Characterization of the *Drosophila* protein arginine methyltransferases DART1 and DART4

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The role of arginine methylation in *Drosophila melanogaster* is unknown. We identified a family of nine PRMTs (protein arginine methyltransferases) by sequence homology with mammalian arginine methyltransferases, which we have named DART1 to DART9 (*Drosophila* arginine methyltransferases 1–9). In keeping with the mammalian PRMT nomenclature, DART1, DART4, DART5 and DART7 are the putative homologues of PRMT1, PRMT4, PRMT5 and PRMT7. Other DART family members have a closer resemblance to PRMT1, but do not have identifiable homologues. All nine genes are expressed in *Drosophila* at various developmental stages. DART1 and DART4 have arginine methyltransferase activity towards substrates, including histones and RNA-binding proteins. Amino acid analysis of the methylated arginine residues confirmed that both DART1 and DART4 catalyse the formation of asymmetrical dimethylated arginine residues and they are type I arginine methyltransferases. The presence of PRMTs in *D. melanogaster* suggest that flies are a suitable genetic system to study arginine methylation.

Key words: arginine methylation, cell cycle, *Drosophila*, methyltransferase.

INTRODUCTION

Protein arginine methylation is a post-translational modification in which one or two methyl groups are added to the guanidino nitrogen atoms of arginine [1]. There are two major classes of enzymes responsible for protein arginine methylation: type I enzymes promote the formation of ADMAs (asymmetrical ω-N^G,N^G-dimethylated arginine residues) and type II enzymes catalyse the formation of SDMAs (symmetrical ω-N^G,$N^{G'}$-dimethylated arginine residues). Arginine methylation has been involved in various cellular processes [1,2]. These include transcriptional regulation [3–5], nuclear trafficking [6–9], pre-mRNA splicing [10] and protein–protein interactions [11,12]. Type I methyltransferases include PRMT1 (protein arginine methyltransferase 1), PRMT4/CARM1 (co-activator-associated arginine methyltransferase), PRMT6 and PRMT7 [3,13–15]. PRMT5 alone is known to be a type II enzyme [16,17]. So far, heterogeneous nuclear ribonucleoproteins as well as other RNA-binding proteins and histones have been the main groups of proteins recognized as substrates for methylation [1].

PRMT1 primarily methylates arginine-glycine-rich sequences and these sequences are abundant in RNA-binding proteins [13,18,19]. Moreover, PRMT1 has also been shown to methylate histone H4 [20,21] and STAT1 (signal transducer of transcription 1) [5]. Mice that are null for PRMT1 die early during embryogenesis, but the growth of PRMT1−/− embryonic stem cells is unaffected [22]. The arginine methylation of histone H4 by PRMT1 as well as the methylation of histone H3 by PRMT4/CARM1 is supposed to contribute to the histone code hypothesis [23]. CARM1 has been identified as a co-activator for nuclear receptors [3,24–26], the myogenic transcription factor MEF2C (myocyte enhancer factor 2C) [27] and β-catenin [28]. CARM1 has also been shown to methylate PABP1 [poly(A)$^+$-binding protein 1] [29], the RNA-binding protein HuR [30] and the transcriptional cofactor p300/CBP [p300/CREB (cAMP-response-element-binding protein)-binding protein] [31]. A consensus methylation site for CARM1 has not been identified. Thus it is difficult to predict substrates on the basis of amino acid sequence. Mice null for CARM1 die perinatally and are smaller in size [32].

In the present study, we report the identification of a family of nine PRMTs in *Drosophila*, which we have named DART1 to DART9 (*Drosophila* arginine methyltransferases 1–9), and we show that all of them are expressed during *Drosophila* development. Biochemical evidence shows that that DART1 and DART4 are type I arginine methyltransferases. Our results suggest that DART1 and DART4 are the *Drosophila* homologues of PRMT1 and PRMT4/CARM1. In addition, we have identified VASA and SQUID as substrates of DART1.

