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Tombola, a tesmin/TSO1-family protein, regulates transcriptional activation in the *Drosophila* male germline and physically interacts with Always early

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During male gametogenesis, a developmentally regulated and cell type-specific transcriptional programme is activated in primary spermatocytes to prepare for differentiation of sperm. The *Drosophila aly*-class meiotic-arrest loci (*aly*, *comr*, *achi/vis* and *topi*) are essential for activation of transcription of many differentiation-specific genes, and several genes important for meiotic cell cycle progression, thus linking meiotic divisions to cellular differentiation during spermatogenesis. Protein interaction studies suggest that the *aly*-class gene products form a chromatin-associated complex in primary spermatocytes. We identify, clone and characterise a new *aly*-class meiotic-arrest gene, *tombola* (*tomb*), which encodes a testis-specific CXC-domain protein that interacts with Aly. The *tomb* mutant phenotype is more like that of *aly* and *comr* mutants than that of *achi/vis* or *topi* mutants in terms of target gene profile and chromosome morphology. *tomb* encodes a chromatin-associated protein required for localisation of Aly and Comr, but not Topi, to chromatin Reciprocally, *aly* and *comr*, but not *topi* or *achi/vis*, are required to maintain the normal localisation of Tomb. *tomb* and *aly* might be components of a complex paralogous to the *Drosophila* dREAM/Myb-MuvB and *C. elegans* DRM transcriptional regulatory complexes.

KEY WORDS: Spermatogenesis, Transcription, SynMuv, Differentiation, CXC

INTRODUCTION

Differential control of gene expression during development is crucial for specification and maintenance of differentiated cell types. One of the most dramatic gene expression switches occurs in primary spermatocytes during spermatogenesis; many genes are active only in these cells. Some are germline-specific homologues of ubiquitously expressed genes [e.g. *β-Tub85D* (Kemphues et al., 1979) and testis-specific proteasome components (Ma et al., 2002)], others are spermatogenesis-specific proteins [e.g. the protamines that replace histones to tightly package sperm DNA (Jayaramaiah Raja and Renkawitz-Pohl, 2005)].

In both mammals and insects, germline stem cells divide to produce spermatogonia. After further mitotic amplification divisions (four in *Drosophila melanogaster*), spermatogonia become primary spermatocytes, committed to differentiation (reviewed by Fuller, 1993). This developmental transition results in transcriptional activation in primary spermatocytes of a large suite of genes required for meiosis and spermiogenesis. In *Drosophila*, transcription stops before the meiotic divisions, so transcripts for late-acting proteins are made pre-meiotically (Olivieri and Olivieri, 1965). Meiotic-arrest mutant testes accumulate primary spermatocytes, but lack later stages of spermatogenesis because mutant primary spermatocytes fail to initiate transcription of many genes whose products are required after meiosis. Meiotic-arrest mutants also fail to express some meiotic gene products; *always early* (*aly*)-class gene products differ from *cannonball* (*can*)-class in their regulation of certain cell cycle genes (Lin et al., 1996; White-Cooper et al., 1998). Through their function in controlling production of cell cycle and differentiation gene products, the meiotic-arrest genes coordinate the independent processes of meiosis and spermatid morphogenesis.

The *aly* class of meiotic-arrest genes have a broader target range than the *can* class. Four *aly*-class and five *can*-class meiotic-arrest loci have been described (Ayyar et al., 2003; Hiller et al., 2004; Hiller et al., 2001; Jiang and White-Cooper, 2003; Perezgazga et al., 2004; Wang and Mann, 2003; White-Cooper et al., 2000; White-Cooper et al., 1998). *aly* encodes one of two *Drosophila* homologues of the *C. elegans* synMuvB gene *lin-9*, the other homologue being *mip130* (Beitel et al., 2000; White-Cooper et al., 2000). *cookie monster* (*comr*) encodes a novel protein of unknown function (Jiang and White-Cooper, 2003). *achintya/vismay* (*achi/vis*) and *matotopetli* (*topi*) encode sequence-specific DNA-binding proteins (Ayyar et al., 2003; Perezgazga et al., 2004; Wang and Mann, 2003). Aly, Comr and Achi/Vis proteins co-immunoprecipitate from testis extracts; Topi was identified in a yeast two-hybrid screen for Comr interactors (Perezgazga et al., 2004; Wang and Mann, 2003). Despite the interactions between *aly*-class gene products, the *aly* and *comr* mutant phenotypes are subtly different from those of *topi* and *achi/vis*. Aly and Comr nuclear localisations are mutually dependent, whereas these proteins require *topi* and *achi/vis* for their concentration on chromatin. *aly* or *comr* (but not *topi* or *achi/vis*) mutants display defects in chromatin organisation. Finally, a small subset of genes are much more dependent on *topi* and/or *achi/vis* than on *aly* or *comr* for their transcription (Jiang and White-Cooper, 2003; Perezgazga et al., 2004).

To find further transcriptional regulators in primary spermatocytes, we screened for Aly-binding proteins. We have identified and characterised a new *Drosophila* meiotic-arrest gene, *tombola* (*tomb*), which is expressed specifically in testis. *tomb* encodes the second *Drosophila* member of the tesmin/TSO1 CXC-domain protein family, the other being Mip120, a subunit of the same complex as Mip130. We show that Tomb complexes with Aly and Comr. We identify a *tomb* mutant and show that *tomb* mutant testes have an *aly*-class meiotic-arrest

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phenotype more like that of *aly* and *comr* than of *topi* and *achi/vis* mutants. Aly and Comr proteins fail to associate with chromatin in the absence of *tomb* function. Topi protein also localises to chromatin in wild-type and *achi/vis* primary spermatocytes, but not in *aly*, *comr* or *tomb* mutant cells. Concentration of ectopically expressed EGFP-Tomb on chromatin in the nucleus is normal in *achi/vis* or *can*-class mutants, but is altered in *aly* and *comr* mutants.

MATERIALS AND METHODS

Fly husbandry and strains

Flies were raised on standard corn meal (or wheat meal) sucrose agar at 25°C. Visible markers and balancer chromosomes are described in Flybase (FlyBase Consortium, 1999). *P[GS]12862/CyO* was from the Kyoto *Drosophila* Stock Centre. Mutant alleles used were aly^{5}, $comr^{Z1340}$, $achi^{Z3922}vis^{Z3922}$, $topi^{Z3-2139}$, $nht^{Z2-5946}$. w^{1118} or *red e* were used as wild-type controls.

Deficiency mapping and P-element excision

Df(2L)cl-h3/SM6b and *Df(2L)cl-h1/CyO, amos*$^{Roi-1}$ (Bloomington *Drosophila* Stock Centre) were crossed to *P[GS]12862/CyO*. Excision of the *P[GS]12862* insertion was carried out by crossing *w; P[GS]12862/CyO,Δ2-3* males to *w; Tft/CyO* females, recovering individual white-eyed progeny and back-crossing to *w; Tft/CyO* to establish stocks. Excision lines were analysed by testis squashing and PCR and sequencing of the ORF. Females were crossed to their balanced brothers to test for fertility; male fertility was tested by crossing to virgin w^{1118} females. The testis phenotype was scored by phase contrast microscopy after dissection and squashing.

Yeast two-hybrid screen and analysis

An Aly(C-terminus)–Gal4-DNA-Binding Domain [Aly(C)-DB] fusion construct was made by subcloning the ORF (equivalent to amino acids 275 to 534) from a full-length *aly* cDNA clone into pGBKT7. We generated and screened a testis cDNA-Gal4-Activation Domain (AD) fusion protein library using the Matchmaker Library Construction and Screening Kit (Clontech) as previously described (Perezgazga et al., 2004). Colonies were picked from SD –Ade –His –Leu –Trp selection plates after 7 days. One million independent co-transformants yielded 90 colonies that grew under selective conditions and were blue in the presence of X-α-Gal. To test for interaction between Tomb and Comr, AH109 yeast cells were co-transformed with pGADT7-Rec-Tomb and pGBKT7-Comr or pGBKT7-CG15031 (CG15031 was another clone isolated in the yeast two-hybrid screen) as a negative control. Transformed cells were plated on SD –Ade –His –Leu –Trp selection plates containing X-α-Gal.

