twin, a *CCR4* homolog, regulates cyclin poly(A) tail length to permit *Drosophila* oogenesis

Jason Z. Morris[1,*], Amy Hong[2], Mary A. Lilly[2] and Ruth Lehmann[1,†]

[1]Developmental Genetics Program, Department of Cell Biology, The Skirball Institute and Howard Hughes Medical Institute, NYU School of Medicine, New York, NY 10016, USA
[2]Cell Biology and Metabolism Branch, National Institutes of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA
*Present address: Department of Natural Sciences, Fordham College at Lincoln Center, New York, NY 10023, USA
†Author for correspondence (e-mail: lehmann@saturn.med.nyu.edu)

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Summary

Cyclins regulate progression through the cell cycle. Control of cyclin levels is essential in *Drosophila* oogenesis for the four synchronous divisions that generate the 16 cell germ line cyst and for ensuring that one cell in each cyst, the oocyte, is arrested in meiosis, while the remaining fifteen cells become polyploid nurse cells. Changes in cyclin levels could be achieved by regulating transcription, translation or protein stability. The proteasome limits cyclin protein levels in the *Drosophila* ovary, but the mechanisms regulating RNA turnover or translation remain largely unclear. Here, we report the identification of *twin*, a homolog of the yeast CCR4 deadenylase. We show that *twin* is important for the number and synchrony of cyst divisions and oocyte fate. Consistent with the deadenylase activity of CCR4 in yeast, our data suggest that Twin controls germ line cyst development by regulating poly(A) tail lengths of several targets including *Cyclin A* (*CycA*) RNA. *twin* mutants exhibit very low expression of Bag-of-marbles (Bam), a regulator of cyst division, indicating that Twin/Ccr4 activity is necessary for wild-type Bam expression. Lowering the levels of CycA or increasing the levels of Bam suppresses the defects we observe in *twin* ovaries, implicating CycA and Bam as downstream effectors of Twin. We propose that Twin/Ccr4 functions during early oogenesis to coordinate cyst division, oocyte fate specification and egg chamber maturation.

Key words: *Drosophila*, *twin*, CCR4, Oogenesis, CycA, Bam

Introduction

Cell cycle regulation is central to the morphogenesis of organs and tissues and to terminal differentiation of cells. In eukaryotes, cell cycle transitions are driven by the successive activation and inactivation of cyclin-dependent kinases (cdks). In *Drosophila*, Cyclin E associates with Cdk2 to promote S phase. CycA, CycB and CycB3 sequentially associate with Cdk1 to promote initiation and progression of M phase (Lee and Orr-Weaver., 2003). Just as in yeast and mammals, *Drosophila* cyclins are regulated at the protein level via proteasome-mediated degradation (Doronkin et al., 2003; Echard and O'Farrell, 2003; Ohlmeyer and Schupbach, 2003; Sigrist et al., 1995). At the RNA level, *Drosophila* and other eukaryotes use E2F and other factors to regulate cyclin transcription (Duronio et al., 1996). Vertebrates also regulate cyclins A1, A2 and B1 via polyadenylation of mRNA, which enhances the stability and translational efficiency of RNA (Fuchimoto et al., 2001; Groisman et al., 2002).

During *Drosophila* oogenesis, the cell cycle is tightly regulated to coordinate egg chamber production. At the beginning of oogenesis, in the germarium, cystoblasts undergo four rounds of synchronous, incomplete cell divisions to generate cysts of 16 germ cells interconnected by ring canals. Two cells, the pro-oocytes, share ring canal connections with four neighbors. One of these cells, the oocyte, arrests in Prophase I of meiosis and condenses its chromosomes into a spherical karyosome. The other cells all become polyploid nurse cells and produce mRNA and proteins, such as Orb, that are trafficked to the oocyte (Spradling, 1993). Elevation of CycA and of CycE can induce the cyst to undergo a fifth division (Doronkin et al., 2003; Lilly et al., 2000; Ohlmeyer and Schupbach, 2003). CycE/Cdk2 kinase activity also directs germ cell fate. Increasing CycE/Cdk2 kinase activity by removing the Cdk-inhibitor *dacapo* directs oocytes to take on nurse cell characteristics. A mutation that reduces CycE/Cdk2 kinase activity levels results in more cells with oocyte character (Hong et al., 2003; Lilly and Spradling, 1996). Study of *bag of marbles* (*bam*) further illustrates the close relationship between cell cycle control and differentiation in the female germ line. Consistent with its role in germ cell differentiation, loss of *bam* results in uncontrolled stem cell proliferation without differentiation (McKearin and Ohlstein, 1995), and ectopic *bam* causes stem cell loss (Ohlstein and McKearin, 1997). A role for Bam in cell cycle control is revealed by the finding that reduction of *bam* gene copy number suppresses the extra cyst division induced by excess cyclin activity (Lilly et al., 2000).

The fusome, a vesicular organelle that contains cytoskeletal proteins such as Hu Li Tai Shao (Hts), coordinates cell cycle and cell fate regulation in the cysts. With each cyst division,

the fusome branches and extends through the ring canals connecting new daughter cells to the cyst (de Cuevas et al., 1996; Lin et al., 1994). One of the daughters always inherits more fusome material than the others and this cell has been posited to differentiate as the oocyte (de Cuevas and Spradling, 1998; Deng and Lin, 2001; Lin and Spradling, 1995; McKearin, 1997). During the cyst divisions, the fusome associates with one pole of each mitotic spindle to regulate the division plane and the number and synchrony of divisions (de Cuevas et al., 1997; McKearin, 1997). Loss of Hts eliminates the fusome and disrupts synchrony and number of the cyst divisions as well as oocyte specification (Lin et al., 1994; Yue and Spradling, 1992; Zaccai and Lipshitz, 1996). CycA colocalizes with the fusome during the M-phase of cystoblast divisions (Lilly et al., 2000) and may help the fusome to coordinate the number and timing of divisions. Components of the proteasome are also localized to the fusome, where they may play a role in CycE turnover (Doronkin et al., 2003; Ohlmeyer and Schupbach, 2003).

In this study, we describe the molecular identification and phenotypic characterization of *twin*, a gene required for synchronous cyst divisions, oocyte fate specification and cyst maturation. We show that *twin* encodes the *Drosophila* homolog of the yeast *ccr4* gene. *ccr4* (carbon-catabolite-repression) was first identified in *S. cerevisiae* as a regulator of RNA levels of the alcohol-dehydrogenase II gene (Denis, 1984). Although CCR4 protein was previously shown to associate with basal transcription machinery (Liu et al., 1998), recent data demonstrate that CCR4 catalyzes the degradation of poly(A) tails in yeast and flies (Chen et al., 2002; Tucker et al., 2002; Temme et al., 2004). We show that in *twin* germaria, CycA is misexpressed and *cycA* mRNA has a longer poly(A) tail. Furthermore, decreasing the dose of *cycA* suppresses *twin* egg chamber degradation. We also find that *twin* germaria accumulate less cytoplasmic Bam protein. Genetically increasing the dose of *bam* suppresses *twin* cyst division and oocyte fate specification defects. We conclude that Twin/Ccr4 is required for the synchrony and number of cyst divisions and the oocyte/nurse cell fate decision. We propose that Twin/Ccr4 exerts control via regulation of poly(A) tail length of mitotic cyclins and by indirectly affecting the expression of others factors required for germline differentiation, such as Bam.

