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—Original Article—

Functional characterization of testis-brain RNA-binding protein, TB-RBP/Translin, in translational regulation

Kanako OYAMA[1, 2], Tadashi BABA[1–3] and Shin-ichi KASHIWABARA[1–3]

[1)]*Ph.D. Program in Human Biology, School of Integrative and Global Majors, University of Tsukuba, Ibaraki 305-8577, Japan*
[2)]*Faculty of Life and Environmental Sciences, University of Tsukuba, Ibaraki 305-8572, Japan*
[3)]*Life Science Center for Survival Dynamics, Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, Ibaraki 305-8577, Japan*

Abstract. Testis-brain RNA-binding protein (TB-RBP/Translin) is known to contribute to the translational repression of a subset of haploid cell-specific mRNAs, including *protamine 2* (*Prm2*) mRNA. Mutant mice lacking TB-RBP display abnormal spermatogenesis, despite normal male fertility. In this study, we carried out functional analysis of TB-RBP in mammalian cultured cells to understand the mechanism of translational repression by this RNA-binding protein. Although the amino acid sequence contained a eukaryotic translation initiation factor 4E (EIF4E)-recognition motif, TB-RBP failed to interact with EIF4E. In cultured cells, TB-RBP was unable to reduce the activity of luciferase encoded by a reporter mRNA carrying the 3'-untranslated region of *Prm2*. However, λN-BoxB tethering assay revealed that the complex of TB-RBP with its binding partner, Translin-associated factor X (TRAX), exhibits the ability to reduce the luciferase reporter activity by degrading the mRNA. These results suggest that TB-RBP may play a regulatory role in determining the sequence specificity of TRAX-catalyzed mRNA degradation.

Key words: mRNA degradation, Spermatogenesis, Testis-brain RNA-binding protein (TB-RBP)/Translin, Tethering reporter assay, Translin-associated factor X (TRAX)

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Spermatogenesis is a highly specialized process of male germ cell differentiation consisting of three major phases: mitotic proliferation of spermatogonia, two meiotic divisions of spermatocytes, and drastic morphological remodeling during spermiogenesis, the haploid phase of spermatogenesis. The process is accomplished by a controlled program of stage-specific gene expression, which is regulated at the transcriptional, post-transcriptional, and translational levels [1–5]. For instance, the mRNAs encoding proteins required for the formation of flagellum and nuclear compaction are postmeiotically transcribed in round spermatids and stored for several days until translated in elongating/elongated spermatids [1, 2, 4, 5]. The 3'-untranslated regions (3'-UTRs) are responsible for storage and delayed translation of haploid cell-specific mRNAs [1, 2, 4, 5]. Several *trans*-acting factors, including testis-brain RNA-binding protein (TB-RBP), Y-box RNA-binding proteins 2 and 3 (YBXs 2 and 3), and a double-stranded RNA-binding protein PRBP/TARBP2, were identified as the proteins binding to the specific sequences conserved in the 3'-UTR of postmeiotic mRNAs [1, 2, 4, 5]. Although depletion of these proteins affects spermatogenesis, it has not been reported whether spermatid-specific mRNAs are prematurely translated in round spermatids of the mutant mice [6–9]. Thus, their contributions to translational regulation remain elusive.

TB-RBP, a mouse ortholog of human Translin (now widely used in many organisms, including mouse, and hereafter referred to as TB-RBP/Translin), is ubiquitously expressed, with high levels in the testis and brain. TB-RBP/Translin has been implicated in many biological processes, including chromosomal translocation, mRNA transport, processing of small RNAs and tRNAs, cell proliferation, and behavior [10]. The protein was initially identified as a protein binding to specific sequences of *protamine 2* (*Prm2*) mRNA, known as Y and H elements, which are conserved among a number of mRNAs from testis and brain, including those for PRMs 1 and 2, A-kinase anchoring protein 4 (AKAP4), and glyceraldehyde 3-phosphate dehydrogenase-S (GAPDS) [11–14]. In the brain, TB-RBP-binding elements in the 3'-UTRs of mRNAs encoding myelin basic protein and calcium- or calmodulin-dependent protein kinase IIa have been identified [15, 16]. TB-RBP/Translin forms a complex with its binding partner, Translin-associated factor X (TRAX), which shares an extensive sequence homology with TB-RBP/Translin [17, 18]. Previous studies have shown that TB-RBP/Translin is a single-stranded DNA- and RNA-binding protein, whereas TRAX possesses endoribonuclease activity [19].

Mouse TB-RBP is considered as a candidate protein responsible for the storage of haploid cell-specific mRNAs and their delayed translation, based on its ability to repress the translation of human growth hormone mRNA carrying the 3'-UTR of *Prm2* in rabbit reticulocyte lysate and of *Gapds* mRNA in wheat germ extract [12,

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Correspondence: S Kashiwabara (e-mail: kashiwabara.shin.fw@u.tsukuba.ac.jp)

13]. Premature translation of sperm nuclear proteins PRM1 and TNP2 results in the arrest of spermiogenesis because of precocious nuclear condensation and in the production of sperm with impaired head morphology and reduced motility, respectively [20, 21]. In addition, sperm with abnormal head were seen in mutant mice lacking a heterogenous nuclear ribonucleoprotein CBF-A/Hnrnpab, which binds to the specific sequence of *Prm2* distinct from Y and H elements and participates in delayed translation of the mRNA [22]. In contrast, in *Tbrbp*-null mice, spermatogenesis is only partially completed, but the sperm show no obvious abnormalities in shape and motility and hence normal male fertility, despite the substantial vacuolation of seminiferous tubules and reduced sperm count [7]. These findings raise the possibility that TB-RBP is not involved in translational repression of postmeiotic mRNAs *in vivo,* or that it alone is not sufficient to repress the translation of mRNAs. In this study, we characterized the function of TB-RBP in mRNA regulation, using a tethering reporter assay in cultured cells, and found that TB-RBP may be involved in mRNA degradation.

