—Original Article—

Adenylation by testis-specific cytoplasmic poly(A) polymerase, PAPOLB/TPAP, is essential for spermatogenesis

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Abstract. The testis-specific cytoplasmic poly(A) polymerase PAPOLB/TPAP is essential for spermatogenesis. Although this enzyme is responsible for poly(A) tail extension of a subset of mRNAs in round spermatids, the stability and translational efficiency of these mRNAs are unaffected by the absence of PAPOLB. To clarify the functional importance of this enzyme's adenylation activity, we produced PAPOLB-null mice expressing a polyadenylation-defective PAPOLB mutant (PAPOLB[D114A]), in which the catalytic Asp at residue 114 was mutated to Ala. Introducing PAPOLB[D114A] failed to rescue PAPOLB-null phenotypes, such as reduced expression of haploid-specific mRNAs, spermiogenesis arrest, and male infertility. These results suggest that PAPOLB regulates spermatogenesis through its adenylation activity.
Key words: Cytoplasmic polyadenylation, PAPOLB, Poly(A), Spermatogenesis

P oly(A) tails present at the 3'-end of eukaryotic mRNAs are central in the life of mRNAs by regulating stability, translation initiation and termination, and degradation [1–4]. Thus, controlling poly(A) tail length is one of the post-transcriptional regulations of gene expression. In mammalian cells, primary transcripts are generally cleaved within 30 nucleotides (nt) downstream of the polyadenylation signal, AAUAAA, followed by the addition of ~250 nt poly(A) tails by poly(A) polymerase α (PAPOLA) in a co-transcriptional manner [5, 6]. Besides nuclear polyadenylation, cytoplasmic polyadenylation occurs during oocyte maturation and early embryogenesis, where transcription is entirely quiescent. Maternal mRNAs transcribed in earlier stages are deadenylated upon their exit from the nucleus ($\sim A_{10}$) and are stored in a translationally inactive state. During specific developmental stages, subsets of mRNAs undergo cytoplasmic poly(A) tail extension ($\sim A_{200}$) as a prerequisite for translational activation [7–11].

Spermatogenesis is a specialized cellular differentiation process that produces spermatozoa, which comprises spermatogonial proliferation, two meiotic divisions, and drastic morphological remodeling during spermiogenesis, the haploid phase of spermatogenesis. This process is accomplished by a controlled, stage-specific gene expression program regulated at the transcriptional, post-transcriptional, and translational levels [12–17]. At least two distinct patterns of poly(A) tail length changes have been described during spermatogenesis.

Haploid-specific mRNAs encoding structural proteins required for the formation of flagella and highly compacted nuclei are transcribed in round spermatids and are stored as translationally inert messenger ribonucleoprotein particles (mRNPs) with long poly(A) tracts ($\sim A_{180}$) for several days. Subsequent translational activation of the mRNAs during later spermiogenesis accompanies the generation of partially deadenylated species ranging from A_{150} to A_{30} [18, 19]. Conversely, poly(A) tails of some mRNAs are 50–150 nt longer in round spermatids than in meiotic pachytene spermatocytes [13, 20–23].

We previously reported that ablating the testis-specific cytoplasmic poly(A) polymerase PAPOLB/TPAP results in impaired spermiogenesis and male infertility, which can be recovered by transgenic re-expression [23, 24]. This enzyme is responsible for cytoplasmic poly(A) tail extension of certain transcription factor mRNAs in round spermatids [23]. However, additional poly(A) tail elongation appeared not to be responsible for either stability or translational efficiency of the substrate mRNAs (Fig. 1) [23]. These findings prompted the hypothesis that PAPOLB polyadenylation activity is dispensable for spermatogenesis. To address this, we produced and analyzed PAPOLB-null mice expressing a polyadenylation-defective PAPOLB mutant.

Materials and Methods

Plasmid construction

Expression plasmids encoding N-terminally FLAG- and HA-tagged PAPOLB and polyadenylation-defective PAPOLB[D114A] mutant, in which Asp at position 114 (one of the three putative catalytic Asp residues) was replaced with Ala, were constructed as follows. The cDNA fragment encoding the entire open reading frame of PAPOLB was amplified by polymerase chain reaction (PCR) using the primers 5'-GGAATTCATGATGCCATTTGCGTGACC-3' and 5'-TCCTCGAGCTAGACTCCTAGTATAGGATTGG-3',

