Developmentally Regulated Mitochondrial Fusion Mediated by a Conserved, Novel, Predicted GTPase

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Summary

The Drosophila melanogaster fuzzy onions (fzo) gene encodes the first known protein mediator of mitochondrial fusion. During Drosophila spermatogenesis, mitochondria in early postmeiotic spermatids aggregate, fuse, and elongate beside the growing flagellar axoneme. fzo mutant males are defective in this developmentally regulated mitochondrial fusion and are sterile. fzo encodes a large, novel, predicted transmembrane GTPase that becomes detectable on spermatid mitochondria late in meiosis II, just prior to fusion, and disappears soon after fusion is complete. Missense mutations that alter conserved residues required for GTP binding in other GTPases inhibit the fusogenic activity of Fzo in vivo but do not affect its localization. Fzo has homologs of unknown function in mammals, nematodes, and yeast.

Introduction

Mitochondria undergo regulated fusion in many cell types (Skulachev, 1990; Kawano et al., 1995). Serial sections from rodent skeletal muscle (Kirkwood et al., 1986), lymphocytes (Rancourt et al., 1975), liver (Brandt et al., 1974), spinal ganglion cells (Hayashida, 1973), and the yeast S. cerevisiae (Stevens, 1981) have revealed that mitochondria within a cell can exist as a giant branched reticulum. Skulachev (1990) proposed that mitochondrial reticula support coordinated and efficient ATP production by allowing widespread transmission of membrane potential to oxygen- or nutrient-poor regions of a cell. Mitochondrial fusion is developmentally regulated in rat diaphragm muscle (Bakeeva et al., 1981), liver (Smith, 1931), Drosophila spermatids (Fuller, 1993), and many single-celled eukaryotes (Kawano et al., 1995). In S. cerevisiae, mitochondria fuse after mating (Dujon, 1981) and during meiosis and sporulation (Stevens, 1981). Mitochondria in Physarum polycephalum fuse during plasmodium formation and sporulation if a linear mitochondrial plasmid is present (Takano et al., 1994). Neither the fusion mediator in P. polycephalum nor any other mitochondrial fusogen has been identified.

Protein mediators of membrane fusion in other contexts are known. Hemagglutinin mediates fusion of endocytosed viruses to cells (Hernandez et al., 1996). In the secretory pathway, docking specificity between vesicles and organelles is provided by integral membrane SNARE proteins on each compartment (Rothman, 1996). Either the ATPase NSF (Rothman, 1996) or a yet unidentified protein (Mayer et al., 1996) could alter SNARE conformation and trigger bilayer fusion. Vesicle trafficking also requires Rab GTPases, which appear to regulate SNARE assembly during vesicle docking (Pfeffer, 1996).

We have investigated molecular requirements for mitochondrial fusion in Drosophila spermatogenesis. Mitochondria undergo dramatic morphogenetic changes during spermatid differentiation in Drosophila (Fuller, 1993). In early postmeiotic spermatids, mitochondria aggregate beside each haploid nucleus and fuse into exactly two giant mitochondrial derivatives that wrap around each other to form the spherical Nebenkern (Figure 1A). The Nebenkern resembles an onion slice when viewed in cross section by transmission electron microscopy (TEM; Figure 1D); hence the term “onion stage” refers to early round spermatids. Serial section analysis confirmed that the onion stage Nebenkern contains two topologically distinct compartments (Tates, 1971). During flagellar elongation, the two mitochondrial derivatives unfurl from each other and elongate beside the growing axoneme (Tates, 1971; Tokuyasu, 1974; Figure 1A).

Here, we describe identification of the fuzzy onions (fzo) gene, which encodes the first known mediator of mitochondrial fusion. fzo mutant males are sterile and have defects in postmeiotic fusion of mitochondria. fzo encodes a novel, predicted transmembrane GTPase associated with spermatid mitochondria during the time of fusion. Missense mutations in conserved GTPase motifs eliminated or reduced Fzo function but did not affect its localization. Fzo has homologs in mammals, nematodes, and yeast and is the first member of known function in a novel family of large, predicted, multidomain GTPases.