Construction of deletion analysis plasmids

PCR products for Tomb deletion derivatives (amino acid residues: 1-73; 1-136; 73-243; 136-243) and full-length Tomb were subcloned into pACT2. Co-transformation of AH109 yeast cells was with pGBKT7-Aly(C) or pGBKT7-Kr(Zn-finger) as the negative control (Perezgazga et al., 2004). Transformed cells were plated on SD –Ade –His –Leu –Trp/X-α-Gal plates.

RT-PCR expression analysis

For semi-quantitative RT-PCR, total RNA was extracted from dissected testes with Trizol (Invitrogen) and resuspended in RNAse-free water (three testes-worth per μl). First-strand cDNA was generated from 4 μl of this sample using oligo-dT primers with the SuperScript II Reverse Transcriptase System (Invitrogen). cDNA derived from 0.18 testes (0.3 μl of RT reaction) was used for each RT-PCR reaction and amplified with Taq DNA polymerase (Qiagen) with 24 amplification cycles. Genomic DNA from wild-type flies was used as a positive PCR control, and a no-reverse-transcriptase (no-RT) reaction on wild-type RNA served as a negative control. For RT-PCR from various developmental stages, total RNA was extracted with Trizol, cDNA prepared as above, and PCR amplification carried out for 30 cycles. For re-amplification, 0.5 μl of the first PCR product was used as the template for a further 30-cycle PCR reaction.

Mapping the 5′ and 3′ ends of *tomb*

A 3′ RACE Kit was used following the manufacturer's (Invitrogen) instructions. The RACE products were either directly sequenced, or were subcloned into pGEM-T-Easy for sequencing. For the 5′ end, RT-PCR was performed using a series of primers upstream of the ATG, paired with a 3′ primer within the coding sequence.

Co-expression and co-immunoprecipitation from tissue culture cells and testis extracts

The full-length *tomb* ORF was subcloned into the mammalian tissue culture expression vector HA-tagged pCDEF3. The full-length *aly* ORF and Kruppel (Kr) zinc-finger region (Perezgazga et al., 2004) were similarly subcloned into FLAG-tagged pCDEF3. 293T human kidney cells were co-transfected with plasmids for expression of HA-Tomb and FLAG-Aly, or HA-Tomb and FLAG-Kr(Zn-finger), respectively, with lipofectamine 2000 reagent (Invitrogen). Co-immunoprecipitation was as previously described (Perezgazga et al., 2004).

Testes dissected from EGFP-Tomb-expressing flies were homogenised in lysis buffer (50 mM Tris-HCl pH 7.5-8.0, 0.5% Triton X-100, 150 mM NaCl, protease inhibitors) (146 testes, 500 μl buffer used), incubated with ethidium bromide (400 μg/ml) for 30 minutes at 4°C, then cleared by centrifugation. 20 μl was retained as the 'input' sample, the remainder was pre-cleared with protein G-sepharose, then incubated with mouse anti-GFP (Roche) and precipitated with protein G-sepharose. Beads were washed, then bound proteins were eluted by boiling in SDS sample buffer. Wild-type testes were processed in parallel as a negative control.

GFP fusion construct

The *tomb* ORF was subcloned in frame into pUAST-EGFP (Parker et al., 2001). Numerous independent P-element-mediated insertions were recovered using standard transformation protocols after injection of w^{1118} embryos. EGFP-Tomb fusion protein expression was driven using Bam-Gal4-VP16, which expresses just before the onset of meiotic-arrest gene expression and functions in all the mutant backgrounds (Chen and McKearin, 2003). Bam-GAL4-VP16 (on chromosome 3) was recombined with a third-chromosome UAS-EGFP-Tomb insertion, and the chromosome was used homozygous to express tagged protein in testes homozygous for second chromosome male steriles (*tomb*, *achi/vis*, *comr*, *nht*). Bam-GAL4-VP16 was recombined with aly^{5} to allow expression from a homozygous second chromosome-linked UAS-EGFP-*tomb* insertion in this mutant background.

Generation of the anti-Topi antibody

Anti-peptide antibodies were raised by Moravian-Biotechnology. The synthesised oligopeptide KNNPTKPIFSDTYL from the Topi C-terminus was coupled to BSA and used to immunise two rats. The staining patterns for these sera were indistinguishable.

Microscopy and immunofluorescence

Live testes were dissected, squashed in 2 μg/ml Hoechst 33342 in testis buffer (183 mM KCl, 47 mM NaCl, 10 mM Tris pH 6.8) and examined by phase contrast and fluorescence microscopy. Images were captured with a Q-imaging Retiga 1300 monochrome CCD camera linked to an Olympus BX50 microscope using Openlab software (Improvision) or on a JVC KY-F75U three-colour CCD camera with KY-Link software, and imported into Photoshop (Adobe). Aly, Comr and Topi proteins were visualised by indirect immunofluorescent staining using rabbit anti-Aly (1:2000), rabbit anti-Comr (1:1000) or rat anti-Topi (1:1000) antibodies detected with FITC-conjugated secondary antibodies (Jackson), as described (Jiang and White-Cooper, 2003; White-Cooper et al., 2000). DNA was co-stained with propidium iodide. Cells were imaged using a Bio-Rad Radiance Plus confocal microscope mounted on a Nikon E800.

RNA in situ hybridisation

Dig-labelled antisense probes for *Cyclin B*, *Mst87F* and *polo* were generated as previously described (White-Cooper et al., 1998). To synthesise RNA probes for *CG3330*, *CG3927* and *CG12907*, we generated 400-600 bp RT-PCR products using total testis RNA as template. For *tomb*, the PCR amplified the entire ORF. The 3′ PCR primers included a T3 RNA

polymerase promoter site for in vitro transcription of dig-labelled antisense RNA probes. In situ hybridisation was carried out as described (White-Cooper et al., 1998). Primer sequences are available on request.

RESULTS

A tesmin-family CXC-motif protein, Tombola, interacts with Aly and Comr

We conducted a yeast two-hybrid screen to identify proteins that act with Aly to control transcriptional activation in *Drosophila* primary spermatocytes. Using the C-terminal half of Aly as bait we recovered seven independent clones of *CG14016*; we named this gene *tombola* (*tomb*) based on the testis phenotype – mutant testes resemble a tube full of balls, as in the lottery game. Co-immunoprecipitation of transiently expressed tagged proteins from tissue culture cells confirmed the interaction between Aly and Tomb proteins. 293T cells were co-transfected to express HA-tagged Tomb (HA-Tomb) and FLAG-tagged Aly (FLAG-Aly). Immunoprecipitation with anti-FLAG antibodies, followed by western blotting with anti-HA antibodies showed that Tomb co-immunoprecipitated with Aly (Fig. 1A). This was confirmed with the reciprocal experiment – immunoprecipitation with anti-HA followed by blotting with anti-FLAG. We detected no co-immunoprecipitation in cells co-expressing HA-Tomb and FLAG-Kr [FLAG fused to the five-zinc-finger motif region of Kruppel (Perezgazga et al., 2004)]. To test the in vivo interaction between Aly and Tomb, and to test whether DNA was implicated in the interaction, we made extracts from EGFP-Tomb-expressing testes (see below), incubated the extracts with ethidium bromide, and immunoprecipitated using anti-GFP. We found that Aly co-immunoprecipitated with EGFP-Tomb, showing that the interaction occurs in testes, and is DNA independent (Fig. 1B). To define the Aly-interaction region of Tomb, we generated deletion constructs and tested their ability to interact in the two-hybrid system. The Aly-interaction domain is found in the C-terminal half of Tomb (amino acids 136-243).

Since *aly* and *comr* have identical mutant phenotypes, we suspect that these gene products probably act together in a complex, but we have not detected direct interaction between these proteins. We tested the ability of Tomb to bind Comr by two-hybrid analysis. Yeast co-transformed with Tomb-AD and Comr-DB grew under selective conditions, demonstrating that Comr can interact with Tomb. The Tomb-Aly and Tomb-Comr interactions were specific, as yeast co-transformed with Tomb-AD and CG15031-DB were unable to grow under selective conditions. Co-expression and co-immunoprecipitation experiments in tissue culture cells confirmed that FLAG-Tomb can interact with HA-Comr (data not shown).

