LETTER

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Maternal mRNA deadenylation and decay by the piRNA pathway in the early *Drosophila* embryo

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Piwi-associated RNAs (piRNAs), a specific class of 24- to 30-nucleotide-long RNAs produced by the Piwi-type of Argonaute proteins, have a specific germline function in repressing transposable elements. This repression is thought to involve heterochromatin formation and transcriptional and post-transcriptional silencing[1–6]. The piRNA pathway has other essential functions in germline stem cell maintenance[7] and in maintaining germline DNA integrity[8–10]. Here we uncover an unexpected function of the piRNA pathway in the decay of maternal messenger RNAs and in translational repression in the early embryo. A subset of maternal mRNAs is degraded in the embryo at the maternal-to-zygotic transition. In *Drosophila*, maternal mRNA degradation depends on the RNA-binding protein Smaug and the deadenylase CCR4[11–13], as well as the zygotic expression of a microRNA cluster[14]. Using mRNA encoding the embryonic posterior morphogen Nanos (Nos) as a paradigm to study maternal mRNA decay, we found that CCR4-mediated deadenylation of *nos* depends on components of the piRNA pathway including piRNAs complementary to a specific region in the *nos* 3′ untranslated region. Reduced deadenylation when piRNA-induced regulation is impaired correlates with *nos* mRNA stabilization and translational derepression in the embryo, resulting in head development defects. Aubergine, one of the Argonaute proteins in the piRNA pathway, is present in a complex with Smaug, CCR4, *nos* mRNA and piRNAs that target the *nos* 3′ untranslated region, in the bulk of the embryo. We propose that piRNAs and their associated proteins act together with Smaug to recruit the CCR4 deadenylation complex to specific mRNAs, thus promoting their decay. Because the piRNAs involved in this regulation are produced from transposable elements, this identifies a direct developmental function for transposable elements in the regulation of gene expression.

In *Drosophila* embryos, Nos is expressed as a gradient that emanates from the posterior pole and organizes abdominal segmentation[15]. The majority of *nos* mRNA is distributed throughout the bulk cytoplasm, translationally repressed[16] and subsequently degraded during the first 2–3 h of development. This repression is essential for head and thorax segmentation[16,17]. A small amount of *nos* transcripts, localized at the posterior pole of the embryo, escapes degradation and is actively translated, giving rise to the Nos protein gradient. *nos* mRNA decay in the bulk cytoplasm depends on the CCR4–NOT deadenylation complex and its recruitment onto *nos* by Smaug (Smg). This contributes to translational repression in the bulk of the embryo and is required for embryonic antero-posterior patterning[13].

Smg has been suggested to be not the only activator of *nos* mRNA decay during early embryogenesis[11,12]. Zygotically expressed miRNAs have been reported to activate maternal mRNA deadenylation in zebrafish embryos[18] and decay in *Drosophila* embryos[14]. We investigated the potential involvement of other classes of small RNAs in mRNA deadenylation and decay before zygotic expression. Because piRNAs are expressed maternally in the germ line and are present in early embryos[19,20], we analysed the possible role of the piRNA pathway in maternal mRNA deadenylation. Piwi, Aubergine (Aub) and Ago3 are specific Argonaute proteins[1,3,21,22], Armitage (Armi) and Spindle-E (Spn-E) are RNA helicases, and Squash (Squ) is a nuclease[2,10,23,24] involved in piRNA biogenesis and function. Poly(A) test assays were performed to measure *nos* mRNA poly(A) tail length in embryos spanning 1-h intervals during the first 4 h of embryogenesis. In contrast to the progressive shortening of *nos* mRNA poly(A) tails observed in wild-type embryos correlating with mRNA decay during this period, *nos* poly(A) tail shortening was affected in embryos from females mutant for the piRNA pathway (herein referred to as mutant embryos) (Fig. 1a and Supplementary Figs 1a, 2 and 12). This defect in deadenylation correlated with higher amounts of *nos* mRNA in mutant embryos, as quantified by reverse transcription–quantitative PCR (RT–qPCR) (Fig. 1b). *In situ* hybridization revealed stabilized *nos* mRNA in the bulk cytoplasm of mutant embryos where it is normally degraded in the wild type (Fig. 1c and Supplementary Fig. 1b). Consistent with previous data showing that *nos* mRNA deadenylation is required for translational repression[13], defective deadenylation in mutant embryos resulted in the presence of ectopic Nos protein throughout the embryo (Fig. 1d and Supplementary Fig. 1c). The presence of Nos in the anterior region results in the repression of *bicoid* and *hunchback* mRNA translation and in affected head skeleton. Consistent with previously mentioned defects[7], we found that the *piwi*^{1} mutant embryos that were able to produce a cuticle had head defects (Fig. 1e).

