The poly(A) polymerase GLD2 is required for spermatogenesis in Drosophila melanogaster

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SUMMARY
The DNA of a developing sperm is normally inaccessible for transcription for part of spermatogenesis in many animals. In Drosophila melanogaster, many transcripts needed for late spermatid differentiation are synthesized in pre-meiotic spermatocytes, but are not translated until later stages. Thus, post-transcriptional control mechanisms are required to decouple transcription and translation during spermatogenesis. In the female germline, developing germ cells accomplish similar decoupling through poly(A) tail alterations to ensure that dormant transcripts are not prematurely translated: a transcript with a short poly(A) tail will remain untranslated, whereas elongating the poly(A) tail permits protein production. In Drosophila, the ovary-expressed cytoplasmic poly(A) polymerase WISPY is responsible for stage-specific poly(A) tail extension in the female germline. Here, we examine the possibility that a recently derived testis-expressed WISPY paralog, GLD2, plays a similar role in the Drosophila male germline. We show that knockdown of GlD2 transcripts causes male sterility, as GLD2-deficient males do not produce mature sperm. Spermatogenesis up to and including meiosis appears normal in the absence of GLD2, but post-meiotic spermatid development rapidly becomes abnormal. Nuclear bundling and F-actin assembly are defective in GLD2 knockdown testes and nuclei fail to undergo chromatin reorganization in elongated spermatids. GLD2 also affects the incorporation of protamines and the stability of dynamin and transition protein transcripts. Our results indicate that GLD2 is an important regulator of late spermatogenesis and is the first example of a Gld-2 family member that plays a significant role specifically in male gametogenesis.

KEY WORDS: Spermatogenesis, Poly(A) polymerase, Gld-2, Drosophila

INTRODUCTION
Spermatogenesis is a tightly controlled developmental process that requires the stage-specific production of proteins. In animals, spermatogenesis begins when a diploid germline cell produced from the testis stem cell niche undergoes differentiation and proliferation through mitosis and meiosis to form many haploid spermatocytes. Post-meiotic development, called spermiogenesis, is a series of morphological changes that will determine the final shape and form of the mature sperm, which can vary greatly among taxa. One important phenomenon that is seen in spermatogenesis in many species is that transcription is silenced for part of the process: for example, transcription cannot occur after nuclear condensation in mice (Braun, 1998) and there is some evidence for transcriptional silencing during meiosis in Drosophila (Vibransovski et al., 2009). In such cases, any proteins that must be translated during the transcriptionally silent period must be synthesized from mRNAs that were transcribed earlier but remain untranslated until the appropriate stage of development. Furthermore, some transcripts needed for late spermiogenesis, such as those of don juan (Santel et al., 1997) and Mst87F (Schäfer et al., 1990), are synthesized in spermatocytes, although they are not translated until much later.

Spermatogenesis in Drosophila melanogaster is well described. Testis gonial cells originating from germline stem cell divisions undergo synchronous mitosis and meiosis with incomplete cytokinesis, resulting in a cyst of 64 round, haploid spermatids after the completion of meiosis (Fuller, 1993; Fuller, 1998) (Fig. 1). The spermatids undergo morphological changes, including flagellum extension and nuclear reshaping within the syncytium, until spermatid individualization occurs. The cells exit the testis as mature sperm.

During the final stages of spermatogenesis in Drosophila, as in many other invertebrate and vertebrate species, chromatin reorganization events cause the spermatid nuclei to become tightly compacted (Lewis et al., 2003). Histones associated with spermatocyte chromatin are ultimately exchanged for protamines, allowing the nucleus to condense up to 200 fold (Fuller, 1993). Two genes encoding protamines have been identified in Drosophila (Mst35Ba, or Protamine A, and Mst35Bb, or Protamine B) (Raja and Renkawitz-Pohl, 2006). Additionally, the gene Tpl94D demonstrates functional homology to mammalian transition proteins, which bind chromatin as an intermediate step between histone-based and protamine-based chromatin organization (Rathke et al., 2007). Therefore, nuclear compaction in Drosophila occurs as a two-step process: histones are first displaced by transition proteins, and transition proteins are later exchanged for protamines.

Soon after protamine incorporation, the spermatids in a cyst become separated from one another in a process called individualization. During this process, a cone-like structure composed of cross-linked F-actin assembles around each nucleus in the cyst. The 64 cones in the cyst move as a unit down the length of the sperm tails, simultaneously pushing out excess cytoplasm and wrapping each spermatid in an individual membrane (Fabrizio et al., 1998; Noguchi and Miller, 2003). The separated, mature sperm then roll into coils and exit the testis to be stored in the seminal vesicle (Tokuyasu et al., 1972).
In *Drosophila*, there are many examples of transcripts that are synthesized in spermatocytes but are not translated until after meiosis, to such an extent that transcriptional activity in the developing *Drosophila* sperm cell was previously thought to be predominantly limited to early spermatocytes and spermatagonia (Olivieri and Olivieri, 1965; Schäfer et al., 1990; Fuller, 1998; Rathke et al., 2007; Barreau et al., 2008). However, recent evidence demonstrates that transcriptional activity occurs post-meiotically as well (Barreau et al., 2008; Vibranovski et al., 2010). For those transcripts that remain quiescent until post-meiotic stages, a translational control mechanism must be in place.