The *tomb* genomic region is complex (Fig. 2A). The *tomb* ORF is embedded within, but in the opposite orientation to, the 3′ UTR of *CG31989*, which is predicted to encode a conserved protein (CapD3) of unknown function. As no *tomb* cDNA clones have been sequenced, we mapped the 5′ and 3′ ends by RACE and RT-PCR. The 5′ end of *tomb* overlaps the 3′ end of the adjacent gene *CG14015*. Translation of the *tomb* ORF gave a 243 amino acid, 26 kD conceptual protein with a theoretical pI of 9.4. The predicted Tomb protein contains a nuclear localisation signal and a CXC motif of the tesmin/TSO1 family (Fig. 2B). Tesmin has been described in vertebrates (it also known as Mtl5/MTL5 in mouse and human), whereas TSO1 is from *Arabidopsis*, indicating that this domain is conserved between animals and plants. The only other *Drosophila* tesmin/TSO1 CXC-domain protein, Mip120, has been found in a complex with the second *Drosophila lin-9* (*aly*) homologue, Mip130 (Beall et al., 2002; Korenjak et al., 2004; Lewis et al., 2004). A second tesmin/TSO1 CXC-domain protein, which we refer to as tesmin-like (tesl), was found in humans and mouse. *C. elegans* has a single member of this family, LIN-54 (JC8.6), sea urchin and *Ciona* each have one homologue. Including TSO1, the *A. thaliana* genome has 11 tesmin/TSO1 family members.

Comparison of the tesmin/TSO1-domain proteins revealed that *tomb* was unusual in only having a single CXC domain (Fig. 2C). All other family members have either two domains (vertebrate tesl, worm LIN-54 and plant TSO1), or one and a half CXC domains (vertebrate tesmin and plant SOL2). These domains were separated by a conserved, 42 amino acid spacer in animals (50 amino acids in plants). The first and second CXC domains contain several residues in common; however, they are distinguished by characteristic amino acids conserved within repeat 1 or 2, but not between repeats (Fig. 2C). The one and a half CXC-domain proteins lack the N-terminus of the first domain, whereas *tomb* has only the second CXC domain. *E(z)* CXC-like domains fall into a separate family.

We also identified a 52 amino acid additional region of homology between the animal proteins near the C-terminus (31% identity, 50% similarity between *mip120* and human tesmin (*hs-tes*); 33% identity and 42% similarity between *tomb* and *hs-tes*). Although the primary sequence conservation is low these sequences are strongly predicted to form a helix-coil-helix secondary structure (PSIpred) (McGuffin et al., 2000) (Fig. 2D). The Aly-interaction domain of Tomb includes this conserved motif but not the CXC domain.

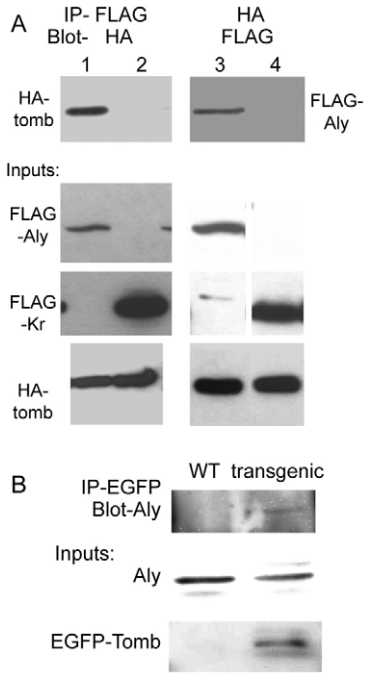

Fig. 1. Aly and Tomb proteins interact. (**A**) 293T cells expressed HA-tagged Tomb and FLAG-tagged Aly (lanes 1 and 3) or Kr(Zn-fingers) (control, lanes 2 and 4). Binding was assessed by immunoprecipitating with anti-FLAG and blotting with anti-HA (lanes 1 and 2, top panel), or vice versa (lanes 3 and 4, top panel). Tomb and Aly co-immunoprecipitated; control assays showed no co-immunoprecipitation. Protein expression was assessed by western blotting of cell lysate (lower three panels). (**B**) EGFP-Tomb was immunoprecipited from Bam-GAL4-VP16, UAS-EGFP-Tomb transgenic testes and blotted with anti-Aly. Wild type was used as the negative control; the lower two panels show expression controls.

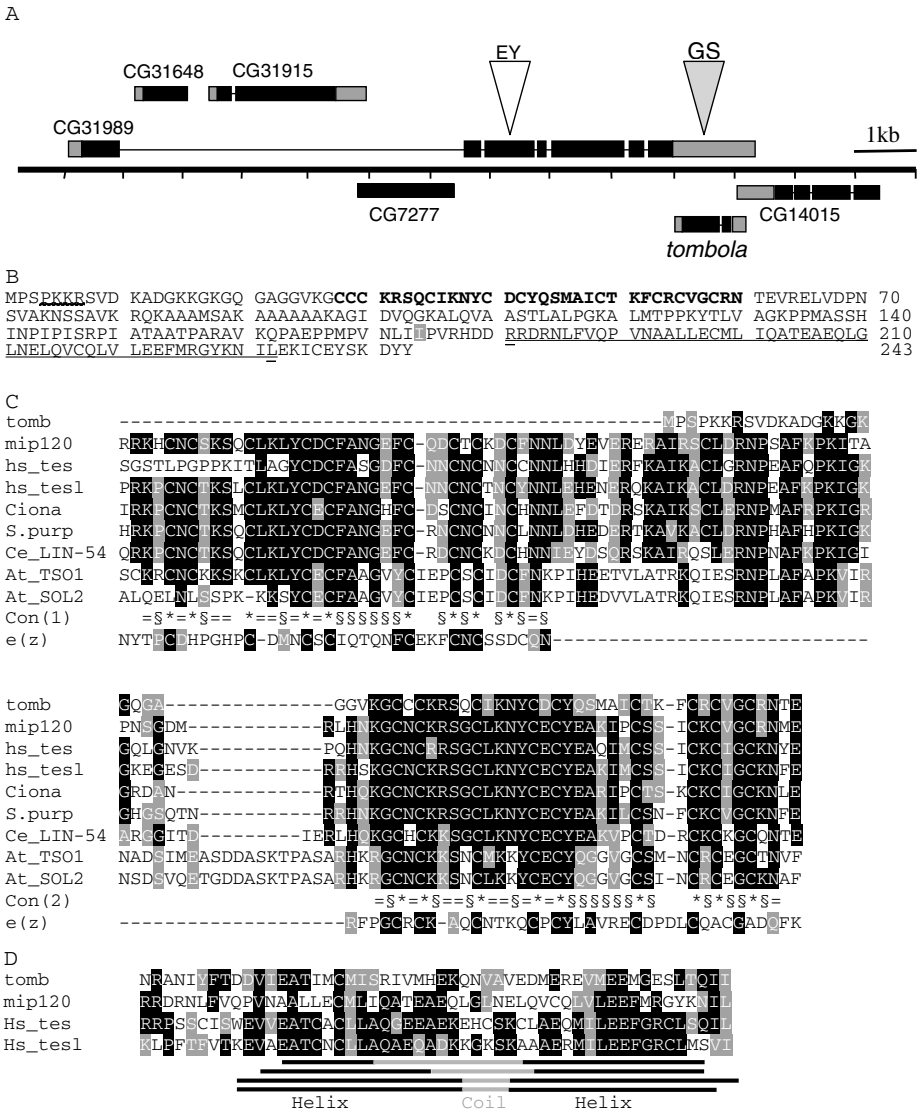

Fig. 2. The *tombola* region and analysis of predicted protein sequence. (**A**) *tombola* genomic region adapted from FlyBase. The *tomb* 5′ UTR is 130-191 bp long; the 3′ UTR is 71 bp long. Black boxes, coding sequence; grey boxes, UTRs; inverted triangles, insertion sites of *P[EY]00456* (EY) and *P[GS]12862* (GS). (**B**) Predicted sequence of Tomb protein. Thick underline, predicted nuclear localisation signal; bold, CXC region; light grey box, *P[GS]12862* insertion site; thin underline, C-terminal conserved region. (**C**) Alignments of CXC domains and spacer from *tomb*; *Drosophila mip120*; human (hs) tesmin (tes) and tesmin-like (tesl); *Ciona intestinalis* (*Ciona*); *Strongylocentrotus purpuratus* (*S. purp*); *C. elegans LIN-54*; *Arabidopsis* (At) *TSO1* and *SOL2*. Con(1) and Con(2) indicate amino acids in the first and second CXC domains: *, conserved Cys; =, residues conserved in both CXC domains; §, residues conserved within CXC(1) or CXC(2), but which differ between the domains. The *E(z)* Cys-rich region is shown as an outgroup. (**D**) Alignment and predicted secondary structure (beneath) of the animal tesmin-family protein C-termini. Predictions of secondary structure are shown in the same order as the sequences (i.e. the first line is the Tomb secondary structure prediction). Black lines indicate high confidence helix predictions; the intervening region (grey line) has either no strong structural prediction (Tomb), or a strong coil prediction (Mip120, Hs-tes, Hs-tesl).

