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The THO complex is required for nucleolar integrity in *Drosophila* spermatocytes

Sungjin Moon[1], Bongki Cho[1,*], Su-Hong Min[1,†], Daekee Lee[2] and Yun Doo Chung[1,‡]

SUMMARY

The THO complex is a conserved multisubunit protein complex that functions in the formation of export-competent messenger ribonucleoprotein (mRNP). Although the complex has been studied extensively at the single-cell level, its exact role at the multicellular organism level has been poorly understood. Here, we isolated a novel *Drosophila* male sterile mutant, *garmcho* (*garm*). Positional cloning indicated that *garm* encodes a subunit of the *Drosophila* THO complex, THOC5. Flies lacking THOC5 showed a meiotic arrest phenotype with severe nucleolar disruption in primary spermatocytes. A functional GFP-tagged fusion protein, THOC5-GFP, revealed a unique pattern of THOC5 localization near the nucleolus. The nucleolar distribution of a testis-specific TATA binding protein (TBP)-associated factor (tTAF), SA, which is required for the expression of genes responsible for sperm differentiation, was severely disrupted in mutant testes lacking THOC5. But THOC5 appeared to be largely dispensable for the expression and nuclear export of either tTAF target mRNAs or tTAF-independent mRNAs. Taken together, our study suggests that the *Drosophila* THO complex is necessary for proper spermatogenesis by contribution to the establishment or maintenance of nucleolar integrity rather than by nuclear mRNA export in spermatocytes.

KEY WORDS: THO complex, Meiotic arrest, *Drosophila*, Spermatogenesis, Nucleolus

INTRODUCTION

During spermatogenesis, the coordinated action of many gene products is essential for proper differentiation of sperm. In *Drosophila*, ~25% of all genes expressed in the testis are testis-specific or testis-enriched, and most of these genes are transcribed in primary spermatocytes and stored until needed (for a review, see White-Cooper, 2010). Testis-specific gene regulation programs might ensure the coordinated expression of this large number of genes from transcription to translation. In *Drosophila*, two distinct classes of meiotic arrest genes, aly-class and can-class genes, represent testis-specific transcription regulation modules. The can-class genes, *cannonball* (*can*), *spermatocyte arrest* (*sa*), *meiosis I arrest* (*mia*) and *no hitter* (*nht*), encode the testis-specific TBP-associated factors (tTAFs), suggesting that their products form a testis-specific TFIID complex in primary spermatocytes (Hiller et al., 2004; Hiller et al., 2001). Interestingly, tTAFs, together with Polycomb group (PcG) proteins, mainly localize to a subcompartment of the nucleolus, rather than to euchromatin. Lack of tTAFs not only disrupts the nucleolar localization of PcG proteins but also causes PC to accumulate at tTAF target promoters. These findings suggested that tTAFs might also antagonize the Polycomb repressor complex (PRC1) to control the coordinated transcription of target genes (Chen et al., 2005).

Most aly-class gene products form tMAC, a testis-specific meiotic arrest complex paralogous to Myb-MuvB (Beall et al., 2007). The aly-class gene products are mainly localized to euchromatin in primary spermatocytes, and this localization is essential for their function, suggesting that the major role of the tMAC complex is transcriptional activation of testis-specific genes (Jiang et al., 2007; Jiang and White-Cooper, 2003; Wang and Mann, 2003; White-Cooper et al., 2000). Thus, it now seems evident that the coordinated transcription of testis-specific genes is regulated by two specific complexes: tTAFs and tMAC. However, little is known about post-transcriptional regulation in the testis. Here, we show that the THO complex, an evolutionarily conserved complex involved in the co-transcriptional formation of export-competent messenger ribonucleoproteins (mRNPs) (for reviews, see Aguilera, 2005; Reed, 2003; Reed and Cheng, 2005), is a novel regulator of *Drosophila* spermatogenesis.

The THO complex was first found in budding yeast, *Saccharomyces cerevisiae*, as a multisubunit protein complex composed of Hpr1, Tho2, Mft1 and Thp2. Yeast cells that lack the THO complex show transcription impairment and transcription-dependent hyper-recombination phenotypes, implying that the THO complex connects transcription elongation to mitotic recombination (Chávez et al., 2000). Subsequent studies showed that the THO complex, together with the mRNA export adaptor proteins Yra1 and Sub2, forms a larger complex called TREX (transcription-export complex), which is required for the co-transcriptional export of bulk mRNAs (Huertas and Aguilera, 2003; Jensen et al., 2003; Jimeno et al., 2002; Rondón et al., 2003; Sträßer et al., 2002). Metazoans also have a functional homolog of the THO complex, but its subunit composition and function are slightly different from those of budding yeast (Masuda et al., 2005; Rehwinkel et al., 2004). The metazoan THO complex lacks Mft1 and Thp2, but contains three other subunits, THOC5, THOC6 and THOC7, instead. Unlike budding yeast, metazoan cells require the THO complex for nuclear export of only a subset of transcripts (Farny et al., 2008; Mancini et al., 2010; Rehwinkel et al., 2004), but almost nothing is known about the common features of the target transcripts. Although it is now clear that, at the single-cell level, a role of the THO complex in mRNP biosynthesis is

[1]Department of Life Sciences, University of Seoul, Seoul, 130-743, Korea.
[2]Department of Molecular Life Sciences, Ewha Womans University, Seoul, 120-720, Korea.

*Present address: Department of Anatomy and Division of Brain Korea 21 Biomedical Science, College of Medicine, Korea University, Seoul, 136-701, Korea
†Present address: Department of Cell Biology, Skirball Institute, NYU School of Medicine, New York, NY 10016, USA
‡Author for correspondence (ydchung@uos.ac.kr)

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conserved throughout evolution from yeast to human (for a review, see Jimeno and Aguilera, 2010), its exact role in various types of cells at the multicellular organism level is still largely unknown. Recent studies using conditional knockout or a hypomorphic mutant mouse model have provided evidence that the THO complex has specific roles in cell differentiation during development (Mancini et al., 2010; Wang et al., 2009; Wang et al., 2007). It has also recently been reported that the *Drosophila* THO complex is required for normal development through collaboration with E(Y)2 (or ENY2), a multifunctional protein important for transcription activation and mRNA export (Kopytova et al., 2010).

Here, we report that a novel meiotic arrest gene, *garmcho* (*garm*), encodes the *Drosophila* THOC5 homolog. Flies lacking THOC5 showed complete male sterility with a meiotic arrest phenotype. Interestingly, unlike any other known meiotic arrest mutants, the nucleolar structure was severely disrupted in *garm* mutant primary spermatocytes. Both tTAF and PC proteins were abnormally distributed, whereas the expression and nuclear export of the three tTAF target mRNAs examined were mainly unaffected in mutant primary spermatocytes. Taken together, our data provide additional evidence that the THO complex is involved in a specific cell differentiation program, *Drosophila* spermatogenesis, probably by participating in the establishment or maintenance of nucleolar integrity in spermatocytes.

MATERIALS AND METHODS

Drosophila stocks and husbandry

The *thoc5*^{1} mutation was originally isolated by Kernan et al. (Kernan et al., 1994). The *thoc5* allele *PBac{RB}thoc5*^{e00906}, the *thoc6* allele *PBac{RB}thoc6*^{e00298} and all fly stocks for genetic mapping were obtained from the Bloomington Stock Center (Indiana University, IN, USA). The *thoc7* allele *P{XP}thoc7*^{d05792} was obtained from the Exelixis collection at the Harvard Medical School. The flies expressing SA-GFP and PC-GFP were kindly provided by Xin Chen in the Fuller Laboratory (Stanford University, CA, USA). *sa*^{1} and *mia*^{1} flies were kindly provided by Helen White-Cooper (Oxford University, UK). GFP-Nopp140-RGG flies (McCain et al., 2006) were kindly provided by Patrick J. DiMario (Louisiana State University, LA, USA). Flies were reared on standard medium at 23-25°C.

