Blanks, a nuclear siRNA/dsRNA-binding complex component, is required for *Drosophila* spermiogenesis

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Small RNAs and a diverse array of protein partners control gene expression in eukaryotes through a variety of mechanisms. By combining siRNA affinity chromatography and mass spectrometry, we have identified the double-stranded RNA-binding domain protein Blanks to be an siRNA- and dsRNA-binding protein from *Drosophila* S2 cells. We find that Blanks is a nuclear factor that contributes to the efficiency of RNAi. Biochemical fractionation of a Blanks-containing complex shows that the Blanks complex is unlike previously described RNA-induced silencing complexes and associates with the DEAD-box helicase RM62, a protein previously implicated in RNA silencing. In flies, Blanks is highly expressed in testes tissues and is necessary for postmeiotic spermiogenesis, but loss of Blanks is not accompanied by detectable transposon derepression. Instead, genes related to innate immunity pathways are up-regulated in *blanks* mutant testes. These results reveal Blanks to be a unique component of a nuclear siRNA/dsRNA-binding complex that contributes to essential RNA silencing-related pathways in the male germ line.

RNAs are well suited as specificity factors for gene regulatory pathways because of their ability to recognize target nucleic acids by base pairing. Organisms ranging from bacteria to humans have systems to generate small RNAs and use the sequence information therein to program a variety of biological outputs (1–3). In eukaryotes, an explosion in the discovery of small (~20–30 nucleotides) RNA pathways (4) has illuminated the many ways in which different organisms orchestrate small RNA biogenesis, specify their targets, and evolve small RNA cofactors such as Argonaute proteins.

Deep sequencing experiments in fungi, nematodes, plants, insects, and mammals have revealed the presence of genome-encoded siRNAs (1, 4–6). In most cases, part of this endogenous siRNA (endo-siRNA) population targets transposon RNAs for silencing. Endo-siRNAs are also born from convergent mRNA transcripts and hairpin RNA structures. Among the endo-siRNAs derived from protein-coding genes, few studies have noted an enrichment in any given gene ontology (GO) group. However, *Caenorhabditis elegans* endo-siRNA profiles showed enrichment for genes involved in spermatogenesis (7).

Small RNA silencing pathways are broadly required for fertility in metazoans. *Drosophila* spermatogenesis requires expression of the proteins Piwi, Aub, and Ago3, which are members of the Piwi subclass of Argonaute proteins (8, 9). Aub and Ago3 cooperate to generate Piwi-associated RNAs (piRNAs) and in the male germ line function mainly to silence transposons in stem, gonialblast, and spermatogonia cells (9–12). In contrast, Piwi expression in the testes is limited to somatic cells proximal to germ line stem cells (12–14). Endo-siRNAs and microRNAs (miRNAs) have also been detected in the testes, and these small RNA classes generally partition into the Ago-subclass Argonaute proteins Ago2 and Ago1, respectively (15).

In *Drosophila* sperm development, germ line stem cells divide asymmetrically to give rise to a gonialblast and a renewed germ line stem cell (16). The gonialblast undergoes four rounds of mitosis to produce a cyst of 16 primary spermatocytes connected by cytoplasmic bridges. After 3 d of growth, the spermatocytes undergo meiosis to yield a syncytial bundle of 64 round spermatids that elongate, differentiate, and individualize into mature sperm. Because expression of Piwi proteins is restricted to the stem-cell niche, gonialblasts, and spermatogonia (12), other small RNA pathways likely support later stages of spermatogenesis. However, the siRNA cofactors Dicer2, Ago2, and R2D2 appear to be unnecessary for male fertility. Therefore, although small RNA-mediated silencing is critical for early stages of spermatogenesis, it is unclear if such a requirement persists or changes during the later stages of sperm development.

Among the central players in *Drosophila* RNA silencing pathways are RNase III enzymes (17–20), double-stranded RNA-binding domain (dsRBD) proteins (21–24), and Argonaute superfamily proteins (8–10, 25, 26). In addition to these and other known silencing factors, it is possible that undiscovered proteins contribute to silencing in specific subcellular locations and tissues. Here we report a screen for siRNA duplex-binding proteins in which we identified the nuclear dsRBD protein CG10630, which we have renamed Blanks for reasons described below. We show that Blanks contributes to RNAi activity in S2 cells, in accordance with findings from a recent screen for RNAi pathway components (27). Interestingly, Blanks is part of a dsRNA-binding complex that is distinct from previously described RNA-induced silencing complexes (RISCs). The nuclear localization and composition of the Blanks-containing complex suggests that this protein performs a unique silencing-related function. In flies, Blanks expression is restricted to postmitotic spermatocytes and persists through meiosis until the onset of nuclear shaping. Loss of Blanks causes complete male sterility marked by a sperm individualization defect and the up-regulation of genes related to innate immunity pathways. These results suggest that a unique branch of the small RNA regulatory network is critical for late stages of sperm development.

Results

Identification of the Double-Stranded-RNA-Binding Protein Blanks. To identify RNA silencing factors we performed siRNA affinity chromatography from *Drosophila* S2 cell extracts (28) using a biotinylated siRNA complimentary to *Pp*-luciferase (luc) (18, 29) (Fig. 1*A*). Analysis of the siRNA-affinity-purified samples re-

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vealed proteins that specifically bound to the siRNA affinity column (Fig. 1*B*, *Upper*). Immunoblotting confirmed that the siRNA affinity column successfully enriched for the known siRNA-binding factor Dicer2 (Fig. 1*B*, *Lower*).

