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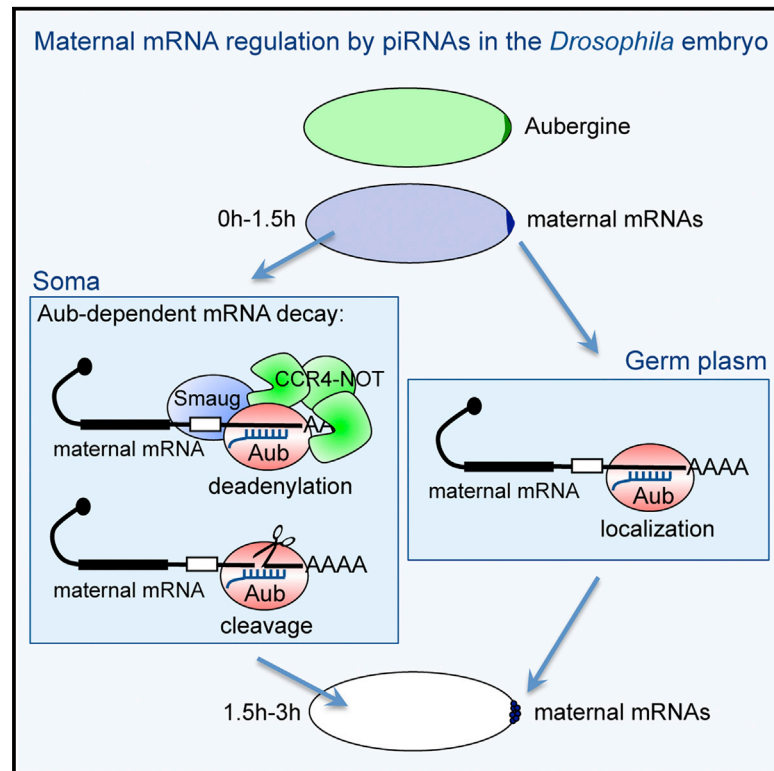


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Aubergine iCLIP Reveals piRNA-Dependent Decay of mRNAs Involved in Germ Cell Development in the Early Embryo

Graphical Abstract



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In Brief

Using iCLIP, Barckmann et al. identify several hundred maternal mRNAs that interact with Aub in early embryos. A number of these mRNAs undergo Aub-dependent destabilization in the soma during the maternal-to-zygotic transition and encode germ cell determinants.

Highlights

- Aub binds to maternal mRNAs in early *Drosophila* embryos
- Interaction between Aub and maternal mRNAs depends on piRNAs
- *aub* mutants are defective in mRNA decay during the MZT
- Aub-dependent unstable mRNAs encode germ cell determinants

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Aubergine iCLIP Reveals piRNA-Dependent Decay of mRNAs Involved in Germ Cell Development in the Early Embryo

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SUMMARY

The Piwi-interacting RNA (piRNA) pathway plays an essential role in the repression of transposons in the germline. Other functions of piRNAs such as post-transcriptional regulation of mRNAs are now emerging. Here, we perform iCLIP with the PIWI protein Aubergine (Aub) and identify hundreds of maternal mRNAs interacting with Aub in the early *Drosophila* embryo. Gene expression profiling reveals that a proportion of these mRNAs undergo Aub-dependent destabilization during the maternal-to-zygotic transition. Strikingly, Aub-dependent unstable mRNAs encode germ cell determinants. iCLIP with an Aub mutant that is unable to bind piRNAs confirms piRNA-dependent binding of Aub to mRNAs. Base pairing between piRNAs and mRNAs can induce mRNA cleavage and decay that are essential for embryonic development. These results suggest general regulation of maternal mRNAs by Aub and piRNAs, which plays a key developmental role in the embryo through decay and localization of mRNAs encoding germ cell determinants.

INTRODUCTION

In most species, the first steps of embryonic development depend on maternally loaded mRNAs and proteins. The control of development then switches from the maternal to the zygotic genome during the maternal-to-zygotic transition (MZT), during which maternal mRNAs are massively degraded (De Renzi et al., 2007). In *Drosophila*, the RNA-binding protein Smaug (Smg) plays a major role in this general maternal mRNA decay (Chen et al., 2014; Tadros et al., 2007). Smg binds to mRNAs

containing Smg recognition elements (SREs) and induces their deadenylation and decay by recruiting the CCR4-NOT deadenylation complex (Semotok et al., 2005; Zaessinger et al., 2006). Previously, we reported that the Piwi-interacting RNA (piRNA) pathway cooperates with Smg for maternal mRNA deadenylation and decay, prior to zygotic transcription. The piRNA pathway plays an essential role in the regulation of the posterior determinant Nanos (Nos) (Rouget et al., 2010).

piRNAs are a specific class of small 23–30 nt non-coding RNAs loaded into Argonaute proteins of the PIWI clade (Guzardo et al., 2013; Ishizu et al., 2012). A prominent function of the piRNA pathway is the repression of the expression and transposition of transposable elements (TEs) in the germline. A large proportion of piRNAs ($\approx 70\%$) derives from TE sequences in *Drosophila* ovaries. These piRNAs target TE mRNAs through complementarity and guide their cleavage by the cytoplasmic PIWI proteins Aubergine (Aub) and Argonaute 3 (Ago3) bound to the piRNAs.

Recently, further evidence has emerged for additional functions of the piRNA pathway (Peng and Lin, 2013; Watanabe and Lin, 2014; Weick and Miska, 2014). Variable fractions of piRNAs are produced from protein-coding mRNAs, with a bias toward 3' UTR, both in mouse testes and in *Drosophila* ovaries and early embryos. The function of these genic piRNAs remains to be investigated, although their production might decrease the levels of mRNAs from which they originate (Robine et al., 2009; Saito et al., 2009).

Pachytene piRNAs, which account for more than 95% of piRNAs in the adult mouse testis, do not derive from TE sequences but mostly from intergenic regions (Beyret et al., 2012; Li et al., 2013). Recently, pachytene piRNAs have been shown to mediate global mRNA decay in spermatocytes and spermatids (Goh et al., 2015; Gou et al., 2014; Watanabe et al., 2015; Zhang et al., 2015). Pachytene piRNAs loaded into MIWI, the mouse homolog of Aub, target spermiogenic mRNAs with imperfect base pairing and induce their decay either through

MIWI-dependent cleavage or by assembling a complex containing CAF1, a deadenylase in the CCR4-NOT complex.

Another compelling example of mRNA regulation by a piRNA has been reported for sex determination in *Bombyx mori*, where a female-specific piRNA plays a vital role by inducing cleavage of a cellular mRNA involved in masculinization of the embryos (Kiu-chi et al., 2014).

In the *Drosophila* embryo, piRNAs target *nos* maternal mRNA and contribute to its decay in the somatic region. *Nos* is expressed as a gradient emanating from the posterior pole of the embryo and is essential for abdominal segmentation and germ cell development. *nos* mRNA is present throughout the embryo, although it is translationally repressed and degraded in the somatic part whereas stabilized and translated in the germ plasm (i.e., the cytoplasm localized at the posterior pole that is required for germ cell specification). Two piRNAs produced from TEs target the *nos* 3' UTR with imperfect base pairing and guide interactions with Aub and Ago3, which in turn recruit the CCR4-NOT deadenylation complex, together with Smg. These interactions lead to *nos* mRNA translational repression and decay in the soma and are required for embryonic patterning (Rouget et al., 2010).

Here, we use Aub iCLIP (individual-nucleotide resolution UV crosslinking and immunoprecipitation) (König et al., 2010) to address a potential general role of Aub and piRNAs in maternal mRNA regulation in the early embryo. This approach identifies several hundred mRNAs that directly interact with Aub in 0- to 2-hr embryos. Gene-expression profiling reveals that one-third of these mRNAs undergo decay at the MZT in wild-type embryos and are stabilized in embryos from *aub* and *spn-E* mutant females. The RNA helicase Spn-E has a prominent role in germline piRNA production (Malone et al., 2009). Strikingly, Aub- and Spn-E-dependent unstable mRNAs are enriched in mRNAs that are stabilized in the germ plasm and that encode germ plasm components involved in germ cell specification and development. This reveals a role of Aub in the localization of these mRNAs to the germ plasm through their selective decay in the somatic part of the embryo. Bioinformatic analyses, as well as iCLIP with a PAZ-domain mutant form of Aub that is unable to load piRNAs, are consistent with the requirement of piRNAs in Aub-mRNA interactions. In addition, base pairing of maternal mRNAs with piRNAs can induce the production of genic piRNAs by cleavage, in a process similar to the ping-pong occurring with TEs, which leads to functional mRNA downregulation.

