Post-transcriptional regulation of the meiotic Cdc25 protein Twine by the Dazl orthologue Boule

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Boule, a *Drosophila* orthologue of the vertebrate Dazl fertility factors, is a testis-specific regulator of meiotic entry and germline differentiation. Mutations inactivating either Boule, which is an RNA-binding protein, or Twine, which is a Cdc25-type phosphatase, block meiotic entry in males. Here we show that *twine* and *boule* interact genetically. We also find that protein expression from *twine* messenger RNA correlates with cytoplasmic accumulation of Boule and is markedly reduced by *boule* mutations. Remarkably, heterologous expression of Twine rescues the *boule* meiotic-entry defect, indicating that the essential function of Boule at the transition from G2 to M phase during meiosis is in the control of Twine translation.

Spermatogenesis occurs through a complex pattern of cell growth, cell division and cell differentiation. These processes must be precisely coordinated for an organism to produce functional gametes and be successful in reproduction. As such, spermatogenesis provides a model system for understanding how cell division occurs in the context of a complex developmental pathway, as well as for understanding how meiotic and mitotic regulation differ in higher eukaryotes.

The need for distinct regulation of meiosis and mitosis arises from fundamental differences between the two types of cell cycle. Whereas mitotic divisions are equational and preceded by a round of DNA synthesis (S phase), the first division of meiosis is reductional and the second ensues without an intervening S phase. These differences presumably underlie the evolution in both invertebrates and vertebrates of mitosis- and meiosis-specific components of the cell-cycle machinery, as well as distinct modes of regulation of these components.

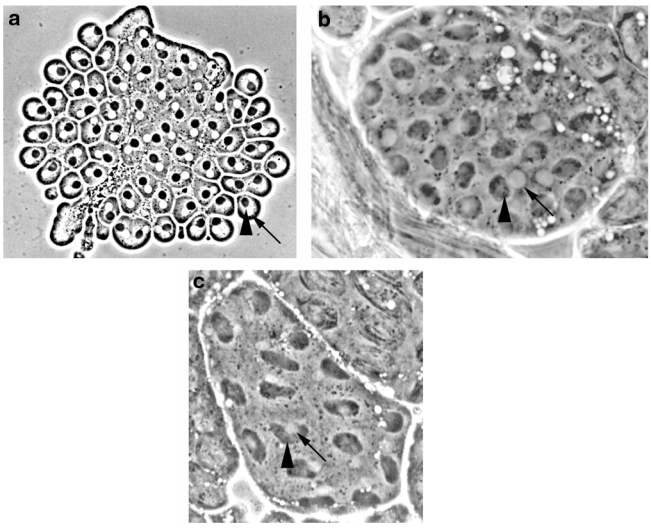

Figure 1 **Phenotypic analysis of meiotic defects in *twine* spermatocytes.** Photographs are of unfixed testis contents viewed by phase-contrast microscopy. In each panel, an arrow points to a single, pale nucleus and an arrowhead to an equally sized, phase-dense mitochondrial derivative in the same cell. **a**, Wild-type, 64-spermatid cyst. **b**, twe^{Z0758} mutant, 32-cell cyst, reflecting the execution of only a single meiotic division. **c**, twe^{Z0758}/twe^{Z0758}; bol^{1}/TM3 mutant, 16-cell cyst; neither meiotic division has occurred. **b** and **c** are at the same magnification (×400), twice that of **a**. The difference in nuclear size between **b** and **c** is not significant but instead reflects variation between the samples with regard to the stage of spermatid differentiation.

In *Drosophila*, there are both a mitotic and a meiotic Cdc25-type phosphatase. The mRNA encoding Twine, the meiotic Cdc25, can be detected early in the spermatocyte growth phase; however, expression of a reporter gene containing *lacZ* fused to *twine* is not detected until much later, just before meiotic entry[1–3]. Twine is thus subject to translational regulation.

Twine is required for meiotic entry in males: spermatocytes lacking *twine* function fail at the G2/M transition and, as a result, carry out incomplete differentiation of tetraploid spermatids. This phenotype is also produced by conditional alleles encoding the cyclin-dependent kinase Cdc2 (refs 1,2,4), as well as by mutations in two other genes, *boule* and *pelota*, that encode proteins predicted to interact with RNA[5,6]. The role of such RNA-binding proteins at the G2/M transition is unclear, but may be related to the need for specialized regulation of the meiotic divisions.

