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# Histone-derived piRNA biogenesis depends on the ping-pong partners Piwi5 and Ago3 in *Aedes aegypti*

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## ABSTRACT

The piRNA pathway is of key importance in controlling transposable elements in most animal species. In the vector mosquito *Aedes aegypti*, the presence of eight PIWI proteins and the accumulation of viral piRNAs upon arbovirus infection suggest additional functions of the piRNA pathway beyond genome defense. To better understand the regulatory potential of this pathway, we analyzed in detail host-derived piRNAs in *A. aegypti* Aag2 cells. We show that a large repertoire of protein-coding genes and non-retroviral integrated RNA virus elements are processed into genic piRNAs by different combinations of PIWI proteins. Among these, we identify a class of genes that produces piRNAs from coding sequences in an Ago3- and Piwi5-dependent fashion. We demonstrate that the replication-dependent histone gene family is a genic source of ping-pong dependent piRNAs and that histone-derived piRNAs are dynamically expressed throughout the cell cycle, suggesting a role for the piRNA pathway in the regulation of histone gene expression. Moreover, our results establish the Aag2 cell line as an accessible experimental model to study gene-derived piRNAs.

## INTRODUCTION

Small RNA-guided gene regulation has come to light as a major, widely conserved mechanism across almost all eukaryotes (1). PIWI-interacting RNAs (piRNAs) are a class of ~25–30 nt small RNAs that associate with the PIWI subclass of Argonaute proteins to form gene regulatory piRNA induced silencing complexes (piRISCs). Specificity of piRISC is mediated by base-pairing between the piRNA sequence and a target RNA (2). In flies, primary piRNAs

are processed from long single-stranded RNA precursors and are loaded into the PIWI proteins Piwi and Aubergine. These piRNAs show a strong bias for uridine at their 5' end (1U). In the presence of target RNA, piRNA amplification by the so-called ping-pong loop is initiated: antisense primary piRNAs mediate cleavage of the target and the 3' cleavage fragment is processed into secondary piRNAs, which are loaded into a different PIWI protein, called Argonaute 3 (Ago3). This mechanism is at the origin of the ping-pong signature of piRNAs: a 1U bias for antisense piRNAs and a bias for adenosine at position 10 (10A) for sense piRNAs (2–4).

The piRNA pathway has been defined as an RNA-based defense system against transposon activity and many studies have addressed its role in maintaining genome stability in the germline (5). Despite this conserved function across species, increasing evidence suggests the presence of piRNAs and PIWI proteins in non-germline tissues (6,7). Expression of PIWI proteins in somatic tissues has been linked to stem cell renewal, maintenance and regeneration in several primitive organisms (1,8–12). Somatic piRNAs have been cloned from *Drosophila* (2,13,14), rhesus macaque and mouse tissues (2–4,15–16). Moreover, accumulation of piRNAs and PIWI proteins has been detected in human somatic and cancer cells (5,17,18). However, the biogenesis and functions of somatic PIWI proteins and their associated piRNAs remain largely unexplored.

Previously, we and others identified an additional class of somatic piRNAs: virus-derived piRNAs in *Aedes* mosquitoes (6,7,19–24). As the vector for several pathogenic human viruses, including dengue virus, Zika virus and chikungunya virus, *Aedes aegypti* is one of the most medically important mosquito species. Its genome encodes eight PIWI family members (Piwi1–7 and Ago3), of which Ago3 clusters with *Drosophila* Ago3, whereas Piwi1–7 form clades distinct from *Drosophila* Piwi and Aubergine (25). We recently used the piRNA competent Aag2 cell line to investigate viral and transposon-derived

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piRNA biogenesis. Aag2 cells express the same PIWI genes present in adult mosquitoes and are fully competent in producing piRNAs via the ping-pong amplification mechanism (19). We demonstrated that Piwi5 and Ago3 are the core proteins of the viral piRNA ping-pong amplification loop, whereas additional PIWI proteins are involved in transposon-derived piRNA biogenesis (20).

*A. aegypti* has a large genome size (~1.4 Gb) of which half is composed of transposable elements (TEs) (26). However, only 19% of the sequenced piRNAs map to TEs, suggesting that the remainder of small RNAs could arise from other genomic loci (27). Indeed, an increasing number of studies indicate that piRNAs may also arise from cellular non-coding and protein-coding genes in different animal species (5,27–30). Gene-derived piRNAs generally derive from the 3' untranslated regions (UTRs) and are produced in a ping-pong independent fashion in *Drosophila* ovaries, murine testes, *Xenopus* eggs and *Anopheles gambiae* germline (3,31–33).

Core histone proteins (H2A, H2B, H3, H4) are highly conserved proteins that play essential structural and functional roles in genome packaging and gene regulation in all eukaryotes. The organization of histone genes in one or more clusters is conserved from yeast to human. Although clustering is important for their transcriptional activation at the G1/S phase transition of the cell cycle, histone transcripts are also regulated post-transcriptionally (34). Devoid of a poly(A) tail, replication-dependent histone mRNAs end in a highly conserved stem-loop (SL) structure that is responsible for their cell cycle regulated degradation at the end of the S phase. Conserved *cis* regulatory elements in histone mRNA, such as the SL motif and the purine-rich histone downstream element (HDE), and the machinery for histone mRNA 3' end processing are conserved in *A. aegypti* (35).

Here, we present evidence that specific PIWI proteins produce genic piRNAs in *A. aegypti* Aag2 cells. We show that coding sequences of replication-dependent histone genes are a major source of 3' end-modified piRNAs, which accumulate in an Ago3-Piwi5 and ping-pong dependent fashion. Our results imply a new link between the piRNA pathway and histone gene expression and establish the Aag2 cell line as an experimental model to study genic piRNAs.

## MATERIALS AND METHODS

### Transfection of Aag2 cells

For immunoprecipitation (IP) analyses, Aag2 cells were transfected with expression plasmids encoding individual PIWI proteins (see Supplementary data). For knockdown experiments, Aag2 were transfected with dsRNA using XtremeGENE HP (Roche) according to the manufacturer's instructions. To increase knockdown efficiency, Aag2 cells were re-transfected at 48 h after the first transfection. Where indicated, cells were infected with a Sindbis virus recombinant expressing GFP from a second subgenomic promoter (SINV-GFP). Unless stated differently, samples were harvested at 48 h post infection.

### RNA and protein detection

For a detailed description of the experimental procedures for northern blot, RT-qPCR, strand specific RT-PCR, stem-loop RT-qPCR, IP, western blot and small RNA deep-sequencing, see Supplementary Data. Oligonucleotide sequences are presented in Supplementary Table S1.

