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Anti-proliferative protein Tob negatively regulates CPEB3 target by recruiting Caf1 deadenylase

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Tob is a member of the anti-proliferative protein family, which functions in transcription and mRNA decay. We have previously demonstrated that Tob is involved in the general mechanism of mRNA decay by mediating mRNA deadenylation through interaction with Caf1 and a general RNA-binding protein, PABPC1. Here, we focus on the role of Tob in the regulation of specific mRNA. We show that Tob binds directly to a sequence-specific RNA-binding protein, cytoplasmic polyadenylation element-binding protein 3 (CPEB3). CPEB3 negatively regulates the expression of a target by accelerating deadenylation and decay of its mRNA, which it achieves by tethering to the mRNA. The carboxyl-terminal RNA-binding domain of CPEB3 binds to the carboxyl-terminal unstructured region of Tob. Tob then binds Caf1 deadenylase and recruits it to CPEB3 to form a ternary complex. The CPEB3-accelerated deadenylation was abrogated by a dominant-negative mutant of either Caf1 or Tob. Together, these results indicate that Tob mediates the recruitment of Caf1 to the target of CPEB3 and elicits deadenylation and decay of the mRNA. Our results provide an explanation of how Tob regulates specific biological processes.

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Introduction

The 3′ poly(A) tail has an important role in the regulation of both the stability and translational efficiency of eukaryotic mRNA (for a review, see Jacobson, 1996). The poly(A) tail interacts with the 5′ cap to synergistically activate translation by circularizing the mRNA (Doel and Carey, 1976; Gallie, 1998). Shortening of the tail, termed deadenylation, is the rate-limiting step of mRNA decay and an important event in the regulation of mRNA stability (Decker and Parker, 1993). Thus, the regulation of deadenylation constitutes a pivotal mechanism of post-transcriptional control of gene expression.

Deadenylation is mediated by two major cytoplasmic deadenylase complexes, Pan2–Pan3 and Caf1–Ccr4 (Daugeron *et al*, 2001; Tucker *et al*, 2001; Yamashita *et al*, 2005). Pan2–Pan3 has been identified in both yeast (Sachs and Deardorff, 1992) and mammals (Uchida *et al*, 2004), and consists of the catalytic subunit Pan2 and regulatory subunit Pan3 (Sachs and Deardorff, 1992; Uchida *et al*, 2004). Caf1–Ccr4 was originally implicated in transcription (Denis and Chen, 2003) and both of its subunits have the catalytic activity of a deadenylase (Chen *et al*, 2002; Tucker *et al*, 2002; Thore *et al*, 2003; Viswanathan *et al*, 2004; Bianchin *et al*, 2005). We previously found that the termination of translation triggers mRNA deadenylation (Hosoda *et al*, 2003), and proposed an initiation mechanism of mRNA decay: after translational termination, the termination complex eRF1–eRF3 is released from a poly(A)-binding protein, PABPC1, and in turn the two mRNA deadenylase complexes, Pan2–Pan3 and Caf1–Ccr4, bind PABPC1 to degrade the poly(A) tail of the mRNA (Funakoshi *et al*, 2007). We also found that the anti-proliferative protein Tob mediates recruitment of Caf1 to PABPC1 and functions in the general mechanism of mRNA decay, where it binds to Caf1 and PABPC1 by using the amino-terminal BTG domain and carboxyl-terminal PAM2 motif, respectively. Tob is a multifunctional protein involved not only in cell-cycle regulation (Suzuki *et al*, 2002) but also in embryonic development (Xiong *et al*, 2006), osteogenesis (Yoshida *et al*, 2000), T-cell activation (Tzachanis *et al*, 2001), spermatogenesis (Ellis and Kimble, 1995), and learning and memory (Jin *et al*, 2005; Wang *et al*, 2006). However, its role in mRNA deadenylation with respect to biological activities remains unclear.

In opposition to deadenylation, cytoplasmic polyadenylation is involved in some aspects of translational activation, in which cytoplasmic polyadenylation element-binding protein (CPEB) binds to a *cis*-acting element (CPE) in the 3′UTR of the target mRNAs and constitutes a binding platform for a poly(A) polymerase (Richter, 2007). In vertebrates, three additional CPEB-like sequence-specific RNA-binding proteins, CPEB2, CPEB3 and CPEB4, have a similar structure in which the carboxy-terminal region is composed of two RNA recognition motifs (RRMs) and two zinc fingers (Kurihara *et al*, 2003; Theis *et al*, 2003). In spite of the sequence similarity, however, CPEB2–4 constitute a different class of RNA-binding protein. CPEB2–4 do not bind the CPE but recognize a RNA secondary structure with a U-rich sequence (Huang *et al*, 2006). CPEB-like proteins seem to regulate translation in a polyadenylation-independent manner, as CPEB3 neither interacts with CPSF nor requires the AAUAAA hexanucleotide for translational activation (Huang *et al*, 2006).

Here we have identified factors, CPEBs, which provide specificity to the actions of Tob. Tob binds to the sequence-specific RNA-binding protein CPEB3 and mediates the inter-

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action between CPEB3 and Caf1 deadenylase. Thus, the binding of CPEB3 to the target mRNA recruits Caf1 to the target, which leads to the deadenylation and decay of the mRNA. Our results provide an explanation of how Tob regulates specific biological processes.

Results

Tob binds to the CPEBs

We have previously shown that Tob binds to the general RNA-binding protein PABPC1 and recruits Caf1 deadenylase to initiate mRNA decay (Funakoshi *et al*, 2007). However, observations that Tob is involved in a specific set of phenomena including learning/memory and bone morphogenesis suggest that it might preferentially control specific mRNAs. In an attempt to better understand the functional specificity of Tob in mRNA decay, we searched interactome databases for a factor that defines this specificity. We found *orb2* from a two-hybrid-based protein interaction database of the *Drosophila* proteome (Giot *et al*, 2003) as a candidate for such a determinant. Since the sequence-specific RNA-binding proteins CPEB2–4 are known as a human orthologue of the *Drosophila orb2*, we first examined the possible interaction between CPEB3 and Tob by co-immunoprecipitation–western blot analysis.

When lysate of COS-7 cells expressing HA–CPEB3 and Flag–Tob was immunoprecipitated with anti-Flag antibody, the precipitated fraction contained HA–CPEB3 (Figure 1A). A similar result was obtained with CPEB4 instead of CPEB3 (Figure 1B). The binding was also observed *in vivo*. When lysate of rat whole brain was immunoprecipitated with anti-CPEB3 antibody, Tob co-precipitated with CPEB3 (Figure 1C). The interaction seemed not to be mediated by RNA as the binding experiments were performed in the presence of RNase A. The ability of Tob to interact directly with CPEB3 was further confirmed using the GST-pull down assay. GST–Tob and MBP–CPEB3 or MBP–CPEB4, which had been prepared from *Escherichia coli* with >95% purity (Figure 1D), were mixed and pulled down by Glutathione Sepharose beads. Western blot analysis with anti-MBP antibody confirmed the presence of MBP–CPEB3 or MBP–CPEB4 (Figure 1E). MBP–CPEBs were not detected in the control experiment with GST.

Next, we examined if the binding is specific to Tob among the Tob/BTG family members, since BTG2 was also demonstrated to function as a general regulator of mRNA deadenylation (Mauxion *et al*, 2008). Recombinant GST-fused proteins for Tob/BTGs and MBP–CPEB3 were purified from *E. coli*, and a GST-pull down assay was performed as above. MBP–CPEB3 co-purified with Tob but not with BTG1, BTG2 or BTG3 (Figure 1F). These results indicate that CPEB3 specifically binds to Tob.

Characterization of Tob–CPEB3-binding regions

To identify the CPEB3-binding sites in Tob, deletion mutants were generated and expressed in *E. coli* as GST-fused proteins (Figure 2). The GST–Tob mutant proteins purified from *E. coli* were mixed with lysate of COS-7 cells expressing Flag–CPEB3, and the interaction between Tob and CPEB3 was determined by GST-pull down assay.

Carboxyl-terminal deletion mutants of Tob with truncations up to amino acid 218 bound to CPEB3 (Figure 2A, lanes 3 and 4), while deletions past amino acid 161 (lanes 5 and 6) abolished the binding. On the other hand, amino-terminal deletion mutants of Tob with truncations up to amino acid 160 (lanes 7 and 10) interacted with CPEB3, whereas deletions past amino acid 219 (lane 12) abolished the binding. These results indicate that the region consisting of amino acids 161–218 is required for the binding. Furthermore, regions 161–218 retained the ability to bind CPEB3 (lane 11) demonstrating that amino acids 161–218 are required and sufficient for the binding. Thus, CPEB3 binds to a region between the amino-terminal BTG domain (Caf1-binding region) and a carboxyl-terminal PAM2 motif (PAM2-C; the primary PABP-binding site) (Figure 2B).

We next examined Tob-binding sites in CPEB3. Lysate of COS-7 cells expressing Flag–CPEB3 fragments was mixed with GST–Tob (1–285) that had been purified from *E. coli*, and the interaction between CPEB3 and Tob was determined by GST-pull down assay (Figure 3). The carboxyl-terminal RNA-binding domain (RBD) (417–685), which is comprising two RRMs and two zinc fingers (Zif), but not the amino-terminal fragment (1–416) interacted with GST–Tob (1–285) (Figure 3A, lanes 6 and 8). Deletions of Zif (lane 4), RRM1 (lane 10), RRM2 (lane 14) and RRM1,2 (lane 12) of CPEB3 disabled the interaction with Tob. These results indicate that the entire region encompassing the RBD of CPEB3 is the minimal domain required for binding Tob (Figure 3B).

CPEB3 regulates the amount of its target mRNA

We and others have shown that Tob recruits the deadenylase Caf1 to the general RNA-binding protein, PABP (Ezzeddine *et al*, 2007; Funakoshi *et al*, 2007). This led us to speculate that Tob also recruits Caf1 to the sequence-specific RNA-binding proteins, CPEBs. This possibility was examined with co-immunoprecipitation experiments. As shown in Figure 4, when the lysate of COS-7 cells expressing Flag–Caf1 and HA–CPEB3 was immunoprecipitated with anti-Flag antibody, HA–CPEB3 was not co-precipitated. This suggests endogenous Tob to be a limiting factor. Consistent with this idea, when Myc–Tob was additionally expressed with Flag–Caf1 and HA–CPEB3, HA–CPEB3 was co-purified with Flag–Caf1. These results indicate that Tob mediates the interaction between CPEB3 and Caf1 to form a ternary complex.

