A double-stranded RNA binding protein required for activation of repressed messages in mammalian germ cells

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Chromatin packaging in mammalian spermatozoa requires an ordered replacement of the somatic histones by two classes of spermatid-specific basic proteins, the transition proteins and the protamines[1]. Temporal expression of transition proteins and protamines during spermatid differentiation is under translational control, and premature translation of protamine 1 leads to precocious nuclear condensation and sterility[2]. We have previously suggested that the double-stranded (ds) RNA binding protein Prbp (encoded by the gene *Tarbp2*) functions as a translational regulator during mouse spermatogenesis[3]. Here we show that Prbp is required for proper translational activation of the mRNAs encoding the protamines. We generated mice that carry a targeted disruption of *Tarbp2* and determined that they were sterile and severely oligospermic. Using immunohistological analysis, we determined that the endogenous *Prm2* mRNA and a reporter mRNA carrying protamine 1 translational-control elements were translated in a mosaic pattern. We showed that failure to synthesize the protamines resulted in delayed replacement of the transition proteins and subsequent failure of spermiation. The timing of Prbp expression suggests that it may function as a chaperone in the assembly of specific translationally regulated ribonucleoprotein particles.

We isolated *Tarbp2* in a screen for cDNAs encoding proteins that bind to the testis-specific *Prm1* mRNA (ref. 3). Prbp contains three copies of the dsRNA-binding motif[4] (dsRBM) and is highly expressed in differentiating male germ cells. Prbp binds, *in vitro*, to a conserved stem-loop structure in the *Prm1* 3′ UTR, a sequence required for translational control of *Prm1* mRNA *in vivo*[5]. Orthologues of Prbp have been isolated from humans[6,7] and amphibians[4]. The human orthologue, TRBP, acts synergistically with the HIV-1 Tat protein to transactivate the viral long terminal repeat[6] (LTR), and is believed to be an important host factor during HIV-1 infection[6,8].

We deleted *Tarbp2* coding exons 3–9, including those encoding its dsRBMs, by gene targeting in embryonic stem cells (J.Z., manuscript submitted). In a mixed C57BL/6J×129/Sv background, *Tarbp2*$^{-/-}$ mice were significantly smaller than their littermates and usually died at the time of weaning. In some mice, perinatal lethality was rescued with a dietary supplement. Although the animals never grew to normal size, some appeared healthy up to 6–10 months of age.

Tarbp2$^{-/-}$ males of reproductive age showed normal mating behaviour and formed copulation plugs in wild-type females; however, the females did not become pregnant. *Tarbp2*$^{-/-}$ males have severe oligospermia, with epididymal sperm counts reduced to less than 0.5% (Table 1). Using histological analysis, we determined that the epididymis mostly contained degenerating spermatids (Fig. 1*b*). The few mature spermatozoa that were found had a range of morphological abnormalities (Fig. 1*d–g*), although normal sperm were occasionally observed. The infertility observed was probably not due to effects of body weight on hormonal regulation of spermatogenesis, as levels of FSH and LH were within the normal range in four animals tested (Table 1).

We weighed the testes from mutant animals and determined that they were reduced relative to controls, but the ratio of testis to total body weight was the same or greater than control littermates (Table 1), suggesting that spermatogenesis does occur in mutants. Examination of the testes by histological analysis showed that initiation of spermatogenesis and progression through meiosis was normal (Fig. 1*h–k*). We noted the appearance during spermiogenesis, however, of multinucleated giant cells (symplasts) that contained, in some instances, more than 12 spermatid nuclei (Fig. 1*j*, arrows). We also observed defects in the differentiation of elongated spermatids, which included abnormal nuclear morphogenesis. Spermiation, the release of spermatozoa during stage VIII, was incomplete in many tubules, resulting in phagocytosis of the abnormal cells by the Sertoli cells in stages IX–XII (Fig. 1*i*). Loss of Prbp function also resulted in increased apoptosis in individual spermatids and in some symplasts (data not shown).