The piRNA pathway has a role during early oogenesis in preventing DNA damage, possibly through the repression of transposable element transposition. DNA double-strand breaks arising in mutants of the piRNA pathway correlate with affected embryonic axis specification, and this developmental defect is suppressed by mutations in the Chk2 DNA-damage signal transduction pathway[9,10]. We found that defects in *nos* mRNA deadenylation and decay observed in *aub* or *armi* mutants were not suppressed by Chk2 (*mnk*^{P6}) mutations, indicating that these defects did not result from activation of the Chk2 pathway earlier during oogenesis (Supplementary Fig. 3a–c). Moreover, affected deadenylation of *nos* mRNA in piRNA pathway mutants did not depend on *oskar* (Supplementary Fig. 3d).

We addressed a potential direct role of the piRNA pathway in the regulation of *nos* mRNA deadenylation and decay in the embryo. Aub and Piwi accumulate in the pole plasm and in pole cells of the embryo[25,26]. However, we found lower levels of Aub and Piwi throughout the entire embryo (Fig. 2a and Supplementary Figs 4 and 5). Ago3 was also present throughout the embryo (Supplementary Fig. 6a, c). Aub and Ago3 were found in the cytoplasm and accumulated in discrete foci, a distribution similar to that of CCR4 and Smg (Fig. 2b and Supplementary Fig. 6b). CCR4 and Smg were reported to partially colocalize in small cytoplasmic foci[13]. Aub and Ago3 also partially

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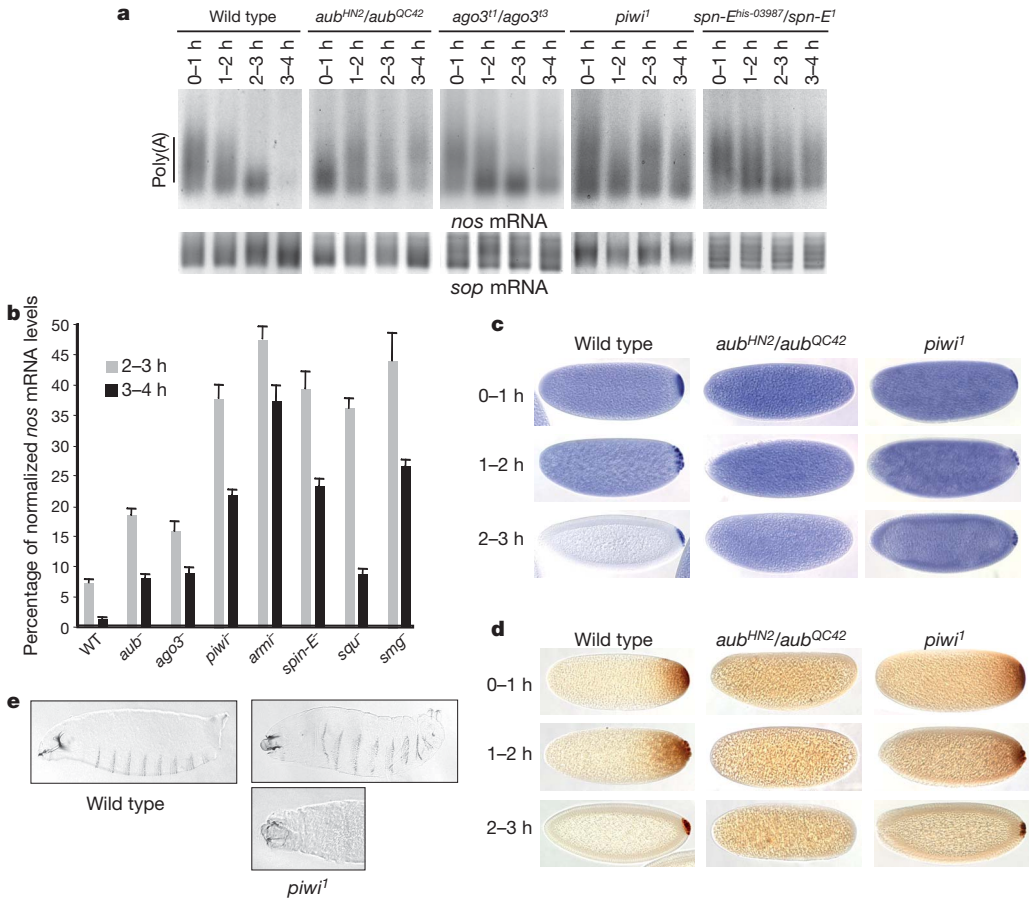