Transgenic constructs and germline transformation

For genomic rescue experiments, an 8255-bp *Hin*dIII fragment, a 7922-bp *Spe*I/*Kpn*I fragment and a 7418-bp *Bam*HI/*Eco*RV fragment were subcloned from the P1 clone DS06185 (*Drosophila* Genome Resource Center, Bloomington, IN, USA) into pCaSpeR4 vector to give H3, Sp-K and BRV rescue constructs, respectively. To construct the THOC5-GFP fusion gene, the Sp-K genomic rescue construct was digested by *Mlu*I, which generates a unique cut at the third base of the 545th codon (tyrosine). After filling the digested ends using Klenow enzyme, the full-length open reading frame without initiator methionine of enhanced GFP (eGFP) was inserted by blunt end ligation. The resulting encoded protein has the eGFP between Y545 and A546 of THOC5. A cDNA clone corresponding to mouse *Thoc5* was purchased from the 21C Frontier Human Gene Bank of KRIBB (Daejeon, South Korea) (Clone # mMU008032). A 2.2-kb full length cDNA was isolated by double digestion with *Eco*RI and *Xho*I, and subcloned into pUAST vector to generated *UAS-mThoc5* construct. Germline transformation of *w*^{1118} flies was carried out as described by Rubin and Spradling (Rubin and Spradling, 1982). To induce the expression of mouse *Thoc5*, a *da-GAL4* driver (Bloomington #8641) was used.

RT-PCR analysis

Total RNA was extracted from dissected testes with Trizol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions and dissolved in RNase-free water. First-strand cDNA was generated with equal amounts of RNA from each genotype using the SuperScript III Reverse Transcriptase System (Invitrogen) and oligo(dT) primers. PCR amplification was carried out using the following primer sets: *rp49*, 5′-TACAGGCCCAAGATCGTGAA-3′ and 5′-ACCGTTGGGGTTGGTGAG-3′; *bol*, 5′-CCGCAGAGATGCACAAAATA-3′ and 5′-TGAAGGTGGGTAGATGGC-3′; *CycB*, 5′-GGACAGCCACTGGAAGAAAC-3′ and 5′-GAACTGCAGGTGGACTTC-3′; *twe*, 5′-CGCCAAGGATTTGGCAATC-3′ and 5′-CTGGGATACATGCTTAGGC-3′; *dj*, 5′-CCCACTTTTATACGGCCTCA-3′ and 5′-CGCACTTCGTCTTCTTCTCC-3′; *fzo* 5′-GCCATACCACCAGCTGTTTT-3′ and 5′-AAAGAGATTTGGACGCGAGA-3′; *Mst87F*, 5′-TCCGACTTGTCAAACCGATA-3 and 5′-GCAGCAAGGGTATCCACAAT-3′.

RNA in situ hybridization

To make digoxigenin (DIG)-labeled riboprobes, cDNAs corresponding to the coding region of each gene were reverse transcribed, amplified by using the same primer sets described above, and cloned into pBlueScript KS(+). The riboprobes were synthesized using DIG RNA Labeling Kit (Roche Applied Science, Penzberg, Upper Bavaria, Germany) according to the manufacturer's instructions. For RNA in situ hybridization to testis squashes, testes from newly eclosed males of desired genotypes were dissected in PBS [10 mM $NaPO_4$ (pH 7.2), 150 mM NaCl], squashed at –80°C and the entire sample was immersed into methanol for 5 minutes at –80°C followed by 2 minutes in acetone at –80°C under RNase-free conditions. Fixed samples were washed three times (5 minutes each) with PBS and prehybridized with hybridization buffer (50% formamide, 5× SSC, 100 μg/ml ssDNA, 1× Denhardt's, 100 μg/ml tRNA and 10% dextran sulfate) at 55-60°C for 1 hour. After hybridization with a DIG-labeled riboprobe at 55-60°C for overnight, the sample was washed twice with 1× SSC and then twice with 0.1× SSC at 55-60°C for 30 minutes each. Then, the sample was incubated with Cy3-conjugated anti-DIG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 hour at room temperature. After three washes (5 minutes each) with PBS, the samples were imaged under an LSM510 confocal laser microscope (Carl Zeiss, Oberkochen, Germany). Whole-mount RNA in situ hybridization was performed as previously described (White-Cooper et al., 1998) with minor modifications. Hybridization and post-hybridization washing conditions were the same as those used for testis squashes.

Antibody staining and microscopy

Testes from newly eclosed males of desired genotypes were dissected in PBS, squashed at –80°C and the entire sample was immersed into methanol for 5 minutes at –80°C followed by 2 minutes in acetone at –80°C. Fixed samples were washed three times (5 minutes each) with PBST (0.1% Triton X-100 in PBS) and then blocked with blocking solution (2% bovine serum albumin and 2% normal goat serum in PBS) for 1 hour at room temperature. The samples were then incubated with primary antibodies at 4°C overnight, then washed three times (5 minutes each) with PBST and incubated with secondary antibodies for 2 hours at room temperature. After three washes (5 minutes each) with PBST, the samples were mounted with 80% glycerol and imaged with Axiovert 200M or LSM510 confocal laser microscope (Carl Zeiss).

Primary antibodies used in this study were: anti-THOC7 (Kim et al., 2011) at 1:1000, anti-HPR1 (Rehwinkel et al., 2004) at 1:100, anti-THO2 (Rehwinkel et al., 2004) at 1:100, anti-THOC5 (Kopytova et al., 2010) at 1:100 and anti-Fibrillarin (38F3; Santa Cruz, sc-56676) at 1:50. The secondary antibodies Alexa 546-conjugated goat anti-rabbit (1:500) and Alexa 488-conjugated goat anti-mouse (1:500) were purchased from Molecular Probes. Chromatin was visualized by Hoechst 33258 (Sigma-Aldrich, St Louis, MO, USA).

Co-immunoprecipitation and western blot analysis

Testes from one-day-old flies were dissected and homogenized in 600 μl lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Triton X-100, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. For each antibody, the equivalent of ~100 testis pairs was used to co-immunoprecipitate proteins. Protein extract corresponding to the equivalent of two testis pairs was retained as the 'input' sample, and the remainder was incubated overnight with 1 μl of anti-serum at 4°C and pulled down with protein A/G-agarose beads (Santa Cruz Biotechnology). After washing the beads with lysis buffer, the bound proteins were eluted by boiling in SDS sample buffer. Rabbit IgG was used as a negative control. For western

blotting, newly eclosed male flies were homogenized and boiled with SDS-PAGE sample buffer. After fractionation by SDS-PAGE, the proteins were transferred to PVDF membrane (BioRad, USA). The blots were then blocked with 5% non-fat milk in TBST [50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Triton X-100] and incubated overnight with primary antibody at 4°C. The following primary antibodies were used at the concentration indicated: anti-HPR1 (1:1000, rat), anti-THO2 (1:500, rat), anti-THOC5 (1:1000), anti-THOC7 (1:1000, rabbit), mAb JLA20 (1:200). Horseradish peroxidase (HRP)-coupled secondary antibodies (1:10,000-50,000; Jackson ImmunoResearch Laboratories) were used for ECL detection.

RESULTS

A novel meiotic arrest mutant, *garm*, shows abnormal nucleolar structure

During spermatogenesis in *Drosophila*, a germline stem cell located at the apical tip of the testes divides asymmetrically to produce one spermatogonium, which then undergoes four rounds of mitosis to produce 16 primary spermatocytes in a cyst. After DNA synthesis and growth, the mature primary spermatocytes undergo meiosis to produce 64 spermatozoa in each cyst (Fig. 1A). One of the EMS (ethylmethanesulfonate)-induced mutants, *garm*^{1}, isolated from a screen for uncoordinated flies (Kernan et al., 1994) had a male sterile phenotype. When the live squashed testes were examined under phase contrast, the mutant testes lacked mature spermatids, instead being full of arrested mature primary spermatocytes (Fig. 1C).