Results

fzo Is Required for Developmentally Regulated Mitochondrial Fusion

Mutations in fzo cause male sterility and defects in mitochondrial fusion during Nebenkern formation. In fzo early spermatids, mitochondria aggregate, forming misshapen Nebenkerns as viewed by phase contrast microscopy (Figure 1C, arrowhead), and fail to fuse into two giant mitochondria. Instead, many smaller mitochondrial fusion derivatives unfurl from each other at the onion stage (Figure 1E). Despite the prior defect in fusion, mitochondria unfurl and elongate in fzo mutants. Early elongating mitochondria in fzo mutants appear fragmented (Figure 1G, arrowhead) compared to wild type (Figure 1F). At later stages, each fzo spermatid has many elongating mitochondria (Figures 1I and 1K), rather than the normal two elongating mitochondrial derivatives (Figures 1H and 1J). Thus the defects in fzo mutant spermatids appear specific for mitochondrial fusion, as mitochondrial aggregation, membrane wrapping, and elongation all occur. In addition, spermatid mitochondria in fzo mutants take up rhodamine 123 as in wild type (not shown), indicating presence of a membrane potential and suggesting that fzo mutations probably do not grossly affect respiration.
Two ethylmethane sulfonate (EMS)-induced alleles, \textit{fzo}^1 \textit{fzo}^2, were characterized. \textit{fzo}^1/\textit{fzo}^1, \textit{fzo}^1/\textit{fzo}^2, \textit{fzo}^1/\textit{Df}(3R)P2O, and \textit{fzo}^2/\textit{Df}(3R)P2O flies showed identical phenotypes, suggesting that both mutations are strong loss-of-function alleles. The severity of the phenotype was consistent among all spermatids in all testes observed. The \textit{fzo}^1 and \textit{fzo}^2 mutations did not noticeably affect female fertility or overall viability.

\textbf{\textit{fzo} Encodes a Novel, Conserved, Predicted Transmembrane GTPase}

The \textit{fzo}^1 mutation was mapped by recombination to an 11 kb genomic region using visible markers (Figure 2A) and restriction fragment length polymorphisms (RFLPs) (Figure 2B; see Experimental Procedures). A 12 kb deficiency (\textit{Df}(3R)P2O) generated by imprecise excision of a nearby P element (\textit{cnc-03871}) failed to complement \textit{fzo} mutations, consistent with the RFLP mapping data (Figure 2B). cDNA clones corresponding to transcripts from the \textit{fzo} region were isolated from a testis cDNA library and all represented a single transcription unit; the largest cDNAs were 2.4 kb. A single copy of a 4 kb genomic fragment containing the candidate locus plus 1 kb upstream and approximately 500 bp downstream restored fertility and normal mitochondrial morphogenesis to \textit{fzo} mutant males.

Sequence analysis of the \textit{fzo} cDNA revealed a complete open reading frame (ORF) encoding a predicted protein of 718 amino acids (Figure 2C) with flanking AT-rich sequences and a single consensus translational start site (Cavener and Ray, 1991). Database searches identified related predicted proteins (see GenBank entries for references) in \textit{C. elegans} (U29244, ORF 14, 28% identity), \textit{S. cerevisiae} (Z36048, 19% identity), and mammals. Human expressed sequence tags (ESTs) homologous to \textit{fzo} appear to derive from two different genes. We sequenced the cDNA clone from which a human fetal brain EST (T06373) was derived and showed that

(A) Diagram of wild-type mitochondrial morphogenesis. Nuclei, open; mitochondria, closed. Left to right: mitochondrial aggregation; onion stage; early elongation; mid-elongation. A protein body of unknown function appears in nuclei of elongating spermatids.

