

Post-meiotic transcription in *Drosophila* testes

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Post-meiotic transcription was accepted to be essentially absent from *Drosophila* spermatogenesis. We identify 24 *Drosophila* genes whose mRNAs are most abundant in elongating spermatids. By single-cyst quantitative RT-PCR, we demonstrate post-meiotic transcription of these genes. We conclude that transcription stops in *Drosophila* late primary spermatocytes, then is reactivated by two pathways for a few loci just before histone-to-transition protein-to-protamine chromatin remodelling in spermiogenesis. These mRNAs localise to a small region at the distal elongating end of the spermatid bundles, thus they represent a new class of sub-cellularly localised mRNAs. Mutants for a post-meiotically transcribed gene (*scotti*), are male sterile, and show spermatid individualisation defects, indicating a function in late spermiogenesis.

KEY WORDS: *Drosophila*, Spermatid, Transcription, RNA localisation, Spermatid individualisation

INTRODUCTION

In *D. melanogaster* spermatogenesis (reviewed by Fuller, 1993), the primary spermatogonium undergoes four mitotic divisions, each with incomplete cytokinesis. Two somatic cyst cells, analogous to mammalian Sertoli cells, encapsulate each spermatogonium, and thus, later, 16 spermatocytes, and, after meiosis, 64 inter-connected spermatids. After meiosis, morphological changes, including axoneme assembly and mitochondrial and membrane remodelling, transform round spermatids into mature 2-mm-long motile sperm. These post-meiotic events are believed to be driven by translation, as spermiogenesis genes are typically transcribed in primary spermatocytes, the mRNAs are then stored in spermatids and translated during elongation (reviewed by Schäfer et al., 1995).

In mammalian early spermatids, transcription is readily detected (by ³H-Uridine incorporation) and continues until chromatin compaction (Kierszenbaum and Tres, 1975; Monesi, 1965). Mammalian spermatocytes transcribe genes required during meiosis or in early spermatids, whereas mRNAs for spermiogenesis proteins are transcribed post-meiotically (Schultz et al., 2003), with some under additional translational control (reviewed by Braun, 1998). Radiographic studies showed no detectable transcription in *Drosophila* spermatids (Gould-Somero and Holland, 1974; Olivieri and Olivieri, 1965); however, RNA polymerase II activity in *Drosophila* spermatid nuclei suggests that these cells actively transcribe genes (Rathke et al., 2007).

Spermatid elongation generates dramatic cellular asymmetry, with nuclei at one end, and axonemes extending towards the distal end, where sister cells are connected via ring canals (Hime et al., 1996). During individualisation, each spermatid is stripped of excess cytoplasm and organelles, and is separated from sister spermatids by an individualisation complex that progresses from the heads along to the tails, a process dependent on apoptotic pathway activation (Arama et al., 2003). Spermatids have a unique, highly compact, non-nucleosomal chromatin organisation. Histones are removed from spermatid chromosomes and replaced by other small basic

proteins, initially transition proteins, then protamines or protamine-like proteins (reviewed by Braun, 2001; Oliva, 2006). *Drosophila* have at least one transition protein and several protamine-like proteins (Jayaramaiah Raja and Renkawitz-Pohl, 2005; Rathke et al., 2007).

Here, we provide compelling evidence for the transcription of 24 genes in *Drosophila* spermatids, activated by at least two regulatory pathways. In *Drosophila*, transcription shuts off in late primary spermatocytes, then (for a few loci) is reactivated mid-elongation, just before histone-to-protamine chromatin remodelling. At least one post-meiotically expressed gene, *scotti*, is required for normal actin cone progression during spermatid individualisation, and thus for male fertility. mRNAs encoded by all of the post-meiotically transcribed genes are localised to the extreme distal ends of the elongating cells; thus, they represent a novel class of sub-cellularly localised mRNAs.

MATERIALS AND METHODS

Drosophila strains and culture

Flies were raised on standard cornmeal sucrose agar at 25°C. Visible markers and balancer chromosomes are described in FlyBase (Crosby et al., 2007). Wild type was *w¹¹¹⁸*. Protamine-EGFP flies were from Renate Renkawitz-Pohl (Jayaramaiah Raja and Renkawitz-Pohl, 2005) and H2A-mRFP1 flies from Jürgen Knoblich (IMBA, Vienna, Austria).

