Development 130, 2853-2865

doi:10.1242/dev.00510

Requirement for two nearly identical TGIF-related homeobox genes in *Drosophila* spermatogenesis

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Accepted 21 March 2003

SUMMARY

The genetic analysis of spermatogenesis in *Drosophila melanogaster* has led to the identification of several genes that control the onset of meiosis, spermatid differentiation, or both. We described two tightly linked and nearly identical homeobox genes of the TGIF (TG-interacting factor) subclass called *vismay* and *achintya* that are essential for spermatogenesis in *Drosophila*. In flies deficient for both genes, spermatogenesis is blocked prior to any spermatid differentiation and before the first meiotic division. This suggests that *vismay* and *achintya* function at the same step as two previously characterized meiotic arrest genes, *always early* and *cookie monster*. Consistent with this idea, both *always early* and *cookie monster* are still expressed in flies deficient in *vismay* and *achintya*. Conversely, Vismay and Achintya proteins are present in *always early* mutant testes. Co-immunoprecipitation experiments further suggest that Vismay and Achintya proteins exist in a complex with Always early and Cookie monster proteins. Because Vismay and Achintya are likely to be sequence-specific DNA binding factors, these results suggest that they help to specify the spermatogenesis program by recruiting or stabilizing Always early and Cookie monster to specific target genes that need to be transcriptionally regulated during testes development.

Key words: Spermatogenesis, Meiosis, Homeobox genes, TGIF, TALE genes, *Drosophila melanogaster*

INTRODUCTION

The homeodomain is a DNA-binding motif present in a large number of eukaryotic transcription factors. To date, over 1,000 homeodomain proteins from over 100 different organisms have been described, making this family of DNA binding proteins one of the largest in biology (Banerjee-Basu et al., 1999; Banerjee-Basu et al., 2000; Gehring et al., 1994). Many homeodomain-containing factors, such as members of the Hox family, play critical roles in animal development (Cavodeassi et al., 2001; Hobert and Westphal, 2000; Lawrence and Morata, 1994; McGinnis and Krumlauf, 1992; Panganiban and Rubenstein, 2002; Trainor and Krumlauf, 2001). Perhaps in part because of their central role in animal development, many human diseases and genetic disorders are linked to mutations in homeodomain proteins (Abate-Shen, 2002; Buske and Humphries, 2000; Goodman and Scambler, 2001; Wallis and Muenke, 2000). Moreover, changes in the expression patterns, target genes and functions of homeodomain proteins are also thought to be a driving force in animal evolution (Mann and Carroll, 2002; Vervoort, 2002).

Homeodomain proteins are classified into different groups depending upon the sequence of the homeodomain and immediately flanking amino acids, and the presence of other protein domains elsewhere in the protein (Gehring et al., 1994). One group of homeodomain proteins, called the TALE (for three amino acid loop extension) group, has an additional three amino acids separating the first and second alpha helices, resulting in a 63, instead of the more typical 60, amino acid homeodomain (Burglin, 1997). The TALE group is noteworthy for several reasons. Two TALE subgroups, MEIS and PBC, function as cofactors for the Hox homeodomain proteins (Mann and Affolter, 1998). At least for the regulation of some Hox target genes, a Hox/PBC/MEIS trimer is critical (Ferretti et al., 2000; Jacobs et al., 1999; Ryoo et al., 1999). In the PBC subgroup, the TALE-specific amino acids are Leu Ser Asn, which form part of a hydrophobic pocket that is directly contacted by the peptide Tyr-Pro-Trp-Met present in many Hox proteins (Passner et al., 1999; Piper et al., 1999). In addition to their role as Hox cofactors, the MEIS and PBC genes *homothorax* (*hth*) and *extradenticle* (*exd*) carry out several additional functions during *Drosophila* development. These genes are important for eye and antennal development and help to form the proximodistal axis in the thoracic appendages (Abu-Shaar and Mann, 1998; Bessa et al., 2002; Casares and Mann, 1998; Gonzalez-Crespo and Morata, 1996; Pai et al., 1998; Wu and Cohen, 1999). The Iroquois genes, which constitute a third TALE subgroup, are also important for patterning the appendages, as well as in neural and organ development (Cavodeassi et al., 2001).

The TG interacting factors (TGIF) make up another interesting TALE subgroup. The founding member of this subgroup, TGIF, can be recruited to DNA directly, by binding to its target site, or indirectly, by virtue of its interaction with

Smad proteins, which are key transcription factors that are activated by TGFβ signaling (Bertolino et al., 1995; Sharma and Sun, 2001; Wotton et al., 2001; Wotton et al., 1999a; Wotton et al., 1999b; Wotton and Massague, 2001). In humans, mutations in TGIF cause holoprosencephaly (HPE), a severe genetic disorder affecting craniofacial development (Gripp et al., 2000; Wallis and Muenke, 2000). HPE is also caused by mutations in the Nodal pathway, which is related to TGFβ, thus linking TGIF with Nodal signaling in vivo (Gripp et al., 2000; Wallis and Muenke, 2000). TGIF appears to be a transcriptional repressor protein with at least two repressor domains that recruit either histone deacetylases (HDACs) or C-terminal binding protein (CtBP), a general corepressor (Sharma and Sun, 2001; Wotton et al., 2001; Wotton et al., 1999a; Wotton et al., 1999b; Wotton and Massague, 2001).

The genome sequence of the fruit fly, *Drosophila melanogaster*, predicts the existence of eight TALE genes: *exd* (a PBC gene), *hth* (a MEIS gene), three linked Iroquois genes (*mirror*, *araucan* and *caupolican*), two tightly linked TGIF-like genes, *vismay* (*vis*) and *achintya* (*achi*), and one predicted gene, CG11617 (Adams et al., 2000; Misra et al., 2002). Of these, only CG11617 and the two TGIF-like genes have not yet been characterized. To analyze a potential role for the TGIF-like genes in *Drosophila* development, we generated a deficiency that uncovers both *vis* and *achi*. Surprisingly, flies homozygous for this deletion are viable, suggesting that these genes play no essential role in embryonic or larval development. However, homozygous males are sterile. Further analysis shows that in the absence of *vis* and *achi*, spermatogenesis is blocked prior to the meiotic divisions with no signs of differentiation, placing these genes in the category known as male meiotic arrest genes. Genomic rescue and epistasis experiments suggest that Vis and Achi are redundant transcription factors that function at the same step in spermatogenesis as two other meiotic arrest genes, *always early* (*aly*) and *cookie monster* (*comr*) (Jiang and White-Cooper, 2003; White-Cooper et al., 2000). Moreover, Aly and Comr co-immunoprecipitate with Vis and/or Achi, suggesting that these proteins may function together as a complex to control normal male meiosis and spermatid differentiation.

MATERIALS AND METHODS

Fly stocks

A stock carrying EP(2)2107, a P element inserted close to the 5′ end of *achintya* (Fig. 1C), was obtained from Exelexis/BDGP. Stocks carrying aly^{1}, sa^{1} or $CycB^{2}$ were ordered from Bloomington stock center. aly^{2} and can^{12} mutant stocks were kindly provided by Helen White-Cooper. All stocks were raised at 25°C, at which temperature these alleles behave as null mutations (Jiang and White-Cooper, 2003; White-Cooper et al., 2000; White-Cooper et al., 1998).

Generation and identification of a deficiency that removes *vis* and *achi*

Although the EP(2)2107 insertion is homozygous viable, some sterility is observed in this stock. Among six males tested, two did not give any progeny after being crossed to *yw* females for 10 days. The four fertile males yielded an average of 56 progeny.

To generate a deletion of *vis* and *achi*, we employed a P-element-mediated male recombination strategy (Preston et al., 1996). We screened for recombination between a proximal marker (*hook* or *aristaless*) and EP(2)2107, which is marked by the mini *white* gene. Out of six recombinant lines, one deleted *vis* and *achi*. Because of its resulting male sterile phenotype, we named this deficiency Df(2R)*pingpong* (Fig. 1C), and refer to it here simply as *pingpong*. The other recombinants did not disrupt *vis* or *achi* as determined by PCR mapping, and they are homozygous viable and fertile. Other potential lethals on the Df(2R)*pingpong* chromosome were removed by meiotic recombination.

In the *pingpong* deficiency the original EP insertion is still present. We therefore mapped the deletion molecularly by P-element-mediated plasmid rescue. The sequence at the deletion junction is as follows: GTAAGTGTATGTGTGTGTGGAAGCCCTGAT**TCATGATGAAA-TAACATAAGGTGGTCCCGT** (genomic sequences are in normal type and P-element sequences are in bold type).

Approximately 40 kb was deleted (Fig. 1C). The absence of *vis*, *achi*, *CG8824*, *CG30044* and *CG12370* was verified by Southern analysis and PCR mapping (data not shown).

