in the genome. Additionally, we found that the 6mA signal was much more enriched in the gene body of transposons when compared with the upstream and downstream regions (Figure 7E). To link the 6mA modification with transposon expression, we employed wild-type and *DMAD* mutant samples to perform RNA-seq analysis. Global expression profiling analysis revealed that transposons with 6mA peaks express significantly higher in *DMAD* mutant ovary than do those in wild-type (Figure 7F).

We next performed qRT-PCR assays and measured levels of transposon transcripts in wild-type and *DMAD* mutant ovaries, respectively. As shown in Figure 7G, loss of *DMAD* led to an increase in levels of most of the transposon transcripts that we chose to evaluate. Particularly, *Idefix*, *Het-A*, *Tart*, and *Copia* were significantly increased in the *DMAD* mutant, compared with the control. Importantly, the 6mA DNA immunoprecipitation followed by qPCR assays further confirmed that more 6mA modification occurs on the transposon regions in *DMAD* mutant ovaries than in wild-type ovaries (Figures 7H, 7I, and S7A–S7D). Taken together, our findings suggest that *DMAD*-mediated 6mA demethylation is correlated with transposon expression.

DISCUSSION

In this study, we find that 6mA is present in the *Drosophila* DNA at a relatively high level at the very earliest embryonic stages but at low levels at the late embryonic stages. Moreover, we show that the dynamic change of 6mA modification during embryonic development may involve an active demethylation event, a process that is primarily regulated by the *Drosophila* *DMAD* protein. Importantly, *DMAD* is essential for *Drosophila* development and tissue homeostasis, perhaps partially by suppressing adenine methylation and transposon expression in ovary. Thus, our study suggests a potential role

of the *DMAD*-6mA regulatory axis in controlling development in higher eukaryotes.

Adenine Methylation in *Drosophila*

To date, studies examining 6mA as a biologically relevant, methylated DNA base have mainly been limited to bacteria, although it is well known that 6mA is also present in the genomic DNA of several unicellular eukaryotes (Wion and Casadesús, 2006). Previous studies have reported that, while 5mC is enriched in genomes of many higher eukaryotes, particularly mammals, a signal for 6mA has not been detected (Ratele et al., 2006). Because the important function of 5mC modification in mammals has attracted much interest in the field of the epigenetic control, the issue of whether adenine methylation occurs in general and its related roles in higher eukaryote DNA has remained largely unresolved. The previous failure to detect 6mA in higher-eukaryote DNA could be that its level is so low in eukaryotes that it was undetectable with the technology used in previous reports (Lawley et al., 1972; Vanyushin et al., 1970). However, adenine methylation might occur in a tissue-specific or in a developmentally regulated manner in higher-eukaryote cells (Raddatz et al., 2013), and low levels of 6mA could be controlled by a tight negative regulatory mechanism mediated by 6mA demethylases. Thus, searching for specific 6mA demethylases is important for understanding the potential role of adenine methylation in higher-eukaryote cells. In this study, we show that the *Drosophila* *DMAD* directly catalyzes 6mA demethylation in our biochemical assays, suggesting that it is likely an 6mA demethylase. Moreover, our functional assays show that loss of *DMAD* leads to strong developmental defects and significantly increases the abundance of 6mA modification in DNA. These findings bring an insight into understanding the potential function of 6mA modification in development and tissue homeostasis in higher eukaryotes. In this study, we find that *DMAD*-mediated 6mA demethylation is correlated with transposon suppression in ovary, indicating that 6mA modification as an epigenetic mark likely regulates gene expression. However, the possibility of other *DMAD*-mediated processes contributing to normal development cannot be completely ruled out and warrants further investigation.

The discovery that loss of *DMAD* causes a dramatic increase of 6mA abundance in adult tissues opens an interesting possibility of the existence of potential 6mA methylases in flies for DNA 6mA methylation. Our in vitro enzymatic assays revealed that *Drosophila* nuclear extracts possess both methylation and

(B and C) The average 6mA signal strength of all common peaks (B) and *DMAD* mutant unique peaks (C). 6mA signal was stronger in *DMAD* ovary mutant sample than in wild-type ovary sample.

