Development 134, 157-165 (2007) doi:10.1242/dev.02698

Histone methylation is required for oogenesis in *Drosophila*

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SET domain proteins are histone lysine methyltransferases (HMTs) that play essential roles in development. Here we show for the first time that histone methylation occurs in both the germ cells and somatic cells of the *Drosophila* ovary, and demonstrate in vivo that an HMT, the product of the *eggless* (*egg*) gene, is required for oogenesis. Egg is a SET domain protein that is similar to the human protein SETDB1 and its mouse ortholog ESET. These proteins are members of a small family of HMTs that contain bifurcated SET domains. Because depletion of SETDB1 in tissue culture cells is cell-lethal, and an ESET mutation causes very early peri-implantation embryonic arrest, the role of SETDB1/ESET in development has proven difficult to address. We show that *egg* is required in the *Drosophila* ovary for trimethylation of histone H3 at its K9 residue. In females bearing an *egg* allele that deletes the SET domain, oogenesis arrests at early stages. This arrest is accompanied by reduced proliferation of somatic cells required for egg chamber formation, and by apoptosis in both germ and somatic cell populations. We propose that other closely related SET domain proteins may function similarly in gametogenesis in other species.

KEY WORDS: Histone, Methylation, Oogenesis, *Drosophila*

INTRODUCTION

Histones are subject to extensive post-translational modifications (Iizuka and Smith, 2003). The best studied of these histone modifications include the methylation of arginine (R) and lysine (K) residues, acetylation of K residues and phosphorylation of Serine (S) and Threonine (T) residues. The nucleosomal diversity created by these modifications forms the basis of the 'histone code' hypothesis, which proposes that chromatin domains, defined by local combinatorial signatures of histone modifications, have distinct outcomes for chromatin structure and gene expression (Strahl and Allis, 2000; Jenuwein and Allis, 2001).

Of the various modifications made to histones, methylation is one of the most complex, both in terms of the nature of the signal and its biological consequences (Lachner and Jenuwein, 2002). Both the site of methylation and the degree of methylation affect the biological outcome of methylation. For instance, histone H3 methylation at H3K4 (lysine at residue 4 in histone H3), H3K36 and H3K79 is usually associated with gene activation, whereas methylation at H3K9 and H3K27 leads to gene repression, although exceptions to these rules exist (Martin and Zhang, 2005). Furthermore, K residues can be subject to different degrees of methylation (mono-, di- or trimethylation), with different consequences for the cell (Rice et al., 2003). These methyl marks serve as binding sites for proteins that assemble complexes that in turn regulate chromatin structure and gene transcription (Daniel et al., 2005).

In 2000, the first histone lysine methyltransferase (HMT) was identified, Suv39h1, and its enzymatic activity was mapped to its SET domain (Rea et al., 2000). The SET domain takes its name from the three *Drosophila* genes in which it was first recognized: *Su(Var)3-9*, *Enhancer of zeste* (*E(z)*), and *trithorax* (*trx*). SET domain proteins have been identified and studied in yeast, plants, worms, flies and mammals. These proteins play important roles in development (Marguerron et al., 2005), and misexpression of HMTs occurs in some human cancers (Santos-Rosa and Caldas, 2005).

Gene silencing in germ cells is a widespread phenomenon, and recent studies have begun to examine the contributions of histone modifications to germ cell gene regulation (Pirrotta, 2002). In *Caenorhabditis elegans* and *Drosophila*, formation of the embryonic primordial germ cells is accompanied by changes in histone acetylation and methylation (Schaner et al., 2003), and two maternally expressed SET domain proteins, MES-2 and MES-4, are required in *C. elegans* for the viability and proliferation of the germline (Capowski et al., 1991; Kelly and Fire, 1998; Fong et al., 2002; Bender et al., 2004). Less is known about histone modifications in the adult gonad. We show here that a SET domain protein, the product of a *Drosophila* gene that we have named *eggless* (*egg*), is required for oogenesis at early stages of egg chamber formation.

Egg is very similar to the human protein SETDB1 and its mouse ortholog ESET (Schultz et al., 2002; Yang et al., 2002). These proteins belong to a small subfamily of HMTs that contain bifurcated SET domains – that is, SET domains interrupted by insertions of novel stretches of amino acids (Harte et al., 1999; Alvarez-Venegas and Avramova, 2002). The *egg* mutations we isolated have provided us with the opportunity to study the role of this subfamily of HMTs in a developmental context. We show in vivo that Egg catalyzes trimethylation of histone H3 at its K9 residue (H3K9), and that this modification is present in both germ and somatic cells during oogenesis. In the absence of Egg-catalyzed histone methylation, oogenesis arrests at early stages. Egg chamber formation is defective, and egg chambers never fully bud off from the germarium. We show that apoptotic cell death and reduced somatic cell proliferation are likely underlying causes of this early arrest.

MATERIALS AND METHODS

Fly stocks

The genotypes of the *egg* mutant females used in these studies were *cn bw* egg^{1473}/*Df(2R)Dll-Mp* or *cn bw* egg^{235}/*Df(2R)Dll-Mp*, and either a w^{1118} or a *Canton S* strain was used as an egg^{+} control. Flies were grown at either 25°C or room temperature on cornmeal-molasses medium supplemented with live yeast.

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Accepted 16 October 2006

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EMS mutagenesis screen

A standard ethyl methanesulfonate (EMS) mutagenesis screen was performed to isolate female-sterile and lethal mutations uncovered by the chromosome 2R deficiency *Df(2R)Dll-Mp*(60E1-2;60E6). Males from a *cn bw* isoline were fed EMS (0.025 mol/l) in a 1% sucrose solution, and mated en masse to *Tft*/*CyO* virgin females. *cn bw*/*Tft* F_1 males were mated singly to *Df*(2R)*Dll-Mp*/*SM6* virgins, and the *cn bw*/*Df*(2R)*Dll-Mp* F_2 progeny were screened for lethality or female sterility. In total 3500 mutagenized second chromosomes were screened, and 45 lethal or female sterile mutations were recovered. Inter se crosses placed these 45 mutations into seven complementation groups. One complementation group consisted of 13 mutations in the gene we named *eggless*, described in this report.

Genomic rescue experiments to identify the *egg* gene

A *Drosophila* genomic library (courtesy of Jym Mohler, Barnard College, New York, NY, USA) was screened using a cDNA for the *Exu-associated protein* (*Eap*) gene, which maps near *egg*. A 9 kb genomic fragment (GC64N), from one of the clones obtained in this screen, detected three mRNAs (3.9, 1.1 and 1.4 kb) on northern blots of ovary RNA. These mRNAs were mapped within GC64N as shown in Fig. S1 in the supplementary material. The entire GC64N 9 kb fragment, and internally deleted or truncated DNA fragments (NΔ1-NΔ4), were subcloned into pCaSpeR4 and transformed into flies. Each genomic fragment was then crossed into *egg*/*Df*(2R)*Dll-Mp* backgrounds and tested for its ability to rescue *egg*-associated female sterility. The rescue pattern of these constructs (see Fig. S1 in the supplementary material) mapped *egg* to the region defined by NΔ2 and NΔ4.