The function in meiotic entry of the RNA-binding protein encoded by *boule* is of particular interest, as *boule* is closely related to both the human Y-linked *Deleted in Azoospermia* (DAZ) gene and the more broadly conserved, autosomal *DAZ*-like (*Dazl*) genes. Deletions in *DAZ* are strongly associated with male infertility[7,8]; targeted inactivation of the murine *Dazl* gene results in both male and female sterility[9]. Furthermore, the *Xenopus* Xdazl protein, which binds RNA *in vitro*, rescues meiotic entry in *boule* mutant flies, consistent with a conservation in function among family members in diverse species[10].

Given that the Boule protein is required for meiotic entry, is a putative RNA-binding protein, and localizes to the cytoplasm before meiosis[11], we have speculated that the similarity in phenotype between *boule* and *twine* mutants might reflect a role for Boule in regulating *twine* translation. Here we provide evidence that the activity of wild-type *boule* is required for efficient translation of *twine* mRNA, and show that the meiotic-entry failure in *boule* mutants can be overcome by heterologous expression of Twine.

Results

A hypomorphic *twine* mutant executes only one meiotic division. During normal *Drosophila* spermatogenesis, the two meiotic divisions occur sequentially and synchronously within a cyst of 16

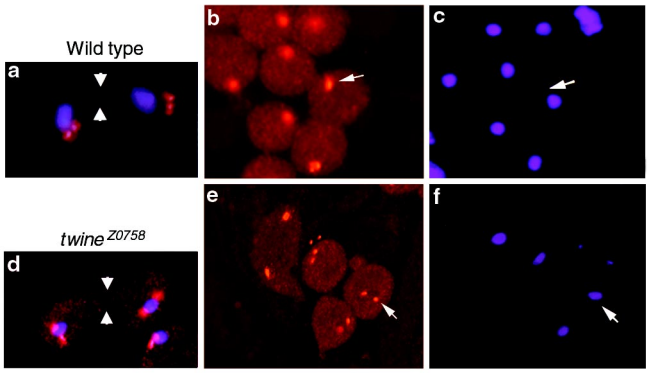

Figure 2 **Centrosome-cycle analysis in *twine* spermatocytes. a–c**, Wild-type and **d–f**, *twe*^{Z0758} cells. **a**, **d**, Cells at telophase of meiosis I, stained for centrosomin (red) and DNA (purple). Arrowheads indicate the position of the cleavage furrow in each cell. **b**, **e**, Centrosomin in post-meiotic spermatids, with **c**, **f**, paired DNA images. Arrows indicate the position of centrosomes in the paired images. In the wild type (**b**, **c**), each cell contains a single centrosome, which will contribute to the basal body. In *twe*^{Z0758} mutants (**e**, **f**), each cell contains two foci of centrosomin, suggesting that meiosis II did not occur.