(D) Percentage of 6mA enrichment peaks located in transposon regions. 6mA peaks were significantly located in transposon regions.

(E) The average 6mA signal strength on all 6mA peak-related transposons. The 6mA signal was enriched in transposon body.

(F) Accumulative distribution of expression levels of 6mA peak-related transposons in wild-type and *DMAD* mutant ovary samples. The p value represent Wilcoxon rank sum test.

(G) qRT-PCR experiments were performed to measure the transposon expression levels in wild-type control and *DMAD* mutant ovary.

(H) The 6mA-enriched regions are found in the *Het-A* transposon region in the indicated chromosome, in which *DMAD* mutant samples show higher enrichment when compared with that of wild-type samples.

(I) qPCR experiments were performed to confirm the 6mA-enriched regions indicated in (H) when *DMAD* mutant samples were compared with that of wild-type. In this assay, the corresponding regions IPed by IgG were used for normalization. The experiments were carried out by triplicates, and the standard deviations were calculated by Excel. See also Figure S7.

demethylation enzymatic activities for 6mA base modification. Thus, it is likely that the potential 6mA methylase(s) and DMAD act antagonistically to maintain the proper modification of 6mA in flies. It would be interesting to identify specific 6mA methylases in the future.

Another question is whether DMAD is involved in the DNA damage process and repair DNA methylation lesions. In this study, we have measured the levels of 1mA, 3mC, 3mA, and m6G because 1mA and 3mC are predominant forms of base damage in single-stranded DNA, and 3mA and m6G are products in double-stranded DNA damage (Lindahl et al., 1988; Trewick et al., 2002). Our results revealed that loss of DMAD did not cause apparent increase in levels of these bases. Additionally, methyl iodide treatment did not cause apparent upregulation of 6mA levels but led to a dramatic increase in levels of m6G and 3mC in fly genomic DNA (Figures S3G–S3I). Thus, our findings strongly argue that 6mA comes from enzymatic installation rather than as a byproduct of DNA damage.

The Role of DMAD in 6mA Demethylation in *Drosophila*

The controversy over 6mA in mammalian DNA is similar to that of 5mC modification in *Drosophila* and has been discussed for a long time (Raddatz et al., 2013). A recent work suggested that the *Drosophila* genome lacks a defined 5mC pattern (Raddatz et al., 2013). In this study, we find that no evidence supports oxidation of 5mC in *Drosophila*. Although DMAD can catalyze the 5mC oxidation in *in vitro* enzymatic reactions, the *in vivo* studies revealed no difference in levels of 5mC and 5hmC detected in DNA from the wild-type and *DMAD* mutant flies, revealing that the DMAD has no role in catalyzing 5mC oxidation *in vivo*.

A broadly accepted concept is that DNA base modification through methylation plays evolutionarily conserved epigenetic roles in a wide array of organisms from bacteria to animals, although the underlying mechanisms might be different among species (Wion and Casadesús, 2006). From an evolutionary perspective, since its DNA is not methylated at cytosine, *Drosophila* likely uses other types of methylated bases, such as 6mA, to fulfill the function of 5mC in mammals. The discovery that DMAD possesses enzymatic activity for 6mA demethylation, as well as the identification of its role in suppressing 6mA modification *in vivo*, support that DMAD functions as an 6mA demethylase in *Drosophila*.

All AlkB family members contain a core catalytic domain called the double-stranded β-helix (DSBH) fold (Shen et al., 2014). Our results suggest that the DSBH domain in DMAD is essential for its function in regulating 6mA demethylation in flies. It would be interesting to search for DSBH-domain-containing dioxygenases responsible for 6mA demethylation in mammals in the future. Members of Tet family proteins, without a doubt, are attractive candidates.