To identify additional factors that were specifically bound to the siRNA affinity purification, we trypsinized the affinity purifications and performed tandem liquid chromatography (LC) and MS (LC–LC–MS/MS). As expected, our analysis detected multiple unique Dicer2 peptides from the siRNA affinity preparations but not from negative controls. Additionally, our MS analysis of the siRNA affinity purifications identified multiple unique peptides encoded by the candidate gene *CG10630*, which we chose to rename *blanks* to reflect its essential role in sperm development (see below). Much like the RNAi factor genes *loqs* and *r2d2*, *blanks* encodes a protein with multiple predicted dsRBDs (Fig. 1*C*). The *Drosophila* genome also includes a closely linked paralogue of *blanks* (*CG12493*), with which it shares 55% amino acid identity and 67% similarity. We generated polyclonal antibodies specific to each paralogue using unique fragments of recombinant protein (Fig. S1 *A* and *B*). Immunoblot analysis with our anti-Blanks antibody confirmed that Blanks is specifically enriched on siRNA duplex affinity columns but not on a similar column complexed with single-stranded siRNA or on streptavidin alone (Fig. S1*C*). Endogenous CG12493 expression could not be detected in S2 cells (Fig. S1*B*).

To test if Blanks plays a functional role in RNAi, we performed a standard dual-luciferase assay for RNAi factors in S2 cells (Fig. 1*D*). As a positive control, we treated S2 cells with dsRNAs targeting Dicer2 and R2D2. Simultaneous knockdown of Dicer2 and R2D2 resulted in a derepression of the *Pp*-luc reporter at three different concentrations of *Pp*-luc dsRNA trigger, which confirmed that this assay effectively detects RNAi factors in S2 cells in our hands. Knockdown of Blanks likewise resulted in a derepression of luciferase activity at three different concentrations of *Pp*-luc dsRNA trigger (Fig. 1*D*) but failed to enhance luciferase expression in the absence of *Pp*-luc dsRNA (Fig. S1*D*). Consistent with these data, a recent screen for RNAi factors showed that Blanks is important for RNAi in two different siRNA reporter assays (27). Collectively, these results suggest that Blanks functions directly or indirectly in S2 cells as an RNAi factor.

Much like the dsRBD proteins Loqs and R2D2, Blanks can associate with siRNAs and is necessary for efficient RNAi. Unlike Loqs and R2D2, however, Blanks and CG12493 contain predicted bipartite nuclear localization signals (Fig. 1*B*). To assess the localization of these proteins, we generated N-terminal EGFP fusions of Blanks, CG12493, Loqs, and R2D2 and transiently expressed them in S2 cells. Whereas Loqs and R2D2 localized to cytoplasmic foci, Blanks and CG12493 both localized to the nucleus (Fig. S2 *A–D*). Immunofluorescent staining of fixed S2 cells (Fig. S2 *E* and *F*) and adult fly tissues (see below) also revealed strong nuclear partitioning of endogenous Blanks. Intriguingly, these results suggest that Blanks contributes to RNAi from within the nucleus.

Blanks Is Part of a Short Interfering Ribonucleoprotein (siRNP) Complex That Is Distinct from Canonical RISC. To begin characterizing Blanks-containing complexes, we fractionated S2 cell extracts by gel filtration and anion exchange chromatography (Fig. 2 *A* and *B*). Immunoblotting revealed a Blanks-containing peak (Fig. 2 *A* and *B*) that indicates that Blanks resides in a complex of ~400–500 kDa. We performed native polyacrylamide gel-shift assays with two radiolabeled siRNAs (19-nt duplexes with 2-nt 3′ overhangs) of unrelated sequence, and either whole S2 cell lysates or ion exchange Blanks fractions. Crude S2 cell lysates formed the previously described siRNP complexes R2D2–Dicer2 initiator

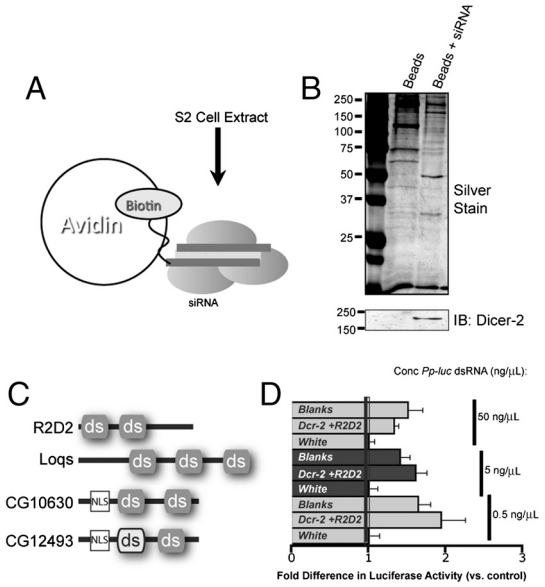

Fig. 1. Proteomic identification of the unique dsRBD protein Blanks from *Drosophila* S2 cells. (*A*) Schematic of the siRNA affinity experiment from S2 cell extracts. (*B*) S2 cell protein extracts were subjected to siRNA affinity chromatography with either streptavidin (SA) beads (beads) or SA beads in complex with biotinylated *Pp*-luc siRNA (beads + siRNA). The affinity preparations were analyzed by silver staining (*Upper*), immunoblotting for Dicer2 (*Lower*), or mass spectrometry. (*C*) Predicted domain maps of the dsRBD proteins R2D2, Loqs, CG10630 (identified from affinity preparations in *B*), and CG12493 (an apparent paralogue of CG10630). (*D*) Dual-luciferase assay for siRNA silencing in S2 cells. Cells were transfected with dsRNAs targeting either *white*, *dicer2* and *r2d2*, or *CG10630*. Cells were later transfected with plasmids expressing *Pp*-luc and *Renilla* luciferase, along with dsRNA targeting *Pp*-luc. The standardized *Pp*-luc activity was plotted as the average fold difference between *white* gene knockdowns and *dicer2/r2d2* or *CG10630* knockdowns. Error bars indicate the standard deviation of the mean.

