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Article

The Sm-Protein Methyltransferase, Dart5, Is Essential for Germ-Cell Specification and Maintenance

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Summary

Background: The C-terminal tails of spliceosomal Sm proteins contain symmetrical dimethylarginine (sDMA) residues in vivo. The precise function of this posttranslational modification in the biogenesis of small nuclear ribonucleoproteins (snRNPs) and pre-mRNA splicing remains largely uncharacterized. Here, we examine the organismal and cellular consequences of loss of symmetric dimethylation of Sm proteins in *Drosophila*.
Results: Genetic disruption of *dart5*, the fly ortholog of human *PRMT5*, results in the complete loss of sDMA residues on spliceosomal Sm proteins. Similarly, *valois*, a previously characterized grandchildless gene, is also required for sDMA modification of Sm proteins. In the absence of *dart5*, snRNP biogenesis is surprisingly unaffected, and homozygous mutant animals are completely viable. Instead, Dart5 protein is required for maturation of spermatocytes in males and for germ-cell specification in females. Embryos laid by *dart5* mutants fail to form pole cells, and Tudor localization is disrupted in stage 10 oocytes. Transgenic expression of Dart5 exclusively within the female germline rescues pole-cell formation, whereas ubiquitous expression rescues sDMA modification of Sm proteins and male sterility.
Conclusions: We have shown that Dart5-mediated methylation of Sm proteins is not essential for snRNP biogenesis. The results uncover a novel role for *dart5* in specification of the germline and in spermatocyte maturation. Because disruption of both *dart5* and *valois* causes the specific loss of sDMA-modified Sm proteins and studies in *C. elegans* show that Sm proteins are required for germ-granule localization, we propose that Sm protein methylation is a pivotal event in germ-cell development.

Introduction

Pre-messenger-RNA splicing, a hallmark feature of eukaryotic cells, is carried out by a large ribonucleoprotein (RNP) complex called the spliceosome. Numerous gene products are therefore dedicated to the task of building functional spliceosomes, the cellular machines that mediate the removal of intronic sequences. Small nuclear RNPs (snRNPs), central components of the spliceosome, are assembled in a highly orchestrated and sequential manner, involving maturation steps in both the nucleus and cytoplasm of the cell [1]. The U1, U2, U4, and U5 spliceosomal snRNPs each contain a common set of seven core Sm proteins—SmB/B′, SmD1, SmD2, SmD3, SmE, SmF, and SmG [1]. These proteins bind to a common sequence motif within the U snRNAs and form a heteroheptameric ring structure [1].

Assembly of the Sm ring takes place in the cytoplasm and, in vivo, requires the activity of the survival of motor neurons (SMN) protein complex [2–5]. Mutations that reduce the level of SMN, the central member of this complex, result in a human neurogenetic disorder called spinal muscular atrophy (SMA) [6]. Importantly, cells from SMA patients display a reduced capacity for Sm core assembly [7]. Collectively, the available data are consistent with the idea that SMA results from a general reduction in snRNP biogenesis, with motor neurons being particularly susceptible to reduced snRNP levels. However, the possibility that SMN functions in a novel cell-specific pathway has not been conclusively ruled out.

Three of the seven core Sm proteins, SmB/B′, SmD1, and SmD3, contain symmetric dimethylarginine (sDMA) residues within their C-terminal tails [8, 9]. The enzymes that catalyze this posttranslational modification are called protein arginine methyltransferases (PRMTs) and have been placed into two categories—type I and type II [10]. Type I enzymes mediate the more common modification, asymmetric dimethylarginine (aDMA). Type II enzymes are responsible for the less frequent sDMA modification [10]. To date, the only known type II enzymes are PRMT5 and PRMT7, each of which is capable of methylating Sm proteins in vitro [11–13]. Reduction of PRMT5 levels by RNA interference (RNAi) correlates with a decrease in the level of Sm-protein methylation in vivo [14]. Furthermore, PRMT5 associates, along with MEP50/WD45 and pICln, in a complex that contains Sm proteins in vivo [15–17]. Both MEP50 and pICln can directly bind to Sm proteins, thus making a strong case for involvement of the PRMT5 complex in Sm-protein methylation [15–17]. It is not currently known whether PRMT7 plays any role in Sm-protein methylation, and binding partners for PRMT7 have not been described.

The precise role of Sm-protein methylation in snRNP biogenesis remains a poorly understood topic. In vitro, SMN protein preferentially binds to C-terminal peptides, derived from SmD1 and SmD3, that contain sDMA but not aDMA residues [18]. The prevailing view holds that sDMA modification of Sm proteins serves to recruit SMN, thus facilitating efficient transfer of Sm proteins from the PRMT5 complex to the SMN complex for assembly of the Sm core [4, 15, 16]. A prediction that follows from this interpretation is that symmetric dimethylation of Sm proteins is a requirement for efficient snRNP biogenesis. We explored this hypothesis in vivo, with *Drosophila melanogaster*. For these experiments, we used a fly strain containing an insertion in the *dart5* gene, the fly ortholog of human *PRMT5* [19]. Lysates

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prepared from homozygous mutant flies display a complete and specific loss of sDMA modification of Sm proteins. Surprisingly, homozygous disruption of *dart5* was not lethal, and the expected number of progeny was recovered. Using additional molecular assays, we found that spliceosomal snRNP biogenesis was similarly unaffected. Instead, we found that *dart5* males were completely sterile, with defects in spermatogenesis. In contrast to the males, the homozygous mutant females were fertile. However, the progeny obtained from homozygous *dart5* mothers were sterile and agametic. Consistent with this finding, embryos from *dart5* females were devoid of pole cells, the germline precursors. This is reminiscent of the classic "grandchildless" phenotype described for a number of genes such as *tudor*, *vasa*, and *valois* [20, 21]. Interestingly, it was recently shown that *valois* is the *Drosophila* ortholog of human *MEP50/WD45* [22, 23]. Like their human counterparts PRMT5 and MEP50, the Valois and Dart5 (also known as Capsuléen) proteins were recently shown to associate in the fly [15, 17, 22]. Plausibly, these two gene products may function in a related and perhaps overlapping pathway that contributes to germ-cell specification. Notably, we found that, similar to the *valois* mutant phenotype, Tudor protein was mislocalized in *dart5* mutant ovaries. On the basis of these and other findings, we propose that sDMA modification of Sm proteins represents a critical step in the specification and maintenance of the germ-cell lineage.

Results and Discussion

Dart5 Is Required for Symmetric Arginine Dimethylation of Spliceosomal Sm Proteins

Reduction of PRMT5 levels in HeLa cells is correlated with a decrease in the sDMA modification of Sm proteins [14]. We tested this finding in *Drosophila* Schneider 2 (S2) cells by RNAi with double-stranded RNA (dsRNA) targeting *dart5*. After 72 hr of dsRNA treatment, the cells were transfected with GFP-SmB overnight, and total-cell lysates were immunoprecipitated with GFP antibodies. The methylation status of the newly synthesized GFP-SmB was analyzed by western blotting with the sDMA monoclonal antibody Y12 [9]. As shown in Figure 1A, the level of Y12 reactivity for GFP-SmB was greatly reduced in the presence of *dart5* dsRNA but not control dsRNA. This analysis demonstrates that Dart5, like its human counterpart PRMT5, is important for the sDMA modification of Sm proteins in cultured cells.