RNA in situ hybridisation

RT-PCR products (400–600 bp) were generated from total testis RNA. 3' PCR primers included a T3 RNA polymerase promoter for the in vitro transcription of digoxigenin (DIG)-labelled RNA probes. In situ hybridisation was as described previously (White-Cooper et al., 1998). Primer sequences are available on request.

Isolation of RNA from individual cysts or bundles

Testes were dissected from *w¹¹¹⁸* or the young male progeny of *w; Mst35Ba-egfp × w; H2A-mRFP1*, in testis buffer (183 mM KCl, 47 mM NaCl, 10 mM Tris pH 6.9), transferred onto a siliconised slide, opened with tungsten needles and examined using an Olympus BX50 upright microscope with a long working distance 10× objective. Individual photographed cysts were transferred into 100 µl lysis buffer using a pulled-out Pasteur pipette; total RNA was extracted according to manufacturer's instructions (RNAqueous-Micro kit, Ambion, Warrington, UK).

Q-RT-PCR

First-strand cDNA was synthesised using oligo(dT)₂₀ and SuperScript III Reverse Transcriptase (Invitrogen, Paisley, UK) in a 20 µl reaction. Each PCR contained 0.33 µl cDNA, 10 µl 2× Power SYBR Green PCR Master

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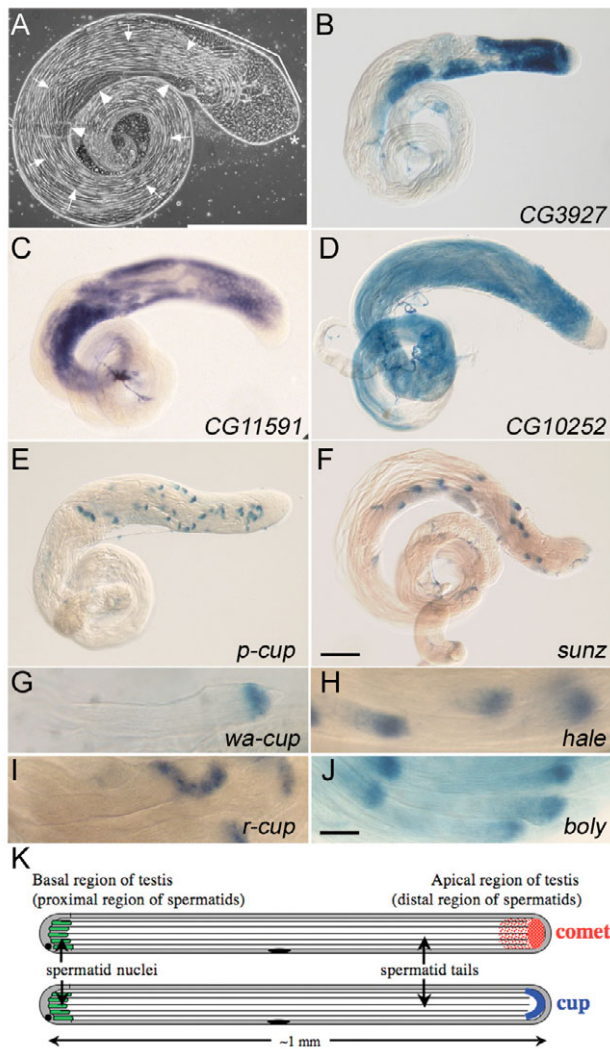


Fig. 1. RNA in situ hybridisation reveals comet and cup transcript patterns. (A) *Drosophila* testis viewed by phase contrast. Asterisk, apical tip; bracket, spermatocytes; arrowheads, early spermatids; arrows, elongating spermatid bundles. (B-D) Control genes. (E,F) Typical cup and comet localisation. Scale bar: 100 μ m for to A-F. (G-J) Typical cup localisation at spermatid distal ends. Scale bar: 25 μ m. (K) Diagram of a spermatid bundle showing the location of comet and cup transcripts relative to spermatid nuclei, and cyst cells (grey).