tomb expression is testis-specific

We investigated the *tomb* developmental expression profile by RT-PCR. *tomb* is entirely included within the *CG31989* 3′ UTR; however, they are encoded on opposite strands. *tomb* contains a 62 bp intron, whereas the *CG31989* 3′ UTR lacks introns, allowing us to distinguish the transcripts. *tomb* transcript was detected only in testis (Fig. 3A). Unspliced products, derived from *CG31989* transcripts, were not produced from the testis sample, but were found after re-amplification in gonadectomised adults (both males and females) and embryos (0-16 hours) (data not shown).

We determined the testes *tomb* expression pattern by RNA in situ hybridisation, and found that *tomb* is highly expressed in early primary spermatocytes (arrow in Fig. 3C), with transcript

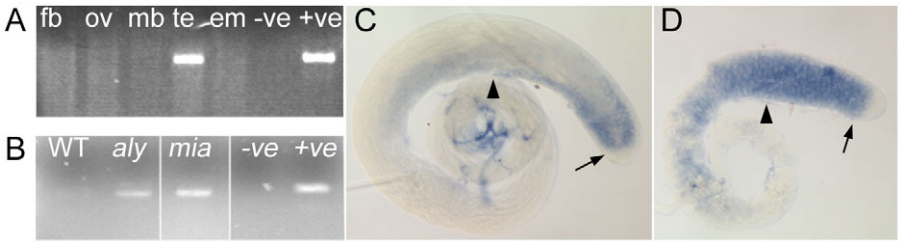

Fig. 3. *tomb* expression is primary spermatocyte-specific. (**A**) RT-PCR of *tomb* ORF from female bodies lacking ovaries (fb), ovaries (ov), male bodies lacking testes (mb), testes (te) and 0-16 hour embryos (em). Negative control (–ve) was without reverse transcriptase. Positive control (+ve) was gDNA. (**B**) Semi-quantitative RT-PCR on testis RNA showed just detectable levels of transcript in wild type (WT), and slightly elevated levels in the meiotic-arrest mutants *aly* and *mia*. Controls as in A. (**C**,**D**) RNA in situ hybridisation. (C) In wild type, *tomb* expression was exclusively detected in primary spermatocytes. Early primary spermatocytes showed robust staining (arrow); mRNA levels gradually declined as spermatocytes matured (arrowhead). (D) *tomb* mRNA expression levels in *aly* mutant early primary spermatocytes was similar to wild type (arrow); however, levels did not decline as spermatocytes matured (arrowhead).

abundance declining as primary spermatocytes mature (arrowhead in Fig. 3C). The *tomb* expression pattern in testes was essentially identical to the other *aly*-class meiotic-arrest loci. We found, using RT-PCR, that *tomb* was expressed in other meiotic-arrest mutants (*aly*, *comr*, *achi/vis*, *topi*, *mia*, *sa*, *nht*), indicating that *tomb* expression does not depend on the activity of any known meiotic-arrest gene (*aly* and *mia* shown, Fig. 3B). Some elevation of the *tomb* transcript level was seen in mutant testes by RT-PCR. This apparent increase in transcript abundance was not due to uniformly increased expression; rather, the transcript appeared specifically more abundant in mutant than wild-type mature primary spermatocytes (Fig. 3D).

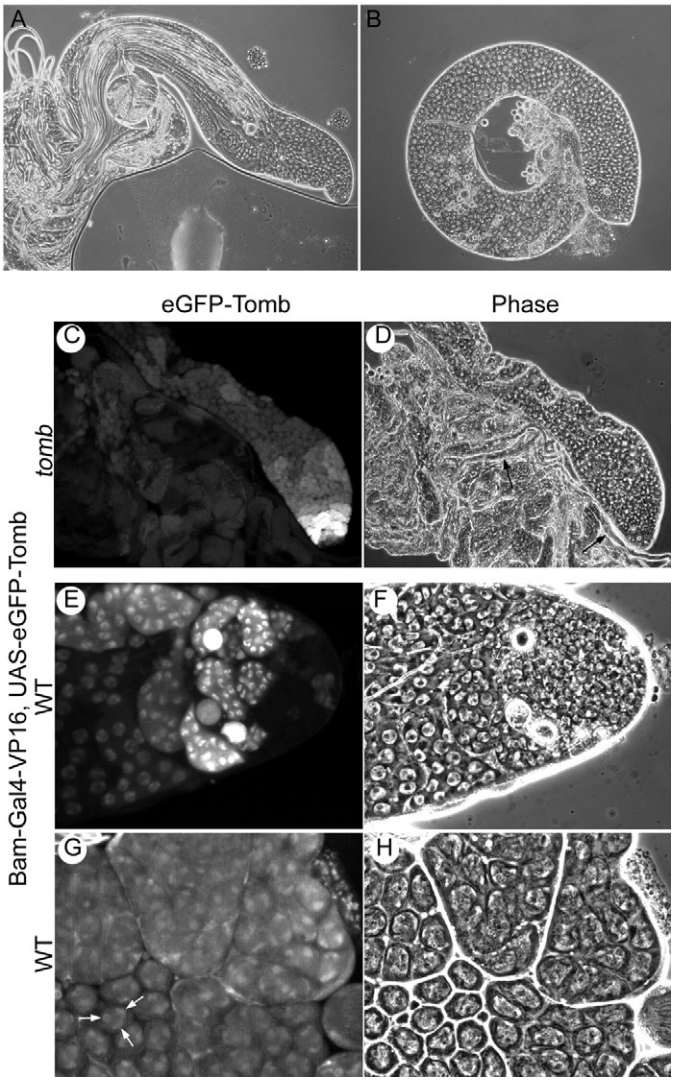

Fig. 4. EGFP-Tomb rescues the *tomb* meiotic-arrest mutant, and localises to chromatin in wild-type primary spermatocytes. (**A**,**B**) Phase contrast of wild-type (A) and $tomb^{GS12862}$ (B) testes. Primary spermatocytes occupy most of the apical end. Elongating spermatid bundles are seen inside, and spilling out from, the wild-type testis, whereas $tomb^{GS12862}$ testes contain only stages up to mature primary spermatocytes. (**C**,**D**) EGFP-Tomb expression rescues the *tomb* meiotic-arrest defect; extensive spermatid elongation is apparent (D, arrows). (**E-H**) EGFP and phase contrast of Bam-Gal4-VP16, UAS-EGFP-Tomb testes. The driver promotes strong expression in early primary spermatocytes (E,F); expression declines as spematocytes mature (G,H). In primary spermatocytes, EGFP-Tomb was predominantly chromatin associated: each nucleus had three prominent labelled regions corresponding to the major chromosome bivalents (G, arrows).

tomb is a meiotic-arrest gene

To further characterise *tomb* function, we searched P-element mutagenesis databases (BDGP, Baylor, Cambridge and Kyoto) and found a potential *tomb* mutant allele in the Kyoto P-collection (Toba et al., 1999). Inverse PCR and sequencing of the flanking DNA of *P[GS]12862* confirmed that the element was inserted in codon 174 of *tomb*. The *P[GS]12862* line was homozygous viable, but male sterile. Homozygous females were initially semi-sterile; however, this phenotype was later lost from the stock.

