CUP promotes deadenylation and inhibits decapping of mRNA targets

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Supplemental Materials and methods

Functional assays and dsRNA interference

RNA interference was performed as described (Behm-Ansmant et al. 2006). Cells were depleted on days 0 and 4 and transfected on day 6. In all experiments, cells were collected three days after transfection. Firefly and Renilla luciferase activity were measured using the Dual-Luciferase Reporter Assay System (Promega). Northern blotting was performed as described (Behm-Ansmant et al. 2006). RNase H (USB, Cleveland, Ohio) digestion, using a (dT)₁₅ oligonucleotide was performed according to the manufacturer’s instructions. To measure mRNA half-lives, transfected cells were treated with actinomycin D (5 µg/ml final concentration) three days after transfection and harvested at the indicated time points.

RT-PCR analysis

Cells treated with GST, Bruno or Smaug dsRNA were re-suspended in TriFast reagent (PeqLab). Total RNA samples were isolated according to the manufacturer’s protocol. After DNaseI (Fermentas) treatment for 1 hour at 37°C, first-strand cDNA was synthesized using M-MuLV reverse transcriptase (Fermentas) and random primers. PCR reactions were performed using primers specific for Bruno or Smaug and PABPC1, which served as an internal reference. The primer sequences used were: Smaug forward
primer (5’-GAAGACGCGCGCTCTAAC-3’) and reverse primer (5’-TTTGGACAAACTAACTTCAG-3’); Bruno forward primer (5’-TTCGGGACAAAAACGGACAG-3’) and reverse primer (5’-AATGGCCGCTAGATTCTGGAC-3’); and PABPC1 forward primer (5’-AGCTCTTCACCAATGTATATG-3’) and reverse primer (5’-TTCCTTTGGGCGAAAGCAACGTAC-3’). PCR products were visualized in a 1% agarose gel stained with ethidium bromide.

**Supplemental Figure Legends**

**Figure S1.** CUP-mediated translation and deadenylation requires binding to the mRNA target. (A–C) The experiment described in Fig. 1 was performed with a F-Luc reporter lacking the BoxB hairpins in the 3’ UTR. To this end, S2 cells were transfected with a mixture of three plasmids: one expressing the F-Luc reporter (lacking the BoxB hairpins), another expressing the Renilla luciferase (R-Luc) as a transfection control and a third plasmid expressing λN-HA or λN-HA-CUP as indicated. (A) Firefly luciferase activity was normalized to Renilla luciferase and set to 100 in cells expressing λN-HA. (B) F-Luc mRNA levels were normalized to those of Renilla mRNA and set to 100 in cells expressing λN-HA. Mean values ± standard deviations from three independent experiments are shown. (C) Northern blot analysis of RNA samples corresponding to those shown in Panel B. (D) CUP mutants were tested for nonspecific effects using the F-Luc reporter lacking the BoxB hairpins as described above. Mean values ± standard deviations from three independent experiments are shown. (E) The interactions of wild-
type or the indicated mutant CUP proteins with GFP-eIF4E was analyzed as described in Fig. 2G. An HA-tagged version of maltose-binding protein (MBP) served as negative control. CUP mutant Δ4E-T carries a deletion of a 4E-T homology motif in the Mid region (amino acids 580–600; see Dostie et al. 2000). This deletion does not affect eIF4E binding or CUP activity (not shown).

**Figure S2.** Tethered CUP competes with eIF4G for eIF4E-binding. (A–C) S2 cells were transfected with a mixture of four plasmids: one expressing the F-Luc-5BoxB reporter; another expressing V5-tagged eIF4G or GST; a third expressing λN-HA or λN-HA-CUP; and a fourth plasmid expressing *Renilla* luciferase (R-Luc). (A) Firefly luciferase activity was normalized to that of the *Renilla* luciferase. The normalized values of F-Luc activity were set to 100 in the presence of λN-HA for each condition. (B) RNA samples were immunoprecipitated using V5 antibodies. The levels of the F-Luc-5BoxB reporter (each normalized to R-Luc mRNA) in the inputs and immunoprecipitates (IP) were analyzed by RT-qPCR. For each condition, the normalized values of F-Luc mRNA levels were set to 1 in the presence of λN-HA (black bar). Mean values ± standard deviations from three independent experiments are shown. (C) The efficacy of the immunoprecipitations was examined by Western blotting. The non-canonical 4E-BM2 is required for CUP-mediated mRNA stabilization. (D–F) A tethering assay using wild-type CUP or the N-terminal domain only (N-term) was performed as described in Fig. 1. The protein fragments that contained the 4E-BM2 stabilized the F-Luc-5BoxB mRNA, leading to a 2-fold increase in mRNA levels. In
contrast, mutants carrying alanine substitutions of two residues in the 4E-BM2 (Mut2 and Mut1+2) did not stabilize F-Luc5BoxB mRNA.