Materials and methods

Dissection, fixation, and staining, and microscopy

Ovaries were dissected, fixed and stained as previously described (Morris et al., 2003) with the following modifications: all fixations for BamC and anti-cyclin antisera in 4% formaldehyde. No serum was used with the primary antibody in these samples: Anti-Cyclin A, 1:600; anti-Cyclin B, 1:2000; anti-ORB, 1:1:1 of block, 6H4, 4H8 antibodies; anti-Bam, 1:500; 1B1, 1:20. Phalloidin (1 μl) was added to primary antibody incubation for ring canal labeling. Ovary RNA in situ hybridizations performed as described previously (Ephrussi et al., 1991). Embryo RNA in situ hybridizations was performed as described by Lehmann and Tautz (Lehmann and Tautz, 1994).

Comparison and quantitation of *twin* phenotypes

Counts are from stages 3-10. We did not count chambers within the germaria because, especially in *twin* germaria, it is difficult to determine number and fate of cells in immature chambers. All crosses were kept at 25°C except for the initial quantitation of the *twin* phenotypes (Fig. 1) which were at room temperature to reveal the full range of phenotypes.

The *twin* phenotypes are age and temperature sensitive. Whenever comparing flies of different genotypes, we used sisters of the same cross and bottle, of similar age, dissected on the same day. The only exceptions were when we could not use sisters of the same cross (i.e. Oregon R BAMC staining and comparisons of *cycA* $twin^{ry3}$*/Df* to $twin^{ry3}$*/Df*). In these cases, we dissected and stained flies grown in identical conditions on the same day.

P-insertion site identification

Genomic DNA was digested with *Bam*HI and *Bgl*II. The DNA was then ligated and PCR was performed with the Expand Hi Fidelity kit using primers extending outward from the P element ends. Sequencing ABI sequencing of the fragments and analysis with Editview, DNAstar (Lasergene) and BLAST (Altschul et al., 1997) followed.

Northern analysis

RNA was isolated from $twin^{ry3}$ homozygous and wild-type ovaries using Trizol. The RNA was then poly(A) purified using the PolyATract mRNA Isolation System III (Promega), and was run out on an agarose gel, blotted and probed using standard methods.

Mutagenesis, screening, allele sequencing and sequence analysis

We mutagenized *ru h th st sr e ca* males with EMS. Mutants were tested for failure to complement the *ry5* allele for female sterility. *ry5* is viable in trans to deficiency and fertile in males, so null alleles can be isolated in such a screen and recovered via male progeny. ESTs were ordered from the Berkeley *Drosophila* Genome Project and were ABI-sequenced from ends. New primers were generated from each round of sequencing to generate the next round.

PAT assays

As described by Salles and Strickland (Salles and Strickland, 1999). RNA was prepared from ovary extracts of young (1- and 2-day-old) adults using TRIZOL. Very young *twin* and wild-type adults had comparably little development of mid- and late-stage egg chambers. Total RNA (100 ng) used for first-strand synthesis. PCR reactions performed with expand Hi-Fidelity kit. ^{32}P dCTP was added to 10 mM cold dNTP stock before setting up PCR reactions, and a fraction of the PCR product was tested on a gel to permit approximately equal loading of hot PCR product for the experiment. Samples were run on 6% acrylamide gels, dried down and exposed as autoradiographs.

Results

Twin is required for multiple processes in early oogenesis

twin mutant females are nearly sterile, and their ovaries show defects in cyst division, cyst survival and germ cell fate. We quantitated frequencies of egg chamber degradation, cyst division defects and oocyte specification defects in *twin* alleles in trans to deficiency, allowing us to place the alleles in a series: *S1>ry3~ry5>S3* (Table 1). The stronger alleles in the series also caused more egg chamber death within the germarium (not shown). At room temperature, 27% of $twin^{S1}$*/Df* egg chambers degrade between stages 3 and 10 (Fig. 1B, Table 1), 41% of egg chambers contain fewer than 16 germ cells, (Fig. 1C,D,G, Table 1), and 41% of egg chambers fail to specify an oocyte (Fig. 1D,G, Table 1). Twenty-nine percent of the egg chambers were defective in both germ cell number and oocyte specification (Fig. 1D, Table 1). We also observed chambers

Table 1. The range of egg chamber phenotypes of the *twin* alleles at room temperature*

Genotype	*n*	% Wild type	% Degrading	% Division defect (less than four, one oocyte)	% Oocyte defect (four divisions, no oocyte)	% Division and (less than four oocyte defect divisions, no oocyte)	% Other†
$twin^{S1}/Df$	199	18	27	12	12	29	2
$twin^{ry3}/Df$	393	28	37	5	13	11	6
$twin^{ry5}/Df$	156	34	31	3	4	16	12
$twin^{S3}/Df$	322	73	14	0	3	0	10

*All the twin alleles exhibit more severe phenotypes at higher temperatures. These data were generated at room temperature to assay the greatest range of phenotypes. All other phenotypic characterization is performed at 25°C and shows a shift towards more severe phenotypes.

†This category includes more than one oocyte, too many cyst divisions and oocytes in the center of egg chambers.

containing an oocyte at the center rather than the posterior (Fig. 1E,G), chambers with two oocytes (Fig. 1F) and chambers containing cells with more than four ring canals, indicating that these cells divided more than four times (Fig. 1G, fifth ring canal in a different plane). Each allele exhibited stronger mutant phenotypes at higher temperatures (not shown). *twin* cysts contain any number of germ cells from one to more than 25. All alleles displayed the full range of phenotypes, indicating either that none of the alleles are nulls or that *twin* activity is not absolutely required for egg chamber development.

The cell division defects in *twin* egg chambers are very striking. In contrast to other mutants defective in counting the number of cyst divisions, such as *orb*, *encore* and *tribbles* (which maintain synchrony of division) (Deng and Lin, 2001; Hawkins et al., 1996; Johnston, 2000; Lantz et al., 1994; Mata et al., 2000; McKearin and Christerson, 1994; Seher and Leptin, 2000; Van Buskirk et al., 2000), the number of germ cells in *twin* egg chambers did not generally correspond to a power of two (Fig. 1C). Germ cell numbers in adjacent chambers did not generally add up to 8, 16 or 32 cells, arguing that encapsulation defects cannot explain this phenotype. Finally, every cell in each cyst was connected to at least one neighbor by a single ring canal, arguing against germ cell death as the cause of unusual germ cell number (Fig. 1G). We conclude that *twin* affects the synchrony and number of cyst divisions.

When egg chambers contained oocytes, these oocytes appeared normal and contained the most ring canals. We observed chambers containing three germ cells, including an oocyte (not shown) as well as rare chambers specifying two oocytes (Fig. 1F). In all cases, the oocytes condensed their chromosomes into a normal karyosome and accumulated oocyte specific factors such as Orb protein (Fig. 1C) and *osk* mRNA (not shown). We observed two oocytes in chambers containing too many, too few or exactly 16 germ cells. In these cases, both oocytes appeared normal, even to the extent that they attracted migrating follicle cells in stage 9 egg chambers.