The mutant phenotype of *P[GS]12862* could be due to disruption of the function of *tomb* or *CG31989* or both, or could be unrelated to the P-insertion. We tested the contribution of *CG31989* to the phenotype using *P[EY]00456*, a P-element insertion in the *CG31989* ORF. *P[EY]00456* mutant flies were homozygous viable and male and female fertile, as were *P[GS]12862/P[EY]00456* trans-heterozygotes, indicating that the phenotype of *P[GS]12862* was not due to *CG31989* loss-of-function. The male fertility defect of *P[GS]12862* was uncovered by both Df(2L)cl-h3 and Df(2L)cl-h1, which delete 25D2-3;26B2-5 and 25D4;25F1-2, respectively (*tomb* is at 25E5). *P[GS]12862*/Df females were fully fertile, confirming that the male and female fertility defects of *P[GS]12862* were separable. Transposase-mediated excision of *P[GS]12862* resulted in full reversion of the mutant phenotype, indicating that the male sterility is caused by the insertion into *tomb*.

Phase contrast examination of squash preparations of $tomb^{GS12862}$ homozygous or $tomb^{GS12862}$/Df testes revealed that *tomb* is a meiotic-arrest gene. *tomb* testes contained morphologically normal stages of spermatogenesis, up to and including mature primary spermatocytes, but no meiotic division or post-meiotic stages (Fig. 4A,B).

Tomb protein is concentrated on chromatin in primary spermatocytes

We expressed an EGFP-Tomb fusion protein in primary spermatocytes using a Bam-GAL4-VP16 driver, and found that tagged Tomb protein was able to rescue the meiotic-arrest phenotype of $tomb^{GS12862}$ homozygous males. This confirmed that the expressed protein is functional, and provided final confirmation that the meiotic-arrest phenotype is due to loss of *tomb* function (Fig. 4C,D). When expressed in a wild-type background, EGFP-tagged Tomb protein was initially both nuclear and cytoplasmic (at lower levels) in early primary spermatocytes. In more mature primary spermatocytes, EGFP-Tomb was restricted to the nucleus and concentrated on chromatin (Fig. 4E-H): three brightly labelled major chromosome bivalents apposed to the nuclear membrane were visible in every nucleus.

tomb is *aly*-class

aly-class meiotic-arrest mutant primary spermatocytes fail to express *Cyclin B* mRNA, whereas *can*-class mutants express normal levels of *Cyclin B* mRNA (White-Cooper et al., 1998). *tomb* mutant testes did not accumulate significant levels of *Cyclin B* mRNA (Fig. 5I-L), so *tomb* is *aly*-class. RNA in situ hybridisation confirmed that *tomb*, like all known meiotic-arrest genes, is also required for expression of spermatid differentiation genes, including *Mst87F* (Fig. 5E-H). *tomb* mutant testes again resembled other meiotic-arrest loci in that transcription was not completely blocked; for example, they accumulated *polo* transcripts normally (Fig. 5A-D).

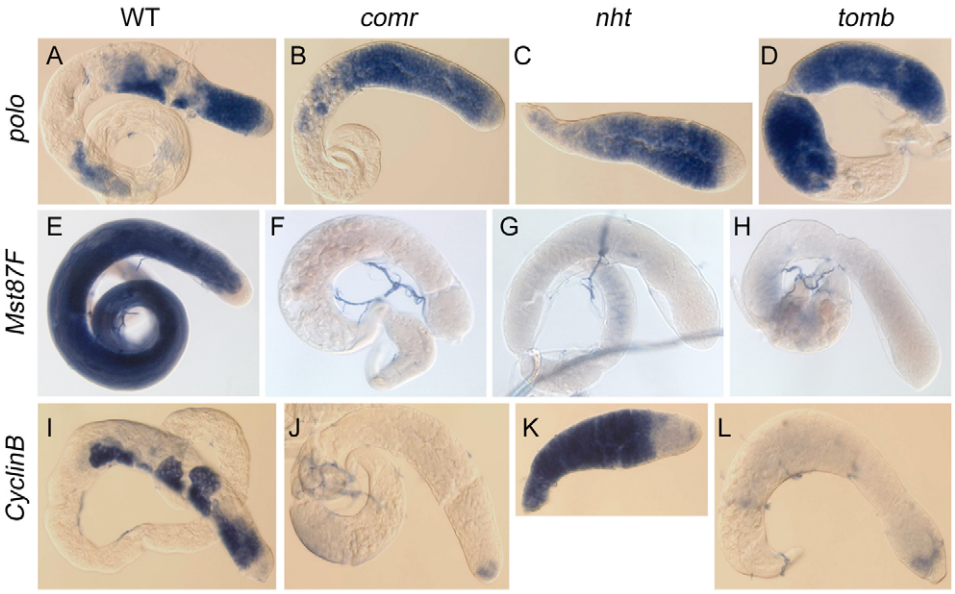

Fig. 5. *tomb* is an *aly*-class meiotic-arrest gene. Diagnostic RNA in situ hybridisations using probes for *polo* (**A-D**), *Mst87F* (**E-H**) and *Cyclin B* (**I-L**). $tomb^{GS12862}$ testes (D,H,L) were more like the *aly*-class mutant *comr* (B,F,J) than the *can*-class mutant *nht* (C,G,K). The testes shown in C and K broke near the seminal vesicles during processing. (A,E,I) Wild-type control.

aly-class mutants fall into two subgroups based on primary spermatocyte DNA morphology (Ayyar et al., 2003; Jiang and White-Cooper, 2003; Lin et al., 1996; Perezgazga et al., 2004). Hoechst 33342 labelling revealed that the *tomb* DNA chromosomes were somewhat condensed and fuzzy, like *aly* or *comr* mutants, rather than more condensed and away from the nuclear envelope as seen in *achi/vis* or *topi* mutants (Fig. 6A-D′). *achi/vis* and *topi* also differ slightly from *aly* and *comr* in their target gene specificities (Perezgazga et al., 2004). Although all genes that depend on *aly* or *comr* for expression also depend on *achi/vis* and/or *topi*, there are a few genes, including *CG3927* and *CG12907*, whose transcription depends on *achi/vis* and *topi* but not on *aly* or *comr*. Several other genes, including *CG3330*, depend on all the *aly*-class meiotic-arrest genes to some extent for their expression, but differ between *aly* or *comr* and *achi/vis* or *topi* in that their expression is undetectable in testes from the latter two mutants, but is detected at very low levels in *aly* or *comr* testes. The *tomb* phenotype was indistinguishable from that of *aly* or *comr* with respect to expression of *CG3927*, *CG12907* and *CG3330* (Fig. 6E-P). Phenotypic comparison data are summarised in Table 1.

Aly, Comr and Topi proteins mislocalise in *tomb* mutant testes

The *tomb* phenotype is also more like *aly* and *comr* than like *achi/vis* with respect to Topi localisation. Immunofluorescence revealed that Topi, like the other *aly*-class meiotic-arrest proteins, localises to primary spermatocyte nuclei, and concentrates on chromatin (Fig. 7A-C). *topi* mutant testes showed no staining, confirming the antibody specificity (data not shown). Topi protein was nuclear, but less concentrated on chromatin in *aly* and *comr* mutant spermatocytes (Fig. 7D-F, *comr* data not shown), indicating that *aly* and *comr* functions are not required for Topi's nuclear localisation or DNA binding per se, but are required for efficient accumulation of Topi on chromatin. In *achi/vis* mutants, Topi localisation was similar to wild type, being nuclear and more concentrated on

Table 1. Summary of phenotypic characteristics of *tomb*, *aly*, *comr*, *topi* and *achi/vis*

	Wild type	*tomb*	*aly* and *comr*	*achi/vis* and *topi*
Chromosome morphology in primary spermatocytes				
	Partially condensed, adjacent to nuclear membrane	Fuzzy, adjacent to nuclear membrane	Fuzzy, adjacent to nuclear membrane	Partially condensed, NOT adjacent to nuclear membrane
Expression of:				
polo	High	High	High	High
Cyclin B	High	OFF	OFF	OFF
Mst87F	High	OFF	OFF	OFF
CG3330	High	Low	Low	OFF
CG12907	High	High	High	OFF
CG3927	High	High	High	Low
Localisation of:				
Aly (or Comr)	Nuclear, on chromatin	Nuclear, NOT on chromatin	Cytoplasmic	Nuclear, on chromatin
Topi	Nuclear, on chromatin	Nuclear, NOT chromatin enriched	Nuclear, NOT chromatin enriched	Nuclear, on chromatin
Tomb	Nuclear, on chromatin		Nuclear, on chromatin initially; unstable	Nuclear, on chromatin

