DRMT4 (*Drosophila* Arginine Methyltransferase 4) Functions in *Drosophila* Oogenesis

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Abstract

DRMT4 (*Drosophila* Arginine Methyltransferase 4) is an arginine methyltransferase in *Drosophila* (Boulanger et al. 2004). It shows the highest identities with mammalian PRMT4/CARM1 (Protein Arginine Methyltransferase 4) (59% identity, 75% similarity). HPLC analysis demonstrated that DRMT4 belongs to the type I class of methyltransferases (Boulanger et al. 2004), meaning that DRMT4 catalyzes asymmetrical dimethylarginine formation. A polyclonal antibody against DRMT4 was generated and used to study DRMT4 expression using western blots and immunostainings. In order to study *DRMT4* function in *Drosophila* using genetic methods, we created three kinds of *DRMT4* transgenes: a genomic *DRMT4* under its own control, a genomic *DRMT4-GFP* fusion gene and a *cDNA DRMT4* under UAS control. We investigated DRMT4 localization in wild type flies using the *DRMT4-GFP* transgenic line and immunostaining. In embryos, DRMT4 signal is predominantly found in the cytoplasm until cycle 13 and then in the nuclei when gastrulation starts. In larvae it is mainly expressed in the imaginal discs and in the larval brain. During oogenesis, DRMT4 is found in the cytoplasm of follicle cells and in the nuclei of nurse cells and oocytes. The presence of elevated *DRMT4* mRNA in ovaries and early embryos suggests that *DRMT4* is mainly maternal (Boulanger et al. 2004). To investigate the role of *DRMT4* during oogenesis, we isolated two EMS induced female sterile mutants, *E0467* and *E6115*. As assessed by western blot analysis the DRMT4 protein levels are severely reduced in both mutants compared with wild type flies. The complete genomic *DRMT4* transgene rescued the female sterile phenotype.

Moreover, targeted expression of *DRMT4* in the germline cells, but not in the somatic cells, rescued the female sterile phenotype of *E0467* and *E6115*. This indicates that DRMT4 in the germline cells is crucial for *Drosophila* oogenesis.

Résumé

DRMT4 (*Drosophila* Arginine Methyltransferase 4) est une méthyltransférase arginine de *Drosophila*. Elle est le plus proche en séquence de la protéine des mammifères PRMT4/CARM1 (Protein Arginine Methyltransferase 4) (59% identité, 75% similarité). Une analyse par HPLC a démontré que DRMT4 appartient à la classe de méthyltransférases de type I (Boulanger et al. 2004), ce qui signifie qu'elle catalyse la formation de diméthylarginines assymétriques. Un anticorps polyclonal dirigé contre la protéine DRMT4 a été produit et utilisé afin d'analyser l'expression de DRMT4 á l'aide de Western-blots et d'immunofluorescence.

Afin d'étudier la fonction de *DRMT4* dans la drosophile par une approche génétique, nous avons créé trois différents transgènes de *DRMT4*: la séquence génomique de *DRMT4* sous son propre contrôle, une fusion *DRMT4* génomique – *GFP*, et l'ADNc de *DRMT4* sous le contrôle de *UAS*. Nous avons étudié la localisation de la protéine DRMT4 dans les mouches sauvages en utilisant la lignée transgénique *DRMT4-GFP* et l'immunofluorescence. Dans les embryons, le signal est détecté majoritairement dans le cytoplasme jusqu'au treizième cycle, puis dans les noyaux dès le début de la gastrulation. Dans les larves, la protéine est principalement exprimée dans les disques imaginaux et dans le cerveau. Au cours de l'ovogenèse, DRMT4 se trouve dans le cytoplasme des cellules folliculaires et dans les noyaux des cellules nourrices et des ovocytes. La présence d'importantes quantités d'ARNm de *DRMT4* dans les ovaires et dans les jeunes embryons suggère que cet ARNm est principalement d'origine maternelle

(Boulanger et al. 2004). Pour mieux comprendre le rôle de *DRMT4* au cours de l'ovogenèse, nous avons isolé deux mutations EMS femelles stériles, *E0467* et *E6115*. L'analyse par Western-blot montre que, dans les deux mutants, les niveaux de DRMT4 sont bien inférieurs à ceux présents dans les mouches sauvages.

Le transgène contenant la séquence génomique complète de *DRMT4* permet d'éliminer le phénotype stérile des femelles mutantes. De plus, l'expression ciblée de *DRMT4* dans les cellules de la lignée germinale uniquement et pas dans les cellules somatiques permet de retablir la fertilité des femelles *E0467* et *E6115*. Cela indique que la présence de DRMT4 dans les cellules de la lignée germinale est cruciale pour le bon déroulement de l'ovogenèse de la drosophile.

Introduction

Drosophila Oogenesis

1. Oocyte determination

Drosophila ovaries are composed of ovarioles. Each ovariole has a germarium at its tip and older egg chambers in the adjacent more posterior region. At the anterior tip of the germarium, two to three germ line stem cells divide to produce a cystoblast (Spradling 1993b). Cystoblasts undergo four cycles of cell division to form a 16-cell cyst. Several genes have been found to function early in oogenesis for stem cell maintenance and for division and differentiation of the germ-line cells. Bam (Bag of marbles) exists as a fusome component and *bam* mutant germ cells are blocked in differentiation, they are trapped as mitotically active, stem-cell like cells (McKearin and Ohlstein 1995). In *encore* mutants, one extra round of cystocyte division is caused and 32 cell follicles are produced (Hawkins et al. 1996).

Then, the cystocytes begin their differentiation into either nurse cells or oocyte fate. During this process, a single cystocyte develops a microtubule-organizing center (MTOC) and forms a polarized microtubule network that extends into all 16 cells (Theurkauf et al. 1993). The single cell of the cyst that contains the MTOC develops into the oocyte. In *Bicaudal-D* and *egalitarian* mutants, oocyte-specific mRNAs fail to accumulate and the cystocytes differentiate into 16-nurse-cells without any oocyte (Suter and Steward 1991; Theurkauf et al. 1993; Ran et al. 1994). During normal oogenesis, the majority of

the spectrosomal material is inherited by only one of the two daughter cells at the first cystoblast division. During the following divisions, the spectrosome grows from this cystocyte into the other cells to form the fusome (Lin and Spradling 1995). It was suggested that the asymmetric inheritance of the spectrosome determines which of the two daughter cells will give rise to the cell that becomes the oocyte (Lin and Spradling 1995; Grunert and St Johnston 1996).

In *par-1* null mutants, the oocyte is initially specified but loses its character as the egg chamber leaves the germarium and ultimately adopts a nurse cell fate (Cox et al. 2001; Huynh et al. 2001). Determination of oocyte fate is accompanied by the translocation of BicD, the MTOC and the centrioles from the anterior to the posterior of the oocyte, constituting the first sign of polarity within the oocyte itself (Pare and Suter 2000; Clegg et al. 2001; Huynh et al. 2001). The fact that the posterior shift of oocyte specific markers does not take place in *par-1* mutants in which oocyte identity eventually gets lost suggests that there might be a causal relationship between translocation and maintenance of oocyte fate (reviewed in (Riechmann and Ephrussi 2001)).

2. Oocyte development

Once the oocyte is determined, the remaining 15 nurse cells undergo endoreduplication of DNA and supply the oocyte with materials through cytoplasmic bridge called the ring canals (reviewed in (Spradling 1993a)). The microtubule cytoskeleton is required for essential steps at different stages of oogenesis (Koch and Spitzer 1983; Theurkauf et al. 1993). Tavosanis and

Gonzales studied the centrosomal component γ-tubulin during *Drosophila* oogenesis. They found that females carrying the weaker double mutant combination of γ-tubulin mutant alleles are sterile, but lay a few eggs. Their ovaries are much reduced in size and contain only a few egg chambers (Tavosanis and Gonzalez 2003).

Patterning of the *Drosophila* egg requires cooperation between the germline cells and surrounding somatic follicle cells. The anteroposterior (A-P) polarity of the oocyte is established when Gurken is localized to the posterior of the oocyte and signals to the overlying follicle cells via *torpedo* to adopt a posterior follicle cell fate (Gonzalez-Reyes et al. 1995; Roth et al. 1995). These follicel cells then signal back to the oocyte, resulting in the establishment of it's A-P polarity (Theurkauf et al. 1992). In 2003, Gupta and Schüpbach identified a new gene, *phosphocholine cytidylyltransferase* (CCT), which is involved in follicle cell patterning. Mutations in *Cct1* result in a number of oogenesis defects, including a loss of germline stem cell maintenance, mispositioning of the oocyte, and a shortened operculum (Gupta and Schupbach 2003).

In order to identify more genes required for oogenesis, Morris *et al* carried out a clonal screen using the *Flp-FRT-ovoD* system and identified eight new genes, *omelet, soft boiled, hard boiled, poached, fried, over easy, sunny side up* and *benedict*. They found that these mutants exhibited phenotypes in oocyte specification or early development (Morris et al. 2003).

In short, there are hundreds of genes involved in *Drosophila* oogenesis. According to their diverse functions, they can be divided into several groups:

genes required for the maintenance and division of germ-line stem cells, e.g. *bag of marbles* (McKearin and Ohlstein 1995); genes for oocyte determination and formation of the anterior-posterior axis, e.g. *vasa* (Lasko and Ashburner 1988); genes for establishing dorsal-ventral polarity, e.g. *gurken* (Neuman-Silberberg and Schupbach 1993); genes affecting the cytoskeleted transport, e.g. *Bicaudal-D* (Suter and Steward 1991); genes influencing the cell cycle, e.g. *cyclin E* (Masrouha et al. 2003).

GAL4/UAS system in *Drosophila*

1. GAL4 and UAS

GAL4 is a regulator of gene expression in the yeast *Saccharomyces cerevisiae* (Laughon et al. 1984; Laughon and Gesteland 1984; Oshima et al. 1984). It encodes an 881 amino acids protein whose functions in DNA binding and transcriptional activation have been well studied (Ptashne 1988). The DNA binding activity of GAL4 maps to the first 74 residues, while its transcriptional activation function maps to two regions around residues 148–196 and 768–881 (Ma and Ptashne 1987). GAL4 regulates transcription by directly binding to the UAS (Upstream Activating Sequences) elements (Giniger et al. 1985). These binding sites are analogous to an enhancer element defined in multicellular eukaryotes, and they are essential for the transcriptional activation of these GAL4-regulated genes (Giniger et al. 1985). GAL4 can function in a wide variety of systems to activate transcription from UAS elements (Kakidani and Ptashne 1988; Ma et al. 1988; Webster et al. 1988). In 1988, Fischer *et al.* demonstrated

that GAL4 expression was capable of stimulating transcription of a reporter gene under UAS control in *Drosophila,* and that the expression of GAL4 in *Drosophila* initially appeared to have no deleterious phenotypic effects (Fischer et al. 1988).

2. GAL4/UAS system is a tool for targeted gene expression in *Drosophila*

In 1993, Brand and Perrimon described the use of the GAL4/UAS system for targeted gene expression in *Drosophila* (Brand and Perrimon 1993). They created a pUAST vector that contains five tandemly arrayed and optimized GAL4 binding sites. In my study, this pUAST vector was used to construct a recombinant vector in which the cDNA of interest is cloned behind the UAS control elements. The UAS construct and the GAL4 driver are maintained in different lines. In order to start transcription of the gene of interest, transcription from UAS needs to be initiated by GAL4. For this purpose, the responder lines (UAS with targeted gene) are crossed with the driver lines in which GAL4 is expressed in the desired pattern. The resulting progeny then expresses the gene in a pattern that reflects the GAL4 expression pattern of the respective driver.

Recently, Duffy summarized several strengths of this bipartite approach (Duffy 2002). 1) Because the responder gene is in a silent state in the UAS line as long as it is not crossed to the GAL4 driver, the gene is not toxic, not lethal, and does not reduce viability. Therefore, it is a convenient system in which to study the function of toxic or oncogenic genes. 2) To date, a lot of GAL4 lines have been generated and GAL4 can be expressed in a particular pattern in each line.

Since the expression pattern of the responder gene is dependent on the GAL4 expression pattern, it is possible to target gene expression in a variety of spatial and temporal fashions or in specific tissue. Then the effects of mis-expression of some specific gene may be observed.

3. Rescue of a *Drosophila* mutant phenotype using the GAL4/UAS system

As the GAL4/UAS system can target gene expression in specific patterns, it has been widely used to rescue mutant phenotypes in *Drosophila.* In 1995 Lawrence *et al.* used the GAL4/UAS system to rescue the repeated clusters of *even-skipped* expression cells. They made embryos in which the Wingless protein is uniformly expressed (Lawrence et al. 1995). In 1997, a UAS-α_{PS2} transgene was used to rescue the embryonic lethality caused by the lack of $\alpha_{PS2.}$ The transgenic line was crossed with the *24B* GAL4 line in which *GAL4* is expressed in the embryonic mesoderm starting at stage 10 (Martin-Bermudo et al. 1997). Svendsen *et al.* (Svendsen et al. 2000) were able to rescue the anterior compartment *combgap* phenotype by expressing additional *cubitus interruptus* using the GAL4/UAS system. It suggested that the Combgap protein could be a direct regulator of *cubitus interrupus* transcription. Another study showed that the expression of *fru* transgenes rescues the *eve* phenotype in *fru* mutants (Song et al. 2002). In this case, *fru* transgene expression was driven by the *sca-GAL4 and* the *elav-GAL4* drivers.

Instead of the cDNA clone, the use of the GAL4 system also works with genomic transgenes. In 1999, Dezazzo *et al.* inserted a genomic fragment of amn^{+} into pUAST. With the use of a *UAS-*amn^{+} transgene, the memory defects of amn^{28A} were completely rescued (DeZazzo et al. 1999). The amn^{28A} is a mutant allele caused by the insertion of a *GAL4* enhancer trap transposon. As an inherent *GAL4* insertion already existed in the amn^{28A} mutation, they just crossed amn^{28A} females to *UAS-* amn^{+} male.

In summary, it is possible to rescue phenotypes caused by mutated genes using the GAL4/UAS system. Basically, the *GAL4*-driven expression pattern should be similar to that of the endogenous, wild type gene.

Arginine Methyltransferases

1. Protein arginine methylation

Covalent modifications of amino acid residues are very important for protein function. There are various different types of post-translational modifications, such as methylation and phosphorylation. One of them is the S-adenosylmethionine (AdoMet or SAM) -dependent methylation. It commonly occurs on carboxyl groups of glutamate, leucine, and isoprenylated cysteine, or on the side-chain nitrogen atoms of lysine, arginine, and histidine residues (Kakimoto et al. 1975; Clarke 1993). Protein arginine methylation is found in most cells of higher eukaryotic species (Paolantonacci et al. 1986) as well as in lower eukaryotes (Yarlett et al. 1991; Schurter et al. 2001). In contrast, to date it has not yet been found in prokaryotic organisms. There are two distinct classes of

protein arginine N-methyltransferases, type I and type II. The type I enzyme catalyzes the formation of N^G-monomethylarginine and asymmetric N^G, N^G-dimethylarginine; whereas the type II enzyme catalyzes the formation of N^G-monomethylarginine and symmetric N^G,$N^{G'}$- dimethylarginine residues (Fig.1). Several *in vivo* substrates have been identified for the Type I (asymmertrically methylating) enzyme, including hnRNP A1, Fibrillarin, and Nucleolin. In contrast, only few *in vivo* substrates for the Type II (symmetrically methylating) methyltransferase are known. They include myelin basic protein (Gary and Clarke 1998) and two RNA-binding proteins, spliceosomal snRNP proteins SmD1 and SmD3 (Brahms et al. 2000). Recently, Boisvert *et al.* identified over 200 new proteins that are putatively arginine methylated. They used anti- asymmetrical or symmetrical dimethylated arginine antibodies for isolating proteins and microcapillary reverse-phase liquid chromatography for analysis (Boisvert et al. 2003)

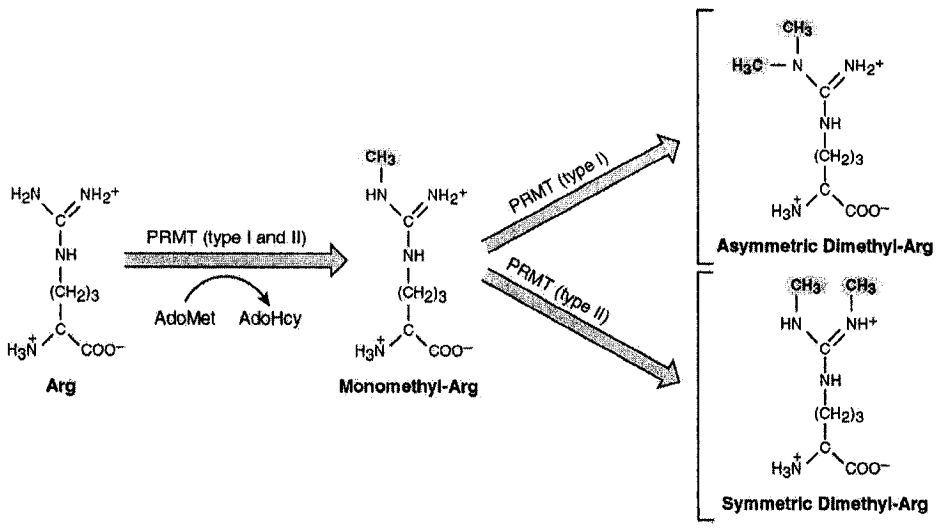


Figure 1. Chemistry of arginine methylation. Molecular structure of arginine, and mono- and di-methylargininie. Type I and II protein arginine methlytransferases catalyze asymmetric and symmetric dimethylation, respectively. (From Zhang, Y. and Reinberg, D. Genes Dev. 15: 2343-60, 2001)

2. The family of Protein arginine N-methyltransferases --PRMTs

The family of mammalian Protein arginine N-methyltransferases (PRMTs) includes protein arginine methyltransferases (PRMT)-1, -2, -3, -4, -5, -6 (Lin et al. 1996; Chen et al. 1999; Pollack et al. 1999; Frankel and Clarke 2000; Frankel et al. 2002), and the yeast hnRNP arginine methyltransferase 1 (HMT1) (Henry and Silver 1996). Except for PRMT4, these arginine methyltransferases share a highly conserved catalytic domain, the RGG domain (Zhang and Reinberg 2001; Kouzarides 2002). However, the substrates of PRMT4, which is also called the coactivator-associated arginine methyltransferase 1 (CARM1), do not harbor RGG motifs (Lee and Bedford 2002). The methylation motif in CARM1/PRMT4

substrates, PRBP1 and histone H3, is APR. The only type II PRMT identified to date is PRMT5/JBP1, the Janus kinase-binding protein (Pollack et al. 1999; Branscombe et al. 2001; Rho et al. 2001).

1.1 PRMT1

PRMT1 was initially identified in a yeast two-hybrid experiment. It was found to interact with the immediate-early gene product TIS21 and the anti-proliferative protein BTG1 (Lin et al. 1996). A recent study showed that PRMT1 is a major histone H4-specific methyltransferase (Wang et al. 2001). PRMT1 has been implicated in regulating multiple cellular processes by methylating proteins. These processes include nuclear-cytoplasmic transport (Russell and Tollervey 1992; Henry and Silver 1996; Lee et al. 1996; Shen et al. 1998; McBride et al. 2000), interferon-mediated signal transduction (Schindler and Darnell 1995; Abramovich et al. 1997; Mowen et al. 2001), and transcriptional activation involving histone methylation (Lin et al. 1996; Strahl et al. 2001; Wang et al. 2001).