Many cell types, including oocytes and neurons, achieve translational regulation through adjusting the length of the poly(A) tail in the cytoplasm (Galili et al., 1988; Preiss et al., 1998; Benoit et al., 2008; Cui et al., 2008; Kwak et al., 2008). A long poly(A) tail promotes translation of the transcript through recruitment of translation initiation factors, whereas a transcript with a short poly(A) tail remains untranslated or is degraded. Most mRNAs are extensively polyadenylated in the nucleus; however, for some transcripts that will be held in an untranslated state for a period of time, poly(A) tail modifications occur outside the nucleus (Kim and Richter, 2006). In *Xenopus*, transcripts destined for post-transcriptional poly(A) tail adjustment contain two consensus sequences in their 3'UTR: a cytoplasmic polyadenylation element (CPE) and the hexamer AAUAAA, which recruit a complex of proteins that alter poly(A) tail length (Mendez and Richter, 2001; Pique et al., 2008). In *Xenopus*, CPE is bound by CPE-binding protein (CPEB) (Hake and Richter, 1994; Stebbins-Boaz et al., 1996). The cleavage and polyadenylation specificity factor (CPSF) binds to the hexamer (Dickson et al., 1999; Mendez et al., 2000). CPEB and CPSF recruit a cytoplasmic poly(A) polymerase (PAP) and a deadenylase, both of which work on the transcript simultaneously (Kim and Richter, 2007). However, the deadenylase is slightly more efficient than the PAP, so the net effect is a poly(A) tail that remains short. Upon a signal to activate translation, CPEB is phosphorylated, causing the deadenylase to dissociate from the complex; the PAP is then free to elongate the poly(A) tail (Kim and Richter, 2007).

PAPs that act in the cytoplasmic complex differ from nuclear PAPs. The Gld-2 (germline development 2) family of cytoplasmic PAPs has been described in *C. elegans*, *Xenopus* and *Drosophila* (Wang et al., 2002; Barnard et al., 2004; Benoit et al., 2008; Cui et al., 2008). Whereas nuclear PAPs contain a catalytic domain and an RNA-binding domain, Gld-2 family members have only a catalytic domain (Bard et al., 2000; Martin et al., 2000). For RNA specificity, Gld-2 associates with an RNA-binding protein, typically a Gld-3, to form a heterodimer that acts as a cytoplasmic PAP (Wang et al., 2002).

Gld-2 family members have been shown to play roles in oogenesis in several organisms. In worms, a Gld-2 homolog is involved in the mitosis/meiosis decision to make both male and female germ cells (Kadyk and Kimble, 1998). In *Drosophila*, the X-linked Gld-2 homolog *wispy* (*wisp*) is necessary for oogenesis and egg activation (Benoit et al., 2008; Cui et al., 2008). WISP is present in ovaries but not testes and is necessary for the completion of meiosis in oocytes. WISP has been shown to polyadenylate transcripts of *cortex*, which is required for proper meiotic progression (Benoit et al., 2008). WISP also polyadenylates several developmental transcripts, the protein products of which are needed for early embryogenesis, including *bicoid, Toll* and *torso* (Benoit et al., 2008; Cui et al., 2008).

The *Drosophila* genome contains an autosomal paralog of *wisp* called *Gld2*. Previous studies of *Gld2* have demonstrated a role in long-term memory and show that GLD2 acts as a PAP in vitro (Kwak et al., 2008). Here, we show that *Gld2* is expressed in the male, but not female, germline. It is required for the completion of spermatogenesis, specifically for the elongation and individualization stages. In GLD2 knockdown testes, the first disruption observed is post-meiotic, at the onset of spermatocyst elongation. In these testes, the nuclei in developing cysts scatter and basal bodies are not observed near nuclei. F-actin-containing individualization complexes do not assemble and nuclear compaction does not complete. Additionally, protamines are not incorporated and transcripts for both *dynamin (shibire – FlyBase)* and the transition protein are undetectable. Our findings indicate that *Gld2* arose from duplication of the *wisp* locus, and that this derived paralog was likely maintained in the genome owing to its essential role in spermatogenesis.
GLD2 in Drosophila spermatogenesis

MATERIALS AND METHODS

Evolutionary analysis

Orthologs of wisp and GLD2 within the Drosophila genus were identified from sequences obtained from The Drosophila 12 Genomes Consortium (Clark et al., 2007). Homologs in other insect species were identified via best reciprocal BLASTp hits between non-redundant protein databases at the NCBI BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi), using either GLD2 or WISP protein sequence as the query.

Drosophila stocks

All strains were raised on standard yeast-glucose media at 22°C. Oregon R P2 (Allis et al., 1977) was used as the wild-type stock. The stock containing GAL4-VP16 (Chen and McKernin, 2003), which expresses a GAL4 driven by the bag of marbles (bam) promoter, was kindly provided by C. Baker and M. T. Fuller (Stanford University, Palo Alto, CA, USA). The stock carrying an X-linked transgenic protamine-B-eGFP transgene (Manier et al., 2010) was kindly provided by J. Belote (Syracuse University, Syracuse, NY, USA). Lines carrying UAS-driven snapback RNA against GLD2 (CG5732) transcripts were obtained from the Vienna Drosophila RNAi Center (VDRC ID#52042 and ID#51605) (Dietzl et al., 2007). Both lines carry the same construct, and prediction programs detect no off-targets. We crossed virgin females from the VDRC line to males carrying bam-GAL4-VP16 to achieve knockdown of GLD2 specifically in the gonads of the progeny. To observe Protamine B in knockdown and control flies, we crossed protamine-B-eGFP, P[ubP-GAL4]/L1T/TM3;Sh,vg females to males from the VDRC line using methods described by Avila and Wölfner (Avila and Wölfner, 2009); the Sv progeny were the knockdown group and their Sh siblings were the control group. Expression of the protamine-B-GFP construct was confirmed by RT-PCR.

Fertility tests

To assess fertility, 3- to 5-day-old GLD2 knockdown males and females created by the cross described above were singly mated to 3- to 5-day-old wild-type males or females. In vials, single pairs were observed until completion of mating, and the male of each couple was then removed. The females from each mating were transferred to new vials for three consecutive 24-hour periods. The number of eggs laid during each period and the number of progeny resulting from those eggs were recorded.