### Cell cycle analysis by flow cytometry

Sub-confluent Aag2 cells were treated with 0.2 mM Hydroxyurea (HU, Sigma) for 24 h and subsequently released by changing the medium. At 0, 2, 4, 6 and 8 h post release (hpr), cells were harvested, washed with phosphate buffered saline, fixed overnight with ice-cold 70% ethanol at 4°C and stained in Staining buffer (50 µg/ml propidium iodide, 50 µg/ml Ribonuclease A, 3.8 mM tri-sodium citrate dehydrate, 0.1% Triton X-100) overnight at 4°C in the dark. Intracellular DNA content was then analyzed by flow cytometry on a BD FACSCalibur. FlowJo software was used for the analyses.

### Statistical analysis

Experiments were repeated as biological replicates as indicated in the figure legends and data are expressed as mean ± standard deviation (SD). Unpaired two-tailed student's *t*-tests were used to determine statistical significance. A *P*-value of <0.05 was considered statistically significant. Graphs were plotted and statistical analyses were performed using GraphPad Prism (version 6.00 for Mac, GraphPad Software, La Jolla CA, USA).

## RESULTS

### *A. aegypti* coding genes are a source of piRNA-sized small RNAs

We have previously shown that different combinations of PIWI proteins can generate either transposon-derived or viral piRNAs in the *A. aegypti* Aag2 cell line (20,21). Although it has been proposed that protein-coding genes may also be sources of piRNAs in *A. aegypti* (27), the biogenesis mechanism and the PIWI proteins involved remained to be defined.

To characterize the piRNA repertoire in Aag2 cells and explore its dependency on specific PIWI proteins, we analyzed our previous small RNA sequencing data from Sindbis virus (SINV)-infected Aag2 cells, upon RNAi-mediated knockdown or immunoprecipitation (IP) of Ago3, Piwi4, 5 and 6 (20). In addition to the expected siRNA and miRNA populations (21–23 nt), a piRNA-sized population of small RNAs (25–30 nt) accumulated in control knockdown libraries (Supplementary Figure S1A).

We mapped small RNA reads to the SINV genome and to the genomes of viruses known to persistently infect Aag2 cells. The remaining reads were then mapped to the *A. aegypti* genome (AaegL3) and 25–30 nt reads were assigned to different categories of transcripts (Table 1). Most reads mapped to unannotated regions of the *A. aegypti* genome (40%). As previously indicated (27), TE-derived piRNAs represented only a modest fraction (~24%) of the piRNA-like reads. Approximately 26% of reads mapped to other

repeated regions in the *A. aegypti* genome. Among the remaining 25–30 nt piRNA-like reads, ~8% mapped to annotated *A. aegypti* coding genes (Table 1) and were selected for further analyses. Mapping to individual transcripts, a total of 339 protein-coding genes produced at least ten piRNA-sized reads per million mapped reads.

### Different combinations of PIWI proteins produce distinct sets of genic piRNAs

We next investigated genic piRNA dependence on and association with specific PIWI proteins. To this end, fold changes of piRNA-sized reads in sense or antisense orientation from individual PIWI knockdown or IP libraries were compared to the control libraries (dsLuc or GFP-IP, respectively). The global levels of sense, genic 25–30 nt RNA reads were specifically reduced in Ago3 knockdown (Supplementary Figure S1B) and enriched in Ago3 IP (Supplementary Figure S1C), whereas the antisense reads were reduced by both Ago3 and Piwi5 knockdowns (Supplementary Figure S1B) and enriched in Piwi5 and Piwi6 IP (Supplementary Figure S1C).

To better characterize genic piRNA-like reads based on their PIWI protein dependency, we analyzed the changes in small RNA levels for individual coding genes upon PIWI knockdown and IP. We performed hierarchical clustering of the top 50 piRNA producing coding genes and we classified them into six groups based on their dependency on specific PIWI proteins (Figure 1A, left panel and 1B). In addition, we calculated the sense or antisense bias of the reads relative to the annotated transcriptional orientation of the loci (Figure 1A, central panel). Furthermore, we evaluated the nucleotide bias at each position of small RNA sequences within the six defined groups to determine if genic piRNA-like reads contained the characteristic 1U/10A ping-pong signature (Figure 1A, right panel).

A large number of genes fall in group II and III, which contained piRNA-like reads that were dependent on and enriched in Piwi5 and Piwi6, indicating that these PIWI proteins are directly responsible for their production, classifying these small RNAs as *bona fide* genic piRNAs. BLAST analyses of the predicted gene products identified eight loci that seem to be of viral origin (group II: AAEL007844, AAEL007866, AAEL009873, AAEL017001; group III: AAEL000976, AAEL00991, AAEL00997, AAEL001003 and AAEL009870). Such non-retroviral integrated RNA virus elements (NIRVs) are likely integrated into the host genome by spurious reverse transcription and integration events by retrotransposons and have been proposed to be a catalog of previous viral encounters in the mosquito genome (36,37). These Piwi5/Piwi6 dependent viral-like piRNAs were 1U-biased and in antisense orientation to the annotated gene. The viral-like piRNAs from group II have previously been identified as a source of piRNAs in adult *A. aegypti* (27). Strikingly, four out of five viral-like loci of group III (AAEL000976, AAEL00991, AAEL00997 and AAEL001003) are clustered on the *A. aegypti* genome in a 6761 bp window.

Group IV and V included genic piRNA-like reads that depend on the ping-pong partners Ago3 and Piwi5. Accordingly, these genes predominantly produced piRNAs from

the sense strand, which were enriched in Ago3 and showed a 10A bias (Figure 1A and B). These features resemble *A. aegypti* viral piRNAs that are produced in a ping-pong dependent manner (20,21). Like group II and III, group IV piRNA levels were reduced upon Piwi4 knockdown, but they were depleted in Piwi4 IP. This pattern resembled our previous observations for TE-derived piRNAs (20), suggesting an indirect role for Piwi4 in piRNA biogenesis also in genic piRNA production.

Group I comprised three genes characterized by an increase in piRNA expression upon Piwi4 knockdown and a depletion in Piwi4 IP, suggesting an indirect effect of Piwi4 on piRNA accumulation. Group VI consisted of a heterogeneous group of six genes, overall distinguished by a loss of piRNAs upon Piwi4 and Ago3 knockdown and an association with Ago3. The strong nucleotide bias for group I (sense and antisense) and VI (antisense) members was caused by the predominance of individual sequences. Together, these results suggest that different combinations of PIWI proteins mediate the biogenesis of genic piRNAs from subsets of protein-coding genes.