Sequencing and northern blot analysis

Genomic DNA was prepared from hemizygous egg^{235} or egg^{1473} flies as described in Mansfield et al. (Mansfield et al., 2002). Amplified DNA from four to six independent PCR reactions using *egg*-specific primers was pooled, purified (Qiagen), and used as template for sequencing with 15 primers designed at intervals of 200-300 bp encompassing the entire *egg* coding sequence. (The sequence of these primers is available on request.) Sequencing was performed on an ABI 3100 capillary sequencer at the Herbert Irving Comprehensive Cancer Center's DNA Analysis and Sequencing Facility (Columbia University). The breakpoints of the deletion identified in egg^{1473} were confirmed by sequencing a cloned 350 bp DNA fragment spanning the deletion breakpoints. Two embryo cDNAs, LD15023 (3.9 kb) and LD09692 (3.5 kb) were obtained from the Drosophila Genome Research Center (DGRC; Bloomington, Indiana), and sequenced in full. LD15023 is the longest cDNA, and contains a poly(A) tail as well as an in-frame AUG start codon. LD09692 is identical to LD15023, except that it is 285 bp shorter at its 5′ end.

Northern analysis was performed following the methods of Mansfield et al. (Mansfield et al., 2002), with poly(A+)RNA isolated from hand-dissected ovaries using Ambion's MicroPoly(A)Pure small scale mRNA purification kit. One microgram of RNA was electrophoresed through a 1% agarose-formaldehyde gel and transferred to a Magna NT nylon filter. Probes consisted of ^{32}P-labeled PCR fragments from either the 5′ end (covering exons 1-3) or 3′ end (covering exons 6 and 7) of *egg*.

Egg antibody production and western analysis

Anti-Egg polyclonal antiserum was raised in rats (Cocalico Biological) against a bacterially expressed GST fusion protein containing Egg residues 79-315. The DNA encoding these Egg residues was PCR-amplified from an *egg* cDNA, LD09692, with primers containing overhanging restriction sites (*Eco*RI or *Xho*I) and cloned into the pGEX-4T-1 vector. The GST-Egg fusion protein was expressed in *Escherichia coli* BL21 cells and purified using a MicroSpin GST Purification Module (Amersham). The identity and purity of the fusion protein were determined by western blot using an anti-GST antibody. The antibody was affinity purified by incubating Egg antisera with Cyanogen Bromide (CNBR) Sepharose beads (Amersham) coupled to purified GST-Egg and eluting the affinity purified Egg antibodies with 100 mmol/l glycine, pH 2.5, followed by dialysis into PBS pH 7.2 and addition of an equal volume of glycerol. The specificity of the Egg antisera and affinity purified Egg antibodies were confirmed by the altered levels or patterns of Egg signals observed by antibody labeling of *egg* mutant ovaries (data not shown) and the recognition of a truncated Egg mutant protein (from egg^{235}) on western blots.

To prepare ovary extracts for western blots, hand-dissected ovaries were homogenized in RIPA Buffer (150 mmol/l NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, 50 mmol/l Tris pH 8.0, 5 mmol/l EDTA) with protease inhibitors (Complete Protease Inhibitor Cocktail, Roche), followed by the addition of an equal volume of 2× sample buffer (120 mmol/l Tris pH 6.8, 4% SDS, 20% glycerol, 0.006% Bromophenol Blue, 50 mmol/l DTT). Samples were boiled for 15 minutes, loaded and electrophoresed through a 9% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (MSI) in transfer buffer (39 mmol/l glycine, 48 mmol/l Tris Base, 0.04% SDS, 20% methanol) using a TE42 transfer apparatus (Hoefer Scientific). The membrane was blocked for several hours in 5% non-fat dry milk in PBST (0.1% Tween-20 in PBS). Affinity-purified Egg antibody, 1:170 in 3% BSA in PBST, was incubated with the membrane overnight at 4°C. The secondary antibody, HRP-conjugated goat anti-rat, was hybridized at 1:5000 in 3% BSA in PBST, for 2 hours at room temperature. All washes were done using PBST. Enhanced chemiluminescence (ECL) was used for detection, following company protocols (Amersham).

Whole-mount antibody labeling and microscopy

Ovaries were dissected from flies held on yeasted food, within 1-2 days after eclosion. The fixation and antibody-labeling protocol follows methods reported in Moon and Hazelrigg (Moon and Hazelrigg, 2004). Primary and secondary antibodies were diluted in 0.1% BSA in PBTx (1× PBS, 0.1% Triton X-100) and before DNA staining with propidium iodide, ovaries were RNaseA treated (1 mg/ml) for 2 hours at 37°C.

The following antibodies were used to label ovaries: rat anti-Egg (1:1000) and anti-Vasa (1:500, A. Ephrussi); rabbit anti-Vasa (1:1000, R. Lehmann), anti-H3K9me2 (1:1000, Upstate), anti-H3K9me3 (1:1000, Upstate), anti-phosphorylated Histone H3 (Ser10) (1:100, Upstate) and anti-cleaved Caspase-3 (1:500, Cell Signaling Technology). Mouse monoclonal antibodies obtained from the Developmental Studies Hybridoma Bank included anti-Orb (1:10, P. Schedl) and anti-Fas III (1:10; C. Goodman). FITC- or Rhodamine-conjugated secondary antibodies included goat anti-rat, rabbit or mouse $F(ab')_2$ fragments, and were used at 1:500 or 1:1000 (Jackson ImmunoResearch).

Microscopy was performed on an inverted Olympus IX71 confocal microscope with Fluoview software. Images were analyzed using Image J software (W. Rasband, NIH, Bethesda, MD, http://rsb.info.nih.gov/IJ/).