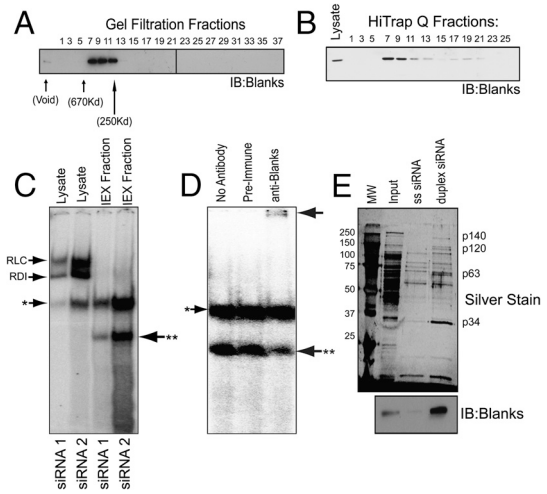

Fig. 2. The Blanks siRNA-binding complex is distinct from known forms of RISC. S2 cell lysates were fractionated by gel filtration (*A*) or ion-exchange chromatography (*B*) and the Blanks-containing fractions were identified by immunoblotting. (*C*) S2 whole lysate or ion-exchange fractions from *B* were incubated with two different siRNAs (siRNA 1 or siRNA 2). The positions of the RDI complex and the RLC are indicated. The unique siRNP formed by the Blanks ion-exchange peak is labeled with two stars (**). The ribonucleoprotein marked with a single star (*) denotes a background complex that only forms in S2 cell extracts. (*D*) The Blanks-containing ion exchange peak fractions from *B* were incubated with radiolabeled siRNA and either no antibody, preimmune antibody, or anti-Blanks antibody. The position of the supershifted siRNP is denoted by an arrow. (*E*) Affinity chromatography of Blanks peak-containing fractions from *B* with biotinylated *Pp*-luc guide strand alone or biotinylated *Pp*-luc siRNA duplex. Input and purifications were either silver stained (*Upper*) or immunoblotted (*Lower*) with anti-Blanks antibodies.

(RDI) and RISC loading complex (RLC) (Fig. 2*C*) (30). The Blanks ion exchange peak (which lacked RDI/RLC assembly activity) formed a distinct, faster-migrating siRNP with both siRNAs (Fig. 2*C*). Importantly, this fast-migrating siRNP is specific for siRNAs because it was not detected in the presence of radiolabeled let-7 miRNA (Fig. S3*A*). Interestingly, the Blanks ion exchange peak formed complexes with blunt-ended 21-nt and (to a lesser extent) 30-nt *Pp*-luc RNA duplexes (Fig. S3 *B* and *C*), suggesting that the Blanks complex dsRNA-binding activity is not restricted to canonical siRNAs.

To test whether Blanks is a component of the fast-migrating siRNP complex, we treated the siRNP with antibodies against Blanks. Incubation of the Blanks peak fraction assembly reactions with anti-Blanks antibodies, but not preimmune serum, supershifted the siRNP (Fig. 2*D*). Collectively, these results strongly suggest that Blanks is associated with an siRNP complex that is unlike the previously described RDI or RLC complexes.

To identify additional components of the Blanks complex we performed siRNA affinity chromatography of the ion exchange Blanks peak fractions. Several proteins were enriched in the siRNA duplex affinity sample compared to the single-stranded control, including Blanks (Fig. 2*E*). Likewise, LC–MS/MS tandem mass spectrometry of the siRNA and guide strand affinity purifications showed that Blanks peptides were specifically enriched in the duplex siRNA affinity sample (Table S1). Additionally, the duplex-specific purification contained peptides from RM62 (a putative RNA helicase and silencing factor associated with chromatin insulators) (31, 32), CG6133 (an apparent orthologue of the mammalian NSUN2 RNA cytosine methyltransferase), and Xrn2 (a 5′–3′ exoribonuclease), strongly suggesting that they are components of the Blanks complex (Fig. 2*E* and Table S1). Additional proteins representing common background contaminants and lower-confidence identifications were also present in our sample (Table S1). These results confirm that the Blanks complex is distinct from previously described RISCs but is associated with a factor (RM62) that is both nuclear and implicated in RNA silencing.

Blanks Expression is Enriched in the Adult Male Germ Line. Previous microarray studies indicate that *CG10630*/*blanks* mRNA is primarily expressed in adult male testes and in S2 cells (33). We used RT-PCR to confirm that testes are the only significant source of *blanks* mRNA in adult flies (Fig. 3*A*), because *blanks* mRNA is undetectable in RNA samples collected from adult male carcasses after dissection of the testes. Expression is also weak but detectable in embryo through pupal stages of mixed gender, and in growing S2 cells. Western blotting detects a band of the predicted molecular weight (35.6 kDa) only in adult testes tissue and S2 lysates (Fig. 3*B*).

Interestingly, in adult male flies, Blanks protein expression is limited to germ line cells when visualized by immunofluorescence microscopy, as shown by costaining with the cytoplasmic germ line marker Vasa (Fig. 3*C*). As in S2 cells, Blanks is restricted to the nucleus in testes (Fig. 3 *C* and *D*); however, the protein is further confined to nuclear subdomains that exclude the bulk of the condensing chromosomal bivalents of the premeiotic primary spermatocytes (Fig. 3*E*). Blanks is also excluded from the spheroid, phase-dense nucleolus. Notably, Blanks protein is not detected in germ line stem cells or mitotically dividing gonialblasts and spermatogonia. Rather, expression begins at the primary spermatocyte stage after the mitotic divisions and persists through both rounds of meiosis into the "onion" stage at the onset of spermatid differentiation (Fig. S4*A*).

