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Arginine methyltransferase Capsuléen is essential for methylation of spliceosomal Sm proteins and germ cell formation in *Drosophila*

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Although arginine modification has been implicated in a number of cellular processes, the in vivo requirement of protein arginine methyltransferases (PRMTs) in specific biological processes remain to be clarified. In this study we characterize the *Drosophila* PRMT Capsuléen, homologous to human PRMT5. During *Drosophila* oogenesis, catalytic activity of Capsuléen is necessary for both the assembly of the nuage surrounding nurse cell nuclei and the formation of the pole plasm at the posterior end of the oocyte. In particular, we show that the nuage and pole plasm localization of Tudor, an essential component for germ cell formation, are abolished in *csul* mutant germ cells. We identify the spliceosomal Sm proteins as in vivo substrates of Capsuléen and demonstrate that Capsuléen, together with its associated protein Valois, is essential for the synthesis of symmetric di-methylated arginyl residues in Sm proteins. Finally, we show that Tudor can be targeted to the nuage in the absence of Sm methylation by Capsuléen, indicating that Tudor localization and Sm methylation are separate processes. Our results thus reveal the role of a PRMT in protein localization in germ cells.

KEY WORDS: *Drosophila* oogenesis, Protein arginine methyltransferase, Pole plasm, Nuage, *capsuléen*

INTRODUCTION

Establishment and maintenance of the germline is an essential process for all sexually reproducing organisms. Germ cells, which carry the genetic information to the next generation, are simultaneously totipotent and highly specialized (Wylie, 1999). All germ cells throughout the animal kingdom contain in their cytoplasm a distinct cloud-like structure termed the nuage (Eddy, 1975).

One system with a high potential for understanding the assembly and role of the nuage is *Drosophila* oogenesis. The *Drosophila* egg chamber consists of a germ line cyst generated from a single cystoblast by four successive mitotic divisions and surrounded by a monolayer of somatic follicle cells. Due to incomplete cytokinesis, the germ cells remain connected to each other through specialized cytoplasmic bridges. The oocyte derives from one of the germ cells, while the remaining 15 cells differentiate into nurse cells (Spradling, 1993) (Fig. 2A). The nuage is concentrated in the perinuclear cytoplasm of nurse cells and can be associated with nuclear pores. It appears as a dense fibrous organelle unbound by membrane and often associated with mitochondrial clusters (Mahowald, 1971). Moreover, the nuage interfaces with sponge bodies, which are abundant RNA-rich particles present in the cytoplasm of nurse cells and, to a lesser degree, in the oocyte (Wilsch-Bräuninger et al., 1997).

The majority of the components identified in the nuage are also present in the pole plasm of the oocyte, and more particularly in the polar granules. The pole plasm constitutes the determinant that is both necessary and sufficient to induce germ cell formation during early embryogenesis (Illmensee and Mahowald, 1974). The first step in pole plasm formation is the transport of *oskar* (*osk*) transcripts synthesized in nurse cell nuclei to the posterior pole of stage 8 oocytes (Ephrussi et al., 1991; Kim-Ha et al., 1991). At this location Osk is synthesized and serves as an anchor to initiate polar granules assembly (Ephrussi and Lehmann, 1992; Smith et al., 1992; Snee and Macdonald, 2004). In addition to Osk the polar granule components include Vasa (Vas) (Hay et al., 1988; Lasko and Ashburner, 1990), which interacts directly with Osk (Breitwieser et al., 1996), Tudor (Tud) (Bardsley et al., 1993) and a number of transiently localized factors that are mainly necessary for *osk* mRNA transport and translation. During late oogenesis and early embryogenesis, the polar granules are maintained at the posterior pole. At the time of blastoderm formation, they are sequestered in the pole cells, the primordial germ cells of the fly, in which they coalesce into a smaller number of large particles (Mahowald, 1968), ultimately disappear and are replaced by the nuage (Mahowald, 1971). This structure appears to evolve from components of the pole plasm and persists only in established germ cells. The only identified nuage-specific component absent from the polar granules is Maelstrom (Mael) which shuttles between the nucleus and the cytoplasm (Findley et al., 2003).

Among these proteins, Vas plays a cardinal role in the formation of the nuage (Findley et al., 2003). In *vas* ovaries the nurse cells are devoid of nuage at the ultrastructural level (Liang et al., 1994). The function of Tud in the nuage remains unknown but Tud may play a role in the assembly or modification of specific RNP complexes, as indicated by its requirement for the transfer of mitochondrial ribosomal RNAs from the mitochondria to the polar granules (Amikura et al., 2001). The presence of shared components reinforces the view that the nuage and the polar granules are closely related structures, in which components, such as Vas and Aubergine (Aub), may dissociate from the nuage to reassemble into the polar granules (Snee and Macdonald, 2004).

The mechanisms by which nuage components become assembled at the perinuclear region of the nurse cells remains, however, to be identified. Here we report that the catalytic activity

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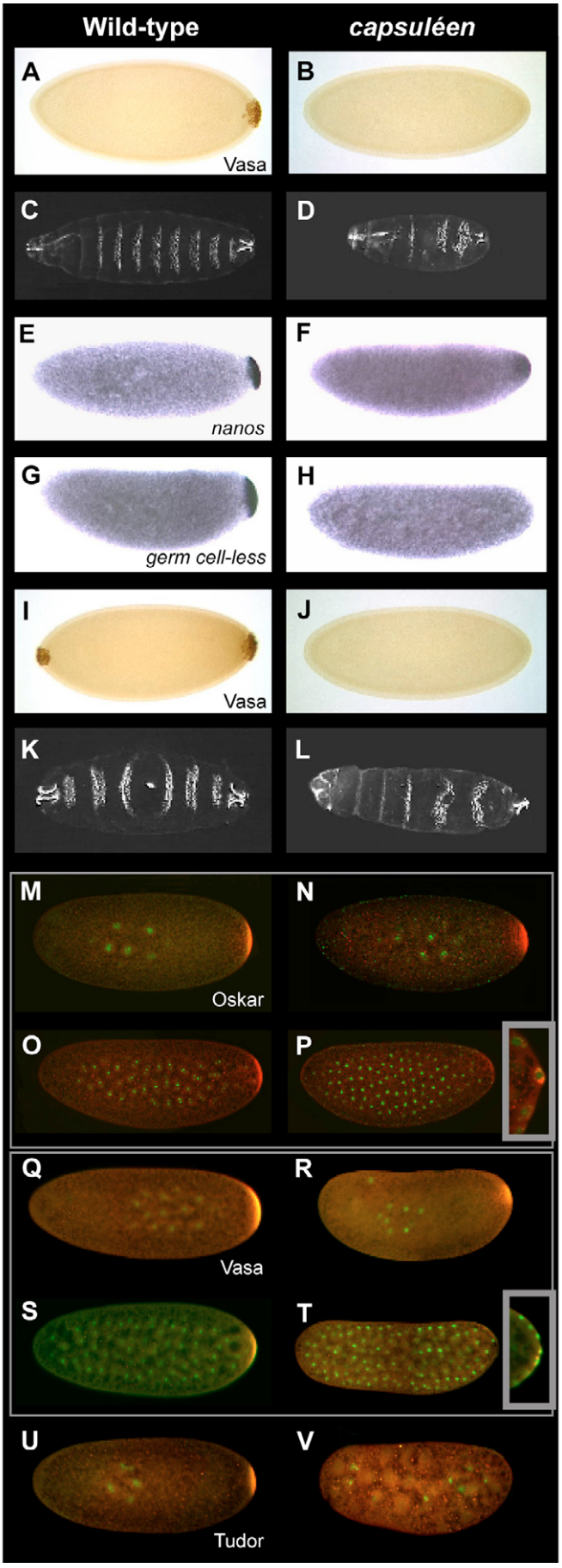