A

B
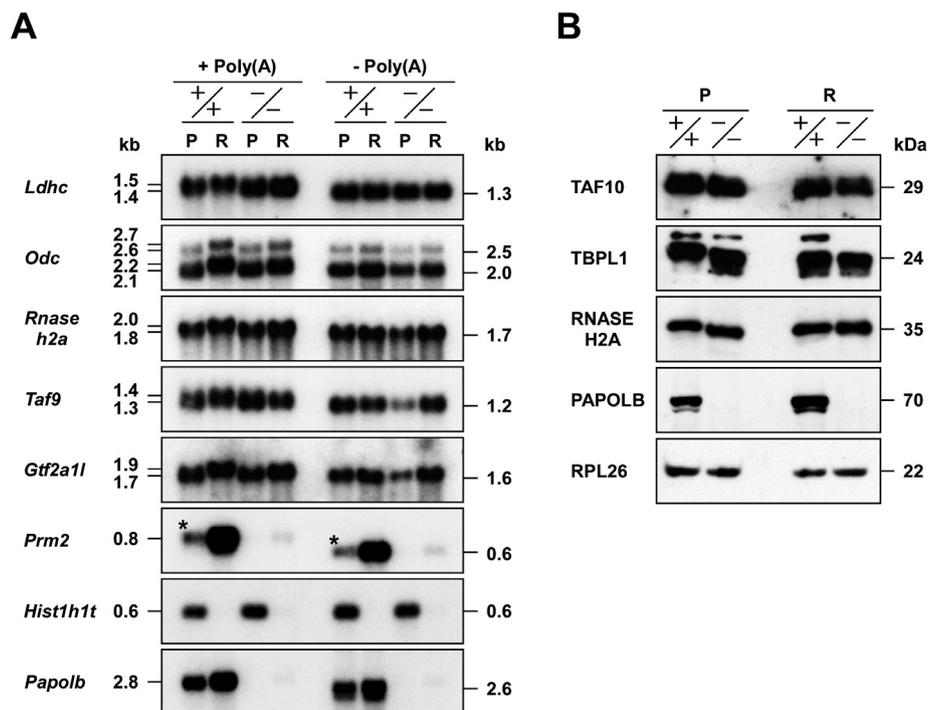
Fig. 1. Additional poly(A) tail extension by PAPOLB and its effect on mRNA metabolism. Pachytene spermatocytes (P) and round spermatids (R) of wild-type (+/+) and PAPOLB-null (–/–) mice were purified by unit gravity sedimentation on 2–4% BSA gradients. (A) Northern blot analysis. Total RNAs were incubated with RNase H in the presence [–Poly(A)] or absence [+Poly(A)] of oligo(dT)$_{15}$ and subjected to Northern blot analysis with the cDNA probes indicated. *Hist1h1t* and *Prm2* were used as meiosis- and haploid-specific markers, respectively. Asterisks represent *Prm2* mRNA derived from round spermatids contaminated during germ cell fractionation. (B) Immunoblot analysis. Protein extracts were analyzed by immunoblotting using the antibodies indicated. RPL26 was used as a control.

and the cloned mouse cDNA as a template [22]. After digestion with *Eco*RI and *Xho*I, the fragment was inserted into pcDNA3-FLAGHAN, which is designed to express the protein with N-terminal FLAG and HA tags (PAPOLB/pcDNA3-FLAGHAN). The plasmid encoding FLAG- and HA-tagged PAPOLB^{D114A} (PAPOLB^{D114A}/pcDNA3-FLAGHAN) was generated by PCR-based mutagenesis using the primer pair (mutated nucleotides are underlined): 5'-CGAAAGGTGCAG<u>C</u>TATCGATGCTTTG-3' and 5'-CAAAGCATCGATA<u>G</u>CTGCACCTTTCG-3' and PAPOLB/pcDNA3-FLAGHAN as a template.

Polyadenylation assay

Poly(A) polymerase activity was measured as previously described [22]. Briefly, recombinant enzymes were produced in rabbit reticulocyte lysates using a TNT T7 Quick Coupled Transcription/Translation system (Promega, Madison, WI, USA). PAPOLB/pcDNA3-FLAGHAN or PAPOLB^{D114A}/pcDNA3-FLAGHAN (0.5 µg) was used as a template in a 50-µl reaction mixture. The polyadenylation assay was carried out in 100 µl of 50 mM Tris/HCl, pH 8.3, 7% glycerol, 0.3% polyvinyl alcohol, 40 mM KCl, 0.5 mM MnCl$_2$, 25 mM (NH$_4$)$_2$SO$_4$, 0.25 mM dithiothreitol (DTT), 50 units of RNase OUT (Thermo Fischer Scientific, Waltham, MA, USA), 0.1 mM ATP, 4 µl of [α-^{32}P]ATP (29.6 TBq/mmol; PerkinElmer, Waltham, MA, USA), 4 µg of oligo(A)$_{12}$, and 12 µl of *in vitro* translation

product. The mixtures were incubated at 30°C and aliquots (5 µl) of reaction mixtures were spotted onto Whatman DE-81 papers, dried, and washed five times with 0.1 M sodium phosphate buffer, pH 7.0, and once with ethanol. The incorporation of radiolabeled AMP was quantified by a liquid scintillation counter (Beckman Coulter, Indianapolis, IN, USA).