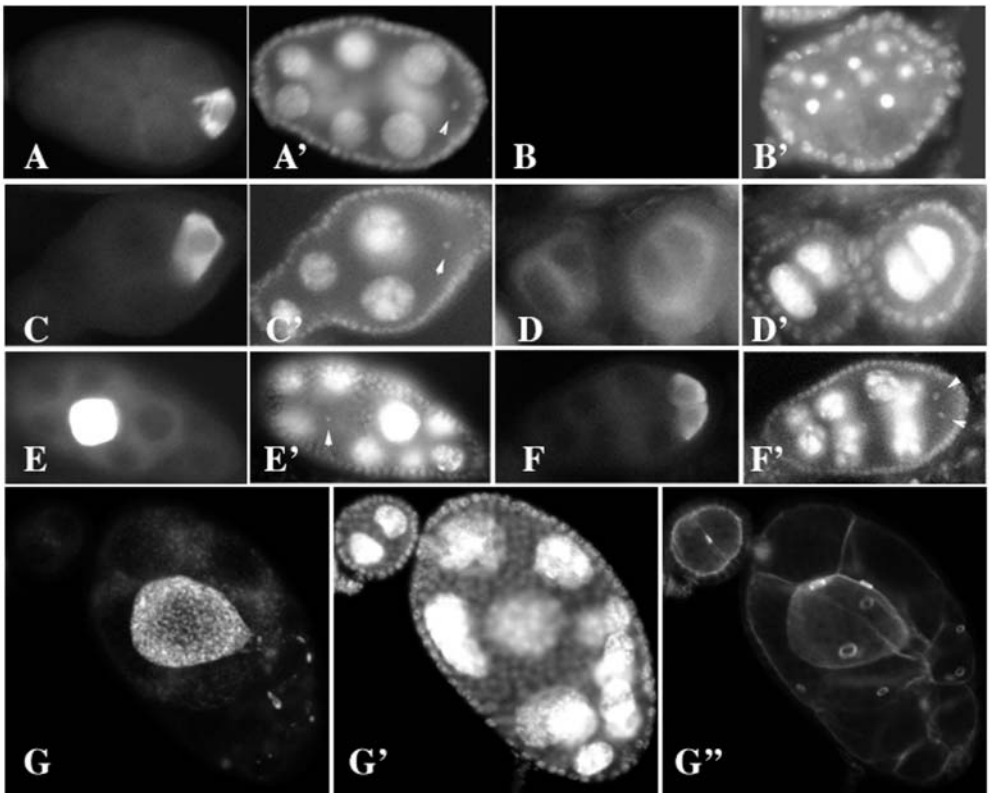

Fig. 1. *twin* egg chambers are defective in germ cell number and oocyte specification. Anterior is leftwards in all panels. (A-G) Anti-ORB antibody. (A′-G′) DAPI. Arrowheads in A′,C′,E′,F′ indicate the karyosomes in the oocytes. (G″) Phalloidin. A *twin* mutant ovariole typically contains egg chambers exhibiting many of these phenotypes. (A,A′) Wild-type chambers have 15 polyploid nurse cells and a posterior oocyte containing Orb and a condensed karyosome. (B,B′) $twin^{ry5}/Df$. Many *twin* egg chambers die. These chambers do not stain with Orb antiserum and they have bright, punctate DAPI staining. Egg chamber death occurs in the germarium as well (not shown). (C,C′) $twin^{S3}/Df$ egg chamber with five nurse cells and a normal oocyte. (D,D′) $twin^{S3}/twin^{ry5}$ egg chamber with two nurse cells and no oocyte. (E,E′) A $twin^{S3}/twin^{ry5}$ egg chamber with an otherwise normal oocyte in the center. (F,F′) $twin^{S3}/Df$ egg chamber containing 14 nurse cells and two normal oocytes. (G,G′,G″) $twin^{ry3}/twin^{ry5}$ egg chamber with ~17 nurse cells and an oocyte in the center that has undergone five divisions. Five ring canals are present but one is not easily seen in this focal plane.

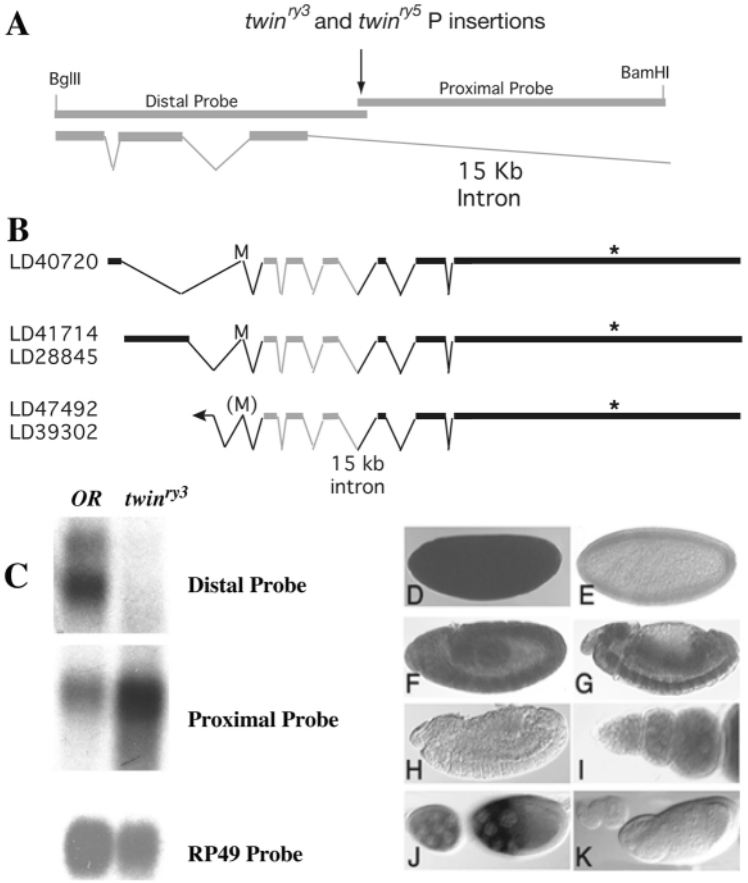

Fig. 2. Mapping, cloning and expression of *twin*. (A) Genomic region around the *ry3* and *ry5* P-element insertions. The insertions (black arrow) fall in a 15 kb intron of *ccr4*. Distal probe: 1.5 kb of genomic DNA isolated from inverse PCR generated from the 5′ end of the *ry3* or the 3′ end of the *ry5* strains. Proximal probe: 1.5 kb of genomic DNA isolated from inverse PCR generated from the 3′ end of the *ry3* or the 5′ end of the *ry5* strains. The three exons of *ccr4* included in the distal probe are shown. The proximal probe lies in the 15 kb intron. (B) Different splice forms of *Drosophila ccr4* represented by ESTs isolated by the *Drosophila* Genome Project. The three exons preceding the 15 kb intron containing the P insertions are shown in gray. M, putative start methionine; (M), start methionine less certain because there is no in-frame stop codon upstream in the EST isolated; *, stop codon. (C) Northern blot of poly(A)-selected RNA from wild-type and $twin^{ry3}/twin^{ry3}$ ovaries. The distal and proximal probes are as described in A. RP49 was used as a loading control. The proximal probe seems to be upregulated in $twin^{ry3}$ homozygotes, but this is unlikely to contribute to the *twin* phenotype. The alleles are all recessive and phenotypically similar, and the point mutants have missense mutations in *ccr4* and not in the predicted ORFs of the intron. (D-K) mRNA in situ hybridization. Anterior is leftwards in all panels. (D-G,I-J) *ccr4* antisense probe. (H,K) *ccr4* sense negative control. (D) Maternal *ccr4* is present at very high levels in early embryos. (E) Expression is quite reduced in the blastoderm. (F,G) Expression is general, with strong staining in the central nervous system. (H) No staining observed in sense control. (I,J) Expression is present throughout the ovary, with stronger expression in germ cells than in soma. Expression is particularly strong in late (stage 10 and later) nurse cell. Staining in germarium region 1 was not strong, but this tissue is refractory to in situ hybridization. (K) No staining observed in sense control.