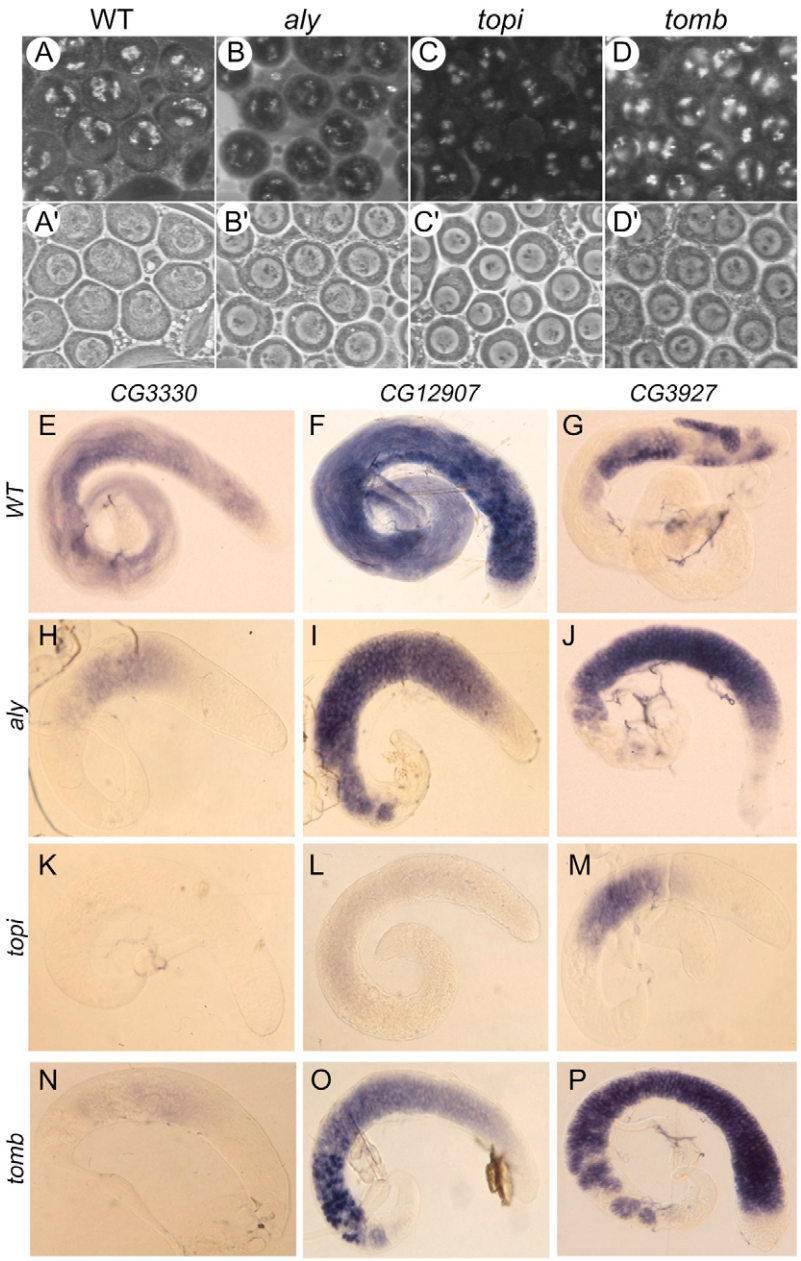

Fig. 6. *tomb* is more like *aly* than *topi*. (**A-D**) Hoechst 33342 labelling of primary spermatocyte DNA in live squashes and (**A′-D′**) corresponding phase contrast images. In wild type (A,A′), the three major bivalents are decondensed, adjacent to the nuclear envelope. Chromosomes in *aly* (B,B′) mutant primary spermatocytes are apposed to the nuclear envelope, but fuzzier and less well defined than in the wild type. Chromosomes in *topi* (C,C′) mutant primary spermatocytes are partially condensed, and not close to the nuclear envelope. (D,D′) *tomb* mutant primary spermatocyte chromosomes resemble those in *aly* mutants rather than those in wild type or *topi* mutants. (**E-P**) RNA in situ hybridisations. *CG3330* (E,H,K,N), *CG12907* (F,I,L,O) and *CG3927* (G,J,M,P) in wild type (E-G), *aly* (H-J), *topi* (K-M) and *tomb*^{GS12862} (N-P). In wild type, *CG3330* message (E) persisted from primary spermatocytes until mid-elongation spermatids. *CG3330* transcript was undetectable in *topi* testes (K), whereas *aly* and *tomb* testes had low levels of transcript (H,N). *CG12907* was expressed in wild-type primary spermatocytes (F) and persisted to late elongation. This transcript was not detected in *topi* mutant testes (L); levels in *aly* and *tomb* spermatocytes (I,N) were similar to wild type. *CG3927* in wild type was detected only in primary spermatocytes (G). *aly* and *tomb* testes showed robust expression of this gene (J,P), whereas *topi* testes showed low levels of *CG3927* transcript (M).

chromatin. Thus, Topi nuclear localisation and chromatin accumulation are independent of *achi/vis* function (Fig. 7G-I). Topi protein localisation in *tomb* and *aly* mutant spermatocytes were indistinguishable (Fig. 7J-L). Therefore *tomb*, like *aly* and *comr*, is required for accumulation of Topi protein on chromatin.

Aly and Comr proteins both localise to chromatin in wild-type primary spermatocytes; however, if either gene is mutant, the other protein remains cytoplasmic (Jiang and White-Cooper, 2003). By contrast, mutation of *achi/vis* or *topi* does not prevent nuclear translocation of Aly and Comr, although these proteins fail to concentrate on chromatin and show a uniform nuclear localisation in *achi/vis* or *topi* mutant spermatocytes (Ayyar et al., 2003; Perezgazga et al., 2004). Immunofluorescence revealed that in *tomb* mutant spermatocytes, Aly and Comr proteins were localised to the nucleus, but were excluded from chromatin (Fig. 7M-R). Therefore, *tomb* function is not required for nuclear import of Aly and Comr, but is required to load these proteins onto chromatin.

Tomb protein requires Aly and Comr for stability

When expressed in *achi/vis* (Fig. 8A,C), or *nht* (a *can*-class meiotic-arrest gene, data not shown) mutant testes, EGFP-tagged Tomb protein also localised to primary spermatocyte nuclei. The protein was concentrated on chromatin, but was also found throughout the nucleoplasm. Therefore, the functions of *achi/vis* and the *can*-class genes are not required to establish or maintain the correct subcellular localisation of Tomb, although they might be required to enhance the association of Tomb with chromatin.