Fig. 1. Embryonic phenotype of *csul*^{P}. (**A-D**) The *csul* gene belongs to the 'posterior-grandchildless' group. Immunohistochemical detection (horseradish peroxidase) of Vas protein (A,B) and cuticle preparations of first instar larvae (C,D) in wild-type (A,C) and *csul*^{P} (B,D). Anterior is to left. Pole cell formation and abdominal patterning are defective in *csul*^{P}. (**E-H**) *csul* activity is required for the localization of maternal determinants. Distribution of *nos* (E,F) and *gcl* (G,H) transcripts detected by whole-mount in situ hybridization in wild-type (E,G) and *csul*^{P} (F,H) embryos. In *csul*^{P} the accumulation of *nos* mRNA is reduced at the posterior pole (F), whereas *gcl* mRNA is evenly distributed (H). (**I-L**) *csul*^{P} suppresses the bicaudal phenotype induced by *osk-bcd3'UTR*. Immunohistochemical staining of Vas protein (I,J) and cuticle preparations of first instar larvae (K,L) in *osk-bcd3'UTR* (I,K) and *csul*^{P}; *osk-bcd3'UTR* (J,L). Although the cuticle has a normal polarity in *csul*^{P}; *osk-bcd3'UTR*, the head is malformed, indicating that the embryo contains a residual amount of *nos* activity at the anterior pole. (**M-P**) Distribution of Osk protein in wild-type (M,O) and *csul*^{P} (N,P) embryos. Immunofluorescent detection of Osk (red). DNA detected using Oli-Green (green). After nuclear cycle 6, the level of Osk is strongly reduced at the posterior pole in *csul*^{P} embryos (P). In older *csul*^{P} embryos (insert in P) trace amounts of Osk can be detected at the posterior pole. (**Q-T**) Distribution of Vas protein in wild-type (Q,S) and *csul*^{P} (R,T) embryos. Immunofluorescent detection of Vas (red); DNA (green). Similar to Osk, Vas staining is strongly reduced in the pole plasm of *csul*^{P} embryos (T) and is rarely detected at the posterior pole of syncytial embryos (insert in T). (**U**,**V**) Distribution of Tud protein in wild-type (U) and *csul*^{P} (V) embryos. Immunofluorescent detection of Tud (red); DNA (green). No localized Tud staining is observed in *csul*^{P} embryos.

of the Capsuléen (Csul) protein-arginine methyltransferase is required for the localization of specific components of the nuage and pole plasm, and in particular of Tud.

MATERIALS AND METHODS

Molecular biology

Plasmid constructs were generated by PCR amplification of the relevant DNA segments (High Fidelity PRC Master; Roche), which were subcloned into appropriate vectors. *vas*, *tud*, *nos*, *gcl* and *SmD3* cDNA plasmids were kindly provided by P. Lasko, R. Boswell, R. Lehmann, T. Jongens, and H. Schenkel, respectively. The plasmids pBS1479 (C-TAP) and pBS1761 (N-TAP) were obtained from Cellzome (Heidelberg). *SmB* and *SmD1* cDNAs (BDGP EST project clones LD14049 and RE39488, respectively) were provided by the Drosophila Genomics Resource Center (DGRC), Bloomington, IN, USA. Interstitial deletions in *csul* were prepared using a QuickChange site-directed mutagenesis kit (Stratagene) with the modifications of Makarova et al. (Makarova et al., 2000). Details of primers and cloning strategies will be provided upon request.

GST pull-down assay

Fragments of *tud* and *SmB* cDNAs were cloned into pCITE-4 (Novagen). Tud and SmB polypeptides were synthesized using the TNT Coupled Reticulocyte Lysate System (Promega) in the presence of unlabeled amino acids. Full-length SmB proteins were synthesized from the *LD14049* cDNA plasmid in the presence of [^{35}S]methionine (Amersham). To co-translationally inhibit sDMA synthesis, 0.3 mM S-adenosylhomocysteine (Sigma) was included in the reaction. GST pull-down assays were performed as previously described (Anne and Mechler, 2005).

Immunocytochemistry

A GST-Csul fusion protein construct containing a 442-amino-acid peptide corresponding to amino acid residues 168-609 of Csul was prepared by cloning the *Bam*HI-*Xho*I fragment of the *csul* cDNA in the *pGEX-4T2* GST fusion vector (Pharmacia) and expressed in the bacterial strain BL21(DE3).

Anti-Csul polyclonal antibodies raised in rabbits against the GST-Csul fusion protein were purified by affinity chromatography on a protein A-agarose column (Roche) and preadsorbed on proteins extracted from *Escherichia coli*.

For immunostaining, primary antibodies were anti-Osk from rabbit, anti-Vas from rabbit and rat (gifts from P. Lasko and A. Nakamura, respectively), anti-Tud from rabbit (TUD56; a gift from S. Kobayashi), anti-Mael from rabbit (a gift from S. Findley), anti-Nos from rat (a gift from R. Wharton), mouse monoclonal anti-HA (clone 16B12; BAbCO), anti-Sm (clone Y12; NeoMarkers), and anti-dimethyl-arginine (SYM10 and ASYM24) from rabbit (Upstate). DNA was visualized by staining with Oli-Green (Molecular Probes). Biotinylated and Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used at 1:200.

RESULTS

Maternal-effect phenotype of *csul*

The *csul*^{P} mutation was identified in a screen for maternal-effect mutations induced by a *P-[white*$^{+}$*]* transposon. The *P*-element insertion was mapped to 52F on chromosome 2 (data not shown). Following *P*-element excision, we recovered numerous (92/150) fertile *white* flies producing fully viable and fertile progeny, indicating that the *P*-insert was the cause of the maternal-effect phenotype (see below). Moreover, we isolated a series of viable *white* females producing embryos similar to those laid by *csul*^{P} females. These alleles resulted from a partial excision of the *P-[white*$^{+}$*]* transposon and are named *csul*^{RM}.

Homozygous *csul*^{P} females produce embryos (*csul*^{P} embryos) displaying defects in abdominal patterning and lacking pole cells (Fig. 1B). Flies hatching from *csul*^{P} larvae are fully agametic. The severity of the abdominal defects is variable, ranging from the presence of only one to two abdominal segments (Fig. 1D) to a fully normal abdomen. On the basis of the absence of pole cells and the occurrence of abdominal defects, we classified *csul*^{P} as belonging to the 'posterior-grandchildless' class of mutations (Schüpbach and Wieschaus, 1986).

To determine whether *csul* acts in the posterior-grandchildless pathway, we investigated two gene transcripts, *nanos* (*nos*), the posterior determinant (Wang and Lehmann, 1991), and *germ cell-less* (*gcl*), specifically involved in pole cell formation (Jongens et al., 1992). The posterior localization of their transcripts in preblastoderm embryos depends on this pathway. In one half of *csul*^{P} embryos, *nos* mRNA was absent or barely detectable (data not shown), whereas in the other half it was correctly localized, albeit in much lower amounts than in wild-type embryos (Fig. 1E,F). Analysis of the Nos protein revealed a Nos gradient emanating from the posterior pole in only 15 percent of *csul*^{P} embryos, whereas Nos remains undetected in the other embryos (data not shown). In situ hybridization showed that *gcl* mRNA is evenly distributed in *csul*^{P} embryos instead of being localized to the posterior pole (Fig. 1G,H). From these data we conclude that *csul* is required for the posterior localization of at least two transcripts, *nos* and *gcl*.