To determine whether Prbp is involved in *Prm1* translational repression, we examined the expression of Prm1 in *Tarbp2*$^{-/-}$ testis sections by immunohistochemistry. Premature translation of *Prm1* mRNA was not observed in late round and elongating spermatids (data not shown). Inability to detect premature translation of *Prm1* mRNA was expected, as we have previously shown

Table 1 • Comparison of *Tarbp2*$^{-/-}$ and *Tarbp2*$^{+/-}$ males

Line	Age (weeks)	Genotype	Body weight (g)	Testis weight (mg)	Testis/body ($\times10^{-3}$)	Sperm count ($\times10^{6}$)	Sperm count/ testis weight (10^{4}/mg)	Het/ homo	Blood FISH (ng/ml)	Het/ homo	Blood LH (ng/ml)	Het/ homo
47-5	13.1	+/–	40.5	123.8	3.06	25.5	20.60	10.0	14.412	1.34	0.490	1.57
		–/–	21.5	70.3	3.27	1.45	2.06		10.762		0.313	
5-3	11.0	+/–	32.5	99.1	3.05	19.5	19.68	35.8	3.487	1.09	0.340	0.90
		–/–	15.8	54.1	3.42	0.3	0.55		3.207		0.376	
7-2	10.4	+/–	35.0	108.7	3.11	24.5	22.54	10.6	3.943	1.05	0.388	0.73
		–/–	18.0	75.0	4.17	1.6	2.13		3.749		0.529	
7-2	9.3	+/–	23.7	71.5	3.02	14.0	19.58	279.7	6.915	1.89	0.568	1.71
		–/–	18.7	73.0	3.90	0.05	0.07		3.652		0.332	

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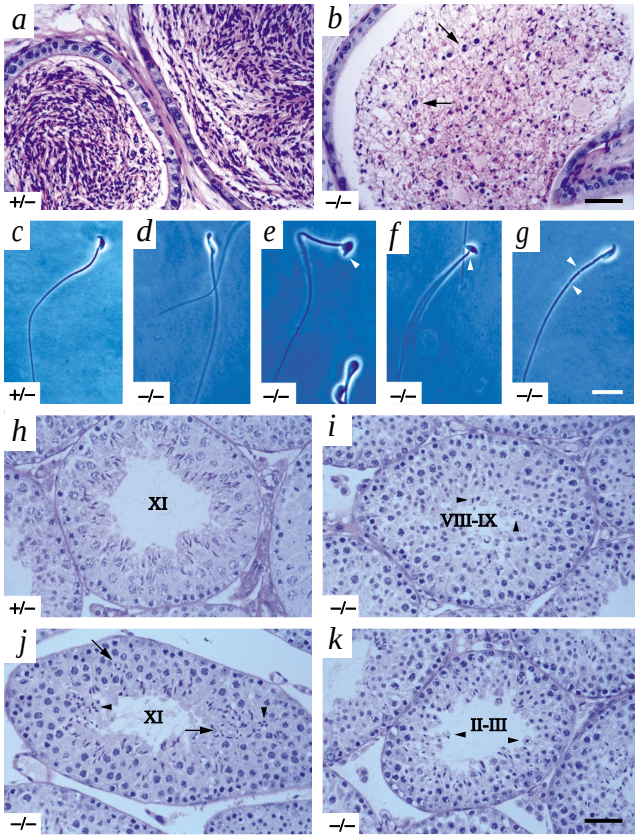