Materials and Methods

Antibodies

Antibodies against murine TB-RBP, murine YBX3, and human TRAX were raised and affinity-purified as described in "Supplementary Materials and Methods" (online only). Rabbit polyclonal antibodies against eukaryotic translation initiation factor 4E (EIF4E) and EIF4G1 were prepared as described previously [23, 24]. Rabbit polyclonal antibody against human ribosomal protein L26 (RPL26; IHC-00093) was purchased from Bethyl Laboratories (Montgomery, TX, USA). Mouse monoclonal antibody against β-actin (ACTB; 5441) and rat monoclonal antibody against hemagglutinin peptide (HA; 11867423001) were from Sigma-Aldrich (St. Louis, MO, USA), whereas horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Construction of reporter plasmids

psiCHECK-2, a dual-luciferase reporter plasmid harboring firefly (*F-Luc*) and renilla (*R-Luc*) luciferase genes, was purchased from Promega (Madison, WI, USA). psiCHECK-2 carrying *R-Luc* with *Prm2* 3'-UTR (R-Luc-Prm2 3'-UTR/psiCHECK-2), 6 × YH elements (R-Luc-6 × YH/psiCHECK-2), and 6 × BoxB sequence (R-Luc-6 × BoxB/psiCHECK-2) were constructed as described in "Supplementary Materials and Methods." To facilitate the detection of the reporter mRNAs on Northern blots, F-Luc/pcDNA3 was produced by cloning *F-Luc* derived from pGL3-basic (Promega) into pcDNA3 (Thermo Fisher Scientific, Waltham, MA, USA). Similarly, *R-Luc* from pGL4.73[hRluc/SV40] (Promega) was inserted into pcDNA3, together with 6 × BoxB sequence, to generate R-Luc-6 × BoxB/pcDNA3. By the use of pcDNA3 vector, expression of the reporter mRNAs is driven by a strong cytomegalovirus promoter.

Construction of effector plasmids

The cDNA fragments spanning the entire coding region of mouse TB-RBP (Met^{1}–$Lys^{C\text{-}term.}$) and human TRAX (Met^{1}–$Ser^{C\text{-}term.}$), and a region containing silencing domain of murine TNRC6A (Val^{1334}–$Met^{C\text{-}term.}$) were amplified by polymerase chain reaction (PCR), using a cDNA library of mouse testis or human embryonic kidney (HEK) 293T cell line. The amplified fragments were cloned into either pcDNA3-HAC, designed to express proteins with C-terminal HA tag, or λN/pcDNA3-HAC that expresses proteins with a λ phage N-peptide and a HA tag at N and C termini, respectively [23].

Cell culture and transfection

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (high glucose, FUJIFILM Wako, Tokyo, Japan) supplemented with 10% (v/v) EquaFETAL (Atlas Biologicals, Fort Collins, CO, USA), 100 U/ml penicillin, and 0.1 mg/ml streptomycin, at 37°C under 5% CO_2. Cells were co-transfected with reporter and effector plasmids, using a Perfectin transfection reagent (Genlantis, San Diego, CA, USA) or polyethyleneimine MAX (PEI Max; Polysciences, Warrington, PA, USA).

Dual-luciferase reporter assay

After 24-h transfection, the cells were lysed in 1 × passive lysis buffer (Promega), and F-Luc and R-Luc activities were measured using a dual-luciferase reporter assay system (Promega) in a CentroPRO LB 962 microplate luminometer (Berthold, Bad Wildbad, Germany). R-Luc activity was normalized to F-Luc activity to assess the sequence-specific function of effector proteins.

Generation of TB-RBP/Translin knock-out HEK293T cells

The sgRNA sequence targeting human TB-RBP/Translin (5'-CGTGGAGCTGCAGGGCTTTTTGGC-3') was designed using CRISPR direct (https://crispr.dbcls.jp/). The sequence was inserted into Cas9-sgRNA co-expression plasmid, pSpCas9(BB)-2A-Puro (PX459; Addgene plasmid #62988, Watertown, MA, USA). HEK293T cells in a 10-cm dish were transfected with 8 μg of plasmid and incubated for 24 h. An aliquot of the transfected cells was cultured in the presence of 1 μg/ml puromycin. Drug-resistant colonies were individually transferred to 24-well plates, expanded, and analyzed for mutation by genome sequencing and immunoblotting with anti-TB-RBP antibody. Clonal cell lines transfected with PX459 empty plasmid were used as control.

Preparation of protein extracts

Male C57BL/6 mouse tissues (4-month-old) and transfected HEK293T cells were homogenized at 4°C in buffer A containing 20 mM Tris/HCl, pH 7.5, 0.15 M NaCl, and 0.5% Nonidet P-40, supplemented with 0.5 mM dithiothreitol (DTT), 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF), in a Teflon-glass homogenizer (750 rpm, 10 strokes). The homogenates were centrifuged at 13,400 × *g* for 10 min at 4°C to remove cellular debris. Protein concentration of the supernatants was determined using a Coomassie protein assay reagent kit (Thermo Fisher Scientific).

m7GTP cap-agarose pull-down assay

Tissue extracts (0.5 mg/ml in buffer A) were pre-cleared with a 20-μl bed volume of Sepharose 4B (GE Healthcare, Piscataway, NJ, USA) and incubated at 4°C for 1 h with 7-methyl GTP (m7GTP)-Sepharose beads (10-μl bed volume, GE Healthcare). After extensive washing with the same buffer, the bound proteins were detected by

immunoblot analysis, as described previously [23].