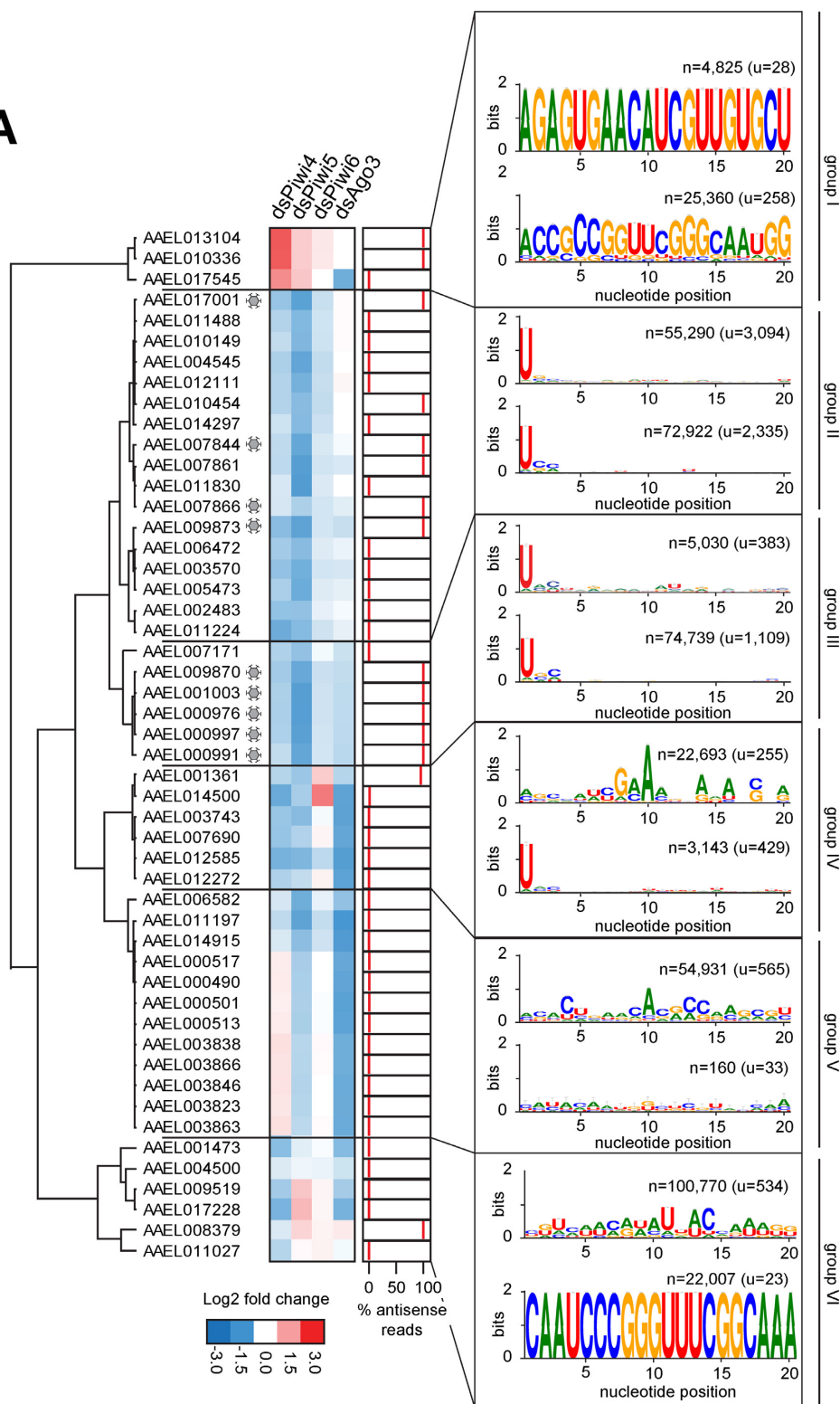
### Sense and antisense histone 4-derived piRNAs accumulate in an Ago3/Piwi5 ping-pong-dependent fashion

We have previously shown that Ago3 and Piwi5 process viral RNA substrates in a ping-pong-dependent manner to generate antisense, U1 biased, Piwi5-bound piRNAs and sense, 10A biased, Ago3-bound piRNAs (20). To further study the role of the ping-pong partners Ago3 and Piwi5 in control of host gene expression, we first analyzed representative genes from group IV (AAEL012272, AAEL007690, AAEL003743) and group V (AAEL011197, AAEL14915, AAEL006582) (Supplementary Figure S2). Interestingly, for all genes a similar small RNA distribution was observed: piRNA-sized reads accumulated as hotspots in exons, almost exclusively in a sense orientation to the host transcript (Supplementary Figure S2A and B). Despite the absence of antisense piRNA-sized reads accumulating from those loci, low levels of minus strand-derived 21 nt reads were detected, suggesting the existence of antisense transcripts (Supplementary Figure S2B). Nonetheless, RT-qPCR analyses indicate that mRNA steady-state levels remained largely unchanged in Ago3 and Piwi5 knockdown, despite the associated decrease in piRNA levels (Figure 1A and Supplementary Figure S2C).

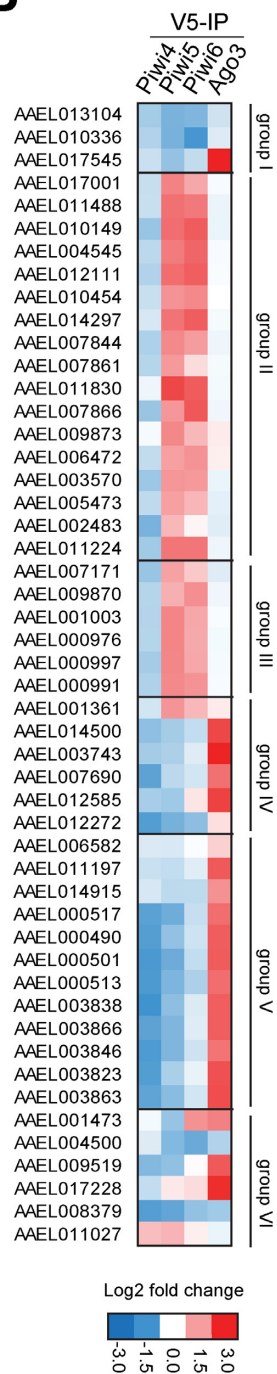
Among the genes that produce Ago3/Piwi5-dependent piRNAs in group V, we found nine members of the histone 4 (H4) gene family (AAEL000517, AAEL000490, AAEL000501, AAEL000513, AAEL003838, AAEL003866, AAEL003846, AAEL003823 and AAEL003863). H4 forms the central core nucleosome with histone 3 (H3) and interacts with DNA and all other core histones (34). In addition to coding an evolutionary conserved protein, H4 genes display a strikingly high conservation at the nucleotide level (38). The *A. aegypti* genome encodes fifteen almost identical H4 genes. Among those, thirteen display the unique features of replication-dependent histone genes in metazoans (Supplementary Figure S3A). They encode mRNAs ending in a conserved SL sequence, rather than a poly(A) tail, their 3' end



