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The testis-specific proteasome subunit Prosα6T of *D. melanogaster* is required for individualization and nuclear maturation during spermatogenesis

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Most regulated proteolysis in eukaryotes is carried out by the 26S proteasome. This large, multisubunit complex comprises a catalytic core particle (20S proteasome) and a regulatory particle (19S regulator) capping each end. In *Drosophila*, about a third of the 32 proteasome subunits are found to have testis-specific isoforms, encoded by paralogous genes. Here, we characterize in detail the spermatogenic expression of the core particle subunit Prosα6 (Pros35) and its testis-specific isoform Prosα6T. Using GFP-tagged transgenes, it is shown that whereas the Prosα6 subunit is expressed in early stages of spermatogenesis, gradually fading away following meiosis, the testis-specific Prosα6T becomes prominent in spermatid nuclei and cytoplasm after meiosis, and persists in mature sperm. In addition, these subunits are found in numerous 'speckles' near individualization complexes, similar to the previously described expression pattern of the caspase Dronc (Nedd2-like caspase), suggesting a link to the apoptosis pathway. We also studied the phenotypes of a loss-of-function mutant of *Prosα6T* generated by targeted homologous recombination. Homozygous males are sterile and show spermatogenic defects in sperm individualization and nuclear maturation, consistent with the expression pattern of Prosα6T. The results demonstrate a functional role of testis-specific proteasomes during *Drosophila* spermatogenesis.

KEY WORDS: Apoptosis, Individualization, Proteasome, Spermatogenesis

INTRODUCTION

The proteasome is the protein degrading machine of the ubiquitin-mediated proteolytic pathway, which has been implicated in many cellular processes, including cell cycle progression, transcriptional regulation, signal transduction, and cell fate determination (Glickman and Ciechanover, 2002). This pathway also carries out an important quality control function by ridding cells of defective polypeptides. The highly conserved 26S proteasome is composed of two subcomplexes: a 20S catalytic core particle (CP) and 19S regulatory particles (RP) capping each end. The CP consists of two identical outer α-rings and two identical inner β-rings, each composed of seven evolutionarily related subunits (α1-7 and β1-7) that stack to form a hollow cylinder (Gröll et al., 2000; Unno et al., 2002). The RP includes a ring of six homologous AAA-family ATPase subunits that function to unfold protein substrates and translocate them into the CPs inner chamber. The functions of most of the twelve other RP subunits are less well understood but they include binding and removing polyubiquitin chains from proteins targeted for destruction (Glickman et al., 1998).

In addition to these integral proteasomal subunits, a number of more loosely, or transiently, associated proteins have recently been identified (Glickman and Raveh, 2005). These auxiliary proteins include ubiquitin-binding proteins that facilitate the delivery of substrates to the proteasome, ubiquitin ligases that build polyubiquitin chains onto the target proteins, and deubiquitinating enzymes that remove these tags (Schmidt et al., 2005).

The variety of proteasome-associated proteins suggests that proteasomes may be dynamic complexes with some diversity in composition. There is, however, another level of compositional diversity, as represented by the structurally heterogeneous proteasomes found in some species (Belote and Zhong, 2005). For example, in mammals, during the anti-viral immune response, γ-interferon induces the synthesis of three new β-type subunits which take the places of their corresponding conventional subunits to form 'immunoproteasomes' (Kloetzel, 2004) that are more efficient at producing peptide antigens for MHC class-I mediated antigen presentation. In *Arabidopsis* and rice, many of the proteasome subunits are represented by more than one gene, suggesting a high degree of structural heterogeneity in which different proteasome complexes are composed of different isoforms of several of the subunits (Fu et al., 1999; Yang et al., 2004; Oguchi et al., 2001; Shibahara et al., 2004). In this case, however, the functional significance of this proposed heterogeneity is unknown.

One of the most striking examples of this phenomenon is seen in *Drosophila melanogaster*, where about a third of the 26S proteasome subunits have two or three alternative isoforms encoded by paralogous genes (Yuan et al., 1996; Ma et al., 2002; Belote and Zhong, 2005). Molecular analyses have shown that, in every case, one form of each subunit is expressed in both sexes at all developmental stages examined (these will be referred to as conventional proteasome subunits), whereas all of the additional isoforms are testis specific. This represents a unique example of developmental regulation of alternative proteasome subunit expression, and suggests that there might be a specialization of proteasome function during spermatogenesis. Expression pattern studies of the testis-specific subunit Prosα3T, using GFP-tagged reporters, found that this subunit is first detected during meiosis and becomes conspicuous during the late stages of spermiogenesis (Ma et al., 2002). By contrast, Prosα3 (also known as Pros29 – FlyBase), representing the conventional counterpart of Prosα3T, is expressed somatically and in the germline at the early stages of spermatogenesis, but gradually disappears after meiosis, and is not detected in mature sperm (Ma et al., 2002). Although the expression patterns of all testis-specific proteasome subunit genes have not been

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analyzed in detail, preliminary studies of a few of them suggest that they are expressed in a similar way (Yuan et al., 1996) (L.Z., unpublished; X. Li and J.M.B., unpublished). The large number of proteasome subunit isoform genes, and the collective shift of the expression patterns between the conventional subunit genes and their testis-specific counterparts, suggest that there might be a testis-specific proteasome that is dynamically assembled during *Drosophila* spermatogenesis. If so, what is its functional significance?

Here we characterize in detail the spermatogenic expression of the α-type subunit Prosα6 (also known as Pros35 – FlyBase) and its testis-specific isoform Prosα6T. We also address the question of the functional significance of testis-specific proteasome subunits by studying the phenotypes of a knockout mutant of *Prosα6T*. Mutants are male-sterile, with disruption of actin cone movement during sperm individualization, and abnormal nuclear maturation and morphology. These findings establish a necessary role of at least one of the testis-specific proteasome subunits in *Drosophila* spermatogenesis.

MATERIALS AND METHODS

Fly strains

The *Df(2L)ED784* and *His2AvGFP* stocks were obtained from the Bloomington Stock Center (stock nos 7421 and 5941, respectively). The *y w; [ry$^+$ 70FLP]4 [v$^+$, 70 I-SceI]2B Sco/S^2 CyO* and *w^{1118}; [ry$^+$, 70FLP] 10* lines were provided by Kent Golic (University of Utah).

