TRF4 Is Involved in Polyadenylation of snRNAs in *Drosophila melanogaster*[▽]

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The *Saccharomyces cerevisiae* poly(A) polymerases Trf4 and Trf5 are involved in an RNA quality control mechanism, where polyadenylated RNAs are degraded by the nuclear exosome. Although Trf4/5 homologue genes are distributed throughout multicellular organisms, their biological roles remain to be elucidated. We isolated here the two homologues of Trf4/5 in *Drosophila melanogaster*, named DmTRF4-1 and DmTRF4-2, and investigated their biological function. DmTRF4-1 displayed poly(A) polymerase activity in vitro, whereas DmTRF4-2 did not. Gene knockdown of DmTRF4-1 by RNA interference is lethal in flies, as is the case for the *trf4 trf5* double mutants. In contrast, disruption of DmTRF4-2 results in viable flies. Cellular localization analysis suggested that DmTRF4-1 localizes in the nucleolus. Abnormal polyadenylation of snRNAs was observed in transgenic flies overexpressing DmTRF4-1 and was slightly increased by the suppression of DmRrp6, the 3'-5' exonuclease of the nuclear exosome. These results suggest that DmTRF4-1 and DmRrp6 are involved in the polyadenylation-mediated degradation of snRNAs in vivo.

The addition of poly(A) tails to mRNA 3' ends is important in many aspects of mRNA metabolism. The conventional role of a poly(A) tail in eukaryotes, which are added by a canonical nuclear poly(A) polymerase (PAP), is threefold: (i) stabilization of RNA, (ii) facilitation of export from the nucleus, and (iii) stimulation of the subsequent translation (7, 33, 55). However, recent studies have changed the traditional view of polyadenylation in eukaryotic RNA metabolism. In particular, a number of noncanonical PAPs were discovered in yeast, worms, and vertebrates. These noncanonical PAPs, which contain a catalytic NTP transferase and PAP-associated domains similar to canonical PAPs, are localized either in the nuclei or cytoplasm and are thought to catalyze polyadenylation of specific RNAs (1, 23, 34, 39, 45).

Schizosaccharomyces pombe Cid1 and Cid13 are cytoplasmic noncanonical PAPs. Cid1 is required for the S-M checkpoint control, including migration from mitosis to meiosis (47), while Cid13 participates in DNA replication and genome maintenance by specifically regulating *suc22* mRNA, which encodes a subunit of ribonucleotide reductase (39). In addition, very recent studies have shown that Cid1 has poly(U) polymerase activity in addition to PAP activity (24, 36). *Caenorhabditis elegans* GLD-2, initially identified as a gene involved in the control of germ line development, is a cytoplasmic noncanonical PAP that promotes the transition from mitosis to meiosis. GLD-2 enzyme activity is stimulated by forming a heterodimeric PAP with an RNA-binding protein GLD-3 (45). Subsequently, GLD-2 homologues were identified in verte-

brates, including frogs, mice, and humans. Vertebrate GLD-2 proteins are important for meiotic maturation of oocytes and are probably involved in the activation of many mRNAs throughout early development (37). In addition, it has been suggested that mammalian GLD-2 are responsible for localized cytoplasmic polyadenylation of neuronal mRNAs at synapses (52). Hs4, one of the human noncanonical PAPs (Hs1-Hs5), was identified as the mitochondrial PAP (hmtPAP) required for mitochondrial RNA polyadenylation (31, 42).

In *S. cerevisiae*, Trf4 and its redundant homologue Trf5 were identified as nuclear noncanonical PAPs (9, 14, 16). These proteins were initially reported to possess DNA polymerase activity in vitro and were classified as the fourth essential DNA polymerase, DNA polymerase σ (48, 49). Unlike cytoplasmic noncanonical PAPs, Trf4 functions in a nuclear RNA surveillance pathway as part of the TRAMP complex, which activates RNA for exosome-mediated degradation by the addition of a poly(A) tail (9, 20, 21, 25, 43, 53). Trf4 interacts with an RNA helicase (Mtr4) and one of two functionally redundant putative RNA-binding proteins (Air1 or Air2) to form the Trf4/Air1/Mtr4 polyadenylation (TRAMP) complex. This mechanism resembles the role of polyadenylation in bacterial RNA turnover (6, 8, 22, 27, 32). Recent studies show that Trf4 is involved in the regulation of histone mRNA levels for the maintenance of genome stability (35). Furthermore, Trf4 is responsible for the control of the ribosomal DNA (rDNA) copy number by degradation of cryptic transcripts from telomeric and rDNA spacer regions (15). In *S. pombe*, Cid14 has been identified as the functional homologue of Trf4 and Trf5. It appears that this enzyme polyadenylates pre-rRNAs to trigger RNA processing by the exosome (51). In addition, Cid14 is also involved in the elimination of a variety of RNA targets to regulate heterochromatic gene silencing, meiotic differentiation, and maintenance of genomic integrity (2, 46). A similar polyadeny-

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TABLE 1. Oligonucleotide sequences used in this study

Oligonucleotide ^a	Sequence (5'–3')	Target
PM01	CCGGAATTCATGGATCCATTTGTTGGCTGGT	DmTRF4-1
PM02	TCTAGACTATCGCTCGTCACGCTGCT	DmTRF4-1
PM03	ACGCGTCGACATGATGATGATGATGATGAAGCTTTCGCTCGT CACGCTGCTGAT	DmTRF4-1
PM04	TGTGTCTAGATCGCTCGTCACGCT	DmTRF4-1
PM05	CTCGAGTTCTTCGAACCTTA	DmTRF4-1
PM06	GAATTCATCGTTGTAGTCGTCGTTAC	DmTRF4-1
PM07	CTCGAGCTGACGCCAGGCAATGATAT	DmTRF4-1
PM08	TCTAGAATCGTTGTAGTCGTCGTTAC	DmTRF4-1
PM09	CGAAACGGTCGCGTGTTCGC	DmTRF4-1
PM10	TTGTGGCGGGTTGTTGCTGG	DmTRF4-1
PM11	CCGTTTGAGTTCTTGTGCTGTG	Actin5C
PM12	CATGATGGAGTTGTAGGTGGTC	Actin5C
PM13	CCGGAATTCATGTGTGACGAAGCGAATCC	DmTRF4-2
PM14	CCCAAGCTTATTCATCTTAAGGTTTGCAAGGTCG	DmTRF4-2
PM15	TGGACTTGGATGTGGACAAG	DmRrp6
PM16	ACGGAGTACATGTCATCATCC	DmRrp6
PB01	TCGGGACGGCGCGAACGCCA	U1 snRNA
PB02	GTAATGCAATACCGGGCCAA	U2 snRNA
PB03	GTAATGGAACGGTATTTACGCTC	U4 snRNA
PB04	GACTCATTAGAGTGTCTCTC	U5 snRNA
PB05	GTGGAACGCTTCACGATTTTG	U6 snRNA
PB06	GTCCAGCTTCTTGAAGCCAATG	RpS29

^a Oligonucleotides PB01 to PB06 were used as probes.

lation-mediated degradation was observed in the catabolism of pre-mRNAs in human cells, but the PAP involved in the pathway is still obscure (50).

