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dBre1/dSet1-dependent pathway for histone H3K4 trimethylation has essential roles in controlling germline stem cell maintenance and germ cell differentiation in the *Drosophila* ovary

Tao Xuan, Tianchi Xin, Jie He, Jieqiong Tan, Yin Gao, Shiyun Feng, Lin He, Gengchun Zhao, Mingfa Li *

MoE Key Laboratory of Developmental Genetics and Neuropsychiatric Diseases, Bio-X Institutes, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, 200240 Shanghai, PR China

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ABSTRACT

The *Drosophila* ovarian germline stem cells (GSCs) constantly experience self-renewal and differentiation, ensuring the female fertility throughout life. The balance between GSC self-renewal and differentiation is exquisitely regulated by the stem cell niche, the stem cells themselves and systemic factors. Increasing evidence has shown that the GSC regulation also involves epigenetic mechanisms including chromatin remodeling and histone modification. Here, we find that dBre1, an E3 ubiquitin ligase, functions in controlling GSC self-renewal and germ cell differentiation via distinct mechanisms. Removal or knock down of dBre1 function in the germline or somatic niche cell lineage leads to a gradual GSC loss and disruption of H3K4 trimethylation in the *Drosophila* ovary. Further studies suggest that the defective GSC maintenance is attributable to compromised BMP signaling emitted from the stem cell niche and impaired adhesion of GSCs to their niche. On the other hand, *dBre1-RNAi* expression in escort cells causes a loss of H3K4 trimethylation and accumulation of spectrosome-containing single germ cells in the germarium. Reducing *dpp* or *dally* levels suppresses the germ cell differentiation defects, indicating that dBre1 limits BMP signaling activities for the differentiation control. Strikingly, all phenotypes observed in *dBre1* mutant ovaries can be mimicked by RNAi-based reduced expression of dSet1, a *Drosophila* H3K4 trimethylase. Moreover, genetic studies favor that *dBre1* interacts with *dSet1* in controlling GSC maintenance and germ cell differentiation. Taken together, we identify a dBre1/dSet1-dependent pathway for the H3K4 methylation involved in the cell fate regulation in the *Drosophila* ovary.

Introduction

Renewal and homeostasis of many adult tissues including hematopoietic system, skin and gut are mainly dependent on stem cells that continuously experience self-renewal and directed differentiation throughout the life of an animal. The adult stem cells are often anchored in specialized microenvironments called niches, and exquisitely regulated by local signals from the niche, intrinsic factors in the stem cells, and systemic factors such as insulin and the steroid hormone (Ables and Drummond-Barbosa, 2010; Hsu and Drummond-Barbosa, 2009, 2011; König et al., 2011; LaFever and Drummond-Barbosa, 2005; Wong et al., 2005). Unraveling how this regulation occurs will gain more insights into the fundamental biological mechanisms governing the tissue maintenance and regeneration.

The *Drosophila* ovary provides an in vivo model system for studying adult stem cell behavior and regulation. Each ovariole, a basic structural unit of the ovaries, consists of the anteriorly located germarium and a string of progressively matured egg chambers. At the anterior tip of each germarium, two to three germline stem cells (GSCs) form a single GSC unit with a number of somatic niche cells such as terminal filament cells (TFs) and cap cells, closely apposed to the stem cells (Fig. 1A) (Lin, 2002; Spradling et al., 2001, 2008). GSCs constantly undergo the asymmetric division by which one daughter cell remaining in contact with the GSC niche retains stem cell identity, whereas the other is displaced away from the niche, acquiring cystoblast (CB) fate. The CBs further divide with incomplete cytokinesis to consecutively produce 2-cell, 4-cell, 8-cell and 16-cell germline cysts (Spradling, 1993). GSCs and the differentiating descendant cells are covered by escort cells (ECs), a group of somatic cells lying adjacent to cap cells (Decotto and Spradling, 2005). While TFs and cap cells constitute the GSC niche, ECs have recently been defined as the germ cell differentiation niche (Kirilly et al., 2011).

* Corresponding author. Fax: +86 21 34205709.
E-mail addresses: mfli@sjtu.edu.cn, mfli64@hotmail.com (M. Li).

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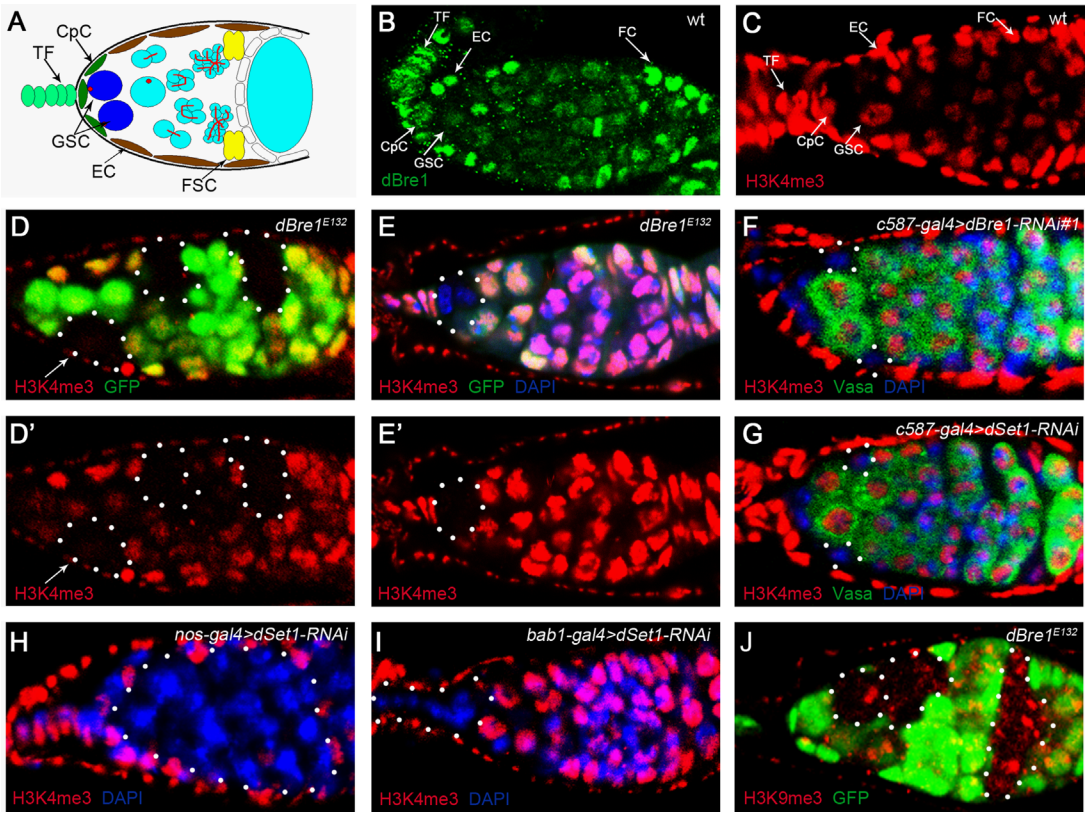

Fig. 1. The bulk histone H3K4 trimethylation in the *Drosophila* ovary is dependent on dBre1 and dSet1: (A) a schematic diagram of wild type germarium with different cell types: germline stem cells (GSCs) (dark blue) and surrounding somatic cells including terminal filaments (TFs) (light green), cap cells (CpCs) (dark green), escort cells (ECs) (brown) and follicle stem cells (FSCs) (yellow). (B–I) Wild type control (B, C) and mutant germaria carrying clones homozygous for $dBre1^{E132}$ (D and E) or expressing *dBre1-RNAi#1* (F) or *dSet1-RNAi* (G–I) under the control of specific *gal4* driver stained for dBre1 (B) or trimethylated H3K4 (C–I). (B) Expression of *dBre1* is evident in almost all cells of germarium, predominantly in TFs, CpCs, ECs and follicle cells (FCs). (C) H3K4me3 staining is ubiquitously present in all cell types. (D and E) H3K4 trimethylation is barely detectable in the germline clones (D, D′) or CpC clones (E, E′) homozygous for $dBre1^{E132}$. Note that the clones are outlined with white dots; arrow indicates GSC. (F–I) Loss of H3K4 trimethylation is evident in the ECs expressing a *dBre1-RNAi#1* (F) or *dSet1-RNAi* (G) transgene. Meantime, similar results are observed in the germ cells (H) or the TFs and CpCs (I) in which *dSet1* is knocked down using the RNAi-based approach. Note that RNAi-expressing cells are marked with broken lines. (J) As a control, H3K9me3 staining is present in the germline clones (broken lines) homozygous for $dBre1^{E132}$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

It is known that the GSC niche plays an instructive role in maintaining self-renewal of the stem cells by preventing their differentiation. Cap cells in the niche produce BMP-like signal molecule Decapentaplegic (Dpp) for activating BMP signaling pathway in GSCs to prevent differentiation via silencing expression of differentiation-promoting genes such as *bag-of-marbles* (*bam*) (Chen and McKearin, 2003a, 2003b; McKearin and Ohlstein, 1995; Rojas-Ríos et al., 2012; Song et al., 2004; Xie and Spradling, 1998). The niche-controlled GSC self-renewal is largely dependent on both niche maintenance and signal output from the niche in the adult ovary. In the case of niche size control, Notch signaling has been shown to be required for maintaining cap cells and thus GSCs in adulthood (Hsu and Drummond-Barbosa, 2011; Song et al., 2007). In parallel, JAK/STAT signaling pathway positively regulates *dpp* expression in cap cells, thereby determining the signal output level for the control of GSC self-renewal (Lopez-Onieva et al., 2008; Wang et al., 2008a). Meanwhile, regulation of BMP signal output can occur in the level of morphogen diffusion that is modulated by *dally*, a glypican-encoding gene. By repressing *dally* transcription, EGFR pathway in ECs acts to limit BMP signaling activities to GSCs in the anterior tip of the germarium (Guo and Wang, 2009; Hayashi et al., 2009; Liu et al., 2010; Schulz et al., 2002). Remarkably, the range of BMP signaling activity precisely determines the balance between GSC self-renewal and differentiation, as exemplified by the differential response of GSCs vs. CBs one cell diameter away from the stem cells to BMP signals (Eliazer et al., 2011; Guo and Wang, 2009; Harris et al., 2011; Hayashi et al., 2009; Liu et al., 2010; Wang et al., 2008b, 2011; Xia et al., 2010, 2012). Besides those extrinsic factors from the niche, intrinsic ones in the stem cells required for responding to niche-derived BMP signals are also essential for GSC maintenance (Chen et al., 2009, 2010; Jiang et al., 2008; Sun et al., 2010; Xi et al., 2005). In addition, DE-cadherin-mediated adhesion between cap cells and GSCs is required for anchoring GSCs in the niche, thus contributing to continuous self-renewal of GSCs (Song et al., 2002).