In order to understand the function of *dart5* in an organismal context, we obtained from the Exelixis collection (stock number e00797) a piggyBac insertion allele that we shall refer to as *dart5-1*. The predicted genomic structure of the fruit fly *dart5* gene and the location of the insertion site within exon 2 are illustrated in Figure 2A. The insertion site, which was confirmed by sequencing, indicated that the position of the piggyBac element should result in production of a truncated protein, because of the presence of multiple in-frame stop codons (Figure 2A). Note that the putative truncated polypeptide is missing the entire conserved methyltransferase domain. Surprisingly, homozygous *dart5-1* animals are completely viable and survive to adulthood. We therefore analyzed the level of Dart5 protein in wild-type

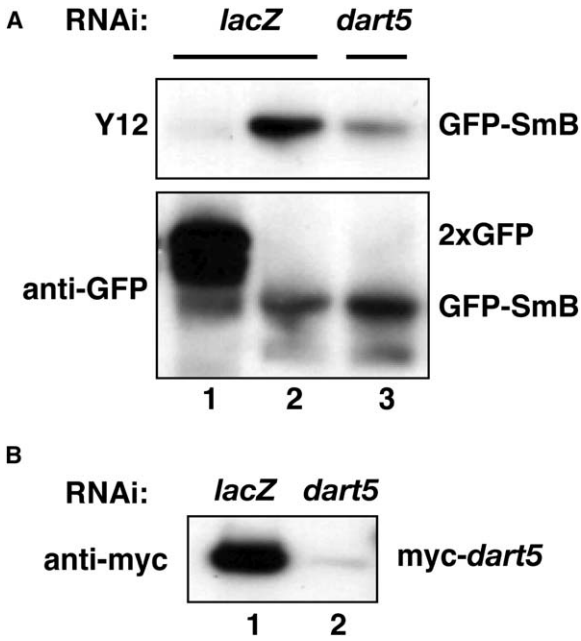

Figure 1. Dart5 Is Required for sDMA Modification of SmB in Schneider Cells

(A) Schnieder cells (1.0×10^6) were treated with 15 μg of either lacZ (lanes 1 and 2) or *dart5* dsRNA (lane 3) for 72 hr. Subsequently 2× GFP-NLS (lane 1) or GFP-SmB (lanes 2 and 3) were transfected into the cells. Twenty-four hours after transfection, the cells were harvested, lysates were prepared, and the GFP-tagged proteins were immunoprecipitated by using a polyclonal GFP antibody. The methylation status of the immunoprecipitated proteins was analyzed with sDMA antibody Y12 (top blot). Note that in the presence of *dart5* dsRNA, the level of Y12 reactivity for GFP-SmB was reduced. The blot was stripped and reprobed with a monoclonal GFP antibody to verify the loading (bottom blot).

(B) As a control for the RNAi, Schneider cells (1.0×10^6) were treated with 15 μg of either lacZ (lane 1) or *dart5* dsRNA (lane 2) for 72 hr. Subsequently, myc-*dart5* was transfected into the cells. Twenty-four hours after transfection, the cells were harvested, and lysates were prepared and analyzed by western blotting with a polyclonal myc antibody.

and *dart5-1* homozygotes (herein referred to as *dart5-1* mutants) by western blotting. Consistent with the predicted size of the Dart5 protein, we detected in wild-type lysates a prominent band of 70 kDa that was not observed in the mutants (Figure 2B). A shorter band of ~35 kDa was sometimes detected only in the mutant lysates, but its presence was inconsistent (data not shown). Importantly, excision repair of the transposon correlated with the re-emergence of this 70 kDa band (Figure 2B). On the basis of these results, we conclude that the *dart5-1* insertion does not produce any full-length protein.

To determine whether the Sm proteins were sDMA modified in the mutants, we analyzed the *dart5-1* adult lysates with two sDMA-specific antibodies, Y12 and SYM10 (Figure 2C) [9, 14]. In *Drosophila*, SYM10 recognizes a number of proteins, including SmB, SmD1, and SmD3, whereas Y12 primarily detects SmB. We confirmed the identity of the Sm proteins in the lysates by probing purified snRNP proteins with the SYM10 antibody (Figure 2D). Note that in the mutant lysates, the Sm proteins fail to react with either SYM10 or Y12

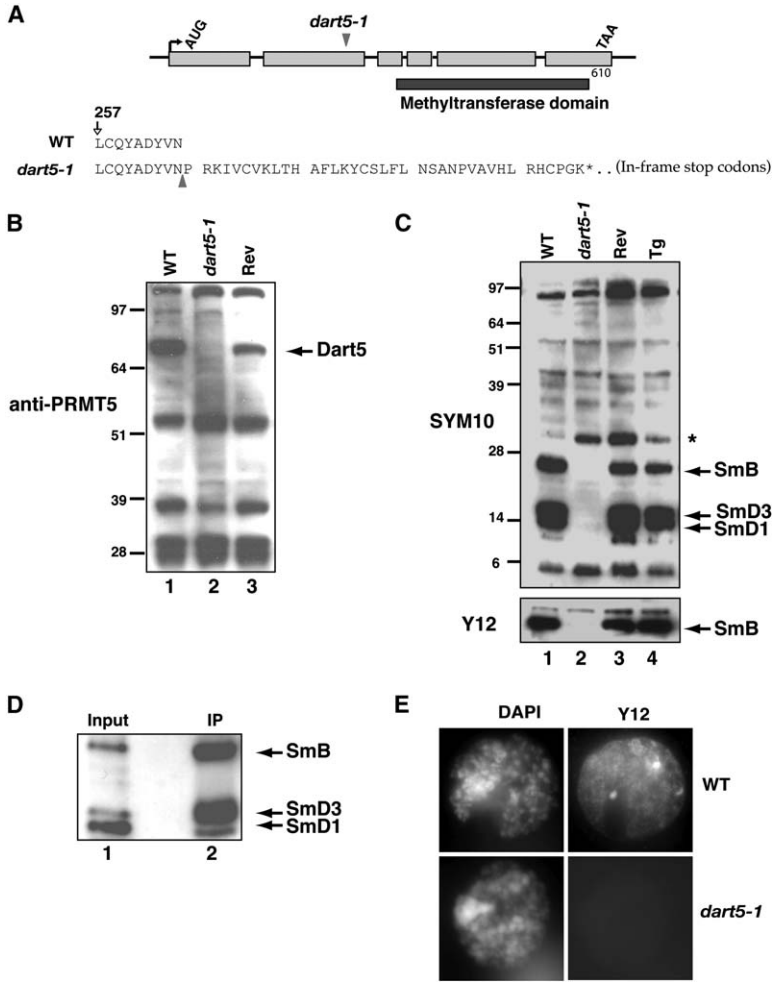

Figure 2. Dart5 Is Required for Symmetric Arginine Dimethylation of Sm Proteins in the Fly

(A) Predicted genomic organization of the *dart5* gene. The arrow indicates the position of the piggyBac element in *dart5-1*. The shaded box denotes the location of the highly conserved methyltransferase domain. Indicated below is the amino acid sequence of the wild-type and mutant *dart5* alleles. The transposon insertion site (arrowhead) and the in-frame stop codon (asterisk) are indicated.

(B) Western blotting of lysates from three adult strains: wild-type (WT, lane 1), *dart5-1* mutant (lane 2), and excision repair (Rev, lane 3). The lysates were analyzed with a PRMT5 antibody. Note the presence in the wild-type sample (arrow, lane 1) of a 70 kDa band that is consistent with the predicted size of Dart5. This band is absent in the mutant lysates (lane 2) and present in the revertants generated by excision repair of the transposon (lane 3).

(C) Lysates from four adult strains—wild-type (WT, lane 1), *dart5-1* (lane 2), an excision-repair allele (Rev, lane 3), and a *dart5-1* strain expressing a wild-type myc-*dart5* transgene (Tg, lane 4)—were analyzed by western blotting. The transgene was expressed under control of the low-level, constitutively expressed *armadillo* promoter by using the UAS-GAL4 system. Blots were probed with sDMA-specific antibodies SYM10 (top blot) and Y12 (bottom blot). Note that SmB, SmD1, and SmD3 (arrows) were not sDMA modified in the *dart5-1* lysates (lane 2). However, modification of these proteins was restored upon excision repair of the transposon (lane 3) or by rescue of the *dart5-1* mutant with a wild-type transgene (lane 4). The intensity of the cross-reacting band indicated by the asterisk in the SYM10 blot was inconsistent and failed to correlate with the presence or absence of Dart5 activity.

(D) Lysates were prepared from wild-type 0–1 hr embryos, and snRNPs were purified with the TMG antibody. The immunoprecipitate (lane 2) and load (lane 1) were probed with SYM10. SmB, SmD1, and SmD3 are indicated by arrows.

(E) Gut-cell nuclei from wild-type and *dart5-1* mutant adult flies were stained with the sDMA antibody Y12. Note the absence of nuclear staining by Y12 in the mutant tissues. The nuclei were counterstained with DAPI.

(Figure 2C, compare lanes 1 and 2). This phenotype was confirmed by immunostaining gut cells from *dart5-1* mutant flies (Figure 2E). Importantly, the sDMA modification of Sm proteins was restored by excision repair of the *dart5-1* piggyBac allele or by expression of a wild-type transgene in the mutant background (Figure 2C, lanes 3 and 4, respectively). Thus, Dart5 is essential for the sDMA modification of Sm proteins in the fly.