Immunoprecipitation assay

Antibodies (2–3 μg) were incubated with Protein A agarose beads (25-μl bed volume; Thermo Fisher Scientific) in 1 ml of buffer A at 4°C for 1 h. After washing with the same buffer, the antibody-bound beads were mixed with testicular extracts (1 mg/ml) that had been pre-cleared with Sepharose 4B (20-μl bed volume) and rocked at 4°C for 1 h. After centrifugation, the pellet was washed extensively with buffer A, suspended in 50 μl of the same buffer, mixed with 25 μl of 3 × Laemmli buffer, and analyzed by immunoblotting.

RNA immunoprecipitation assay

Antibodies (2 μg) were incubated with Protein A agarose beads (25-μl bed volume) in 1 ml of RIPA buffer (50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid, 0.5 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.5 mM PMSF) at 4°C for 1 h. After washing with the same buffer, the beads were rocked for 4 h with testicular extracts (2 mg/ml in RIPA buffer) pre-cleared with Protein A agarose beads (25-μl bed volume). After extensive washing with RIPA buffer containing 1 M NaCl, the beads were suspended in 200 μl of RIPA buffer. RNA was extracted from 160 μl suspension, using ISOGEN LS kit (Nippon Gene, Toyama, Japan), followed by cDNA synthesis with PrimeScript II 1st strand cDNA synthesis kit (TAKARA Bio, Otsu, Japan). A portion of the synthesized cDNA was analyzed by PCR using gene-specific primers.

Northern blot analysis

Total RNA was extracted from transfected HEK293T cells and from fractions of sucrose gradient ultracentrifugation (see "Supplementary Materials and Methods"), using ISOGEN and ISOGEN LS kits (Nippon Gene), respectively. The RNA samples were denatured with glyoxal, electrophoresed on agarose gels, and transferred to Hybond-N^+ nylon membranes (GE Healthcare). The blots were hybridized with ^{32}P-labeled DNA fragments and detected by autoradiography.

Statistical analysis

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

Ethics statement

All animal experiments were approved and conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" at the University of Tsukuba (Permit numbers: 16-008, 17-219, 18-352, 19-352).

Results

Mouse TB-RBP is reported to repress the translation of postmeiotic, translationally delayed mRNAs, including *Prm2* and *Gapds*, in *in vitro* translation assays [12, 13]. Translation is initiated by the binding of EIF4F, which comprises mRNA cap-binding protein EIF4E and scaffold protein EIF4G, to the m7G cap structure. EIF4E-binding proteins (4E-BPs) are a class of translational regulators that share a conserved EIF4E-recognition motif (YxxxxLϕxx, where ϕ is a hydrophobic residue, and x is any amino acid) found in EIF4G [25–27]. Consequently, 4E-BPs compete with EIF4G for binding to EIF4E, thereby inhibiting translation initiation. A search for the amino acid sequence of mouse TB-RBP identified a potential EIF4E-recognition motif, YLSGVLILA, at residues 140–148 (Fig. 1A). To elucidate the mechanism of translational repression by TB-RBP, we examined whether TB-RBP interacts with EIF4E, using immunoprecipitation assay. When the testicular extracts were immunoprecipitated with antibody against EIF4E, EIF4G1 was found in the immunoprecipitation complex, whereas TB-RBP was not (Fig. 1B). Moreover, EIF4E was not included in the protein complex immunoprecipitated with anti-TB-RBP antibody (Fig. 1B). We further tested the interaction of TB-RBP with EIF4E by an m7GTP cap pull-down assay of protein extracts from selected tissues, including testis and brain. In contrast to EIF4E and the associated EIF4G1, TB-RBP was not detected in the cap-binding complex (Fig. 1C). These results indicate that the conserved sequence found in TB-RBP is incapable of interacting with EIF4E and that even if TB-RBP acts as a translational repressor, the mechanism is distinct from that mediated by 4E-BPs.

Previous studies examined the effect of TB-RBP on translation, using *in vitro* translation of synthesized mRNAs in rabbit reticulocyte lysate and wheat germ extract in the presence of testicular protein fraction containing TB-RBP and of recombinant TB-RBP expressed in *Escherichia coli*, respectively [12, 13]. To study the effect of TB-RBP on translation in greater details, we carried out luciferase reporter assays in HEK293T cells. When a psiCHECK-2 reporter plasmid harboring both *R-Luc* with 3'-UTR of *Prm2* and a control *F-Luc* (R-Luc-Prm2 3'-UTR/psiCHECK-2) was transfected into HEK293T cells, together with a pcDNA3-based effector plasmid encoding TB-RBP with HA tag at the C terminus (TB-RBP/pcDNA3-HAC), no significant decrease in the normalized R-Luc activity was observed, compared with the control cells co-transfected with empty vector (Fig. 2A). TB-RBP is reported to bind Y and H elements in the 3'-UTRs of the haploid cell-specific mRNAs, including *Prm2* [11, 12]. To enhance the association of TB-RBP with the reporter mRNA, 3'-UTR of *Prm2* was replaced with six tandem repeats of Y and H elements (6 × YH) and assayed. Despite the increase in the binding sites, introduction of TB-RBP had no impact on the R-Luc activity (Fig. 2B). Next, we checked whether TB-RBP interacts with postmeiotic mRNAs by immunoprecipitating the RNA from testicular extracts (Fig. 2C). While another RNA-binding protein, YBX3, reported to bind a distinct sequence element in the 3'-UTRs of postmeiotic mRNAs [28], was indeed associated with *Prm1* and *Prm2* mRNAs, no detectable TB-RBP was bound to the mRNAs (Fig. 2C). These results indicate that TB-RBP is unable to associate with the known target mRNAs, raising the possibility that TB-RBP is not involved in mRNA regulation.