By contrast, EGFP-Tomb protein localisation was altered when expressed in *comr* (Fig. 8B,D) or *aly* (data not shown) mutant testes. The fusion protein was able to localise to nuclei and chromatin of early primary spermatocytes. However, as spermatocytes matured, the nuclear staining was lost, so that in late primary spermatocytes only very weak, cytoplasmic EGFP fluorescence could be detected. We conclude that *aly* and *comr* functions are not required for the localisation of Tomb to the

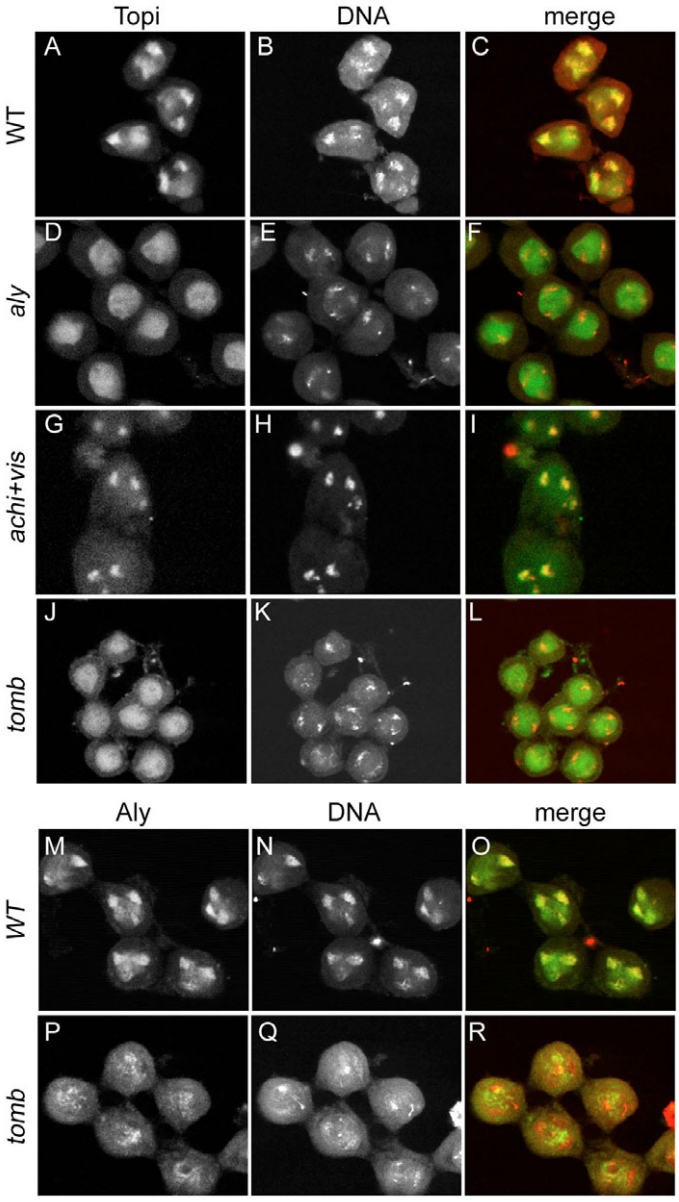

Fig. 7. Aly and Topi mislocalise in *tomb* testes. (**A-L**) Anti-Topi immunostaining (A,D,G,J, green) and DNA staining (B,E,H,K, red) in mature primary spermatocytes. In wild-type primary spermatocytes (A-C), Topi protein was predominantly chromatin associated. In *achi/vis* cells (G-I), Topi staining was distributed throughout the nucleus, but was brighter on chromatin, whereas the nuclear Topi staining in *aly* (D-F) and *tomb* (J-L) cells was less concentrated on chromatin. (**M-R**) Anti-Aly immunostaining (M,P, green) and DNA staining (N,Q, red) in mature primary spermatocytes. In wild type (M-O), Aly protein was nuclear and concentrated on chromatin. Aly protein was nuclear, but excluded from chromatin in *tomb* cells (P-R).

nucleus or chromatin per se, but are required to maintain the nuclear concentration of Tomb by preventing either nuclear export or Tomb degradation.

DISCUSSION

The meiotic-arrest genes of *Drosophila* regulate a developmental transition and associated gene expression switch, during which many hundreds of genes whose products are required during sperm

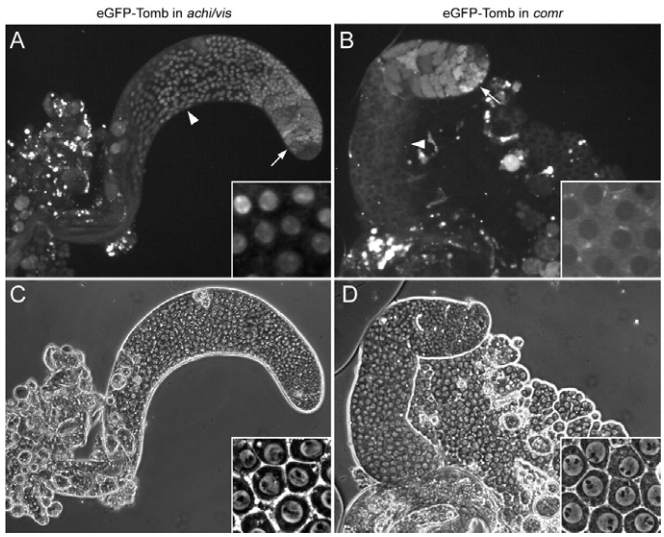

Fig. 8. Tomb nuclear localisation in late primary spermatocytes depends on *comr*, but not *achi/vis*. EGFP (**A**,**B**) and phase contrast (**C**,**D**) of *achi/vis; Bam-Gal4-VP16, UAS-EGFP-Tomb* (A,C) or *comr; Bam-Gal4-VP16, UAS-EGFP-Tomb* (B,D) whole testes and (insets) mature primary spermatocytes. Initially, the EGFP-Tomb localisations in *achi/vis* and *comr* are indistinguishable (A,B, arrows). Nuclear EGFP-Tomb was retained in *achi/vis*, but lost from *comr* mature spermatocytes (A,B, arrowheads). Arrested *achi/vis* spermatocytes had nuclear EGFP-Tomb (A, inset), whereas arrested *comr* spermatocytes had low levels of exclusively cytoplasmic fusion protein (B, inset).

formation are upregulated (Andrews et al., 2000; Parisi et al., 2004). Most meiotic-arrest genes described to date have been identified through classical genetics. To find additional gene products that act with those already isolated, we undertook a reverse genetics approach; we identified *tomb* while screening for proteins that could bind Aly in a yeast two-hybrid system.

Pathway of assembly and localisation of an *aly*-class meiotic-arrest complex

The *tomb* predicted protein contains a tesmin/TSO1-family CXC domain that probably mediates DNA binding. Other tesmin/TSO1-family members have either two full CXC domains, or one truncated domain and one full domain, separated by a conserved spacer. Tomb is exceptional in having a single CXC domain and no spacer sequence. In addition to the CXC domain, we identified a second region of homology shared between *tomb* and the other animal tesmin/TSO1 CXC-domain-containing proteins. This C-terminal domain has conserved secondary structure, and might be responsible for the Tomb-Aly interaction.

Direct interactions have been demonstrated between Comr and Topi, whereas Aly, Comr and Achi/Vis have been found in a complex in vivo (Perezgazga et al., 2004; Wang and Mann, 2003). Here, we additionally show that Aly and Comr can interact with Tomb. In support of our interaction data, Beall et al. (Beall et al., 2007) have purified a complex of proteins containing Aly, Topi, Comr, Tomb and other factors from *Drosophila* testes extracts and these components were not detected in ovary-specific extracts. The known *aly*-class meiotic-arrest gene products localise primarily on chromatin in wild-type primary spermatocytes, although Aly and Tomb are also detected at significant levels in early primary spermatocyte cytoplasm. Only when all five *aly*-class gene

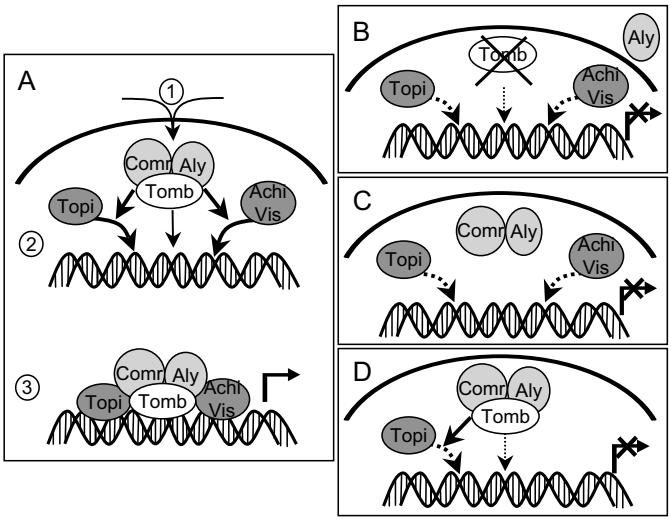

Fig. 9. A model for assembly of the Aly-class meiotic-arrest proteins at target promoters. (**A**) Normal assembly of an *aly*-class gene product complex is regulated at several steps. (1) Aly-Comr interaction facilitates their nuclear translocation (or possibly prevents nuclear export). Tomb, Topi and Achi/Vis proteins localise constitutively to the nucleus, and can bind with low affinity to target promoters. (2) Nuclear Aly and Comr bind to and stabilise Tomb, then interact with Topi and Achi/Vis to facilitate cooperative DNA binding. (3) Transcriptional activation requires tight association of all five components with DNA. (**B**) In *comr* (or *aly*) spermatocytes, Aly (or Comr) remains cytoplasmic, Tomb protein is destablised and Topi and Achi/Vis only weakly interact with DNA; transcription is not activated. (**C**) In *tomb* mutants, Aly and Comr are stable in the nucleus, but cannot promote Topi and Achi/Vis association with DNA; transcription is not activated. (**D**) In *achi/vis* (or *topi*) mutants, the complex is not efficiently associated with DNA; transcription is not activated.