Rescue constructs

Genomic DNA fragments containing either *vis* or *achi* were generated by PCR using a BAC clone (24H9, Research Genetics/BDGP) as the template. The primers used to generate the genomic rescue fragments of *vis* or *achi* are:

5′-vis cccggctatcagatacccgg
3′-vis tcactgcgtaagcctctgcc
5′-achi gcagcatccatgcttccact
3′-achi gttggaggtgcgatcgtcac

P{*vis*} contains a 4.7 kb fragment extending from 362 bp upstream of the first exon to 1563 bp downstream of the last exon of *vis*. P{*achi*} contains a 4.8 kb fragment ranging from 900 bp upstream of the first exon to 700 bp downstream of the last exon of *achi*. These PCR products were cloned into a shuttle vector and sequenced for verification. Finally, *vis* or *achi* rescue fragments were cloned into the pP{W8} vector (FlyBase) and transgenic flies were generated by standard methods. Although P{*achi*} and, to some extent, P{*vis*} rescue the infertility of Df(2R)*pingpong* males, Df(2R)*pingpong*; P{*achi*} and Df(2R)*pingpong*; P{*vis*} flies are not as healthy as wild type.

tub-AchiS2 and *tub*-AchiL were constructed by cloning the *Eco*RI/*Xba*I fragments of LD25085 and LP02076 (from BDGP EST collection), respectively, into an *α1-tubulin* (*tub*; *αTub84B* – FlyBase) cassette described previously (Chen and Struhl, 1996; Zecca et al., 1995).

Antibodies and immunofluorescence

A polyclonal antibody was raised against 6xHis-AchiS (full length) in guinea pigs (Cocalico Biologicals, Inc., Reamstown, PA). No staining above background was observed in Df(2R)*pingpong* embryos, imaginal discs or germline tissues. In contrast, testes from either Df(2R)*pingpong*; P{*vis*} or Df(2R)*pingpong*; P{*achi*} males showed a normal expression pattern, demonstrating that this antibody recognizes both Vis and Achi proteins (data not shown). We therefore refer to the immunoreactivity as Vis/Achi when both genes are present. Rabbit anti-Aly and rabbit anti-Comr antibodies were generously provided by Helen White-Cooper. Rabbit antibodies against Cyclin A or Cyclin B were gifts from Christian Lehner and David Glover. Mouse monoclonal antibody against Polo was a gift from Claudio Sunkel. All secondary antibodies were purchased from Jackson ImmunoReseach Laboratories.

Embryos and ovaries were fixed and immunostained according to published protocols (Patel, 1994; Verheyen and Cooley, 1994). Third instar larval discs were dissected in PBS and fixed in 4% formaldehyde. Adult testes were dissected in PBS, frozen in liquid nitrogen before being fixed by either the methanol/acetone method or in 4% paraformaldehyde (Bonaccorsi et al., 2000). Using the paraformaldehyde fixation we observed Vis/Achi throughout the testes, including in mitotic cells at the apical tip (shown in Fig. 3); the methanol/acetone fixation revealed a more limited Vis/Achi pattern in

which only the primary spermatocytes were labeled. Anti-Achi antibody was used at 1:1000, Aly antibody at 1:2000, Comr antibody at 1:1000, Cyclin A antibody at 1:600, Cyclin B antibody at 1:2000 and Polo antibody at 1:40 dilutions. All secondary antibodies were diluted to 1:400. To stain DNA with propidium iodide (Molecular Probes), 100 μg/ml of RNAse A was included in the secondary antibody incubation.

In situ hybridization

We carried out RNA in situ hybridization according to the protocol described by White-Cooper et al. with the following modifications (White-Cooper et al., 1998). Prehybridization, hybridization and washes were all done at 60°C, except for *twine* in situs, which were at 55°C. Hybridized Dig-RNA probes were detected by AP-conjugated anti-Dig antibody at a 1:2500 dilution. To ensure equivalent conditions for both wild-type and *pingpong* mutant testes (easily distinguished by their morphology), they were kept in the same incubation well in all the in situ experiments. cDNA clones of *boule* and *mst87F* were kindly provided by Helen White-Cooper. Digoxigenin-labeled RNA probes were synthesized by in vitro transcription according to manufacturer's instruction (Roche).

BrdU labeling

Testes were dissected in Ringer's solution and bathed in 100 mg/ml BrdU (Sigma) in Ringer's. After labeling for 60 minutes, testes were washed 3 times in PBT (PBS + 0.1% Triton X-100) and subsequently fixed in 4% formaldehyde/PBT for 30 minutes. To make labeled DNA more accessible to immunodetection, fixed testes were treated with 50 U/ml DNAseI (Roche) for 60 minute at 25°C (Gonczy and DiNardo, 1996). BrdU incorporation was detected by 1:100 mouse anti-BrdU (Becton Dickinson) and followed by FITC-conjugated secondary antibody.

Western analysis

Whole-testes lysates were prepared by putting dissected testes directly into 2× SDS sample buffer then vortexing vigorously. Adult carcasses were saved and ground in 2 ×SDS sample buffer. Primary antibodies were used at 1:7,500-10,000 for AchiS, at 1:10,000 for Aly, and at 1:5,000 for Comr. Signals were detected by a secondary antibody conjugated to peroxidase followed by the ECL reaction (Amersham). After antibody probing, the blot was stained with GelCode Blue (Pierce) to reveal the loading profile. For these experiments, wild-type extracts were generated from flies in which the original EP insertion was precisely excised, which is genetically most similar to the Df(2R)*pingpong* stock.

Co-immunoprecipitation

20-30 testes of each genotype were ground up in 100 μl RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 0.5% NP40, and 1 mM PMSF) containing a protease inhibitor cocktail (Roche) and 2.5 mM Na_3VO_4 (Sigma). Testes extracts and α-Achi antibody at 1:500 dilution were mixed and incubated at 4°C overnight. 10 μl of proteinA/G-agarose beads (Santa Cruz Biotechnology) was added and incubated for an additional 3 hours. Samples were then washed three times and all bound proteins were eluted with 2× SDS sample buffer.

RESULTS

vis and *achi* encode members of the TGIF subgroup of TALE homeodomain proteins

vis (CG8821) and *achi* (CG8819) are both predicted to encode TALE homeodomain proteins (Fig. 1A). Analysis of genomic and cDNA sequences suggests that both genes have the potential to encode two protein products, which we refer to as long (L) and short (S) isoforms (Fig. 1A,B). The long and short forms of both Vis and Achi, which have been directly observed by immunoblot analysis (see below), differ in length because of the presence or absence of an alternatively spliced exon (Fig. 1B). Blast and ClustalW analyses indicate that Vis and Achi are almost identical. For example, AchiS and VisS are predicted to be identical in 406 of 424 residues (96% identity). In addition, the alternatively spliced exons in Vis and Achi are 129 residues in length and are predicted to be 100% identical to each other. The high degree of identity between these proteins is unusual for homeobox genes.

Blast and ClustalW analyses also reveal that the mammalian proteins most similar to Vis and Achi are human TGIF and TGIF2 (Fig. 1A). The similarity to TGIF and TGIF2 is most obvious in the homeodomain (TGIF is ~78% identical to Achi), but the identity extends to residues C-terminal to the homeodomain. A protein predicted from the genome sequence of the mosquito, *Anopheles gambiae*, shows several additional regions with similarity to Vis and Achi. Other members of the TGIF subgroup have homology to Vis and Achi primarily within the homeodomain (Fig. 1A).

vis and *achi* are required for male fertility

vis and *achi* are located on the right arm of chromosome 2 in the *Drosophila* genome, in cytological position 49A. Starting with a P element (EP(2)2107) inserted close to the 5′ end of *achi*, we used P element-mediated male recombination to generate a ~40 kilobase deletion that removes both *vis* and *achi* (Fig. 1C). Because this deficiency deletes two nearly identical genes and results in male sterility, we named it Df(2R)*pingpong* and refer to it here simply as *pingpong*. In addition to deleting *vis* and *achi*, *pingpong* also removes four additional genes (Fig. 1C). However, homozygous flies carrying this deficiency are viable, suggesting that none of these genes are required for embryonic or larval development. Moreover, *pingpong* homozygous females are fertile and can give rise to viable *pingpong* progeny, eliminating the possibility that any of these genes have an essential maternal function. The only highly penetrant phenotype observed in *pingpong* homozygous flies is male sterility which can be rescued by transgenes carrying genomic regions for either *vis* (P{*vis*}) or *achi* (P{*achi*}) (Fig. 1C and see below). Thus, we conclude that *vis* and *achi* encode redundant functions required for male fertility.