METHODS

RNA isolation

Samples of embryos (< 4 h old and 4–20 h old), larvae, males, females and ovaries were collected and homogenized in 6 M urea and 3 M LiCl. RNA was precipitated overnight at 4 °C. The

Abbreviations used: ADMA, asymmetrical ω-N^G,N^G-dimethylated arginine residue; CARM1, co-activator-associated arginine methyltransferase 1; DART, *Drosophila* arginine methyltransferase; GST, glutathione S-transferase; MBP, myelin basic protein; ω-MMA, ω-monomethylarginine; PABP, poly(A)$^+$-binding protein; PRMT, protein arginine methyltransferase; SDMA, symmetrical ω-N^G,$N^{G'}$-dimethylated arginine residue.

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The nucleotide sequences for DART1 to DART9 have been deposited in the GenBank® Nucleotide Sequence Database under the accession numbers AE003688, AE003577, AE003708, AE003685, AE003807, AE003702, AE003459, AE003630, AE003702.

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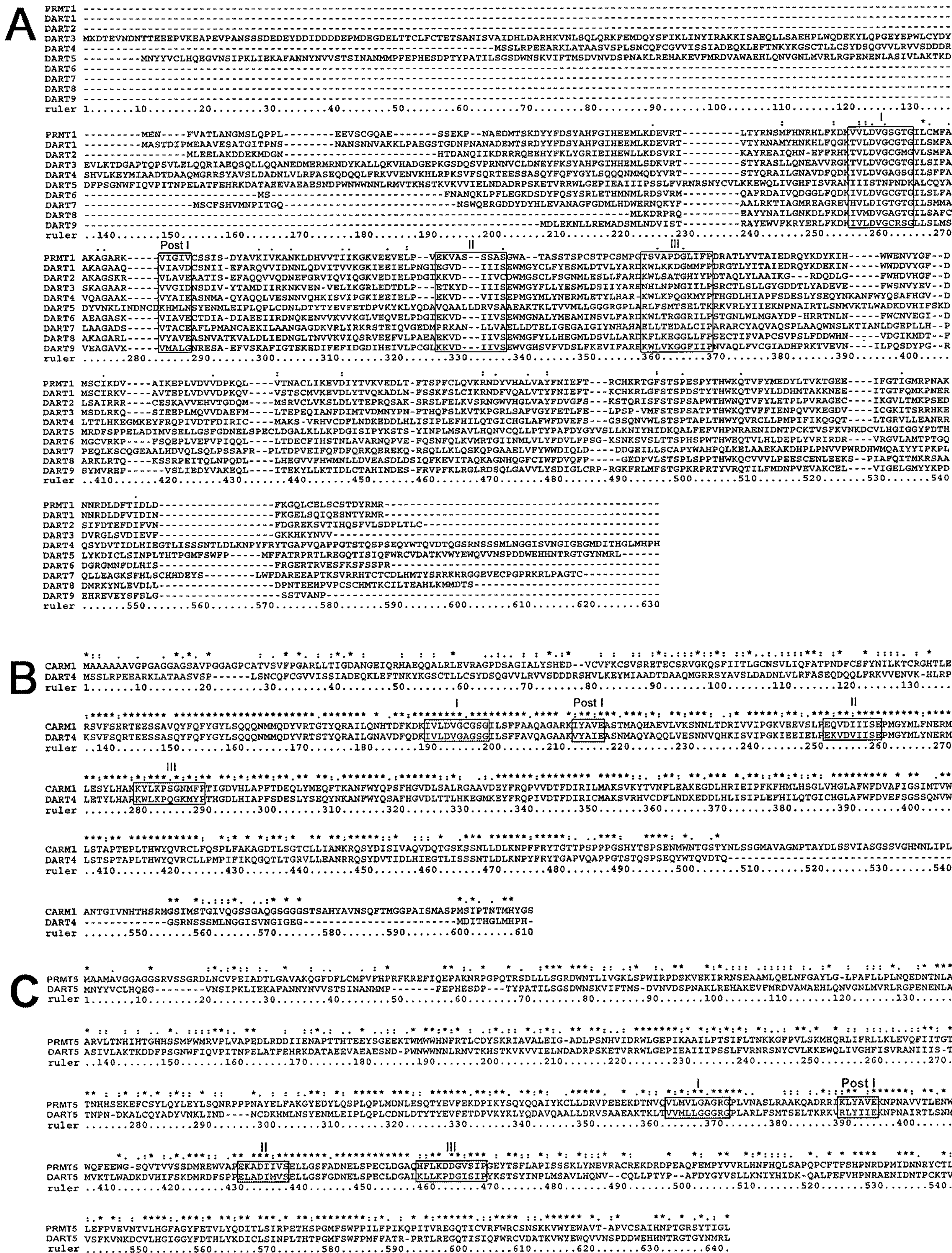