Figure 1 | The piRNA pathway is required for *nos* mRNA deadenylation and decay as well as translational repression in the bulk cytoplasm of the embryo. **a**, **b**, Poly(A) test assays and RT–qPCR of *nos* mRNA. Mutant females of the indicated genotypes were crossed with wild-type males. The *sop* mRNA was used as a control in **a**. **b**, Levels of *nos* mRNA in 2–3-h and 3–4-h embryos. WT, wild type. Mean value of three quantifications, error bars correspond to standard deviation (s.d.). **c**, *In situ* hybridizations of *nos* mRNA. **d**, Immunostaining of embryos with anti-Nos antibody. **e**, Cuticle preparations of $piwi^1$ embryos showing head defects (rudimentary head skeleton (top), head skeleton replaced by a hole (bottom)). **c**–**e**, Magnification of images is ×20. Two per cent of embryos from $piwi^1$ germline clones produced a cuticle ($n = 1{,}060$); among those, 22/23 had head defects. No embryos from aub^{N11}/aub^{HN2} ($n = 1{,}230$) or aub^{QC42}/aub^{HN2} ($n = 813$) females produced a cuticle.

colocalized with Smg and CCR4 in the bulk of syncytial embryos, in both cytoplasmic foci and a diffusely distributed cytoplasmic pool (Fig. 2b and Supplementary Fig. 6b). Importantly, the distributions of CCR4 and Smg depended on the piRNA pathway, as they were strongly affected in *aub* and *spn-E* mutant embryos. Although global amounts of CCR4 and Smg did not decrease in mutant embryos, CCR4 foci strongly increased in size, whereas Smg foci decreased in size or disappeared (Fig. 2c, d). This suggests that subsets of CCR4 and Smg foci have different functions and that deadenylation may take place diffusely in the cytoplasm. These results demonstrate a functional link between CCR4-mediated deadenylation and the piRNA pathway.

Co-immunoprecipitation experiments showed that Aub co-precipitated Smg, CCR4 and Ago3 in the absence of RNA, indicating the presence of these proteins in a common complex (Fig. 3a and Supplementary Fig. 7a, b). Smg also co-precipitated CCR4, Aub and Ago3 (Fig. 3b and Supplementary Fig. 7c); however, Piwi was not found to co-precipitate Smg or CCR4 (data not shown). Importantly, Smg, CCR4 and Ago3 also co-precipitated with Aub in osk^{54} mutant embryos that are defective in pole plasm assembly[27], indicating the presence of this complex outside the pole plasm (Fig. 3a). Next we showed that *nos* mRNA co-precipitated with Aub in both wild-type and osk^{54} embryos. The amount of *nos* mRNA was similar in Aub and Smg immunoprecipitates (Fig. 3c).

These findings show that the Argonaute proteins Aub and Ago3 associate with Smg and the CCR4 deadenylase complex to directly regulate *nos* mRNA in the bulk cytoplasm of early embryos.

The *nos* 3′ untranslated region (UTR) contains Smg-binding sites located in its 5′-most region (referred to as the translational control element (TCE))[16]. We searched for piRNAs sequenced from early embryos and presumed capable of targeting *nos* 3′ UTR based on their sequence complementarity. Notably, a specific region located in the 3′-most part of the 3′ UTR could be targeted by over 200 copies of piRNAs originating from two transposable elements, *412* and *roo* (Fig. 4a and Supplementary Fig. 8). piRNAs complementary to *nos* 3′ UTR were visualized by northern blots. In addition, piRNAs predicted to target *nos* 3′ UTR co-immunoprecipitated with Aub (Fig. 4b). We used *nos* genomic transgenes deleted for different parts of the 3′ UTR[16] to address the requirement of the corresponding regions for *nos* mRNA deadenylation. We have shown previously that the TCE (nucleotides 1–184) is required for *nos* mRNA poly(A) tail shortening, consistent with the role of Smg in this process[13]. Deletion of region 184–403 (*nos(Δ1)*) had no effect, whereas poly(A) tails from the transgene deleted for the region 403–618 (*nos(Δ2)*) were elongated in 3–4-h embryos (Fig. 4c and Supplementary Fig. 12). This could indicate regulation by the miRNA predicted by miRBase to target this region. Deletion of 618–844 in the *nos* 3′ UTR (*nos(Δ3)*) had a strong effect on *nos* deadenylation (Fig. 4c and Supplementary Figs 9 and 12). Consistent with this, *nos* mRNA levels produced by this transgene remained mostly stable (Fig. 4d). This resulted in defects in embryo patterning: a total of 35% ($n = 1{,}894$) of embryos from *nos(Δ3)* females did not hatch and among them 86% ($n = 28$) showed head skeleton defects (Fig. 4e). We next deleted specific sequences complementary to

