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Human *BOULE* gene rescues meiotic defects in infertile flies

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Defects in human germ cell development are common and yet little is known of genes required for germ cell development in men and women. The pathways that develop germ cells appear to be conserved broadly, at least in outline, in organisms as diverse as flies and humans beginning with allocation of cells to the germ cell lineage, migration of these cells to the fetal gonad, mitotic proliferation and meiosis of the germ cells, and maturation into sperm and eggs. In model organisms, a few thousand genes may be required for germ cell development including meiosis. To date, however, no genes that regulate critical steps of reproduction have been shown to be functionally conserved from flies to humans. This may be due in part to strong selective pressures that are thought to drive reproductive genes to high degrees of divergence. Here, we investigated the micro- and macro-evolution of the *BOULE* gene, a member of the human *DAZ* (*deleted in azoospermia*) gene family, within primates, within mammals and within metazoans. We report that sequence divergence of *BOULE* is unexpectedly low and that rapid evolution is not detectable. We extend the evolutionary analysis of *BOULE* to the level of phyla and show that a human *BOULE* transgene can advance meiosis in infertile *boule* mutant flies. This is the first demonstration that a human reproductive gene can rescue reproductive defects in a fly. These studies lend strong support to the idea that *BOULE* may encode a key conserved switch that regulates progression of germ cells through meiosis in men.

INTRODUCTION

Many genes with direct and specific roles in reproduction are remarkable for their poor conservation even between closely related species. Although rapid evolution of reproductive genes may be relevant to speciation (1,2), the pathways that develop germ cells appear, in contrast, to be conserved broadly at least in outline. From flies to humans, reproductive pathways begin in the early embryo with allocation of cells to the germ cell lineage and migration of these cells to the somatic gonad. The pathways continue with programs of mitotic proliferation, entry and progression through meiosis, and finally culminate with maturation of immature germ cells into fully-differentiated and functional sperm and eggs (3). The molecular mechanisms that coordinate these pathways may also be conserved between distant species and yet no genes that comprise or regulate these critical reproductive processes have been shown to be functionally conserved from flies to humans. Here we show that one gene that is a candidate for functional conservation is the human *BOULE* gene, a member of the *DAZ* gene family (4,5).

The *DAZ* gene family was first identified in a search for Y chromosome genes whose deletion causes infertility. Y chromosome deletions occur in 10–15% of men with spermatogenic defects, and among the genes absent from such chromosomes are members of the *deleted in azoospermia* (*DAZ*) family (5). The *DAZ* genes encode germ cell-specific RNA binding proteins and form a cluster of four genes on the Y chromosome (5–7). In addition, humans have a *DAZ-Like* (*DAZL*) gene on chromosome 3 (6,7). More recently, we identified another autosomal *DAZ* homolog, *BOULE*. It was identified as a DAZ-binding protein in a two-hybrid screen and its interaction with DAZ has been confirmed by co-immuno-precipitation (F. Moore and R. A. Reijo Pera, unpublished data). Homologs of the *DAZ* gene family have also been identified throughout metazoans, where they are inevitably expressed only in the germ cell lineage (8–12). Comparative sequence analysis indicated that *BOULE* represents the ancestral sequence for the *DAZ* family (4) and our recent identification and analysis of *Boule* genes from chimpanzee and Old World monkeys is consistent with this suggestion (Fig. 1).

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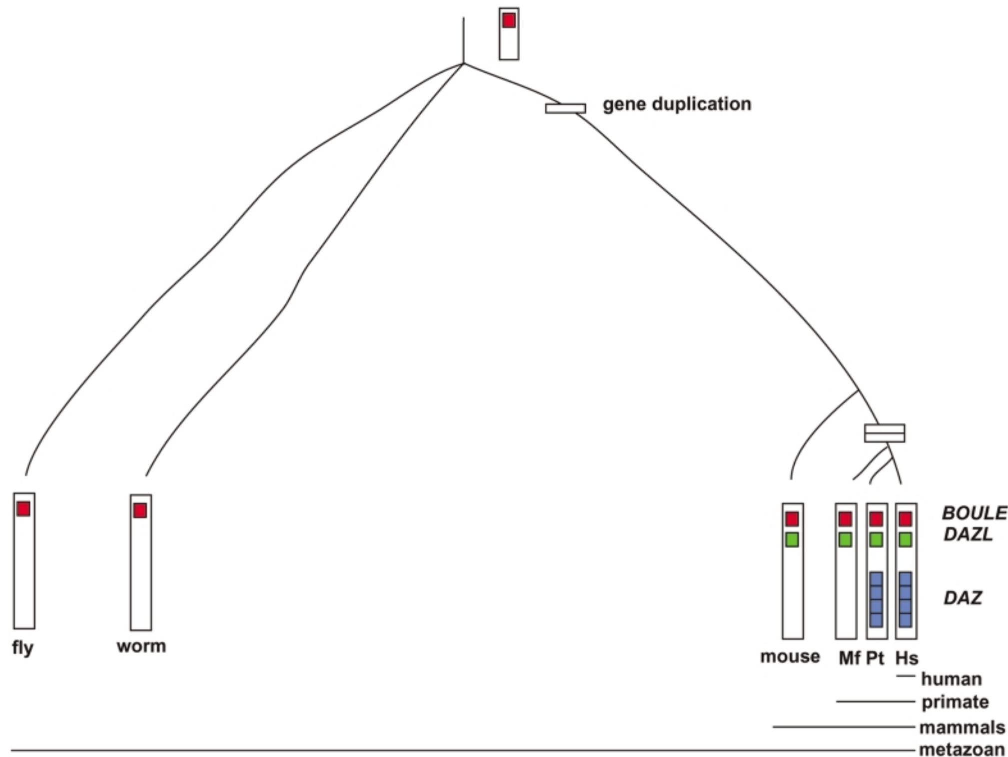

