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Meiotic cell cycle requirement for a fly homologue of human *Deleted in Azoospermia*

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INFERTILITY resulting from a severe defect in sperm production affects 2% of men worldwide[1,2]. Of these men with azoospermia, the absence of sperm in semen, one in eight carry *de novo* deletions for a specific region of the Y chromosome[3–5]. A candidate gene for the Y-chromosome azoospermia factor (AZF) has been identified and named *Deleted in Azoospermia* (*DAZ*)[5]. Here we describe the cloning and characterization of the *Drosophila* gene *boule*, which is a homologue of *DAZ*. The two genes encode closely related proteins that contain a predicted RNA-binding motif, and both loci are expressed exclusively in the testis. Loss of *boule* function results in azoospermia; meiotic divisions are blocked, although limited spermatid differentiation occurs. Histological examination of *boule* testes with cell-cycle markers indicates that the primary defect is at the meiotic G2/M transition. These results support the hypothesis that DAZ is the human AZF, and indicate that Boule and DAZ have an essential meiotic function in fly and human spermatogenesis.

The *boule* (*bol*) gene was identified during screening for transposon-induced, male-sterile mutations in *Drosophila*[6]. Sequencing of several *boule* complementary DNAs indicated that *boule* encoded a protein of 228 amino acids that contained a single ribonucleoprotein (RNP)-type RNA-binding domain[7,8] (Fig. 1*a–c*). Database comparisons indicated that the putative RNA-binding domain of the Boule protein is most similar (42% identity) to that of the human DAZ protein and a closely related mouse protein, Dazla[9]. Boule also has considerable sequence similarity (33% identity) to a second region of DAZ and Dazla (Fig. 1*d*). This region contains a motif, termed a DAZ repeat, that is present once in the mouse sequence and seven times in the human protein[5,9]. Strikingly, the positions of the RNP domain and the first DAZ repeat are conserved among the fly, mouse and human proteins (Fig. 1*b*). The human *DAZ* gene maps to a 500-kilobase region of Yq that is frequently deleted in azoospermic men; the mouse gene, like *boule*, is autosomal.

Northern analysis of RNA from adult male and female flies demonstrated that *boule* expression is limited to males (Fig. 2*a*). The *boule* transcript is absent from flies lacking a germ line, indicating that expression is testis specific (Fig. 2*a*). In *bol*^{1} homozygotes this transcript, which is normally 3.0 kb in length, is truncated to just 1.1 kb (Fig. 2*b*, lane 6); in *bol*^{2} flies the messenger RNA is severely reduced in abundance (Fig. 2*b*, lane 8). The defect in spermatogenesis seen with either allele in *trans* to a deletion is indistinguishable from that seen with *boule* homozygotes, suggesting that the phenotype of the *boule* gene could be brought about by a strong or complete loss of gene function.

To demonstrate that *boule* is active in the germ line, we performed transposon-mediated germ line transformation. The protein-coding region of *boule* was cloned into a P-element vector carrying 5′ sequences from the *Drosophila* β2-tubulin locus that direct expression in postmitotic germ cells of the testis[10]. When introduced into *boule* mutant flies, this construct resulted in meiotic products in more than half of the spermatid cysts. Furthermore, all spermatid cysts underwent extensive, albeit incomplete, spermiogenesis (data not shown). This partial rescue observed with the *boule* transgene suggests that the locus has a dosage-sensitive role in male germ-cell development. Indeed, minor, but reproducible, defects in spermatogenesis are seen in the testes of males bearing only one wild-type copy of *boule*.