(B±K) Phase contrast (B, C, and F±I) and transmission electron (D, E, J, and K) micrographs of wild-type (B, D, F, H, and J) and \textit{fzo}^1 (C, E, G, I, and K) spermatids. Arrows indicate spermatid nuclei in (B), (C), and (F)-I). Spermatids differentiate together in cysts of 64 cells; in (B), (C), and (F)-I), some appear syncytial due to opening of cytoplasmic bridges between cells during sample preparation. (B and C) Onion stage spermatids with Nebenkerns (arrowheads) adjacent to nuclei. (D and E) Cross sections of onion stage Nebenkerns. Individual mitochondria in \textit{fzo} (E) appear to wrap around each other, as do the two giant mitochondrial derivatives in wild type (D). (F and G) Early elongation stage spermatids with unfurling and elongating mitochondrial derivatives (arrowheads). Note the fragmented appearance of mitochondria in the mutant. (H and I) Mid-elongation stage spermatids with elongating mitochondrial derivatives (arrowheads). (J and K) Cross sections of elongating spermatids. In wild type (J), each axoneme (arrow) is associated with one major (large arrowhead) and one minor (small arrowhead) mitochondrial derivative. In the mutant (K), each axoneme (arrow) is associated with many mitochondrial derivatives, roughly half of which display dark paracrystalline material characteristic of major mitochondrial derivatives. Scale bars: 10 \mu m (B, C, and F-I); 2 \mu m (D and E); 0.5 \mu m (J and K).
Predicted GTPase Mediates Mitochondrial Fusion

Figure 2. Molecular Cloning of fzo

(A) Genetic, cytological, and molecular map of the fzo region on chromosome 3R. Stippled, numbered boxes represent polytene chromosome bands; open bars are deficiencies, with (+) indicating complementation of fzo and (−) indicating failure to complement. The distal breakpoint of Df(3R)EB6 is at +2 kb on a genomic phage walk (Mohler et al., 1991) that extends 90 kb distal from hh. (B) Molecular map of the fzo region of the genomic walk, with coordinates 0–20 indicated in kb. Deficiencies are represented as in (A). Restriction sites: (R), EcoRI; (X), XbaI; and (Z), Xhol. Thick line on molecular map represents genomic DNA used to screen testis cDNA library.

(C) Predicted amino acid sequence (single letter amino acid code) of the fzo gene product, with regions matching GTPase motifs in boldface. Underlined region, predicted transmembrane domain (TMpred, Hofmann and Stoffel, 1993). Italics, large hydrophobic region including the predicted transmembrane domain and 13 adjacent uncharged residues (see Figure 3D). Lowercase, predicted coiled-coil regions with probability scores above 0.4 (Lupas et al., 1991; see Figure 3C):

it encodes an incomplete predicted protein with 35% identity to Fzo (Figure 3B). Heart (AA248162, AA248083), fibroblast (W49736), and other brain ESTs (R20140, T37724) are virtually identical to regions of this cDNA, while ESTs from liver/spleen (H58349) and pancreas (AA155601) seem to originate from a different gene and together encode a 125 residue peptide (not shown) 56% identical to the human brain gene product. Both human isoforms are 28% identical to Fzo in this C-terminal region. Four overlapping mouse ESTs (W41601,
AA199015, AA212845, AA052806) together encode 211 amino acids with 22% identity to the Fzo carboxyl terminus and 84% identity to the analogous region from the human brain homolog (not shown). A predicted protein from the thermophilic bacterium C. saccharolyticum (L18965 ORF 6) is 11% identical to Fzo and 24% identical to the S. cerevisiae homolog.

The region of highest homology between Fzo and its human fetal brain, C. elegans, and S. cerevisiae homologs (50%, 49%, and 20% identity to Fzo, respectively) is a 186 amino acid region containing four completely conserved motifs found in virtually all GTPases (G1±G4 in Figure 3A; Bourne et al., 1991). The C. saccharolyticum predicted protein also contains these motifs (Figure 3A). Outside the individual motifs there is no significant similarity to any known GTPase. However, spacing between GTPase motifs, their N-terminal placement, and overall predicted protein size are reminiscent of the dynamin family (Warnock and Schmid, 1996). The G2 motif, a conserved threonine (Bourne et al., 1991), has not been defined in dynamins. Both the Fzo and dynamin families have a conserved threonine exactly 20 residues beyond the G1 motif that we propose represents G2 (Figure 3A).