The physical interaction between Caf1, Tob and CPEB3 suggests these factors to be involved in the regulation of CPEB3's target mRNA. A previous study found that CPEB3 binds to the 3′UTR of AMPA receptor (GluR2) mRNA and negatively regulates its expression (Huang *et al*, 2006). Although GluR2 mRNA is the only known target of CPEB3 at present, other targets must exist, as CPEB3 is ubiquitously expressed in cells and tissues (Theis *et al*, 2003). To gain more insight into the role of CPEB3 in the control of such genes, we first performed a CAT reporter assay with COS-7 cells. As shown in Figure 5A, CAT mRNA was appended with the 3′UTR of GluR2 and the level of CAT protein co-expressed with various amounts of CPEB3 was analysed. The steady-state level of CAT protein was reduced to ∼10% by CPEB3 when it was appended with GluR2 3′UTR, whereas it was not significantly affected in the control experiment with hGH 3′UTR (Figure 5B and C). Ectopically expressed CPEB3 was confirmed to form a complex with endogenous Tob and Caf1 in COS-7 cells (Supplementary Figure S3).

The effect of CPEB3 on steady-state levels of CAT mRNA was also examined. As reported previously, two types of

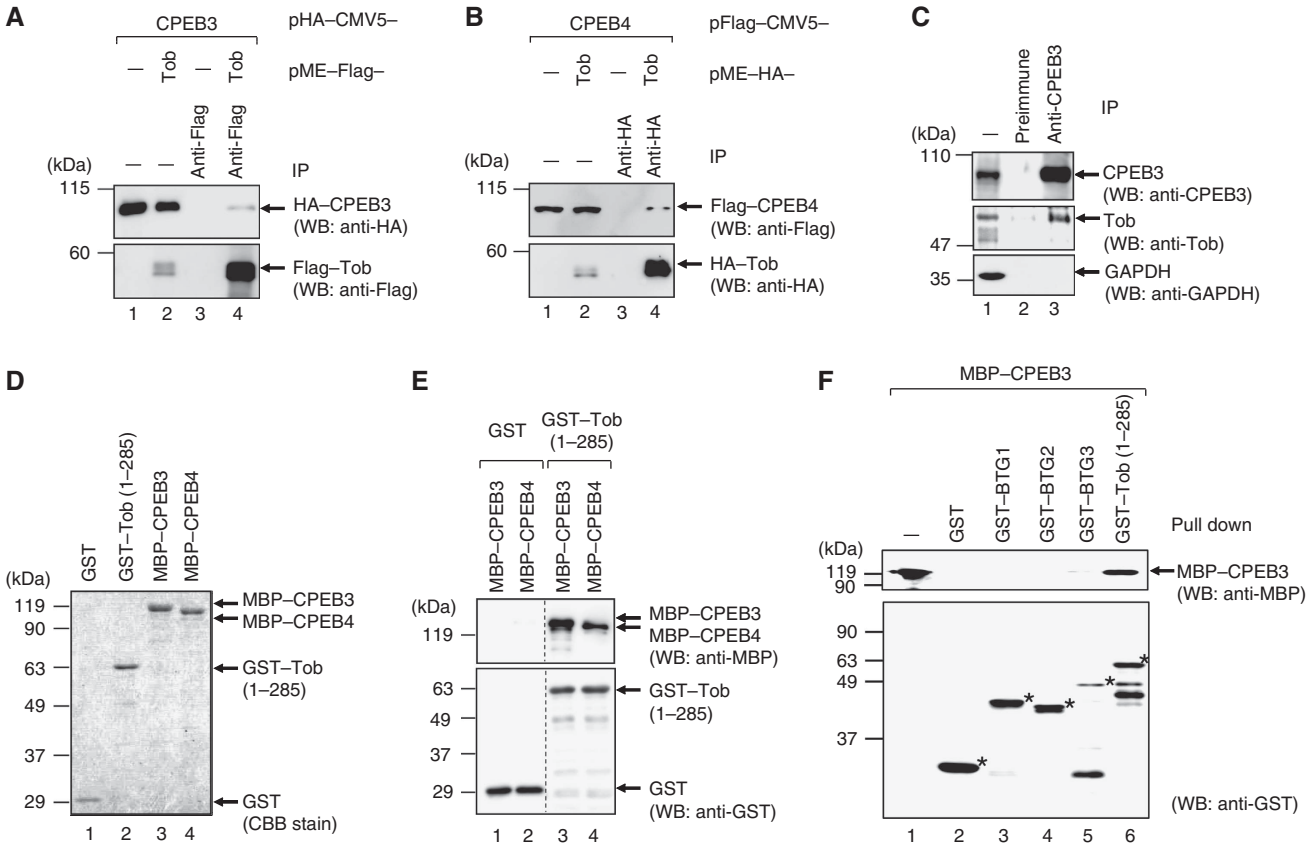

Figure 1 CPEB directly binds Tob *in vitro* and *in vivo*. (**A**) COS-7 cells were transfected with pHA–CMV5–CPEB3 and either pME–Flag (lanes 1 and 3) or pME–Flag–Tob (lanes 2 and 4). The cell extracts were subjected to an immunoprecipitation assay (IP) using anti-Flag antibody. The immunoprecipitates (lanes 3 and 4) and inputs (lanes 1 and 2, 10% of the amount immunoprecipitated) were analysed by western blotting (WB) using the indicated antibodies. (**B**) COS-7 cells were transfected with pFlag–CMV5–CPEB4 and either pME–HA (lanes 1 and 3) or pME–HA–Tob (lanes 2 and 4). The cell extracts were subjected to an immunoprecipitation assay (IP) using anti-HA antibody. The immunoprecipitates (lanes 3 and 4) and inputs (lanes 1 and 2, 10% of the amount immunoprecipitated) were analysed as in (**A**). (**C**) Rat brain extract was immunoprecipitated (IP) using anti-CPEB3 antibody or, to control for non-specific IP, preimmune serum. The immunoprecipitates (lanes 2 and 3) and input (lane 1, 10% of the amount of immunoprecipitated) were analysed by western blotting using the indicated antibodies. (**D**) Coomassie-stained gel of purified recombinant GST (lane 1), GST–Tob (1–285) (lane 2), MBP–CPEB3 (lane 3) and MBP–CPEB4 (lane 4). (**E**) GST (lanes 1 and 2) or GST–Tob (1–285) (lanes 3 and 4) was immobilized on Glutathione Sepharose resin. The resins were incubated with either MBP–CPEB3 (lanes 1 and 3) or MBP–CPEB4 (lanes 2 and 4), and bound proteins were analysed by western blotting with the indicated antibodies. The dotted lines indicate the places where cutting and pasting of the gel images have been made. The gel images are from the same gel. (**F**) GST or GST–Tob/BTG was immobilized on Glutathione Sepharose resin (lanes 2–6). The resin was incubated with MBP–CPEB3 (lane 1), and bound proteins were analysed by western blotting with the indicated antibodies. GST and GST-fused BTG1, BTG2, BTG3 and Tob are shown by asterisks.

mRNA were produced from the CAT reporter gene appended with the 3′UTR of GluR2 (Huang *et al*, 2006). CPEB3 reduced the level of both the long and short forms of mRNA to ∼40%, without affecting CAT mRNA with hGH 3′UTR (Figure 5D and E). These results suggest that CPEB3 regulates the expression of its target gene at least in part at the level of mRNA stability.

CPEB3 regulates deadenylation and decay of its target mRNA through Caf1

To determine whether CPEB3 negatively regulates the expression of its target gene by promoting deadenylation and decay of the mRNA, β-globin reporter mini gene (BGG (1–39)) mRNA was appended with GluR2 3′UTR and analysed for the stability and poly(A) tail shortening of the mRNA (Figure 6A). T-REx-HeLa cells were co-transfected with β-globin reporter mini gene (BGG (1–39)) appended with GluR2 3′UTR, a reference plasmid expressing 5xFlag–GST–CAT as a transfection/loading control and either the CPEB3 expression vector or its control, and the levels of the β-globin mRNA were examined after transcription was shut off. As in the case of the CAT reporter, two types (long and short forms) of mRNA were produced from BGG (1–39) with GluR2 3′UTR. Steady-state mRNA levels of both forms were reduced ∼80% (Figure 6B) and their half-lives were significantly decreased from 2.4 to 1.6 h (short form) and from 2.6 to 1.9 h (long form) by the presence of CPEB3 (Figure 6C and E), with most of the poly(A) tails shortened to A_0 even at time 0 (Figure 6C). The expression of the CPEB3 was confirmed by western blotting (Figure 6D). Also, ectopically expressed CPEB3 was confirmed to form a complex with endogenous Tob and Caf1 in HeLa cells (Supplementary Figure S3).