Most meiotic arrest mutants can be divided into two classes, 'aly-class' and 'can-class' (White-Cooper et al., 1998). The aly-class mutants have abnormal chromatin morphology; chromosomes appear fuzzy and less condensed compared with wild type. By contrast, chromosomes of can-class mutants have an almost normal shape (Fig. 1H). To investigate whether *garm* falls into one of the classes, the mutant spermatocytes were carefully observed under phase microscope. Unlike any other known mutants, *garm* showed abnormally strong condensation of three major bivalent chromosomes (Fig. 1G). Under phase contrast, a prominent dark nucleolus was easily seen in each wild-type primary spermatocyte (Fig. 1F; for schematic drawing, see Fig. 1A). In the mutant cells, however, the phase-dark structure was severely disorganized (Fig. 1G). Fibrillarin, which marks a fibrillar compartment of the nucleolus, showed that the disorganization of the phase-dark structure was due to the nucleolar disruption (Fig. 2). In each wild-type spermatocyte, the anti-Fibrillarin antibody stained a single dot-shaped structure that correlates with the phase-dark structure (arrowheads in Fig. 2A,C). By contrast, in the mutant mature spermatocytes not only were the signals reduced and fragmented, but also some phase-dark spots were not associated with Fibrillarin-rich spots (Fig. 2D,E). The reduction and dis-localization of Fibrillarin immunoreactivities were also seen in the immature mutant spermatocytes (Fig. 2B). At this stage, some Fibrillarin-rich spots were not associated with the phase-dark spots (arrows in Fig. 2B), although the phase-dark spots appeared relatively normal in shape at this stage, revealing that the nucleolar disruption was not due to the secondary effect of the meiotic arrest. All these results suggest that *garm* is a novel class of meiotic arrest gene.

garm encodes THOC5, a subunit of the *Drosophila* THO complex

Genetic recombination mapping, deficiency complementation tests and P-element-mediated male recombination mapping revealed that *garm* is located within a ~20-kb interval between two P-element insertions, EP(2)0489 and EP(2)0316, on the second chromosome (Fig. 3A). To find out the molecular identity

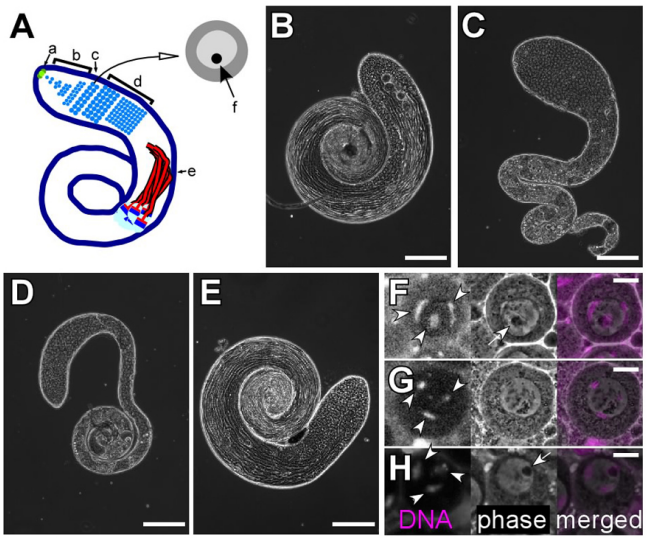

Fig. 1. *garm* is a novel meiotic arrest mutant. (**A**) Schematic of spermatogenesis. Germline stem cells at the apical tip of the testis produce spermatogonia by asymmetric division (a). The spermatogonia undergo mitosis (b) then become spermatocytes (c). After extensive growth in volume, the spermatocytes undergo the two meiotic divisions, producing haploid spermatids (d) which form flagella and elongate in bundles (e). In the pre-meiotic spermatocytes, a dot-shaped nucleolus (f) is clearly seen under a phase contrast microscope. (**B-E**) Phase contrast images of live *Drosophila* testis. Wild-type testis (B) contained all stages of germ cells, whereas *garm*^{1} (C) or *garm*^{1}/*thoc5*^{e00906} (D) had primary spermatocytes but no post-meiotic cells. Transgenic introduction of a genomic construct (Sp-K, shown in Fig. 3) rescued the meiotic arrest phenotype of *garm*^{1} (E). (**F-H**) Combined images of DNA staining (magenta) and phase contrast of mature primary spermatocytes from wild-type (F), *garm*^{1} (G) or *sa*^{1} (H) flies. The phase-dark spherical structure that represents the nucleolus is indicated by arrows in F and H. Chromosomes are indicated by arrowheads in F-H. Scale bars: 100 μm in B-E; 10 μm in F-H.

of *garm* in this extremely gene-dense region (16 genes in ~30-kb interval), we generated transgenic flies harboring three different genomic DNA constructs. Only one construct, Sp-K, could rescue the mutant phenotypes (Fig. 1E, Fig. 3G,H), identifying two candidate genes for *garm*: *CG3803* and *thoc5*. Genomic DNA sequencing showed that *garm*^{1} has an in-frame deletion of 255 bp in exon5 of *thoc5* (Fig. 3B), and a western blot did indeed detect a truncated band with a molecular weight of ~60 kDa (see Fig. S1 in the supplementary material), suggesting *garm* as a gene that encodes THOC5, a THO subunit. A fly strain, *thoc5*^{e00906}, harboring a piggyBac transposon in the first exon of *thoc5* was obtained from the Bloomington Stock Center. Both *thoc5*^{e00906} homozygotes and *garm*^{1}/*thoc5*^{e00906} trans-heterozygotes (Fig. 1D) revealed complete male sterility and the meiotic arrest phenotype, confirming that *garm* encodes THOC5. Thus, we renamed *garm*^{1} as *thoc5*^{1}.

As the THO complex is conserved from yeast to human (for a review, see Reed and Cheng, 2005), we hypothesized that mouse Thoc5 could replace its *Drosophila* homolog. Indeed, when mouse Thoc5 was expressed in the *thoc5*^{1} mutant background, the mutant phenotypes were rescued; the phase-dark nucleolar shape was normal, chromosome condensation was much similar to wild type although its shape appeared somewhat abnormal, and the elongating spermatid flagella were seen (Fig. 3I-K).

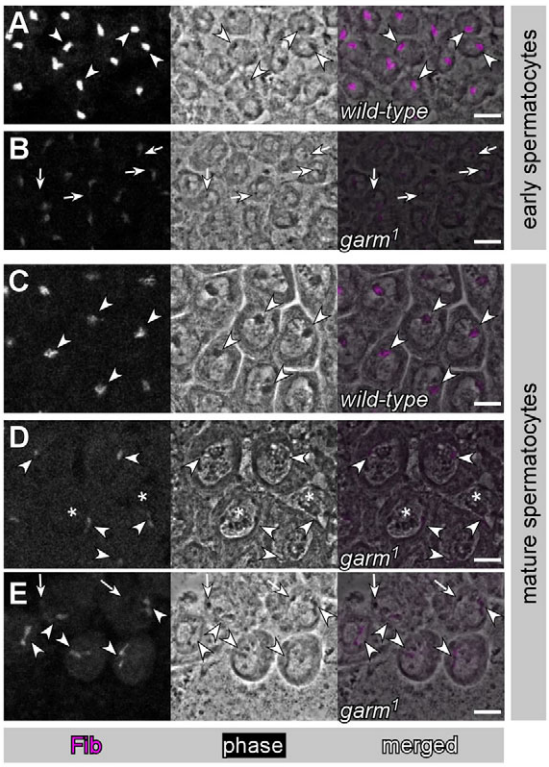

Fig. 2. Nucleolar disruption in *Drosophila garm* mutant testis. (**A**,**B**) Combined images of anti-Fibrillarin staining (magenta) with phase contrast in early primary spermatocytes of wild-type (A) or *garm*^{1} (B) testis . The phase-dark spot was highly enriched with Fibrillarin in wild type (arrowheads in A). In *garm*^{1}, some phase-dark spots (arrows in B) were lacking Fibrillarin signal. (**C-E**) Combined images of anti-Fibrillarin staining (magenta) with phase contrast in mature primary spermatocytes of wild-type (C) or *garm*^{1} (D,E) testis. The subnuclear structures marked by Fibrillarin are indicated by arrowheads in C-E. In some cells, the Fibrillarin signals were split (asterisks in D, arrowheads in E). Many phase-dark structures were Fibrillarin-free in the mutant cells (D, arrows in E). Scale bars: 10 μm.