products are present is full chromatin-binding activity achieved. There are subtle differences in *aly* and *comr* phenotypes as compared with *achi/vis* and *topi*. Most notably, *achi/vis* and *topi* have broader ranges of target genes than *aly* and *comr* (Perezgazga et al., 2004). We have previously shown that the nuclear localisations of Aly and Comr are mutually dependent, i.e. Aly remains cytoplasmic in *comr* mutants and vice versa (Jiang and White-Cooper, 2003). We have also shown that *topi* and *achi/vis* act later in the localisation pathway, both gene products being required for the efficient loading of Aly and Comr onto chromatin (Ayyar et al., 2003; Perezgazga et al., 2004). We can now place *tomb* into the pathway of complex assembly and activity (Fig. 9). We propose that Tomb, Achi/Vis and Topi enter the nucleus independently, whereas Aly and Comr can only become (or remain) nuclear as a complex. Topi and Achi/Vis probably have inherent sequence-specific DNA-binding activity, which allows them to localise independently, albeit inefficiently, to their targets. Like Mip120, Tomb might also have DNA-binding activity. When in the nucleus, Aly and Comr interact with Tomb; this complex then promotes Topi and Achi/Vis interactions with target promoters. Tomb protein is destabilised in the absence of Aly and Comr; hence, the phenotypes of *tomb*, *aly* and *comr* mutants are identical with respect to target gene expression levels. DRM, a complex containing the proteins encoded by the *C. elegans aly* and *tomb* homologues (*lin-9* and *lin-54*), has recently been described (Harrison et al., 2006). Formation of the DRM complex was sensitive to loss of *lin-9* or *lin-54*, just as *aly* and *tomb* are crucial for formation of the *aly*-class gene product complex in testis. Mammalian tesmin is cytoplasmic in early pachytene cells, and normally translocates to the nucleus during late pachytene and diplotene stages of male meiosis, in a similar manner to fly *aly* and *tomb* (Matsuura et al., 2002; Sutou et al., 2003).

Relationship between *aly*-class meiotic-arrest genes and other transcriptional regulators in primary spermatocytes

modulo (*mod*), which encodes *Drosophila* nucleolin, has recently been implicated in transcriptional activation of spermiogenesis genes (Mikhaylova et al., 2006). *mod*-null mutants are lethal, but a viable weak allele is male sterile. Mod was shown to bind sequence elements in certain testis-specific promoters. Many, but not all, *mod* target genes are also meiotic-arrest gene targets. An alternative form of Mod, expressed only in testis, has an acidic N-terminal domain that probably allows Mod to act as a transcriptional activator. The *can*-class meiotic-arrest genes, which encode testis-specific homologues of the basal transcription factor complex $TF_{II}D$ (testis TAFs), might activate transcription by sequestering the polycomb repressor complex away from active chromatin, i.e. they might activate genes by repressing a repressor (Chen et al., 2005; Hiller et al., 2004; Hiller et al., 2001). In normal primary spermatocytes, Pc and testis TAFs are primarily nucleolar, although the proteins are also detected uniformly on chromatin. ChIP analysis revealed that Sa protein binds promoters of target genes in primary spermatocytes, suggesting a direct transcriptional activator role for testis TAFs (Chen et al., 2005).

The *aly*-class meiotic-arrest mutant phenotype is most easily explained in terms of transcriptional activation rather than through the repression of a repressor. The *aly*-class gene products accumulate on chromatin in primary spermatocytes in transcriptionally active regions, and not in the nucleolus. Their function depends on the chromatin localisation. In addition, lack of testis TAF gene activity results in low (but readily detectable) levels of target gene expression, whereas expression of many target genes in *aly*-class mutant testes is undetectable.

A testis-specific dREAM/Myb-MuvB complex?

tomb and *mip120* (*CG6061*) are the only *Drosophila* tesmin/TSO1 CXC-motif proteins. Likewise, *aly* and *mip130* (*twit*, *CG3480*, *EG86E4.4*) are the only *Drosophila* homologues of *lin-9* (White-Cooper et al., 1998). Mip120 and Mip130 have been described as components of the dREAM/Myb-MuvB complex found in embryos and tissue culture cells (Beall et al., 2002; Korenjak et al., 2004; Lewis et al., 2004). The dREAM complex contains, in addition to Mip120 and Mip130, Myb, Caf1p55, Dp, Mip40, E2F2 and Rbf or Rbf2 (Korenjak et al., 2004). The MybMuvB complex was purified independently and contains all the subunits of the dREAM complex as well as several additional proteins including Rpd3, Lin-52 and l(3)MBT (Lewis et al., 2004). dREAM/Myb-MuvB regulates DNA replication at chorion gene amplification origins in ovarian follicle cells (Beall et al., 2004; Beall et al., 2002; Cayirlioglu et al., 2001; Frolov et al., 2001). In addition to this role in controlling developmentally regulated DNA replication, the dREAM/Myb-MuvB complex acts as a transcriptional repressor, primarily of genes involved in differentiation (Korenjak et al., 2004; Lewis et al., 2004). This transcriptional repressor role is also developmentally regulated as there are different transcriptional targets for Rbf2 and E2F2 in ovaries, early embryos and S2 tissue culture cells (Stevaux et al., 2005).

DRM, a complex containing the *C. elegans* homologues of the dREAM subunits has recently been described (Harrison et al., 2006). The genes encoding DRM components act together in the SynMuvB genetic pathway that regulates vulval development redundantly with the SynMuvA and SynMuvC pathways [see the following studies (Ceol and Horvitz, 2004; Ceol et al., 2006; Poulin et al., 2005) and references therein; see Lipsick (Lipsick, 2004) for commentary]. All the dREAM/Myb-MuvB genes are also conserved in mammals, and recently LIN9, the human homologue of *aly/Mip130*, has been shown to have tumour suppressor activity and to work in concert with Rb to promote differentiation (Gagrica et al., 2004). LIN9, LIN54 (human Mip120) and hMip40 are all also capable of binding directly to Rb (Korenjak et al., 2004).

Drosophila E2f2- and *Rbf2*-null mutants are viable and male fertile, but E2F2 females have reduced fertility (Cayirlioglu et al., 2001; Frolov et al., 2001; Stevaux et al., 2005), whereas *Myb*-, *Dp*- and *Rbf*-null mutants are lethal (Duronio et al., 1995; Manak et al., 2002; Royzman et al., 1997) and males mutant for weak *Dp* alleles are sterile (Duronio et al., 1998) but do not show a meiotic-arrest phenotype. Thus, the mutant phenotypes of the DNA-binding subunits *dE2F2*, *Rbf*, *Rbf2*, *Dp* and *Myb* are not consistent with them functioning in testes with *aly* and *tomb* to activate gene expression. Indeed, *Rbf2* function in ovaries is implicated in repression of some testis-specific genes (Stevaux et al., 2005).

There is remarkable evolutionary conservation of the interaction between dREAM/Myb-MuvB gene products in somatic tissues in mammals, flies and worms. We suggest that gene duplications in *Drosophila* of *lin-54* (*tomb/mip120*), *lin-9* (*aly/mip130*) and *lin-52* (*CG12442/lin52*) (J.J., K.D. and H.W.-C., unpublished), has led to the evolution of a complex paralogous to the dREAM/MybMuvB complex, but using different DNA-binding subunits, dedicated to testis-specific transcriptional regulation.

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