To confirm that the observed effect of CPEB3 was mediated through its binding to the 3′UTR, a tethering strategy based on the coat protein of bacteriophage MS2 was utilized. T-Rex-HeLa cells were transfected with a plasmid expressing BGG (1–39) mRNA appended with MS2-binding sites (BGG (1–39)-MS2bs) and a reference plasmid expressing 5xFlag–EGFP as a transfection/loading control, with or without a

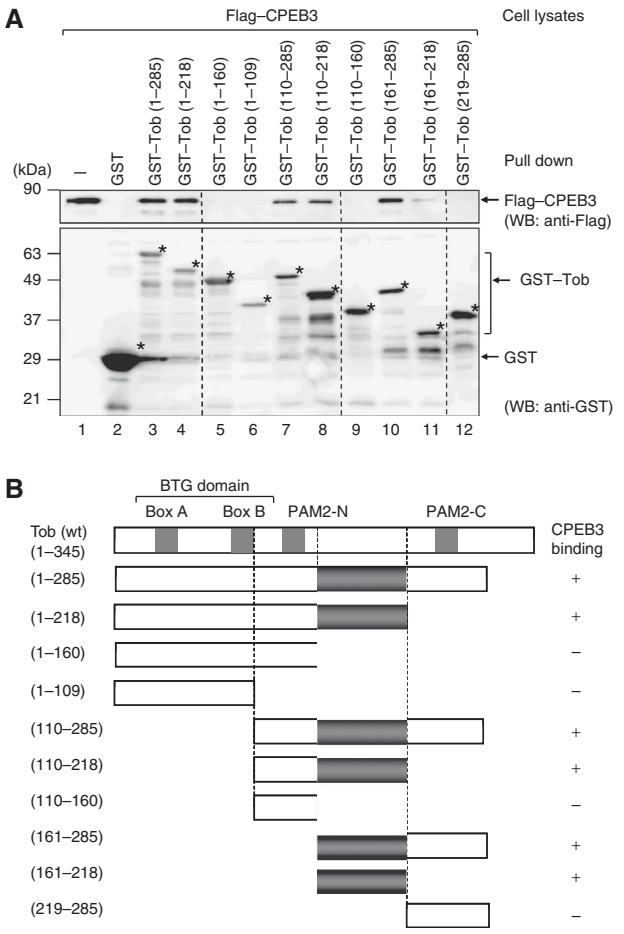

Figure 2 Identification of the CPEB3-binding site in Tob. (**A**) Purified recombinant GST-fused Tob fragments (lanes 3–12) were mixed with lysate of COS-7 cells expressing Flag–CPEB3 (lane 1) and GST-pull down assays were performed. The bound proteins were detected by western blotting using anti-Flag (upper panel) and anti-GST (lower panel) antibodies. GST and GST-fused Tob fragments are shown by asterisks. The dotted lines indicate the places where cutting and pasting of the gel images have been made. The gel images are from the same gel. (**B**) Schematic diagram of the GST–Tob fragments with a summary of the Tob–CPEB3 interaction results. The conserved BTG domain consisting of Box A and Box B, and both the primary (PAM2-C) and the cryptic (PAM2-N) PABP-binding sites are indicated as grey boxes. Black boxes represent the region containing CPEB3-binding site in Tob.

plasmid expressing MS2-fused CPEB3 (Figure 7A). The kinetics of the deadenylation and decay of the mRNA were monitored using a tetracycline regulatory transcriptional pulse-chase approach as described previously (Funakoshi *et al*, 2007). BGG (1–39)-MS2bs mRNA showed slow decay kinetics with a half-life of 4.8 h (Figure 7B, lanes 1–5). In this case, CPEB3 alone had no effect on the deadenylation or decay of the mRNA (Figure 7B, lanes 11–15). On the other hand, MS2–CPEB3 markedly increased the rate of deadenylation and reduced the half-life of the mRNA from 4.8 to 2.2 h (Figure 7B, lanes 6–10). The expression of the CPEB3 protein was confirmed by western blotting (Figure 7C). Thus, when tethered to mRNA, CPEB3 is able to recapitulate the accelerated deadenylation of its target mRNA.

Finally, we determined whether the CPEB3-accelerated deadenylation of mRNA is catalysed by Caf1. For this purpose, we applied a dominant-negative approach. T-Rex-HeLa cells were co-transfected with the BGG (1–39)-MS2bs reporter plasmid, a reference plasmid expressing 5xFlag–EGFP as a transfection/loading control and a plasmid expressing MS2–CPEB3, with or without a plasmid expressing a nuclease-deficient Caf1 mutant in which a catalytically essential aspartate residue was mutated to alanine (Caf1 D161A). The Caf1 mutant drastically reduced the rate of deadenylation and decay of the CPEB3-tethered mRNA (Figure 8A). In sharp contrast, a Pan2 mutant (Pan2 D1083A) that had no deadenylase activity (Uchida *et al*, 2004) showed no apparent inhibition. The expression of comparable amounts of Pan2 D1083A and Caf1 D161A was confirmed by western blotting (Figure 8C). Collectively, these results indicate that CPEB3 negatively regulates the gene expression of its targets by activating Caf1-catalysed deadenylation.

As a control, we also performed the same experiment in the absence of MS2–CPEB3 (Supplementary Figure S1A). The β-globin reporter mRNA showed slow and biphasic decay kinetics typical for general mRNAs, where a Pan2-dependent deadenylation occurred in the first phase (0–2 h) and a Caf1-dependent deadenylation with a concomitant decay of the mRNA body occurred in the second phase (>4 h) (Supplementary Figure S1C). Dominant-negative mutants Pan2 D1083A and Caf1 D161A reduced the rate of deadenylation at the first and second phases, respectively. Thus in the absence of CPEB3, the β-globin reporter mRNA exhibited a deadenylation and decay kinetics equivalent to the general mRNA, while tethering CPEB3 to the mRNA caused a shift to a rapid deadenylation and decay kinetics.

Tob mediates deadenylation accelerated by CPEB3

The above results strongly suggest that Tob mediates CPEB3-accelerated mRNA deadenylation by recruiting Caf1 to CPEB3's target. To test this possibility, we utilized both dominant-negative and siRNA-mediated knocking down strategies. T-Rex-HeLa cells were co-transfected with BGG (1–39)-MS2bs reporter plasmid, a reference plasmid expressing 5xFlag–EGFP as a transfection/loading control, and a plasmid expressing MS2–CPEB3, with or without a plasmid expressing the Tob-deletion mutant Tob (1–160), which contains the Caf1-binding region (BTG domain) but not CPEB3-binding region, or Tob (110–218), which contains the CPEB3-binding region but not Caf1-binding region. The deadenylation and decay kinetics were monitored as above. CPEB3-accerelated deadenylation was partially repressed by each of the deletion mutants in a dominant-negative manner (Figure 8B). The expression of the proteins was confirmed by western blotting (Figure 8C and D). As a control, we also performed the same experiment without a plasmid expressing MS2–CPEB3 (Supplementary Figure S1B). In this case the BGG (1–39)-MS2bs mRNA exhibited decay kinetics similar to the general mRNA, and the deletion mutant Tob (110–218), which contains the PABPC1-binding sites (both PAM2-N and PAM2-C) but not the Caf1-binding region (BTG domain), reduced the rate of deadenylation of β-globin reporter mRNA mainly in the second phase (Supplementary Figure S1C, right). This result is consistent with the previous report showing that Tob functions in the second phase of deadenylation of general mRNA (Funakoshi *et al*, 2007). In contrast, Tob (1–160), which has the Caf1-binding region and PAM2-N, did not

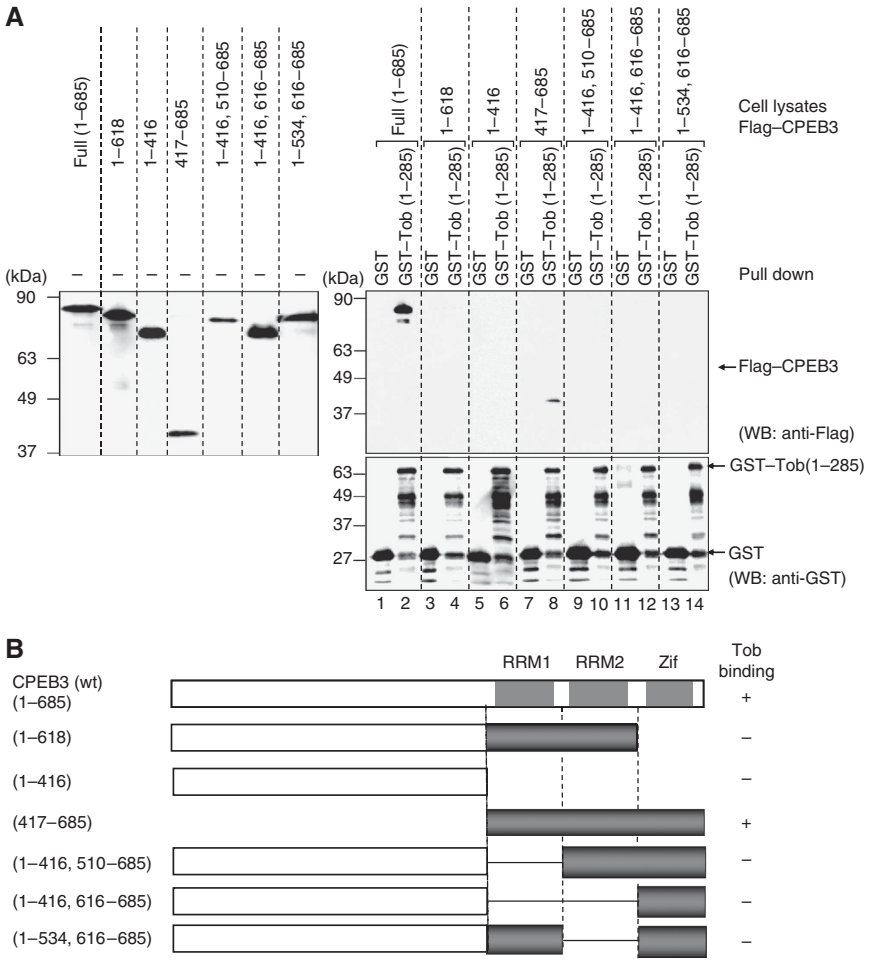

Figure 3 Identification of the Tob-binding site in CPEB3. (**A**) Purified recombinant GST (right panel, lanes 1, 3, 5, 7, 9, 11 and 13) or GST-fused Tob (1–285) (right panel, lanes 2, 4, 6, 8, 10, 12 and 14) was mixed with lysate of COS-7 cells expressing Flag–CPEB3 fragments (left panel), and GST-pull down assays were performed as in Figure 2A (right panel). (**B**) Schematic diagram of the Flag–CPEB3 fragments with a summary of the Tob–CPEB3 interaction results. Black boxes represent the region containing Tob-binding site in CPEB3.