Like genetic factors, epigenetic mechanisms involving chromatin remodeling and histone modification are equally important for adult stem cell regulation. Increasing evidence has demonstrated that the control of GSC maintenance and differentiation in the *Drosophila* ovary requires epigenetic contributions (Buszczak et al., 2009; Eliazer et al., 2011; Maines et al., 2007; Wang et al., 2011; Xi and Xie, 2005). In one hand, chromatin remodeling factors such as ISWI and Stonewall are essential for maintaining GSC self-renewal cell-autonomously in a BMP/Bam-dependent or -independent manner respectively. On the other hand, Lsd1, a H3K4 demethylase in the *Drosophila* ovary, has recently been shown to promote the germ cell differentiation non-autonomously presumably through repressing *dpp* expression. A more recent study identified *Drosophila* histone H3K9 trimethylase Eggless (Egg) as an essential regulator controlling GSC self-renewal and differentiation in the ovaries. Although GSC regulation at epigenetic level is evident, the underlying mechanisms remain to be further explored.

Drosophila Bre1 (*dBre1*) encodes an E3 ubiquitin ligase required for the monoubiquitination of histone H2B both in vitro and

in vivo (Hwang et al., 2003; Mohan et al., 2010; Wood et al., 2003). This post-translational modification subsequently mediates histone H3K4 or H3K79 trimethylation in a specific cellular context (Bray et al., 2005; Mohan et al., 2010). Further, the dBre1-dependent histone methylation has been linked to cell signaling in development such as Notch and Wnt pathways. In the present study, we investigated the potential role of dBre1 in the oogenesis. Molecular and genetic studies revealed a H3K4 methylation pathway in the ovaries by which dBre1 and dSet1, a *Drosophila* H3K4 trimethyltransferase, control GSC maintenance and germ cell differentiation via distinct mechanisms.

Material and methods

Fly strains and genetics

All *Drosophila* stocks were maintained and crossed at 25 °C according to standard procedures. The Canton S (CS) strain was used as wild type.

The following mutant alleles, transgenes and enhancer trap lines were used in this study: *dBre1*^{E132} and *dBre1*^{P1541} (Bray et al., 2005), *UAS-dBre1-RNAi#2* and *UAS-dBre1-RNAi#3* (Mohan et al., 2010) *UAS-dBre1-RNAi#1* and *UAS-dSet1-RNAi* (Tsinghua Fly Center), *hsFlp; FRT80B,ubi-GFP*, *w;FRT80B,his-RFP* and *nos-gal4.NGT* (Bloomington *Drosophila* Stock Center), *hsFlp;FRT79D,his-GFP/TM3,Sb* (gift from Zhao-hui Wang), *UAS-shotgun* (Sanson and Vincent, 1996), *Dad-lacZ* (Tsuneizumi et al., 1997), *c587-gal4* (Kai and Spradling, 2003), *bamP-GFP* (Chen and McKearin, 2003a), *c587-gal4,UAS-Flp* (Wang et al., 2008a), *bab1-gal4* (Bolívar et al., 2006), *dally*^{gem} (Nakato et al., 1995), *UAS-dally-RNAi* (Vienna *Drosophila* RNAi Center, VDRC), *dpp*^{hr4} (Wharton et al., 1996), *UAS -shmiR-dpp2* (Haley et al., 2008), *Mad*^{12} (Sekelsky et al., 1995).

Genetic mosaics were generated by FLP/FRT-mediated mitotic recombination (Xu and Rubin, 1993). To obtain *dBre1* mutant GSC clones, 2-day-old female flies bearing *hsFlp/+;; FRT80B,ubi-GFP/FRT80B,dBre1*^{E132} or *hsFlp/+;; FRT79D,his-GFP/FRT79D,dBre1*^{P1541} were heat-shocked at 37 °C for 60 min twice per day for consecutive 3 days. *hsFlp/+;; FRT80B, ubi-GFP/FRT80B* or *hsFlp/+;; FRT79D, his-GFP/FRT79D* flies were used as controls respectively. Ovaries were dissected for quantification of GSC clones on days 2, 6, 10, 14 and 18 after induction of heat shock. For generating cap cell clones, we used *c587-gal4*-driven *UAS-FLP* in combination with *FRT80B, dBre1*^{E132} or *FRT79D, dBre1*^{P1541} for cell type specific targeting. Ovaries were dissected for quantification of cap cell clones on days 2, 7 and 14 after eclosion.

RNAi-based knock down experiments were performed by Gal4/UAS binary system (Brand and Perrimon, 1993).

Antibodies and immunofluorescence

Ovary dissections and antibody staining were performed as described elsewhere (Li et al., 2008).

The following primary antibodies were used in this work: mouse anti-α-spectrin [1:20 DSHB 3A9(323 or M10-2)], rat anti-Vasa (1:20 DSHB anti-vasa), rabbit anti-Vasa (1:200 Santa Cruz), mouse anti-Bam (1:20 DSHB Fly Bag-of -Marbles), mouse anti-Lamin C (1:20 DSHB LC28.26), rat anti-DE-cadherin (1:10 DSHB DCAD2), mouse anti-Armadillo (1:20 DSHB N2 7A1 ARMADILLO), mouse anti-βgal (1:10 DSHB 40-1a), rabbit anti-βgal (1:1000 Cappel), rabbit anti-H3K4me3 (1:200 Cell Signaling), rabbit anti-H3K9me3 (1:200 Millipore), rabbit anti-dBre1(1:500 gift from C. P. Verrijzer), rabbit anti-pMad (1:2000 gift from E. Laufer), rabbit anti-phosphorylated ERK1/2 (1:50 Cell Signaling). Secondary antibodies conjugated with Alexa Fluor 488, 546 (Molecular Probes) were used at 1:1000 dilutions. DAPI (Molecular Probes) was used to visualize the nuclei. Confocal images were captured on Zeiss LSM 510 META laser scanning microscope or Leica TCS SP5 laser confocal microscope and processed in Adobe Photoshop.

Quantification for GSCs, cap cells and UGCs

We used anti-Lamin C antibodies for labeling terminal filaments and cap cells in anterior tip of germaria, and differentiated between these two cell types based on their relative position and morphology. GSCs were identified by their juxtaposition to cap cells and the presence of the anteriorly anchored spectrosomes in the cells. The spectrosome-containing single germ cells in the germarium which are located away from terminal filaments and cap cells were classified as UGCs.

Quantitative RT-PCRs

RNA was isolated from bab1-gal4/+,bam^{BG}, bab1-gal4 > dBre1-RNAi#1,bam^{BG}, bab1-gal4 > dSet1-RNAi,bam^{BG}, c587-gal4/+, bam^{BG}, c587-gal4 > dBre1-RNAi#1,bam^{BG} and c587-gal4 > dSet1-RNAi,bam^{BG} mutant ovaries using TRIzol reagents (Invitrogen). 1 μg total RNA was treated with DNase and subjected to a RevertAid first strand cDNA synthesis system (Fermentas) for preparing cDNA. To amplify dpp and dally mRNA, real-time PCR was conducted using 1.5 μl of cDNA as a template with the following conditions: 95 °C for 3 min followed by 40 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °Cfor 30 s with a final cycle at 72 °C for 10 min. Relative mRNA levels were quantified with Mastercycler ep realplex^4 PCR systems (Eppendorf) by using SYBR Green (Invitrogen). These quantitative RT-PCRs were normalized to the internal gene control rp49.

The primers used for amplifying *dpp* and *dally* mRNA are as follows:

dpp forward: 5′- AGCCGATGAAGAAGCTCTACG
dpp reverse: 5′- ATGTCGTAGACAAGCACCTGGTA
dally forward: 5′-TGACTTGCACGAGGACTAC
dally reverse: 5′-TAATACGACTCACTATAGGG
rp49 forward: 5′-TCCTACCAGCTTCAAGATGAC
rp49 reverse: 5′-CACGTTGTGCACCAGGAACT

Statistical analysis

χ^2-test and Mann–Whitney test were used to calculate the *p*-values.

Results

dBre1 and dSet1 are essential for the bulk H3K4 trimethylation of the germ cells and somatic cells in the germaria

Considering that dBre1 mediates the monoubiquitination of histone H2B and indirectly trimethylation of H3K4 or H3K79 in certain *Drosophila* tissues, we sought to determine whether dBre1 has a similar role in the ovaries. To this end, we first examined the expression pattern of *dBre1* in the tissue using specific antibodies raised against dBre1 (van der Knaap et al., 2010). Immuno-staining of the wild type ovaries revealed that *dBre1* is ubiquitously expressed in almost all cell types, predominantly in the somatic cells of the GSC niche, ECs and the epithelial follicle cells (Fig. 1B). We further tested if H3K4 trimethylation in the ovarian cells is dependent on dBre1 function. Wild type ovaries or ovaries bearing *dBre1* mutant clones generated by the FLP/FRT-mediated mitotic recombination in an otherwise heterozygous background were

stained with H3K4 trimethylation-specific antibodies. As shown in Fig. 1C, staining signals for trimethylated H3K4 were present in the nuclei of all cell types. By contrast, H3K4 trimethylation was barely detectable in the germline clones homozygous for *dBre1*^{E132} or *dBre1*^{P1541} located in the germarium (Fig. 1D and D′) (*dBre1*^{E132}: 100%, $n=26$; *dBre1*^{P1541}: 100%, $n=32$). Likewise, loss of H3K4 trimethylation was evident in the GSC niche clone cells mutant for *dBre1* (Fig. 1E and E′) (*dBre1*^{E132}: 100%, $n=35$). Meanwhile, a *dBre1-RNAi* transgene *VALIUM22-dBre1-RNAi* (*dBre1-RNAi#1*) from the Tsinghua Fly Center of China (also available from Bloomington *Drosophila* Stock Center) was employed for this epigenetic assay. Prior to the test, we validated specificity of this transgene in down-regulation of the endogenous *dBre1* expression. For this purpose, we targeted expression of the transgene in posterior compartments of the wing imaginal discs by using *en-gal4* driver and examined *dBre1* expression in the epithelial cells. The indirect immunoflurescence assay showed a remarkable reduction of dBre1 in those cells (Fig. S1, A, A′). A similar reduction was observed in the ovarian follicle cells expressing the *dBre1-RNAi#1* transgene (Fig. S1B, B′), indicating that this RNAi transgene can specifically knock down the endogenous *dBre1*. Consistent with the observations in *dBre1* loss-of-function mutations, expressing this *dBre1-RNAi#1* transgene under the control of *c587-gal4* (Kai and Spradling, 2003) led to the absence of H3K4 trimethylation in the ECs (Fig. 1F) (100%, $n=62$). As a control, loss or down regulation of dBre1 function caused no change in H3K9 trimethylation in the mutant cells (Fig. 1J and data not shown). These data indicate that dBre1 is required cell autonomously for the bulk H3K4 trimethylation of both germ cells and the somatic cells in the germarium.