Localization of THOC5-GFP in primary spermatocytes

To determine the subcellular location of THOC5, we generated transgenic flies expressing a GFP-tagged fusion protein, THOC5-GFP. This construct was able to rescue the *thoc5* mutant phenotypes, suggesting that it is functional (see Fig. S2 in the supplementary material). Under a fluorescence microscope, THOC5-GFP was detected mainly in the nuclei, but it showed a unique localization pattern depending on the developmental stage (Fig. 4). In early stage primary spermatocytes, THOC5-GFP was highly concentrated as a single dot near the nucleolus (Fig. 4B). As the primary spermatocyte matured, however, the signal dispersed and surrounded the nucleolus as a ring shape (Fig. 4C). DNA staining with Hoechst 33258 showed that a significant amount of GFP signal also overlapped with the chromosomes in mature spermatocytes (Fig. 4C). To test whether other THO components localize similarly to THOC5-GFP in spermatocytes, we immunostained spermatocytes with antibodies for other *Drosophila* THO subunits. All subunits examined showed a similar localization, which matched the THOC5-GFP signal, suggesting that they do indeed compose a stable complex (Fig. 5A-F). This was further confirmed by a co-immunoprecipitation assay using testis extract, in which all subunits examined were co-immunoprecipitated with each other (Fig. 5G).

The tTAF SA and Polycomb proteins are abnormally distributed in *thoc5* mutant spermatocytes

During *Drosophila* spermatogenesis, tTAFs encoded by can-class meiotic arrest genes are known as the key players in terminal differentiation for coordinated transcriptional activation of several spermiogenesis-related genes. One crucial role of tTAFs in spermatogenesis is counteracting repression by the Polycomb group (PcG) transcriptional regulatory complex (Chen et al., 2005). It has been proposed that this might be achieved by sequestering the PcG proteins into nucleoli, because nucleoli are enriched with both tTAFs and PcG proteins, and recruitment of PcG to nucleoli requires tTAFs (Chen et al., 2005). Because the nucleolar structure is abnormal in *thoc5* mutant spermatocytes, we wondered whether the nuclear distributions of tTAF and PcG proteins are altered in mutant cells. Thus, we used GFP-tagged fusion proteins, SA-GFP and PC-GFP, to examine the localization of tTAF and PcG proteins, respectively. As reported previously (Chen et al., 2005), SA-GFP started to be expressed soon after initiation of spermatocyte differentiation, and its expression was maintained throughout the remainder of the primary spermatocyte stage (Fig. 6A-B). In mature spermatocytes, consistent with the report by Chen et al., the nucleolus was highly enriched with SA-GFP with faint signals near the chromatin regions (Fig. 6B). The nucleolar accumulation occurred normally from the initial phase of SA-GFP expression (Fig. 6A,A′). In *thoc5* mutants, however, the SA-GFP-enriched spots were fragmented and scattered (Fig. 6C-D), suggesting that the THO complex might be required for retaining SA in the nucleolus. Interestingly, the SA-GFP-enriched spots overlapped with neither Fibrillarin-rich spots nor chromosomes in *thoc5* mutants (arrows in Fig. 6D). More interestingly, the scattered distribution of SA-GFP in *thoc5* mutants was observed from the initial phase of its expression, although nucleolar disruption was not so evident at this stage (Fig. 6C,C′). This suggested that the abnormal distribution might not be a secondary effect caused by nucleolar disruption.

The location of PC-GFP in mature wild-type primary spermatocytes was comparable to that of SA-GFP; it was concentrated in the nucleolus as well as chromatin with scattered speckles in the nucleoplasm (Fig. 6E). In mutant cells, however, PC-GFP was relatively evenly distributed throughout the nucleus, although some GFP-rich areas were still seen in some cells (Fig. 6F,G). Taken together, these data suggested that THOC5 is necessary for proper distribution of both SA and PC, although it is unclear whether THOC5 is directly involved in the localization of SA and PC or is indirectly required for retaining them in the proper subnucleolar domain by organizing or maintaining nucleolar integrity in a similar manner to ALY (Metcalf and Wassarman, 2007).

The expression and nuclear export of mRNAs in *thoc5* mutant testis

Abnormal distribution of SA and PC raised the question of whether tTAF target genes are properly expressed in *thoc5* mutant testis. To address this question, we examined the mRNA levels of three tTAF target genes, *don juan* (*dj*), *fuzzy onions* (*fzo*) and *Male-specific RNA 87F* (*Mst87F*), in adult testis by RT-PCR. Unexpectedly, there were no significant differences between wild type and *thoc5*