Outside the GTPase domain, the human fetal brain, C. elegans, and S. cerevisiae homologs are 30%, 21%, and 19% identical to Fzo, respectively, and all share several predicted structural features. All have a predicted transmembrane domain near the carboxyl terminus (Figures 3B and 3C) embedded in a large (≈34 amino acids) uncharged region interrupted by 1–3 basic residues (Figure 3D). The homologs have predicted coiled-coil regions flanking the predicted transmembrane domain (Figures 3B and 3C). All four homologs are acidic overall between the amino terminus and the transmembrane domain, with predicted isoelectric points (pI) near 5, and basic in the carboxy-terminal tail, with predicted pIs near 9 (Figure 3E).

Fzo Is Associated with Mitochondria during a Short Time Period Spanning Fusion

Antibodies raised against a fusion protein containing the C-terminal 115 residues of Fzo stained onion stage Nebenkerns brightly in wild-type spermatids (Figure 4A, arrow). Fzo was undetectable or present at greatly reduced levels in fzo1/fzo1 or fzo2/Df(3R)P2O testes, respectively (Figures 4B and 4C). A wild-type fzo transgene in a fzo1/fzo1 mutant background restored detectable Fzo protein to the Nebenkern (Figure 4D).

The Fzo protein was associated with mitochondria in wild-type spermatids during a narrow developmental window corresponding to the time that Fzo function is required. Mitochondria align on the spindle equator throughout meiotic divisions (Fuller, 1993), but the Fzo protein was not detected on mitochondria until the last stages of meiosis II (Figures 4E and 4F). In postmeiotic haploid spermatids, Fzo was associated with aggregat-
Fzo is a novel, predicted GTPase

The Drosophila fzo gene encodes a predicted GTPase required for mitochondrial fusion during spermatogenesis and detected on mitochondria during a short time spanning the fusion event. Fzo contains four motifs common to virtually all known GTPases (Boume et al., 1991) and conserved among Fzo homologs from mammals to yeast. Fzo is the first to be assigned a function in this new family of large, predicted transmembrane GTPases.

Mutations predicted to diminish guanine nucleotide binding did not affect localization of Fzo to mitochondria but eliminated or reduced its ability to mediate mitochondrial fusion. The fzoR249L mutation, predicted to disallow key hydrogen bonds with the GTP β and γ phosphates (Pai et al., 1990; Noel et al., 1993), caused a severe loss of function phenotype (Figure 5B). In contrast, the fzoK99T mutation appeared to allow some mitochondrial fusion to occur (Figure 5C), though not enough to restore normal sperm morphology or motility. The Fzo arginine 249 is part of the G4 motif and is predicted to contact the ribose moiety of GTP (Pai et al., 1990; Theobald et al., 1993) and conserved among Fzo homologs from mammals to yeast. Fzo is the first to be assigned a function in this new family of large, predicted transmembrane GTPases.

Conserved Residues in the GTP Domain Are Required for Fzo Function but Not for Targeting of the Protein to Mitochondria

Mitochondrial fusion appears to require the predicted Fzo GTP-binding activity. Missense mutations that alter conserved residues (Figure 3A) required in other GTPases for guanine nucleotide binding (Sigal et al., 1986; Der et al., 1988; van der Bliek et al., 1993) were introduced into the fzo genomic rescue construct. Neither the fzoR249L or fzoK99T mutated transgene (in one or two copies) could restore fertility or sperm motility to fzo mutant males. The fzoR249L transgene had no detectable effect on the subcellular mutant phenotype (Figure 5B), while the fzoK99T transgene appeared to allow some mitochondrial fusion (Figure 5C). Neither mutant transgene showed a dominant effect in a wild-type background, even in multiple copies (not shown). The proteins encoded by the fzoR249L and fzoK99T mutated transgenes were properly localized to spermatid mitochondria (Figures 5D and 5E) with wild-type timing of appearance and disappearance (not shown).