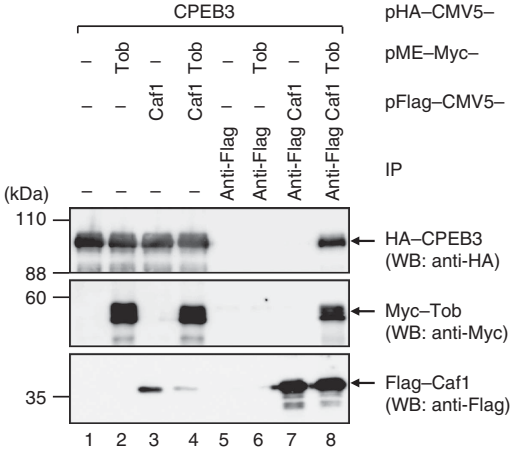

Figure 4 Tob mediates interaction between CPEB3 and Caf1. COS-7 cells were transfected with expression plasmids encoding HA–CPEB3, Myc–Tob, and Flag–Caf1 in the indicated combinations. The cell extracts were subjected to an immunoprecipitation assay using anti-Flag antibody. The immunoprecipitates (lanes 5–8) and inputs (lanes 1–4, 10% of the amount immunoprecipitated) were analysed by western blotting using the indicated antibodies.

affect the rate of deadenylation. As the PAM2-N binds weakly but significantly to PABPC1 (Ruan *et al*, 2010), Tob (1–160) can bind both Caf1 and PABPC1, and is expected to mediate the binding between Caf1 and PABPC1 (and not to act as a dominant-negative mutant for the deadenylation of general mRNA).

For knocking down experiments, T-REx-HeLa cells were co-transfected with the BGG (1–39)-MS2bs reporter plasmid, a reference plasmid expressing 5xFlag–EGFP as a transfection/loading control, a plasmid expressing MS2–CPEB3 and either Tob/Tob2 siRNA or its control siRNA, and a transcriptional pulse-chase analysis was performed. In this study, we have knocked down Tob2 as well as Tob, as the highly homologous paralogue of Tob is also expressed in T-REx-HeLa cells and is able to bind CPEB3 (Supplementary Figures S2C and E). Tob/Tob2 siRNAs reduced the level of both Tob and Tob2 mRNAs to ~20% (Supplementary Figure S2C). Comparable reduction was observed in the amount of Tob protein (Supplementary Figure S2D). In a control experiment without MS2–CPEB3, knocking down Tob/Tob2 reduced the rate of deadenylation of the reporter mRNA mainly at the second phase (Supplementary Figure S2A, compare lanes 3–7 with 13–17, Supplementary Figure S2F). In the presence of MS2–CPEB3, CPEB3-accelerated deadenylation of the reporter mRNA was significantly suppressed by Tob/Tob2 siRNA compared with a control experiment with luciferase siRNA (Supplementary Figure S2A, compare lanes 8–12 with lanes 18–22) to a level nearly comparable

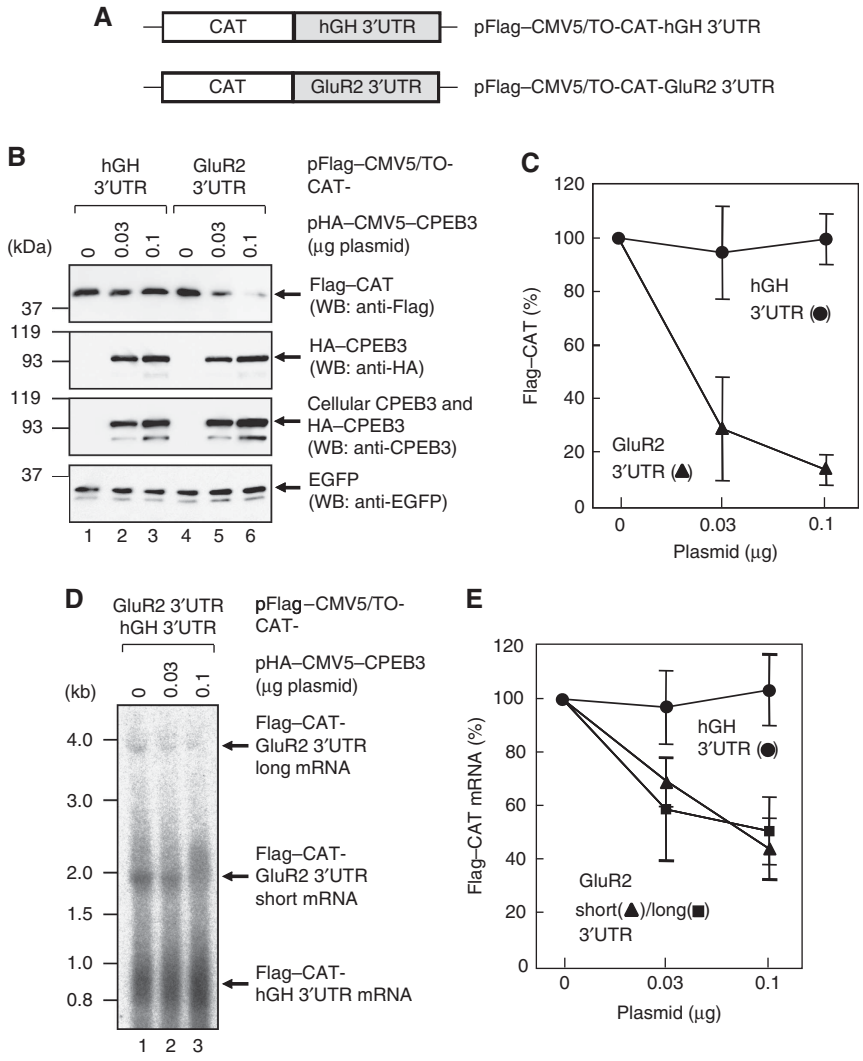

Figure 5 CPEB3 reduces abundance of CAT mRNA with GluR2 3′UTR. (**A**) Scheme of the reporter constructs: pFlag–CMV5/TO-CAT-GluR2 3′UTR and its control pFlag–CMV5/TO-CAT. (**B**) COS-7 cells were transiently transfected with the indicated amounts of pHA–CMV5–CPEB3, pEGFP–C1 reference plasmid expressing EGFP and either pFlag–CMV5/TO-CAT (lanes 1–3) or pFlag–CMV5/TO-CAT-GluR2 3′UTR (lanes 4–6). The cell extracts were subjected to western blot analyses with the indicated antibodies. EGFP served as a transfection/loading control. (**C**) The amount of Flag–CAT protein was measured and plotted against the amount of pHA–CMV5–CPEB3, where the amount of Flag–CAT protein without pHA–CMV5–CPEB3 was defined as 100%. Error bars represent the s.d. of three independent experiments. (**D**) COS-7 cells were transfected with the indicated amounts of pHA–CMV5–CPEB3, pEGFP–C1, pFlag–CMV5/TO-CAT and pFlag–CMV5/TO-CAT-GluR2 3′UTR. Total RNA was prepared from the cells and Flag–CAT mRNA was detected by Northern blotting. (**E**) The amount of Flag–CAT mRNA was measured and plotted as in (**C**).

to that observed without MS2–CPEB3 (lanes 13–17). These results are consistent with the results obtained with dominant-negative mutants of Tob (Supplementary Figure S1B). Also, mRNA half-lives analysed by quantifying the levels of mRNA in Supplementary Figure S2A showed that knocking down Tob/Tob2 repressed CPEB3-accelerated decay rate (Supplementary Figure S2B, compare closed triangle with open triangle) to a level nearly comparable to that observed without CPEB3 (open circle). From these findings, we conclude that Tob/Tob2 mediate CPEB3-accelerated deadenylation and decay.

Finally, we examined if Tob/Tob2 as well as CPEB3/4 regulate endogenous GluR2 mRNA in neuroblastoma SK-N-SH cells by performing RNAi knockdown experiments. We used SK-N-SH cells, since GluR2, CPEB3/4, Tob/Tob2 and Caf1 were all expressed in this cell line (Figure 9C and D), and endogenous CPEB3–Tob–Caf1 ternary complex was detected when lysate of SK-N-SH cells was immunoprecipitated with anti-CPEB3 antibody (Figure 9E). SK-N-SH cells were transfected with either Tob/Tob2 siRNAs or a control luciferase siRNA, and GluR2 mRNA was analysed by real-time quantitative RT–PCR. As is often the case with neural cells, the transfection efficiency is low (~60%) in SK-N-SH cells, and both Tob and Tob2 mRNAs were reduced by Tob/Tob2 siRNAs respectively to 45 and 46% compared with the control siRNA in three independent experiments (Figure 9B). In this condition, the half-life of the GluR2 mRNA but not GAPDH mRNA (control) was significantly increased by two-fold (Figure 9A). Similar results were obtained with CPEB3/4 siRNAs (Figure 9A and B). From these results, we conclude that Tob/Tob2 as well as CPEB3/4 regulate GluR2 mRNA expression in neuroblastoma cells.