**Figure S3.** Interactions between CUP and decapping factors. (A–D) Cell lysates were immunoprecipitated using anti-HA (A), anti-GFP (B) or anti-V5 (C) antibodies. HA-MBP, GFP and R-Luc-V5 served as negative controls. (A, B) CUP does not co-immunoprecipitate endogenous EDC4 or HA-tagged DCP2. Endogenous eIF4E served as a positive control for the immunoprecipitations. (C) V5-tagged XRN1 does not interact with CUP, whereas V5-GFP tagged eIF4E co-immunoprecipitated CUP under the same conditions. (D) Wild-type CUP or CUP mutants do not interact with EDC4, XRN1 or DCP2. Endogenous eIF4E served as a positive control for the immunoprecipitations.

**Figure S4.** CUP represses bound mRNAs independently of decapping activators. (A–C) S2 cells were treated with the indicated dsRNAs on days 0 and 4. Control cells were treated with an unrelated dsRNA targeting GFP or GST. On day 6, cells were cotransfected with a mixture of three plasmids: one expressing the F-Luc-5BoxB mRNA, another expressing *Renilla* luciferase (R-Luc) and a third expressing λN-HA or λN-HA-CUP. Firefly luciferase activity was normalized to that of *Renilla* luciferase. For each condition, the normalized values of F-Luc activity were set to 100 in cells expressing λN-HA (black bars). Mean values ± standard deviations from three independent experiments are shown.
Figure S5. CUP represses bound mRNAs independently of Bruno and Smaug. (A–F) S2 cells were treated with the indicated dsRNAs on days 0 and 4. Control cells were treated with an unrelated dsRNA targeting Neomycin (Neo) or GST. On day 6, cells were cotransfected with a mixture of three plasmids: one expressing the F-Luc-5BoxB mRNA, another expressing Renilla luciferase (R-Luc) and a third expressing λN-HA, λN-HA-CUP or λN-HA-CUP Mut1. Firefly luciferase activity was normalized to that of Renilla luciferase. For each condition, the normalized values of F-Luc activity were set to 100 in cells expressing λN-HA. (A, D) Mean values ± standard deviations from three independent experiments are shown. (B, E) Northern blot analysis of RNA samples corresponding to those shown in panels (A) and (D), respectively. F-Luc-5Box mRNA containing (A\textsubscript{n}) or lacking a poly(A) tail (A\textsubscript{0}) was loaded as reference. The dashed red line indicates the position of the deadenylated F-Luc-5BoxB mRNA. (C, F) The efficacy of Bruno or Smaug depletion was analyzed by semi-quantitative RT-PCR. PABPC1 mRNA served as a loading control. In lanes 1–4, PCR reactions were performed with dilutions of the cDNA sample isolated from control cells to estimate the efficacy of the depletion. Note that Bruno is expressed at very low levels in S2 cells and the Bruno oligos give non-specific amplification products.
Supplemental Material

Igreja et al. Supplemental Figure 2

A Protein levels

B RT-qPCR

C Western blot

D Protein levels

E F-Luc-5BoxB mRNA

F Northern blot
Igreja et al. Supplementary Figure 3

A  HA-CUP - EDC4 interaction

B  GFP-CUP - DCP2-V5 interaction

C  GFP-CUP - XRN1-V5 interaction

D  GFP-CUP - decapping factors interaction
Igreja Supplementary Figure 4

A  Protein levels

B  Protein levels

C  Protein levels
Igreja Supplementary Figure 5

A  Protein levels

B  Northern blot

C  RT-PCR

D  Protein levels

E  Northern blot

F  RT-PCR