1.2 PRMT2

The gene encoding PRMT2 was identified by screening EST (expressed sequence tag) databases (Katsanis et al. 1997). Recently, PRMT2 was also identified as a new ERα (estrogen receptor α)-binding protein using yeast two-hybrid screening. The potential methyltransferase activity of PRMT2 appeared to

be related to its coactivator function (Qi et al. 2002). However, whether PRMT2 possesses protein arginine methyltransferase activity remains to be demonstrated.

1.3 PRMT3

PRMT3 was identified in a yeast two-hybrid screen using PRMT1 as a bait (Tang et al. 1998). PRMT3 can function as monomer and is predominantly localized in the cytoplasm (Tang et al. 1998). A unique feature of PRMT3 is the presence of a zinc finger at its amino terminus (Zhang and Reinberg 2001). This zinc finger is required for recognizing the RNA-associated substrates in RAT1 cell extracts and it plays an important role in determining substrate specificity of PRMT3 (Frankel et al. 2002).

1.4 PRMT4

CARM1/PRMT4 was identified in a yeast two-hybrid screen using the carboxy-terminal domain of GRIP1 as a bait (Chen et al. 1999); for details see next chapter. CARM1 has been shown to methylate histone H3, the poly(A)-binding protein 1 (PABP1) (Lee and Bedford 2002), the RNA-binding protein HuR (Li et al. 2002), and the transcriptional cofactors CBP/p300 (Xu et al. 2001). Therefore, it seems to be implicated in the activation of gene expression. Recently, Xu *et al.* found that CARM1 acts on chromatin as a member of NUMAC complex *in vivo*. They identified the the NUMAC complex (nucleosomal methylation activator complex) in the process of purifying endogenous CARM1-interacting proteins (Xu et al. 2004).

The *Drosophila* homologue of CARM1/PRMT4, DART4, has been shown to methylate histone H3 and PABP1 *in vitro* (Boulanger et al. 2004). However, in 1988 Desrosiers and Tanguay demonstrated that histone H3 is only methylated on lysine residues and not arginine under normal growth temperature conditions (Desrosiers and Tanguay 1988). To clarify this controversy, a genetic approach would help. *Drosophila* is a good genetic model to study this.

1.5 PRMT5

PRMT5 was first identified as a human homologue of the *Schizosaccharomyces pombe* Shk1 kinase-binding protein 1, SKB1 (Gilbreth et al. 1998). Later it was identified as a Jak (Janus kinase) –binding protein (JBP1) in a yeast two-hybrid screen using Jak2 as bait (Pollack et al. 1999). PRMT5 is localized predominantly in the cytoplasm (Rho et al. 2001). It is able to methylate myelin basic protein, fibrillamin, and histone H2A and H4 *in vitro* (Pollack et al. 1999; Lee et al. 2000; Rho et al. 2001).

1.6 PRMT6

PRMT6 was found as a novel gene on chromosome 1 in searching the human genome for protein arginine N-methyltransferase (PRMT) family members. It codes for an apparent methyltransferase (Frankel et al. 2002) and it has a catalytic core sequence common to other PRMT enzymes. In addition, PRMT6 displays automethylation activity; it is the first PRMT to do so (Frankel et al. 2002).

CARM1/PRMT4

1. Identification of CARM1

Coactivator-associated arginine methyltransferase 1 (CARM1) is a recently discovered secondary transcriptional coactivator. It can cooperate with p160 transcriptional coactivators (Chen et al. 1999). In 1999, Chen *et al* identified CARM1 in a yeast two-hybrid screen from a 17-day mouse embryo cDNA library. It was found to bind the carboxyl-terminal domain of the glucocorticoid receptor interacting protein 1 (GRIP1). GRIP1 is a member of the p160 family of coactivators (Chen et al. 1999). Comparing the sequence homology between CARM1 and members of the PRMT family suggested that CARM1 may possess methyltransferase activity. The CARM1 protein is a 608 amino acid, 66 kDa, protein. Its 3.8 kb mRNA is also widely, but not evenly, expressed in adult mouse tissues (Chen et al. 1999).

2. Substrates and function of CARM1

2.1 Histone H3

Protein CARM1 was first found to preferentially methylate histone H3 *in vitro* (Chen et al. 1999). It binds to the carboxyl-terminal region of p160 coactivators and enhances transcriptional activation by nuclear receptors. To further evaluate the significance of H3 methylation, Schurter et al. later studied the site specificity of its modification by CARM1 (Schurter et al. 2001). The mapping of the residues demonstrated that histone H3 methylation occurred

specifically on Arg2, Arg17, and Arg26. In addition, CARM1 methylates the carboxyl terminus of histone H3 at one or more of the four arginine (128/129/131/134) residues (Schurter et al. 2001).

Recently, Bauer *et al* and Ma *et al* confirmed that methylation of histone H3 by CARM1 exists *in vivo* (Ma et al. 2001; Bauer et al. 2002). In both studies, an antibody that specifically recognizes the CARM1 methylated form of histone H3 was developed. They used a histone H3 peptide containing asymmetric dimethylarginine at position 17. *Bauer et al.* showed that CARM1 was recruited to the active promoter of the estrogen receptor-regulated *ps2* gene (Bauer et al. 2002). *Ma et al.* found that CARM1 was associated with GRIP1 to enhance the activation of mouse mammary tumor virus (MMTV) promoters, and these coactivator functions required the methyltransferase activity of CARM1 (Ma et al. 2001).

In addition to activate the promoters of the *ps2* gene and the MMTV virus, CARM1 is also necessary for muscle differentiation (Chen et al. 2002). It was demonstrated that CARM1 and GRIP-1 (a member of the p160 family), cooperatively stimulate the activity of myocyte enhancer factor-2C (MEF2C). CARM1 is expressed in somites during embryogenesis and in the nuclei of muscle cells. Furthermore, inhibition of CARM1 prevented differentiation by eliminating the expression of the transcription factors, myogenin and MEF2 that initiate differentiation (Chen et al. 2002).

It is well known that CARM1 can function *in vitro* and *in vivo* as the coactivator of the p160 family. In addition, a recent study demonstrated that

CARM1 cooperates with other types of coactivator proteins as well. It was shown that CARM1 binds to β-catenin and can function in synergy with β-catenin and p300 as coactivator for the androgen receptor (AR) (Koh et al. 2002).

2.2. CBP/p300

In addtion to histones, it has been shown that CARM1 can methylate the KIX domain of CBP (CREB Binding Protein), at least *in vitro* (Xu et al. 2001). CARM1 methylated an arginine residue of CBP. The methylation site is essential for stabilizing the structure of the KIX domain, which mediates CREB recruitment. Methylation of KIX by CARM1 blocks CREB activation by disabling the interaction between KIX and the kinase inducible domain (KID) of CREB (Xu et al. 2001).

In addition to the KIX domain of CBP, it has also been shown that CARM1 methylates CBP on a conserved arginine residue outside the KIX domain *in vitro* and *in vivo* (Chevillard-Briet et al. 2002). The functional importance of the methylation is further suggested by the finding that GRIP-1- and steroid hormone-dependent transcriptional activation was not detected when the methylation site in CBP was mutated by a point mutation (Chevillard-Briet et al. 2002).

2.3. PolyA-binding protein

Another substrate for CARM1 was recently identified using arrayed high-density protein membranes (Lee and Bedford 2002). The predominant CARM1

substrate identified in this screen was PABP1. Somewhat surprisingly, it was found that the methylation motif was not GGRGG as in PRMT1 substrates. Instead, the most strongly methylated arginine residues are in the RPAAPR motif. This motif is similar to Arg17 (APR) site in histone H3. The same authors also demonstrated that PABP1 is indeed methylated *in vivo* (Lee and Bedford 2002).

2.4. RNA-binding Protein HuR

Mammalian Hu proteins are a family of highly conserved RNA-binding proteins with homology to the *Drosophila* protein ELAV (embryonic lethal/altered visual system) (Antic and Keene 1997; Keene 1999) and Rbp9 (Kim and Baker 1993). The Hu protein family has four members: HuR (also called HuA), HuB (previously called Hel-N1), HuC, and HuD (Szabo et al. 1991; Levine et al. 1993; Liu et al. 1995; Ma et al. 1996). The last three are neuronal proteins, whereas HuR is ubiquitously expressed (Ma et al. 1996; Okano and Darnell 1997; Keene 1999).

Recently, it was reported that HuR can be specifically methylated by CARM1 (Li et al. 2002). The major HuR methylation site is Arg217. Arg217 is located in the region between the second and third of the three HuR RNA recognition motif domains. Antibodies against a methylated HuR peptide were used to demonstrate *in vivo* methylation of HuR. HuR methylation increased in cells that overexpressed CARM1. The methylation of endogenous HuR increased during lipopolysaccharide stimulation of macrophages. This stimulation can lead to HuR-mediated stabilization of tumor necrosis factor mRNA. Thus,

CARM1may play a role in post-transcriptional gene regulation by methylating HuR (Li et al. 2002).

Protein Methylation in *Drosophila*

Evidences to date showed that protein methylation in *Drosophila* may occur on lysine, proline or arginine side chains of histone (Desrosiers and Tanguay 1988; Boulanger et al. 2004), as well as isoaspartyl residues (Bennett et al. 2003).

Protein L-isoaspartyl methyltransferases (PIMTs) catalyze the transfer of methyl groups from S-adenosylmethionine (AdoMet) to isoaspartyl residues (Kagan et al. 1997; Ichikawa and Clarke 1998). In *Drosophila melanogaster*, overexpression of PIMT in transgenic flies extends the normal life span, suggesting that protein damage can be a limiting factor for longevity (Bennett et al. 2003).

However, most published data focus on lysine methylation of histones. Histone methylation in *Drosophila* occurs on lysine 4 (Byrd and Shearn 2003; Sedkov et al. 2003), 9 (Schotta et al. 2002), and 27 (Muller et al. 2002) of H3 and lysine 20 of H4 (Fang et al. 2002; Nishioka et al. 2002).

1. Lysine methylation in *Drosophila*

To date, lysine methylation in *Drosophila* has only been found to occur on histones. Recently, Sedkov *et al.* demonstrated that TRR (Trithorax-related) is a histone methyltransferase which is capable of trimethylating lysine 4 of histone

H3 (H3-K4) (Sedkov et al. 2003). *trr* encodes the SET domain protein and acts upstream of *hedgehog* (*hh*) in the progression of the morphogenetic furrow. It is also required for retinal differentiation (Sedkov et al. 2003). ASH1 protein (Absent, Small or Homeotic discs 1), which is a *Drosophila* trithorax group protein, is required for essentially all H3-K4 methylation *in vivo* (Byrd and Shearn 2003).

Su(var)3-9 encodes a histone methyltransferase (HMTase), which selectively methylates lysine 9 of histone H3 (H3-K9) (Czermin et al. 2001). Association of SU(VAR)3-9 with heterochromatic regions in *Drosophila* was recently demonstrated (Schotta and Reuter 2000). In 2002, Schotta et al found that SU(VAR)3-9 HMTase plays a central role in heterochromatin-induced gene silencing in *Drosophila* (Byrd and Shearn 2003).

In *Drosophila* ESC-E(Z) is a protein complex that functions in a cooperative manner to maintain long-term gene silencing (Simon and Tamkun 2002). It was found that this complex contains methyltransferase activity against lysine 27 of histone H3 (H3-K27) (Czermin et al. 2002). ESC-E(Z) complex mediated H3-K27 methylation is required for Hox gene silencing (Muller et al. 2002).

So far, only 2 residues on histone H4 are known to be methylated, arginine 3 (H4-R3) and lysine 20 (H4-K20). H4-R3 methylation is mediated by PRMT1 in humans (Wang et al. 2001). The enzyme responsible for H4-K20 methylation was not known until 2002. In 2002, Fang *et al* purified and cloned a novel human SET domain-containing protein, named SET8, which specifically methylates H4-K20

(Fang et al. 2002). The *Drosophila* SET8 homolog has the same substrate specificity as its human counterpart. Disruption of *SET8* in *Drosophila* reduces levels of H4-K20 methylation *in vivo* and results in lethality (Fang et al. 2002).

2. Arginine methylation in *Drosophila*

To date, there are very few studies on arginine methylation in *Drosophila.* In 1988, Desrosiers and Tanguay demonstrated that heat shock induces new site-specific methylation of arginine residues in histone H3 in *Drosophila* Kc III cells (Desrosiers and Tanguay 1988). They found that histone H3 is only methylated on lysine residues under normal growth temperature condition (Desrosiers and Tanguay 1988). Recently, DART4 has been found to be type I arginine methyltransferase which catalyses the formation of asymmetrical dimethylarginines and (Boulanger et al. 2004). Boulanger *et al* also showed that DART4 methylates histone H3 and PABP1 *in vitro*. However, this is inconsistent with the fact that histone H3 is only methylated on lysine under normal conditions (Desrosiers and Tanguay 1988). We will clarify this controversy. DRMT4 is used as a synonym of DART4 in this thesis. We are trying to gain insight into the *in vivo* functions of arginine methylation in *Drosophila* by using of *Drosophila* arginine methyltransferase 4 (DRMT4) mutants.

Materials and Methods

Plasmid Construction

1. Genomic rescue construct

A 5.9 kb NheI/BamHI fragment including DRMT4 and parts of the flanking two genes were cloned into pSL1180. Then this vector was then digested with BspmII and AgeI to remove 1 kb of non-essential sequence. A self-ligation was done as BspmII and AgeI produce compatible ends. Next, the *pSL1180+4.8kb* recombinant vector was cut with NotI and BamHI to get the 4.8 kb fragment containing the *DRMT4* gene which was then inserted into the modified pCaSpeR transformation vector (Fig. 6A).

2. DRMT4-GFP construct

To construct the *DRMT4-GFP* fusion transgene with the *DRMT4* promoter, *pSL1180+4.8kb* (described above) was digested with EcoRI and SpeI, creating a 219 bp fragment. The 219 bp EcoRI/SpeI fragment including the third *DRMT4* intron and extending slightly beyond the translational stop codon was cloned into pBSKS+ This *pBSKS+-219bp* clone was transformed into the E.coli cj236 strain in order to produce ssDNA. Site-directed mutagenesis (Kunkel 1985) was performed in order to introduce a BglII site just before the stop codon of *DRMT4* using the primer 5' TTA TGC ATC CGC ACA AAG ATC TTA GGG GGG CGG TGG AT 3'. After transformation into E.coli JM101 strain, the *pBSKS+-225bp* clone with the presumed BglII site was purified and sequenced in

order to confirm that a BglII site was introduced and no other mutation had been created. The BamHI fragment from GFP S65T (Heim et al. 1995), cloned into pRSET, was inserted into this unique BglII site and a clone with the correct orientation was selected. To restore the full *DRMT4* coding sequence, the fusion fragment (240bp+GFP) was cloned back into *pSL1180+4.8kb* using EcoRI and SpeI (Fig. 7A). Finally, the NotI/BamHI fragment of the resulting fusion gene was inserted into the NotI and BamHI sites of a modified pCaSpeR transformation vector. The fusion gene was then introduced into flies by P-element mediated transformation.

3. UAS-cDNA construct

In order to study the effect of ectopic expression or over-expression of *DRMT4*, I made the *pUAS-cDRMT4* construct. The *DRMT4* cDNA sequence (called *cDRMT4*) was already cloned in pBSKS+ (ordered from Resgen company, Clone ID: GM01306). In order to prepare the recombinant plasmid, an EcoRI site in the 3'UTR had to be removed from *cDRMT4* using a short ds-linker 5' AAT TGC GGC CGC 3' that was inserted into the sticky end of EcoRI (Fig. 8A). Then put the SpeI/HindIII (EcoRI$^-$) back to the cDRMT4 sequence. The final step was to digest pUAST and (*pBSKS+-cDRMT(EcoRI$^-$)*) using EcoRI and XhoI and to ligate the cDNA fragment into the modified pUAST vector. The fusion plasmid was then ready for injection.

Fly Transformations

1. DNA Preparation

Constructed plasmids were transformed into *E.coli DH5α* to be amplified. DNA extraction was done using the QIAprep Miniprep Kit. In order to get high DNA concentration, I used the amount of injection buffer required for one column to wash several columns in the last step. The injection buffer was prepared as follows: 40 µl of injection buffer, 0.1mM Na-phosphate pH6.8 and 5mM KCl. The DNA solution ready to be injected contained 150 µg/ml helper plasmid (*pUCHSΔ2-3*) and 500-800 µg/ml constructed plasmid.

2. P-element mediated transformation

2.1 Flies

Freshly eclosed *yw* flies were put into bottles containing standard fly food supplemented with live yeast. To stimulate their egg production, they were kept under non-crowded conditions and transferred to fresh food every other day. After a minimum of one week they can be used for egg laying. For this, flies were transferred from bottles into a plastic beaker with an apple juice plate at the bottom. After verifying that the plates were dry, I put a very thin layer of yeast paste in the middle of the plate. Egg lay plates were changed every 30 min. at room temperature.

2.2 Injection

Embryos were collected using a brush into a basket and rinsed with water. Embryos were dechorionated by bathing the basket in 50% household bleach for 2 min. The dechorionated eggs were dried on absorbent paper, washed carefully with tap water, and line up on a piece of apple juice agar while keeping them moist. Too old or too soft eggs were discarded. Then, the eggs were picked up

with a cover slip covered with double-coated tape and dehydrated for 5-10 min on silica gel (the actual time depends on the humidity and varies with weather conditions). In order to stop dehydration, the embryos were covered with Halocarbon oil. After injection, I put the cover slip onto a large apple juice plate, let them age for 2 days at 18°C. The hatched larvae were picked up carefully into a food vial containing yeast and incubate them at 25°C. Flies hatched 10-12 days after they were injected.

2.3 Mapping

I crossed each injected fly separately with 3-4 *yw* flies. After 10-12 days at 25°C, the transgenic flies showing a w^+ phenotype (red eye color) were obtained. Mapping crosses were done as follows: male transformants were selected to be crossed to *yw* virgin. If all the male progeny are white eyed and all the female progeny are red eyed, the insertion is on the 1st (X-) chromosome. Otherwise, the transformants were crossed to the double balancer flies *w;Sp/SM1;PrDr/TM3,Sb*, and progeny with red eyes, *CyO* and *Sb* phenotypes was seleced to be crossed back to *yw*. In the progeny of this latter cross, if flies with red eyes and *CyO* are missing, the insertion is on the 2nd chromosome. If flies with red eyes and *Sb* are missing, the insertion is on the 3rd chromosome.