RESULTS

The *Drosophila eggless* gene encodes an HMT similar to mammalian SETDB1/ESET

We identified the *Drosophila* gene *eggless* (*egg*) in a genetic screen for EMS-induced lethal and female-sterile mutations uncovered by *Df(2R)Dll-Mp* (see Materials and methods). All 13 *egg* mutations isolated in this screen were female-sterile. In females bearing strong alleles (8), oogenesis was arrested at early stages, while weak alleles (5) were associated with mid-late stage oogenesis arrest. Some alleles also reduced viability, indicating that *egg* has other developmental functions in addition to its role in oogenesis. Rescue experiments, using DNA fragments from a region of DNA contained within the *Df(2R)Dll-Mp* deficiency (see Materials and methods), identified a 9 kb DNA fragment that contains the *egg* gene, and the position of *egg* within this fragment was mapped more precisely using a set of truncated and internally deleted fragments (see Fig. S1 in the supplementary material). Probes made from genomic DNA from this region hybridized to a single 3.9 kb band on northern blots of poly(A+) RNA from adult ovaries (Fig. 1A). Using these probes, we isolated three overlapping cDNAs from a *Drosophila* ovary cDNA library. Sequence analysis of these cDNAs, as well as two embryo cDNAs obtained from the DGRC, yielded the proposed *egg* transcript structure shown in Fig. 1C. The Berkeley *Drosophila*

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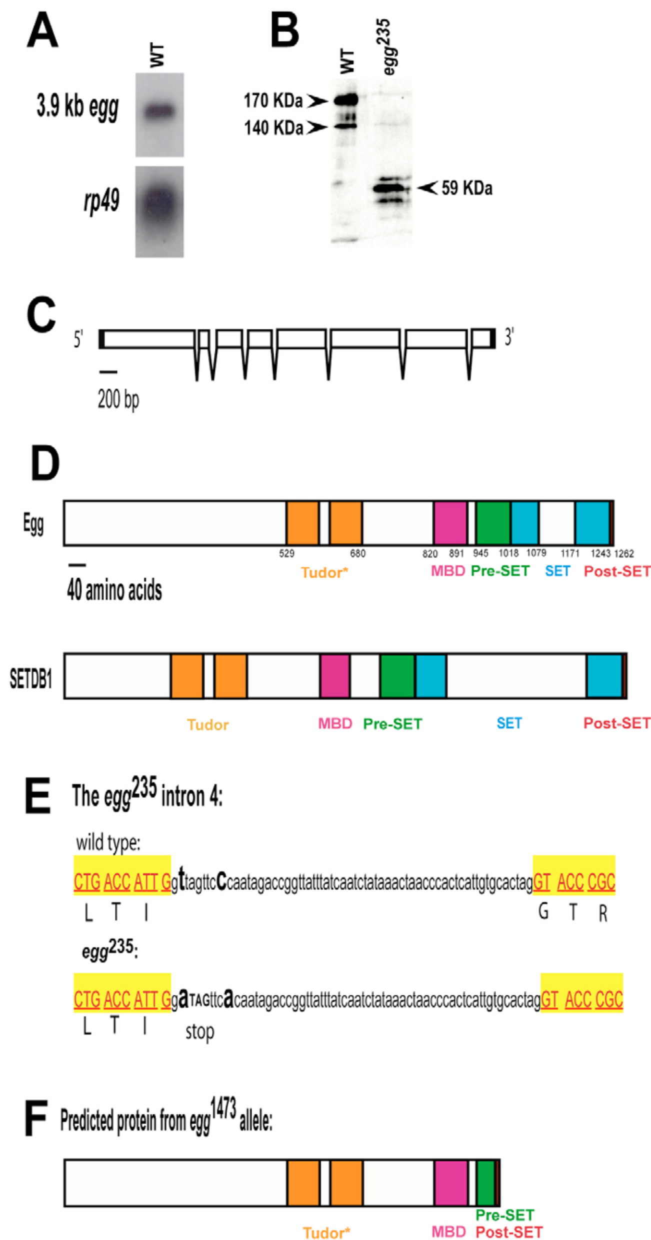

Fig. 1. The *Drosophila eggless* (*egg*) gene encodes a SET domain protein similar to human/mouse SETDB1/ESET. (**A**) Northern blot of ovary poly(A+) RNA hybridized with a probe made from the 5′ end of the *egg* gene. A 3′ probe labels the same 3.9 kb band. (**B**) Western blot of ovary extracts, labeled with an antibody to Egg. Two major protein bands, ~170 and 140 kDa, are detected in wild-type ovaries. These proteins are strongly reduced in egg^{235} ovaries, and replaced with a smaller ~59 kDa protein. (**C**) Proposed structure of the *egg* transcript. This structure is based on the assembly of the complete sequences of three overlapping ovary cDNAs and two embryonic cDNAs. (**D**) The predicted protein structures of Egg and SETDB1, showing the identified protein domains within each protein. (**E**) The egg^{235} allele contains two nucleotide substitutions near the 5′ end of intron 4 (shown in large bold letters). Failure to splice out this intron introduces a premature stop codon (shown in uppercase letters). (**F**) The predicted protein product of the egg^{1473} allele. This allele contains an 856 bp in-frame deletion that removes the entire SET domain. WT, wild type.

Genome Project (BDGP) annotation of this region of the genome (version 4.3) predicted two genes, CG30422 and CG30426, but our analysis of the above cDNAs, including one that is full length, shows that the correct gene structure is as diagrammed in Fig. 1C.

Conceptual translation of this transcript predicts the Egg protein diagrammed in Fig. 1D. Egg contains a bifurcated SET domain, a methyl DNA-binding domain (MBD), and two Tudor-like domains (Tudor*). The SET domain is the catalytic domain of a family of HMTs that methylate histones on lysine residues (Rea et al., 2000) (reviewed by Lachner and Jenuwein, 2002). Egg also contains characteristic cysteine-rich Pre- and Post-SET domain regions that are necessary for enzymatic activity (Xiao et al., 2003). Egg is the sole *Drosophila* protein to contain a bifurcated SET domain (Alvarez-Venegas and Avramova, 2002; Mis et al., 2006), an unusual disruption of the SET domain that characterizes its closest relative, human/murine SETDB1/ESET (Schultz et al., 2002; Yang et al., 2002). *C. elegans* also has a bifurcated SET domain protein, MET-2, of unknown function. Based on sequence comparisons, the SET domain of Egg falls into the Su(var)3-9 family of SET domains, which have specificity for methylating the K9 residue of histone H3 (Alvarez-Venegas and Avramova, 2002; Mis et al., 2006).

The predicted length of Egg (1262 amino acids) is close in size to SETDB1 (1291 amino acids) and ESET (1307 amino acids). A comparison of Egg and SETDB1 revealed that these proteins are 44% identical in the pre-SET plus first half of the SET domain, and 76% identical in the second half of the SET domain plus the post-SET domain. The MBD domains are less conserved (29% identical). While the putative Tudor domains in Egg were predicted with subthreshold E-values by the SMART protein domain identification program [http://smart.embl.de/], the sequence similarity with SETDB1 in this region is high (44% identical). The amino acid segment that divides the SET domain of Egg is much shorter than the segment that interrupts the SET domain of SETDB1 or ESET (91 amino acids in Egg compared to 338 in SETDB1 and 337 in ESET) but is close in size to the corresponding segment of MET-2 (103 amino acids).