When there were two oocytes they were often both at the posterior of the chamber (Fig. 1F), although they were also found at opposite ends of the chamber (not shown). Because cyst division and oocyte specification defects occur independently in *twin* mutants, Twin may couple these processes in the wild type to ensure exactly one oocyte forms in each 16 cell egg chamber.

In spite of the striking defects observed in *twin* ovaries, most other tissues in *twin* animals are apparently unaffected. *twin/Df* flies are viable and males are fertile. Within the ovary, the defect is restricted to the germline. Follicle cells form normal epithelia and migrate properly. In germline clones, *twin* mutant germ cells exhibit mutant phenotypes in an otherwise heterozygous background (data not shown), indicating that the functioning of *twin* is required in the germline.

twin encodes a homolog of yeast CCR4, a deadenylase

To determine how *twin* affects germline development, we molecularly identified the gene using two P-element insertion alleles, $twin^{ry3}$ and $twin^{ry5}$ (Spradling, 1993). We reverted the mutant phenotype by precise excision of the P-elements, confirming that the insertions caused the *twin* defects. *twin* maps to chromosomal region 95F and the *twin* alleles failed to complement *Df(3R)CRB87-4*. Inverse PCR and sequencing of the distal and proximal genomic DNA flanking the P-element insertions showed that the $twin^{ry3}$ and $twin^{ry5}$ transposons were only offset by 1 bp from each other and in opposite orientations. Using BLAST analysis we found ESTs that matched the distal flanking region (Fig. 2A,B). These ESTs included several splice isoforms of a *Drosophila ccr4* homolog that differ in their 5′UTRs but not in their coding regions (Fig. 2B). Sequence analysis showed that the P-insertions as well as the P-element proximal DNA fell in a 15 kb intron of the *ccr4* gene.

We hybridized labeled distal and proximal flanking DNA to northern blots containing mRNA from ovaries isolated from OR flies and $twin^{ry3}$ homozygous flies (Fig. 2C). A 3 kb doublet detected by the distal probe and by *ccr4* ESTs was strongly reduced in $twin^{ry3}$ homozygous ovaries. Overexposing the blot showed very weak expression. A 1.5 kb band detected by the proximal probe lies within the 15 kb intron of *ccr4*. The genome project and Genefinder do not predict this transcript. The transcript was not altered in size or reduced in levels in the $twin^{ry3}$ ovaries (Fig. 2C).

We next wanted to see if *ccr4* is expressed in a pattern consistent with the role of *twin* in oogenesis. Consistent with FlyBase reports we find that *ccr4* RNA is expressed throughout embryogenesis. Newly deposited embryos show very strong maternal deposition of *ccr4* RNA (Fig. 2D). The maternal transcript is rapidly degraded, but the germline and soma exhibit low-level uniform expression in the syncytial blastoderm (Fig. 2E) and throughout embryogenesis (Fig. 2F,G). *ccr4* is expressed throughout oogenesis, most strongly in the germline but also in the soma (Fig. 2I,J,K) (see Temme et al., 2004).

S. cerevisiae CCR4 has three functional domains (Fig. 3A) (Malvar et al., 1992). The N-terminal region is rich in

glutamine and asparagine. The central region contains five leucine-rich repeats (LRR), which are crucial for dimerization with CAF1/POP1, an adapter protein that facilitates the association of CCR4 with other proteins and is required for deadenylation of targets (Clark et al., 2004). The Twin LRR domain contains three and a half repeats (Fig. 3A,B) which are most similar to the repeats shown to be essential to yeast CCR4 function (Clark et al., 2004). The C-terminal domain of *S. cerevisiae* CCR4 has 3′-5′ exoribonuclease and ssDNA exonuclease activity, and it catalyses the poly(A)-deadenylation reaction (Tucker et al., 2002; Tucker et al., 2001; Chen et al., 2002; Draper et al., 1994; Malvar et al., 1992). The catalytic domain of Twin shares extensive homology with *S. cerevisiae* CCR4. Blast analysis of the *Drosophila* genome showed two other predicted *ccr4* homologs in *Drosophila*, *angel* and *Dnocturnin* (Fig. 3A). Angel (Kurzik-Dumke and Zengerle, 1996) and Dnocturnin share extensive homology with the CCR4 catalytic domain, although Dnocturnin is missing an asparagine shown to be essential for catalysis (Chen et al., 2002). Neither protein possesses an LRR repeat region (Fig. 3A); Twin is therefore the best candidate to encode the *Drosophila* CCR4 homolog. Consistent with a prominent role of Twin in deadenylation, Temme et al. (Temme et al., 2004) showed that *Drosophila* CCR4 catalyzes deadenylation when purified from extracts. Partial depletion of CCR4 either from tissue culture or flies led to an overall increase in bulk poly(A)tail length and more specifically affected the poly(A)tail length and stability of heat-shock, *hsp 70*, RNA.