Transgenic construct

The 7.1-kbp transgenic construct was prepared as follows. The 2.0-kbp *Sal*I-*Bam*HI and the 3.0-kbp *Hin*dIII-*Eco*RI fragments at the 5'- and 3'-ends, respectively, were cut from the wild-type *Papolb* transgenic construct [24]. The 0.9-kbp *Bam*HI-*Apa*I fragment was prepared by restriction digestion of a 1.0-kbp fragment amplified by PCR using the primers 5'-TTGTCAGCAACAGGAGTGAAACTGG-3' and 5'-AAGGGCCCCTCTAGCTCCCGCGCCG-3'. The 1.2-kbp *Apa*I-*Hin*dIII fragment encoding the N-terminal region of FLAG- and HA-tagged PAPOLB^{D114A} was prepared by digesting a 2.0-kbp PCR product amplified by using the primers 5'-AAGGGCCCGCCACCATGGACTACAAGGACGATGATG-3' and 5'-TCCTCGAGCTAGACTCCTAGTATAGGATTGG-3' and PAPOLB^{D114A}/pcDNA3-FLAGHAN as a template. The 2.0-kbp *Sal*I-*Bam*HI and the 0.9-kbp *Bam*HI-*Apa*I fragments were cloned into pBluescriptII SK (+) digested with *Sal*I and *Apa*I, whereas the 1.2-kbp *Apa*I-*Hin*dIII and the 3.0-kbp *Hin*dIII-*Eco*RI fragments were

inserted into the *Apa*I and *Eco*RI sites of pBluescriptII SK (+) followed by digestion with *Apa*I and *Not*I. The resulting 2.9-kbp *Sal*I-*Apa*I and the 4.2-kbp *Apa*I-*Not*I fragments were cloned into *Sal*I and *Not*I sites of pBluescriptII SK (+). The 7.1-kbp *Sal*I-*Not*I fragment was purified by agarose gel electrophoresis after restriction digestion.

Generation of transgenic mice

The 7.1-kbp *Sal*I-*Not*I fragment was injected into male pronuclei of C57BL/6 fertilized eggs at the Laboratory Animal Resource Center, University of Tsukuba. Transgenesis was verified by PCR of tail DNA using the primers 5'-ATGGACTACAAGGACGATGATG-3' and 5'-CGAAGCTTGCACCTTTCGTGTGTACTCC-3'. Screening of 72 male pups identified five mice positive for the transgene. The founder mice were bred with C57BL/6 females (Japan SLC, Shizuoka, Japan) to produce F1 heterozygotes. Two lines of transgenic mice, designated as Tg16 and Tg66, were maintained by mating with C57BL/6 mice. To produce PAPOLB-deficient mice ($Papolb^{-/-}$) expressing PAPOLB^{D114A}, Tg16 males were initially crossed with $Papolb^{-/-}$ females. The resulting $Tg16^{+/-}/Papolb^{+/-}$ F1 males were further mated with $Papolb^{-/-}$ females to obtain $Tg16^{+/-}/Papolb^{-/-}$ F2 mice.

Antibodies

Antibodies against mouse RNASEH2A and mouse TAF10 were produced in rabbits by immunizing with recombinant proteins followed by affinity-purification. Briefly, $6 \times$ His- and thioredoxin (TRX)-tagged mouse RNASEH2A at residues Met^{45}-Leu^{301} or the entire region of mouse TAF10 was produced in *Escherichia coli* BL21 (DE3). The recombinant proteins were purified on a Ni-NTA His column (Merck Millipore, Billerica, MA, USA), emulsified with Freund's complete or incomplete adjuvant (Becton Dickinson, Franklin Lakes, NJ, USA), and injected intradermally into female New Zealand White rabbits (Japan SLC) [25]. Each antiserum was fractionated with ammonium sulfate (0–40% saturation), followed by immunoaffinity chromatography on a Sepharose 4B (GE Healthcare, Piscataway, NJ, USA) column conjugated with the same protein region fused to glutathione *S*-transferase (GST) [26]. The anti-PAPOLB antibody was prepared as described previously [22]. A mouse monoclonal antibody against mouse A-kinase anchor protein 4 (AKAP4; 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). A mouse monoclonal antibody against β-actin (ACTB; A5441), rabbit polyclonal antibody against human ribosomal protein L26 (RPL26; IHC-00093), rabbit polyclonal antibody against human TATA-binding protein-like 1 (TBPL1; GTX104694), and rat monoclonal antibody against hemagglutinin peptide (HA; 11867423001) were obtained from Sigma-Aldrich (St. Louis, MO, USA), Bethyl Laboratories (Montgomery, TX, USA), GeneTex (Hsinchu, Taiwan), and Roche Life Science (Indianapolis, IN, USA), respectively.

Northern blot analysis

Total RNAs were prepared from mouse testicular tissues or purified populations of spermatogenic cells [27] using ISOGEN kit (Nippon Gene, Toyama, Japan). RNase H digestion of poly(A) RNA in the presence of oligo(dT)$_{15}$, which generates deadenylated mRNA, was carried out as described previously [22]. Briefly, total RNAs (10 μg) were incubated in a 50-μl mixture containing 40 mM Tris/HCl, pH 7.8, 4 mM MgCl$_2$, 100 mM KCl, 1 mM DTT, 4% glycerol, 1 unit of RNase H (TAKARA Bio., Otsu, Japan), and 0.5 μg of oligo(dT)$_{15}$. After incubation at 37°C for 1 h, the RNAs were extracted with phenol/chloroform, and precipitated with ethanol. The RNA samples (2–5 μg) were denatured with glyoxal, separated on agarose gels, and transferred onto Hybond-N$^+$ nylon membranes (GE Healthcare) [22]. The blots were probed with ^{32}P-labeled cDNA fragments.