We sequenced two *egg* alleles that are members of the strong class of alleles. The first, egg^{235}, contained two nucleotide substitutions at the 5′ end of intron 4 (Fig. 1E). Failure to splice out this intron would yield an mRNA with an in-frame stop codon just within the intron, encoding a truncated protein lacking any of the identifiable domains of Egg. The second allele, egg^{1473}, contained an internal 856 bp in-frame deletion. The predicted protein product of egg^{1473} lacks the entire SET domain (Fig. 1F).

Egg is expressed during oogenesis

We raised a polyclonal antibody to a unique N-terminal segment of Egg (see Materials and methods). This antibody labels two protein bands on ovary western blots, ~170 kDa and ~140 kDa (Fig. 1B). Both proteins were absent or strongly reduced in egg^{235} ovaries, replaced by a smaller truncated protein, indicating that both are *egg* products. We used this antibody to label ovaries to determine the expression pattern and subcellular distribution of Egg during oogenesis (Fig. 2).

Drosophila ovaries consist of 15-20 ovarioles that hold egg chambers in progressive stages of development (Spradling, 1993). These egg chambers contain germ cells derived from germ stem cells located in the germarium, a structure at the tip of each ovariole. At their anterior ends (region 1), each germarium houses two to three

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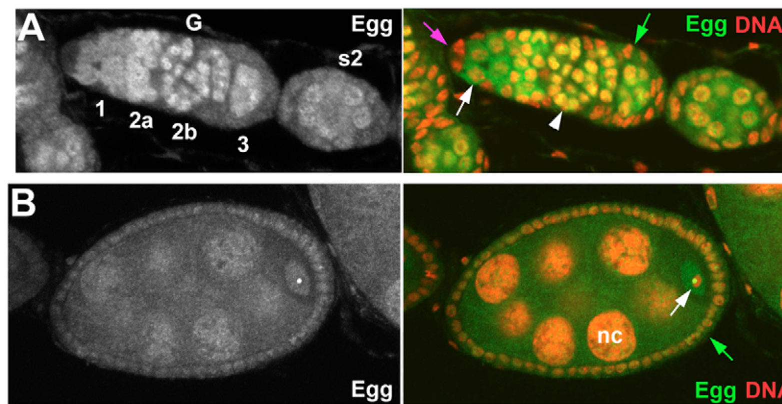

Fig. 2. Expression of Egg protein during oogenesis. Ovaries labeled with an antibody to Egg. The left panels show Egg alone, and the right panels show Egg (green) and DNA (red). (**A**) Germarium and stage 2 egg chamber. The regions of the germarium are labeled. In germ stem cells (white arrow), cystoblasts and dividing cystocytes, the protein is both cytoplasmic and nuclear. In 16-cell germline cysts, Egg is concentrated in the germ cell nuclei (white arrowhead). Egg is not detected in somatic cells at the tip of the germarium (magenta arrow), but is present at low levels in prefollicular cells, and follicle cells of stage 1 egg chambers (green arrow). (**B**) Stage 8 egg chamber. In mid- and late stages of oogenesis, Egg is present throughout the nurse cell nuclei, and localizes to distinct foci associated with the DNA in the oocyte nucleus (white arrow). Egg expression increases in the follicle cells, where the protein is nuclear (green arrow). G, germarium; nc, nurse cell nuclei; s2, stage 2 egg chamber.

germ stem cells (GSCs) that divide asymmetrically to produce another GSC, which replenishes the GSC population, and a cystoblast (CB). The CB undergoes four incomplete mitotic divisions (the dividing cells are called cystocytes) to yield a 16-cell germline cyst; one of these 16 germ cells becomes the oocyte, and the other 15 germ cells form the nurse cells. After germline cysts are formed, they become encapsulated (in region 2 of the germarium) by somatic prefollicular cells to form an egg chamber. At the posterior end of the germarium (region 3) lies a single stage 1 egg chamber, ready to bud off from the germarium and proceed through the rest of oogenesis.

Egg was expressed most strongly at early stages of oogenesis, in germ cells in the germarium (Fig. 2A). In germ stem cells and dividing germline cyst cells, the protein was present at roughly equal levels in the cytoplasm and the nucleus, but after 16 cell germline cysts had formed Egg accumulated preferentially in the nucleus. We did not detect Egg in somatic cells at the tip of the germarium, including the terminal filament, inner sheath cells, or cap cells, but the protein was present at low levels in somatic cells in regions 2 and 3 of the germarium, including the prefollicular cells and the follicle cells of stage 1 egg chambers. Soon after egg chambers budded off the germarium, Egg levels increased in the follicle cells (Fig. 2A). By mid-oogenesis (Fig. 2B) Egg decreased in the nurse cells, while protein levels continued to increase in the follicle cells. In the oocyte nucleus, Egg was present throughout the nucleoplasm and also localized to one to three distinct subnuclear sites associated with the chromosomes (Fig. 2B).

Loss of the Egg SET domain causes early oogenesis arrest

Both of the strong alleles with sequences we report here, egg^{235} and egg^{1473}, had similar oogenesis phenotypes. As egg^{235} may express low levels of Egg (Fig. 1B), by inefficient splicing of the fourth intron, it may provide some *egg* function. However, as egg^{1473} lacks the entire SET domain (Fig. 1F), it provided us with the opportunity to determine the consequences to oogenesis of eliminating the HMT function of *egg*.

Oogenesis arrested very early in egg^{1473} females (Fig. 3). (In all cases, egg^{1473} refers to the genotype egg^{1473}/*Df(2R)Dll-Mp*.) egg^{1473} ovaries were tiny, consisting of germaria that did not bud off normal egg chambers. Many egg^{1473} germaria lacked recognizable regions and instead appeared teardrop-shaped, full of replicating germ cells (see below) and germline cysts that formed no apparent egg chambers (Fig. 3A, middle panels). Disc-shaped germline cysts, which form as germ cells, are encapsulated by prefollicular cells in region 2b of the germarium, were absent in *egg* germaria. In some cases, *egg* germaria had an incompletely budded egg chamber at their posterior ends (Fig. 3A, bottom panel).

Labeling with an antibody to Fasciclin III (Fas III), a membrane-associated protein present in prefollicle and follicle cells, demonstrated that these somatic cell types were present in egg^{1473} germaria (Fig. 3B). However, these cells were reduced in number and failed to completely encapsulate germline cysts. In the partially encapsulated cysts that did exist, germ cells showed at least partial differentiation as nurse cells and oocytes, as judged by the chromosome morphology of the nurse cells, and the accumulation of Orb protein in the oocyte (not shown). In most cases the nurse cells at the posterior end of the mutant germaria had endoreplicated DNA, typical of later stage nurse cell nuclei (Fig. 3A,B).