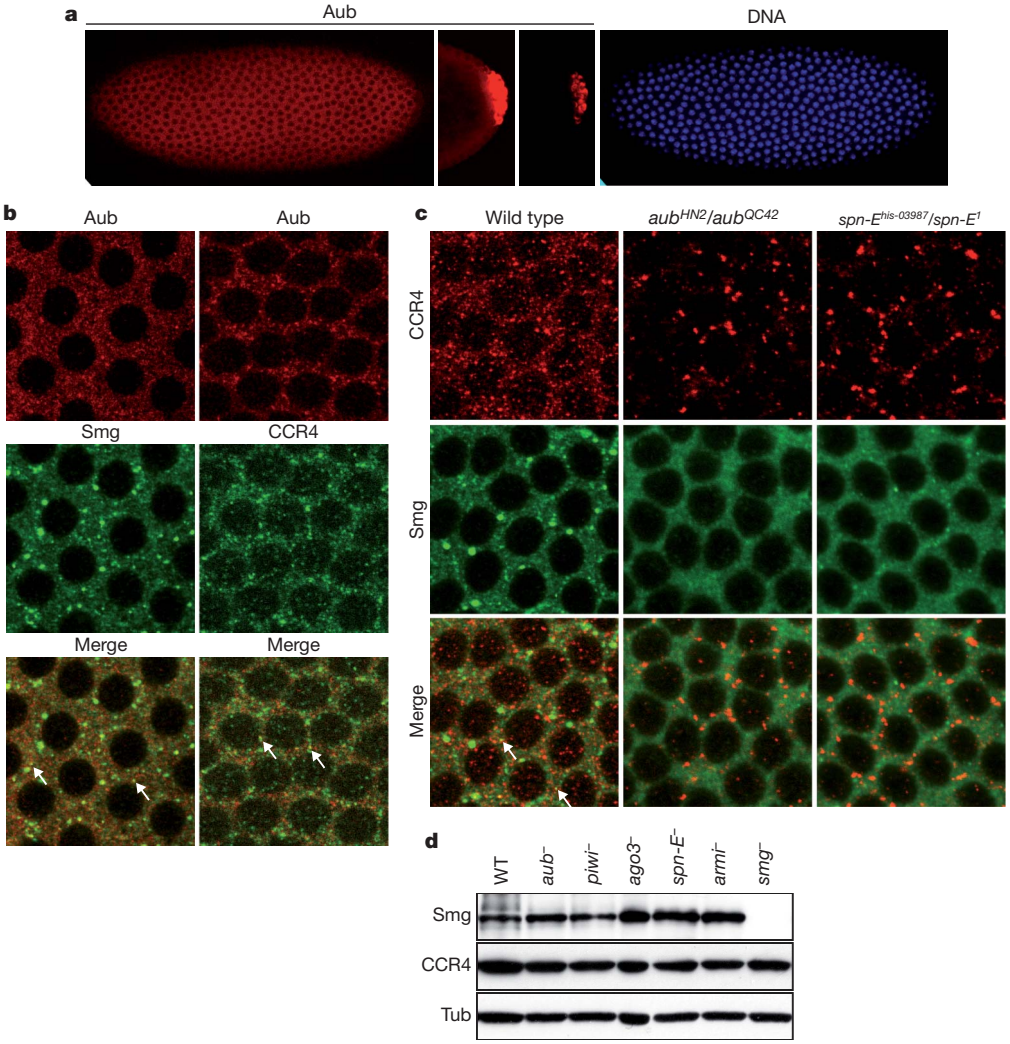
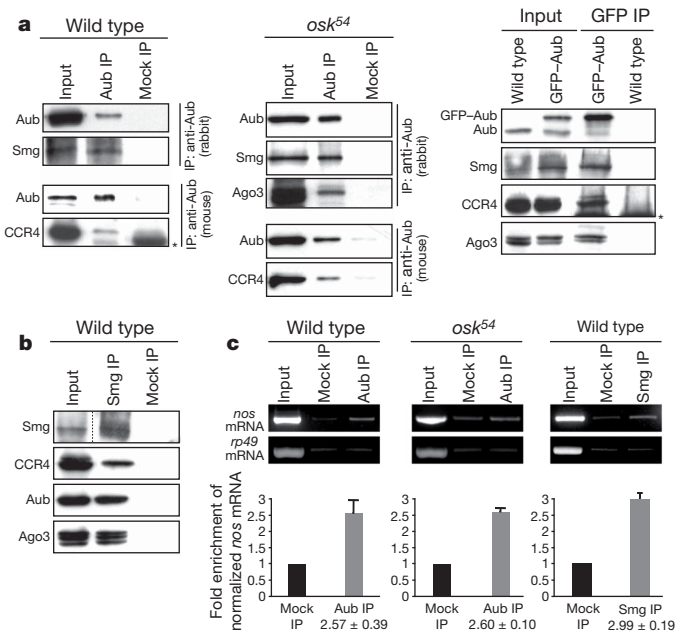


