Supplementary_4

Materials and methods

Fly strains and expression vectors

Standard *Drosophila* techniques were used to generate transgenic lines from the following constructs: For the *hsp83*(5’-3’UTRs) lines, a 338 bp fragment from the 5’ UTR and a 197 bp fragment from the 3’ UTR of *cyt-c-d* were PCR amplified from the BDGP’s EST clone LP05614 and subcloned in a subsequent order into the *Xho*I + *Eco*RI and *Bam*HI + *Not*I sites, respectively, of the CasperR-HSP83 vector (Horabin and Schedl, 1993) to obtain the pHSP83(5’-3’UTRs) vector. For the *hsp83*(5’-3’UTRs)-*cyt-c-d* and *hsp83*(5’-3’UTRs)-*cyt-c-p* lines, we PCR amplified the *cyt-c-d* and *cyt-c-p* ORFs from the BDGP’s EST clones LP05614 and RH17228, respectively, digested the PCR products with *Eco*RI + *Bgl*II, and ligated them into the *Eco*RI + *Bam*HI sites of the pHSP83(5’-3’UTRs) vector. For the *UAS-cyt-c-d* and *UAS-cyt-c-p* lines, the clones LP05614 and RH17228, respectively, were digested with *Eco*RI and *Xho*I (for *cyt-c-d*) or *Acc*65I (for *cyt-c-p*), and the fragments containing the *cyt-c-d* and *cyt-c-p* coding regions were ligated into the *Eco*RI and *Xho*I or *Acc*65I sites, respectively, of the pUASt vector (Brand *et al*, 1993).

Genetic screen of the “Zuker” male sterile collection lines

A list of more than one thousand male sterile lines with defects during late spermatogenesis was obtained from Barbara Wakimoto (University of Washington). These flies were obtained from Charles Zuker (University of California at San Diego), and for each line the testes of at least three homozygous males were dissected in testis buffer (10mM Tris-Hcl [pH 6.8], 183 mM KCl, 47 mM NaCl, 1 mM EDTA, and 1 mM PMSF) and placed in fixative made of 4% formaldehyde in PBX (PBS + 0.1% Triton X-100) in a MultiScreen 96 well filter plates (MADVN6510, Millipore), standing on ice. After dissections, the plate was rocked for 20 min at RT. Solutions in all the wells are being changed simultaneously using vacuum filtration (Millipore). Testes were washed three times in PBX for 10 min, blocked with PBS/BSA (1% BSA in PBS) for 45 min, incubated with 100 µl of the CM1 antibody (diluted 1:75 in PBS/BSA) overnight at 4°C,
and washed three times with PBX for 10 min. Testes were incubated with 100 µl of the secondary antibody (biotinylated universal antibody diluted 1:50 in PBS/BSA, Vectastain, Vector) for 1 hr, washed three times in PBX for 10 min, and the colorimetric assay was developed using the Vectastain kit (Vector) according to manufacture’s recommendations and DAB (Fast DAB tablet set, Sigma). The reaction was stopped by washing twice with 120 mM Tris-HCl.

**Antibody staining**

CM1 antibody staining of young (0-2 day old) adult testes was carried out (as described in Arama *et al.*, 2003) using a rabbit polyclonal anti-Cleaved Caspase-3 (Asp175) antibody (CM1, Cell Signaling Technology, Cat. # 9661) diluted 1:75. The only exceptions are that the subsequent TRITC-phalloidin (Sigma) incubation for staining the ICs was carried out for 5 min in room temperature, and the slides were subsequently rinsed twice for 10 min in PBS.

To generate the anti-Cyt-c-d antibody, four peptides corresponding to the entire cytochrome C-d protein length have been synthesized, purified, conjugated to KLH, and injected together to the same rabbits. Part of the 2nd bleed has been purified on an affinity column coupled to a mixture of all the four peptides (Alpha Diagnostic International). The anti-Cyt-c-d antibody (diluted 1:10 for the affinity purified or 1:400 for the non purified) was used to stain young adult testes (essentially as described in Hime *et al.*, 1996). Secondary anti-rabbit antibodies were purchased from Jackson ImmunoResearch Laboratories.

**Mitochondrial staining**

Wild-type or *dj*-GFP transgenic fly testes were dissected and fixed in formaldehyde (as described in Arama *et al.*, 2003). Following fixation the slides were rinsed in PBS, and the tissues were incubated for 20 min in PBS containing 200 nM of the MitoFluor Red 589 probe (Molecular Probes). Then, the slides were washed once with PBS, and mounted in Vectashield mounting medium with DAPI (Vector Laboratories).
Isolation of genomic DNA and PCR

Genomic DNA was isolated from 25-50 adult flies using the High Pure PCR Template Preparation Kit (Roche). Two µg of genomic DNA were used to amplify the cyt-c-d or cyt-c-p coding regions from wild-type and cyt-c-dZ2-1091 homozygotes in a PCR reaction. The pairs of primers used are indicated above for the comparative RT-PCRs. PCR reactions were carried out using DyNAzyme EXT DNA polymerase (Finnzymes), according to the manufacturer instructions. The products were purified using the High Pure PCR Product Purification Kit (Roche), concentrated by evaporation, and sequenced in the Rockefeller University sequencing facility. A similar procedure was carried out to confirm the presence of the transgenes (Figure 2F).

DEVDase activity assay

124 testes were dissected from newly eclosed wild-type or cyt-c-dZ2-1091 homozygote males, collected into 1.5 ml Eppendorf tubes, standing on ice and containing 150 µl of testis buffer (10 mM Tris-HCl [pH 6.8], 183 mM KCl, 47 mM NaCl, 1 mM EDTA, and 1 mM PMSF), homogenized using a Pellet Pestle Motor (Kontes), and subsequently equally divided into two tubes. Either Z-VAD (20 µM final concentration; Enzyme Systems Products) or DMSO was added to each tube, and the samples were transferred to a 96 well assay white plate (Costar #3610, Corning Inc), and allowed to incubate for 10 min at RT. Caspase-Glo 3/7 reagent (Promega) was added to a final volume of 200 µl and the signal was detected with a multiwell plate reader (SPECTRA max M2, Molecular Devices). Luminescence readings were obtained every two minutes; therefore, each time interval in the figure represents an average of five readings. Three similar experiments were performed that gave similar results.

Examination of testis contents by phase-contrast microscopy

Testes from newly eclosed wild-type or plnZ2-0516 homozygote males were placed on a glass slide with a drop of PBS. The testes were torn open and gently squashed under the weight of a cover slip and were taken immediately to visualize under the microscope.
Ultrastructural studies

Wild-type and *pln* mutant testes were dissected in chilled glutaraldehyde (2.5% in 0.1 M cacodylic buffer, pH 7.4) and fixed on ice. The testes were post-fixed in 1% osmium tetroxide in the same buffer on ice, dehydrated in graded concentrations of ethanol, stained *en* bloc with uranyl acetate, and embedded in Epon. For the transmission electron microscopy (TEM), ultra-thin sections were cut on a Reichert-Jung Ultracut E microtome and poststained with uranyl acetate and lead citrate. Sections were examined and photographed on a JEOL100CXII at 80 kV.

References


Horabin JI, Schedl P (1993) Sex-lethal autoregulation requires multiple cis-acting elements upstream and downstream of the male exon and appears to depend largely on controlling the use of the male exon 5' splice site. *Mol Cell Biol* **13**: 7734-7746