The appearance of the wild-type *boule* transcript during the life

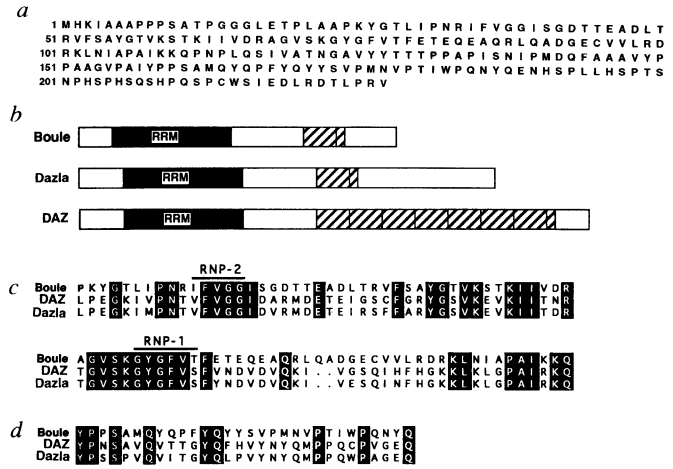

FIG. 1 Comparison of structure and sequence for Boule and DAZ proteins. *a*, Predicted Boule protein sequence. *b*, Domain organization of Boule and DAZ proteins. Scale drawings are of Boule, Dazla, (a mouse DAZ-like protein[9]) and the human protein DAZ[5]. RNA-recognition motif (RRM) RNA-binding domains and DAZ motifs are indicated by black and shaded boxes, respectively. *c*, *d*, Alignment of the RRM (*c*) and DAZ (*d*) domains of boule, DAZ and Dazla. Highlighted amino acids are identical in all three proteins. In *c*, the RNP domains are indicated by lines above the sequence. *d*, Alignment of the 24 amino-acid DAZ motifs and a 5 amino-acid extension found in Boule, DAZ (last repeat) and Dazla.
METHODS. Bacteriophage M13 sequencing was performed with an Amersham Sequenase kit. Sequences were assembled using AssemblyLIGN (IBI/Kodak). Homology searches used the BLASTP program[24].

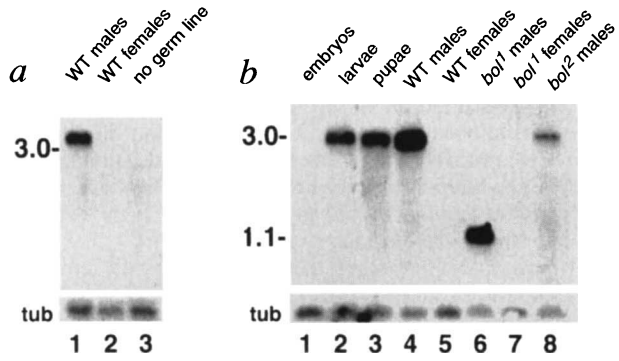

FIG. 2 Northern analysis of *boule* transcripts. Autoradiographs are of northern blots (2 μg of poly(A)$^+$ RNA per lane) probed with *boule* cDNA (top), then stripped and reprobed with *α1 tubulin* as a loading control (bottom). Indicated to the left are *boule* transcript sizes in kilobases. *a*, Lane 1, wild-type (WT) males; 2, wild-type females; 3, males and females lacking a germ line. *b*, Lane 1, wild-type embryos; 2, wild-type larvae; 3, wild-type pupae; 4, wild-type males; 5, wild-type females; 6, bol^1 males; 7, bol^1 females; 8, bol^2 males.

METHODS. The *boule* locus extends over more than 50 kb in polytene interval 66F on the third chromosome, and is uncovered by *Df(3L)29A6* (ref. 25). To generate the chromosomal walk for *boule*, a phage (EMBL3) genomic library (supplied by J. Tamkun) was screened with genomic probes flanking the bol^2 P-element insertion site. The *boule* cDNAs were isolated from both a testis library (Bluescript vector) (provided by T. Hazelrigg) and an adult male library (λZAPII vector) (provided by T. Karr). RNA isolation from flies and germ line transformation followed standard techniques[26]. Flies lacking a germ line were generated as the progeny of females homozygous for the $oskar^{301}$ mutation[27]. The P-element testis expression vector has been described previously[10].

cycle coincides with the onset of testis development and spermatogenesis[11] (Fig. 2*b*, lanes 1–4). Consistent with this pattern of expression, *boule* seems to be required for spermatogenesis only, because *boule* homozygotes have no visible defects, *boule* females are fertile, and viability in both sexes is normal. In humans, the function and expression of *DAZ* also seem to be limited to the testis.

The defects in spermatogenesis that are associated with an absence of *boule* or *DAZ* function have substantial similarities. Spermatocytes are formed in *boule* homozygotes, but fail to undergo meiotic divisions. No meiotic figures are observed, and the products of meiosis, 64-cell spermatid cysts, are absent. In individuals deleted for the *DAZ*-containing region of Yq, the postmeiotic stages of the spermatogenesis are similarly rare or absent. Some affected men display maturation arrest, in which spermatogenesis is completely, or nearly completely, stopped at the end of meiotic prophase. Others have a 'Sertoli-cell only' condition, which is defined by the lack of germ cells in testis biopsy. However, such 'Sertoli-cell only' testes frequently contain foci of germ cells in maturation arrest[5,12]. Indeed, it has been postulated that germ-cell degeneration may be a secondary consequence of meiotic arrest in humans[13].

