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An ancient satellite repeat controls gene expression and embryonic development in *Aedes aegypti* through a highly conserved piRNA

Rebecca Halbach[1], Pascal Miesen[1], Joep Joosten[1], Ezgi Taşköprü[1], Inge Rondeel[1], Bas Pennings[1], Chantal B.F. Vogels[2,3], Sarah H. Merkling[4,5], Constantianus J. Koenraadt[2], Louis Lambrechts[4,5], and Ronald P. van Rij[1]

[1] Department of Medical Microbiology, Radboud University Medical Center, Radboud Institute for Molecular Life Sciences, P.O. Box 9101, 6500 HB Nijmegen, the Netherlands

[2] Laboratory of Entomology, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, the Netherlands

[3] Current affiliation: Department of Epidemiology of Microbial Diseases, Yale School of Public Health, 60 College Street, New Haven, CT 06510, USA

[4] Insect-Virus Interactions Group, Department of Genomes and Genetics, Institut Pasteur, 75015 Paris, France

[5] Evolutionary Genomics, Modeling and Health, Unité Mixte de Recherche 2000, Centre National de la Recherche Scientifique, 75015 Paris, France

Correspondence: ronald.vanrij@radboudumc.nl

Abstract

Tandem repeat elements such as the highly diverse class of satellite repeats occupy large parts of eukaryotic chromosomes. Most occur at (peri)centromeric and (sub)telomeric regions and have been implicated in chromosome organization, stabilization, and segregation[1]. Others are located more dispersed throughout the genome, but their functions remained largely unknown. Satellite repeats in euchromatic regions were hypothesized to regulate gene expression *in cis* by modulation of the local heterochromatin, or *in trans* via repeat-derived transcripts[2,3]. Yet, due to a lack of experimental models, gene regulatory potential of satellite repeats remains largely unexplored. Here we show that, in the vector mosquito *Aedes aegypti*, a satellite repeat promotes sequence-specific gene silencing via the expression of two abundant PIWI-interacting RNAs (piRNAs). Strikingly, whereas satellite repeats and piRNA sequences generally evolve extremely fast[4-6], this locus was conserved for approximately 200 million years, suggesting a central function in mosquito biology. Tandem repeat-derived piRNA production commenced shortly after egg-laying and inactivation of the most abundant of the two piRNAs in early embryos resulted in an arrest of embryonic development. Transcriptional profiling in these embryos revealed the failure to degrade maternally provided transcripts that are normally cleared during maternal-to-zygotic transition. Our results reveal a novel mechanism by which satellite repeats regulate global gene expression *in trans* via piRNA-mediated gene silencing, which is fundamental to embryonic development. These findings highlight the regulatory potential of this enigmatic class of repeats.

Main

Even though satellite repeats have been discovered nearly 60 years ago[7,8], and comprise a substantial portion of eukaryotic genomes, little is known about the functions of this class of repetitive DNA. Many satellite repeats are actively transcribed, and some of them produce small interfering (si)RNAs required for heterochromatin formation and maintenance[9-16]. Around two-thirds of the genome of *Aedes aegypti*, the most important vector for arthropod-borne viruses like dengue, Zika, and yellow fever virus, consists of repetitive elements[17] (Extended Data Fig 1A), making this mosquito an interesting model to study these sequences. We analysed small RNAs in somatic (female carcass) and germline (female ovaries) tissues as well as the somatic *Ae. aegypti* cell line Aag2. Even though satellite repeats constitute less than 10 % of the genome, they were not only highly covered by siRNAs, but especially by PIWI-interacting (pi)RNAs (Extended Data Fig 1A). piRNAs are a class of small RNAs that protect animal genomes from harmful parasitic elements like transposons[18]. In the fruit fly *Drosophila melanogaster*, piRNAs are mostly derived from transposon-rich genomic regions termed piRNA clusters[19], and predominately restricted to germline tissues[19]. In contrast, in *Ae. aegypti*, piRNAs are also highly abundant in non-germline tissues, and piRNAs from transposable elements (TEs) are underrepresented compared to the abundance of these elements in the genome[20], especially in the soma (Extended Data Fig 1A). Instead, we found satellite-repeat derived piRNAs to be highly overrepresented in somatic tissues. Intriguingly, approximately three-quarters of satellite-repeat derived reads in the soma, and half of the reads in the germline and Aag2 cells stem from only two individual sequences that map to a repeat locus on chromosome 3. This locus is about 3.5 kb in size and consists of 20 full repeats and one disrupted repeat unit organized in a head-to-tail array (Fig 1A). These two highly abundant satellite-derived small RNAs were 30 and 29 nucleotides in size, respectively (Extended Data Fig 1B), and resistant to β-elimination, suggesting that they are 2'-*O*-methylated at their 3' end, a common feature of mature PIWI-bound piRNAs[21-23] (Fig 1B). We named these two sequences tapiR1 and 2 (tandem repeat-associated piRNA1/2).

Expression of tapiR1 and 2 was ubiquitous in both somatic and germline tissues of adult mosquitoes (Extended Data Fig 2A). In *Ae. aegypti*, the PIWI gene family has expanded to seven PIWI genes (Piwi2-7 and Ago3) compared to three in flies[24]. Immunoprecipitation (IP) in Aag2 cells of the aedine PIWI proteins that are expressed both in the soma and gonads (Piwi4-6 and Ago3)[25-27] followed by northern blotting or deep sequencing indicates that both tapiR1 and tapiR2 exclusively associate with Piwi4 (Fig 1C, Extended Data Fig 2B, C, Supplementary Fig S1A,B). Indeed, only knockdown of Piwi4, but not of other PIWI or Argonaute (AGO)-clade genes reduced tapiR1 and 2 levels (Extended Figure 2D,E, Supplementary Fig S1C,D). Thus far, the piRNA repertoire and function of Piwi4 remained unclear. Piwi4 neither associates with TE nor virus-derived piRNAs[26],

yet was linked to piRNA biogenesis from transposons[26] and to antiviral defense[28,29]. As nearly 90 % of Piwi4-associated small RNAs only comprise tapiR1, and, to a much lower extent, tapiR2 (Fig 1C), we hypothesize that tapiR1 not only dominates the piRNA repertoire, but also shapes downstream functions of Piwi4.

Satellite repeats are one of the fastest evolving parts of eukaryotic genomes. Except for a few examples[30-32], most satellite repeats display high sequence divergence between species, and can even be species-specific[6,33,34], akin to piRNAs[4,5]. Hence, we were surprised to find that the identified tandem repeat locus in *Ae. aegypti* is conserved in the closely related Asian tiger mosquito *Ae. albopictus*, and even in the more distantly related southern house mosquito *Culex quinquefasciatus* (Extended Data Fig 3A). This locus is, however, not present in the genome assembly of the malaria vector *Anopheles gambiae*. The tandem repeat locus differed in the number of monomers across species, and the monomers exhibited substantial length and sequence divergence, both between species and between monomers within one species. However, the sequences in the monomers that give rise to tapiR1 and 2, as well as a downstream T were by far more conserved than the overall monomer, suggesting that these sequences are under extensive selective constraints (Fig 1D, Extended Data Fig 3B-C). We further analysed whether expression of the two repeat locus-derived piRNAs is conserved among different mosquitoes, including species for which no genome assembly is available. We analysed 18 different mosquito species from 6 different genera (*Aedes*, *Culex*, *Culiseta*, *Coquillettidia*, *Toxorhynchites*, and *Anopheles*), as well as *Culicoides nubeculosus*, a biting midge that also transmits arboviruses but is only distantly related to mosquitoes, and the fruit fly *Drosophila melanogaster*. Strikingly, even though piRNAs are usually not conserved even between closely related species[4,5], we detected both tapiR1 and 2 in all five genera of the Culicinae subfamily of mosquitoes (Fig 1E, Extended Data Fig 3E). In line with the absence of the repeat locus in the *Anopheles gambiae* genome, we did not observe tapiR1 or 2 expression in this subfamily of mosquitoes, nor in the two non-mosquito species. These observations suggest that the locus evolved in the late Triassic after divergence of the Anophelinae from the Culicinae subfamily of mosquitoes (229-192 mya[35]), but before further divergence of the culicine genera (226-172 mya[35]). This establishes this repeat locus as one of the very few ancient and deeply conserved satellite repeats that have hitherto been described[30-32,36]. Conservation of the locus over million years of mosquito evolution strongly suggests important and conserved functions for the locus and its associated piRNAs.