After meiosis, differentiating spermatid bundle nuclei condense and elongate (16). As with many other proteins, Blanks levels decline sharply during this process. This decline is likely because of protein degradation after nuclear export because compact foci of cytoplasmic Blanks can occasionally be seen at the base of the needle-shaped nuclei in elongating spermatids undergoing cytoplasmic extrusion during individualization (Fig. S4*B*). No Blanks protein is detectable in mature sperm. An essentially identical expression and localization pattern is seen for the Blanks paralogue, CG12493 (Fig. S4 *C*–*E*).

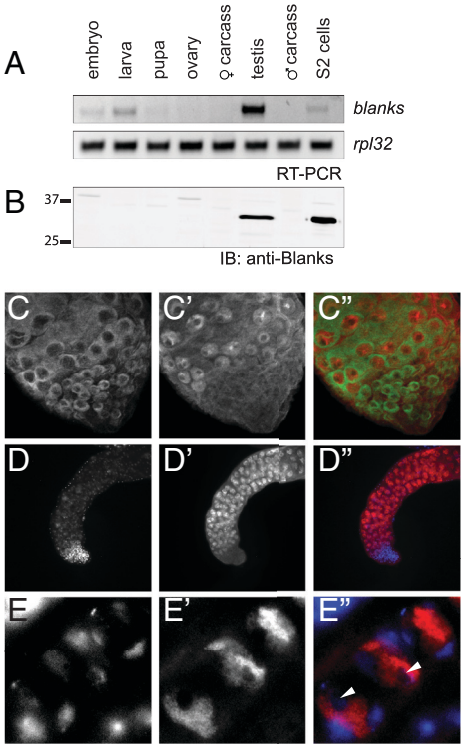

Fig. 3. Blanks expression is restricted to the male germ line. (*A*) Blanks mRNA is most abundant in adult testes, as assayed by RT-PCR. *Rpl32* mRNA was used as a loading control. (*B*) Blanks protein is only detected in testes and S2 cells. An anti-Blanks Western blot of 50 mg total protein from the tissues indicated in *A* is shown. The arrow indicates the Blanks-specific band. (*C* and *D*) Blanks is expressed in primary spermatocyte nuclei. Immunofluorescence microscopy stained with the cytoplasmic germ line-specific marker anti-Vasa, *C*; anti-Blanks, *C*′; merge, *C*″. Blanks is first visible after mitotic divisions in the enlarged nuclei of primary spermatocytes (*D*′), which are distinguishable by weaker DAPI costaining (*D*; merge, *D*″). (*E*) Magnified image of primary spermatocytes. Anti-Blanks staining (*E*′) is restricted to nuclear subdomains that are mutually exclusive with the bulk DAPI-stained (*E*) chromosomal bivalents of the premeiotic spermatocytes. White triangles in the merged image (*E*″) mark nucleoli, which also exclude Blanks staining.

Blanks is Required for Spermiogenesis. The testes-specific expression profile of Blanks suggests that it could play a role in male fertility. To investigate this possibility we obtained a mutant allele, $blanks^{KG00804}$ (34). This allele has a SUPor-*P* element inserted 29 nt downstream of the predicted *blanks* transcriptional start site (Fig. S5*A*). RT-PCR and quantitative Western blotting revealed a ~10-fold and >400-fold reduction in *blanks* mRNA and protein levels, respectively, in the homozygous mutants (Fig. 4*A* and Fig. S5*B*). We found that males homozygous for the *P* element insertion are sterile, whereas females are fertile. Precise excision of the *P* element resulted in fertile males, confirming that the *blanks* lesion caused the recessive male sterility.

We also assessed the levels of the apparent paralogue, CG12493, in the *blanks* mutant testes. Intriguingly, although *CG12493* mRNA abundance was unchanged in the *blanks* mutant (Fig. S5*C*), steady-state protein levels were diminished ~10-fold (Fig. 4*A*), suggesting that Blanks is at least partially required for CG12493 translation or stability. In contrast, an *EP* element-induced allele ($CG12493^{G4506}$) with an insertion at the −1 position

relative to the *CG12493* transcription start site causes dramatic reduction of *CG12493* mRNA and protein levels in testes (Fig. S5 *E* and *F*), whereas *blanks* mRNA and protein are unaffected. $CG12493^{G4506}$ homozygous mutants are viable, and males exhibit wild-type fertility levels until late in adulthood (~30 d). Aged $CG12493^{G4506}$ homozygous males possess a smaller than normal seminal vesicle and reduced mature sperm numbers (Fig. S5*G*).

DAPI nuclear staining of dissected testes revealed that the $blanks^{KG00804}$ homozygous males lack mature sperm in their seminal vesicle (Fig. 4*B*), which provided inspiration for the name *blanks*. Importantly, precise excision of the *P* element restored Blanks protein levels as well as mature sperm in the seminal vesicle (Fig. S5*J*). Additionally, flies expressing a dsRNA targeting both Blanks and CG12493 showed a reduction in seminal vesicle sperm levels (Fig. S5 *M* and *N*). Intriguingly, most stages in the male germ line lineage appear to be unaffected in *blanks* flies: Spermatocyte development occurs normally through all mitotic divisions and both rounds of meiosis, producing phenotypically wild-type cysts of 64 onion-stage round spermatids (Fig. S5*H*). Nuclear elongation and axoneme development are also grossly normal. However, syncitial spermatid bundles fail to fully individualize, resulting in nonmotile, decomposing spermatid cysts (Fig. 4*C*). Rhodamine–phalloidin staining revealed asynchronous migration of the actin-rich individualization complexes (ICs) during individualization (Fig. 4*D* and Fig. S5*I*), suggesting that *blanks* mutants are unable to coordinate IC movement along the axoneme. Precise excision of the *P* element restored the apparent synchrony of the actin-rich IC (Fig. S5 *K* and *L*).