The 3'–5' exonuclease Rrp6, a constituent of the exosome, plays a key role in nuclear exosome-dependent degradation (30). *S. cerevisiae* strains lacking Rrp6 accumulate polyadenylated snRNAs, snoRNAs, and rRNAs in a ScTrf4-dependent manner (53). These findings suggest that the noncoding RNAs, such as snRNAs, snoRNAs, and rRNAs, are the main targets of ScTrf4 during polyadenylation-mediated degradation.

The process of mRNA splicing is a major posttranscriptional event that is essential for the maturation of mRNAs in eukaryotes. The reaction is accomplished by spliceosomes, which primarily consist of RNA-protein complexes called small nuclear ribonucleoproteins (snRNPs). The snRNPs contain U1, U2, U4, U5, and U6 small nuclear RNAs (snRNAs) and bind to the pre-mRNA in a specific order to align the splice sites for cleavage. First, the U1 and U2 snRNPs bind to the 5' end of the intron and the branch site close to the 3' end of the intron, respectively, generating the prespliceosome. Then, the preformed U4/U6-U5 tri-snRNP, in which the U4 and U6 snRNAs are base paired, join the complex and form the mature spliceosome. The mature spliceosome induces a major structural change that results in the dissociation of the U1 and U4 snRNPs, allowing U6 snRNP to interact with sequences close to the 5' splice site and U5 snRNP interacts with nucleotides at the end of the first exon, just before the 5' splice junction. This is the active spliceosome, which catalyzes 5' cleavage and lariat formation. U5 snRNP remains attached to the end of the first exon and also begins to interact with the beginning of the second exon, thus bringing the two exons together. The ligated exons are released from the spliceosome, but the lariat intron remains bound. The spliceosome then disassembles, releasing the lariat, which is linearized and degraded. The snRNPs are subsequently recycled (19, 28).

Although the Trf4 family of proteins is conserved from yeast to humans, their biological role in multicellular organisms is poorly understood. We analyzed here the biological roles of *Drosophila* Trf4 homologs (DmTRF4). Our data suggest that in addition to ScTrf4/5 one DmTRF4 is also involved in the RNA surveillance system.

MATERIALS AND METHODS

Drosophila stocks. Fly stocks were cultured at 25°C on standard food. The Canton-S fly was used as the wild-type (WT) strain and *w¹¹¹⁸* was used as a control in certain experiments. The following GAL4 drivers were used. *Act5C-GAL4* drives GAL4 under the control of the constitutive Actin5C promoter. *GMR-GAL4* drives GAL4 under the control of the eye-specific glass multimer reporter (11). *ey-GAL4* drives GAL4 in the pattern of the eyeless gene (13, 18). *MS1096-GAL4* drives GAL4 in the dorsal compartment of the wing imaginal disc (3). *Hsp70-GAL4* induces GAL4 by heat shock. The heat shock involved incubating the third-instar larvae at 37°C for 3 h.

P-element insertion mutant for the *CG17462* gene, *CG17462^{NP2505}*, and for the *DmRrp6* gene, *DmRrp6⁶⁰⁷⁰⁰¹*, were obtained from the *Drosophila* Genetic Resource Center, Kyoto Institute of Technology (Kyoto, Japan) and the Exelixis Collection at the Harvard Medical School (Boston, MA), respectively.

Establishment of transgenic flies. The pUAS-DmTRF4-1 was constructed from the entire open reading frame of DmTRF4-1. This was achieved by PCR using the primer pair PM01/PM02 (Table 1) with cDNA (EST clone RE04457) as a template. The amplified product was then subcloned into pUAST transformation vector. The pUAS-Dmtrf4-1 IR was constructed from nucleotides 1540 to 2015 of DmTRF4-1 cDNA cloned into pUAST as an inverted repeat with an interruption of the DmTRF4-1 third intron. The PCR was performed using cDNA and genome-DNA as a template with the primer pairs PM05-PM06 and PM07-PM08, respectively (Table 1). The two amplified products were then subcloned into pUAST vector in a head-to-head orientation. P-element transformation was performed by using a standard method. Several independent lines were established for the pUAS-DmTRF4-1 and pUAS-Dmtrf4-1 IR, respectively. All crosses were performed at 28°C, except crossing *Hsp70-GAL4*.

RNA purification, Northern blotting, and reverse transcription-PCR (RT-PCR) analysis. Total RNAs were extracted by using TRIzol (Invitrogen, Carlsbad, CA) from *Drosophila melanogaster* bodies at several different stages, from Kc cells, and from the treated third-instar larvae in respective experiments, treated with DNase I (TaKaRa Bio., Inc., Kyoto, Japan) and then purified with phenol-chloroform. Poly(A)⁺ RNA was purified from total RNA using

PolyAtract mRNA isolation kit according to the manufacturer's protocols (Promega, Madison, WI). Then, 1 μ g of poly(A)⁺ RNA was hybridized to 300 ng of oligo(dT)₁₈ in 25 mM Tris-HCl (pH 7.9), 1 mM EDTA, and 50 mM NaCl by slow cooling from 68 to 30°C. The mixture was adjusted to 53 mM Tris-HCl (pH 7.9), 25 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, and 30 μ g of bovine serum albumin (BSA)/ml and digested for 1 h at 37°C with 60 U of RNase H (TaKaRa Bio., Inc.).