Mix (Applied Biosystems, Warrington, UK), and 100 nM of each gene-specific primer, in a 20 μ l final volume. Primers amplified cDNA only, as one of each pair spanned an exon-exon junction. Negative controls (testis RNA without reverse transcriptase) were performed for every primer pair in every PCR plate. Q-PCR reactions in 96-well thin-wall white plates (BIOplastics, Braintree, UK) were run in a Chromo4 with Opticon Monitor Software (GRI, Braintree, UK). Q-PCRs were run in triplicate and the internal reference control (CG10252) was quantified for each sample.

soti mutant generation

soti deletions were generated by FLP-mediated recombination between PBac[WH]f02605 inserted 2162 bp 3' of *soti* and P[XP]d01837 inserted 468 bp 5' of *soti* (Parks et al., 2004; Thibault et al., 2004). All five independent deletion lines were confirmed by *soti* ORF PCR, and were viable but male sterile.

FITC-Phalloidin staining of testes

Visualisation of actin cones in mutant and wild-type testes was carried out as described previously (White-Cooper, 2004).

Table 1. Comet and cup gene names, symbols and CG identifiers

Gene	Symbol	CG number	PCR
Comet genes			
<i>schumacher-levy</i>	<i>schuy</i>	CG17736	Yes*
<i>hale-bopp</i>	<i>hale</i>	CG7570	Yes*
<i>sungrazer</i>	<i>sunz</i>	CG15179	Yes [†]
<i>solwind</i>	<i>sowi</i>	CG15178	Yes [†]
<i>borrelly</i>	<i>boly</i>	CG30362	Yes*
<i>comas sola</i>	<i>cola</i>	CG30363	Yes*
<i>hug-bell</i>	<i>hubl</i>	CG30364	
<i>spacewatch</i>	<i>spaw</i>	CG30365	
<i>whipple</i>	<i>whip</i>	CG34218	
<i>swift-tuttle</i>	<i>swif</i>	CG30366	Yes*
<i>scotti</i>	<i>soti</i>	CG8489	Yes [†]
<i>Pglym87</i>		CG17645	
Cup genes			
<i>calcutta-cup</i>	<i>c-cup</i>	CG15623	Yes [†]
<i>walker-cup</i>	<i>wa-cup</i>	CG10113	Yes [†]
<i>ryder-cup</i>	<i>r-cup</i>	CG10998	Yes [†]
<i>davis-cup</i>	<i>d-cup</i>	CG14387	Yes [†]
<i>presidents-cup</i>	<i>p-cup</i>	CG12993	Yes [†]
<i>world-cup</i>	<i>w-cup</i>	CG7363	
<i>stanley-cup</i>	<i>s-cup</i>	CG30044	
<i>tetleys-cup</i>	<i>t-cup</i>	CG31858	
<i>flyers-cup</i>	<i>f-cup</i>	CG9611	
<i>heineken-cup</i>	<i>h-cup</i>	CG6130	
<i>mann-cup</i>	<i>m-cup</i>	CG11896	
<i>oo18 RNA-binding protein</i>	<i>orb</i>	CG10868	

*Genes resembling *hale* in Q-RT-PCR transcript profiling.

[†]Genes resembling *sunz* in Q-RT-PCR transcript profiling.

RESULTS AND DISCUSSION

Drosophila 'comet' and 'cup' transcripts are specifically detected in spermatid bundles

Many genes with unknown functions have testes-specific expression. To determine when during spermatogenesis these proteins are made, we examined the transcript patterns of >1200 genes by in situ hybridisation (www.fly-ted.org). The expression of spermiogenesis genes in primary spermatocytes, and the storage of transcripts for later use during spermiogenesis, means that the translation of specific mRNAs in *Drosophila* spermatids correlates well with their disappearance, as translation exposes stored mRNAs to the RNA degradation machinery (Schäfer et al., 1995). The in situ hybridisation results will be described in detail elsewhere; in summary, 529 of the 553 mRNAs detected in spermatids were transcribed in primary spermatocytes, persisted in the spermatid cytoplasm, and were degraded at various stages in elongation (Fig. 1C,D). Unexpectedly, we found 24 germ-line expressed genes that did not conform to this pattern (Table 1; see also Table S1 in the supplementary material). We subdivided these on the basis of subtle differences in transcript localisation patterns, and refer to the genes collectively as 'comets and cups'.