Screening the female sterile mutant collection for DRMT4 mutants

The following strains were used for the screen. Two 3rd chromosome deficiency lines: *+;+;Df(3R)by62/TM1* (Bloomington stock #1893) and *+;+;Df(3R)by10/TM3,Sb* (Bloomington stock #1931). A large collection (1709 lines) of 3rd chromosomal EMS female sterile mutants was obtained from Charkes Zuker (Howard Hughes Medical Institute). The genotype of these lines is: *+;bw;st,EMS/TM6B.* The *TM6B* balancer contains the dominant marker, *Hu.* The screen was performed by crossing each EMS induced female sterile line separately over the two deficiency lines: *+;+;Df(3R)by62/TM1* and *+;+;Df(3R)by10/TM3.* Their female progeny *Df(3R)by62/st,EMS* and *Df(3R)by10/st,EMS* was then tested for sterility (Fig. 2). The expectation for a mutation in *DRMT4* is that the EMS chromosome is female sterile over *Df(3R)by62,* but fertile over *Df(3R)by10.* 726 lines of the collection were screened through and two *DRMT4* candidate mutant lines were obtained *E0467* and *E6115.*

Antibody Generation

To generate antibodies against DRMT4, the cDNA clone (ID: GM01306, Resgen company) was digested with KpnI and EcoRI and the resulting 0.7 kb fragment was subcloned into pBSKS+. A NdeI/BamHI fragment containing 0.6 kb of the *DRMT4* cDNA was inserted into the expression vector pET3b and expressed in the BL21(DE3) E.coli strain. A 22 KD polypeptide was purified using the Bio-RAD Prep Cell system. This purified protein was injected into rabbits to produce polyclonal antiserum, which was affinity purified with the help

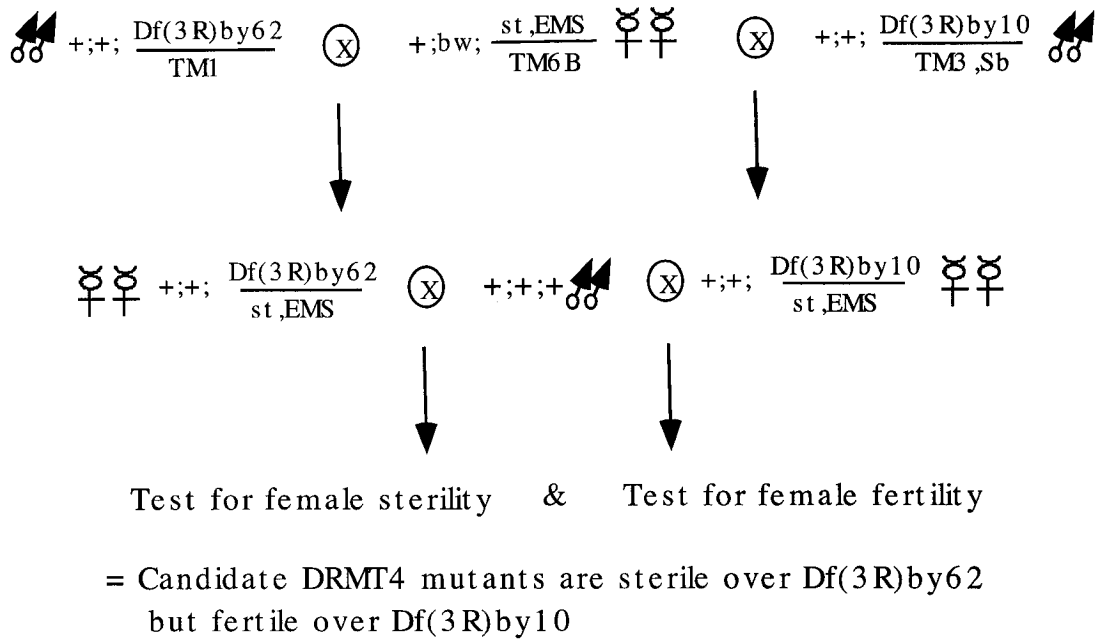


Figure 2. Crossing scheme for screening the candidate *DRMT4* mutants.

of a DRMT4-MBP fusion protein coupled to CNBr-activated Sepharose 4B beads (Amersham Pharmacia Biotech, 17-0430-01).

Western Blotting

Ovaries, embryos, larvae and whole fly extracts were prepared as follows: ovaries were dissected in Ringer's buffer under a Leica stereo microscope. The dissected ovaries were transferred to a 1.5 ml tube and immediately frozen on dry ice. They were then homogenized in 2x SDS loading buffer on ice. Embryos were collected and aged on apple juice plates at 25°C. Aged embryos were washed, dried and weighed. Dried embryos were homogenized in 2x SDS loading buffer (100μg/μl). Larvae and whole flies were collected directly and immediately frozen on dry ice. Frozen larvae or flies were grinded quickly and carefully in 2x SDS loading buffer. The samples in 2x SDS loading buffer were boiled for 10 min, sonicated 180 sec, boiled again for 10 min and then centrifuged. The supernatants were loaded in gels. Protein samples were resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose membranes at 250 mA for 1 hour, and probed for DRMT4 using the Rabbit anti-DRMT4 serum at 1:500- 1:1,000. As a loading control, blots were probed with anti-eIF4a antibody at a dilution of 1:1,000 (Styhler et al. 2002), or anti-α-tubulin at 1:500 (Sigma). Horseradish peroxidase conjugated anti-rabbit IgG were used at a dilution of 1:2000. Staining with ECL™ Western Blotting Detection Reagents (Amersham) was performed for 1 min.

Immunostainning

1. Embryo immunostaining

Staged embryos were dechorionated with 50% bleach, washed with tap water, and fixed for 20 min in a mixture (1:1) of heptane and PBS containing 4% paraformaldehyde. Fixed embryos were devitellinized in a mix (1:1) of heptane and methanol/EGTA (90% methanol; 10% 0.5M EGTA, pH8.0) for 10-20 min. Devitellinized embryos were washed with methanol/EGTA for 2-3 times and blocked in PBST +0.1% BSA for 3-4 hours (with changes of buffer in between). Rabbit polyclonal anti-DRMT4 antibodies were used at a dilution of 1:500. The anti DRMT4 antibodies were detected by staining with Oregon-green488-conjugated anti rabbit secondary antibodies (Molecular Probes).

2. Ovary Immunostaining

Ovaries were dissected in Ringers, partially separated into ovarioles, and transferred into 1.5 ml Eppendorf tubes. The buffer was replaced by fixation buffer (200 µl 4% paraformaldehyde; 600 µl heptane; 20 µl DMSO) and ovaries were fixed for 20 min at RT. Subsequently, the buffer was removed and ovaries were rinsed 3 times with PBST and washed 2 times for 5 min. They were then blocked 3-4 times for 1 hour in PBSBT (PBST + 0.1% Triton + 1% BSA) and subsequently incubated with antibodies in PBSBT overnight at 4°C on a wheel and 2 hours at RT. Rinses and washes were performed as above. Incubation with the fluorescent 2^{nd} antibodies was done in the dark in PBSBT overnight at 4°C on a wheel and 2 hours at RT. This procedure was again followed by 3 rinses and 3

washes for 20 min with PBST. The samples were then incubated for 20 min with PBST+1μM Yo-Pro-1 for confocal analysis or Hoechst for regular fluorescent microscopy. This DNA staining was terminated with two rinses and two washes of 20 min with PBST. Ovarioles were then mounted in 70% glycerol for microscopy.

Immunoprecipitation (IP)

1. Measurement of the Antibody Concentration

A BSA sample buffer of 1mg/ml was prepared. On a 10% SDS-PAGE gel, different amounts of BSA were loaded separately in sample buffer on 5 lanes as follows: 0.5, 1, 2, 4, 6 μl. 2 μl of antibody solution was also loaded on another lane. At the end of the run, the gel was stained with Gelcode® Blue (PIERCE Co.) and the intensity of the IgG heavy chain band of the antibody was compared to the different amounts of BSA to estimate the antibody concentration.

2. Immunoprecipitation

In a 1.5 ml Eppendorf tube, 20 whole female flies were ground in 100 μl IP buffer (150 mM NaCl, 2% Triton X-100, 1x PBS, 1x protease inhibitor (Roche: Protease Inhibitor Cocktail Tablets)) on ice. 100 μl IP buffer was then used to rinse the pestle to make the volume up to 200 μl. The extract was incubated on a wheel at 4 °C for 10 min. and then centrifuged at 12,000 rpm at 4 °C for 10 min. The supernatant was transferred into a new tube and was centrifuged again. The supernatant was then transferred into a 1.5 ml Eppendorf

tube and 10 μg of mono and dimethyl arginine antibody (Abcam Co.) was added. The reaction was incubated on a wheel at 4 °C o/n. Next, 10 μl Protein G sepharose were added and the incubation was continued on wheel at 4°C for 3-4 hour. Beads were spun down and the supernatant was kept to check for efficient removal of the target protein. The beads were washed 3x with 1x PBS and once with water, transferred to a new tube in order to prevent contamination with non-precipitated proteins that stick to the wall. 50 ~100 μl 2x SDS sample buffer was then added and the mixture was boiled for 10 min. 50 μl supernatant and 50 μl 2x SB were also boiled for 10 min and used as control. The samples were then analyzed on a gel.

Results

DRMT4 has the best sequence similarity to mammalian PRMT4/CARM1

DRMT4/CG5358 (Drosophila Arginine Methyltransferase 4) maps to the cytological region 85F4 (Fig. 3). The *DRMT4* genomic sequence contains 3 introns and encodes a predicted polypeptide of ~60 KD. It shows sequence similarities to arginine methyltransferases and is therefore a putative *Drosophila* arginine methyltransferase. The highest sequence identity is with mammalian PRMT4/CARM1 (identities=59%, positives=75%; Fig. 4). HPLC analysis has demonstrated that DRMT4 (DART4) belongs to the type I enzyme class as it catalyzes the formation of asymmetric dimethyl-arginines (Boulanger et al. 2004).

Different *DRMT4* transgenes are expressed in flies

1. Preparation and specificity of anti-DRMT4 antibody

In order to analyze the function of *DRMT4*, a rabbit anti-DRMT4 antibody was generated against the C-terminal part of DRMT4. In wild type embryo and ovary extracts, the antiserum recognized a specific band of about 60 KD which is the predicted size for DRMT4 (Fig. 5).

2. Genomic *DRMT4* DNA transgene

In order to identify potential *DRMT4* mutants with a female sterile phenotype, I made a genomic rescue construct that I transformed into flies. I then

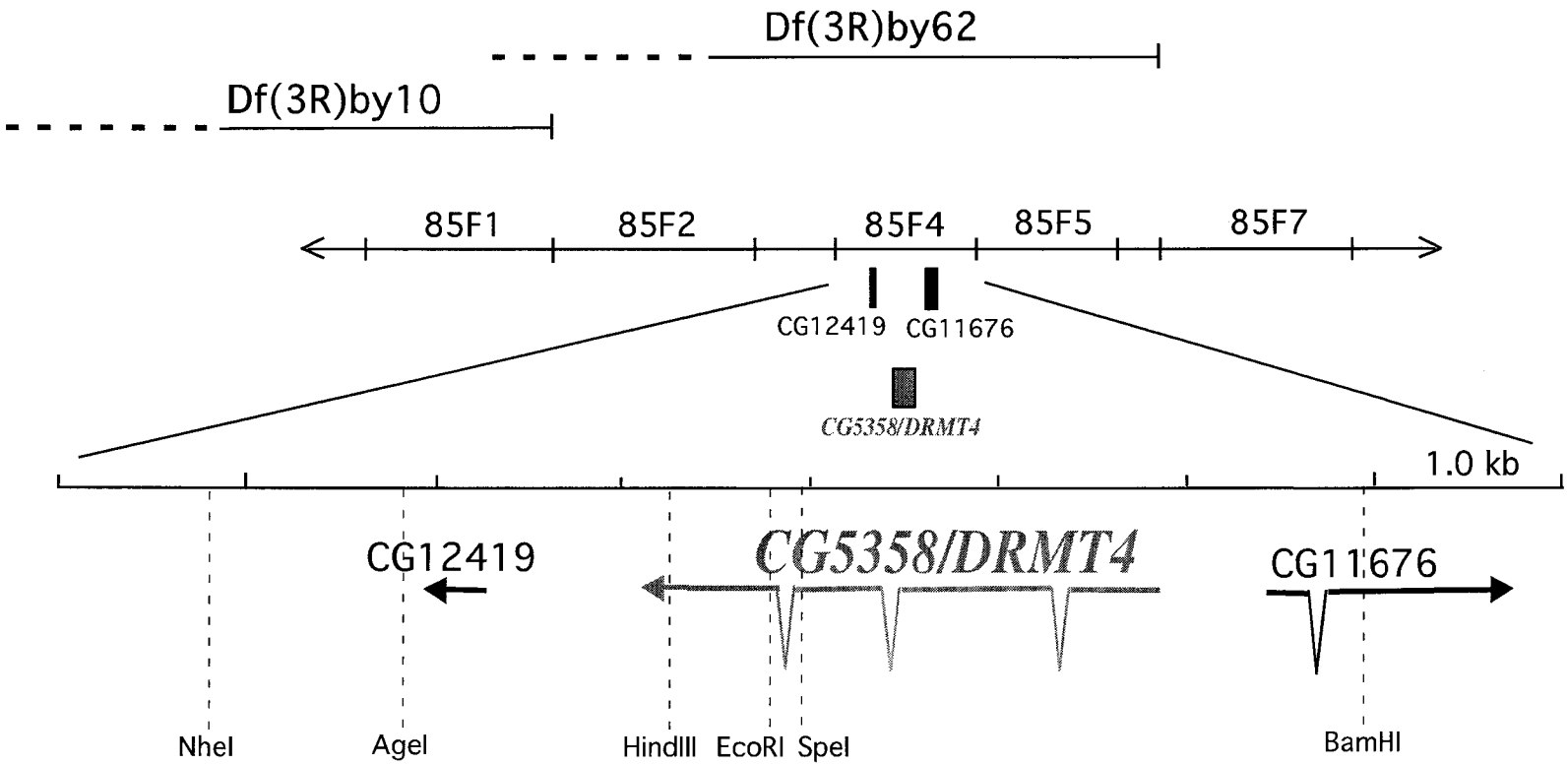


Figure 3. *DRMT4* genomic region. Three transcription units, *DRMT4/CG5358, CG1249* and *CG11676* are found in a 12kb stretch of DNA in region 85F4 (flybase). The deficiency *Df(3R)by10* uncovers region 85F4, whereas deficiency *Df(3R)by10* doesn't. These two deficiency lines were used to screen for *DRMT4* mutants.
The restriction enzyme sites indicated were used in *DRMT4* constructs.

Figure 4. Protein sequence alignment of DRMT4 and human CARM1 (Coactivator-associated arginine methyltransferase-1).
The AdoMet binding regions are underlined.

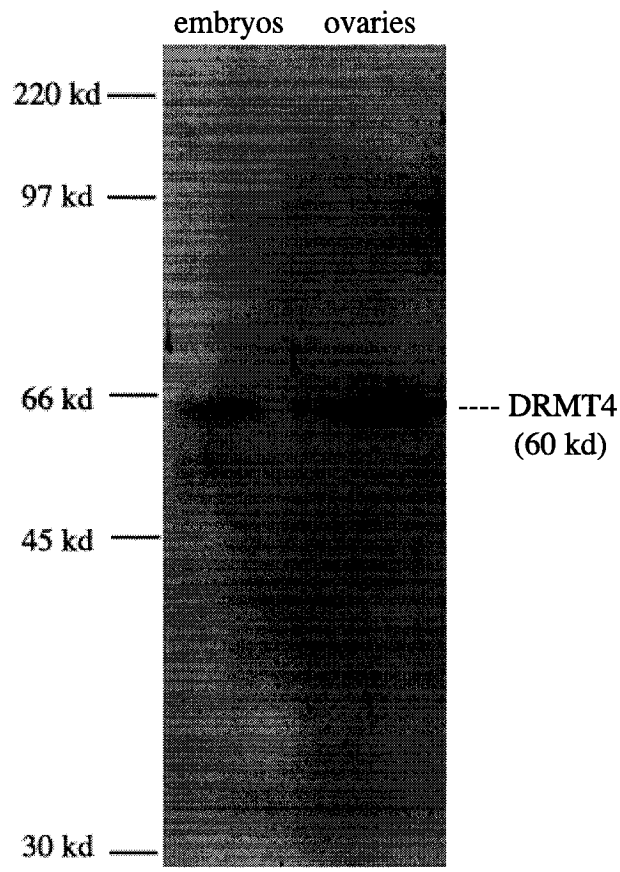


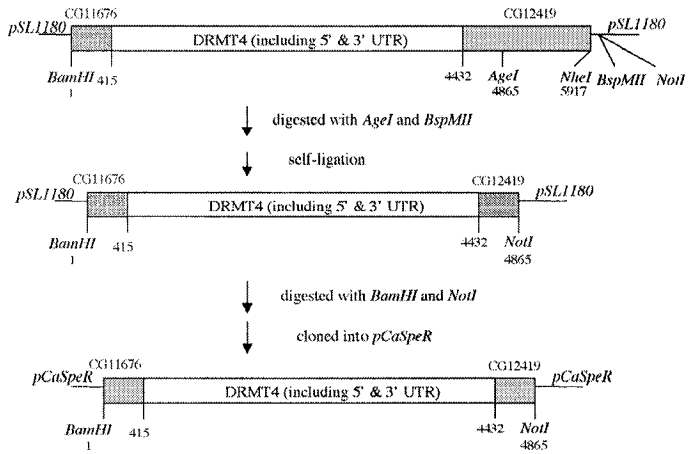
Figure 5. Western blot of DRMT4 protein with antiserum in OregenR embryo and ovary extracts.

tested whether this rescue construct can rescue the candidate *DRMT4* mutations. The *pSL1180+4.8kb* recombinant vector containing the *DRMT4* gene was created (Fig. 6A). Then it was cut with NotI and BamHI to clone the 4.8 kb fragment containing the *DRMT4* gene into the modified pCaSpeR transformation vector. The design of the construct is straightforward because of its close vicinity to the 5' end of the neighboring genes (Fig. 3), which in the vast majority of cases defines the limit of the promoter region. I transformed this construct into flies by P-element mediated transformation and got one transgenic line. With the mapping crosses, I found that it had inserted in the 3rd chromosome. A subsequent western blot showed higher levels of DRMT4 in the transgenic line than in the untransformed parental control flies (Fig. 6B).

3. *DRMT4* genomic DNA-GFP fusion transgene

To further ascertain its function, I made a *DRMT4-GFP* fusion construct under its own promoter (Fig. 7A). After microinjection, I got 2 different lines and both of them had the insertion on the second chromosome. Then I checked the expression of the transgene by Western Blot. The result showed that the *DRMT4-GFP* fusion gene was expressed at almost the same level as the native *DRMT4* (Fig. 7B). Furthermore, observation under the fluorescent light confirmed this assessment (Fig. 11, 12). Even though we also developed an antibody against DRMT4, GFP fluorescence may give us more accurate information about the localization of DRMT4 and we can perform a dynamic analysis because the GFP signal is directly visible in living cells and requires no fixation, substrates or coenzymes.

A.



B.

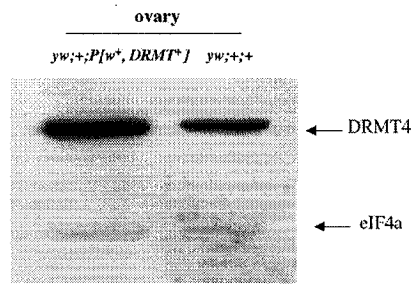


Fig. 6 Construction and expression of the genomic *DRMT4* transgene.
(A). Cloning scheme for the genomic *DRMT4* transgene construct.
(B). Expression of the genomic *DRMT4* transgene in *yw* flies.
Ovary extracts were used for the western blots. eIF4a is a loading control.