Phase-contrast microscopy

Testes were dissected in cold Ringer’s solution (Ashburner, 1989) and squashed in a drop of solution on a slide under the weight of a cover slip. Squashes were examined under phase-contrast using a Zeiss Axioskop compound microscope. Images were captured using Hamamatsu ORCA-ER camera equipment and software.

Fluorescence microscopy

To detect actin individualization cones and nuclei, testes of 3- to 5-day-old males were prepared for phalloidin staining as described (Fabrizio et al., 1998). For immunostaining of GLD2, an affinity-purified polyclonal antibody was generated using a 329 amino acid region (residues 480-808) of the protein as antigen and previously described methods (Cui et al., 2008). Testes were dissected in cold PBS (10 mM sodium phosphate buffer pH 7.4, 150 mM NaCl). Dissected testes were then squashed and fixed in 4% paraformaldehyde in PBS as described (Fabrizio et al., 1998). Squash preparations were then washed with PBS, blocked, and stained as described (Hurst et al., 2003). A 1:1000 dilution of primary GLD2 antibody or a 1:500 dilution of primary γ-Tubulin antibody (γGTU88, Sigma, St Louis, MO, USA) was used, and a 1:1000 dilution of Alexa Fluor 488 anti-rabbit or anti-mouse secondary antibody (Invitrogen, Carlsbad, CA, USA) was used. Squashes were incubated with propidium iodide for 30 minutes or DAPI for 5 minutes at room temperature following secondary antibody incubation. To detect monomeric G-actin, squashes were fixed and washed as described above. Squashes were incubated with Alexa Fluor 488-conjugated Dnase I (Invitrogen) for 1 hour at room temperature and then washed with PBS.

Coverslips with a drop of anti-fade mounting solution (10 mM N-propyl gallate in 75% glycerol) were placed on stained squash preparations. Preparations were examined using a Leica TCS SP2 confocal microscope or a Zeiss Axioskop compound microscope.

RNA extraction and RT-PCR

Total RNA was extracted from hand-dissected testes of 3- to 5-day-old males using TRIzol (Invitrogen) and reverse transcribed using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. The GoTaq PCR system (Promega, Madison, WI, USA) was used for PCR analysis of cDNA.

Western blot

For each sample prepared for western blot analysis, testes from five 3-day-old males or ovaries from two 3-day-old females were dissected in cold PBS and homogenized by rapid pipetting in sample buffer. Samples were boiled for 5 minutes and run on a 7.5% SDS-polyacrylamide gel, and proteins were transferred to an Immobilon membrane (Millipore). The membrane was then blocked with 5% milk in TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20) for 1 hour before adding primary GLD2 antibody (1:1000) overnight at 4°C. The membrane was then washed four times in TBST and incubated with HRP-linked secondary antibody (Jackson Laboratories, Bar Harbor, ME, USA) for 1 hour at room temperature. ECL-Plus (Amersham) was added to the membrane as per the manufacturer’s instructions to detect the signal.

Poly(A) tail assay

Poly(A) tail (PAT) assays were performed as described (Salles and Strickland, 1999). For PAT assay of low-abundance transcripts, nested PAT PCR experiments were performed in which the first PCR was run using a gene-specific primer 500-600 bp upstream of the 3’ end of the transcript of interest, plus the anchor primer; the product from this reaction was used as template in a second PAT PCR using a primer 200-400 bp upstream of the 3’ end of the transcript of interest, plus the anchor primer. Products were resolved on a 2% agarose or 8% polyacrylamide gel. For primer sequences, see Table S1 in the supplementary material.

Yeast two-hybrid analysis

Yeast two-hybrid analysis was performed as described (Cui et al., 2008). Briefly, we cloned full-length coding sequences from Glld2 and Bic-C into vectors of the Matchmaker yeast two-hybrid system (Clontech, Mountain View, CA, USA). In-frame fusions of each coding region were generated in both the DNA-binding domain vector pGBK7 and the activation domain vector pGADT7. Yeast cells co-transformed with pGBK7 and pGADT7 derivatives were grown on –Trp –Leu synthetic medium and tested for growth on –Trp –Leu –His and –Trp –Leu –His –Ade synthetic media.

RESULTS

Evolutionary history of Glld2 and wisp

The genus Drosophila can be divided into subgenera, and genome sequences are available for two of these: Sophophora (which contains D. melanogaster) and Drosophila (which contains species that diverged from D. melanogaster ~63 million years ago) (Tamura et al., 2004). We identified wisp and Glld2 orthologs in species from both subgenera. Available gene expression data show that the wisp orthologs in D. melanogaster, D. mojavensis and D. virilis have female-biased expression, whereas the Glld2 orthologs from D. melanogaster, D. simulans, D. sechellia, D. yakuba, D. pseudoobscura and D. mojavensis have male-biased expression (Zhang et al., 2007). This is consistent with the conservation of the oogenesis and spermatogenesis functions of WISP (Benoit et al., 2008; Cui et al., 2008) and Glld2 (see below), respectively, across the entire genus. All other insect species with sequenced genomes (including Anopheles gambiae and Aedes aegypti, which are the closest relatives to Drosophila with completely sequenced genomes) possess only a single ortholog of wisp/Glld2, based on best reciprocal BLAST searches. Therefore, a duplication event occurred...
Fig. 2. GLD2 is expressed in the testes and is not detectable in the female. (A) RT-PCR for Gld2 shows expression in male Drosophila larvae, pupae and adult stages, whereas expression is not detected in the female. Rpl32 is used as a positive control. (B) Western blot with anti-GLD2 shows that expression is robust in the testes, but is not detected in the remainder of the male (carcass) or in whole females. (C) Western blots with anti-GLD2 and anti-WISP show that GLD2 is expressed in testis but not ovary, and that WISP is expressed in ovary but not in testis. Membranes were probed with anti-α-Tubulin (TUB) as a loading control. (D) Immunostaining of whole testis squashes using anti-GLD2 shows the extent of GLD2 expression. (E, F) GLD2 is first detected in primary spermatocytes at the distal tip of the testis (E) and persists at the distal ends of spermatogenic cysts in late stages of spermiogenesis (F). Arrowheads in E indicate the boundary of the region of the testis that contains weakly labeled primary spermatocytes. Arrows in F indicate the distal ends of elongated spermatogenic cysts. Scale bars: 40 μm.