Comet and cup transcripts were detected at very low levels in primary spermatocytes by RNA in situ hybridisation (Fig. 1E,F; see Fig. S1 in the supplementary material), and were barely detected in early elongation spermatids. However, robust signals, with striking subcellular localisations, were evident in more elongated spermatids. Spermatid nuclei are located at one end of these elongated cells, in the basal-most region of the testis; comet and cup mRNAs were localised to the distal ends of the spermatids, in subtly different patterns (Fig. 1G-K). 'Comet' mRNAs localised into a ball shape at the ends of spermatid bundles, trailing away proximally to

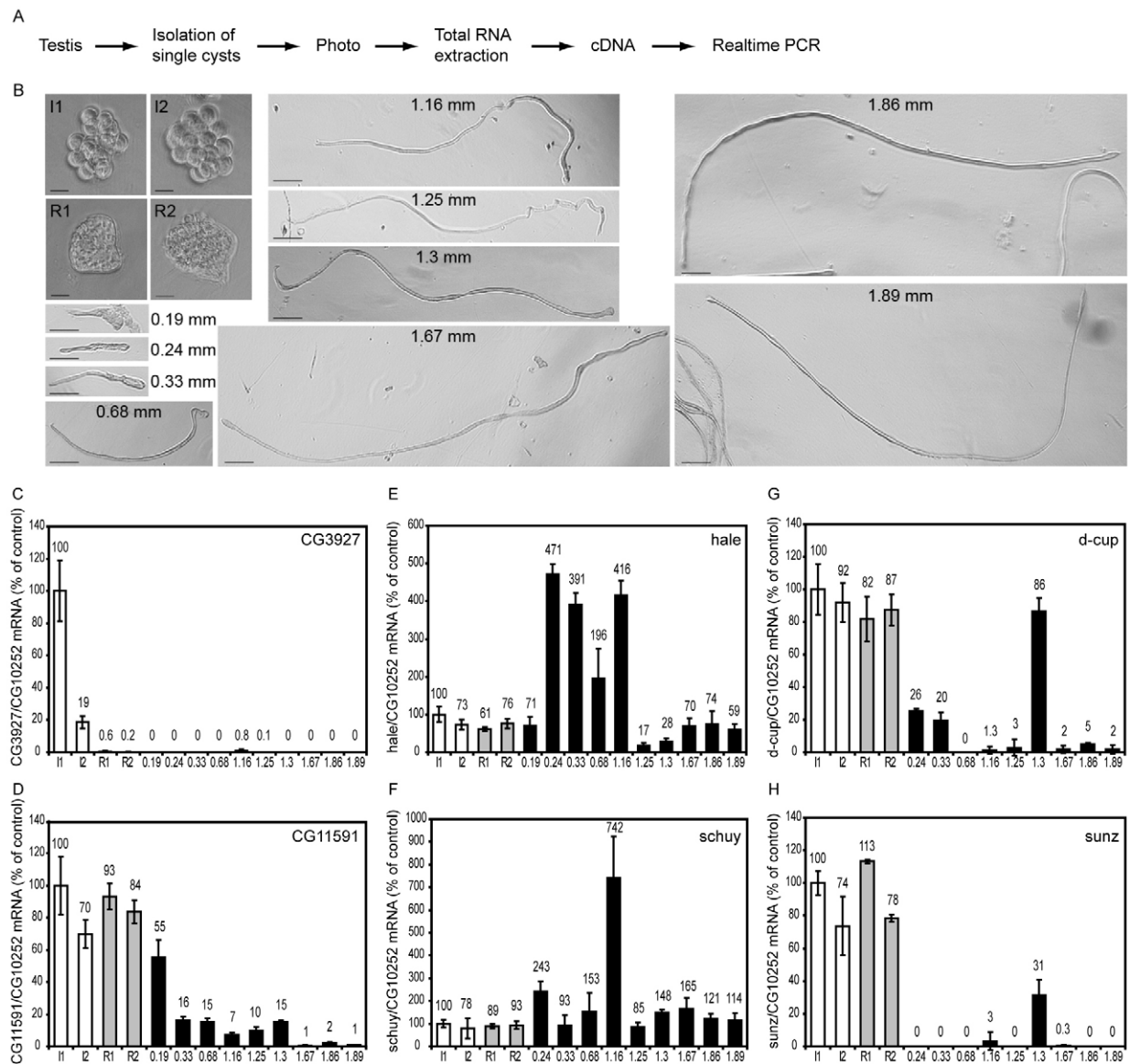


Fig. 2. Q-RT-PCR confirms post-meiotic transcription. (A) Individual cyst Q-RT-PCR assay schematic. (B) Cysts used for RNA extraction, from primary spermatocytes (I1, I2) and round spermatids (R1, R2; scale bars: 25 μ m), and from elongating spermatids (0.19–1.89 mm; scale bars: 100 μ m). (C,D) Control Q-RT-PCRs. (E–H) Peak of expression in spermatids confirms post-meiotic transcription of comet and cup genes. *hale* (E) and *schuy* (F) were expressed in more bundles than were *d-cup* (G) and *sunz* (H).

a less abundant, speckled distribution (Fig. 1H,I). ‘Cup’ transcripts localised in shallow cup-like shapes at the ends of spermatid bundles (Fig. 1G,I).

Quantitative RT-PCR confirms post meiotic transcription