Valois Is Required for Dart5 Expression and sDMA Modification of Sm Proteins in Vivo

In HeLa cells, PRMT5 associates in a complex along with MEP50/WD45 [15, 16]. Bacterially produced PRMT5 has little or no methyltransferase activity [16]. However, when the PRMT5 complex, including MEP50, was purified from HeLa cells, it was able to effectively methylate Sm proteins [15, 16]. The recent cloning of the fruit fly *valois* gene showed that it is the *Drosophila* ortholog of human *MEP50/WD45* [22, 23]. Furthermore, like its human counterparts, Valois was found to associate with Dart5 in fly lysates [15, 16, 22]. As such, we tested whether Valois, like Dart5, was required for sDMA modification of Sm proteins in vivo by probing lysates derived from homozygous mutant (vls^3) flies with sDMA antibodies. As shown in Figure 3A, Sm proteins in the vls^3 lysate failed to react with either SYM10 or Y12 antibodies. Rather unexpectedly, we found that *valois* mutant lysates did not contain detectable Dart5 protein (Figure 3B). Because Dart5 and Valois interact in vivo, it is likely that complex formation is required for stability of Dart5. Most important, our findings suggest that phenotypes previously attributed to the loss of Valois may actually be due to codepletion of Dart5.

Symmetric Dimethylation of Sm Proteins Is Not Required for snRNP Biogenesis

After methylation by the PRMT5 complex, the Sm proteins are assembled in the cytoplasm by the SMN complex onto newly transcribed snRNAs that have been exported from the nucleus [1, 24]. Once the Sm core

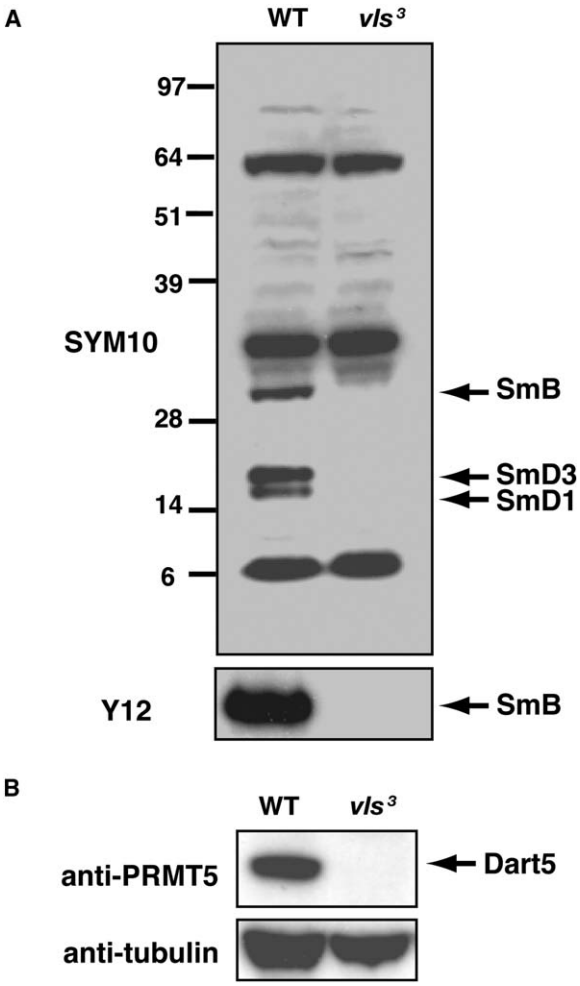

Figure 3. Valois Is Required for sDMA Modification of Sm Proteins and for Dart5 Expression

(A) Lysates were prepared from wild-type (lane 1) and *vls*^{3} (lane 2) adult flies. The lysates were analyzed with sDMA-specific antibodies, SYM10 (top panel) and Y12 (bottom panel). Note that the bands corresponding to SmB, SmD1, and SmD3 are specifically absent from the *vls*^{3} lysates (lane 2).

(B) Lysates were prepared from wild-type (lane 1) and *vls*^{3} (lane 2) adult flies. Western analysis was performed with the PRMT5 antibody (top panel). In the absence of Valois activity, Dart5 expression is undetectable. The blot was stripped and reprobed with a tubulin antibody to control for loading (bottom panel).

has been assembled, the 5′ cap of the snRNA is hypermethylated to form a trimethylguanosine (TMG) moiety by a protein called Tgs1, again in collaboration with the SMN complex [25]. The snRNPs are then imported back into the nucleus, where the final steps of snRNP maturation take place, including the binding of various snRNP-specific proteins [1, 24]. Because the PRMT5 complex is thought to facilitate Sm core assembly [4, 15, 16], it was quite surprising that the *dart5-1* allele was fully viable. Heterozygous intercrosses yielded the expected number of homozygous mutant flies. We therefore analyzed lysates of both wild-type and *dart5-1* adults for defects in snRNP biogenesis by assaying three different stages of RNP assembly. As shown in Figures 4A–4C, the amounts of U1 and U2 snRNA that coprecipitated with SmF, TMG cap, or SNF (a U1- and U2-specific protein) antibodies were analyzed by northern blotting and found to be indistinguishable between the wild-type and *dart5-1* lysates.

We also monitored snRNP import into the nucleus in the absence of Sm sDMA modification by processing tissues from the *dart5-1* mutant flies for immunofluorescence with the TMG antibody. A block in snRNP biogenesis should result in the cytoplasmic accumulation of snRNPs. However, in both wild-type tissues and those from *dart5-1* mutant flies, snRNPs were predominantly localized in the nucleus, consistent with proper snRNP maturation (Figure 4D). Furthermore, when *dart5-1* homozygous females were mated to heterozygous males, we again obtained homozygous progeny in the expected numbers. Thus the mutant animals can survive for two generations in the complete absence of *dart5* function. Although it is possible that the kinetics of snRNP biogenesis might be affected by loss of sDMA modification, the viability of the adult animal for two successive generations suggests that such a kinetic reduction, if it exists, is inconsequential. On the basis of these observations, we conclude that sDMA modification of Sm proteins is required for neither snRNP biogenesis nor pre-mRNA splicing.

Methylation of Sm Proteins by Dart5 Is Required for Efficient Interaction with SMN

Previously, human SMN was shown to preferentially bind to sDMA-modified Sm proteins in vitro [8, 15, 18]. We therefore tested, using RNAi, whether this preference held true in *Drosophila* cells. *Drosophila* S2 cells were incubated with *dart5* dsRNA and subsequently transfected with plasmids expressing GFP-SmB and FLAG-dSMN. The association between SMN and SmB was monitored by immunoprecipitating GFP-SmB and then examining the level of coprecipitated FLAG-dSMN. As shown in Figure 4E, *dart5* dsRNA treatment significantly reduced the SMN-SmB protein interaction. Given the recent findings that human PRMT5 cooperates with SMN in the cytoplasmic assembly of Sm cores [4, 15, 16], it is curious that snRNP biogenesis was unaffected in *Drosophila*, despite a decrease in the apparent affinity of SMN for Sm proteins.

Mutations that result in reduced levels of SMN protein are correlated with the human disease spinal muscular atrophy (SMA) [6]. This disorder is characterized by degeneration of spinal motor neurons followed by atrophy in the limbs and trunk [26, 27]. In contrast to the well-characterized SMA phenotypes, *dart5-1* mutants display no obvious locomotor defects at either larval or adult stages (data not shown). Furthermore, consistent with normal snRNP assembly and function, analysis of the thoracic musculature in adult *dart5* mutants revealed no obvious degeneration or disorganization (data not shown). It is possible that, under conditions where SMN protein is limiting, Dart5-mediated sDMA modification of Sm proteins might be required for snRNP assembly. Future studies will be required to address this question.