Figure 1 For legend see facing page

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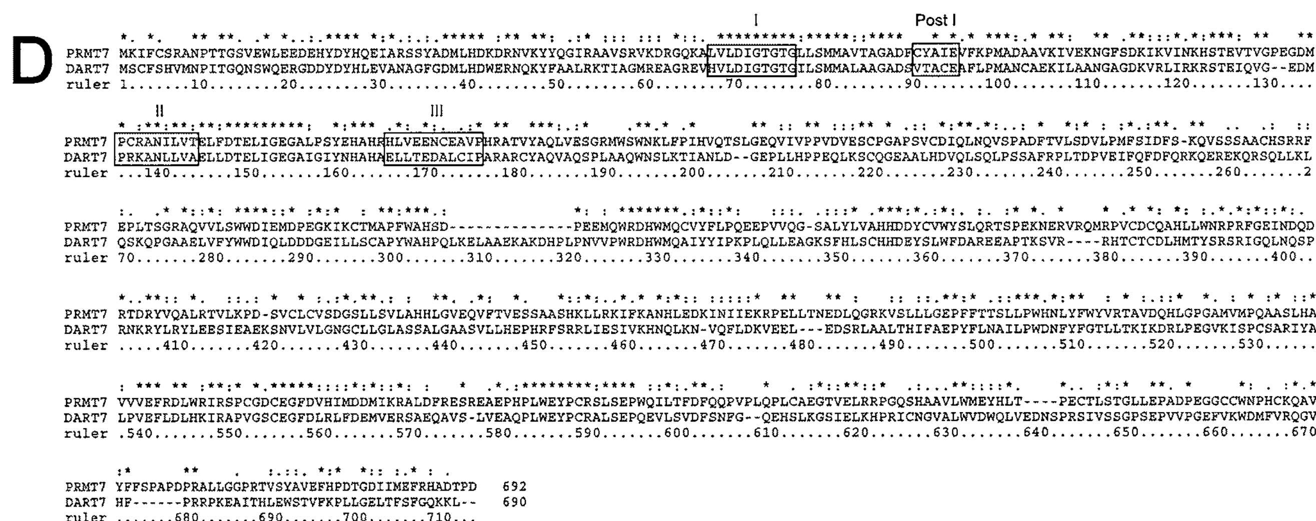

Figure 1 ***Drosophila*** **PRMTs**

Amino acid sequence alignment of (**A**) the DART family with human PRMT1, (**B**) DART4 with murine PRMT4/CARM1, (**C**) DART5 with human PRMT5 and (**D**) DART7 with PRMT7. The conserved domains, which are common to all methyltransferases, are indicated by boxed regions. Domain I contains the *S*-adenosyl-methionine-binding site.

samples were centrifuged for 30 min at 4 °C and resuspended again in the urea/LiCl solution. After a second centrifugation, the pellets were resuspended in 10 mM Tris/HCl (pH 7.5) containing SDS and proteinase K. The mixtures were incubated for 20 min at 37 °C, followed by two phenol/chloroform extractions. The RNA was then precipitated overnight in ethanol and 0.1 M NaCl at − 80 °C and was resuspended in water at a concentration of 2 μg/μl.

Developmental Northern-blot analysis

The isolated RNA (10 μg) was diluted in a solution containing 50 % (v/v) formamide, 5 % (v/v) formaldehyde, 0.5 × Mops buffer and RNA-loading dye [50 % (v/v) glycerol, 1 mM EDTA and 0.4 % Bromophenol Blue]. The RNA was separated on a 1 % formaldehyde gel and transferred on to a nitrocellulose membrane. The membrane was blocked with hybridization buffer [100 μg/ml single-stranded DNA, 10 × Denhardt's (0.02 % Ficoll 400/0.02 % polyvinylpyrrolidone/0.02 % BSA), 50 % formamide, 5 × SSC (0.75 M NaCl/0.075 M sodium citrate) and 0.5 % SDS] for 2 h. We amplified 200-bp-long sequences by PCR using the DNA primers atgtccagcctgcgacccgaggagg and cgtcgtctgacacaacacggaggac. ^{32}P-labelled DNA probes recognizing the different DART RNA sequences were generated from the PCR products using random primers and the Klenow enzyme (Amersham Biosciences). The probes were diluted to 1 × 10^6 counts/ml in the hybridization buffer and incubated overnight with the membrane. The membrane was washed extensively first with 2 × SSC/0.1 % SDS and then with 0.1 × SSC/0.1 % SDS at 60 °C for 20 min and exposed for 3 days.