Comet and cup expression patterns are extremely unusual. The obvious explanation for the abrupt mRNA appearance during spermatid differentiation is post-meiotic transcription. Alternatively, the transcripts could be present earlier, but either (1) diffuse or (2) masked, so undetectable by in situ hybridisation.

To verify the post-meiotic transcription, and to determine its timing with respect to cellular differentiation events, we developed a single-cyst quantitative reverse transcription PCR (Q-RT-PCR) protocol. Testes were dissected, and individual cysts isolated, photographed, and staged according to morphology; total RNA was

then isolated and first strand cDNA synthesised (Fig. 2A). Each cyst yielded cDNA for 60 Q-RT-PCR reactions. Testis-specific control genes were chosen. The *CG10252* transcript conforms to the conventional pattern for a late elongation protein (Fig. 1D) and *CG10252* protein is detected in mature sperm (Dorus et al., 2006). *CG3927* was detected exclusively in primary spermatocytes; *CG11591* was expressed in primary spermatocytes and the signal disappeared from mid-elongation spermatids (Fig. 1B,C). *CG3927* and *CG11591* controlled for cyst visual staging. For each cyst, expression levels of both staging controls and up to eight test genes were compared with the internal control *CG10252*. Fig. 2 shows a typical experiment, expression levels were normalised to primary spermatocyte cyst I1. Consistent with the in situ hybridisation patterns, *CG3927* transcript was only detected in primary spermatocytes. *CG11591* transcript was highest in primary spermatocytes, and persisted into early elongation stage spermatids.