These results reveal a general role for Aub and piRNAs in the regulation of maternal mRNAs in the embryo. This piRNA-dependent regulation plays a key role in the decay and localization of mRNAs involved in germ cell development.

RESULTS

Aub Directly Binds Maternal mRNAs in Embryos

To identify maternal mRNAs directly interacting with Aub, we performed iCLIP of GFP-Aub in 0- to 2-hr embryos using anti-GFP antibodies. This technique allows mapping RNA-protein interactions at individual nucleotide resolution (König et al., 2010). The GFP-Aub transgene (Harris and Macdonald, 2001) was able to rescue the *aub* mutant maternal phenotypes of embryonic

lethality and fused dorsal appendages to a large extent, and it was not detrimental when expressed in wild-type embryos (Figure S1A). We performed three independent iCLIP experiments (Figure 1A). The protein-RNA complexes were absent when anti-GFP was replaced with rabbit serum, as well as when UV crosslinking was omitted, indicating that the protein-RNA complexes purified in the experiment were covalent complexes depending on UV. Crosslinked RNA was reverse transcribed and PCR amplified, and the resulting DNA was submitted to high-throughput sequencing. Sequence duplicates due to PCR amplification were eliminated by removing identical sequences linked to the same random barcode primer. High-throughput sequencing of the three biological replicates using Illumina HiSeq 2000 generated 32.9, 36.3, and 26.1 million reads, which identified, after removal of PCR duplicates 651,699, 265,646 and 2,620,195 reads mapping to the *Drosophila* genome, respectively (Table S1). Each of these reads represents a uniquely crosslinked RNA molecule.

Although crosslink efficiency was variable between replicates, the reproducibility of crosslinks was validated by a peak of crosslink positions reproduced in an independent experiment at the same exact position and with an offset of a few nt (Figure S1B). A similar offset by a few nt has previously been observed in iCLIP experiments. It was proposed to result from contacts of the protein to more than 1 nt of the RNA and/or from the steric hindrance of the remaining peptide on the RNA, which might cause an imprecise termination of reverse transcription (König et al., 2010). We also determined that the reproducibility between experiments increased with the incidence of crosslinks at the same position within one experiment (cDNA count), showing that the strongest crosslink sites were the most reproducible (Figure 1B).

Because Aub is thought to directly interact with piRNAs and mRNAs, sequence reads from both types of molecules were expected to be present in GFP-Aub iCLIPs. This technique is based on the truncation of cDNAs at the crosslink site; therefore, the recovery of complete piRNAs in Aub iCLIPs was not expected. However, length analysis of reads mapping to piRNA clusters revealed peaks of 23- to 27-nt reads, suggesting the presence of full-length piRNAs in GFP-Aub iCLIPs (Figures 1C and S1C). Analysis of nt distribution of reads 23–29 nt in length identified a 1U bias (Figures 1D and S1D), in agreement with a proportion of full-length piRNAs being recovered in GFP-Aub iCLIPs. These piRNAs would be crosslinked through their 5' phosphate, consistent with the interactions between this phosphate and the MID domain of Argonaute proteins (Schirle and MacRae, 2012). The presence of reads corresponding to piRNAs validated the ability of GFP-Aub iCLIP to identify RNA molecules specifically interacting with Aub.

The mapping of unique-mapped and multi-mapped reads to the genome identified both sequences related to TE piRNAs (i.e., reads mapping to piRNA clusters and TEs referred to as piRNA reads), as well as sequences corresponding to cellular mRNAs (Figure S1E; Table S1). The proportion of reads in each of these categories was variable between iCLIP replicates, due in part to size variations in the RNA-protein complexes selected from the gels (Figure 1A). piRNA reads were enriched in short size reads (86%–96% of piRNA reads were 12–29 nt in length,

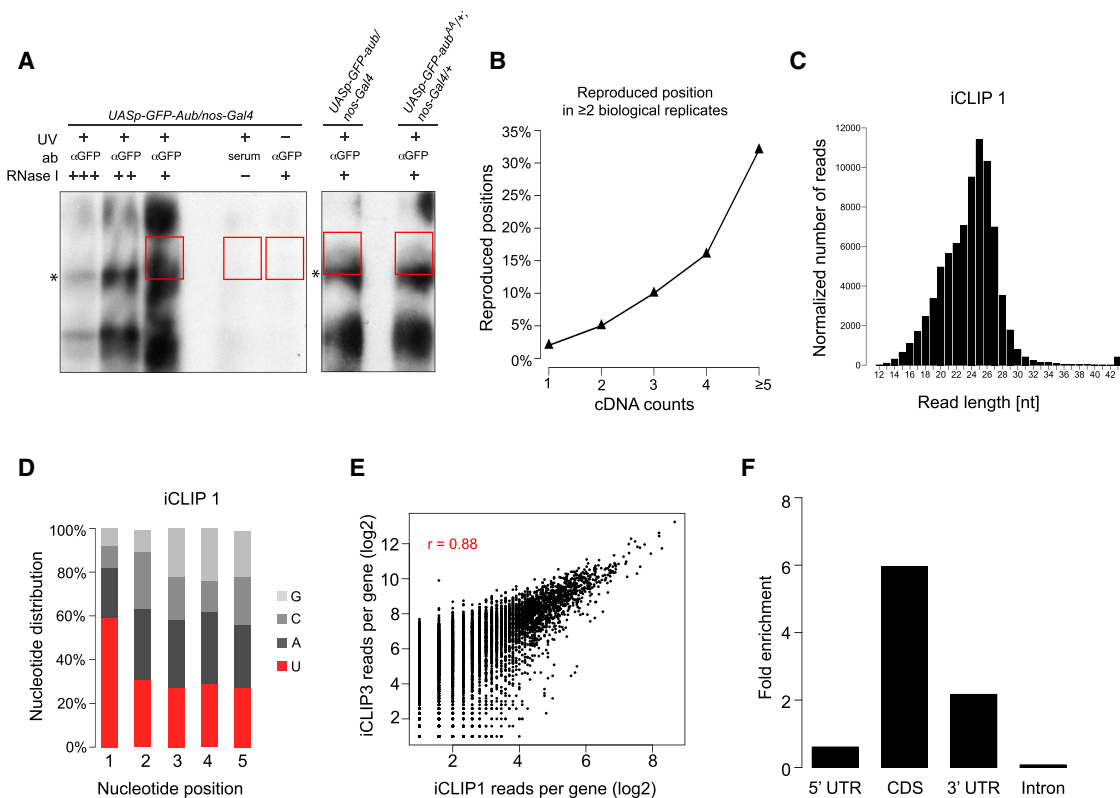


Figure 1. Aub iCLIP Reveals Direct Interactions of Aub with piRNAs and Cellular mRNAs

(A) 32 P-labeled Aub-RNA complexes showing decreasing size with increasing amounts of RNase I (+ to +++). No complexes were formed in the absence of UV or when rabbit serum was used for immunoprecipitation. The gel on the right represents an independent experiment in which embryos expressing GFP-Aub and GFP-Aub^{AA} were used in parallel. The asterisks indicate the size of Aub, whose presence was validated by western blot. The red squares indicate the regions of the membrane cut out for RNA extractions.

(B) Reproducibility of crosslink positions. Graph of the percentages of crosslinked nt with a given cDNA count that were reproduced in at least two biological replicates.

(C) Size distribution of reads from iCLIP1 that map to piRNA clusters.

(D) nt distribution in 23- to 29-nt reads mapping to piRNA clusters. The 1U bias is consistent with a proportion of these reads corresponding to full-length piRNAs.

(E) Plots of the number of uniquely mapped reads per gene in two iCLIP biological replicates. Each dot represents one gene. Pearson's correlation coefficient (r) is indicated.