Immunoblot analysis

Testicular tissues or purified germ cells were homogenized at 4°C in 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, supplemented with 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM DTT, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF), using a Teflon-glass homogenizer (750 rpm, 10 strokes). After incubation at 4°C for 4 h, the homogenates were centrifuged at $13,400 \times g$ for 10 min at 4°C. The supernatant solutions were used as protein extracts. Protein concentration was determined by using a Coomassie protein assay reagent kit (Thermo Fisher Scientific). Protein samples (5 μg) were subjected to sodium dodecyl sulfate-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). The immunoreactive bands 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 by 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, Osaka, Japan), the slides were observed under a DM IRBE microscope (Leica Microsystems, Wetzlar, Germany).

Statistical analysis

Data are presented as mean ± SEM (n ≥ 3), unless stated otherwise. The Student's *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

We previously found that PAPOLB catalyzes additional poly(A) tail extension of specific transcription factor mRNAs in round spermatids [23]. To examine whether cytoplasmic polyadenylation by PAPOLB is specific for transcription factor mRNAs, we analyzed the lactate dehydrogenase C subunit (*Ldhc*) and two forms of

ornithine decarboxylase (*Odc*) mRNAs. Northern blot analysis showed that these mRNAs are ~100 nt longer in round spermatids than in pachytene spermatocytes of wild-type mice (*Papolb*^{+/+}, +/+), as described previously [21, 28]. In contrast, no (for *Ldhc*) or slight (for *Odc*) increase in mRNA length was observed in round spermatids of PAPOLB-null mice (*Papolb*^{−/−}, −/−). After RNase H digestion in the presence of oligo(dT)$_{15}$, each mRNA decreased in length and their size was uniform among four cell types, indicating that the size differences are due to poly(A) tail elongation (Fig. 1A). Extensive analysis revealed that the ribonuclease H2 A subunit (*Rnaseh2a*), a male germ cell-specific paralog of general transcription factor IIA α/β subunit (*Gtf2a1l/Alf/TfIIaτ*), and TATA-binding protein-associated factor subunit 9 (*Taf9*) mRNAs are also regulated by PAPOLB. These results indicate that PAPOLB is capable of extending poly(A) tails of different mRNA classes. The levels of substrate mRNAs in round spermatids of *Papolb*^{−/−} mice were comparable to those in *Papolb*^{+/+} mice, despite the incomplete poly(A) tail extension (Fig. 1A). We next examined protein levels encoded by two previously identified target mRNAs (*Taf10* and *Tbpl1*) and *Rnaseh2a* mRNA. Though it has long been believed that there is a positive correlation between poly(A) tail length and translational efficiency [9], the amount of TAF10, TBPL1, and RNASEH2A protein in round spermatids was similar between *Papolb*^{+/+} and *Papolb*^{−/−} mice (Fig. 1B). Sucrose gradient analyses also showed that additional polyadenylation of *Tbpl1* and *Rnaseh2a* mRNAs in round spermatids did not greatly increase the ratio of mRNAs associated with translationally active polyribosomes (Supplementary Fig. 1: online only), as was reported for *Ldhc* mRNA [21]. Collectively, these results suggest that additional poly(A) tail extension by PAPOLB is not responsible for enhancing either stability or translation of the mRNAs examined.

The results described above led us to speculate that PAPOLB regulates spermiogenesis independently of its polyadenylation activity. To address this possibility, we sought to examine whether PAPOLB-null phenotypes can be recovered by transgenically introducing polyadenylation-defective PAPOLB. First, we tested whether Asp at residue 114 (Asp[114]), one of three putative catalytic Asp residues conserved in the DNA polymerase β-like nucleotidyltransferase superfamily [22], is essential for polyadenylation activity. The N-terminally FLAG- and HA-tagged PAPOLB[D114A] mutant (hereafter, abbreviated as PAPOLB[D114A]), where Asp[114] was mutated to Ala, was assayed for non-specific polyadenylation activity [22]. When the same amount of *in vitro* translation product was analyzed, wild-type PAPOLB was capable of adding AMP residues on oligo(A)$_{12}$, whereas PAPOLB[D114A] mutant only showed activity comparable to background levels (Fig. 2A and B). We thus generated transgenic mice expressing PAPOLB[D114A] under control of the *Papolb* promoter (Fig. 2C). Immunoblot analysis of testicular extracts from two transgenic mouse lines, designated as Tg16 and Tg66, detected a 72-kDa protein band reactive to anti-HA antibody, which corresponds to FLAG- and HA-tagged PAPOLB[D114A] (Fig. 2D). Because Tg16 male mice produced mutant protein at levels equivalent to endogenous 70-kDa PAPOLB, we mainly used Tg16 for further experiments.