Plasmid constructions and generation of transgenic flies

The construction of the *Prosα6T-GFP* reporter gene and generation of transgenic flies were previously described by Ma et al. (Ma et al., 2002). For the construction of the *Prosα6-GFP* reporter gene, a recombinant λ-phage containing the *Pros35* gene (encoding the Prosα6 proteasome subunit) was isolated as described by Ma et al. (Ma et al., 2002), and an 8.0 kb *Bam*HI fragment was cut out of this and subcloned into pGEM4 (Promega) to give pGEM4/Prosα6-8.0B. Site-directed mutagenesis, with primers Pros35Not5-(5′-AGCAGCGTCCAGCGGCCGCTAGGCATT-TATAG-3′) and Pros35Not3-(5′-CTATAAATGCCTAGCGGCCGCTG-GACGCTGCT-3′), was carried out to create a *Not*I site at the 3′ end of the *Pros35* open reading frame (ORF) using the Quik Change Site-directed Mutagenesis Kit (Stratagene), yielding pGEM4/Prosα6-8.0B-Not. A *Not*I GFP cassette was made by PCR amplification of pEGFP (Invitrogen) using the following primers: EGFP Not5′-(GCGCGGCCGCCATGGT-GAGCAAGGGCGAGGAGCTGTTCAC-3′) and EGFP Not3-(5′-AAG-GGCCCGTACGGCCGACTAGTAGGCCTA-3′). This PCR product was cloned into pGEM-T Easy and then cut out with *Not*I and ligated into the newly created *Not*I site of pGEM4/Prosα6-8.0B-Not. A 4.4 kb *Pst*I-*Eco*RV fragment containing the GFP-tagged *Pros35* gene was shuttled into pBlueScript KS+ (Stratagene), and then removed using *Pst*I and *Xho*I and subcloned into the transformation vector pW8 to give pW8/Prosα6-GFP.

For the generation of flies expressing fluorescently tagged protamine, a BAC clone (RPCI-98 33.C.24) of the genomic region containing the *Mst35Ba* (*Protamine A*) gene was obtained (Hoskins et al., 2000), and a 6.0 kb *Nru*I-*Asp*700 fragment, containing the *ProtA* gene, was subcloned into pBlueScript to give pBS/ProtA6.0NA. To create the GFP fusion, site-directed mutagenesis was done to create a unique *Nde*I site at the C terminus of the ProtA coding region yielding pBS/ProtA-Nde. A GFP *Nde*I cassette was created by PCR amplification of pEGFP. This was inserted in-frame into the *Nde*I site of pBS/ProtA-Nde and the fusion construct then subcloned into pW8 to give pW8/ProtA-GFP.

A testis-specific expression vector, pW8/TS1, was created by modifying a genomic clone, pW8/Prosα3T-2.8, containing the testis-specific *Prosα3T* gene coding region flanked by 0.8 kb of upstream sequences and 1.1 kb of downstream sequences (Ma et al., 2002). Using site-directed mutagenesis, a *Kpn*I site was inserted just before the start codon, and a *Not*I site was inserted just before the stop codon. The *Prosα3T* coding region was then replaced by sequences between the *Kpn*I and *Not*I sites of the multiple cloning region of pBlueScript KS+, yielding pW8/TS1. The *Prosα6* coding region was PCR amplified from genomic DNA and inserted into the *Not*I site of pW8/TS1 to give pW8/TS1-Prosα6.

The various pW8 constructs were introduced into the genome by P-element transformation methods, with *w^{1118}* as the host, and several transgenic lines established for all constructs.

Generation of the *Prosα6T^1* mutant by homologous recombination

For the construction of the *Prosα6T* targeting donor plasmid, two oligonucleotides, 5′-GGCCTAGGGATAACAGGGTAAT-3′ and 5′-GGCCATTACCCTGTTATCCCTA-3′, were annealed to create an I-*Sce*I cutting site flanked by 5′-GGCC-3′ overhangs. This was used to replace the GFP-*Not*I cassette in pBS/Prosα6T-GFP. To eliminate two unwanted *Pst*I sites flanking the *Prosα6T* gene, this plasmid was digested with *Hinc*II, ligated, digested with *Eco*RV and *Sma*I, and ligated. To create a frameshift in the 5′ region of the *Prosα6T* ORF, the resulting plasmid was digested with *Pst*I, the 3′ overhangs polished using the Strategene Polishing Kit (Strategene), and treated with ligase to yield plasmid pBS/Prosα6T-KO-SceI. The insert was cut out with *Hin*dIII and *Xho*I and cloned into the corresponding sites of plasmid pBS(-Not), provided by Kent Golic. The insert was then cut out with *Not*I and cloned into the transformation vector pTV2, also provided by Kent Golic. The resulting plasmid, pTV2-Prosα6T-KO-SceI, was then introduced into the fly genome by *P*-element germline transformation.

Flies with an X-linked *TV2-Prosα6T-KO-SceI* targeting donor transgene were crossed to *y w; [ry$^+$ 70FLP]4 [v$^+$, 70 I-SceI]2B Sco/S^2 CyO* mates and 0- to 3-day-old F1 larvae were heat-shocked at 38°C for 90 minutes. Mosaic-eyed daughters were then crossed to *w^{1118}; [ry$^+$, 70FLP]10* males, and non-mosaic red-eyed F2 offspring, potentially representing homologous recombinants, were selected and crossed to *w; Bl/CyO* to establish balanced lines. Of the 79 candidate F2 flies, only one had the *w^{hs}* marker on chromosome *2*, as expected for the homologous recombinant. Curly-winged flies from this line were crossed with *w^{1118}; Bl/CyO; P{v[+t1.8]=hs-I-CreI.R}1A Sb^1/TM6* flies and F1 larvae heat-shocked at 36°C for 60 minutes. Resulting mosaic eyed, Curly-winged females were then crossed with *w^{1118}*; *Bl/CyO* males and selected for white-eyed, Curly-winged, non-Bristle offspring, which were the potential *Prosα6T* mutants. Separate lines were established for each candidate mutant, balanced over *CyO*, and the *Prosα6T* gene PCR amplified and checked by *Pst*I digestion and DNA sequencing. Out of 11 candidate mutant lines, only one was found to have the desired mutation.

Male fertility tests

Individual males were placed with two virgin *w^{1118}* females in culture vials for 7 days at 25°C. The flies were then removed and all resulting F1 progeny were counted. At least six vials were set up and scored for each genotype tested.

Immunofluorescence staining

Alexa Fluor 635 phalloidin, Alexa Fluor 532 phalloidin, Alexa Fluor 488 phalloidin, Alexa Fluor 633 rabbit anti-mouse IgG, Alexa Fluor 532 rabbit anti-mouse IgG, Alexa Fluor 488 rabbit anti-mouse IgG, and TO-PRO-3 were obtained from Molecular Probes (Invitrogen). The anti-Prosα7 monoclonal (clone MCP72) antibody was from Biomol (Exeter, UK). The anti-active Dronc (also known as Nedd-2 like caspase – FlyBase) antibody was generously provided by Bruce Hay (California Institute of Technology), and the CM1 antibody was a gift from Maya Bader and Hermann Steller (The Rockefeller University). For DNA and actin double staining, testes from newly eclosed males were dissected in phosphate-buffered saline (PBS, 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.2) and then fixed with EM grade 4% paraformaldehyde (Electron Microscope Sciences) in PBST (PBS+0.3% Triton X-100) for 1 hour at room temperature. After washing with PBST, the testes were blocked with 10% fetal bovine serum (FBS) and 0.5 mg/ml RNaseA in PBST at room temperature for 1 hour. Then the testes were washed once in PBST, and twice in PBS, followed by incubation with 1 μM TO-

PRO3 and 4 units/ml Alexa Fluor phalloidin in PBS for 30 minutes in the dark. They were then rinsed three times in PBS, and mounted in ProLong mounting solution (Molecular Probes). For anti-Prosα7, active Dronc and CM1 antibody staining, after fixing and washing as described above, the testes were blocked with 10% FBS in PBST at room temperature for 1 hour, and then incubated with anti-Prosα7 antibody, diluted 1:500 in PBST + 10% FBS, at 4°C overnight. After washing in PBST, the testes were incubated with secondary antibody (Alexa Fluor 633 or 532 anti-mouse IgG, 1:1000 dilution in PBST + 10% FBS) at room temperature for 4 hours, rinsed three times in PBST, three times in PBS, and then mounted in ProLong. A Zeiss LSM5 Pascal confocal microscope was used for fluorescence imaging.