Discussion

Fzo Is a Novel, Predicted GTPase

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The fzoR249L and fzoK99T mutations are recessive. Analogous mutations in mammalian dynamins cause dominant-negative phenotypes when expressed in tissue culture cells (Herskovits et al., 1993; van der Bliek et al., 1993). Formation of macromolecular dynamin ring-shaped complexes appears to require GTP binding by all subunits (Warnock and Schmid, 1996). In contrast to dynamins, Fzo molecules may act individually or form complexes in which only some subunits must bind GTP for proper assembly or function.
Models for Fzo Orientation and Function

The conserved overall charge distribution and predicted transmembrane domain of the Fzo protein are consistent with a possible Nout-Cin orientation on mitochondria. Mitochondrial matrix proteins are typically more basic than cytoplasmic isoforms (I ausi, 1995), and inner mitochondrial membrane proteins are generally basic in matrix-residing regions and more acidic in outside regions (Gavel and von Heijne, 1992). Fzo and homologs, when conceptually divided at their transmembrane domains, have amino termini with predicted pIs of 5.3–6 and carboxyl termini with pIs of 8.1–9.3 (Figure 3E), suggesting that the carboxyl terminus may reside in the mitochondrial matrix.

The Fzo protein has eight acidic residues and only one arginine in its first 50 amino acids, making import via a traditional amino-terminal targeting signal unlikely (von Heijne et al., 1989). Although the serine-rich nature of this region could allow mitochondrial targeting despite its acidic residues, Fzo may instead be targeted to mitochondria by an internal basic region just carboxy-terminal to the predicted transmembrane domain (e.g. residues 621–636), as is the S. cerevisiae Bcs1p protein (Felsh et al., 1996).

The predicted transmembrane region of Fzo and homologs, which consists of two blocks of uncharged residues separated by a small region with one or more acidic residues, could potentially span a single membrane twice. Alternatively, as overall charge distribution in the protein suggests compartmental separation of the amino and carboxyl termini, it is plausible, although unprecedented, that Fzo could span both the inner and outer mitochondrial membranes at a contact site. With the C-terminal hydrophobic block in the inner membrane and the charged region in the intermembrane space, the N-terminal hydrophobic block could traverse the outer membrane in a β sheet conformation, which requires fewer hydrophobic residues to span the membrane than an α helix. This putative β sheet region could form lateral hydrogen bonds with other copies of itself or with β sheet transmembrane regions from other outer membrane proteins like porin (De Pinto and Palmieri, 1992). In mammalian tissue culture cells, mitochondrial fusion appears to initiate where inner/outer membrane contact sites on each of two separate mitochondria are apposed (Bereiter-Hahn and Voth, 1994). Stable inner/outer membrane contact sites that are independent of protein import channels have been observed (Glick and Schatz, 1991). Perhaps Fzo acts at such inner/outer membrane contact sites to mediate mitochondrial fusion.

If Fzo spans both membranes with its carboxyl terminus in the matrix, then the predicted GTPase domain and adjacent predicted coiled-coil regions would be oriented toward the cytoplasm. The Fzo protein could act as part of a ligand/receptor pair between separate mitochondria, binding to copies of itself or to other molecules displayed on the outer mitochondrial membrane. Alternatively, the Fzo protein could recruit other molecules to form a complex that links adjacent mitochondria. In either case, GTP binding and hydrolysis by Fzo may regulate the specificity of these interactions, as Rab GTPases seem to regulate formation of the SNARE complex prior to membrane fusion in the secretory and endocytic pathways (Pfeffer, 1996). Alternatively, the Fzo predicted GTPase may have a biomechanical role, as may dynamin GTPases in the formation of endocytic vesicles (Warnock and Schmid, 1996). GTP hydrolysis could cause a fusion-triggering conformational change in Fzo itself or in other recruited proteins, analogous to the mechanism by which hemagglutinin mediates bilayer mixing during fusion of influenza virus to cells (Hernandez et al., 1996). Future work will elucidate the mechanism by which Fzo acts.