Figure 2 | Aub is present in the bulk of the embryo and the piRNA pathway is required for CCR4 and Smg cytoplasmic distributions. **a**, Confocal images of cytoplasmic expression of Aub in the embryo. Syncytial blastoderm embryo at nuclear cycle 11, anterior is to the left. Pole cells of the same embryo, at the same setting (middle Aub panel) and at lower intensity (right Aub panel)[20,25]. 4′,6-Diamidino-2-phenylindole (DAPI) staining (right panel). Magnification, ×20. **b**, Double immunostaining of embryos at nuclear cycles 11/12 with anti-Aub and anti-Smg, or anti-Aub and anti-CCR4. Arrows indicate examples of small foci showing colocalization in **b** and **c** (magnification, ×100). **c**, Smg and CCR4 cytoplasmic distributions are affected in *aub* and *spn-E* mutant embryos. Double immunostaining of embryos at nuclear cycle 11 with anti-CCR4 and anti-Smg. **d**, Western blots of proteins from 0–2-h embryos revealed with anti-Smg and anti-CCR4. α-Tubulin (Tub) was used as a loading control.

412 (15 nucleotides) and *roo* (11 nucleotides) retrotransposon piRNAs (Supplementary Fig. 8). These short deletions, either independently or in combination, affected *nos* mRNA deadenylation (Fig. 4f and Supplementary Fig. 12).



To support further the role of retrotransposon piRNAs in *nos* mRNA regulation, we blocked *412* and *roo* piRNAs by injecting specific 2′-O-methyl anti-piRNA in embryos[28], and recorded cuticles as a functional assay of Nos ectopic synthesis at the anterior pole. Injection of anti-piRNA(*412*) or anti-piRNA(*roo*) resulted in specific head development defects (Fig. 4g).

Together, these results provide strong evidence that an interaction between piRNAs and *nos* mRNA is required for *nos* mRNA deadenylation and translational repression in the first hours of embryogenesis.

We have identified a new function of the piRNA pathway in the regulation of maternal mRNAs. Recently, piRNAs derived from the 3′ UTRs of cellular transcripts have been identified in gonadal somatic cells, although their biological role has not been clarified[29,30]. Here we propose that piRNAs, in complex with Piwi-type Argonaute proteins Aub and Ago3, target *nos* maternal mRNAs and recruit or stabilize the CCR4–NOT deadenylation complex together with Smg (Supplementary

Figure 3 | Aub, Ago3, Smg, CCR4 and *nos* mRNA are present in a common complex in the bulk of the embryo. **a**, Co-immunoprecipitations of Smg, CCR4 and Ago3 with Aub in 0–2-h embryo extracts. Anti-Aub and anti-green fluorescent protein (GFP) were used for immunoprecipitations (IP) in wild-type, *osk*[54] and GFP–Aub-expressing embryos, respectively. The asterisks indicate immunoglobulins. **b**, Co-immunoprecipitations of CCR4, Aub and Ago3 with Smg in 0–2-h wild-type embryo extracts. **c**, Quantification of *nos* mRNA enrichment in Aub and Smg immunoprecipitations. Extracts from 0–2-h wild-type or *osk*[54] embryos were immunoprecipitated with anti-Aub (rabbit), or anti-Smg. For quantifications performed by RT–qPCR, the ratio of *nos* mRNA/*rp49* mRNA was set to 1 in the mock immunoprecipitation. Mean value of three quantifications, error bars correspond to s.d. *rp49* was used as a control mRNA.