Figure 1. Phylogenetic overview of the *DAZ* gene family with emphasis on species with identified *BOULE* homologs. There is a single member of the gene family in invertebrates, which is more closely related to human *BOULE* than to either *DAZL* or *DAZ* based on sequence and expression pattern (4). *DAZL* probably arose via duplication of ancestral *Boule* and *DAZ* arose via duplications of *DAZL* in the primate lineage. The lines indicate the scope of our evolutionary analyses of the *BOULE* gene: variation of *BOULE* gene within human population, divergence in primate lineage between human, chimpanzee (Pt) and Old World Monkey (Mf), divergence within lineages of mammals and divergence across metazoans.

To explore the function of *BOULE* further, we examined the sequence variation of *BOULE* in humans, close primate relatives and other mammals, and we asked whether *BOULE* is subject to the rapid evolution typical of reproductive genes in recent evolutionary history, and also whether human *BOULE* might be functionally conserved through distant evolutionary history, from humans to flies.

RESULTS

Variation of the *BOULE* gene in humans

To examine sequence variation of *BOULE* within humans, we sequenced DNA samples obtained from 164 infertile men. Although the population we studied was diverse and encompassed many of the ethnicities in the northern California area, no nucleotide variation, synonymous or non-synonymous, was detected in the coding region or 3′ untranslated region (UTR) of *BOULE*. Instead, we found just three nucleotide substitutions—two in intron 3 and one in intron 10. We further verified the lack of variation in *BOULE* in an independent survey of the ethnically diverse NIH Coriell human reference DNA panel (containing 100 individuals); no variation other than that in introns 3 and 10 was detected. The nucleotide diversity of human BOULE (pi) was calculated as 3.5×10^{-4}, lower than many other human genes (13). The low variation is mainly due to the absence of any polymorphism in exon sequences as intronic sequences exhibit a normal SNP frequency and nucleotide diversity (Table 1). Tajima's statistic test (Table 1) shows that polymorphism frequency in *BOULE* region is not significantly different from expectation under neutral theory ($P < 0.10$). Further comparison of human *BOULE* polymorphism/divergence between human and Old World monkeys (*Macaca fascicularis*) with those of other human genes by HKA test is also insignificant (data not shown), suggesting low variation of human *BOULE* is most likely due to strong function constraint on the *BOULE* gene.

Molecular evolution of the *BOULE* gene in primates and other mammals

As many reproductive proteins have been shown to undergo rapid evolution in diverse lineages including primate and human lineages (1), we decided to determine if any rapid evolution is detectable in BOULE protein during primate and mammalian evolution. We compared the *Boule* sequences of human, chimpanzee, Old World monkey, mouse and rat and tallied the non-synonymous substitution (K_a) and synonymous substitution (K_s) rates. A ratio of K_a:$K_s > 1$ suggests positive selection. Results indicated that none of the lineage comparisons demonstrate detectable positive selection (Table 2).

Table 1. The sequence variation of coding, non-coding and intronic regions of human *BOULE* from 164 men

	Length (bp)	Polymorphic sites	Single-nucleotide polymorphism frequency	π	θ	Tajima's *D*
Total length	2046	3	1/682 bp	0.00035 (0.00002)	0.00023 (0.00014)	0.78127
Coding region	849	0	0	0	NA	NA
Non-coding region	325	0	0	0	NA	NA
Intronic	872	3	1/291 bp	0.00082 (0.00004)	0.00054 (0.00032)	0.78127

These results are in sharp contrast to evolutionary analysis of other members of the *DAZ* gene family that indicated that the *DAZ* and *DAZL* homologs have diverged rapidly in primates (14,15). Instead, the *BOULE* gene appears to be strongly conserved. It is of interest to note that ka/ks ratio are 2–3-fold different between rat–human and mouse–human, which is indicative of functional change in the mouse and rat lineages. Identification of complete *BOULE* cDNA sequences in rat and other mammalian species and further analyses are needed to provide a complete picture of changes of evolutionary rate of *BOULE* genes during mammalian evolution.