We quantitated the role of *vis* and *achi* in male fertility by comparing the fertility of *pingpong* males with *pingpong*; P{*vis*} and *pingpong*; P{*achi*} males. We crossed individual males to wild-type females and counted the number of progeny after 15 days. In this assay wild-type (*yw*) males yielded an average of 132 progeny/male (n=2). *pingpong* males were completely infertile (0 progeny; n=26). In contrast, *pingpong*; P{*achi*} males were nearly as fertile as wild type (100.5 progeny/male; n=10) and *pingpong*; P{*vis*} males were partially rescued (27.8 progeny/male; n=16). Although both P{*vis*} and P{*achi*} appear to generate a wild-type Vis/Achi expression pattern in the male germline (see below and data not shown) these results suggest that *achi* is better able to rescue the mutant phenotype than *vis*.

Vis and Achi are widely expressed during development

To analyze the *vis* and *achi* expression patterns we generated

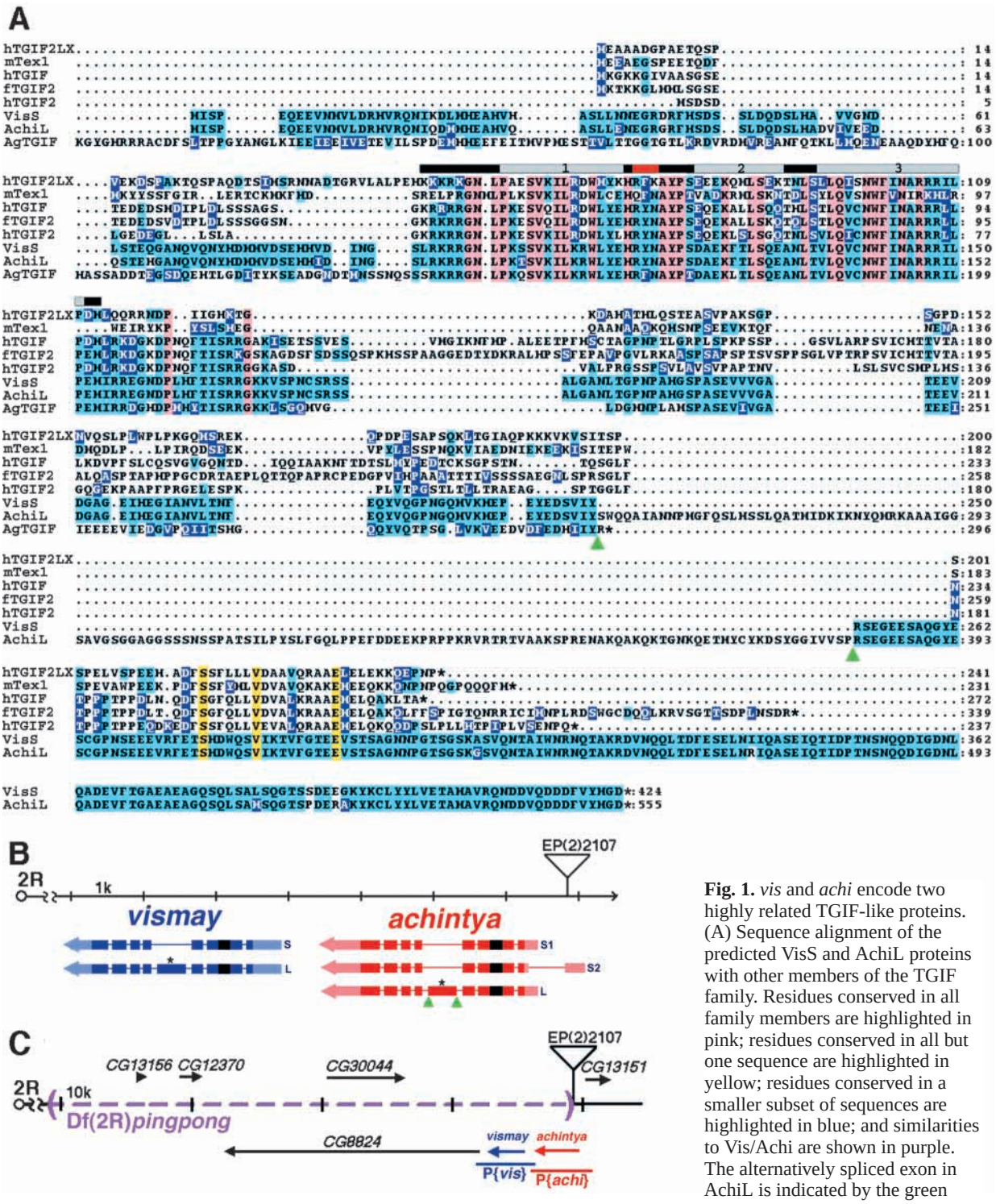

Fig. 1. *vis* and *achi* encode two highly related TGIF-like proteins. (A) Sequence alignment of the predicted VisS and AchiL proteins with other members of the TGIF family. Residues conserved in all family members are highlighted in pink; residues conserved in all but one sequence are highlighted in yellow; residues conserved in a smaller subset of sequences are highlighted in blue; and similarities to Vis/Achi are shown in purple. The alternatively spliced exon in AchiL is indicated by the green arrowheads (also in B). * marks the predicted end of each protein. hTGIF, human TGIF; hTGIF2, human TGIF2; hTGIF2LX, human TGIF2-like on X; mTex1, mouse Tex1; fTGIF, *Fugu* TGIF; AgTGIF, *Anopheles gambiae* TGIF. The extent of the homeodomain and three alpha helices (1, 2, 3) is indicated above the sequence by the black and grey bars; the red box marks the three TALE residues in between helices 1 and 2. The residues 'AYP', also in the loop between helices 1 and 2, are one of the hallmarks of the TGIF family. (B) Genomic organization of the *vis/achi* locus. cDNAs predicted to encode *vis*S (SD01238), *achi*S2 (LD25085) and *achi*L (LP02076) have been sequenced; transcripts for *vis*L and *achi*S1 are predicted from the analysis of the genome sequence. The alternatively spliced exons are indicated with an asterisk. Lighter shading indicates non-coding sequences, and the black boxes indicate the homeoboxes in *vis* and *achi*. (C) Df(2R)*pingpong* is a deletion (indicated by the dashed line) of *vis*, *achi* and four neighboring genes. The extent of the two rescue P elements for *vis* (P{*vis*}) and *achi* (P{*achi*}) are indicated.

a polyclonal antibody against AchiS. Using this antibody, we detected nuclear expression in nearly all cells and stages of development, including cells in the testes (Fig. 2). The specificity of this antibody was confirmed by observing only background staining in the *pingpong* deficiency (Fig. 2B and data not shown). Moreover, a wild-type testes staining pattern is generated by either the P{*vis*} or P{*achi*} transgenes (data not shown and see below) demonstrating that this antibody recognizes both Vis and Achi proteins.

Because of the male sterile phenotype of homozygous *pingpong* flies we examined the Vis/Achi expression pattern in more detail in wild-type testes and compared its pattern to that of Aly, a chromatin-associated protein that is required for male meiosis in *Drosophila* (Fig. 3) (White-Cooper et al., 2000). At the most apical tip of the testes are somatic 'hub' cells, germline stem cells, and mitotically dividing spermatogonia (Gonczy et al., 1992). Further from the tip is a zone of larger primary spermatocytes organized into 16-cell cysts. These cells, which grow in volume ~25-fold over a period of ~3.5 days, remain in meiotic prophase, a specialized stage of the meiotic cell cycle that precedes the meiotic divisions. Like Aly (White-Cooper et al., 2000), Vis/Achi is most strongly observed in the nuclei of primary spermatocytes (Fig. 3A-C). In addition, weaker nuclear Vis/Achi staining is also observed in the mitotically dividing cells at the apical tip of the testes (Fig. 3B). This is not a background staining because it is not observed in the *pingpong* deficiency, which still have these cells (data not shown and Fig. 4B). In addition, we also observed weak Aly staining at this stage (Fig. 3B).

The various stages of meiosis can be identified by the state of the chromosomes, as revealed by a fluorescent DNA dye such as propidium iodide (Fig. 3). At the large primary spermatocyte stage, when both Aly and Vis/Achi are nuclear, the three major chromosome pairs can be seen as three separate but diffuse signals, indicating that the chromosomes are partially condensed (Fig. 3C). At metaphase of meiosis I the chromosomes are fully condensed and appear as nuclear dots (Fig. 3D). At this stage, Aly appears to associate with the spindle and Vis/Achi is predominantly in the cytoplasm. A similar pattern is seen for both proteins at metaphase of meiosis II (Fig. 3E). Following the second meiotic division spermatid differentiation begins with a stage known as the onion stage, in which each cell has a characteristically round and specialized mitochondrion adjacent to its haploid nucleus. At this stage, the anti-Aly antibody labels a single dot per nucleus that co-localizes with the DNA (Fig. 3F). In contrast, Vis/Achi is observed throughout these cells except that it is excluded from the large and specialized mitochondrion next to each nucleus (Fig. 3F). Finally, at an even later stage of spermatid differentiation, Aly is barely detected above background and Vis/Achi partially co-localizes with the DNA (Fig. 3G). Vis/Achi is not detected in the somatic cyst cells that surround the primary spermatocytes (data not shown).