Developmental Regulation of Fzo

The appearance of Fzo on spermatid mitochondria is developmentally regulated. Antibodies to the Fzo carboxyl terminus detected the protein on mitochondria only around the time of mitochondrial fusion, approximately four days after the initial appearance of fzo mRNA in primary spermatocytes (data not shown). Detection of Fzo was not simply a result of mitochondrial aggregation; mitochondria aggregate in early primary spermatocytes and align on the spindle during meiotic divisions (Fuller, 1993), but Fzo was first detected only after metaphase II. Expression of Fzo could be regulated at the translational level, as is typical of many gene products needed for postmeiotic spermatid differentiation (Schäfer et al., 1995). Alternatively, Fzo could be regulated posttranslationally to allow association with mitochondria or to unmask C-terminal epitopes. As mitochondria elongate, Fzo could be degraded or removed from mitochondrial membranes (becoming diffuse and undetectable), or C-terminal epitopes could be cleaved or masked.

The Fzo Family of Predicted GTPases

Fzo homologs could mediate mitochondrial fusion in other organisms and cell types. Spermatid mitochondria in the nematode X. thersia (and likely in C. elegans) appear to undergo developmentally regulated fusion (Kruger, 1991), perhaps mediated by the nematode Fzo homolog. In the yeast S. cerevisiae, no mutants defective in mitochondrial fusion have been identified. We are currently analyzing whether the S. cerevisiae Fzo homolog plays a role in this process (G. Hermann, K. G. H., M. T. F., and J. Shaw, unpublished data). ESTs from genes encoding the human homologs were derived from brain, heart, pancreas, liver/spleen, and fibroblasts; mitochondrial fusion occurs in mammalian liver (Smith, 1931; Brandt et al., 1974) and may occur in neurons (Hayashida, 1973) and fibroblasts (Johnson et al., 1980). Mammalian spermatid mitochondria do not fuse but form structurally distinct contacts (Olson and Winfrey, 1992), which seem to allow connection between mitochondrial matrices (Zorov et al., 1990). Similar connections are also seen in rat cardiac tissue (Bakeeva et al., 1983) and may require protein mediators similar to those needed for full mitochondrial fusion in other cells. Analysis of possible roles of Fzo homologs in other organisms will allow assignment of a general function for this new family of large multidomain GTPases.
Experimental Procedures

Fly Strains and Culture

Flies were grown on standard media at 25°C. Visible markers and balancer chromosomes are described in FlyBase (FlyBase Consortium, 1996) unless otherwise noted. Oregon R was used as the wild-type strain. Df(3R)M95A (94D; 95A3) and Df(3R)E86 (94C2–5; 94E3) are described in Reuter et al. (1986) and Mohler et al. (1995). Df(3R)P2O was generated by mobilizing a ry-element P element associated with the cnc locus (Mohler et al., 1995) using the a2–3 chromosomal source of transposase (Roberts et al., 1988). ry progeny were picked, and 200 independent mutagenized chromosomes were tested for failure to complement fzo. Molecular breakpoints of Df(3R)P2O were mapped by Southern blot analysis (Sambrook et al., 1989). Df(3R)P2O failed to complement mutations in cnc and was therefore not homozygous viable.

fzo was isolated in a screen for EMS-induced recessive male sterile mutations by J. Hackstein (1991). fzo was isolated in a screen of 1799 EMS-treated third chromosomes, as described in Lin et al. (1996), except that mutagenized chromosomes were tested for failure to complement fzo. Fertility and viability tests of the allelic combinations fzo/fzo, fzo/fzo, fzo/Df(3R)P2O, and fzo/Df(3R)P2O were as in Lin et al. (1996). The fzo chromosome carried a secondary lethal.