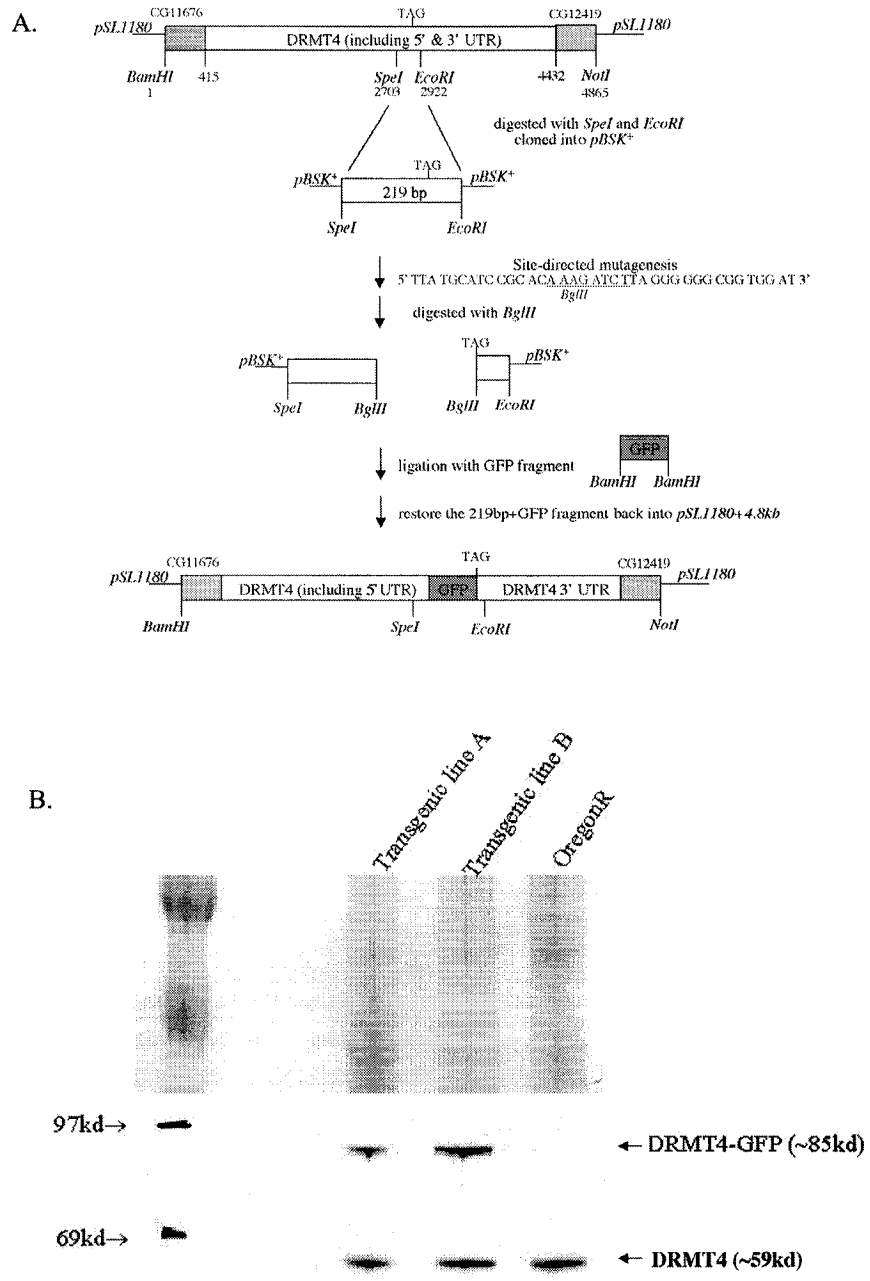


Figure 7. Construction and expression of the gemomic *DRMT4* and *GFP* fusion transgene.
(A). Cloning scheme for the *DRMT4-GFP* fusion construct, the final fragment was then cloned into the pCasSpeR vector.
(B). Expression of *DTMT4-GFP* in transgenic flies.
Total female extracts were used for the western blot.

4. *UAS- cDRMT4* construct

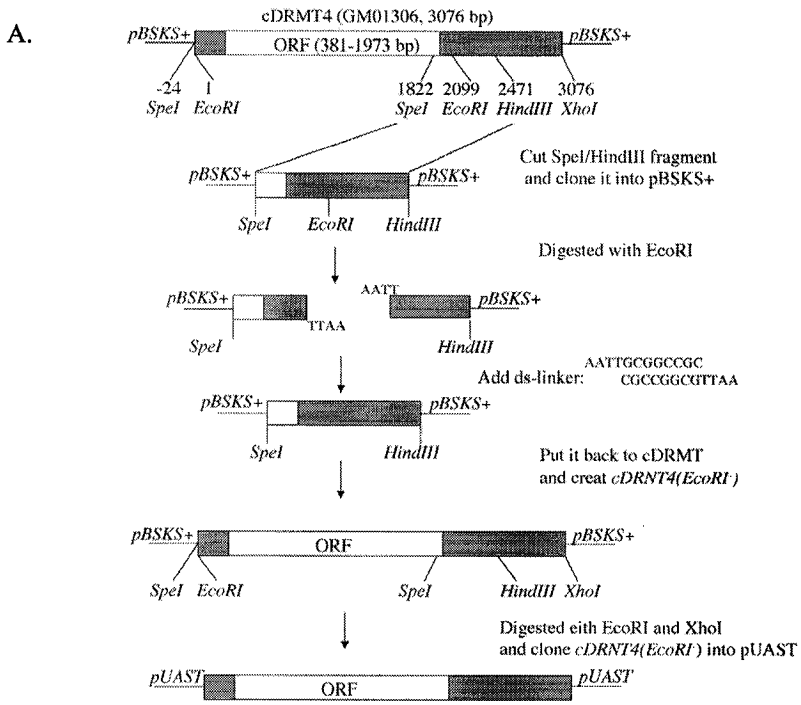
The *UAS/GAL4* system is a very useful tool to study the over-expression and ectopic expression of specific genes in *Drosophila*. Therefore I made a *UAS-cDRMT4* (cDNA sequence of DRMT4) construct. I cloned the complete cDNA sequence into the pUAST vector such that cDNA expression is under *UAS* control and can be driven by different *GAL4* lines (Fig. 8A). I transformed this construct into *yw* flies and got 3 transgenic lines. After mapping crosses, I found that their insertions are on the first, second and third chromosome, respectively.

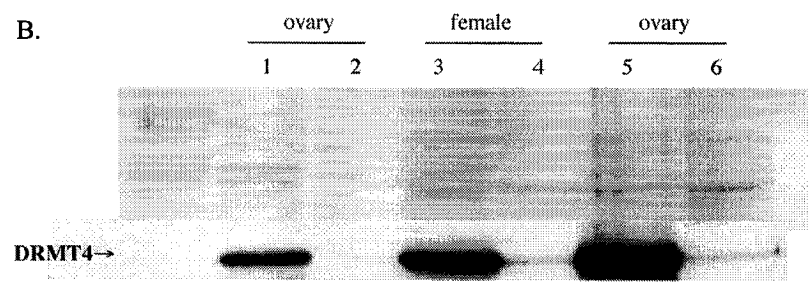
In order to test the activity of the *UAS-cDRMT4* construct, I crossed the transgenic lines to a *GAL4* line driven by a α-tubulin promoter. Using the offspring from this cross I then performed a western blot with extracts from ovaries and total adult females. The results showed that the DRMT4 protein was strongly over-expressed compared to *UAS-cDRMT4* transformants that were not activated by *GAL4* (Fig. 8B). This indicates that the construct is correct and the transgene can be induced and used to study the effects of over-expression and tissue specific expression

DRMT4 in wild type background

1. DRMT4 in embryos

In order to be able to study the expression and cellular localization of DRMT4 by immunostaining, a polyclonal antibody was generated against DRMT4. In embryo stainings, I found that DRMT4 localizes to the cytoplasm during the early stages (Fig. 9a), and later it accumulates in the cytoplasm of pole





1, 3, UAS-cDRMT4/+; +; P[w^{+mC}=tubP-GAL4]/+
2, 4, UAS-cDRMT4/+; +; TM3,Sb/+
5, +; UAS-cDRMT4/+; P[w^{+mC}=tubP-GAL4]/+
6, +; UAS-cDRMT4/+; TM3,Sb

Figure 8. Construction and expression of the *DRMT4* cDNA transgene.
(A). Cloning scheme for the *DRMT4* cDNA construct.
(B). Expression of the *DRMT4* cDNA transgene was induced by an α–tubulin-GAL4 driver.

cells during cycle 13 (Fig. 9b). During gastrulation, the signal becomes nuclear (Fig. 9c). This nuclear localization coincides with the activation of zygotic transcription. CARM1, the mammalian homologue of DRMT4, has been demonstrated to methylate the RNA binding protein HuR, the polyA-binding protein and histone H3, and to function in transcription. Therefore DRMT4 probably has similar functions during embryogenesis. It might activate some zygotic genes by methylating RNA binding proteins that specifically bind those zygotic transcripts and/or might methylate histone H3 and hereby alter chromatin structure.

2. DRMT4 in larvae

Different stages of larvae were collected according to the synchronization of larvae development. A long-standing problem in the practice of *Drosophila* biology has been obtaining samples of homogenously staged larvae. The problem is that even under optimal conditions of culture, the variation in growth rate of larvae is such that absolute age is a poor indicator of developmental stage. First-instar larvae collected at hatching within 30 min may vary by as much as 6 hr in developmental stage by the mid-L3 (Fristrom and Mitchell 1965). Synchronization at the molts from L1 to L2 and L2 to L3 is the only practical method for collecting staged L2 (Ashburner 1989). In my work, 0 to 1 hour old larvae were collected as the first-instar. The third-instar larvae can be distinguished by their behavioral change. Before pupariation, the larvae leave the food medium and begin to wander, usually on the walls of the culture vials. Pupariation occurs up to 100 hours or more (4-5 days) after egg hatching.

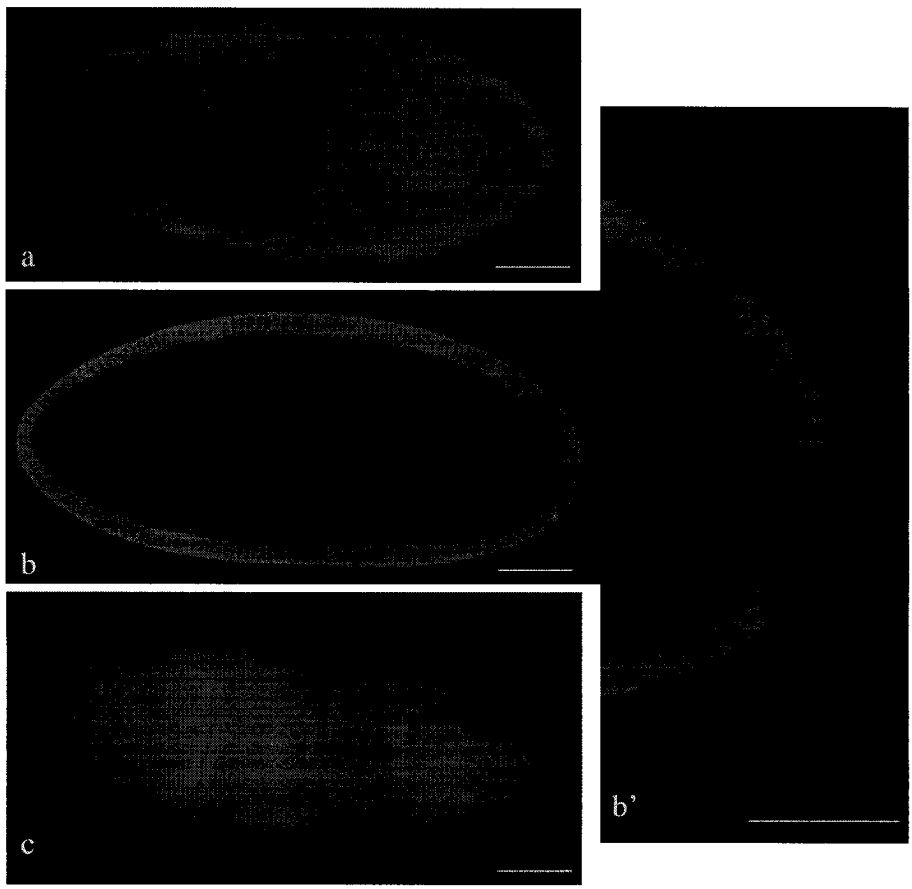


Figure 9. Localization of DRMT4 in wild type embryos.
(a). DRMT4 localizes to the cytoplasm during early stages.
(b). During germ cell formation DRMT4 accumulates in the cytoplasm of pole cells.
(c). During gastrulation, anti-DRMT4 signal is found in the nuclei.
(a), (c) are from immunostainings. (b) shows a GFP signal in living embryos.
(b') is a magnified picture of (b). Scal bars represent 1 mm.

Generally, between the moltings, the second and third instars last about 24 hours and 48 hours, respectively. The second instar is thus selected as 1-2 days after hatching from the egg. Second-instar larvae were collected around 40 hours (~2 days) old. Western Blot (Fig. 10) showed that DRMT4 expression levels increase with development. There is much more DRMT4 protein in 3rd instar larvae than the 1st instar.

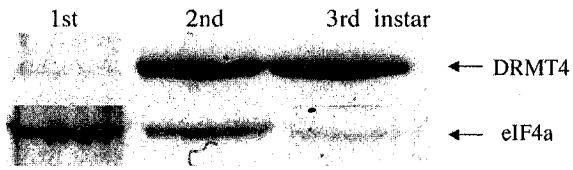


Figure 10. Expression level of DRMT4 in different larval stages.
The amount of DRMT4 increases with growth. Total larval extracts were used. eIF4a was used as a loading control.

Interestingly, in the DRMT4-GFP transgenic lines I found that DRMT4-GFP shows the strongest fluorescence in the brain and in imaginal discs (Fig. 11). This is consistent with the Western blot results because the DRMT4 protein level increases with the development of the imaginal discs and the brain. To understand in more detail the localization of DRMT4 in discs, costaining with a nuclear marker will be done in the future.

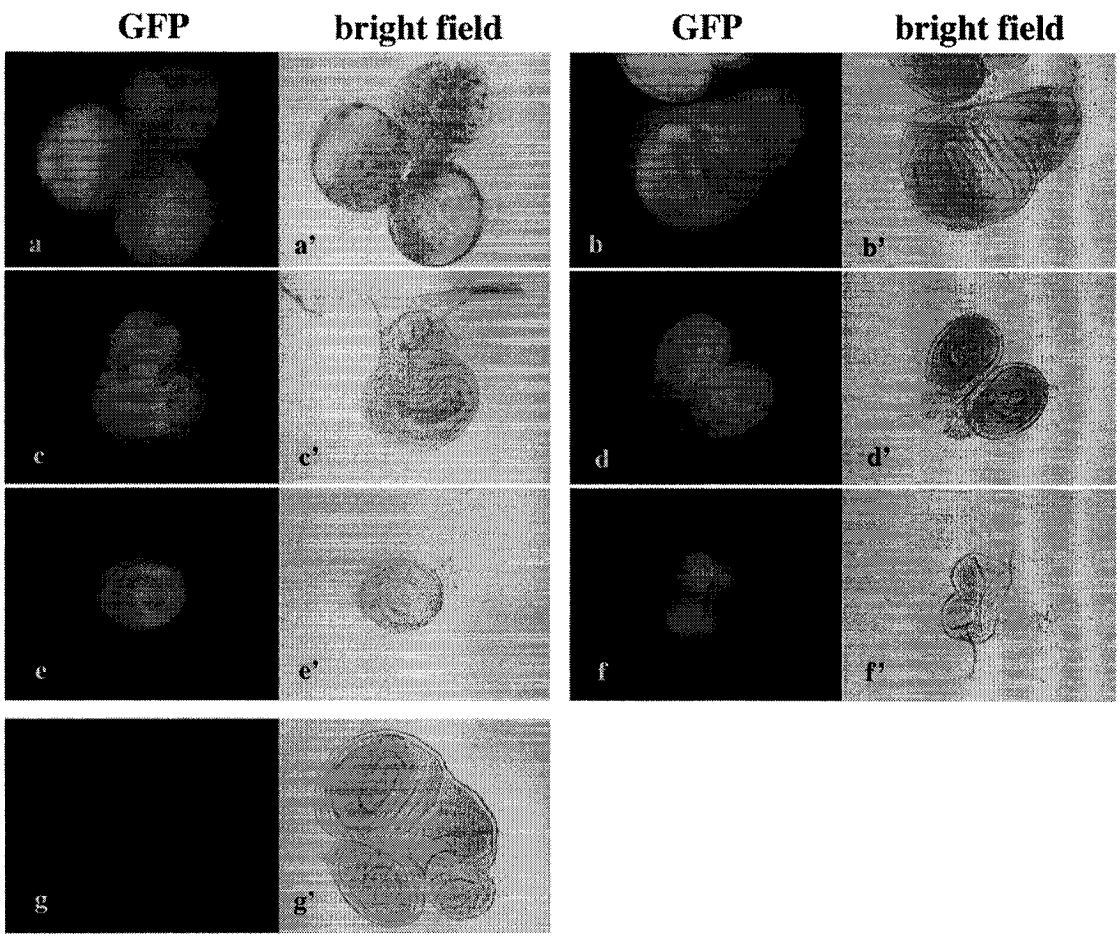


Figure 11. Overview of DRMT4-GFP signal in transgenic larvae of the genotype *yw;P[w+,DRMT4-GFP];+*.
a, a', brain; b,b', wing disc; c,c', eye disc; d,d', leg disc; e,e' haltere disc; f,f' genital disc; g,g', wing, leg and haltere discs of a *yw;+;+* control fly.

During metamorphosis, most of the larval tissues die and are resorbed, providing building blocks for subsequent development. The remaining larval tissues are specialized imaginal discs, which differentiate to form the organs of the adult insect. In the early stage of larval development, the imaginal discs are small and difficult to identify. Prior to metamorphosis, the discs undergo a dramatic increase in mass and become patterned while they grow (Weinkove and Leevers 2000). At the wandering third instar stage, the discs are big and easy to isolate. As the imaginal discs are very important in the development of several kinds of organs, such as eyes, wings, legs, etc. and the DRMT4-GFP shows a strong signal in the imaginal discs, the DRMT4 protein probably functions in different developmental pathways. Furthermore, the expression in brain may relate to the ELAV expression, a HuR homologue.

3. DRMT4 in ovaries

I found that the DRMT4-GFP signal accumulates in the somatic follicle cells and the germ line throughout oogenesis (Fig. 12). This is confirmed by immunostaining of *OregonR* ovaries with anti-DRMT4 antibody (Fig. 13). We also found that germ line accumulation of the DRMT4-GFP signal is mainly seen in the nuclei of nurse cells and oocytes (Fig. 12). This could indicate that DRMT4 may function in these nuclei.