Egg mediates trimethylation of histone H3 at its K9 residue

As ESET/SETDB1 methylates histone H3 at lysine 9 (H3K9) (Schultz et al., 2002; Yang et al., 2002; Wang et al., 2003; Yang et al., 2003), we examined histone methylation in egg^{1473} ovaries to determine if Egg also methylates H3K9. Histone methylation patterns in *Drosophila* oogenesis have not previously been described, so we first determined the patterns of H3K9 methylation in wild-type ovaries using antibodies that specifically recognize either the dimethylated or trimethylated forms of H3K9 (Fig. 4). We found that both dimethyl H3K9 (H3K9me2) and trimethyl H3K9 (H3K9me3) were present in germ cell and somatic cell nuclei during oogenesis, and occurred with distinct patterns. In the germarium the H3K9me2 signal was strong in both the somatic and germ cells, and

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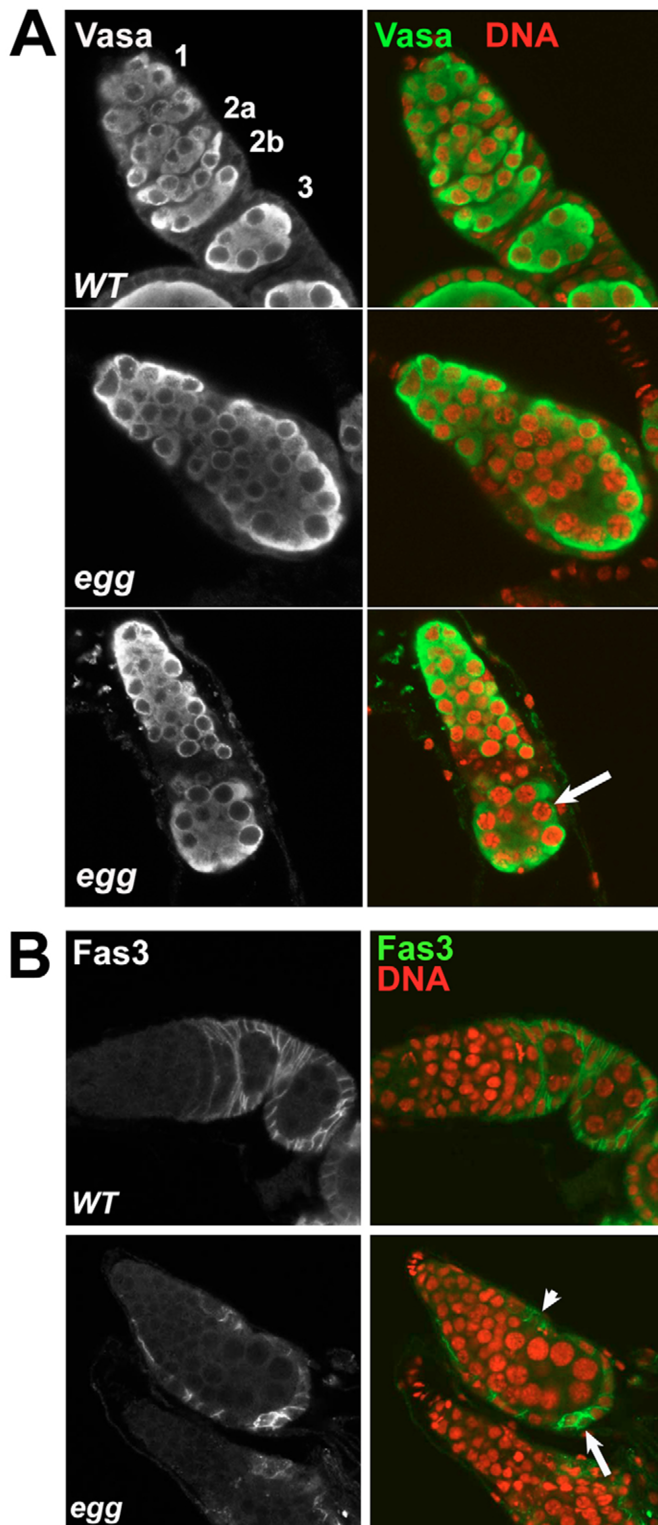

Fig. 3. Oogenesis arrest in *egg* mutants. (**A**) Ovaries labeled for the germ-cell-specific protein Vasa (green) and a DNA dye (red). Regions of the wild-type germarium are indicated in the top panel. egg^{1473} ovaries consist of germaria that fail to bud off normal egg chambers. These germaria often exhibit no recognizable regions and are filled with germ cells and germline cysts that do not form apparent egg chambers (middle panels). In some cases an incompletely budded single egg chamber lies at the posterior end of the mutant germarium (white arrow; bottom panel). (**B**) Ovaries labeled with an antibody to Fas III (Fas3, green) and a DNA dye (red). Incomplete encapsulation of germline cysts occurs in egg^{1473} germaria, and the prefollicular (arrowhead) and follicle cells (arrow) appear reduced in number. WT, wild type.

as oogenesis proceeded the signal remained high in the somatic cells of egg chambers, while germ cell signal decreased (Fig. 4A). By contrast, while H3K9me3 could be detected in both the germ cells and somatic cells of the ovary, it was always more abundant in the germ cells (Fig. 4B). Not every somatic cell showed signal, and no clear pattern of H3K9me3 was present in the soma. The H3K9me3 signal was strongest in the germarium, and decreased as oogenesis proceeded. In the oocyte, strong H3K9me2 and H3K9me3 signals were present in the germinal vesicle, the oocyte nucleus, throughout most of oogenesis.

In egg^{1473} ovaries, H3K9me3 was absent throughout the germarium (Fig. 5A). Sometimes faint H3K9me3 was detected in germ and somatic cells located at the posterior end of the germarium, although at very reduced levels compared with controls. This weak H3K9me3 signal indicates the existence of a separate minor HMT with trimethylase activity that may function at later stages of oogenesis. The H3K9me2 pattern did not change in egg^{1473} ovaries (Fig. 5B), although the H3K9me2 signal sometimes appeared stronger in mutant cells. This apparent increase could occur if H3K9me2 failed to be converted to H3K9me3. We conclude that *egg* is required for trimethylation, but not dimethylation, of histone H3 at the K9 residue. *egg* mediates trimethylation of H3K9 in both the germ and somatic cells of the germarium, and is the major HMT conferring H3K9me3 in these cells.

Reduced somatic cell proliferation and apoptotic cell death in *egg* ovaries

The failure of egg^{1473} egg chambers to bud off the germarium suggests that encapsulation of germline cysts by somatic cells is not normal in *egg* ovaries. This was confirmed by Fas III antibody labeling, which showed that the prefollicle and follicle cell populations were reduced in egg^{1473} germaria (Fig. 3B). To determine the proliferative capacities of the germ and somatic cells of *egg* ovaries, we labeled wild-type or egg^{1473} germaria with an antibody to phosphorylated H3 (Ser 10), a marker of mitotic cells (Fig. 6) (Sauve et al., 1999). Ovaries were simultaneously labeled with an antibody to Vasa, to distinguish whether observed mitoses were occurring in germ cells or somatic cells.