(F) Fold enrichment of reproduced crosslinks in gene regions, relative to the size of the corresponding regions in the whole genome.

See also Figure S1 and Tables S1–S3 and S4.

in iCLIP1–3), and the higher proportion of long reads in iCLIP3 (84% >29 nt versus 33% in iCLIP2 and 17% in iCLIP1) correlated with a smaller proportion of piRNA reads.

We focused on cellular mRNAs and identified a positive correlation (Pearson's correlation coefficient $r = 0.88$) between the numbers of unique-mapped reads per gene in independent iCLIP experiments, indicating that the specificity of GFP-Aub iCLIP also applied to cellular mRNAs (Figure 1E). To differentiate robust reproducible from transient Aub-mRNA interactions, we considered crosslink sites with a cDNA count of at least five within an iCLIP experiment. Crosslink sites were reproduced with an offset of a few nt (Figure S1B); therefore, this cDNA count was scored within clusters of ± 5 nt of each crosslink position (Änkö et al., 2012). These criteria identified 1,778, 1,473, and 3,744 genes showing at least one cluster with a cDNA count greater than or equal to five in iCLIP1, 2, and 3, respectively

(Table S2). Reproducibility between iCLIP biological replicates was then analyzed, and clusters with greater than or equal to five cDNA counts reproduced in at least two independent iCLIP experiments were selected (Table S3). This identified 1,594 reproduced clusters and a total of 634 genes (Table S4; Figure S1F). Among these genes, only five (*ari-1*, CG3812, CG8765, CG30497, and CG45186) had a TE insertion in the vicinity (± 60 nt) of crosslink sites (see below), indicating that interactions between Aub and maternal mRNAs were largely independent of TE insertions. Analysis of reproduced crosslink distribution along genes revealed an enrichment in coding sequences and 3' UTR and a depletion in 5' UTR (Figure 1F).

We conclude that GFP-Aub iCLIP in early embryos allows specific identification of RNA directly bound by Aub. This includes complete piRNAs as well as several hundred maternal mRNAs.

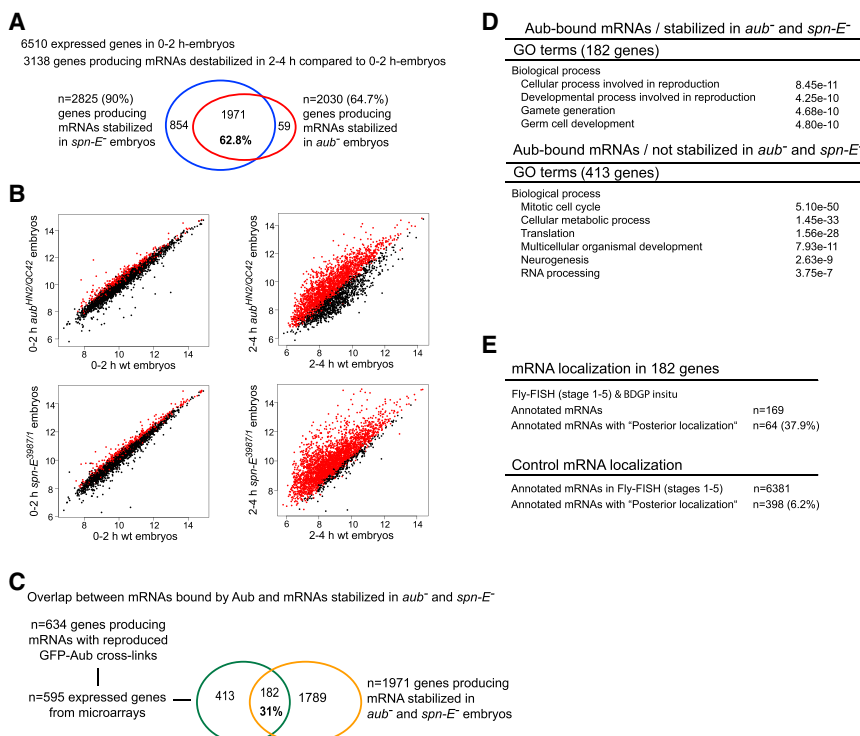


Figure 2. Aub Binds Maternal mRNAs Involved in Germ Cell Development

(A) Summary of mRNA profiling in wild-type, *aub*^{H²}/*aub*^{QC42}, and *spn-E*³⁸⁸⁷¹/*spn-E*¹ embryos using microarrays, including the Venn diagram of mRNAs stabilized in *aub* and *spn-E* 2- to 4-hr mutant embryos.

(B) Plots comparing unstable mRNAs (3,138 genes), in wild-type and *aub* or *spn-E* mutant embryos, at 0–2 hr and 2–4 hr. Each dot represents one gene; red dots represent upregulated genes in mutant embryos.

(C) Venn diagram of mRNAs interacting with Aub and mRNAs stabilized in *aub* and *spn-E* mutant embryos.

(D) GO term analysis of mRNAs bound by Aub, stabilized (top) or not (bottom) in *aub* and *spn-E* mutant embryos.

(E) mRNAs bound by Aub and stabilized in *aub* and *spn-E* mutant embryos accumulate at the posterior pole. Annotation from Fly-FISH and BDGP insitu expression patterns were used to determine mRNA localization.

See also Table S4.

mRNAs Interacting with and Destabilized by Aub Are Involved in Germ Cell Development

Because Aub binding to *nos* mRNA leads to its decay in the somatic part of the embryo (Rouget et al., 2010), we sought to identify maternal mRNAs whose decay during the MZT depended on Aub or the piRNA pathway. We used microarrays to identify unstable mRNAs in wild-type embryos by comparing the transcriptomes derived from 0- to 2-hr and 2- to 4-hr embryos. mRNAs from 6,510 genes were present in 0- to 2-hr embryos, consistent with about half of *Drosophila* genes being maternally expressed (Thomsen et al., 2010). Microarray analyses identified 3,138 genes whose mRNAs were significantly destabilized in 2- to 4-hr embryos (fold change 1.2–151), representing 48.2% of maternally expressed genes. We then analyzed which of these unstable mRNAs are stabilized in embryos from *aub* or *spn-E* mutant mothers (*aub* or *spn-E* mutant embryos) by comparing mutant and wild-type embryonic transcriptomes. In 0- to 2-hr embryos, 547 and 571 genes produced unstable mRNAs that were stabilized in *aub* or *spn-E* mutant embryos, respectively. In 2- to 4-hr embryos, the number of stabilized mRNAs in *aub* and *spn-E* mutants increased (Figures 2A and 2B). This is consistent with maternal mRNA decay being more prominent after 2 hr of development (Thomsen et al., 2010) and with the role of Aub and the piRNA pathway in this decay, both directly and indirectly through developmental defects in the mutants, affecting mRNA decay.

The overlap between mRNAs bound by Aub and those stabilized in *aub* and *spn-E* mutant embryos identified mRNAs directly regulated by Aub. Among the 595 genes that were maternally expressed from microarray analysis and reproducibly crosslinked in iCLIP experiments, 182 (31%) produced mRNAs stabilized in

aub and *spn-E* mutant embryos (Figure 2C; Table S4). Strikingly, Gene Ontology (GO) term enrichment analysis using FlyMine (<http://www.flymine.org>)

with a p value < 0.05 (Benjamini corrected) identified terms related to “germ cell development” as strongly enriched in these mRNAs (Figure 2D). Maternal mRNAs involved in germ cell development often accumulate at the posterior pole of the embryo, raising the possibility that Aub-mediated mRNA degradation could contribute to subcellular mRNA localization. We used Fly-FISH (<http://fly-fish.cabr.utoronto.ca>; Lécuyer et al., 2007) and BDGP expression patterns (BDGP insitu; <http://insitu.fruitfly.org/cgi-bin/ex/insitu.pl>) to obtain information on the localization patterns of mRNAs bound and regulated by Aub. Among the 182 genes, 169 were annotated in Fly-FISH or BDGP insitu and 64 (37.9%) produced mRNAs that localized in the germ plasma or primordial germ cells (Figure 2E; Table S4). This represents a high enrichment of posteriorly localized mRNAs as compared to posteriorly localized mRNAs among all maternal mRNAs (6.2%; Figure 2E). Moreover, a number of these genes are known determinants of germ cell specification or development, such as *oskar* (*osk*) (Ephrussi et al., 1991; Kim-Ha et al., 1991), *germ cell-less* (*gcl*) (Jongens et al., 1994), *polar granule component* (*pgc*) (Hanyu-Nakamura et al., 2008), and *Hsp83* (Song et al., 2007), in addition to *nos* (Table S4).