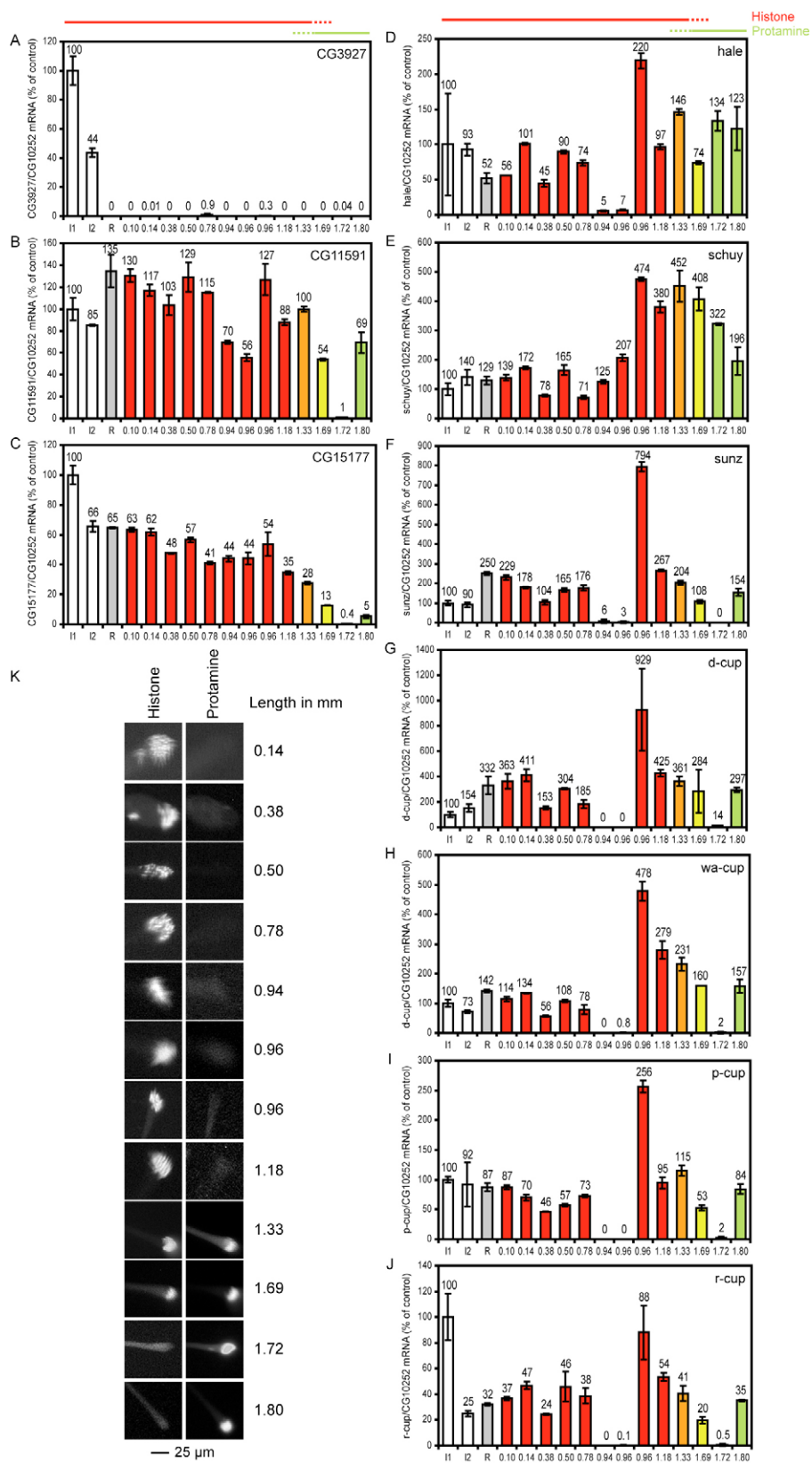


Fig. 3. Comet and cup transcription precedes the histone-protamine switch. Bars above graphs and colours within graphs indicate H2A-mRFP (histone, red) and Mst35Ba-EGFP (protamine, green; overlap orange/yellow) detection. (A-C) Control Q-RT-PCRs. (D-J) Comet and cup Q-RT-PCR expression profiles show that expression initiates in cysts negative for protamine. (K) Nuclear fluorescence in elongating cysts.

The 13 comet and cup gene transcripts assayed by isolated-cyst Q-RT-PCR showed broadly similar profiles in Q-RT-PCR assays (Figs 2, 3; see also Figs S2, S5, S6 in the supplementary material). All transcripts were detected in primary spermatocytes and round

spermatids. *sunz*, *sowi*, *soti*, *c-cup*, *d-cup*, *wa-cup*, *p-cup* and *r-cup* were low or not detected in very short elongating cysts, but were detected at high levels in a few longer spermatid cysts. *hale*, *schuy*, *boly*, *cola* and *swif* were detected at a basal level in almost all cysts,

but were much more abundant in a few mid-elongation bundles. From these differences, we infer two separate regulatory modules activating post-meiotic gene expression, with the *hale* group being transcribed in more cysts than the *sunz* group. Spermatid length measurements give good staging of the relative differentiation states of cysts from a single testis, but the exact length of spermatids expressing comets and cups varied between testes. The initial low-level signal in primary spermatocytes, the dip in signal intensity in early spermatids, then the dramatic appearance in later spermatids conclusively demonstrate that there is post-meiotic transcription in *Drosophila* testes.