after the most recent common ancestor (MRCA) of Drosophila and mosquitoes, but prior to the MRCA of the genus Drosophila, to give rise to either wisp or Gld2 (depending on which locus is ancestral and which is derived). A phylogenetic reconstruction of the evolutionary relationships of the insect protein coding sequences supports this hypothesis (see Fig. S1 in the supplementary material). Furthermore, the wisp/Gld2 ortholog in A. gambiae has female-biased expression, based on microarray data from whole males and females (Marinotti et al., 2005). This led us to hypothesize that the ancestral germline function of this gene family is in ovaries, and that the testis function of GLD2 (see below) is derived.

We examined the conservation of flanking genes on both local and global scales to determine whether wisp or Gld2 is the ancestral locus. On the local scale, wisp is in close proximity to Upf1, and the A. gambiae wisp/Gld2 homolog is in close proximity to the mosquito ortholog of Upf1. Assuming conserved syntenies, this suggests that wisp is the ancestral copy. On a more global scale, the A. gambiae homolog is located on chromosome 2R. This arm has homology to both the X chromosome and 3R of D. melanogaster (see Fig. S2A in the supplementary material). D. melanogaster wisp is X-linked and Gld2 is found on 3R. However, the local region of A. gambiae 2R that contains the wisp/Gld2 homolog is enriched for genes that are X-linked in D. melanogaster (see Fig. S2B in the supplementary material). This provides further evidence that wisp is the ancestral copy. Additionally, Gld2 is missing introns that are found in both wisp and the other insect homologs (see Fig. S3 in the supplementary material), suggesting that Gld2 might have arisen via the retrotransposition of wisp from the X chromosome to the autosome. These data all support the hypothesis that wisp is the ancestral copy of this cytoplasmic PAP family and that Gld2 is the derived copy.

GLD2 has the ability to bind the Drosophila Gld-3 homolog BIC-C

Sequence analysis predicts that GLD2 is a cytoplasmic PAP. Like its Drosophila paralog WISP and other Gld-2 family members (Kwak et al., 2004; Kwak and Wickens, 2007), GLD2 does not contain any predicted RNA recognition motifs. In C. elegans, binding of GLD-2 to mRNA is mediated by an RNA-binding protein, GLD-3, through formation of a heterodimer (Wang et al., 2002). The Drosophila Gld-3 homolog is encoded by Bicaudal C (Bic-C). Previous studies have shown that the Drosophila cytoplasmic PAP WISP, a Gld-2 family member, can interact with BIC-C in yeast two-hybrid assays (Cui et al., 2008); co-immunoprecipitation experiments from ovary extracts suggest that this interaction is RNA-dependent in Drosophila (Benoit et al., 2008).

Using a yeast two-hybrid assay, we found that GLD2 can interact with BIC-C. GLD2 in an activation domain (AD) fusion can interact with BIC-C in a binding domain (BD) fusion, but not with the BD alone. We observed interaction between GLD2 and BIC-C only in this orientation. The two-hybrid interaction we observed between GLD2 and BIC-C suggests that GLD2 might function, analogous to WISP and other Gld-2 family members, within a protein complex with an RNA-binding subunit. BIC-C is expressed in the testis as well as in the ovary (Chintapalli et al., 2007), so GLD2 has the opportunity to associate with BIC-C, supporting the case for the role of GLD2 as a PAP during spermatogenesis.

D. melanogaster GLD2 is expressed in the testes and localizes to elongating spermatogenic cysts

To determine the normal expression pattern of Gld2, we performed RT-PCR and immunoblots of RNA and protein from dissected tissues as well as from whole wild-type flies. Gld2 transcripts were detected in larvae, pupae and adults. The signal was robust in males, whereas expression was barely detectable in whole females (Fig. 2A). Western blotting with affinity-purified GLD2 antibody revealed GLD2 in the testes of the adult, and did not detect GLD2 in the remainder of the male or female body (Fig. 2B). Gld2 has previously been reported to be expressed in, and to function in, the Drosophila nervous system (Kwak et al., 2008). Apparently, this expression was below the detection limits of our western blots but we believe that it is the source of the low levels of expression that we see in RT-PCR of whole females. We did not detect GLD2 in the ovaries of wild-type females (Fig. 2C).
We further analyzed GLD2 expression in specific cells and developmental stages by immunostaining adult testes. GLD2 localized to the cytoplasm in primary spermatocytes throughout meiosis and in early round spermatids, consistent with its proposed action as a cytoplasmic PAF in the male germline (Fig. 2D). GLD2 was first detected in early spermatocytes, with robust expression in mid-primary spermatocytes (Fig. 2E). In later stages of spermatogenesis, GLD2 was detectable in elongated spermatogenic cysts and was specifically localized to the distal end of the cysts (Fig. 2F).

**GLD2 is necessary for late spermatogenesis**

To assess whether GLD2 function is necessary in the testes, we examined flies expressing dsRNA against Gld2 transcripts specifically in the germline. The efficiency of GLD2 knockdown was confirmed by RT-PCR (not shown) and by western blot (Fig. 3A); we did not detect GLD2 protein in these animals. We analyzed the GLD2 knockdown phenotype using two different RNAi lines (see Materials and methods) as well as two different drivers: bam-GAL4-VP16 and tubulin-GAL4. All four combinations of RNAi line and driver gave the same phenotype in our experiments (data not shown). The results reported here are from the RNAi progeny of a cross between VDRC line #52042 and our experiments (data not shown). The results reported here are from the RNAi progeny of a cross between VDRC line #52042 and bam-GAL4-VP16, unless otherwise stated.