RESULTS

Spermatogenic expression patterns of Prosα6 and Prosα6T

To investigate the roles of the testis-specific proteasome subunit Prosα6T, we analyzed in detail its expression and subcellular localization patterns during spermatogenesis. In normal spermatogenesis, stem cells located at the apical tip of the coiled testis divide to produce spermatogonial cells that undergo four cycles of mitosis, each yielding sixteen primary spermatocytes that are interconnected, as a result of incomplete cytokinesis, and surrounded by two somatic cyst cells (Lindsley and Tokuyasu, 1980; Fuller, 1993). Following a growth phase in which the cyst increases in size 25-fold, the 16 primary spermatocytes of each cyst undergo meiosis, resulting in a cyst of 64 spherical, haploid spermatids, each containing a prominent round nucleus and a phase-dark structure, the nebenkern, consisting of fused mitochondria.

In the later stages of sperm development, known as spermiogenesis, the spermatids undergo tremendous elongation. The nuclei condense and experience a dramatic shape change to assume dense, needle-shaped configurations. The spermatids undergo individualization, during which the syncytial spermatid bundle is resolved into 64 separate sperm cells, and excess cytoplasmic contents are removed and accumulated into a 'waste bag' that is subsequently degraded and reabsorbed. In the final stage of spermiogenesis, the sperm coil up, the cyst ruptures, and the mature sperm move into the seminal vesicle for storage.

In the present study, we have examined the spermatogenic expression patterns of Prosα6 and Prosα6T in detail, using GFP-tagged reporter transgenes. Similar to the expression pattern of Prosα3-GFP, Prosα6-GFP is seen in both cytoplasm and nuclei of spermatogonia, spermatocytes and early stage spermatids. During spermiogenesis, the Prosα6-GFP signal begins to fade in elongated sperm bundles and condensed nuclei and is undetected in individualized, mature sperm (Fig. 1A-H). By contrast, Prosα6T-GFP is not observed in pre-meiotic stages, but becomes prominent in the cytoplasm and nuclei of 64-cell spermatid cysts. The GFP fluorescent signal persists in the elongated and condensed spermatid nuclei and also in spermatid tail bundles and can be seen in the mature sperm heads and tails (Fig. 1A′-H′).

In addition to being expressed in elongated nuclei and distributed along spermatid tail bundles, Prosα6T-GFP also aggregates as 'speckles' at certain positions along the sperm bundles (Fig. 2A). To better discern the precise localization of these speckles, Prosα6T-GFP testes were fixed and incubated with Alexa-Fluor-phalloidin and TO-PRO3 to stain actin and nuclei, respectively (Fig. 2B). In the elongated spermatid bundles, Prosα6T-GFP speckles were found near the individualization complex (IC), a cytoskeletal-membranous complex containing a cluster of 64 actin-rich structures, called actin cones, that mediate sperm individualization. During this process, the IC moves caudally down the bundle, eliminating all cytoplasmic bridges and

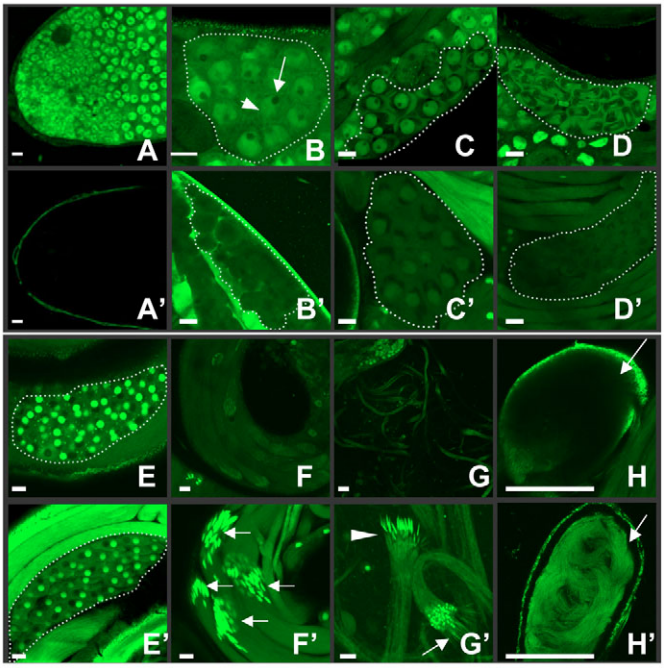

Fig. 1. The expression patterns of Prosα6-GFP and Prosα6T-GFP during spermatogenesis. (**A-H**) Prosα6-GFP; (**A′-H′**) Prosα6T-GFP. (A,A′) Apical ends of testes showing early spermatogonial stages. (B,B′) 16-cell primary spermatocyte cysts (dotted lines, also in C-E). (B) Arrow, nucleolus; arrowhead, nucleus. (C,C′) Late primary spermatocyte cysts. (D,D′) Cysts undergoing meiosis I. (E,E′) 64-cell onion-stage spermatid cysts. Nuclei are brightly fluorescing and nebenkerns are dark. (F,F′) Elongated spermatid bundles. Nuclear bundles are brightly fluorescing in F′ (arrows). (G,G′) Spermatid bundles undergoing individualization. In G′ the arrowhead indicates elongated and condensed nuclei and the arrow indicates spermatid bundles at the onset of individualization. (H,H′) Seminal vesicles containing stored mature sperm (arrows). Scale bars: 20 μm in A-G,A′-G′; 150 μm in H,H′.

pushing the excess cytoplasm and organelles into a 'cystic bulge' that eventually becomes the waste bag. As the IC progresses down the bundle, each spermatid becomes sheathed in its own plasma membrane (Tokuyasu et al., 1972; Noguchi and Miller, 2003). The relative positions of the speckles and the actin cones depend on the position of the IC along the bundle. When the IC is first assembled around the elongated and condensed nuclei, the speckles are seen in front of the actin cones. As the IC moves away from the nuclei, the speckles first overlap the actin cones and then lag behind them. Similar speckles are also found in Prosα3-GFP, Prosα3T-GFP and Prosα6-GFP sperm bundles, although speckles associated with the conventional subunits, Prosα3-GFP and Prosα6-GFP, are fainter than those of their testis-specific counterparts (Fig. 2C-E).