In *Drosophila*, dSet1 has been shown to be the main H3K4 trimethyltransferase throughout development (Ardehali et al., 2011; Hallson et al., 2012; Mohan et al., 2011). This prompted us to identify if dSet1 has also an essential role in the global H3K4 trimethylation in the ovaries. We chose to examine whether the UAS/GAL4 binary system-based reduced expression of *dSet1* could affect the bulk H3K4 trimethylation in the ovarian cells. For this experiment, a *dSet1-RNAi* transgene *VALIUM20-dSet1-RNAi* (*dSet1-RNAi*) was ordered from Tsinghua Fly Center of China (also available from Bloomington *Drosophila* Stock Center) and molecularly characterized for its targeting specificity. RT-PCR assay revealed that expressing the transgene can significantly down regulate *dSet1* transcription in the 3rd instar larvae, whereas causing no change in the expression of other three *Drosophila* H3K4 trimethylase encoding genes (Fig. S2). After validating the RNAi transgene's on-targeting, we performed immuno-staining of the mutant ovaries in which expression of the *dSet1-RNAi* transgene is driven by either *nos-gal4* or *bab1-gal4* or *c587-gal4*. As predicted, reduced expression of *dSet1* in the germ cells or somatic cells removed the bulk H3K4 trimethylation in a cell-autonomous manner (Fig. 1G–I). Taken together, the bulk H3K4 trimethylation in *Drosophila* ovaries is dependent on activities of both dBre1 and dSet1.

Disruption of dBre1 function in the germ cells causes a defective GSC maintenance

dBre1 mediates the histone modification and is required for cell signaling in certain developmental contexts of *Drosophila*. Given the essential role of dBre1 in the bulk H3K4 trimethylation of the ovarian cells, we asked whether dBre1 and the H3K4 methylation mediated by dBre1 execute regulatory roles during *Drosophila* oogenesis. In the adult ovary, gametogenesis begins with asymmetric cell division of the GSCs located at the anterior tip of the germarium, producing a self-renewing stem cell and a differentiating daughter cell. The self-renewing property of GSCs enables female flies to continuously generate mature eggs throughout life. A number of studies have recently identified a few epigenetic factors involved in GSC maintenance (Ables and Drummond-Barbosa, 2010; Buszczak et al., 2009; Maines et al., 2007; Wang et al., 2011; Xi and Xie, 2005). Therefore, we chose to investigate the potential role of dBre1 in GSC self-renewal. As the mutant flies homozygous for *dBre1* alleles showed lethality at embryonic stages, we employed the well-established clonal analysis for determining whether dBre1 is required cell-autonomously for the maintenance of GSCs (Xie and Spradling, 1998). For this purpose, the *dBre1* mutant GSC clones were generated by the FLP/FRT techniques and the percentage of the marked mutant GSCs were measured at different time points after clonal induction (ACI). The marked GSCs either in the wild type or *dBre1* mutant background were identified by the absence of GFP expression and the presence of an anteriorly anchored spectrosome (Fig. 2A–C). As shown in Fig. 2E, the majority (87.7%) of the marked control GSCs detected 2 days ACI remained unchanged 18 days ACI. By contrast, the rate of *dBre1*^{E132} mutant GSC clones declined rapidly from 36.3% ($n=215$) to 0.97% ($n=206$) during the 18-day period. Similarly, loss of the marked mutant GSCs was observed in another weaker allele of *dBre1*, *dBre1*^{P1541} (Fig. 2E). These data suggested that *dBre1* is essential for maintaining GSCs intrinsically. To validate this observation, we further disrupted dBre1 function in the germ cells by expressing *nos-gal4*-driven *dBre1-RNAi#1* transgene and analyzed if knockdown of the germline *dBre1* leads to a defective GSC maintenance. Consistently, we found that the GSC number per germaruim was dropped from 1.63 ($n=143$) at 2 days after eclosion to 0.73 ($n=156$) at 14 days after eclosion (Fig. 2F–H), albeit the residual GSCs were detectable in one-week old females. The RNAi-based results excluded the possibility that GSC loss observed in the clonal analysis is due to out-competition of the marked *dBre1* mutant GSCs by their neighboring wild type counterparts, providing more evidence indicative of a cell autonomous role of dBre1 in maintaining GSCs during oogenesis.

The failure of GSC maintenance could be caused by either defects in cell proliferation, cell death or aberrant differentiation. To distinguish between these possibilities, we first measured the relative division rate for marked wild type or *dBre1* mutant GSCs respectively according to the standard procedure (Xie and Spradling, 1998). The results showed that the relative division rate for the marked mutant GSCs is comparable with that for the control GSCs which is close to 1.0, suggesting no contribution of defective cell proliferation to the GSC loss induced by removal of dBre1 function in the cells. Next, we tested whether apoptosis contributes to loss of the marked *dBre1* mutant GSCs. TUNEL assay revealed that there is no apoptotic signal in all examined *dBre1* mutant GSC clones ($n=104$) (data not shown). Together with the observation that the lost marked *dBre1* mutant GSCs can develop into differentiated germline cysts both in the germarium and egg chambers (Fig. 2D), these studies favored the idea that loss of the mutant GSCs is induced presumably by precocious differentiation.

As the niche-derived BMP signaling plays an instructive role in GSC self-renewal through modulating the *bam* silencing, we then determined whether the removal of dBre1 function in GSCs could lead to a defect in responding to BMP/Dpp signal from the niche cells. In the wild type germarium, active BMP signaling is restricted to GSCs, as indicated by the presence of high levels of phosphorylated Mad (pMad) in the cells. In our experiments, staining signals for pMad were significantly reduced in the marked *dBre1* mutant GSCs (*dBre1*^{E132}: 65.7%, $n=67$; *dBre1*^{P1541}: 80.9%, $n=47$), while high level of pMad was evident in the neighboring wild type counterparts (Fig. 3A and A′). In the meantime, we obtained similar results by examining expression of the BMP pathway reporter line *Dad-lacZ* in the marked *dBre1* mutant GSCs

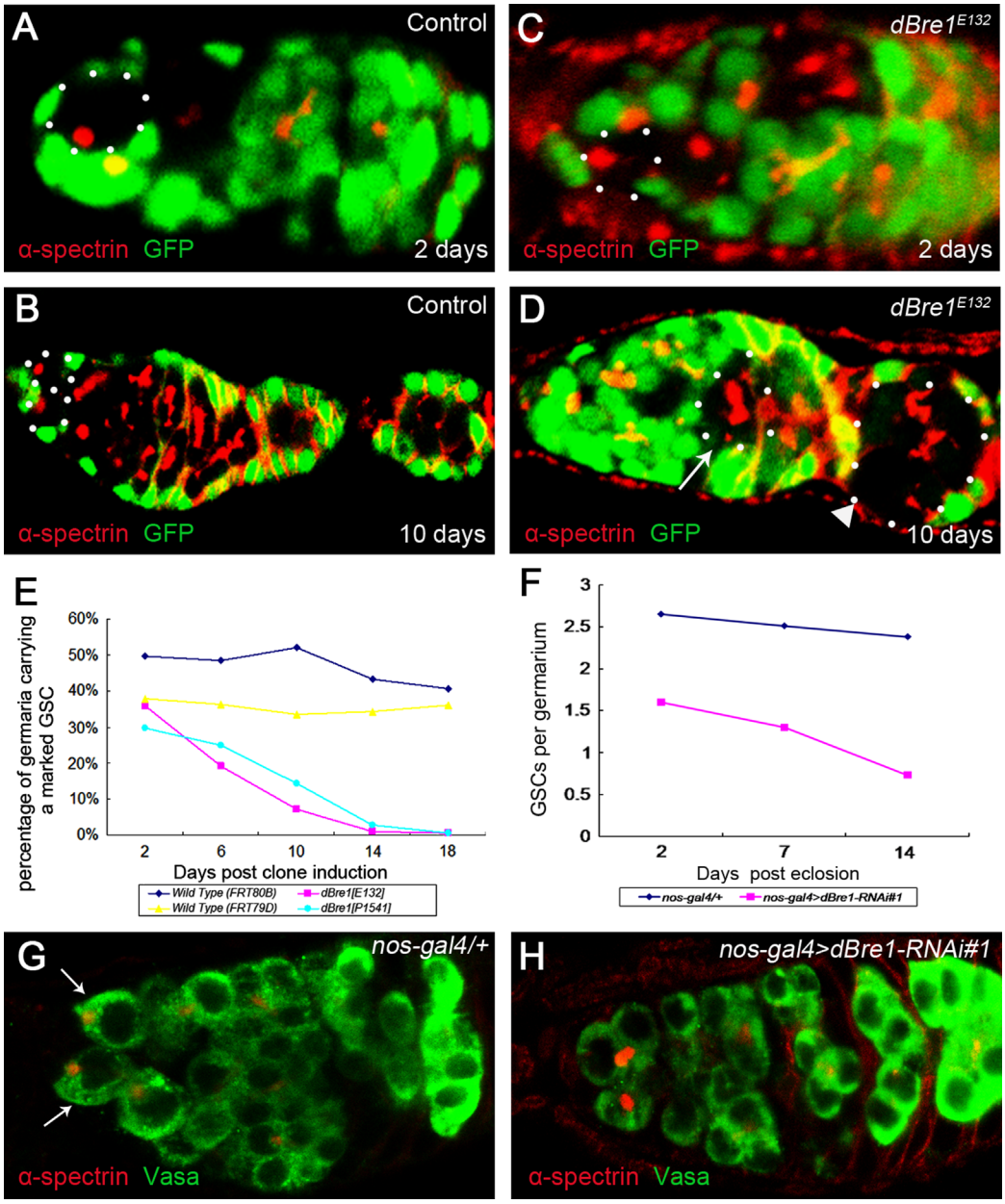

Fig. 2. *dBre1* is required intrinsically for GSC maintenance: (A–D, G, H) The control (A, B, G) and mutant germaria bearing germline clones homozygous for $dBre1^{E132}$ (C, D) labeled by the absence of the nuclear GFP (green) or expressing *dBre1-RNAi#1* (H), stained for α-spectrin (red) (A-D) or α-spectrin and Vasa (G, H). Marked wild type and mutant GSCs (broken lines) are identified by loss of GFP expression and the presence of a spectrosome on their anterior side (A–C). (A, B) Marked GSCs are observed in the germaria of wild type female flies at 2 days (A) or 10 days (B) after clonal induction (ACI). (C, D) A $dBre1^{E132}$ GSC clone (C) is present in the germarium at 2 days ACI, while the mutant GSC clone (D) is lost, as evidenced by the presence of marked germline cysts in the germarium (broken lines and arrow) and egg chamber (broken lines and arrowhead) at 10 days ACI. (E) Graph showing the percentage of negatively GFP-marked GSC clones detected from two *dBre1* null alleles and wild type FRT controls over a 18-day period ACI. (F) Graph showing that a gradual GSC loss is induced by knocking down *dBre1* in the germ cells. (G, H) The germarium expressing *nos-gal4*-driven *dBre1-RNAi#1* contains no GSC (H), while two GSCs (arrows) are present in the control germarium (G). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 3B and B′) ($dBre1^{P1541}$, 72.1%, $n=43$). These observations suggested that loss of dBre1 function compromises BMP signaling activity in the GSCs. To further explore the relationship between dBre1 and BMP signaling in GSC maintenance, we performed genetic interaction studies on *dBre1* and *Mad*. As shown earlier (Fig. 2F), *nos-gal4*-driven expression of *dBre1-RNAi#1* in germ cells caused a remarkable decrease in GSC number per germarium from 2 days to 14 days after eclosion. Strikingly, the GSC number decline was significantly enhanced by introducing one copy of Mad^{12} allele into the *dBre1* knock down background (Fig. 3C). Given that heterozygous Mad^{12}germaria had a constant GSC number during the testing period (Fig. 3C), we proposed that *dBre1* interacts genetically with *Mad* in maintaining GSCs.