Northern blotting, for the experiments shown in Fig. 1, was carried out as described previously (41). Full-length *DmTRF4-1* or *-2* was used as the specific probe. Full-length *ribosomal protein 49* (*Rp49*) cDNA was used as a control. For the experiments shown in Fig. 7, RNA [5 μ g of total RNA, 1 μ g of purified poly(A)⁺ RNA, and 1 μ g of deadenylated RNA] was separated in a 6% polyacrylamide gel (19:1) containing 8 M urea, electrophoretically transferred to nylon membrane (Hybond-N⁺; Amersham) in 0.5× TBE (1× TBE is 89 mM Tris-borate and 2 mM EDTA) at 3.3 mA/cm² for 2 h, and then UV cross-linked to the membrane by using Stratalinker (Stratagene, La Jolla, CA). Membranes were hybridized in ULTRAhyb-Oligo (Ambion, Austin, TX) using oligonucleotide DNA probes (Table 1) that were 5' end labeled using T4 polynucleotide kinase (TaKaRa Bio) and [γ -³²P]ATP (GE Healthcare Bioscience, Piscataway, NJ). After two washes in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate [pH 7.0]) plus 0.5% sodium dodecyl sulfate, the membrane was exposed to BioMax MS-1 (Kodak, Rochester, NY).

In the RT-PCRs for the experiments shown in Fig. 3, 5, and 6, first-strand cDNA was synthesized from 5 μ g of total RNA by using the SuperScript First-Strand synthesis system (Invitrogen) with oligo(dT)₁₂₋₁₈ and then amplified using the specific primer pairs PM09-PM10, PM11-PM12, PM13-PM14, and PM15-PM16 (Table 1). PCR products were visualized by staining with ethidium bromide after agarose gel electrophoresis. Quantification was analyzed by using a BAS-3000 imaging analyzer (Fuji Film, Tokyo, Japan).

Purification of recombinant DmTRF4-1 WT, mutant, and DmTRF4-2 proteins. The entire open reading frames of *DmTRF4-1* and *DmTRF4-2* cDNA were amplified by PCR using the *DmTRF4-1* cDNA clone (RE04457) and the *DmTRF4-2* cDNA clone (LP06848) with the primer pairs PM01-PM03 and PM13-PM14, respectively (Table 1). Note that in each case the 3'-oligonucleotide primer encodes an in-frame His tag. The amplified DNA products were then subcloned into the pGEX 6P-1 expression vector (GE Healthcare Bioscience). The resulting construct was then transformed into *Escherichia coli* BL21(DE3) (Novagen, Madison, WI). One colony was picked and incubated in 10 ml of Terrific broth medium containing 1% (wt/vol) glucose and 50 μ g of ampicillin/ml. The culture was grown overnight at 30°C and transferred into 1 liter of fresh Terrific broth medium containing 1% (wt/vol) glucose and 50 μ g of ampicillin/ml. The cells were grown at 30°C until the *A*₆₀₀ value reached 0.5 and cooled on ice for 30 min. The culture was then incubated with 100 μ M IPTG (isopropyl- β -D-thiogalactopyranoside) at 16°C for 16 h. The cells were harvested by centrifugation at 4°C and washed with cold phosphate-buffered saline. After centrifugation, the cells were frozen in liquid nitrogen. The frozen cells were thawed in an ice bath and resuspended in 7 ml of His tag binding buffer (50 mM NaH₂PO₄, 300 mM NaCl, 5 mM imidazole, 10% [vol/vol] glycerol, 0.01% [vol/vol] Nonidet P-40 [pH 8.0])/g containing 1 μ g/ml each of pepstatin A and leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mg of lysozyme/ml. The suspension was incubated on ice for 40 min. After an equal volume of His tag binding buffer was added, the cell lysate was centrifuged for 30 min at 15,000 × g at 4°C. After filtration through a 0.45- μ m-pore-size polyvinylidene difluoride membrane, the supernatant was loaded onto 1 ml of a Ni-NTA agarose column equilibrated with His tag binding buffer, washed with 50 ml of His tag wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 30 mM imidazole, 10% [vol/vol] glycerol, 0.01% [vol/vol] Nonidet P-40 [pH 8.0]), and eluted with 10 ml of His tag elution buffer (50 mM NaH₂PO₄, 100 mM NaCl, 200 mM imidazole, 10% [vol/vol] glycerol, 0.01% [vol/vol] Nonidet P-40 [pH 7.7]). After being mixed with 30 ml of TEMG buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 5 mM 2-mercaptoethanol, and 10% glycerol) containing 0.4 M NaCl, the eluted material was loaded onto a 0.3 ml glutathione-Sepharose 4B column equilibrated with TEMG buffer containing 0.4 M NaCl. The column was washed with 50 ml of TEMG buffer containing 2 M NaCl and then with 10 ml of TEMG buffer containing 0.2 M NaCl. Protein was eluted from the column with TEMG buffer (adjusted to pH 8.0) containing 10 mM glutathione (reduced form). The fractions containing protein were collected, frozen in liquid nitrogen, and then stored at -80°C until use.

The DmTRF4-1 mutant (GST-DmTRF4-1 DD328,330AA) was overexpressed and purified as for the WT protein.

PAP assay. PAP assays were performed in 20- μ l reaction mixtures containing 250 to 500 ng of the purified protein, 2.5 fmol of 5'-end-labeled oligo(A)₁₅, 0.5 mM ATP, 0.5 mM MnCl₂, 25 mM Tris-HCl (pH 7.9), 20 mM KCl, 10% glycerol, 0.01 mM EDTA, 0.1 mg of BSA/ml, 1 mM dithiothreitol, 0.02% Nonidet P-40,

and 5 U of RNA Guard (Promega). As indicated in the figure legends, in some experiments we modified the components of the above reaction in the following ways: 5 mM MgCl₂ replaced MnCl₂ (see Fig. 2C, lanes 2 and 6); both 0.5 mM MnCl₂ and 5 mM MgCl₂ were added at the same time (see Fig. 2C, lanes 4 and 8); and the reaction mixture contained 0.5 mM concentrations of either GTP, UTP, or CTP instead of ATP (see Fig. 2D). Assay mixtures were assembled on ice and incubated at 30°C for the times indicated and stopped by the addition of loading buffer (20 μ l) containing 95% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol FF, and 0.5 mM EDTA. The reaction products were resolved on 20% polyacrylamide gels containing 8 M urea. After drying, the gel was exposed to BioMax MS-1 film (Kodak).