Both Tg16 male and female mice grew normally and were apparently normal in behavior, body weight, and health condition. When male fertility was tested by mating with wild-type females, the average litter size from Tg16 males (8.2 ± 0.4 pups/litter, n = 5) was comparable to that of wild-type matings (8.4 ± 0.4 pups/litter, n = 5, Fig. 3A). The testicular weights of Tg16 and wild-type littermates were also similar (90.3 ± 5.3 and 91.7 ± 4.0 mg in Tg16 and wild-type mice, respectively, n = 6, Fig. 3B). Histological analysis indicated that spermatogenesis and sperm migration, respectively, in the testis and epididymis of Tg16 mice were unaffected (Supplementary Fig. 2: online only). Normal progression of spermatogenesis to elongating/elongated spermatids in transgenic mice was also evidenced by the unaltered expression of postmeiotic mRNAs and the presence of translation-coupled deadenylated mRNA species (0.4 kb for *Prm1* and *Tnp1* and 0.6 kb for *Prm2* and *Tnp2*, Fig. 3C) [18, 19]. Furthermore, the abundance of four testis-specific proteins, PRM2, TNP2, PGK2, and AKAP4, was similar among wild-type and two transgenic mouse lines (Fig. 3D). These results indicate that the transgenic expression of PAPOLB[D114A] in wild-type mice has no dominant-negative effect on spermatogenesis.

We then crossed Tg16 male mice with *Papolb*^{−/−} females. The resulting *Tg16*^{+/−}/*Papolb*^{+/−} males were successively mated with *Papolb*^{−/−} females to produce *Tg16*^{+/−}/*Papolb*^{−/−} mice. Northern blot analysis of testicular tissues using a *Flag* probe revealed transgene expression in *Tg16*^{+/−}/*Papolb*^{−/−} (Tg16/KO) but not in *Papolb*^{+/−} (HET) and *Papolb*^{−/−} (KO) mice (Fig. 4A). When hybridized with a *Papolb*-specific probe, the signal intensity of *Papolb*[D114A] mRNA was approximately two-fold stronger than that of the endogenous *Papolb* mRNA in *Papolb*^{+/−} testes. Correspondingly, the 72-kDa FLAG- and HA-tagged PAPOLB[D114A] in *Tg16*^{+/−}/*Papolb*^{−/−} testis was more abundant than 70-kDa wild-type PAPOLB in *Papolb*^{+/−} testis (Fig. 4B). To examine whether introducing polyadenylation-defective PAPOLB[D114A] is able to restore male infertility of PAPOLB-null mice, *Tg16*^{+/−}/*Papolb*^{−/−} males were mated to wild-type females. In contrast to wild-type male mice (8.7 ± 0.4 pups/litter, n = 6), *Tg16*^{+/−}/*Papolb*^{−/−} and *Papolb*^{−/−} males failed to produce any pregnancies, despite the formation of copulation plugs in females mated (n = 6, Fig. 4C). Testes of *Tg16*^{+/−}/*Papolb*^{−/−} and *Papolb*^{−/−} animals were similar in weight (55.1 ± 1.2 and 56.1 ± 2.8 mg in *Tg16*^{+/−}/*Papolb*^{−/−} and *Papolb*^{−/−} mice, respectively, n = 5), but were 42% smaller than those of heterozygous mice (94.5 ± 2.8 mg, n = 5, Fig. 4D). Histological analysis showed disordered spermatogenesis in *Tg16*^{+/−}/*Papolb*^{−/−} mice, including the complete absence of late haploid cells (Supplementary Fig. 3: online only). As a consequence, cauda epididymis of *Tg16*^{+/−}/*Papolb*^{−/−} mice contained no detectable sperm but a small number of round-shaped cells possibly corresponding to degenerated spermatogenic cells. These data indicate that transgenic introduction of PAPOLB[D114A] into PAPOLB-null mice fails to rescue abnormal spermatogenesis and infertility, suggesting that the polyadenylation activity of PAPOLB is indispensable for spermiogenesis.