It is interesting to note that Azzouz et al. [28] recently hypothesized that the SMN protein has two modes of Sm binding: a high-affinity binding mode that requires methylation for interaction with the C-terminal tails of the Sm proteins and a lower-affinity one that

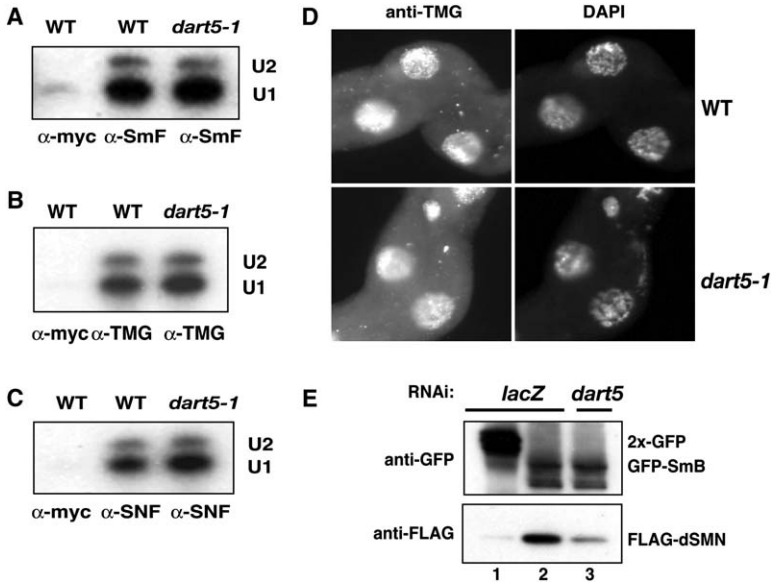

Figure 4. Symmetric Arginine Dimethylation of Sm Proteins Is Dispensable for snRNP Biogenesis

(A) Sm core assembly was analyzed in wild-type (lanes 1 and 2) and *dart5-1* mutant lysates (lane 3) with an SmF antibody (lanes 2 and 3). The myc antibody served as a control for specificity (lane 1). The bound RNAs were extracted, precipitated, and analyzed by northern blotting with probes specific for U1 and U2 snRNAs.

(B) TMG capping was analyzed in wild-type (lanes 1 and 2) and *dart5-1* mutant lysates (lane 3) with a TMG antibody (lanes 2 and 3). Controls and blotting were as in panel (A).

(C) The association of SNF, a U1- and U2-snRNP-specific protein, was analyzed in wild-type (lanes 1 and 2) and *dart5-1* mutant lysates (lane 3) with an SNF antibody (lanes 2 and 3). Controls and blotting were as in panel (A).

(D) Gut tissues from wild-type and *dart5-1* mutant adult flies were processed for immunofluorescence with a TMG cap antibody. Note that the nuclear staining of snRNPs by the TMG antibody was unaffected in the *dart5-1* tissues. The nuclei were counterstained with DAPI.

(E) Schnieder cells (1.0×10^6) were treated with 15 μg of either lacZ (lanes 1 and 2) or *dart5* dsRNA (lane 3) for 72 hr. Subsequently, 2×GFP-NLS (lane 1) or GFP-SmB (lanes 2 and 3) was transfected. In addition, FLAG-SMN was cotransfected into the cells. Twenty-four hours after transfection, cells were harvested, lysates were prepared, and the GFP-tagged proteins were immunoprecipitated with a polyclonal GFP antibody. The immunoprecipitated proteins were analyzed with FLAG and GFP monoclonal antibodies. In the presence of *dart5* dsRNA, the level of the SMN-Sm interaction was significantly reduced.

recognizes Sm folds of these proteins in a methylation-independent fashion. Our observation that snRNP biogenesis is unaffected by loss of *dart5* (Figures 4A–4D) is consistent with the existence of the two binding modes. We conclude that either the residual level of the SMN-Sm interaction, observed in our assay (Figure 4E), is sufficient to mediate snRNP biogenesis in vivo or that such an interaction is not required for snRNP assembly in the fly.

Dart5 Is Required for Male Fertility

Although *dart5-1* male flies display no obvious SMA-like phenotypes, they are completely sterile. Upon initial examination, testes from *dart5-1* males looked similar in size and structure to control testes (data not shown). However, preparation of testis squashes revealed several defects in spermatocyte maturation (Figure 5). In *Drosophila*, spermatocyte maturation takes place within cysts. In later stages, each cyst contains 64 spermatocytes that are connected by cytoplasmic bridges. Individualization is a coordinated process that involves a variety of cytoskeletal components that enclose each spermatid within its own plasma membrane. Also, during the process of individualization, sperm nuclei undergo a drastic change in shape from a rounded to an elongated, needle-like form [29]. In comparison to wild-type testes, which contain numerous sperm bundles undergoing maturation in an organized manner, *dart5-1* testes contained many fewer bundles. Furthermore, there was a dramatic loss of organization among the *dart5-1* sperm bundles (Figure 5). Additionally, the average bundle in *dart5-1* testes contained far fewer spermatid nuclei than in the wild-type control. Motile sperm were found in great numbers in the seminal vesicles of wild-type testes. In contrast, the seminal vesicles of *dart5-1* males were almost completely devoid of sperm (Figure 5). This phenotype is due to the loss of *dart5*, because the male fertility and all of the aforementioned spermatogenesis defects can be rescued by ubiquitous expression of UAS-myc-*dart5* under the control of the *armadillo*-GAL4 driver (data not shown).

Dart5 Is Required for Germ-Cell Specification

Unlike *dart5-1* males, mutant females are fertile, albeit with slightly reduced fecundity. When crossed to wild-type males, approximately 65% of the embryos from *dart5-1* mutant mothers hatched into larvae that developed normally into adult flies. Cuticle preparations revealed that the remaining 35% of the embryos displayed a variety of segmentation defects, as compared to wild-type (Figures 6A and 6B). However, analysis of the embryos that developed normally revealed that 100% were completely agametic, and thus sterile. As such, *dart5*, like *valois*, is a grandchildless gene [20]. A defining feature of grandchildless mutations is a failure to form primordial germ cells. Early in development of *Drosophila* embryos, specialized cells called pole cells are formed at the posterior of the organism. During the course of development, pole cells migrate from their posterior location to eventually colonize the developing gonad. All of the germ cells in the adult animal are derived from these pole cells. As such, formation of pole cells is one of the earliest events in the specification of the germline [30]. We therefore examined *dart5-1* embryos for pole-cell formation. Consistent with the grandchildless phenotype, we found these embryos to be completely devoid of pole cells (Figures 6E and 6F). This phenotype was rescued by specifically driving the

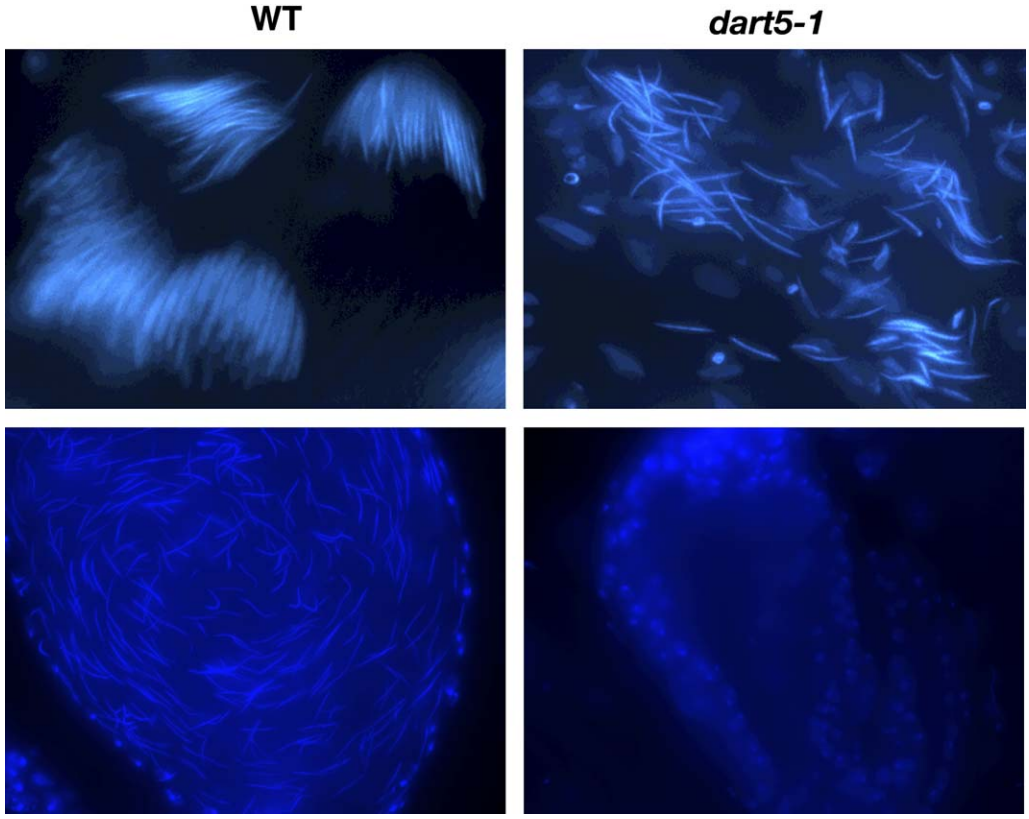

Figure 5. Dart5 Is Required for Proper Spermatocyte Maturation

Testes were dissected from wild-type and *dart5-1* adult male flies, fixed in formaldehyde, and counterstained with DAPI (blue). Shown in the top panel is a section of the testis in which sperm bundles are matured. Note that in comparison to the wild-type control, the *dart5-1* testis is characterized by a general lack of spermatid organization within the bundles. The bottom panel represents the seminal vesicles from wild-type and *dart5-1* testes. Unlike the wild-type seminal vesicle, which contains numerous mature sperm, the *dar5-1* seminal vesicle is devoid of sperm.