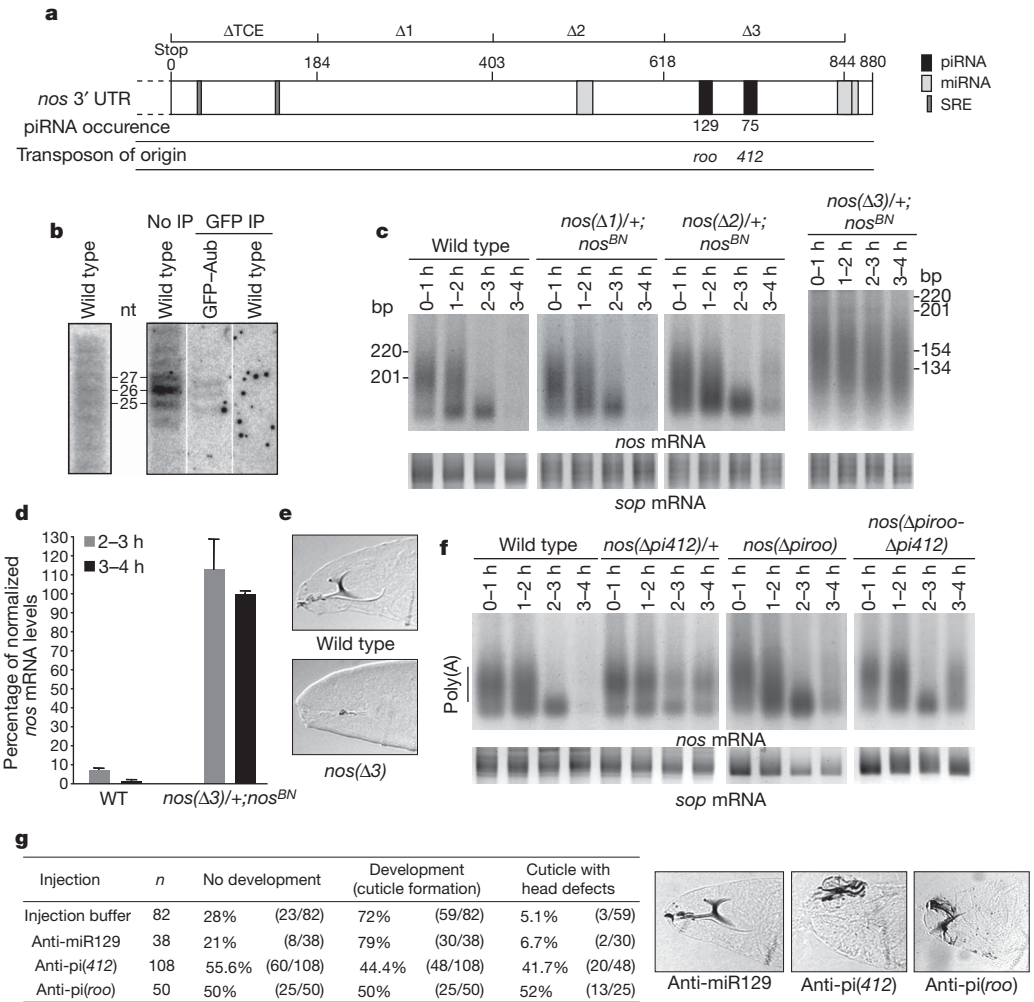


Figure 4 | piRNAs target a specific region in *nos* 3′ UTR that is required for *nos* mRNA deadenylation. **a**, Schematic representation of the *nos* 3′ UTR. The regions deleted in *nos* genomic transgenes (*nos(Δ)*) are indicated on the upper line[16]. The Smg recognition elements (SRE) and the piRNA and miRNA target sites are indicated. Predictions of miRNA-targeted regions are from miRBase (miR-31a, miR-314 and miR-263b from proximal to distal). piRNA occurrences in the data sets[19,20] are indicated. **b**, Northern blots of 0–2-h embryos probed with riboprobes corresponding to the sense *nos* 3′ UTR (position 403–844) (left) and to the antisense *412* piRNA (right). Anti-GFP immunoprecipitations (IP) were performed using wild-type and GFP–Aub-expressing embryos. **c**, *nos* poly(A) test assays. For *nos(Δ3)* the fragment amplified in the poly(A) test is shorter than the fragment amplified in the other *nos* poly(A) tests (Supplementary Fig. 9). bp, base pairs; nt, nucleotides. **d**, Quantification of *nos* mRNA levels from the *nos(Δ3)* transgene by RT–qPCR. Mean value of three quantifications, error bars correspond to s.d. **e**, Cuticle preparations of embryos from *nos(Δ3)* females (lack of head skeleton). **e**, **g**, Magnification, ×20. **f**, *nos* poly(A) tests from embryos containing *nos* genomic transgenes in which sequences complementary to *412* piRNA, *roo* piRNA, or both sequences have been deleted. The *sop* mRNA was used as a control in **c** and **f**. **g**, Injection of 2′-O-methyl anti-piRNA in embryos. Control injections were with injection buffer alone or with the irrelevant anti-miR129. Examples of cuticles following injections of anti-miR129 (wild-type head skeleton), anti-pi(*412*) and anti-pi(*roo*) (affected head skeleton).

Fig. 10). These interactions induce rapid mRNA deadenylation and decay. Thus, activation of mRNA deadenylation represents a new direct mechanism of action for the piRNA pathway with an essential developmental function during the first steps of embryogenesis.