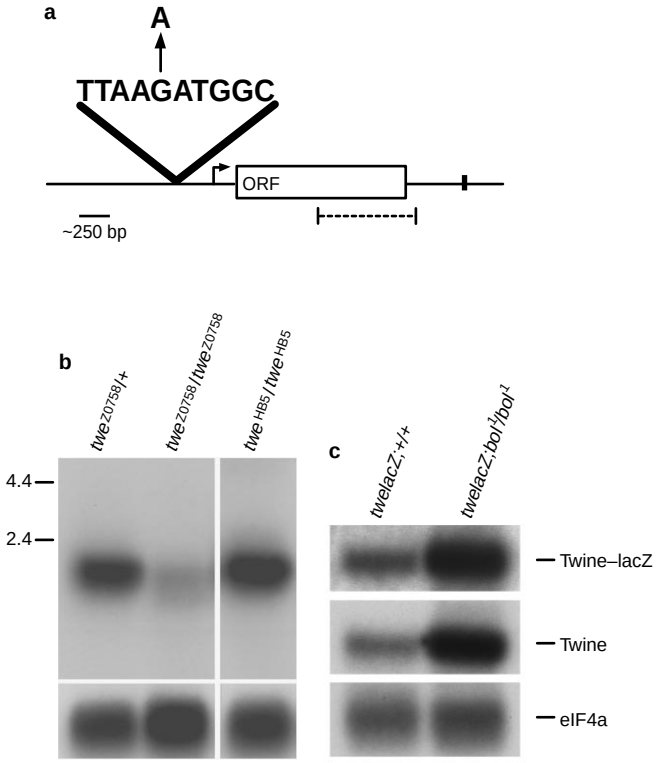

Figure 3 **Molecular genetic analyses of *twine* expression. a**, The *twine* locus. In *twe*^{Z0758}, a single base substitution was detected 418 base pairs upstream of the *twine* open reading frame. Vertical lines indicate the boundaries of the transcriptional unit. Dashed line indicates sequences removed in the *lacZ* reporter fusion[3]. Scale is approximate. bp, base pairs. **b**, **c**, Northern analysis of *twine* and *twine–lacZ*. Each lane was loaded with poly(A)$^+$ RNA from ~100 testes. The probe was cDNA for *twine* (upper panels) or *eIF4a*, a loading control (bottom panels). **b**, Lane 1, *twe*^{Z0758}/CyO; lane 2, *twe*^{Z0758}/*twe*^{Z0758}; lane 3, *twe*^{HB5}/*twe*^{HB5}. **c**, Lane 1, *twine–lacZ/twine–lacZ*; +/+ (wild-type for *boule*); lane 2, *twine–lacZ/twine–lacZ*; *bol*^{1}/*bol*^{1}.

interconnected germ cells, generating 64 spermatids (Fig. 1a). When meiotic entry is blocked, for example by the absence of *twine* or *boule* function, 16-cell cysts persist[1,2,6]. A partial loss of *twine* function brought about by the *twe*^{ZO758} allele results in cysts of 32 cells (Fig. 1b). Thus the *twe*^{Z0758} mutation results in the occurrence of only a single meiotic division in the male germ line.

Analysis of the centrosome cycle provides evidence that the single division occurring in *twe*^{Z0758} homozygotes is a first meiotic (MI) division. In the wild type, centrosomes separate at telophase of MI. As a result, the centrosome-binding protein centrosomin is found in two distinct foci at each pole of the spindle[12] (Fig. 2a). During the second meiotic division, the centrosomes segregate, such that post-meiotic spermatids each contain a single focus of centrosomin staining (Fig. 2b,c). In *twe*^{Z0758} homozygotes, the centrosomes separate, as in wild-type MI, indicating that the single division is an MI division (Fig. 2d–f).

A 3.7-kilobase genomic region that fully rescues the loss-of-function *twe*^{HB5} mutant defines the *twine* gene and its regulatory regions[1]. To determine the nature of the *twe*^{Z0758} mutation, we sequenced this genomic region from homozygous mutant males. We detected a single G-to-A transition 418 base pairs upstream of the *twine* open reading frame (Fig. 3a). The position of this mutation indicates that it may affect a transcriptional regulatory element. To test this hypothesis, we measured *twine* mRNA levels in wild-type and *twe*^{Z0758} mutant testes. The abundance of *twine* mRNA was reduced by at least tenfold in *twe*^{Z0758} testes compared with wild-type testes (Fig. 3b). The *twe*^{Z0758} mutation thus apparently acts by reducing *twine* transcription and thereby lowering the level of Cdc25 phosphatase activity available at meiotic entry.

Boule is required for Twine protein expression. By assaying the progress of spermatogenesis in the *twe*^{Z0758} genetic background, we identified the fertility factor *boule* as a candidate *in vivo* regulator of *twine*. Spermatocytes from *twe*^{Z0758}/*twe*^{Z0758} males with only a single wild-type copy of *boule* fail to enter meiosis (Fig. 1c). Moreover, these cells do not carry out the G2/M transition; unlike wild-type or *twe*^{Z0758}/*twe*^{Z0758} spermatocytes, these cells fail either to relocalize cyclin A from the cytoplasm to the nucleus or to form bipolar spindles (data not shown). Such a phenotype is more severe than that of *twe*^{Z0758}/*twe*^{HB5} flies, in which meiotic entry does occur, and is very similar to that of flies homozygous for loss-of-function alleles of either *twine* or *boule*[1,2,6,13].