The most striking effect of GLD2 knockdown in the germline is that the knockdown males are completely sterile, whereas the knockdown females display normal fertility in our assays, even though the same dsRNA is produced in their germlines (data not shown). Wild-type females mated to GLD2 knockdown males produced a normal number of eggs, but no eggs from these matings hatched. The reciprocal cross, in which GLD2 knockdown females were mated to wild-type males, produced normal numbers of eggs and progeny, indicating that females do not require GLD2 for fertility. The same fertility phenotypes were observed when GLD2 RNAi was driven globally using a tubulin-GAL4 driver as when GLD2 RNAi was driven exclusively in the male and female germlines using a bam-GAL4-VP16 driver.

To determine why GLD2 knockdown males are infertile, we examined sperm development in knockdown testes. Phase-contrast images of GLD2 knockdown testis squashes showed that, in spermatocytes and spermatocytes, mitosis and meiosis proceed normally, producing spermatogenic cysts that contain 64 spermatids after completion of meiosis II (Fig. 3B, C). Each early spermatid contained one nucleus and one associated mitochondrial derivative, indicating no obvious morphological defects during meiosis and mitochondrial development. However, spermatids within elongated cysts showed several abnormalities. Testes and seminal vesicles from GLD2 knockdown flies were devoid of mature or motile sperm, thus resulting in male sterility. Instead, fully elongated cysts in GLD2 knockdown males still contained syncytial spermatids, indicating that spermatogenic individualization did not occur (Fig. 3D, E). Bulges of material that accumulate along the tails of the elongated spermatids were evident in the squash preparations of GLD2 knockdown testes to a considerably higher degree than in control squashes prepared in the same way (Fig. 3D, E; these images are typical of what we see in the control and knockdown, respectively). Bulges of similar appearance have been observed in testis squash preparations of mutants that fail to complete spermatogenic individualization (Fabrizio et al., 1998). We speculate that these bulges are cytoplasmic aggregates that have been freed from the cyst during squash preparation and that, because the GLD2 knockdown spermatid tail is not contained within a membrane, the droplet of cytoplasm can adhere to it.

To determine the genesis of the individualization defect, we stained testis squash preparations from knockdown and wild-type males with DAPI and Rhodamine-conjugated phalloidin in order to detect nuclear bundles and actin individualization complexes, respectively. In wild type, individualization complexes appear as groups of cone-like structures composed of F-actin that assemble around bundled nuclei; each nucleus is associated with one actin cone. During the individualization process, these actin cones move as a complex down the length of the cyst, wrapping each spermatid in an individual membrane (Tokuyasu et al., 1972; Noguchi and Miller, 2003). In the wild type, cysts isolated from ruptured testis tissue contained all 64 spermatid nuclei at their apical ends, and many of these nuclear bundles were closely associated with assembled individualization complexes (Fig. 4A). By contrast, the spermatogenic cysts of GLD2 knockdown males never contained bundled nuclei or assembled F-actin-containing individualization complexes. Instead, the nuclei were unevenly scattered throughout the cyst, without any tendency for greater density at the apical end (Fig. 4B). A scattered nuclear phenotype is common in many individualization mutants (Fabrizio et al., 1998) and has usually been attributed to malformation of the actin individualization complexes. It has been proposed that in those mutants, a malformed individualization complex will drag nuclei along as the complex moves down the length of the cyst (Fabrizio et al., 1998).
GLD2-deficient pupae. Tissues were dissected 3–4 days after pupation and stained for nuclei and GLD2 localization, as had been done with the adult tissues. In wild-type preparations, we observed nuclear bundling at one end of the elongating cysts at the onset of elongation (Fig. 4E). By contrast, we never observed such nuclear bundles in GLD2 knockdown preparations from pupae. These preps showed nuclear scattering within cysts even at the earliest stages of spermatocyst elongation examined (Fig. 4F). Thus, spermatogenetic defects in the GLD2 knockdown first appear in the early stages of post-meiotic spermatid morphogenesis, and nuclear scattering does not result from malformed actin cones (in contrast to other mutants that exhibit nuclear scattering). It is possible that the spermatogenetic defects we observed in late spermatogenic cysts, such as in actin cone formation and subsequent individualization, result from the improper completion of these early events.

**Basal body docking is defective in GLD2-deficient spermatogenesis**

Because knockdown of GLD2 resulted in nuclear scattering, and because nuclear scattering has previously been described in mutants affecting basal body docking (Texada et al., 2008; Anderson et al., 2009), we examined basal body docking in the absence of GLD2. After completion of meiosis, haploid, round spermatids normally undergo differentiation events before entering the spermatid elongation period. One important event is the formation of the basal body, which is derived from the centriole. During meiosis II, the centrosome undergoes reductive division so that each post-meiotic spermatid nucleus is associated with only one centriole. Upon spermatid differentiation, soon after meiosis, the centriole inserts into a groove in the nuclear membrane at the base of the nucleus (a process called basal body docking), and becomes the spermatid basal body (Tokuyasu, 1974; Fuller, 1993). To assess whether basal bodies could form and dock properly in the absence of GLD2, we compared staining patterns of γ-Tubulin in the GLD2-deficient in the testes of GLD2-deficient and control adult flies.

