—Original Article—

# Functional compensation for the loss of testis-specific poly(A)-binding protein, PABPC2, during mouse spermatogenesis

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**Abstract.** Mouse testes contain several isoforms of cytoplasmic poly(A)-binding proteins (PABPCs), including ubiquitous PABPC1 and testis-specific PABPC2/PABPt. PABPC2 is characterized by its absence from translationally active polyribosomes and elongating spermatids. To elucidate the function of PABPC2 in spermatogenesis, we produced mutant mice lacking PABPC2. The PABPC2-null mice showed normal fertility. The processes of spermatogenesis and sperm migration in the testes and epididymides, respectively, were normal in the mutant mice. When the involvement of PABPC2 in translational regulation of haploid-specific mRNAs was examined, these mRNAs were correctly transcribed in round spermatids and translated in elongating spermatids. Moreover, immunoblot analysis revealed low abundance of PABPC2 relative to PABPC1 in spermatogenic cells. These results suggest that PABPC2 may be either functionally redundant with other PABPCs (including PABPC1) or largely dispensable for translational regulation during spermiogenesis.
**Key words:** Mouse, mRNA metabolism, PABPC2, Poly(A), Spermatogenesis

Spermatogenesis is a highly specialized process of cellular differentiation for the production of spermatozoa, in which diploid spermatogonia proliferate and divide meiotically to generate haploid round spermatids that are in turn transformed into spermatozoa. The precise regulation of spermatogenesis requires a controlled program of stage-specific gene expression regulated at the transcriptional, post-transcriptional, and translational levels [1–6]. Drastic morphological changes, such as the formation of a flagellum and a highly compacted nucleus, are accomplished during spermiogenesis: the haploid phase of spermatogenesis. Transcription of the haploid genome ceases midway through spermiogenesis, concomitantly with the onset of chromatin condensation [7]. Accordingly, mRNAs encoding structural proteins required for cellular remodeling are transcribed in early haploid spermatids and stored as translationally inert messenger ribonucleoprotein particles (mRNPs). Subsequent translational activation of the repressed messages in late spermatids accompanies poly(A) shortening [8, 9]. On the other hand, poly(A) tails of some mRNAs are known to be longer in early haploid spermatids than in meiotic spermatocytes [10–14]. Thus, the mRNA metabolism plays a critical role in spermiogenesis.

Poly(A) tails of eukaryotic mRNAs are post-transcriptionally added to the 3′ end of mRNAs in the nucleus. After the transport of mRNA to the cytoplasm, the cytoplasmic forms of poly(A)-binding proteins (PABPCs) associate with the poly(A) tails. In mice, five PABPCs— PABPC1, PABPC2/PABPt, PABPC4/iPABPC, PABPC1L/ ePABPC, and PABPC5— have been identified to date [15]. *Pabpc1* is ubiquitously expressed in mammalian cells, whereas expression of intronless *Pabpc2* is exclusive to spermatogenic cells [16, 17]. PABPC4 and PABPC1L are essential for erythroid differentiation and oocyte maturation, respectively [18, 19]. Moreover, X-linked PABPC5 lacks the C-terminal domain present in the other PABPC members. Among these five PABPCs, PABPC1 has been extensively studied in relation to various parameters of mRNA metabolism, including mRNA stabilization, cytoplasmic polyadenylation and deadenylation, translation initiation and termination, and microRNA-associated regulation [15, 20–22]. In spermatogenesis, PABPC1 is implicated in the ablation of PABPC1-interacting proteins, including DAZL, BOULE, and PABPC-interacting protein 2A (PAIP2A), which results in spermatogenic defects at different stages [5, 6].