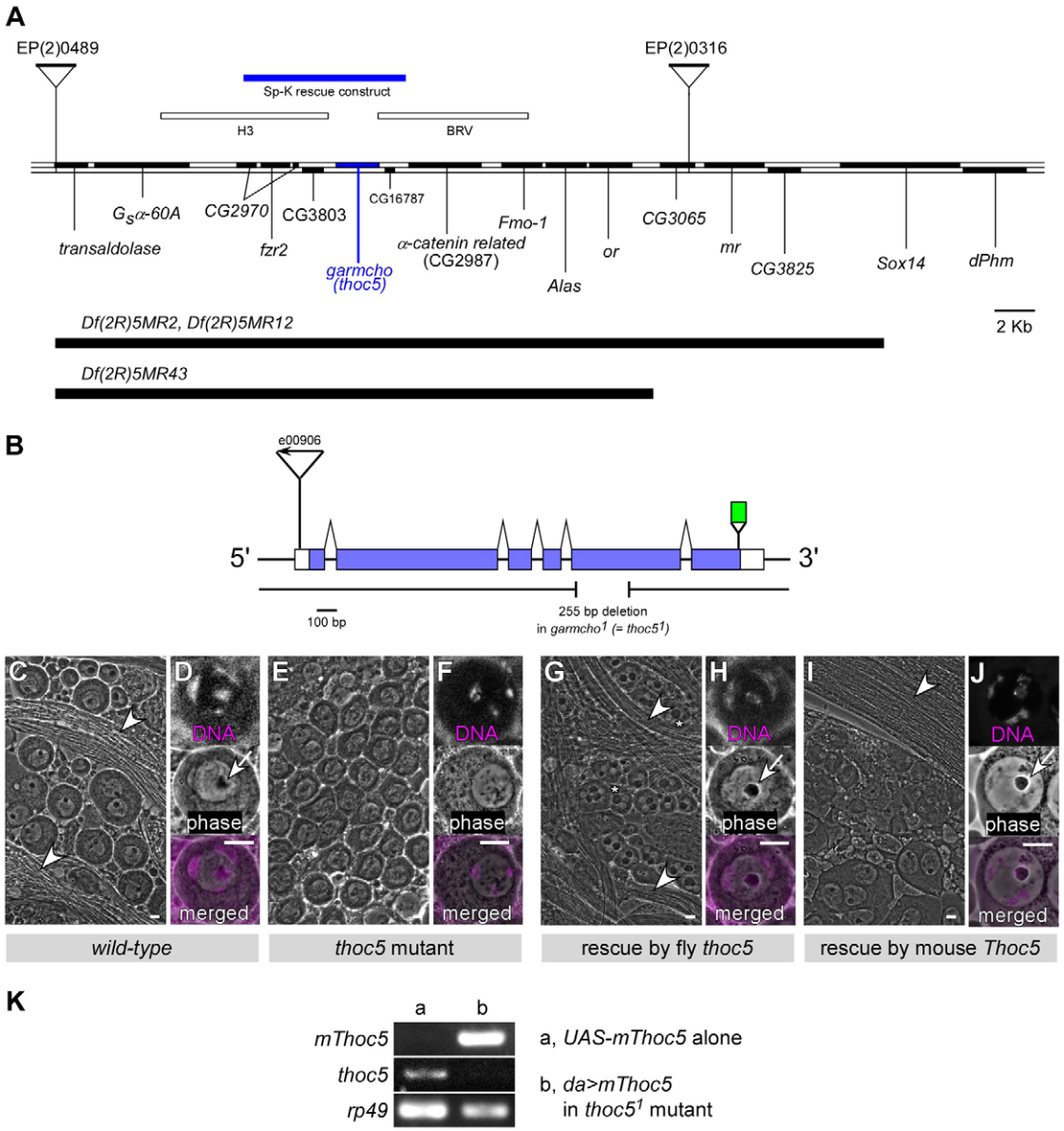

Fig. 3. *garm* encodes the *Drosophila* homolog of THOC5, a subunit of the THO complex. (**A**) Map of the 60A-B region. Three deficiency chromosomes [*Df(2R)5MR2*, *Df(2R)5MR12* and *Df(2R)5MR4*] generated by P element-mediated male recombination uncovered the *garm*^{1} mutation. The deletion end-points of each deficiency chromosomes were mapped by inverse PCR, and the deleted regions are represented by the black bars below. The shortest deficiency, *Df(2R)5MR43*, located *garm* in an ~30-kb interval. Of three genomic rescue constructs within that region (indicated by the boxed bars above), only Sp-K (blue boxed bar) could rescue the *garm* mutant phenotype. Genomic DNA sequencing and complementation tests with *PBac{RB}thoc5*^{e00960} finally revealed that *garm* encodes THOC5. (**B**) Schematic of the *garm* (*thoc5*) gene. The structural gene consists of six exons (indicated by boxes). The open reading frame is indicated by purple shading. In *PBac{RB}thoc5*^{e00960}, a piggyBac transposon is inserted in the first exon *thoc5*. A 255-bp deletion found in the *garm*^{1} (*thoc5*^{1}) mutant is indicated under the gene model. A full-length open reading frame of eGFP, which was inserted near the C-terminus to make a THOC5-GFP fusion protein, is indicated by the green box. (**C-J**) Mouse *Thoc5* can replace the function of fly *thoc5*. A full-length cDNA of mouse *Thoc5* was expressed by the GAL4-UAS system in a *thoc5* mutant background. (C,E,G,I) Phase contrast images of live testis squash. Elongating spermatid flagella were seen in the wild type (arrowheads in C). In *thoc5* mutant testis, however, only mature spermatocytes were seen (E). Expression of fly *thoc5* (G) or mouse *Thoc5* (I) restored the elongating spermatid flagella (arrowheads). Some cytokinesis defects were rarely seen in the rescued flies (asterisks in G). (D,F,H,J) Combined images of DNA staining and phase contrast. The phase-dark nucleolar structures are indicated by arrows in D, H and J. Genotypes: *thoc5*^{1}*/CyO* in C,D; *thoc5*^{1} in E,F; *thoc5*^{1}*; p{Sp-K}* in G,H; *thoc5*^{1}*; da-Gal4/UAS-mThoc5* in I,J. (**K**) RT-PCR analysis confirmed that mRNA of mouse *Thoc5* was expressed in the *thoc5*^{1} mutant background. Genotypes: *thoc5*^{1}*/Cy; UAS-mThoc5/+* in lane a; *thoc5*^{1}*; da-Gal4/UAS-mThoc5* in lane b. Scale bars: 10 μm.

mutants (Fig. 7A). We also failed to detect any changes in the level of other male sterile tTAF-independent genes: *boule* (*bol*), *Cyclin B* (*CycB*) and *twine* (*twe*). Whole-mount RNA in situ hybridization to testis also showed no significant differences in the levels of the three tTAF target mRNAs examined (Fig. 7B-D). These data suggest that the expression of tTAF target genes, as well as tTAF non-target genes, during *Drosophila* spermatogenesis might be largely independent of the THO complex.

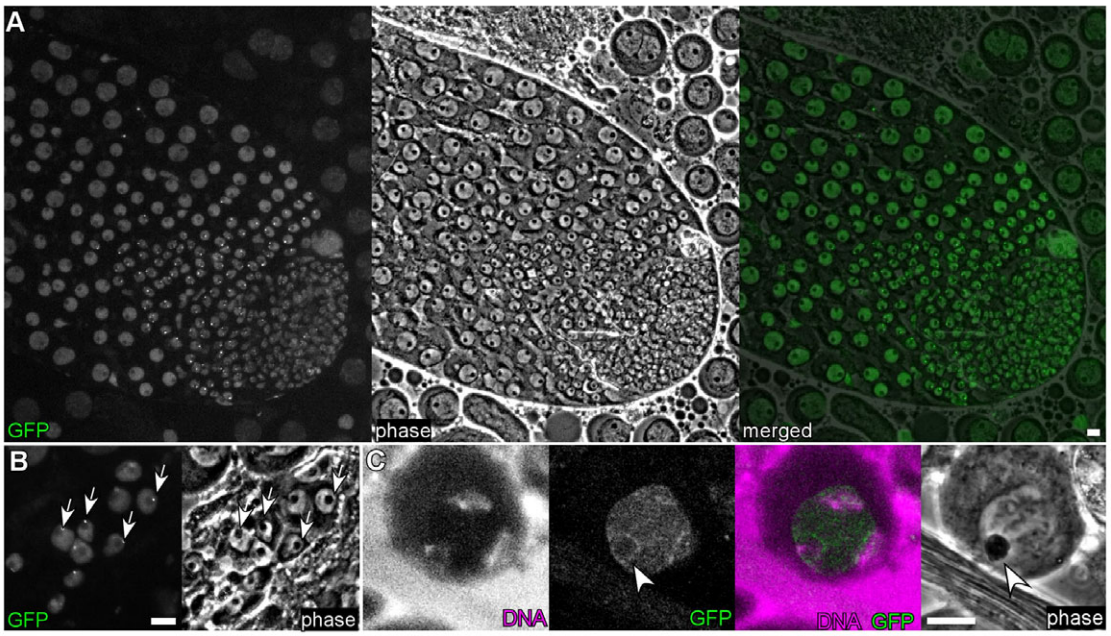

Fig. 4. The peri-nucleolar region in developing spermatocytes is enriched with THOC5-GFP. Localization of THOC5-GFP in live testis squash. THOC5-GFP was detected in nuclei at all stages of developing germ cells (**A**) but its subnuclear distribution changed dynamically during development. At the early primary spermatocyte stage, GFP signal was observed near nucleoli as a single dot (arrows in **B**), and then distributed as a ring shape (arrowhead in **C**) around nucleoli at the late stage of primary spermatocytes. Scale bars: 10 μm.

As it has been well established that the THO complex plays an important role in nuclear mRNA export, we next examined whether nuclear export of mRNAs is affected in *thoc5* mutants by in situ hybridization to testis squashes using fluorescence-labeled riboprobes.

Unexpectedly, all transcripts we examined were mainly detected in the cytoplasm of *thoc5* mutant spermatocytes, suggesting that they are exported from the nucleus independently of the THO complex (Fig. 8). Interestingly, one significant alteration was found in the distribution of a tTAF target mRNA, *dj*. In wild-type spermatocytes, unexpectedly, the nucleus was highly enriched with *dj* transcript, with weak signal in the cytoplasm (Fig. 8A). In the nucleus, *dj* transcript was accumulated at certain compartments near the nucleolus (arrowheads in Fig. 8A). No detectable signals were seen either by a *dj* sense probe in wild-type testis (Fig. 8C) or by a *dj* antisense probe in a tTAF mutant (*mia*) spermatocyte (see Fig. S3 in the supplementary material), suggesting that the signal is genuinely revealing *dj* transcripts. In *thoc5* mutant cells, however, *dj* transcript was detected mainly in the cytoplasm (Fig. 8B). This result raised the question of whether the alteration in mRNA distribution is general to all tTAF target mRNAs. But two other tTAF target mRNAs, *fzo* and *Mst87F*, showed no significant differences in their distribution between wild type and *thoc5* mutants (Fig. 8D-I), suggesting that the alteration is specific to *dj*.