The satellite repeat locus overlaps with the 3'UTR of the gene AAEL017385 (LOC23687805) of unknown function in the current genome annotation (Fig 1A). This organization suggests that one or more splice variants of AAEL017385 are the source of the piRNAs, and that expression of this gene and piRNA biogenesis might be closely linked. However, knockdown of the different splice

isoforms did not reduce expression of tapiR1 (Extended Data Fig 3F, Supplementary Fig S1E), arguing against this possibility. Rapid amplification of 3' ends (RACE) of AAEL017385 transcripts revealed transcription termination sites directly upstream of the first tapiR1 and tapiR2 repeat, respectively (Extended Data Fig 3G). In addition, we detected a putative tapiR precursor only of ~500 nt which is much smaller than full-length AAEL017385 transcripts (~3.6-5.4 kb) (Extended Data Fig 3H), despite the whole repeat locus being covered by RNA sequencing reads (Extended Data Fig 3I). Even though we cannot exclude that this is a processing intermediate rather than the full-length precursor transcript, and that some AAEL017385 transcripts overlap with the satellite repeat, our data strongly suggest that the two piRNAs and AAEL017385 are expressed from the different transcriptional units. In support, the tapiR repeat locus is not associated with AAEL017385 orthologues in *Ae. albopictus* (AALF011179) and *Cx. quinquefasciatus* (CPIJ011773). We propose that the satellite repeat locus is transcribed from an unknown upstream or internal promoter.

We next characterized the sequence requirements for target silencing by tapiR1, as its expression is approximately one log higher compared to tapiR2 in Aag2 cells. Using a luciferase reporter harbouring a fully complementary target site in the 3' UTR, we validated that this piRNA is able to target RNAs *in trans*. The reporter was silenced more than 35-fold compared to a control reporter with a partially inverted target site (Fig 2A). Addition of an antisense oligonucleotide (AO) complementary to tapiR1, but not tapiR2, relieved silencing in a concentration-dependent manner (Extended Data Fig 4A), confirming that the observed effect is mediated by the piRNA in a sequence-specific fashion. Treatment with the tapiR1 AO did not relieve silencing of a tapiR2 reporter (Extended Data Fig 4A), and reduced only expression of tapiR1, but not tapiR2 (and *vice versa*) (Extended Data Fig 4B), indicating that the antisense oligonucleotides are specific to the mature piRNAs, and do not, or only to a minor extent, target the tapiR1/2 precursor. Additionally, AO treatment did not influence microRNA (miRNA) stability (Extended Data Fig 4B). Unlike most miRNAs[37], silencing was not dependent on the position of the target site in the mature mRNA, and was efficient in the open reading frame and both the 5' and 3' UTR (Fig 2B, Extended Data Fig 5A). In contrast, silencing was strongly reduced when the target site was located in an intron (Fig 2B), or in a non-translated transcript expressed from a polymerase III promoter (Extended Data Fig 5B). During the course of our study we noticed that the firefly and *Renilla* luciferase genes, which we used as reporter and normalization control, respectively, contain potential target sites for tapiR1. Using reporter assays, we observed that *Renilla* luciferase is indeed potently suppressed by tapiR1 and firefly luciferase slightly (Extended Data Fig 5C-E), yet, mutating these target sites did not affect our conclusions (Extended Data Fig 5F,G).

To assess targeting requirements for tapiR1, we introduced mismatches in the piRNA target sites. Three consecutive mismatches were tolerated unless they were located in the t1 to t9 region of the target (the nucleotides expected to based-pair to piRNA positions 1 to 9) (Fig 2C, Extended Data Fig 6A), and single mismatches only impaired silencing at positions t3 to t7 (Extended data Fig 6A,B), reminiscent of a microRNA seed[37] and comparable to the piRNA seed in *C. elegans*[38,39]. In contrast to observations with slicer (cleavage)-competent Argonautes including Drosophila PIWI proteins[40,41], basepairing at the potential Argonaute cleavage site between nucleotides t10 and t11 was dispensable for silencing.

A mismatch at position t1 did not alter silencing, suggesting that the first nucleotide is anchored in a binding pocket of Piwi4, similar to other Argonaute proteins[42-44]. Unexpectedly, a mismatch at position t2 was tolerated as well. This was, however, only the case when the rest of the target site was perfectly complementary, but not when the target contained mismatches outside of the seed (Extended Data Fig 6C). We further noticed that, in contrast to *C. elegans*[38], G:U wobble pairs were not tolerated inside the seed and had the same effect as a mismatch at the same position (Extended Data Fig 6D). Whereas the 5' seed region is normally absolutely required for targeting[38,39,41,42,45,46], the 3' part of the piRNA might increase specificity and efficiency of the targeting. For this reason we further assessed the extent of complementarity needed for tapiR1-mediated silencing. Introduction of increasing numbers of mismatches at the 3' end did not interfere with silencing when at least half of the piRNA could basepair with the target site (Fig 2D), indicating that the 3' part of the piRNA is not necessarily required, yet the seed region alone not sufficient for silencing.

Taken together, these results suggest that tapiR1 needs relatively low sequence complementarity to efficiently silence targets, a pattern that is more similar to target requirements of miRNAs than those of piRNAs studied thus far[38,41,45,46]. There are no constraints regarding the position of the target site on the mature mRNA, and as a consequence, tapiR1 may target a wide range of different cellular RNAs that perfectly basepair to the seed with additional matches outside the seed. Even though tapiR1 targeting requirements resemble those of miRNAs, the results are unlikely to be due to the piRNA being funnelled into the miRNA pathway. First, target silencing of different tapiR1 reporters, or tapiR1 target genes *in vitro* and *in vivo*, is dependent on Piwi4, but not on Ago1 (Extended Data Fig 8B,C, Extended Data Fig. 10H). Second, tapiR1 biogenesis itself is not dependent on Ago1 (Extended Data Fig 2E), and, third, tapiR1 is 2'-*O*-methylated (Fig 1C), a feature of siRNAs and mature PIWI-bound piRNAs, but not Ago1-associated miRNAs.

Given the extreme conservation of the tapiR repeat locus and the efficiency of target reporter silencing, we hypothesized that these piRNAs regulate cellular gene expression. Some satellite repeats can influence gene expression by modulation of the local chromatin environment *in cis*[47,48],

or have been hypothesized to induce silencing of genes with homologous repeat insertions[31]. In contrast, the tapiR locus has the potential to silence expression of a broad range of remote genes *in trans*, independent of repeat insertions in target genes, and thus, to regulate diverse and highly complex cellular processes.

To test this idea, we used RNAHybrid[49] to predict tapiR1 target sites and verified these sequences in luciferase reporter assays. Twelve out of 23 sites in protein-coding genes, lncRNAs, or transposable elements were indeed sufficient to support suppression of the reporter (Extended Data Fig 7). Encouraged by these results, we then blocked tapiR1-mediated silencing with the tapiR1 AO in Aag2 cells and assessed global gene expression by RNAseq two days after treatment. Intriguingly, expression of 134 genes, amongst which many long non-coding (lnc)RNAs, was significantly increased up to ~450 fold compared to the treatment with a control oligonucleotide (Fig 2E, Supplementary Table 1). Transposons were not globally affected, although some elements were up-regulated as well, up to around 850-fold (Extended Data Fig 8A, Supplementary Table 2). Expression of differentially expressed genes and a transposable element was increased in a concentration-dependent manner upon tapiR1 AO treatment as measured by RT-qPCR (Fig 2F), validating our RNAseq results. As expected, knockdown of Piwi4, but not Ago1, led to derepression of target gene expression and reporters carrying cellular target sites (Extended Data Fig 8B,C). Yet, tapiR2 AO treatment did not relieve silencing of these target genes (Extended Data Fig 8D), confirming again that the tapiR1 AO is specific for the mature piRNA.

Our results indicate that tapiR1 directly and strongly regulates expression of a subset of cellular genes and transposons in a sequence-dependent manner. Moreover, these data indicate that tapiR1 only needs limited basepairing to mediate silencing. Yet, computationally predicted tapiR1 target genes were not upregulated as a group upon tapiR1 AO treatment (Extended Data Fig 8E) and tapiR1 AO modulated genes were not enriched for predicted target sites. Moreover, the minimum free energy of piRNA/target duplexes was not predictive for effect sizes of tapiR1-mediated silencing, although we note that some of the strongest predicted targets were functional in a reporter context and among the genes with the strongest response upon tapiR1 AO treatment (Extended Data Fig 8F). Thus, similar to miRNAs[50], factors beyond Watson-Crick base-pairing seem to be essential to define *bona fide* target genes for tapiR1.

Argonaute proteins in complex with their bound small RNAs repress target RNAs in a sequence-specific manner, however, the mechanism of silencing depends on the nature of Argonaute protein (Extended Data Fig 9A). Whereas metazoan miRNA-Argonaute complexes can interfere with translation, or destabilize their targets by inducing deadenylation and decapping, siRNA-Argonaute complexes, as well as PIWI proteins cleave (slice) their target RNAs. The cleavage products are then either quickly degraded by cellular RNA degradation pathways, or, in the case of the ping-

pong amplification loop in the piRNA pathway, processed into secondary piRNAs[51]. Furthermore, the *Drosophila* Piwi protein induces transcriptional silencing at target loci in the nucleus.