expression of a UAS-myc-*dart5* transgene in the female germline with a *nanos*-GAL4 driver (Figures 6G and 6H). Furthermore, the pole cells formed in the transgeneic flies were functional, as indicated by their ability to migrate and populate the developing gonad (Figures 6I and 6J). When the flies were incubated at 22ºC, the rescue frequency was approximately 30%. The transgene was constructed with a pUAST vector. Although GAL4-driven expression by this vector is tight, expression in the female germline is rather inefficient [31]. Given that the goal of the experiment was to determine whether germline-only expression of Dart5 was sufficient to restore pole-cell formation, we chose this vector because the level of GAL4-independent expression is very low. The caveat of this strategy, however, was that we obtained incomplete rescue of pole-cell formation. Because the activity of GAL4 can be enhanced at higher temperature [31], we found that incubation of the embryos at 29ºC increased the frequency of rescue to approximately 70%, consistent with a GAL4-dependent effect. Importantly, those embryos that formed pole cells were capable of developing into fertile adults.

Tudor Localization Is Defective in *dart5-1* Ovaries

Pole-cell formation is directed by a number of gene products that act in a sequential and hierarchical manner. One of the earliest players in the germ-cell specification pathway is *oskar* [32]. During oogenesis, *oskar* mRNA and protein are localized to the posterior of the developing oocyte [33]. The localization of Oskar protein is essential for proper formation of the posterior pole plasm, an event that is required not only for germ-cell specification, but also for abdominal patterning [32]. In *valois* mutants, the localization of *oskar* mRNA and protein in the oocyte is not disrupted [22, 23]. However, late in oogenesis, there is a drastic reduction in the level of Oskar protein in *valois* mutant ooctytes [22, 23]. In contrast to the *valois* phenotype, the level of Oskar protein in *dart5-1* embryos remained relatively unchanged. In wild-type and *dart5-1* preblastoderm embryos, Oskar was localized to the posterior of the embryo at relatively equivalent levels (Figures 7G, 7H, 7K, and 7L). In wild-type blastoderm embryos, Oskar localized to the pole cells (Figures 7I and 7J). However, in *dart5-1*embryos, pole cells are absent, yet Oskar was still detected at the posterior (Figures 7M and 7N).

Another factor required for pole-cell formation is Vasa, a DEAD-box protein [34]. Vasa acts downstream of Oskar in the germ-cell specification pathway [30]. In *valois* mutant oocytes, the localization of Vasa was not disrupted [22, 23]. However, Vasa is absent from blastoderm-stage embryos laid by *valois* mutant females [22, 23]. To ascertain whether Dart5 and Valois participate in the same pathway in pole-cell formation, we examined the localization of Vasa protein in embryos laid by *dart5-1* females. Similar to the *valois* mutant phenotype,

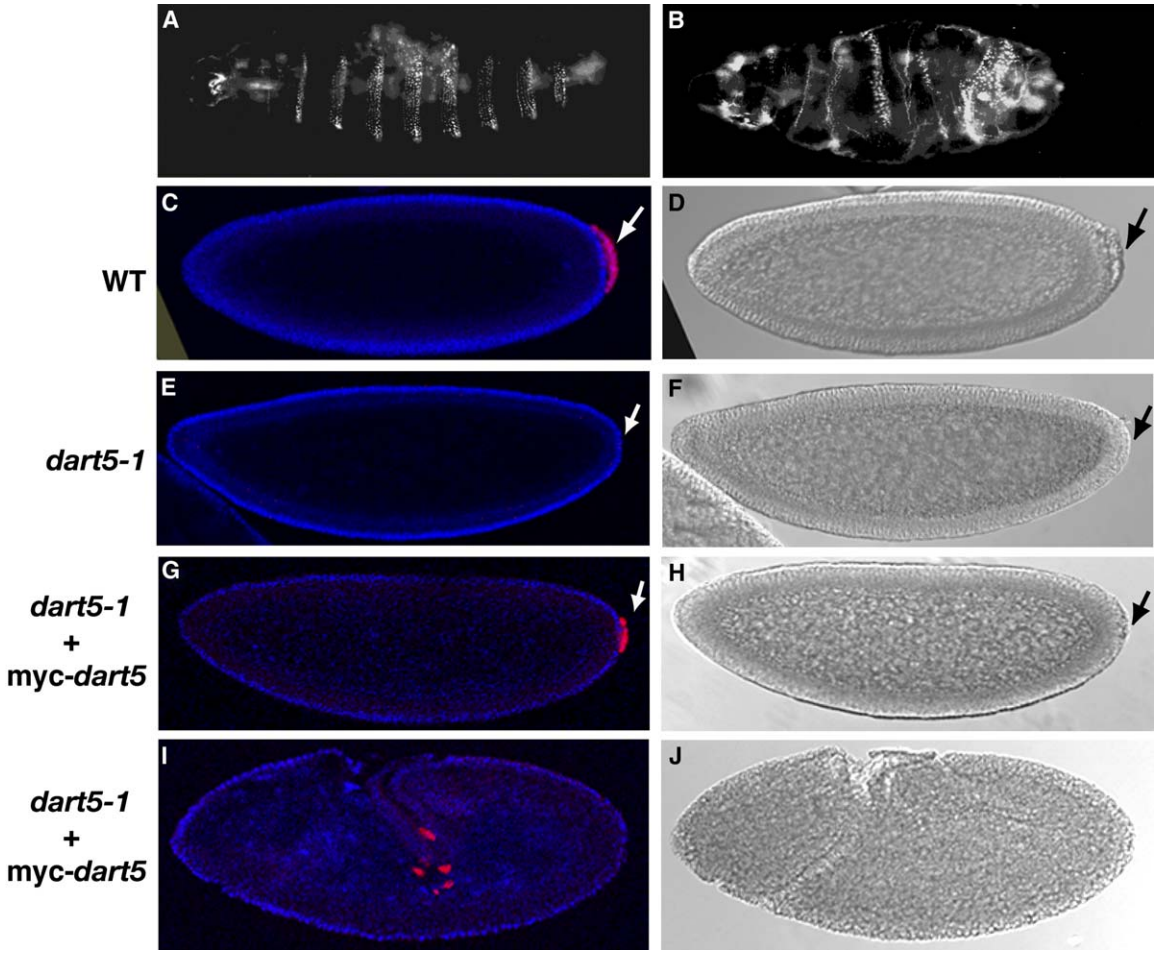

Figure 6. Dart5 Is a Grandchildless Gene

The *dart5-1* embryos displayed a hatching frequency of 65% (n > 100). The embryos that fail to hatch display a variety of abdominal defects; compare A (wild-type) to B (*dart5-1*). Pole-cell formation was examined in wild-type and *dart5-1* embryos. The embryos were immunostained for Vasa (red) and counterstained with DAPI (blue). In contrast to wild-type blastoderm embryos (C and D), *dart5-1* blastoderm embryos (E and F) did not form pole cells (arrows). In addition, Vasa, which normally localizes to the posterior and within the pole cells (arrow, [C]), was absent in *dart5-1* blastoderm embryos (E). Pole-cell formation and Vasa localization was rescued in the *dart5-1* mutants by specifically expressing myc-*dart5* in the female germline (G and H). The myc-*dart5* transgene was expressed under the control of the *nanos* driver by using the UAS/GAL4 system. Furthermore, the pole cells thus formed were functionally active, as indicated by their ability to migrate and colonize the developing embryonic gonad (I and J).

we observed an absence of Vasa staining in *dart5-1* blastoderm embryos (Figures 6C and 6D). However, we found that in *dart5-1* and vls^3 preblastoderm embryos, the localization and intensity of Vasa staining in the posterior of the embryo were comparable to those of wild-type embryos (Figures 7A–7F). Therefore, between these two developmental stages, the absence of Dart5 and Valois activity from the embryo results in the disappearance of Vasa. It is possible that, in the absence of pole-cell formation, Vasa is destabilized and hence does not accumulate in the posterior of the developing embryo.