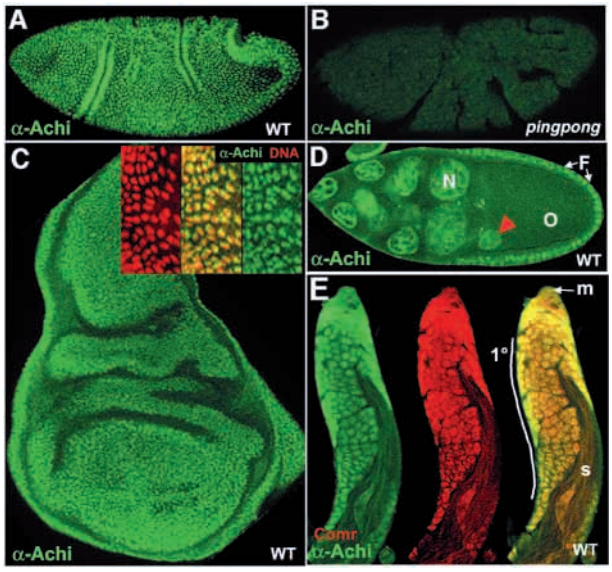

Fig. 2. Vis and Achi are widely expressed proteins. Immunofluorescent images of Vis/Achi expression (green) in an embryo (A), a wing imaginal disc (C), an egg chamber (D), and a testis (E). All images are of wild-type tissues except for B which shows a Df(2R)*pingpong* homozygous mutant embryo. Only background staining is observed, confirming the specificity of the antibody. In C, the inset shows detail of wing disc expression co-stained with propidium iodide (red) to highlight the nuclei. In D, the nurse cell (N), follicle cell (F) and oocyte (O) nuclei are indicated (red arrowhead, oocyte nucleus). In E, the testes are co-stained for Comr (red), which has a very similar expression pattern to Vis/Achi in the testes. Three stages observed in wild-type testes, primary (1°) spermatocytes, elongating spermatids (s) and mitotically dividing germ cells (m) are indicated.

Spermatogenesis in Df(2R)*pingpong* males arrests before the first meiotic division

The male sterile phenotype, together with its strong nuclear expression in primary spermatocytes, suggested that *vis* or *achi* may be required for meiosis. We therefore examined *pingpong* and *pingpong*; P{*achi*} testes by phase contrast microscopy and by staining the chromosomes with propidium iodide. In contrast to wild type, *pingpong* mutant testes are much smaller and are blocked before the first meiotic division (Fig. 4). Specifically, no evidence of any elongated or onion stage spermatids are observed in the *pingpong* mutant (Fig. 4A-D). Instead, the testes are filled with cells that appear to remain at the primary spermatocyte stage. 16-cell cysts are still present, suggesting that the four mitotic divisions proceed normally in this mutant. However, the cells are smaller and not as round as in wild type (Fig. 4C,D). Far from the apical tip of the testes cells appear to degenerate. In addition, some of the chromosomes fail to fully condense in the absence of Vis and Achi (Fig. 4F,H). Typically, we observe three spots of DNA per cell, but the appearance of these spots ranges from diffuse (partially decondensed) to fully condensed (Fig. 4H). This phenotype indicates that the normally synchronized events leading to chromosome condensation fail in the *pingpong* mutant. Furthermore, these results suggest that although the *pingpong* mutant initiates meiosis in the male, meiosis is blocked before the first meiotic division, probably prior to the G_2 to M transition. In addition to being blocked in meiosis, *pingpong* testes do not show any signs of spermatid differentiation, such as the distinctive onion stage cysts or spermatid elongation.

We also used bromodeoxyuridine (BrdU) labeling to determine if the 16-cell cysts in *pinpong* testes undergo DNA

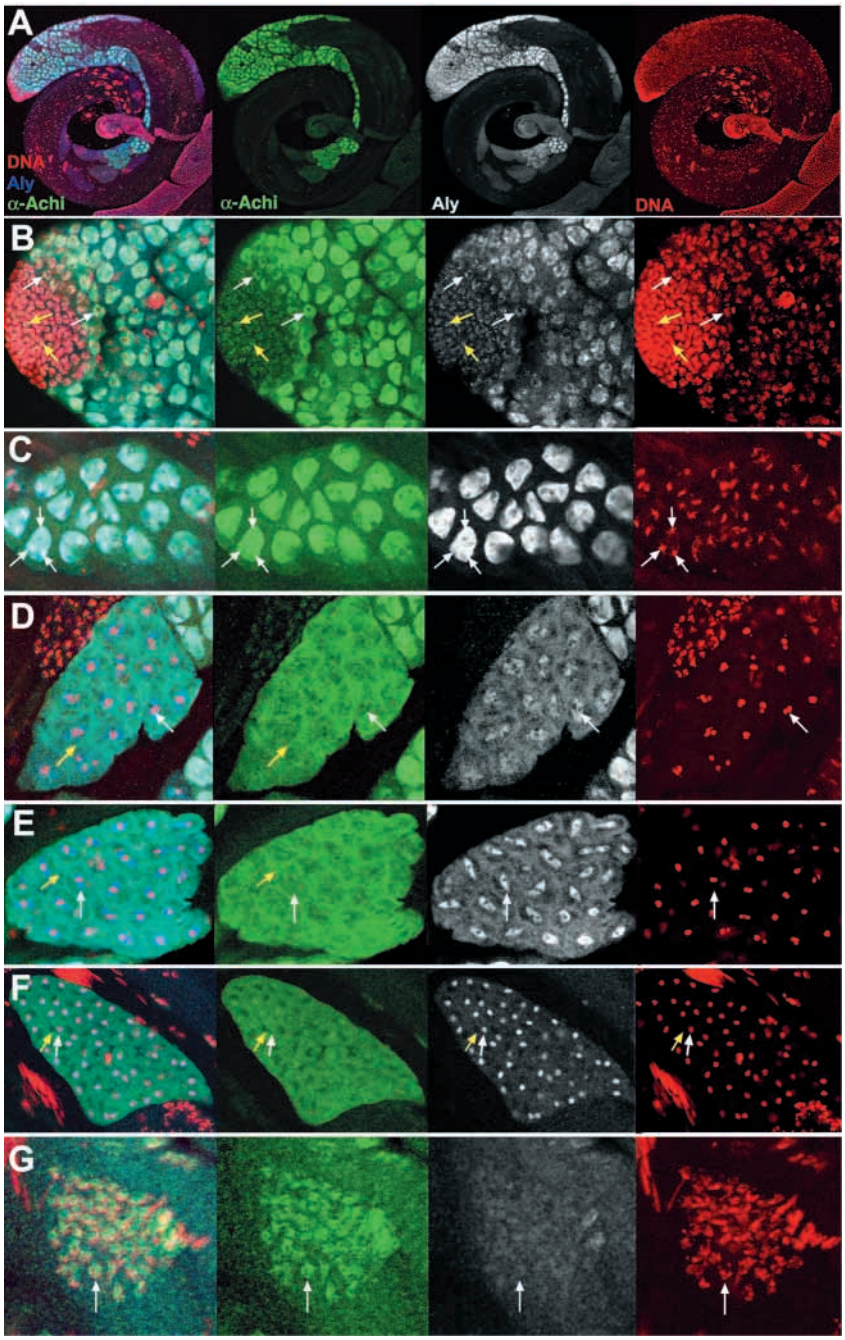

Fig. 3. Vis/Achi expression during male meiosis. All images are of wild-type testes stained for Vis/Achi (green), Aly (blue or white) and DNA (red). (A) Low magnification view of an entire testis. Vis/Achi and Aly are most highly expressed in the primary spermatocytes. (B) High magnification view of the apical tip and immature primary spermatocyte stages. Weak Aly and Vis/Achi staining is observed in the nuclei of the mitotically dividing cells (yellow arrows) and in the nuclei of immature primary spermatocytes (white arrows). (C) A cyst with mature primary spermatocytes. The DNA is present as three diffusely staining regions per nucleus (white arrows), which correspond to the three main chromosomes bivalents in *Drosophila*. Both Aly and Vis/Achi are highly expressed in these nuclei. (D) A cyst in metaphase of meiosis I. The DNA is compacted into 1-3 dots per nucleus. Aly appears associated with the spindle (white arrow) and Vis/Achi appears to be primarily cytoplasmic (yellow arrow). (E) A cyst in metaphase of meiosis II. The DNA, Aly and Vis/Achi signals are similar to that seen at metaphase of meiosis I (D). (F) An 'onion stage' cyst. Meiosis is completed, and spermatid differentiation has begun by the 64 haploid progeny. Nuclei (white arrows) are closely paired to round, specialized mitochondria (yellow arrows). At this stage, the DNA is present in one dot per nucleus, Aly is tightly associated with the DNA (also in one dot/cell) and Vis/Achi is observed throughout the cell, but is excluded from the mitochondria. (G) At a later stage in spermatid differentiation, the DNA is less compact and is associated with Vis/Achi (arrow) whereas only background Aly staining is observed.