To confirm this, we performed a λN-tethering assay, which is used for the functional analysis of RNA-binding proteins with unknown binding specificity [29]. In this assay system, an RNA reporter construct for *R-Luc* contains a six tandemly repeated sequence coding for the 19-nucleotide BoxB hairpins in the 3'-UTR of its mRNA

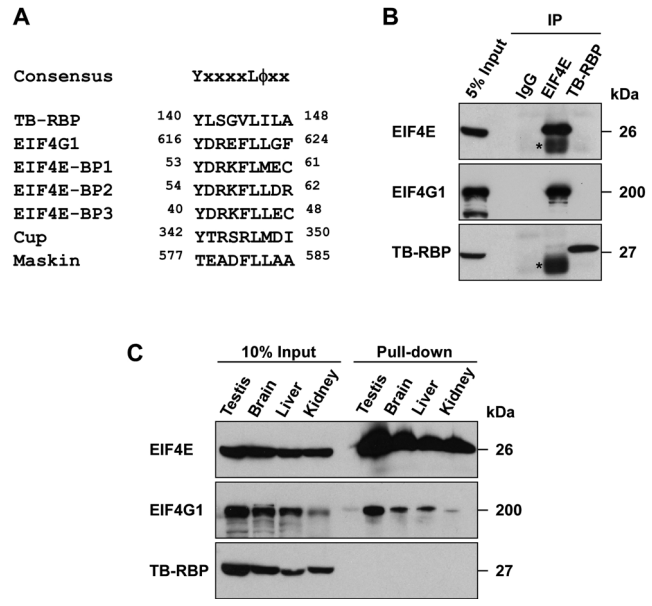

Fig. 1. Characterization of the eukaryotic translation initiation factor 4E (EIF4E)-recognition motif in testis-brain RNA-binding protein (TB-RBP) (A) Alignment of sequence motifs conserved in EIF4E-binding proteins. Amino acid sequences of murine TB-RBP, EIF4G1, EIF4E-BP1, EIF4E-BP2, EIF4E-BP3, *Drosophila* Cup, and *Xenopus* Maskin were aligned. (B) Immunoprecipitation analysis. Testicular protein extracts (1 mg) were immunoprecipitated (IP) with antibody against EIF4E or TB-RBP (3 μg), and analyzed by immunoblotting using antibodies indicated. One-fifth of the immunoprecipitates was loaded in each lane. Preimmune IgG was used as a negative control. The asterisks represent non-specific signals. (C) m7GTP cap-agarose pull-down assay. Cap-agarose beads (10-μl bed volume) were incubated with protein extracts (0.5 mg) from indicated mouse tissues, washed, and analyzed by immunoblotting. One-fifth of the pull-down products was loaded in each lane.

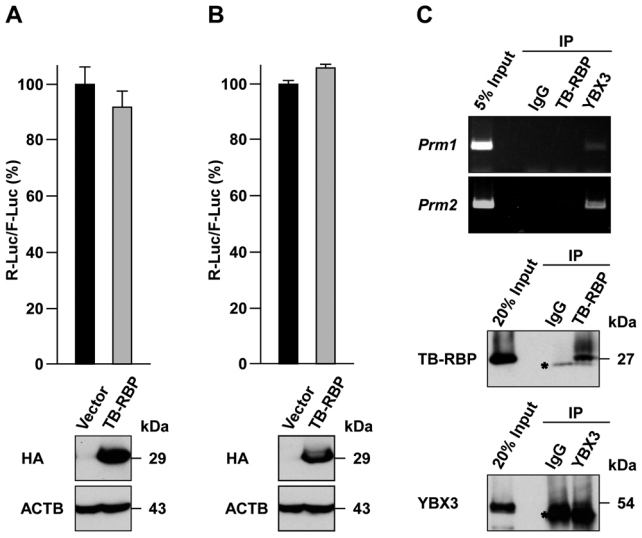

Fig. 2. Interaction of testis-brain RNA-binding protein (TB-RBP) with 3'-UTR of postmeiotic mRNAs (A) Luciferase reporter assay using *R-Luc* carrying 3'-UTR of *Prm2*. Reporter plasmid psiCHECK-2 (50 ng), in which *Prm2* 3'-flanking region was inserted downstream of *R-Luc* (R-Luc-Prm2 3'-UTR/psiCHECK-2), was introduced into HEK293T cells (24-well plates) along with an effector pcDNA3 plasmid encoding C-terminally HA-tagged TB-RBP (TB-RBP/pcDNA3-HAC, 400 ng). Cell lysates were subjected to luciferase assay and immunoblot analysis using anti-HA antibody. β-Actin (ACTB) was used as loading control. R-Luc activity was normalized to F-Luc activity to assess sequence-specific effect of TB-RBP. The normalized R-Luc activity, when co-transfected with empty vector (pcDNA3-HAC), was set to 100. Error bars indicate the mean ± SD with $n = 3$. (B) Luciferase assay using *R-Luc* carrying 6 × YH elements. Luciferase assay was carried out as described in (A), except that *R-Luc* carrying six tandem repeats of Y and H elements (R-Luc-6 × YH/psiCHECK-2) was analyzed. Error bars indicate the mean ± SD with $n = 3$. (C) RNA immunoprecipitation of testicular extracts. Testicular protein extracts (2 mg) were immunoprecipitated with antibody (2 μg) against TB-RBP or YBX3. Immunoprecipitated RNAs were extracted, purified, and amplified by RT-PCR using gene-specific primers indicated. An aliquot of the immunoprecipitates was also analyzed by immunoblotting using antibodies indicated. The asterisks represent non-specific signal in the middle panel and IgG heavy chain in the bottom panel, respectively.