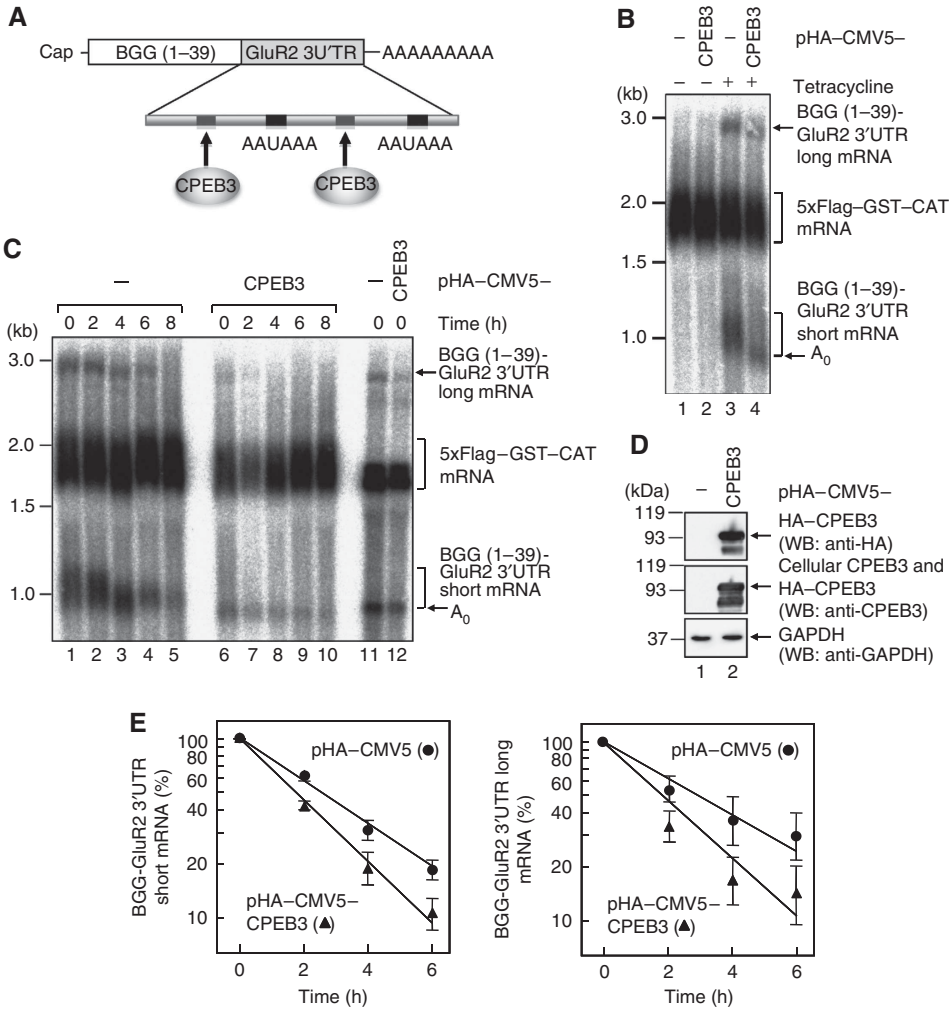

Figure 6 CPEB3 controls mRNA deadenylation. (**A**) Schematic representation of the BGG (1–39)-GluR2 3′UTR mRNA. CPEB3 binds to multiple regions throughout the length of the GluR2 3′UTR but the precise sites are not known. (**B**) Steady-state levels of BGG (1–39)-GluR2 3′UTR mRNA were analysed by Northern blotting (upper panel). T-REx-HeLa cells were transiently transfected with the BGG (1–39)-GluR2 3′UTR reporter plasmid, pCMV–5xFlag–GST–CAT reference plasmid and either pHA–CMV5 (lanes 1 and 3) or pHA–CMV5–CPEB3 (lanes 2 and 4) and treated with 0 (lanes 1 and 2) or 50 ng/ml (lanes 3 and 4) tetracycline for 12 h before harvesting. 5xFlag–GST–CAT mRNA served as a transfection/loading control. Results are representative of three independently performed experiments. (**C**) T-REx-HeLa cells were co-transfected with the BGG (1–39)-GluR2 3′UTR reporter plasmid, pCMV–5xFlag–GST–CAT reference plasmid and either pHA–CMV5 (lanes 1–5 and 11) or pHA–CMV5–CPEB3 (lanes 6–10 and 12). At 8 h post-transfection, BGG (1–39)-GluR2 3′UTR mRNA was induced to express by treatment with tetracycline for 12 h, and cells were harvested at the specified time after the transcription was shut off. BGG (1–39)-GluR2 3′UTR short mRNA half-lives of 2.4 and 1.6 h were calculated from lanes 1–5 and 6–10, respectively. BGG (1–39)-GluR2 3′UTR long mRNA half-lives of 2.7 and 1.8 h were calculated from lanes 1–5 and 6–10, respectively. To mark the deadenylated (A_0) RNA, BGG (1–39)-GluR2 3′UTR mRNA that was induced by treatment with tetracycline for 12 h was digested with RNase H in the presence of oligo(dT) (lanes 11 and 12). 5xFlag–GST–CAT mRNA served as a transfection/loading control. Results are representative of three independently performed experiments. (**D**) Total cell lysate was analysed by western blotting using anti-HA, anti-CPEB3 or anti-GAPDH. (**E**) The levels of the long (right panel) and short (left panel) forms of the BGG (1–39)-GluR2 3′UTR mRNA were quantified with the level of the mRNA from 0 h time point defined as 100%. Results are the average of three independently performed experiments.

Discussion

We and others have previously identified Tob as a general regulator of mRNA. Tob binds to the general RNA-binding protein PABPC1 and mediates recruitment of Caf1 to the PABPC1-bound poly(A) tail of mRNA to promote deadenylation (Ezzeddine *et al*, 2007; Funakoshi *et al*, 2007), which is triggered by the translation termination-coupled dissociation of the termination factor eRF3 from PABPC1 (Funakoshi *et al*, 2007). This finding answers how the poly(A) tail of mRNA is generally shortened during translation to initiate mRNA decay. However, an important question related to the functional specificity of Tob in various biological processes remained unanswered. In this study, we have identified factors, CPEBs, which provide specificity to the actions of Tob. Tob directly binds to the sequence-specific RNA-binding proteins and recruits Caf1 deadenylase to the target mRNA to negatively regulates its gene expression (Figure 10). Thus, Tob has a dual role in mRNA deadenylation, acting as both a general regulator via PABPC1 and a transcript-specific regulator via CPEB3. In this study, β-globin reporter mRNA with MS2-binding sites in its 3′UTR showed slow and biphasic deadenylation where Pan2–Pan3 and Tob–Caf1–Ccr4 function via PABPC1, respectively, in the first and second phases of the deadenylation (Supplementary Figures S1A and C) as

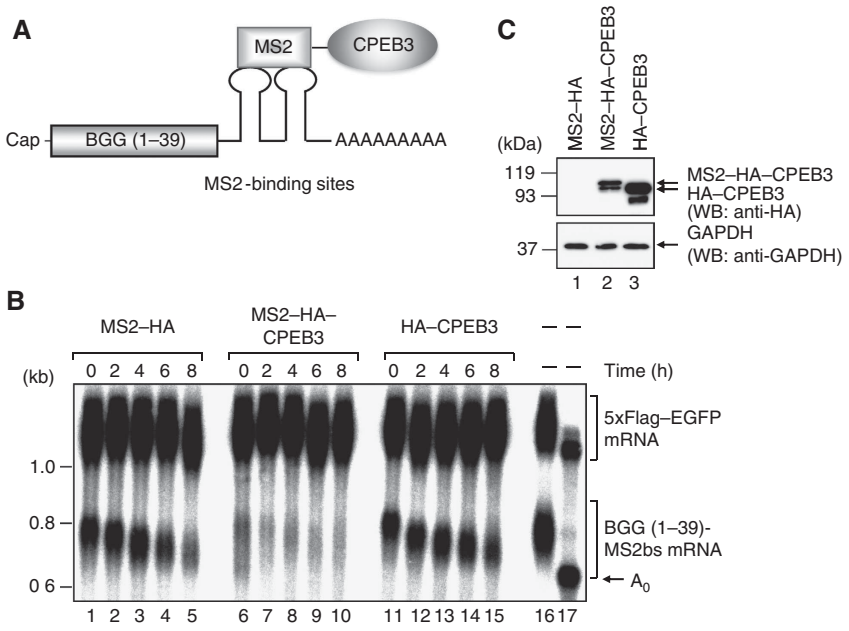

Figure 7 Tethering CPEB3 to the mRNA accelerates deadenylation. (**A**) Schematic representation of the Flag–CMV5/TO-BGG (1–39)-MS2bs mRNA, where a cassette comprising eight MS2-binding sites was inserted about 30 nucleotides downstream of the termination codon. (**B**) T-REx-HeLa cells were co-transfected with the pFlag–CMV5/TO-BGG (1–39)-MS2bs reporter plasmid, pCMV–5xFlag–EGFP reference plasmid and either pMS2–HA (lanes 1–5, 16 and 17), pMS2–HA–CPEB3 (lanes 6–10) or pHA–CMV5–CPEB3 (lanes 11–15). One day later, BGG (1–39)-MS2bs mRNA was induced to express by treatment with tetracycline for 2.5 h, and cells were harvested at the specified time after the transcription was shut off (lanes 1–15). mRNA half-lives of 5.4, 1.8, and 5.9 h were calculated from lanes 1–5, 6–10 and 11–15, respectively. BGG (1–39)-MS2bs mRNA was induced to express by treatment with tetracycline for 12 h to analyse steady-state BGG (1–39)-MS2bs mRNA (lane 16). To mark the deadenylated (A_0) RNA, BGG (1–39)-MS2bs mRNA that was induced to express by treatment with tetracycline for 12 h was digested with RNase H in the presence of oligo(dT) (lane 17). 5xFlag–EGFP mRNA served as a loading control (lower panel). Results are representative of three independently performed experiments. (**C**) Total cell lysate was analysed by western blotting using anti-HA or anti-GAPDH.

previously reported for general mRNAs (Yamashita *et al*, 2005; Funakoshi *et al*, 2007). In the presence of MS2–CPEB3, however, the kinetics of the same reporter mRNA switched to a rapid deadenylation where Tob–Caf1 functions via CPEB3 (Figures 7B, 8A and B). These results suggest that the transcript-specific regulation by Tob via interaction with CPEB3 is dominant over the general regulation via PABPC1. In this study, we have identified Caf1 as the deadenylase responsible for CPEB3-mediated deadenylation. Dominant-negative mutant of Caf1 (Caf1 D161A) but not that of Pan2 (Pan2 D1083A) repressed CPEB3-accelerated deadenylation almost completely to a level comparable to that observed in the absence of CPEB3 (compare lanes 13–17 in Figure 8A with lanes 6–10 in Supplementary Figure S1A). However, we cannot totally rule out the possibility that other deadenylases might also be involved in the regulation of CPEB3 target mRNAs. In contrast, CPEB is known to bind directly to PARN deadenylase and regulates deadenylation of its target mRNA that contains CPEs in processes, including germ cell development (Kim and Richter, 2006). In spite of the sequence similarity between CPEB and CPEB3, both proteins differ in their binding specificities for RNA and also in their requirement of CPSF and AAUAAA cis element for translation activation. Therefore, it is interesting to assume that CPEB and CPEB3 differentially utilize the two deadenylases for the regulation of the target mRNAs.

The physical interaction between Tob and CPEBs observed in this study might support their functional relationship defined by elegant genetics in *Caenorhabditis elegans*. Fertilization of the germline (FOG)-1 and FOG-3, which are the orthologues of CPEBs and Tob, respectively, in *C. elegans*, have been identified as key factors that control the decision of germ cells to become sperm or oocytes. Mutations in either *fog-1* or *fog-3* cause germ cells to become oocytes rather than sperm (Barton and Kimble, 1990; Ellis and Kimble, 1995). Thus, both FOG-1 and FOG-3 are thought to be directly responsible for causing spermagenesis at the end of the sex determination pathway. Taking our observations into account, FOG-1 and FOG-3 might also bind to form a complex and control the expression of specific mRNAs pivotal for the germline development.