Colocalization of Blanks with the RNA Silencing Factor RM62 in the Male Germ Line. Our analyses from S2 cells suggested that Blanks is part of a complex that includes RM62, a silencing factor (32) that is part of chromatin insulators (31). Notably, *rm62* mutants have been reported to exhibit male fertility defects (35). We observed a nearly perfect overlap in the localization of RM62 and Blanks in the spermatocyte nuclei (Fig. S5*O*), consistent with the possibility that testis Blanks is a component of a complex similar to that observed in S2 cells.

Analysis of Gene Expression in *blanks* Mutant Testes. The RNAi pathway phenotype of Blanks in S2 cells together with its observed expression pattern in adult flies suggested that it might participate in small RNA-mediated silencing of transposable elements in the male germ line. Unexpectedly, quantitative RT-PCR assays revealed no significant differences in the levels of several transposon-derived mRNAs between the $blanks^{KG00804}$ homozygotes and heterozygotes (Fig. S6*A*). Furthermore, *stellate* mRNA, which is derepressed in many RNA silencing mutants (9, 14, 21), was unchanged in the mutant (Fig. S6*A*). To probe more deeply into changes in mRNA expression that arise in the absence of Blanks, we performed an Affymetrix microarray gene expression analysis (from three biological replicates) on mRNA collected from the testes of *blanks* homozygous mutants and *blanks*/*TM3* heterozygous fertile controls. Interestingly, genes up-regulated in the absence of Blanks were significantly enriched for GO terms associated with the innate immune response and related stress pathways (Table 1). Quantitative PCR results also supported this observation (Fig. S6*B*).

Discussion

Here we report the characterization of Blanks, a nuclear, testes-specific siRNA- and dsRNA-binding protein that is critical for sperm maturation in *Drosophila*. Flies lacking Blanks are phenotypically wild type except for the inability of males to produce viable sperm. In S2 cells, *blanks* knockdown results in an RNAi loss-of-function phenotype and Blanks protein appears to be associated with a unique siRNA- and dsRNA-binding complex containing the silencing factor RM62, the 5′–3′ exonuclease Xrn2, and the putative nucleic acid methyltransferase CG6133. Spermatid bundles lacking Blanks fail to individualize and are not deposited in the seminal vesicle. Colocalization of Blanks and RM62 in the spermatid nucleus, together with their cofractionation in S2 cells, suggests that Blanks and RM62 are part of a unique nuclear complex that is necessary for sperm development.

Many retrotransposons are essentially endogenous retroviruses that can form virus-like particles (VLPs) in the germ line. We found no evidence for an up-regulation of transposon RNA levels in a *blanks* mutant. However, similar to *C. elegans* RNAi-defective mutants (36), *blanks* mutant testes exhibit an up-regulation of genes involved in innate immunity. Derepression of the retroelement lifecycle is often associated with sterility. Therefore, our results suggest that the Blanks complex may help to suppress the retroelement lifecycle in the male germ line during later stages of sperm development, though in a manner that is not associated with suppression of RNA levels. Furthermore, loss of Blanks may result in innate immunity activation by hyperactivity of selfish genetic elements. Consistent with our hypothesis, retrotransposon VLPs are observed in the cystic cytoplasm of partially individualized spermatids (37).

What is the molecular function of the apparent Blanks/RM62/Xrn2/CG6133 complex? In testes there exists a two-tiered transposon immune system composed of both siRNA components and

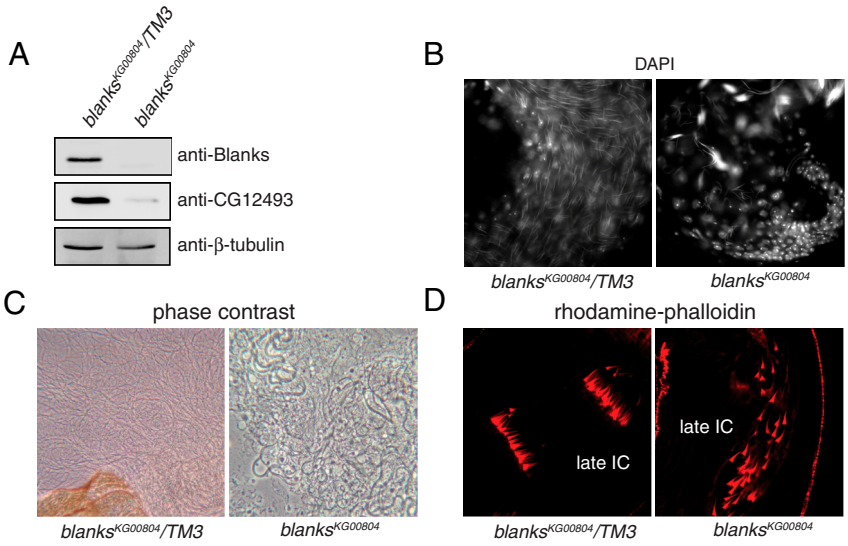

Fig. 4. A *P* element-induced allele suppresses *blanks* expression and disrupts sperm individualization. (*A*) Blanks protein is not detectably expressed in $blanks^{KG00804}$ homozygous testes. Loss of Blanks protein in *blanks* homozygotes is also accompanied by a ~10-fold reduction of CG12493 protein. (*B*) Images of DAPI-stained *blanks* heterozygous (*Left*) and homozygous (*Right*) mutant seminal vesicles. Elongated sperm nuclei are largely absent in the homozygote. (*C*) Phase-contrast images of ruptured seminal vesicles of *blanks* heterozygous (*Left*) and homozygous (*Right*) mutant testes. Thousands of individualized motile sperm are liberated from heterozygous testes, but only detritus and decaying bundles of unindividualized spermatid cysts are seen in the homozygous mutants. (*D*) Late ICs stained by rhodamine–phalloidin (red). Control ($blanks^{KG00804}$/*TM3*) ICs migrate in unison along the axoneme, extruding excess cytoplasm from elongated spermatids (*Left*). The *blanks* ICs lose synchrony after progressing caudally along the axoneme.