The remarkably efficient silencing by Piwi4-tapiR1 raises questions about its mechanism of action. The ability of tapiR1 to efficiently silence mRNAs with target sites anywhere in the transcript but in introns (Fig 2B) suggest a post-transcriptional rather than a transcriptional mode of action.

Most confirmed target sites harbor a bulge at the t10 or t11 sites when basepaired to tapiR1 (Extended Data Fig 7A), which is thought to sterically prohibit cleavage of the target[43]. Additionally, basepairing of these nucleotides is dispensable for silencing (Fig 2C, Extended Data Fig E6B), arguing against cleavage-induced silencing. In agreement, we were unable to detect a slice product that would be the result of a Piwi4 cleavage event for a representative tapiR1 target genes by 5' RACE, despite the fact that we readily detected slice product when targeting the same sites with siRNAs (Extended data Fig 9B). Additionally, we did not observe secondary piRNA generation for confirmed target genes (Extended Data Fig 9C), supporting the notion that Piwi4-tapiR1 complexes neither slice targets, nor are involved in the ping-pong amplification loop.

We then tested the ability of tapiR1 to interfere with cap-dependent and cap-independent translation. Both a firefly luciferase reporter translated in a cap-dependent manner, as well as an Renilla Luc reporter translated from the cricket paralysis virus 5' internal ribosomal entry site (IRES) encoded on the same mRNA[52] were efficiently silenced to a similar extent (Extended Data Fig 9D). As miRNA-induced silencing complexes inhibit translation of cap-dependent, but not IRES-driven cap-independent reporters, these results distinguish the mechanism of Piwi4 from that of miRNA-mediated translational inhibition[53-55]. In line with that, tapiR1 AO treatment and Piwi4 knockdown resulted in substantial changes of target mRNA levels, as assessed by RNAseq and qPCR in Aag2 cells and *in vivo* in embryos (Figure 2E,F, Extended data Fig 7B-D, Figure 3E), arguing that Piwi4-tapiR1 complexes regulate RNA stability rather than interfere with protein translation. We thus performed an RNAi knockdown screen of genes encoding components of major cellular RNA decay pathways. However, only few factors had a minor effect on expression of a tapiR1 reporter (Extended Data Fig 9E), and none of these were reproducibly involved in silencing of established tapiR1 target genes, as measured by RT-qPCR (Extended Data Fig 9F, Supplementary Figure S1F,G). It is of note that tapiR1-mediated silencing was also independent of the CCR4/NOT deadenylase complex (Extended Data Fig 9E,F) that is important both for miRNA-mediated target silencing[56-58], and piRNA-mediated RNA decay in *Drosophila melanogaster* embryos[59]. The CCR4/NOT complex catalyses removal of poly(A) from mRNAs which initiates subsequent degradation[60]. In line with that, we did not consistently observe elongation of poly(A) tails of target RNAs upon AO treatment (Extended Data Fig 9G-I), while being able to detect even the small elongation of Sindbis virus (SINV) RNA upon infection in cells (from 37nt to a mean

length of 70nt)[61] (Extended Data Fig. 9J). Together, these results indicate that Piwi4-tapiR1 efficiently destabilize target mRNA at the post-transcriptional level, but the exact mechanism of silencing remains elusive.

Expression of satellite repeats is often regulated in a developmental or stage-specific manner[31,62], thus we analysed the expression pattern of tapiR1 and 2 throughout the mosquito life cycle. tapiR1 and 2 were not expressed during the first three hours of embryonic development of *Ae. aegypti* (Fig 3A), but could be detected in all subsequent life stages (Extended Data Fig 10A,B). Likewise, Piwi4 is expressed in all analysed tissues, but is already present in early embryos (Extended Data Fig 10C,D). The very beginning of embryonic development is characterized by a transcriptionally quiescent zygotic genome, and the first mitotic divisions are exclusively driven by maternally deposited transcripts and proteins. Maternal-to-zygotic transition (MZT) is marked by the degradation of these maternal transcripts and concomitant zygotic genome activation[63]. Destabilization of maternal transcripts initially occurs through maternal decay activities, and later, after onset of zygotic transcription also by zygotic components[63,64]. One of the decay mechanisms involves zygotically expressed miRNAs, for example miR-430, miR-427, and the miR-309 cluster in zebrafish[65], *Xenopus*[66], and flies[67], respectively. Since tapiR1 is expressed early during zygotic genome activation in *Ae. aegypti* embryos (Extended Data Fig 10E, Supplementary Fig S1H) and has strong gene silencing potential, we hypothesized that tapiR1 is involved in the zygotic degradation pathway and necessary for embryonic development. To test this idea we injected either tapiR1-specific AO or control AO into early pre-blastoderm *Ae. aegypti* embryos (before zygotic genome activation and expression of tapiR1) (Fig 3B), and assessed their development using a discrete scoring scheme (Supplementary Fig 1H). Strikingly, more than 90 % of all tapiR1 AO-injected embryos were arrested early in development, whereas about half of all control embryos showed obvious signs of developmental progression (Fig 3C). In accordance, only a small fraction of tapiR1 AO-injected embryos hatched (Fig 3D) and continued to develop as larvae, indicating that tapiR1-deficiency impedes development. RNA sequencing from tapiR1 AO or control AO-injected embryos 20.5 h after injection revealed massive deregulation of cellular transcripts (Fig 3E, Extended Data Fig 10F, Supplementary Table 3, 4). Expression of 205 genes, among which 44 lncRNAs, as well as few transposable elements was increased up to around 1000 and 500 fold in tapiR1 AO-treated embryos, respectively. We confirmed these results, and the dependency of these genes on Piwi4 for a selection of target genes by RT-qPCR upon tapiR1 AO and Piwi4 dsRNA injection (Extended Data Fig 10G,H). Target genes with predicted tapiR1 target sites were more strongly up-regulated than genes without predicted site, as indicated by a shift of the cumulative distribution of RNA fold changes (Fig 3F). Additionally, a subset of these genes was also found to be deregulated in tapiR1 AO-treated Aag2 cells (Extended Data Fig 10I). These findings show that

tapiR1 controls regulatory circuits by direct gene targeting *in vivo,* and that this function is essential for embryonic development, likely by promoting mRNA turnover of a subset of maternal transcripts. In line with this conclusion, confirmed target genes are down-regulated after the onset of tapiR1 expression (Fig 3G), and tapiR1 targets are overrepresented in transcripts that are maternally provided and degraded during MZT (Fig 3H).

piRNAs have been shown to promote degradation of *nanos*[59] and other transcripts involved in germ cell development[68]. Yet, this was dependent on transposon-derived piRNAs, and rather depicts a re-purposing of the existing piRNA pool, which is, due to its large targeting potential, ideal to be used to degrade a large number of transcripts. In contrast, we propose that, analogous to abundant miRNAs in other animals[65-67], Culicinae mosquitoes have evolved a specific piRNA to destabilize a defined set of maternally deposited transcripts in early embryonic development. To our knowledge, this is the first example of sequence-specific gene silencing by transcriptional products from a satellite repeat *in trans,* and underlines the regulatory potential of tandemly repeated DNA.

Methods

Cell culture

Aedes aegypti Aag2 cells were cultured in Leibovitz's L-15 medium (Invitrogen) supplemented with 10 % heat-inactivated Fetal Bovine Serum (PAA Laboratories), 2 % Tryptose Phosphate Broth Solution (Sigma Aldrich), 1x MEM Non-Essential Amino Acids (Invitrogen) and 50 U/ml penicillin/ streptomycin (Invitrogen) at 25 °C. Aag2 cells are a widely-used non-clonal cell line of probably embryonic origin[69] that expresses all somatic aedine PIWI proteins and produces both primary and secondary piRNAs via ping-pong dependent amplification[25,26]. For all experiments, cells were seeded the day before and used at 70-80 % confluency.

Mosquito rearing

Injections of embryos for RNA sequencing and northern blotting presented in Fig 3A,G were performed using a Cell fusion agent virus-free, isofemale *Aedes aegypti* strain called Jane. This strain was initiated from a field population originally sampled in the Muang District of Kamphaeng Phet Province, Thailand[70], and reared for 26 generations at 28±1°C, 75±5 % relative humidity, 12:12 hour light-dark cycle. Embryos were hatched under low pressure for 30-60 min. Larvae were grown in dechlorinated tap water and fed fish food powder (Tetramin) every two days. Adults were maintained in cages with constant access to a 10 % sucrose solution. Female mosquitoes were fed on commercial rabbit blood (BCL) through a membrane feeding system (Hemotek Ltd.) using pig intestine as membrane. For AO injections, female mosquitoes were transferred to 25 °C and 70 % humidity for at least two days before forced to lay eggs, and embryos were then placed back to 28 °C immediately after the injection. For the time-course experiment in Fig. 3A,G, embryos were kept at 25 °C during the course of the experiment. All other injection experiments were conducted with *Ae. aegypti* Liverpool strain, reared at 28 ±1 °C, 70 % humidity as described above, however, fed on human blood (Sanquin Blood Supply Foundation, Nijmegen, The Netherlands), and maintained at 28 °C throughout experiments.