Cell culture, plasmid construction, and transfection. S2 cells were cultured in Schneider's *Drosophila* medium (Invitrogen) containing 10% heat-inactivated fetal bovine serum at 25°C. The expression vector for V5-tagged DmTRF4-1 WT and mutant was constructed by subcloning the *DmTRF4-1* coding region, using the primer pair PM01-PM04, into pAc5.1/V5-His A (Invitrogen). All transfections and establishment of the stable cell lines were performed in accordance with the manufacturer's protocols (Invitrogen).

Immunofluorescence analysis. S2 cells were placed on poly-(L-lysine)-coated coverslips and fixed with 4% paraformaldehyde in NaCl/P_i for 10 min at room temperature. After several washes with NaCl/P_i, the cells were treated with methanol for permeabilization. The samples were incubated with primary antibodies, mouse monoclonal anti-V5 antibody (Invitrogen) and rabbit polyclonal antifibrillarin antibody (Abcam, Cambridge, MA) (used as a nucleolar marker), at 4°C overnight and then treated for 1 h with the secondary antibodies Alexa546 anti-mouse immunoglobulin G and Alexa488 anti-rabbit immunoglobulin G (Molecular Probes, Eugene, OR). Samples were also counterstained with DAPI (4',6'-diamidino-2-phenylindole). The preparations were observed under a fluorescence microscope, and the data were collected using a charge-coupled device camera (Nikon, Chiyoda, Japan).

Oligonucleotide sequences. The oligonucleotide sequences used in the present study as primers and probes are shown Table 1.

RESULTS

The *Drosophila* genome contains two homologues of *S. cerevisiae* Trf4. A search of the *Drosophila* genome database (Flybase [http://flybase.bio.indiana.edu/]) identified two genes homologous to ScTrf4, which are named CG11265 and CG17462 (*DmTRF4-1* and *DmTRF4-2*, respectively). The predicted protein product of *DmTRF4-1* is composed of 1,001 amino acids and contains a unique sequence with no homology to any known protein in both the N-terminal and the C-terminal regions (Fig. 1A). In contrast, *DmTRF4-2* encodes a predicted protein of 407 amino acids (Fig. 1A). The Trf4/5 family is a divergent member of the DNA polymerase β -nucleotidyltransferase superfamily (25, 43, 53), which includes CCA-adding enzymes, DNA polymerases, and eukaryotic nuclear canonical PAPs. The catalytic domain, consisting of the nucleotidyltransferase domain and PAP-associated domain, is well conserved among the Trf4/5 family members. We compared the sequences of these domains from ScTrf4, DmTRF4-1, and DmTRF4-2. DmTRF4-1 and DmTRF4-2 display 36% identity (55% similarity) and 31% identity (50% similarity) with ScTRF4, respectively (Fig. 1A and B). The catalytic domains of DmTRF4-1 and DmTRF4-2 display 48% identity (65% similarity). We performed Northern blot analysis to analyze the expression patterns of these genes over the range of developmental stages. Both *DmTRF4* mRNAs were highly transcribed in unfertilized eggs and in the early embryonic stages from 0 to 4 h (Fig. 1C).

DmTRF4-1, but not DmTRF4-2, exhibits PAP activity in vitro. To test whether DmTRF4-1 and DmTRF4-2 possess PAP activity, we induced expression of a glutathione S-transferase (GST) fusion protein containing DmTRF4-1 and DmTRF4-2 with a 3' terminal His tag (GST-DmTRF4-1

FIG. 2. DmTRF4-1, but not DmTRF4-2, exhibits PAP activity. (A) Purified GST fusion protein containing WT DmTRF4-1 (GST-DmTRF4-1 WT; lane 1) and mutant DmTRF4-1 (GST-DmTRF4-1 DD328,330AA; lane 2), in which aspartate residues at positions 328 and 330 have been changed to alanine, and WT DmTRF4-2 (GST-DmTRF4-2 WT; lane 3) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5 or 12.5% polyacrylamide gel and stained with Coomassie brilliant blue (1.0 μ g of each). The positions of the purified proteins are indicated by an asterisk. (B) DmTRF4-1 has PAP activity, but DmTRF4-2 has no activity in vitro. GST-DmTRF4-1 WT (lane 2 to 3) (250 ng), GST-DmTRF4-1 DD328,330AA (lane 4) (500 ng), and GST-DmTRF4-2 WT (lane 5 to 6) (350 ng) were incubated with 5'-end-labeled oligo(A)₁₅ in the presence of 0.5 mM Mn²⁺ at 30°C. A control reaction with no enzyme is shown in lane 1. (C) Mg²⁺ versus Mn²⁺ dependence. GST-DmTRF4-1 WT (250 ng) was incubated with 5'-end-labeled oligo(A)₁₅ in the presence of either Mg²⁺ (5 mM; lane 2), Mn²⁺ (0.5 mM; lane 3), or both Mg²⁺ and Mn²⁺ ions (5 and 0.5 mM, respectively; lane 4) at 30°C for 30 min. GST-DmTRF4-2 WT (350 ng) was incubated with 5'-end-labeled oligo(A)₁₅ in the presence of either Mg²⁺ (5 mM; lane 6), Mn²⁺ (0.5 mM; lane 7), or both Mg²⁺ and Mn²⁺ ions (5 and 0.5 mM, respectively; lane 8) at 30°C for 30 min. A control reaction with no cations is shown in lanes 1 and 5. (D) Nucleotide substrate dependence. GST-DmTRF4-1 WT (250 ng) was incubated with 5'-end-labeled oligo(A)₁₅ in the presence of either ATP (lane 1), GTP (lane 2), CTP (lane 3), or UTP (lane 4) at 30°C for 30 min. All PAP polymerase reaction products were resolved on a 20% polyacrylamide-8 M urea gels and visualized by using autoradiography.