To explore the mechanism by which *boule* acts as an enhancer of *twe*^{Z0758}, we assayed Twine expression in genetic backgrounds deficient for *boule*, using a reporter construct[3]. Expression of the Twine reporter protein is dramatically reduced in a *boule* mutant compared with in the wild type (Fig. 4a,b). This reduction occurs at the level of protein, and not RNA, accumulation, as the amounts of *twine–lacZ* and endogenous *twine* RNA present are not reduced but are instead increased in the absence of *boule* (Fig. 3c).

Given that, in wild-type flies, *twine* RNA accumulates well before the onset of meiosis[1,2,11], these data indicate both that Twine is translationally regulated and that efficient Twine translation requires Boule. Support for this hypothesis is provided by a study of the patterns of Boule localization and Twine expression in developing spermatocytes. We have reported previously[11] that Boule protein translocates from the nucleus to the cytoplasm just before the first meiotic division, and is required in the cytoplasm for meiotic entry. Boule relocalization is coincident with the first detectable expression of the Twine reporter (Fig. 4c,d), consistent with cytoplasmic Boule being essential in the translation of Twine protein.

Heterologous Twine expression rescues the *boule* meiotic-entry defect. If *boule* mutants fail in meiotic entry because of inadequate accumulation of Twine protein, heterologous expression of Twine should drive meiotic entry in a *boule* mutant. To test this prediction, we expressed Twine in a *boule*-independent manner by placing *twine* under the control of the spermatocyte-specific β2-*tubulin* (β2) gene promoter and the 5′ and 3′ untranslated sequences of the β2-*tubulin* gene (Fig. 5a)[14,15]. Expression of *twine* in this context is sufficient to restore fertility to either *twe*^{Z0758} or *twe*^{HB5} mutant males (data not shown).

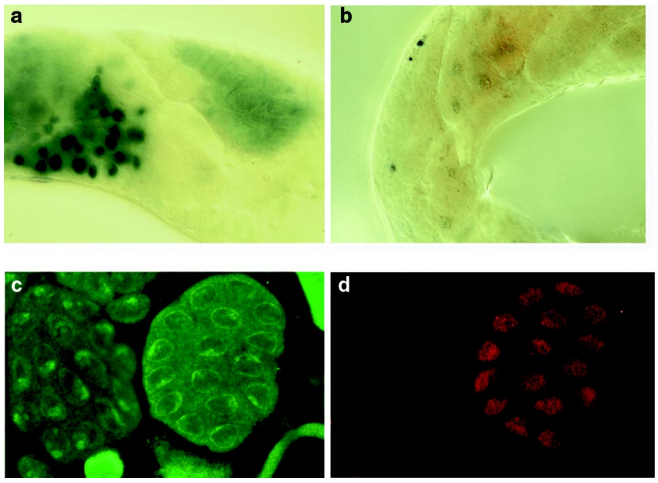

Figure 4 **Expression of the *twine–lacZ* transgene and *boule* in wild-type and mutant testes. a**, **b**, Expression of *twine–lacZ* in wild-type and *boule* mutant testes. **a**, Wild-type testes. Upper right, β-galactosidase (LacZ) staining of late primary spermatocytes. Lower left, punctate staining is of spermatids. **b**, bol^1/bol^1 mutant testes. Primary spermatocytes do not stain. Faint staining is of spermatids. **c**, **d**, Coordinate timing of Boule cytoplasmic accumulation and Twine–LacZ expression. **c**, Nuclear accumulation of Boule is prominent in the 16-cell cyst on the left, and cytoplasmic Boule accumulation is promiinent in the cyst on the right. **d**, Anti-β-galactosidase shows that Twine–LacZ protein accumulates only in the cyst that shows Boule cytoplasmic staining (right).