To test whether these speckles are really proteasome-related structures, and not merely artifactual aggregates of GFP-tagged proteins, testes were immunostained with an antibody directed against Prosα7 (also known as Prosalpha7 – FlyBase), a conventional proteasome subunit that has no testis-specific isoform. As shown in Fig. 2F, similar speckles were detected. In addition, immunostaining testes of Prosα3T-GFP (not shown) or Prosα6T-GFP transgenic flies with anti-Prosα7 antiserum shows colocalization of the GFP and antibody signals (Fig. 2G-I), confirming that these speckles are indeed proteasome-related

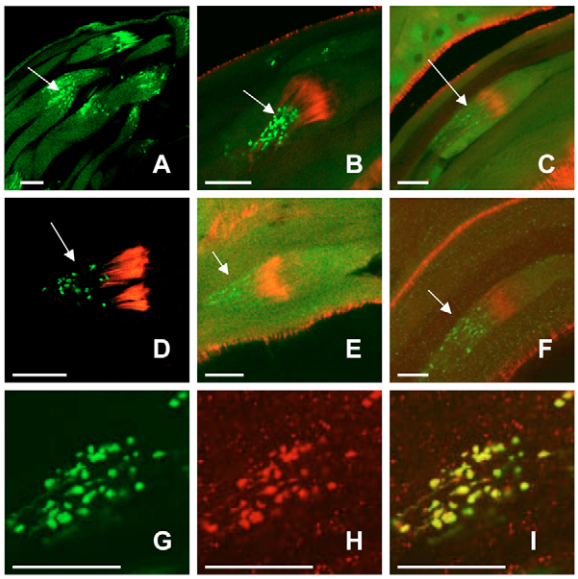

Fig. 2. Proteasomal 'speckles' associated with the individualization complex. (**A**) Elongated spermatid bundles showing Prosα6T-GFP speckles (arrows, green). (**B-E**) Speckles (green) are located close to the actin cones (red): (B) Prosα6T-GFP, (C) Prosα6-GFP, (D) Prosα3T-GFP, (E) Prosα3-GFP. (**F**) Wild-type IC stained with anti-Prosα7 antibody (green) and Alexa Fluor 635 phalloidin (red). (**G-I**) Prosα6T-GFP (G, green) in an IC stained with anti-Prosα7 antibody (H, red). (I) The merged image of G and H with the yellow signal indicating colocalization. Scale bars: 20 μm.

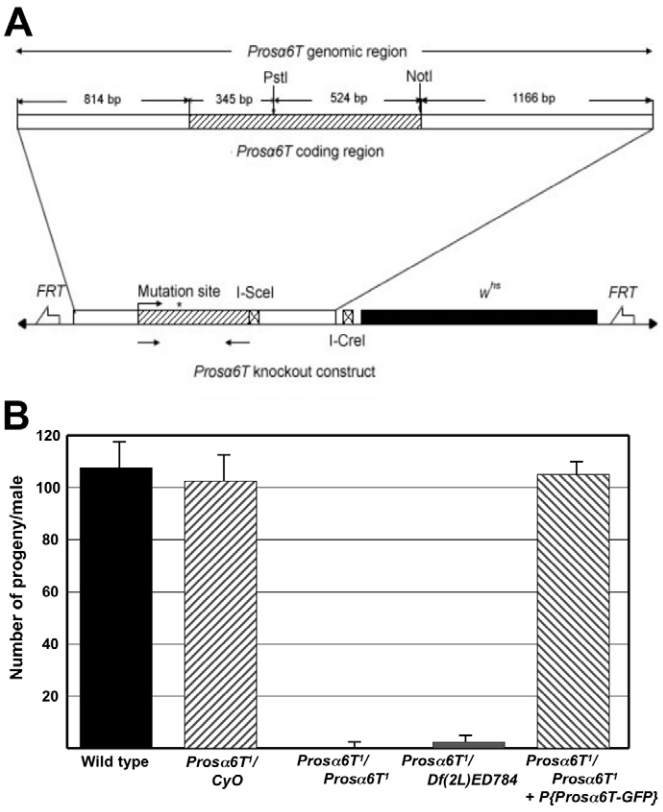

Fig. 3. Generation of the *Prosα6T¹* mutant. (**A**) *Prosα6T* knockout construct. The bent arrow to the left of the *Prosα6T* coding region (hatched area) indicates the transcription direction. The two arrows beneath the *Prosα6T* coding region indicate the positions of PCR primers used to verify the targeted *Prosα6T* mutation. (**B**) Results of male fertility tests showing mean number of progeny per male±s.e.m.

structures. The subcellular nature of these structures is not known, and there are no obvious correlative structures in the published transmission electron micrographs of individualization complexes (Tokuyasu et al., 1972).

Prosα6T¹ is a recessive male sterile mutant

The expression patterns of Prosα6T and other testis-specific proteasome subunits suggest that there might be crucial roles for the proteasome during the late stages of sperm development. To investigate this, we generated a knockout mutant of the *Prosα6T* gene (also known as *CG5648*, map position 34A11), using targeted homologous recombination (Rong et al., 2002). A four base pair deletion was generated in the *Prosα6T* coding region to produce a frame-shift mutation at codon 115 (out of 289), with a translation stop occurring 41 codons later (Fig. 3A). Candidate mutation lines were examined by PCR amplification, restriction analysis and sequencing and one was shown to carry the desired mutant allele, which was named *Prosα6T¹*.

Because *Prosα6T* is expressed only in testes it was expected that the *Prosα6T¹* mutant would be homozygous viable, but that it might affect male fertility. Indeed, this was the case. Homozygous mutants exhibited normal viability, but the males were sterile (Fig. 3B). That this male-sterility is due to *Prosα6T¹*, and not caused by some inadvertently induced mutation, is shown by the observations that (1) the deficiency chromosome *Df(2L)ED784*, a deletion of the *Prosα6T* gene region, fails to complement this mutant, and (2) the male sterility can be completely rescued by the *Prosα6T-GFP* transgene (Fig. 3B).

Homozygous *Prosα6T¹* mutant testes are of normal size and shape, but there are no mature sperm in the seminal vesicle (Fig. 4A-D). Phase-contrast microscopy of testis squashes showed that all stages up through spermatid elongation were indistinguishable in *Prosα6T¹* mutants and wild type (Fig. 4E-L). However, there appeared to be defects in sperm individualization, and few, if any, mature, motile sperm were evident. The phenotype of *Prosα6T¹/Df(2L)ED784* hemizygotes is indistinguishable from that of *Prosα6T¹* homozygotes, suggesting that this mutant is a strong loss-of-function allele (not shown).

Individualization complexes are disrupted in *Prosα6T¹* mutants

The presence of proteasome-associated speckles in the IC of elongated sperm bundles, together with the observation that the *Prosα6T¹* mutant testes are unable to process elongated sperm bundles to mature sperm, suggest that at least one major function of the proteasome during spermatogenesis is to facilitate sperm individualization. To investigate this possibility, we stained wild-type and *Prosα6T¹* testes with Alexa Fluor phalloidin to visualize the actin cones in the IC.