Germ cell mitoses were observed as single cells dividing (GSC or CB divisions) or as multiple nuclei dividing together (cystocytes): two, four or eight nuclei in a group. Examples of all categories of germ cell divisions were observed in egg^{1473} germaria in a frequency not significantly different from control germ cells (Fig. 6C). Although there was a slight increase in the number of mutant germaria with no germ cell mitoses, this increase was not statistically significant.

By contrast, mitotic frequencies in the somatic cells (identified by lack of Vasa labeling) of egg^{1473} germaria showed a marked and statistically significant ($P<0.001$; $n=21$) decrease compared with wild-type somatic cells (Fig. 6D). Of 21 control germaria counted, 18 contained at least one mitotic somatic cell and frequently had

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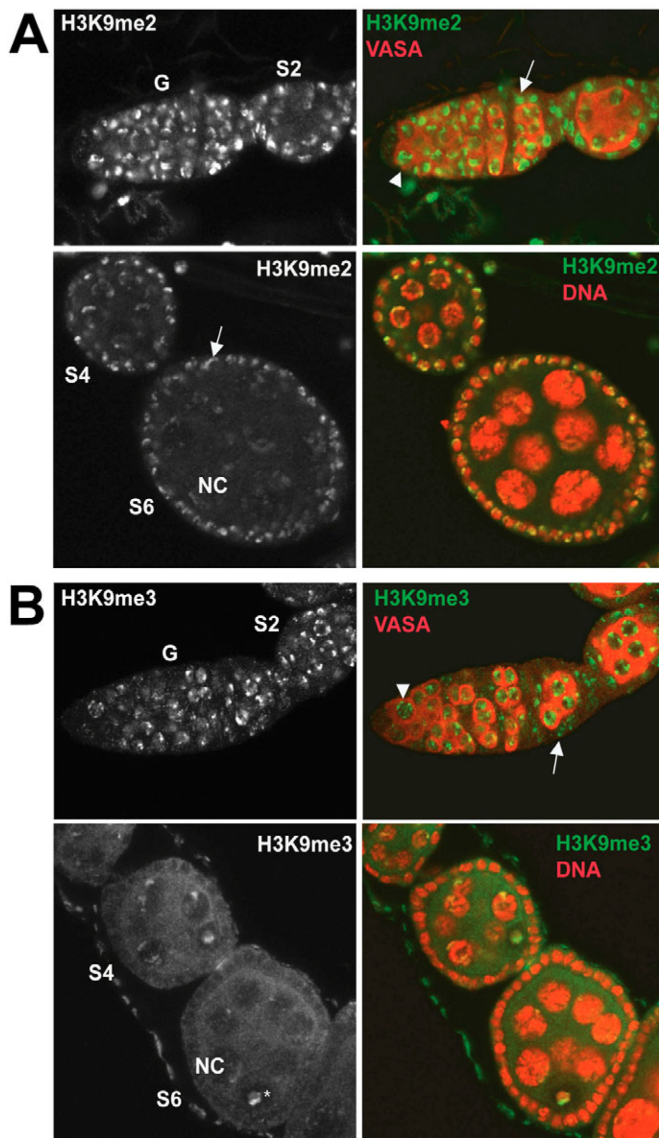

Fig. 4. Patterns of Histone H3 Lysine 9 (H3K9) methylation during oogenesis. Wild-type ovaries labeled with antibodies to dimethyl-H3K9 (H3K9me2) or trimethyl-H3K9 (H3K9me3), and an antibody to Vasa, which labels germ cells, or a DNA dye. Left panels, methylated H3K9 alone; right panels, merged images of H3K9me2 or H3K9me3 (green) and Vasa (red) or DNA (red). (**A**) H3K9me2 is present in both the germ cells and somatic cells in the germarium and newly budded egg chambers (upper panels). As oogenesis proceeds (stage 4 and 6 egg chambers in bottom panels), the H3K9me2 signal increases in the somatic cells relative to the germ cells. A nurse cell nucleus, germ stem cell nucleus (arrowhead) and follicle cell nuclei (arrows) are indicated. (**B**) In the germarium (top panels) H3K9me3 is present in both the germ cells and somatic cells, with higher levels apparent in the germ cells. A germ stem cell (arrowhead) and somatic cells (arrow) are indicated. In later stages (stage 4 and 6 egg chambers in bottom panels), H3K9me3 persists in the germ cells but is reduced in somatic cells. The signal is relatively weak in nurse cell nuclei compared with the oocyte nucleus (*). G, germarium; NC, nurse cell nuclei; S2/S4/S6, stage 2/4/6 egg chambers.

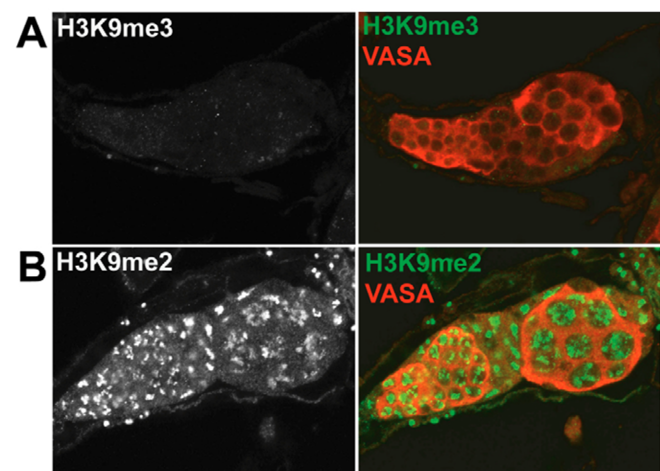

Fig. 5. *egg* is required for trimethylation but not dimethylation of H3K9 in germ and somatic cells during oogenesis. Left panels, methylated H3K9 alone; right panels, merged images of H3K9me3 or H3K9me2 (green) and Vasa (red). (**A**) H3K9me3 is absent or severely reduced in both the germ and somatic cell nuclei of egg^{1473} germaria. (**B**) High levels of H3K9me2 are present in both germ and somatic cell nuclei of egg^{1473} germaria.

multiple somatic cells simultaneously undergoing mitosis (we observed a maximum of four). In wild-type germaria, somatic cell mitoses were observed in regions 2b and 3 of the germarium, where dividing prefollicle and follicle cells lie, and also in single somatic cells about midway through the germarium, near the 2a/b border, where the somatic stem cells reside (Zhang and Kalderon, 2001). By contrast, only two of 21 egg^{1473} germaria contained a single mitotic somatic cell. In both cases, the mitotic cells were located at the posterior end of the germarium. These observations indicate that the proliferation of prefollicle and follicle cells, and possibly also somatic stem cells, is strongly reduced in egg^{1473} ovaries.