The posterior-grandchildless group comprises a relatively small number of genes hierarchically organized with *osk* orchestrating the activity of the other genes. As ectopic expression of *osk* induces pole cell and abdomen formation at the anterior of the embryo (Ephrussi and Lehmann, 1992), we tested whether expression of an *osk-bcd3′UTR* transgene would induce pole cells formation in *csul*^{P} (Fig. 1I-L). No pole cell could be detected in *csul*^{P}; *osk-bcd3′UTR* blastoderm embryos. Cuticle examination revealed the absence of duplicated abdominal structures and a marked atrophy of the head in the majority of the larvae, indicating a downregulation of *osk* activity. We therefore suggest that *csul* is essential for pole plasm assembly and acts downstream of *osk*.

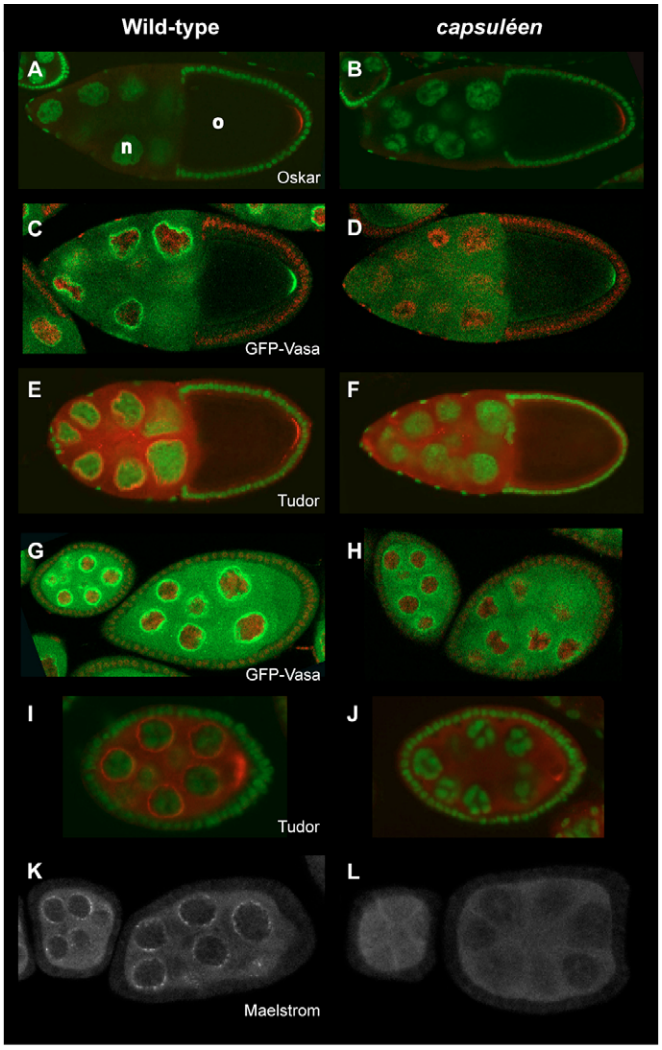

Fig. 2. *csul* activity contributes to nuage and pole plasm assembly during oogenesis. Distribution of Osk (**A**,**B**), GFP-Vas (**C**,**D**,**G**,**H**), Tud (**E**,**F**,**I**,**J**), and Mael (**K**,**L**) proteins in stage 10 egg chambers (A-F) and previtellogenic egg chambers (G-L). Left and right columns show wild-type and *csul*^{P} egg chambers, respectively. (A,B) Osk immunostaining (red) and DNA (green). Osk is correctly synthesized and positioned at the posterior pole of *csul*^{P} oocytes. (C,D,G,H) GFP-Vas (green) and DNA staining (red). GFP-Vas is only detected in the nuage of previtellogenic *csul*^{P} egg chambers and disappears in later stages of egg chamber development. In comparison with wild type, the amount of GFP-Vas is markedly reduced in the pole plasm of *csul*^{P} stage 10 oocytes. (E,F,I,J) Tud immunostaining (red) and DNA (green). Although Tud is synthesized in *csul*^{P} egg chambers, it is absent both from nuage and pole plasm. (K,L) Mael immunostaining. Mael localization in the nuage is abolished in *csul*^{P} egg chambers. o, oocyte; n, nurse cell nucleus.

Distribution of pole plasm components in early *csul* embryos

We then determined whether *csul* activity is needed for the localization of Osk and Vas (Fig. 1M-T). We found that Osk and Vas are present at the posterior pole of early syncytial *csul*^{P} embryos, albeit at a reduced level for Vas. However, starting from nuclear cycle 6, Osk and Vas signals significantly decrease and are nearly completely absent from the posterior pole of late syncytial