synthesis before arresting. As seen (Fig. 4I,J), BrdU-labeled 16-cell cysts were observed in both wild-type and *pingpong* testes. Thus, the block in meiosis occurs after DNA synthesis but before the first meiotic division.

vis and *achi* act in parallel with *aly* and *comr*

The genetic analysis of spermatogenesis in *Drosophila* has identified several genes that are required for the normal progression through meiosis in males (Fuller, 1998). Two of these genes, *aly* and *cookie monster* (*comr*) can be distinguished from the others because they are required for the expression of *twine*, which encodes a cdc25-like phosphatase, and *boule*, an ortholog of the human gene *Deleted in Azoospermia* (Alphey et al., 1992; Eberhart et al., 1996; Jiang and White-Cooper, 2003; White-Cooper et al., 2000; White-Cooper et al., 1998). In contrast, the gene *cannonball* (*can*) is also required for meiosis in males, but is not required for the expression of *twine* or *boule* mRNAs (Hiller et al., 2001; White-Cooper et al., 1998). To gain additional insight into the *pingpong* mutant phenotype we stained mutant testes for several markers known to be expressed in testes, including *twine* and *boule*. Like *aly* and *comr*, but unlike *can* mutant testes, the *pingpong* mutant does not express *twine* or *boule* mRNAs (Fig. 5A-D). Also like *aly* and *comr* mutants, *pingpong* testes do not express *mst87F*, a gene that is required for spermatid differentiation (Kuhn et al., 1988) (Fig. 5E,F).

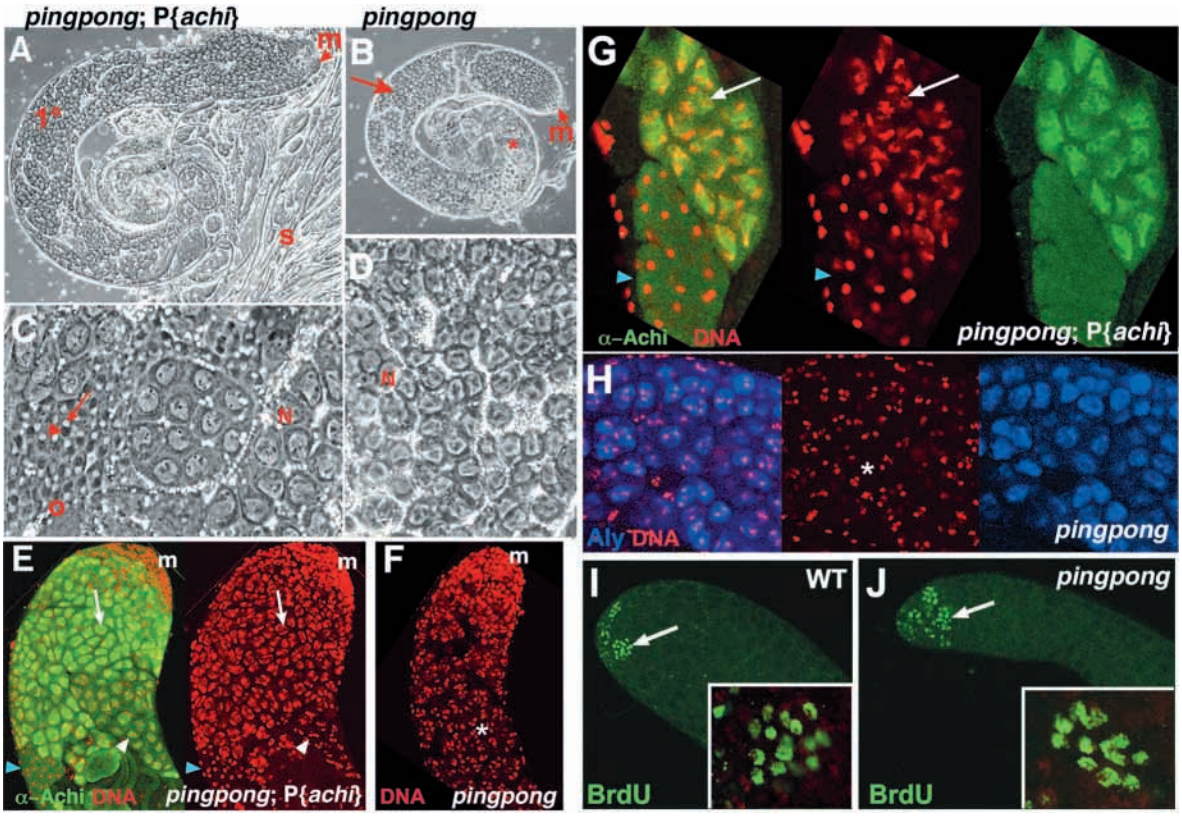

Fig. 4. Df(2R)*pingpong* spermatocytes arrest prior to meiosis I. (A,C) Low and high magnification phase contrast images, respectively, of a testis from *pingpong*; P{*achi*} males, which is indistinguishable from wild type. Several stages can be observed, including the mitotically dividing spermatogonia (m), large primary spermatocytes (1°), mature spermatids (s), and the onion stage (o), in which small white nuclei (arrow) and round, dark specialized mitochondria (arrowhead) can be seen. N, a primary spermatocyte nucleus. (B,D) Low and high magnification phase contrast images, respectively, of a testis from a *pingpong* mutant male. These images are at the same magnification as their wild-type counterparts (A,C). Note that the entire testis is filled with primary spermatocytes but that these cells are not as round or large as in the wild type (arrow in B). The testes shown in A-D were gently squashed with a cover slip to generate thin specimens. The '*' in B indicates the basal region in which cells are degenerating. (E,G) *pingpong*; P{*achi*} testes stained for Vis/Achi (green) and DNA (red). In the wild type, several stages can be observed, including cysts in which the DNA is in three diffuse regions per nucleus (mature primary spermatocytes, white arrows), cysts in which the DNA is partially condensed (white arrowhead), and cysts in which the DNA is fully condensed (blue arrowhead). The levels of nuclear Vis/Achi are highest in the mature primary spermatocyte stage and gradually decrease as the cells approach the G_2/M transition. (F,H) *pingpong* mutant testes stained for DNA (red) and Aly (blue, H only). In the mutant, some chromosomes appear to be partially condensed and others appear fully condensed (*). Aly is still observed in nuclei in the mutant. The images in E and F, and G and H, are at the same magnification and are directly comparable. (I,J) *pingpong*/CyO (I) and *pingpong* (J) testes labeled for BrdU incorporation (green) during DNA synthesis. BrdU-labeled 16-cell cysts were observed in both the wild type and mutant. The insets show an example of a labeled cyst at higher magnification. Not all 16 cells are visible in a single focal plane.

These results suggest that *vis* and *achi* function at a similar step as *aly* and *comr*.

To determine if *vis* and *achi* act upstream of or in parallel with *aly* and *comr* we stained *pingpong* mutant testes with antibodies for the *aly* and *comr* gene products, which are also highly expressed in primary spermatocytes (Jiang and White-Cooper, 2003; White-Cooper et al., 2000). Strikingly, both Aly and Comr were observed in the nuclei of most cells in *pingpong* mutant testes (Fig. 4H and Fig. 5G-J). Thus, *vis* and *achi* are not required for the expression or nuclear localization of these genes. Conversely, Vis/Achi proteins are still observed in *aly* mutant testes (as is Comr) (Fig. 5K,L). Consistent with these results, Vis and Achi are also expressed and nuclear in testes mutant for *spermatocytes arrest* (*sa*) and *can*, genes that appear to act downstream of *aly* and *comr* (Fig. 5M-P). Thus, neither *sa*, *can*, or *aly* are required for the expression or nuclear localization of Vis or Achi. Taken together, these data suggest that Vis and Achi function in spermatogenesis in parallel with Aly and Comr.