In egg^{1473} ovaries stained with DNA dyes, we noted the presence of pycnotic nuclei, suggestive of apoptotic cell death. To address this possibility, we labeled ovaries with an antibody against cleaved Caspase-3, a marker of apoptotic cells (Fig. 7) (Gilboa and Lehmann, 2006). We observed abundant levels of apoptosis in egg^{1473} germaria: of 24 egg^{1473} germaria, 21 showed positive labeling with the antibody to cleaved Caspase-3, whereas only 1 of 27 control germaria was labeled. While apoptosis was observed in both the germ and somatic cells of egg^{1473} germaria, it was predominantly seen within the somatic cells (Fig. 7). Similar results were obtained using the TUNEL method for detecting apoptosis (not shown). The majority of somatic apoptotic cells reside in the posterior region of egg^{1473} germaria, in cells found between incompletely budded egg chambers and anterior germ cells. The follicle cells associated with the incompletely budded egg chamber were also frequently undergoing apoptosis. Apoptotic somatic cells at the anterior of egg^{1473} germaria (including terminal filament and cap cells) were never observed (n=24). The death and reduced proliferation of somatic cells that give rise to follicle cells and stalk cells provides an explanation for the failure of *egg* mutants to bud off fully encapsulated egg chambers.

DISCUSSION

Egg is a member of a small class of SET domain proteins that contain bifurcated SET domains (Alvarez-Venegas and Avramova, 2002; Mis et al., 2006), and is most similar to the human protein SETDB1 and its mouse ortholog ESET (Schultz et al., 2002; Yang et al., 2002). While functional rescue experiments have not yet

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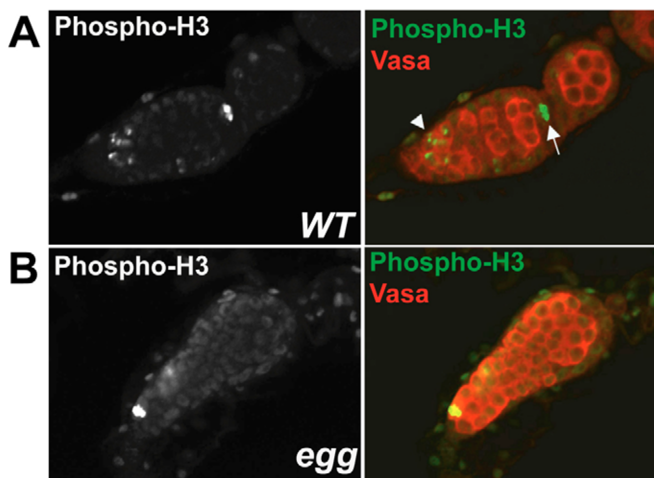

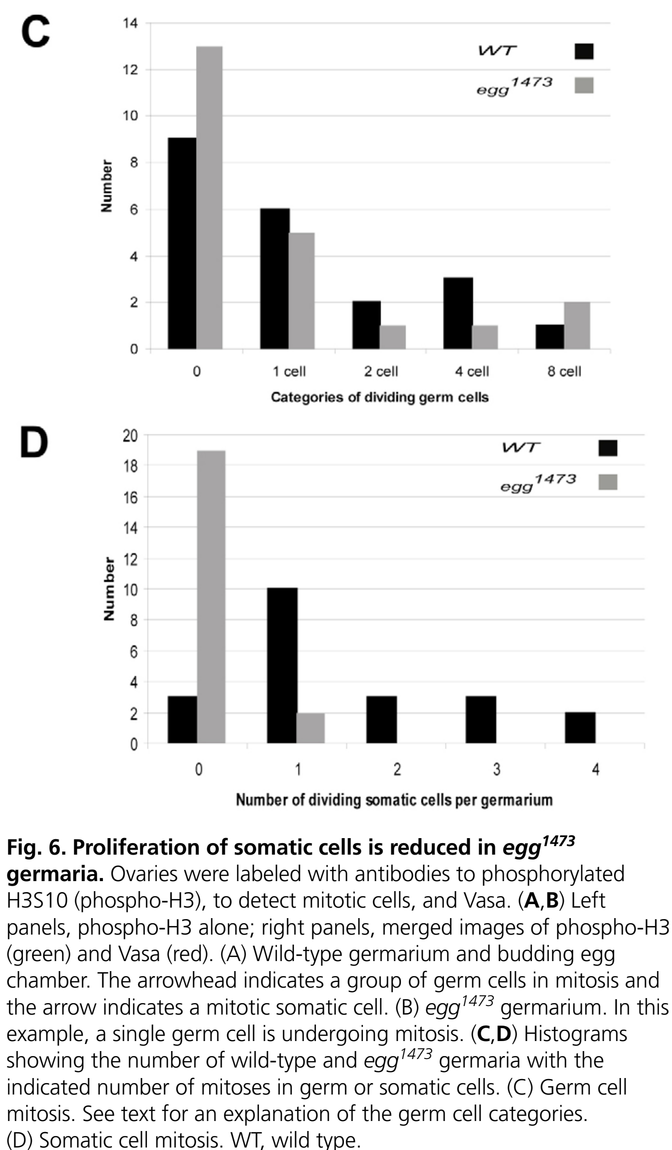

Fig. 6. Proliferation of somatic cells is reduced in *egg*^{1473} germaria. Ovaries were labeled with antibodies to phosphorylated H3S10 (phospho-H3), to detect mitotic cells, and Vasa. (**A**,**B**) Left panels, phospho-H3 alone; right panels, merged images of phospho-H3 (green) and Vasa (red). (A) Wild-type germarium and budding egg chamber. The arrowhead indicates a group of germ cells in mitosis and the arrow indicates a mitotic somatic cell. (B) *egg*^{1473} germarium. In this example, a single germ cell is undergoing mitosis. (**C**,**D**) Histograms showing the number of wild-type and *egg*^{1473} germaria with the indicated number of mitoses in germ or somatic cells. (C) Germ cell mitosis. See text for an explanation of the germ cell categories. (D) Somatic cell mitosis. WT, wild type.

shown if the mammalian proteins can substitute for Egg in vivo, the similarity in size, overall domain architecture and high sequence similarity within their shared domains suggests that they are likely to be functional orthologs.