In wild-type testes, γ-Tubulin stains centrosomes throughout meiotic phases and stains basal bodies associated with nuclei in elongated spermatids (Fig. 5A). In GLD2 knockdown testes, centrosomal γ-Tubulin staining was seen in meiotic spermatocytes (data not shown), but γ-Tubulin was not consistently detected at or near the nuclei of elongated spermatids (Fig. 5B). This indicates that, in the GLD2 knockdown, centrioles are unable to attach to the nuclear membrane after meiosis, resulting in post-meiotic

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Fig. 4. Nuclei are scattered throughout spermatogenic cysts and individualization cones do not form in GLD2 knockdown testes. (A, B) Testes squashes were fixed and stained with DAPI and with Rhodamine-conjugated phalloidin to detect nuclei (blue) and F-actin individualization cones (red), respectively. (A) In wild-type Drosophila controls, individualization cones assemble around bundled nuclei in late spermatogenic cysts. (B) In GLD2 knockdown testes, nuclei of late spermatogenic cysts are scattered uniformly throughout the cyst (the extent of the cyst is visible from the phase-contrast overlay) and individualization cones are absent. (C, D) Higher magnification shows that nuclei in late spermatogenic cysts of the knockdown (D) are noticeably less condensed than those of wild type (C). Nuclei shown in D are representative of the most highly condensed nuclei that we see in knockdown testes. (E, F) Examination of pupal testes with propidium iodide staining shows that nuclei begin to bundle at the onset of cyst elongation in the wild type (E), whereas at early elongation stages in the GLD2 knockdown the nuclei are already scattered (F). The early elongation cyst is outlined. Scale bars: 10 μm.

However, in such mutants, even in cysts containing scattered nuclei, there is a higher density of nuclei at the apical end. This pattern suggests that nuclear bundles are initially formed in these mutants, but later become scattered by malformed individualization complexes. The phenotype of GLD2 knockdown cysts differs from this: since individualization complexes are apparently never assembled, nuclear scattering cannot be due to the previously described dragging effect. Instead, because the nuclei are scattered uniformly across the length of the spermatogenic cyst, they were unlikely to have been bundled in the initial stages of spermiogenesis and, as such, defects might be apparent even before the onset of individualization complex assembly. To test this, we examined preceding spermatogenic stages for defects in the absence of GLD2.

Because many post-meiotic spermatogenic cysts within adult Drosophila testes have already undergone substantial flagellar extension and cyst elongation, the smaller, early post-meiotic cysts are difficult to identify in adults. In order to observe cysts that are just beginning to elongate, we examined testes of wild-type and...
spermatids that lack basal bodies at the nucleus. When the full length of the testis was examined, some intermittent γ-Tubulin staining was detected in the GLD2 knockdown, but the staining did not appear to be associated with nuclei, nor did it appear as distinct puncta, in contrast to the wild-type situation. It is unclear what happens to centrosomal derivatives in the elongated cysts of GLD2 knockdown testes. The presence of axonemes raises the possibility that centrosome-derived structures are intact, at least in early spermatids, as the basal body is required for axoneme assembly (Fuller, 1993). However, our confocal microscopy scans of late spermatogenic cysts of knockdown testes failed to detect distinct γ-Tubulin staining, which would have indicated the presence of properly formed basal bodies; we did detect such basal body staining in the wild type (see Fig. S4 in the supplementary material). Thus, basal body docking does not occur in the absence of GLD2, indicating that the differentiation of the centrosome to the basal body is defective, or that the basal body is otherwise unable to dock at the nuclear envelope.

**Nuclear compaction is disrupted in GLD2-deficient spermatogenesis**

DAPI staining indicated that, in addition to the nuclear scattering phenotype, nuclei in elongated spermatocytes of GLD2 knockdown testes were less condensed than those of the wild type (Fig. 4C,D), suggesting a nuclear compaction defect. We found that the nuclei that were the most condensed in GLD2 knockdown testes had not progressed past the canoe stage (Fig. 4D). A possible explanation for this arrest is that the translation of nuclear compaction proteins could require GLD2. To investigate whether spermatids in GLD2 knockdown flies are able to exchange histones for protamines, we examined spermatid nuclei of GLD2 knockdown males carrying a protamine-B-eGFP construct. In this cross, snapshot RNA against Gld2 was driven by tubulin-GAL4. Whereas many spermatid nuclear bundles and all nuclei of free-swimming sperm in control testes showed a clear GFP signal (Fig. 6A,B), there was no GFP signal detectable anywhere in the knockdown testis (Fig. 6C,D). However, RT-PCR showed the presence of protamine-B-eGFP transcripts, indicating that the construct is present and transcribed in these flies (Fig. 6E). Therefore, we performed PAT assays for Protamine A and Protamine B RNAs on control and knockdown flies. We found no difference in the poly(A) tail length of these protamine transcripts between the two groups (Fig. 6F). Thus, it is likely that the protamines are regulated post-transcriptionally in the *Drosophila* testes, but they are not direct targets of GLD2.

Although no effect of GLD2 on protamine transcripts was observed, we did note that the poly(A) tail of the Protamine A and Protamine B transcripts was ~30 nucleotides longer in larval testes than in adult testes (Fig. 6F), a previously unreported finding for *Drosophila* spermatogenesis. Larval testes are composed mostly of primary spermatocytes and have not yet developed the protamine-based nuclear configurations that are seen in adult testes (Fuller, 1993). Therefore, the difference in protamine poly(A) tail length between larval and adult testes indicates that decondensation accompanies spermatid differentiation and might promote protamine translation. Although this seems contradictory to what is known about the role of the poly(A) tail in regulating translational efficiency, decondensation has been known to accompany translation in some cases. In mice, for example, transition protein and protamine transcripts are synthesized in primary spermatocytes and are translationally repressed for several days. The translationally inactive, non-polysome-bound forms of the mouse protamine transcripts are considerably longer than the active polysome-bound forms, and this difference in transcript length reflects differences in the length of the poly(A) tail (Kleene et al., 1984).