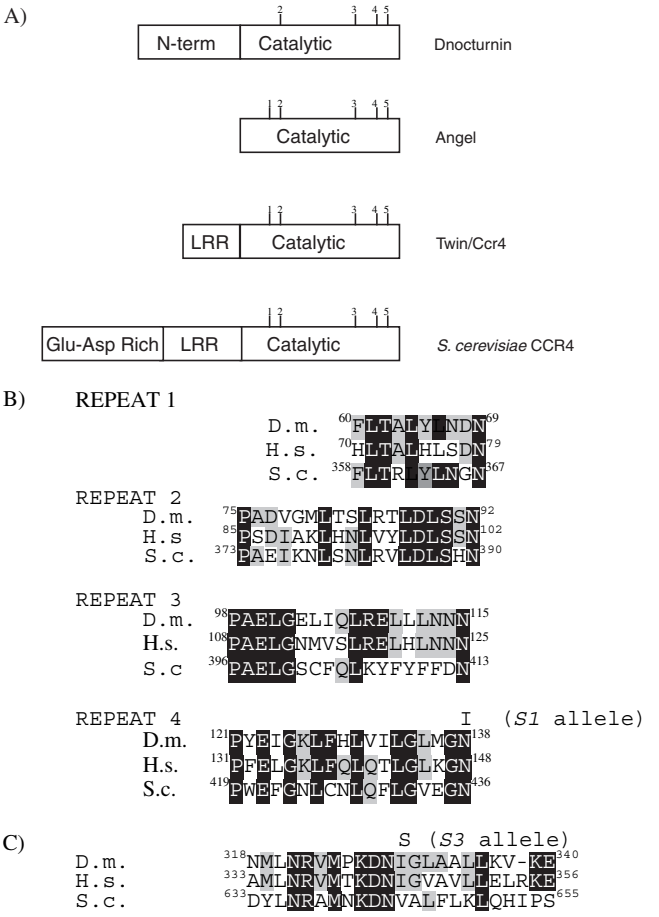

Fig. 3. The *S1* and *S3* alleles disrupt highly conserved amino acids in *Drosophila* Ccr4. (A) Cartoon showing the domains present in DNOCTURNIN, ANGEL and TWIN/CCR4, as well as *S. cerevisiae* CCR4. The LRR domains are not present in ANGEL and DNOCTURNIN. Five amino acids, corresponding in *Drosophila* CCR4 to N199, E243, D412, D486 and H526 have been shown to be essential for catalysis in *S. cerevisiae* (numbered 1-5) (Chen et al., 2002). (B,C) Black boxes: amino acids identical in all three species. Gray boxes: amino acids identical in two of the three species. Numbers designate the position of amino acids in their primary sequences. (B) Alignment of the leucine-rich repeat regions (LRRs) of CCR4 from *Drosophila*, humans and *S. cerevesiae. S. cerevisiae* has five LRRs, humans and *Drosophila* have half of the first LRR and the second, third and fourth LRRs. Deletions of yeast LRRs 4 and 5 have no effect (Clark et al., 2004). The invariant asparagine at the end of repeat 3 is translated as an isoleucine in the *twin*^{S1} allele. (C) Alignment of a region of the sequence between catalytic amino acids 2 and 3 from A, including the site of the *S3* lesion. An isoleucine conserved in humans and *Drosophila* is translated as a serine in the *twin*^{S3} allele. *S. pombe* also has an isoleucine at this position (not shown), although *S. cerevisiae* has a valine.

In order to conclusively determine that the *twin* phenotypes are due to disruption of *Drosophila ccr4*, we carried out an EMS mutagenesis screen for mutants that failed to complement the *ry5* allele. We screened 2749 independent mutants and isolated two lines, *twin*^{S1} and *twin*^{S3}, that failed to complement *twin*^{ry5}, *twin*^{ry3}, *Df(3R)CRB87-4* and each other. Sequencing of *ccr4* showed that the *S1* mutation results in the substitution of an isoleucine for a highly conserved asparagine in the last LRR repeat (Fig. 3B). This mutation could therefore prevent Twin from deadenylating mRNAs. The *S3* mutation, the weakest *twin* allele, changes an isoleucine to a serine within a conserved motif of the deadenylase catalytic domain (Fig. 3C). This amino acid is conserved but has not been shown to be essential for catalysis in yeast. Because all *twin* alleles affect the *ccr4* transcript, we conclude that *twin* encodes *Drosophila ccr4*.

Misregulation of mitotic cyclins contributes to *twin* defects

twin mutants exhibit defects in the divisions of the cystoblast. Such defects have been observed in mutants directly affecting cell cycle regulators (Mata et al., 2000) and in mutants affecting the integrity of the fusome, which co-localizes with CycA in a cell cycle-dependent manner (de Cuevas et al., 1996; Lilly et al., 2000; Lin et al., 1994). Since CCR4 in *S. cerevisiae* regulates gene expression, we examined *twin* mutants for changes in cyclin regulation in the germarium (Fig. 4A-F). Staining of wild-type or *twin*^{S1}/+ germaria showed that, as reported previously, CycA is expressed in the dividing cysts of germarium region 1 and is downregulated in the post-mitotic cysts from region 2 on (Fig. 4A,C). In *twin*^{S1}*/Df* ovaries, CycA was ectopically expressed throughout the germarium, including what should be the postmitotic regions (Fig. 4B). Similar, but less strong misexpression was also observed in *twin*^{ry3}*/Df (*Fig. 4D). Somatic expression appeared unaffected. We also analyzed the expression of CycB, which acts together with CycA in promoting entry into mitosis (Knoblich and Lehner, 1993; Knoblich et al., 1994). CycB is misexpressed in a pattern similar to that of CycA in *twin* germaria (Fig. 4E,F), although in contrast to the very consistent CycA misexpression phenotype, not all germaria misexpressed CycB. CycB misexpression may be an indirect result of reduced Twin

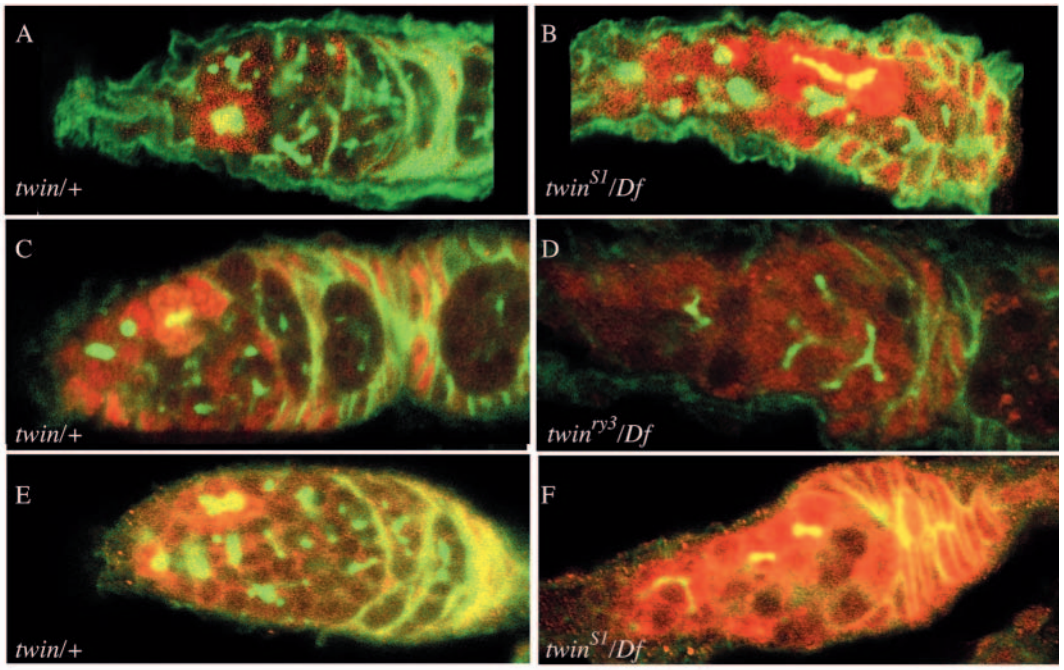

Fig. 4. CycA and CycB are misregulated in *twin*. Anterior is leftwards in all panels. The pair in each row was stained and photographed in parallel. (A-D) CycA, red; fusome (1B1), green. (E,F) CycB, red; fusome, green. (A) Wild-type (*twin*/+) chamber showing a cyst in region 1 expressing CycA. Some of the CycA co-localized with the fusome, which appears yellow. Posterior to this cyst are several highly branched fusomes. (B) *twin*^{S1}*/Df* germarium showing very strong CycA staining throughout. CycA colocalizes with some fusomes even towards the posterior of the germarium (yellow). Fusomes are present, and contain branches. (C) Wild-type (*twin*/+) germarium showing CycA in two cysts in region 1. One of these cysts colocalizes CycA to the fusome. (D) *twin*^{ry3}*/Df* germarium showing CycA staining throughout. The misexpression phenotype is not as prominent as in the strongest allele, S1. The fusomes are clearly visible and highly branched. (E) Wild-type (*twin*/+) germarium showing CycB in two cysts in region 1. Both of these cysts show CycB co-localizing with the fusome. (F) *twin*^{S1}*/Df* showing very strong CycB staining throughout germarium. The unstained regions are sites of dying cysts. CycB colocalizes with the fusome in all the cysts. Staining with anti-CycE antisera did not reveal a difference between *twin/Df* and *twin*/+ germaria (not shown).

activity or CycB may have other important regulators in addition to Twin. We stained ovaries with antibodies raised against the S-phase cyclin CycE, and did not detect a difference between *twin*^{S1}*/Df* and *twin*/+ germaria (not shown). Our findings suggest that Twin is required for downregulating CycA expression in the germarium and, to a lesser extent, expression of CycB.