Murine *Pabpc2* is a retroposed paralogue gene that originates from *Pabpc1* [16, 23]. Previously, we found that PABPC1 and PABPC2 are complexed with each other and associate nonspecifically with mRNAs during spermatogenesis [17]. Both PABPC proteins also interact with several translation-associated factors, including eukaryotic translation initiation factor 4G (eIF4G), and are capable of enhancing translation of a reporter mRNA *in vitro*. Despite these functional similarities, PABPC2 differs from PABPC1 in the distribution among spermatogenic cells and polyribosomes and in the expression pattern during spermatogenesis [17]. In the present study, to uncover the function of PABPC2, we produced mutant mice lacking PABPC2 and analyzed the effects of the PABPC2 knockout on mRNA metabolic pathways.

## Materials and Methods

### Generation of PABPC2-null mice

A targeting vector containing a 1.1-kbp expression cassette of the neomycin resistance gene *neo* (pMC1neopA; Stratagene, La Jolla, CA, USA), which was flanked by approximately 8.0- and 1.6-kbp genomic regions of *Pabpc2* at the 5′ and 3′ ends, respectively, was constructed as described previously [14]. The herpes simplex virus thymidine kinase gene (HSV-*tk*) was inserted into the 1.6-kbp *Pabpc2* genomic region at the 3′ end (Fig. 1A). After electroporation of the targeting vector (which had been linearized by digestion with *Not*I) into mouse D3 embryonic stem (ES) cells, homologous recombinants were selected using G418 and ganciclovir, as described previously [14]. Five ES cell clones carrying the targeted mutation were isolated from approximately 300 doubly-resistant colonies, and three clones were injected into blastocysts of C57BL/6 mice (Japan SLC, Shizuoka, Japan). The chimeric male mice that were born were crossed with C57BL/6 females to create heterozygous (*Pabpc2*$^{+/-}$) mutant mice. Homozygous (*Pabpc2*$^{-/-}$) mice were obtained by mating the heterozygous males and females.

### PCR genotyping

Genomic DNAs of ES cell clones and mouse tails were amplified by PCR using three primers: 5′-ATGGATGACGAGACCCTGAATG-3′ (G16, see Fig. 1A), 5′-GCGCTGCGAATCGGGAGCGGCGATACC GT-3′ (Neo), and 5′-GGTCTCTGGTCAGTTTAAACAGTTGGG-3′ (G17). The PCR program consisted of 35 cycles of 94°C for 30 sec, 64°C for 1 min, and 72°C for 2 min. Approximately 1.3- and 2.1-kbp DNA fragments were produced by the primer sets G16/G17 and Neo/G17, respectively.

### Southern blot analysis

Genomic DNA samples (10 µg) were digested with *Bam*HI and *Hin*dIII, subjected to electrophoresis in agarose gels, and transferred onto Hybond-N$^+$ membranes (GE Healthcare, Piscataway, NJ, USA). The blots were hybridized with a $^{32}$P-labeled *Hin*cII-*Sph*I DNA fragment (Fig. 1A), as described previously [14].

### Northern blot analysis

Total RNA samples (5 µg) of mouse testicular tissues were prepared using the ISOGEN Kit (Nippon Gene, Toyama, Japan). The RNA samples were denatured with glyoxal, separated on agarose gels, and transferred onto Hybond-N$^+$ nylon membranes (GE Healthcare) [13]. The blots were probed with $^{32}$P-labeled DNA fragments.

### Reverse transcription polymerase chain reaction (RT-PCR)

Total RNAs (1 µg) were digested with RQ1 RNase-free DNase (Promega, Madison, WI, USA) and reverse-transcribed in the presence of an oligo(dT)$_{20}$ primer using a SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA, USA). A portion of the synthesized cDNA was amplified by PCR using the following sets of primers: *Pabpc2*, 5′-GGAATTCGCCACCATGGCCTCGCTCTATG-3′ and 5′-CCTGCCAAAGAGGCCATTCA-3′; *Actb*, 5′-GGTGACAGC ATTGCTTCTGTG-3′ and 5′-GGAATTCAAGTCAGTGTACAG GCCAG-3′. The PCR program consisted of 30 cycles of 98°C for 10 sec, 55°C for 15 sec, and 68°C for 1 min.