A



B



**Figure 1.** Genic piRNAs depend on different combinations of *Aedes aegypti* PIWI proteins. (A) Relative changes in piRNA abundance of the top-50 piRNA producing protein-coding genes upon PIWI knockdown. Left panel: heatmap showing the relative piRNA abundance in the indicated knockdown libraries compared to the control knockdown (dsLuc). These data were used to generate the hierarchical clustering and assign individual genes to specific groups (I–VI). The virus symbol indicate annotated genes of viral origin. Middle panel: antisense bias, defined as the percentage of antisense 25–30 nt reads that map to genic sequences. Right panel: nucleotide bias at each position of the 25–30 nt small RNA reads mapping to the sense (upper panels) and antisense coding gene sequences (lower panels). All reads of three independent experiments were combined to generate sequence logos using the Weblogo3 program. *n*, number of reads; *u*, number of unique sequences. (B) Heatmap showing the relative piRNA abundance in the indicated Piwi IP libraries over the control IP (GFP).

**Table 1.** Annotation of 25–30 nt small RNA reads in Aag2 cells

| Mapping                             | Annotation           | Number of reads<br>(three libraries) | Percentage of total<br>reads | Percentage of AagL3<br>mapping reads |
|-------------------------------------|----------------------|--------------------------------------|------------------------------|--------------------------------------|
| Total                               |                      | 11 717 519                           | 100                          |                                      |
| SINV                                |                      | 166 152                              | 1.42                         |                                      |
| AaDV2                               |                      | 12                                   | 0.0001                       |                                      |
| MXV                                 |                      | 5839                                 | 0.05                         |                                      |
| CFAV                                |                      | 5284                                 | 0.05                         |                                      |
| <i>A. aegypti</i> genome<br>(AagL3) | Total                | 8 945 062                            | 76.34                        | 100                                  |
|                                     | TEfam                | 2 177 498                            | 18.58                        | 24.34                                |
|                                     | Other repeats        | 2 299 843                            | 19.63                        | 25.71                                |
|                                     | Non repeated         |                                      |                              |                                      |
|                                     | Protein-coding genes | 701 882                              | 5.99                         | 7.85                                 |
|                                     | Non-coding genes     | 153 705                              | 1.31                         | 1.72                                 |
|                                     | Shared               | 34 075                               | 0.29                         | 0.38                                 |
|                                     | Unannotated          | 3 578 059                            | 30.54                        | 40.00                                |
| Unmapped                            |                      | 2 595 170                            | 22.15                        |                                      |

Small RNA reads from control SINV-GFP infected Aag2 cells (luciferase dsRNA treated) were mapped to the indicated viral genomes and the *A. aegypti* genome. *A. aegypti* specific 25–30 nt RNAs were assigned to different categories of transcripts (Transposable Elements annotated in the TEfam database; other repeats; protein-coding or non-coding genes). SINV, Sindbis virus; AaDV2, Aedes aegypti densovirus; MXV, mosquito X virus; CFAV, cell fusing agent virus.

formation is directed by a purine-rich sequence known as HDE, they lack introns and they are clustered with the other core histone genes (H2A, 2B and 3) in the genome (Supplementary Figure S3B). All replication-dependent H4 genes can be a source of genic piRNAs (Supplementary Figure S3A). The remaining two H4 genes (AAEL011999 and AAEL013709) are not clustered and possess a canonical polyadenylation signal at the 3' end, typical of replication-independent H4 replacement variants (39).

We mapped small RNA reads to the H4 genes and analyzed their size profile and distribution across the open reading frame (ORF) (Figure 2A and B). Both sense and antisense small RNAs ranged in size from 25 to 30 nt, resembling the size distribution of piRNAs. The vast majority of H4 small RNA reads derived from the sense strand of the gene (Figure 2A and B) and displayed a 10A nucleotide bias (Figure 2C).

To confirm our data, we compared our dataset to a publicly available small RNA sequence dataset from dsGFP transfected Aag2 cells (40) (Supplementary Figure S4) and found a strong correlation in abundance of gene-derived piRNAs in both datasets ( $r_s = 0.75$ ;  $P < 0.001$ , Supplementary Figure S4A). Moreover, in the Haac *et al.* dataset, H4-derived piRNAs accumulate with a similar size profile and ping-pong signature as in our dataset (Supplementary Figure S4B and C).

H4 piRNAs predominantly derived from the sense strand in the second half of the H4 ORF and were dominated by a few, highly abundant sequences (Figure 2A, in red). Nonetheless, antisense 29 nt small RNA reads were also detectable (Figure 2A, in blue). Moreover, the 10-nt overlap between small RNAs that mapped to opposite strands suggests that the ping-pong amplification loop mediates biogenesis of these small RNAs (Supplementary Figure S5A). In agreement, both sense and antisense piRNAs were reduced in Ago3 or Piwi5 depleted cells (Figure 2D). Moreover, sense piRNAs were specifically enriched in Ago3, whereas antisense reads were preferentially bound by Piwi5 (Figure 2E). These results indicate that H4-derived