EXPERIMENTAL PROCEDURES

Drosophila Strains

Fly stocks used in this study were maintained under standard culture conditions. The *w¹¹¹⁸* strain was used as the host for all P-element-mediated transformations. Strains P{tubP-gal80ts}, P{tubP-gal4}, and P{nosP-gal4:vp16}

have been maintained in the Chen lab. P{UASp-HA:DMAD} was generated in this study; DMAD¹, DMAD², DMAD^{del-CXXC}, and DMAD^{del-CD} were generated by the CRISPR/Cas system in this study. See the [Extended Experimental Procedures](#) for a more detailed protocol for generation of DMAD null alleles using CRISPR/Cas system.

Immunohistochemistry

Ovaries were prepared for immunohistochemistry as described previously (Yang et al., 2007). See the [Extended Experimental Procedures](#) for a more detailed protocol.

Gene Knockdown in *Drosophila* Embryos

The dsRNA fragments corresponding to DMAD and gfp mRNAs were synthesized in a PCR reaction and then fused to the T7 RNA polymerase binding site at both 5' and 3' ends, which were used to generate the DMAD and gfp dsRNA *in vitro* by using the RiboMAX Large Scale RNA kit (Promega) following the manufacturer's instructions. The DMAD or gfp dsRNA (1 μg/μl) was injected into *w¹¹¹⁸* embryos. The embryos were incubated at room temperature for turnover of the target protein.

Purification of Nuclear Extracts and Genomic DNA

Nuclear extracts were extracted from embryos or adult tissues using Minute Cytoplasmic and Nuclear Extraction Kit (Invent Biotech). Genomic DNA was extracted with Wizard genomic DNA purification Kit (Promega) following the manufacturer's instructions.

Anti-DMAD Antibodies

The anti-DMAD antibody was generated by immunizing rabbit and mouse with the recombinant protein GST-DMAD (amino acids 959–1108) produced in *E. coli*.

Immunodepletion Experiments

For immunodepletion experiments, 10 μl of protein A/G beads were mixed with 200 μl of hypotonic buffer (plus 0.02% CHAPS, 0.1 mM PMSF). This solution was mixed with 10 μg rabbit anti-DMAD antibody or rabbit IgG and was rotated for about 1 hr using a head-to-tail roller at 4°C. Embryonic nuclear extracts were obtained as described above and were subjected to immunoprecipitation using protein A/G beads treated with antibodies at 4°C for 2 hr. Subsequently, samples were centrifuged and supernatants were collected and used for *in vitro* 6mA demethylation assays.

Quantitative Real-Time PCR Analysis

qRT-PCR experiments were performed as described previously (Huang et al., 2014). See the [Extended Experimental Procedures](#) for a more detailed protocol.

Dot Blot Assay

Different amounts of standard DNA either containing the base dA or 6mA and fly genomic DNA were used for dot blot assay. See the [Extended Experimental Procedures](#) for a more detailed protocol.

UHPLC-MRM-MS/MS Analysis

Genomic DNA was enzymatically digested into single nucleosides by a mixture of DNaseI, calf intestinal phosphatase, and snake venom phosphodiesterase I at 37°C for 12 hr. After the enzymes were removed by ultrafiltration, the digested DNA was subjected to UHPLC-MS/MS analysis (Yin et al., 2013). HPLC fractionation of *Drosophila* 6mA and UHPLC-QTOF-MS/MS analysis are shown in the [Extended Experimental Procedures](#) with a more detailed protocol.

In Vitro 6mA Demethylation

Calf thymus (CT) dsDNA was methylated by Dam methyltransferase following the manufacturer's instructions. The detailed protocol for 6mA demethylation can be seen in the [Extended Experimental Procedures](#).

Statistical Analysis

Data are presented as the mean +SEM from at least three independent experiments. Student's t test was used for comparison of two independent groups. For all tests, a p < 0.05 was considered statistically significant.