Protein analysis

Samples of embryos, larvae, males, females and ovaries were collected and homogenized in lysis buffer [1 % Triton X-100 (Roche)/150 mM NaCl/20 mM Tris/HCl, pH 7.5/100 μg/ml PMSF/1 μg/ml aprotinin]. The lysates were centrifuged for 10 min at 16 000 ***g*** and 2 × Laemmli buffer was added to the supernatant. The samples were heated at 100 °C for 20 min and separated on SDS/polyacrylamide gels. Immunoprecipitations were performed by the method described previously [33]. *In vitro* methylation assays were performed as described previously using histones H3 and H4, MBP (myelin basic protein; Sigma), GST (glutathione S-transferase)–SmB C-terminal region [11], GST–PABP [29], GST–p300 Kix domain [31,32], GST–SQUID S [34] and GST–VASA [35]. GST–DART1 was amplified from cDNA clone LD38136 with the primers 5′-AAGGAATTCCA-ATGGCCAGCACAGACATTC-3′ and 5′-AAAGAATTCCTAG-CGCATGCGGTATGTGT-3′. GST–DART4 was obtained by subcloning DART4 from LD30574 into the *Bam*HI and *Eco*RI sites of pGEX using the DNA primers 5′-AAAGGATCCATG-TCCAGCCTGCGACCC-3′ and 5′-AAAGAATTCCTAGTGCG-GATGCATAAG-3′. Anti-DART1 and anti-DART4 rabbit polyclonal antibodies were generated by using KPNERNNRDLDFVI-DINF and KISVNGIGEGMDITHGLMHPH peptides coupled with keyhole-limpet haemocyanin as the antigens. The presence of methylated arginine residues was detected by the method described previously [17].

Immunostaining of egg chambers

Ovaries from 1–2-day-old flies were dissected and fixed in 4 % (w/v) paraformaldehyde. The ovaries were washed three times with PBST (PBS with 0.2 % Tween 20) and blocked for 4 h in PBSBT (PBS with 1 % BSA/0.2 % Triton X-100/0.2 % Tween 20). The ovaries were incubated overnight at 4 °C with the DART4 antibody (1:200) in PBSBT, followed by incubation with a secondary antibody conjugated with the green fluorescent dye Alexa 488 (1:200; Molecular Probes, Eugene, OR, U.S.A.) for 2 h at room temperature (22 °C). The slides were visualized using a Zeiss LSM-510 confocal microscope.

RESULTS AND DISCUSSION

We identified a family of nine putative arginine methyltransferases by sequence homology using FLYBASE and named them DART1 to DART9 (Figure 1). Typical arginine methyltransferase domains with *S*-adenosyl-methionine-binding sites [1] are highly conserved (Figure 1A). DART1 (AE003688) has the highest homology with PRMT1 (Table 1), DART4 (AE003685) with PRMT4/CARM1 (Figure 1B, Table 1) and DART5 (AE003807) with PRMT5 (Figure 1C, Table 1). Other family members DART2, DART3, DART6, DART8 and DART9 (AE003577, AE003708,

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Table 1 Percentage similarity/identity between the DART family and mammalian PRMTs

	PRMT1	PRMT2	PRMT3	PRMT4	PRMT5	PRMT6	PRMT7
DART1	84/67	55/34	64/47	53/35	None	54/36	40/25
DART2	63/41	54/36	59/38	52/33	None	54/37	46/26
DART3	56/37	53/33	56/37	51/29	39/26	53/35	40/23
DART4	66/41	52/37	50/33	72/56	40/23	51/38	36/24
DART5	None	None	None	42/28	58/41	None	None
DART6	57/31	52/33	58/37	51/32	None	52/35	45/29
DART7	51/35	49/28	38/19	50/29	None	48/29	55/36
DART8	67/48	55/38	51/35	52/37	49/33	54/40	39/25
DART9	50/28	45/26	49/28	42/23	None	44/24	None