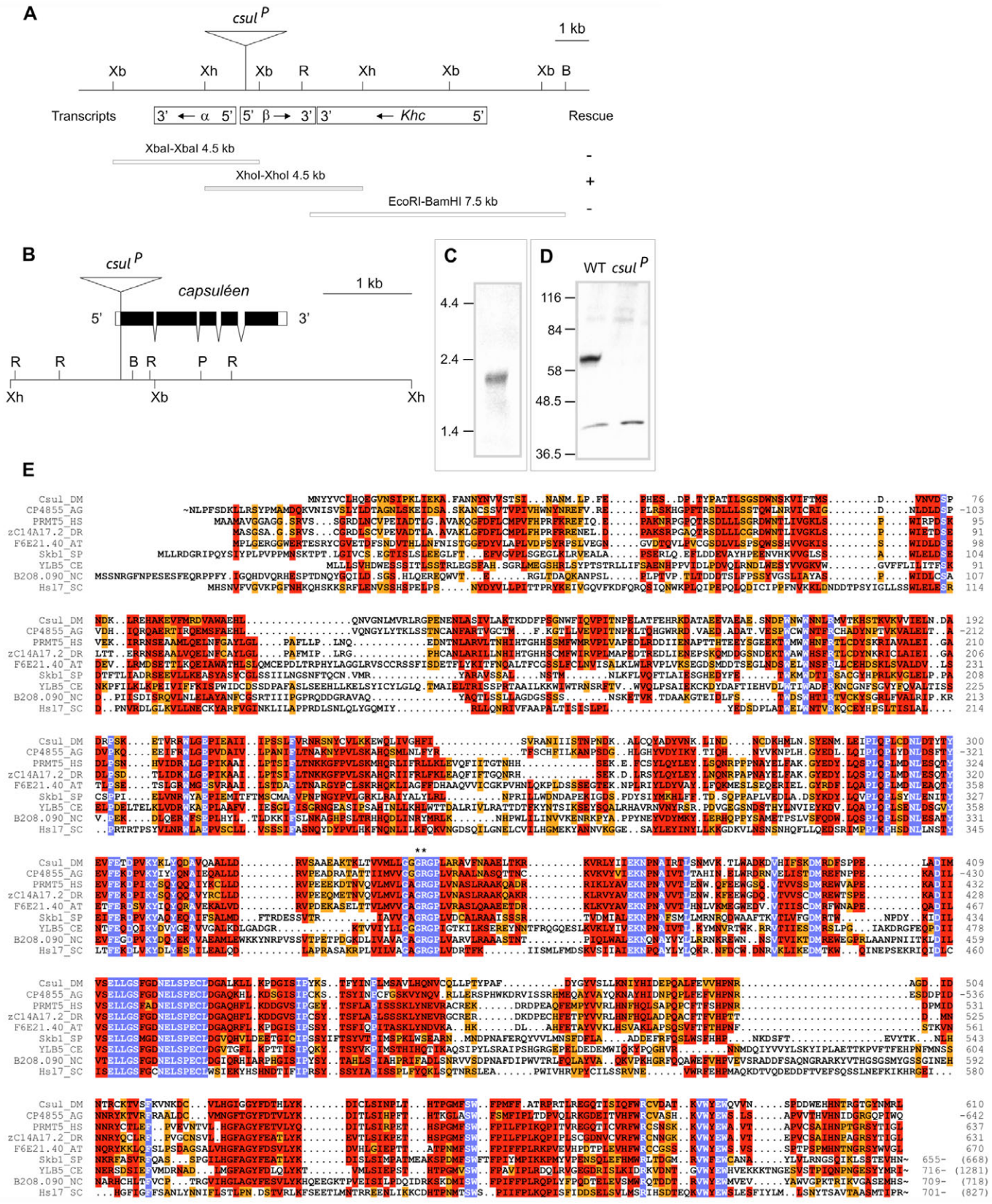

Fig. 3. See next page for legend.

Fig. 3. Molecular analysis of the *csul* locus. (**A**) Restriction map of the genomic DNA covering the *csul* locus with the location of the *P* element insertion in $csul^{P}$. Below the map are shown three transcription units in this locus and their orientation, as well as the three genomic fragments used for constructing transgenes. Transcription orientation of the *fidipidine* (α), *csul* (β), and *Kinesin heavy chain* (*Khc*) genes is indicated by arrows. The analysis of complementation of the $csul^{P}$ mutation by transgenes shown on the right demonstrates that the β gene corresponds to *csul*. (**B**) Restriction map of the *csul* gene, with alignment of the *csul* transcript. Exons are drawn as boxes and the putative open reading frame is indicated in black. (B) *Bam*HI; (P) *Pst*I; (R) *Eco*RI; (Xb) *Xba*I; (Xh) *Xho*I. (**C**) Northern blot showing a single ovarian *csul* transcript of ~2 kb. RNA marker sizes (in kb) are indicated on the left. (**D**) Western blot detection of Csul in wild-type and $csul^{P}$ ovarian protein extracts using anti-Csul antibodies. A band of ~65 kDa (arrow) is detected in wild-type but not in $csul^{P}$. Protein load in each well was normalized by using anti-actin antibodies (data not shown). The anti-Csul antibodies recognize another protein of ~38 kDa unrelated to Csul. (**E**) Alignment of the amino acid sequences of Csul and homologous proteins performed using the Pileup program of the Wisconsin Package (Genetics Computer Group) reveals that *csul* encodes a protein-arginine methyltransferase similar to human PMRT5. Gaps in the amino acid sequence, indicated by dots, were introduced for optimal alignment. At each position of the alignment, residues identical in all sequences are background-shaded blue, and functionally conserved (i.e. more than half of the amino acids of a column) residues with strong or weak similarities are background-shaded red and orange, respectively. The asterisks over the positions 243 and 244 indicate the substitutions made in the $csul^{G343A;R344L}$ transgene. GenBank accession number sequences are as follows: CP4855_AG: EAA14767; PRMT5_HS: AF167572; zC14A17.2_DR: CAD60861; F6E21.40_AT: T10666; Skb1_SP: U59684; YLB5_CE: U10402; B208.200_NC: T49572; Hsl7p_SC: U65920.

$csul^{P}$ embryos. In a few pre-blastoderm embryos we detected Osk- and Vas-stained particles closely associated with nuclei at the posterior pole (inserts in Fig. 1P,T). These structures may represent remnants of polar granules, similar to those detected in hypomorphic *tud* mutant embryos (Hay et al., 1990). In contrast to Osk and Vas, no posterior accumulation of Tud could be detected in $csul^{P}$, even in very early embryos (Fig. 1V). From these data we conclude that *csul* activity mediates Tud and (to some degree) Vas accumulation and the maintenance of Osk and Vas at the posterior pole of the embryo.

csul-dependent pole plasm assembly during oogenesis

As pole plasm assembly occurs during oogenesis we investigated the distribution of pole plasm components in $csul^{P}$ egg chambers. *osk* mRNA and Osk protein distributions appear normal (Fig. 2A,B; data not shown) and western blot analysis of ovarian protein extracts revealed similar levels of Osk proteins and phosphorylation of the short Osk isoform in $csul^{P}$ and wild-type egg chambers (data not shown). We then used a *GFP-Vas* transgene (Sano et al., 2002) to monitor the distribution of Vas. GFP-Vas is absent from the pole plasm in the majority of stage 10 $csul^{RM}$ egg chambers (data not shown). Only a minority of $csul^{RM}$ oocytes display a small amount of GFP-Vas at the posterior pole (Fig. 2D). Immunostaining of $csul^{P}$ egg chambers using anti-Vas antibodies showed a similar distribution (data not shown). By comparison with early embryos, the smaller number of stage 10 oocytes displaying a posterior localization of Vas indicates that Vas may accumulate in the pole plasm during late $csul^{RM}$ oogenesis to become detectable during early embryogenesis.

By contrast, we found that Tud fails to concentrate at the posterior pole in $csul^{P}$ oocyte of stage 10 egg chambers (Fig. 2F) indicating that *csul* activity is essentially required during oogenesis for the localization of Tud, and to a lesser degree for that of Vas, in the pole plasm.

csul-dependent nuage assembly

As a number of pole plasm components localize in the nuage, we analyzed their pattern of distribution in *csul* egg chambers. GFP-Vas shows a normal, albeit weak, nuage localization in $csul^{RM}$ nurse cells during early oogenesis (Fig. 2H). Subsequently, GFP-Vas progressively fades away and is barely detected in the nuage of stage 10 egg chambers (Fig. 2D). By contrast, no Tud is detected in the nuage in $csul^{P}$ egg chambers, although Tud accumulates in mutant oocytes (Fig. 2J) and transiently localizes at their anterior margin in stage 6/7 egg chambers (data not shown). In wild-type nurse cells, Mael displays a punctate perinuclear distribution (Fig. 2K), which is not detected in $csul^{P}$ egg chambers (Fig. 2L). Hence, *csul* is needed for the perinuclear localization of Mael, Tud and Vas, indicating that *csul* contributes to the assembly of the nuage.

Molecular characterization of the *csul* gene

To gain insight into *csul* function, we isolated the gene by inverse PCR amplification of a genomic DNA fragment adjacent to the *P-[white^{+}]* insertion site. This fragment was used to isolate DNA segments overlapping the *P*-element which in turn were used to recover cDNA clones. Northern blot analysis using genomic DNA probes from both sides of the *P*-insert revealed transcripts of ~2kb in size. Alignment of the cDNA sequences with genomic DNA revealed two types of transcripts oriented in opposite directions. The *P*-element was inserted in one of the transcription units, 51 bp downstream of its 5′ end and 7 nucleotides downstream of the putative translation initiation site. This transcript was assigned to the *csul* gene, extending from the *P*-insert down to the adjacent *Kinesin heavy chain* (*Khc*) gene. The 3′ end of *Khc* is located 5 nucleotides downstream of the 3′ end of *csul*. To validate the identification of the *csul* gene, we generated a transgene and tested its ability to complement *csul* mutations. We found that the fertility of $csul^{RM}$ females was restored by a single copy of the *Xho*I-*Xho*I *P-[csul]* transgene (Fig. 3A). Constructs containing either the downstream *Khc* (Saxton et al., 1991) or the upstream *fidipidine* (data not shown) genes failed to restore fertility to $csul^{RM}$ females.