The expression of cell cycle regulators in *pingpong* mutant

The lack of *twine* expression in *pingpong* mutant testes is consistent with it causing a meiotic arrest phenotype (Courtot et al., 1992). In addition to *twine*, meiosis is also controlled by the availability of Cyclins A and B, which are required to activate Cyclin dependent kinase 1 (Cdk1) (Knoblich and Lehner, 1993; Lehner and O'Farrell, 1990; Sigrist et al., 1995). Both Cyclins A and B are normally expressed during male spermatogenesis, at high levels in the mitotically dividing cells at the apical tip of the testes, at lower levels during the primary spermatocyte stage, and at higher levels in 16-cell cysts just

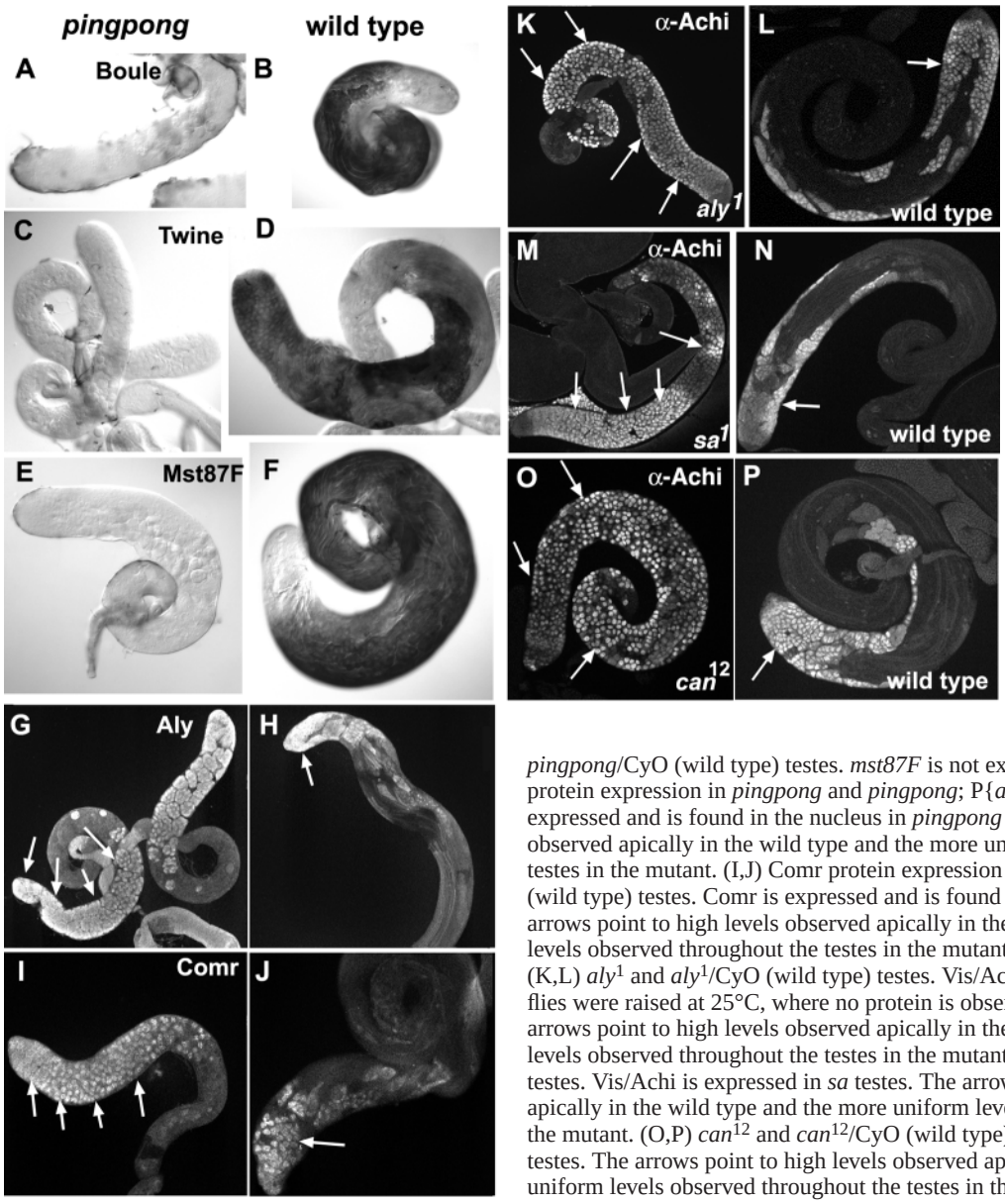

Fig. 5. Relationship between *vis*, *achi* and other meiotic arrest genes. (A,B) *boule* in situ hybridization in *pingpong* and *pingpong*/CyO (wild type) testes. *boule* is not expressed in *pingpong* testes. (C,D) *twine* in situ hybridization in *pingpong* and *pingpong*; P{*achi*} (wild type) testes. *twine* is not expressed in *pingpong* testes. (E,F) *mst87F* in situ hybridization in *pingpong* and *pingpong*/CyO (wild type) testes. *mst87F* is not expressed in *pingpong* testes. (G,H) Aly protein expression in *pingpong* and *pingpong*; P{*achi*} (wild type) testes. Aly is expressed and is found in the nucleus in *pingpong* testes. The arrows point to high levels observed apically in the wild type and the more uniform levels observed throughout the testes in the mutant. (I,J) Comr protein expression in *pingpong* and *pingpong*; P{*achi*} (wild type) testes. Comr is expressed and is found in the nucleus in *pingpong* testes. The arrows point to high levels observed apically in the wild type and the more uniform levels observed throughout the testes in the mutant. (K-P) Vis/Achi protein expression. (K,L) *aly*^{1} and *aly*^{1}/CyO (wild type) testes. Vis/Achi is expressed in *aly* testes. The *aly*^{1} flies were raised at 25°C, where no protein is observed (White-Cooper et al., 2000). The arrows point to high levels observed apically in the wild type and the more uniform levels observed throughout the testes in the mutant. (M,N) *sa*^{1} and *sa*^{1}/CyO (wild type) testes. Vis/Achi is expressed in *sa* testes. The arrows point to high levels observed apically in the wild type and the more uniform levels observed throughout the testes in the mutant. (O,P) *can*^{12} and *can*^{12}/CyO (wild type) testes. Vis/Achi is expressed in *can* testes. The arrows point to high levels observed apically in the wild type and the more uniform levels observed throughout the testes in the mutant.

prior to the G_2 to M transition (Fig. 6A,C). The cyclins are rapidly degraded at the end of metaphase (Lin et al., 1996; White-Cooper et al., 1998). We therefore examined cyclin levels in *pingpong* mutant testes (Fig. 6A-D). Although there is some variation between individual testes, in general we observed intermediate levels of both Cyclin A and Cyclin B throughout *pingpong* testes (Fig. 6A-D). Cyclin A and B levels persisted, although at lower levels, up to the point when the cells appear to degenerate. In addition, although there is a transient nuclear localization of both Cyclins prior to cell division in the wild type, both Cyclins were predominantly observed in the cytoplasm in the *pingpong* mutant. Therefore, unlike in the wild type, Cyclin levels are not modulated in the *pingpong* mutant, consistent with a block prior to the G_2/M transition. In addition, the levels of Polo, a protein kinase required for cytokinesis during meiosis (Herrmann et al., 1998), indicate that in *pingpong* testes the block occurs prior to the first meiotic division (Fig. 6E,F).

Either AchiL or AchiS is sufficient to partially rescue Df(2R)*pingpong*

The characterization of *vis* and *achi* cDNAs suggested the existence of two isoforms that differ because of the presence or absence of an alternatively spliced exon (Fig. 1A,B). To analyze the protein products derived from *vis* and *achi* we used the anti-Achi antibody in immunoblot experiments. Two bands with apparent molecular masses of ~60 kDa and ~80 kDa were detected in whole lysates of wild-type testes (Fig. 7A). In contrast, only a ~60 kDa band was detected in wild-type ovaries. Neither band was detected in the testes or ovaries from the *pingpong* mutant, suggesting that both are derived from *vis* and *achi* (Fig. 7A). Furthermore, based on the sizes of proteins observed in *pingpong*; P{*vis*} or *pingpong*; P{*achi*} flies we deduce that VisL and AchiL both migrate at ~80 kDa and both VisS and AchiS migrate at ~60 kDa (Fig. 7B). This assignment was confirmed by expressing the AchiL or AchiS cDNAs under the control of the *α1-tubulin* promoter in Df(2R)*pingpong* flies