The consistent phenotype of *boule* mutants, together with the accessibility of spermatogenesis in *Drosophila*[14,15], allowed us to define more precisely the point at which *boule* mutations disrupt the meiotic cell cycle. Close examination of mature *boule* spermatocytes indicates that they have a wild-type morphology[6]. Furthermore, BrdU labelling shows that the mitotic and premeiotic S phases occur as in wild-type flies (data not shown). Comparison of the localization of cyclin A in *boule* germ cells with that in wild-type flies supports the conclusion that the meiotic prophase is normal in *boule* germ cells. In the wild type, cyclin A is exclusively cytoplasmic during the extended premeiotic G2 phase (Fig. 3*a*, arrow), but begins to enter nuclei at the transition between G2 and M phases (Fig. 3*a* inset, arrow). Cyclin A is for a short while present only in nuclei, before being degraded early in metaphase[16]. In *boule*, cyclin A is also initially excluded from nuclei, but then translocates into nuclei as chromosomes condense (arrow in Fig. 3*b*, arrowheads in Fig. 3*d*).

Although the meiotic prophase appears wild type in *boule* germ cells, subsequent stages are aberrant. Cyclin A, which in wild-type flies degrades rapidly after nuclear translocation, persists in *boule* nuclei (Fig. 3*c*). Chromosomes remain at the nuclear periphery (Fig. 3*e*), and centrosome separation occurs, but the centrosomes do not reach the poles and do not nucleate asters (data not shown). The nuclear lamina fails to break down in *boule* spermatocytes (Fig. 4). The simplest interpretation of these results is that the transition to metaphase does not occur in *boule* mutants. It remains possible, however, that loss of *boule* function alters the timing of events during spermatogenesis.

Spermiogenesis, which is the differentiation of spermatid structures that follows meiosis in the wild type, continues to a limited extent in *boule*. In particular, mitochondria begin to form into specialized nebenkern structures in *boule* tetraploid spermatids. A

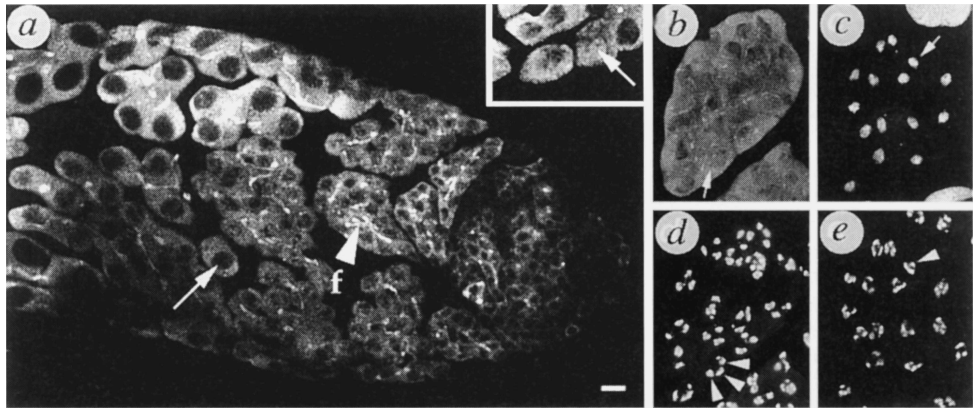

FIG. 3 Cyclin A localization in wild-type and *boule* mutant germ cells. Fixed testis contents were stained with monoclonal cyclin A antibody (*a*–*c*) or the DNA-binding dye Hoechst (*d*, *e*); scale bar, 10 μm. *a*, Wild type. The distal 10% of the testis is shown, with the tip to the right. Cyclin A is excluded from the nuclei of the growth-phase spermatocytes (arrow), and is concentrated in the branched fusome[28,29] that interconnects the developing spermatocytes within a 16-cell cyst (f, arrowhead). The inset shows cyclin A translocation into the nuclei of mature spermatocytes (arrow) late in meiotic prophase; it becomes exclusively nuclear, and is then rapidly degraded in metaphase of meiosis I[14]. *b*–*e*, bol^1 homozygotes. Paired images of cyclin A localization (*b*, *c*) and chromosome condensation (*d*, *e*). *b*, *d*, Mature *boule* spermatocytes. Cyclin A has begun to move into the nuclei of these cells (*b*, arrow) and the chromosomes have partly condensed (*d*, arrowheads). *c*, *e*, *boule* tetraploid spermatids. Cyclin A is concentrated in the nuclei of these cells (*c*, arrow), although the chromosomes remain at the nuclear periphery (*e*, arrowhead).