Considering that *bam* silencing in GSCs is dependent on active BMP signaling, we further tested if reduced BMP signaling due to lack of dBre1 function causes de-repression of *bam* in GSCs. As shown in Fig. 3D and D′, the absence of *bam* expression was evident in the marked *dBre1* mutant GSCs, suggesting that dBre1 is dispensable for BMP signaling-mediated *bam* repression in GSCs.

In sum, we found that dBre1 is required intrinsically for active BMP signaling in GSCs, hence functioning in the control of GSC self-renewal.

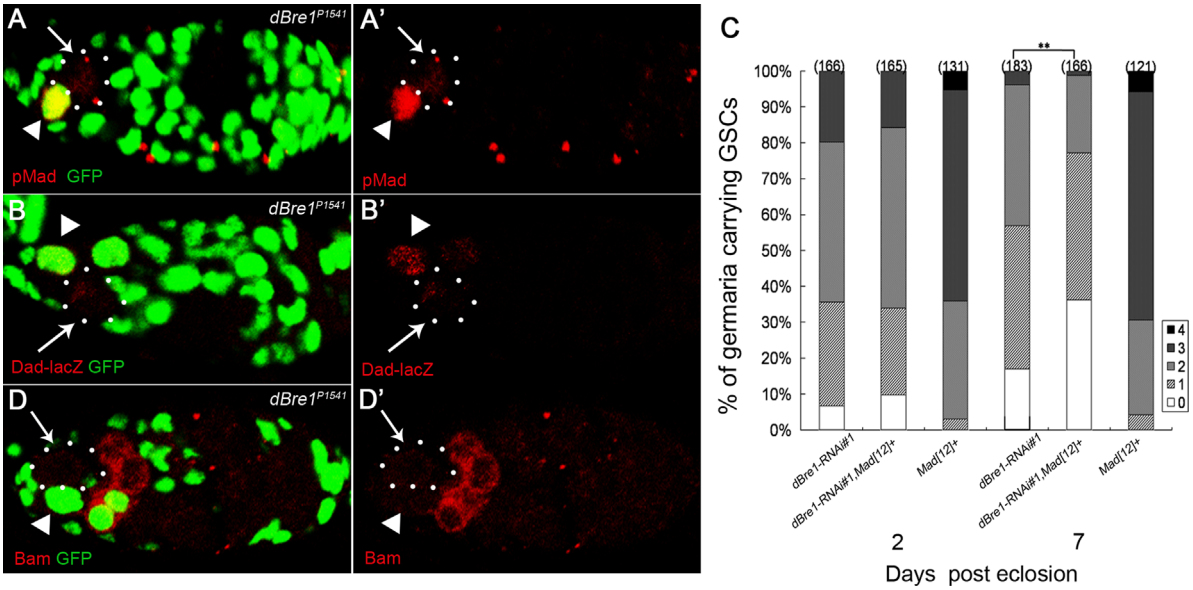

Fig. 3. Loss of dBre1 function in GSCs impairs BMP signaling pathway: (A-B′, D, D′) Germaria containing the marked GSCs homozygous for *dBre1*^{P1541} (A-B′, D, D′) labeled by the absence of the nuclear GFP (A, B, D), stained for pMad (A, A′) or β-gal (B, B′) or Bam (D, D′). (A-B′) High levels of pMad are evident in the wild-type GSC (arrowhead in A, A′). However, staining signal for pMad is remarkably reduced in the neighboring mutant GSC (broken lines and arrow in A, A′). Likewise, *Dad-lacZ* expression is down-regulated in the mutant GSC (broken lines and arrow in B, B′), compared with its wild type counterpart (arrowhead in B, B′). (C) Quantitation of GSCs per germarium in *dBre1* knock down alone or with one copy of *Mad* null allele at 2 and 7 days after eclosion. The statistical analysis suggests a genetic interaction of *dBre1* with *Mad* in maintaining GSCs. Germaria are categorized by the number of GSCs per germarium. Number in parentheses indicates the amount of germaria scored. $^{**}p < 0.01$. (D, D′) *bam* silencing is still present in the mutant GSCs based on Bam staining (arrows). Note the absence of Bam in the wild type GSC (arrowheads).

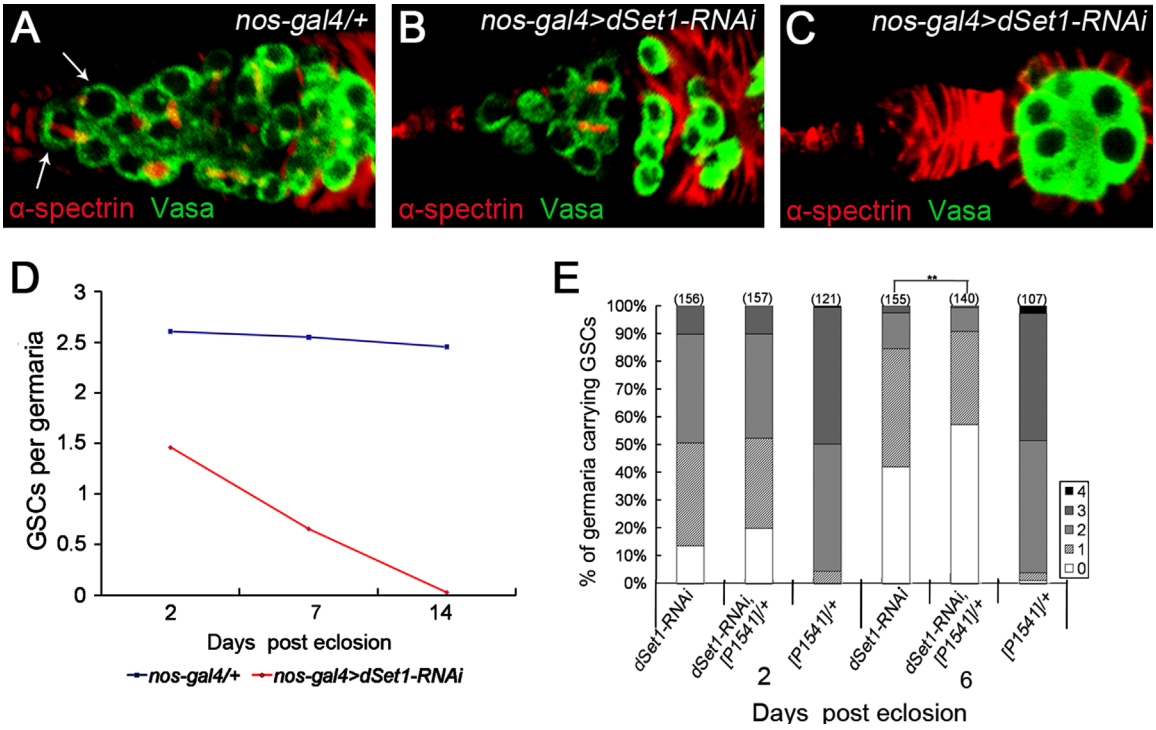

Fig. 4. Knocking down *dSet1* in the germ cells leads to a gradual GSC loss that can be genetically modified by *dBre1* heterozygosity: (A–C) The control germarium (A) and germaria expressing *nos-gal4*-driven *dSet1-RNAi* (B, C) stained for α-spectrin (red) and Vasa (green). GSCs are absent in the *dSet1* knock down germaria (B, C), while two GSCs are present in the control germarium (arrows in A). (D) Graph shows that knocking down *dSet1* in the germ cells causes a gradual GSC loss over two weeks after eclosion. (E) Quantitation of GSCs per germarium in *dSet1* knock down alone or with one copy of *dBre1* null allele at 2 and 6 days after eclosion. The statistical analysis suggests a genetic interaction of *dBre1* with *dSet1* in maintaining GSCs. Germaria are categorized by the number of GSCs per germarium. Number in parentheses indicates the amount of germaria scored. $^{**}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

dBre1 genetically interacts with dSet1 in maintaining GSCs

We demonstrated that dBre1 is essential for H3K4 trimethylation in ovarian germ cells, and plays an important role in maintaining GSCs. To causatively link dBre1-mediated H3K4 methylation to GSC maintenance, we then investigated dSet1, the main *Drosophila* H3K4 trimethyltransferase for a role in controlling GSC self-renewal. RNAi-based knock down of *dSet1* in