Fig. 1 Epididymal and testicular histology in *Tarbp2*$^{-/-}$ mice. Epididymis from *Tarbp2*$^{+/-}$ (***a***) and *Tarbp2*$^{-/-}$ (***b***) mice are shown. *Tarbp2*$^{-/-}$ mice have mostly undifferentiated and degenerating spermatids. Arrows indicate multinucleated symplasts. ***c***, Normal (*Tarbp2*$^{+/-}$) spermatozoon. *d–g*, Abnormal spermatozoa. ***d***, Degenerating head. ***e***, Blunted acrosome with flagellum misattached. ***f***, Flagellum attached to equatorial region of sperm head. ***g***, Gaps in the midpiece of the flagellum. ***h***,***i***,***j***,***k***, Testicular histology. Arrows in *Tarbp2*$^{-/-}$ sections indicate multinucleated symplasts. Arrowheads indicate individual condensed nuclei of developmentally arrested and degenerating spermatids. Roman numerals indicate approximate spermatogenic stages[14–16]. *a*,*b*,*h–k*, Scale bar, 40 μm; *c–g*, scale bar, 20 μm.

that there are non-overlapping, redundant elements in the *Prm1* 3′ UTR which control its translation[9]. The first of these elements maps to the 5′ half of the *Prm1* 3′ UTR (Fig. 2*a*; ref. 10). The second element maps to the 3′ half of the *Prm1* 3′ UTR and binds Prbp *in vitro*[3]. We constructed a transgene (*Prm1*$_{69–156}$) containing the *Prm1* promoter, the gene encoding the human growth hormone reporter (*GH1*) and sequence encoding the 3′-most 88 nt of the *Prm1* 3′ UTR, and expressed it under *Prm1*-like translational control in *Tarbp2*$^{+/+}$ and *Tarbp2*$^{+/-}$ mice (Fig. 2*b*,*d*). We then crossed the *Prm1*$_{69–156}$ transgene into *Tarbp2*$^{-/-}$ animals to determine if Prbp absence results in premature translation of the transgene. Using immunohistochemical detection of growth hormone, we showed that the transgenic mRNA was not prematurely translated in *Tarbp2* mutants (Fig. 2*c*), but translational activation at the expected stage was affected. Staining of GH1 showed a mosaic pattern, with some cells expressing the transgene at the correct stage (Fig. 2*e*, arrow), whereas in other cells at the same stage the transgenic mRNA remained translationally repressed (Fig. 2*e*, arrowhead). Mosaic expression of the transgene in elongated spermatids suggests that Prbp functions in translational activation, not translational repression.

Failure to translate *Prm1*$_{69–156}$ transgenic mRNA at the appropriate stage was not due to absence of its mRNA in those cells. *In situ* hybridization showed that the transgenic mRNA was present in cells in which translational activation failed to occur (Fig. 3*a*,*b*). Translational activation actually resulted in degradation of the transgenic mRNA (Fig. 3*a*,*b*, arrows), suggesting that failure to translate the mRNA leads to its enhanced stability.

We did not observe translational activation of *Prm1*$_{69–156}$ transgenic mRNA in a subset of elongated spermatids in all appropriately staged seminiferous tubules, suggesting the presence of two distinct populations of spermatids. To determine if mosaic expression was specific to the transgene or was a more general feature of the *Tarbp2*$^{-/-}$ phenotype, we assayed expression of endogenous transition protein 2 (Tnp2) and Prm2. Correct translational activation of Tnp2 occurred in all appropriately staged spermatids in both wild-type (Fig. 3*c*) and mutant animals (Fig. 3*d*), suggesting that there is no general defect in translational activation in mutants. Similar to *Prm1*$_{69–156}$ transgenic mRNA, Prm2 expression was also mosaic