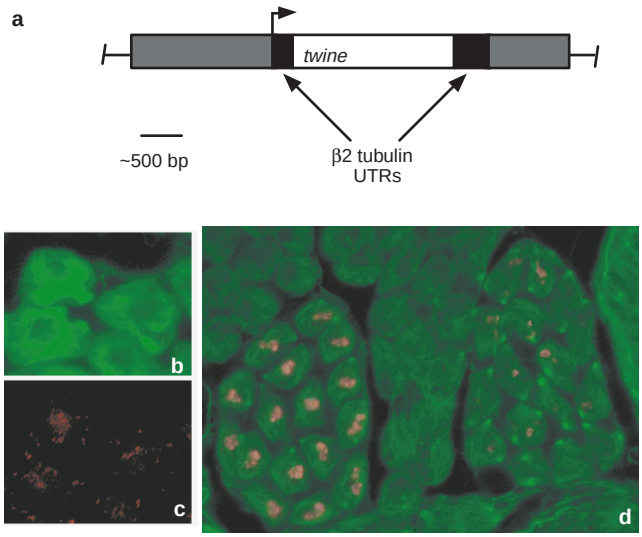

Figure 5 **Restoration of meiotic entry in *boule* mutants by heterologous Twine expression. a**, Line drawing of the β2–*twine* construct. Shaded areas represent the β2-*tubulin* genomic sequence; black areas represent the β2-*tubulin* transcriptional start and untranslated region (UTRs); open bar represents *twine* coding sequence. **b**, **c**, Immunostained bol^1/bol^1 testes, showing anti-tubulin staining in green and staining for phosphohistone-H3-positive chromosomes in orange. **d**, Immunostained β2-*twine*; bol^1/bol^1 testes. Metaphase of meiosis. The presence of the meiotic spindle (anti-tubulin staining, green) and phosphohistone-H3-positive chromosomes (orange) indicates that meiotic entry and the metaphase transition have occurred. These features are not visible in the *boule* mutant (**b**, **c**).

Introduction of the β2–*twine* construct into a *boule* mutant drives meiotic entry, with some cells completing at least one division in all individuals studied. Meiotic spindles, which are absent in *boule* mutant males lacking the β2–*twine* transgene (Fig. 5b,c), are easily apparent in transformed lines (Fig. 5d). Although the observed rescue could result from *twine* RNA overexpression, this seems unlikely. First, amounts of endogenous *twine* mRNA are already quite high in *boule* mutants. Second, the rescue is not dose dependent and the presence of either one or two copies of the β2–*twine* transgene has no deleterious effect in the wild type. Furthermore, the activity of the β2–*twine* construct in a *boule* mutant does not reflect a general ability to drive meiotic entry. For example, although the male sterile phenotype produced by mutation of the *pelota* gene closely resembles that produced in *twine* or *boule* mutants[5], the β2–*twine* construct does not restore meiotic entry in a *pelota* male (data not shown). Thus, the β2–*twine* transgene appears to specifically compensate for the defect in endogenous Twine translation in a *boule* mutant, thereby restoring meiotic entry.

Discussion

Our results allow us to order the function of Boule relative to Twine, placing Boule upstream of the core cell-cycle machinery. They further indicate that the failure in meiotic entry of *boule* mutants is due to a failure to express wild-type levels of Twine protein. Boule cannot be required to stabilize the *twine* mRNA, as the *twine* message is abundant in a genetic background lacking *boule* activity. Instead, Boule is likely to play a part in Twine translation.

Given that Boule contains an RNA-recognition motif (RRM), we believe that Boule could influence Twine expression through direct binding to the *twine* mRNA. Twine is not, however, the sole target of Boule activity, as *boule* mutants show defects in spermatid differentiation that are absent in *twine* mutant males[6]. Taken together, these data indicate that Boule may be required for both meiosis and spermatid differentiation, and may have a role in coordinating these two events.

The *boule* and *twine* gene products act downstream of a second set of genes required for meiotic entry. The products of these genes, which include *spermatocyte arrest* (*sa*) and *meiosis I arrest* (*mia*), are also required for the expression of Twine protein but not of *twine* mRNA[3]. We have found that mutations in these genes result in a failure to accumulate Boule protein (M. Cheng and S. Wasserman, unpublished observations). This inability of *sa* and *mia* mutants to express Boule and therefore Twine presumably contributes to their failure to initiate meiotic divisions.

