H3K36 Trimethylation-Mediated Epigenetic Regulation is Activated by Bam and Promotes Germ Cell Differentiation During Early Oogenesis in Drosophila

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ABSTRACT
Epigenetic silencing is critical for maintaining germline stem cells in Drosophila ovaries. However, it remains unclear how the differentiation factor, Bag-of-marbles (Bam), counteracts transcriptional silencing. We found that the trimethylation of lysine 36 on histone H3 (H3K36me3), a modification that is associated with gene activation, is enhanced in Bam-expressing cells. H3K36me3 levels were reduced in flies deficient in Bam. Inactivation of the Set2 methyltransferase, which confers the H3K36me3 modification, in germline cells markedly reduced H3K36me3 and impaired differentiation. Genetic analyses revealed that Set2 acts downstream of Bam. Furthermore, orb expression, which is required for germ cell differentiation, was activated by Set2, probably through direct H3K36me3 modification of the orb locus. Our data indicate that H3K36me3-mediated epigenetic regulation is activated by bam, and that this modification facilitates germ cell differentiation, probably through transcriptional activation. This work provides a novel link between Bam and epigenetic transcriptional control.

KEY WORDS: Drosophila, Germ cell, Differentiation factor, Histone modification, Transcriptional control

INTRODUCTION
Post-translational modifications to core histone proteins are proposed to regulate essential cellular functions, including transcriptional activation and repression. For instance, histone H3 methylation at lysine 4 (H3K4) and at lysine 36 (H3K36) are usually associated with gene activation, whereas methylation of lysine 9 (H3K9) and lysine 27 (H3K27) are associated with gene repression. Several histone modifications play fundamental roles in the maintenance of embryonic stem cells, particularly with respect to their developmental potential (Bloushtain-Qimron et al., 2009; Jiang et al., 2011); histone modifications are also associated with the maintenance of stem cells in adult tissues (Buszczak et al., 2009).

In the adult Drosophila ovary, the germline stem cells (GSCs) at the tip of the germline are maintained in their niche. After GSC division, the daughter cell that is displaced from the niche becomes a cystoblast, and subsequently differentiates into a 16-cell cyst interconnected by the branched fusome: 1 germ cell develops into the oocyte and the other 15 germ cells form nurse cells. The bone morphogenetic protein (BMP)-like molecules produced from the niche maintain GSCs by directly repressing bag-of-marbles (bam), which encodes a key differentiation factor (Ohlstein and McKearin, 1997; Chen and McKearin, 2003b). When a cystoblast exits the niche, the Bam produced in the cystoblast antagonizes the Nanos/Pumilio translational repressor complex to promote differentiation (Li et al., 2009). In addition to BMP signalling, epigenetic silencing is essential for GSC maintenance. The functions of scrawny and eggless, both of which encode histone-modifying enzymes that are associated with gene silencing, are required for GSC maintenance (Buszczak et al., 2009; Wang et al., 2011). However, the mechanisms by which epigenetic regulation promotes differentiation, and by which Bam counteracts gene silencing remain unclear. We found that the levels of trimethylation of H3K36 (H3K36me3) in cystoblasts were enhanced by Set2 methyltransferase. Set2 acted downstream of bam and promoted differentiation. Furthermore, Set2 activated orb expression, which is required for cyst differentiation. Our results indicate that H3K36me3 in cystoblasts is developmentally controlled by bam, and that this modification facilitates cystoblast differentiation, probably through transcriptional activation.

MATERIALS AND METHODS

Fly stocks
The wild-type strain used was Oregon-R. Set21/FM7 was a gift from Dr. M. Kuroda. bam10/TM3 was a gift from Dr. D. M. McKearin. orbdec/+; UAS-Set2IR, v111 P(FRT)101, P(ubi-GFP FRT101), P(MKRS, hs-FLP 86E), and P(hs-Gal4) were obtained from the Bloomington Stock Center. A2BP1C25661 was obtained from the Drosophila Genetic Resource Center. UAS-Set2 RNAi (106459) was obtained from the Vienna Drosophila RNAi Center (VDRC). All stocks were maintained at 25°C or at room temperature in standard Drosophila medium unless otherwise noted.