Fifteen mRNAs were selected to validate the role of Aub and Spn-E in their destabilization using qRT-PCR (Figures 3A, 3B, S2A, and S2B). In piRNA pathway mutants, TE deregulation induces developmental defects through activation of the Chk2-dependent DNA-damage checkpoint. We verified that mRNA stabilization recorded in *aub* and *spn-E* mutant embryos did not result from developmental defects, either depending or not on activation of the checkpoint. qRT-PCR experiments were performed using either *mnk aub* and *mnk spn-E* double-mutant

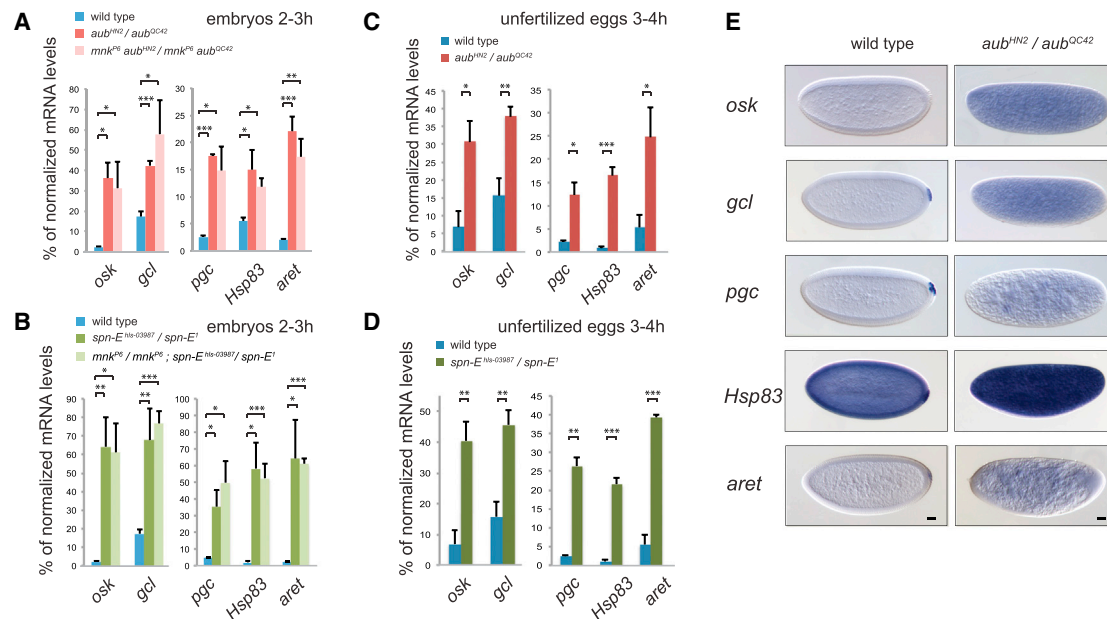


Figure 3. Validation of Maternal mRNA Regulation by Aub

(A–D) mRNA quantification using qRT-PCR in wild-type and mutant 2- to 3-hr embryos (A and B) or 3- to 4-hr unfertilized eggs (C and D). *RpL32* was used as a control mRNA for normalization. The levels of mRNA at 2 to 3 hr or 3 to 4 hr were normalized to the levels at 0 to 1 hr set to 100% for each genotype. The genotypes of females are indicated. Females were either crossed or not with wild-type males. Means are from two to four biological replicates. The error bars represent SE. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ with a one-tailed Student's *t* test.

(E) In situ hybridization of mRNAs analyzed in (A)–(D) in 2- to 4-hr wild-type and *aub* mutant embryos. The scale bars represent 30 μ m.

See also Figures S2 and S7.

embryos (*mnk* encodes the Chk2 kinase) or unfertilized wild-type and mutant eggs. mRNA stabilization in *mnk aub* and *mnk spn-E* double mutants and in *aub* and *spn-E* unfertilized eggs was consistent with the role of Aub and the piRNA pathway in the degradation of these mRNAs, independently of embryonic developmental defects (Figures 3A–3D and S2). mRNA stabilization throughout *aub* mutant embryos was also validated using in situ hybridization experiments for five mRNAs (Figure 3E).

Among the 413 mRNAs that are bound by Aub, but not stabilized in *aub* and *spn-E* mutant embryos, 18% ($n = 74$) were destabilized in wild-type embryos from the microarray analysis and 61% ($n = 254$) were stable according to the study of maternal mRNAs at the MZT (Thomsen et al., 2010) (Table S4). mRNAs from these 413 genes were enriched in GO terms involved in cell cycle, metabolic processes, and developmental processes occurring during later embryogenesis, such as neurogenesis or organ development (Figure 2D).

These results show that Aub directly binds a number of maternal mRNAs involved in germ cell development, which accumulate in the germ plasm and primordial germ cells. Aub participates in their localization by promoting their decay in the somatic part of the embryo. Aub also binds to a set of stable mRNAs and might mediate their regulation through a different mechanism.

Implication of piRNAs in the Binding of Aub to Maternal mRNAs

We have shown previously that Aub and Smg form a complex involved in *nos* mRNA destabilization in the somatic part of the

embryo (Rouget et al., 2010). Smg binds to stem-loop structures, SREs which contain the consensus motif CNGGN₀₋₄ in the loop (Chen et al., 2014). Consistent with Aub and Smg co-regulating mRNAs that undergo Aub-dependent destabilization, calculation of SRE scores in Aub-bound mRNAs revealed an enrichment of SREs in mRNAs stabilized in *aub* and *spn-E* mutant embryos, with a median SRE score of 17 ($n = 182$ genes), compared to mRNAs that were not stabilized in mutant embryos whose median SRE score was 6.1 ($n = 413$ genes).

As a first approach to determine whether Aub is loaded with piRNAs for its role in maternal mRNA regulation, we examined whether piRNAs were present in Aub-Smg complexes by analyzing small RNAs that coprecipitated with Smg in 0- to 2-hr embryos. microRNAs were enriched in Smg immunoprecipitates (Figures 4A and 4B; Table S5), consistent with the interaction of Smg with Argonaute 1 in early embryos (Pinder and Smibert, 2013). piRNAs were also found in Smg immunoprecipitates and displayed the same features as piRNAs present in total embryos: a 1U bias and a bias toward antisense sequences for piRNAs matching TEs (Figures 4C, 4D, and S3). Because these are the same features as those of piRNAs loaded into Aub (Brennecke et al., 2007), these results indicate that at least a fraction of Aub in complex with Smg was loaded with piRNAs.

We then investigated the potential of embryonic piRNAs to target Aub-bound mRNAs in the vicinity of crosslinks. We analyzed potential piRNA targeting within ± 60 nt from the cross-link sites, using varying complementarities. This identified a number of mRNAs potentially targeted by piRNAs for each