In wild-type testes, actin cones have a characteristic triangular shape and they move in a coordinated fashion (Fig. 5A,B), with a few actin cones only occasionally falling out of synchronization (Fig. 5B, arrow). This fine coordination is disrupted in the *Prosα6T¹* mutants, and becomes more extreme as the actin cones move further down the spermatid bundles. The initial assembly of the actin cones, and their triangular shape are not affected in the mutants, indicating that the movement, and not the formation, of the IC, is

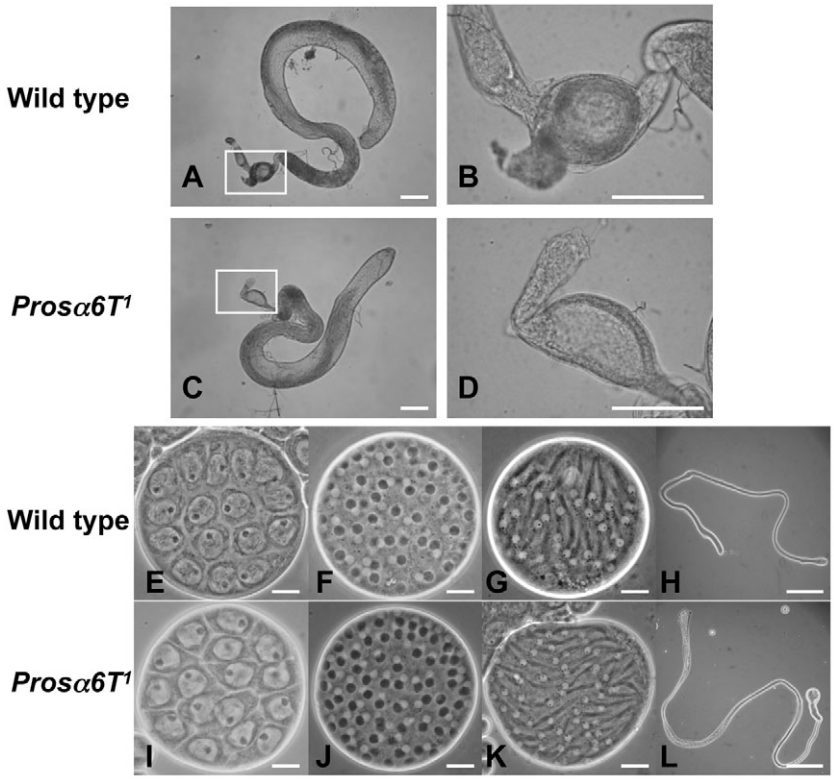

Fig. 4. Phase-contrast microscopy of whole testes and pre-individualization stages of spermatogenesis of wild-type and *Prosα6T¹* mutant males. (**A**,**C**) Wild-type and *Prosα6T¹* testes. (**B**,**D**) Higher magnification of the seminal vesicle in A and C (boxed), respectively. (**E-L**) Comparison of different spermatogenic stages of wild-type (E-H) and *Prosα6T¹* (I-L) testes. (E,I) Primary spermatocyte cysts at the 16-cell stage. (F,J) Onion (64-cell) stage spermatid cysts. (G,K) Early stage of elongating spermatid cysts. (H,L) Fully elongated spermatid cyst bundles. Scale bars: 150 μm in A-D,H,L; 10 μm in E-G,I-K.

malfunctioning (Fig. 5C,D). In addition, the proteasome-related speckles that are seen in the IC's of wild-type testes are absent in the *Prosα6T¹* mutants (Fig. 5E,F).

Prosα6T¹ affects caspase activation

The pattern of proteasome speckles associated with the IC is very reminiscent of similar speckles detected when testes are immunostained for the active form of the apoptotic protein Dronc (see Huh et al., 2004). In that study, the punctate pattern of active Dronc staining was found localized within the IC, with the speckles first appearing ahead of the actin cones, and then transitioning to a position behind them as the IC progresses caudally along the spermatid bundle during individualization. This dynamic is very similar to what we see here for the proteasomal speckles.

In an attempt to confirm if the proteasome-related speckles are the same as the previously described Dronc speckles, and to test the hypothesis that the activation of Dronc requires the function of testis-specific proteasomes, we used anti-active Dronc antiserum to immunostain testes from wild type, *Prosα3T-GFP*, *Prosα6T-GFP* and *Prosα6T¹* flies. In wild-type testes, although we could not reproduce the speckled pattern of immunostaining as found by Huh et al. (Huh et al., 2004), probably because of the loss of the effectiveness of the anti-active Dronc antiserum over time in storage

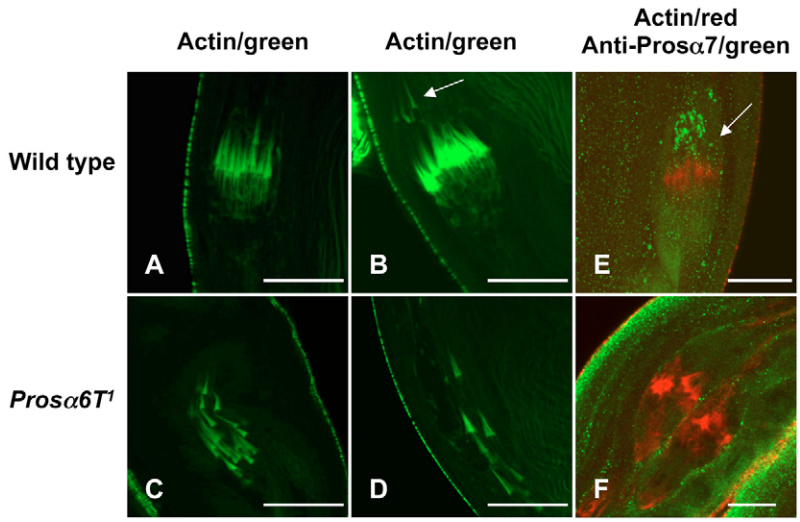

Fig. 5. The individualization complexes are disrupted in *Prosα6T¹* mutant testes. (**A-D**) Testes stained with Alexa-Fluor-phalloidin (green) showing actin cones of the ICs. (A,B) Actin cones (green) in wild-type ICs move synchronously, although occasionally a few actin cones lag behind (arrow). (C,D) In *Prosα6T¹* testes, actin cone synchronization is disrupted. The further the ICs move along the tail bundles, the more scattered the actin cones become (compare D with C). (**E**,**F**) Wild-type (E) and *Prosα6T¹* (F) testes stained with anti-Prosα7 antibody (green) and Alexa-Fluor-phalloidin (red). Speckles (arrow) can be observed in the wild-type testis, whereas they are absent in the *Prosα6T¹* testis. Two cystic bulges are shown in F. The actin cones are disorganized as a result of the mutation. Scale bars: 20 μm.