Tob is a multifunctional protein involved not only in spermatogenesis but also in cell-cycle regulation (Suzuki *et al*, 2002), embryonic development (Xiong *et al*, 2006), osteogenesis (Yoshida *et al*, 2000) and T-cell activation (Tzachanis *et al*, 2001). Recent findings demonstrate that Tob also functions in learning and memory. First, Tob is expressed in rat hippocampal neurons. When Tob is knocked down in the hippocampal CA1 region, long-term potentiation as well as spatial learning and memory were impaired (Jin *et al*, 2005). Second, Tob is also expressed in the cerebellum and is implicated in the acquisition of motor skills (Wang *et al*, 2006). CPEB3 is also expressed in the hippocampus and cerebellum in the mouse brain (Theis *et al*, 2003), and controls the expression of GluR2 (Huang *et al*, 2006). Recently, a highly conserved short intronic sequence within human CPEB3 was identified as a ribozyme whose self-cleavage activity regulates the expression of CPEB3. An

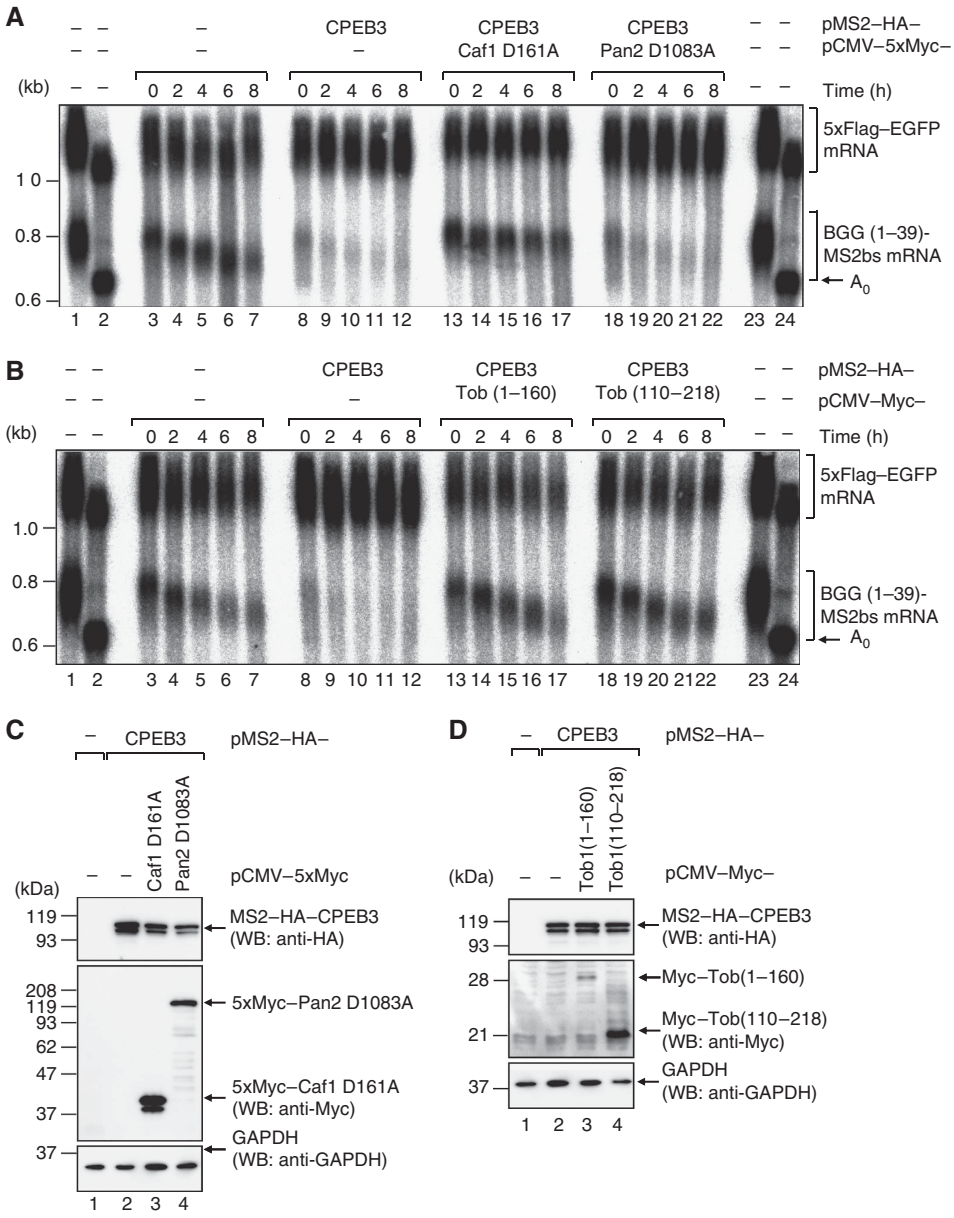

Figure 8 CPEB3-accelerated deadenylation is mediated by Caf1 and Tob. (**A**) T-REx-HeLa cells were co-transfected with the pFlag–CMV5/TO-BGG (1–39)-MS2bs reporter plasmid, pCMV–5xFlag–EGFP reference plasmid, pMS2–HA–CPEB3 (lanes 8–22) and either pCMV–5xMyc–Caf1 D161A (lanes 13–17) or pCMV–5xMyc–Pan2 D1083A (lanes 18–22). As a control, cells were transfected with pMS2–HA and pCMV–5xMyc (lanes 3–7). The transcriptional pulse-chase analysis was performed as described in Figure 7B. mRNA half-lives of 4.9, 1.9, 5.4 and 2.0 h were calculated from lanes 3–7, 8–12, 13–17 and 18–22, respectively. Steady-state BGG (1–39)-MS2bs mRNA (lanes 1 and 23) and the deadenylated (A_0) mRNA (lanes 2 and 24) were analysed as in Figure 7B. 5xFlag–EGFP mRNA served as a transfection/loading control. Results are representative of two independently performed experiments. (**B**) T-REx-HeLa cells were co-transfected with the pFlag–CMV5/TO-BGG (1–39)-MS2bs reporter plasmid, pCMV–5xFlag–EGFP reference plasmid, pMS2–HA–CPEB3 (lanes 8–22), and either pCMV–Myc–Tob (1–160) (lanes 13–17) or pCMV–Myc–Tob (110–218) (lanes 18–22). As a control, cells were transfected with pMS2–HA and pCMV–Myc (lanes 3–7). The transcriptional pulse-chase analysis was performed as described in Figure 7B. mRNA half-lives of 4.3, 2.2, 5.1 and 4.0 h were calculated from lanes 3–7, 8–12, 13–17 and 18–22, respectively. Steady-state BGG (1–39)-MS2bs mRNA (lanes 1 and 23) and the deadenylated (A_0) mRNA (lanes 2 and 24) were analysed as in Figure 7B. 5xFlag–EGFP mRNA served as a transfection/loading control. Results are representative of two independently performed experiments. (**C**, **D**) Total cell lysate was analysed by western blotting using anti-HA, anti-Myc or anti-GAPDH antibody.

analysis of a single-nucleotide polymorphism in the ribozyme sequence revealed that CPEB3 is associated with episodic memory (Vogler *et al*, 2009). Moreover, the Drosophila orthologue of CPEB3, orb2, was demonstrated using genetics to function in long-term courtship memory (Keleman *et al*, 2007). From these results, it is tempting to speculate that Tob functions with CPEB3 in learning and memory at least in part by regulating the expression of the AMPA receptor GluR2. GluR2 mRNA is the only known target of CPEB3 at present, however, it is reasonable to assume that CPEB3 and Tob regulate other mRNAs as both proteins are ubiquitously expressed in cells and tissues. In addition, we also found CPEB4 as the binding partner of Tob. CPEB3 and CPEB4 are >95% identical in their RBD, and Tob binds to the highly