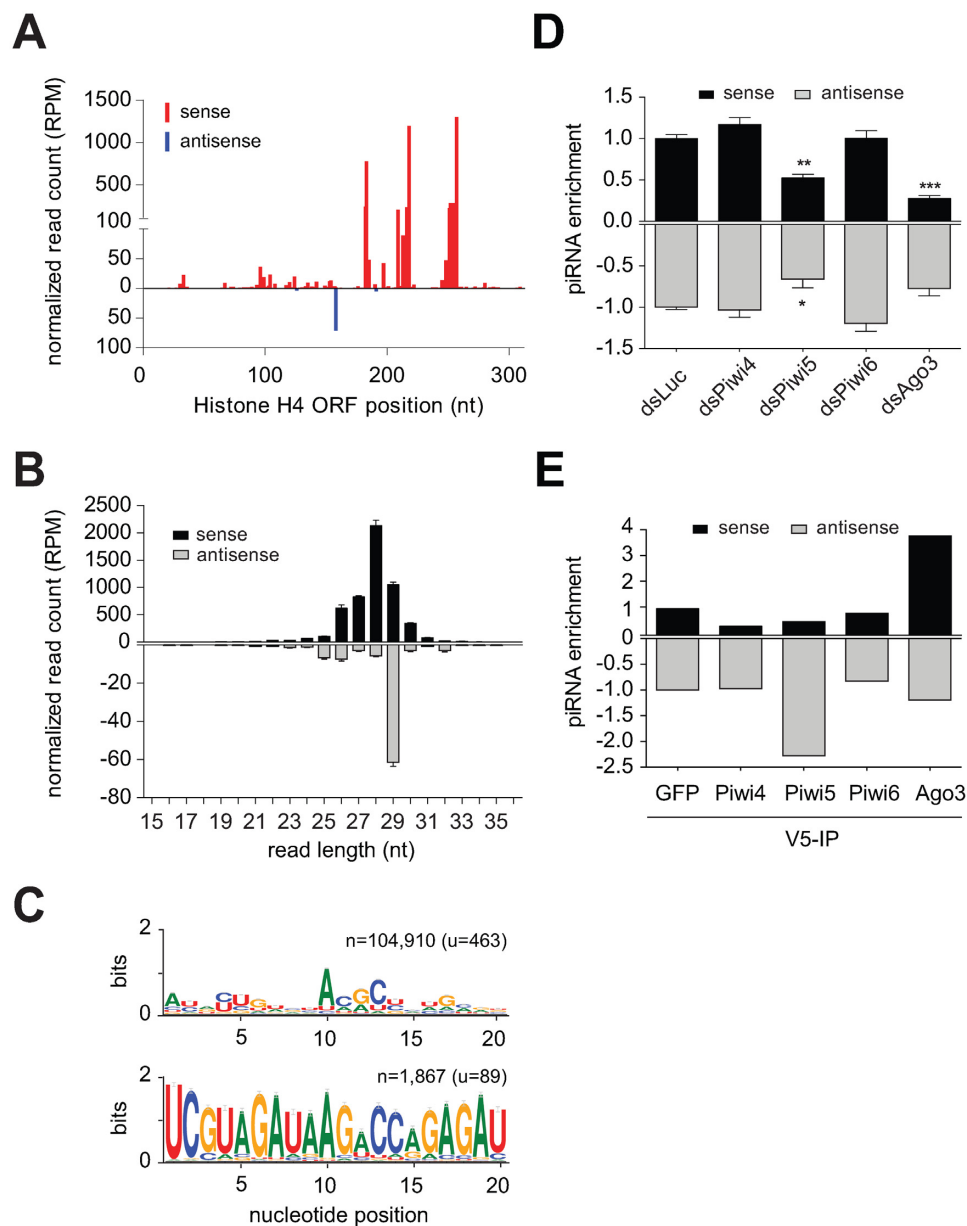
25–30 nt small RNAs are ping-pong dependent piRNAs (H4piRNAs).

#### Sense histone 4-derived piRNAs are 3' end-modified and associate with Ago3

To confirm these findings in a sequencing-independent manner, we extracted total RNA from mock and SINV-infected Aag2 cells in different PIWI knockdown conditions and analyzed H4 sense piRNA accumulation levels by northern blot using a mix of four DNA probes (Figure 3A). The presence of individually cloned H4 sense piRNAs was verified using each of the four DNA probes separately (Supplementary Figure S5B). Consistent with the sequencing results (Figure 1A, group V and Figure 2D), Ago3 and Piwi5 knockdown specifically reduced H4piRNA levels (Figure 3A and Supplementary Figure S5B and C). As expected, H4piRNAs are independent of Ago1 and Ago2, which are the effector proteins in the microRNA and siRNA pathways, respectively (Supplementary Figure S5C). Our results showed that H4piRNAs accumulate as abundant and discrete RNA molecules independently of virus infection. For this reason, further analyses have been performed in uninfected Aag2 cells. In agreement with the sequencing data (Figure 1B, group V and Figure 3B), we confirmed that sense H4piRNAs are specifically enriched in Ago3 upon GFP-Trap IP of a GFP-Ago3 fusion protein (Figure 3B and C; Supplementary Figure S5D).

The PAZ domain of PIWI proteins is known to recognize the 3'-end of piRNAs (41,42), which invariantly carries a 2'-O-methyl group (1). Also, *in vitro* studies in silkworm BmN4 cells established that the 3' end modification takes place on piRNA precursors that are loaded into PIWI proteins (43). We thus performed a beta elimination assay on total RNA extracted from Aag2 cells and confirmed the presence of a modification at the H4piRNA 3' terminus, consistent with 2'-O methylation (Figure 3D).

The ping-pong cycle predicts that antisense piRNAs are produced from antisense transcripts. Although their accumulation was low compared to sense piRNAs, we could also detect antisense H4piRNAs by northern blot (Figure 3E).