Smg is a general factor for mRNA decay during early embryogenesis[12]. Because Aub and Ago3 are present in a complex with Smg in early embryos, a proportion of Smg mRNA targets could be regulated by the piRNA pathway. Consistent with this, other maternal mRNAs that are destabilized during early embryogenesis are targeted by abundant piRNAs and their deadenylation depends on the piRNA pathway (Supplementary Fig. 11).

These piRNAs involved in gene regulation are generated from transposable element sequences. Although transposable elements have been described to be essential for genome dynamics and evolution, their immediate function within an organism has remained rather elusive. This study provides evidence for a co-evolution between transposable elements and the host genome and reveals the direct developmental function of transposable elements in embryonic patterning, through the regulation of gene expression.

METHODS SUMMARY

RNA and proteins were manipulated using methods described previously and reported in Methods.

Embryo injections. Injections of embryos were performed laterally with 400 μM of 2′-O-methyl oligonucleotides as reported previously[28]. The injection buffer was 0.5 mM $NaPO_4$, 5 mM KCl. Sequences of 2′-O-methyl oligonucleotides are indicated in Methods.

Bioinformatics. A total of 29,108,987 piRNAs sequenced from 0–1-h embryos (GSM286613 and GSM286604 data sets[19]) and from 0–2-h embryos (GSM327625, GSM327626, GSM327627, GSM327628 and GSM327629 data sets[20]) was blasted against *nos* 3′ UTR using the following parameters: a National Center for Biotechnology Information (NCBI) blast with an *E* value of 100 and a 14-nucleotide match and a Washington University (WU)-blast with an *E* value of 10 and an 11-nucleotide match. Regions potentially targeted by piRNAs with an occurrence of less than ten were not considered.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions C.R. and C.P. designed and performed the experiments, analysed the data and contributed equally to the study. A.-C.M. contributed to the generation of DNA constructs, B.F. contributed to poly(A) test assays in Fig. 1. A.B., N.R. and E.C.L. performed the bioinformatic analyses. A.P. performed northern blots. M.S. designed the study, analysed data and wrote the paper. All authors discussed the results and commented on the manuscript.

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METHODS

***Drosophila* stocks and genetics.** The w^{1118} stock was used as a control. Mutant stocks were: *w; Sp* aub^{NT1} *bw/CyO*, aub^{HN2} cn^1 bw^1*/CyO*, aub^{QC42} cn^1 bw^1*/CyO* (ref. 31), mnk^{P6}, mnk^{P6} aub^{HN2}*/CyO*, mnk^{P6} aub^{QC42}*/CyO* (ref. 9), mnk^{P6}*; armi*^{1}*/SM6-TM6B*, mnk^{P6}*; armi*$^{72.1}$*/SM6-TM6B* (ref. 9), *piwi*^{1} *neo-FRT40A/CyO* (ref. 7), *spn-E*^{1}*/TM3*, ry^{506} *spn-E*$^{hls\text{-}03987}$ *e/TM3* (ref. 32), *armi*^{1}*/TM3*, *armi*$^{72.1}$*/TM3* (ref. 23), cn^1 bw^1 squ^{HE47}*/CyO*, cn^1 bw^1 squ^{PP32}*/CyO* (ref. 10), *bw; st ago3*^{t1}*/TM6B Tb*, *bw; st ago3*^{t3}*/TM6B Tb* (ref. 22), osk^{54} *spn-E*^{1}*/TM3*, osk^{54} *spn-E*$^{hls\text{-}03987}$ *e/TM3*. *piwi*^{1} mutant embryos are from germline clones that were induced with two 1.5-h heat shocks at 37 °C during the second- and third-instar larval stages, using the flippase-dominant female sterile technique[33]. *smg* mutants were *smg*^{1} and a deficiency overlapping *smg*, *Df(Scf*^{R6}*)* (ref. 17). The nos^{BN} mutant does not produce *nos* mRNA[34]. osk^{54} is a null allele. GFP–Aub was expressed following crosses between the germline driver *nos-Gal4:VP16* (ref. 35) and *UASp-GFP-Aub* (ref. 25). *nos(Δ)* stocks are transgenic lines containing a *nos* genomic transgene in which different parts of the 3′ UTR have been removed[16]. *nos(Δ)* stocks were a gift from R. Wharton. The *nos(Δpi412)* and *nos(Δpiroo)* transgenes, in which 15 nucleotides (TATATTTATTCAATT) and 11 nucleotides (AACACACATAT) were deleted, respectively, were generated as follows. The pBSKS-R5561 (containing a 5.7-kb *nos* genomic fragment, a gift from R. Wharton) was used as a template for PCR reactions to produce the deletion. For each construct, two PCR reactions were performed using the following primers: for *nos(Δpi412)*, 5′-CATTCCGATC AAAGCTGGGTTAACC (primer 1) and 5′-AAATTGATCAATGGTAAACAA TAACATATATATAT, which contains the 15-nucleotide deletion; and 5′-TA TATATATATATATATGTTATTGTTTACCATTGATCAATTT, which contains the 15-nucleotide deletion, and 5′-CTCCACCGCGGTGGCGGCCGC (primer 2). For *nos(Δpiroo)*, primer 1 and 5′-TATATATATATATATATATAGGAAATGAA TACTTGCGATACA, which contains the 11-nucleotide deletion; and 5′-TG TATCGCAAGTATTCATTTCCTATATATATATATATATATA, which contains the 11-nucleotide deletion and primer 2. For each construct, the two PCR products were annealed and used as a template for a third PCR reaction using primers 1 and 2. This third PCR product containing either the 15-nucleotide or the 11-nucleotide deletion surrounded by the restriction sites BglII and NotI was cloned into the TAcloning vector (pCRII) (Invitrogen) and sequenced. For the *nos(Δpiroo-Δpi412)* transgene, the PCR generating the *Δpiroo* deletion was done using pCRII containing the *nosΔpi412* deletion as a template. The BglII–NotI fragment containing the deletion was used to replace the BglII–NotI fragment in the original pBSKS-R5561. An EcoRI–NotI fragment containing the whole genomic fragment with the deletion was cloned into the pCaSpeR4. Transformant stocks were produced by BestGene.