Comet and cup genes are expressed before most of the DNA has been loaded with transition proteins or protamines

In *Drosophila*, bulk histone removal initiates in the early ‘canoe’ stage of nuclear remodelling, and protamine deposition is complete by late canoe stage (Jayaramaiah Raja and Renkawitz-Pohl, 2005). To determine comet and cup transcription timing with respect to chromatin reorganisation, we staged cysts via combined fluorescent fusion-protein localisation and spermatid-length measurements. We isolated cysts from flies co-expressing Mst35Ba-GFP (protamine-GFP) and H2A-mRFP1 (histone-GFP). Protamine accumulation initiates before all histones have been removed, as some nuclei fluoresced both red and green (Fig. 3K). Transcription of comet and cup genes was detected in mid-elongation cysts that were positive for histone and negative for protamine (Fig. 3D-J). Thus, comet and cup transcription occurs just before the deposition of protamines. Some comet and cup mRNAs were also detected in cysts positive for protamine-GFP. This could be due to ongoing transcription, or to message stability. The recently described active transcription in spermatids (Rathke et al., 2007) coincides with the comet and cup gene transcription peak.

We repeated these experiments using cysts isolated from flies co-expressing Tpl94D-GFP (transition protein) and H2A-mRFP1. Initiation of post-meiotic comet and cup gene expression was found in cysts lacking nuclear Tpl94D (see Fig. S2B in the supplementary material), indicating that comet and cup gene transcription initiates before the deposition of transition protein, while chromatin is presumably still nucleosomal.

Rfx is not required for expression of the comet and cup genes

Rfx is a winged-helix transcription factor important for ciliogenesis gene expression. In testes, Rfx protein was detected transiently in canoe-stage spermatid nuclei only (Vandaele et al., 2001), and thus was an excellent comet and cup candidate transcription factor. All comet and cup genes tested (*hale*, *schuy*, *cola*, *soti*, *c-cup*, *w-cup*, *p-cup* and *wa-cup*) by in situ hybridisation to *Rfx*²⁵³/*Rfx*⁴⁹ (Dubruille et al., 2002) mutant testes were indistinguishable from wild type (see Fig. S3 in the supplementary material). *Rfx*⁴⁹ is a null, whereas *Rfx*²⁵³ lacks DNA-binding activity. Thus, despite the intriguing localisation of Rfx to spermatid nuclei, its X-box binding function is not required for comet or cup expression.

scotti is required for spermatid individualisation

We deleted approximately 4 kb of genomic DNA, including the entire *scotti* (*soti*, a comet) ORF, by FLP-mediated recombination between flanking FRT-containing transposons. *soti* homozygous mutants were viable and female fertile, but male sterile. Phase contrast microscopy indicated no gross defects in *soti* testes organisation or spermatid elongation; however, empty seminal

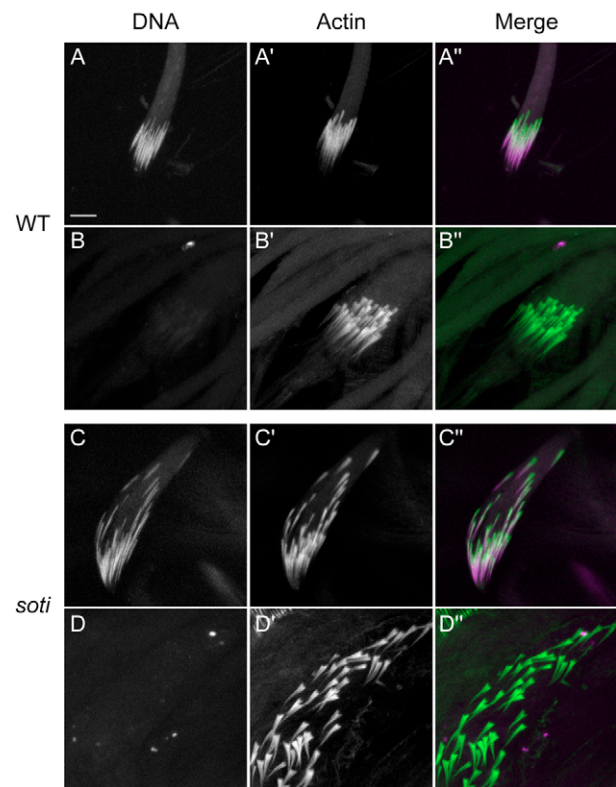


Fig. 4. *soti* is required for normal individualisation complex progression.