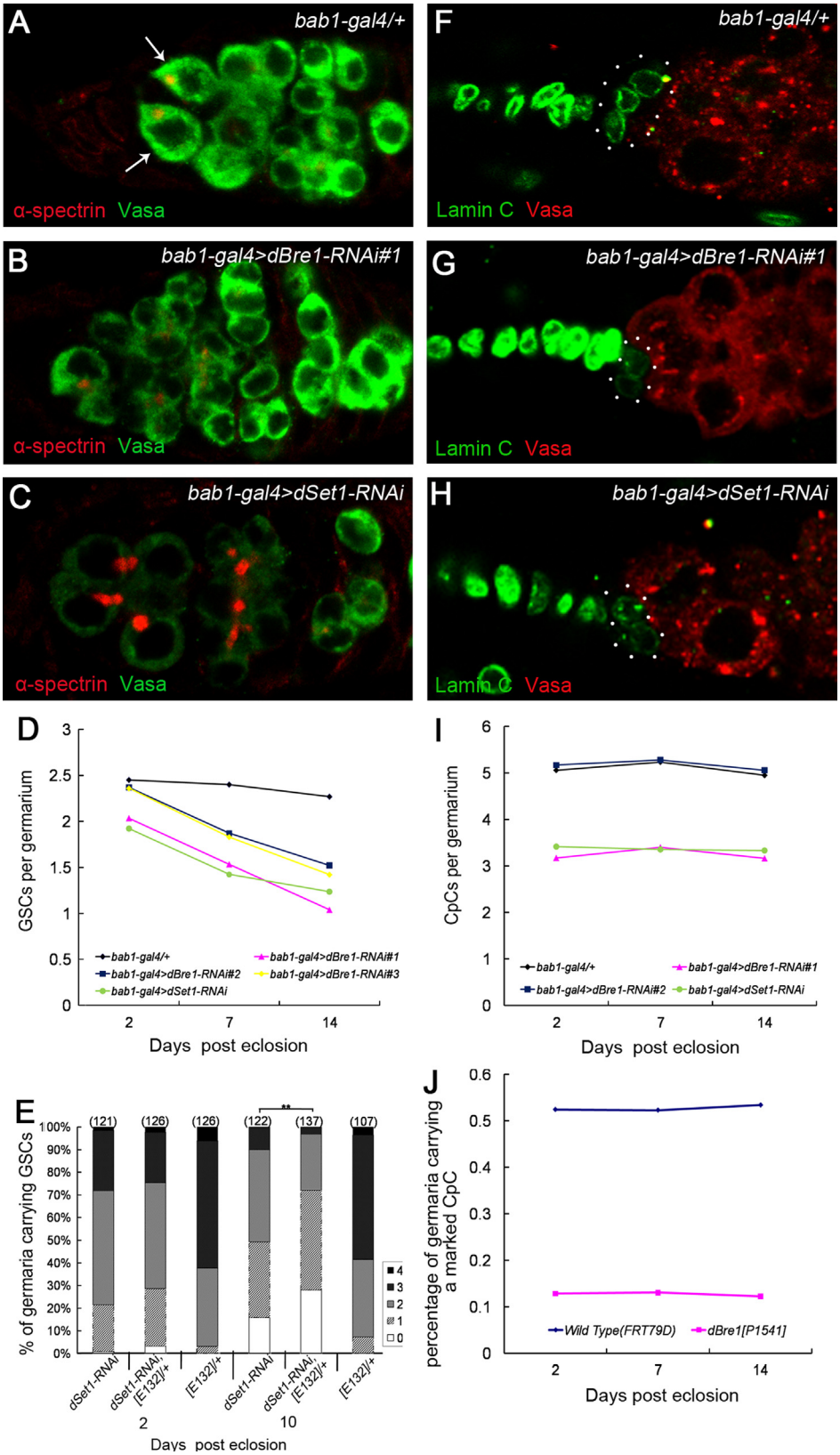

Fig. 5. dBre1/dSet1 function extrinsically in controlling GSC self-renewal: (A–C, F–H) The control (A, F) and mutant germaria expressing *dBre1-RNAi#1* (B, G) or *dSet1-RNAi* (C, H) under the control of *bab1-gal4*, stained for Vasa and α-spectrin (A–C) or Vasa and Lamin C (F–H). (A–C) GSCs are lost in the germarium in which *dBre1* (B) or *dSet1* (C) is knocked down in the niche cells, while two GSCs are present in the control (arrows in A). (D) Graph shows that knocking down *dBre1* or *dSet1* in the niche leads to a significant drop of GSC number over a 2-week period after eclosion, whereas the GSC number in the germarium expressing *bab1-gal4* alone remains stable throughout this period. Note that similar results are obtained using three independent *dBre1-RNAi* transgenic lines. (E) Quantitation of GSCs per germarium in *dSet1* knock down alone or with one copy of *dBre1* null allele at 2 and 10 days after eclosion. The statistical analysis shows that $dBre1^{E132}$ heterozygosity significantly increases the GSC loss induced by *dSet1* knock down, while GSC maintenance is not affected in heterozygous $dBre1^{E132}$ germarium. Germaria are categorized by the number of GSCs per germarium. Number in parentheses represents the amount of germaria scored. (F–H) Cap cells (broken circles) are visualized in the control (F), and *dBre1* or *dSet1* knock down germarium (G, H). (I) Graph shows that cap cells are well maintained in *dBre1* or *dSet1* knock down germaria over a 2- week period after eclosion, Note that reduced number of cap cells is observed in the germaria of newly eclosed mutant flies expressing *dSet1-RNAi* or one of three *dBre1-RNAi* transgenes. (J) Graph showing the percentage of negatively GFP-marked CpC clones detected from one *dBre1* null allele and the FRT control over a 14-day period ACI. Marked CpCs lacking *dBre1* are well maintained over the period, albeit the mutant flies eclose with fewer CpCs. $^{**}p < 0.01$.

the germ cells was performed for this purpose. Consistent with the observation that dSet1 is required for the bulk H3K4 trimethylation during oogenesis (Fig. 1G–I), expression of *dSet1-RNAi* under the control of *nos-gal4* induced a progressive and sharp decline in GSC number per germarium within 14-day period after eclosion (Fig. 4A–D). These data clearly showed that dSet1 has a similar role with dBre1 in maintaining GSCs. Given similar mutant phenotypes and shared biochemical pathway for the H3K4 trimethylation (Dover et al., 2002; Schneider et al., 2005; Sun and Allis, 2002; Wood et al., 2003), we assumed that dBre1 and dSet1 act in a common pathway for controlling GSC self-renewal. To test this scenario, we further analyzed the possible genetic interactions between *dBre1* and *dSet1* in GSC maintenance. As depicted in Fig. 4D, *nos-gal4*-driven expression of *dSet1-RNAi* caused a remarkable decrease in GSC number per germarium from 2 days to 6 days after eclosion. Remarkably, heterozygosity for $dBre1^{P1541}$ allele exacerbated GSC loss in the *dSet1* knock down germaria (Fig. 4E), suggesting a genetic interaction of *dBre1* with *dSet1*. Taken together, these studies identified an in vivo dBre1/dSet1-dependent pathway for the trimethylation of histone H3K4 that is required intrinsically for GSC maintenance.

dBre1/dSet1 function in the niche to regulate GSC self-renewal

GSC maintenance involves both intrinsic and extrinsic factors. To explore the possibility that dBre1/dSet1 function non-autonomously in GSC self-renewal, we next tested whether down-regulation of *dBre1* or *dSet1* expression in the stem cell niche could impair GSC maintenance. For these experiments, we knocked down *dBre1* or *dSet1* expression in the niche comprising mainly TFs and cap cells using the *UAS-RNAi* in combination with *bab1-gal4* driver, and quantified GSCs at different time points after eclosion. Interestingly, reduced expression of either *dBre1* or *dSet1* caused a significant drop of GSC number per germarium during the 14-day period after eclosion (Fig. 5A–D). In the case of *dBre1*, we tested three independent *dBre1-RNAi* transgenes including *dBre1-RNAi#1*, *dBre1-RNAi#2* and *dBre1-RNAi#3* (Mohan et al., 2010), and obtained consistent data (Fig. 5D). All these results indicate that both dBre1 and dSet1 are required in the niche for controlling GSC maintenance. To identify the functional relationship between dBre1 and dSet1 in this process, we further studied for genetic interactions of *dBre1* with *dSet1*. As revealed in Fig. 5E, heterozygosity for $dBre1^{E132}$ remarkably enhanced the GSC loss induced by *bab1-gal4*-driven expression of *dSet1-RNAi*, implying that dBre1 acts in concert with dSet1 to extrinsically control GSC self-renewal. Combined with the finding that both dBre1 and dSet1 are required for the bulk H3K4 trimethylation in the GSC niche (Fig. 1E and I), the studies above not only identified a non-autonomous role of dBre1 and dSet1 in maintaining GSCs, but provided more evidence indicative of a dBre1/dSet1-dependent pathway responsible for the H3K4 methylation involved in GSC self-renewal as well.

Maintenance of the niche size and regulation of the niche signaling output in the adult ovary are important for controlling GSC self-renewal. To unravel how dBre1/dSet1 function in the niche to control GSC maintenance, we firstly examined whether down-regulation of *dBre1* or *dSet1* expression in somatic cells of the niche affects the niche maintenance. In this experiment, *dBre1-RNAi* or *dSet1-RNAi* was expressed under the control of *bab1-gal4*, and the niche size was determined based on cap cell counting. Clearly, cap cells were properly maintained over time in either *dBre1* or *dSet1* knock down ovaries (Fig. 5F–I). To further assess the cap cell maintenance, we performed a FLP/FRT-based clonal analysis. As revealed in Fig. 5J, the rate of *dBre1* mutant cap cell clone per germarium remained unchanged over 14-day period after eclosion. Together, these results suggest that dBre1/dSet1 are dispensable for maintenance of the niche size in the adult ovary. Given that the niche-derived BMP signals promote GSC self-renewal via repressing differentiation, we next tested if dBre1/dSet1 in the niche act in controlling the BMP signaling output. For this purpose, we knocked down the expression of *dBre1* or *dSet1* in the niche cells, and then analyzed BMP signaling activity in the GSCs. pMad staining intensity in the GSCs located in the mutant germarium was significantly reduced, compared with that in the control (Fig. 6A–C), indicating that BMP signaling is compromised by localized down-regulation of *dBre1* or *dSet1* expression in the niche. The compromised BMP signaling output in the niche mutant backgrounds could be due to a defect either in *dpp* expression and/or Dpp morphogen diffusion. To distinguish these possibilities, we quantified expression of *dpp* and *dally*, a glypican-encoding gene that facilitates BMP signal diffusion, in the niche-specific *dBre1* or *dSet1* knockdown ovaries. As depicted in Fig. 6D and E, reduced level of *dally*, but not *dpp* mRNA was evident in the mutant ovaries, suggesting that impaired *dally* expression accounts for the BMP signaling defects, and presumably the GSC loss phenotypes induced by the niche-specific *dBre1* or *dSet1* knockdown.

The DE-cadherin complex-mediated cell–cell adhesion between cap cells and GSCs plays an essential role in anchoring the stem cells in the niche, ensuring the continuous self-renewal of GSCs (Song et al., 2002). To test if dBre1/dSet1 activities in cap cells are required for this process, we generated cap cell clones homozygous for $dBre1^{E132}$ or $dBre1^{P1541}$ and analyzed the accumulation of DE-cadherin complex at the cap cell–GSC junction. Intriguingly, the marked mutant cap cells had much less DE-cadherin complex accumulation at the junction than the neighboring wild type cap cells in the same niche, as indicated by reduced expression of DE-cadherin ($dBre1^{E132}$: 47.6%, $n=42$) and Armadillo (Arm) ($dBre1^{E132}$: 51.0%, $n=49$; $dBre1^{P1541}$: 48.8%, $n=41$) (Fig. 7B, B′, J, J′). These results suggest that dBre1 is required in cap cells for maintaining the adhesion of the stem cells to their niche, presumably contributing to promoting GSC self-renewal. To prove this assumption, we first examined if *bab1-gal4*-driven expression of *dBre1-RNAi#1* or *dSet1-RNAi* can mimick the phenotype of *dBre1* loss-of-function mutation, disrupting the cell–cell adhesion. As depicted in Fig. 7C–F and K–M, knocking down *dBre1* or *dSet1* alone in cap cells led to a loss of accumulation of DE-cadherin (dBre1: 55.2%, $n=143$; dSet1: 60.2%, $n=98$) and Arm (dBre1: 45.2%, $n=168$; dSet1: 52.9%, $n=170$) at cap cell–GSC junction. Further, introducing one copy of $dBre1^{E132}$ significantly increased the penetrance of defective DE-cadherin complex accumulation induced by *bab1-gal4*-driven expression of *dSet1-RNAi* (Fig. 7F), implying that *dBre1* functions with *dSet1* in the same pathway in maintaining GSC–niche junction. We then tested whether the disrupted accumulation of DE-cadherin complex at GSC–niche junction contributes to the GSC loss. To this end, we targeted *UAS-shotgun* (*UAS-shg*) or *UAS-Arm* for expression in *dBre1* or *dSet1-RNAi*-mediated knock down cap cells. Remarkably, the forced expression of DE-cadherin or Arm slowed the GSC loss elicited by *bab1-gal4*-driven expression of *dBre1-RNAi#1* or *dSet1-RNAi* (Fig. 7G, H, and data not shown). Taken together, these studies indicate that dBre1/dSet1 in the niche function in maintaining DE-cadherin-mediated adhesion of GSCs to the niche, thereby contributing to the stem cell maintenance.

dBre1/dSet1 are required in ECs for controlling GSC-derived cell differentiation through limiting BMP signaling within the germarium