However, immunostainings of ovaries revealed a signal that was largely cytoplasmic in the nurse cells during the early stages until stage 10A of oogenesis (Fig. 13a, b, c). The nuclear signal becomes apparent in the nurse cells at around

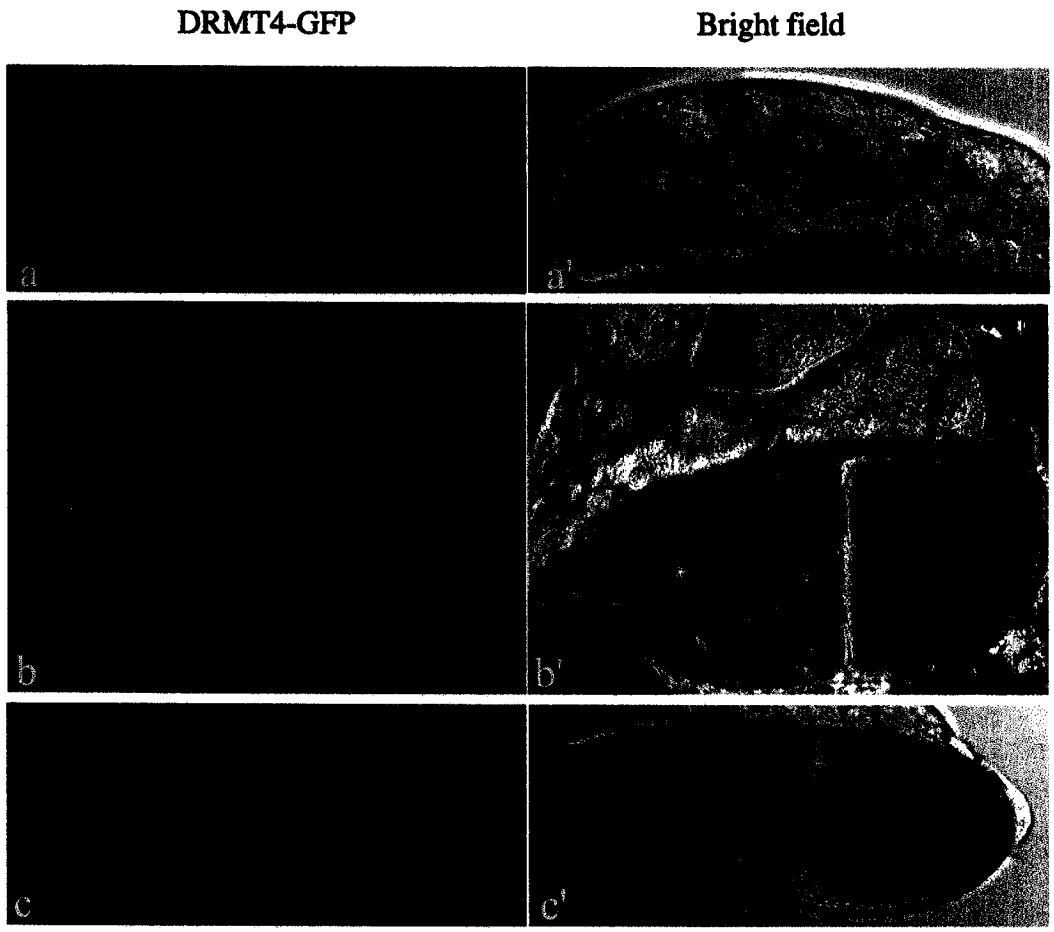


Fig. 12 DRMT4-GFP in transgenic ovaries.
DRMT4-GFP signal was found in the cytoplasm of follicle cells (a) and nuclei of the nurse cells (b) and the oocytes (c) through out oogenesis. fc, follicle cells; nc, nurse cells; oo, oocytes.

stage 10B (Fig. 13d). This is inconsistent with the fact that DRMT4-GFP localizes in the nuclei of nurse cells throughout oogenesis (Fig. 12).

Both the ovary immunostaining using anti-DRMT4 antibody and the analysis of DRMT4-GFP distribution showed that in follicle cells, the DRMT4 signal is predominantly cytoplasmic (Fig. 12a and Fig. 13b). However, using a different antibody, Boulanger et al showed that DRMT4 (DART4) localizes to the nuclei of follicle cell (Boulanger et al. 2004). Because the cytoplasmic staining is seen with two different techniques, it is more likely to represent the normal distribution pattern. However, it is still possible that the distribution is dynamic and that the different antibodies have different specificity for the nuclear and the cytoplasmic DRMT4.

Isolation of *DRMT4* mutants

In the early work of this project, we focused on *DRMT4*'s function in oogenesis because we thought that *DRMT4* interacts genetically with *Bic-D,* which plays an important role in oocyte formation and patterning (Suter and Steward 1991; Ran et al. 1994; Swan and Suter 1996). We thus expected *DRMT4* to be essential for oogenesis. In order to identify candidate mutant alleles of *DRMT4*, we set up the cross shown in Figure 2. We crossed a collection of third chromosomal, EMS induced female sterile mutants (*+;bw;st,EMS/TM6B*) with two deficiency lines. The proximal break points of both deficiencies are near one another, 85D11-14 for *Df(3R)by62* and 85D08-12 for *Df(by62)by10*. However, *Df(3R)by62* extents slightly more distal than *Df(by62)by10* (85F6 vs 85F1). The

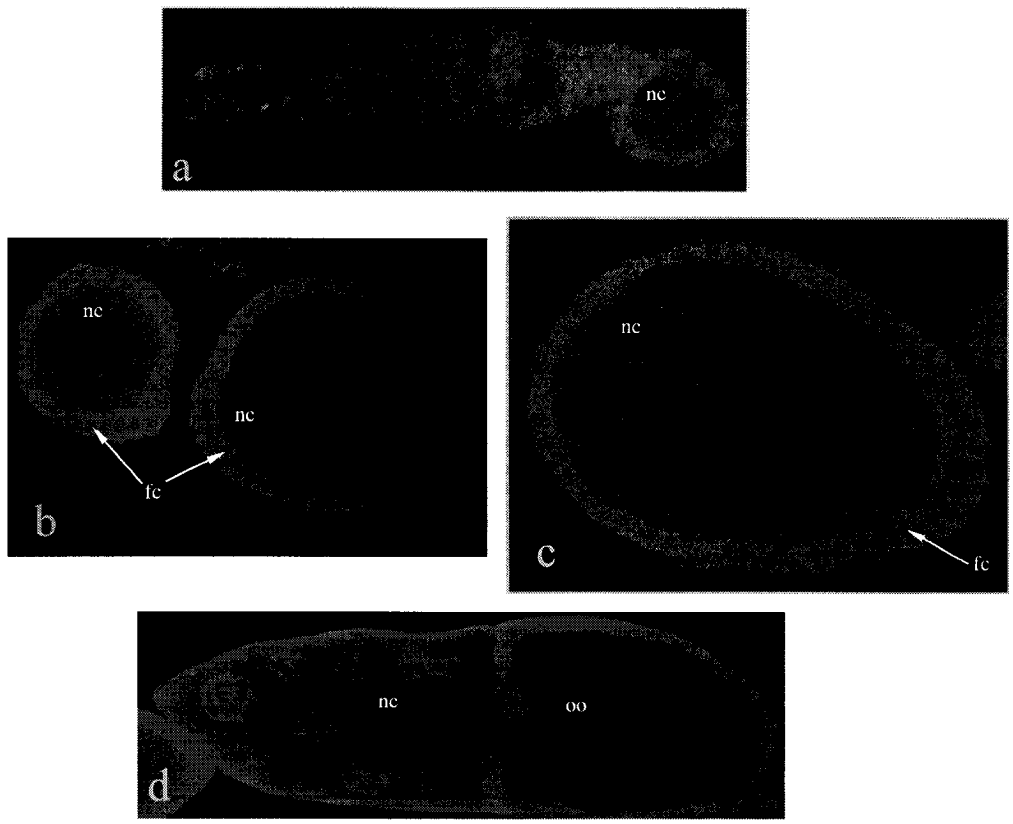


Figure 13. Immunostaining of *OregonR* ovaries usig anti-DRMT4 antibodies. DRMT4 signal localizes to the cytoplasm of follicle cells (a, b, c) and to the nuclei of the oocytes (d) throughout oogenesis. In nurse cells, DRMT4 signal is seen in the cytoplasm before stage 10 (a, b, c) and in the nuclei in the later stages (d). fc, follicle cells; nc, nurse cells; oo, oocytes.

mapping position of the *DRMT4* gene, 85F4, is in the narrow region between 85F1 and 85F6 (Fig. 3). Therefore, a female sterile phenotype over *Df(3R)by62,* but not *Df(3R)by10,* should map to the 85F1 to 85F6 region. Such a mutation is thus a good candidate for a *DRMT4* allele. In this manner we identified two female sterile lines, *E0467* and *E6115*, as *DRMT4* candidates. In order to find out whether the female sterile phenotype is really caused by a mutation in the *DRMT4* gene, I performed first a Western blot to see if DRMT4 expression levels are lower in the mutants or whether the mobility of DRMT4 is altered. The results of this experiment showed that DRMT4 levels are reduced in the *E0467* and the *E6115* lines (Fig. 14).

In order to confirm that the female sterile phenotype is indeed caused by defects in *DRMT4* expression, a rescue of the mutant phenotype was attempted. I performed the rescue cross using the *DRMT4* genomic construct. Because the insertion site in the transgenic line is on the third chromosome, I attempted to recombine the transgene onto the mutant chromosome. However, no such recombinant chromosome was detected among 121 individual crosses, indicating that the insertion site is very close to the *DRMT4* gene. I next performed a rescue experiment with the *DRMT4-GFP* transgenic line on another chromosome and this one rescues the female sterile phenotype successfully (Fig. 15). This demonstrates that the phenotype is indeed caused by a defect in *DRMT4*. In addition, this result indicates that the *DRMT4-GFP* transgene is functional in the transgenic line.

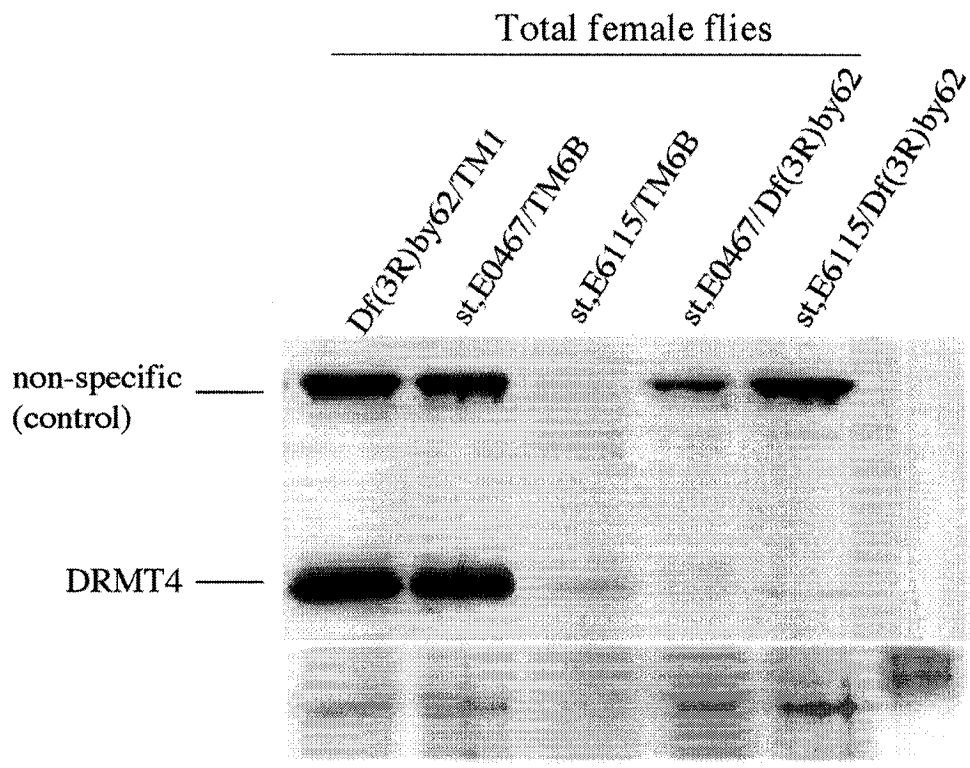


Figure 14. The amounts of DRMT4 protein are reduced in the two candidate *DRMT4* mutants *E0467* and *E6115*.

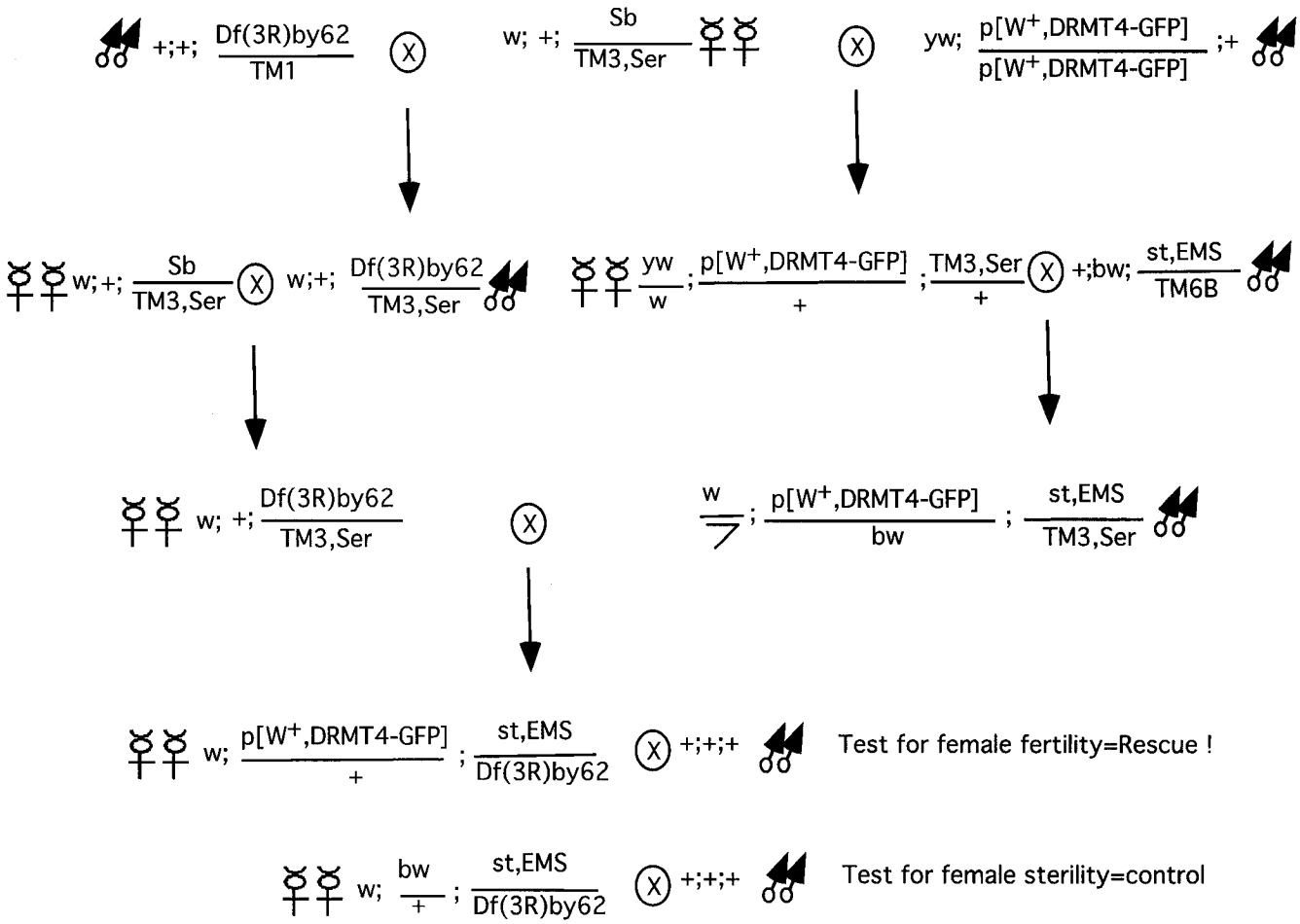


Figure 15. Rescue cross for the female sterile mutant *st,EMS/Df(3R)by62* using the *DRMT4-GFP* transgenic line. EMS refers to the two EMS alleles *E0467* and *E6115*. *Df(3R)by62* is a deficiency that uncovers the *DRMT4* region.

DRMT4 functions in oogenesis

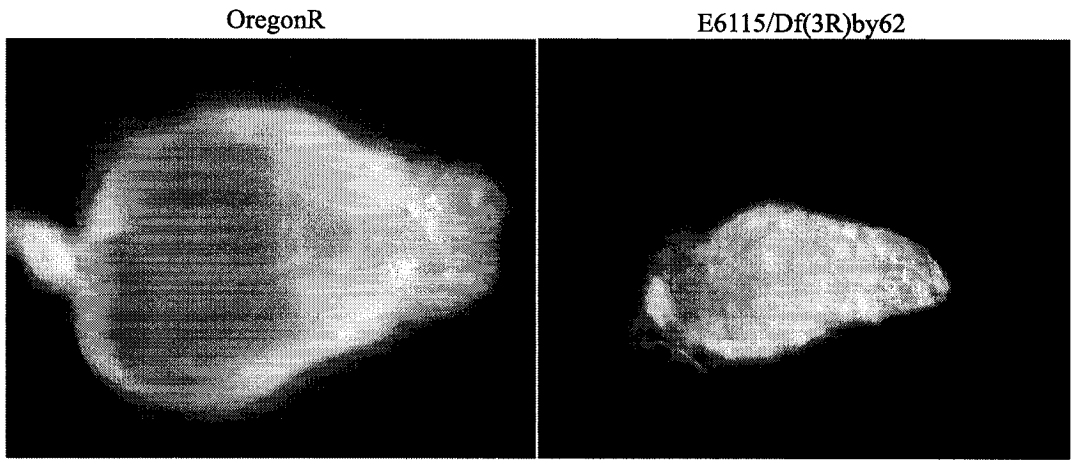
1. The phenotype of *DRMT4* mutant ovaries

Since the phenotype of *DRMT4* mutants is female sterility, we wanted to identify the oogenesis defects. I fed the mutants and wild type *OregonR* female flies for 48 hours after they eclosed at 25°C. I then dissected their ovaries and found that the development of the mutant ovaries is arrested (Fig. 16A). Later stage oocytes are not present. Even in older ovaries, oogenesis generally does not proceed beyond stage 9. Western Blot using whole female fly extracts showed that *vasa* expression levels are lower in the *DRMT4* mutants compared to the wild type control (Fig. 16B). This is consistent with the phenotype of the *E0467* and *E6115* mutant ovaries because Vasa is a germ line-specific protein that accumulates strongly in older egg chambers. Therefore, the decreased Vasa protein levels in *DRMT4* mutants are probably due to the fact that ovaries are under-developed. In addition, I found that in *E0467/Df(3R)by62* ovaries, some egg chambers contain more than 16 nurse cell nuclei (Fig. 17). These defects in oogenesis indicated that *DRMT4* functions in more than one pathway of ovary development.

2. Germ line specific expression of *DRMT4* rescues the oogenesis phenotype

DRMT4 was found to accumulate in the nuclei of oocytes and nurse cells, but in addition also in the somatic follicle cells (Fig. 12, 13). Because reduced function of *DRMT4* interferes with normal development of the germ line part of

A.