Alignment of cDNA and genomic DNA sequences revealed that *csul* is composed of five exons (Fig. 3B). The *csul* transcript has a size of about 2 kb (Fig. 3C) and potentially encodes a 610 amino-acid protein (accession number: AJ002740). In vitro translation of a full-length cDNA produced a single polypeptide with an apparent molecular mass of ~67 kDa corresponding to the predicted molecular mass of Csul (data not shown). Polyclonal antibodies were raised against the carboxyl-terminal half of bacterially synthesized Csul. On western blots of ovarian and embryonic protein extracts, these antibodies reacted with a protein band displaying a molecular mass of ~65 kDa, consistent with the expected mass of Csul (Fig. 3D). No such protein was detected in ovarian extracts of $csul^{P}$ females. However, long exposure of the immunoblot revealed a faint band in the 65 kDa range, suggesting that $csul^{P}$ females may produce a low level of Csul, possibly by use of an in-frame initiation codon present in the *P-white^{+}* inverted

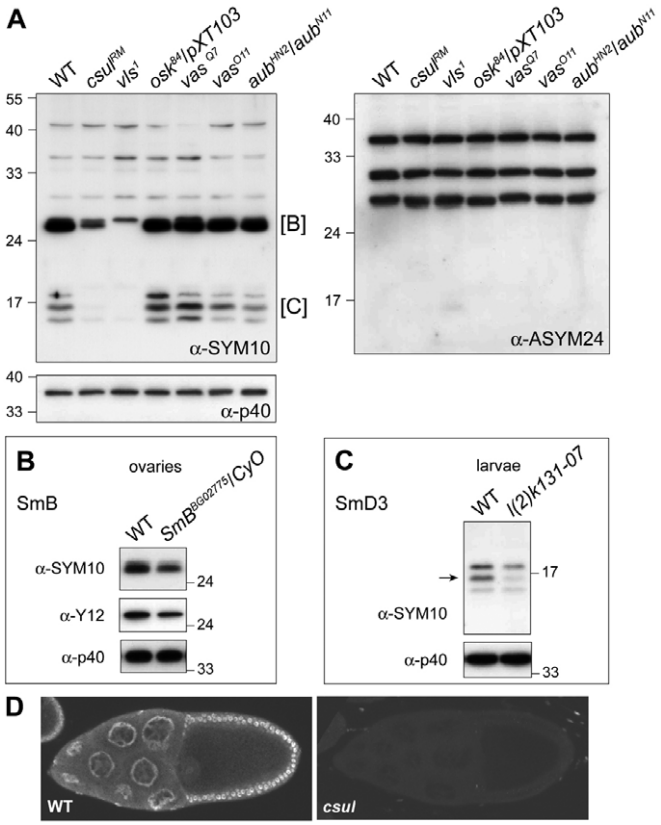

Fig. 4. Symmetrical methylation of SmB and SmD3 proteins is dependent on Csul and Vls. (**A**) Csul and Vls are required for sDMA synthesis. Protein extracts of wild-type (WT), *csul*^{RM}, *vls*^{1}, *osk*^{84}/*pXT103*, *vas*^{Q7}, *vas*^{O11} and *aub*^{HN2}/*aub*^{N11} ovaries were separated by SDS-PAGE, blotted and probed with α-SYM10 (left panel) and α-ASYM24 antibodies (right panel). Anti-ribosomal p40 antibodies (α-p40) were used as a loading control (left panel, lower blot). The intensity of four SYM10-reactive protein bands in the range of 15 to 26 kDa was strongly reduced in *csul*^{RM} and *vls*^{1} protein extracts. No change of the intensity of the ASYM24-reactive bands was observed in any mutant background. [B] and [C] indicate the position of protein bands shown in B and C, respectively. (**B**) SmB is symmetrically dimethylated in vivo. Ovarian protein extracts of wild-type and *SmB*^{BG02775}/*CyO* females were separated by SDS-PAGE, blotted and probed with α-SYM10 and α-Y12 antibodies. By comparison to wild type, the intensity of the SYM10/Y12-reactive protein band is significantly reduced in *SmB*^{BG02775}/*CyO*. (**C**) SmD3 is symmetrically dimethylated in vivo. Protein extracts of wild-type and homozygous *SmD3*$^{l(2)k131-07}$ larvae were separated by SDS-PAGE, blotted and probed with α-SYM10 antibodies. Arrow indicates the position of SmD3. (**D**) SmB immunostaining using α-Y12 antibodies. sDMA-SmB localizes in the nuclei of the nurse cells and somatic follicular cells of wild-type egg chambers but is undetected in *csul*^{RM} egg chambers.

repeat and located 16 nucleotides upstream of the insertion site (data not shown). This finding suggests that *csul*^{P} is a hypomorphic allele.

Csul acts as a protein methyltransferase

Csul protein sequence was found to exhibit significant similarity to protein-arginine methyltransferases (PRMTs), which catalyse the formation of symmetric di-methyl arginyl (sDMA) residues (Fig. 3E). Csul homologues can be identified in organisms ranging from the budding yeast *Saccharomyces cerevisiae* to humans (Ma, 2000).

To ascertain that Csul acts as a methyltransferase, we investigated whether amino acid substitutions in the catalytic domain of the enzyme would inactivate its function. Csul family proteins contain a conserved core region characterized by motif I [GXGRG], which, together with the Post-I motif, forms the *S*-adenosyl-L-methionine binding module (Ma, 2000). As the GXGRG motif is highly conserved in the Csul homologs and is required for protein methylation (Pollack et al., 1999) we selected this region for mutational analysis, substituting the conserved residues Gly^{343} and Arg^{344} with Ala and Leu residues, respectively.

Wild-type *csul* and *csul*$^{G343A;R344L}$ gene sequences were fused to the tandem affinity purification (TAP) (Rigaut et al., 1999) tag, cloned into a transformation vector and transgenic strains were generated. After introducing the transgenes in a *csul*^{RM} genetic background we determined, by western blot, that they both express at similar level (data not shown) and examined their ability to abolish the *csul*^{RM} maternal-effect embryonic phenotype. *csul-TAP* and *TAP-csul* transgenes rescue the development of *csul*^{RM} embryos, whereas the *csul*$^{G343A;R344L}$*–TAP* transgene shows no rescue activity (data not shown and Fig. 7D). These results strongly suggest that *csul* encodes a methyltransferase whose activity requires an intact catalytic domain.