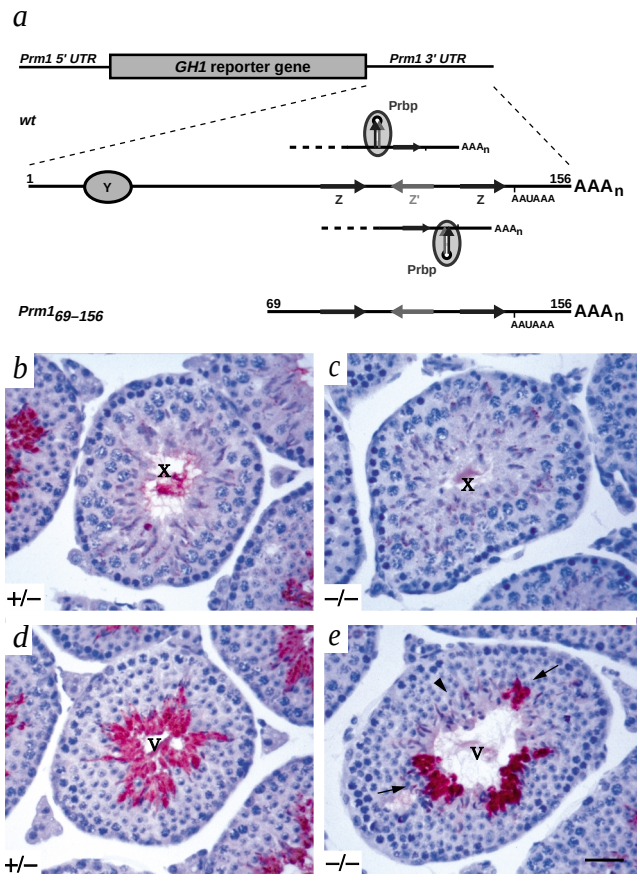

Fig. 2 Mosaic patterns of translation in *Tarbp2*$^{-/-}$ testis. ***a***, Structure of chimaeric transgenic mRNAs containing the *Prm1* 5′ UTR, *GH1* coding sequence and 156 (WT) or 88 (*Prm1*$_{69–156}$) nt of *Prm1* 3′ UTR. Within the 5′ end of the 3′ UTR is a binding site for a sequence-specific Y box protein (Y; H. Davies *et al.*, manuscript submitted). Within the 3′-most 88 nt is a nearly perfect direct repeat (Z) and its complement (Z′), which base pair to form 2 alternative stem-loop structures[3]. Prbp binds to the 3′-most 88 nt of the *Prm1* 3′ UTR (ref. 3), and to RNA containing a single copy of the repeat and its complement *in vitro* (unpublished data). The full-length 156-nt *Prm1* 3′ UTR confers translational delay on *GH1* mRNA *in vivo*[5], as does a transgenic mRNA containing the first 37 nt of the *Prm1* 3′ UTR (which harbours the binding site for the Y box protein) fused to the 3′-most 23 nt (ref. 9). ***b***,***c***, Proper translational delay of *Prm1*$_{69–156}$ transgenic mRNA in elongating spermatids of stage-X seminiferous tubules in *Tarbp2*$^{+/-}$ and *Tarbp2*$^{-/-}$ testes. ***d***, Proper translational activation of *Prm1*$_{69–156}$ in elongated spermatids of wild-type stage V seminiferous tubule. ***e***, Mosaic pattern of translational activation of *Prm1*$_{69–156}$ in *Prm1*$^{-/-}$ stage V seminiferous tubule. Arrows indicate immunopositive elongated spermatids. Arrowhead indicates immunonegative elongated spermatids. Scale bar, 30 μm.