and GST-TRF4-2) in *Escherichia coli*. Soluble GST-DmTRF4-1 and -2 were purified on Ni-NTA agarose and glutathione-Sepharose columns (Fig. 2A, lanes 1 and 3). Purified GST-DmTRF4-1 displayed PAP activity (Fig. 2B, lanes 2 to 3). To confirm the absence of *E. coli* PAP enzymes, we constructed a DmTRF4-1 inactive mutant (GST-DmTRF4-1 DD328,330AA) in which the aspartate residues 328 and 330 were replaced with alanine (Fig. 1B). These residues correspond to the conserved amino acids essential

for catalysis in many polymerase β families (29). The GST-DmTRF4-1 DD328,330AA was expressed and purified by the same procedures as for the WT protein (Fig. 2A, lane 2). The GST-DmTRF4-1 DD328,330AA showed no PAP activity (Fig. 2B, lane 4), indicating that GST-DmTRF4-1 protein was responsible for the enzyme activity and not a coprecipitating contaminant. GST-DmTRF4-1 requires Mn²⁺ for its PAP activity rather than Mg²⁺ (Fig. 2C, lanes 1 to 4). Furthermore, GST-DmTRF4-1 showed a high degree of

FIG. 3. *DmTRF4-1* knockdown and *DmTRF4-2* knockout mutants. (A) Physical map of the genomic location of *DmTRF4-2* (CG17462). In CG17462^{NP2505}, P-element was inserted in the 5'-untranslated region of the CG17462 gene. Black and gray arrows indicate predicted genes. (B) RT-PCR was performed for total RNAs from WT (lane 1) and *DmTRF4-2* mutant (CG17462^{NP2505}; lane 2) third-instar larvae. Expression of *Act5C* was used as an internal control. The cycle numbers used are indicated. (C) RT-PCR was performed for total RNAs from *Act5C-GAL4/+* (control; lane 1) and *Act5C-GAL4/UAS-Dmtrf4-1 IR* (*DmTRF4-1* KD; lane 2) third-instar larvae. Expression of *Act5C* was used as an internal control. The cycle numbers are indicated. The relative amount of the *DmTRF4-1* transcript in *Act5C-GAL4/UAS-DmTRF4-1 IR* to that in *Act5C-GAL4/+*, normalized to *Act5C* mRNA, was also quantified graphically. The data represent the mean of three independent measurements. Error bars indicate $\pm$ the standard deviation. (D) Knockdown of the *DmTRF4-1* level by ubiquitous expression of the *DmTRF4-1* double-stranded RNAs in living flies (*Act5C-GAL4/UAS-Dmtrf4-1 IR*) caused third-instar-larval or early pupal lethality. Few transgenic flies survived until the late pupal stage, but when this happened the body of the pupa was significantly darkened. Note the darkened imago in the *DmTRF4-1* knockdown mutant, as indicated by the arrowhead (*DmTRF4-1* KD; panel a). Early pupal lethality was not observed in control flies (*Act5C-GAL4/+*) (Control; panel b).

selectivity for ATP incorporation into poly(A) RNA, whereas GTP, CTP, and UTP were incorporated very poorly (Fig. 2D). GST-DmTRF4-2 displayed no PAP activity in the presence of either Mn^{2+} or Mg^{2+} (see Fig. 2B, lanes 5 to 6; Fig. 2C, lanes 5 to 8).

Although it was initially reported that ScTrf4 is inactive in the absence of accessory proteins (25, 43, 53), a recent study showed that Trf4 and Trf5 exhibit significant PAP activity in isolation (14). Similarly, GST-DmTRF4-1 does not require any accessory proteins for PAP activity in vitro.

***DmTRF4-1*, but not *DmTRF4-2*, is essential for development in *D. melanogaster*.** We searched for disruption mutants in the Gal4 Enhancer Trap Insertion Database (GETDB [http://flymap.lab.nig.ac.jp/~dclust/getdb.html]). The *DmTRF4-1* knockout mutant was not stocked, but a strain with a disrupted *DmTRF4-2* was found. In this mutant, CG17462^{NP2505}, the P element was inserted in the 5'-untranslated region of the *DmTRF4-2* (CG17462) gene (Fig. 3A). We performed semi-quantitative PT-PCR and confirmed that CG17462^{NP2505} was a null mutant of *DmTRF4-2* (Fig. 3B). The CG17462^{NP2505} ho-

mozygous mutants were viable and fertile despite lacking *DmTRF4-2*, indicating that *DmTRF4-2* is not essential for viability. To address the in vivo function of *DmTRF4-1*, we established transgenic fly lines carrying *UAS-Dmtrf4-1 IR*, which causes an RNA interference-induced knockdown of the *DmTRF4-1* transcript when crossing a *GAL4* driver (Fig. 3C and 5A). The ubiquitous knockdown of *DmTRF4-1* driven by *Act5C-GAL4* caused third-instar-larval or early pupal lethality (Fig. 3D), which is reminiscent of *trf4 trf5* double mutants (5). These results indicate that *DmTRF4-1* is essential for development in *D. melanogaster* and that *DmTRF4-2* is unable to complement *DmTRF4-1*.

Our experiments using the recombinant proteins and the transgenic flies suggested that *DmTRF4-1*, like ScTrf4/5, might play a crucial role in RNA quality control. In contrast, *DmTRF4-2* appears to be inessential, although we cannot rule out the possibility that the protein is also involved in the same pathway. Therefore, we focused on the characterization of *DmTRF4-1* in the present study.

FIG. 4. Subcellular localization of DmTRF4-1 in living S2 cells. The S2 cells overexpressing V5-tagged DmTRF4-1 DD328,330AA was immunostained with anti-V5 antibody (red) (A and A') and antifibrillarin (green) (B and B'), as a nucleolar marker, and stained with DAPI (blue) (C and C'). The merge is shown in (D and D'). The lower panels are a high-magnification view.

DmTRF4-1 is a nucleolar protein. To investigate the subcellular localization of DmTRF4-1, we attempted to generate Schneider 2 (S2) cells expressing V5-tagged DmTRF4-1 WT under the control of an *Actin5C* promoter. Unfortunately, we were unable to obtain the desired cell line. This was probably caused by the high-level expression of V5-tagged DmTRF4-1 WT being toxic to S2 cells. We then attempted to visualize the subcellular localization of V5-tagged DmTRF4-1 DD328,330AA mutant by using immunofluorescence microscopy. We found that DmTRF4-1 DD328,330AA overlapped with the nucleolar marker, fibrillarin (Fig. 4), suggesting that the DmTRF4-1 is a nucleolar protein. It is widely thought that Trf4 family members are nuclear protein (44). However, our findings are consistent with the recent observation that *S. pombe* Cid14 is a nuclear protein enriched in the nucleolus (51).