Experiments presented in Extended data Fig 2A, and Extended data Fig 10A,B were performed with the *Ae. aegypti* Rockefeller strain, obtained from Bayer AG, Monheim, Germany. The mosquitoes were maintained at 27±1 °C with 12:12 hour light:dark cycle and 70 % relative humidity, as described before[71]. For the bloodfeeding experiment mosquitoes were offered human blood (Sanquin Blood Supply Foundation, Nijmegen, The Netherlands), and five engorged females were selected and sacrificed at each of the indicated time points.

Mosquitoes used in Fig 1F were either different laboratory-reared, or wild-caught species: *Aedes aegypti* Liverpool strain, *Culex pipiens, Anopheles coluzzii, An. quadriannulatus, An. stephensi* mosquitoes, *Culicoides nubeculosus* biting midges, and *D. melanogaster* w^{1118} flies were laboratory

strains. The mosquitoes were deep-frozen and stored at -80 °C until use. *Ae. albopictus*, *Ae. cantans*, *Ae. intrudens*, *Ae. pullatus*, *Ae. cinereus*, *Ae. vexans*, *Cx. pusillus*, *Culiseta morsitans*, *Coquillettidia richiardii*, *An. maculipennis*, *An. claviger*, and *An. coluzzii* were wild-caught individuals collected in different regions in Italy, Sweden, or the Netherlands between July 2014 and June 2015[72]. Species were identified at the species level, and stored at -20 °C for a maximum of two years.

Gene knockdown

Double-stranded RNA was generated by *in vitro* transcription of T7 promoter-flanked PCR products with T7 RNA polymerase. Primer sequences are given in Supplementary Table S5. The reaction was carried out at 37°C for 3 to 4 h, then heated to 80 °C for 10 min and gradually cooled down to room temperature to facilitate dsRNA formation. The dsRNA was purified with the GeneElute Total RNA Miniprep Kit (Sigma Aldrich).

Aag2 cells were seeded in 24-well plates the day before the experiment, and transfected with X- tremeGENE HP Transfection reagent (Roche) according to the manufacturer's instructions, using a ratio of 4 µL reagent per µg of dsRNA. The transfection medium was replaced after 3 h with fully supplemented Leibovitz-15 medium. For Extended Data Fig. 2D,E, Extended Data Fig 3F, and Extended Data Fig 8B, cells were harvested after 48h; in other experiments, the knockdown was repeated 48 h after the first transfection and cells were then harvested after 24 h. Knockdown was confirmed by RT-qPCR. For the RNAi screen shown in Extended Data Fig 9F, a luciferase reporter harbouring a target site of AAEL001555 in the 3' UTR, and a *Renilla* luciferase reporter were co-transfected with the dsRNA during the second transfection.

For injection of embryos with dsRNA, engorged female *Ae. aegypti* Liverpool mosquitoes were allowed to lay eggs for 45 min. Embryos were desiccated for 1.5 min, covered with Halocarbon oil (Sigma Aldrich) and injected with 500ng/µL dsRNA with the Pneumatic PicoPump PV820 (World Precision Instruments) with 30 psi inject pressure. Injected embryos were then transferred to a wet Whatman paper and kept at 28 °C and 80 % humidity for 21 h. Per experiment, 30-60 embryos were injected per condition.

RNA isolation

RNA from cells and mosquitoes was isolated with Isol-RNA lysis buffer (5PRIME) according to the manufacturer's instructions. Briefly, 200 µL chloroform was added to 1 mL lysis buffer, and centrifuged at 16,060 x g for 20 min at 4 °C. Isopropanol was added to the aqueous phase, followed by incubation on ice for at least one hour, and centrifugation at 16,060 x g for 10 min at 4 °C. The

pellet was washed three to five times with 85 % ethanol and dissolved in RNase free water. RNA was quantified on a Nanodrop photospectrometer.

Periodate treatment and β-elimination

Total RNA was treated with 25 mM $NaIO_4$ in a final concentration of 60 mM borax and 60 mM boric acid (pH 8.6) for 30 min at room temperature. In the control, $NaIO_4$ was replaced by an equal volume of water. The reaction was quenched with glycerol and β-elimination was induced with a final concentration of 40 mM NaCl for 90 min at 45 °C. RNA was ethanol precipitated and analysed by northern blot.

Generation of antibodies

Custom-made antibodies (Eurogentec) against endogenous PIWI proteins were generated by immunization of two rabbits per antibody with a mix of two unique peptides (Ago3: TSGADSSESDDKQSS, IIYKRKQRMSENIQF; Piwi4: HEGRGSPSSRPAYSS, HHRESSAGGRERSGN; Piwi5: DIVRSRPLDSKVVKQ, CANQGGNWRDNYKRAI; Piwi6: MADNPQEGSSGGRIR, RGDHRQKPYDRPEQS). After 87 days and a total of four immunizations (t=0, 14, 28, 56 days), sera of both rabbits were collected, pooled, and purified against each peptide separately. Specificity of the antibody was confirmed by Western blotting of Aag2 cells stably expressing PTH (Protein A, TEV cleavage site, 6x His-tag)-tagged PIWI[27] upon knockdown of the respective PIWI protein, or a control knockdown (dsRLuc).

Immunoprecipitation and western blotting

Aag2 cells were lysed with RIPA buffer (10 mM Tris-HCl, 150 mM NaCl, 0.5 mM EDTA, 0.1 % SDS, 1 % Triton-X-100, 10 % DOC, 1x protease inhibitor cocktail), supplemented with 10 % glycerol and stored at -80 °C until use. The IP was performed with custom-made antibodies against Piwi4-6 and Ago3 (1:10 dilution) at 4 °C for 4 h on rotation. Protein A/G Plus beads (Santa Cruz) were added at a dilution of 1:10 and then incubated overnight at 4 °C on rotation. Beads were washed 3 times with RIPA buffer, and half was used for RNA isolation and protein analysis each. For RNA extraction, beads were treated with proteinase K for 2 h at 55 °C and isolated with phenol-chloroform extraction. Equal amounts of RNA for input and IPs were then analysed by northern blotting. For western blotting, the IP samples were boiled in 2x Laemmli buffer for 10 min at 95 °C, separated on 7.5 % SDS-polyacrylamide gels, and blotted on 0.2 µm nitrocellulose membranes (Bio-Rad) in a wet blot chamber on ice. Membranes were blocked for 1 h with 5 % milk in PBS-T (137 mM NaCl, 12 mM phosphate, 2.7 mM KCl, pH 7.4, 0.1 % (v/v) Tween 20) and incubated with PIWI-specific (dilution 1:1000) and Tubulin primary antibodies (rat anti-Tubulin alpha, MCA78G,

1:1000, Sanbio) overnight at 4 °C. The next day, membranes were washed three times with PBS-T and incubated with secondary antibodies conjugated to a fluorescence dye (IRDye 800CW conjugated goat anti rabbit, 1:10,000, Li-Cor, and IRDye 680LT conjugated goat anti rat, 1:10,000, Li-Cor) for 1 h at room temperature in the dark. After washing three times in PBS-T, signal was detected with the Odyssey-CLx Imaging system (Li-Cor). Alternatively, *Ae.aegypti* Liverpool embryos were bead-beaten in RIPA buffer supplemented with 1mM PMSF (Sigma Aldrich), and boiled in 1x Laemmli buffer. Western blotting was performed as described above, however, the membrane was blocked in 5 % BSA in PBS-T, and incubated with pSer2 PolII (rabbit anti-RNA polymerase II CTD YSPTSPS (phosphor S2), ab5095, 1:1000, abcam), and Tubulin primary antibodies.