Table 2. Synonymous (K_s) and nonsynonymous (K_a) nucleotide substitution rate for coding sequence of human and mammalian *BOULE*

	Substitutions	Total sites	K_a	K_s	K_a:K_s
Human/chimp	1	308	0.000	0.0102	0.000
Human/OWM[a]	2	308	0.000	0.0203	0.000
Human/mouse	80	1084	0.0347	0.257	0.135
Chimp/mouse	2	308	0.0389	0.290	0.134
Old World monkey/mouse	55	653	0.0300	0.266	0.113
Rat/mouse	9	244	0.0062	0.130	0.0477
Rat/human	30	244	0.0831	0.221	0.377

Chimpanzee DNA sequence was amplified and sequenced using human *BOULE* primers that anneal to intron sequences and span the exons. Chimpanzee sequence is exons 2, 3 and 7; rat sequence is from a genomic clone (exons 9 and 10). Old World monkey (*M.fascicularis*) *BOULE* cDNA sequence is from GenBank (AB074454).

[a]There is one deletion of 63 amino acids in the carboxyl terminus of OWM BOULE. Rat *Boule* sequence was inferred from a genomic clone (AC103419, *Rattus norvegicus* clone CH230-145A24) using mouse *Boule* sequence as a query. K_a and K_s values were calculated using K-Estimator version 5.5 (28).

Functional conservation of the *BOULE* gene in humans and flies

Given the conservation of the *BOULE* genes in humans, primates and other mammals, we next explored the extent of conservation of *BOULE* in metazoans. As shown in Figure 1, homologs of the *DAZ* gene family exist throughout metazoans with those in the fly and worm being the most distant from humans. *Drosophila boule* encodes a protein 42% similar to the human BOULE protein (4). Mutations of fly *boule* cause infertility in males due to arrested meiosis (12,16). To test whether human *BOULE* can function in flies, we generated five independent lines of flies that carry a human *BOULE* transgene. We then compared testis histology of wild-type flies to that of *boule* mutant flies carrying either a fly or human transgene and to infertile *boule* mutant flies without a transgene (Fig. 2). In wild-type flies, sperm were continuously produced, and cells at all stages of spermatogenesis were present in adult testis (Fig. 2A and B). In contrast, testis in *boule* mutant flies produced no sperm and had germ cells that were arrested before meiosis. The mutant testis contained only somatic cells, germ line stem cells and arrested and degenerating cysts of premeiotic spermatocytes; no mature spermatids were observed (Fig. 2C and D). The meiotic defect observed in fly *boule* mutants was alleviated by addition of a fly *boule* transgene, as reported previously (12), and spermatogenic cells progressed beyond the meiotic block to form postmeiotic spermatids (Fig. 2E and F). Remarkably, the human *BOULE* transgene also rescued the meiotic defects in *boule* mutant flies (Fig. 2G and H). Progression of meiosis in flies carrying the human *BOULE* transgene was morphologically indistinguishable from that observed with the fly *boule* transgene. Note, however, that fertility was not restored by either transgene, a complication that we attribute to the use of the testis-specific beta-2 tubulin promoter in the transgene constructs. This promoter is the only suitable testis-specific promoter now available (17).

Molecular analysis of rescued spermatogenesis in transgenic flies

In order to confirm that human *BOULE* effectively substitutes for fly *boule* in meiosis at a molecular level, we compared gene expression in testes from wild-type flies, *boule* mutant flies, and flies carrying the human or fly *boule* transgenes. Microarrays with DNA representing 14 000 putative transcripts were probed (Fig. 3). In all, 2585 genes were found to be highly induced in testes in comparison to other tissues (Fig. 3A). Expression of 12 of these testis-specific genes was reduced or absent in *boule* mutants ($P < 0.05$). Of these 12, the expression of all except one of these genes was restored in flies carrying either the fly or human transgene (Fig. 3B). Expression of the *boule* gene, which was absent in the mutant, was detected in flies carrying the fly transgene, but not the human *BOULE* transgene. This lack of fly *boule* gene expression in flies rescued by human *BOULE* further confirmed that meiotic rescue was accomplished by the heterologous human gene. Of further interest, note that expression of the *twine* gene, the proposed downstream target of RNA-binding by the Boule protein, was detected in the mutant, wild-type and transgenic flies, consistent with the concept that regulation of *twine* by Boule occurs post-transcriptionally (18). In summary, it is clear that at the level of histology and entire testes transcript