To ascertain whether PAPOLB[D114A] is inactive *in vivo*, we compared the poly(A) tail length of target mRNAs. Northern blot analysis of total testicular RNAs revealed that the sizes of three substrate mRNAs were identical between *Tg16*^{+/−}/*Papolb*^{−/−} and *Papolb*^{−/−} testes, but were approximately ~100 nt smaller than those in *Papolb*^{+/−} mice (Fig. 5A). Poly(A) tail removal by RNase H digestion in the presence of oligo(dT)$_{15}$ diminished respective mRNAs to the same length among *Papolb*^{+/−}, *Papolb*^{−/−}, and *Tg16*^{+/−}/*Papolb*^{−/−} testes, indicating
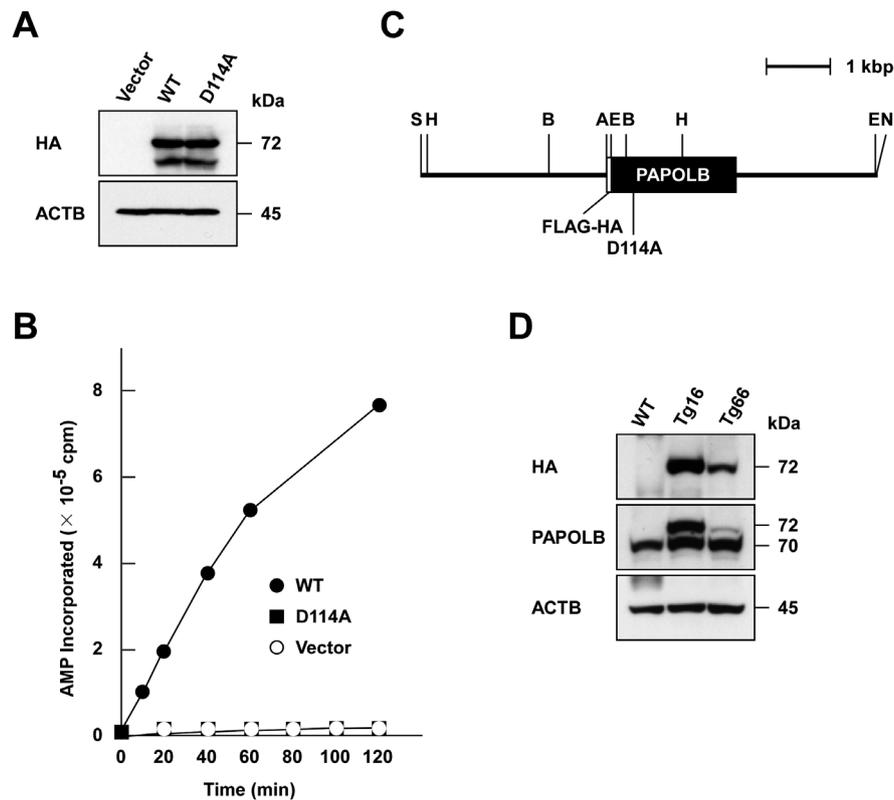
Fig. 2. Generation of transgenic mice expressing polyadenylation-defective PAPOLB[D114A]. (A) Immunoblot analysis of proteins synthesized *in vitro*. N-terminally FLAG- and HA-tagged PAPOLB (WT) and PAPOLB[D114A] (D114A) were synthesized in rabbit reticulocyte lysates and were analyzed by immunoblotting using anti-HA antibody. ACTB was used as a control. (B) Polyadenylation assay. The polyadenylation activity of wild-type PAPOLB (closed circle) and PAPOLB[D114A] (closed square) was monitored by measuring the incorporation of AMP from [α-^{32}P] ATP into an oligo(A)$_{12}$ primer at the incubation times indicated. Empty vector (open circle) was used as a control. (C) Physical map of the transgenic construct. The protein-coding regions of FLAG and HA tags and PAPOLB[D114A] are indicated by open and filled boxes, respectively. The restriction enzymes are denoted as follows: A, *Apa*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; N, *Not*I. (D) Immunoblot analysis. Testicular protein extracts of wild-type (WT) and two transgenic mouse lines (Tg16 and Tg66) were probed with anti-HA and anti-PAPOLB antibodies. ACTB served as a control.

that the size decrease was attributed to defective polyadenylation (Fig. 5A). We next investigated if spermiogenesis arrest in *Tg16*^{+/–}/*Papolb*^{–/–} mice accompanies impaired testicular mRNA expression. The amount of *Pgk2* and five postmeiotic mRNAs was significantly decreased in *Tg16*^{+/–}/*Papolb*^{–/–} and *Papolb*^{–/–} mice compared with levels in *Papolb*^{+/–} mice (Fig. 5B). In addition, deadenylated forms of *Tnp1*, *Tnp2*, *Prm1*, and *Prm2* mRNAs (0.4 kb for *Tnp1* and *Prm1* and 0.6 kb for *Tnp2* and *Prm2*), which are characteristic of late haploid cells, were undetectable in mutant mice lacking wild-type PAPOLB. Consistent with the absence of poly(A)-shortened mRNA forms [18, 19], four proteins PGK2, AKAP4, TNP2, and PRM2 were totally absent in *Tg16*^{+/–}/*Papolb*^{–/–} and *Papolb*^{–/–} mice (Fig. 5C). These results further support that spermatogenesis is arrested prior to the spermatid elongation stage in *Tg16*^{+/–}/*Papolb*^{–/–} mice.