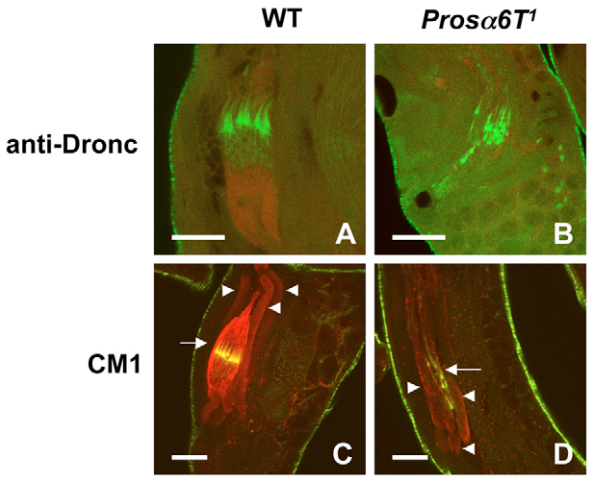

Fig. 6. Caspase activation is affected in *Prosα6T¹*. (**A**,**B**) Anti-active Dronc staining (red) is observed in cystic bulges in wild-type testes (A), whereas it is absent in *Prosα6T¹* testes (B). (**C**,**D**) Visualization of active Ice with anti-cleaved-caspase-3 antibody (CM1; red). CM1 staining is observed in the cystic bulge (arrow) and elongated spermatid bundles (arrowheads) in wild-type testes (C). However, CM1 staining is not detected in the cystic bulge (arrow) in *Prosα6T¹* testes (D), and is weaker in elongated spermatid bundles (arrowheads) compared with wild type. Actin cones were visualized by Alexa-Fluor-phalloidin staining (green). Scale bars: 20 μm.

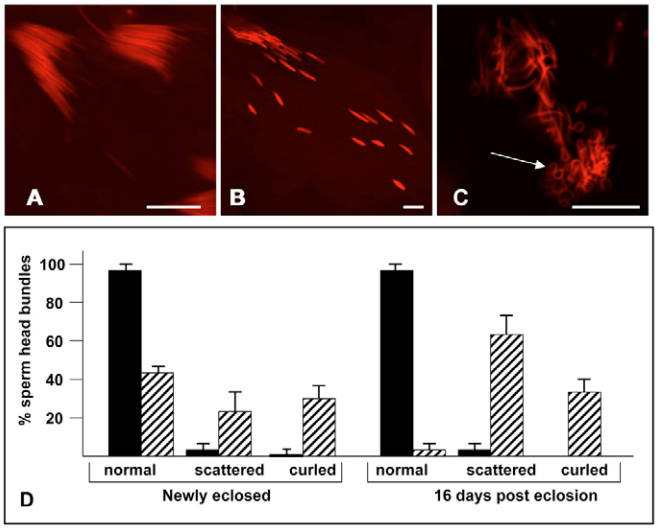

Fig. 7. *Prosα6T¹* mutant testes show abnormalities in nuclear bundles. Wild-type or *Prosα6T¹* spermatid nuclei stained with TO-PRO3. (**A**) Wild-type nuclei are long, fully condensed and remain in register. (**B**) Some of the *Prosα6T¹* nuclei are not fully condensed and they are often scattered. (**C**) Spermatid nuclei of the mutant are often contorted and frequently form circles (arrow). (**D**) Proportion of normal, scattered and curled spermatid nuclear bundles in young (black bars) versus old (hatched bars) flies. Scale bars: 20 μm.

(B. Hay and J. Huh, personal communication), we did observe active Dronc staining just ahead of the IC in sperm bundles undergoing individualization (Fig. 6A). Importantly, this staining is absent in the *Prosα6T¹* testes, suggesting that the testis-specific proteasome is required for the activation of Dronc (Fig. 6B). In wild-type testes, active Dronc is responsible for activating the downstream effector caspase Ice (Huh et al., 2004). To test whether *Prosα6T¹* is required for Ice activation, we immunostained wild-type and mutant testes with the CM1 antibody, which is specific for the active form of Ice (Dorstyn et al., 2002). As expected from the previous results of Arama et al. (Arama et al., 2003), in wild-type testes, active Ice is prominently observed in the cystic bulge and uniformly along non-individualized spermatid bundles (Fig. 6C). By contrast, the signal is significantly reduced, although not eliminated, in mutant *Prosα6T¹* testes (Fig. 6D).

Prosα6T¹ mutants show nuclear abnormalities in spermatid bundles

The prominent Prosα6T-GFP and Prosα3T-GFP signals in spermatid nuclei suggest that the proteasome might also play a role in the dramatic nuclear morphological changes during spermiogenesis. Normally, the spherical spermatid nuclei elongate and condense to form long, needle-shaped structures during and after spermatid bundle elongation (Lindsley and Tokuyasu, 1980). During this shape change and the subsequent individualization process, nuclei in the same bundle tend to remain in register (Fig. 7A). However, in *Prosα6T¹* mutants, some nuclei do not fully condense, some are scattered, and some lose the rigid, needle-like shape and appear curled, and in many cases, contorted into circles (Fig. 7B,C). Although there are a few normal looking sperm nuclear bundles in the *Prosα6T¹* mutant testes, their number decreases dramatically in older males. In accordance with this, the number of scattered nuclei increases with time (Fig. 7D).

Histone removal is delayed in the *Prosα6T¹* mutant but protamine incorporation occurs normally

In addition to the morphological changes of nuclei during spermiogenesis, the spermatid chromatin undergoes dramatic molecular changes as well. Some, if not all, of the somatic histones become replaced by the small, highly basic protamines, ProtA and ProtB (also known as Mst35Ba and Mst35Bb, respectively – FlyBase), and a sperm-specific linker histone-related protein Mst77F, during the process of sperm nuclear elongation and condensation (Jayaramaiah Raja and Renkawitz-Pohl, 2005; Rathke et al., 2007). The timing of the histone-protamine transition overlaps the expression patterns of Prosα3T and Prosα6T, raising the possibility that the somatic histones are degraded by testis-specific proteasomes to set the stage for recruiting the protamines and Mst77F to the sperm chromosomes. To investigate whether this transition is disrupted in the *Prosα6T¹* mutant, a GFP-tagged histone His2AvD transgene (Clarkson and Saint, 1999) was crossed into the *Prosα6T¹* mutant background. In testes of heterozygous wild-type controls, the H2AvD-GFP signal is prominent in spermatocyte and spermatid nuclei of pre-elongation stages of spermatogenesis, but fades away in the elongating and condensing spermatid heads, and completely disappears in fully condensed sperm nuclei (Fig. 8A-F). The overall pattern of H2AvD-GFP expression in homozygous mutants is similar to that in the wild type, indicating that there is no dramatic alteration in histone replacement. However, there is a slight, but noticeable, delay in the disappearance of the histone signal in the mutant. That is, the H2AvD-GFP signal is seen to persist longer in the elongating and partially condensed nuclei of the mutant (compare Fig. 8D with J), although it does eventually disappear as the nuclei reach full condensation, and it is not seen at all in the abnormal curled nuclei (Fig. 8K-L).