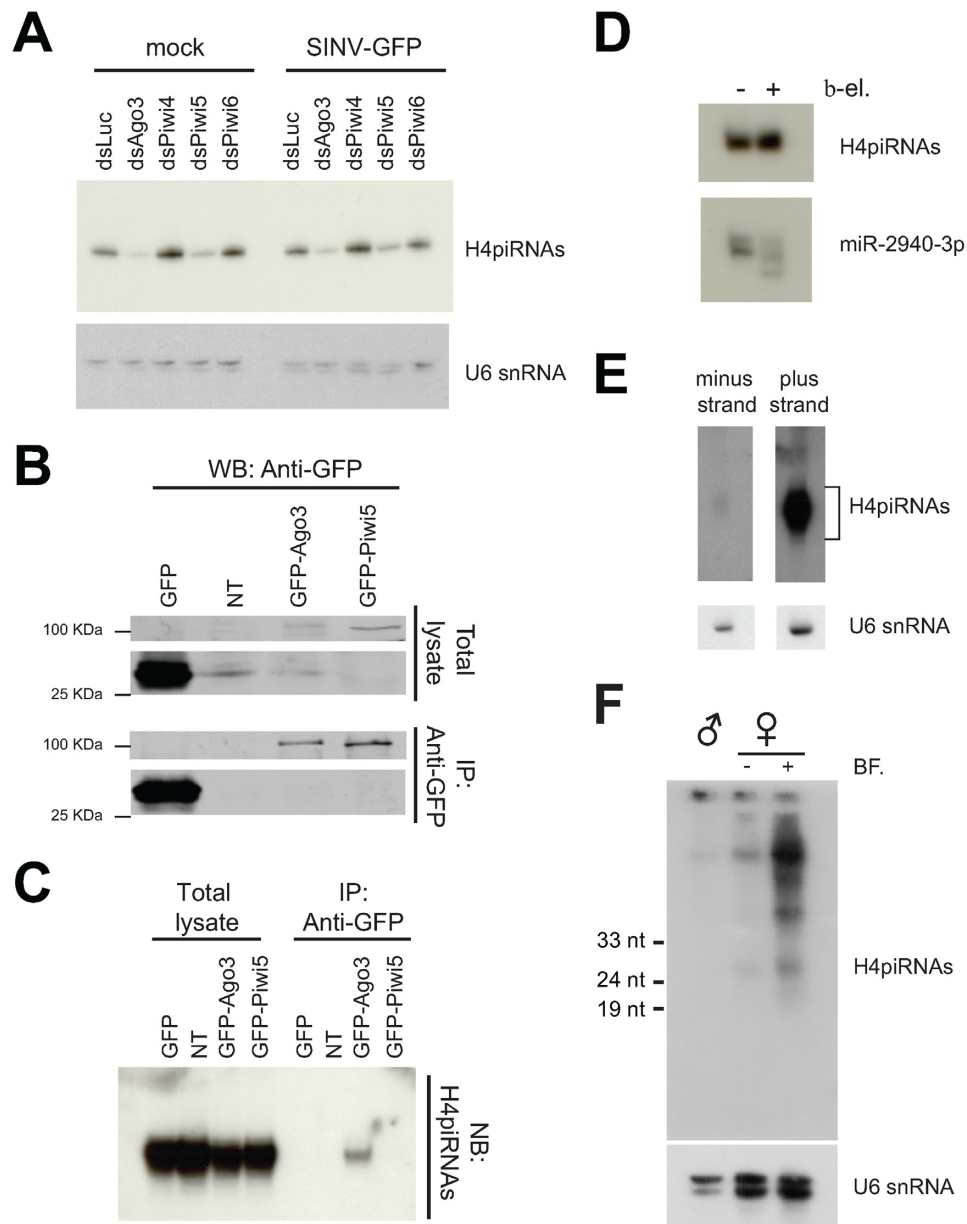


**Figure 2.** H4piRNA biogenesis depends on Ago3 and Piwi5 dependent ping-pong amplification. (A) Distribution of 25–30 nt sense (red) or antisense (blue) RNA reads across the H4 open reading frame (ORF). The average count (three control libraries) of 5' ends of reads at each nucleotide position are shown. (B) Size profile of small RNA reads derived from H4 genes. Black and gray bars indicate sense and antisense reads, respectively. (C) Nucleotide bias at each position of the 25–30 nt small RNA reads mapping to H4 genes in sense (upper panel) and antisense (lower panel) orientation. All reads of three independent libraries were combined to generate the sequence logo; *n*, number of reads; *u*, number of unique sequences. (D and E) Relative abundance of 25–30 nt sense (black) and antisense (gray) H4piRNA reads in the indicated (D) PIWI knockdown or (E) PIWI IP libraries. Bars in panel (D) are the mean  $\pm$  SD of three independent small RNA libraries. Two-tailed student's *t*-test was used to determine statistical significance (\**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001; \*\*\*\**P* < 0.0001).

We reasoned that a transcript that is antisense to the H4 mRNA could serve as a potential precursor for these antisense H4piRNAs. We therefore established a strand-specific RT-PCR to specifically detect sense and antisense RNAs (Supplementary Figure S6A). As expected, we readily amplified cDNA from the sense strand, which corresponds to the H4 mRNA. In addition, we detected specific RT-PCR products from the antisense strand, indicating that anti-

sense H4 transcripts accumulate in Aag2 cells (Supplementary Figure S6B).

Although not ranking among the top-50 piRNA producing genes, the other core histone genes, H2A, H2B and H3, were also sources of Ago3 and Piwi5-dependent piRNAs in Aag2 cells (Supplementary Figure S7A–C). In addition to piRNAs, these core histone genes produced low levels of 21 nt sense and antisense reads, indicating that these genes also produced antisense transcripts.



**Figure 3.** H4-derived sense piRNAs associate with Ago3. (A) Northern blot analysis of sense H4piRNAs upon knockdown of the indicated PIWI/AGO transcripts in mock and SINV-GFP infected Aag2 cells. H4piRNAs were detected using a pool of four probes. U6 snRNA serves as loading control. (B) GFP-trap immunoprecipitation (IP) on Aag2 cells expressing GFP-Ago3, GFP-Piwi5, or, as a negative control, GFP. Immunoprecipitates and total lysates were analyzed by western blot using anti-GFP antibodies. The complete western blot is shown in Supplementary Figure S5. NT, non-transfected. (C) Northern blot analysis of sense H4piRNAs in RNA isolated from IPs of the indicated GFP-tagged proteins, using a strand-specific riboprobe complementary to the H4 mRNA. (D) Beta-elimination on total RNA of Aag2 cells followed by northern blot of H4piRNAs and miR-2940-3p. H4piRNAs were detected using a pool of four DNA oligonucleotide probes. (E) Northern blot for antisense (left) and sense (right) H4piRNAs in total RNA extracted from Aag2 cells. Strand-specific riboprobes complementary to either the sense or antisense strand of the H4 gene were used. U6 snRNA serves as loading control. All RNA samples were analyzed on high resolution 17.5% polyacrylamide gel. (F) Northern blot analyses of H4-piRNAs in male, female, and blood-fed (BF) female *Aedes aegypti* mosquitoes. A pool of four DNA oligonucleotide probes was used to detect H4-piRNAs. U6 snRNA serves as a loading control.



To analyze whether histone-derived piRNAs are produced *in vivo*, we analyzed H4-derived small RNAs from adult mosquitoes by northern blot (Figure 3F) and observed that H4piRNAs accumulate in adult mosquitoes and that their levels were higher in blood-fed female mosquitoes than in non-blood-fed ones (Figure 3F). Moreover, small RNA sequencing data from adult mosquitoes contained histone-derived small RNAs in the size range of 26–29 nt, which were predominantly sense to histone transcripts and derived from the second half of the ORF (Supplementary Figure S8A–D).

Together, our results show that sense H4piRNAs are highly abundant molecules and that they are 3' end modified, 10A biased, loaded in Ago3 and expressed in an Ago3 and Piwi5-dependent manner. An H4 antisense transcript is the likely source of Piwi5-dependent, 1U biased, antisense H4piRNAs that initiates the ping-pong amplification loop.

### Histone-derived piRNAs accumulate during the cell cycle

High expression of the intronless, non-polyadenylated, replication-dependent histone genes is specifically required when DNA is being synthesized. Highly cell cycle-regulated activation of transcription, coupled with tight control of mRNA stability, causes a rapid increase in histone mRNA abundance as cells enter S phase and a rapid decrease at the end of the S phase (34). H4piRNAs specifically derive from the replication-dependent H4 genes in class I and II (Supplementary Figure S3A) rather than from the constitutively expressed H4 replacement variants (class IV). For this reason, we hypothesized that H4piRNAs production and function was linked with DNA replication and the cell cycle.

