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RESEARCH COMMUNICATION

# A novel, noncanonical mechanism of cytoplasmic polyadenylation operates in *Drosophila* embryogenesis

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**Cytoplasmic polyadenylation is a widespread mechanism to regulate mRNA translation that requires two sequences in the 3′ untranslated region (UTR) of vertebrate substrates: the polyadenylation hexanucleotide, and the cytoplasmic polyadenylation element (CPE). Using a cell-free *Drosophila* system, we show that these signals are not relevant for *Toll* polyadenylation but, instead, a "polyadenylation region" (PR) is necessary. Competition experiments indicate that PR-mediated polyadenylation is required for viability and is mechanistically distinct from the CPE/hexanucleotide-mediated process. These data indicate that *Toll* mRNA is polyadenylated by a noncanonical mechanism, and suggest that a novel machinery functions for cytoplasmic polyadenylation during *Drosophila* embryogenesis.**

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Oocyte maturation and early development in many organisms occurs in the absence of transcription. Developmental progression at these times depends largely on differential translation of maternal mRNAs, and cytoplasmic polyadenylation is a major component of this control. In general, mRNAs with a short poly(A) tail remain translationally silent, while elongation of the poly(A) tail in the cytoplasm results in translational activation. Most of the accumulated knowledge on cytoplasmic polyadenylation derives from studies in oocytes of *Xenopus* (for review, see Belloc et al. 2008; Radford et al. 2008). Two *cis*-acting sequences in the 3′ untranslated region (UTR) of substrate mRNAs are essential for this process: the conserved polyadenylation hexanucleotide —also required for nuclear polyadenylation—with the structure A(A/U)UAAA, and the U-rich cytoplasmic polyadenylation element (CPE), which generally consists of $U_{4–5}A_{1–3}U$. The hexanucleotide is recognized by the multisubunit complex CPSF (cleavage and polyadenylation specificity factor), and the CPE is recognized by CPEB, a protein with a dual function that acts as a switch between translational repression and activation. In immature oocytes, CPEB represses translation by recruiting a set of factors that functionally block the two ends of the mRNA. On the one hand, CPEB recruits Maskin (or 4E-T in growing oocytes), which in turn binds to eIF4E and prevents its recognition by eIF4G during translation initiation (Stebbins-Boaz et al. 1999; Minshall et al. 2007). On the other hand, CPEB recruits the deadenylase PARN, which keeps the poly(A) tail short (Kim and Richter 2006). Upon meiotic maturation, CPEB phosphorylation results in eviction of PARN and enhanced recruitment of CPSF. Together, CPEB and CPSF recruit the cytoplasmic poly(A) polymerase GLD-2, leading to elongation of the poly(A) tail and translational activation (Barnard et al. 2004). The distance between the CPE and the hexanucleotide dictates the timing and extent of polyadenylation (Piqué et al. 2008). Additional elements reported to function early during oocyte maturation are the U-rich polyadenylation response elements (PREs), which bind the protein Musashi (Charlesworth et al. 2002, 2006).

CPEB is a conserved family of four members in vertebrates that, in addition to oocyte maturation, contribute to the regulation of local protein synthesis at synapses that underlies long-term changes in synaptic plasticity (for review, see Richter 2007). In *Drosophila*, the CPEB1 homolog Orb plays a role in mRNA localization and regulates the polyadenylation of *oskar* and *cyclin B* mRNAs during oogenesis (Chang et al. 1999; Castagnetti and Ephrussi 2003; Benoit et al. 2005). Orb2, the homolog of CPEB2-4, is required for long-term memory, but its role in cytoplasmic polyadenylation has not been demonstrated (Keleman et al. 2007). Other conserved factors that contribute to cytoplasmic polyadenylation during *Drosophila* oogenesis are the canonical poly(A) polymerase Hiiragi, and the GLD-2 homolog Wispy (Wisp) (Juge et al. 2002; Benoit et al. 2008; Cui et al. 2008).

Cytoplasmic polyadenylation also occurs during embryogenesis, but the sequences and factors responsible for polyadenylation at these times remain poorly understood. In *Drosophila*, translation of the transcripts encoding Bicoid, Toll, and Torso is activated by cytoplasmic polyadenylation in early embryogenesis, and this activation is required for appropriate axis formation (Sallés et al. 1994; Schisa and Strickland 1998). How this polyadenylation occurs is intriguing, as Orb is barely detectable in early embryos (Vardy and Orr-Weaver 2007). Furthermore, no *cis*-acting elements responsible for cytoplasmic polyadenylation have been described yet in this organism. Therefore, an important question is whether similar signals, factors, and mechanisms operate for cytoplasmic polyadenylation in different biological settings. To address this question, we used an in vitro cytoplasmic polyadenylation system derived from *Drosophila* early embryos. Using *Xenopus cyclin B1* (*CycB1*) mRNA as a substrate, we found that the canonical cytoplasmic polyadenylation signals—the CPE and the hexanucleotide—function in *Drosophila*. Surprisingly, however, these sequences are not necessary for *Toll* polyadenylation. Rather, a region of the 3′ UTR that we term the "polyadenylation region" (PR) is required. Consistently, competition experiments indicate

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that PR-mediated polyadenylation is mechanistically distinct from the CPE/hexanucleotide-mediated process, implying that a novel machinery for cytoplasmic polyadenylation operates during *Drosophila* embryogenesis.