B.

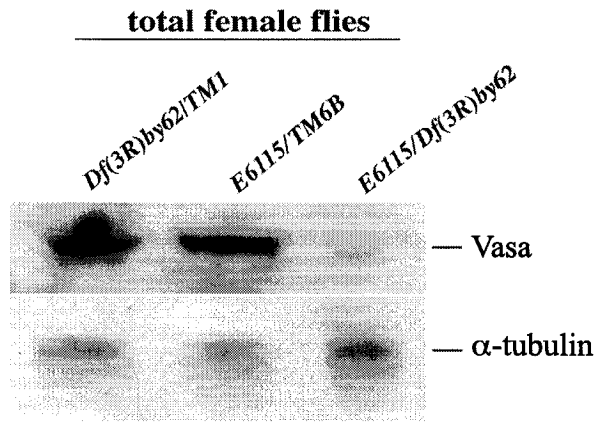


Figure 16. Under-developed ovaries of *E6115* mutants.
A. Ovary overview of wild type and *E6115/Df(3R)by62* mutants.
B. Western blot using anti-Vasa antibodies shows the reduced expression of the germ cell marker Vasa.
The other mutant line *E0467/Df(3R)by62* has similar ovarian defects.

E0467/Df(3R)by62

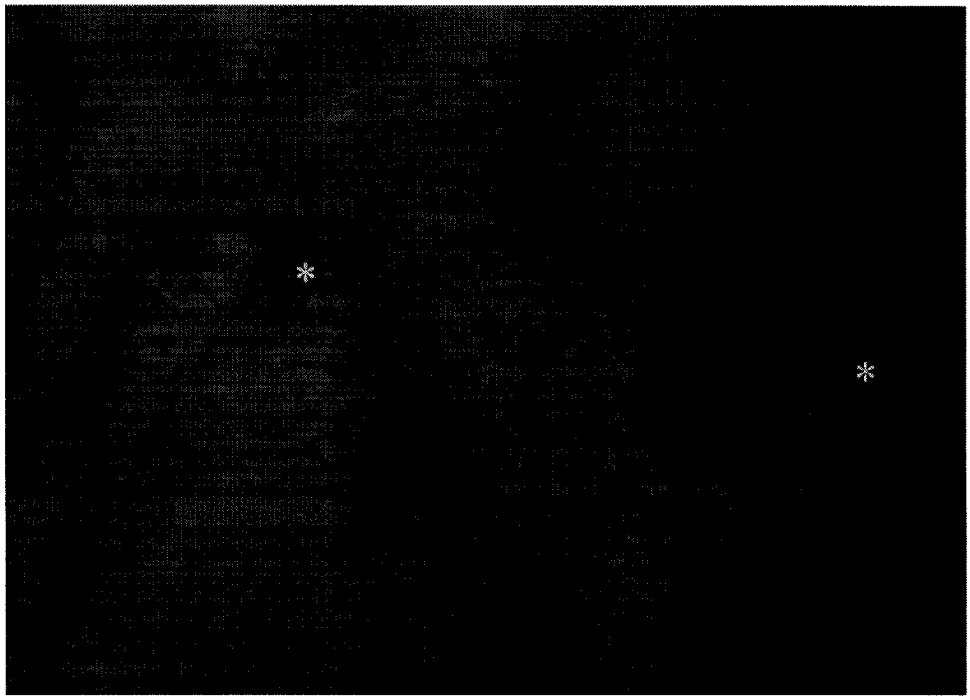
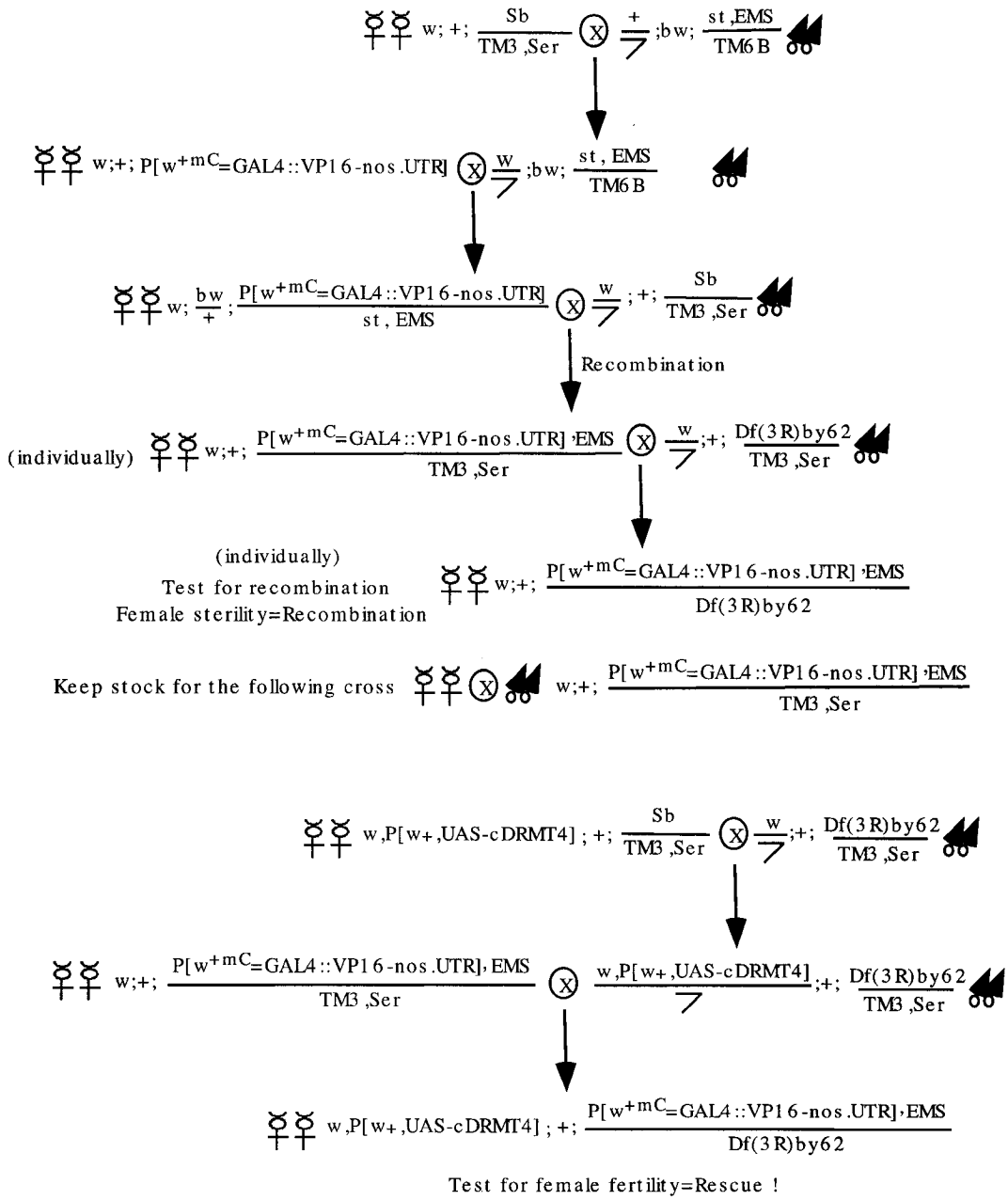


Figure 17. *E0467/Df(3R)by62* egg chambers with more than 16 nurse cell nuclei. Such egg chambers are indicated by yellow asterisks.

the ovary (Fig. 16, 17), we hypothesized that that expression of *DRMT4* in the germ line is crucial for *Drosophila* oogenesis. In order to test this, I attempted to rescue the female sterility of *E0467/Df(3R)by62* and *E6115/Df(3R)by62* by targeting expression of *DRMT4* to the germ line (Fig. 18) and to the follicle cells separately. I used a nos-GAL4 construct (Bloomington stock number: 4937) to drive *DRMT4* expression in germ cells and I targeted *DRMT4* expression to follicle cells by crossing my *pUAST-cDRMT4* transgenic line with a *GAL4* line that is expressed in the follicle cells (Bloomington stock number: 3732 and 3750). Because expression of *DRMT4* in germ cells rescues the female sterility of both *E0467* and *E6115*, while expression of *DRMT4* in follicle cells does not, germ line expression of *DRMT4* must be essential for normal oogenesis.

3. ELAV is methylated *in vivo* in *Drosophila*

We have investigated the basic expression pattern of DRMT4 in *Drosophila* and found that the *DRMT4* gene is crucial for *Drosophila* oogenesis. To further understand the *in vivo* functions of *DRMT4*, I plan to identifiy substrates and downstream targets of DRMT4. The mammalian PRMT4/CARM1 has been shown to methylate histone H3 (Chen et al. 1999; Ma et al. 2001; Bauer et al. 2002), CBP/p300 (Xu et al. 2001), RNA-binding protein HuR (Li et al. 2002) and PABP (polyA-binding protein) (Lee and Bedford 2002). Because DRMT4 shows high identities with PRMT4/CARM1, it probably has similar substrate specificities. *in vitro* methylation assays have shown that DRMT4 can methylate histone H3, PABP and p300 (Boulanger et al. 2004). I tried to identify the *in vivo* targets of DRMT4 using immunoprecipitation (IP). We hypothesized



Fiure 18. Rescue cross of the female sterile mutant *EMS/Df(3R)by62* using the *UAS-cDRMT4* transgenic line.
UAS-cDRMT4 is a cDNA construct of *DRMT4* in which the cDNA sequence (cDRMT4) was put under the control of UAS promoter.
[GAL4::VP16-nos.UTR] can drive UAS-gene expression in the germ line.

that Rbp9 is the predicted target because Rbp9 is the homologue of HuR, a substrate of PRMT4/CARM1 (Li et al. 2002) in mammals, and because *Rbp9* is required for regulation of cystocyte differentiation and oocyte determination during oogenesis (Kim-Ha et al. 1999). However, even though Y. Kim-Ha promised to send anti Rbp9 antibodies, we have not received them yet.

I was able to analyze methylation of ELAV, which is another *Drosophila* HuR homologue (Kim and Baker 1993). IP was done using anti mono- and di-methyl arginine antibodies (Abcam Co.). Whole female fly extracts from the two *DRMT4* mutants and wild type flies were used, and Western Blot was performed using anti-ELAV antibodies (gift from Yong Rao). From the results we concluded that ELAV is indeed methylated on one or more arginine residues *in vivo* (Fig. 19).

In figure 19, a band at around 75 KD seen in wild type background (*Df(3R)by62/TM1*) was lost in the *E0467* and *E6115* mutants. This is possibly because the normal expression of the protein is affected in the *DRMT4* mutants. Another unknown protein was seen in the *E0467* sample. The fact that it was detected in the IP pellet and the supernatant may indicate that methylated and unmethylated forms of this protein exist *in vivo*. This band is only seen in the *E0467* line and may thus be a product of a gene that is usually silent at this stage, but becomes activated as the result of the mutation in the *DRMT4* gene. There is ample evidence demonstrating that some gene silencing is due to histone methylation (reviewed in (Czermin and Imhof 2003)). On the other hand, lack of

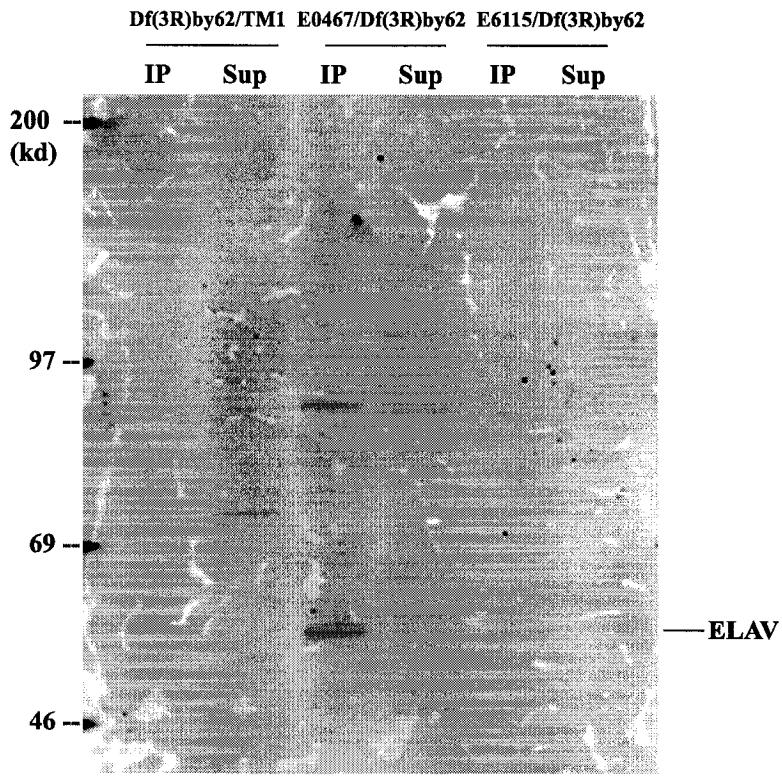


Figure 19. ELAV is methylated *in vivo*.
Total female protein extracts were immunoprecipitated with mouse anti-(mono- and dimethyl-) arginine antibodies.
The western blot was probed with anti-ELAV antibodies generated in rat.

modification of the RNA binding proteins could also cause the appearance of a new protein.

We showed that ELAV is methylated *in vivo* and its methylation state seems normal in *DRMT4* mutants (Fig. 19). One possibility is that the methyltransferase responsible for ELAV arginine methylation is not DRMT4, because there are 9 putative arginine methyltransferases in *Drosophila* (Boulanger et al. 2004). The other possibility is that the methylation of ELAV by DRMT4 is not affected in the *DRMT4* mutants *E0467* and *E6115*, which are not null mutants. Because these two lines have only germ line defects, it is possible that the remaining DRMT4 activity is sufficient to methylate ELAV.

Discussion

The *E234* line is not a *DRMT4* mutant

Previous data showed that the *E234* line (Bloomington stock number: 1931), *+; +; E234/TM3,Sb,Ser*, acts as a dominant enhancer of the *Bic-D* phenotype (Thuy Nguyan, Master thesis, McGill University). *E234* is a chromosome with a P-element insertion in the 5'UTR or promoter of *DRMT4*. Therefore, we had hypothesized that the *E234* line is a *DRMT4* mutant and that *DRMT4* possibly interacts with *Bic-D*. The *Bic-D* phenotype is the 16-nurse-cell egg chamber that fails to differentiate an oocyte (Suter and Steward 1991). We therefore tested whether the mutation causes a reduced expression of *DRMT4*. However, Western Blot analysis revealed normal expression levels of DRMT4 in the *E234* line (data not shown). In addition, the hatch rate of hemizygous (*E234/Df(3R)by62*) flies is wild type, indicating that either *DRMT4* is normal in the *E234* line, or that it is a non-essential gene. Furthermore, the *DRMT4* transgene was not able to rescue the enhancement of the *Bic-D* phenotype caused by the *E234* line. Therefore, the enhancement of the *Bic-D* phenotype must be due to another mutation on the *E234* chromosome. Because the P-element insertion does not appear to disrupt the *DRMT4* gene and because it does not cause the genetic interaction with *Bic-D*, the *E234* line is not suitable to study the function of *DRMT4* and it is not suitable to study the *Bic-D* pathway.

Nevertheless, I continued studying the functions of *DRMT4* in *Drosophila* oogenesis, because I had already developed some useful tools and because

DRMT4 is a very interesting gene that is presently attracting considerable interest. Therefore, its functional analysis in a genetic model system seems attractive.

Bicaudal-D (Bic-D) and Vasa protein localizations are not affected in the *DRMT4* mutants

We demonstrated that defects in *DRMT4* expression cause female sterility. Then we tried to figure out its oogenesis related partners. Bic-D and Vasa were selected for testing because both of them are crucial in oogenesis and because antibodies against them are at hand. *Bic-D* has a role in oocyte differentiation (Suter and Steward 1991). Ovary immunostaining of *E0467* and *E6115* mutants using anti-Bic-D antibody 1B11 (Suter and Steward 1991) showed that the Bic-D signal accumulated in the determined oocytes during the early stages of oogenesis as in the *OregonR* control (data not shown). Thus, in *DRMT4* mutants, oocyte differentiation is not affected in the early stages. Western Blot results showed that *Bic-D* expression levels are also the same as in the wild type control (data not shown). Together with the immunostaining data, this indicates that *Bic-D* is probably not a downstream gene of *DRMT4*.

I also studied *vasa* expression and Vasa localization in *DRMT4* mutants. Western blots using whole female fly extracts showed that *vasa* expression levels are decreased in the *E0467* and *E6115* mutants (Fig. 16B). The decreased Vasa protein levels observed in *DRMT4* mutants could be an indirect result of the smaller mutant ovaries. I also did ovary immunostainings with anti-Vasa antibodies (gift from Paul Lasko). *Drosophila* Vasa protein localizes during early

oogenesis to the perinuclear region of the nurse cells (nuage) and later, starting around stage 10, to the pole plasm at the posterior end of the oocyte (Liang et al. 1994). In *E0467* and *E6115*, Vasa localizes normally to the perinuclear nuage (data not shown). Because the mutant ovaries do not develop beyond stage 9, late Vasa localization could not be assessed. The localization of Vasa to the nuage particles is independent of the pole plasm assembly pathway (Liang et al. 1994). Therefore, even though the perinuclear localization in nurse cells appears normal, it is still possible that *DRMT4* has an essential function on proteins involved in Vasa posterior localization.

DRMT4 as a zygotic expression activator

Drosophila has a period of transcriptional quiescence that occurs during the early embryonic nuclear division cycles. In *Drosophila* embryos, the maternal/zygotic transition (MZT) in cell cycle control normally follows mitosis 13 (Edgar and O'Farrell 1989). Also after the 13^{th} nuclear division, cell membranes form between the nuclei and the embryo cellularizes. As soon as cellularization is complete, at about 3.5 h after fertilization, gastrulation begins (Costa 1993).

Immunostaining of *OregonR* embryos using anti-DRMT4 antibodies revealed a cytoplasmic DRMT4 signal in the preblastoderm embryos and a nuclear one after the onset of gastrulation (Fig. 9). This nuclear localization coincides with the activation of zygotic transcription. CARM1, the mammalian homologue of DRMT4, has been demonstrated to methylate histone H3, RNA

binding protein HuR and polyA-binding protein and it functions in gene transcription (Lee and Bedford 2002; Li et al. 2002). The observed immunostaining is consistent with *DRMT4* having a similar function during *Drosophila* embryogenesis. It may activate zygotic gene expression by methylating RNA-binding proteins which specifically bind those zygotic transcripts. The methylation could be a signal, which directs or activates its RNA binding activity. Then the RNA binding proteins' methylation may therefore influence further gene expression by facilitating RNA/protein interactions. The observed nuclear translocation could also reflect a general role of DRMT4 in nucleosome remodelling at the MZT analogous to the function of CARM1 in hormone signaling (Xu et al. 2004).