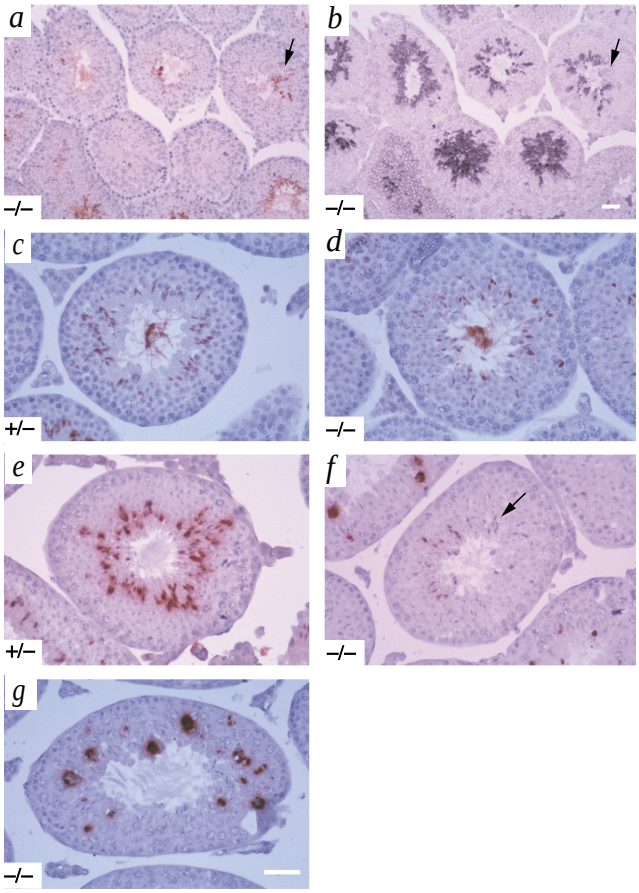

Fig. 3 *In situ* hybridization and immunohistochemistry of GH1, Tnp2 and Prm2. ***a***, Immunolocalization of GH1 in *Tarbp2*$^{-/-}$ mice transgenic for *Prm1*$_{69–156}$. ***b***, Detection of *GH1* mRNA by *in situ* hybridization in an adjacent tissue section. Arrows (*a*,*b*) point to a seminiferous tubule mosaic for the expression of GH1. Reciprocal patterns of protein and mRNA are seen. Regions of the tubule in (*a*) that lack GH1 protein are positive for the mRNA in (*b*), and regions of the tubule in (*a*) that stain positive for the protein show reduced mRNA in (*b*). Loss of mRNA in (*b*) presumably reflects its degradation as a consequence of its translation[17]. ***c***,***d***, Immunolocalization of Tnp2 in control and *Tarbp2*$^{-/-}$ testis, respectively. ***e***,***f***, Immunolocalization of Prm2 in control and mutant testis, respectively. ***g***, Expression of Prm2 in degenerating symplasts in *Tarbp2*$^{-/-}$ testis. *a*,*b*, Scale bar, 20 μm; *c*–*g*, scale bar, 40 μm.

in mutant (Fig. 3*f*) but not wild-type (Fig. 3*e*) testis. Failure to express Prm2 at the appropriate time resulted in prolonged retention of Tnp2 in later-staged elongated spermatids (data not shown). *Prm2* was translated in some later-stage cells, because we detected Prm2 in multinucleated symplasts present in later-stage seminiferous tubules (Fig. 3*g*).

To determine if the mosaic patches of translation overlapped between the *Prm1*$_{69–156}$ transgene and endogenous *Prm2*, we performed double-label immunofluorescence. The pattern of translational mosaicism was the same for Prm2 and GH1 (Fig. 4*a*–*h*), suggesting that translation of a group of target mRNAs may be affected in each clone. We were unable to assay mosaicism of endogenous *Prm1* mRNA due to inability of our Prm1 antibody to recognize the Prm1 protein in elongated spermatids[2]. To examine whether translational activation of endogenous *Prm1* mRNA occurred in mutants, we measured Prm1 protein levels in extracts prepared from whole adult testes by acid-urea gel electrophoresis. Prm1 accumulates to approximately the same levels as Prm2 in sonication-resistant spermatid nuclei (Fig. 5), showing that endogenous *Prm1* mRNA is translated. We believe that endogenous *Prm1* mRNA is also translated in a mosaic pattern; indirect support for this comes from *in situ* hybridization patterns of *Prm1*, *Prm2* and *Prm1*$_{69–156}$. In serial sections, all three mRNAs show strong signals in the same mosaic patches where Prm2 and GH1 are not detected, but in patches of active translation all three mRNAs show weaker signals (data not shown). As seen for *Prm1*$_{69–156}$ transgenic mRNA (Fig. 3*a*,*b*), activation of translation induces degradation of the mRNA, presumably because mRNA degradation is coupled with active translation.