An abnormal morphogenesis induced by the overexpression of DmTRF4-1 is partially rescued by suppression of DmRrp6. To address the function of DmTRF4-1 in vivo, we established transgenic fly lines carrying *UAS-DmTRF4-1*, where overexpression of *DmTRF4-1* is induced by crossing a GAL4 driver. Ubiquitous overexpression of *DmTRF4-1* using the *Act5C-GAL4* driver caused embryonic lethality. In addition, flies overexpressing *DmTRF4-1* in which knockdown of *DmTRF4-1* was induced using the *Act5C-GAL4* driver were also inviable (data not shown). The knockdown of *DmTRF4-1* using the *Hsp70-GAL4* driver, which transiently induced the transcript of the GAL4 gene by heat shock, decreased the amount of the DmTRF4-1 transcripts (Fig. 5A, lane 2), whereas the overexpression of *DmTRF4-1* using the *Hsp70-GAL4* driver increased the amount of the DmTRF4-1 transcripts (Fig. 5A, lane 3). The simultaneous overexpression and knockdown of *DmTRF4-1* using the *Hsp70-GAL4* driver almost restored the amount of the DmTRF4-1 transcripts close to its normal level (Fig. 5A, lane 4). Moreover, knockdown of *DmTRF4-1* posterior to the morphogenetic furrow (MF) of the eye disc using the *GMR-GAL4* driver suppressed rough eye phenotypes induced by overexpression of *DmTRF4-1* (Fig. 5Bc and d), although a phenotype was not detectable by knockdown of *DmTRF4-1* using this driver. These data suggest that the *UAS-DmTRF4-1* and *UAS-*

Dmtrf4-1 IR lines specifically regulate the amount of *DmTRF4-1* transcript. When the *UAS-DmTRF4-1* strain was mated with the *MS1096-GAL4* strain and the *ey-GAL4* strain, respectively, the progeny flies carrying both the GAL4 driver and *UAS-DmTRF4-1* displayed a shrunken wing phenotype and a headless phenotype (Fig. 5C and D), whereas a phenotype was not detectable by knockdown of DmTRF4-1 using these drivers. These data suggest that overexpression of *DmTRF4-1* induced an abnormal morphogenesis.

Previous studies demonstrated that the RNAs polyadenylated by ScTrf4 are exposed to exosome-mediated degradation (9, 20, 21, 25, 43, 53). Rrp6, a 3'-5' exonuclease, plays a key role in this degradation machinery (30). The homozygote of the *DmRrp6*⁷⁰⁷⁰⁰¹, where the P-element is inserted into the second intron of the *DmRrp6* gene (Fig. 6A), is inviable. The heterozygote of the *DmRrp6*⁷⁰⁷⁰⁰¹ expressed approximately half the amount of *DmRrp6* transcript compared to the WT (Fig. 6B). We crossed the flies overexpressing *DmTRF4-1* with the *DmRrp6* mutant. A rough eye phenotype caused by overexpression of *DmTRF4-1* was partially rescued in the *DmRrp6* heterozygous background, whereas *DmRrp6* heterozygous mutants displayed no rough eye phenotype (Fig. 6C).

Overexpression of DmTRF4-1 caused accumulation of polyadenylated snRNAs. It is possible that an aberrant polyadenylation of RNA substrates by DmTRF4-1 interferes with the morphogenetic process. To address this possibility, we examined the polyadenylation of RNAs from third-instar larvae of *W* (control), heterozygous *DmRrp6*⁷⁰⁷⁰⁰¹ (*DmRrp6* KD), *UAS-Dmtrf4-1* IR (*DmTRF4-1* KD), *UAS-Dmtrf4-1* IR and heterozygous *DmRrp6*⁷⁰⁷⁰⁰¹ (*DmTRF4-1* KD and *DmRrp6* KD), *UAS-DmTRF4-1* (*DmTRF4-1* OE), *UAS-DmTRF4-1*, and heterozygous *DmRrp6*⁷⁰⁷⁰⁰¹ (*DmTRF4-1* OE and *DmRrp6* KD) lines crossed with the *Hsp70-GAL4* driver. Polyadenylated RNAs were purified from total RNAs using oligo(dT)-immobilized beads [poly(A)⁺ RNA fraction]. We found that the band mobility of snRNAs was shifted up to approximately 500 nucleotides in the poly(A)⁺ RNA fraction from the transgenic flies overexpressing *DmTRF4-1* (*DmTRF4-1*

FIG. 5. Effects of overexpression of the *DmTRF4-1* in *D. melanogaster*. (A) RT-PCR was performed for total RNAs from *Hsp70-GAL4/+* (control; lane 1), *Hsp70-GAL4/UAS-Dmtrf4-1 IR* (DmTRF4-1 KD; lane 2), *Hsp70-GAL4/+; UAS-DmTRF4-1/+* (DmTRF4-1 OE; lane 3), and *Hsp70-GAL4/UAS-Dmtrf4-1 IR; UAS-DmTRF4-1/+* (DmTRF4-1 KD and OE; lane 4) third-instar larvae incubated for 3 h at 37°C. Expression of *Act5C* was used as an internal control. The cycle numbers are indicated. The relative amount of the *DmTRF4-1* transcript in each sample to that in *Hsp70-Gal4/+*, normalized to *Act5C* mRNA, was also quantified graphically. The data represent the mean of three independent measurements. Error bars indicate + the standard deviation. (B) The transgenic fly lines crossed the *GMR-GAL4* driver, which induced *GAL4* posterior to the MF of the eye disc. No phenotype was observed in control flies (*GMR-GAL4/+; +; +*) (control; panel a) and knockdown flies DmTRF4-1(*GMR-GAL4/+; UAS-Dmtrf4-1 RI/+; +*) (DmTRF4-1 KD; panel b). Overexpression of the *DmTRF4-1* caused a rough eye phenotype (*GMR-GAL4/+; +; UAS-DmTRF4-1/+*), as indicated by the arrowhead (DmTRF4-1 OE; panel c). Knockdown of the *DmTRF4-1*, while overexpressing *DmTRF4-1*, suppressed a rough eye phenotype induced by overexpression of the *DmTRF4-1* (*GMR-GAL4/+; UAS-Dmtrf4-1 RI/+; UAS-DmTRF4-1/+*) (DmTRF4-1 KD and OE; panel d). (C) Overexpression of *DmTRF4-1* in the wing imaginal disc using the *MS1096-GAL4* driver caused an underdeveloped wing (*MS1096-GAL4/+; +; UAS-DmTRF4-1/+*), as indicated by the arrowhead (DmTRF4-1 OE; panel a). No phenotype was observed in control flies (*MS1096-GAL4/+*) (control; panel b). (D) Overexpression of *DmTRF4-1* in the whole eye imaginal disc using the *ey-GAL4* driver resulted in a headless phenotype, as indicated by the arrowhead, and caused lethality at the pupal stage (*ey-GAL4/+; UAS-DmTRF4-1/+*) (DmTRF4-1 OE; panel a). No phenotype was observed in control flies (*ey-GAL4/+*) (control; panel b). These were the contents of the dissected pupa.