## Results and Discussion

### *A* Drosophila *cell-free system for cytoplasmic polyadenylation*

Cytoplasmic polyadenylation has been observed in oocytes and early embryos of *Drosophila*. Maximal polyadenylation of *Toll* and *bicoid* mRNAs occurs at ~90 min of development (Sallés et al. 1994). To study cytoplasmic polyadenylation, we first tested whether extracts from 90-min embryos could recapitulate this process. Similar extracts obtained from nonstaged embryos have been shown previously to support translation (Gebauer et al. 1999). Because translation is the consequence of cytoplasmic polyadenylation for *Toll* and *bicoid*, we tested whether polyadenylation of these substrates in embryonic extracts could occur under translation conditions. We incubated nonadenylated full-length *bicoid* mRNA and the 3′ UTR of *Toll* in staged 90-min embryo extracts. In addition, we used the mRNA encoding the ribosomal protein Sop as a negative control, as this transcript contains a canonical hexanucleotide and has been shown to undergo nuclear but not cytoplasmic polyadenylation (e.g., see Benoit et al. 2008). After incubation, we measured the length of the poly(A) tail by the PCR-based poly(A) test (PAT) assay. We found that both *bicoid* and *Toll* RNAs gained a poly(A) tail of ~150 nucleotides (nt) while *sop* mRNA remained nonadenylated, indicating that this system recapitulates the cytoplasmic polyadenylation process (Fig. 1A). *Toll* mRNA was selected for further studies because it was consistently polyadenylated more efficiently than *bicoid* mRNA in the cell-free system.

To evaluate whether cytoplasmic polyadenylation resulted in increased translation, we first tested the correlation between both processes in a time-course experiment. We fused the 3′ UTR of *Toll* to the firefly luciferase ORF to yield the Luc-toll transcript. Translation of this transcript closely paralleled polyadenylation of *Toll* 3′ UTR (Fig. 1B, cf. the left panel and the Luc-toll curve in the right panel). In addition, treatment of the mRNA with the chain elongation inhibitor cordycepin (3′-deoxyadenosine) dramatically reduced the efficiency of translation, decreasing it to the levels of nonadenylated luciferase mRNA (Fig. 1B, right panel). These data show that both cytoplasmic polyadenylation and polyadenylation-dependent translation can be recapitulated in 90-min *Drosophila* embryo extracts.

### *The canonical cytoplasmic polyadenylation signals function in* Drosophila

The *cis*-acting elements for cytoplasmic polyadenylation in *Drosophila* are unknown. To test whether the CPE and the hexanucleotide were recognized as polyadenylation elements in this organism, we analyzed the polyadenylation of the best-characterized vertebrate substrate, *CycB1*. The 3′ UTR of this transcript contains three CPEs, one of them overlapping with the hexanucleotide (Fig. 1C), and has been shown to undergo strong polyadenylation at a late time during *Xenopus* oocyte maturation and in early embryos (Groisman et al. 2002; Piqué et al. 2008). The 3′ UTR of *CycB1* was polyadenylated in the *Drosophila* cell-free system and was sufficient to confer polyadenylation when fused to a CAT reporter (Fig. 1C). Importantly, mutation of the hexanucleotide (Fig. 1C, left panel) or the CPEs (Fig. 1C, right panel, CPE0) completely abrogated polyadenylation, indicating that the vertebrate cytoplasmic polyadenylation signals function in *Drosophila*. These results suggest that a canonical cytoplasmic polyadenylation machinery exists in *Drosophila* embryos. Intriguingly, however, poly(A) tail length control must occur in the absence of Orb, which is undetectable in early embryos, and PARN, which is not conserved in *Drosophila*.