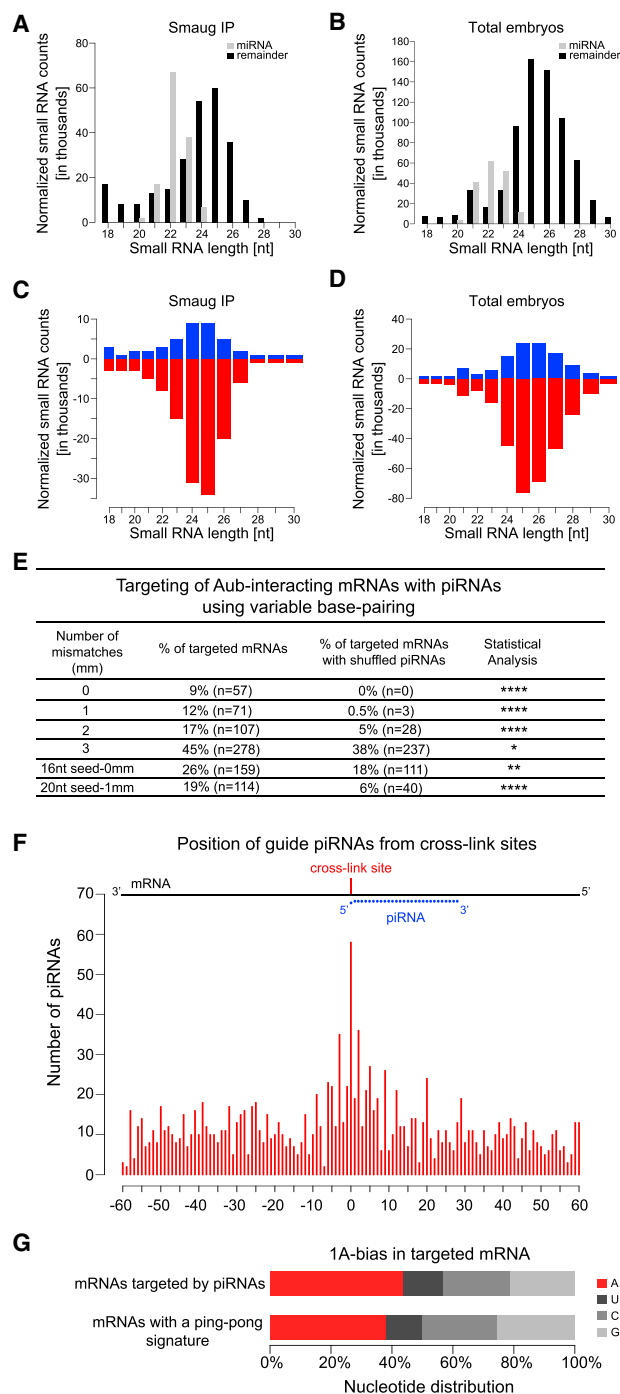


Figure 4. Smaug Is in Complex with piRNAs, and Embryonic piRNAs Have the Potential to Target Aub-Crosslinked mRNAs with Imperfect Base Pairing

(A and B) Length distribution of small RNAs that either coimmunoprecipitated with Smaug in 0- to 2-hr embryos (A) or were present in 0- to 2-hr embryos (B). miRNAs were identified by their sequences.

(C and D) Length distribution of TE-derived small RNAs in sense (blue) and antisense (red) orientations.

(E) Capacity of embryonic piRNAs to target Aub-bound mRNAs with different complementarities within ± 60 nt of crosslink sites. The first nt of piRNAs was not considered in the base pairing. Base pairing with 16-nt or 20-nt seed in-

complementarity (Figure 4E). To address whether this targeting could result from random complementarity, we performed negative controls consisting of the same targeting with shuffled piRNA sequences. For each complementarity, the number of mRNAs targeted by shuffled piRNA sequences was significantly lower than that of mRNAs targeted by piRNAs, suggesting a potential for embryonic piRNAs to target Aub-crosslinked mRNAs. Measurements of the distance between Aub crosslink sites and the 5' end of guide piRNAs revealed a peak of piRNAs overlapping Aub crosslink sites (0–29 nt) and within a few nt (–1 to –6 nt; Figure 4F). The peak at 0 nt suggested Aub potential interaction with the first nt of the sequence targeted by the guide piRNA. Strikingly, this result is consistent with recent data showing Aub capacity to select a specific nt (A) at this first position (Wang et al., 2014). We therefore analyzed the nt distribution at the first targeted position and identified a 1A bias, in agreement with the reported Aub preference for 1A on the target mRNA (Figure 4G).

Together, these data support the notion that piRNAs are involved as guides for the interaction of Aub with maternal mRNAs.

Unloaded Aub Is Defective for Maternal mRNA Binding

To confirm the role of piRNAs in the binding of Aub to maternal mRNAs, we produced a mutant form of Aub unable to load piRNAs. The 5' and 3' ends of small RNAs interact with the MID and PAZ domains of Argonaute proteins, respectively (Elkayam et al., 2012; Schirle and MacRae, 2012). We replaced by alanines two conserved tyrosines involved in the interaction with piRNAs, in the PAZ domain of the *GFP-Aub* transgene (Y345A and Y346A; Figure 5A). The resulting mutant transgene *GFP-Aub^{AA}* could not rescue the maternal effect embryonic lethality and dorso-ventral patterning defects of *aub* mutants (Figure S1A). We verified that *GFP-Aub^{AA}* was unable to bind piRNAs by performing GFP immunoprecipitations in 0- to 2-hr embryos (Figure 5B). Strikingly, in contrast to *GFP-Aub*, *GFP-Aub^{AA}* neither accumulated in the nuage, a structure involved in piRNA biogenesis that surrounds nurse cell nuclei in ovaries, nor in the germ plasm of oocytes and embryos (Figures 5C, S4A, and S4B). This shows that Aub accumulation in the nuage and germ plasm requires its loading with piRNAs. We performed an iCLIP experiment with *GFP-Aub^{AA}*, concomitantly with *GFP-Aub* iCLIP3. A low proportion of reads corresponded to piRNAs and mRNAs, whereas a high proportion of reads corresponded to rRNAs, suggesting that the sequences recovered in *GFP-Aub^{AA}* iCLIP were mostly unspecific (Table S1; Figure S1E). Consistent with this, the reproducibility of crosslinks

indicates that nt 2–16 and 2–20 of piRNAs were considered, respectively. Statistical analysis was performed using the Fisher's exact test.

(F) Position of guide piRNAs from the crosslink sites. The distance between the 5' end of piRNAs and the crosslink site was calculated; a schematic representation is at the top of the graph. The number of piRNAs is indicated for each distance. The complementarities shown in (E) were used.

(G) nt distribution of the first nt in the mRNA targeted by the guide piRNA for Aub-bound mRNAs either potentially targeted by piRNAs as in (E; top) or showing a ping-pong signature (bottom). The 1A bias in target mRNAs is consistent with Aub preference for A at the first position of targeted mRNAs. See also Figure S3 and Table S5.

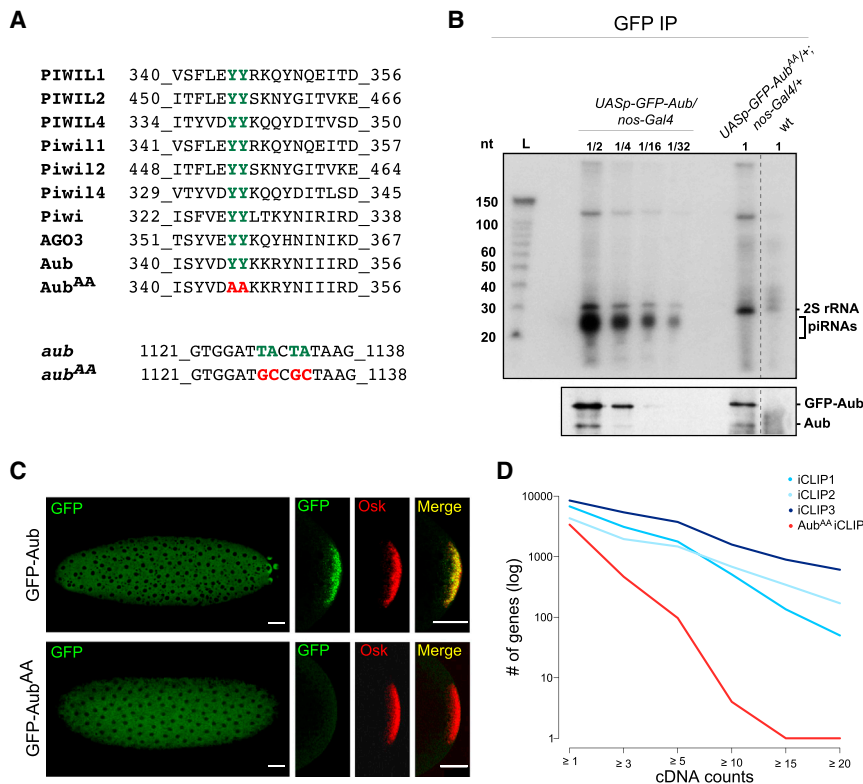


Figure 5. iCLIP with Unloaded Aub Produces a Low Number of Reproducible Crosslinks

(A) Portion of the PAZ domain in PIWI proteins from human, mouse, and *Drosophila*. The two conserved tyrosine residues involved in the binding of the piRNA 3' end are in green. These residues, Y345 and Y346 in Aub, were replaced by alanines in GFP-Aub^{AA} (in red). The mutations introduced in the DNA sequence are indicated in red.