Cell cycle progression can be arrested at the transition step between G1 and S phase (G1/S) using hydroxyurea (HU) which reversibly induces replication stalling by nucleotide depletion and inhibition of DNA synthesis. To test our hypothesis, Aag2 cells were synchronized with 0.2 mM HU for 24 h and the cell cycle was subsequently reinitiated by removal of the drug. The cells were harvested at different time points post-release and their cell cycle distribution was assessed by flow cytometry based on DNA content (Figure 4A and B). As expected, H4 mRNA levels increased upon entry into S phase at 2 h post release (hpr) and rapidly dropped when cells progressed through the G2/M phase (Figure 4C). Likewise, the other core histone mRNAs showed the same dynamics (Supplementary Figure S9A).

To quantify H4piRNA accumulation, we set up stem-loop (SL) RT-qPCR assays for four individual H4piRNAs (named A–D, Supplementary Figure S5B). The previously observed H4piRNA reduction upon Ago3 and Piwi5 knockdown (Figure 3A) was recapitulated by the SL-qPCR assay (Supplementary Figure S9B), thus validating the approach. We then analyzed H4piRNA accumulation in asynchronous and synchronized cells and found that H4piRNAs are dynamically expressed throughout the cycle with a peak in expression at 4 hpr, with a slight delay compared to the peak of H4 mRNA expression (Figure 4C and D). Together, our results indicate that Piwi5 and Ago3 produce histone-derived piRNAs and suggest that piRNAs contribute to histone mRNA turnover during the cell cycle.

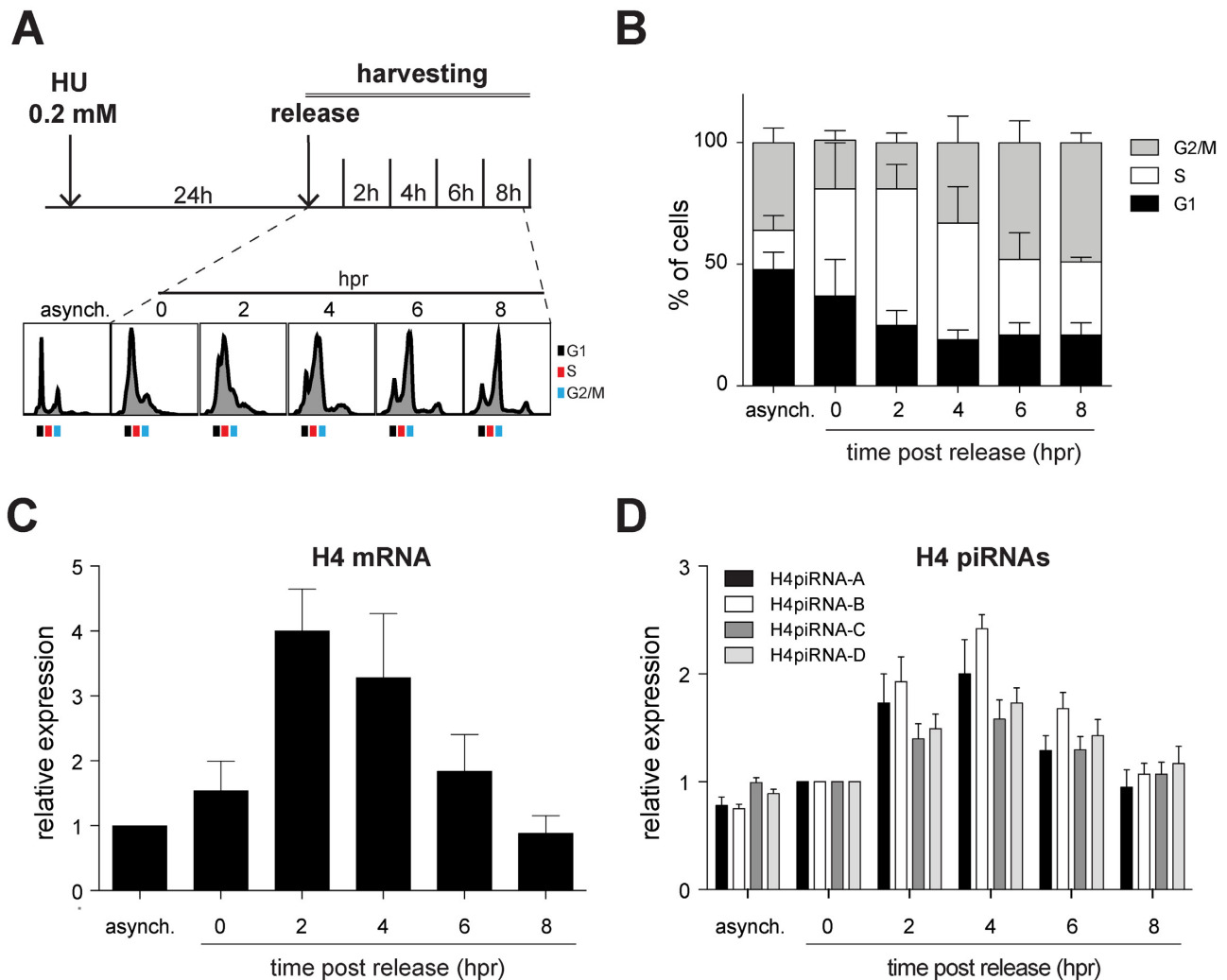
## DISCUSSION

Ten years have passed since the first identification of piRNAs by several independent laboratories (44–48). In addition to the canonical function in protecting germline integrity, recent evidence imply broader roles for PIWI/piRNAs as regulators of gene expression in both germline and somatic tissues (6,7). *Aedes* mosquitoes display an expansion of the PIWI gene family, several of which are expressed in the soma (25,49). In this study, we characterized the biogenesis of gene-derived piRNAs in the *A. aegypti* Aag2 cell line, which express the same PIWI genes as are expressed in somatic tissues of adult mosquitoes. We find that replication-dependent histone genes produce piRNAs in a ping-pong dependent manner, suggesting that piRNAs can be involved in dynamic regulation of mRNA expression in the soma. Moreover, since cell culture has a higher experimental amenability than adult mosquitoes, our work establishes Aag2 cells as an accessible and relevant model to study gene-derived piRNAs.

We identified several classes of genic piRNAs that depend on different combinations of PIWI proteins. Among these, class IV and V genic piRNAs are dependent on the ping-pong proteins Ago3 and Piwi5 and accumulate as hotspots in exonic sequences along the gene body. This distinguishes them from gene-derived piRNAs reported before in other species, which generally derive from the 3' UTRs and are generated in a ping-pong independent fashion (3,31–33). Class IV and V genes do not seem to share biological and molecular functions, nor do they share structural similarities. For example, group IV and V included canonical spliced host genes that are expressed as polyadenylated transcripts, as well as replication-dependent histone genes that produce unspliced, non-polyadenylated mRNAs.