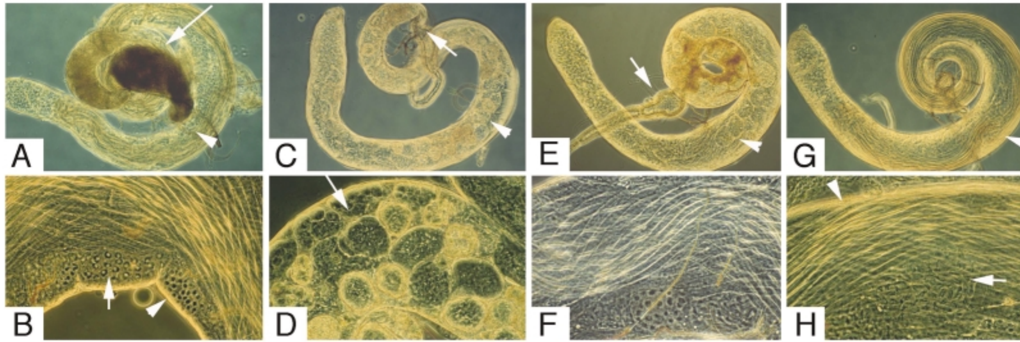

Figure 2. Human *BOULE* transgene rescues meiotic defects in infertile flies. Shown are histologic preparations of testes from wild-type flies (**A**, **B**); *boule* mutant homozygotes (**C**, **D**); *boule* mutant homozygotes carrying a fly *boule* transgene (**E**, **F**); and *boule* mutant homozygotes carrying a human *BOULE* transgene (**G**, **H**). The testis is composed of a testis tubule and a seminal vesicle. All cellular stages of spermatogenesis are visible in histologic preparations of the testis tubule in adult wild-type testis: postmeiotic spermatid bundles are readily observed (arrowhead in A), and the seminal vesicle (arrow in A) is filled with many mature sperm. Cysts going through meiosis (arrow in B) and postmeiotic cysts (arrowhead in B) are also observed. In *boule* homozygotes, there are no meiotic cysts or post-meiotic spermatid bundles. The testis tubule is filled with cysts of arrested spermatocytes (arrowhead in C and arrow in D) and degenerating cysts. No sperm are observed in the seminal vesicle (arrow in C). In contrast, flies carrying the human *BOULE* gene contain many postmeiotic spermatid bundles in the testis tubule (arrowhead in G), as in wild-type. Higher magnification of the testis indicates the presence of spermatid bundles (arrowhead in H) and cysts just finishing meiosis (arrow in H). Identical rescue is observed in flies carrying fly *boule* transgenes (12). A–D: magnification, ×160; E–H: magnification, ×450.

profiling, progression of meiosis in flies carrying a human *BOULE* transgene was as complete as and indistinguishable from that observed with the fly *Boule* gene. Direct comparison of the ability of the human *BOULE* to regulate its downstream target, *twine*, might further confirm these results. However, taken together, both our micro- and macro-evolutionary analyses indicate that the *BOULE* gene encodes an unusually conserved reproductive protein whose function as a meiotic regulator has been maintained throughout metazoans.

DISCUSSION

Human *BOULE* is the ortholog of fly *Boule*

The similar functionality of human and fly *Boule* suggests that the human *BOULE* gene is the ortholog of fly *boule* and probably functions in men as it does in flies to regulate meiosis. This suggestion is supported by the functional analysis described above and by sequence comparison. The human BOULE protein is more closely related to fly Boule than is DAZ or DAZL. Human BOULE and fly Boule share 42% similarity (and 30% identity) throughout the protein sequence and 80% similarity at the amino acid level in the RNA-binding motif. In contrast, human DAZ and fly Boule share just 19% similarity and 13% overall identity. Moreover, DAZ and fly Boule share just 59% similarity in the RNA-binding motifs; similar comparisons are observed with DAZL and fly Boule (4). In addition to sharing greater homology to each other than to DAZ or DAZL, fly *boule* and human *BOULE* also have identical expression patterns; they are expressed exclusively at meiosis but not in germplasm or germline stem cells (4). In contrast, other members of the *DAZ* gene family such as *Xdazl* in frogs, a member of the *DAZ* gene family in frogs that can confer partial rescue of defects in *boule* mutant flies (8,9), is expressed in germplasm and is required for primordial germ cell development.