Table 1. GO term enrichment for genes ≥2-fold up-regulated in *blanks*^{KG00804} mutant testes relative to *blanks*^{KG00804}/*TM3* heterozygotes

Gene	Fold induction	Antibacterial humoral response	Defense response	Response to stress	Immune response	GO term *p* value
		1.27E-11	1.56E-10	2.24E-07	3.00E-07	
CecC	6.4	x	x	x	x	
Def	4.0	x	x	x	x	
AttA	2.7	x	x	x	x	
AttB	2.7	x	x	x	x	
AttD	7.3	x	x	x	x	
Mtk	3.4	x	x	x	x	
PGRP-SA	2.1	x	x	x	x	
DptB	3.2	x	x	x	x	
IM3	2.9	x	x	x	x	
IM2	2.8		x	x		
PGRP-SB1	2.8		x	x	x	
ninaD	3.1		x	x		
Tsf1	3.8		x	x		
rpr	2.0			x		
TotA	2.8			x		
Fst	2.3			x		

The Benjamani–Hochberg multiple hypothesis correction *p* value is displayed for each GO classification associated with the gene products.

piRNA factors (2). The piRNAs and endo-siRNAs share overlapping transposon targets. However, the tissue expression pattern of the piRNA cofactors in the male germ line is restricted to the stem-cell niche and early mitotic stages, with little to no apparent expression in later stages of spermatogenesis (12). The expression pattern of Blanks suggests that a dsRNA-based transposon defense system may help suppress selfish genetic elements during and after meiosis. Blanks participates in an siRNA/dsRNA-binding complex unlike those described previously, and our proteomic analyses provide no evidence for the presence of an Ago protein in this complex. These observations suggest that the Blanks complex is not a canonical form of RISC. However, loss of Blanks compromises RNAi in S2 cells, suggesting that the Blanks siRNP can directly or indirectly contribute to specific substrate targeting.

Blanks is highly expressed in testes and S2 cells but is greatly reduced or absent in other tissues. S2 cells do not resemble cells from testes, but exhibit an aneuploid male (2X;4A) genotype (38, 39). Importantly, many *Drosophila* cell lines, including S2 cells, have a vastly expanded transposon population (40, 41). As a result S2 cells may have come under selective pressure to suppress selfish elements through mechanisms that include the activation of normally germ line-restricted factors such as Blanks.

Our data suggest a critical role for siRNAs or dsRNAs in male fertility. Curiously, previous work has shown that Dicer2, which generates most if not all siRNAs from dsRNA precursors (17), is dispensable for male fertility despite its role in the endo-siRNA pathway within the male germ line (15). One possible explanation for these observations could be the redundant involvement of Dicer1, which has previously been implicated in both the miRNA and siRNA pathways (17). Thus, the small RNAs that function with Blanks conceivably could be provided by either Dicer, or both.

It is presently unclear whether the Blanks siRNP is identical in both testes and S2 cells. Intriguingly, protein levels of the Blanks paralogue, CG12493, were dramatically reduced in *blanks*^{KG00804} mutant testes despite no significant change in mRNA expression. One possible explanation is that CG12493 and Blanks interact directly in testes, and this association is necessary for CG12493 protein stability. A reciprocal relationship is not apparent because Blanks protein levels are unaffected by the *CG12493* disruption and essentially no CG12493 protein or mRNA is detectable in S2 cells. The *CG12493* mutant male fertility phenotype is significantly milder than that of *blanks*, suggesting that it may carry out a supplementary role.

Components of the Blanks siRNP. Our biochemical purifications of the Blanks siRNP also yielded RM62, Nsun2, and Xrn2. RM62 has an established role in RNA silencing (32). Nsun2 and Xrn2 have not been implicated in *Drosophila* siRNA silencing. However, in *Saccharomyces cerevisiae*, the predicted orthologue of Nsun2 (Ncl1p) is involved in the control of transposon proliferation (42). Ncl1p methylates tRNAs in yeast (43). It is currently unknown if Ncl1p can target additional substrates for methylation. Interestingly, Ncl1p and the Xrn2 orthologue Rat1p interact genetically (44), suggesting that the Blanks complex may support an evolutionarily conserved mechanism. Our results also indicate that Blanks is capable of binding a variety of RNA duplex forms. It remains unclear whether bound RNA represents a target or a cofactor of the Blanks complex, and what relation this binding has (if any) to canonical RISC.

Blanks and Spermiogenesis. Numerous roles have been described for dsRNA-binding proteins in reproductive processes in organisms from fungi to humans, and most of these processes overlap mechanistically with small RNA silencing (45, 46). Currently, the known small RNA-mediated silencing pathways in *Drosophila* that impinge upon gamete generation function at the earliest stages of germ line development. Genes encoding components of the miRNA machinery such as Loqs and Ago1 act at the level of germ line stem cell self-renewal in females (21, 47, 48), and a Dicer1-dependent role for R2D2 has been suggested in somatic follicle cells (49). A role for the miRNA pathway in sperm development has not yet been fully investigated, though *loqs* hypomorphic males are incompletely fertile and show a derepression of *stellate* (21). The piRNA and repeat-associated siRNA-mediated silencing pathway is essential for both germ line establishment in the embryo (50) and later for germ line stem cell (GSC) self-renewal, and mutants in this pathway are depleted of GSCs (8, 9). In contrast the *blanks* male-sterile phenotype appears during spermiogenesis, which is the final stage of sperm development. Transposon and *stellate* silencing are grossly unaffected in *blanks* mutants, and the early germ line and somatic lineages appear phenotypically wild type. Furthermore, Blanks expression overlaps with that of piRNA cofactors Aub, Ago3, and Piwi only in the first few hours of the primary spermatocyte stage (12), making it unlikely that Blanks is an obligate piRNA pathway component.