Interestingly, the *dart5* phenotype is very similar to that of embryos containing mutations in a gene called *tudor* [21, 35]. Tudor acts downstream of Vasa in the germ-cell specification pathway. To examine the effect of the *dart5* mutation on Tudor levels, we probed lysates from wild-type and *dart5-1* ovaries with a Tudor antibody (Figure 8A). In comparison to wild-type ovarian lysates, the level of Tudor was decreased in the *dart5-1* mutant. In contrast, the level and methylation status of Sm proteins was not affected in *tudor* mutants (Figure 8B). It was recently shown that Valois not only interacts with Tudor, but is also required for proper localization of Tudor in the oocyte [22]. In the absence of Valois, Tudor does not accumulate within the nurse-cell nuage and is absent from the polar plasm of stage 10 oocytes [22]. However, in the same oocytes, Oskar and Vasa remain properly localized [22]. Thus the first defect observed in *valois* mutants is the disappearance of Tudor. Only at later time points in the development of the organism do the Oskar and Vasa phenotypes manifest themselves. Because *valois* mutants affect the levels of Dart5 (Figure 3), we examined the localization of Tudor in *dart5-1* mutant ovaries. In comparison to wild-type egg chambers, Tudor was not as prominent within the nurse-cell nuage (Figures 8C, 8D, 8G, 8G′, and 8H); however, an occasional accumulation of

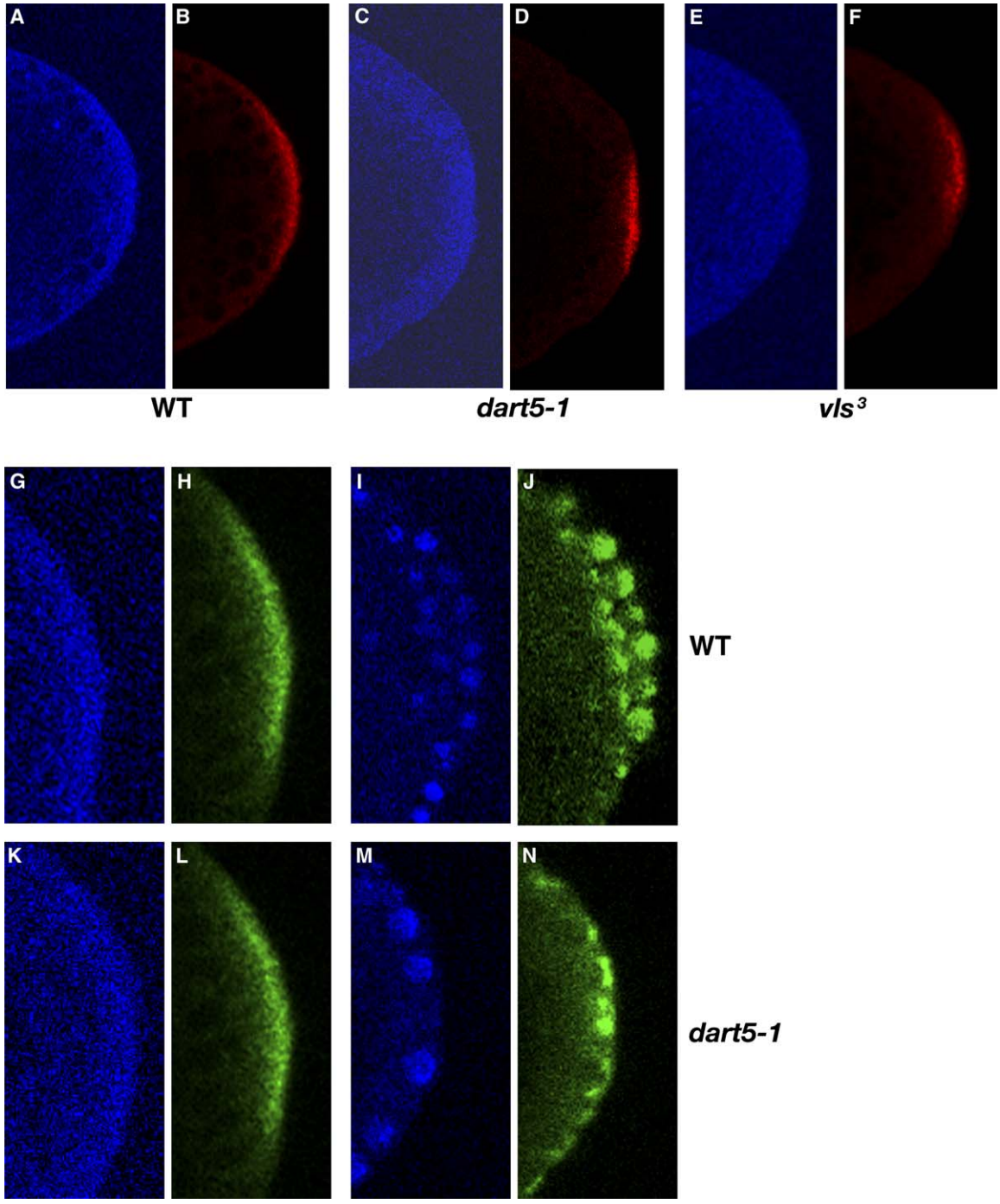

Figure 7. *dart5* Acts Downstream of *oskar* and *vasa* in the Germ-Cell Specification Pathway

The localization of Vasa (red) was examined in preblastoderm wild-type (A and B), *dart5-1* (C and D), and vls^3 embryos (E and F). Note that at this developmental time point, the posterior localization of Vasa was maintained in the mutants. Similarly, the localization of Oskar (green) was examined in preblastoderm wild-type (G and H) and *dart5-1* (K and L) embryos. The localization of Oskar was identical between the two alleles. In addition, the localization of Oskar was also examined in wild-type (I and J) and *dart5-1* (M and N) blastoderm embryos. In wild-type embryos, Oskar was found at the posterior and also localized to the pole cells (I and J). In contrast, no pole cells were found in *dart5-1* embryos (M and N and also Figures 5C and 5D); however, Oskar remained localized to the posterior pole in these embryos (M and N). All embryos were counterstained with DAPI (blue).

protein could be detected in this region (Figure 8G′, arrow). Although accumulation within the nuage was somewhat defective, localization of Tudor to the posterior pole plasm of stage 10 oocytes was significantly inhibited (Figures 8E–8J). Thus, consistent with the *valois* mutant phenotype, Tudor localization was disrupted in *dart5-1* mutant egg chambers.

On the basis of these findings, it appears that Valois and Dart5 primarily affect Tudor. As a consequence, later-stage defects in pole-cell formation and Vasa