OE) and *DmTRF4-1* in the heterozygous *DmRrp6*^{f07001} background (DmTRF4-1 OE and DmRrp6 KD) (Fig. 7A to E, middle panels, lanes 11 and 12). In addition, an increase in the elongated snRNAs (1.6- to 2.5-fold) was observed in the DmTRF4-1 OE and DmRrp6 KD compared to that in the DmTRF4-1 OE. We next treated the poly(A)⁺ RNA fractions with oligo(dT) and RNaseH [poly(A)⁺ RNA fraction]. The

RNase H enzyme hydrolyzes the 3'-terminal phosphodiester bonds of RNA hybridized to DNA. This resulted in a dramatic decrease in the length of snRNAs (Fig. 7A to E, right panels, lanes 17 and 18). These data indicate that the overexpressed DmTRF4-1 catalyzes the extraordinary polyadenylation of snRNAs and that the abnormally polyadenylated snRNAs by overexpression of DmTRF4-1 are digested in a DmRrp6-de-

FIG. 6. *DmRrp6* mutant suppresses a rough eye phenotype induced by the overexpression of *DmTRF4-1*. (A) Physical map of the genomic location of *DmRrp6*. In *DmRrp6*¹⁰⁷⁰⁰¹, the P-element was inserted in the second intron of the *DmRrp6* gene. Black and gray arrows indicate predicted genes. (B) RT-PCR was performed for total RNAs from WT (lane 1) and *DmRrp6* mutant (*DmRrp6*¹⁰⁷⁰⁰¹; lane 2) third-instar larvae. A homozygote of the *DmRrp6*¹⁰⁷⁰⁰¹ is inviable. Expression of *Act5C* was used as an internal control. The cycle numbers used are indicated. The relative amount of the *DmRrp6* transcript in *DmRrp6*¹⁰⁷⁰⁰¹ to that in the WT, normalized to *Act5C* mRNA, was also quantified graphically. The data represent the mean of three independent measurements. Error bars indicate $\pm$ the standard deviation. (C) *DmRrp6* mutation suppresses the phenotype induced by overexpression of *DmTRF4-1*. No phenotype was observed in the WT fly (control; panel a) and *DmRrp6* mutant (*DmRrp6*¹⁰⁷⁰⁰¹) (*DmRrp6* KD; panel b). Overexpression of *DmTRF4-1* posterior to the MF of the eye disc caused a rough eye phenotype (*GMR-GAL4/+; +; UAS-DmTRF4-1/+*) (*DmTRF4-1* OE; panel c). *DmRrp6* mutation slightly suppressed the rough eye phenotype induced by overexpression of *DmTRF4-1* (*GMR-GAL4/+; +; UAS-DmTRF4-1/DmRrp6*¹⁰⁷⁰⁰¹) (*DmTRF4-1* OE and *DmRrp6* KD; panel d). Note that the rough eye phenotype is indicated by arrowheads.

pendent manner. In contrast, no aberrant polyadenylation of snRNAs was detectable in the poly(A)⁺ RNA fraction from the other flies (Fig. 7A to E, middle panels, lanes 7 to 10), whereas snRNAs were abundant in the total RNA fractions.

This is probably because the polyadenylated snRNAs were subject to immediate degradation under normal conditions. Several reports indicate that noncoding RNAs and tRNAs are targets for polyadenylation by *Saccharomyces cerevisiae* Trf4

FIG. 7. Overexpression of *DmTRF4-1* causes extraordinary snRNA polyadenylation, which is *DmRrp6* dependent. Total RNA (5 μ g) (total; left panels), purified poly(A)⁺ RNA (1 μ g) [poly(A)⁺; middle panels], deadenylated RNA (1 μ g) [poly(A)⁻; right panels] were isolated from third-instar larvae of *Hsp70-GAL4/+* (control; lanes 1, 7, and 13), *Hsp70-GAL4/+*; *DmRrp6^{07001/+}* (*DmRrp6* KD; lanes 2, 8, and 14), *Hsp70-GAL4/UAS-Dmtrf4-1 IR* (*DmTRF4-1* KD; lanes 3, 9, and 15), *Hsp70-GAL4/UAS-Dmtrf4-1 IR*; *DmRrp6^{07001/+}* (*DmTRF4-1* KD and *DmRrp6* KD; lanes 4, 10, and 16), *Hsp70-GAL4/+*; *UAS-DmTRF4-1/+* (*DmTRF4-1* OE; lanes 5, 11, and 17), *Hsp70-GAL4/+*; *UAS-DmTRF4-1/DmRrp6⁰⁷⁰⁰¹* (*DmTRF4-1* OE and *DmRrp6* KD; lanes 6, 12, and 18) after induction of *GAL4* expression by heat shock for 3 h. Specific radiolabeled probes (Table 1) for *U1* (A), *U2* (B), *U4* (C), *U5* (D), *U6* (E), *snRNA* and *Ribosomal protein S29* (*RpS29*), as a loading control (F), were hybridized and detected by autoradiography. The migration positions of the regular snRNA and polyadenylated snRNA are indicated on the right of the panels. The ratio of the polyadenylated snRNAs in *Hsp70-GAL4/+*; *UAS-DmTRF4-1/DmRrp6⁰⁷⁰⁰¹* (*DmTRF4-1* OE and *DmRrp6* KD) to those in *Hsp70-GAL4/+*; *UAS-DmTRF4-1/+* (*DmTRF4-1* OE) are shown in lane 12. Each band intensity was quantified by using a BAS-3000 imaging analyzer and normalized to *RpS29* mRNA.