### Antibodies

Affinity-purified rabbit polyclonal antibodies against the 13- and 12-residue peptides specific for mouse PABPC1 and PABPC2, respectively, were prepared as described previously [17]. A polyclonal antibody capable of recognizing both mouse PABPC1 and PABPC2 (hereafter called the anti-PABPC1/2 antibody) was also prepared using a 6 × His- and thioredoxin (TRX)-tagged fragment of the PABPC1 protein (amino acid residues 53–363) as an antigen. The 311-residue sequence is 88% identical between PABPC1 and PABPC2 [16]. Briefly, the recombinant PABPC1 was expressed in *Escherichia coli* and purified on a Ni-NTA His column (Merck Millipore, Billerica, MA, USA). The purified protein (400 µg) was emulsified with Freund's complete (Becton Dickinson, Franklin Lakes, NJ, USA) or incomplete adjuvant (Wako, Osaka, Japan) and injected into female New Zealand White rabbits (Japan SLC) [17]. The antisera were fractionated with ammonium sulfate (0–40% saturation) followed by immunoaffinity chromatography on a Sepharose 4B (GE Healthcare) column conjugated with the 311-residue fragment of PABPC1 protein fused to glutathione *S*-transferase (GST), as described previously [24]. A mouse monoclonal antibody against murine A-kinase anchor protein 4 (AKAP4/AKAP82/PRKA4; sc-135827) and goat polyclonal antibodies against human phosphoglycerate kinase 2 (PGK2; sc-133905), human protamine 2 (PRM2; sc-23104), and mouse transition protein 2 (TNP2; sc-21106) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Mouse monoclonal anti-β-actin (ACTB; A5441) and rabbit polyclonal anti-His tag antibodies (PM032) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Medical & Biological Laboratories (Nagoya, Japan), respectively.

### Immunoblot analysis

Testicular tissues were homogenized at 4°C in 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5% Nonidet P-40, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, and 0.5 mM phenylmethanesulfonyl fluoride, using a Teflon-glass homogenizer (750 rpm, 10 strokes). After incubation at 4°C for 4 h, the homogenates were centrifuged in a microcentrifuge at 13,400 × *g* for 10 min at 4°C. The supernatant solution was used as protein extracts. Protein concentration was determined by means of the Coomassie Protein Assay Reagent Kit (Thermo Fisher Scientific). Protein samples (5 µg) were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Merck Millipore). After blocking with 2% skim milk or gelatin, the blots were probed with primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The immunoreactive proteins were visualized by an ECL or an ECL Prime Western Blot Detection Kit (GE Healthcare).