DRMT4 in imaginal discs and brain development

The pairs of imaginal discs of *Drosophila melanogaster* are the precursors of the adult fly organs and an unpaired genital disc will form the reproductive structures. Discs increase in size by cell division during laval develoment. The onset of proliferation of eye discs first starts around 13-15 hours after hatching. Most discs reinitiate proliferation during the second and third larval instar. During larval stages, the cells of the imaginal disc primordia undergo extensive growth and proliferation, increasing in number by three orders of magnitude (Bryant 1978). In the third instar, each disc has a characteristic size, shape and pattern of folds and is easy to identify. Larval neurons then connect the brain to the major discs (the eye discs and the leg discs) (Jan et al. 1985; Tix et al. 1989).

Drosophila melanogaster is an important model organism to study brain functions (Posey et al. 2001) such as how the brain receives and sends information to the other parts of the body and how it thereby directs behaviors. The brain is involved in motor coordination (Ilius et al. 1994), the processing of visual information (Bausenwein et al. 1992), olfactory learning (Heisenberg et al. 1985), memory (Davis 1996) and other complex behaviors.

In the *DRMT4-GFP* transgenic lines I found that DRMT4-GFP shows the strongest fluorescence in the brain and in imaginal discs (Fig. 11). In addition, the Western Blot analysis showed that the DRMT4 expression levels increase with development. There is much more DRMT4 protein in 3rd instar larvae than the 1st instar (Fig. 10). Therefore, the DRMT4 protein level increases with the development of the imaginal discs and the brain. Since imaginal discs and brain are responsible for the development of several *Drosophila* adult organs, it appears that *DRMT4* may be involved in a broad range of different pathways.

DRMT4 in *E0467* and *E6115* mutants

Using Western Blots we found that DRMT4 accumulation is dramatically decreased in *E0467* and *E6115* mutants (Fig. 14). This could either indicate defects in the expression of *DRMT4* or it could simply be because of the lack of late oogenesis in the mutants. I then investigated the *DRMT4* expression pattern in the *E0467* and *E6115* ovaries by means of immunostainings using anti-DRMT4 antibodies. Because the mutant flies are female sterile and do not lay eggs, immunostaining could not be performed on embryos of such mutants. Upon

staining of mutant ovaries. I can see a strong DRMT4 signal and I found that its localization pattern is similar to that in the wild type flies (data not shown). In the ovary staining, the DRMT4 pattern looks normal. However, even when I repeated this immunostaining under the same conditions, the brightness of the signals still varied. Therefore, at present we cannot discriminate between these two possibilities. It will be interesting to determine the molecular nature of the EMS alleles.

Functional pathway of *DRMT4* during *Drosophila* oogenesis

During *Drosophila* oogenesis, DRMT4 localizes to the nuclei of oocytes, to nuclei or cytoplasm of nurse cells and to the cytoplasm of follicle cells (Fig. 12, 13). Nurse cells are responsible for providing the oocyte with some of the nutrient materials. The *DRMT4* mutants *E0467* and *E6115* show defects in oogenesis. They are female sterile and the development of the egg chambers arrests at stage 9. We were able to show that *DRMT4* is required in the germ line to progress beyond this developmental block because expression of an evidently functional *DRMT4* cDNA under the control of a germ line specific *GAL4* driver was able to rescue the mutant phenotype, whereas the expression under the control of a follicle cell specific *GAL4* driver was not (Fig. 18).

We then tried to find out how *DRMT4* functions during oogenesis. DRMT4 is an arginine methyltransferase, which functions in post-translational protein modification (Boulanger et al. 2004). The mammalian homologue of DRMT4, PRMT4/CARM1, can methylate diverse proteins, including histone H3,

RNA-binding protein HuR and PABP1, and it plays a role in gene expression (Li et al. 2002). DRMT4 presumably functions similarly in flies. In the germline nuclei, DRMT4 may for instance methylate specific RNA-associated proteins and, through regulating these contribute to their regulation of gene expression. In the *E0467* and *E6115* mutants methylation of these substrates may be disrupted, thereby preventing the function of the RNA binding proteins.

Towards the identification of DRMT4 targets, we first analyzed the *DRMT4* expression pattern. DRMT4 protein is expressed throughout almost all *Drosophila* stages, including embryos, larvae and oogenesis (Fig. 9-13). We therefore focused on the RNA-binding proteins that are expressed during oogenesis and are the homologues of known targets of PRMT4/CARM1. Rbp9 emerged as a candidate for two reasons: (1) Rbp9 belongs to the same protein family as HuR, which is a substrate of PRMT4/CARM1 (the homologue of DRMT4). (2) *Rbp9* has been demonstrated to be required for *Drosophila* oogenesis (Kim-Ha et al. 1999). In severe *Rbp9* mutants, cystocyte differentiation is arrested and egg chambers never develop beyond stage 6. In *E0467* and *E6115* point mutants, the ovary development is arrested at stage 9. Despite that, there are some resemblances between the phenotypes. Some *Rbp9* alleles have abnormal egg chambers with more than 15 nurse cells. We also observe occasional egg chambers with more than the normal 16 germ cells (Fig. 17).

PRMT4/CARM1 has a nuclear function (Ma et al. 2001) and DRMT4 localizes to the nuclei of nurse cells and oocytes during oogenesis, and it moves from the embryonic cytoplasm to the nuclei at the time these nuclei become

transcriptionally active (Fig. 8). It is thus possible that *DRMT4* also has a nuclear function. We further know that during oogenesis, *DRMT4* has a function in the germ line. *Drosophila* Rbp9 is a putative RNA binding protein (Kim and Baker 1993) and probably functions in mRNA stabilization as its HuR homologue (Peng et al. 1998). HuR has been described as a target of PRMT4/CARM1 and it shuttles between the nucleus and cytoplasm (Fan and Steitz 1998). Rpb9 was detected in the cytoplasm of ovaries (Kim-Ha et al. 1999). One possibility is therefore that DRMT4 initially methylates Rbp9 (and/or other RNA-related proteins) in the nuclei before their binding to the mRNA. Then the methylated Rbp9 may bind to mRNAs and transport them to the cytoplasmic compartment. Rbp9 binding may provide protection for the mRNA during and after their export. Once in the cytoplasm Rbp9 may then disassociate from the RNA so that ribosomes can bind to it and start translation. The disassociated Rbp9 might degrade or shuttle back to the nuclei to be reused (Fig. 20). In *E0467* and *E6115*, the binding to the RNA would be disrupted and then some proteins required for oogenesis would not be expressed normally. However, future work is needed to test this model.

We also found evidence that DRMT4 itself needs to be activated by a factor that acts upstream of it. We over-expressed DRMT4 using a strong promoter (α-tubulin promoter) that is active in most tissues (Bloomington stock number: 5138) (Lee and Luo 1999). The α-tubulin promoter allows such a strong general expression (Fig. 8). Despite this, animals in which *DRMT4* expression was driven by this construct did not show obvious defects, indicating that strong

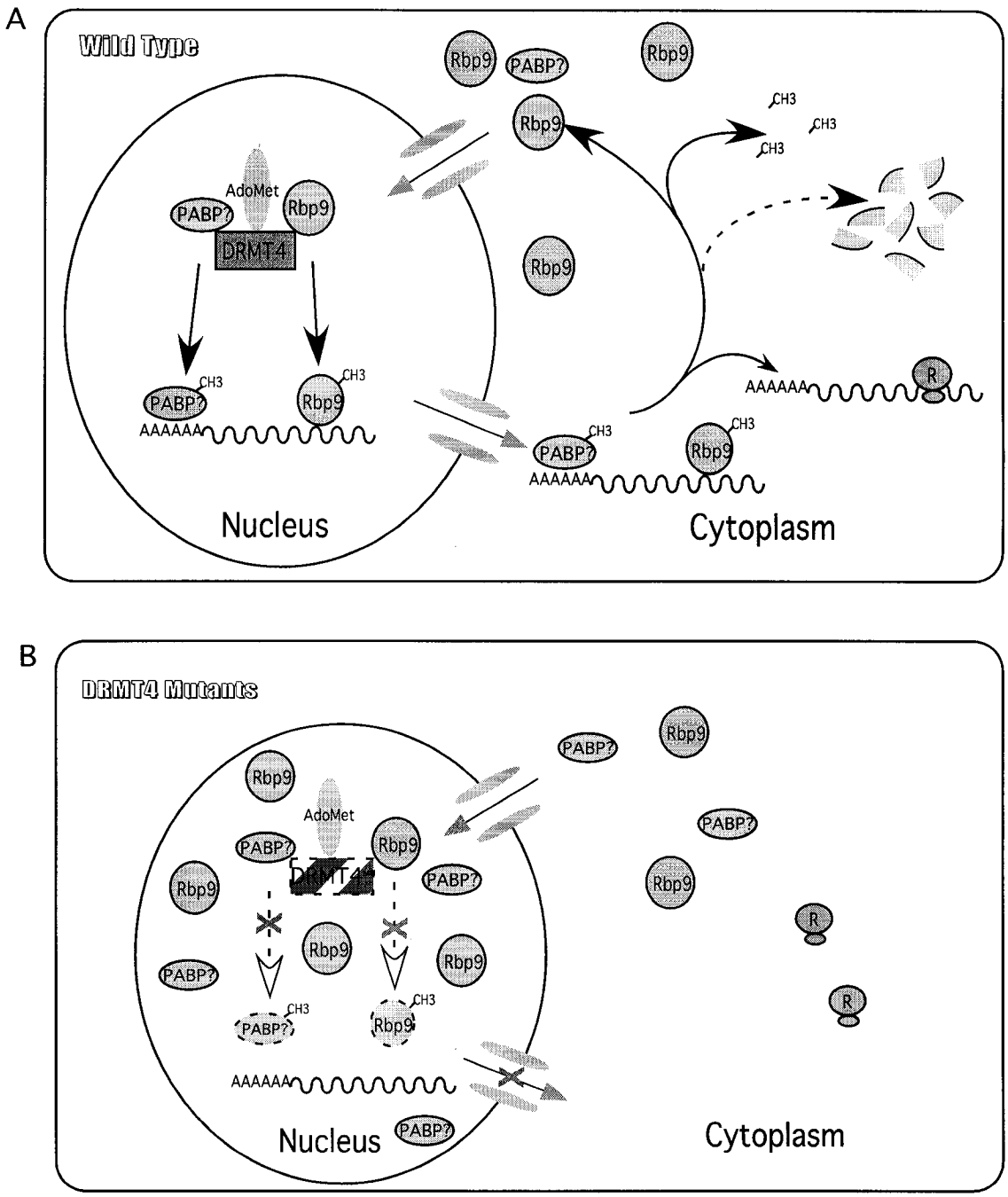


Figure 20. Model for arginine methylation by DRMT4 in germcells during *Drosophila* oogenesis.
(A) In wild type nuclei, the methylation of Rbp9 by DRMT4 may provide a signal to bind specific mRNAs that are then exported to the cytoplasm for gene expression. Once in the cytoplasm, Rbp9 proteins are released and re-enter the nucleus.
(B) In *DRMT4* mutant nuclei, the methylation of Rbp9 by DRMT4 is disrupted, which leads to Rbp9 getting trapped in the nuclei and to the failure of expression of specific genes.
PABP is polyA-binding protein which might also be the target of DRMT4.
AdoMet is the methyl donor.

expression of DRMT4 does not cause any deleterious effects, at least as long as it is not activated. It will be interesting to identify this hypothetical activator.

Summary

DRMT4, a *Drosophila* arginine methyltransferase, is a newly studied gene. The family of arginine methyltransferases is presently attracting considerable interest because of their newly described functions. We have shown here that DRMT4 is expressed in embryos, larvae and adults. These findings suggest that *DRMT4* might be involved in multiple developmental pathways. Here we focus on its role in oogenesis. DRMT4 is essential for normal oogenesis and we have identified two DRMT4 mutants that show a female sterile phenotype. This mutant phenotype can be rescued with different DRMT4 transgenes. Targeted expression of *DRMT4* in germline cells (but not in the somatic follicle cells) can also rescue the female sterile mutants. This indicated that DRMT4 functions in the germline cells. However, the substrate of DRMT4 is not known yet. Future efforts should concentrate on testing candidate substrates. We have developed some useful tools: several *DRMT4* transgenic lines and an anti-DRMT4 antibody. They will be used for addressing the remaining questions.

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References

Abramovich, C., B. Yakobson, J. Chebath, and M. Revel. 1997. A protein-arginine methyltransferase binds to the intracytoplasmic domain of the IFNAR1 chain in the type I interferon receptor. Embo J 16: 260-6.

Antic, D. and J.D. Keene. 1997. Embryonic lethal abnormal visual RNA-binding proteins involved in growth, differentiation, and posttranscriptional gene expression. Am J Hum Genet 61: 273-8.

Ashburner, M. 1989. Drosophila A Laboratory Handbook. Cold Spring laboratory Press, New York.

Bauer, U.M., S. Daujat, S.J. Nielsen, K. Nightingale, and T. Kouzarides. 2002. Methylation at arginine 17 of histone H3 is linked to gene activation. EMBO Rep 3: 39-44.

Bausenwein, B., A.P. Dittrich, and K.F. Fischbach. 1992. The optic lobe of Drosophila melanogaster. II. Sorting of retinotopic pathways in the medulla. Cell Tissue Res 267: 17-28.

Bennett, E.J., J. Bjerregaard, J.E. Knapp, D.A. Chavous, A.M. Friedman, W.E. Royer, Jr., and C.M. O'Connor. 2003. Catalytic implications from the Drosophila protein L-isoaspartyl methyltransferase structure and site-directed mutagenesis. Biochemistry 42: 12844-53.

Boisvert, F.M., J. Cote, M.C. Boulanger, and S. Richard. 2003. A Proteomic Analysis of Arginine-methylated Protein Complexes. Mol Cell Proteomics 2: 1319-30.

Boulanger, M.C., T. Branscombe Miranda, S. Clarke, M. DiFruscio, B. Suter, P. Lasko, and S. Richard. 2004. Characterization of Drosophila protein arginine methyltransferases DART1 and DART4. Biochem J Pt. *in press.*

Brahms, H., J. Raymackers, A. Union, F. de Keyser, L. Meheus, and R. Luhrmann. 2000. The C-terminal RG dipeptide repeats of the spliceosomal Sm proteins D1 and D3 contain symmetrical dimethylarginines, which form a major B-cell epitope for anti-Sm autoantibodies. J Biol Chem 275: 17122-9.

Brand, A.H. and N. Perrimon. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118: 401-15.

Branscombe, T.L., A. Frankel, J.H. Lee, J.R. Cook, Z. Yang, S. Pestka, and S. Clarke. 2001. PRMT5 (Janus kinase-binding protein 1) catalyzes the formation of symmetric dimethylarginine residues in proteins. J Biol Chem 276: 32971-6.

Bryant, P.J. 1978. Pattern formation in imaginal discs. In The genetics and biology of Drosophila. (ed. M. Ashburner), pp. 230-336. Academic Press, London.

Byrd, K.N. and A. Shearn. 2003. ASH1, a Drosophila trithorax group protein, is required for methylation of lysine 4 residues on histone H3. Proc Natl Acad Sci U S A 100: 11535-40.

Chen, D., H. Ma, H. Hong, S.S. Koh, S.M. Huang, B.T. Schurter, D.W. Aswad, and M.R. Stallcup. 1999. Regulation of transcription by a protein methyltransferase. Science 284: 2174-7.

Chen, S.L., K.A. Loffler, D. Chen, M.R. Stallcup, and G.E. Muscat. 2002. The coactivator-associated arginine methyltransferase is necessary for muscle differentiation: CARM1 coactivates myocyte enhancer factor-2. J Biol Chem 277: 4324-33.

Chevillard-Briet, M., D. Trouche, and L. Vandel. 2002. Control of CBP co-activating activity by arginine methylation. Embo J 21: 5457-66.

Clarke, S. 1993. Protein methylation. Curr Opin Cell Biol 5: 977-83.

Clegg, N.J., S.D. Findley, A.P. Mahowald, and H. Ruohola-Baker. 2001. Maelstrom is required to position the MTOC in stage 2-6 Drosophila oocytes. Dev Genes Evol 211: 44-8.

Costa, M., Sweeton, D. and Wieschaus, E. 1993. Gasrulation in Drosophila: Cellular Mechanism of Morphogeetic Movements. In The Development of Drosophila melanogaster (ed. M. Bate), pp. 425-467. Cold Spring Harbor Laboratory Press, New York.

Cox, D.N., B. Lu, T.Q. Sun, L.T. Williams, and Y.N. Jan. 2001. Drosophila par-1 is required for oocyte differentiation and microtubule organization. Curr Biol 11: 75-87.

Czermin, B. and A. Imhof. 2003. The sounds of silence--histone deacetylation meets histone methylation. Genetica 117: 159-64.

Czermin, B., R. Melfi, D. McCabe, V. Seitz, A. Imhof, and V. Pirrotta. 2002. Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell 111: 185-96.

Czermin, B., G. Schotta, B.B. Hulsmann, A. Brehm, P.B. Becker, G. Reuter, and A. Imhof. 2001. Physical and functional association of SU(VAR)3-9 and HDAC1 in Drosophila. EMBO Rep 2: 915-9.

Davis, R.L. 1996. Physiology and biochemistry of Drosophila learning mutants. Physiol Rev 76: 299-317.

Desrosiers, R. and R.M. Tanguay. 1988. Methylation of Drosophila histones at proline, lysine, and arginine residues during heat shock. J Biol Chem 263: 4686-92.

DeZazzo, J., S. Xia, J. Christensen, K. Velinzon, and T. Tully. 1999. Developmental expression of an amn(+) transgene rescues the mutant memory defect of amnesiac adults. J Neurosci 19: 8740-6.

Duffy, J.B. 2002. GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis 34: 1-15.

Edgar, B.A. and P.H. O'Farrell. 1989. Genetic control of cell division patterns in the Drosophila embryo. Cell 57: 177-87.

Fan, X.C. and J.A. Steitz. 1998. Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. Embo J 17: 3448-60.

Fang, J., Q. Feng, C.S. Ketel, H. Wang, R. Cao, L. Xia, H. Erdjument-Bromage, P. Tempst, J.A. Simon, and Y. Zhang. 2002. Purification and functional characterization of SET8, a nucleosomal histone H4-lysine 20-specific methyltransferase. Curr Biol 12: 1086-99.

Fischer, J.A., E. Giniger, T. Maniatis, and M. Ptashne. 1988. GAL4 activates transcription in Drosophila. Nature 332: 853-6.

Frankel, A. and S. Clarke. 2000. PRMT3 is a distinct member of the protein arginine N-methyltransferase family. Conferral of substrate specificity by a zinc-finger domain. J Biol Chem 275: 32974-82.

Frankel, A., N. Yadav, J. Lee, T.L. Branscombe, S. Clarke, and M.T. Bedford. 2002. The novel human protein arginine N-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity. Journal of Biological Chemistry 277: 3537-43.

Fristrom, J.W. and H.K. Mitchell. 1965. The preparative isolation of imaginal discs from larvae of Drosophila melanogaster. J Cell Biol 27: 445-8.

Gary, J.D. and S. Clarke. 1998. RNA and protein interactions modulated by protein arginine methylation. Prog Nucleic Acid Res Mol Biol 61: 65-131.

Gilbreth, M., P. Yang, G. Bartholomeusz, R.A. Pimental, S. Kansra, R. Gadiraju, and S. Marcus. 1998. Negative regulation of mitosis in fission yeast by the shk1 interacting protein skb1 and its human homolog, Skb1Hs. Proc Natl Acad Sci U S A 95: 14781-6.