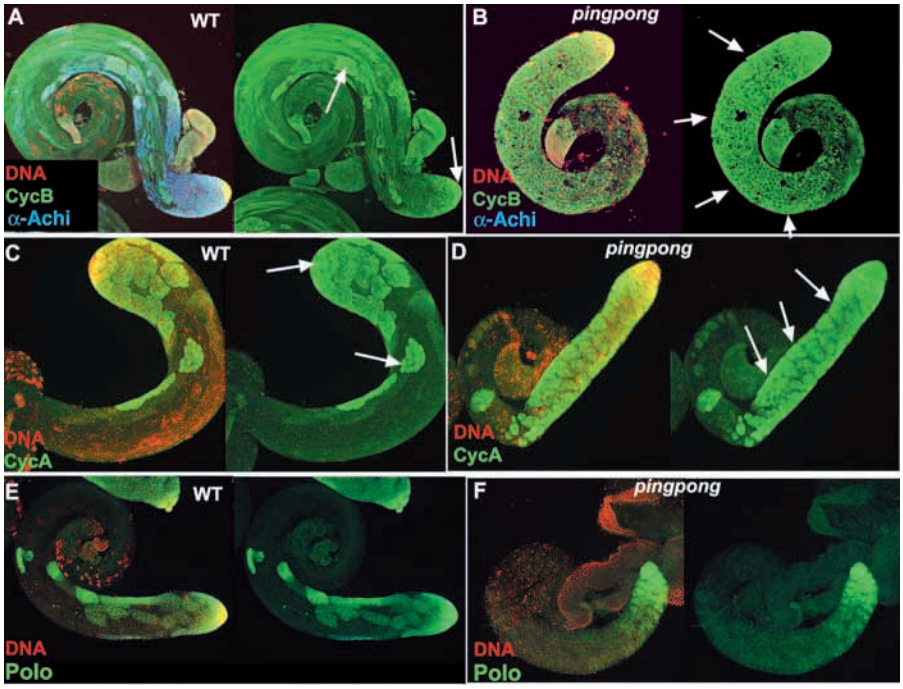

Fig. 6. Expression of cell cycle regulators in *pingpong* testes. (A,B) Wild-type (A) and *pingpong* (B) testes stained for DNA (red), CycB (green) and Vis/Achi (blue). In the *pingpong* mutant CycB is expressed throughout the testes at intermediate levels and is cytoplasmic. Loss of staining is only observed far from the apical tip of the testes where cells are degenerating. The arrows point to the high levels of CycB staining in wild type and more uniform staining in the mutants. (C,D) Wild-type (C) or *pingpong* (D) testes stained for DNA (red) and CycA (green). In the *pingpong* mutant CycA is expressed throughout the testes and is primarily cytoplasmic. Loss of staining is only observed at the basal end of the testes where the cells are degenerating. The arrows point to the high levels of CycA staining in wild type and more uniform staining in the mutants. (E,F) Wild-type (E) or *pingpong* (F) testes stained for DNA (red) and Polo (green). Staining is high at the apical tip in both wild type and the mutant. In the mutant, the levels gradually decrease in cells farther from the apical tip whereas in the wild type, Polo levels are high in older (metaphase) 16-cell cysts.

(Fig. 7B and data not shown). Furthermore, in wild-type flies the slower migrating forms (AchiL and VisL) are testes specific because only the ~60 kDa species was observed in male or female somatic tissues (Fig. 7A). Although additional bands were observed in wild-type extracts, they were still present in extracts derived from Df(2R)*pingpong* flies, suggesting that they are not encoded by *vis* or *achi* (data not shown).

We also tested if either AchiL or AchiS2 is sufficient to rescue the *pingpong* male infertility phenotype by ubiquitously expressing these isoforms under the control of the *tub* promoter. Flies containing either *tub*-AchiL or *tub*-AchiS in an otherwise wild-type background appear normal. In addition, although the long isoforms are testes specific, both Df(2R)*pingpong*; *tub*-AchiL and Df(2R)*pingpong*; *tub*-AchiS2 males had normal appearing testes, as demonstrated by both phase contrast microscopy and immunostaining with several markers (Fig. 7C,D and data not shown). However, in both cases these males were only weakly fertile (Df(2R)*pingpong*; *tub*-AchiL males gave rise to an average of 10 progeny/male; n=24). One explanation for this result is that the *tub* promoter fails to provide accurate levels or timing of Achi expression to fully rescue *pingpong* sterility. However, males in which both long and short forms of Achi are co-expressed exhibit better fertility (Df(2R)*pingpong*; *tub*-AchiL; *tub*-AchiS2), suggesting that expression of both isoforms is required for complete rescue (data not shown).

Vis/Achi, Aly and Comr exist in a complex in wild-type testes

Many of the phenotypes we observe in the *pingpong* deficiency are also observed in *aly* and *comr* mutants (Jiang and White-Cooper, 2003; White-Cooper et al., 2000; White-Cooper et al., 1998). In addition, immunolocalization studies suggest that Vis, Achi, Aly and Comr proteins are co-expressed in the nuclei of primary spermatocytes. These observations prompted us to test if these proteins may be present as a complex in wild-type testes. We tested this by carrying out immunoprecipitation (IP) experiments with the anti-Achi antibody and determining if either Aly or Comr is co-immunoprecipitated. Interestingly, both Aly and Comr can be co-immunoprecipitated with Vis/Achi from wild type, but not from *pingpong* testes (Fig. 8). These results suggest that Vis and Achi proteins are present in a complex with Aly and Comr during wild-type testes development.

DISCUSSION

These results suggest that the *vis* and *achi* genes encode at least four highly related and partially redundant TGIF-like homeodomain proteins that are required for spermatogenesis in *Drosophila*. The block in spermatogenesis occurs during the primary spermatocyte stage, which is a growth phase prior to either meiotic division. No evidence of spermatid differentiation is observed in a deficiency that removes both genes, suggesting that the block in development affects both meiosis and differentiation. Below, we discuss three main points relevant to these results, specifically, potential mechanisms of Vis/Achi action, the similarities and differences between Vis, Achi and mammalian TGIFs, and a potential role for TGIF-like proteins in mammalian spermatogenesis.

vis and *achi* encode homeodomain proteins required for male meiosis in *Drosophila*

Previous work has identified a handful of meiotic arrest genes that are required for the completion of meiosis in males (Fuller, 1998; Hiller et al., 2001; Jiang and White-Cooper, 2003; Lin et al., 1996; White-Cooper et al., 2000; White-Cooper et al., 1998). Some of these genes, including *aly* and *comr*, have been proposed to work at an early step in

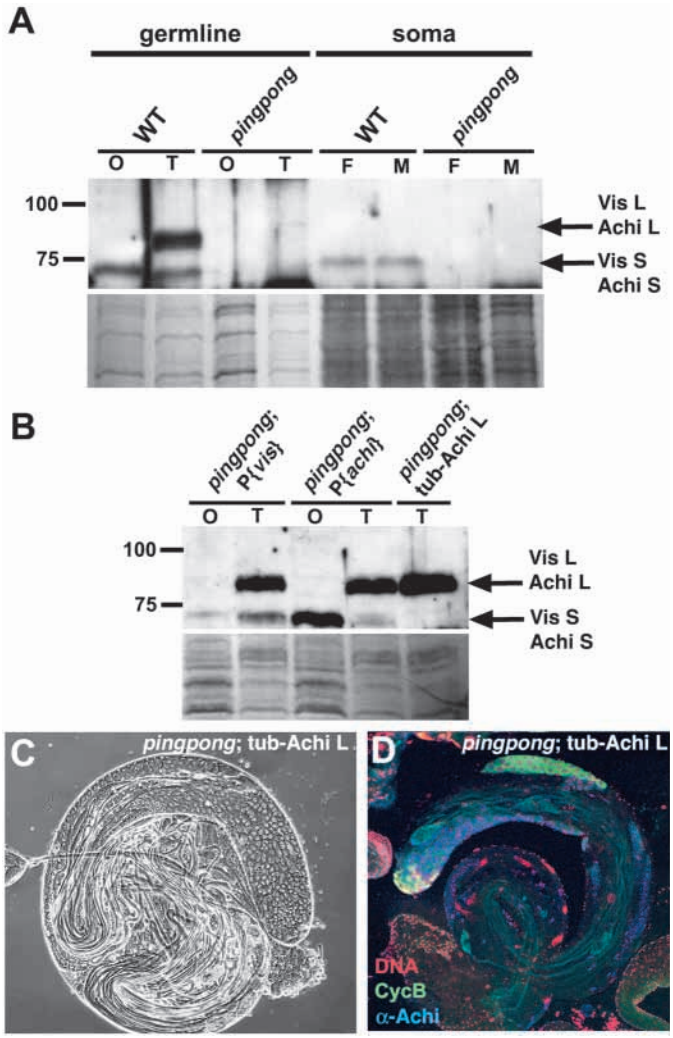

Fig. 7. AchiL can rescue *pingpong* function. (A,B) Immunoblot analysis of Vis/Achi proteins in wild type (WT), *pingpong*, *pingpong*; P{*vis*}, *pingpong*; P{*achi*}, and *pingpong*; *tub*-AchiL. Germline (O, ovaries; T, testes) and somatic tissues (soma; F, female adult soma; M, male adult soma) were analyzed. In extracts from wild-type testes, two bands are observed, the lower of which is also observed in ovaries. In both male and female somatic tissues a single band is observed that appears to migrate slightly slower than the ~60 kDa germline band; the reason for this difference is not known. However, none of these bands are observed in extracts from the *pingpong* mutant, confirming that they are derived from these genes. These images were cut just above a background band that was present in all lanes. The lower panels in both A and B show each blot stained for total proteins to illustrate that similar amounts of lysate were loaded in each lane (see also Methods). (C,D) *pingpong*; *tub*-AchiL testes appear normal by phase contrast microscopy and by immunostaining for Vis/Achi (blue), DNA (red) and CycB (green).