Spermatid individualization in *Drosophila* is a process that requires the concerted action of caspases (51, 52), actomyosin systems (53–55), proteasomes (56), and other machineries (57). To our knowledge, Blanks is a unique RNA silencing-related protein shown to be important to this process. However, Pavelec et al. (58) have recently demonstrated a requirement for silencing factors during various stages of amoeboid sperm development in *C. elegans*. It seems unlikely that Blanks, a nuclear dsRNA-binding protein, would directly interact with the largely cytoplasmic (or extracellular) cadre of proteins implicated in individualization. It is also doubtful that Blanks acts during individualization per se, because the protein rapidly disappears after meiosis and during nuclear elongation. We suspect that Blanks sets the stage for this final phase of sperm development by helping to coordinate earlier gene expression and that it likely does so via a unique branch of the set of small RNA silencing pathways. Intriguingly, the mammalian spermatid perinuclear RNA-binding protein has a dsRBD with 50% amino acid similarity to a Blanks dsRBD and plays an important role in male fertility (59). Our results suggest that Blanks takes part in a conserved dsRNA-based regulatory mechanism during spermiogenesis that is critical for male fertility.

Experimental Procedures

S2 cell extracts were prepared, subjected to siRNA affinity chromatography, and analyzed as described (28). RNAi assays in S2 cells were also as described (28). Rabbit antisera were raised against full-length Blanks and residues 1–95 of CG12493 expressed in *Escherichia coli*. Native gel analyses were as reported previously (18, 30). Microscopic examination of testes were as described (60). Mutant *Drosophila* lines ($blanks^{KG00804}$ and $CG12493^{G4506}$) were obtained from the Bloomington *Drosophila* Stock Center. For further details on materials and methods, see *SI Materials and Methods*.