(9, 21, 25, 43, 53). Thus, we also tested the polyadenylation of several kinds of snoRNAs and tRNAs. However, no signal could be detected in the present study (data not shown).

DISCUSSION

In this study, we identified two kinds of Trf4 homologue genes from *D. melanogaster*, *DmTRF4-1* and *DmTRF4-2*, which are expressed with the same pattern during development. Only *DmTRF4-1* displays substantial PAP activity in vitro, and transgenic flies in which the expression of *DmTRF4-1* is either up-regulated or downregulated were inviable. These results suggest that *DmTRF4-1* is an essential PAP, as well as ScTrf4 and ScTrf5, and that the *DmTRF4-1* expression level is strictly regulated in vivo. In contrast, *DmTRF4-2* did not polyadenylate RNA substrates. Previous studies indicated that ScTrf4 alone has no polyadenylation activity and suggested that ScTrf4 is the catalytic subunit of the TRAMP complex in which the Air1 and Air2 proteins confer poly(A) addition activity to the Trf4 subunit by providing the RNA-binding domain (25, 43, 53). It is likely that various accessory proteins may allow Trf4 to target different substrates in different situations. Therefore, it is possible that *DmTRF4-2* proteins may show PAP activity through interaction with accessory proteins. However, because disruption of *DmTRF4-2* does not generate an abnormal phenotype, the biological role of *DmTRF4-2* is presumably complemented by other proteins.

Our experiments suggest that *DmTRF4-1* is localized in the nucleoli and reveal that overexpression of *DmTRF4-1* induces an extraordinary polyadenylation of snRNAs. Recently, Cid14 was identified as a nucleolar protein (51). Furthermore, it was reported that polyadenylated RNAs accumulate in the nucleoli of yeast cells lacking either Rrp6 or Mtr4 (4), suggesting that polyadenylation-dependent exosome-mediated degradation mainly occurs in nucleoli. In addition, it has been found that the vertebrate snRNAs are transiently transferred to the nucleoli (12, 26), where snRNAs undergo common internal modification, including pseudouridylation and 2'-O-methylation (54). These observations suggest that *DmTRF4-1* catalyzes polyadenylation of snRNAs in the nucleoli, where snRNAs are exposed to posttranscriptional modification.

We found that overexpression of *DmTRF4-1* in the eye disc causes the rough eye phenotype, which is partially rescued by the suppression of Rrp6. This phenotype might result from abnormal degradation of the polyadenylated snRNAs caused by constitutive overexpression of *DmTRF4-1*, leading to disruption of normal RNA splicing. Suppression of *DmRrp6* might moderate the rough eye phenotype by inhibiting the degradation of the polyadenylated snRNAs, although it is unclear whether the polyadenylated snRNAs retain a normal function. We detected polyadenylated snRNAs only in the *DmTRF4-1*-overexpressing background, probably because the rate of polyadenylation by overexpressed *DmTRF4-1* exceeds that of *DmRrp6*-mediated degradation. Unfortunately, we could not examine the polyadenylation of snRNAs using the *DmRrp6* null mutant because *DmRrp6* is an essential gene in *Drosophila*. However, enhanced accumulation of polyadenylated snRNAs in the *DmTRF4-1* overexpressing and heterozygous *DmRrp6*¹⁰⁷⁰⁰¹ background suggests that the snRNAs polyadenylated by *DmTRF4-1* are digested in the *DmRrp6*-

dependent pathway. In contrast, we could not detect polyadenylation of snoRNAs and tRNAs even under conditions where the transcription of *DmTRF4-1* was stimulated. In yeast, the elongated RNAs were detectable only in the *rrp6*-null mutant. Polyadenylation was tested by transient overexpression of *DmTRF4-1* using the *Hsp-GAL4* driver. We cannot rule out the possibility that these noncoding RNAs and tRNAs are targets of polyadenylation catalyzed by *DmTRF4-1* under normal conditions. Therefore, ubiquitous overexpression of *DmTRF4-1* might cause an abnormal polyadenylation of RNAs other than snRNAs that could not be detected by the transient overexpression. Such polyadenylation might lead to the rough eye phenotype.

Trf4 and GLD-2 proteins are often considered as a single family. However, we propose these proteins should be classified into two distinct families; the nuclear/nucleolar noncanonical PAP family and the cytoplasmic noncanonical PAP family. In fact, it seems more likely that each member is conserved, respectively, in eukaryotes, except *S. cerevisiae*, in which the candidate gene of cytoplasmic noncanonical PAP, as a homologue of GLD-2, does not exist (40). Indeed, a homology search of the *Drosophila* genome sequence database shows CG5732 and CG15737 are similar to GLD-2 (data not shown). Thus, we speculate that CG5732 and/or CG15737 may play a role as a cytoplasmic noncanonical PAP. We suggest that various PAPs, including the Trf4 family, GLD-2 family, mitochondrial PAP and nuclear canonical PAP, play important roles in diverse biological processes, in different cellular locations.

Here, we focused on the function of *DmTRF4-1* in RNA surveillance. However, a previous study using two-hybrid screening showed that *DmTRF4-1* (CG11265) might interact with several components of the Hedgehog signaling (Hh) pathway, including suppressor of fused protein and kinesin-like protein Costal2 (10). The Hh signaling pathway is important for development, which regulate the transcription of specific target genes, resulting in the control of growth and differentiation (17, 38). Therefore, it is possible that *DmTRF4-1* might participate not only in an RNA quality control but also in the Hh pathway, although we have never tested whether this protein interacts with the Hh factors.

In summary, we identified the *Drosophila* homologues of ScTrf4/5, named *DmTRF4-1* and *DmTRF4-2*. Only *DmTRF4-1* possesses PAP activity, and both the *DmTRF4-1* knockdown and the overexpressing mutants were inviable, suggesting that transcription of this gene must be strictly regulated for development. *DmTRF4-1* is localized in nucleoli, and the overexpression of *DmTRF4-1* induced an extraordinary polyadenylation of snRNAs. Moreover, enhanced accumulation of the polyadenylated snRNAs was observed by suppression of *DmRrp6*. From these data, we suggest that *DmTRF4-1* plays an important role in the regulation of RNA quality in vivo.

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