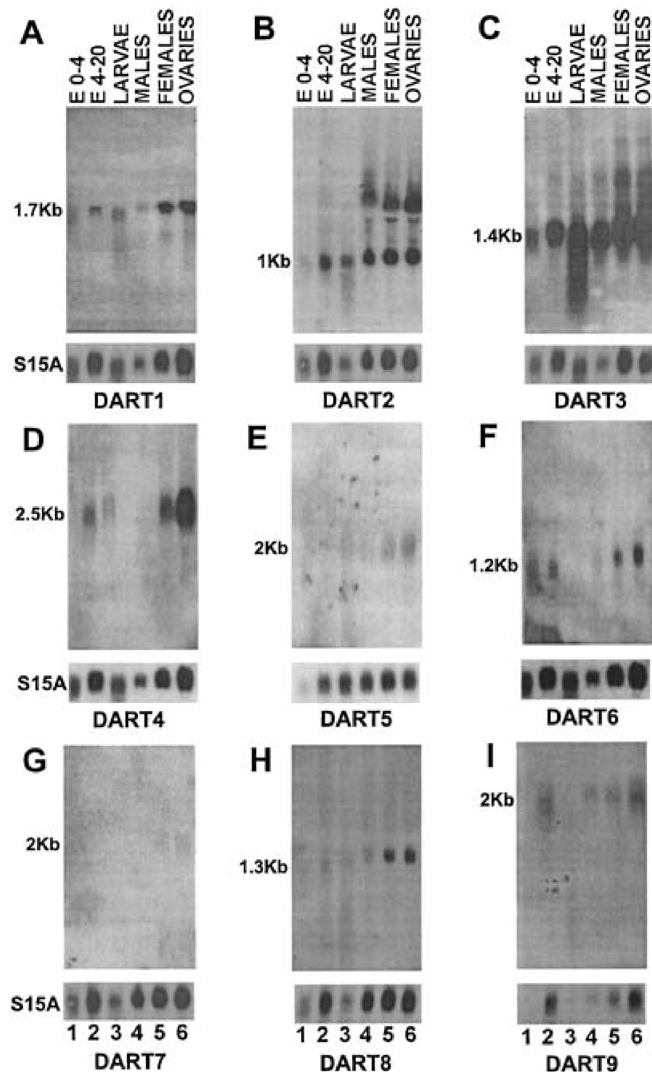

Figure 2 mRNA expression of the DART family in *Drosophila*

RNA isolated from selected stages and organs were analysed by Northern blotting. A probe against RNA of the ribosomal protein S15A was used to monitor loading efficiency.

AE003702, AE003630 and AE003702 respectively) share the highest similarity with PRMT1. DART7 (AE003459) has the highest homology with PRMT7 (Figure 1D, Table 1), a methyltransferase supposed to be involved in cell sensitivity to DNA-damaging agents [15]. A clear PRMT6 homologue was not identified [17].