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1. Carthew RW, Sontheimer EJ (2009) Origins and mechanisms of miRNAs and siRNAs. *Cell* 136:642–655.
2. Malone CD, Hannon GJ (2009) Small RNAs as guardians of the genome. *Cell* 136:656–668.
3. Marraffini LA, Sontheimer EJ (2010) CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nat Rev Genet* 11:181–190.
4. Ghildiyal M, Zamore PD (2009) Small silencing RNAs: An expanding universe. *Nat Rev Genet* 10:94–108.
5. Drinnenberg IA, et al. (2009) RNAi in budding yeast. *Science* 326:544–550.
6. Mosher RA, et al. (2009) Uniparental expression of PolIV-dependent siRNAs in developing endosperm of Arabidopsis. *Nature* 460:283–286.
7. Ruby JG, et al. (2006) Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in *C. elegans*. *Cell* 127:1193–1207.
8. Cox DN, et al. (1998) A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. *Genes Dev* 12:3715–3727.
9. Li C, et al. (2009) Collapse of germ line piRNAs in the absence of Argonaute3 reveals somatic piRNAs in flies. *Cell* 137:509–521.
10. Brennecke J, et al. (2007) Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. *Cell* 128:1089–1103.
11. Gunawardane LS, et al. (2007) A slicer-mediated mechanism for repeat-associated siRNA 5′ end formation in *Drosophila*. *Science* 315:1587–1590.
12. Nishida KM, et al. (2007) Gene silencing mechanisms mediated by Aubergine piRNA complexes in *Drosophila* male gonad. *RNA* 13:1911–1922.
13. Saito K, et al. (2009) A regulatory circuit for piwi by the large Maf gene traffic jam in *Drosophila*. *Nature* 461:1296–1299.
14. Vagin VV, et al. (2006) A distinct small RNA pathway silences selfish genetic elements in the germ line. *Science* 313:320–324.
15. Czech B, et al. (2008) An endogenous small interfering RNA pathway in *Drosophila*. *Nature* 453:798–802.
16. Davies EL, Fuller MT (2008) Regulation of self-renewal and differentiation in adult stem cell lineages: Lessons from the *Drosophila* male germ line. *Cold Spring Harbor Symp Quant Biol* 73:137–145.
17. Lee YS, et al. (2004) Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* 117:69–81.
18. Pham JW, Pellino JL, Lee YS, Carthew RW, Sontheimer EJ (2004) A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in *Drosophila*. *Cell* 117:83–94.
19. Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409:363–366.
20. Lee Y, et al. (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425:415–419.
21. Forstemann K, et al. (2005) Normal microRNA maturation and germ line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. *PLoS Biol* 3:e236.
22. Jiang F, et al. (2005) Dicer-1 and R3D1-L catalyze microRNA maturation in *Drosophila*. *Genes Dev* 19:1674–1679.
23. Liu Q, et al. (2003) R2D2, a bridge between the initiation and effector steps of the *Drosophila* RNAi pathway. *Science* 301:1921–1925.
24. Saito K, Ishizuka A, Siomi H, Siomi MC (2005) Processing of pre-microRNAs by the Dicer-1-Loquacious complex in *Drosophila* cells. *PLoS Biol* 3:e235.
25. Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* 293:1146–1150.
26. Okamura K, Ishizuka A, Siomi H, Siomi MC (2004) Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes Dev* 18:1655–1666.
27. Zhou R, et al. (2008) Comparative analysis of argonaute-dependent small RNA pathways in *Drosophila*. *Mol Cell* 32:592–599.
28. Gerbasi VR, Golden DE, Hurtado SB, Sontheimer EJ (2010) Proteomic identification of *Drosophila* siRNA-associated factors. *Mol Cell Proteomics*, 9 pp:1866–1872.
29. Nykanen A, Haley B, Zamore PD (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* 107:309–321.
30. Pham JW, Sontheimer EJ (2005) Molecular requirements for RNA-induced silencing complex assembly in the *Drosophila* RNA interference pathway. *J Biol Chem* 280:39278–39283.
31. Lei EP, Corces VG (2006) RNA interference machinery influences the nuclear organization of a chromatin insulator. *Nat Genet* 38:936–941.
32. Ishizuka A, Siomi MC, Siomi H (2002) A *Drosophila* fragile X protein interacts with components of RNAi and ribosomal proteins. *Genes Dev* 16:2497–2508.
33. Chintapalli VR, Wang J, Dow JA (2007) Using FlyAtlas to identify better *Drosophila* melanogaster models of human disease. *Nat Genet* 39:715–720.
34. Bellen HJ, et al. (2004) The BDGP gene disruption project: Single transposon insertions associated with 40% of *Drosophila* genes. *Genetics* 167:761–781.
35. Buszczak M, et al. (2007) The carnegie protein trap library: A versatile tool for *Drosophila* developmental studies. *Genetics* 175:1505–1531.
36. Welker NC, Habig JW, Bass BL (2007) Genes misregulated in *C. elegans* deficient in Dicer, RDE-4, or RDE-1 are enriched for innate immunity genes. *RNA* 13:1090–1102.
37. Rachidi M, Lopes C, Benichou JC, Hellio R, Maisonhaute C (2005) Virus-like particle formation in *Drosophila* melanogaster germ cells suggests a complex translational regulation of the retrotransposon cycle and new mechanisms inhibiting transposition. *Cytogenet Genome Res* 111:88–95.
38. Schneider I (1972) Cell lines derived from late embryonic stages of *Drosophila* melanogaster. *J Embryol Exp Morphol* 27:353–365.
39. Zhang Y, et al. (2010) Expression in aneuploid *Drosophila* S2 cells. *PLoS Biol* 2: e1000320.
40. Maisonhaute C, Ogereau D, Hua-Van A, Capy P (2007) Amplification of the 1731 LTR retrotransposon in *Drosophila* melanogaster cultured cells: Origin of neocopies and impact on the genome. *Gene* 393:116–126.
41. Potter SS, Brorein WJ, Jr, Dunsmuir P, Rubin GM (1979) Transposition of elements of the 412, copia and 297 dispersed repeated gene families in *Drosophila*. *Cell* 17:415–427.
42. Aye M, et al. (2004) Host factors that affect Ty3 retrotransposition in *Saccharomyces cerevisiae*. *Genetics* 168:1159–1176.
43. Motorin Y, Grosjean H (1999) Multisite-specific tRNA:m5C-methyltransferase (Trm4) in yeast *Saccharomyces cerevisiae*: Identification of the gene and substrate specificity of the enzyme. *RNA* 5:1105–1118.
44. Chernyakov I, Whipple JM, Kotelawala L, Grayhack EJ, Phizicky EM (2008) Degradation of several hypomodified mature tRNA species in *Saccharomyces cerevisiae* is mediated by Met22 and the 5′–3′ exonucleases Rat1 and Xrn1. *Genes Dev* 22:1369–1380.
45. Lau NC (2010) Small RNAs in the animal gonad: Guarding genomes and guiding development. *Int J Biochem Cell Biol* 42:1334–1347.
46. Matzke MA, Birchler JA (2005) RNAi-mediated pathways in the nucleus. *Nat Rev Genet* 6:24–35.
47. Hatfield SD, et al. (2005) Stem cell division is regulated by the microRNA pathway. *Nature* 435:974–978.
48. Park JK, Liu X, Strauss TJ, McKearin DM, Liu Q (2007) The miRNA pathway intrinsically controls self-renewal of *Drosophila* germ line stem cells. *Curr Biol* 17:533–538.
49. Kalidas S, et al. (2008) *Drosophila* R2D2 mediates follicle formation in somatic tissues through interactions with Dicer-1. *Mech Dev* 125:475–485.
50. Harris AN, Macdonald PM (2001) Aubergine encodes a *Drosophila* polar granule component required for pole cell formation and related to eIF2C. *Development* 128:2823–2832.
51. Arama E, Bader M, Rieckhof GE, Steller H (2007) A ubiquitin ligase complex regulates caspase activation during sperm differentiation in *Drosophila*. *PLoS Biol* 5:e251.
52. Muro I, et al. (2006) The *Drosophila* caspase Ice is important for many apoptotic cell deaths and for spermatid individualization, a nonapoptotic process. *Development* 133:3305–3315.
53. Desai BS, Shirolikar S, Ray K (2009) F-actin-based extensions of the head cyst cell adhere to the maturing spermatids to maintain them in a tight bundle and prevent their premature release in *Drosophila* testis. *BMC Biol* 7:19–36.
54. Noguchi T, Frank DJ, Isaji M, Miller KG (2009) Coiled-coil-mediated dimerization is not required for myosin VI to stabilize actin during spermatid individualization in *Drosophila* melanogaster. *Mol Biol Cell* 20:358–367.
55. Noguchi T, Miller KG (2003) A role for actin dynamics in individualization during spermatogenesis in *Drosophila* melanogaster. *Development* 130:1805–1816.
56. Zhong L, Belote JM (2007) The testis-specific proteasome subunit Prosalpha6T of D. melanogaster is required for individualization and nuclear maturation during spermatogenesis. *Development* 134:3517–3525.
57. Hicks JL, Deng WM, Rogat AD, Miller KG, Bownes M (1999) Class VI unconventional myosin is required for spermatogenesis in *Drosophila*. *Mol Biol Cell* 10:4341–4353.
58. Pavelec DM, Lachowiec J, Duchaine TF, Smith HE, Kennedy S (2009) Requirement for the ERI/DICER complex in endogenous RNA interference and sperm development in *Caenorhabditis elegans*. *Genetics* 183:1283–1295.
59. Pires-daSilva A, et al. (2001) Mice deficient for spermatid perinuclear RNA-binding protein show neurologic, spermatogenic, and sperm morphological abnormalities. *Dev Biol* 233:319–328.
60. Singh SR, Hou SX (2008) Immunohistological techniques for studying the *Drosophila* male germ line stem cell. *Methods Mol Biol* 450:45–59.