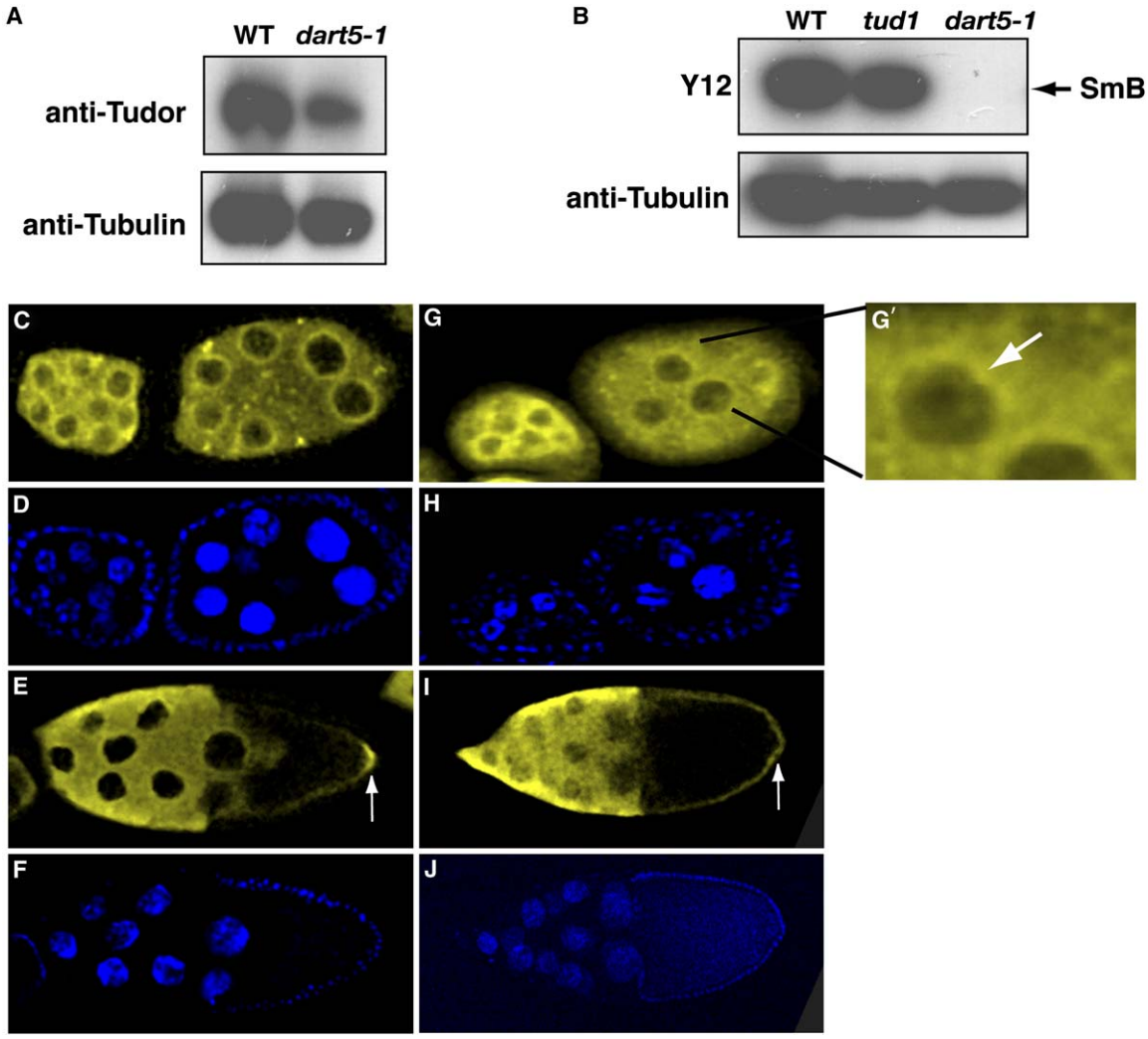

Figure 8. Dart5 Is Required for Proper Localization of Tudor to the Nuage and Pole Plasm

(A) Lysates were prepared from wild-type (lane 1) and *dart5-1* ovaries (lane 2). The lysates were probed with a Tudor antibody (top panel). Note that the level of Tudor is significantly decreased in the *dart5-1* lysates. The blot was reprobed with a tubulin antibody (bottom panel) to serve as a loading control.

(B) Lysates were prepared from wild-type (lane 1) and *tud-1* ovaries (lane 2). The lysates were probed with sDMA antibody Y12 (top panel). The level and methylation status of SmB protein was unaffected. The blot was reprobed with a tubulin antibody (bottom panel) to serve as a loading control.

(C–J) The localization of Tudor was examined in wild-type and *dart5-1* mutant ovaries. Note that in intermediate-stage *dart5-1* egg chambers, prominent localization of Tudor to the nurse-cell nuage is disrupted (G and H). However, a slight accumulation of Tudor to the vicinity of the nuage could still be detected (G′). In contrast to wild-type oocytes, Tudor was not enriched at the pole plasm in stage 10 *dart5-1* oocytes ([I and J], arrows).

localization can be observed in *dart5-1* embryos (Figures 6 and 7). However, it is quite likely that *dart5* and *valois* have functions in addition to Tudor regulation, given that males mutant for *dart5* and *valois* display fertility defects (Figure 5 and data not shown). In contrast, *tudor* males are completely fertile [35]. Thus, in addition to germ-cell specification, Dart5 and Valois play downstream roles in germ-cell development in males. Other differences exist as well. In contrast to the *dart5-1* phenotype, Oskar protein is not found at the posterior in blastoderm stage *tudor* null embryos [35]. Furthermore, only about 15% of *tudor* null embryos hatch [35], whereas the hatching frequency of eggs derived from *dart5-1* mothers was much higher (65%).

Although Dart5 activity is required for Tudor function, *dart5* does not fit the mold of a classical posterior-group gene. In order to be placed directly in the germ-cell specification pathway, upstream of *tudor*, the *dart5* phenotype should be at least as strong as that of *tudor*. It is not. Similarly, mutations in *vasa* do not have an appreciable effect on Dart5 activity, as measured by Sm-protein methylation (data not shown). Thus we propose a revised model (see Figure S1 in the Supplemental Data available online) of the germ-cell specification pathway, wherein *dart5* (and *valois*) primarily affect Tudor localization, resulting in a loss of pole-cell formation. However, unlike *oskar* and *vasa* mutations, somatic patterning appears to be relatively unaffected. Because 15% of

tudor null embryos develop normally, Thomson and Lasko have suggested that Tudor is not directly required for posterior patterning [35]. However, *tudor* null embryos contain fewer polar granules than wild-type embryos and never form pole cells [35]. Thus a fully functional pole plasm may be required for stabilizing the level or maintaining the localization of factors involved in establishing the body plan. In such a scenario, it is not surprising that a subset of *tudor* null embryos display patterning defects [35]. Given that mutation of *dart5* appears to compromise Tudor function, a small fraction of *dart5-1* embryos also display patterning defects.

The elevated hatching frequency of *dart5-1* as compared to *tudor* null embryos and the residual accumulation of Oskar in *dart5-1* blastoderm embryos suggest that a partially functional pole plasm is formed in the absence of Dart5. However, this level of functionality is insufficient to mediate germ-cell specification, given that 100% of the embryos that develop are agametic. Because Tudor is only modestly reduced in *dart5-1* mutant ovaries (Figure 8A) in comparison to its complete absence from *tudor* null ovaries [35], it is logical that the *dart5-1* phenotype would be less severe than the *tudor* null phenotype. Although Tudor was not enriched at the posterior pole in *dart5-1* oocytes (Figure 8I), neither was it completely absent from this location (data not shown). As such, the residual level of Tudor, along with properly localized Oskar and Vasa, might be sufficient to assemble a partially functional pole plasm in the oocyte.

Valois and Dart5

Given their in vivo association, it is not surprising that *valois* and *dart5* share many phenotypes: absence of pole cells, male sterility, and loss of Sm-protein sDMA residues. Despite the similarity of the mutant phenotypes, there are a few differences worth noting. For instance, the spermatocyte maturation defect was less severe in the *valois* mutant as compared to *dart5-1* (data not shown). Additionally, unlike the *dart5-1* mutant, the vls^3 mutant displayed a rather strong maternal-effect lethal phenotype. This result is consistent with a previous finding that *valois* mutants displayed pleiotropic defects during cellularization [36]. In addition, whereas *valois* mutants affect the level of Oskar protein in ovaries, there is no apparent Oskar defect in *dart5-1* mutants. Thus Valois may have additional functions outside of its complex with Dart5.

Sm Proteins, Germ-Cell Specification, and the Tudor Connection

In this report, we identified Sm proteins as in vivo targets of Dart5. Furthermore, we showed that Valois is also required for the sDMA modification of Sm proteins and proper expression of Dart5. In the absence of Dart5 and Valois, germ-cell specification, but not general snRNP biogenesis, is disrupted. These observations point to a model whereby Sm proteins, or more precisely symmetrical arginine dimethylation of Sm proteins, play a critical role in germ-cell specification. Consistent with this hypothesis, Sm proteins are thought to play a specific role, unrelated to splicing, in P granule integrity germ-cell specification in *C. elegans* [37, 38]. P granules are structurally and functionally related to the nuage of *Drosophila*. Like the *Drosophila* nuage, P granules are RNA rich and contain a number of proteins that have critical roles in germ-cell development. Importantly, Valois is localized to, and is required for, the proper formation of the nurse-cell nuage in *Drosophila* [22]. Another prominent component of the *Drosophila* nuage is Tudor [22, 39]. In mouse spermatocytes, Mouse-Tudor-Repeat gene1 (MTR-1) is localized to the nuage and specifically associates with Sm proteins therein [40]. Furthermore, the nuage of *Xenopus* oocytes was also shown to specifically contain Sm proteins [41]. It will therefore be of great interest to determine whether Sm proteins are components of the nuage in *Drosophila*. In the absence of Dart5 activity, prominent Tudor localization to the nuage is disrupted. Sm-protein methylation may therefore be required for maintaining proper integrity of the *Drosophila* nuage. In order to more fully explore this hypothesis, ultrastructural analyses will be required.