BOULE as a regulator of the human meiotic cell cycle

Based on our findings and studies from *Drosophila* and mouse, we propose a hypothetical model of how BOULE might regulate the human meiotic cell cycle (Fig. 4A). In *Drosophila*, Boule is a post-transcriptional regulator of a *CDC25* homolog called *twine*, a cell cycle regulator that is required for the G2-M transition in the meiotic cell cycle during spermatogenesis (18). *Twine* encodes a phosphatase that regulates activity of the MPF (maturation promoting factor) complex and consequently is the gatekeeper of the meiotic cell cycle, allowing orderly progression of meiotic cell cycle events (18–21). Studies in the mouse implicate MPF in regulation of the G2-M transition during spermatogenesis, as well as two Cdc25 homologs, Cdc25A and Cdc25C, that are expressed at the same time and in the same place as BOULE (4,22,23). In contrast to model organisms, no genes that regulate entry into meiosis in human spermatogenesis have been identified. Here we show that the human *BOULE* transgene can accomplish the task of meiotic cell cycle regulation usually performed by fly *Boule* and hence function to regulate MPF in the G2-M transition. We therefore suggest that human *BOULE* is also probably a meiotic regulator in humans, and may function as the master regulator that controls the G2-M transition and progression through meiosis in men (Fig. 4A). Although our data did not indicate directly that human *BOULE* regulates meiosis through post-transcriptional regulation of *twine* in rescued flies, the unaffected level of twine mRNA from *boule* mutants and rescued *boule* mutants is consistent with such a mode of regulation. Study of spermatogenic phenotypes and expression of *BOULE* and *Cdc25* in human patients defective in *BOULE* or *Cdc25* or in mouse mutant models of *Boule* and *cdc25* could provide the ultimate evidence for or against this proposed model.

Differential evolution of reproductive proteins

Reproductive traits are known to evolve rapidly at both morphological and molecular levels (2). In some species, such

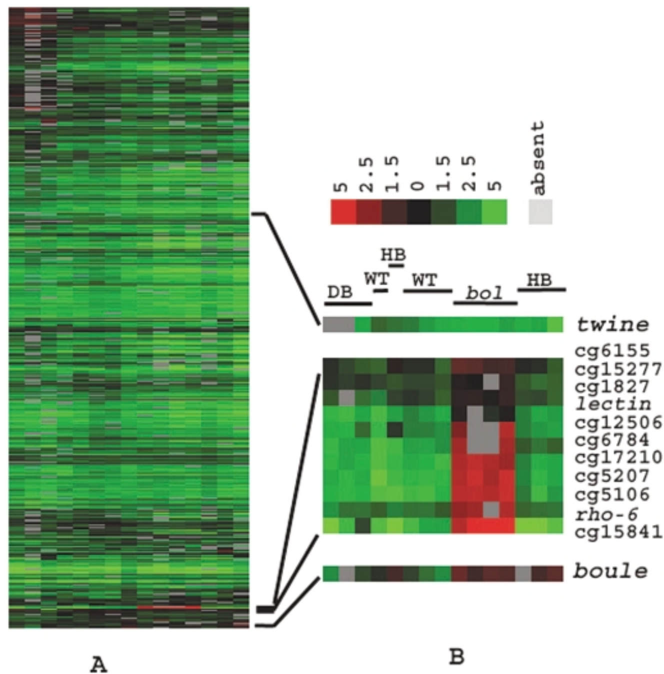

Figure 3. Gene profiling of testes from wild-type (WT), *boule* mutants (*bol*), and *boule* mutants carrying either a fly (DB) or human (HB) transgene. (**A**) The subcluster of genes with preferential expression in testes. Columns represent different experiments and rows indicate genes. RNA was dissected from testes of WT (four experiments), *bol* (four experiments), DB (three experiments) and HB (four experiments) flies. Dyes were switched in half of the experiments for the same condition in four-experiment sets, or at least one experiment was dye-switched in three-experiment sets. Green indicates higher expression levels in testis relative to reference samples and red indicates lower levels. The numbers in the color code bar indicate corresponding folds of induction. (**B**) Enlargement of (A) showing the expression profiles of *twine*, *boule* and *boule*-dependent genes. The expression level of five genes (cg6155, cg15277, cg1827, *lectin* and cg12506) were reduced in *bol* flies relative to WT, DB or HB flies, while expression levels of the other seven genes (cg6784, cg17210, cg5207, cg5106, *rho-6* and cg15841) were significantly reduced or abolished in *bol* mutants. Expression levels of these genes in WT, DB and HB testis were equivalent.