(*R-Luc*-6 × BoxB), whereas *F-Luc* does not, and therefore, serves as a control. A 23-residue λN-peptide, capable of recognizing the BoxB structure in *R-Luc*-6 × BoxB, was fused to the N terminus of TB-RBP with HA tagged on its C terminus (λN-TB-RBP-HAC) (Fig. 3A). The silencing domain of TNRC6A (λN-TNRC6A-HAC), which is responsible for the mRNA degradation and translational repression mediated by miRNAs [30, 31], was used as a positive control. When λN-TB-RBP-HAC was co-expressed in HEK293T cells, the activity of R-Luc encoded by *R-Luc*-6 × BoxB was significantly reduced (~30% of control, Fig. 3B). As reported previously, drastic decrease of the R-Luc activity was observed upon introduction of λN-TNRC6A-HAC, despite the lower level of protein expression (Fig. 3B). To assess the sequence-specific effect of TB-RBP on the reporter mRNA, we substituted 1 × BoxB for 6 × BoxB sequences and carried out the luciferase assay. As shown in Fig. 3C, the decrease in the R-Luc activity was dependent on the number of BoxB sequences. We also tested the specificity of the assay, using TB-RBP with or without λN-peptide. The R-Luc activity decreased only when the λN-peptide was fused to TB-RBP (Supplementary Fig. 1: online only), establishing the specificity of the tethering assay. Finally, the R-Luc activity proportionately reduced with increasing amount of λN-TB-RBP-HAC (Fig. 3D). These results indicate that TB-RBP, when bound to mRNAs, negatively regulates mRNAs by repressing their translation and/or through their degradation. To determine how TB-RBP reduces the R-Luc activity, we examined the distribution of *R-Luc*-6 × BoxB mRNA in the presence of λN-fused TB-RBP, on sucrose density gradient (Supplementary Fig. 2: online only). If translation is inhibited at the initiation step, the association of ribosomes with the mRNA is likely to be compromised by the introduction of λN-fused TB-RBP. When the reporter plasmid was co-transfected with λN-pcDNA3/HAC empty vector (Supplementary Fig. 2, left panels), both *R-Luc*-6 × BoxB and *F-Luc* mRNAs were mostly associated with actively translating polyribosomes. The distribution of *R-Luc*-6 × BoxB mRNA, as well as that of *F-Luc*

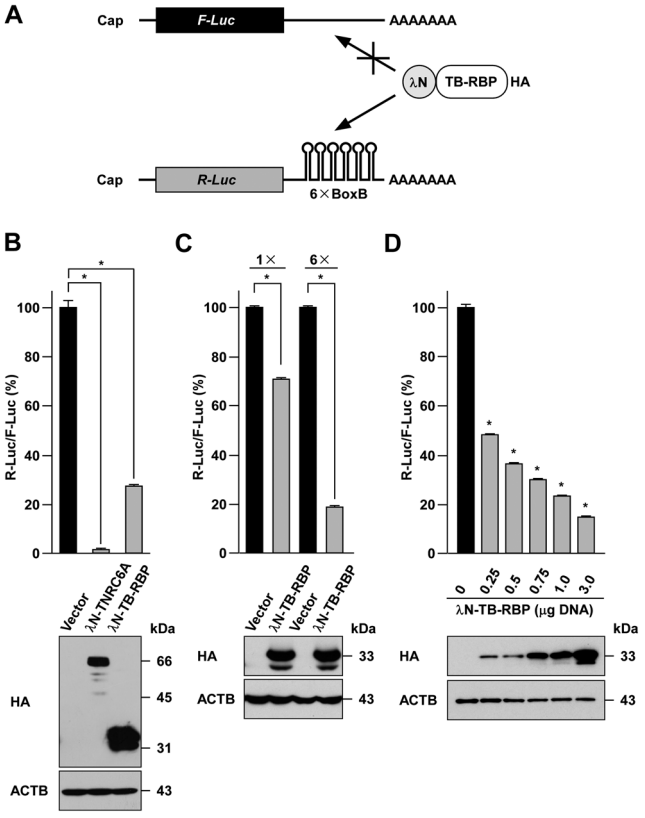

Fig. 3. λN-BoxB tethering assay (A) Schematic representation of the λN-tethering assay. *R-Luc* mRNA carrying six BoxB hairpin sequences in the 3'-UTR (*R-Luc*-6 × BoxB) was used to examine the sequence-specific function of testis-brain RNA-binding protein (TB-RBP). The C-terminally HA-tagged TB-RBP, fused to the λN-peptide at the N terminus (λN-TB-RBP-HAC), specifically binds to the BoxB sequences in the 3'-UTR of *R-Luc*-6 × BoxB mRNA but not to *F-Luc* mRNA without the BoxB sequences. C-terminal HA tag was included for detection on immunoblots. (B) Luciferase assay using *R-Luc* carrying 6 × BoxB sequence. Reporter plasmid psiCHECK-2 (50 ng) carrying six tandem repeats of BoxB hairpin sequence downstream of the open reading frame of *R-Luc* (R-Luc-6 × BoxB/psiCHECK-2) was co-transfected with an effector pcDNA3 plasmid encoding TB-RBP with an N-terminal λN-peptide and a C-terminal HA tag (λN-TB-RBP/pcDNA3-HAC, 400 ng) into HEK293T cells grown in 24-well plates. The silencing domain of TNRC6A fused to λN-peptide (λN-TNRC6A-HAC) was used as a positive control. The normalized R-Luc activity when transfected with empty vector (λN/pcDNA3-HAC) was set to 100. Error bars indicate the mean ± SD with $n = 3$. * represents $P < 0.01$. Expression of effector proteins was detected by immunoblotting with anti-HA antibody. The sizes of molecular weight markers are shown on the right. ACTB served as loading control. (C) Luciferase assay using *R-Luc* carrying different numbers of BoxB sequences. The sequence-specific effect of λN-fused TB-RBP was analyzed using *R-Luc* with 1 × or 6 × BoxB sequences. Error bars indicate the mean ± SD with $n = 3$. * represents $P < 0.01$. Expression of λN-TB-RBP-HAC was detected by anti-HA antibody. ACTB was used as loading control. (D) Dose-dependent effects. HEK293T cells grown on 6-cm dish were co-transfected with 1.5 μg each of two reporter plasmids carrying sequences for *R-Luc*-6 × BoxB and *F-Luc* mRNAs (R-Luc-6 × BoxB/pcDNA3 and F-Luc/pcDNA3) and an increasing amount of λN-TB-RBP/pcDNA3-HAC effector plasmid (0–3 μg). λN/pcDNA3-HAC empty plasmid was used to adjust the total amount of transfected DNA (total 3 μg). Error bars indicate the mean ± SD with $n = 3$. * represents $P < 0.01$. Expression of the effector protein was detected by anti-HA antibody. ACTB served as loading control.