Among the core histone genes, H4 produced the most abundant piRNAs. Sense and antisense H4piRNAs are specifically enriched in Ago3 and Piwi5, respectively. This reflects their nucleotide bias: 10A for sense H4piRNAs and 1U for the antisense ones. As a consequence, sense H4piRNA biogenesis seems to rely on the feed-forward ping-pong amplification loop, most likely initiated by cleavage of H4 mRNAs by antisense piRNAs. Although ping-pong-dependent production of genic piRNAs initiated by transposon-derived piRNAs has recently been reported (50,51), to our knowledge H4piRNAs are the first example of genic piRNAs that are produced in an autonomous ping-pong amplification loop.

In metazoans, expression of replication-dependent histone gene is tightly controlled to ensure their massive production as cells enter S phase and their reduction to baseline between the end of the S phase and mitosis. An intriguing possibility would thus be that piRNA–PIWI complexes are regulated by or have a role in the cell cycle. We found that histone mRNAs and histone piRNAs are dynamically expressed during the cell cycle, with the peak of H4piRNA abundance lagging ~2 h behind the peak of histone mRNA expression, suggesting that histone-derived piRNAs are processing products of histone mRNAs. The factors involved in cell cycle-dependent histone mRNA expression and degradation are conserved during evolution, and it is likely that the canonical mechanisms for histone



**Figure 4.** H4piRNAs accumulate during the cell cycle in synchronized Aag2 cells. (A) Schematic representation of the synchronization protocol. Aag2 cells at 50% confluency were treated with 0.2 mM hydroxyurea (HU) for 24 h to induce cell cycle arrest at the G1/S phase. Once released, cells were collected every 2 h, stained with propidium iodide (PI) and quantified for cell cycle distribution by flow cytometry. An example is shown (lower panel). (B) The relative percentage of cells in the G1, S or G2/M phase of the cell cycle was assessed in three independent experiments. Data are presented as means  $\pm$  SD. (C) RT-qPCR analysis of H4 mRNA levels in synchronized cells. Expression is normalized to Lysosomal Aspartic Protease (LAP) levels and is expressed relative to asynchronous cells. Bars represent means  $\pm$  SD of three biological replicates. (D) Stem-loop (SL) RT-qPCR analyses of individual H4piRNAs (shown in Supplementary Figure S5B) in non-synchronized cells and in HU treated, synchronized cells at the indicate time points. Expression is normalized to aae-bantam-3p and presented relative to 0 hpr. Bars represent means  $\pm$  SD of three biological replicates. hpr, hours post release.

mRNA metabolism are responsible for the major changes in histone mRNA levels in *A. aegypti*. However, our results indicate that the piRNA pathway may add an additional layer of dynamic histone mRNA regulation in a narrow window of the cell cycle.

Previous studies have indirectly shown a connection between piRNAs and histone genes in different organisms. For instance, Hiwi2 IP analyses in human somatic cells identified several genes involved in cell growth and proliferation among the piRNA-producing loci (30). Moreover, sense 1U biased piRNA-like molecules from histone mRNAs have been identified by deep sequencing in the cnidarian *Hydra magnipapillata*. During regeneration, downregulation of these piRNA-like molecules leads to a small, but consistent, upregulation of histone transcripts (52). Support for a role of PIWI proteins in the regulation of his-

tone mRNA levels have also been suggested in other species. For example, expression of the histone variant H3.3 during macronuclear differentiation is impaired after PIWI knock-down in the ciliate *Stylonychia lemnae* (53). In the parasitic protozoan *Leishmania*, histone transcripts are upregulated in PIWI null mutants. As PIWI in this species is unable to bind piRNAs due to lack of a typical PAZ domain, this result suggests a role in the stability of histone transcripts independently of piRNAs (54). In fly ovaries, H2B mRNA expression levels are upregulated upon nuclear PIWI elimination (55).

Our analyses indicate that, although all core histone genes produce piRNAs, the majority derive from H4 genes. This is unexpected given that replication dependent histone mRNAs are produced at the same time during the cell cycle. We hypothesize that H4piRNA production not

only depends on relative transcript abundance, but also on other specific features. Compared to other histone genes, H4 genes show the highest level of conservation at the nucleotide level during evolution, suggesting an important role for sequence or structure of their mRNA (38). Indeed, the secondary structure of murine H4 mRNA is crucial for its non-canonical translational initiation mechanism (56). Of note, *A. aegypti* H4piRNAs originate from a region in the mRNA that corresponds to the structural element that is critical for internal translation initiation on the murine transcript. This raises the intriguing possibility that histone mRNA structure or sequence enhances recognition or processing by the piRNA pathway.

Among the most abundant genic piRNAs, we retrieved piRNAs from annotated genes consisting of sequences of RNA viruses that are integrated in the *A. aegypti* genome. Virus-like genic piRNAs are in antisense orientation to the annotated host gene, show a clear 1U nucleotide bias and associate with Piwi5 and Piwi6. We noticed that four of the virus-like loci in group III (AAEL001003, AAEL000976, AAEL00997 and AAEL00991 which are reminiscent of rhabdoviral nucleoprotein sequences) are clustered in the *A. aegypti* genome and may represent something akin to a piRNA cluster.

Sequences with similarity to non-retroviral RNA viruses have been detected in the genome of both *A. aegypti* (27) and *Aedes albopictus* and have been referred to as NIRVs (36,37). Interestingly, vertebrate genomes also contain sequences corresponding to viral fragments. For instance, Endogenous Bornavirus-Like Nucleoprotein elements (EBLNs) are the result of reverse transcription and integration of ancient bornaviral nucleoprotein mRNA in the genome of primates and rodents. A recent study proposed that EBLN-derived piRNAs explains the resistance to bornaviral infections in these host species (57). The majority of these piRNAs are antisense to viral sequences, which would render the primary piRNA pathway capable of slicing viral gene transcripts. Similarly, as virus-like piRNAs are antisense and Piwi5 and Piwi6 associated in *Aedes*, one can envision that piRNAs confer heritable immunity to infection. An invading cognate virus may thus be targeted directly by the host-encoded piRNA, initiating a ping-pong amplification cycle and inducing the production of phased piRNAs to diversify the viral piRNA population, as was recently proposed for transposon-derived piRNAs in *Drosophila* (50,58).

Even though PIWI proteins are well conserved across different organisms, piRNA sequences are generally not conserved during evolution (2). However, given recent examples of piRNAs that regulate coding genes in the soma (29,51,59–61), sequence constraints between piRNAs and their RNA targets may be expected. For example, a recent study has identified Eutherian-Conserved piRNA cluster loci, which most likely have a functional relevance (62). The high conservation and essential function of the histone genes in all eukaryotes suggest that histone piRNAs have key functions not only in *A. aegypti* but also in other species.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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