Northern blot

piRNAs were detected by northern blot analyses, as published in ref.[73]. Briefly, RNA was denatured at 80 °C for 2 min in Gel Loading Buffer II (Ambion) and size-separated on 0.5 x TBE (45 mM Tris-borate, 1 mM EDTA), 7 M Urea, 15 % denaturing polyacrylamide gels. RNA was then blotted on Hybond-NX nylon membranes (GE Healthcare) in a semi-dry blotting chamber for 45 min at 20 V and 4 °C and crosslinked to the membrane with EDC crosslinking solution (127 mM 1-methylimidazole (Sigma-Aldrich), 163 mM N-(3-dimethylaminopropyl)-N´-ethylcarbodiimide hydrochloride (Sigma-Aldrich), pH 8.0) at 60 °C for two hours. Crosslinked membranes were pre-hybridized in ULTRAHyb-Oligo hybridization buffer (Thermo Scientific) for one hour at 42 °C and probed with indicated ^{32}P 5' end-labelled DNA oligonucleotide probes overnight at 42 °C. Membranes were then washed with decreasing concentrations of SCC (300 mM NaCl, 30 mM sodium citrate pH 7.0; 150 mM NaCl, 15 mM sodium citrate; 15 mM NaCl, 1.5 mM sodium citrate) and 0.1 % SDS, and exposed to Carestream BioMax XAR X-Ray films (Kodak). Probe sequences can be found in the Supplementary Table S5.

To detect a putative tapiR precursor transcript, RNA was size-separated on a 0.5 x TBE, 7 M Urea, 6 % denaturing polyacrylamide gel, transferred to Hybond-N+ nylon membranes (GE Healthcare) in a semi-dry blotting chamber for 2 h at 200 mA at 4 °C, UV-crosslinked at 150 mJ, pre-hybridized in ULTRAHyb Ultrasensitive hybridization buffer (Thermo Scientific), and probed with ^{32}P-labeled DNA probes overnight at 42 °C, washed as described above, exposed to a Storage Phosphor Screen GP (Kodak), and developed with the Amersham Typhoon 5 Biomolecular Imager (GE Healthcare). The probe was produced with the Amersham Rediprime DNA Labelling System II (GE Healthcare) from a PCR-amplified sequence of the tapiR locus spanning 1.5 repeat units (~450 nt), that was cloned with flanking T7 sites separated by *Eco*RI and *Zra*I restriction sites into the pUC19 vector.

Reporter cloning and luciferase assay

Reporters were constructed by cloning annealed and phosphorylated oligonucleotides with the indicated tapiR1 or control target sites in the pMT-GL3 vector[74]. This vector encodes the *Photinus pyralis* firefly luciferase (GL3) under a copper-inducible metallothionein promoter. Sense and antisense oligonucleotides (Sigma Aldrich) were annealed by heating to 80 °C, and gradually cooling down to room temperature, phosphorylated with T4 polynucleotide kinase (Roche) at 37 °C for 30 min, purified and then ligated into the pMT-GL3 vector. For cloning of 3'UTR and 5' UTR reporters, the target site or the target site and an upstream *Bam*HI site were cloned into the *Pme*I and *Sac*II, or *Not*I and *Xho*I restriction sites, respectively. ORF reporters were constructed by cloning a Kozak sequence followed by the first 45 nucleotides of luciferase and the target site into *Xho*I and *Nco*I sites. For designing the intron reporters, the first intron of RpS7 (AAEL009496) was cloned behind the duplicated first 45 nt of luciferase in the pMT-GL3 vector, and the original ATG of firefly luciferase was mutated, and 3 stop codons were introduced at the 3' end of the intron, and a *Bsa*BI restriction site was inserted in the first third of the intron of by site directed mutagenesis. Afterwards, oligonucleotides encoding tapiR1 or control target sequences were inserted into *Bsa*BI as described above. IRES-containing reporters were designed by cloning the 5' UTR of cricket paralysis virus amplified from infected S2 cells into *Pme*I and *Sac*II restriction sites of the pMT-GL3 vector with a mutated tapiR1 target site (ΔtapiR1) using the HD In-Fusion cloning kit (Takara). Afterwards oligonucleotide containing tapiR1 or control target sites were cloned into the *Sac*II site in the 3' UTR as described above. RNAPIII reporters were constructed by cloning the *Ae. aegypti* U6 promoter and the GL3 3' UTR including different tapiR1target sites and a series of 6 T's as termination signal into pUC19 with the HD In-Fusion cloning kit (Takara). As normalization control, a part of GL3 ORF was cloned downstream of the U6 promoter. Sequences of the oligonucleotides are provided in Supplementary Table S5. Correct insertion of target sites was confirmed by Sanger sequencing for all clones.

Where indicated, mutated firefly or *Renilla* luciferase versions were used that harbour synonymous mutations destroying the predicted target sites for tapiR1 (firefly luciferase, nt 782: 5'-gagtcgtcttaatgtatagatttgaagaa-3' mutated to 5'-g**t**gtcgt**gc**t**t**atgta**cc**g**g**tt**c**ga**g**ga**g**-3', and *Renilla* luciferase, nt 462 5'-tgaatggcctgatattgaagaa-3' mutated to 5'-tga**g**tggcc**a**gatat**c**ga**g**ga**g**-3'; modified nucleotides in bold). Aag2 cells were seeded in 96-well plates the day before the experiment and transfected with 100 ng of the indicated plasmids and 100 ng pMT-*Renilla*[74] per well, using 2 µL X-tremeGENE HP DNA transfection reagent per 1 µg plasmid DNA according to the manufacturer's instructions. Alternatively, 100 ng reporter plasmid and 100 ng pMT-*Renilla* were co-transfected with the indicated amounts of unlabelled, fully 2'*O*-methylated antisense RNA oligonucleotide using an additional amount of 4 µL X-tremeGENE HP DNA transfection reagent (Roche) per 1 µg

oligonucleotide. Medium was replaced 3 h after reporter plasmid transfection with 0.5 mM $CuSO_4$ in fully supplemented Leibovitz's L-15 medium to induce the metallothionein promoter. 24 h later, cells were lysed in 30 µL Passive lysis buffer (Promega) and activity of both luciferases was measured in 10 µL of the sample with the Dual Luciferase Reporter Assay system (Promega) on a Modulus Single Tube Reader (Turner Biosystems). Firefly luciferase was normalized to *Renilla* luciferase activity. For each construct, at least two to three independent clones were measured in triplicate in order to exclude clonal effects.

RT-qPCR

1 µg of total RNA was treated with DNaseI (Ambion) for 45 min at 37 °C and reverse transcribed using the Taqman reverse transcription kit (Applied Biosystems) according to the manufacturer's protocol. Real-time PCR was performed with the GoTag qPCR Master Mix (Promega) and measured on a LightCycler480 instrument (Roche) with 5 min initial denaturation and 45 cycles of 5 s denaturation at 95 °C, 10 s annealing at 60 °C and 20 s amplification at 72 °C. Starting fluorescence values of specific mRNAs were calculated with linear regression method of log fluorescence per cycle number and LinRegPCR program, version 2015.3, as described in ref. [60].

Stem-loop RT-qPCR

Quantification of tapiR1 piRNA levels were performed similar to miRNA quantification as published ref.[75]. Briefly, 100ng of total RNA was reverse transcribed with 10 pmol stem-loop RT primer using 25u Superscript II reverse transcriptase (Invitrogen) in 1x First Strand buffer, 0.33 mM dNTPs, and 2u RNase inhibitors. cDNA was then measured by Real-time PCR as described above.

3' RACE, and 5' RACE of slicer products

3' Rapid Amplification of cDNA Ends (3' RACE) was performed using the FirstChoice RLM-RACE Kit (Thermo Fischer Scientific) according to the manufacturer's instructions. Amplification products were separated on agarose gel, purified and Sanger sequenced. Slicer products were detected from1 µg total RNA by 5' RACE following to the instructions, but without prior Calf Intestine Alkaline Phosphatase and Tobacco Acid Pyrophosphatase treatment. After amplification by PCR RACE products in the size range of 150-250 nt were purified from agarose gel, and cloned into pUC19 with HD In-Fusion cloning kit (Takara Bio) according to the manufacturer's instructions, and sequenced by Sanger sequencing.

Primer sequences can be found in Supplementary Table S1.

tapiR1/tapiR2 antisense oligonucleotide treatment and injection

Aag2 cells were seeded in 24-well plates the day before the experiment. Cells were treated with 500 nM 5'Cy5-labelled, fully 2'*O*-methylated antisense RNA oligonucleotide in 530 µL medium with 4 µL X-tremeGENE HP DNA transfection reagent (Roche) per 1 µg oligonucleotide. Medium was replaced after 3 h and cells from three independent experiments were harvested 48 h after transfection and prepared for RNA sequencing (see below). For 5' RACE, cells were treated with 200 nM antisense oligonucleotides together with 50 nM of siRNA duplexes, and RNA was harvested 24 h later.

Ae. aegypti Jane embryos were injected with antisense oligonucleotides similar to as described above. However, the engorged female mosquitoes were kept at 25 °C and 70 % humidity and allowed to lay eggs for 45 min. Injection was performed with a FemtoJet 4x (Eppendorf) with 1200 hPa pressure. Injected embryos were then transferred to a wet Whatman paper and kept at 27 °C and 80 % humidity for the indicated times. Per experiment, 50 to 150 embryos were injected per condition.