Although proteins of the Dazl family are required for fertility in several organisms[7,8,9] (T. Karashima, A. Sugimoto and M. Yamamoto, personal communication), little is known about their biochemical function. Given the similarities in sequence and in expression patterns among Boule and other Dazl-family members, and also in the phenotypes induced by mutation of these proteins[6,9,10,16], we suggest that a role in translation or translational control will be a general property of such proteins. Moreover, given that inactivation of the murine Dazl protein results in a proliferation defect[9], it is possible that transcripts encoding Cdc25 proteins will prove to be a general target for Daz-related proteins. □

Methods

Drosophila strains.

The twe^{HB5} and bol^1 mutations have been described previously[1,2,13]. The *ZO758* mutation was obtained from a collection of 12,000 heavily mutagenized and homozygous viable autosomal lines generated in the laboratory of C. Zuker by E. Koundakjian, D. Cowan, B. Hardy and C. Zuker. B. Wakimoto and D. Lindsley identified *ZO758* as producing male-sterile mutants with a defect in spermatogenesis. Recombination mapping revealed that *ZO758* lies at position 51 on the genetic map of the second chromosome; complementation experiments showed that *ZO758* is an allele of *twine*. The twe^{HB5} and twe^{ZO758} alleles were balanced with CyO; bol^1 was balanced with TM3. All crosses were performed at 22 or 25 °C on yeasted cornmeal molasses agar.

DNA manipulations and analyses.

Genomic sequence was obtained by direct sequencing of polymerase-chain-reaction (PCR)-amplified genomic DNA from the twe^{ZO758} stock, as well as from a control line, *Z2146*. For each fragment, four independent PCR reactions were pooled and sequenced directly in both directions using an automated sequencer (Applied Biosystems). Sequences obtained from twe^{ZO758} and strain *Z2146* were compared to each other, as well as to the available genomic sequence (Berkeley *Drosophila* Genome Sequencing project

accession numbers L49408 and AC003701).

The *twine–lacZ* reporter substitutes the *lacZ* gene for a 700-base-pair fragment of the *twine* gene[3], truncating the Twine protein and removing ~60 nucleotides of 3′ untranslated sequence. To generate the β2–*twine* rescue construct, we cloned the *twine* cDNA[2] as a 1.7-kilobase *Eco*R1 fragment from the BS vector into the *Eco*R1 sites of the β2-*tubulin* testis vector[14]. Both the reporter and the rescue constructs were generated in P-element transformation vectors carrying the w^+minigene. P-element vectors were co-injected with a plasmid containing P[ry^+, Δ2–3] into w^{1118} embryos using standard procedures[17].

RNA analysis.

Poly(A)$^+$ RNA was isolated from the testes of young adult males (~30 per sample) using the FastTrac isolation kit (Invitrogen). Northern analysis was performed according to standard methods[18]. Northern blots were hybridized with a DNA probe recognizing the 5′ end of the *twine* open reading frame. The *Drosophila eIF4a* cDNA[19] was used as a probe for loading control.

Analysis of contents of live and fixed testes.

Testes from newly eclosed males were dissected in Ringer's solution and flattened under a coverslip before analysis by phase microscopy[20]. Fixation and immunostaining for all antibodies followed the method of ref. 21. Tubulin was detected with a monoclonal antibody clone DM1 (Sigma) used at 1:500 dilution. The M-phase marker phosphorylated histone H3 was detected with a polyclonal antiserum (Upstate Biotechnologies; 1:200). β-Galactosidase was detected with a polyclonal antiserum (Promega; 1:500). Centrosomin was detected with an affinity-purified polyclonal antiserum (1:500; provided by T. Kaufman). Fluorescently labelled secondary antibodies (Jackson Laboratories) were used at 1:500. DNA was also stained with 1 mg ml^{-1} Hoechst 88842 (Sigma). Fluorescent images were recorded from a Leica DMRXE microscope equipped with a charge-coupled-device (CCD) digital imaging camera (DIC; Hamamatsu). Digital images were converted to Adobe Photoshop for printing.

β-Galactosidase-activity assay.

β-Galactosidase activity in the male germ line was assayed according to ref. 3. Testes from newly eclosed males were stained overnight at 37 °C in X-gal buffer containing 0.01–0.1% X-gal. Testes from wild-type and *bol* homozygotes were stained simultaneously in a common X-gal bath to ensure uniform development. Images were obtained using a Nikon E800 microscope equipped with DIC optics and a Spot CCD camera.

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