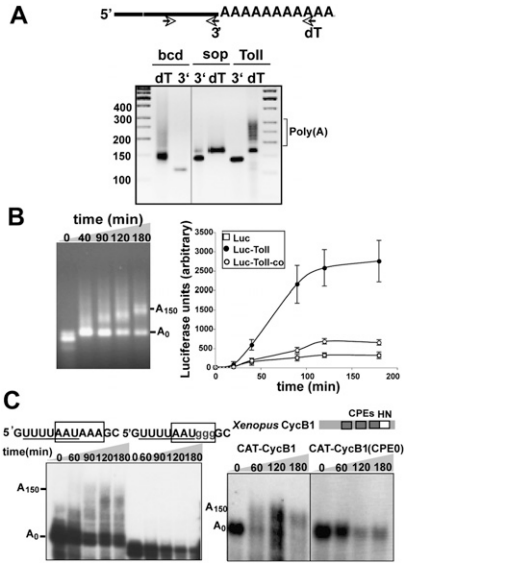


**Figure 1.** The canonical cytoplasmic polyadenylation signals function in *Drosophila*. (*A*) Cytoplasmic polyadenylation in *Drosophila* embryo extracts. Nonadenylated full-length *sop* and *bcd* mRNAs, as well as the 3′ UTR of *Toll*, were incubated in 90-min embryo extracts, and the poly(A) tail was measured using the PAT assay. A schematic representation of the oligonucleotides used in this assay is shown in the *top* panel. For each transcript, a specific 5′ oligonucleotide was combined with either a specific 3′ primer to reveal the size of the nonadenylated RNA (3′ lanes), or with an oligo(dT) anchor to visualize the poly(A) tail (dT lanes). Molecular weight markers are also shown. (*B*) Cytoplasmic polyadenylation promotes translation of *Toll* mRNA. (*Left* panel) Polyadenylation time course of the *Toll* 3′ UTR, as measured by PAT assay. (*Right* panel) A firefly luciferase reporter containing the 3′ UTR of *Toll* (Luc-toll) was incubated in embryo extracts for different times, and the efficiency of translation was determined by measuring the luciferase activity. As controls, the translation efficiencies of nonadenylated Luciferase and cordycepin-treated Luc-toll mRNAs were determined. Curves represent the average of five independent experiments. (*C*) The CPE and the hexanucleotide are functional CPEs in *Drosophila*. (*Left* panel) Polyadenylation of radioactively labeled wild-type and hexanucleotide-mutated *CycB1* 3′ UTRs. The nature of the mutation is indicated in gray lowercase letters. RNAs were separated in a 6% denaturing acrylamide gel and visualized using the PhosphorImager. (*Right* panel) Polyadenylation of CAT reporter mRNAs containing either a wild-type *CycB1* 3′ UTR or a derivative with U-to-G transversions in all three CPEs. RNAs were visualized by Northern blot using a probe against the CAT ORF. A schematic representation of a *Xenopus CycB1* 3′ UTR is shown, with the three CPEs and the polyadenylation hexanucleotide (HN) depicted as gray and white boxes, respectively.

### Toll *mRNA is polyadenylated in a CPE-independent and hexanucleotide-independent fashion*

*Toll* mRNA contains a canonical hexanucleotide, followed by a putative CPE (Fig. 2A). To determine whether these sequences were responsible for polyadenylation, we analyzed the behavior of *Toll* mutant derivatives. To visualize the polyadenylated products, we used Northern blots, which allow a more accurate measurement of the efficiency of polyadenylation as compared with PAT assays. Surprisingly, mutation or deletion of the CPE and/or the hexanucleotide did not affect polyadenylation of *Toll* (Fig. 2A). Treatment with oligo(dT) and RNase H confirmed that the size increase of *Toll* upon incubation was due to polyadenylation (Supplemental Fig. 1). That *Toll* polyadenylation was unaffected by deletion of the CPE and the hexanucleotide was unexpected, as both elements function as polyadenylation signals in *Drosophila* (Fig. 1C), and a single point mutation in the hexanucleotide (AAUAAA to AAGAAA) is sufficient to hinder cytoplasmic polyadenylation in vertebrates (e.g., see McGrew and Richter 1990). Thus, it appeared that *Toll* polyadenylation was independent of the CPE and the hexanucleotide. However, the canonical polyadenylation machinery could, in principle, bind to functional variations of these elements that could pass unrecognized by sequence inspection. To exclude this possibility, we performed competition assays. *CycB1* effectively competed the polyadenylation of radiolabeled *CAT-CycB1* (Fig. 2B, lanes 10–15). Polyadenylation of *Toll* was readily competed by an excess of cold *Toll* 3′ UTR, but, remarkably, not by an excess of *CycB1* (Fig. 2B, lanes 1–9, and see also lanes 16–21, showing the same reactions as lanes 4–9 taken 15 min later). These data argue that polyadenylation of *Toll* is independent of the CPE and the hexanucleotide. Addition of excess *Toll* competitor also destabilized the *Toll* substrate, while addition of *CycB1* did not (Fig. 2B, lanes 16–21). In addition, nonadenylated *CAT-CycB1* was often stabilized in the presence of *CycB1* competitor (Fig. 2B, lanes 13–15), suggesting that the *Drosophila* extracts can be used to monitor both stability and adenylation changes, but that these two processes are not necessarily linked.