ECs have recently been defined as the germline stem cell differentiation niche. Given that *dBre1* is predominantly expressed in ECs (Fig. 1B), we sought to determine if dBre1 is required in ECs for controlling germ cell differentiation. For this purpose, *dBre1-RNAi#1* was expressed under the control of *c587-gal4* driver,

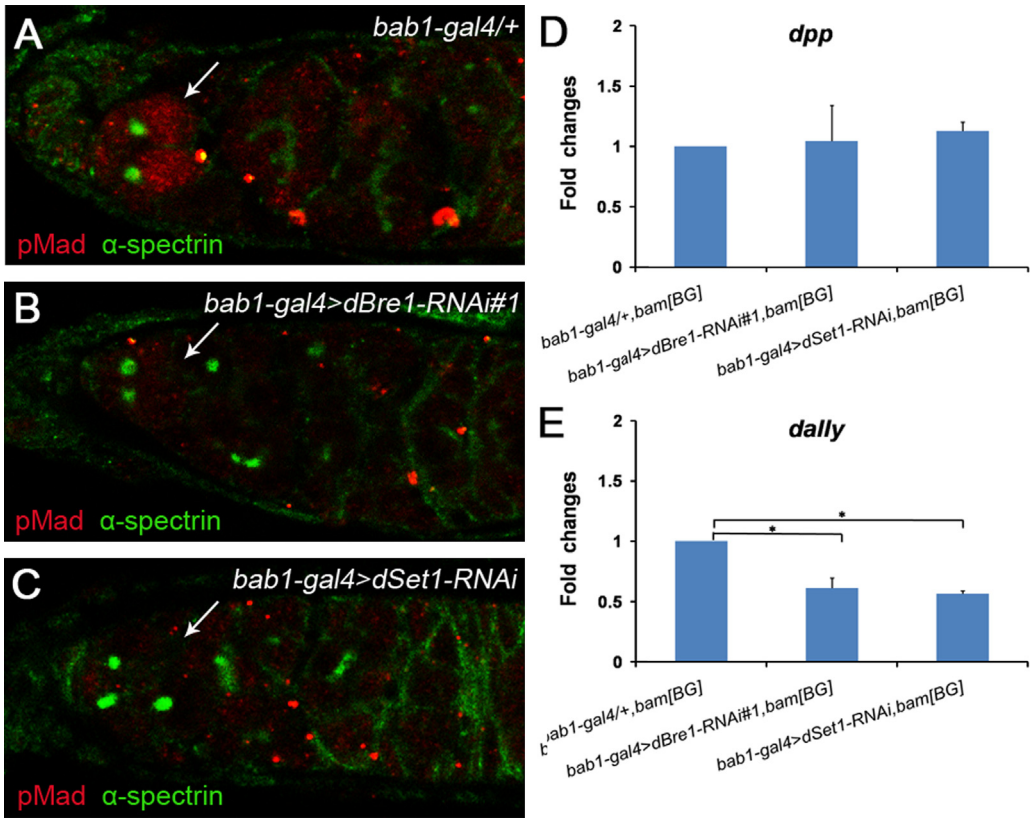

Fig. 6. The BMP signaling output is compromised in the niche-specific *dBre1* or *dSet1* knock down germaria: (A–C) The control (A) and mutant germaria expressing *dBre1-RNAi#1* (B) or *dSet1-RNAi* (C) under the control of *bab1-gal4*, stained for pMad and α-spectrin. Expression of pMad is significantly reduced in the GSCs contained in the mutant germaria (arrows in B and C), compared with the control ones (arrow in A). (D, E) The niche-specific knock down of *dBre1* or *dSet1* does not alter expression of *dpp* mRNA (D), but impairs *dally* transcription (E). Quantitative RT-PCRs are normalized to internal gene control *rp49*, while the value for the *bab1* driver control is designated to 1. All these results are based on three experiments performed independently.

restricting *dBre1* down-regulation to ECs and early follicle cells in adults (Kai and Spradling, 2003; Song et al., 2004). In contrast with the control (Fig. 8A and D), we observed that reduced expression of *dBre1* in ECs results in a remarkable increase of the spectrosome-containing single germ cells classified as undifferentiated germ cells (UGCs) (Kirilly et al., 2011), located away from the niche in the germarium (Fig. 8B and D), indicative of a defective germ cell differentiation. To further characterize the accumulated UGCs, we examined the distribution of pMad and *bamP*-GFP. As stated earlier, pMad, an indicator of active BMP signaling is localized primarily in GSCs, whereas *bamP*-GFP is normally expressed in differentiated germ cells including cystoblasts but not in GSCs (Fig. 8F and I). Significantly, the number of pMad positive cells was increased in the germaria expressing *dBre1-RNAi* in ECs, albeit lower overall levels of pMad were present in the UGCs than control GSCs (Fig. 8F and G). By contrast to that in endogenous GSCs, *bamP-GFP* expression was evident in majority of the accumulated UGCs (85.9%, $n=128$) (Fig. 8J). Thus, we favor that the accumulated UGCs induced by *dBre1* knock down in ECs behave like CBs.

Expanding distribution of pMad in EC-specific *dBre1* knock down germarium suggests that up-regulation of BMP signaling accounts for the germ cell differentiation defects. To test this, we examined if removal of one copy of *dpp* could suppress the defective differentiation. As expected, heterozygous dpp^{hr4} can largely rescue the cell differentiation defect phenotypes, as indicated by a significant drop of UGC number, together with the presence of more germline cysts with branched fusomes (Fig. 9A and C). These data argue strongly that dBre1 acts in ECs to control germ cell differentiation via negatively regulating BMP signaling.

Recently, Lsd1 and Egg have been shown to repress transcription of either *dpp* or *dally*, thus promoting germ cell differentiation (Eliazer et al., 2011; Wang et al., 2011). We, therefore, asked if disruption of dBre1 function in ECs causes a de-repression of *dpp* or *dally* or perhaps both, thereby up-regulating BMP signaling in the germarium. To clarify this question, we performed a RT-PCR-based quantatitive assay. Noticeably, both *dpp* and *dally* mRNA were elevated in the EC-specific *dBre1* knockdown ovaries (Fig. 9D and E), suggesting that upregulation of *dpp* and *dally* expression is responsible for the defective GSC differentiation. These observations on molecular level were further verified by genetic studies. Consistently, expressing *dpp* or *dally-RNAi* in ECs suppressed the penetrance of UGC accumulation in the knock down germaria (Fig. 9C, F, and G). Meanwhile, heterozygous $dally^{gem}$ can partially restore the germ cell differentiation, as shown in Fig. 9F. Together, these results lead us to propose that dBre1 in ECs controls the germ cell differentiation via limiting BMP signaling range through repressing expression of both *dpp* and *dally*. Given that EGFR/MAPK signaling pathway acts in ECs to repress *dally* transcription (Liu et al., 2010), we further investigated a possible role of dBre1 in regulation of the EGFR signaling. Clearly, activation of the EGFR/MAPK pathway still occurs in the ECs expressing *dBre1* RNAi transgene, as evident in the expression of pERK (Fig. 9I and J). Overall, the data presented above suggest that dBre1 in ECs is required for repressing *dally* expression in a EGFR pathway-independent manner.

In parallel, we tested whether dSet1 has a similar role with dBre1 in the control of germ cell differentiation and dBre1/dSet1 act in a common pathway. Like the case of *dBre1*, RNAi-based knock down of *dSet1* expression in ECs causes accumulation of the CB-like UGCs in the germaria (Fig. 8C and D). Moreover, molecular