Our data show that Prbp functions in the temporal translational activation of protamine mRNAs during male germ-cell

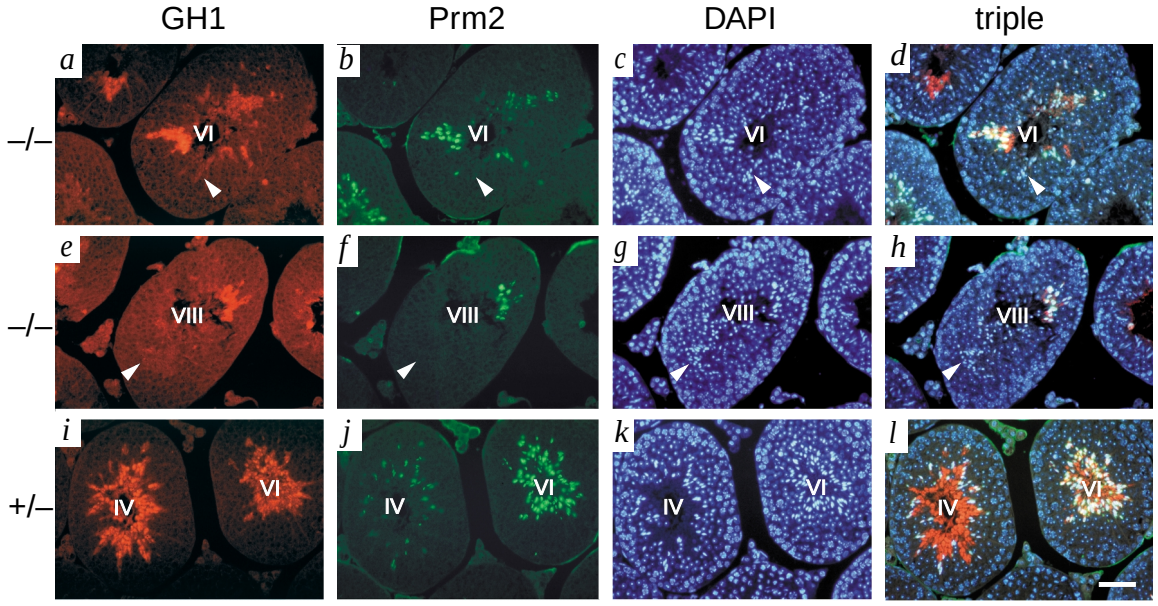

Fig. 4 Mosaic co-localization of GH1 and Prm2. ***a***,***e***,***i***, Localization of GH1. ***b***,***f***,***j***, Localization of Prm2. ***c***,***g***,***k***, DAPI-stained seminiferous tubules. ***d***,***h***,***i***, Photographs taken using a triple band pass filter. Roman numerals represent the stage of the spermatogenic tubule. Arrowheads in (*a*–*h*) designate spermatids not expressing GH1 or Prm2. Scale bar, 60 μm.

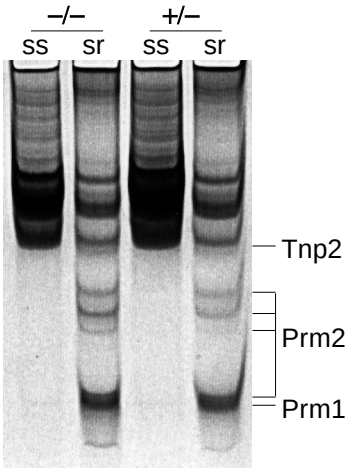

Fig. 5 Analysis of total testis basic proteins. Sonication-sensitive (ss) and sonication-resistant (sr) nuclear extracts were prepared from testes of homozygous mutant (–/–) and heterozygous mutant (+/–) animals as described[2]. Proteins were fractionated by acid-urea polyacrylamide gel electrophoresis and stained with 0.2% napthol blue-black. The positions of Tnp2 and Prm1 and the precursor, processed and mature forms of Prm2 are indicated.