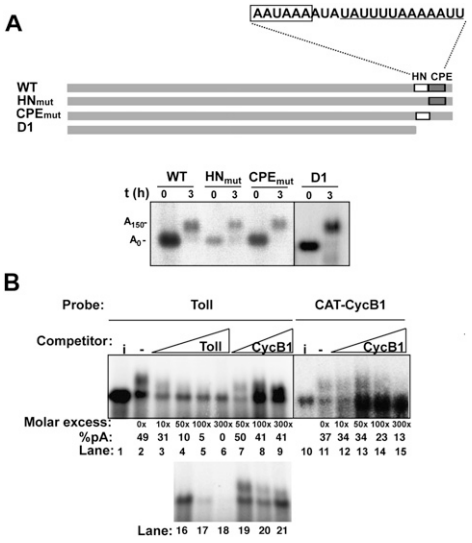


**Figure 2.** Polyadenylation of *Toll* mRNA is independent of the CPE and the hexanucleotide. (*A*, *top* panel) Schematic representation of a *Toll* 3′ UTR (1256 nt) and mutant derivatives. The location and sequence of the putative CPE and the hexanucleotide is detailed. (*Bottom* panel) Polyadenylation of these mRNAs was measured by Northern blot. (*B*) Polyadenylation competition assays. Polyadenylation of a $^{32}$P-labeled *Toll* 3′ UTR or *CAT-CycB1* after addition of excess *Toll* or *CycB1* 3′ UTRs. RNAs were separated in a 1% denaturing agarose gel. Input (i) RNA probes are also shown. The percentage of polyadenylated transcript with respect to total transcript within each lane is indicated (%pA). Lanes *16–21* in the *bottom* panel show samples of the same reactions as lanes *4–9* taken 15 min later.

### *A proximal region in* Toll *3′ UTR directs noncanonical cytoplasmic polyadenylation*

To identify the elements of *Toll* that were responsible for cytoplasmic polyadenylation, we performed mutational analysis. The distal 40% of *Toll* 3′ UTR could be deleted without significant consequences for cytoplasmic polyadenylation (Fig. 3A, fragment D3). Further deletions progressively reduced the efficiency of polyadenylation (fragments D4 and D5). A region of 183 nt within the first half of the 3′ UTR was sufficient to provide detectable levels of polyadenylation (fragment D6), while other regions of the 3′ UTR were not (fragments D5 and D7). We refer to the D6 fragment as the PR. Importantly, deletion of the PR from an otherwise wild-type 3′ UTR severely blocked polyadenylation and translation of *Toll*, indicating that the PR is essential for expression of this mRNA (Fig. 3B).

Although the PR is necessary for polyadenylation, other sequences within the *Toll* 3′ UTR seem to influence both the efficiency of polyadenylation and the length of the poly(A) tail. Deletions downstream from the PR reduce the polyadenylation efficiency, while deletions upstream of the PR reduce the length of the poly(A) tail (Fig. 3A, cf. fragments D3, D4, and D6). This illustrates the complexity and fine regulation of the process, which is likely to be mediated by a complex interplay of multiple activities. Auxiliary elements located both upstream of and downstream from the polyadenylation signal have also been described for nuclear polyadenylation (Chen and Wilusz 1998; Zarudnaya et al. 2003), including sequences and factors that mediate hexanucleotide-independent polyadenylation (Venkataraman et al. 2005).

Elements other than the canonical CPE have been shown previously to stimulate cytoplasmic polyadenylation in other organisms. In *Xenopus* oocytes, the U-rich PRE and TCS (translational control sequence) stimulate the polyadenylation of a number of mRNAs early after progesterone induction (Charlesworth et al. 2002, 2004; Wang et al. 2008). Similarly, poly(U) and poly(C) sequences promote polyadenylation during *Xenopus* embryogenesis (Simon et al. 1992; Paillard et al. 2000), and undefined elements other than the CPE and the hexanucleotide direct polyadenylation of *lamin B1* mRNA in *Xenopus* embryos (Ralle et al. 1999). However, no direct evidence exists that these elements function independently of the canonical polyadenylation machinery. To confirm that the PR is responsible for noncanonical polyadenylation, we performed competition assays. Polyadenylation of *Toll* was competed with the PR, but not with the distal 119-nt fragment of the *Toll* 3′ UTR that contained the CPE and the hexanucleotide (Fig. 4A, lanes 1–6). Conversely, the PR did not compete polyadenylation