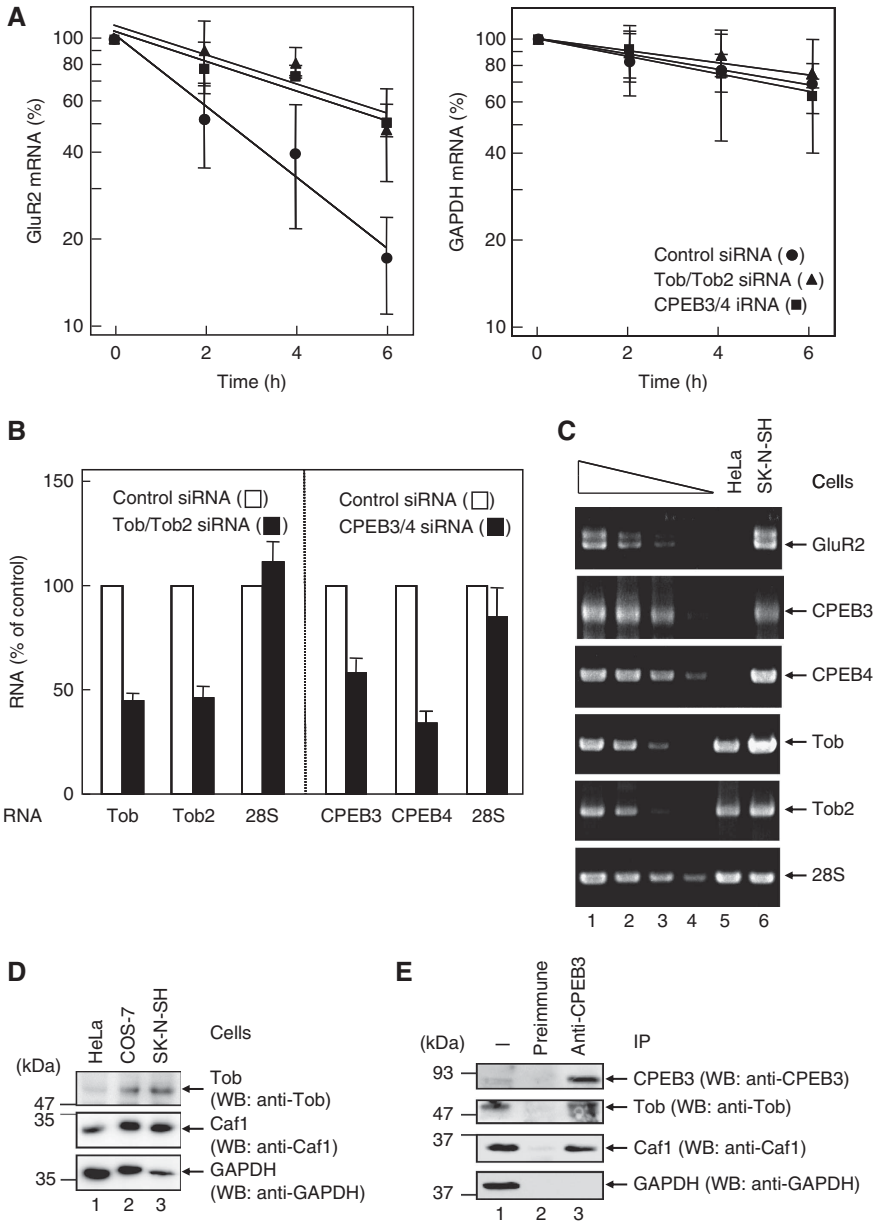

Figure 9 Downregulating Tob or CPEB stabilizes endogenous GluR2 mRNA in SK-N-SH neuroblastoma cells. (**A**) SK-N-SH neuroblastoma cells were transfected with Tob/Tob2 siRNA (closed triangle), CPEB3/CPEB4 siRNA (closed rectangle) or a control luciferase siRNA (closed circle). Cells were harvested at the specified time after the transcription was shut off using actinomycin D. GluR2 mRNA (left panel) and GAPDH mRNA (right panel) were analysed by real-time PCR. The levels of the mRNAs were quantitated, where the level of the mRNAs from 0 h time point was defined as 100%. Error bars represent the s.d. of three independent experiments. (**B**) Downregulation of Tob/Tob2 and CPEB3/CPEB4 in SK-N-SH neuroblastoma cells. Tob mRNA, Tob2 mRNA, CPEB3 mRNA, CPEB4 mRNA and 28S rRNA were analysed by real-time PCR. (**C**) Total RNA that is isolated from HeLa (lane 5) or SK-N-SH (lane 6) cells was analysed by semi-quantitative RT–PCR. GluR2 mRNA, CPEB3 mRNA, CPEB4 mRNA, Tob mRNA, Tob2 mRNA, or 28S rRNA was amplified. The leftmost four lanes, which analysed two-fold dilutions of RNA, show that the conditions used for RT–PCR is semi-quantitative. (**D**) Total cell lysate that is isolated from HeLa (lane 1), COS-7 (lane 2) or SK-N-SH (lane 3) cells was analysed by western blotting using anti-Tob, anti-Caf1 or anti-GAPDH. (**E**) Total cell extract that is isolated from SK-N-SH cells was immunoprecipitated (IP) in the presence of RNase I using anti-CPEB3 antibody or to control for non-specific IP, preimmune serum. The immunoprecipitates (lanes 2 and 3) and inputs (lane 1, 10% of the amount of immunoprecipitated) were analysed by western blotting using the indicated antibodies.

conserved RBD of CPEB4 (data not shown) as in the case of CPEB3 (Figure 3). Although the functional difference between CPEB3 and CPEB4 is not known, Tob could also regulate the target of CPEB4. Thus by identifying targets of CPEB3 and CPEB4 other than GluR2 mRNA, it should be possible to elucidate the regulatory mechanisms underlying other biological processes regulated by Tob.

Materials and methods

Plasmids

To construct pHA–CMV5–CPEB3, pFlag–CMV5–CPEB3 and pCMV–Myc–CPEB3, the full-length ORF was PCR-amplified using the KIAA0940 clone as a template. The resulting fragments were digested with *Eco*RI and *Sal*I, and inserted into pHA–CMV5, pFlag–CMV5 and pCMV–Myc (Clontech), respectively. To generate the

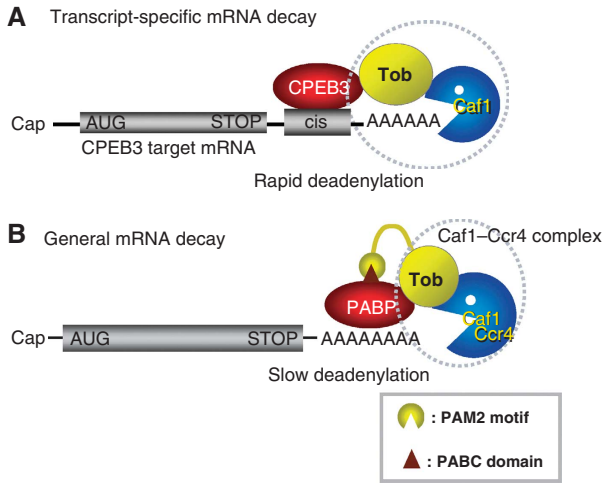

Figure 10 Schematic representation of Tob-mediated mRNA decay. (**A**) Transcript-specific mRNA decay. Tob mediates the recruitment of Caf1 deadenylase to the mRNA bound by the sequence-specific RNA-binding protein CPEB3 to negatively regulates its gene expression. (**B**) General mRNA decay. Tob generally mediates the recruitment of the Caf1–Ccr4 complex to the poly(A) tail of mRNA bound by the general RNA-binding protein, PABPC1. The termination of translation triggers the recruitment of Tob–Caf1–Ccr4 to the mRNA (Funakoshi *et al*, 2007).

pFlag–CMV5–CPEB3 fragments 1–618, 1–416 and 417–685, the corresponding region of CPEB3 was PCR-amplified using pFlag–CMV5–CPEB3 as a template, and the resulting fragment was digested with *Eco*RI and *Sal*I, and inserted into pFlag–CMV5. For the construction of the pFlag–CMV5–CPEB3 fragments 1–416/510–685, 1–416/616–685 and 1–534/616–685, the corresponding region of CPEB3 was amplified by inverse PCR using the primer pairs 5′ AAA GTC GAC TTA ACA GCG AGT GGG AGA ACT 3′ (sense) and 5′ CCA GTG CAA ATT CGA CCA TGG 3′ (antisense), 5′ AAA GTC GAC TTA ACA GCG AGT GGG AGA ACT 3′ (sense) and 5′ GTG CTG GAT GAT CAG ATG TG 3′ (antisense), and 5′ TCT GGG GTC CAA AGG CTG AG 3′ (sense) and 5′ GTG CTG GAT GAT CAG ATG TG 3′ (antisense), respectively, and pFlag–CMV5–CPEB3 as a template. To construct pFlag–CMV5–CPEB4, the full-length ORF was PCR-amplified using the KIAA1673 clone as a template. The resulting cDNA fragment was digested with *Eco*RI and *Sal*I and inserted into pFlag–CMV5. To construct pCMV–Myc–Tob (1–160) and pCMV–Myc–Tob (110–218), the corresponding region of Tob was PCR-amplified, digested with *Eco*RI and inserted into pCMV–Myc. To construct pGEX6P1-BTG1, BTG2 and BTG3, the corresponding full-length cDNAs were amplified by RT–PCR using HeLa total RNA as a template. The resulting fragments were digested with *Eco*RI and *Sal*I and inserted into pGEX6P1 (GE Healthcare). For the construction of the pGEX6P1-Tob fragments 1–285, 1–218, 1–160, 1–109, 110–285, 110–218, 110–160, 161–285, 161–218 and 219–285, the corresponding regions of Tob were PCR-amplified using pME–Flag–Tob as a template. The resulting fragments were digested with *Eco*RI and ligated into pGEX-6P1. To generate pME–Myc–Tob, PCR-amplified Tob was inserted into the *Eco*RI site of pME–Myc. To construct pCMV–5xMyc–Caf1 D161A and pCMV–5xMyc–Pan2 D1083A, Caf1 D161A and Pan2 D1083A cDNA was PCR-amplified with pHA–CMV5–Caf1 D161A and pFlag–CMV5–Pan2 D1083A (Funakoshi *et al*, 2007), respectively, as a template and the resulting cDNAs were inserted into the *Hin*dIII and *Xho*I sites of pCMV–5xMyc. To construct pFlag–CMV5/TO-CAT, CAT cDNA was excised by digestion of pCAT3 control (Promega) with *Hin*dIII and *Xba*I, and the resulting fragment was inserted into pFlag–CMV5/TO. To generate pFlag–CMV5/TO-CAT-GluR2 3′UTR and pFlag–CMV5/TO-BGG (1–39)-GluR2 3′UTR, GluR2 3′UTR was PCR-amplified using HeLa genomic DNA as a template and the primers 5′ GGT CTA GAT GAC CTT GAA TGA TGC C 3′ (sense) and 5′ GGT CTA GAT GGA ATG GTT GGT GAT G 3′ (antisense). The resulting DNA was inserted into the *Xba*I and *Bam*HI sites of pFlag–CMV5/TO-CAT and the *Eco*RI and *Xba*I sites of pFlag–CMV5/TO-BGG (1–39), respectively, by blunt-end ligation.