Immunohistochemistry
Immunostaining was carried out as described (Mukai et al., 2011). Monoclonal antibodies specific for H3K4me1 (CMA301), H3K4me2...
RESULTS AND DISCUSSION

H3K36me3 is associated with cystoblast differentiation

To examine histone modifications in differentiating germ cells, we stained wild-type ovaries using monoclonal antibodies specific for histone modifications (Fig. 1; Kimura et al., 2008). We found that the H3K36me3 histone modification associated with active genes expressed in differentiating cystoblasts (Fig. 1F,G). H3K36me3 signals were increased in the differentiating cystoblasts that expressed the bam reporter gene ([bam-GFP]; Chen and McKearin, 2003a) (Fig. 1H). By contrast, the H3K27me3 modification associated with gene repression accumulated in early germ cells, and its signals decreased as the cells differentiated (Fig. 1E,I). These results suggest that the H3K36me3 levels were upregulated in differentiating cystoblasts. Next, we examined H3K36me3 levels in the ovaries of the third instar larvae and bam86 mutant adult females, both of which contain undifferentiated germ cells. Although H3K27me3 signals were detected in these undifferentiated germ cells, we did not detect strong H3K36me3 signals (Fig. 1J–M). Taken together, these data supported the idea that H3K36me3-mediated epigenetic regulation may be involved in germ cell differentiation.

Set2 is required for both H3K36me3 accumulation and cyst formation

Set2 methyltransferase is responsible for the H3K36me3 modification (Larschan et al., 2007; Stabell et al., 2007). Immunostaining revealed that, in the germarium region, Set2

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**Phenotypic analysis of ovaries**

Clones of mutant cells were generated by FLP-mediated mitotic recombination as described (Mukai et al., 2011). We introduced Set2λ into the chromosome carrying FRT by meiotic recombination. We generated Set2− germline clones by using the Set2λ FRT chromosome. Control germline clones were generated using FRT chromosomes without the mutation. For RNAi knockdown of Set2, nanos-Gal4/+; UAS-Set2IR/+ females were cultured at 30˚C up to adulthood. nanos-Gal4/+ females raised at 30˚C served as controls. Ovaries were processed for immunostaining. A ChIP assay was performed using the wild-type and bam86 mutant ovaries as described (Baxley et al., 2011). Immunoprecipitation was performed using 1 μg of antibody. As a control, normal mouse IgG (Jackson ImmunoResearch Laboratories) was used. Anti-H3K36me3, anti-H3K4me3 (Kimura et al., 2008), and anti-RNA polymerase II (8WG16; Covance) antibodies were used for the ChIP assays. Input DNA, mock-precipitated DNA, and DNA from the ChIP assays were analyzed by PCR. Quantitative PCR analyses were performed using GeneAce SYBR qPCR Mix (Nippon Gene). The sequences of the primers used for the ChIP assays are listed in supplementary material Table S1.
was expressed in most of the germline cells, and that nuclear Set2 levels increased in differentiating cystoblasts (Fig. 2A). To determine whether Set2 participates in H3K36me3 accumulation and differentiation, we inhibited Set2 expression by using an UAS-Set2.IR line (Stabell et al., 2007). Set2 levels in germ cells were reduced by the expression of Set2 RNAi (supplementary material Fig. S1). Specifically, while Set2 signals in differentiating cystoblasts were detected in 100% of control (nanos-Gal4/+; germline clones. The arrow in H indicates fragmented fusomes.

To address whether bam is sufficient for H3K36me3 accumulation, we examined H3K36me3 levels in the ovaries carrying the hs-bam transgene, which is used to ectopically express bam by heat shock treatment (Ohlstin and McKearin, 1997). No GSCs with a strong H3K36me3 signal were observed in germline from wild-type females 1 hour post-heat shock (PHS; n = 42). However, H3K36me3 levels in the GSCs were significantly increased in 3% in Set2I/+/bam86/+/ females (n = 134) females (supplementary material Fig. S3). These data prompted us to explore the mechanism of regulation of Set2 activity by bam.

To determine whether bam expression requires Set2 activity, we examined bam expression in Set2I/- germine clones by immunostaining. Indeed, Set2 activity in germ cells was dispensable for bam expression (supplementary material Fig. S2). Conversely, nuclear Set2 expression in the germ cells was significantly reduced by bam mutation, suggesting that bam is involved in the regulation of Set2 in these cells (Fig. 3D,E). This result is consistent with the observation that H3K36me3 levels were reduced by bam mutation. Moreover, reducing of bam activity by introducing of a single copy of bamI86 dominantly increased the number of germaria with weaker H3K36me3 signals in Set2I/+ flies. Decreased H3K36me3 signals in the cytoblasts were observed in 29% of germaria from the Set2I/+; bam86I/+ females (n = 157), as compared to 3% in Set2I/+/ females (n = 117) and 2% in bam86I/+ females (n = 134) females (supplementary material Fig. S3). These data prompted us to explore the mechanism of regulation of Set2 activity by bam.