To further investigate why nuclei fail to condense fully in the GLD2 knockdown, we tested for the presence of transcripts of the *Drosophila* transition protein Tpl94D (CG31281) by RT-PCR. *Tpl94D* is transcribed in spermatocytes, and the protein appears only transiently during nuclear remodeling, where it facilitates the exchange of histones for protamines (Rathke et al., 2007). Thus *Tpl94D* is a candidate for post-transcriptional regulation at the transcript 3’ sequence. *Tpl94D* transcripts were detectable in wild-type but not in GLD2 knockdown testes (Fig. 7). This could indicate that *Tpl94D* is not transcribed or that its transcript is synthesized but is unstable. Such instability could arise from a
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**Fig. 7. Tpl94D transition protein and dynamin transcripts are absent from GLD2 knockdown testes.** RT-PCR showing that the dynamin (dyn) and Tpl94D transcripts are present in control adult *Drosophila* testes, but absent from GLD2 knockdown testes. Rpl32 is used as a positive control.

poly(A) tail elongation defect; however, because the transcript is not detectable in GLD2 knockdown samples, we could not measure its poly(A) tail length or otherwise test these hypotheses.

**An absence of Dynamin may contribute to the F-actin assembly defect in GLD2-deficient spermatogenesis**

In wild-type adult testes, F-actin individualization complex formation follows protamine incorporation. As described above, actin individualization complexes do not develop in the absence of GLD2. Lack of F-actin at nuclear bundles could be due to a failure to regulate actin transcripts, a failure to stabilize G-actin, or a failure to assemble G-actin into individualization cones in GLD2 knockdown testes. To distinguish between these possibilities, we performed tests at each level.

We examined the expression of two actin genes, *Act42A* and *Act87E*, which are reported to be highly expressed in testis tissue (Chintapalli et al., 2007). We detected both transcripts in wild-type and GLD2 knockdown testes, and PAT assays indicated no change in their polyadenylation state in the absence of GLD2 (see Fig. S5A in the supplementary material). To test whether actin transcripts were being translated, we stained adult testes for monomeric G-actin using Alexa Fluor-labeled DNase I. DNase I binds to G-actin and is an indicator of G-actin levels (Snabes et al., 1981; Philip et al., 1992). There was no discernible difference in the staining pattern of wild-type and GLD2-deficient testes, indicating that G-actin is present in apparently equivalent amounts (see Fig. S5B,C in the supplementary material).

Our data indicate that the loss of F-actin in GLD2 knockdown testes is unlikely to be due to a deficit of available G-actin, but might be attributable to an absence of F-actin assembly machinery. We examined a number of factors known to be involved in F-actin formation, including Profilin (Chickadee – FlyBase) and Dynamin. RT-PCR showed that dynamin mRNA was present in wild-type but not GLD2-deficient testes (Fig. 7). Dynamin has been shown to be necessary in late spermatogenesis for the initiation of assembly of F-actin cones at spermatid nuclei (Ghosh-Roy et al., 2005). Thus, the absence of Dynamin in GLD2-deficient testes might account for the lack of F-actin individualization cones in late-stage spermatids. However, as with the transition protein transcript, we could not determine whether the absence of the dynamin transcript is a direct result of a lack of polyadenylation by GLD2 or is a secondary result of a block to spermatogenesis upstream of Dynamin regulation.

**Candidate targets of GLD2**

Kwak and colleagues have shown that GLD2 is an enzymatically active cytoplasmic PAP in vitro (Kwak et al., 2008). We attempted to identify the target genes through which GLD2 functions during spermatogenesis, choosing candidates based on the phenotype of the GLD2 knockdown; specifically, genes known to be necessary for the affected late spermatogenesis events such as basal body formation, nuclear anchoring, nuclear compaction and individualization. When candidate transcripts were tested for GLD-dependent changes in poly(A) tail length (Table 1), we did not identify any potential targets: all transcripts (with the exception of *Tpl94D* and *dynamin*, see above) were present at equal levels in GLD2 knockdown and control testes, and variation in transcript length between GLD2 knockdown and control samples was not detected.

Transcripts of *Tpl94D* and *dynamin* remain potential targets for polyadenylation by GLD2. If these transcripts are GLD2 targets, then they would presumably remain deadenylated in the cytoplasm in the GLD2 knockdown, in which case they would be likely to be degraded by exonucleases. Unfortunately, we could not test these candidates by PAT assay since their transcripts are undetectable.

Although direct targets of GLD2 were not found among carefully chosen candidate genes, future high-throughput transcriptomic analysis might be needed to reveal GLD2 targets.

**DISCUSSION**

We report here that a testis-expressed cytoplasmic PAP, GLD2, is required for spermatogenesis in *D. melanogaster*. Knockdown of GLD2 in the testes causes widespread defects in post-meiotic spermatogenesis events. In a GLD2 knockdown, the earliest defects are seen in early post-meiotic spermatids, when the basal body fails to dock at the nuclear envelope and the nuclei begin to scatter. Many late-stage events of spermatogenesis are also affected, including protamine translation and F-actin cone formation on individualization stage spermatids. Additionally, GLD2 knockdown affects the stability of *dynamin* transcripts and those of transition protein (*Tpl94D*) in the testes. Interestingly, GLD2-deficient germ cells appear to undergo normal meiosis, in contrast to mutants of other Gld-2 homologs, including the GLD2 paralog WISP (Benoit et al., 2008; Cui et al., 2008).

**GLD2 as a cytoplasmic PAP**

There is indirect evidence that GLD2 acts as a cytoplasmic PAP in the *Drosophila* testes. The GLD2 protein contains a PAP/25A domain, which is shared by all known Gld-2 family proteins (Benoit et al., 2008). Additionally, GLD2 has the ability to elongate poly(A) tails in vitro (Kwak et al., 2008). In the current study, we have shown that GLD2 interacts with the Gld-3 homolog BIC-C in a yeast two-hybrid assay. Furthermore, at least two transcripts are absent in GLD2 knockdown testes, which might be the result of destabilization owing to an inability to elongate their poly(A) tails. Taken together, we conclude that *Drosophila* GLD2 does act as a PAP during spermatogenesis and that the defects seen in its absence are the result of failure of one or more polyadenylation events.