Scoring of embryo development and hatching

Injected embryos were allowed to develop for 2.5 days after injection on a moist Whatman paper and then fixed in 4 % paraformaldehyde for 8 h to overnight. Afterwards, the pigment of the endochorion was bleached with Trpis solution[76] (0.037 M sodium chlorite, 1.45 M acetic acid) for 24 to 48 h. Embryos were washed five times in PBS and images were taken with a EVOS FL imaging system (Thermo Fisher Scientific). Embryos with evident larval segmentation (head, fused thoracical elements and abdomen) were scored as fully developed and embryos without any evident structure of the ooplasm as undeveloped. Individuals that showed first signs of structural rearrangements of the ooplasm, but did not complete larval segmentation were scored as intermediate (see Supplementary Fig S1F). To avoid biases, the scoring was performed blindly. Hatching rate was counted from injected embryos 4 days post injection. Embryos were kept moist for two days and then allowed to slowly dry for the rest of the period. The embryos were transferred to water and then forced to hatch by applying negative pressure for a period of 30 min. The number of hatched L1 larvae was counted immediately afterwards.

Poly(A) tail length (PAT) assay

Ligation-mediated (LM) PAT assay was performed as published in ref.[77], and RACE PAT as published in ref.[78]. Superscript IV Reverse transcriptase (Invitrogen) was used for cDNA synthesis. RACE-PAT products were amplified by PCR with 3 min initial denaturation at 95 °C, 35 cycles of 30s denaturation, 30s annealing at 60 °C and 1 min extension at 72 °C, and final extension for 5

min, while PCR was performed likewise, but with 2 min extension each cycle. PCR products were separated on agarose gel, and stained with ethidium bromide. As control, Sindbis virus poly(A) tails were compared between *in vitro* transcribed RN, produced as described previously ref.[79,80], and total RNA isolated from infected Aag2 cells.

Sequence logo

Repeat monomers from the satellite repeat loci in *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus* were extracted manually from the current genome annotations obtained from VectorBase (*Aedes aegypti* Liverpool AaegL5, *Aedes albopictus* Foshan AaloF1, *Culex quinquefasciatus* Johannesburg CpipJ2). A repeat unit was defined as the sequence starting from the first tapiR1 nucleotide until one nucleotide upstream of the next tapiR1 sequence. Sequences were aligned using MAFFT (v7.397)[81] (with options –genafpair –leavegappyregion --kimura 1 --maxiterate 1000 --retree 1) and the sequence logo was constructed with the R package ggseqlogo[82].

Small RNA sequencing

Small RNAs from Aag2 cells (input) or PIWI immunoprecipitations were cloned with the TruSeq small RNA sample preparation kit (Illumina) according to the manufacturer's instructions. For the input sample, size selected 19-33 nt small RNAs purified from polyacrylamide gel were used to construct the library as described previously[83], whereas IP samples were not extracted from gel but isolated as described before[27]. Libraries were sequenced on an Illumina HiSeq 4000 instrument by Plateforme GenomEast (Strasbourg, France).

mRNA sequencing

RNA was isolated from Aag2 cells 48 h after AO transfection (three independent experiments), or embryos 20.5 h after AO injection (50 embryos pooled per experiment from five independent experiments) with RNAsolv reagent following standard phenol-chloroform extraction. Polyadenylated RNAs were extracted and sequencing libraries were prepared using the TruSeq stranded mRNA Library Prep kit (Illumina) following the manufacturer's instructions, and sequenced on an Illumina Hi-Seq 4000 instrument (2x50 bases).

Analysis of mRNA sequencing

Reads were mapped to the *Ae. aegypti* genome AaegL5 as provided by VectorBase (https://www.vectorbase.org) with STAR (version 2.5.2b)[84] in 2-pass mode: first mapping was done for all samples (options: --readFilesCommand zcat --outSAMtype None --outSAMattrIHstart 0 --outSAMstrandField intronMotif), identified splice junctions were combined (junctions located

on the mitochondrial genome were filtered out, as these are likely false positives), and this list of junctions was used in a second round of mapping (with –sjdbFileChrStartEnd) and default parameters as above. Reads were quantified with the additional option –quantMode GeneCounts to quantified reads per gene. Alternatively, reads were quantified on TEfam transposon consensus sequences (https://tefam.biochem.vt.edu/tefam/get_fasta.php) with Salmon (v.0.8.2)[85], default settings and libType set to "ISR". Statistical and further downstream analyses were performed with DESeq2[86] from Bioconductor. Significance was tested at an FDR of 0.01 and a log2 fold change of 0.5. tapiR1 target sites were predicted with the online tool from RNAHybrid[49] with helix constraints from nucleotide two to seven, and no G:U wobble allowed in the seed. Predictions were made on the AaegL5.1 geneset as provided by VectorBase, and on TEfam transposon consensus sequences. For Fig4H, publicly available sequencing datasets[87] (accession numbers: SRR923702, SRR923826, SRR923837, SRR923853, SRR923704) were mapped and quantified as described above. Genes were categorized on the basis of their expression in embryos at 0-2 h vs. 12-16 h post egg-laying. Genes not detected in the 0-2 h sample were defined as purely zygotic, and genes that did not increase or decrease by more than $\log_2(0.5)$ as maternal stable. Genes that changed in expression by more than $\log_2(0.5)$, $\log_2(2)$, and $\log_2(5)$ from 0-2 h to 12-16 h were categorized as maternal unstable fraction (decreased expression), or as genes that are maternally provided but are transcribed by the zygote in addition to the preloaded maternal pool (increased expression). tapiR1 targets were defined as genes that were significantly upregulated at least two fold in tapiR1 AO injected embryos and harbour a predicted tapiR1 target site (mfe <= -24).
Sequenced libraries are available under BioProject number PRJNA482553. A list of publicly available datasets that were used in this study is provided in Supplementary Table S6.
The code will be made available on GitHub upon publication.

Analysis of small RNA sequencing

3' sequencing adapters (TGGAATTCTCGGGTGCCAAGG) were trimmed from the sequence reads with Cutadapt (version 1.14)[88] and trimmed reads were mapped with Bowtie (version 0.12.7)[89] to the *Aedes aegypti* LVP_AGWG genome sequence AaegL5.1 obtained from VectorBase with at most 1 mismatch. Reads that mapped to rRNAs or tRNAs were excluded from the analyses. Alternatively, 3'sequencing adapters ((NNN)TGGAATTCTCGGGTGCCAAGGC) and three random bases were trimmed from publicly available datasets from *Ae. aegypti* somatic and germline tissues[90] (SRR5961503, SRR5961504, SRR5961505, SRR5961506) and then processed as described above. Oxidized libraries, IPs and input sample were normalized to the total number of mapped reads, all other libraries to the total number of miRNAs (in millions). piRNAs that were at least two fold enriched in a PIWI-IP compared to the corresponding input sample and were present

with at least 10 rpm in the IP sample were considered PIWI-bound. Mapping positions were overlapped with basefeatures and repeatfeatures retrieved from VectorBase and counted with bedtools[91]. Reads that mapped to two or more features were assigned to only one feature with the following hierarchy: open reading frames > non-coding RNAs (incl. lncRNAs, pseudogenes, snoRNAs, snRNAs, miRNAs) > LTR retrotransposons > Non-LTR retrotransposons (SINEs, LINEs, Penelope) > "Cut and paste" DNA transposons > other DNA transposons (Helitrons, MITEs) > satellite and tandem repeat features > DUST > other /unknown repeats. Accordingly, reads that mapped to a repeat feature and an intron or UTR were classified as repeat-derived, whereas all other reads mapping to introns or UTRs were considered as gene-derived. Positions not overlapping with any annotation were summarized as "other". Results were then visualized with ggplot2[92], or Gviz[93] in R.

The code will be made available on GitHub upon publication.

Data availability

Raw sequence data is deposited in the NCBI Sequence Read Archive under the BioProject number PRJNA482553.

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Supplementary Information

Supplementary Table S1: Differentially expressed genes upon tapiR1 AO treatment in Aag2 cells.

Supplementary Table S2: Differentially expressed transposable elements upon tapiR1 AO treatment in Aag2 cells.

Supplementary Table S3: Differentially expressed genes upon tapiR1 AO treatment in embryos.

Supplementary Table S4: Differentially expressed transposable elements upon tapiR1 AO treatment in embryos.

Supplementary Table S5: Oligonucleotide sequences used in this study.

Supplementary Table S6: RNA sequencing datasets used in this study.