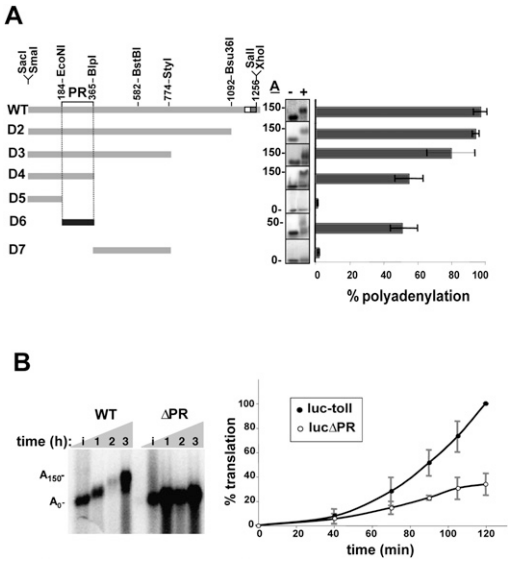


**Figure 3.** Elements required for cytoplasmic polyadenylation of *Toll* mRNA. (*A*, *left* panel) Schematic representation of *Toll* 3′ UTR deletion derivatives. Nucleotide positions are shown according to the annotated *Drosophila Toll* sequence, taking as reference the first nucleotide of the 3′ UTR. The location of unique restriction sites and the PR are indicated. Restriction sites at both ends of *Toll* belong to the vector in which this sequence was cloned. Typical patterns of these RNAs are shown in the *middle* panel, before (−) or after (+) incubation in the *Drosophila* extracts. The sizes of the respective poly(A) tails are indicated. (*Right* panel) Quantification of the efficiency of polyadenylation, measured as the percentage of RNA that was polyadenylated versus the total signal (polyadenylated and nonadenylated) within the "+" lane. Values represent the average of at least three independent experiments. (*B*) The PR is essential for polyadenylation and translation of *Toll*. (*Left* panel) Polyadenylation time course of a wild-type *Toll* 3′ UTR or a mutant derivative lacking the PR. (*Right* panel) Translation efficiencies of reporter mRNAs containing either wild-type or ΔPR *Toll* 3′ UTRs. *Renilla* luciferase mRNA was cotranslated as an internal control. The firefly values were corrected for Renilla expression, and the data are represented relative to the translation of Luc-toll mRNA at the last time point. Curves represent the average of three independent experiments using a single batch of extract.

of *CAT-CycB1* (Fig. 4A, lanes 7–10), while polyadenylation of this transcript was efficiently competed by an excess of *CycB1*, as well as by any fragment of *Toll* containing the CPE and the hexanucleotide, including the full-length *Toll* 3′ UTR (Fig. 4A, lanes 11–18). Consistent with the results of the polyadenylation assays, the PR competed translation of a *Toll* reporter but not of a *CycB1* reporter (Supplemental Fig. 2). The PR competed both polyadenylation and translation less efficiently than the full-length *Toll* 3′ UTR, in agreement with its lower polyadenylation efficiency. The competition results cannot be explained by different affinities of the same factors for the PR compared with the canonical sequences, because neither the PR competes *CycB1* mRNA polyadenylation nor *CycB1* competes *Toll* polyadenylation. Thus, we conclude that polyadenylation of *Toll* is driven by a complex that binds to the PR and differs from the canonical machinery in at least one limiting component.

Previously, a region of the *Toll* 3′ UTR that lies downstream from the PR (located between nucleotides 582 and 774) was shown to promote polyadenylation of this transcript (Schisa and Strickland 1998). In our hands, this region could not compete for polyadenylation of *Toll* (data not shown), suggesting that it does not function for polyadenylation in early embryos. Nevertheless, it should be noted that translation of *Toll* is also required at later times in development, where these sequences and/or the canonical signals could become relevant.

To map more finely the sequences within the PR that were responsible for polyadenylation, we first searched for regions ultraconserved among Drosophilids. In addition, we looked for sequence words within the PR significantly overrepresented in the 3′ UTRs of *Drosophila melanogaster* transcripts. We found two ultraconserved regions and two related sequence words distributed along the PR (Supplemental Fig. 3A,B, observe shadowed regions and words within red boxes). Fragments of the PR containing or lacking these sequences were used in functional competition assays (Supplemental Fig. 3C). The results suggest the existence of a complex element responsible for polyadenylation of *Toll* that is not associated with a simple linear sequence. We speculate that a structure—or multiple redundant, interdependent linear sequences—within the PR are necessary for polyadenylation. Interestingly, the conserved region at the 3′ end of the PR consisting of TGTTATCTGTAAGC behaved as a stabilization element. All fragments containing this region destabilized *Toll* when added in excess, while fragments lacking it did not (Supplemental Fig. 3C).