ACCESSION NUMBERS

All sequencing data are available at NCBI SRA under accession number SRA: SRP055483.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2015.04.018>.

AUTHOR CONTRIBUTIONS

G.Z. generated reagents (KO and transgenic flies and antibodies) and performed genetic and molecular biology experiments. H.H. and G.Z. performed enzymatic assays, and H.H. performed all mass-spec analysis. D.L. and G.Z. constructed libraries for the analysis of DNA-IP-seq and RNA-seq. S.H. performed all bioinformatics.

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REFERENCES

- Bhutani, N., Burns, D.M., and Blau, H.M. (2011). DNA demethylation dynamics. *Cell* **146**, 866–872.
- Cheng, X. (1995). Structure and function of DNA methyltransferases. *Annu. Rev. Biophys. Biomol. Struct.* **24**, 293–318.
- Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., and Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823.
- Feng, S., Jacobsen, S.E., and Reik, W. (2010). Epigenetic reprogramming in plant and animal development. *Science* **330**, 622–627.
- He, Y.F., Li, B.Z., Li, Z., Liu, P., Wang, Y., Tang, Q., Ding, J., Jia, Y., Chen, Z., Li, L., et al. (2011). Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science* **333**, 1303–1307.
- Huang, H., Li, Y., Szulwach, K.E., Zhang, G., Jin, P., and Chen, D. (2014). AGO3 Slicer activity regulates mitochondria-nuage localization of Armitage and piRNA amplification. *J. Cell Biol.* **206**, 217–230.
- Ito, S., Shen, L., Dai, Q., Wu, S.C., Collins, L.B., Swenberg, J.A., He, C., and Zhang, Y. (2011). Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* **333**, 1300–1303.
- Julio, S.M., Heithoff, D.M., Provenzano, D., Klose, K.E., Sinsheimer, R.L., Low, D.A., and Mahan, M.J. (2001). DNA adenine methylase is essential for viability and plays a role in the pathogenesis of *Yersinia pseudotuberculosis* and *Vibrio cholerae*. *Infect. Immun.* **69**, 7610–7615.
- Kriaucionis, S., and Heintz, N. (2009). The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science* **324**, 929–930.
- Lawley, P.D., Crathorn, A.R., Shah, S.A., and Smith, B.A. (1972). Bio-methylation of deoxyribonucleic acid in cultured human tumour cells (HeLa). Methylated bases other than 5-methylcytosine not detected. *Biochem. J.* **128**, 133–138.
- Li, D., Delaney, J.C., Page, C.M., Yang, X., Chen, A.S., Wong, C., Drennan, C.L., and Essigmann, J.M. (2012). Exocyclic carbons adjacent to the N6 of adenine are targets for oxidation by the *Escherichia coli* adaptive response protein AlkB. *J. Am. Chem. Soc.* **134**, 8896–8901.
- Lin, H. (2002). The stem-cell niche theory: lessons from flies. *Nat. Rev. Genet.* **3**, 931–940.
- Lindahl, T., Sedgwick, B., Sekiguchi, M., and Nakabeppu, Y. (1988). Regulation and expression of the adaptive response to alkylating agents. *Annu. Rev. Biochem.* **57**, 133–157.
- Lyko, F., and Maleszka, R. (2011). Insects as innovative models for functional studies of DNA methylation. *Trends Genet.* **27**, 127–131.
- Lyko, F., Ramsahoye, B.H., and Jaenisch, R. (2000). DNA methylation in *Drosophila melanogaster*. *Nature* **408**, 538–540.
- Maiti, A., and Drohat, A.C. (2011). Thymine DNA glycosylase can rapidly excise 5-formylcytosine and 5-carboxylcytosine: potential implications for active demethylation of CpG sites. *J. Biol. Chem.* **286**, 35334–35338.
- Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., and Church, G.M. (2013). RNA-guided human genome engineering via Cas9. *Science* **339**, 823–826.
- Ohlstein, B., Kai, T., Decotto, E., and Spradling, A. (2004). The stem cell niche: theme and variations. *Curr. Opin. Cell Biol.* **16**, 693–699.
- Raddatz, G., Guzzardo, P.M., Olova, N., Fantappié, M.R., Rampp, M., Schaefer, M., Reik, W., Hannon, G.J., and Lyko, F. (2013). Dnmt2-dependent methylomes lack defined DNA methylation patterns. *Proc. Natl. Acad. Sci. USA* **110**, 8627–8631.
- Ratel, D., Ravanat, J.L., Berger, F., and Wion, D. (2006). N6-methyladenine: the other methylated base of DNA. *BioEssays* **28**, 309–315.
- Reisenauer, A., Kahng, L.S., McCollum, S., and Shapiro, L. (1999). Bacterial DNA methylation: a cell cycle regulator? *J. Bacteriol.* **181**, 5135–5139.
- Shen, L., Song, C.X., He, C., and Zhang, Y. (2014). Mechanism and function of oxidative reversal of DNA and RNA methylation. *Annu. Rev. Biochem.* **83**, 585–614.
- Smith, Z.D., and Meissner, A. (2013). DNA methylation: roles in mammalian development. *Nat. Rev. Genet.* **14**, 204–220.
- Spradling, A., Drummond-Barbosa, D., and Kai, T. (2001). Stem cells find their niche. *Nature* **414**, 98–104.
- Stephens, C., Reisenauer, A., Wright, R., and Shapiro, L. (1996). A cell cycle-regulated bacterial DNA methyltransferase is essential for viability. *Proc. Natl. Acad. Sci. USA* **93**, 1210–1214.
- Tahiliani, M., Koh, K.P., Shen, Y., Pastor, W.A., Bandukwala, H., Brudno, Y., Agarwal, S., Iyer, L.M., Liu, D.R., Aravind, L., and Rao, A. (2009). Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* **324**, 930–935.
- Trewick, S.C., Henshaw, T.F., Hausinger, R.P., Lindahl, T., and Sedgwick, B. (2002). Oxidative demethylation by *Escherichia coli* AlkB directly reverts DNA base damage. *Nature* **419**, 174–178.
- Vanyushin, B.F., Tkacheva, S.G., and Belozersky, A.N. (1970). Rare bases in animal DNA. *Nature* **225**, 948–949.
- Wion, D., and Casadesús, J. (2006). N6-methyl-adenine: an epigenetic signal for DNA-protein interactions. *Nat. Rev. Microbiol.* **4**, 183–192.
- Wright, R., Stephens, C., and Shapiro, L. (1997). The CcrM DNA methyltransferase is widespread in the alpha subdivision of proteobacteria, and its

- essential functions are conserved in *Rhizobium meliloti* and *Caulobacter crescentus*. *J. Bacteriol.* 179, 5869–5877.
- Wu, H., and Zhang, Y. (2014). Reversing DNA methylation: mechanisms, genomics, and biological functions. *Cell* 156, 45–68.
- Yang, L., Chen, D., Duan, R., Xia, L., Wang, J., Qurashi, A., Jin, P., and Chen, D. (2007). Argonaute 1 regulates the fate of germline stem cells in *Drosophila*. *Development* 134, 4265–4272.
- Yin, R., Mao, S.Q., Zhao, B., Chong, Z., Yang, Y., Zhao, C., Zhang, D., Huang, H., Gao, J., Li, Z., et al. (2013). Ascorbic acid enhances Tet-mediated 5-methylcytosine oxidation and promotes DNA demethylation in mammals. *J. Am. Chem. Soc.* 135, 10396–10403.
- Zhang, Y., Liu, T., Meyer, C.A., Eeckhoute, J., Johnson, D.S., Bernstein, B.E., Nusbaum, C., Myers, R.M., Brown, M., Li, W., et al. (2008). Model-based analysis of ChIP-Seq (MACS). *Genome Biol.* 9, R137.