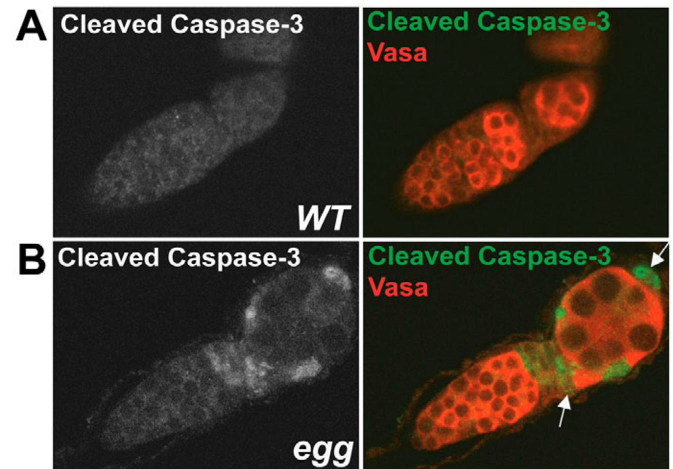

Fig. 7. Abundant apoptotic cell death occurs in *egg*^{1473} germaria. Ovaries were labeled with antibodies to cleaved Caspase-3, a marker of apoptotic cell death, and Vasa. Left panels, cleaved Caspase-3 alone; right panels, merged images of cleaved Caspase-3 (green) and Vasa (red). (**A**) Wild-type germarium. No apoptotic cells are present in this example, and were only rarely seen in our sample. (**B**) *egg*^{1473} germarium. Several apoptotic somatic cells are present in this example (arrows). See text for a full description. WT, wild type.

Experiments with tissue culture cells bearing reporter genes have shown that SETDB1 and ESET negatively regulate gene expression, and that their targets are likely to be euchromatic genes (Schultz et al., 2002; Yang et al., 2003). Despite these important studies, the biological roles of SETDB1 and ESET in development are poorly understood. RNAi knockdown of SETDB1 in tissue culture cells is cell lethal (Wang et al., 2003; Sarraf and Stancheva, 2004), and the very early embryonic lethality of a mouse ESET insertion allele, along with possible maternal contributions of its gene product, has complicated the analysis of its in vivo functions (Dodge et al., 2004). The genetic analysis of *egg* reported here has allowed us to examine the in vivo contributions of this HMT in a developmental context, and to show that Egg plays an essential role in oogenesis. The strong expression of ESET in testes suggests that the mammalian proteins may also play similar roles in gametogenesis (Yang et al., 2002).

Biochemical studies demonstrated that ESET and SETDB1 methylate histone H3 at its K9 residue (Schultz et al., 2002; Yang et al., 2002; Wang et al., 2003; Yang et al., 2003), and we show here that in vivo Egg also has H3K9 HMT activity. Specifically, we found that trimethylation of histone H3K9 occurred during oogenesis, in both the germ cells and somatic cells, in an *egg*-dependent manner (Fig. 5). Egg does not appear to be required for dimethylation of H3K9, as the H3K9me2 signal remained strong in *egg*^{1473} ovaries. These observations suggest a pathway for H3K9 methylation, with Egg catalyzing the addition of a terminal methyl group to H3K9me2, previously established by a separate HMT.

Egg is present in germ cells at the earliest stages of oogenesis, including germ stem cells, cystoblasts, dividing cystocytes and newly formed germline cysts (Fig. 2A). While we have not detected Egg in anterior somatic cells, including terminal filament, cap and interstitial cells, low levels of Egg were present in more posterior somatic cells, including prefollicular cells and follicle cells of stage 1 egg chambers. However, Egg was also expressed in postgermarial egg chambers (Fig. 2B), and is therefore likely to have functions at later stages of oogenesis as well. Of particular interest is the strong

accumulation of Egg in the oocyte nucleus, in distinct subnuclear foci. The oocyte nucleus is arrested at prophase of meiosis I, and is generally transcriptionally quiescent, raising the possibility that Egg could contribute to transcriptional repression in the oocyte and/or meiotic cell cycle control.

Strong *egg* alleles, including *egg*^{1473}, which deletes the entire SET-domain-coding region, caused very early arrest of oogenesis. Mutant ovaries consisted of germaria in which the early stages of egg chamber formation were not clearly demarcated. While proliferating germ cells were present, the existing germline cysts were not fully encapsulated by somatic follicle cells, and did not bud off normally from the germarium.

We have shown that *egg* is required for the proliferation and viability of somatic cells in the germarium, and that a reduction in somatic cell populations is likely to be the cause of the encapsulation and budding defects observed in mutant germaria (Figs 6 and 7). In wild-type germaria, we observed mitosis in prefollicular and follicle cell populations, as well as in single cells located near the 2a/b border, where somatic stem cells reside. As the only examples of somatic cells undergoing mitosis in *egg*^{1473} ovaries were cells positioned at the posterior end of germaria, we think it is likely that *egg* affects proliferation of at least three populations of somatic cells: the somatic stem cells, the prefollicular cells and the follicle cells that surround newly formed egg chambers. *egg* is also required for the viability of both the germ and somatic cells, as apoptotic cell death occurred in both cell types in *egg*^{1473} ovaries.

Several HMTs play roles in cell proliferation, and aberrant HMT expression is in some cases oncogenic (reviewed by Santos-Rosa and Caldas, 2005). Histone methylation can impinge on cell proliferation by either of two routes: by regulating the expression of genes that in turn regulate the cell cycle (Nielsen et al., 2001), or by promoting structural changes in chromosomes necessary for mitosis (Melcher et al., 2000). SETDB1 has recently been shown to function at promoters that are silenced in human cancers, suggesting that it too may normally play a role in regulating cell proliferation (Li et al., 2006).

There are important questions that remain to be answered. We have shown that Egg mediates H3K9 methylation in early oogenesis, and that loss of H3K9me3 has striking biological consequences for oogenesis, but we do not yet know the exact genomic effects of this methylation program. Methylation of H3K9 plays an important role in the formation of heterochromatin domains, and also regulates the expression of individual euchromatic genes (Lachner and Jenuwein, 2002). Our analysis of Egg localization in whole-mount ovaries indicates that it is associated with distinct foci within germ and somatic cell nuclei (Fig. 2A), but the small size of these chromosomes has not allowed us to precisely map these sites. A goal of future work will be to identify the genomic targets of Egg, a necessary and important first step in determining whether Egg regulates euchromatic gene expression or plays a role in establishing heterochromatic domains.

Another important goal of future work is to determine which cell types require *egg* activity during early oogenesis. While we have shown that Egg is expressed in both germ cell and somatic cell populations in the ovary, and mediates H3K9 methylation in both cell types, the fact that numerous germ cell-somatic cell interactions contribute to early oogenesis (Roth, 2001) implies that the functional consequences of perturbations in histone methylation patterns in one cell type could impact on the development of other cells. Thus somatic cell defects could arise from loss of H3K9me3 in germ cells, and vice versa. Future experiments, using clonal analysis and the expression of *egg* transgenes in specific cell types, should allow us to determine unequivocally which cells require the HMT activity of *egg*.

We thank Marty Chalfie, Songtao Jia and Dan Kalderon for comments on the manuscript, Jym Mohler for the *Drosophila* genomic library, and Anne Ephrussi and Ruth Lehmann for antibodies. This work was supported by an NSF grant to T.H., and E.C. was supported by an NIH training grant to the Columbia University's Department of Biological Sciences.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/1/02698/DC1

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