Giniger, E., S.M. Varnum, and M. Ptashne. 1985. Specific DNA binding of GAL4, a positive regulatory protein of yeast. Cell 40: 767-74.

Gonzalez-Reyes, A., H. Elliott, and D. St Johnston. 1995. Polarization of both major body axes in Drosophila by gurken-torpedo signalling. Nature 375: 654-8.

Grunert, S. and D. St Johnston. 1996. RNA localization and the development of asymmetry during Drosophila oogenesis. Curr Opin Genet Dev 6: 395-402.

Gupta, T. and T. Schupbach. 2003. Cct1, a phosphatidylcholine biosynthesis enzyme, is required for Drosophila oogenesis and ovarian morphogenesis. Development 130: 6075-87.

Hawkins, N.C., J. Thorpe, and T. Schupbach. 1996. Encore, a gene required for the regulation of germ line mitosis and oocyte differentiation during Drosophila oogenesis. Development 122: 281-90.

Heim, R., A.B. Cubitt, and R.Y. Tsien. 1995. Improved green fluorescence. Nature 373: 663-4.

Heisenberg, M., A. Borst, S. Wagner, and D. Byers. 1985. Drosophila mushroom body mutants are deficient in olfactory learning. J Neurogenet 2: 1-30.

Henry, M.F. and P.A. Silver. 1996. A novel methyltransferase (Hmt1p) modifies poly(A)+-RNA-binding proteins. Mol Cell Biol 16: 3668-78.

Huynh, J.R., J.M. Shulman, R. Benton, and D. St Johnston. 2001. PAR-1 is required for the maintenance of oocyte fate in Drosophila. Development 128: 1201-9.

Ichikawa, J.K. and S. Clarke. 1998. A highly active protein repair enzyme from an extreme thermophile: the L-isoaspartyl methyltransferase from Thermotoga maritima. Arch Biochem Biophys 358: 222-31.

Ilius, M., R. Wolf, and M. Heisenberg. 1994. The central complex of Drosophila melanogaster is involved in flight control: studies on mutants and mosaics of the gene ellipsoid body open. J Neurogenet 9: 189-206.

Jan, Y.N., A. Ghysen, I. Christoph, S. Barbel, and L.Y. Jan. 1985. Formation of neuronal pathways in the imaginal discs of Drosophila melanogaster. J Neurosci 5: 2453-64.

Kagan, R.M., H.J. McFadden, P.N. McFadden, C. O'Connor, and S. Clarke. 1997. Molecular phylogenetics of a protein repair methyltransferase. Comp Biochem Physiol B Biochem Mol Biol 117: 379-85.

Kakidani, H. and M. Ptashne. 1988. GAL4 activates gene expression in mammalian cells. Cell 52: 161-7.

Kakimoto, Y., Y. Matsuoka, M. Miyake, and H. Konishi. 1975. Methylated amino acid residues of proteins of brain and other organs. J Neurochem 24: 893-902.

Katsanis, N., M.L. Yaspo, and E.M. Fisher. 1997. Identification and mapping of a novel human gene, HRMT1L1, homologous to the rat protein arginine N-methyltransferase 1 (PRMT1) gene. Mamm Genome 8: 526-9.

Keene, J.D. 1999. Why is Hu where? Shuttling of early-response-gene messenger RNA subsets. Proc Natl Acad Sci U S A 96: 5-7.

Kim, Y.J. and B.S. Baker. 1993. The Drosophila gene rbp9 encodes a protein that is a member of a conserved group of putative RNA binding proteins that are nervous system-specific in both flies and humans. J Neurosci 13: 1045-56.

Kim-Ha, J., J. Kim, and Y.J. Kim. 1999. Requirement of RBP9, a Drosophila Hu homolog, for regulation of cystocyte differentiation and oocyte determination during oogenesis. Mol Cell Biol 19: 2505-14.

Koch, E.A. and R.H. Spitzer. 1983. Multiple effects of colchicine on oogenesis in Drosophila: induced sterility and switch of potential oocyte to nurse-cell developmental pathway. Cell Tissue Res 228: 21-32.

Koh, S.S., H. Li, Y.H. Lee, R.B. Widelitz, C.M. Chuong, and M.R. Stallcup. 2002. Synergistic coactivator function by coactivator-associated arginine methyltransferase (CARM) 1 and beta-catenin with two different classes of DNA-binding transcriptional activators. J Biol Chem 277: 26031-5.

Kouzarides, T. 2002. Histone methylation in transcriptional control. Curr Opin Genet Dev 12: 198-209.

Kunkel, T.A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc Natl Acad Sci U S A 82: 488-92.

Lasko, P.F. and M. Ashburner. 1988. The product of the Drosophila gene vasa is very similar to eukaryotic initiation factor-4A. Nature 335: 611-7.

Laughon, A., R. Driscoll, N. Wills, and R.F. Gesteland. 1984. Identification of two proteins encoded by the Saccharomyces cerevisiae GAL4 gene. Mol Cell Biol 4: 268-75.

Laughon, A. and R.F. Gesteland. 1984. Primary structure of the Saccharomyces cerevisiae GAL4 gene. Mol Cell Biol 4: 260-7.

Lawrence, P.A., R. Bodmer, and J.P. Vincent. 1995. Segmental patterning of heart precursors in Drosophila. Development 121: 4303-8.

Lee, J. and M.T. Bedford. 2002. PABP1 identified as an arginine methyltransferase substrate using high-density protein arrays. EMBO Rep 3: 268-73.

Lee, J.H., J.R. Cook, B.P. Pollack, T.G. Kinzy, D. Norris, and S. Pestka. 2000. Hsl7p, the yeast homologue of human JBP1, is a protein methyltransferase. Biochem Biophys Res Commun 274: 105-11.

Lee, M.S., M. Henry, and P.A. Silver. 1996. A protein that shuttles between the nucleus and the cytoplasm is an important mediator of RNA export. Genes Dev 10: 1233-46.

Lee, T. and L. Luo. 1999. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron 22: 451-61.

Levine, T.D., F. Gao, P.H. King, L.G. Andrews, and J.D. Keene. 1993. Hel-N1: an autoimmune RNA-binding protein with specificity for 3' uridylate-rich untranslated regions of growth factor mRNAs. Mol Cell Biol 13: 3494-504.

Li, H., S. Park, B. Kilburn, M.A. Jelinek, A. Henschen-Edman, D.W. Aswad, M.R. Stallcup, and I.A. Laird-Offringa. 2002. Lipopolysaccharide-induced methylation of HuR, an mRNA-stabilizing protein, by CARM1. Coactivator-associated arginine methyltransferase. J Biol Chem 277: 44623-30.

Liang, L., W. Diehl-Jones, and P. Lasko. 1994. Localization of vasa protein to the Drosophila pole plasm is independent of its RNA-binding and helicase activities. Development 120: 1201-11.

Lin, H. and A.C. Spradling. 1995. Fusome asymmetry and oocyte determination in Drosophila. Dev Genet 16: 6-12.

Lin, W.J., J.D. Gary, M.C. Yang, S. Clarke, and H.R. Herschman. 1996. The mammalian immediate-early TIS21 protein and the leukemia-associated BTG1 protein interact with a protein-arginine N-methyltransferase. J Biol Chem 271: 15034-44.

Liu, J., J. Dalmau, A. Szabo, M. Rosenfeld, J. Huber, and H. Furneaux. 1995. Paraneoplastic encephalomyelitis antigens bind to the AU-rich elements of mRNA. Neurology 45: 544-50.

Ma, H., C.T. Baumann, H. Li, B.D. Strahl, R. Rice, M.A. Jelinek, D.W. Aswad, C.D. Allis, G.L. Hager, and M.R. Stallcup. 2001. Hormone-dependent,

CARM1-directed, arginine-specific methylation of histone H3 on a steroid-regulated promoter. Curr Biol 11: 1981-5.

Ma, J., E. Przibilla, J. Hu, L. Bogorad, and M. Ptashne. 1988. Yeast activators stimulate plant gene expression. Nature 334: 631-3.

Ma, J. and M. Ptashne. 1987. Deletion analysis of GAL4 defines two transcriptional activating segments. Cell 48: 847-53.

Ma, W.J., S. Cheng, C. Campbell, A. Wright, and H. Furneaux. 1996. Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein. J Biol Chem 271: 8144-51.

Martin-Bermudo, M.D., O.M. Dunin-Borkowski, and N.H. Brown. 1997. Specificity of PS integrin function during embryogenesis resides in the alpha subunit extracellular domain. Embo J 16: 4184-93.

Masrouha, N., L. Yang, S. Hijal, S. Larochelle, and B. Suter. 2003. The Drosophila chk2 gene loki is essential for embryonic DNA double-strand-break checkpoints induced in S phase or G2. Genetics 163: 973-82.

McBride, A.E., V.H. Weiss, H.K. Kim, J.M. Hogle, and P.A. Silver. 2000. Analysis of the yeast arginine methyltransferase Hmt1p/Rmt1p and its in vivo function. Cofactor binding and substrate interactions. J Biol Chem 275: 3128-36.

McKearin, D. and B. Ohlstein. 1995. A role for the Drosophila bag-of-marbles protein in the differentiation of cystoblasts from germline stem cells. Development 121: 2937-47.

Morris, J.Z., C. Navarro, and R. Lehmann. 2003. Identification and analysis of mutations in bob, Doa and eight new genes required for oocyte specification and development in Drosophila melanogaster. Genetics 164: 1435-46.

Mowen, K.A., J. Tang, W. Zhu, B.T. Schurter, K. Shuai, H.R. Herschman, and M. David. 2001. Arginine methylation of STAT1 modulates IFNalpha/beta-induced transcription. Cell 104: 731-41.

Muller, J., C.M. Hart, N.J. Francis, M.L. Vargas, A. Sengupta, B. Wild, E.L. Miller, M.B. O'Connor, R.E. Kingston, and J.A. Simon. 2002. Histone

methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell 111: 197-208.

Neuman-Silberberg, F.S. and T. Schupbach. 1993. The Drosophila dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein. Cell 75: 165-74.

Nishioka, K., J.C. Rice, K. Sarma, H. Erdjument-Bromage, J. Werner, Y. Wang, S. Chuikov, P. Valenzuela, P. Tempst, R. Steward, J.T. Lis, C.D. Allis, and D. Reinberg. 2002. PR-Set7 is a nucleosome-specific methyltransferase that modifies lysine 20 of histone H4 and is associated with silent chromatin. Mol Cell 9: 1201-13.

Okano, H.J. and R.B. Darnell. 1997. A hierarchy of Hu RNA binding proteins in developing and adult neurons. J Neurosci 17: 3024-37.

Oshima, Y., A. Tohe, and K. Matsumoto. 1984. [Regulatory circuits for gene expression: the metabolism of galactose and phosphate in Saccharomyces cerevisiae]. Tanpakushitsu Kakusan Koso 29: 14-28.

Paolantonacci, P., F. Lawrence, F. Lederer, and M. Robert-Gero. 1986. Protein methylation and protein methylases in Leishmania donovani and Leishmania tropica promastigotes. Mol Biochem Parasitol 21: 47-54.

Pare, C. and B. Suter. 2000. Subcellular localization of Bic-D::GFP is linked to an asymmetric oocyte nucleus. J Cell Sci 113 (Pt 12): 2119-27.

Peng, S.S., C.Y. Chen, N. Xu, and A.B. Shyu. 1998. RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. Embo J 17: 3461-70.

Pollack, B.P., S.V. Kotenko, W. He, L.S. Izotova, B.L. Barnoski, and S. Pestka. 1999. The human homologue of the yeast proteins Skb1 and Hsl7p interacts with Jak kinases and contains protein methyltransferase activity. J Biol Chem 274: 31531-42.

Posey, K.L., L.B. Jones, R. Cerda, M. Bajaj, T. Huynh, P.E. Hardin, and S.H. Hardin. 2001. Survey of transcripts in the adult Drosophila brain. Genome Biol 2: RESEARCH0008.

Ptashne, M. 1988. How eukaryotic transcriptional activators work. Nature 335: 683-9.

Qi, C., J. Chang, Y. Zhu, A.V. Yeldandi, S.M. Rao, and Y.J. Zhu. 2002. Identification of protein arginine methyltransferase 2 as a coactivator for estrogen receptor alpha. J Biol Chem 277: 28624-30.

Ran, B., R. Bopp, and B. Suter. 1994. Null alleles reveal novel requirements for Bic-D during Drosophila oogenesis and zygotic development. Development 120: 1233-42.

Rho, J., S. Choi, Y.R. Seong, W.K. Cho, S.H. Kim, and D.S. Im. 2001. Prmt5, which forms distinct homo-oligomers, is a member of the protein-arginine methyltransferase family. J Biol Chem 276: 11393-401.

Riechmann, V. and A. Ephrussi. 2001. Axis formation during Drosophila oogenesis. Curr Opin Genet Dev 11: 374-83.

Roth, S., F.S. Neuman-Silberberg, G. Barcelo, and T. Schupbach. 1995. cornichon and the EGF receptor signaling process are necessary for both anterior-posterior and dorsal-ventral pattern formation in Drosophila. Cell 81: 967-78.

Russell, I.D. and D. Tollervey. 1992. NOP3 is an essential yeast protein which is required for pre-rRNA processing. J Cell Biol 119: 737-47.

Schindler, C. and J.E. Darnell, Jr. 1995. Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. Annu Rev Biochem 64: 621-51.

Schotta, G., A. Ebert, V. Krauss, A. Fischer, J. Hoffmann, S. Rea, T. Jenuwein, R. Dorn, and G. Reuter. 2002. Central role of Drosophila SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing. Embo J 21: 1121-31.

Schotta, G. and G. Reuter. 2000. Controlled expression of tagged proteins in Drosophila using a new modular P-element vector system. Mol Gen Genet 262: 916-20.

Schurter, B.T., S.S. Koh, D. Chen, G.J. Bunick, J.M. Harp, B.L. Hanson, A. Henschen-Edman, D.R. Mackay, M.R. Stallcup, and D.W. Aswad. 2001.

Methylation of histone H3 by coactivator-associated arginine methyltransferase 1. Biochemistry 40: 5747-56.

Sedkov, Y., E. Cho, S. Petruk, L. Cherbas, S.T. Smith, R.S. Jones, P. Cherbas, E. Canaani, J.B. Jaynes, and A. Mazo. 2003. Methylation at lysine 4 of histone H3 in ecdysone-dependent development of Drosophila. Nature 426: 78-83.

Shen, E.C., M.F. Henry, V.H. Weiss, S.R. Valentini, P.A. Silver, and M.S. Lee. 1998. Arginine methylation facilitates the nuclear export of hnRNP proteins. Genes Dev 12: 679-91.

Simon, J.A. and J.W. Tamkun. 2002. Programming off and on states in chromatin: mechanisms of Polycomb and trithorax group complexes. Curr Opin Genet Dev 12: 210-8.

Song, H.J., J.C. Billeter, E. Reynaud, T. Carlo, E.P. Spana, N. Perrimon, S.F. Goodwin, B.S. Baker, and B.J. Taylor. 2002. The fruitless gene is required for the proper formation of axonal tracts in the embryonic central nervous system of Drosophila. Genetics 162: 1703-24.

Spradling, A.C. 1993a. Developmental genetics of oogenesis. In The Development of Drosophila melanogaster (ed. M. Bate), pp. 1-70. Cold Spring harbor Laboratory Press, NY.

Strahl, B.D., S.D. Briggs, C.J. Brame, J.A. Caldwell, S.S. Koh, H. Ma, R.G. Cook, J. Shabanowitz, D.F. Hunt, M.R. Stallcup, and C.D. Allis. 2001. Methylation of histone H4 at arginine 3 occurs in vivo and is mediated by the nuclear receptor coactivator PRMT1. Curr Biol 11: 996-1000.

Styhler, S., A. Nakamura, and P. Lasko. 2002. VASA localization requires the SPRY-domain and SOCS-box containing protein, GUSTAVUS. Dev Cell 3: 865-76.

Suter, B. and R. Steward. 1991. Requirement for phosphorylation and localization of the Bicaudal-D protein in Drosophila oocyte differentiation. Cell 67: 917-26.

Svendsen, P.C., S.D. Marshall, M. Kyba, and W.J. Brook. 2000. The combgap locus encodes a zinc-finger protein that regulates cubitus interruptus

during limb development in Drosophila melanogaster. Development 127: 4083-93.

Swan, A. and B. Suter. 1996. Role of Bicaudal-D in patterning the Drosophila egg chamber in mid-oogenesis. Development 122: 3577-86.

Szabo, A., J. Dalmau, G. Manley, M. Rosenfeld, E. Wong, J. Henson, J.B. Posner, and H.M. Furneaux. 1991. HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and Sex-lethal. Cell 67: 325-33.

Tang, J., J.D. Gary, S. Clarke, and H.R. Herschman. 1998. PRMT 3, a type I protein arginine N-methyltransferase that differs from PRMT1 in its oligomerization, subcellular localization, substrate specificity, and regulation. J Biol Chem 273: 16935-45.

Tavosanis, G. and C. Gonzalez. 2003. gamma-Tubulin function during female germ-cell development and oogenesis in Drosophila. Proc Natl Acad Sci U S A 100: 10263-8.

Theurkauf, W.E., B.M. Alberts, Y.N. Jan, and T.A. Jongens. 1993. A central role for microtubules in the differentiation of Drosophila oocytes. Development 118: 1169-80.

Theurkauf, W.E., S. Smiley, M.L. Wong, and B.M. Alberts. 1992. Reorganization of the cytoskeleton during Drosophila oogenesis: implications for axis specification and intercellular transport. Development 115: 923-36.

Tix, S., J.S. Minden, and G.M. Technau. 1989. Pre-existing neuronal pathways in the developing optic lobes of Drosophila. Development 105: 739-46.

Wang, H., Z.Q. Huang, L. Xia, Q. Feng, H. Erdjument-Bromage, B.D. Strahl, S.D. Briggs, C.D. Allis, J. Wong, P. Tempst, and Y. Zhang. 2001. Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. Science 293: 853-7.

Webster, N., J.R. Jin, S. Green, M. Hollis, and P. Chambon. 1988. The yeast UASG is a transcriptional enhancer in human HeLa cells in the presence of the GAL4 trans-activator. Cell 52: 169-78.

Weinkove, D. and S.J. Leevers. 2000. The genetic control of organ growth: insights from Drosophila. Curr Opin Genet Dev 10: 75-80.

Xu, W., H. Chen, K. Du, H. Asahara, M. Tini, B.M. Emerson, M. Montminy, and R.M. Evans. 2001. A transcriptional switch mediated by cofactor methylation. Science 294: 2507-11.

Xu, W., H. Cho, S. Kadam, E.M. Banayo, S. Anderson, J.R. Yates, 3rd, B.M. Emerson, and R.M. Evans. 2004. A methylation-mediator complex in hormone signaling. Genes Dev 18: 144-56.

Yarlett, N., A. Quamina, and C.J. Bacchi. 1991. Protein methylases in Trypanosoma brucei brucei: activities and response to DL-alpha-difluoromethylornithine. J Gen Microbiol 137 (Pt 3): 717-24.

Zhang, Y. and D. Reinberg. 2001. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes Dev 15: 2343-60.