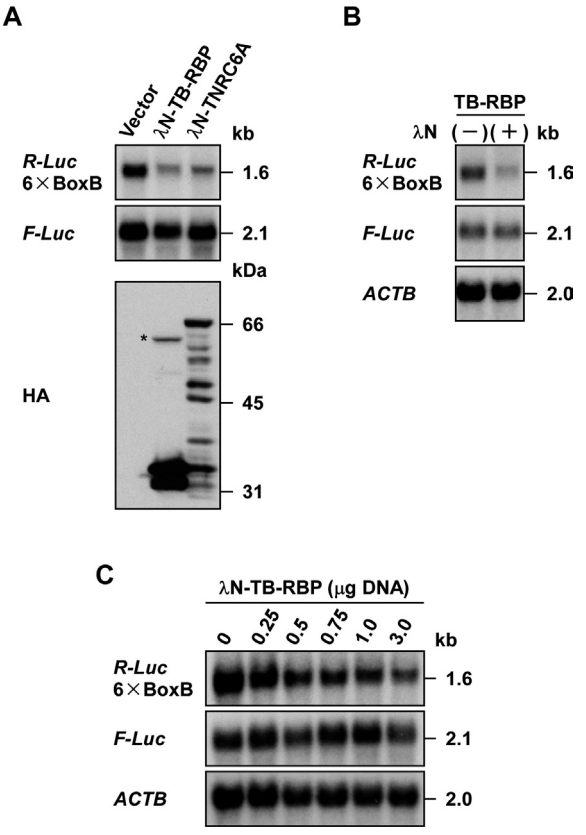

Fig. 4. Effect of testis-brain RNA-binding protein (TB-RBP) on mRNA degradation (A) Decrease in the *R-Luc*-6 × BoxB mRNA by λN-fused TB-RBP. *R-Luc*-6 × BoxB and *F-Luc* reporter plasmids (R-Luc-6 × BoxB/pcDNA3 and F-Luc/pcDNA3, 1.5 μg each) were co-transfected into HEK293T cells (6-cm dish) together with λN-TB-RBP/pcDNA3-HAC effector plasmid (3 μg). Total RNA from transfected cells was analyzed by Northern blotting using *F-Luc* and *R-Luc* DNA probes. Plasmid encoding silencing domain of TNRC6A fused to λN-peptide (λN-TNRC6A/pcDNA3-HAC) and empty vector (λN/pcDNA3-HAC) were used as positive and negative controls, respectively. Expression of effector proteins was examined by immunoblotting with anti-HA antibody. The asterisk denotes a possible dimeric form of λN-TB-RBP-HAC. The molecular weight markers are shown on the right. (B) λN-peptide-dependent effect of TB-RBP. The two reporter plasmids (1.5 μg each) used in (A) were introduced into HEK293T cells (6-cm dish) together with an effector plasmid (3 μg) encoding TB-RBP-HAC with (+) or without (–) λN-peptide. Total RNA from transfected cells was analyzed by Northern blotting using *F-Luc* and *R-Luc* DNA probes. (C) Dose-dependent effect. Total RNA from HEK293T cells shown in Fig. 3D was subjected to Northern blot analysis with *F-Luc* and *R-Luc* DNA probes. *ACTB* served as the loading control.

mRNA, remained unaffected by the presence of λN-TB-RBP-HAC (Supplementary Fig. 2, right panels). These results suggest that the decreased R-Luc activity by TB-RBP is not due to translation inhibition, at least, at the initiation step.

TB-RBP/Translin interacts with its binding partner TRAX, which has endonuclease activity but no RNA-binding activity [17–19]. The complex is known to be involved in the processing of small RNAs

and tRNAs [19, 32, 33], raising the possibility that the reduction of R-Luc activity is caused by mRNA degradation. To examine this possibility, we carried out Northern blot analysis. As shown in Fig. 4A, the level of *R-Luc*-6 × BoxB mRNA was decreased by introduction of λN-TB-RBP-HAC, compared with the control cells co-transfected with empty plasmid, whereas the level of *F-Luc* mRNA remained unaffected. λN-TNRC6A-HAC showed similar effect on *R-Luc*-6 × BoxB mRNA (Fig. 4A), as reported previously [30, 31]. In accordance with the results of luciferase assays (Supplementary Fig. 1 and Fig. 3D), the *R-Luc*-6 × BoxB mRNA was decreased only when TB-RBP was tethered to the mRNA through λN-peptide, and the reduction was dose-dependent (Figs. 4B and 4C). These results suggest that TB-RBP is involved in post-transcriptional regulation by modulating the mRNA levels.