Sm proteins are substrates of the Csul-Vls complex

We used anti-SYM10 antibodies (α-SYM10), which specifically recognize proteins containing multiple sDMA-glycine repeats (Boisvert et al., 2002), to investigate the pattern of methylated proteins extracted from wild-type, *csul*, *valois* (*vls*), *vas*, *aub* and *osk* ovaries. As shown in Fig. 4A, α-SYM10 reacted with five protein bands of a relatively low molecular mass, ranging from 14 to 30 kDa, in wild-type, *osk*, *vas* and *aub* protein extracts. In *csul* and *vls* the intensity of four of these bands was strongly reduced. Probing similar protein blots with the anti-ASYM24 antibodies, which identify proteins containing asymmetric di-methyl arginyl residues (aDMA) (Boisvert et al., 2002), we found no change in the pattern of the reactive protein bands in all extracts. These results corroborate our finding that Csul and Vls are part of a methylosome complex contributing to sDMA synthesis in specific proteins (Anne and Mechler, 2005), and indicate that Vls acts as a co-factor of Csul. Moreover, these data show that Vas, Osk and Aub exert no function in the Csul-dependent synthesis of sDMA residues.

As the size of the *Drosophila* proteins recognized by α-SYM10 corresponds to that of components required for pre-mRNA splicing in human cells (Boisvert et al., 2003), we analyzed whether Sm proteins might be in vivo substrates of Csul. As shown in Fig. 4B,C, making use of available mutations in *Drosophila* genes encoding Sm proteins, we identified SmB and SmD3 as targets of the Csul-Vls complex. In particular we found that the amount of an ~26 kDa α-SYM10 reactive protein was reduced by half in western blots of heterozygous *SmB*^{BG02775} in comparison to wild-type ovarian extracts. A similar reduction in intensity was obtained with the Y12 monoclonal antibody (α-Y12) that specifically recognizes methylated SmB (Paterson et al., 1991). Similarly, comparison of protein extracts of wild-type and homozygous *SmD3*$^{l(2)k131-07}$ larvae (Schenkel et al., 2002) revealed that the α-SYM10 signal of a 16 kDa protein is nearly abolished in the mutant, identifying this protein as SmD3.

To further confirm the Csul-dependent symmetrical methylation of SmB, we stained ovaries using α-Y12. As compared with wild-type ovaries, in which methylated SmB accumulates in the nuclei of the nurse cells and somatic

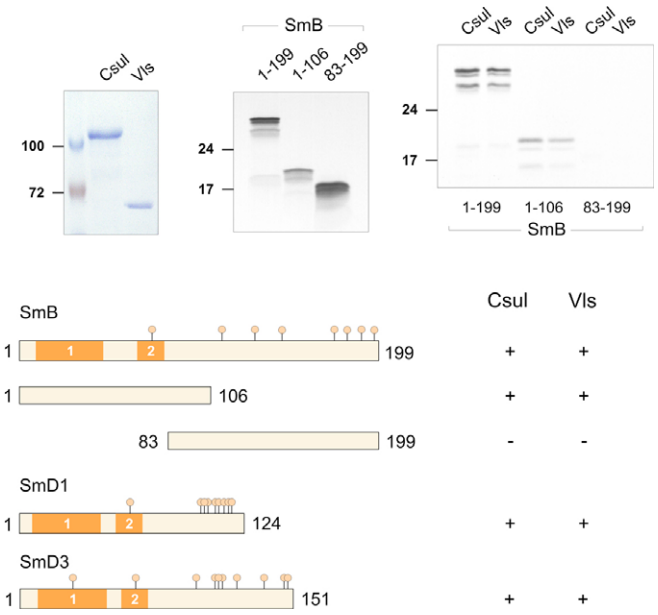

Fig. 5. SmB binds to CsuI and Vls. Left panel: GST-CsuI and GST-Vls proteins stained with Coomassie Blue. Middle panel: [^{35}S]S•Tag-SmB fragments were separated by SDS-PAGE and detected by fluorography. Right panel: Following incubation with GST-CsuI or GST-Vls, the bound [^{35}S]S•Tag-SmB fragments were separated and detected as described above. No binding was detected with GST alone (data not shown). Below these panels the binding results of SmB and derivatives as well as those of SmD1 and SmD3 to CsuI and Vls are summarized graphically. Size and structure of the SmB, SmD1 and SmD3 proteins are depicted with the two Sm domains and the RG dipeptides shown as orange boxes and circles, respectively. N- and C-terminal amino acid residues of the Sm proteins and fragments are indicated.

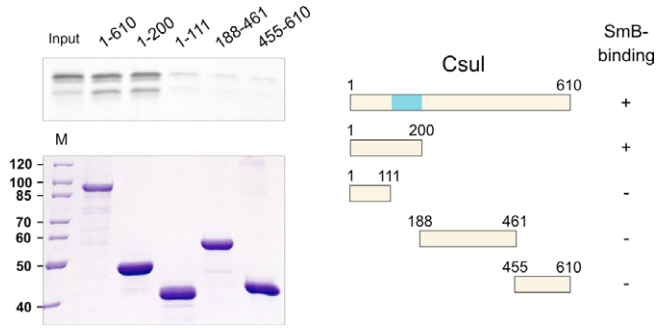

Fig. 6. SmB-binding domain in CsuI. (Upper panel) Full-length GST-CsuI or derivatives were purified from bacteria and incubated with [^{35}S]S•Tag-SmB. Bound [^{35}S]S•Tag-SmB proteins were separated by SDS-PAGE and detected by fluorography. Amino acid numbers are given across the top. 'Input' was one tenth of the synthesized [^{35}S]S•Tag-SmB proteins. The smallest fragment of CsuI showing binding to SmB encompasses amino acids 1-200. (Lower panel) The amount of GST-CsuI polypeptides was visualized by Coomassie Blue staining. (Right) Representation of the GST-CsuI fragments used for the mapping and the summary of the results, with the identified domain required for SmB-binding in CsuI shown in blue.

follicular cells, α-Y12 signal was dramatically reduced in *csul^{RM}* egg chambers (Fig. 4D). Taken together, our data demonstrate that CsuI is required for the symmetrical methylation of SmB in vivo.

CsuI and Vls interact with Sm proteins in vitro

As Sm proteins can be methylated by the CsuI-Vls methylosome, we investigated by binding assays whether these proteins can physically interact together. We found that CsuI and Vls can bind to SmB (Fig. 5), SmD1, and SmD3 (data not shown). We then determined the reciprocal binding domains between CsuI and SmB. First we synthesized ^{35}S-labeled S-tag SmB$^{1\text{-}199}$ (full-length), SmB$^{1\text{-}106}$ (N-terminal moiety with Sm domains) and SmB$^{83\text{-}199}$ (C-terminal moiety with RG repeats) polypeptides and incubated them with GST-CsuI, GST-Vls, or GST alone. We found that the deletion of the RG domain had no effect on SmB binding to CsuI or Vls, indicating that the N-terminal moiety containing the Sm domains mediates SmB interaction with CsuI or Vls. Then, by using a similar approach we assigned the SmB binding domain of CsuI to the N-terminal part (amino acid residues 1-200) (Fig. 6). Further analysis showed that both N- or C-terminal truncations in this domain (fragments 20-610 and 1-111, respectively) prevent SmB binding to CsuI (Fig. 6 and data not shown), indicating that the N-terminal part of CsuI contains at least two domains necessary for interaction with SmB.