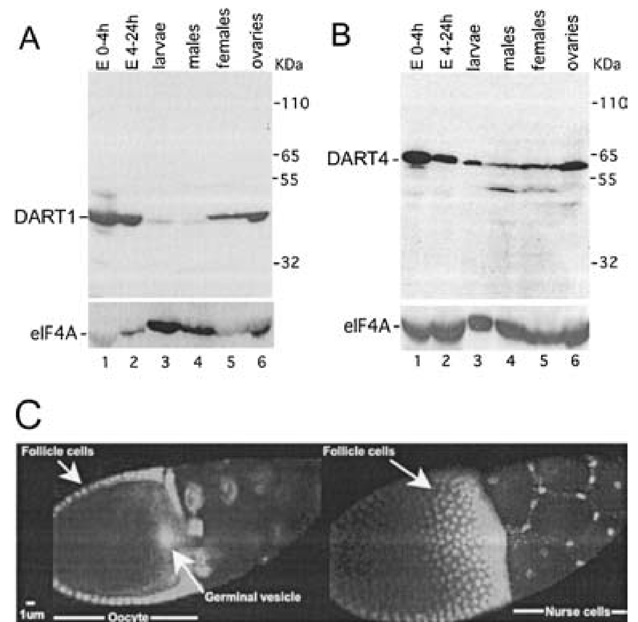

Figure 3 Protein expressions of DART1 and DART4

(**A**) and (**B**) Protein expressions of DART1 and DART4 at different developmental stages are shown. Tissues were homogenized in lysis buffer, separated by SDS/PAGE, transferred on to a nitrocellulose membrane and immunoblotted with anti-DART1, anti-DART4 or anti-eIF4A as a loading control. The presence of DART1 and DART4 immunoreactive proteins, with an approximate molecular mass of 40 and 60 kDa respectively, is shown. In DART4 immunoblots, a weaker band at 50 kDa is also present in adults and the identity of this band is unknown. (**C**) Localization of DART4 in *Drosophila* egg chambers. Ovaries from 1–2-day-old females were dissected and fixed in 4 % paraformaldehyde, blocked and incubated with the anti-DART4 antibody, followed by incubation with a secondary antibody conjugated with Alexa 488. The slides were visualized using a Zeiss confocal microscope.

Northern-blot analysis was performed to determine the expression pattern of the mRNAs encoding DART1 to DART9. DART1 mRNA was abundant in ovaries and was also observed in late embryos, larvae and males (Figure 2A). The mRNA for DART2 was expressed in late embryos, larvae, males and ovaries (Figure 2B). DART3 mRNA is highly expressed at all stages (Figure 2C), suggesting a significant role during development. DART4 mRNA was observed to be mainly present in samples of females and ovaries (Figure 2D). The mRNA for DART5 was expressed only in ovaries (Figure 2E). This may suggest either that DART5 is important during oogenesis or that it is maternally derived. DART6 mRNA is present in embryos and ovaries, suggesting that it plays a role early in development (Figure 2F). The mRNA for DART7 is expressed at low levels in ovaries (Figure 2G). DART8 is predominantly expressed in ovaries, but is also expressed at lower levels in late embryos, larvae and males (Figure 2H). The mRNA for DART9 is expressed in late embryos, males and ovaries (Figure 2I). The presence of the mRNA for all nine homologues of the DART family at various developmental stages suggests that arginine methylation is essential during *Drosophila* development.

Polyclonal peptide antibodies were raised against DART1 and DART4. DART1 migrated with a molecular mass of approx. 40 kDa by SDS/PAGE, which is its predicted mass (Figure 3A). The protein was abundant in ovaries (Figure 3A, lanes 5 and 6), consistent with the Northern-blot analysis in Figure 2(A). Early and late embryos have increased DART1 protein expression, which does not correlate with the mRNA expression data (Figure 2A). Although the reason for this is unknown, we suspect that either DART1 mRNA is unstable in embryos or DART1 is maternally derived (Figure 2A). DART4 has a predicted molecular