To clarify the involvement of TRAX in the degradation of the reporter mRNA, we used the TB-RBP/Translin knock-out HEK293T cell lines, where TRAX is expected to be coordinately lost [7], for reporter assays. Immunoblot analysis revealed that, along with TB-RBP, TRAX also was absent in the knock-out cells (Fig. 5A). When λN-TB-RBP-HAC was co-expressed in the control HEK293T cells (Fig. 5A), R-Luc activity was compromised as described above (Fig. 3). In contrast, no significant reduction of R-Luc activity was found in TB-RBP/Translin knock-out cells (Fig. 5A), suggesting that λN-TB-RBP-HAC is unable to degrade *R-Luc*-6 × BoxB mRNA in the absence of TRAX. We thus examined whether TRAX is capable of degrading *R-Luc*-6 × BoxB mRNA, when tethered to the mRNA. Introduction of λN-TRAX-HAC elicited a dose-dependent decrease of R-Luc activity, not only in the control cells but also in the knock-out cells (Fig. 5B). Collectively, these results suggest that RNA-binding protein TB-RBP is involved in the degradation of bound mRNAs in association with the nuclease subunit, TRAX.

Discussion

Using *in vitro* translation systems, TB-RBP was shown to repress the translation of haploid cell-specific mRNAs, including *Prm2* [12, 13]. Thus, the initial purpose of this study was to elucidate the mechanism of translational repression by TB-RBP. Although TB-RBP contained a sequence motif found in EIF4E-binding proteins, no interaction was observed between TB-RBP and EIF4E (Fig. 1). We reexamined the function of TB-RBP in HEK293T cells, using luciferase reporter assay. Contradictory to the previous reports [12, 13], TB-RBP failed to decrease the activity of reporter mRNA carrying 3'-UTR of *Prm2* (Fig. 2). We were also unable to detect the interaction of TB-RBP with *Prm1* and *Prm2* mRNAs (Fig. 2), despite the fact that the mRNAs were immunoprecipitated with antibody against YBX3, another RNA-binding protein. The reasons for our inability to detect any effect on the reporter mRNAs and the interaction of TB-RBP

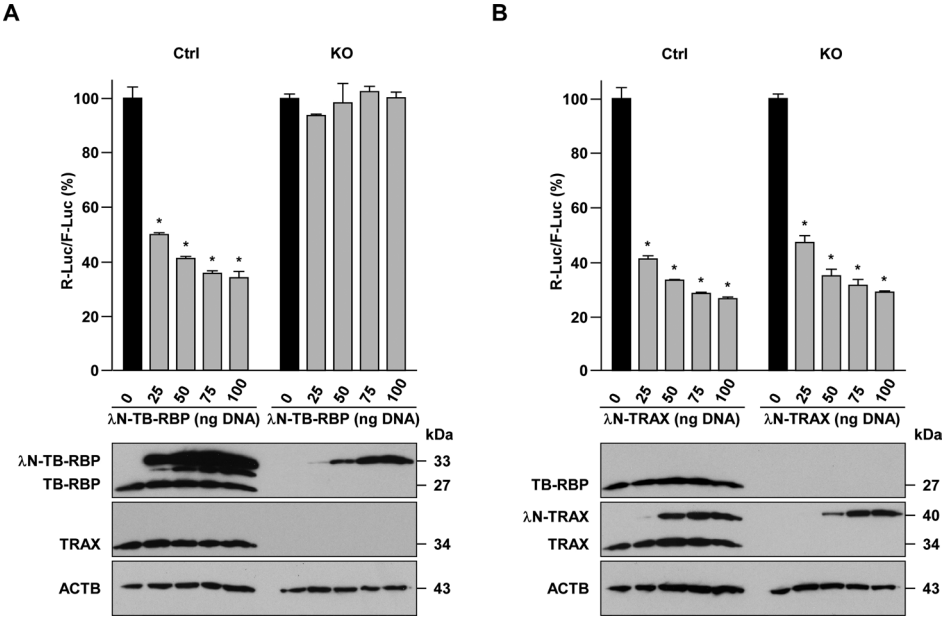

Fig. 5. Translin-associated factor X (TRAX)-dependent effect of testis-brain RNA-binding protein (TB-RBP). (A) Effect of λN-fused TB-RBP on the reporter activity in the absence of TRAX. Tethering reporter assay was carried out in TB-RBP/Translin knock-out cells. Control (Ctrl) and TB-RBP/Translin knock-out (KO) HEK293T cells were grown in 24-well plates and co-transfected with a reporter plasmid (R-Luc-6 × BoxB/psiCHECK-2, 50 ng) and an effector plasmid (λN-TB-RBP/pcDNA3-HAC, 0–100 ng). The normalized R-Luc activity when transfected with empty vector (λN/pcDNA3-HAC) was set to 100. Error bars indicate the mean ± SD with $n = 3$. * represents $P < 0.01$. Exogenously expressed λN-TB-RBP-HAC and endogenous TB-RBP/Translin were detected by immunoblot analysis with anti-TB-RBP antibody. ACTB was used as the loading control. Note that not only TB-RBP/Translin but also TRAX is absent in the KO cells. (B) Reporter assay using λN-fused TRAX. Reporter assay was carried out as described in (A), except that an effector plasmid expressing λN-fused TRAX (λN-TRAX/pcDNA3-HAC, 0–100 ng) was analyzed. Exogenous λN-TRAX-HAC and endogenous TRAX were detected by immunoblotting with antibody against TRAX. ACTB served as the loading control.