(B) Aub^{AA} does not bind piRNAs. Immunoprecipitations (IPs) of GFP-Aub and GFP-Aub^{AA} with anti-GFP from *UASp-GFP-Aub/nos-Gal4* or *UASp-GFP-Aub^{AA}/+; nos-Gal4/+* 0- to 2-hr embryos, followed by 5' end labeling of coprecipitated small RNAs are shown. Serial dilutions of GFP-Aub IP were deposited. IP of wild-type embryos (wt) with anti-GFP was used as a negative control. The levels of Aub IP were controlled using western blot revealed with anti-Aub (lower panel). Note that a low level of Aub coimmunoprecipitates with GFP-Aub. (C) Aub^{AA} does not accumulate in the germ plasm. Confocal images of immunostaining of *UASp-GFP-Aub/nos-Gal4* and *UASp-GFP-Aub^{AA}/+; nos-Gal4/+* embryos with anti-GFP and anti-Osk are shown (right panels, posterior pole). GFP-Aub and GFP-Aub^{AA} are present at similar levels in the somatic part of the embryo, although only GFP-Aub accumulates in the germ plasm. The scale bars represent 30 μ m.

(D) Graph showing the number of genes with reproduced crosslinks (cDNA counts) in ± 5 -nt clusters in the different iCLIP experiments. See also Figure S4 and Tables S1, S2, and S6.

in GFP-Aub^{AA} iCLIP was low (97 genes with cDNA counts greater than or equal to five in ± 5 -nt clusters, as compared to 1,778, 1,473, and 3,744 genes in iCLIP1, 2, and 3, respectively; Table S2; Figure 5D). This low number of reproduced crosslinks with GFP-Aub^{AA} indicates that the majority of interactions between Aub and mRNAs required the loading of Aub with piRNAs. Among the 97 genes that had reproduced crosslinks within Aub^{AA} iCLIP, ten had clusters overlapping those identified as reproduced in Aub iCLIP experiments (Table S6). This could reflect the formation of complexes between GFP-Aub^{AA} and wild-type Aub, which were visualized in GFP immunoprecipitation experiments (Figure 5B); here, loaded Aub would drive the interaction between GFP-Aub^{AA} and maternal mRNAs. Alternatively, these results could reveal a weak potential for Aub to bind mRNAs independently of piRNAs.

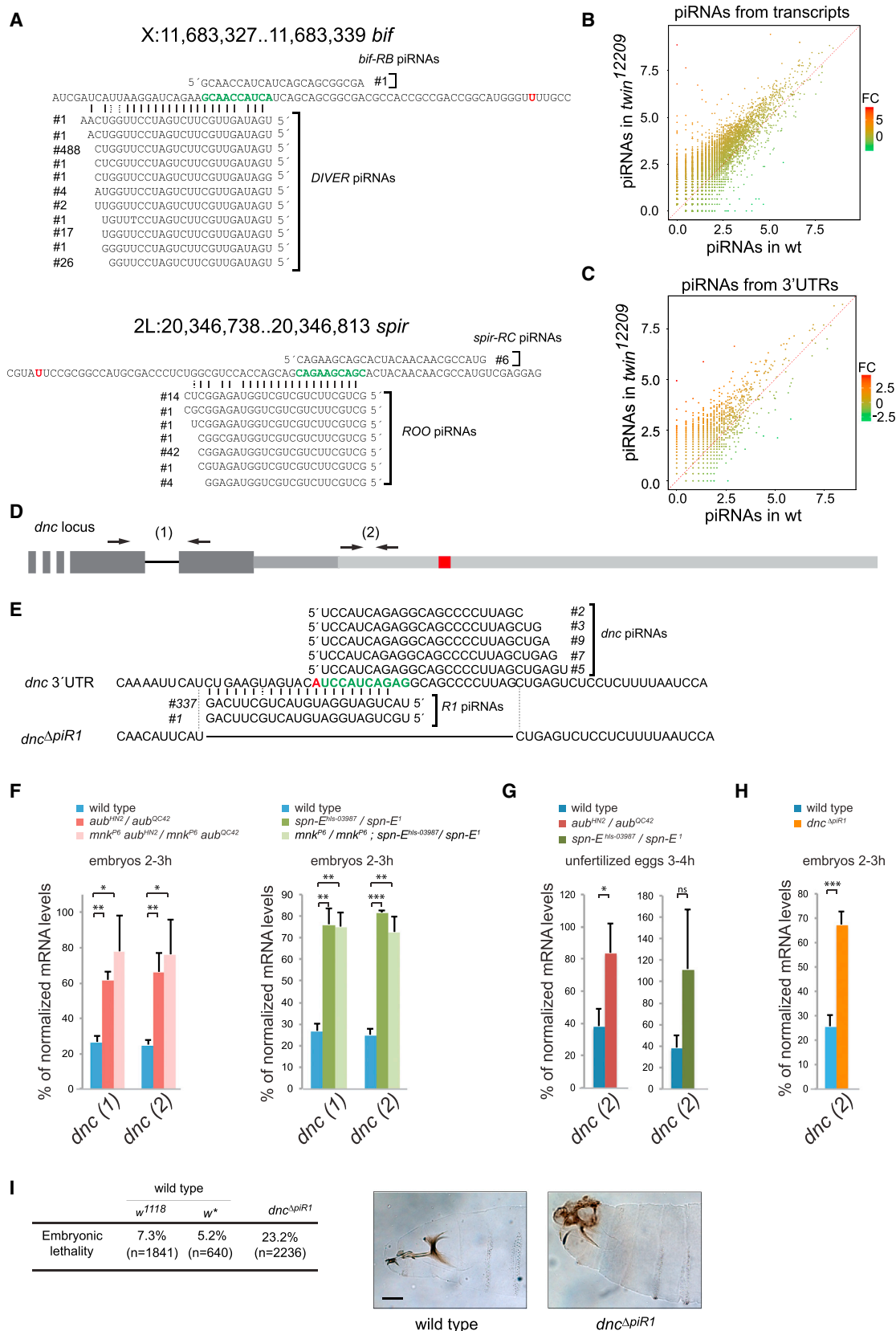
Because GFP-Aub^{AA} does not accumulate in the germ plasm, we performed a control GFP-Aub iCLIP in *osk*⁵⁴ mutant embryos that fail to accumulate GFP-Aub in the germ plasm (Figure S4C). In contrast to in Aub^{AA} iCLIP, the reproducibility of crosslinks in Aub iCLIP in *osk* mutant embryos was similar to that in wild-type embryos (Figure S4D; Table S2). Moreover, in *osk*⁵⁴ embryos, Aub interacted with mRNAs encoding germ cell determinants as it did in wild-type embryos (Figures S4E–S4G). This showed that Aub interaction with these mRNAs could occur in the somatic part of the embryo and did not result from concentration in the germ plasm.

We conclude that the loading of Aub with piRNAs is required for the vast majority of Aub interactions with mRNAs.

Production of Genic piRNAs upon Targeting of mRNAs by piRNAs

In the germline, piRNA biogenesis involves a mechanism known as ping-pong, in which the targeting of an mRNA by a piRNA induces the production of a secondary piRNA. The cleavage reaction catalyzed by PIWI proteins produces a 10-nt overlap between sense and antisense piRNAs, the ping-pong signature. We noticed such ping-pong signatures between piRNAs targeting Aub-bound mRNAs and piRNAs produced by these mRNAs (Figure 6A). Although the occurrence of genic piRNAs was low, these ping-pong signatures indicated the potential of maternal mRNAs to be cleaved by Aub. In total, out of the 634 genes producing mRNAs bound by Aub, 55 showed a ping-pong signature within ± 60 nt from crosslink sites, using piRNA base pairing described in Figure 4E (Table S7). Among the five genes identified as containing a TE insertion within ± 60 nt of crosslink sites, four produced a ping-pong signature and served as a proof of principle of this genic ping-pong (Figure S5).