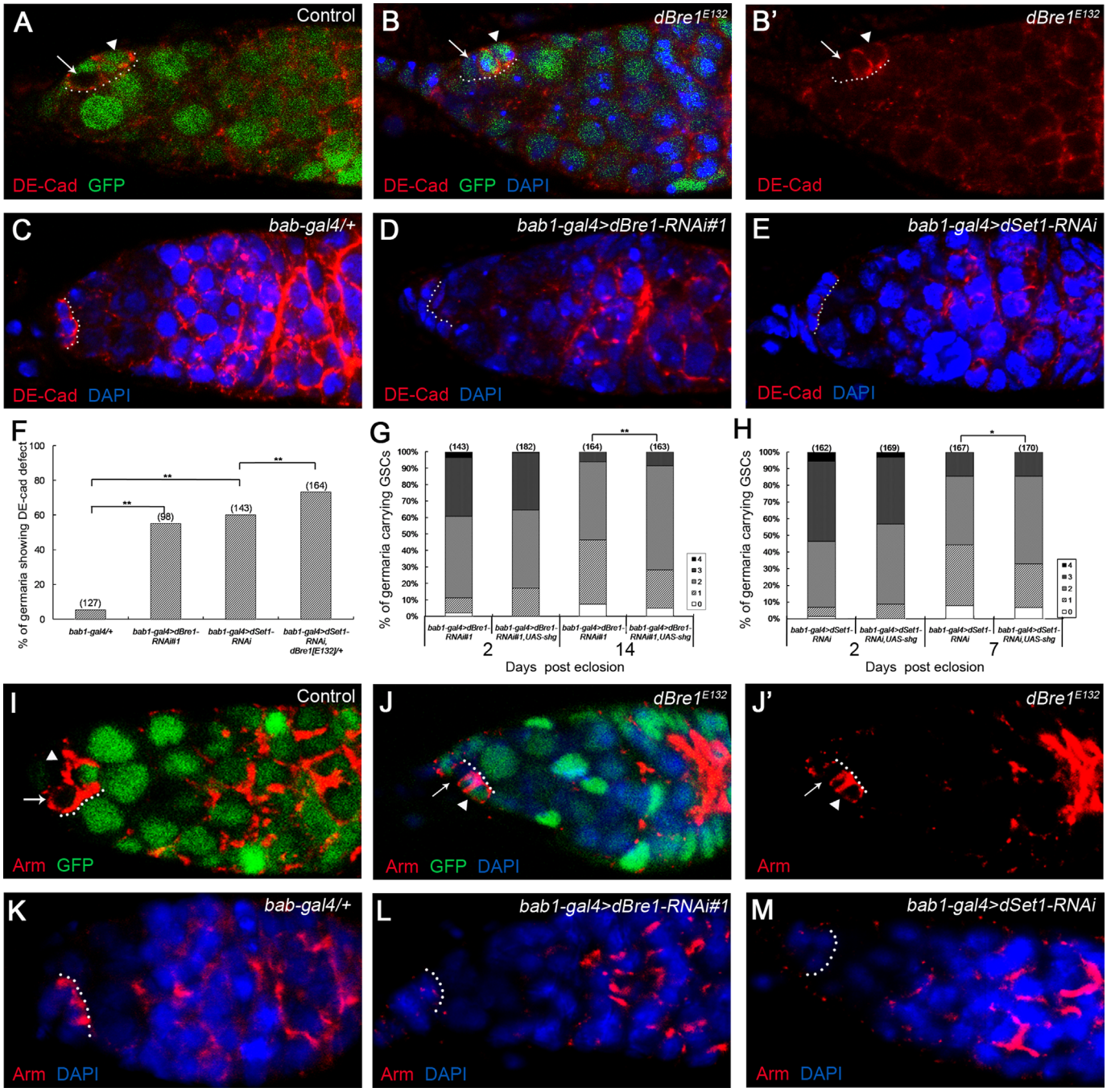

Fig. 7. dBre1/dSet1 are required in cap cells for maintaining the accumulation of DE-cadherin/Armadillo complexes in the niche–GSC junction, and thus controlling GSC self-renewal (A–E, I–M) The control (A, C, I, K) and mutant germaria bearing CpC clones homozygous for *dBre1*^{E132} (B, B′, J, J′) labeled by the absence of the nuclear GFP (green) or expressing *dBre1-RNAi#1* (D, L) or *dSet1-RNAi* (E, M) under the control of *bab1-gal4*, stained for DE-cadherin (DE-Cad, A–E) or Armadillo (Arm, I–M). (A–E, I–M) Loss of DE-cadherin or Arm accumulation in the interface (broken lines) between GSCs and the marked *dBre1* mutant CpC (arrows in B, B′ and J, J′) or CpCs expressing *dBre1-RNAi#1* (D, L) or *dSet1-RNAi* (E, M) is evident. Note that DE-cadherin or Arm accumulation is detectable in the junction between GSCs and internal control CpCs (arrowheads in A, B′ and I, J′, C, K). (F) RNAi-based knock down of *dBre1* or *dSet1* in CpCs significantly causes failure of DE-cadherin to accumulate in CpC-GSC junction. Further, the penetrance of disrupted DE-cadherin accumulation in *dSet1* knock down germaria is greatly increased by introducing one copy of *dBre1*^{E132} allele. (G, H) Niche cell-specific expression of DE-cadherin can partly block the GSC loss elicited by *bab1-gal4*-driven expression of *dBre1-RNAi#1* or *dSet1-RNAi.* Germaria are categorized by the number of GSCs per germarium. Number in parentheses represents the amount of germaria scored. $^{*}p < 0.05$, $^{**}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and genetic studies demonstrated that ectopic BMP signaling in the germarium causatively links *dSet1* knock down to defective germ cell differentiation (Figs. 8H, K; 9B, C), and increased expression of *dpp* and *dally* underlies the differentiation defects elicited by the ectopic BMP signaling (Fig. 9C–F, and H). More importantly, we found that penetrance of disrupted germ cell differentiation caused by reduced expression of *dSet1* in ECs can be promoted by introducing one copy of *dBre1*^{P1541} allele (Fig. 8E). Given that heterozygous germaria for *dBre1*^{P1541} displayed normal germ cell differentiation (data not shown), we identified a common pathway by which dBre1 and dSet1 control the germ cell differentiation.

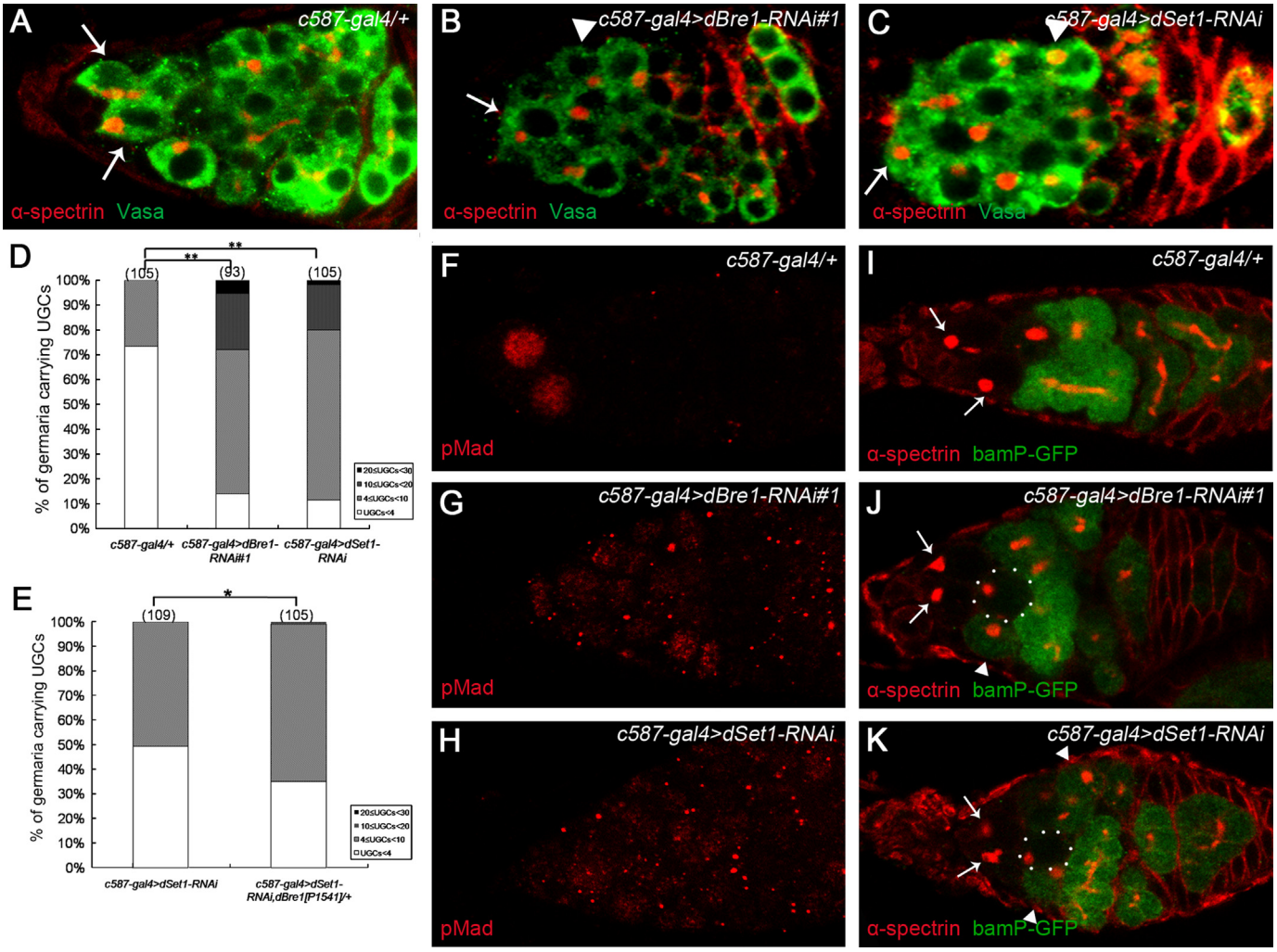

Fig. 8. Reduced expression of *dBre1* or *dSet1* in ECs results in defective germ cell differentiation: (A–C, F–K) The control (A, F, I) and mutant germaria expressing *dBre1-RNAi#1* (B, G, J) or *dSet1-RNAi* (C, H, K) under the control of *c587-gal4*, stained for α-spectrin and Vasa (A–C) or pMad (F-H) or stained/visualized for α-spectrin/GFP (I–K). (A–C) Knocking down *dBre1* or *dSet1* in ECs causes accumulation of spectrosome-containing single germ cells called UGCs in the germaria (arrowheads in B and C). Note that endogenous GSCs are present in both control and mutant germarium (arrows in A–C). (D) Quantitation of UGCs in the control or *dBre1* or *dSet1* knock down germarium at two weeks after eclosion. The statistical analysis reveals a significant increase of the UGC number in the knock down germaria. Germaria are categorized by the number of UGCs per germarium. Number in parentheses represents the amount of germaria scored. (E) Quantitation of UGCs in the germaria expressing *dSet1-RNAi* in ECs alone or in the presence of one copy of *dBre1* mutant allele at 2 days after eclosion. Remarkably, $dBre1^{P1541}$ heterozygosity can enhance the *dSet1* knock down-induced accumulation of UGCs. Germaria are categorized by UGC number per germarium. Number in parentheses represents the amount of germaria scored. (F–K) The number of pMad-positive cells is greatly expanded in either *dBre1* or *dSet1* knock down germarium (G, H). However, staining signals for pMad in the UGCs are not as strong as that of GSCs in the control (G and H, compared with F). Meanwhile, the expression of *bamP-GFP* is evident in the UGCs (arrowhead in J and K). Note that *bamP-GFP* expression is repressed in GSCs (arrow in I–K). The UGCs without *bamP-GFP* signal are outlined with white dots (J, K). $^{*}p < 0.05$, $^{**}p < 0.01$.

Overall, studies here provided evidence that the dBre1/dSet1-dependent pathway in ECs controls germ cell differentiation via limiting BMP signaling range in the germaria. Given the essential role of dBre1 and dSet1 in bulk H3K4 trimethylation of ECs (Fig. 1F and G), we infer that the dBre1/dSet1-mediated epigenetic regulation is important for the control of germ cell differentiation.

Discussion

From yeast to human, Bre1 has been shown to be responsible for the H2B monoubiquitination and, indirectly, for trimethylation of H3K4 or H3K79 both in vitro and in vivo (Bray et al., 2005; Dover et al., 2002; Hwang et al., 2003; Mohan et al., 2010; Ng et al., 2002; Shilatifard, 2006; Sun and Allis, 2002; Wood et al., 2003). In *Drosophila* development, loss of dBre1 function causes either Notch signaling or Wnt signaling defects that are functionally linked to disruption of H3K4 or H3K79 methylation respectively (Bray et al., 2005; Mohan et al., 2010). In the present study, we showed that dBre1 and dSet1 are essential for the bulk H3K4 trimethylation in *Drosophila* ovarian cells. Genetic studies identified a common pathway by which dBre1 and dSet1 control GSC maintenance and germ cell differentiation during oogenesis presumably through modulating the H3K4 methylation. In the case of GSC maintenance, loss of dBre1 or dSet1 function results in defective niche-derived BMP signaling and DE-cadherin-mediated adhesion between GSCs and their niche. On the contrary, dBre1/dSet1 in ECs function in the control of germ cell differentiation via repressing BMP signaling in the germarium. These observations lead us to propose that dBre1/dSet1-dependent pathway controls GSC maintenance and germ cell differentiation via distinct mechanisms. The findings in this study not only provide evidence linking the dBre1/dSet1-mediated histone modification to *Drosophila* oogenesis, but also may help to address the potential function of the H3K4 methylation in adult stem cell regulation in higher organisms.