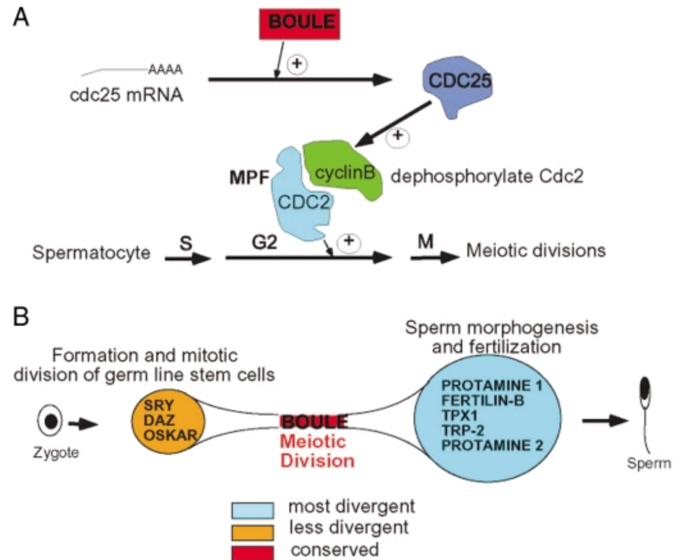

Figure 4. Models of proposed meiotic function of human *BOULE* and evolution of male reproductive genes. (**A**) *BOULE* may act as a key switch in meiotic control by initiating CDC25 protein translation through direct or indirect post-transcriptional regulation of mRNA translation. CDC25 protein then activates the MPF complex by dephosphorylating the CDC2 component of MPF. The activated meiotic cell cycle machinery in turn drives the G2-M transition forward and meiosis progresses. (+) indicates positive regulation. (**B**) Evolutionary model of divergence of genes involved in male reproduction. This model suggests that genes required for meiosis are more likely to be conserved than genes required for sperm morphogenesis or premeiotic development of germ cells. Genes required for postmeiotic spermatid differentiation and fertilization are the most variable (indicated by size of circle); genes required for premeiotic germline development are variable to a lesser extent. In contrast, meiotic genes are the least variable. Hence meiosis is like a bottleneck in the evolution of male gamete production. Evolutionary analysis of some known reproductive genes is consistent with this model (1,2). Examples of those genes in each category are given inside the circles.

as salmon, evolution of reproductive traits may be evident in as few as 12 generations (24). It is therefore surprising that BOULE protein has maintained its function in male reproduction over a period of more than 600 million years of evolution of invertebrates and vertebrates. We suggest that one explanation for this observation is that the proteins that comprise the meiotic machinery may be conserved to a greater degree than are proteins that are required for premeiotic or postmeiotic development (Fig. 4B). This suggestion is supported by several observations. First, premeiotic developmental programs for germ cell formation early in the embryo or fetus may have diverged significantly, even on a microscopic level. In some organisms, such as flies and frogs, germ cell formation requires visible germ plasm (clusters of RNAs and RNA-binding proteins) in the oocytes or eggs (3). In others, visible germ plasm is not present and formation of the germ cell lineage occurs via extrinsic inductive signals (25). Second, in contrast to genes required for premeiotic and postmeiotic germ cell development, those that comprise the meiotic machinery, such as homologs of genes involved in DNA repair, recombination, checkpoint control and other cellular events, are shared in diverse organisms from yeast to humans (26). Although functional conservation of those genes across phyla has not been demonstrated, none of the genes known to function in meiosis have been observed to be of the rapidly evolving type.

In conclusion, little is known about the genetic control of human fertility, yet recent studies suggest that genetic defects contribute to a significant proportion of infertility (5,27). In this work, we demonstrated that one of the genes that may be required for fertility in men may act as a key meiotic regulator as its function is conserved from flies to humans. The human–fly chimeric system described above should serve to further advance our understanding of BOULE function and may facilitate in the direct testing in flies of other genes that are implicated in establishing and maintaining human fertility.

MATERIALS AND METHODS

Sequence analysis

Human DNA samples were obtained from men who reported to the UCSF clinic for analysis of fertility and from samples of the

Coriell collection that represent the diversity of American population. Chimpanzee DNA sequence was amplified and sequenced using human *BOULE* primers that anneal to intron sequences near the intron–exon boundary and span exons. Old World monkey (*M.fascicularis*) *BOULE*, rat and mouse *Boule* sequences were obtained from GenBank. K_a and K_s values were calculated using K-Estimator version 5.5 (28).

Transgenic flies

For transgenic experiments, human *BOULE* was cloned into the *Drosophila* testis-specific vector, beta-2 tubulin, which is expressed during the spermatocyte stage prior to meiosis according to standard protocols (12,18). Twelve independent lines were generated. Five lines with insertions on the second chromosome were crossed into the *boule* mutant background. Testis from 1-day-old *boule* homozygous mutant males with or without human *BOULE* transgenes were dissected into phosphate-buffered saline (12). Phase-contrast images of the intact testis or a section of the testis at a higher magnification were recorded. Full rescue was defined as the presence of an equal or greater percentage of meiotic and postmeiotic cysts than that observed in *boule* mutants carrying *Drosophila boule* transgenes (12,18).