Tudor is the founding member of a family of proteins that contain Tudor domains [42]. Several lines of evidence point to a function for Tudor domains as methyl binding protein modules. First, the SMN protein contains a single Tudor domain, mutation of which causes a significant decrease in binding affinity for Sm proteins [43, 44]. Second, molecular modeling studies suggest that Tudor domains are structurally related to other domains, such as the Chromo domain, that are known to bind methylated proteins [45]. Third, SMN binding to Sm proteins decreases upon loss of methylation [15, 18]. Finally, Cote and Richard [46] recently showed that two other Tudor-domain proteins, splicing factor 30 (SPF30) and Tudor-domain-containing 3 (TDRD3) interact with Sm proteins in a methylation-dependent manner. *Drosophila* Tudor contains 11 such protein motifs [47]. Thus, it is plausible that Tudor interacts with sDMA residues within the C termini of Sm proteins and that this interaction is somehow required for Tudor function and, consequently, for germ-cell development. Experiments designed to examine this hypothesis are ongoing. In this regard, it is noteworthy that disruption of *dart5* affects the levels of Tudor protein and its localization within the egg chamber.

We have shown that symmetrical dimethylation of arginine residues within the Sm proteins is lost upon disruption of *dart5*, the *Drosophila* ortholog of PRMT5. We cannot rule out the possibility that, in the absence of Dart5 activity, Sm proteins might contain other post-translational modifications (e.g., monomethylated or asymmetrically dimethylated arginine residues). However, correlated with the loss of symmetric dimethylation of Sm proteins is a complete failure to develop germ cells in subsequent generations. Expression of myc-tagged Dart5 only in the female germline via a *nanos* driver rescued pole-cell formation in early embryos and Vasa localization to the developing gonad. One interpretation of these observations is that symmetric dimethylation of Sm proteins plays a central role in specifying the germline. The *dart5-1* allele will be a valuable tool in exploring this hypothesis. If Sm proteins do play a role in germ-cell specification, simple mutational or knockout experiments will not be useful in uncovering the mechanism, because these alterations cause somatic-cell lethality. RNAi of Sm proteins in *C. elegans*, while causing a disruption in the localization and integrity of

P granules, was also coupled with embryonic lethality [38]. The available mutations in *Drosophila* Sm proteins are likewise all lethal.

Conclusions

Taken together with the work of Barbee et al. [38], our results suggest that Sm proteins play at least two distinct roles in the organism, one a general function in pre-mRNA splicing and the other in germ-cell specification and maintenance. The *dart5-1* allele is very informative in this regard because it uncouples these two functions: snRNP biogenesis and splicing are ongoing in *dart5-1* homozygotes, but germ-cell specification is disrupted. Given the similar phenotypes of the *dart5* and *valois* mutants, the function of the Tudor domain, the delocalization of Tudor in *dart5-1* egg chambers, and the available data on the localization of Sm proteins to the nuage in several different species, the strongest interpretation favors a critical role for Sm proteins in germ-cell specification. Although we favor this hypothesis, we cannot at this time rule out the possibility that, for example, loss of methylation of some other protein causes the observed phenotypes. Future work should provide much-needed mechanistic insight into this question. In this regard, it will be particularly important to determine whether Sm proteins are components of the nuage and pole plasm in *Drosophila*. If so, it will be most interesting to elucidate whether they are associated with snRNAs or are complexed with a different class of RNA.

Experimental Procedures

Fly Stocks

Oregon-R was used as the wild-type strain. Unless otherwise specified, all balancer chromosomes and dominant markers used in this study were described in [48]. The *dart5-1* stock was obtained from the Exelixis collection, housed at Harvard Medical School (stock number e00797). We note that the original Exelixis stock displayed pleiotropic ovarian phenotypes that are unlinked to the *dart5* mutation. The original stock was isogenized and rebalanced with a CyO-Act5c::GFP balancer. The pleiotropic ovarian phenotypes were not observed in the rebalanced stock. The vls^3 and *tud1* mutants were obtained from the Bloomington Stock Center. The *dart5* transgene was constructed by cloning *dart5* cDNA into the pPUAS-HM vector [49]. The injections and subsequent balancing of the transgenic flies were performed by BestGene. All stocks were cultured on standard cream of wheat-agar medium at room temperature (25ºC ± 1ºC) in half-pint bottles.

Lysate Prep and Precipitations

Schneider cell (S2) soluble lysates were prepared by resuspending the cells in RIPA buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% NP-40, 1 mM EDTA) containing protease inhibitors (Halt protease-inhibitor cocktail kit, Pierce) and passing several times through a 25 guage needle. The lysate was cleared by centrifugation at 10,000 × g for 10 min at 4ºC. Fly lysates were likewise prepared by homogenizing adult flies in RIPA buffer containing protease inhibitors and clearing by centrifugation at 10,000 × g for 10 min at 4ºC. For the methylation assay, GFP-SmB was precipitated from 500 μg S2 cell lysate by using a polyclonal GFP antibody (Abcam) bound to Protein A beads. The snRNP assays were performed by using anti-SmF, anti-TMG, or anti-SNF bound Protein A beads. For these experiments, 100 μg adult fly lysate was used. In order to assess the SMN-Sm interaction, we precipitated GFP-SmB from 500 μg of S2 lysate by using a polyclonal GFP antibody.

Northern Blotting

After the precipitations, RNA was extracted with a (125:24:1) mixture of phenol, choloroform, and isoamyl alcohol. The RNA was subsequently precipitated, run on a 10% polyacrylamide-Urea gel, transferred to a nylon membrane, and probed with ^{32}P-labeled PCR products corresponding to the *Drosophila* U1 and U2 snRNAs.

Antibodies

For immunoprecipitation of GFP-SmB, a polyclonal GFP antibody (Abcam) was used. The immunoprecipitated protein was subsequently analyzed with the monoclonal antibody Y12 (gift of J. Steitz) and a monoclonal GFP antibody (Roche). The efficiency of RNAi-mediated knockdown of myc-Dart5 was assessed with a polyclonal myc antibody (SantaCruz Biotechnology). In order to determine the expression of Dart5 in wild-type and mutant lysates, we used a polyclonal antibody directed against PRMT5 (Upstate). The antibody was created against an N-terminal peptide of PRMT5 that is well conserved in Dart5. The methylation status of the lysates was analyzed with the Sym10 (Upstate) and Y12 sDMA antibodies. Precipitation of Sm cores was accomplished by using an SmF antibody (gift of R. Lührmann). TMG capping of snRNA was analyzed with anti-TMG beads (Calbiochem). SNF was immunoprecipitated with the 4G3 monoclonal antibody (gift of H. Salz). snRNP import into the nucleus was analyzed with a monoclonal TMG antibody (Calbiochem). The SMN-Sm interaction was examined with a monoclonal Flag antibody (Sigma) and a polyclonal GFP antibody (Abcam). The Vasa and Tudor antibodies were kind gifts of P. Lasko. The Oskar antibody was a kind gift of A. Ephrussi.

Immunofluorescence

Immunostaining of embryos was performed as described [50], with minor variations. Anti-rabbit Alexa 594 (Molecular Probes) was used as the secondary antibody. For adult tissues, animals were dissected in 1× PBS and fixed in 4% formaldehyde for 20 min. Anti-mouse FITC (Molecular Probes) was used as the secondary antibody. Immunostaining of ovaries was performed as previously described [51]. Anti-rabbit Alexa 594 (Molecular Probes) was used as the secondary antibody. Images were captured with either a Leica Confocal Scanner TCS SP2 interfaced with Leica Confocal Software or a Leica DM6000 interfaced with Volocity imaging software and a Retiga Exi digital camera.

Testis and Cuticle Preparations

Testes were dissected in 1× PBS and fixed in 4% formaldehyde for 20 min. The samples were subsequently washed in 1× PBS, stained with DAPI, and mounted onto slides. Cuticle preparations of 24–30 hr embryos were performed with standard techniques. Dark-field microscopy was used to image the embryos.

Supplemental Data

Supplemental Data include one figure and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/11/1077/DC1/.

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