Discussion

This study describes the essential role of PAPOLB polyadenylation activity in spermatogenesis. The enzyme is capable of extending

poly(A) tails of not only transcription factor mRNAs but also mRNAs in other classes (Fig. 1). In addition, poly(A) tail extension is unlikely to enhance stability and translational efficiency of the substrate mRNAs examined because the levels of mRNAs and their translation products were not compromised by the absence of PAPOLB (Fig. 1). These observations raised the possibility that PAPOLB possesses a critical function besides poly(A) polymerase activity. Nevertheless, transgenic introduction of polyadenylation-defective PAPOLB[D114A] in PAPOLB-null mice failed to compensate for loss of endogenous PAPOLB (Figs. 4 and 5), implying that spermatogenesis indeed requires cytoplasmic polyadenylation catalyzed by PAPOLB, or at least its terminal nucleotidyltransferase activity.

Efficient translation of eukaryotic mRNAs is accomplished by cap and poly(A) tail synergy mediated by three proteins: cytoplasmic poly(A)-binding protein (PABPC) bound to poly(A) tails associates with the scaffold protein eukaryotic initiation factor (EIF) 4G, which is also capable of interacting with EIF4E, a cap-binding protein. This "closed-loop" structure is thought to be critical for efficient translation by promoting ribosome recycling and/or enhancing
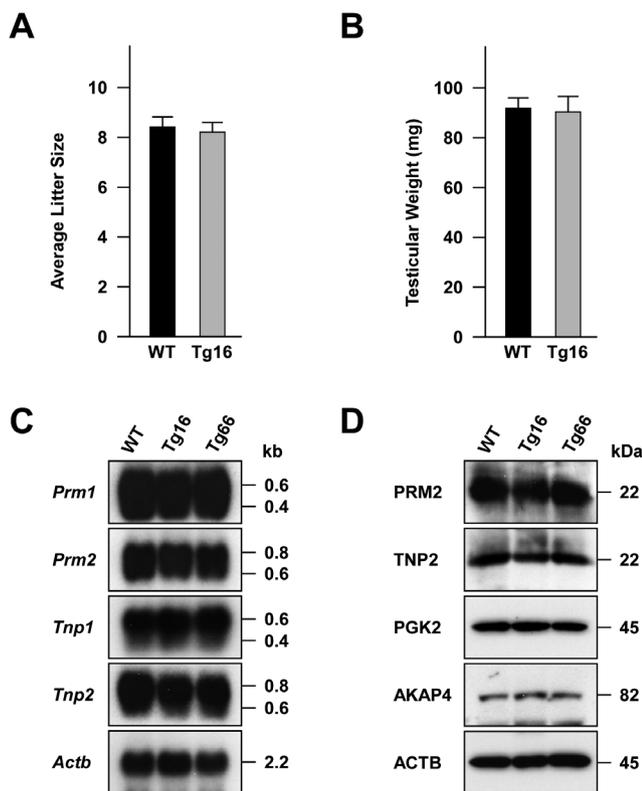
Fig. 3. Characterization of transgenic mice expressing PAPOLB[D114A]. Three- to four-month-old mice were analyzed. (A) Fertility of male mice. Wild-type (WT) and Tg16 males were mated with 8-week-old C57BL/6 females and the pups were counted (5 litters). (B) Testicular weight. Testicular tissues of WT and Tg16 mice were weighed (n = 6). (C) Northern blot analysis. Total testicular RNAs of wild-type (WT) and two transgenic mouse lines (Tg16 and Tg66) were subjected to Northern blot analysis with the haploid-specific cDNA probes indicated. *Actb* served as a control. (D) Immunoblot analysis. Testicular protein extracts were examined by immunoblotting with the antibodies indicated. ACTB served as a control.

Fig. 4. Generation and characterization of PAPOLB-null mice expressing PAPOLB[D114A]. Three- to four-month-old mice were used for analysis. (A) Northern blot analysis. Total testicular RNAs from *Papolb*^{+/−} (HET), *Papolb*^{−/−} (KO), and *Tg16*^{+/−}/ *Papolb*^{−/−} (Tg16/KO) mice were subjected to Northern blot analysis using *Flag* and *Papolb* cDNA probes. *Actb* served as a control. (B) Immunoblot analysis. Testicular protein extracts were probed with anti-PAPOLB and anti-HA antibodies. ACTB was used as a control. Band marked with an asterisk likely represents PAPOLB[D114A] lacking FLAG and HA tags, which was translated from the initiation codon of *Papolb*. (C) Fertility of male mice. Wild-type (WT) and *Tg16*^{+/−}/*Papolb*^{−/−} (Tg16/KO) males were crossed with 8-week-old C57BL/6 females and the pups were counted (6 litters). (D) Testicular weight. Testicular tissues of *Papolb*^{+/−} (HET), *Papolb*^{−/−} (KO), and *Tg16*^{+/−}/*Papolb*^{−/−} (Tg16/KO) mice were weighed (n = 5).