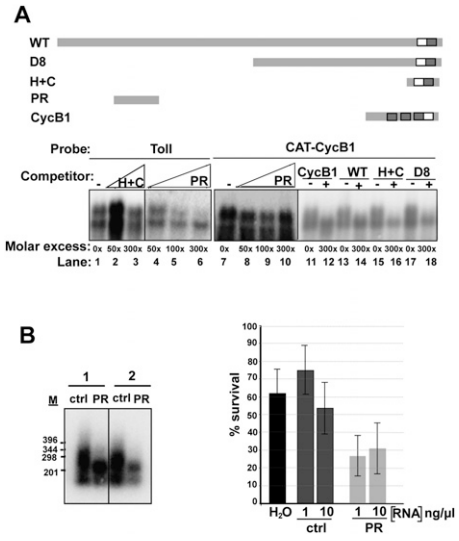


**Figure 4.** The PR is required for noncanonical cytoplasmic polyadenylation in vitro and in vivo. (*A*) Polyadenylation competition experiments using the RNAs schematically represented in the *top* panel as competitors. Experiments were performed as indicated in the legend for Figure 2B. (*B*) Excess PRs disrupt polyadenylation of endogenous *Toll* and reduce viability. (*Left* panel) Embryos (0–30 min) were microinjected with different amounts of the PR or an unrelated RNA of similar length (159 nt) at a concentration of 10 ng/μL. Samples were collected 1 h after injection, the RNA was extracted, and the poly(A) tail length was tested by PAT assay. Amplified products were visualized by Southern blot using a random-primed probe against the *Toll* 3′ UTR. The results for two independent injections are shown. (*Right* panel) Embryos were microinjected with RNA solutions at different concentrations or with water as control, and survival was scored as the percentage of hatched embryos as indicated in the Materials and Methods. The average of at least three independent injections of 100 embryos per injection is shown.

Importantly, fragment 9 (F9) strongly destabilized *Toll* but did not compete for polyadenylation, showing that polyadenylation and stability of *Toll* are separable processes.

We next wanted to test the relevance of the PR in vivo. We performed in vivo competition experiments by injecting wild-type early embryos with either the PR or an unrelated RNA of the same length as control. We then tested polyadenylation of endogenous *Toll* mRNA and survival of injected embryos, as timely expression of *Toll* is essential for early development. The results showed that the PR specifically competed polyadenylation of endogenous *Toll* and reduced the viability of early embryos (Fig. 4B). These results indicate that PR directs noncanonical polyadenylation in vitro and in vivo.

The *Toll* polyadenylation mechanism described here may affect a variety of mRNAs. Recent in silico EST database analysis indicates an incidence of the hexanucleotide A(A/U)UAAA of 60%–70%, suggesting that a significant fraction of mRNAs lack consensus hexanucleotide signals and may undergo AAUAAA-independent polyadenylation (for review, see MacDonald and Redondo 2002). In addition, activation of *Drosophila pgc* (polar granule component) mRNA in early embryos appears independent of Orb (Rangan et al. 2009).

Which factors could be involved in polyadenylation of *Toll*? Mutations in *cortex* and *grauzone* were identified genetically to affect polyadenylation of *Toll* (Lieberfarb et al. 1996). Cortex is an activator of the anaphase-promoting complex, and mutations in this gene prevent the completion of meiosis (Chu et al. 2001). On the other hand, Grauzone is a transcription factor necessary for activation of Cortex (Harms et al. 2000). Thus, Cortex and Grauzone may affect polyadenylation of *Toll* indirectly by precluding the normal initiation of embryogenesis. Similarly, embryos from *wisp* mutant mothers are defective in polyadenylation of several maternal mRNAs, including *Toll* (Cui et al. 2008). Wisp is present until ~2 h of development and, therefore, could be directly involved in polyadenylation of *Toll*. However, Wisp is also required for expression of Cortex (Benoit et al. 2008), so it is unclear to what extent the observed effects on *Toll* polyadenylation are due to primary defects in Cortex expression. Direct biochemical dissection using the cell-free polyadenylation system, combined with *Drosophila* genetics, will allow us to decipher the components of both the CPE-dependent and CPE-independent cytoplasmic polyadenylation machineries.

## Materials and methods

### *Extract preparation*

Extracts were prepared from staged 90-min embryos as described in Gebauer et al. (1999). To stage embryos, collecting trays were exchanged every 90 min, three times, and the third batch of trays was used to prepare extracts. After preparation, extracts were adjusted to 10% glycerol, snap-frozen in liquid nitrogen, and stored at −80°C.

### *In vitro polyadenylation and translation*

Reactions using 90-min embryo extracts were assembled as described in Gebauer et al. (1999), without tRNA. In these reactions, both polyadenylation and translation could be observed. Typically, 0.01 pmol of substrate mRNA was used in the reaction. The use of small amounts of substrate is relevant, as we found that polyadenylation is saturable in this system. To account for batch–to–batch differences in the polyadenylation kinetics and efficiency, polyadenylation of each RNA construct was tested in different batches of extract, carrying in parallel the full-length *Toll* 3′ UTR as a positive control. In some cases, *Renilla* mRNA was cotranslated as an internal control. After incubation, the translation efficiency was determined by measuring the luciferase activity using the Dual Luciferase Assay System (Promega), and firefly luciferase values were corrected for *Renilla* expression.