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**Fig. 2. Set2 is required for H3K36me3 accumulation and cyst formation.** (A) An ovariole was double-stained for Set2 (magenta) and Vas (green). (A') Set2 channel is shown alone. Nuclear Set2 levels increased in differentiating cystoblasts (arrows). (B,C) Control (nos-Gal4/+ (B) and nos-Gal4>UAS-Set2.IR (C) ovarioles were double-stained for H3K36me3 (magenta) and Vas (green). (B',C') H3K36me3 channel is shown separately. (D,E) nos-Gal4/+ (D) and nos-Gal4>UAS-Set2.IR (E) ovarioles were double-stained for 1B1 (magenta), which labels spectrosome (arrowheads) and fusome (arrows), and Vas (green). (D',E') 1B1 channel is shown separately. (F–H) Ovarioles containing control (F) and Set2I/- females (G,H) were double-stained for H3K36me3 (F,G, magenta), or 1B1 (H, magenta) and GFP (green). (F'–H') GFP channel is shown separately. (F',G'). H3K36me3 channel is shown separately. (H') 1B1 channel is shown alone. An absence of GFP marks the clones. The arrow in H indicates fragmented fusomes.
differentiation induced by bam. When bam+ was ectopically expressed by heat shock, GSC differentiation was induced as previously reported (Ohlstein and McKearin, 1997). In 71% of ovaries from hs-bam flies dissected 24 hours PHS, we found that differentiating cysts, instead of GSCs, occupied the tip of germaria (n=79; Fig. 3H). By contrast, when both bam and Set2 RNAi were ectopically expressed, GSC loss was significantly suppressed (19.6%, n=189; P<0.02) (Fig. 3LJ). These data suggest that Set2 activity is regulated by Bam, and that Set2 acts downstream of bam and promotes differentiation.

We found that nuclear Set2 levels were increased in differentiating cystoblasts (Fig. 2A). Furthermore, nuclear Set2 levels in germ cells were reduced by bam mutation (Fig. 3E). We speculated that bam may regulate Set2 nuclear localization. Therefore, we examined whether bam expression is sufficient for Set2 nuclear accumulation. We investigated the subcellular localization of Set2 in hs-bam flies cultured at 30°C (see Materials and Methods). First, we examined H3K36me3 levels in the GSCs. H3K36me3 levels in GSCs were increased in 36% of the germaria from the hs-bam females (n=84), as compared to 6% in wild-type females (n=79, P<0.01; supplementary material Fig. S4A,B). This result suggests that the ectopic expression of bam is sufficient for H3K36me3 accumulation. Next, we investigated Set2 subcellular localization in GSCs of hs-bam females cultured at 30°C. Nuclear Set2 levels in GSCs were increased in 54% of the germaria from the hs-bam females (n=65), as compared to 12% in wild-type females (n=79, P<0.01; supplementary material Fig. S4C,D). These results suggest that bam promotes the nuclear accumulation of Set2.

**Set2 function is required for the proper activation of orb expression in cysts**

To understand the mechanism by which Set2 regulates germ cell differentiation, we analyzed the genetic interaction between Set2 and the differentiation genes A2BP1 and orb, both of which are required for cyst differentiation (Lantz et al., 1994; Tastan et al., 2010). Reduction of Set2 activity by introduction of a single dose of Set21+ dominantly increased the number of germaria exhibiting a differentiation defect in orb dec+ flies (Fig. 4A–C). In 24% of germaria from the Set2+/+; orb dec females, fragmented fusomes were observed (n=132), as compared with 4% in orb dec+/+ females (n=73) and 7% in Set2+/+ females (n=106). By contrast, the reduction of Set2 activity did not significantly affect cyst formation in A2BP1 K06465+ ovaries (data not shown). These results implied that Set2 function is required to specifically regulate orb expression and promote cyst formation. To confirm this, we examined orb expression in Set2– cyst clones. Deletion of Set2 led to the delayed activation of orb. Although 74% of the control cyst clones located at the boundary of germarium regions 1 and 2a initiated orb expression (n=50), only 31% of Set2– cyst clones expressed orb (Fig. 4D,E; n=62, P<0.001). Most (61%) of the Set2– cyst clones in germarium region 2b recovered orb expression (n=62). These observations suggest that Set2 was required for the proper activation of orb in differentiating cysts. Next, we investigated the H3K36me3 state of the orb locus in the ovaries. ChIP assays demonstrated that the H3K36me3 enrichment in the 3′-UTR region of orb was significantly higher than in the 5′-UTR region (Fig. 5B–D). It has been reported that the H3K36me3 modification exhibits a 3′-bias, such that H3K36me3 is preferentially enriched at the 3′ regions of actively transcribed genes (Larschan et al., 2007; Barski et al., 2007). Our results support the idea that orb expression in differentiating cysts is controlled in part by H3K36me3-mediated epigenetic regulation.