The mRNA for *bol*, which is tTAF independent, was also seen mostly in the cytoplasm of both wild-type and *thoc5* cells, with no significant difference in its distribution between wild type and *thoc5* (Fig. 8J-L). Taken together, these results suggest that the THO complex is mostly not required for mRNA export per se, but is required for regulating the distribution of certain RNAs in *Drosophila* spermatocytes.

DISCUSSION

Here, we isolated and characterized a novel *Drosophila* meiotic arrest mutant, *garm*. The phenotypes of *garm* were different from those of other meiotic arrest mutants. First, the chromosomes were more condensed in arrested mutant spermatocytes than in wild-type spermatocytes. In other known meiotic arrest mutants, spermatocytes have chromosomes either similar to or less condensed than wild type (for a review, see White-Cooper, 2010). Second, unlike other mutants, the nucleoli were severely disrupted in *garm* mutant primary spermatocytes. Taken together, these findings suggest that *garm* represents a novel class of meiotic arrest gene, and that its gene product might play a role distinct from that of other meiotic arrest gene products, which are involved in the transcription of target genes (for a review, see White-Cooper, 2010). Indeed, THOC5 (encoded by *garm*) is a subunit of the THO complex, which is an evolutionarily conserved protein complex required for mRNP biosynthesis. So far, *garm* (*thoc5*) is the only meiotic arrest gene whose gene product is likely to be involved in a post-transcriptional step.

THOC5 is likely to function in spermatocytes as a subunit of the THO complex. First, all subunits of the THO complex examined were not only colocalized in spermatocytes but also co-immunoprecipitated with each other, suggesting that they form a stable complex in spermatocytes (Fig. 5). Second, in mutant spermatocytes lacking one of the THO subunits, not only was the localization of other THO subunits disrupted, but also their expression level was significantly reduced (see Figs S4, S5 in the supplementary material). Third, hypomorphic mutants of both *thoc6* and *thoc7* appeared to show mild disruption of the nucleoli, although they did not show the typical meiotic arrest phenotype (see Fig. S5B-E in the supplementary material).

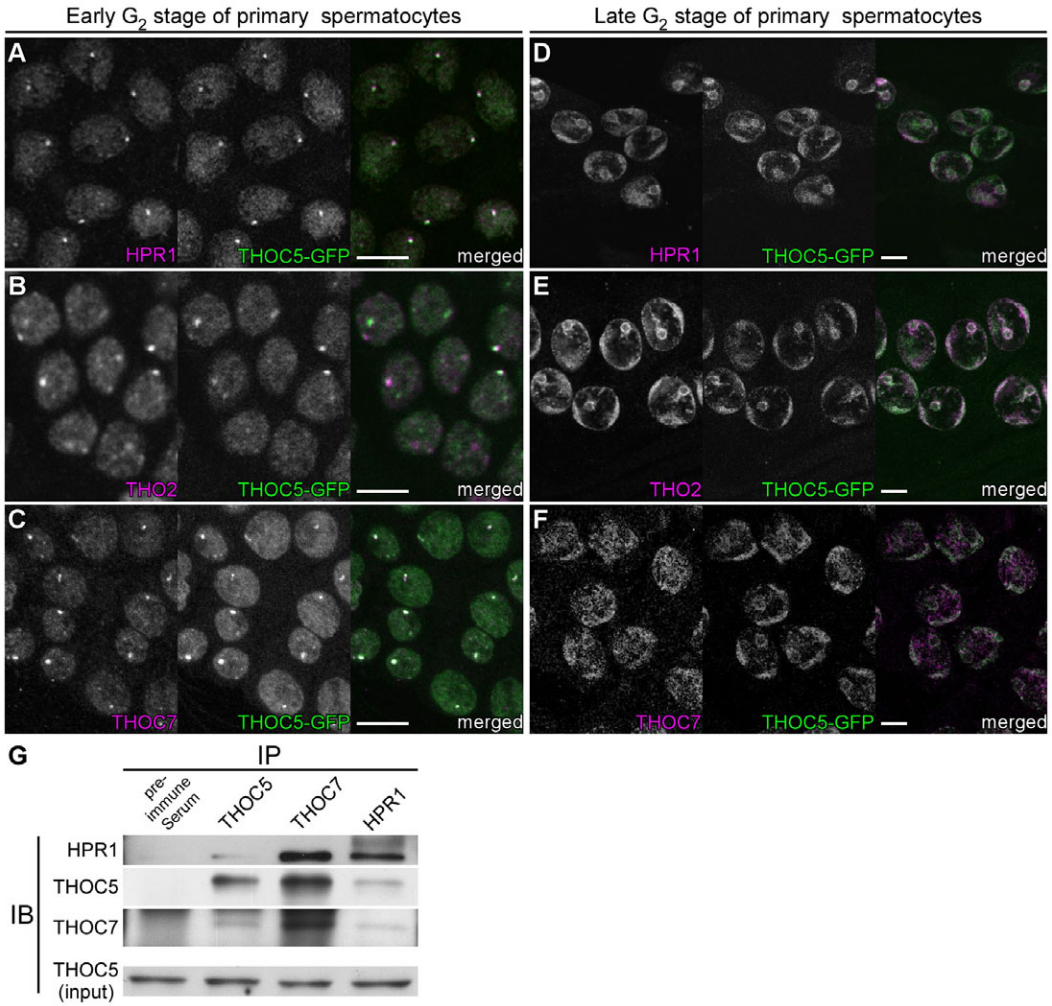

Fig. 5. THO subunits form a stable complex in *Drosophila* testis. (**A**,**D**) Double staining with THOC5-GFP (green) and anti-HPR1 antibody (magenta) in primary spermatocytes at early stage (A) or late stage (D). (**B**,**E**) Double staining with THOC5-GFP (green) and anti-THO2 antibody (magenta) in primary spermatocytes at early (B) or late (E) stage. (**C**,**F**) Double staining with THOC5-GFP (green) and anti-THOC7 antibody (magenta) in primary spermatocytes at early (C) or late (F) stage. All THO subunits examined were colocalized with THOC5-GFP at both early and late stages of primary spermatocyte. (**G**) Western blot analysis showed that HPR1, THOC5 and THOC7 co-immunoprecipitated with each other. Protein extract corresponding to the equivalent of two testis pairs was loaded as input. IB, immunoblot; IP, immunoprecipitation. Scale bars: 10 μm.

What is the specific role of the THO complex in *Drosophila* spermatogenesis? The finding that the nucleolar structure was severely disrupted in *thoc5* mutants suggests that the THO complex might be required for the proper organization of the nucleolus. In accordance with this, all subunits of the THO complex accumulate at the peri-nucleolar region in pre-meiotic spermatocytes. Recent progress in our understanding of the nucleolus suggests that not only does it function as the ribosome-producing factory, but it also regulates mitosis, cell-cycle progression and proliferation, many forms of stress response and biogenesis of multiple ribonucleoprotein particles (for a review, see Boisvert et al., 2007). Consistent with these non-traditional functions, many proteins unrelated to ribosome assembly are found in the nucleolus (Andersen et al., 2005; Andersen et al., 2002; Pendle et al., 2005; Scherl et al., 2002). Moreover, the integration of many different sources of protein-protein interaction data showed that the spliceosomal complex is one of the major protein complexes in human nucleolus (Hinsby et al., 2006), suggesting that the spliceosomes are structural components of the nucleolus. Thus, we speculate that the nucleolar disruption found in *thoc5* spermatocytes might be caused by malformation of the spliceosomal complex, because the THO complex associates with splicesomal proteins independently of transcription (Cheng et al., 2006; Masuda et al., 2005). One of the common phenotypes caused by nucleolar disruption in mammalian cells is the p53-mediated cellular stress response, which includes cell-cycle arrest and apoptosis (Rubbi and Milner, 2003; Yuan et al., 2005). To test whether the meiotic arrest phenotype seen in this study was also mediated by p53, we examined genetic interactions between *thoc5* and *p53*. However, lack of p53 failed to suppress the meiotic arrest phenotype caused by *thoc5* (data not shown), suggesting that p53 is not required for the meiotic arrest phenotype seen in *thoc5* mutant spermatocytes. This also suggested, if nucleolar disruption was the cause of the meiotic arrest, that some other unknown pathways are required for cell-cycle arrest in *Drosophila* spermatocyte. Alternatively, the THO complex might simply be required for the nuclear export of mRNAs for meiotic cell-cycle regulators, such as *CycB* or *twine*.