This suggested two possible levels of mRNA regulation by Aub that involved either the recruitment of the CCR4-NOT deadenylation complex as previously described for *nos* mRNA (Rouget et al., 2010) or endonucleolytic cleavage. We reasoned that, if Aub could destabilize mRNAs by involving either of these two mechanisms, one might be favored through impairment of the other. We therefore used the CCR4 deadenylase mutant *twin* and examined whether the levels of piRNAs produced from transcripts would increase when deadenylation is reduced. We sequenced small RNA libraries generated from wild-type



(legend on next page)

embryos and embryos from *twin* mutant females (Table S5; Figure S6). piRNAs from transcripts and transcript 3' UTRs were more abundant in *twin* mutant than in wild-type embryos (Figures 6B and 6C). This is consistent with both possible modes of action for Aub—activator of deadenylation or endonuclease—in mRNA regulation.

We sought to functionally validate the targeting of *dunce* (*dnc*) mRNA by piRNAs from the *R1* TE, which induces a ping-pong signature. *R1* piRNA target site overlapped Aub crosslink in *dnc* 3' UTR (Figures 6D and 6E). *dnc* mRNA was stabilized in *aub* and *spn-E* mutant embryos and unfertilized eggs and in *mnk aub* and *mnk spn-E* double-mutant embryos (Figures 6F and 6G), suggesting a direct role of Aub in *dnc* mRNA destabilization in the embryo. *dnc* encodes a cyclic AMP (cAMP)-specific phosphodiesterase acting in cAMP catabolism. A tight regulation of cAMP levels is required for female fertility; a decrease in cAMP (e.g., in *Gprk2* mutants) leads to maternal effect alteration of embryonic anterior development, resulting in cuticle holes (Bellen and Kiger, 1988; Lannutti and Schneider, 2001; Schneider and Spradling, 1997). We used the CRISPR-Cas9 genome-editing system (Bassett et al., 2013; Port et al., 2014) to delete a short region including the *R1* piRNA target site in the *dnc* 3' UTR, resulting in *dnc* ^{Δ piR1} mutant (Figure 6E). *dnc* mRNA was stabilized in *dnc* ^{Δ piR1} mutant embryos, as compared to wild-type embryos (Figure 6H). Moreover, *dnc* ^{Δ piR1} homozygous females exhibited 23.2% maternal effect embryonic lethality and unhatched embryos developed cuticles with an anterior hole that resemble those produced by mutants with reduced levels of cAMP (Figure 6I) (Schneider and Spradling, 1997).

Taken together, these results show that the targeting of mRNAs by piRNAs loaded into Aub can induce Aub-dependent mRNA cleavage and destabilization that are required for embryonic development.

DISCUSSION

The function of the piRNA pathway in the regulation of TE expression is well established (Guzzardo et al., 2013; Ishizu

et al., 2012). In contrast, other functions of this pathway in gene regulation are only now emerging and remain poorly understood. Here, we provide evidence for a widespread role of Aub and piRNAs in maternal mRNA decay in the early *Drosophila* embryo during the MZT. An essential biological function of this regulation is to participate in the localization of specific mRNAs involved in germ cell specification and development through their destabilization in the somatic part of the embryo. Furthermore, Aub directly binds another set of maternal mRNAs, which are stable and appear to be required at later steps of embryogenesis.

PIWI proteins are loaded with piRNAs, which serve as guides to target mRNAs by complementarity; this RNA-RNA association underlies PIWI protein mode of action in TE regulation. One important question concerning the role of Aub in cellular mRNA regulation was whether piRNAs were also involved. In mouse, MIWI has been shown to interact massively with cellular mRNAs in spermatids based on CLIP experiments (Gou et al., 2014; Vourekas et al., 2012; Zhang et al., 2015), and it was proposed to bind spermiogenic mRNAs independently of piRNAs in round spermatids (Vourekas et al., 2012). However, more-recent reports have validated the implication of pachytene piRNAs in the regulation of mRNAs involved in spermatogenesis during spermatocyte and spermatid stages (Goh et al., 2015; Gou et al., 2014; Watanabe et al., 2015; Zhang et al., 2015). piRNAs guide MIWI-dependent cleavage or deadenylation of these mRNAs, leading to their destabilization.

We find that unloaded Aub has a very weak capacity to bind mRNAs in iCLIP experiment, indicating that the majority of Aub interactions with cellular mRNAs depend on piRNAs. This result is strengthened by a number of mRNAs showing a ping-pong signature close to Aub crosslinks, thus implicating their targeting by piRNAs. The functional validation of one of these mRNAs establishes a role for piRNA targeting in mRNA destabilization and embryonic development.

How could Aub achieve a specific mRNA regulation? Maternal mRNAs are produced in nurse cell nuclei in the ovary and must transit through the nuage during their export to the cytoplasm.

Figure 6. Production of Genic piRNAs with a 10-nt Overlap upon Targeting of mRNAs by piRNAs

(A) Examples of Aub-bound mRNAs showing a ping-pong signature within ± 60 nt from crosslink sites. The crosslinked nt are in red. The 10-nt overlap between sense and antisense piRNAs is in green. Guide TE piRNAs are below the sequence, and piRNAs produced from the mRNA are above. piRNA occurrences are indicated.

(B and C) Plots comparing the numbers of piRNAs from transcripts (B) or 3' UTRs (C) in wild-type and *twin*¹²²⁰⁹ mutant embryos. Each dot represents a transcript (B) or a 3' UTR (C); the fold change (FC) color code is on the right.

(D) Schematic representation of the *dnc* 3' region. Exons are represented as dark gray boxes, the intron as a black line, and the short and long 3' UTRs as gray and light gray thick lines, respectively. The primer sets (1) and (2) are indicated by arrows. The red box represents the *R1* piRNA target site.

(E) Sequence of the *dnc* 3' UTR region targeted by *R1* piRNAs. Legend is as in (A). Although the crosslink at this nt (red) was reproduced with cDNA counts of 5, 1, and 2 in iCLIP1, iCLIP2, and iCLIP3, respectively, this reproducibility was below the threshold applied to select Aub-interacting mRNAs in Table S4. The sequence of the *dnc* ^{Δ piR1} mutant obtained following CRISPR-Cas9 mutagenesis is shown. The mutation corresponds to a 35-nt deletion spanning the *R1* piRNA target site. (F and G) *dnc* mRNA quantification using qRT-PCR and the primer sets (1) and (2) in wild-type and mutant 2- to 3-hr embryos or 3- to 4-hr unfertilized eggs. *RpL32* was used as a control mRNA. The levels of mRNA at 2 to 3 hr or 3 to 4 hr were normalized to the levels at 0 to 1 hr set to 100% for each genotype. The genotypes of females are indicated; females were crossed or not with wild-type males. Means are from two to five biological replicates. The error bars represent SE. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, non-significant using the one-tailed Student's *t* test.

(H) *dnc* mRNA quantification using qRT-PCR and the primer set (2) in wild-type and *dnc* ^{Δ piR1} embryos. *dnc* ^{Δ piR1} mothers were crossed with wild-type males. Legend as in (F) and (G). Means are from three and six biological replicates for wild-type and *dnc* ^{Δ piR1} embryos, respectively.

(I) Lethality of embryos coming from *dnc* ^{Δ piR1} mothers crossed with wild-type males. The *w*^{*} stock was generated following similar crosses as those used in the recombination to obtain the *dnc* ^{Δ piR1} mutant and served as a wild-type stock with a matched genetic background. Example of cuticle of *dnc* ^{Δ piR1} embryos showing a hole in the anterior region. 84.6% ($n = 39$) of unhatched embryos showed similar anterior defects. The scale bar represents 30 μ m.

See also Figures S5 and S6 and Tables S5 and S7.