Microarray analysis

For each experiment 10–15 gonad pairs were dissected from flies (1–4 days after eclosion) and washed several times in Ringer solution (per liter: 6.5 g NaCl, 0.14 g KCl, 0.2 g Na_2HPO_4, 0.12 g $CaCl_2$, 0.01 g NaH_2PO_4). TRNA for the reference sample was extracted with Trizol reagent (Invitrogen Inc.) from adult flies and poly(A) RNA was purified using the Oligotex mRNA Mini Kit (Qiagen Inc.). RNA preparation, amplification, *in vitro* (IVT) transcription and data analysis were described (29) with the following modifications. Experimental and reference RNA were amplified with only one round of RT and subsequent IVT. For data analysis, only genes that qualified with a combined median intensity >150 above background in both channels in at least 80% of the repeated experiments were included in the analysis. A threshold of >1.74 ($=0.8$ of the $\log_2$ transformed ratios) in at least three out of 10 experiments was chosen for all comparisons. Spotted cDNA microarrays were produced as described at www.microarrays.org/protocols.html. In brief, the Easy-to-Spot *Drosophila* ORF PCR Primer Set (Incyte Genomics Inc.) with 14 151 primer pairs to unique open reading frames and additional control sequences was used for PCR amplification. The amplicons represent non-intronic sequences that range from 100 to 600 bp (with 40% between 450 and 600 bp). All PCR products were analyzed on agarose gels and a success rate of $>94\%$ was estimated. Reactions were arrayed into 384-well plates and printed on poly-L-lysine-coated glass slides using a linear servo arrayer and ArrayMaker version 2 control software. Slides were then processed as described at http://www.microarrays.org/protocols.html. We indirectly labeled the hybridization probes by incorporation of amino-allyl modified nucleotides in a first strand cDNA reverse transcription reaction. Monofunctional Cy5 or Cy3 dye (Amersham Inc.) was subsequently coupled to the reactive residues. Independently generated samples were used for hybridizations for all experiments, and dye labeling was reversed to avoid systematic bias. All experimental probes were compared with a reference sample of total adult flies with an equal proportion of male and female flies.

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REFERENCES

1. Wyckoff, G.J., Wang, W. and Wu, C.-I. (2000) Rapid evolution of male reproductive genes in the descent of man. *Nature*, **403**, 304–309.
2. Swanson, W.J. and Vacquier, V.D. (2002) The rapid evolution of reproductive proteins. *Nat. Rev. Genet.*, **3**, 137–144.
3. Starz-Gaiano, M. and Lehmann, R. (2001) Moving towards the next generation. *Mech. Devl.*, **105**, 5–18.
4. Xu, E.Y., Moore, F.L. and Reijo Pera, R.A. (2001) A gene family required for human germ cell development evolved from an ancient meiotic gene conserved in all metazoans. *Proc. Natl Acad. Sci. USA*, **98**, 7414–7419.
5. Reijo, R., Lee, T.Y., Salo, P., Alagappan, R., Brown, L.G., Rosenberg, M., Rozen, S., Jaffe, T., Straus, D., Hovatta, O. *et al*. (1995) Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. *Nat. Genet.*, **10**, 383–393.
6. Saxena, R., Brown, L.G., Hawkins, T., Alagappan, R.K., Skaletsky, H., Reeve, M.P., Reijo, R., Rozen, S., Dinulos, M.B., Disteche, C.M. *et al*. (1996) The *DAZ* gene cluster on the human Y chromosome arose from an autosomal gene that was transposed, repeatedly amplified and pruned. *Nat. Genet.*, **14**, 292–299.
7. Saxena, R., Vries, J.W.A.d., Repping, S., Alagappan, R.K., Skaletsky, H., Brown, L.G., Ma, P., Chen, E., Hoovers, J.M.N. and Page, D.C. (2000) Four *DAZ* genes in two clusters found in the *AZFc* region of the human Y chromosome. *Genomics*, **67**, 256–267.
8. Houston, D.W. and King, M.L. (2000) A critical role for *Xdazl*, a germ plasm-localized RNA, in the differentiation of primordial germ cells in *Xenopus*. *Development*, **127**, 447–456.
9. Houston, D.W., Zhang, J., Maines, J.Z., Wasserman, S.A. and King, M.L. (1998) A *Xenopus DAZ-like* gene encodes an RNA component of germ plasm and is a functional homologue of *Drosophila boule*. *Development*, **125**, 171–180.
10. Johnson AD, B.R., Drum, M. and Masi, T. (2001) Expression of axolotl dazl RNA, a marker of germ plasm: widespread maternal RNA and onset of expression in germ cells approaching the gonad. *Devl. Biol.*, **234**, 402–415.
11. Ruggiu, M., Speed, R., Taggart, M., McKay, S.J., Kilanowski, F., Saunders, P., Dorin, J. and Cooke, H. (1997) The mouse *Dazla* gene encodes a cytoplasmic protein essential for gametogenesis. *Nature*, **389**, 73–77.
12. Eberhart, C.G., Maines, J.Z. and Wasserman, S.A. (1996) Meiotic cell cycle requirement for a fly homologue of human *Deleted in AZoospermia*. *Nature*, **381**, 783–785.
13. Cargill, M., Altshuler, D., Ireland, J., Sklar, P., Ardlie, K., Patil, N., Shaw, N., Lane, C.R., Lim, E.P., Kalyanaraman, N. *et al*. (1999) Characterization of single-nucleotide polymorphisms in coding regions of human genes. *Nat. Genet.*, **22**, 231–238.
14. Bielawski, J.P. and Yang, Z. (2001) Positive and negative selection in the *DAZ* gene family. *Mol. Biol. Evol.*, **18**, 523–529.