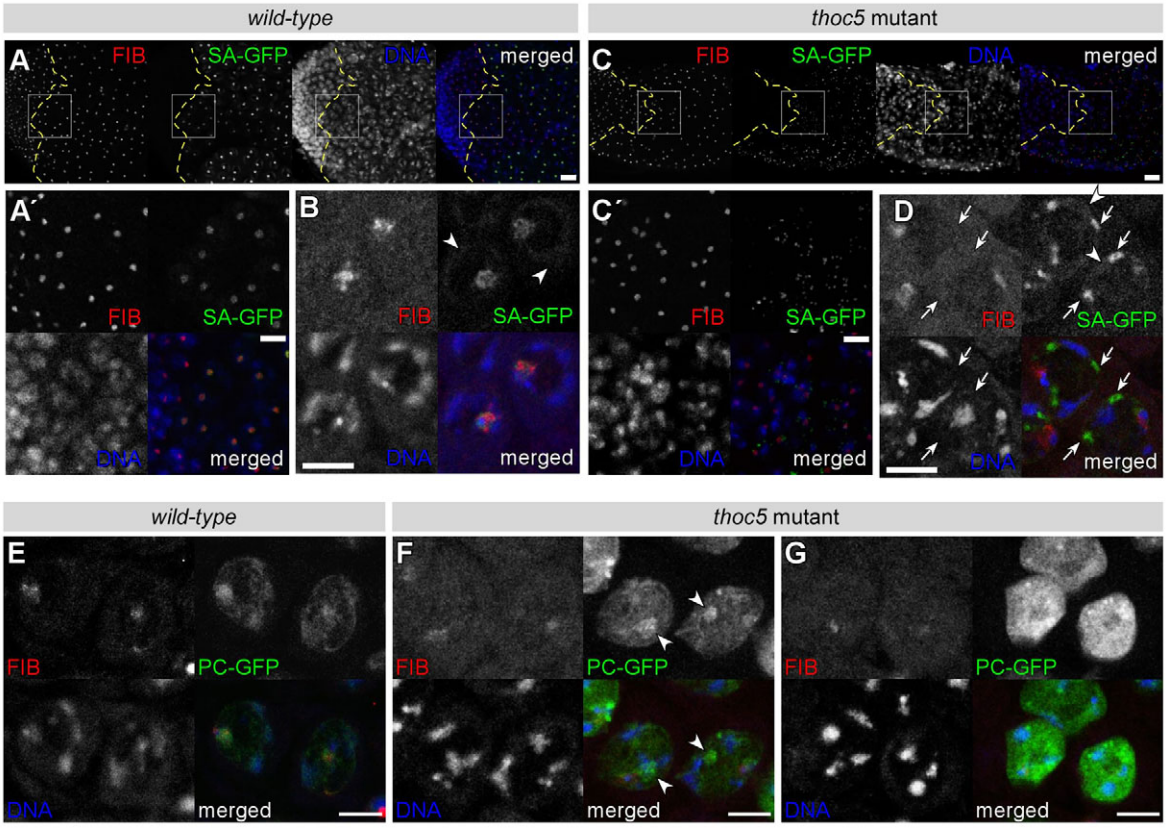

Fig. 6. Abnormal distribution of SA-GFP and PC-GFP in *thoc5* mutants. (**A-D**) SA-GFP localization in primary spermatocytes of wild type (A-B), or *thoc5*^{1}/ *thoc5*^{e00906} (C-D). SA-GFP appeared in the nucleolus of early stage wild-type primary spermatocytes, and the signal got stronger as the cells grew (A). In mature wild-type primary spermatocytes (B), the nucleolus was enriched with SA-GFP (labeled by Fibrillarin; red), and faint signals in chromatin (arrowheads) were also seen. However, SA-GFP was scattered from the initial phase of its expression in *thoc5* mutants (C,C′). In mature mutant spermatocytes (D), scattered GFP signals (arrows) overlapped with neither Fibrillarin (red) nor chromatin (blue), although faint signals in chromatin still remained (arrowheads). The onset of SA-GFP expression is indicated by the dashed lines in A and C. Enlarged images of the area marked by the squares in A and C are shown in A′ and C′, respectively. (**E-G**) Distribution of PC-GFP in primary spermatocytes of wild type (E), or *thoc5*^{1}/ *thoc5*^{e00906} mutants (F,G). PC-GFP (green) co-labeled with anti-Fibrillarin (red) and DNA (blue). PC-GFP was normally concentrated in the nucleolus and chromatin (E). In the mutant cells (F), PC-GFP-enriched regions (arrowheads in F) overlapped neither Fibrillarin nor DNA. In some mutant cells, PC-GFP was dispersed throughout the nucleus (G). Scale bars: 20 μm in A,C; 10 μm in all other panels.

The finding that PC localization to the nucleolus requires tTAFs raised a hypothesis that the nucleolus acts as a sequestering compartment for counteracting transcriptional silencing by PcG proteins in *Drosophila* spermatocytes (Chen et al., 2005). If this is the case, transcription of tTAF target genes might be affected in *thoc5* mutant spermatocytes because the nucleolar localizations of both PC and the tTAF SA are abnormal in the *thoc5* mutant. To our surprise, however, tTAF target genes were transcribed; and even more surprisingly, their transcripts were still exported to cytoplasm in *thoc5* mutant spermatocytes, suggesting that the nucleolar localization of tTAFs and PcG proteins is not essential for counteracting transcriptional silencing by PcG proteins. In addition to this, a male-sterile gene, *bol*, which is a target of another testis-specific meiotic arrest complex, tMAC, was also expressed and exported independently of THOC5. Taken together, these results suggest that the meiotic arrest phenotype in *thoc5* might not be caused by the failure of the canonical THO function, mRNA export.

The finding that *dj* transcript normally appeared to be retained in the nucleus at the pre-meiotic stage of spermatogenesis is very interesting. The transcription of *dj* mRNA is known to be initiated in early spermatogenesis, but its translation is normally delayed until meiotic divisions are completed (Santel et al., 1997). Although it has been reported that translational repression of *dj* mRNA is mediated by the TRE (*dj* translational repression element) located at the 5′-UTR (Blumer et al., 2002), we speculate that nuclear retention of *dj* mRNA might be an additional mechanism by which *dj* mRNA is translationally suppressed. In mammalian cells, a novel regulation mechanism of gene expression through RNA nuclear retention has been recently proposed (Chen and Carmichael, 2009; Prasanth et al., 2005). In this model, certain mRNAs containing elements for adenosine-to-inosine editing within their 3′-UTR are retained in nuclear paraspeckles, and may be released when the demand for their protein products increases. Although it is unclear whether *Drosophila* has a similar regulatory mechanism, the nuclear retention of *dj* mRNA in pre-meiotic spermatocytes might be a sign of the existence of such a mechanism. Further studies are required to clarify this issue. From the finding that nuclear mRNA retention is not generally applicable to other tTAF target genes, we can not completely rule out the possibility that the *dj* probe might cross-hybridize with some other nuclear RNAs concentrated in a certain nuclear structure, such as the Y-loop. However, this is unlikely because the signal was absent in *mia* mutant spermatocytes in which *dj* is not expressed;