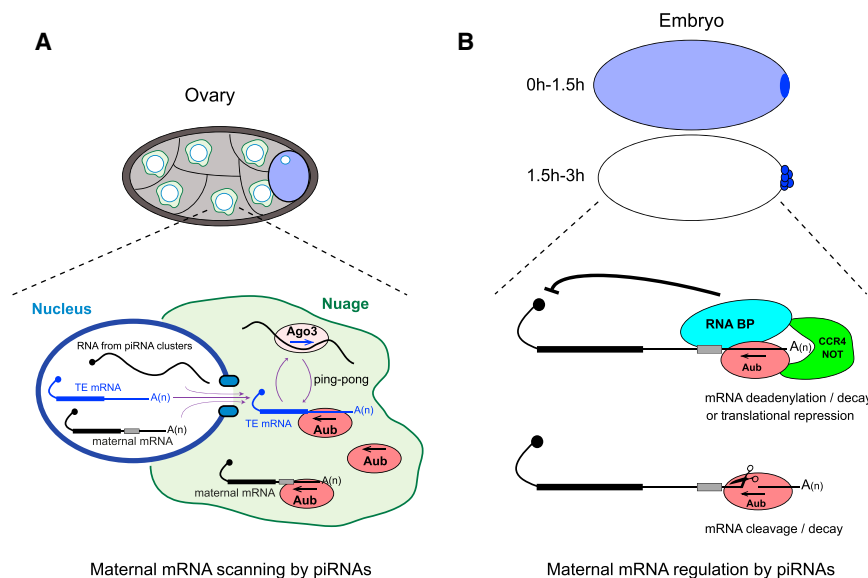


Figure 7. Model of Maternal mRNA Regulation by Aub and piRNAs

(A) The model proposes that maternal mRNAs, as TE mRNAs, would be scanned by piRNAs in the nuage during their export to the cytoplasm, in the nurse cells of the ovary. This would lead to Aub interaction with maternal mRNAs when imperfect base pairing with piRNAs is found but would not result in strong decay, although mRNA cleavage by Aub might be possible.

(B) Once loaded into the embryo, maternal mRNAs would interact with additional RNA-binding proteins (e.g., Smg) that would participate with Aub, either in the recruitment of the CCR4-NOT complex, resulting in massive mRNA decay, or in translational repression. mRNA cleavage by Aub would also contribute to mRNA destabilization.

We propose that, in the nuage, maternal mRNAs undergo a general scanning by piRNAs loaded into Aub, as in the case of TE mRNAs, because mRNAs from TEs and cellular genes might not be discriminated (Figure 7). TE mRNAs are highly targeted by piRNAs, whereas the targeting of maternal mRNAs should be more modest due to the lack of perfect base pairing with piRNAs. Aub binding to maternal mRNAs based on imperfect base pairing with piRNAs could contribute to regulation, as it has been validated for different PIWI proteins (Gou et al., 2014; Lee et al., 2012; Rouget et al., 2010). However, multiple interactions might be required to achieve effective regulation. Additional proteins might also contribute to potentiate piRNA-mediated regulation. Although Aub is expected to bind maternal mRNAs in the nurse cells, these mRNAs are not massively degraded in the ovary, whereas they are in the embryo. This strongly suggests that decay requires another component expressed in the embryo. We determined that SREs are enriched in mRNAs bound and destabilized by Aub in the embryo. This is consistent with the presence of piRNAs in Smg mRNPs and suggests that Smg, which is specifically expressed in early embryos, cooperates with Aub for the destabilization of these maternal mRNAs.

Our data reveal two possible mechanisms of mRNA regulation by Aub (Figure 7): the first involves Aub-dependent mRNA deadenylation through the recruitment of the CCR4-NOT complex, which we previously reported for *nos* mRNA (Rouget et al., 2010). Interestingly, this mechanism involving deadenylation and mRNA decay factors also underlie some aspect of piRNA-dependent repression of TE mRNAs (Lim et al., 2009). The second mechanism based on the endonucleolytic activity of Aub leads to the production of genic piRNAs. Both mechanisms are also implicated in cellular mRNA regulation by MIWI (Goh et al., 2015; Gou et al., 2014; Watanabe et al., 2015; Zhang et al., 2015). Our results reveal increased levels of genic piRNAs in mutant embryos for the CCR4 deadenylase, suggesting that both mechanisms might compete for the repression of the same mRNAs.

Aub is expressed throughout the whole syncytial embryo (Mani et al., 2014; Rouget et al., 2010) and accumulates in the germ plasm, where it is involved in primordial germ cell (PGC) formation (Harris and Macdonald, 2001). This function might be partly indirect because *aub* mutant embryos that do not develop PGCs also show DNA fragmentation resulting from earlier defects during oogenesis (Khurana et al., 2010). However, our results propose a more-direct function of Aub in PGC formation: we uncover a new role for Aub in the direct binding of a set of maternal mRNAs involved in PGC specification and development, which could underlie this Aub developmental function. Stabilization of these mRNAs in *aub* mutant embryos and unfertilized eggs indicates that Aub is involved in the decay of these mRNAs in the somatic part of the embryo. It is likely that the interaction between Aub and mRNAs encoding PGC determinants is maintained in the germ plasm where Aub accumulates, even if it does not lead to mRNA decay. In particular, Aub has been shown to colocalize with *nos*, *pgc*, and *gcl* mRNAs in RNA granules in PGCs (Rangan et al., 2009). Consistent with this, ectopic localization of Aub at the anterior pole of embryos through anterior localization of Osk (Ephrussi and Lehmann, 1992) resulted in the recruitment of mRNAs encoding PGC determinants at the anterior pole (Figure S7). The mechanism behind this switch in Aub function between soma and germ plasm in the early embryo remains to be addressed.

Strikingly, we found that unloaded Aub does not accumulate in the germ plasm, implying that Aub is loaded with piRNAs when it binds PGC determinant mRNAs in the germ plasm. Therefore, Aub would have two inter-related functions in PGC biology: the localization in the germ plasm of mRNAs encoding PGC determinants and the maternal transfer of a pool of piRNAs in the PGCs to start piRNA production and function in TE repression in the future germ cells.

It is likely that the general decay of maternal mRNAs by Aub and piRNAs in the somatic part of the embryo plays a substantial role in embryonic patterning. Indeed, ectopic anterior expression of PGC determinants such as *osk* or *pgc* is known to prevent anterior-posterior patterning of the embryo (Ephrussi and

Lehmann, 1992; Hanyu-Nakamura et al., 2008). We show here that piRNA-dependent regulation of *dnc* mRNA is important for embryonic development. This is consistent with the role of piRNA-dependent regulation of *nos* mRNA for embryonic patterning (Rouget et al., 2010). In both these cases, however, preventing this regulation did not lead to complete embryonic lethality, indicating that piRNA-dependent regulation would be partly redundant with other levels of post-transcriptional regulation and involved in fine-tuning gene expression. Because piRNA populations can evolve rapidly within and between species, mRNA regulation by piRNAs would also change rapidly, thereby providing a potential basis for adaptive responses.

EXPERIMENTAL PROCEDURES

CRISPR-Cas9 Genome Editing

The sgRNA was designed to target the *R1* piRNA target site in the long 3' UTR of *dnc* mRNA. The sgRNA was produced as previously described (Bassett et al., 2013) with the following modifications. PCR was performed with Super-prime Accu Mix I (Invitrogen) using the annealed primers dncCRISPR-F, which contains the *dnc* target sequence, and sgCRISPR-R. In vitro transcription from 1 μ g of the obtained PCR fragment was performed with T7 RNA polymerase (Roche). The sgRNA was extracted with acid phenol chloroform (Ambion), precipitated, dissolved in injection buffer, and stored at -80°C . Two hundred picoliters of a 1 $\mu\text{g}/\mu\text{l}$ sgRNA solution was injected in 0–20 min old *act5C-cas9* embryos (Port et al., 2014). Surviving flies were crossed with a stock containing the FM7c balancer. Offspring were screened by T7 endonuclease I assay as described previously (Hwang et al., 2013), and mutations were determined by sequencing. The *act5C-cas9* transgene was removed from *dnc* mutant stocks by recombination with a *w⁺* stock before further analysis.

ACCESSION NUMBERS

Deep-sequencing and microarray data generated in this study have been deposited to the NCBI GEO and are available under accession number GEO: GSE70778.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.07.030>.

AUTHOR CONTRIBUTIONS

B.B., J.D., C.P., F.P., and T.G. performed biological experiments; S.P., C.A., and T.C. performed bioinformatic analyses; G.B. and J.-P.D. performed microarrays; M.S. conceived the study; B.B., S.P., J.D., and M.S. analyzed the data and wrote the manuscript; and all authors discussed the manuscript.

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