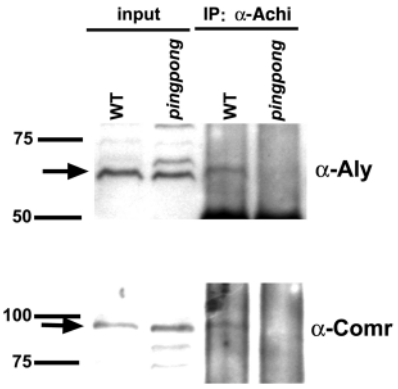

Fig. 8. Vis/Achi co-immunoprecipitates with Aly and Comr. Extracts from wild-type (WT) or *pingpong* testes were immunoprecipitated (IP) with anti-Achi antibody, run on SDS-PAGE gels, and sequentially probed with the anti-Aly antibody (top panel), anti-Comr antibody (lower panel), and anti-Achi antibody (not shown). The two lanes on the left show the input samples. Both Aly and Comr were observed in the IP from WT, but not *pingpong*, extracts (arrows).

spermatogenesis because they are required for both meiosis and spermatid differentiation. In contrast, other meiotic arrest genes, such as *boule*, *twine* and *polo*, block only some aspects of the spermatogenesis program (Eberhart et al., 1996; Fuller, 1998; Herrmann et al., 1998; Hiller et al., 2001; Lin et al., 1996). Our analysis of the Df(2R)*pingpong* mutant phenotype suggests that *vis* and *achi* act at the same or earlier step as *aly* and *comr*. Most significantly, we found that, like *aly* and *comr*, the expression of *boule*, *twine* and *mst87f* mRNAs is absent in the *pingpong* mutant. In addition, the mutant phenotype is more consistent with an early block in spermatocyte development because no sign of spermatid differentiation can be observed in the Df(2R)*pingpong* mutant. Also like *aly* and *comr*, the Df(2R)*pingpong* mutant arrests prior to the G_2 to M transition of meiosis I. Moreover, based on our ability to label 16-cell cysts in the Df(2R)*pingpong* mutant with BrdU, the block in meiosis apparently occurs after DNA synthesis.

Despite the many similarities to *aly* and *comr*, our results also suggest that *vis* and *achi* play a role in spermatogenesis that is different from these genes. First, unlike Aly or Comr, the Vis and Achi proteins are homeodomain-containing proteins of the TGIF subclass, making it likely that they have the ability to bind DNA in a sequence-specific manner. In contrast, *aly* encodes a chromatin-associated protein that is related to the *C. elegans lin-9* gene and *comr* encodes a novel nuclear protein (Jiang and White-Cooper, 2003; White-Cooper et al., 2000). Thus, of the known meiotic arrest genes, *vis* and *achi* are the best candidates for encoding sequence-specific transcription factors necessary for the regulation of specific genes during *Drosophila* spermatogenesis.

Our results further suggest, however, that there are additional transcription factors required for male meiosis in *Drosophila* that have yet to be identified. First, we found that *vis* and *achi* are not required for the expression of *aly* or *comr*, indicating that there are other factors that activate the expression of these genes in the testes. Second, *vis* and *achi* are widely expressed during development. Thus, the expression of these genes cannot be sufficient to trigger the male meiosis program. Instead, these results suggest that Vis and Achi must work together with other factors during testes development. Two of these factors are likely to be Aly and Comr, an idea that is supported by our ability to co-immunoprecipitate Vis/Achi, Aly, and Comr with an anti-Achi antibody. However, it is probable that there are additional, currently unknown

factors that activate *aly* and *comr* expression and that work with Vis/Achi in primary spermatocytes.

Similarities and differences to mammalian TGIFs

Mammalian TGIF is known to interact with three co-repressors, CtBP, Sin3 and HDAC1, and TGIF2 interacts with HDAC1 (Melhuish et al., 2001; Wotton et al., 2001; Wotton et al., 1999a; Wotton et al., 1999b; Wotton and Massague, 2001). These interactions, together with the ability of TGIF to antagonize TGFβ-mediated gene activation, strongly suggest that both TGIF and TGIF2 are transcriptional repressors. However, the sequences in TGIF and TGIF2 required for the interactions with these co-repressors map to sequences that are poorly, if at all, conserved in Vis or Achi. Thus, it is unclear at present if Vis/Achi recruit these or other co-repressors. However, we have shown that Vis/Achi proteins interact with Aly in vivo. Interestingly, an *aly* homolog in *C. elegans*, *lin-9*, has been genetically linked to components of the NURD complex, which is a chromatin remodeling complex with both ATPase and HDAC activities (Solari and Ahringer, 2000; Unhavaithaya et al., 2002; von Zelewsky et al., 2000; Xue et al., 1998). Moreover, the NURD complex has been implicated in gene repression. Thus, for some targets Vis/Achi may repress transcription indirectly, by helping to recruit Aly or stabilize its association with chromatin. Aly, in turn, may be able to recruit or activate a NURD-like complex, resulting in repression.

We observe a loss of expression of specific target genes in the Df(2R)*pingpong* mutant, suggesting that Vis/Achi directly or indirectly activate these genes. However, as yet, none of the TGIF family members have been shown to activate transcription. Thus, it remains an open question whether Vis/Achi play a direct role in the activation of genes such as *boule* and *twine* or, alternatively, if it acts indirectly by repressing the expression of a repressor of these genes.

Another well characterized feature of the mammalian TGIF protein is its ability to directly interact with Smad2, a transcription factor that is activated by TGFβ signaling (Wotton et al., 1999a). The interaction between TGIF and Smad2 is thought to modulate the response to TGFβ from gene activation to repression. Interestingly, *Drosophila* spermatogenesis requires TGFβ signaling (Matunis et al., 1997). However, this pathway, which utilizes the receptor encoded by *punt* and the transcription factor encoded by *schnurri*, is active in the somatic cyst cells that surround the germline spermatocytes. Once this pathway is activated, the somatic cyst cells are thought to release a second, unknown signal that limits the proliferation of the underlying germline cells (Matunis et al., 1997). At present, there is no known TGFβ-like pathway activated in primary spermatocytes, where Vis/Achi are maximally expressed. However, the available data do not rule out that a TGFβ-like pathway, which functions independently of *punt* and *schnurri*, may be active in these cells.

There are also connections between TGIF factors and epidermal growth factor (EGF) signaling. Specifically, TGIF and TGIF2 have consensus MAPK phosphorylation sites that are phosphorylated in response to EGF signaling, and phosphorylation appears to stabilize these proteins (Lo et al., 2001; Melhuish et al., 2001). Although the same phosphorylation sites do not appear to be conserved in Vis/Achi, it will nevertheless be interesting to determine if the activity and/or stability of Vis/Achi are modulated by a Ras/MAPK pathway that may be activated in primary spermatocytes.

Potential role of mammalian TGIFs in spermatogenesis

In summary, our results demonstrate that *vis* and *achi* play a critical role in spermatogenesis in *Drosophila*, but that they are dispensable for other aspects of fly development. The highly restricted role for these homeobox genes contrasts with the widespread roles for the other TALE group homeobox genes in the fly, including *exd*, *hth* and the Iro-C genes. It also contrasts with the apparently important role that the TGIF genes play in TGFβ and Nodal signaling in vertebrates. It is possible that the original function of this gene family was in male meiosis but that they duplicated in vertebrates, allowing some family members to evolve into modulators of TGFβ signaling. Consistent with this idea, there are two TGIF family members, Tex-1 and TGIFLX (TGIF-like on X) that have recently been found to be specifically expressed in mouse or human testes (Blanco-Arias et al., 2002; Lai et al., 2002). Given the many other similarities between fly and mammalian spermatogenesis (Fuller, 1998; Zhao and Garbers, 2002), it is plausible that these genes play an analogous role in mammalian spermatogenesis as the *vis* and *achi* genes play in *Drosophila*.

We thank Rob White and Helen White-Cooper for sharing unpublished results and Minx Fuller for advice. We also thank Siu-Kwong Chan, Steve Dinardo, Minx Fuller, David Glover, Christian Lehner, Pat O'Farrell, Claudio Sunkel, and Helen White-Cooper for sending fly stocks and antibodies. We thank members of the lab for advice throughout this project and Brian Gebelein, Joaquim Culi and Laura Johnston for comments on the manuscript. This work was supported by a grant from the NIGMS to R.S.M.

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