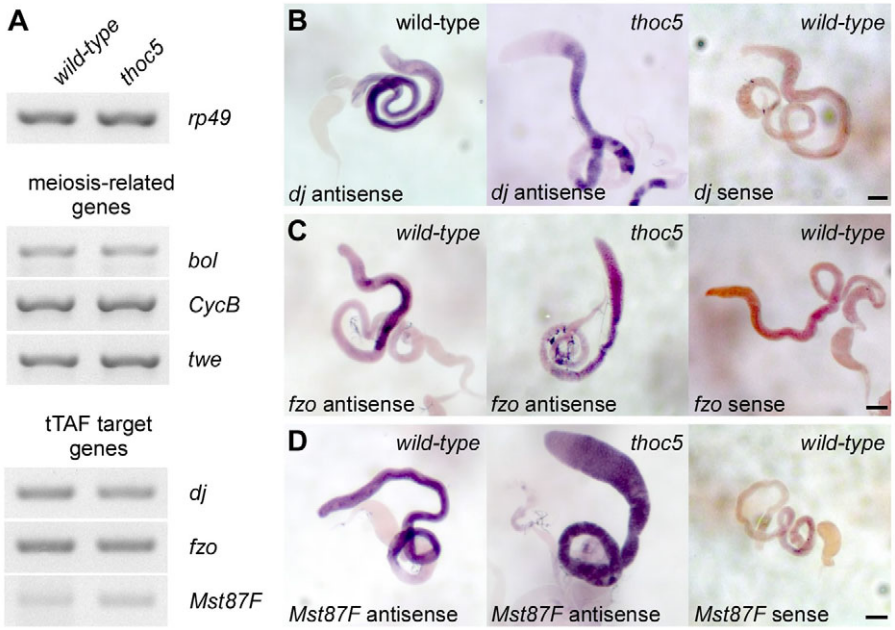

Fig. 7. Expression of three tTAF target genes and three other male sterile genes is unaffected in *thoc5* mutant. (**A**) The expression levels of tTAF target genes (*dj*, *fzo*, *Mst87F*) or tTAF independent male sterile genes (*bol*, *CycB*, *twe*) were analyzed by RT-PCR. All the genes examined showed no significant changes in their expression level between wild type and *thoc5* mutants. *rp49* was used as input control. (**B-D**) Whole-mount RNA in situ hybridization to testis in wild type or *thoc5* mutants. The overall expression pattern of all three tTAF target mRNAs was similar in wild type and *thoc5* mutants. Scale bars: 100 μm.

moreover, the nuclear *dj* message was still detectable in the testes of *XO* males, which lack the Y-loop (see Fig. S3 in the supplementary material). A previous report (Santel et al., 1997) that showed a strong cytoplasmic signal of *dj* mRNA by whole-mount in situ hybridization with colorimetric detection is also inconsistent with our result. However, we also failed to detect a clear signal of *dj* massages in the nucleus (see Fig. 7) by a similar method. Thus, the discrepancy was probably due to methodological differences. Whether the signal detected by a *dj* anti-sense probe represents genuine *dj* message or not, nuclear retention of this RNA appears to be dependent on the THO complex.

The THO complex is not a testis-specific protein complex. Why, then, might the testis be more sensitive to loss of THOC5 than other tissues are? There are possible reasons for this. First, THOC5 might not be essential for the function of the THO complex. In the *thoc5* mutant, a significant amount of HPR1 was still detected in the nucleus, although its level was greatly reduced (see Fig. S4 in the supplementary material). Interestingly, the residual HPR1 was mainly located near the chromatins rather than the nucleolus (see Fig. S4 in the supplementary material). Thus, the residual subunits might still have some degree of activity, and this residual activity might be sufficient for most cells, but not enough for other cells, such as spermatocytes. Second, in addition to mRNP biogenesis, the THO complex might have a non-canonical function in the spermatocytes, such as the organization and/or maintenance of nucleolar structure, and possibly the localization of some nuclear proteins and RNAs. Third, spermatogenesis requires the coordinated expression of a large number of genes. To ensure that

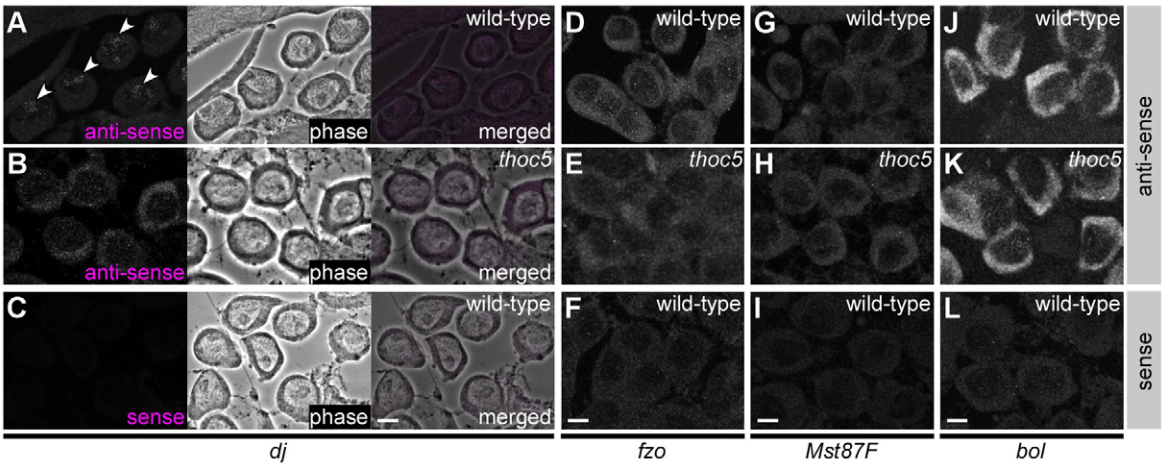

Fig. 8. Comparison of the distribution of mRNAs related to spermatogenesis in primary spermatocytes from wild type and *thoc5* mutants. (**A-C**) In situ hybridization to *dj* mRNA in primary spermatocytes of wild type and *thoc5*^{1} mutants. In wild type, a significant amount of *dj* transcript was accumulated within the nucleus (arrowheads in A) but no detectable nuclear signals of *dj* transcript were seen in *thoc5* mutants (B). (**D-I**) The distribution of *fzo* (D-F) or *Mst87F* (G-I) mRNAs showed cytoplasmic accumulation in both wild type (D,G) and *thoc5* mutants (E,H). (**J-L**) The distribution of transcript for *bol*, a tTAF non-target gene, showed no significant difference between wild type (J) and *thoc5* mutants (K). No specific signals were detected by the sense probes for each transcript (C,F,I,L). Scale bars: 10 μm.

the spermatocytes regulate gene expression in a coordinated manner from transcription to nuclear export, the THO complex might still have a role, although we failed to detect its specific target mRNA.

In summary, disruption of THOC5 caused severe defects in the primary spermatocyte nucleus, including nucleolar disruption, abnormal distribution of proteins (SA and PC) and an RNA transcript (*dj*), suggesting that it has a role in the establishment or maintenance of subnuclear structure in *Drosophila* primary spermatocytes. The main causative factor of meiotic arrest in *thoc5* mutants might be the disruption of subnuclear structure rather than the defect in nuclear mRNA export.

In addition to the meiotic arrest phenotype reported here, the *thoc5* mutant also has other phenotypes, including wing bubble and uncoordinated behavior (S.M. and Y.D.C., unpublished). Our recent work also showed that longevity and tolerance to environmental stress were significantly reduced in the THO mutants (Kim et al., 2011). This suggests that the role of the THO complex is not limited to spermatogenesis, but is also important for other types of cells. When we examined whether a similar nucleolar defect is seen in other cell types, we found no similar defect in other cell types except the salivary gland cells (see Fig. S6 in the supplementary material). This suggests that the specific role of the THO complex is different depending on the cell type. It will be interesting to clarify the specific roles of the THO complex in other types of cells.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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