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Author contributions

R.H., P.M., and R.P.v.R designed the experiments and analysed the data. R.H. performed the computational analyses and most of the experiments, except for PIWI-IPs for small RNA sequencing (J.J. and E.T.), design and validation of PIWI antibodies (B.P.), and tissue isolations and blood feeding experiment (C.B.F.V. and C.J.K.). C.B.F.V. and C.J.K. provided wild-caught mosquito samples. I.R. assisted with the experiments, and S.H.M. and L.L. helped with optimizing embryo injections. R.H. and R.P.v.R. wrote the paper. All authors read and contributed to the manuscript.

Author information

The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to R.P.v.R. (ronald.vanrij@radboudumc.nl).

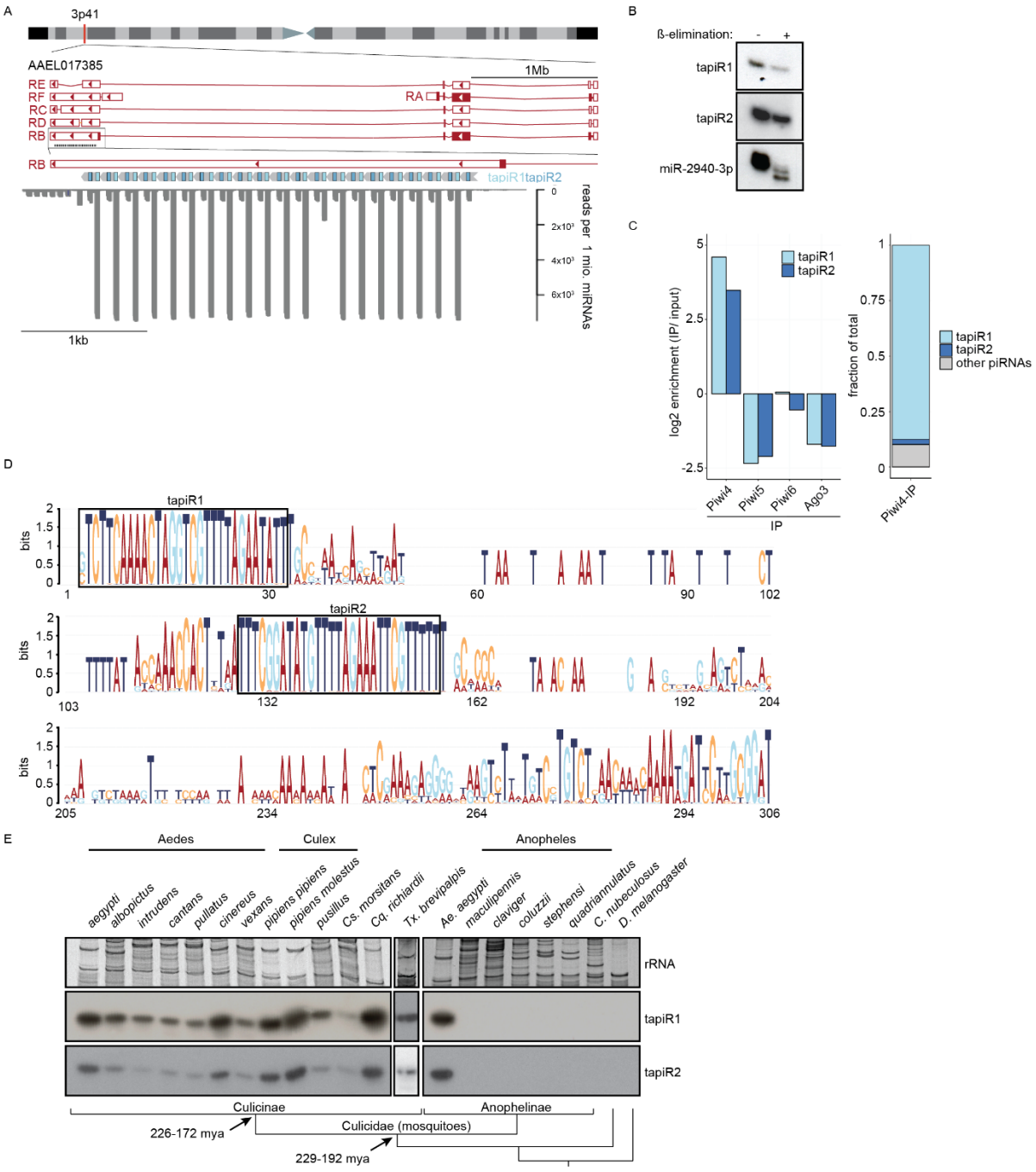


Figure 1: An evolutionary conserved satellite repeat produces piRNAs that associate with Piwi4. (A) Top panel: Current annotation of the gene AAEL017385 and its splice variants (RA-RF) on chromosome 3, with the position of the satellite repeat locus and tapiR1 and 2 sequences indicated. Fill boxes represent open reading frames. Bottom panel: Read coverage of the satellite repeats locus in Aag2 cells (small RNAs per million mapped miRNAs).
(B) Small RNA nothern blot of tapiR1 and 2 upon ß-elimination in Aag2 cells. miR-2940-3p serves as positive control for the treatment.

(C) Enrichment or depletion of tapiR1 and 2 in small RNA sequencing libraries of the indicated PIWI-IP compared to input samples (left panel), and fraction of tapiR1 and 2 of total Piwi4-enriched reads (2-fold, right panel).

(D) Sequence conservation of the satellite repeat monomers. All individual repeat monomers from *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* were used to generate the sequence logo. Boxes highlight the position of tapiR1 and 2 in the monomer.

(E) Northern blot analysis of tapiR1/ 2 in the indicated mosquito species (genera *Aedes, Culex, Culiseta, Coquillettidia*, and *Anopheles*) and other insects (*Culicoides* and *Drosophila*). Ethidium bromide-stained rRNA serves as loading control. For comparison, *Ae. aegypti* was included twice. Schematic representation of the phylogenetic relationships based on ref.[35] are indicated in the bottom panel. Bar lengths are arbitrary and do not reflect evolutionary distances.

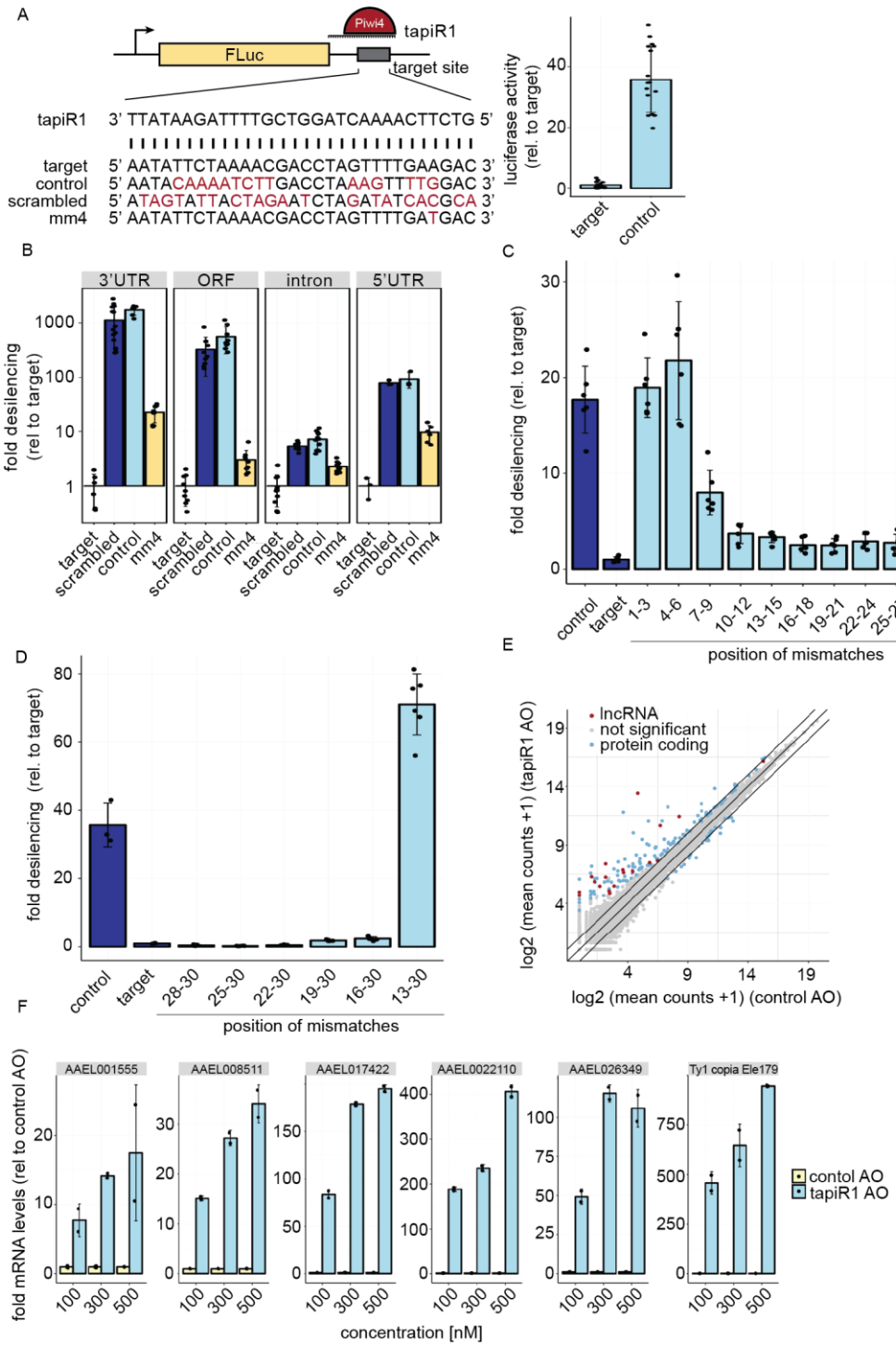


Figure 2: tapiR1 silences target RNAs *in trans* through seed-mediated base-pairing.