### Histological analysis

Testicular and epididymal tissues were fixed with Bouin's fixative and embedded in paraffin. Paraffin sections (4-µm thick) were prepared in a MICROM HM340E (Microedge Instruments, White Rock, BC, Canada), mounted on slides, deparaffinized in xylene, and hydrated in a graded ethanol series. After staining with hematoxylin and eosin (Wako), the slides were examined under a DM IRBE microscope (Leica Microsystems, Wetzlar, Germany).
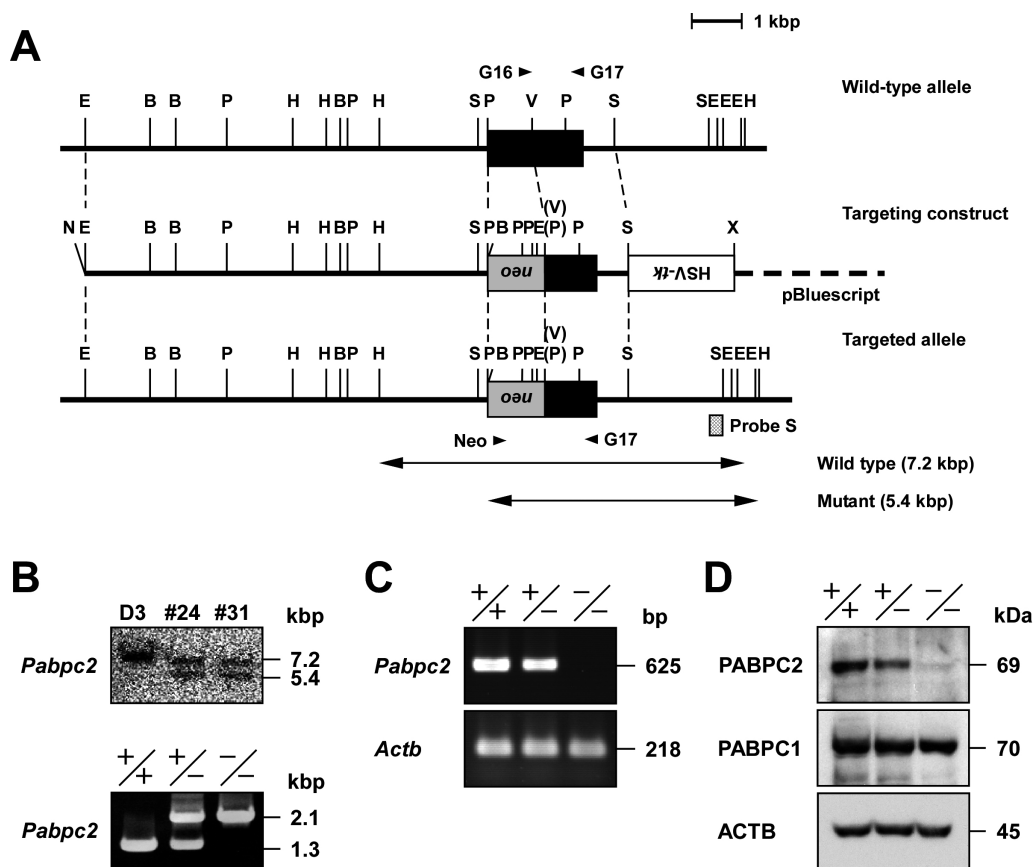
**Fig. 1.** Targeted disruption of mouse *Pabpc2*. (A) Physical maps of *Pabpc2*, targeting construct, and predicted targeted allele. The locations of the protein-coding region in *Pabpc2* and of *neo* are boxed with black and gray colors, respectively. The open box represents HSV-*tk*. The restriction enzyme sites are indicated as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; P, *Pst*I; S, *Sph*I; V, *Eco*RV; and X, *Xho*I. (B) Detection of the targeted allele. Genomic DNAs of original D3 ES cells (D3) and two targeted ES cell clones (#24 and #31) were double-digested with *Bam*HI and *Hin*dIII and subjected to Southern blot analysis (upper panel) using a $^{32}$P-labeled DNA fragment (Probe S in panel A). Tail DNAs of the wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice were analyzed by PCR (lower panel) using primer sets G16/G17 and Neo/G17 (see panel A). (C) RT-PCR analysis. The protein-coding region of *Pabpc2* mRNA was analyzed by RT-PCR in testicular total RNA, with *Actb* mRNA as a control. (D) Immunoblot analysis. Protein extracts of testicular tissues were analyzed by immunoblotting with a PABPC1- or PABPC2-specific antibody. ACTB served as a control.

*Statistical analysis*

The data are presented as mean ± SEM (n ≥ 3), unless stated otherwise. The Student *t*-test was used for statistical analysis; significance was assumed at P < 0.05.

*Ethics statement*

All animal experiments were approved and performed in accordance with the Guide for the Care and Use of Laboratory Animals at the University of Tsukuba (approval numbers 14-022 and 15-015).