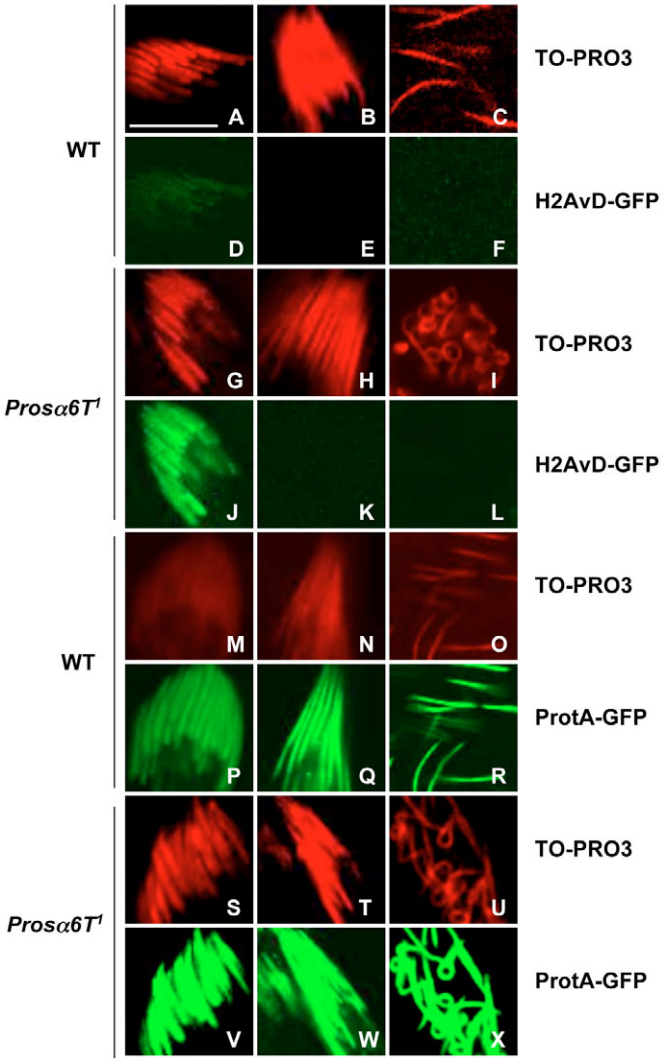

Fig. 8. The *Prosα6T1* mutant delays the disappearance of histone H2AvD but protamine incorporation is not affected. Images are of increasing age, left to right. Wild-type (**A-F**,**M-R**) or *Prosα6T1* (**G-L**,**S-X**) testes expressing either H2AvD-GFP (D-F,J-L; green) or ProtA-GFP (P-R,V-X; green) were stained with TO-PRO3 (A-C,G-I,M-O,S-U; red) to visualize nuclei. In wild-type testes, H2AvD-GFP signal is barely detectable in elongating and condensing nuclei (D), and completely disappears in mature nuclear bundles (E) and nuclei in individualized sperm (F). However, elongating and condensing nuclei in *Prosα6T1* testes still contain significant amounts of H2AvD-GFP (J), although the GFP signal disappears in mature nuclear bundles (K) and curled nuclei (L). ProtA-GFP is highly expressed in elongating and condensing nuclei in wild-type testes (P), and persists in the mature nuclear bundles (Q) and nuclei in individualized sperm (R). In *Prosα6T1* testes, ProA-GFP is observed in elongating and condensing nuclei (V), mature nuclear bundles (W) and curled nuclei (X). Scale bar: 20 μm.

Does this delay in histone removal affect the integration of protamines into the chromosomes? To address this question, a *ProtA-GFP* transgene was introduced into the *Prosα6T1* mutant background. As expected, ProtA-GFP in control heterozygotes is first detected in the elongating nuclei, and it remains prominently visible in the individualized, mature sperm nuclei (Fig. 8M-R). In homozygous *Prosα6T1* mutants, the ProtA-GFP signal is also first seen in the elongating nuclei, and it remains in the scattered and the curled nuclei (Fig. 8S-X). Because of the delay in histone disappearance, there is a more extensive overlap of expression of H2AvD-GFP and ProtA-GFP in mutant testes, but the *Prosα6T1* mutant has no significant effect on the timing and degree of protamine incorporation. Given these results, there does not appear to be a strong link between the sperm nuclear shape defects of the *Prosα6T1* mutant and the histone-protamine transition.

Ectopic expression of Prosα6 in late spermatogenesis can rescue the mutant phenotype of *Prosα6T1*

Given the different expression patterns of the Prosα6 and Prosα6T subunits, one question that arises is: does the testis-specific subunit result in proteasomes that are functionally distinct from proteasomes containing the conventional isoform? To address this, transgenic flies were produced that ectopically express the Prosα6 subunit in late spermatogenesis, in flies that lack the testis-specific Prosα6T subunit. The testis-specific *Prosα6* transgene construct (pW8/*TS1-Prosα6*) was created by replacing the coding region of the cloned testis-specific proteasome gene *Prosα3T* with the coding sequence of *Prosα6* (see Materials and methods), and the transgene was introduced into the *Prosα6T1* mutant background by P-element transformation. It was found that driving testis-specific expression of Prosα6 could completely rescue the sterility of *Prosα6T1* mutant males, and microscopic analysis of spermatogenesis in such flies showed no differences when compared to that of wild type. This result demonstrates the Prosα6 and Prosα6T subunits are functionally similar.

DISCUSSION

One previously unanswered question concerns the functional significance of testis-specific proteasomes. The present study demonstrates, at least for Prosα6T, the testis-specific subunit is required for normal sperm development. Examination of the phenotypic effects of the knockout allele, *Prosα6T1*, demonstrates essential roles for this gene in post-meiotic sperm cell differentiation, most notably in the processes of spermatid individualization and nuclear differentiation, consistent with the observed expression pattern of this subunit. Prosα6-GFP can be detected throughout spermatogenesis, from early spermatogonia to elongating spermatids, but it begins fading away after meiosis, and completely disappears by the end of spermiogenesis. By contrast, the testis-specific subunit Prosα6T-GFP is not detected in early stages until meiosis, after which it becomes prominent in both nuclei and cytoplasm. In elongated spermatids, it persists in the bundled sperm heads and tails, although the GFP signal in nuclei is reduced once nuclear elongation is complete and individualization begins.

Possible roles of testis-specific proteasomes in sperm individualization

The role of the proteasome in sperm individualization may be related to its localization to numerous small ‘speckles’ within the IC, trailing the actin cones as they move along the bundle. We suggest there is a complex of protein degrading machinery that helps in the removal of unneeded cytosolic material, or assists in the release of actin so that movement of the IC can proceed. In *Prosα6T1* mutants, where this does not occur efficiently, the actin cones soon lose their synchronous movement and the IC breaks down, thereby stalling individualization.