Next, we investigated the H3K36me3 status in the orb gene in bam66 mutant ovaries. ChIP assays showed that bam mutation reduced the amount of H3K36me3 in the 3′-UTR region of the orb gene (Fig. 5D). The H3K36me3 modification is linked to transcriptional elongation (Krogan et al., 2003). Therefore, our results suggested that bam activates orb expression through the...
epigenetic control. Additionally, H3K4me3 and RNA polymerase II levels in the 5'UTR region of the orb gene were also reduced by bam mutation (Fig. 5D), implying a role for bam in transcriptional initiation. To investigate this possibility, further investigation will be needed in order to identify the enzymes responsible for H3K4me3 and exploring the interactions between bam and those enzymes.

Our results showed that H3K36me3 levels are regulated by bam. As a cytoplasmic protein, Bam may indirectly regulate Set2 nuclear localization. Set2 exerts its functions through the interactions with cofactors (Fuchs et al., 2012). Understanding the mechanism by which Bam regulates Set2 will require the identification of the cofactors that mediate the nuclear transport of Set2. Our data suggest a link between Bam and epigenetic transcriptional control. Bam may counteract epigenetic silencing in GSCs through H3K36me3-mediated epigenetic regulation. We show that orb expression is activated by epigenetic regulation. Because orb encodes a cytoplasmic polyadenylation element-binding protein, Orb may control translation in differentiating cysts in a polyadenylation-associated manner. Bam antagonizes the Nanos/Pumilio complex, which suppresses the translation of target mRNAs that encode differentiation factors (Li et al., 2009).

Fig. 4. Set2 is required for the proper activation of orb expression in cysts. (A–C) Ovarioles from orb<sup>+</sup>/+ (A), Set2<sup>+</sup>/+ (B) and Set2<sup>−</sup>/+; orb<sup>+</sup>/+ (C) were double-stained for 1B1 (magenta) and Vas (green). (A–C) 1B1 channel is shown separately. Arrows (A',B') indicate branched fusomes. An arrowhead (C') indicates fragmented fusomes. (D,E) Ovarioles containing control (D) and Set2<sup>−</sup> clones (E) were double-stained for Orb (magenta) and GFP (green). (D',E') GFP channel is shown alone. The Orb signal is reduced in the Set2<sup>−</sup> cyst (dotted line in E).

Fig. 5. bam is required for H3K36me3 enrichment in the 3'-UTR region of orb. (A) Schematic representation of the orb locus. (B,C) The H3K36me3 modification is detected in the 5'- and 3'-UTRs of the orb gene by PCR (B) and quantitative real-time PCR (C). (C) Wild-type ovaries were used for a ChIP assay. Input DNA, mock-precipitated DNA, and DNA from the ChIP assay were analyzed by quantitative real-time PCR. Percent input was calculated by using input as standards. Data represent the mean ± s.d. The significance was calculated by comparing the values detected at the 5'- or 3'-UTRs (P<0.05; analysis of variance). (D) The levels of H3K36me3 and H3K4me3 modifications and RNA polymerase II (Pol2) detected in the orb gene 5'- and 3'-UTRs by quantitative real-time PCR. Ovaries dissected from wild-type and bam<sup>−</sup> mutant flies were used for the ChIP assay. The values are expressed as a fold increase relative to the IgG control. The significance was calculated by comparing the values obtained using wild-type and bam<sup>−</sup> mutant ovaries (P<0.05; analysis of variance). All ChIP assays were performed in 3 biological replicates.
However, the identity of the target mRNAs and the mechanisms for transcriptional activation have not yet been elucidated. Because Set2 is required for bam-induced GSC differentiation, studies focused on identifying the genes marked by H3K36me3 and on their epigenetic regulation will aid in the identification of the differentiation genes. Because Set2 is linked to transcriptional elongation (Krogan et al., 2003), differentiation genes in GSCs might be poised for expression, but may be kept awaiting bam expression for full activation. We anticipate that our results will facilitate a better understanding of the epigenetic mechanisms that regulate gametogenesis.

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Competing interests
The authors have no competing interests to declare.

Author contributions
M.M., S.H., H.K. and S.K. designed the experiments, M.M., S.H. and M.S. analyzed data, M.M., S.H. and S.K. wrote the paper.

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