Interaction between CsuI and Tud in vivo is essential for Tud localization

The defective accumulation of Tud in the nuage of *csul^{P}* nurse cells led us to evaluate whether Tud might directly interact with CsuI. As shown in Fig. S1 in the supplementary material, we performed pull-down assays, using tagged fragments of Tud (Golumbeski et al., 1991; Anne and Mechler, 2005). In vitro translated fragments of Tud were incubated with immobilized GST-CsuI proteins. After washing, the bound S•Tag-Tud proteins were revealed by immunodetection. This procedure showed that the JOZ fragment and the 9A1 polypeptide and derivatives display a strong binding, whereas the 3ZS+L fragments exhibit more moderate binding to GST-CsuI whereas GST alone is unable to bind to Tud fragments. We then used Tud9A1-N to map the binding site of Tud in CsuI.

As shown in Fig. 7A, we found that the 9A1-N fragment of Tud can bind to the N-terminal region of CsuI (amino acid residues 1-111). These results suggest that a domain in the N-terminal region of CsuI mediates a direct and specific interaction with Tud. To delimit more precisely the N-terminal domain of CsuI critical for Tud binding, we generated serial N-terminal truncations of CsuI. In this way we identified a critical region for Tud binding between amino acids 60 and 80 (Fig. 7B,C).

We next tested the in vivo relevance of the N-terminal region of CsuI with respect to the localization of Tud in the nuage and the formation of pole cells. For this purpose we constructed four *csul* transgenes each bearing an internal deletion (between amino acids 21-40, 41-60, 61-80 and 81-100, respectively), fused in-frame to the TAP tag, and introduced these transgenes into the *csul^{RM}* genetic background. Immunostaining revealed that the CsuI proteins carrying the deletions Δ21-40 and Δ41-60 could restore Tud localization in the nuage. By contrast, deletions Δ61-80 and Δ81-100 failed to do so (Fig. 7D, left panels). Finally we observed that deletions Δ21-40 and Δ41-60 can give rise to the formation of pole cells and rescue the *csul^{RM}* maternal-effect phenotype, whereas deletions Δ61-80 and Δ81-100 do not (Fig. 7D, right panels). Together with our in vitro interaction results, these findings indicate that the localization of Tud in the nuage requires interaction with CsuI.

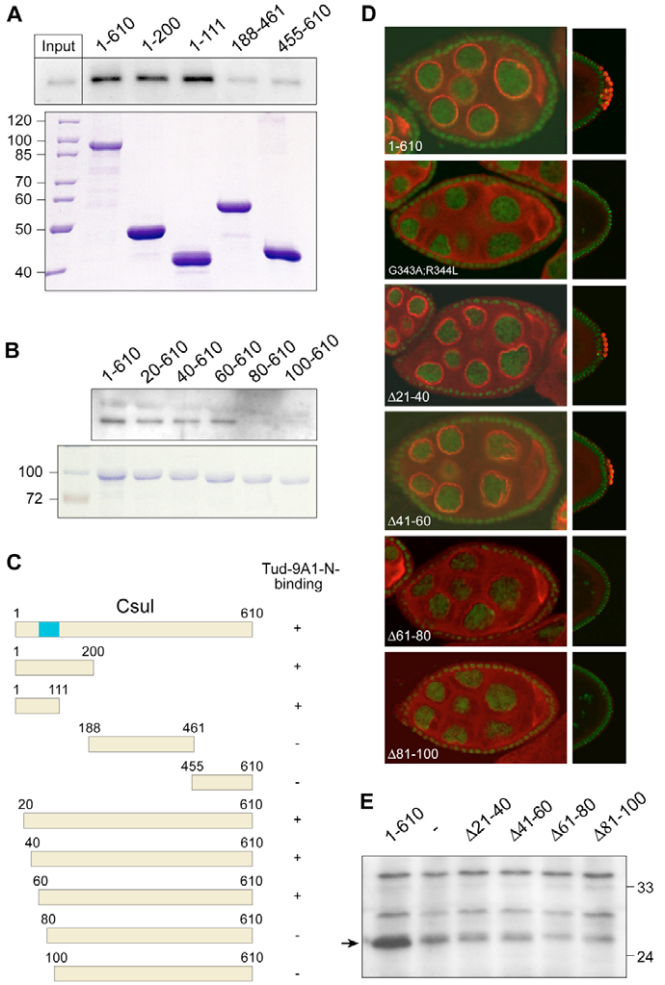

Fig. 7. A domain in the N-terminal region of Csul is essential for the methylation of Sm proteins and localization of Tud. (**A-C**) Identification of the Tud9A1-N-binding domain in Csul. (A) Full-length GST-Csul or derivatives were purified from bacteria and incubated with S•Tag-Tud9A1-N. Bound S•Tag-Tud9A1-N was detected as described in Fig. S1 of the supplementary material (upper panel). The amount of GST-Csul proteins was visualized by Coomassie Blue staining (lower panel). Amino acid numbers are given across the top. 'Input' was one tenth of the S•Tag-Tud9A1-N extract. The smallest fragment of Csul showing binding to Tud9A1-N encompasses the first 111 amino acids of Csul and may thus be distinct from the SmB binding site. (B) Delimitation of the Tud-binding domain using N-terminal truncation of Csul. Detection of S•Tag-Tud9A1-N bound to the N-terminal truncated Csul polypeptides revealed that the sequence following amino acid residue 60 is required for Tud9A1-N binding. (C) Representation of the GST-Csul fragments used for the mapping and the summary of the binding results are indicated on the right. The identified domain of Csul necessary for Tud-binding is shown in blue. (**D**,**E**) Use of interstitial deletions in the N terminus region of Csul to determine the domain necessary for Tud localization in the nuage and sDMA methylation of SmB. (D) The Tud9A1-N-binding domain of Csul is required for Tud localization in the nuage and pole cell formation. Egg chambers and embryos from homozygous *csul*^{RM} females expressing the different transgenes were stained using anti-Tud (red; left column) and anti-Vas (red; right column) antibodies, respectively. DNA staining is in green. The deletions uncovering residues 21-40 and 41-60 can properly target Tud in the nuage and rescue the *csul* phenotype. (E) The N-terminal region of Csul is necessary for sDMA synthesis on SmB protein. Transgenes expressing full-length or modified Csul proteins were introduced into the *csul*^{RM} background and ovarian extracts were prepared from the homozygous females. Western blot analysis using α-SYM10 antibodies indicates that none of the deletion transgenes are able to rescue the methylation of the SmB protein (arrow).

sDMA-Sm proteins can bind to Tud in vitro

As sDMA Sm proteins can bind to the Tudor domain of SMN (Brahms et al., 2001; Selenko et al., 2001) we investigated the requirement of sDMA synthesis for Sm binding to Tud. For this purpose we took advantage of the occurrence of an endogenous type II PRMT activity in the reticulocyte lysate system (Brahms et al., 2001). By using *S*-adenosyl-homocysteine (SAH), which blocks the activity of protein methyltransferases (Brahms et al., 2001), we tested whether SAH could inhibit sDMA synthesis in SmB. Addition of SAH to the reticulocyte lysate significantly reduced the amount of α-SYM10-immunoreactive SmB polypetides, but did not inhibit SmB synthesis (see Fig. S2A in the supplementary material). We next incubated bacterially produced Tud fragments (see Fig. S2B in the supplementary material) with SmB polypeptides synthesized in the presence or absence of SAH. As shown in Fig. S2C in the supplementary material, methylation of SmB promotes its binding to all three tested Tud fragments.