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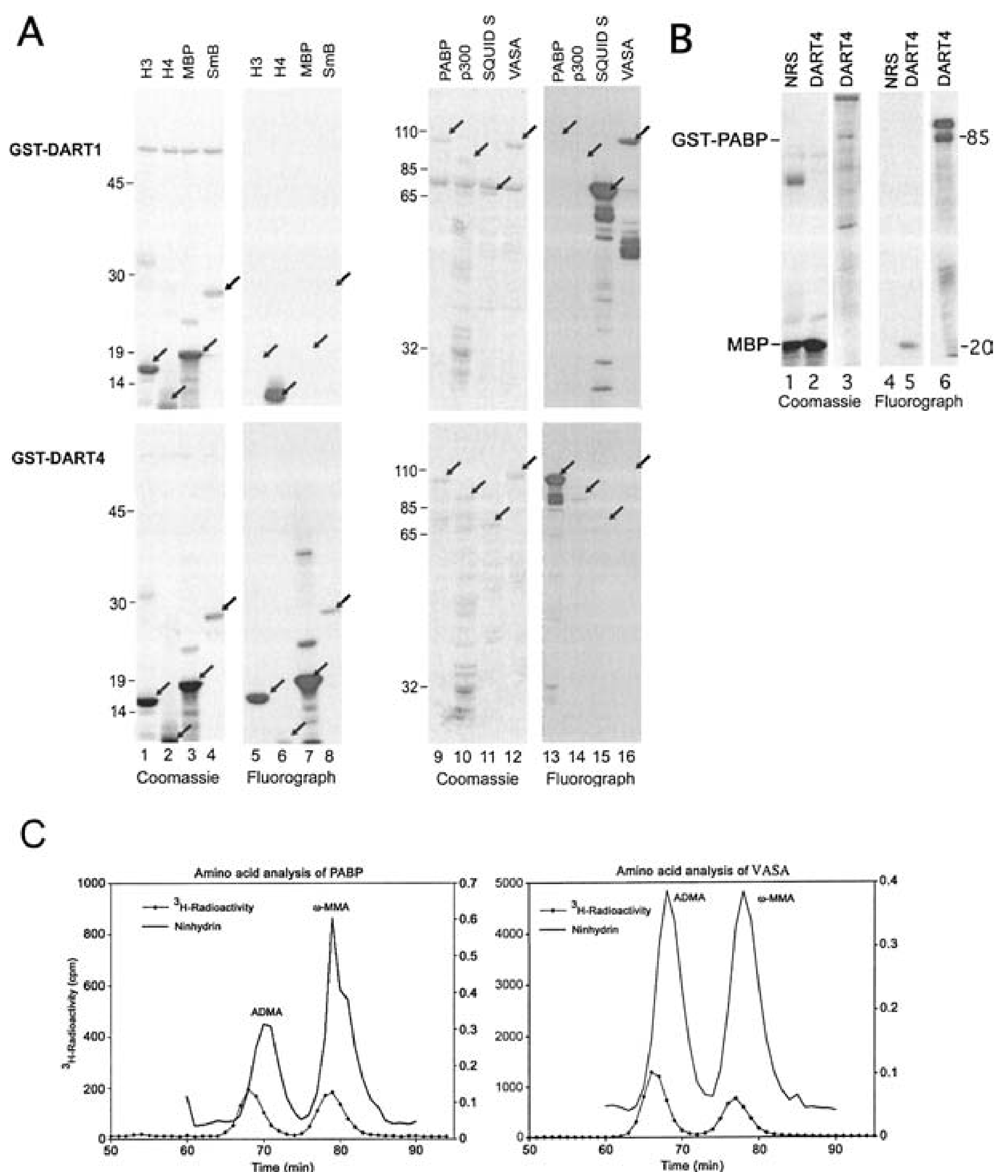

Figure 4 DART1 and DART4, type I arginine methyltransferases

(**A**) Recombinant GST–DART1 or GST–DART4 methylate known mammalian PRMT substrates. *In vitro* methylation reactions with 0.5 μM *S*-adenosyl-L-methyl-methionine were performed with GST–DART1 (upper panels) or GST–DART4 (lower panels). The exogenous substrates included low- (histones H3 and H4, MBP and GST–SmB C-terminal region) and high-molecular-mass substrates (GST–PABP, GST–p300, GST–SQUID S and GST–VASA). The proteins were separated by SDS/PAGE, visualized by Coomassie Blue staining (lanes 1–4 and 9–12) and fluorography (lanes 5–8 and 13–16). Their migration is indicated by arrows. (**B**) S2 cell lysates were immunoprecipitated with either anti-DART4 antibodies or normal rabbit serum (NRS), and *in vitro* methylation assays were performed with MBP or PABP as the exogenous substrate. The proteins were separated on a 12 % (w/v) SDS/polyacrylamide gel, stained with Coomassie Blue and visualized by fluorography. (**C**) PABP methylated *in vitro* by recombinant GST–DART4 (left panel) and VASA methylated *in vitro* by recombinant GST–DART1 (right panel) in the presence of *S*-adenosyl-methionine were acid-hydrolysed separately and analysed using an amino acid cation-exchange column along with ω-MMA and ADMA standards. The standards were visualized by ninhydrin staining and the labelled residues were quantified by scintillation counting.