METHODS. Fluorescent studies were performed using a Zeiss Axiophot microscope. Indirect immunofluorescence of squashed testis contents from males 0–1 days old followed a previous method[30]. Monoclonal antibodies to *Drosophila* cyclin A (from P. O'Farrell) were used undiluted. Cy3-conjugated donkey anti-mouse antiserum (Jackson Labs) was used at a 1:200 dilution.

failure to perform meiosis, accompanied by limited differentiation of tetraploid spermatids, has also been observed for mutations in two other *Drosophila* loci, namely *pelota* and *twine*[17–20]. The biochemical function of *pelota* has not been defined, but *twine* is known to encode the meiosis-specific homologue of Cdc25 phosphatase.

The locus *twine* is an established regulator of M-phase progression. The similarity in phenotype between *boule* and *twine* suggests that *boule*, and by analogy *DAZ* in humans, controls meiotic cell divisions. As putative RNA-binding proteins, Boule and DAZ might be involved in the processing, localization or translation of mRNA[7,8]. Although several other proteins in the RNP family have been implicated in the regulation of spermatogenesis in *Drosophila* and vertebrates, they are generally required during postmeiotic differentiation, a stage in which there is substantial translation of stored mRNAs[21,22].

We have shown that *boule* resembles *AZF* in loss-of-function phenotype, and *DAZ* in both testis-specific pattern of expression and amino-acid sequence. These findings provide strong support for the hypothesis that *DAZ* is *AZF*. Comparative studies of fertile and infertile men indicate that mutations in *DAZ* are extremely frequent, occurring at a frequency of at least 1 in 8,000 men, and that *DAZ* deletions contribute to cases of oligospermia, which is reduced sperm count, as well as azoospermia[5,23]. Future investigations into the function of *DAZ* and *boule* might provide further insight into both the regulation of the meiotic cell cycle and the physiological basis of a significant determinant of human infertility. □

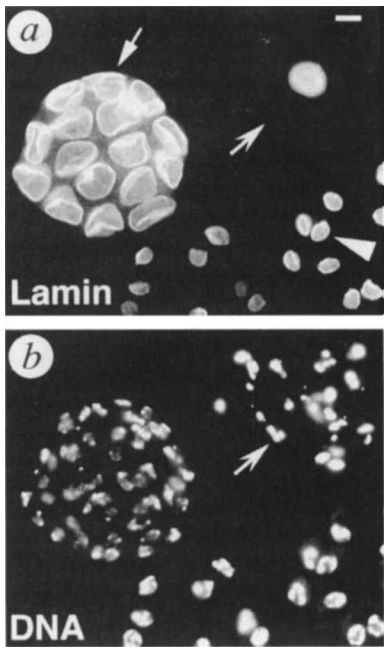

FIG. 4 Persistence of the nuclear lamina in *boule*. Fixed testes contents; spermatocytes and tetraploid spermatids from a *bol*^{1} homozygote. The images are paired, showing staining of the same region with either: *a*, antibodies to nuclear lamin; or *b*, the DNA-binding dye Hoechst. Scale bar, 10 μm. The nuclear lamina is clearly visible in mature *boule* spermatocytes (*a*, arrow). It is still intact in *boule* tetraploid spermatids (*a*, arrowhead) in which the mitochondria have begun to form the spermatid nebenkern. The nuclear lamina is eventually degraded, and is not present in older *boule* tetraploid spermatids (*a*, *b* notched arrows), although the lamina surrounding the large somatic cyst nucleus is still clearly visible (*a*, top right). METHODS. Sample preparation and analysis were as in Fig. 3. Monoclonal anti-lamin antibodies (from D. Glover) were used undiluted.

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Functional receptor for GDNF encoded by the c-*ret* proto-oncogene

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GLIAL-CELL-LINE-DERIVED neurotrophic factor (GDNF) promotes the survival and phenotype of central dopaminergic[1,2], noradrenergic[3] and motor neurons[4–6], as well as various subpopulations of peripheral sensory and sympathetic neurons[7,8]. GDNF is structurally related to members of the transforming growth factor (TGF)-β superfamily[9], several members of which have well-characterized receptor systems[10,11]; however, GDNF receptors still remain undefined. Here we show that GDNF binds to, and induces tyrosine phosphorylation of, the product of the c-*ret* proto-oncogene, an orphan receptor tyrosine kinase, in a GDNF-responsive motor-neuron cell line. Ret protein could also bind GDNF and mediate survival and growth responses to GDNF upon transfection into naive fibroblasts. Moreover, high levels of c-*ret* mRNA expression were found in dopaminergic neurons of the adult substantia nigra, where exogenous GDNF protected Ret-positive neurons from 6-hydroxydopamine-induced cell death. Thus the product of the c-*ret* proto-oncogene encodes a functional receptor for GDNF that may mediate its neurotrophic effects on motor and dopaminergic neurons.