**Results**

To elucidate the function of PABPC2 in spermatogenesis, we produced mice carrying a null mutation of *Pabpc2* using homologous recombination in ES cells (Fig. 1A). A targeting vector was designed to delete PABPC2 by replacing the 871-nucleotide protein-coding region containing the RNA-recognition motifs 1 through 4 with the

*neo* cassette. When genomic DNAs of selected ES cell clones were subjected to Southern blot analysis, a correctly targeted allele yielded an expected band corresponding to a 5.4-kbp *Bam*HI-*Hin*dIII DNA fragment, in addition to a 7.2-kbp *Hin*dIII-*Hin*dIII fragment derived from the wild-type allele (Fig. 1B, upper panel). The genotypes of *Pabpc2*$^{+/+}$, *Pabpc2*$^{+/−}$, and *Pabpc2*$^{−/−}$ mice were also verified by PCR using tail genomic DNA as a template (Fig. 1B, lower panel). Moreover, RT-PCR analysis indicated the loss of functional *Pabpc2* mRNA in *Pabpc2*$^{−/−}$ testes (Fig. 1C). Indeed, testicular extracts of *Pabpc2*$^{−/−}$ mice lacked 69-kDa PABPC2, whereas 70-kDa PABPC1 was normally present (Fig. 1D). These results prove the successful gene knockout of *Pabpc2*.

Intercross between the *Pabpc2*$^{+/−}$ male and female mice yielded an expected Mendelian frequency in the offspring [*Pabpc2*$^{+/+}$: *Pabpc2*$^{+/−}$: *Pabpc2*$^{−/−}$ = 23 (27%): 41 (49%): 20 (24%) for 84 pups from 10 litters]. Both *Pabpc2*$^{−/−}$ males and females apparently demonstrated normal behavior, body weight, and health. When the male fertility

was tested by mating two of each *Pabpc2⁺/⁺* and *Pabpc2⁻/⁻* males with wild-type females, no significant differences were observed between the *Pabpc2⁺/⁺* and *Pabpc2⁻/⁻* males (Fig. 2A). The testicular weights of *Pabpc2⁺/⁺* and *Pabpc2⁻/⁻* mice were also similar (Fig. 2B). Histological analysis indicated that spermatogenesis and sperm migration in the testes and epididymides, respectively, of *Pabpc2⁻/⁻* mice proceeded normally (Fig. 2C). Moreover, *Pabpc2⁻/⁻* epididymal sperm were morphologically indistinguishable from *Pabpc2⁺/⁺* sperm (Fig. 2D). These results suggest that spermatogenesis and fertility are not affected by the loss of PABPC2. It should be noted that the *Pabpc2⁻/⁻* females also showed normal fertility and produced litters of normal size ($8.7 \pm 0.5$ offspring for seven litters).

We next examined the effect of the PABPC2 knockout on mRNA metabolism in spermatogenic cells. Northern blot analysis indicated that the levels of five haploid-specific mRNAs, including *Prm1*, *Prm2*, and *Tnp2* mRNAs, were similar among *Pabpc2⁺/⁺*, *Pabpc2⁺/⁻*, and *Pabpc2⁻/⁻* mice (Fig. 3A). Notably, the abundance of poly(A)-shortened forms of *Prm1*, *Prm2*, and *Tnp2* mRNAs of the sizes 0.4, 0.6, and 0.6 kb, respectively, in the *Pabpc2⁻/⁻* testes was comparable with that in the *Pabpc2⁺/⁺* and *Pabpc2⁺/⁻* testes. Consistent with the previous findings that poly(A) shortening accompanies translational activation of these three mRNAs [8, 9], the PRM2 and TNP2 levels were unaffected by the loss of PABPC2 (Fig. 3B). The levels of PGK2 and AKAP4 were also similar among the *Pabpc2⁺/⁺*, *Pabpc2⁺/⁻*, and *Pabpc2⁻/⁻* testes.

To test whether the loss of PABPC2 elicits precocious translation of *Prm2*, *Tnp2*, *Pgk2*, and *Akap4* mRNAs in round spermatids, we conducted an immunoblot analysis (Fig. 3C). We examined the testicular protein extracts of mice 26 days and 11 weeks after birth because the 26-day-old testes contain no elongating spermatids [6]. Four proteins —PRM2, TNP2, PGK2, and AKAP4— present only in elongating spermatids were absent in the 26-day-old testes of *Pabpc2⁺/⁻* and *Pabpc2⁻/⁻* mice (Fig. 3C). In addition, the 11-week-old *Pabpc2⁺/⁻* and *Pabpc2⁻/⁻* testes contained equal amounts of these four proteins. As expected, the 0.4-kb *Prm1*, 0.6-kb *Prm2*, and 0.6-kb *Tnp2* mRNAs were absent in the 26-day-old *Pabpc2⁺/⁻* and *Pabpc2⁻/⁻* testes but present in the 11-week-old testes (Fig. 3D). Thus, PABPC2 may have no effect on the regulated translation of haploid-specific mRNAs during spermiogenesis.