Polyadenylation was tested by either PAT assay, Northern blot, or direct visualization using radioactively labeled RNAs. For Northern blots, a random-primed probe against the full-length 3′ UTR of *Toll* was used. Radioactively labeled RNAs were resolved in denaturing 6% acrylamide gels and visualized in a PhosphorImager. PAT assays were performed as described by Sallés and Strickland (1995) after treatment of RNA samples with Turbo DNase (Ambion). The gene-specific oligonucleotides used for these assays are described in the Supplemental Material. To amplify endogenous *Toll*, RNA was extracted from embryos using Trizol (Invitrogen), and 150–300 ng of total RNA (100 embryos) were used in the reaction. Amplified products were resolved in 1% agarose gels.

For competition assays, extracts were preincubated for 10 min on ice with increasing amounts of ApppG-capped RNA competitor. The remaining reagents needed for translation were subsequently added, and the reaction was further incubated for 10 min before adding the radioactively labeled substrate mRNA.

### *Plasmids and in vitro transcription*

DNA constructs are detailed in the Supplemental Material. mRNAs were synthesized as described previously (Gebauer et al. 1999). mRNAs lacked a poly(A) tail and contained a $^{7m}$GpppG cap. RNAs used as competitors contained an ApppG cap. Cordycepin was incorporated to the 3′ end of Luc-toll mRNA with yeast poly(A) polymerase (GE Healthcare), following the recommendations of the vendor.

### *Microinjections*

Oregon embryos (0–30 min old) were injected in a ventral–posterior position as described previously (Schisa and Strickland 1998). Embryos were allowed to develop for 72 h at 18°C, and the number of hatched embryos was scored to estimate the percentage of viability. For PAT assays, microinjected embryos were allowed to develop for 1 h before extraction of RNA with Trizol (Invitrogen).

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## References

Barnard DC, Ryan K, Manley JL, Richter JD. 2004. Symplekin and xGLD-2 are required for CPEB-mediated cytoplasmic polyadenylation. *Cell* **119:** 641–651.

Belloc E, Pique M, Mendez R. 2008. Sequential waves of polyadenylation and deadenylation define a translation circuit that drives meiotic progression. *Biochem Soc Trans* **36:** 665–670.

Benoit B, Mitou G, Chartier A, Temme C, Zaessinger S, Wahle E, Busseau I, Simonelig M. 2005. An essential cytoplasmic function for the nuclear poly(A) binding protein, PABP2, in poly(A) tail length control and early development in *Drosophila*. *Dev Cell* **9:** 511–522.

Benoit P, Papin C, Kwak JE, Wickens M, Simonelig M. 2008. PAP- and GLD-2-type poly(A) polymerases are required sequentially in cytoplasmic polyadenylation and oogenesis in *Drosophila*. *Development* **135:** 1969–1979.

Castagnetti S, Ephrussi A. 2003. Orb and a long poly(A) tail are required for efficient oskar translation at the posterior pole of the *Drosophila* oocyte. *Development* **130:** 835–843.

Chang JS, Tan L, Schedl P. 1999. The *Drosophila* CPEB homolog, orb, is required for oskar protein expression in oocytes. *Dev Biol* **215:** 91–106.

Charlesworth A, Ridge JA, King LA, MacNicol MC, MacNicol AM. 2002. A novel regulatory element determines the timing of Mos mRNA translation during *Xenopus* oocyte maturation. *EMBO J* **21:** 2798–2806.

Charlesworth A, Cox LL, MacNicol AM. 2004. Cytoplasmic polyadenylation element (CPE)- and CPE-binding protein (CPEB)-independent mechanisms regulate early class maternal mRNA translational activation in *Xenopus* oocytes. *J Biol Chem* **279:** 17650–17659.

Charlesworth A, Wilczynska A, Thampi P, Cox LL, MacNicol AM. 2006. Musashi regulates the temporal order of mRNA translation during *Xenopus* oocyte maturation. *EMBO J* **25:** 2792–2801.

Chen F, Wilusz J. 1998. Auxiliary downstream elements are required for efficient polyadenylation of mammalian pre-mRNAs. *Nucleic Acids Res* **26:** 2891–2898.

Chu T, Henrion G, Haegeli V, Strickland S. 2001. Cortex, a *Drosophila* gene required to complete oocyte meiosis, is a member of the Cdc20/fizzy protein family. *Genesis* **29:** 141–152.

Cui J, Sackton KL, Horner VL, Kumar KE, Wolfner MF. 2008. Wispy, the *Drosophila* homolog of GLD-2, is required during oogenesis and egg activation. *Genetics* **178:** 2017–2029.

Gebauer F, Corona DF, Preiss T, Becker PB, Hentze MW. 1999. Translational control of dosage compensation in *Drosophila* by Sex-lethal: Cooperative silencing via the 5′ and 3′ UTRs of msl-2 mRNA is independent of the poly(A) tail. *EMBO J* **18:** 6146–6154.

Groisman I, Jung MY, Sarkissian M, Cao Q, Richter JD. 2002. Translational control of the embryonic cell cycle. *Cell* **109:** 473–483.