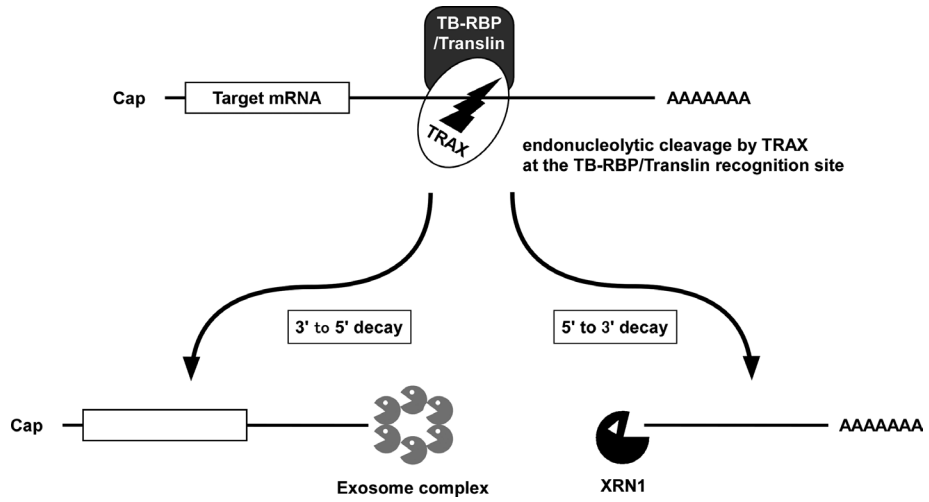

Fig. 6. Proposed mechanism of sequence-specific mRNA degradation initiated by a complex of testis-brain RNA-binding protein (TB-RBP)/Translin and translin-associated factor X (TRAX). TB-RBP/Translin recognizes the specific sequence of target mRNA, followed by the endonucleolytic cleavage by TRAX. The upstream and downstream cleavage products may be degraded rapidly by a complex of 3'-5' exonucleases known as exosome and a 5'-3' exonuclease XRN1, respectively.

with *Prm1* and *Prm2* mRNAs may lie in the differences of reporter assay systems and of the antibodies used.

The present study using λN-tethering reporter assays revealed that TB-RBP is able to decrease luciferase activities, when bound to the reporter mRNA (Fig. 3). The reduced luciferase activities were due to mRNA degradation rather than translation inhibition, at least, at the initiation step, as the λN-fused TB-RBP tethered to the reporter mRNA failed to inhibit the association of mRNA with ribosomes (Fig. 4 and Supplementary Fig. 2). It was also found that mRNA degradation by mouse TB-RBP depends on TRAX in human cell line, HEK293T (Fig. 5). Indeed, mouse TB-RBP is reported to interact with human TRAX [34]. Given that the TB-RBP forms a complex with TRAX in the mouse testis [18] and that the amino acid sequences of TB-RBP/Translin and TRAX are highly conserved between mouse and human (Supplementary Fig. 3: online only), it is most likely that TB-RBP is involved in TRAX-catalyzed mRNA degradation in the mouse testis.

Although TRAX is an endonuclease [19] and acts in a sequence-dependent manner on the reporter mRNA (Fig. 4), the upstream cleavage product of *R-Luc*-6 × BoxB mRNA with an expected size of ~1.0 kb was not detected on the Northern blots using *R-Luc* probe (Fig. 4). The upstream and downstream fragments may be degraded rapidly by a complex of 3'-5' exonucleases known as the exosome and a 5'-3' exonuclease XRN1, respectively, as described for other sequence-specific endonucleases, including the siRNA- and miRNA-directed decay by Ago protein [35, 36]. We propose a model of mRNA degradation initiated by a complex of TB-RBP/Translin and TRAX as depicted in Fig. 6. Although TB-RBP/Translin, complexed with TRAX, has been implicated in the processing of small RNAs and tRNAs [19, 32, 33], our present study provides a possible involvement of this complex in the sequence-specific degradation of mRNA.

Spermatogenesis is governed by precise control of gene expression, which is regulated at the transcriptional, post-transcriptional, and translational levels [1–5]. During spermatogenesis, gene regulation at the post-transcriptional level includes alternative splicing, alternative polyadenylation, and the control of poly(A) tail length [1, 37, 38]. In addition, RNA turnover plays an important role during male germ cell differentiation [39–42]. As mentioned, TB-RBP has been described as a candidate protein responsible for the storage of postmeiotic mRNAs and their delayed translation, based on its ability to repress the translation of specific mRNAs *in vitro* [12, 13]. In the present study, we were unable to detect interaction of TB-RBP with *Prm1* and *Prm2* mRNAs (Fig. 2C). If the TB-RBP complexes were able to associate with spermatid-specific mRNAs, as reported previously [11–14], and if they are involved in mRNA degradation, as suggested by this study, the contribution of TB-RBP to storage and delayed translation of haploid-specific mRNAs is unlikely, because mRNA degradation and mRNA storage are functionally incompatible. In *Tbrbp*-null mice, the sperm count is reduced and the vacuolation of seminiferous tubules becomes more evident with age [7]. Similar defects have been described in mutant mice lacking transcription factor TAF4B, which is essential for the maintenance and proliferation of spermatogonial stem cells [43]. In addition, embryonic fibroblasts from mice lacking TB-RBP exhibit reduced growth rate, compared with those from wild-type and heterozygous littermates [44]. Taken together, although TB-RBP is present throughout spermatogenesis [45], it is conceivable that the protein is responsible for mitotic proliferation of spermatogonia by controlling the levels of specific mRNAs. Further studies, including identification of the mRNAs bound by TB-RBP, are required to clarify the molecular function of TB-RBP.

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