For the construction of pFlag–CMV5/TO-BGG (1–39)-MS2bs, inverse PCR was performed using pFlag–CMV5/TO-BGG (Funakoshi *et al*, 2007) and the primer pair 5′ TCC TCT AGA TAA GCT CGC TTT CTT GCT G 3′ (sense) and 5′ TCC GAA TTC CTA GGT CCA AGG GTA GAC C 3′ (antisense) to generate pFlag–CMV5/TO-BGG (1–39). A fragment with MS2-binding sites was PCR-amplified using pcFLuc-MS2bs (Hosoda *et al*, 2005) as a template, and the primers 5′ TCC TCG AGT CTA GAT GAC CCT TTA GTG AGG GTT AAT GC 3′ (sense) and 5′ TAT CCC GGG CGT AGT CTG GGA CGT CGT ATG GGT AG 3′ (antisense), and ligated into the *Eco*RI and *Xba*I sites of pFlag–CMV5/TO-BGG (1–39). To construct pMS2–HA, inverse PCR was performed using pcFLuc-MS2bs (Hosoda *et al*, 2005) and 5′ TCC TCG AGT CTA GAT GAC CCT TTA GTG AGG GTT AAT GC 3′ (sense) and 5′ TAT CCC GGG CGT AGT CTG GGA CGT CGT ATG GGT AG 3′ (antisense). To construct pMS2–HA–CPEB3, pFlag–CMV5–CPEB3 was digested with *Eco*RI and *Sal*I, and the CPEB3 cDNA fragment was inserted into the *Sma*I site of pMS2–HA by blunt-end ligation. To construct pCMV–5xFlag–GST–CAT, 5xFlag tagged-GST fragment was ligated into the *Sac*I and *Hin*dIII sites of pFlag–CMV5/TO-CAT. To construct pCMV–5xFlag–EGFP, EGFP fragment that was PCR-amplified using pEGFP–C1 (Clontech) was ligated into the *Hin*dIII site of pCMV–5xFlag.

siRNA

CPEB3, CPEB4, Tob, Tob2 and luciferase siRNA consisted of, respectively, 5′ r (CCA UGC UCU UCC AGA ACU U)d(TT) 3′, 5′ r (GCU GGA AUA CUG CCU GAA A)d(TT) 3′, 5′ r (CGU GGA UGA UAA UAA UGA A)d(TT) 3′, 5′ r (GUG CUG UAC CUG GAU GAC A)d(TT) 3′ and 5′ r (CGU ACG CGG AAU ACU UCG A) d(TT) 3′.

Cell culture and DNA/RNA transfection

COS-7 cells, T-REx-HeLa cells (Invitrogen) and SK-N-SH neuroblastoma cells (Riken Cell Bank) were cultured in Dulbecco's modified Eagle's medium (Nissui) supplemented with 5% fetal bovine serum. DNA/RNA transfection was performed using LipofectAMINE2000 (Invitrogen) as described previously (Funakoshi *et al*, 2007).

Immunoprecipitation

The transfected cells were lysed in buffer A (20 mM Tris–HCl (pH 7.5), 50 mM NaCl, 2.5 mM EDTA, 0.5% Nonidet P-40, 1 mM DTT, 0.1 mM PMSF, 2 μg/ml aprotinin and 2 μg/ml leupeptin and either 1 μg/ml RNase A (Sigma) or 50 units/ml RNase I (New England Biolabs)) at 4°C for 30 min. For immunoprecipitation assay using either anti-Flag or anti-HA antibody, the mixture was centrifuged at 15 000 *g* for 20 min, and the supernatant was subsequently incubated with anti-Flag agarose (Sigma) or anti-HA Affinity Matrix (Roche) at 4°C for 1 h. For the assay using anti-CPEB3 antibody, the supernatant was incubated with either anti-CPEB3 (raised against His-tagged CPEB3 (1–441) protein) or preimmune serum, and Protein G Sepharose 4 Fast Flow (GE Healthcare). The resin was then washed three times with buffer A. Bound protein was eluted with SDS–PAGE sample buffer and analysed by western blotting using anti-Flag (Sigma), anti-HA (Roche), anti-Myc (Roche) antibody, anti-CPEB3, anti-Tob (raised against His-tagged Tob (1–110) protein), anti-Caf1 (Ezzeddine *et al*, 2007) or anti-GAPDH (Millipore). For the analysis using rat brain extract, rat brain was homogenized in nine volumes of buffer A without 0.5% Nonidet P-40 using a hand-driven Potter homogenizer and lysed in buffer A at 4°C for 30 min. Immunoprecipitation experiment using anti-CPEB3 antibody was performed as described above.

GST-pull down assay

GST-fused Tob, BTG1, BTG2 and BTG3 were produced in *E. coli* BL21 by adding 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). The cells were harvested and lysed in buffer B (50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM DTT, 0.1 mM PMSF, 2 μg/ml leupeptin and 2 μg/ml aprotinin) and the cell lysate was incubated with 1 mg/ml lysozyme, sonicated at 4°C for 3 min, and centrifuged at 100 000 *g* for 1 h. The supernatant was then incubated with Glutathione Sepharose 4B (GE Healthcare). The resulting resin was mixed with COS-7 cell extract and incubated at 4°C for 2 h with the lysate of COS-7 cells expressing Flag–CPEB3

or purified MBP–CPEB3/CPEB4, and washed three times with buffer B. Bound proteins were eluted with SDS–PAGE sample buffer and analysed by western blotting using anti-MBP (New England Biolabs), anti-GST (Santa Cruz) or anti-Flag antibody.

Purification of recombinant proteins from E. coli

GST-fused Tob and MBP-fused CPEB3 were produced in *E. coli* BL21 by adding 0.1 mM IPTG. The cells were harvested and lysed in buffer B and the recombinant proteins were purified from the lysate. GST–Tob was purified as described previously (Uchida *et al*, 2002). MBP–CPEB3 was purified by using Amylose Resin (New England BioLabs) according to the manufacturer's instructions.

Northern analysis

The transcriptional pulse-chase analysis was performed as described previously (Funakoshi *et al*, 2007). Briefly, 24 h after transfection, cells were treated with 10 ng/ml tetracycline for 2.5 h to induce transcription, washed using phosphate-buffered saline three times to remove tetracycline completely, and harvested at the specified time after transcriptional shut off. Total RNA was isolated with buffer C (50% phenol, 100 mM Na-acetate (pH 4.0), 2 M guanidine-thiocyanate, 12.5 mM Na-citrate (pH 7.0), 0.25% N-lauroyl sarcosine sodium and 0.34% 2-mercaptoethanol), separated by 2.5% agarose gel electrophoresis, and transferred to Biodyne B (Pall Life Sciences) using buffer D (3 M NaCl and 8 mM NaOH). To analyse deadenylated mRNA, 20 μg of the total RNA was incubated with 1 unit of RNase H (Invitrogen) in 20 mM Tris–HCl (pH 7.5), 50 mM NaCl, 20 mM $MgCl_2$, 2 mM DTT, 60 μg/ml BSA and 15 ng/μl oligo$(dT)_{20}$ (Invitrogen) at 30°C for 2 h. Flag–CAT, BGG (1–39), 5xFlag–GST–CAT and 5xFlag–EGFP mRNAs were detected by Northern blotting using [^{32}P]-labeled oligonucleotides: 5′ CTC CTC AGG AGT CAG GTG CAC CAT 3′, 5′ GGT CCA AGG GTA GAC CAC CAG CAG 3′ and 5′ CTT ATC GTC GTC ATC CTT GTA ATC 3′. The transcriptional repression experiment was performed as described above, except that the transfected cells were treated with 50 ng/ml tetracycline for 12 h.

Real-time PCR and semi-quantitative RT–PCR analysis

At 36 h after siRNA transfection, SK-N-SH cells were treated with 10 μg/ml actinomycin D to shut-off transcription for the indicated time. Total RNA was isolated as described above. Reverse transcriptase reactions were performed using SuperScript III Reverse Transcriptase (Invitrogen) with random hexamers according to the manufacturer's instructions. Real-time PCR analysis was performed using StepOne Real-Time PCR system with PowerSYBR Green PCR Master Mix (Applied Biosystems). GluR2 mRNA was amplified using 5′ GGT TAC AAC GTA TAT GGC A 3′ (sense) and 5′ TTC AAG ATA CTG GAT GCC TCT CAC CAC 3′ (antisense). CPEB3 mRNA was amplified using 5′ CAC AAT GAC ATT GAC AAA C 3′ (sense) and 5′ GTA ATA CTG CAG ACA GGT G 3′ (antisense). CPEB4 mRNA was amplified using 5′ CTG CAG CAT GGA GAG ATA G 3′ (sense) and 5′ GGG TCA GAA TGA ACA GAA GAG 3′ (antisense). Tob mRNA was amplified using 5′ TGA CCC AGC CTC ATC AGT G 3′ (sense) and 5′ AGG TCA TTC ACA TTC AAG CC 3′ (antisense). Tob2 mRNA was amplified using 5′ GGC CAG TCA CCC AGC CCT A 3′ (sense) and 5′ CTT CTT CAT CTT AGT GGA G 3′ (antisense). GAPDH mRNA was amplified using 5′ GGT GAA GGT CGG AGT CAA CG 3′ (sense) and 5′ TGG GTG GAA TCA TAT TGG AA 3′ (antisense). 28S rRNA was amplified using 5′ GTA CAC CTG TCA AAC GGT AA 3′ (sense) and 5′ ACC GGC TAT CCG AGG CCA AC 3′ (antisense).

For semi-quantitative RT–PCR analysis, PCR-amplified products were separated by 1.2% agarose gel electrophoresis, detected using ethidium bromide, and quantitated by Image Gauge Ver. 4.23 (Fujifilm). GluR2 mRNA was PCR-amplified using 5′ GGC ATC GCA ACA CCT AAA GGA T 3′ (sense) and 5′ CCA CCA CGC TTT GTC TGT AAC TCG AGT 3′ (antisense). CPEB3 mRNA was amplified using 5′ CTC TAC CTG TGT GTG TCA AGC 3′ (sense) and 5′ ACT CCA GTA CTT GTG GCT GGG TC 3′ (antisense). CPEB4 mRNA was amplified using 5′ GTG TAT CAA GTC CCA CTA TCA 3′ (sense) and 5′ GGG TCA GAA TGA ACA GAA GAG 3′ (antisense). Tob mRNA was amplified using 5′ AAT TCA TGC AGC TTG AAA TCC AAG 3′ (sense) and 5′ AGG TCA TTC ACA TTC AAG CC 3′ (antisense). Tob2 mRNA was amplified using 5′ ATG CAG CTA GAG ATC AAA GTG 3′ (sense) and 5′ TCT TGT GCT TCA GCA GGC TG 3′ (antisense). 28S rRNA was amplified using 5′ GTA CAC CTG TCA AAC GGT AA 3′ (sense) and 5′ ACC GGC TAT CCG AGG CCA AC 3′ (antisense).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: NH and YF designed and performed the majority of the experiments; MH, RY, YA, RM and KO performed the binding experiments; MT provided suggestions and comments; SH conceived and directed the study and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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