Since *twin* mutants exhibit cyst division defects similar to those observed in *hts* mutants, we used 1B1 antiserum to follow fusome morphology during cyst division. As in wild type, 1B1 labeled the fusome and cell cortices in *twin* mutants (Fig. 4A-F). The fusome seemed normal in size and extended throughout the cysts (Fig. 4B,D). We obtained the same results with anti-Spectrin antiserum (not shown). In addition, we observed CycA and CycB co-localizing with the fusome in a subset of cysts throughout the germarium (Fig. 4B,F), which suggests that the fusome retains some normal function and that cysts may be delayed in progressing through mitosis in *twin*. In many *twin* chambers, the fusome had fewer branches (Fig. 4B,F), which we attribute to the fact that *twin* mutant cysts frequently contain fewer cells. Taken together, these data suggest that the *twin* defects in cyst division synchrony and oocyte specification occur in parallel or downstream of fusome integrity, possibly by acting with the fusome to regulate cyclin levels within the cyst.

Our findings show that CycA levels are elevated in *twin.* We analyzed whether reduction in the levels of cyclins affected the *twin* phenotypes. For these experiments we used the *twin*^{ry3}*/Df* allelic combination, whose intermediate phenotype seemed suitable for modification studies. Removing one copy of *cycA* markedly suppressed the number of stage 3-10 chambers that were degrading (Table 2, 51% compared with 70%, a relative suppression of 30%, $P<0.001$), suggesting that excess CycA induces chamber degradation in *twin* (see Discussion, Table 2). We did not observe a significant change in the *twin*^{ry3}*/Df* phenotype when we removed one copy of *cycE* or *cycB* (Table 2). It remains unclear whether a specific stage of the cell cycle is particularly sensitive to loss of Twin function. Staining germaria with anti-phospho-Histone 3 antiserum did not reveal an enrichment of cysts with the condensed chromosomes characteristic of early to mid-M phase (not shown), but *twin* cysts could be delayed elsewhere in the cell cycle. On the basis of the CycA expression and genetic interaction experiments, we conclude that misexpression of CYCA contributes to the *twin* egg chamber phenotype.

Table 2. Genetic interactions of *cycA*, *cycB* and *cycE* with *twin*

Genotype	*n*	% Dying	*P*	% Living chambers without oocyte	*P*	% Living chambers with fewer than 16 cells	*P*
+/+; *twin*^{ry3}*/Df*	327	70	NA	69	<1.0	84	NA
+/+; *cycA twin*^{ry3}*/Df*	181	51	<0.001	69	<1.0	81	<1.0
+/+; *twin*^{ry3}*/Df*	178	70	NA	81	NA	77	NA
cycB/+; *twin*^{ry3}*/Df*	224	71	<1.0	73	<1.0	75	<1.0
cycE/+; *twin*^{ry3}*/Df*	123	78	<1.0	85	<1.0	81	<1.0

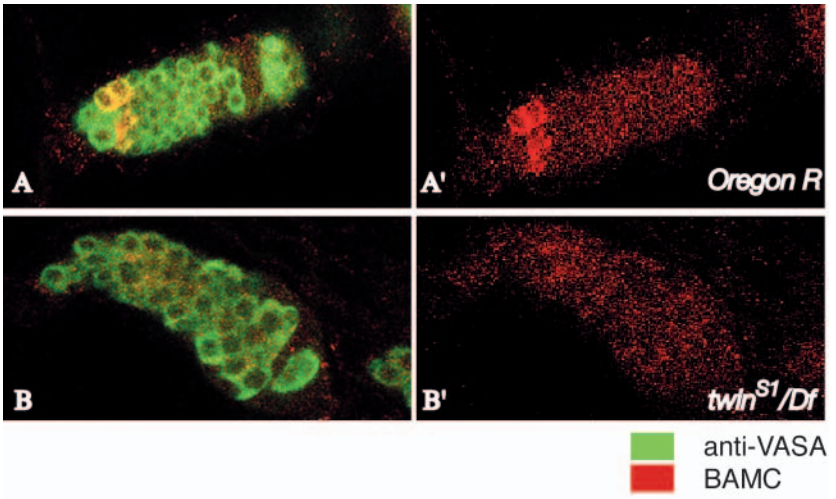

Fig. 5. Cytoplasmic Bam is reduced in *twin* germaria. (A,B) BamC, red; germ cells (Vasa), green. (A′,B′) red channel alone. The BamC antiserum is specific to the cytoplasmic form of Bam (McKearin and Ohlstein, 1995). Bam can also localize to the fusome, but that fraction is not detected by the available antibodies. Vasa is expressed in germ cells. Anterior is leftwards in all panels. All samples were stained and photographed in parallel. (A,A′) Wild-type (OR) germarium showing BamC staining in a cyst in Region 1. All the germ cells express Vasa. (B,B′) *twin*^{S1}*/Df* germarium showing very reduced BamC staining. *twin* heterozygotes exhibit BamC staining in dividing cysts, but also exhibit high background staining (not shown).

Reduced expression of cytoplasmic Bam correlates with *twin* cyst division defects

We assayed expression of Bam in *twin* germaria because Bam is an important determinant of cystoblast division and differentiation. In *bam* mutant ovaries, stem cells divide symmetrically so that the germaria are filled with germline tumors rather than egg chambers (McKearin and Ohlstein, 1995). Precocious expression of Bam in larvae induces all stem cells to differentiate and form cysts (Gilboa and Lehmann, 2004; Kai and Spradling, 2004; Ohlstein and McKearin, 1997). Removing Bam during cyst division can revert differentiating cysts into single stem cell-like cells (Kai and Spradling, 2004). Removing one copy of *bam* suppresses extra cell divisions caused by a pulse of ectopic CycA or by loss of function of *encore*, a gene that has been proposed to promote cyclin protein degradation (Hawkins et al., 1996; Lilly et al., 2000; Ohlmeyer and Schupbach, 2003).

We stained *twin* and wild-type ovaries with BamC antiserum (Fig. 5). In wild-type ovaries, BamC stains the cystoblasts and the dividing cysts but not later stages (McKearin and Ohlstein, 1995) (Fig. 5A,A′). The BamC staining in the dividing cysts of *twin*^{S1}*/Df* ovaries was much fainter (Fig. 5B,B′). Most germaria showed quite weak or undetectable levels of BamC, although some germaria exhibited strong BamC expression. We conclude that Bam is downregulated or not localized properly to the cytoplasm (and thus not detectable by the BamC antiserum) in *twin* mutants.