As described above, murine spermatogenic cells contain at least two isoforms of PABPCs, 70-kDa PABPC1 and 69-kDa PABPC2, that share a relatively high degree of sequence identity (approximately 80%) [16]. Although PABPC1 is distinguished from PABPC2 by the expression pattern during spermatogenesis [17], the levels of expression of these two proteins in spermatogenic cells are still unknown. Accordingly, we prepared the affinity-purified anti-PABPC1/2 antibody capable of recognizing both PABPC1 and PABPC2, using a 311-residue fragment of the PABPC1 protein as an antigen. Specificity of the anti-PABPC1/2 antibody was validated by immunoblot analysis of His-tagged recombinant PABPC1 and PABPC2 proteins (Fig. 4A). Anti-PABPC1 and anti-PABPC2 antibodies specifically recognized the recombinant PABPC1 and PABPC2, respectively, as described previously [17]. Predictably, the anti-PABPC1/2 antibody recognized these two recombinant proteins equivalently. When testicular protein extracts were analyzed by immunoblotting, the anti-PABPC1/2 antibody yielded an immunoreactive band corresponding to both
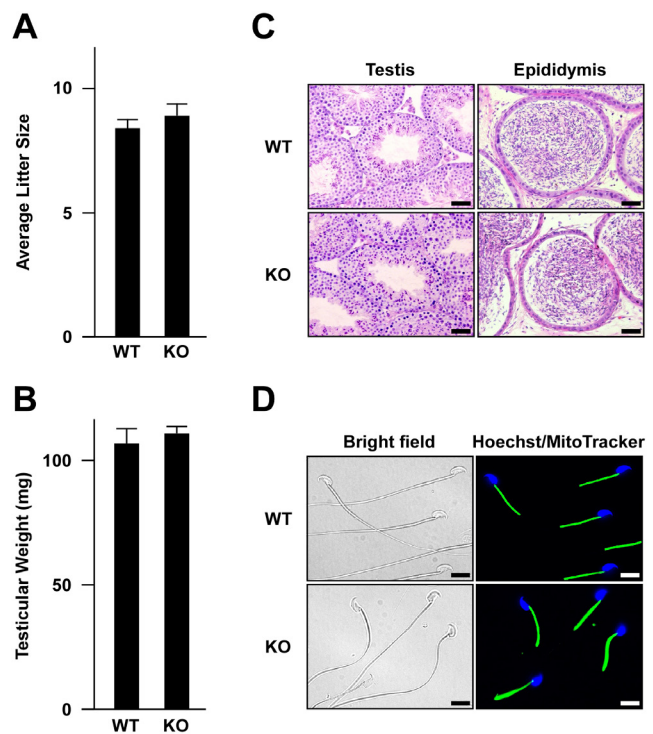


**Fig. 2.** Characterization of the male PABPC2 knockout mice. (A) Fertility of male mice. Ten-week-old wild-type (WT) and PABPC2 knockout (KO) males were mated with 8-week-old C57BL/6 females. Two different KO males were fertile, and the pups born were counted (seven litters). (B) Testicular weight. Testicular tissues of 13-week-old WT and KO mice were weighted (n = 6). (C) Histological analysis. Testicular and epididymal slices were stained with hematoxylin and eosin and examined under a microscope. The scale bar is 50 μm. (D) Sperm morphology. Epididymal sperm of WT and KO mice were double-stained with Hoechst 33342 (blue) and MitoTracker Green FM (green) and then examined. The scale bar is 10 μm.