15. Agulnik, A.I., Zharkikh, A., Tong, H.B., Bourgeron, T.K.K.M. and Bishop, C.E. (1998) Evolution of the *DAZ* gene family suggests that Y-linked *DAZ* plays little, or a limited, role in spermatogenesis but underlines a recent African origin for human populations. *Hum. Mol. Genet.*, **7**, 1371–1377.
16. Castrillon, D.H., Gonczy, P., Alexander, S., Rawson, R., Eberhart, C.G., Viswanathan, S., Di Nardo, S. and Wasserman, S.A. (1993) Toward a molecular genetic analysis of spermatogenesis in *Drosophila melanogaster*: characterization of male-sterile mutants generated by single P element mutagenesis. *Genetics*, **135**, 489–505.
17. Hoyle, H.D., Hutchens, J.A., Turner, F.R. and Raff, E.C. (1995) Regulation of beta-tubulin function and expression in *Drosophila* spermatogenesis. *Devl. Genet.*, **16**, 148–170.
18. Maines, J.Z. and Wasserman, S.A. (1999) Post-transcriptional regulation of the meiotic Cdc25 protein Twine by the Dazl orthologue Boule. *Nat. Cell Biol.*, **1**, 171–174.
19. Sigrist, S., Ried, G. and Lehner, C.F. (1995) Dmcdc2 kinase is required for both meiotic divisions during *Drosophila* spermatogenesis and is activated by the Twine/cdc25 phosphatase. *Mech. Devl.*, **53**, 247–260.
20. Alphey, L., Jimenez, J., White-Cooper, H., Dawson, I., Nurse, P. and Glover, D.M. (1992) twine, a cdc25 homolog that functions in the male and female germline of *Drosophila*. *Cell*, **69**, 977–988.
21. Courtot, C., Fankhauser, C., Simanis, V. and Lehner, C.F. (1992) The *Drosophila* cdc25 homologue *twine* is required for meiosis. *Development*, **116**, 405–416.
22. Liu, D., Liao, C. and Wolgemuth, D.J. (2000) A role for cyclin A1 in the activation of MPF and G2-M transition during meiosis of male germ cells in mice. *Devl. Biol.*, **224**, 388–400.
23. Zhu, D., Dix, D.J. and Eddy, E.M. (1997) HSP70-2 is required for CDC2 kinase activity in meiosis I of mouse spermatocytes. *Development*, **124**, 3007–3014.
24. Hendry, A.P., Wenburg, J.K., Bentzen, P., Volk, E.C. and Quinn, T.P. (2000) Rapid evolution of reproductive isolation in the wild: evidence from introduced salmon. *Science*, **290**, 516–518.
25. Lawson, K.A., Dunn, N.R., Roelen, B.A.J., Zeinstra, L.M., Davis, A.M., Wright, C.V.E., Korving, J.P.W.F.M. and Hogan, B.L.M. (1999) *Bmp4* is required for the generation of primordial germ cells in the mouse embryo. *Genes Devl.*, **13**, 424–436.
26. Kleckner, N. (1996) Meiosis: how could it work? *Proc. Natl Acad. Sci. USA*, **93**, 8167–8174.
27. Reijo, R., Seligman, J., Dinulos, M.B., Jaffe, T., Brown, L.G., Disteche, C.M. and Page, D.C. (1996) Mouse autosomal homolog of *DAZ*, a candidate male sterility gene in humans, is expressed in male germ cells before and after puberty. *Genomics*, **35**, 346–352.
28. Comeron, J.M. (1999) K-Estimator: calculation of the number of nucleotide substitutions per site and the confidence intervals. *Bioinformatics*, **15**, 763–764.
29. Klebes, A., Biehs, B., Cifuentes, F. and Kornberg, T.B. (2002) Expression profiling of *Drosophila* imaginal discs. *Genome Biol.*, **8**, 1–16.
30. Chu, S., Eisen, M., Mulholland, J., Botstein, D., Brown, P.O. and Herskowitz, I. (1998) The transcriptional program of sporulation in budding yeast. *Science*, **282**, 699–705.