the interaction between initiation factors and mRNAs, as well as for protecting mRNAs from deadenylation [29–32]. Given that transcription is entirely silent during oocyte maturation and early embryogenesis, mRNAs encoding proteins required for progression of these processes are transcribed in earlier stages and are stored as deadenylated forms after transport to the cytoplasm [7–11]. These maternal mRNAs with short poly(A) tails ($\sim$A$_{10}$) are translationally incompetent, where PABPC is unable to bind mRNAs, and are later activated by cytoplasmic polyadenylation. However, poly(A) tail extension by PAPOLB differs from the case during oocyte maturation and early embryogenesis in that the substrate mRNAs identified to date already possess poly(A) tract lengths ($\sim$100 nt) sufficient for PABPC-binding. Additional poly(A) tail extension by PAPOLB had no impact on translation and steady-state level of the mRNAs and is thus considered to be non-specific or a side effect in view of function (Fig. 1) [23]. This is consistent with the recent findings that the median length of poly(A) tails is 50–100 nt in mammalian

cells, which is shorter than previously thought (150–250 nt), and that additional poly(A) tail elongation does not further contribute to translational enhancement except on maternal mRNAs [33–35]. Thus, if cytoplasmic polyadenylation by PAPOLB governs spermiogenesis, it is expected that the "true" target(s) possesses maternal mRNA-like short poly(A) tail(s). It is also conceivable that PAPOLB, like noncanonical poly(A) polymerase PAPD4/GLD-2, is involved in 3'-end monoadenylation of certain microRNAs, thereby regulating their stability [36, 37].

It is interesting to note that the poly(A) tails of some mRNAs, including *odc* (Fig. 1A) and *Taf10* [23], are slightly longer in round spermatids than in pachytene spermatocytes of PAPOLB-deficient mice, but are shorter in round spermatids of wild-type mice. These observations imply that poly(A) tail extension of the mRNAs is carried out not only by PAPOLB but also by another poly(A)
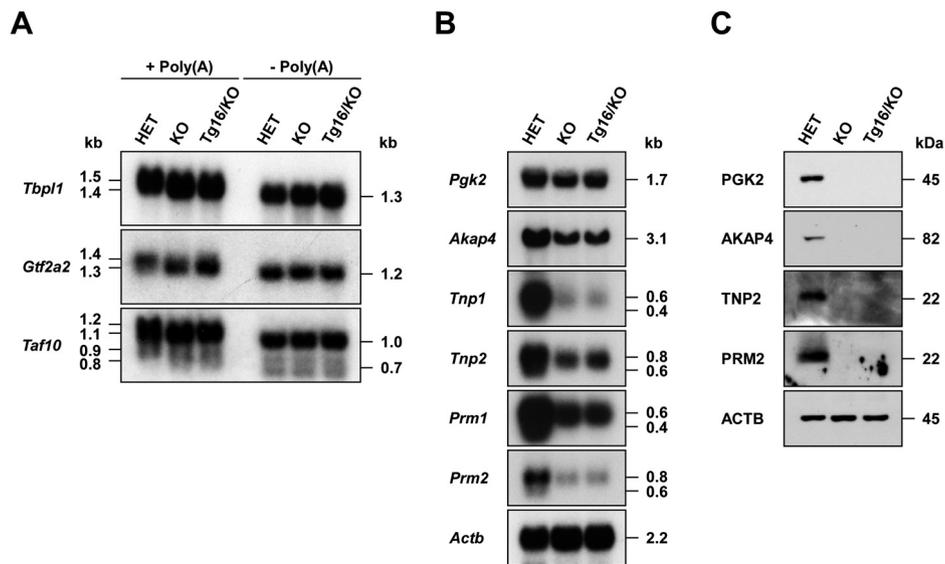
Fig. 5. Poly(A) tail length of substrate mRNAs and expression and translation of haploid-specific mRNAs in *Tg16+/-/Papolb-/-* mice. (A) Poly(A) tail length of target mRNAs. Total testicular RNAs were obtained from *Papolb+/-* (HET), *Papolb-/-* (KO), and *Tg16+/-/Papolb-/-* (Tg16/KO) mice, incubated with RNase H in the presence [–Poly(A)] or absence [+Poly(A)] of oligo(dT)$_{15}$, and subjected to Northern blot analysis with the cDNA probes indicated. (B) Expression of testis-specific mRNAs. Testicular total RNAs were analyzed by Northern blotting using the cDNA probes indicated. *Actb* was used as a control. (C) Translation of testis-specific mRNAs. Testicular protein extracts were subjected to immunoblot analysis with the antibodies indicated. ACTB served as a control.

polymerase, such as PAPD4 [38]. Indeed, poly(A) tails of *Acr*, *Acrbp-W*, *Acrbp-V5*, and *Zpbp1* mRNAs were still elongated in round spermatids even in the absence of PAPOLB (Supplementary Fig. 4: online only) [23]. Identifying the polyadenylation pathway responsible for these processes will be helpful to understand poly(A) tail dynamics during mammalian spermatogenesis.

Although PAPOLB polyadenylation activity is most likely essential for spermatogenesis, we cannot rule out the possibility that Asp^{114} of PAPOLB is involved in another yet unknown activity, as is the case for protein arginine methyltransferase 8 (PRMT8), in which Ser^{120} is required for both arginine methylation and phosphatidylcholine hydrolysis activities [39]. At any rate, further studies, including the identification of the "true" target(s), are necessary to elucidate PAPOLB function.

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