**GLD2 is required for post-meiotic spermatid development**

GLD2 affects many aspects of spermatogenesis in *Drosophila*. First, nuclear anchoring and basal body docking are defective in the absence of GLD2. Both processes occur in early post-meiotic stages of spermatogenesis in wild-type testes; however, in the absence of GLD2, spermatid nuclei scatter throughout spermatogenic cysts and basal bodies cannot assemble at the nuclear envelope. It is possible that, in the GLD2 knockdown, the failure of these events is related. For example, it might be the case that nuclear anchoring cannot occur until basal bodies have docked...
properly, or vice versa. Alternatively, loss of GLD2 might affect formation of the post-meiotic nuclear envelope, which might in turn have negative effects on both nuclear anchoring and basal body docking processes. Other studies of mutants that involve basal body defects have documented nuclear localization disruptions, indicating that these two processes may be linked (Fabian et al., 2010; Texada et al., 2009).

Table 1. Transcripts that tested negative for GLD2-dependent polyadenylation by PAT assay

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<td>Actin</td>
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<td>Basal body docking (Anderson et al., 2009)</td>
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A third defect in GLD2-deficient spermatogenesis occurs at individualization: an actin cone is never detected around late-stage nuclei and the spermatids do not separate from one another. *dynamin* transcripts are missing in GLD2 knockdown testes. The absence of *Dynamin* could account for the lack of actin cones at individualization stage nuclei: previous studies have demonstrated that Dynamin is present throughout the actin cones and that disruption of *Dynamin* function in temperature-sensitive mutants contributes to their instability (Rogat and Miller, 2002). Lack of *dynamin* mRNA could indicate that it is a GLD2 target: lack of *dynamin* polyadenylation by GLD2 could leave the transcript vulnerable to exonucleases in the cytoplasm, resulting in its degradation. Alternatively, it is possible that the absence of Dynamin in the GLD2 knockdown results from a developmental block during late spermatogenesis, at a time before *dynamin* RNA would be present.

What are the targets of GLD2?

GLD2 localization might help to identify its target transcripts. Our immunofluorescence staining experiments showed that in addition to cytoplasmic localization in spermatocytes, GLD2 localizes to the distal ends of elongated spermatogenic cysts. This is where the polarized growth of the cyst occurs in accordance with axoneme extension (Fuller, 1993); additionally, a group of mRNAs that are transcribed post-meiotically have been shown to localize to the distal end of the spermatogenic cyst (Barreau et al., 2008). Interestingly, one of these late-transcribed genes is *orb*, which
encodes the Drosophila ortholog of CPEB, the protein necessary for cytoplasmic polyadenylation in Xenopus (Barnard et al., 2004; Richter, 2007). The presence of both the CPEB ortholog ORB and the cytoplasmic PAP GLD2 at the end of the cyst where growth is occurring might indicate an involvement of GLD2 in late spermatocyst growth. Taken together, these data suggest that the distal end of the cyst might be a major production center for cyst growth, with the necessary mRNAs regulated post-transcriptionally through cytoplasmic polyadenylation.

GLD2 may have arisen through meiotic sex chromosome inactivation

We have reported a role for GLD2 that is essential for male, but not female, gametogenesis. This is a unique finding among the literature describing other Gld-2 homologs, where Gld-2 proteins are necessary for some aspect of oogenesis and egg maturation in Drosophila, Xenopus and mice (Barnard et al., 2004; Benoit et al., 2008; Cui et al., 2008) and for the proliferative stages of gametogenesis in hermaphrodite worms (Kadyk and Kimble, 1998). Drosophila GLD2 plays a role in the male, but not female, germline, and is required in spermatid morphogenesis rather than in proliferative stages. The evidence that GLD2 was retrotransposed to the third chromosome from a duplication of the wisp locus on the X chromosome might give insight to how this unique role for a Gld-2 homolog came about. The phenomenon of meiotic sex chromosome inactivation (MSCI) might have contributed to duplication of the wisp gene and to subsequent retention of GLD2. During spermatogenesis in Drosophila and other animals, the X chromosome is transcriptionally silenced prior to autosomal silencing (Hense et al., 2007; Turner, 2007; Vibranovski et al., 2009). Therefore, genes located on the X chromosome have a limited capacity to encode proteins involved in spermatogenesis. Interestingly, an excess of genes has been retrotransposed from the X to the autosomes, and the autosome-derived copies are hypothesized to allow for the escape from X-inactivation (Betrán et al., 2002; Meisel et al., 2009). The testis-biased expression (Chintapalli et al., 2007) and spermatogenic functions of GLD2 suggest that it was selectively retained because it performs a function unavailable to wisp because of MSCI.

It is interesting that GLD2 is crucial for post-meiotic spermatogenesis in Drosophila, whereas all Gld-2 family members analyzed so far in Drosophila and other species play roles specifically at meiosis. We hypothesize that the function of GLD2 in the male germline reflects its evolutionary origin: duplication of a Gld-2 homolog came about. The phenomenon of meiotic sex chromosome inactivation (MSCI) might have contributed to the X chromosome inactivation (MSCI) might have contributed to the X to the autosomes, and the autosome-derived copies are hypothesized to allow for the escape from X-inactivation (Betrán et al., 2002; Meisel et al., 2009). The testis-biased expression (Chintapalli et al., 2007) and spermatogenic functions of GLD2 suggest that it was selectively retained because it performs a function unavailable to wisp because of MSCI.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.059618/-/DC1

References


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