In addition to this general requirement of proteasome-mediated protein degradation during sperm individualization, there might be a specific role of proteasomes in mediating the activation of caspases to

trigger the spermatogenesis-specific apoptotic pathway that facilitates the individualization process. It has been reported that the active form of caspase Dronc shows a punctate staining pattern associated with the IC, very similar to the proteasome speckles we have observed (Huh et al., 2004). Dronc is activated when bound to Dark, a homolog of the mammalian Apaf1 protein, triggering its autoprocessing (Muro et al., 2004). Normally, its activity is held in check by binding to the inhibitory protein Diap1 [also known as Thread (Th) – FlyBase]. During apoptosis, Diap1 is autoubiquitinylated, through the action of RHG [Rpr, Hid (also known as Wrinkled) and Grim] proteins, and is presumably degraded via the ubiquitin-proteasome pathway (Hays et al., 2002; Holley et al., 2002; Ryoo et al., 2002; Wing et al., 2002; Yoo et al., 2002). Dronc is thus released from the tether of Diap1 and able to cleave and activate the downstream caspases such as the caspase-3-like effector caspase Ice.

In recent years, increasing evidence has indicated that spermatogenesis borrows the apoptotic machinery to achieve non-apoptotic results (Cagan, 2003). Key apoptotic components such as active Dronc, active Ice, and Hid proteins have been found in elongated spermatids (Arama et al., 2003; Huh et al., 2004), and a Ice deletion mutant is defective in spermatid individualization (Muro et al., 2006). It has been shown that Dark- and Hid-dependent activation of Dronc happens in the cystic bulges where individualization takes place (Huh et al., 2004). Furthermore, when Diap1 or dominant-negative (Dn) Dronc is overexpressed using the testis-specific β_2-tubulin promoter, the active Dronc speckles disappear, individualization is defective, and the synchronized movement of the actin cones is disrupted, producing a phenotype that is very similar to what is observed here with the *Prosα6T*^{1} mutant (Huh et al., 2004). These observations, along with the fact that Diap1 is ubiquitinylated during apoptosis (Wilson et al., 2002; Yoo et al., 2002), raise the possibility that testis-specific proteasomes play an important role in degrading DIAP1, which, in turn, triggers Dronc to activate downstream caspases, thus initiating the individualization process. Our observations that in the *Prosα6T*^{1} mutant, active Dronc disappears from the IC and active Ice is significantly reduced is consistent with this model. The fact that active Ice is not completely eliminated suggests that there might be an additional Dronc-independent pathway for Ice activation, as has been suggested by Huh et al. (Huh et al., 2004).

Testis-specific proteasomes might affect individualization by altering cytoskeletal protein dynamics

In *Prosα6T*^{1} mutant testes, coordinated actin cone movement is disrupted. A similar phenotype has been documented for a number of cytoskeletal protein mutations, including *jar*^{1} (myosin VI), *dctex-1*^{1} (*Dlc90F* – FlyBase; *Drosophila* 14 kDa dynein light chain), *ddlc1*^{ins} (*ctp* – FlyBase; 8 kDa *Drosophila* dynein light chain 1), *sw*^{1} (74 kDa intermediate chain) and *MyoV*^{Q1052st} (*didum* – FlyBase; myosin V) (Hicks et al., 1999; Li et al., 2004; Ghosh-Roy et al., 2005; Mermall et al., 2005). The spermatid nuclear bundles in these mutants are also disrupted. Although the mechanism of the actin cone movement has not been fully elucidated, it has been proposed that actin polymerization and depolymerization are the driving force (Noguchi and Miller, 2003). Myosin VI seems to be an actin cone structure stabilizer (Noguchi et al., 2006), and myosin V is required for actin cone formation (Mermall et al., 2005). Here, we demonstrate that the ubiquitin-proteasome pathway is also important in IC movement. It is unclear at this time how proteasomes affect actin cone movement, but it is possible that they facilitate the movement by regulating the protein levels of some key components of the actin cone movement machinery. Alternatively, they could achieve this goal through regulation of the apoptosis pathway, as discussed above. Evidence for the latter possibility is that Dcp1, a caspase activated by Dronc, is required for the cytoskeletal reorganization that occurs during the dumping process of *Drosophila* oogenesis (McCall and Steller, 1998). Another possibility is that proteasome-mediated degradation of numerous cytosolic proteins might achieve the breakdown of cytoplasmic structure within the IC and facilitate the movement of actin cones as they push the digested material into the growing waste bag.

Efficient histone disappearance during spermatogenesis requires Prosα6T but the histone to protamine transition does occur

We observed that in *Prosα6T*^{1} mutants, whereas the histone H2AvD does eventually disappear in the scattered and curled spermatid nuclei, this displacement is slightly delayed. One possible explanation for this delay is that during spermatogenesis the histones may be degraded via the Ub-proteasome pathway to facilitate their removal and replacement by the protamines. In the *Prosα6T*^{1} mutant, the Ub-proteasome pathway may be compromised at this late stage of spermatogenesis, thus inhibiting histone replacement. One possibility is that the mutation affects the removal of the histones from the chromatin. Alternatively, the histones might be removed normally, but then not efficiently degraded.

The incorporation of protamines does not appear to be affected in the *Prosα6T*^{1} mutant nuclei, indicating that the malformed sperm nuclei are not the result of a gross disruption of the histone-protamine transition. From these results, it is not clear why the sperm nuclei exhibit aberrant shapes in the mature spermatid bundles. One possibility is that the abnormal nuclear morphology arises at the end of spermiogenesis, when sperm undergo coiling and abnormal sperm are screened for elimination (Fuller, 1993). In this case, there would not be a direct link between this phenotype and reduced proteasome function in the nucleus.

Why have proteasome genes in *Drosophila* undergone extensive duplication and divergence to testes-specific isoforms?

The proteasome genes of *D. melanogaster* can be divided into those encoding subunits that are expressed throughout development and those whose expressions are limited to the late stages of spermatogenesis. The driving force for the evolution of this situation might be related to the unusual dynamics of gene expression during spermatogenesis (Fuller, 1993; Hiller et al., 2004). That is, because transcription of virtually all genes ceases after the primary spermatocyte stage, and because there is a very large amount of protein degradation occurring during the later stages of spermiogenesis, there might have been strong selective pressure to increase the levels of proteasome subunit expression in these cells. Gene duplication is one way to immediately increase the level of gene expression, and once it happens, one copy would be free to evolve a testis-specific expression pattern to ensure adequate levels of the subunit throughout spermatogenesis. The duplicated gene might then evolve to produce proteasome subunits that are better adapted to carry out the extensive protein breakdown that occurs as the spermatids eliminate their cytoplasm during the individualization process. Importantly, the observation reported here that driving spermatogenic expression of the conventional subunit Prosα6 can functionally substitute for Prosα6T rules out the possibility that the somatic and testis-specific forms of the proteasome have completely distinct functional properties. This situation is analogous to that of

the spermatogenic-specific isoform of cytochrome C (Cyt-c-d), known as Blanks (Bln; FlyBase) (Arama et al., 2006). In that case, it was shown that whereas *bln*$^{-}$ mutants are male sterile, the mutant phenotype can be rescued by driving testis-specific expression of the somatically expressed paralogous gene, *Cyt-c-p*.

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