Confocal maximum brightness projections of the entire cyst depth. DNA is labelled with propidium iodide (A-D, magenta); actin is labelled with FITC-phalloidin (A'-D', green). Scale bar: 10 μ m. (A-B'') In wild-type testes, investment cones assembled above spermatid nuclei (A-A'') and moved along spermatid bundles as a complex (B-B''). (C-D'') In *soti* mutant testes, investment cones assembled normally, but complexes dissociated during individualisation.

vesicles indicated spermiogenesis defects. Within each individualising spermatid cyst, 64 actin-rich investment cones move together as an individualisation complex, pushing ahead a cystic bulge of excess cytoplasm and organelles. This cytoplasm is discarded from spermatid distal ends as a waste bag. Waste bags were completely absent from mutant testes, and cystic bulges were rarely seen. FITC-phalloidin labelling revealed that investment cones formed normally in *soti* mutant males; however, nuclei failed to remain tightly clustered and were displaced distally along the cyst (Fig. 4). Although investment cones progressed away from the nuclei in mutants, investment cone coupling within individualisation complexes was lost, and cones never progressed the full length of mutant spermatids. Thus, *soti* function is required for spermatid individualisation.

Why are the comets and cups transcribed in spermatids?

Post-meiotic transcription, in early spermatids, has been reported for two loci in *Drosophila*, *hsr-omega* and *Hsp70* (Bendena et al., 1991); however, we have been unable to reproduce these findings (see Fig. S5 in the supplementary material). Ninety-six percent of genes whose mRNAs were detected in spermatids are not actively transcribed in these cells (being made in spermatocytes), so what

is special about the exceptional 4% – the comets and cups? These genes are found throughout the euchromatin, including the X chromosome, and their local genomic environments showed no unusual features. Their flanking genes showed no bias towards or away from testis-specific expression in adults (Chintapalli et al., 2007). There are three comet and cup gene clusters, two of which clearly represent gene duplication events (see Table S2 in the supplementary material). The final cluster comprises *hale* and *schuy*; both encode glutamine-rich proteins, but their evolutionary history is unclear. We investigated the expression of all 10 related genes in the CG11635-CG8701 cluster (see Fig. S5A in the supplementary material). *CG11635*, *CG18449*, *CG2127* and *CG8701* were expressed in the conventional spermiogenesis gene pattern – transcribed in primary spermatocytes and stored until late elongation – while *spaw*, *hubl*, *swif*, *cola*, *boly* and *whip* were typical ‘comets’ (see Tables S1, S2 and Fig. S5B,C in the supplementary material). Q-RT-PCR confirmed that the post-meiotic transcription and RNA localisation to distal ends of spermatids were correlated.

In mammals, the transcription of many genes in spermatids has been described, and new reports are frequent (Reynard et al., 2007; Schultz et al., 2003). These mammalian genes typically, although not exclusively, encode components of the mature sperm. By contrast, *Drosophila* comet and cup proteins, with the exception of *Boly* and *Pglym87*, are not sperm components (see Table S1 in the supplementary material) (Dorus et al., 2006). Perhaps comet and cup proteins function, like *Soti*, during spermiogenesis, rather than in sperm. Alternatively, perhaps they are present in sperm but at a very low copy number. mRNAs of several comet and cup gene homologues were transcribed in conventional patterns, and the encoded proteins detected in sperm (see Table S2 in the supplementary material). *orb* (a comet) encodes an RNA-binding protein, potentially anchoring other comet and cup mRNAs. The other comet or cup proteins have no predicted function. *PKD2* encodes a Ca^{2+} -activated non-selective cation channel, and it is intriguing that a *Drosophila* *PKD2* homologue (*Pkd2/Amo*) concentrates at the distal ends of sperm, and is important for sperm function (Watnick et al., 2003). *Sunz*, *Sowi* and *D-cup* are EF-hand-containing proteins, and so could function with *Pkd2* in mediating a Ca^{2+} signal at the spermatid tail tip. This signal could then be transduced along spermatid tails, perhaps via the mitochondrial derivative or the endoplasmic reticulum-derived axonemal sheath, which stretches the length of spermatid tails, activating the apoptotic pathway to synchronise individualisation and ensure normal investment cone progression.

In conclusion, there is significant transcription from several genomic loci in *Drosophila* spermatids, and the post-meiotically expressed transcripts localise to the growing ends of spermatids. This transcription and RNA localisation occurs before spermatid chromatin remodelling, and at least one post-meiotically-expressed gene is required for spermiogenesis.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/11/1897/DC1>

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