PABPC1 and PABPC2 or only PABPC1 in *Pabpc2⁺/⁺*, *Pabpc2⁺/⁻*, and *Pabpc2⁻/⁻* mice (Fig. 4B). Densitometric analysis of the immunoblots (n = 2) indicated that the average ratio of signal intensities was 100: 113: 114 for the *Pabpc2⁺/⁺*, *Pabpc2⁺/⁻*, and *Pabpc2⁻/⁻* testes, respectively. Thus, only a small amount of PABPC2 may be present in spermatogenic cells as compared to PABPC1.

## Discussion

This study describes functional compensation for the loss of mouse PABPC2 in spermatogenic cells. We [17] previously found that PABPC2 is completely or largely absent both in elongating spermatids and in actively translating polyribosomes. These findings raise the possibility that PABPC2 protects haploid-specific mRNAs, including *Prm2* and *Tnp2* mRNAs, from precocious translation in round spermatids. It is also likely that the absence of PABPC2 in elongating spermatids is implicated in deadenylation of the haploid-specific mRNAs as a prerequisite for translation. Nonetheless, our present data indicate that the haploid-specific mRNAs are
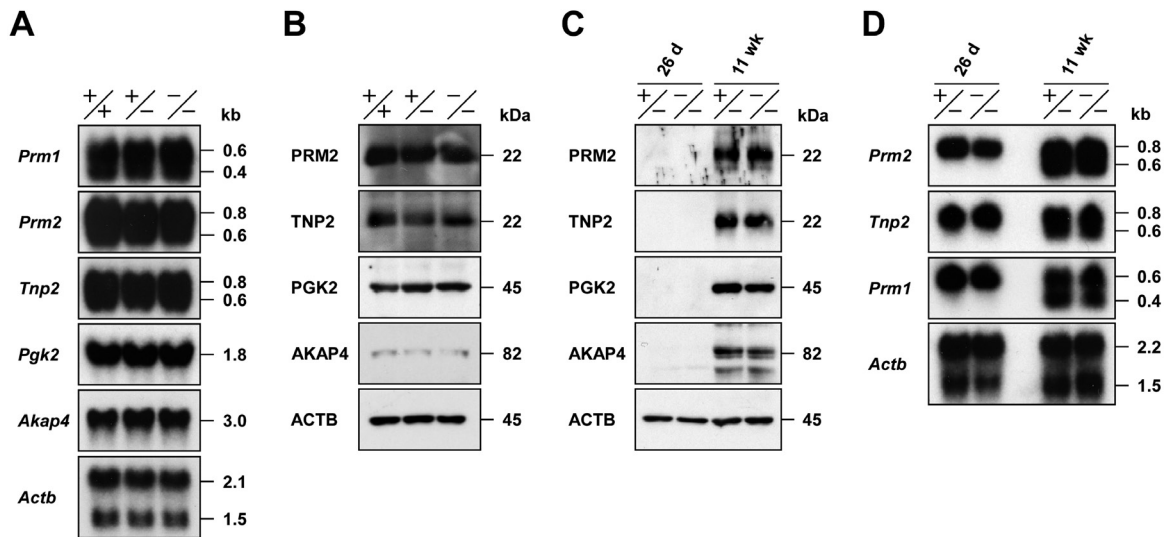
**Fig. 3.** Expression and translation of haploid-specific mRNAs in *Pabpc2*$^{-/-}$ mice. (A) Northern blot analysis. Testicular total RNAs of the wild-type (+/+), heterozygous (+/–), and homozygous (–/–) mice were analyzed by Northern blot hybridization with the cDNA probes indicated. *Actb* served as a control. (B and C) Immunoblot analysis. Testicular protein extracts of 11-week-old mice (B) or 26-day-old (26 d) and 11-week-old mice (C) were analyzed by immunoblotting with the antibodies indicated. ACTB was used as a control. (D) Normal translational control of haploid-specific mRNAs in *Pabpc2*$^{-/-}$ mice. Testicular total RNAs of 26-day-old and 11-week-old mice were analyzed by Northern blot hybridization. *Actb* served as a control.
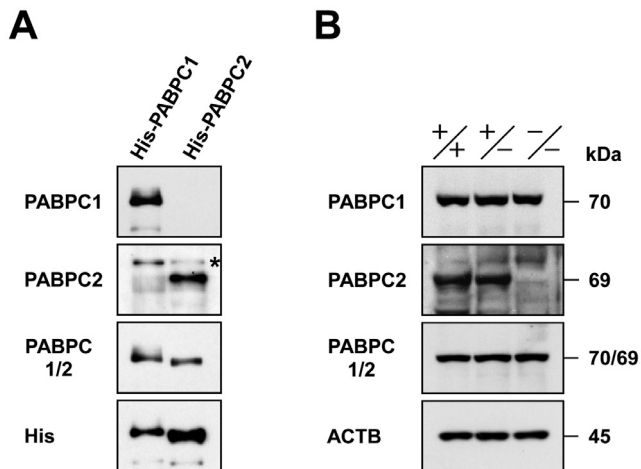