In order to test the functional significance of BamC reduction in *twin* ovaries, we induced expression of *bam* and assayed for suppression of the *twin* phenotype. To avoid alteration of the twin phenotype caused by heat shock, we compared *twin*^{ry3}*/Df* with *hs-bam; twin*^{ry3}*/Df* ovaries grown at 25°C to induce some *bam* expression from the *hs*-promoter. We found that increasing Bam levels suppresses *twin* (Table 3). Fifty percent more chambers contained an oocyte in *hs-bam; twin*^{ry3}*/Df* ovaries than in *twin*^{ry3}*/Df* ovaries (61% compared with 40%, $P<0.001$, Table 3). Comparing the percentage of chambers with no mutant phenotypes to those with oocyte specification or cyst cell number defects showed an even more striking contrast. *hs-bam; twin*^{ry3}*/Df* egg chambers were nearly twice as likely to contain 15 nurse cells and one oocyte than *twin*^{ry3}*/Df* ovaries egg chambers (43% of non-degrading chambers versus 26%, not shown). Taken together, these results suggest that the cell cycle and oocyte specification defects in *twin* are due, in part, to reduced Bam expression.

The *cycA* poly(A) tail is shortened by Twin/Ccr4

twin encodes the *Drosophila* CCR4 homolog. In *S. cerevisiae*, CCR4 is found associated with a large, multi-protein complex and affects RNA levels (Chen et al., 2001; Denis and Malvar, 1990; Verdone et al., 1997). Although CCR4 has been proposed to regulate transcription, recent biochemical experiments strongly support a direct role of CCR4 as the major cytoplasmic deadenylase (Chen et al., 2002; Tucker et al., 2002; Tucker et al., 2001; Temme et al., 2004). In this role, CCR4 regulates mRNA target genes by shortening their poly(A) tails. Short poly(A) tails destabilize RNA and decrease the translational efficiency of mRNAs, leading to lower protein levels. We performed poly(A) tail length (PAT) assays (Salles and Strickland, 1999) to compare the poly(A) tail length of *cycA*, *cycB*, *cycE* and *bam* in total ovary extracts from dissected ovaries of 1- to 2-day-old *twin*^{S1}*/Df* and *twin/+* adult females These qualitative assays use PCR to amplify the whole range of poly(A) tail lengths associated with a particular transcript present in an extract. *cycA* poly(A) tails were significantly longer in *twin*^{S1}*/Df* ovaries than in *twin/+* ovaries (Fig. 6A). Mutant extracts were highly enriched with *cycA* message with poly(A) tails of 40-80 As, while *cycA* poly(A) tails in wild-type extracts predominantly contained 0-20 As. We also observed an upward shift in poly(A) tail length when monitoring *cycB* and *cycE* poly(A) tails in *twin* mutant extracts, although the shift for *cycB* was not as dramatic as the *cycA*

Table 3. Genetic interaction of *hs-bam* with *twin*

Genotype	*n*	% Dying	*P*	% Living chambers without oocyte	*P*	% Living chambers with fewer than 16 cells	*P*
+/+; twin^{ry3}*/Df*	365	50	NA	61	NA	53	<1.0
hs-bam/+; twin^{ry3}*/Df*	283	40	<0.025	40	<0.001	47	

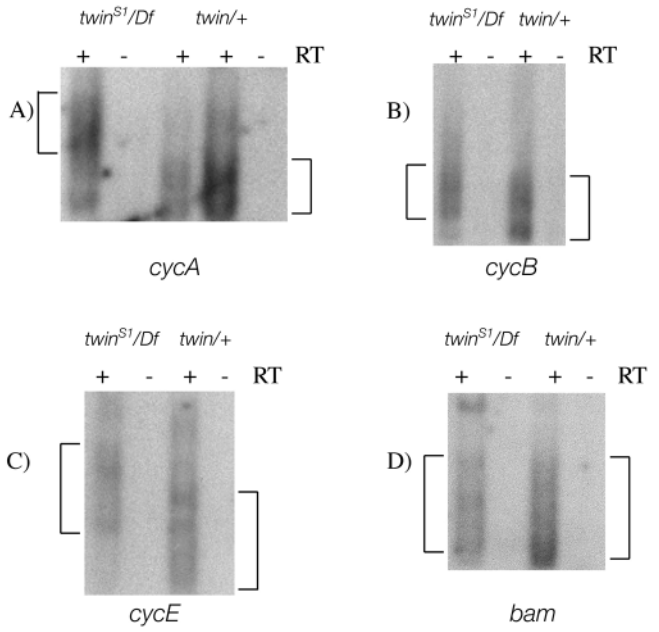

Fig. 6. *cycA*, *cycB* and *cycE* messages have longer poly(A) tails in *twin* mutants. In all panels, + and – refer to the presence or absence of reverse transcriptase (RT) in the reaction. Left brackets show sizes of most abundant poly(A) tails in *twin^{S1}/Df* ovaries. Right brackets show sizes of most abundant poly(A) tails in *twin/+* ovaries. (A) *cycA* mRNA. Two different amounts of wild-type (*twin/+*) RNA are loaded to show the dramatically different distributions of poly(A) tail length in *twin* mutant versus wild type. The predominant class of poly(A) tail lengths in *twin^{S1}/Df* is from 40-80 bp long. The predominant class of poly(A) tail lengths in *twin/+* is from 0-20 bp long. (B) The distribution of lengths of *cycB* poly(A) tails is similar in *twin^{S1}/Df* and *twin/+*. The poly(A) tails may be slightly higher in the *twin* homozygotes. (C) *cycE* poly(A) tails tend to be significantly longer in *twin^{S1}/Df* than in *twin/+*. (D) The distribution of *bam* poly(A) tail length is similar in *twin* mutant versus wild type. Short tails are more abundant in both genotypes and poly(A) tails reach the same maximum length, although there may be more of the very shortest tails in the wild type. A slow-migrating band is more abundant in *twin^{S1}/Df* than in *twin/+*, but as the band is isolated far above the distribution of poly(A) tails and as BamC levels are lower in *twin^{S1}/Df*, this is probably not a reflection of deadenylation defects.

shift (Fig. 6B-C). We did not detect an obvious increase in *bam* poly(A) tail length in *twin* extracts (Fig. 6D). The fact that we do not observe elevated CycE protein levels in spite of the shift in poly(A) tail length in *twin* ovaries suggests that other mechanisms of regulating CycE levels (e.g. proteolysis) are sufficient to compensate for longer *cycE* poly(A) tail length in *twin* mutants.

Taken together, our data strongly suggest that wild-type Twin/Ccr4 deadenylates a number of downstream target mRNAs, including several cyclins. Because CycA levels are elevated in *twin* mutant ovaries and reducing CycA dose suppresses the *twin* egg chamber degradation phenotype, our data support the model that Twin/Ccr4 deadenylation regulates CycA levels, which in turn affects egg chamber growth and maturation.