differentiation, and confirm the importance of translational control in nuclear remodelling and chromatin compaction of the haploid genome. Retention of proper translational repression of *Prm1* and *Prm2* mRNAs suggests that Prbp is not involved in translational repression, or that another factor may compensate for Prbp. The ability of Prbp to inhibit translation *in vitro*[3] presumably reflects the nonspecific RNA-binding properties of this class of dsRNA-binding proteins *in vitro*, and may not accurately reflect normal Prbp function *in vivo*.

We do not know if Prbp acts directly or indirectly on protamine mRNAs. Expression of Prbp in round and elongating spermatids (where protamine RNAs are synthesized and stored) and the inability to detect Prbp in elongated spermatids[3] (where the protamine RNAs are translated) are consistent with Prbp acting as a chaperone in the assembly of a translationally regulated protamine ribonucleoprotein particle. Alternatively, mutation of *Tarbp2* may lead to a defect in spermiogenesis preceding the onset of protamine mRNA translation. Such a defect may lead to activation of a developmental checkpoint, resulting in cessation of translation of a group of target mRNAs. If this is the case, activation of the checkpoint would have to occur after synthesis of the transition proteins, as they are not affected in the mutant; alternatively, *Tnp2* mRNA might not be within the group of target mRNAs regulated by the checkpoint. The mosaicism observed here may reflect a threshold effect that is dependent on another factor whose abundance is variable in the mutant.

Methods

Histological analysis, immunohistochemistry and RNA *in situ* hybridization. For histological evaluation, we fixed dissected tissues in Bouin's (0.2% picric acid and 2% paraformaldehyde in 1×PBS), embedded them in paraffin and sectioned them (2 μm). Sections were deparaffinized in xylene, rehydrated by standard procedures and stained with haematoxylin and eosin or haematoxylin and periodic acid schiff.

We detected Prm2, Tnp2 and GH1 using testes fixed in Carnoy's (60% ethanol, 30% chloroform, 10% acetic acid). Immunohistochemistry was performed on sections (5 μm). We used mouse monoclonal antibodies Hup1N and Hup2B (ref. 11) to detect Prm1 and Prm2, respectively. To detect Prm2, slides were boiled in sodium citrate (0.1 M) for 10 min in a microwave. GH1 was detected using a rabbit polyclonal antibody obtained from the National Hormone and Pituitary Program. We detected Tnp2 using a rabbit polyclonal antibody[12] (kindly provided by S. Kistler). Primary antibodies were applied after the slides had been rehydrated and blocked in 3% goat serum. We washed the slides in PBS, then incubated them with biotinylated secondary antibody and streptavidin conjugated to horseradish peroxidase as suggested by the manufacturer (Zymed Laboratories). Peroxidase activity was visualized with chromogen aminoethyl carbazole. Sections were counterstained with haematoxylin. We used goat-anti-rabbit/Alexa568 and goat-anti-mouse/Alexa488 (Molecular Probes) as secondary antibodies to detect GH1 and Prm2.

We carried out RNA *in situ* hybridization on Carnoy's fixed testes embedded in paraffin, then sectioned them (5 μm). Serial sections were used for immunohistochemistry and RNA *in situ* hybridization. We used a 170-bp *GH1* cDNA fragment and a 140-bp *Prm1* cDNA fragment cloned in pGEM2 plasmid (Promega) to synthesize digoxigenin-labelled RNA probes. RNA *in situ* hybridization was carried out with anti-sense RNA probes as described[13]. The sense probes were used as negative controls.

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