Harms E, Chu T, Henrion G, Strickland S. 2000. The only function of Grauzone required for *Drosophila* oocyte meiosis is transcriptional activation of the cortex gene. *Genetics* **155:** 1831–1839.

Juge F, Zaessinger S, Temme C, Wahle E, Simonelig M. 2002. Control of poly(A) polymerase level is essential to cytoplasmic polyadenylation and early development in *Drosophila*. *EMBO J* **21:** 6603–6613.

Keleman K, Kruttner S, Alenius M, Dickson BJ. 2007. Function of the *Drosophila* CPEB protein Orb2 in long-term courtship memory. *Nat Neurosci* **10:** 1587–1593.

Kim JH, Richter JD. 2006. Opposing polymerase-deadenylase activities regulate cytoplasmic polyadenylation. *Mol Cell* **24:** 173–183.

Lieberfarb ME, Chu T, Wreden C, Theurkauf W, Gergen JP, Strickland S. 1996. Mutations that perturb poly(A)-dependent maternal mRNA activation block the initiation of development. *Development* **122:** 579–588.

MacDonald CC, Redondo JL. 2002. Reexamining the polyadenylation signal: Were we wrong about AAUAAA? *Mol Cell Endocrinol* **190:** 1–8.

McGrew LL, Richter JD. 1990. Translational control by cytoplasmic polyadenylation during *Xenopus* oocyte maturation: Characterization of cis and trans elements and regulation by cyclin/MPF. *EMBO J* **9:** 3743–3751.

Minshall N, Reiter MH, Weil D, Standart N. 2007. CPEB interacts with an ovary-specific eIF4E and 4E-T in early *Xenopus* oocytes. *J Biol Chem* **282:** 37389–37401.

Paillard L, Maniey D, Lachaume P, Legagneux V, Osborne HB. 2000. Identification of a C-rich element as a novel cytoplasmic polyadenylation element in *Xenopus* embryos. *Mech Dev* **93:** 117–125.

Piqué M, Lopez JM, Foissac S, Guigo R, Mendez R. 2008. A combinatorial code for CPE-mediated translational control. *Cell* **132:** 434–448.

Radford HE, Meijer HA, de Moor CH. 2008. Translational control by cytoplasmic polyadenylation in *Xenopus* oocytes. *Biochim Biophys Acta* **1779:** 217–229.

Ralle T, Gremmels D, Stick R. 1999. Translational control of nuclear lamin B1 mRNA during oogenesis and early development of *Xenopus*. *Mech Dev* **84:** 89–101.

Rangan P, DeGennaro M, Jaime-Bustamante K, Coux RX, Martinho RG, Lehmann R. 2009. Temporal and spatial control of germ-plasm RNAs. *Curr Biol* **19:** 72–77.

Richter JD. 2007. CPEB: A life in translation. *Trends Biochem Sci* **32:** 279–285.

Sallés FJ, Strickland S. 1995. Rapid and sensitive analysis of mRNA polyadenylation states by PCR. *PCR Methods Appl* **4:** 317–321.

Sallés FJ, Lieberfarb ME, Wreden C, Gergen JP, Strickland S. 1994. Coordinate initiation of *Drosophila* development by regulated polyadenylation of maternal messenger RNAs. *Science* **266:** 1996–1999.

Schisa JA, Strickland S. 1998. Cytoplasmic polyadenylation of Toll mRNA is required for dorsal-ventral patterning in *Drosophila* embryogenesis. *Development* **125:** 2995–3003.

Simon R, Tassan JP, Richter JD. 1992. Translational control by poly(A) elongation during *Xenopus* development: Differential repression and enhancement by a novel cytoplasmic polyadenylation element. *Genes & Dev* **6:** 2580–2591.

Stebbins-Boaz B, Cao Q, de Moor CH, Mendez R, Richter JD. 1999. Maskin is a CPEB-associated factor that transiently interacts with elF-4E. *Mol Cell* **4:** 1017–1027.

Vardy L, Orr-Weaver TL. 2007. The *Drosophila* PNG kinase complex regulates the translation of cyclin B. *Dev Cell* **12:** 157–166.

Venkataraman K, Brown KM, Gilmartin GM. 2005. Analysis of a noncanonical poly(A) site reveals a tripartite mechanism for vertebrate poly(A) site recognition. *Genes & Dev* **19:** 1315–1327.

Wang YY, Charlesworth A, Byrd SM, Gregerson R, MacNicol MC, MacNicol AM. 2008. A novel mRNA 3′ untranslated region translational control sequence regulates *Xenopus* Wee1 mRNA translation. *Dev Biol* **317:** 454–466.

Zarudnaya MI, Kolomiets IM, Potyahaylo AL, Hovorun DM. 2003. Downstream elements of mammalian pre-mRNA polyadenylation signals: Primary, secondary and higher-order structures. *Nucleic Acids Res* **31:** 1375–1386.