mass of 60 kDa and we indeed observed an approx. 60 kDa protein in samples of embryos, larvae, males, females and ovaries (Figure 3B). These findings suggest that DART4 is ubiquitously expressed. The increased expression of DART4 mRNA in ovaries (Figure 2D) suggests that the transcript is stabilized in this organ or that DART4 is mainly maternally derived. *Drosophila* egg chambers were immunostained and visualized by confocal microscopy. DART4 localized to the germinal vesicle of the oocyte, the nuclei of the follicle and nurse cells (Figure 3C). The ovary, an organ rich in RNA-binding proteins [36], contains DART1 (Figure 3A), consistent with the fact that RNA-binding proteins are major substrates for this enzyme [1]. The presence of DART4 within the nuclei is consistent with a role for this enzyme in transcriptional regulation, as observed with mammalian CARM1 [3,24–26].

In vitro methylation assays using known PRMT substrates and arginine-rich *Drosophila* substrates were performed to confirm that DART1 and DART4 are arginine methyltransferases. Both DART1 and DART4 were expressed as GST-fusion proteins in bacteria and were utilized to methylate histones H3 and H4, the RNA-binding proteins SmB, SQUID [34], VASA [35,37], PABP, the Kix domain of histone acetyltransferase p300 and MBP. DART1 methylated histone H4, SQUID and VASA (Figure 4A, upper panels). DART4 methylated histone H3, MBP, the

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C-terminal region of SmB, PABP and p300 (Figure 4A, lower panels). There also appeared to be some residual methylation of H4 (Figure 4A, lower panel, lane 6). Histone H4 is a known PRMT1 substrate [20,21]. CARM1 substrates include PABP [29], p300 [31] and H3 [3,24–26], and PRMT5 substrates include SmB [12,38] and MBP [16,17]. SQUID (AE003646) and VASA (AE003701) are predicted to be PRMT1 and PRMT5 substrates owing to their RG-rich content. These results demonstrate that DART1 methylates histone H4 and the RG-rich *Drosophila* proteins SQUID and VASA. The methylation of VASA and SQUID by DART1 implies that DART1 has specificity for RG-rich motifs. DART4 has the same specificity as CARM1, since it methylated histone H3, PABP and p300. In addition, DART4 (Figure 4A) and CARM1 (results not shown) methylated SmB and MBP, two substrates previously shown to be type II enzyme-specific.

To characterize further the activity of endogenous DART4, anti-DART4 antibodies were used to immunoprecipitate DART4 from *Drosophila* S2 cells. Immunoprecipitated DART4 was subjected to *in vitro* methylation assays in the presence of MBP or PABP as exogenous substrates. MBP and PABP were methylated in the anti-DART4 immunoprecipitation and not in the control immunoprecipitations (Figure 4B, lanes 5 and 6). These findings confirm that endogenous DART4 has methyltransferase activity.

To confirm that DART1 and DART4 are type I arginine methyltransferases, we performed amino acid analysis on DART1-methylated VASA and DART4-methylated PABP (Figure 4C). Labelled VASA and PABP were subjected to acid hydrolysis, and the amino acids were separated using an amino acid cation-exchange column and compared with methylated arginine standards [17]. The ω-MMA (ω-monomethylarginine) and ADMA standards were visualized by ninhydrin staining. The methylated arginine residues co-migrated with ω-MMA and ADMA (Figure 4C). The SDMA standard migrates in between ω-MMA and ADMA. These results demonstrate that both DART1 and DART4 are type I arginine methyltransferases that generate ω-MMA and ADMA [17]. These observations are consistent with the fact that DART1 and DART4 are homologues of PRMT1 and PRMT4/CARM1.

In the present study, we report the identification of a family of nine PRMTs in *Drosophila*. The DART nomenclature is proposed to distinguish it from the mammalian PRMT family. We have demonstrated that all the family members are expressed at various stages of *Drosophila* development. So far, arginine methyltransferases have been identified only in mammals and yeast and not in *Drosophila*. Therefore the present study is the first one to show arginine methyltransferase activity in *Drosophila* as well as the first one to identify homologues of the methyltransferases in this system. DART1 and DART4 have biochemical properties similar to those of mammalian PRMT1 and PRMT4/CARM1. Both enzymes methylate histones and are type I methyltransferases generating ADMAs. Our results demonstrate that *Drosophila melanogaster* is a suitable model system to study the genetics of arginine methylation.

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