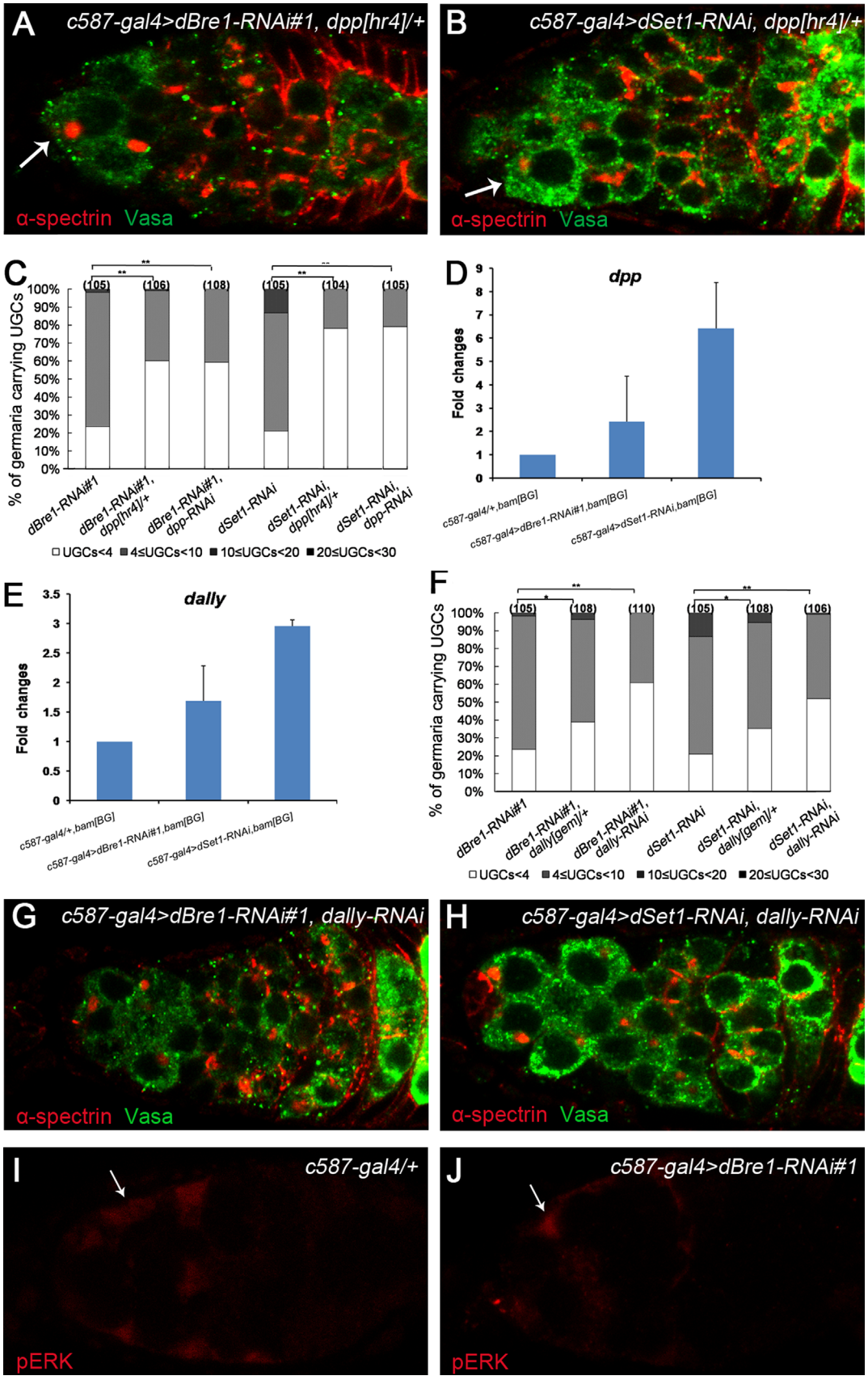

Fig. 9. Knocking down *dBre1* or *dSet1* in ECs increases BMP signaling, causing the defective germ cell differentiation. (A, B, G–J) Germaria expressing *dBre1-RNAi#1* (A, G, J) or *dSet1-RNAi* (B, H) in the presence of one copy of dpp^{hr4} allele (A, B) or with *dally-RNAi* (G, H) in ECs, stained for α-spectrin and Vasa (A, B, G, H) or pERK (I, J). (A-C) dpp^{hr4} heterozygosity or RNAi-based down-regulation of *dpp* expression can suppress the accumulation of UGCs in the *dBre1* or *dSet1* knock down germaria. The quantitative results are shown in (C). (D, E) EC-specific knock down of *dBre1* or *dSet1* leads to increased expression of *dpp* mRNA (D) and *dally* mRNA (E). Quantitative RT-PCRs are normalized to internal gene control *rp49*, while the value for the *c587* driver control is designated to 1. All these results are based on three experiments performed independently. (F–H) RNAi-mediated *dally* knock down or $dally^{gem}$ heterozygosity can partly rescue the germ cell differentiation defects caused by down-regulating *dBre1* or *dSet1* expression in ECs. The quantitative results are summarized in (F). $^{*}p < 0.05$, $^{**}p < 0.01$. (I, J) pERK expression in *dBre1* knock down ECs is comparable with that in the control ones (arrows in I and J).

Identification of a dBre1/dSet1-dependent pathway required for regulation of GSC fate and germline differentiation

Recently, it has been reported that the H3K9 methylation mediated by Eggless, a *Drosophila* histone trimethylase, plays a pivotal role in the control of GSC maintenance and differentiation (Wang et al., 2011). Here, we found that removal or knock down of dBre1 function in specific ovarian cell lineages leads to disruption of H3K4 trimethylation, and defective GSC maintenance or germ cell differentiation. These phenotypes can be mimicked by RNAi-based reduced expression of dSet1, but not other three *Drosophila* H3K4 trimethylases (data not shown). Further genetic studies favored that *dBre1* interacts with *dSet1* in controlling the processes above. Thus, our study identified a dBre1/dSet1-dependent pathway for the H3K4 methylation and provided the first evidence that dBre1/dSet1-linked H3K4 methylation might be functionally implicated in the *Drosophila* ovarian stem cell regulation. Combined with the previous report regarding role of *Drosophila* Lsd1, a specific H3K4 demethylase, in the control of germ cell differentiation in the ovaries (Eliazer et al., 2011), the results in this paper further suggest that the H3K4 methylation/demethylation may represent a general epigenetic mechanism underlying developmental control of stem cells and their derived cell lineages. Hence, it will be of great interest to determine whether and how dBre1/dSet1-dependent methylation pathway functions in self-renewal and differentiation of the adult stem cells in vertebrates.

Each *Drosophila* ovary is composed of 16–20 ovarioles. At the anterior tip of each ovariole lies one GSC unit containing a number of somatic niche cells and 2–3 GSCs anchored in the niche. It is known that formation of the adult ovary requires coordinated regulation of both niche and stem cell precursors throughout the gonad development involving cell proliferation and differentiation (Gancz et al., 2011; Gilboa and Lehmann, 2004, 2006; Zhu and Xie, 2003). In the experiments of this study, we observed that RNAi-mediated knock down of either *dBre1* or *dSet1* expression in the germline or niche significantly decreases number of GSCs in each germarium of the newly enclosed fly ovaries (Figs. 2F, 4D, and 5D), suggesting a defect in GSC formation/establishment. This defect could be attributable to failure of proper niche formation and/or aberrant proliferation of PGCs, the GSC precursors during larval and pupal development. Given that reduced number of cap cells per germarium is evident in the ovaries expressing *dBre1*- or *dSet1-RNAi* under the control of *bab1-gal4* driver (Fig. 5I), we favor that the H3K4 methylation pathway regulates formation of the GSC unit presumably via acting in the somatic niche formation. To address how dBre1/dSet1-dependent pathway acts in the GSC niche development at larval and pupal stages will be the topics of the following paper in our laboratory.

It is noteworthy that dBre1 mediates the H2B monoubiquitination linking H3K79 trimethylation by Dot1, a conserved histone methyltransferase, to Wnt/Wingless signaling in *Drosophila* wing imaginal discs (Mohan et al., 2010). At this time, therefore, it cannot be ruled out that dBre1 functions in *Drosophila* ovaries alternatively through mediating the H3K79 methylation. To test this possibility, we need to screen all H3K79 methylase-encoding genes including *Dot1* for possible mutant phenotypes of defective GSC fate regulation. Further study for genetic interactions between *dBre1* and the identified H3K79 methyltransferase gene may lead to revealing alternative dBre1-dependent methylation pathway involved in *Drosophila* oogenesis.

dBre1/dSet1 control GSC self-renewal and germ cell differentiation via distinct mechanisms

Regulation of GSC maintenance can occur in either the stem cells autonomously or stem cell niche non-autonomously or perhaps both. The present study revealed an involvement of both intrinsic and extrinsic mechanisms in dBre1/dSet1-mediated control of GSC self-renewal. In the case of cell autonomous manner, GSCs lacking dBre1 or dSet1 activity are not able to respond properly to BMP signals emitted from the niche, as indicated by remarkably reduced pMad level and *Dad-LacZ* expression in the stem cells. Likewise, knocking down *dBre1* or *dSet1* in the niche cells compromises the BMP signaling from the niche to GSCs. Besides, dBre1/dSet1 perform the non-autonomous function in controlling GSC self-renewal at least partly through modulating the GSC-niche adhesion. In contrast to the previous reports (Chen et al., 2010; Ji and Tulin, 2012; Song et al., 2002), we excluded a role of GSC dBre1/dSet1 in regulating the adhesion of GSCs to their niche (data not shown). Thus, this study clearly demonstrates that anchoring GSCs in the niche through the cell–cell adhesion for their maintenance requires an extrinsic mechanism involving the cap cell function (Hsu and Drummond-Barbosa, 2009).

Apart from maintaining GSCs, we observed that dBre1/dSet1 regulate the germ cell differentiation in a non-autonomous manner. RNAi-mediated knock down of *dBre1* or *dSet1* in ECs results in accumulation of undifferentiated germ cells behaving like CBs in the germarium according to the expression pattern of pMad and *bamP-GFP*. Like Lsd1 and Eggless (Eliazer et al., 2011; Wang et al., 2011), the genetic rescue experiments indicate that dBre1/dSet1 in ECs promote germ cell differentiation in the germarium by negatively regulating the BMP signaling. Further studies reveal that dBre1/dSet1 in ECs limit BMP signaling through repressing expression of both *dpp* and *dally* in the germarium. Given the findings in this paper that dBre1/dSet1 are essential both intrinsically and extrinsically for maintaining active BMP signaling pathway required for GSC self-renewal, repressing ectopic BMP signaling activity underlying dBre1/dSet1-mediated control of the germ cell differentiation clearly represents a distinct mechanism. It is known that histone H3K4 methylation as an epigenetic marker is exclusively associated with actively transcribed genes (Bernstein et al., 2002; Shilatifard, 2008). Therefore, identification of the target genes regulated by dBre1/dSet1 in the ovaries will be beneficial for elucidating the above distinct mechanisms involved in the control of GSC self-renewal and germ cell differentiation.

Conclusions

dBre1, an E3 ubiquitin ligase, mediates the monoubiquitination of histone H2B which is required for histone H3K4 or H3K79 trimethylation in *Drosophila* development. In this paper, we show that dBre1 is essential for maintaining GSCs and ensuring GSC-derived germ cell differentiation in the *Drosophila* ovary. Further investigation demonstrates that dBre1 functions in GSC maintenance at least partly through modulating BMP signaling response in GSCs and GSC-cap cell adhesion, while dBre1 activities in ECs limit BMP signaling in the germaria, thereby controlling the germ cell differentiation. Genetic studies favor that *dBre1* interacts with *dSet1*, the main *Drosophila* H3K4 trimethylase-encoding gene in controlling GSC self-renewal and germ cell differentiation. Taken with the observation that the bulk H3K4 trimethylation in the *Drosophila* ovarian cells is dependent on both dBre1 and dSet1, we propose that dBre1 and dSet1 constitute a pathway for H3K4 methylation involved in the cell fate regulation in *Drosophila* oogenesis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at: http://dx.doi.org/10.1016/j.ydbio.2013.04.015.

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