Discussion

In *S. cerevisiae* and more recently in *Drosophila*, CCR4 was shown to possess deadenylase activity (Chen et al., 2002; Tucker et al., 2002; Tucker et al., 2001; Temme et al., 2004). It remained unclear whether mutations in CCR4 had specific developmental defects and whether these defects might reveal specific targets sensitive to CCR4 function. Here, we show that *twin* encodes the *Drosophila* homolog of CCR4 and that mutations in *twin* cause defects during early stages of oogenesis. *twin* mutant cysts divide asynchronously and less than four times, oocyte specification is defective and many egg chambers die and degrade. We show that the mitotic cyclins, CycA and CycB, are misexpressed in *twin*, and reducing the gene copy number of *cycA* partially suppresses the *twin* egg chamber degradation phenotype. Furthermore, we show that the poly(A) tails of *cycA*, *cycE* and, to a lesser extent, *cycB* are longer in *twin* extracts, suggesting that Twin/Ccr4 deadenylase activity directly controls the RNA levels of these cell cycle regulators. By contrast, BamC staining is reduced in *twin*. Induction of extra *bam* expression suppresses the cyst division and oocyte fate specification defects in *twin* mutants, implicating low Bam levels as one of the causes of these *twin* phenotypes.

Our *twin* alleles are viable and specifically affect the female germline. In *S. cerevesiae*, *ccr4* mutations are not lethal, although CCR4 is thought to be the main cytoplasmic deadenylase (Denis, 1984). It is possible that *angel* and *Dnocturnin* (CG4796), two other genes with extensive homology to the *ccr4* catalytic domain but lacking the crucial LRR repeats, can partially compensate for loss of Twin function. Alternatively, as our mutations are probably not complete nulls, oogenesis may be more sensitive than the soma to decreased Twin function. Like the ovary, the early embryo relies on precise post-transcriptional gene regulation (Tadros et al., 2003). The mature egg contains high levels of maternally loaded *twin,* consistent with a role for Twin in deadenylation, and probably explaining why *twin* mutants carry out embryogenesis normally.

Control of cyclins in development

Mitotic cells regulate cyclin levels in order to progress through the cell cycle. At the protein level, *Drosophila* regulates CycA, CycB and CycE, via proteasome-mediated degradation (Doronkin et al., 2003; Echard and O'Farrell, 2003; Sigrist et al., 1995). In the *Drosophila* ovary, the novel protein Encore has been proposed to localize components of the proteasome complex to the fusome to regulate CycE (Doronkin et al., 2003; Ohlmeyer and Schupbach, 2003). *encore* mutant cysts undergo an extra cell division and contain 32 cells (Hawkins et al., 1996), probably as a consequence of misexpressing not only CycE, but also CycA (Ohlmeyer and Schupbach, 2003). Other experiments have shown that cyst divisions are sensitive to CycA levels. Adding a brief pulse of CycA by inducing a heat-shock construct can lead to an extra round of cyst division, suggesting that downregulation of CycA is crucial for cell cycle progression (Lilly et al., 2000). Only a small number of cysts respond to such a CycA pulse, suggesting that in the wild type not all germ cells are in a susceptible phase of the cell cycle (G2) during which they can respond to CycA.

Cyclin RNA levels are regulated by control of poly(A) tail

length. In *Xenopus* and mouse oocytes, *cycB* RNA is not translated in the absence of CPEB-mediated poly(A) tail lengthening. Longer poly(A) tails also enhance cyclin translation in *Drosophila* embryos. In the *Drosophila* ovary, Orb, the CPEB homolog, regulates poly(A) tail length and expression of its own RNA and *oskar* RNA (Castagnetti and Ephrussi, 2003; Tan et al., 2001). Consistent with a role for Orb in cyclin regulation and cyst division, *orb* mutant cysts frequently contain eight germ cells (Lantz et al., 1994).

Our data suggest that Twin-mediated deadenylation of cyclin RNA regulates cyst divisions. Cyclin polyadenylation has been well studied, but much less is known about cyclin RNA deadenylation. In *Drosophila*, Nanos and Pumilio have been shown to control deadenylation of *cycB* mRNA in primordial germ cells (Asaoka-Taguchi et al., 1999). Furthermore, *Xenopus* Pumilio interacts with CPEB (Richter and Theurkauf, 2001), and Nanos, Pumilio and Orb/CPEB are all expressed early in *Drosophila* oogenesis (Forbes and Lehmann, 1998). It is intriguing to speculate that Twin may regulate the poly(A) tail lengths in the dividing cyst in conjunction with Nanos, Pumilio and/or Orb.

Twin and Bam

BamC expression is reduced in *twin* germaria; a phenotype we would not predict if Twin directly regulated Bam expression via deadenylation. Indeed, we did not observe a substantial change in *bam* poly(A) tail length in *twin* ovaries. We therefore propose that *bam* is an indirect target of Twin/Ccr4.

Although *bam* is known to control the differentiation of the cystoblast and to promote cyst division, the biochemical role of Bam is unknown. Removing one copy of *bam* suppresses the extra division in cysts lacking *encore* or overexpressing CycA. Our results further implicate Bam in the events of early oogenesis. Increased *bam* expression suppresses not only the cyst division defects observed in *twin* mutants, but also the *twin* oocyte specification defects. Because Twin regulates *cycA* directly and may regulate Bam indirectly, the simplest model would posit that high levels of CycA are sufficient to suppress Bam expression. Two pieces of evidence argue against this model: Bam and CycA are both present at high levels in the dividing cyst; and Bam is required for the fifth cyst division induced by high levels of CycA. In addition, *hs-bam* induces stem cells to develop into normal cysts, indicating that high Bam levels do not disrupt CycA expression. We favor a model by which Bam and CycA act in parallel to each other, downstream of Twin.

Model for *twin* function in the ovary

Although several models could explain our data, we propose that increased mitotic cyclin levels together with low Bam expression cause many of the *twin* phenotypes. If Bam expression were normal, overexpressing cyclins could lead to extra cyst divisions. The low level of Bam in *twin* germaria does not permit continued cell division, yet cyclin levels remain high, delaying cell cycle progression and probably causing the egg chamber degradation we observe in *twin*. This model is consistent with the fact that reducing the copy number of *bam* suppresses the extra cyst division phenotype of *encore* and of *hs-cycA* (Hawkins et al., 1996; Lilly et al., 2000). Corroborating evidence comes from the observation that reducing the gene dose of *cycA* or increasing the dose of *bam* can partially suppress the degradation phenotype. However, there are likely to be other, unidentified targets of *twin* that also contribute to the *twin* phenotype.

twin and *hts* mutants disrupt the number and synchrony of cyst divisions and oocyte specification. This array of defects is not shared by the cell cycle mutants discussed above or by other mutants such as *orb* (Lantz et al., 1994), the M-phase inhibitor *tribbles* or the M-phase activator *string* (Mata et al., 2000), which affect the number but not the synchrony of cyst divisions. Comparison of *twin* and *hts* may therefore be instructive. *hts* cysts have no fusome, and are thought consequently not to coordinate the cyst divisions (Lin et al., 1994; Yue and Spradling, 1992). By contrast, cysts in *twin* mutants contain branched fusomes that are capable of colocalizing with CycA, suggesting the possibility that Twin/Ccr4 gene regulation may mediate the coordination of the cyst divisions with oocyte specification downstream of the fusome.

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