**Fig. 4.** Abundance of PABPC2 in spermatogenic cells. Immunoblot analysis of His-tagged recombinant PABPC1 and PABPC2 proteins (A) or testicular protein extracts from the wild-type (+/+), heterozygous (+/–), and homozygous (–/–) mice (B) was carried out using the antibodies indicated. An asterisk indicates that a nonspecific protein reacted with the anti-PABPC2 antibody. ACTB served as a control.

normally transcribed in round spermatids and translated in elongating spermatids. Thus, PABPC2 may be either functionally redundant with other PABPCs (including PABPC1) or largely dispensable for translational regulation during spermiogenesis. The low abundance of PABPC2 relative to PABPC1 in spermatogenic cells appears to support this notion.

Proteins of the PABPC family perform important functions in vertebrate cells [18, 19, 25]. Despite the presence of PABPC1, depletion of mouse PABPC4/iPABPC results in a change of the steady-state levels of some erythroid mRNAs, leading to inhibition of terminal erythroid maturation [19]. Female mice lacking germ line-specific PABPC1L/ePABPC are infertile; the oocytes fail to mature because protein synthesis is impaired by the abrogated cytoplasmic polyadenylation of maternal mRNAs [18, 26]. The oogenesis-specific defects in the PABPC1L/ePABPC-null mice may be explained by the absence of PABPC1 until four-cell embryos [27], although male germ cells contain both PABPC1 and PABPC1L/ePABPC [28]. As described above, PABPC1 and PABPC2 are both present in pachytene spermatocytes and round spermatids [17]. Thus, even if PABPC2 is involved in the translational mechanism, the loss of PABPC2 in *Pabpc2*$^{-/-}$ spermatogenic cells may be compensated by PABPC1, as in PABPC1L/ePABPC-null spermatogenic cells.

A puzzling question is why functionally redundant *Pabpc2* is expressed in spermatogenic cells. Intronless genes that are specifically expressed in testes, including *Pabpc2*, have arisen from intron-containing progenitor genes by retroposition [23, 29, 30]. It is noteworthy that the retroposition from the X-linked genes has been suggested to be necessary as a compensation mechanism for depletion of somatic isoforms caused by meiotic X chromosome inactivation during spermatogenesis [31]. Indeed, inactivation of genes encoding polyadenylation factor CSTF2T/τCstF-64 or centriole protein CETN1 (centrin 1) results in the spermatogenic arrest [32, 33]. In contrast, *Pabpc2* is believed to be retroposed from autosomal *Pabpc1* [23]. One possible explanation for the exclusive expression of *Pabpc2* in spermatogenic cells may be transcriptional promiscuity induced by a high concentration of RNA polymerase II holoenzyme

in meiotic and early haploid cells; this state of affairs may enable transcription from the gene promoters inactive in other cells [23, 34, 35]. A similar scenario may be applicable to intronless *Pabpc3/ tPabp* exclusively expressed in primate testes [36].

## Acknowledgments

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