Embryo injections. Sequences of 2′-O-methyl oligonucleotides were as follows. anti-pi(*412*), UCGGGCUGACAUAUAUUUAUUCAAUU; anti-pi(*roo*), UCCA AACACACAUAUAUAUAUAAAUA; anti-miR129-1, GCAAGCCCAGACCG CAAAAAG (human miR129-1 is not conserved in *Drosophila*).

RNA. Poly(A) test assays, RT–PCR and RT–qPCR were performed as described previously[13,36], and were made from two-to-four independent RNA preparations. For the *nos(Δ3)* transgene, a different *nos* specific primer was used (5′-GTC GTCGGCTACGCATTCATTGT), as the region normally amplified in *nos* poly(A) test assays is deleted in this transgene. We verified by sequencing that the poly(A) site used in mRNA from this transgene is identical to the one used in *nos* endogenous mRNA. Real-time PCR (qPCR) was performed with the LightCycler System (Roche Molecular Biochemical) using *rp49* as a control mRNA[13]. For quantification of *nos* mRNA in 2–3-h and 3–4-h embryos, the levels were normalized with the levels of *nos* mRNA in 0–1-h embryos that were set to 100% for each genotype. Northern blots were performed as described previously[37]. The sequence of the riboprobe specific to *412* piRNA was 5′-GGGCUGAC AUAUAUUUAUUCAAUU.

RNA *in situ* hybridization and cuticle preparations. Whole-mount *in situ* hybridization and cuticle preparations were performed by standard methods. The probe for *in situ* hybridization was an antisense RNA made from the pN5 *nos* complementary DNA clone.

Antibodies, western blots, immunostaining and immunoprecipitations. Immunoprecipitations were performed as described previously[13] using 0–2-h embryos, and mouse anti-Aub (4D10 (ref. 21)), mock immunoprecipitations: mouse anti-haemagglutinin (12CA5 Developmental Studies Hybridoma Bank, for wild-type embryos) and mouse IgG (sc-2025 Santa Cruz Biotechnology, for osk^{54} embryos); rabbit anti-Aub (Abcam, ab17724), mock immunoprecipitations: rabbit IgG (sc-2027 Santa Cruz Biotechnology); mouse anti-GFP (monoclonal antibody 3E6 Invitrogen); guinea pig anti-Smg (gift from C. Smibert), mock immunoprecipitations: guinea pig pre-immune serum. Protein co-immunoprecipitations were performed in the presence of 0.1 μg μl^{-1} RNase A. Western blots and immunostaining were performed as reported[38,39]. Antibodies for western blots were used at the following dilutions: guinea pig anti-Smg 1:5,000, anti-CCR4 1:1,000[40], anti-Piwi 1:20 (P4D2 (ref. 1)), anti-Aub 1:1,500 (4D10 (ref. 21)) and anti-Ago3 1:500 (9G3 (ref. 21)). Antibodies for immunostaining were used at the following dilutions: rabbit anti-Nos 1:1,000 (gift from A. Nakamura), guinea pig anti-Smg 1:1,000, anti-CCR4 1:300, anti-Aub 1:1,500 (4D10), anti-Piwi 1:1 (P4D2) and anti-Ago3 1:300 (9G3).

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