Light and Electron Microscopy

Light microscopy of live squashed testis preparations was as in Lin pKH3fzo et al. (1996). To assess mitochondrial membrane potential, 10 μg/ml rhodamine 123 (Johnson et al., 1980) was included in the dissection independent. To introduce mutations into the Fzo predicted GTP-binding domain, a 1.8 kb XbaI/BamHI restriction fragment representing the 11±22 on the genomic walk was subcloned into the pKH2 to make pKH3fzo . The resulting 4 kb insert in the pKH3fzo rescue construct contained the genomic region of the 2.4 kb fzo cDNA plus approximately 1 kb 5′ and 500 base pairs 3′.

P Element-Mediated Germline Transformation and Construction of Mutagenized Transgenes

A 3 kb Xhol/XbaI DNA fragment corresponding to +9–+12 on the genomic walk (Figure 2B) was subcloned into the w–marked P element transformation vector pCaSpeR4 (Thummel and Pirrotta, 1992) to make plasmid pKH2. A 1 kb XbaI/EcoRI fragment corresponding to +11–+12 on the walk was subcloned into pKH2 to make pKH3fzo . The resulting 4 kb insert in the pKH3fzo rescue construct contained the genomic region of the 2.4 kb fzo cDNA plus approximately 1 kb 5′ and 500 base pairs 3′.

Mutagenized constructs were selected by altered restriction sites. For each mutagenized construct, a 1.8 kb Xbal/BamHI restriction fragment was ligated with the 10 kb XbaI/BamHI fragment from plasmid pKH3fzo to create pKH3fzo and pKH3fzo. In both cases, the XbaI and BamHI restriction sites used for the final subcloning were regenerated by the ligations, and the reading frame remained unchanged at the mutagenesis and subcloning sites, as shown by detection of mutant proteins with C-terminal-specific antibodies.

Flies were transformed with the fzo and fzo mutant transgenes as above, yielding eight and five independent second chromosome transgene insertions, respectively, all of which were separately introduced into fzo mutant backgrounds by independent assortment. To test for dominant effects, males with one wild-type copy of fzo and as many as four (fzo) and six (fzo) different copies of the mutagenized transgenes were generated by appropriate crosses.

Generation of Anti-Fzo Antibodies

To make an expression construct encoding a fusion protein with a 6-histidine tag and the C-terminal 115 amino acids of Fzo, the 0.5 kb BamHI/HindII restriction fragment from the fzo cDNA (Hindll site from Bluescript SK(–) ) was subcloned into pQE30 (QIAGEN). The fusion protein was expressed in bacteria and purified on a Ni-NTA column with imidazole elution (QIAexpressionist, QIAGEN). The purified fractions were dialyzed in 1.5 M urea, 0.1 M Na phosphate, 0.01 M Tris–HCl, and 500 mM NaCl (pH 7). The fusion protein precipitated at urea concentrations below 4 M. After dialysis, precipitated protein was emulsified in complete Freund’s adjuvant and injected into rabbits using standard schedules for initial (500 mg) and booster (250 mg) injections and for serum collections (Berkeley Antibody Company, Richmond, CA).
Immunofluorescence

Testes were prepared for immunofluorescence staining as in Hime et al. (1996). Slides were incubated at room temperature in PBS (phosphate-buffered saline) washed four times at room temperature in PBS, and incubated for one hour at 37°C in FITC-conjugated anti-rabbit IgG (Jackson Labs) diluted 1:200 in PBS. Slides were washed 4×10 minutes in PBS, with 1 μg/ml DAPI in the second wash, and mounted in 85% glycerol, 2.5% N-propyl gallate. Samples were examined using fluorescence on a Zeiss Axioskop microscope and images collected with a Photometrics cooled CCD camera (courtesy of B. Baker). Emissions from different fluorochromes on the same sample were collected separately and overlaid using Adobe Photoshop.

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References

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GenBank Accession Numbers

The GenBank accession numbers for the fzo cDNA and for the partial cDNA encoding the fzo human fetal brain homolog are U95821 and U95822, respectively.