Tud localization and Sm protein methylation may be unrelated processes, both driven by Csul

To further explore the relationship between Sm methylation and pole plasm function, we investigated the methylation status of Sm proteins in extracts of transgenic *csul*^{RM} ovaries producing Csul proteins with interstitial deletions in the N-terminal region. Western blot analysis using α-SYM10 revealed that all four mutant Csul proteins failed to symmetrically methylate SmB (Fig. 7E). The finding that the Csul proteins containing the two most distal deletions (Δ21-40 and Δ41-60) are still able to restore Tud targeting to the nuage of *csul*^{RM} egg chambers indicates that only the methylation of Sm proteins is impaired. In light of the in vitro binding results (Fig. 6) we envisage that the absence of methylation of Sm proteins is primarily due to a defective binding of SmB to Csul. Whether the two other deletions (Δ61-80 and Δ81-100) only affect the binding of Tud to Csul or structurally compromise other functions of Csul remains an open question but primarily our data show that we can separate Tud localization in the nuage from Sm methylation.

DISCUSSION

Csul is a Type II PRMT

csul encodes a Type II PRMT, which transfers methyl groups from *S*-adenosyl-L-methionine to the guanidinium group of arginyl residues. PRMTs can be divided into two major categories, catalyzing the synthesis of aDMA (Type I) or sDMA (Type II) residues, respectively. The mammalian PRMT5 (Pollack et al., 1999; Lee et al., 2000; Branscombe et al., 2001), homologous to Csul, and the recently identified PMRT7 and PRMT9 (Lee et al., 2005; Cook et al., 2006) are responsible for Type II methylation.

By using α-SYM10 antibodies that recognize proteins harbouring two spaced sDMA-glycine motifs (Boisvert et al., 2002) we identified four major reactive proteins bands as specific targets of Csul. These proteins are distinct from aDMA-containing proteins, whose

methylation is independent of Csul. Among the sDMA proteins, we genetically confirmed that the spliceosomal components SmB and SmD3 are Csul targets. The corresponding mammalian targets have been identified for PMRT5 (Brahms et al., 2001; Meister et al., 2001; Friesen et al., 2001a). As α-SYM10 may only recognize a subset of sDMA proteins methylated by Csul, further proteomic analysis of ovarian Csul complexes may identify additional targets of Csul.

The Csul-Vls and human methylosomes interact with sDMA-proteins and Tudor-domain proteins

As indicated by the physical interaction of Csul with Vls (Anne and Mechler, 2005) and the size of the native Csul complexes, with a molecular mass of ~500 kDa (data not shown), Csul is part of a large protein complex. In the present work we show that Vls, the *Drosophila* homolog of human MEP50, itself a partner of PRMT5 (Friesen et al., 2002), is also required in sDMA synthesis on identical target proteins. However, in the case of pIcln, a component of the human methylosome of yet unknown function (Friesen et al., 2001a), we detected no interaction between *Drosophila* pIcln and Csul in pull-down assays (data not shown). Furthermore, we found that both Csul and Vls interact with the N-terminal moiety of SmB. This is in contrast to PRMT5, which appears to bind to the RG-rich C-terminal domain of Sm proteins (Friesen et al., 2001a). Differences in protein interaction and quaternary structure between the human and *Drosophila* methylosome may reflect divergences in the activities of the methylosome between the two species.

Both human (Friesen et al., 2001a; Meister et al., 2001) and *Drosophila* methylosomes lead to sDMA synthesis on Sm proteins (this study). Similarly to the requirement of sDMA synthesis for the association of human Sm proteins with the SMN Tudor domain (Côté and Richard, 2005), we found that *Drosophila* Sm proteins need to be symmetrically methylated to bind *Drosophila* Tud. The binding of sDMA-Sm to non-overlapping Tud polypeptides indicates that these proteins may bind to several, if not all Tudor domains in Tud.

The association of human SMN protein with the PMRT5 complex suggests direct interactions between PMRT5, MEP50 and SMN (Meister and Fisher, 2002). Similarly, *Drosophila* Tud can directly bind to Csul and Vls (Anne and Mechler, 2005). However, in contrast to Sm, which binds to multiple sites on Tud, Csul and Vls more strongly interact with the N-terminal than the C-terminal moiety of Tud, suggesting a distinct mechanism of association with Tud. Although the specific binding sites of Csul and Vls on Tud remain to be determined, preliminary results indicate that each protein binds to a distinct site.

As this work was being completed, another group reported the identification of the *csul* gene, termed *dart5* (Gonsalvez et al., 2006), and showed that disruption of this gene (mutant e00797 from the Exelixis collection) leads to the absence of sDMA synthesis of spliceosomal Sm proteins without impairing spliceosomal function. This work and ours confirm the previous finding of Khusial et al. (Khusial et al., 2005), indicating that sDMA synthesis on Sm proteins is not required for sRNP assembly and transport, a critical process for *Drosophila* development. In addition, Gonsalvez et al. (Gonsalvez et al., 2006) also characterized the maternal requirement of *csul* for pole cell formation.

Tud localization in the nuage

In addition to their role in sDMA synthesis, Csul and Vls are required for Tud localization in the nuage. Our data indicate that *csul* activity is also necessary for the proper nuage accumulation of Vas. However, despite the occurrence of Vas in the nuage of early *csul* egg chambers, Tud is absent from this structure, suggesting that the activity of Csul in Tud localization is independent from that exerted on Vas.

How the Csul/Vls methylosome directs Tud localization in the nuage remains an open question. The restoration of fertility by mutated *csul* transgenes defective in SmB binding, and hence in sDMA synthesis on SmB, points out the occurrence of a yet unknown protein which should act as a substrate of Csul and specifically function in germline formation. We favour a cytoplasmic association of the Csul/Vls methylosome with this substrate and Tud. Upon methylation the substrate is then transferred to Tud, as indicated by the preferential binding of sDMA-SmB to Tud polypeptides. In our view, the interaction between Csul/Vls, the substrate, and Tud may be critical to position Tud in the vicinity of the site where sDMA synthesis takes place, thus facilitating the association of Tud with the sDMA-protein. A similar model has been proposed for the targeting of high-affinity Sm protein substrates to the SMN complex (Friesen et al., 2001b). Following the docking of the sDMA protein on Tud, the Csul/Vls methylosome is released, and the Tud/sDMA protein complex becomes positioned in the nuage. The docking of the sDMA protein might induce an allosteric change in Tud, increasing its affinity for a component of the nuage.

Finally, although Vas is not properly localized at the perinuclear region of nurse cells in *csul* and *vls* mutant egg chambers we notice that its distribution pattern differs in each mutant. In particular, the level of Vas in the nuage is comparatively smaller in *csul* than in *vls* mutants (Anne and Mechler, 2005), suggesting that Csul acts independently of Vls in the localization of Vas to the nuage. Moreover, the finding that Vls specifically accumulates in the nuage and pole plasm whereas Csul displays a ubiquitous distribution (data not shown) suggests that both proteins may exert additional independent functions.

Although the functional relationship between the nuage and pole plasm remains unresolved, events occurring in the nuage may affect pole plasm formation. In *csul* mutant egg chambers, Tud is absent from both the nuage and the pole plasm and, similarly, a reduced amount of Vas in the nuage correlates with a decreased level of this protein in the pole plasm. However, it has been reported recently that a Tud protein containing the Tudor domains 1 and 6-10 could localize to the pole plasm, albeit at a moderate level compared to full-length Tud, but fail to properly localize to the nuage (Arkov et al., 2006). Additional work on the requirement of Csul for Tud localization in the nuage will be critical for understanding the assembly of this structure, its dynamical relationship with the pole plasm, and the role of arginine methylation in protein targeting.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/1/02687/DC1

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