(A) Schematic representation of the firefly luciferase (FLuc) reporter constructs (left panel) and luciferase assay in Aag2 cells transfected with reporters containing a fully complementary target site to tapiR1, or a control target site.

(B) Luciferase assay of reporters with a fully complementary tapiR1 target site, a target site with a mismatch at position 4 (mm4), or control sequences introduced at different positions in the reporter mRNA.

(C, D) Luciferase assay of tapiR1 reporters harbouring three consecutive mismatches (C), or increasing number of mismatches (D) at the indicated positions of the piRNA target site in the

3’ UTR of firefly luciferase. Firefly luciferase activity was normalized to the activity of a co-transfected *Renilla* luciferase reporter. Indicated are mean, standard deviation, and individual measurements of a representative experiment performed with two to three independent clones per construct and measured in triplicate wells.

(E) log2 expression of mRNAs and lncRNAs in Aag2 cells upon treatment with a tapiR1 specific or control antisense oligonucleotide (AO). Depicted are average read counts in three biological replicates. A pseudo-count of one was added to all values in order to plot values of zero. Diagonal lines indicate a fold change of two. Significance was tested at a false discovery rate (FDR) of 0.01 and a log2 fold change of 0.5 as indicated by coloured dots.

(F) RT-qPCR of tapiR1 target genes upon transfection of Aag2 cells with tapiR1 specific or control AO. Depicted are mean, standard deviation, and individual measurements of one experiment measured in technical duplicates.

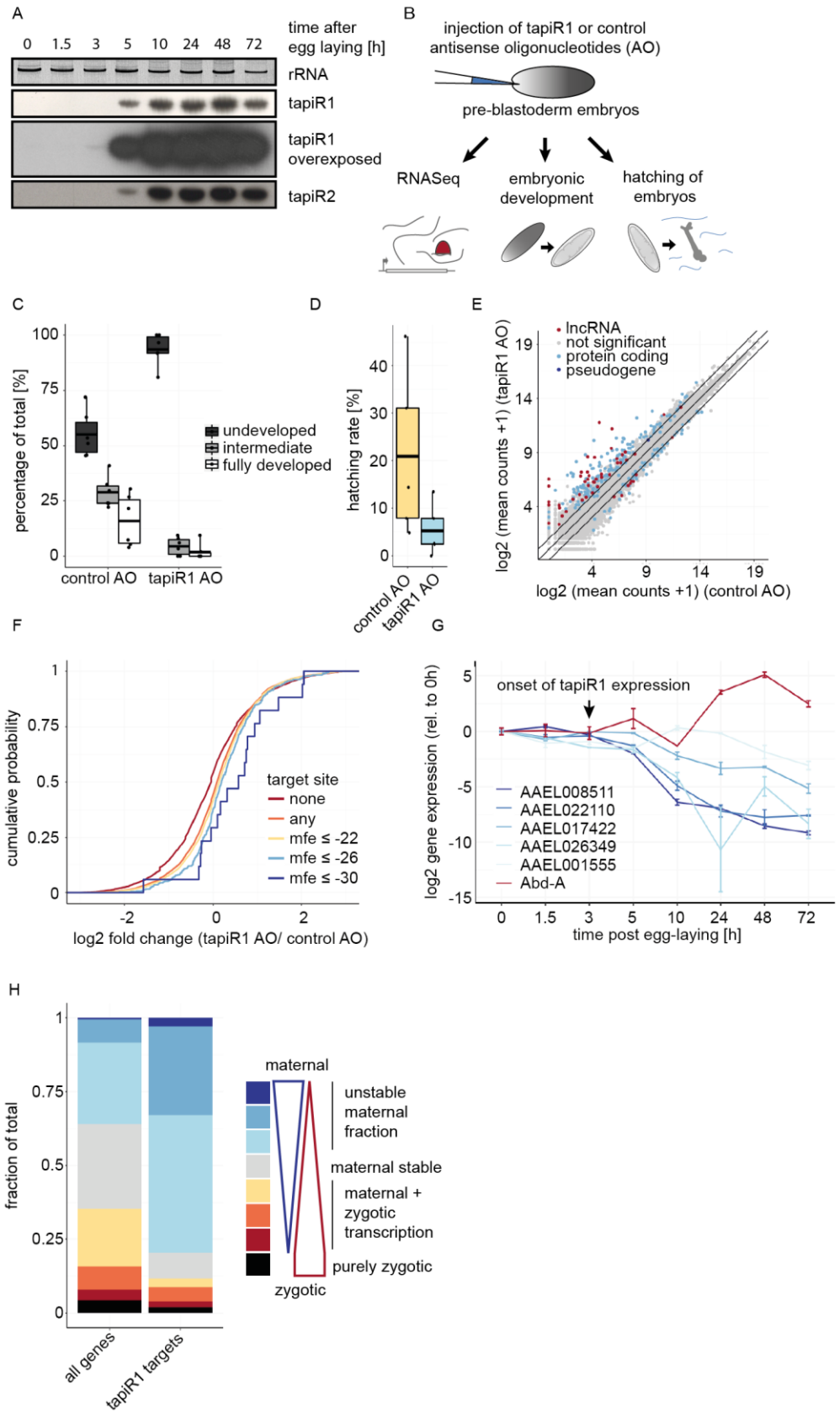
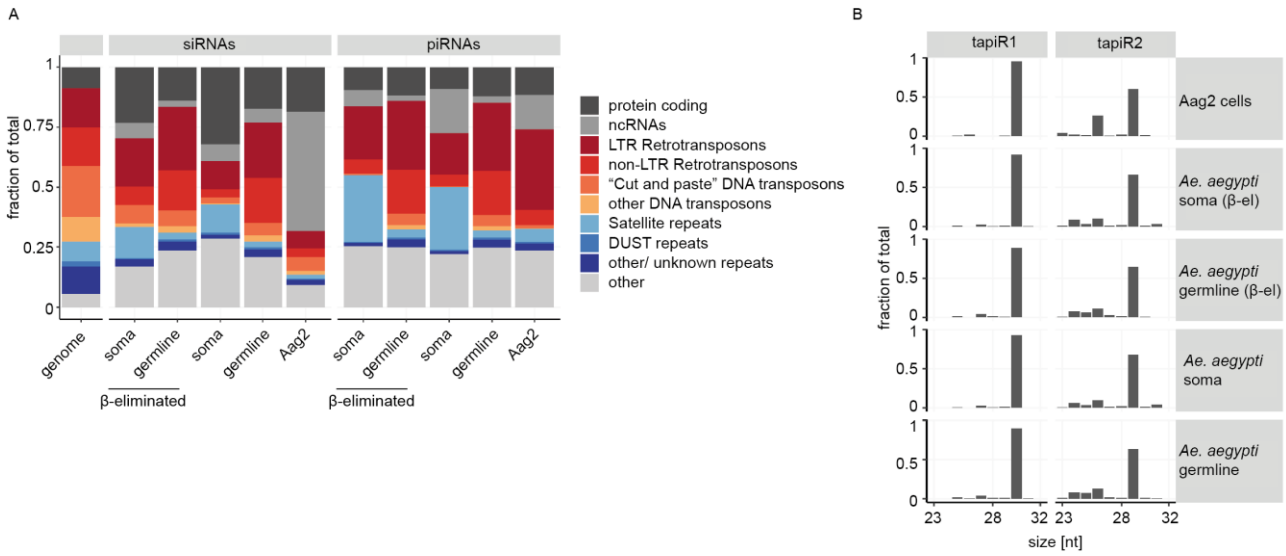


Figure 3: tapiR1 is essential for embryonic development *in vivo* by promoting turnover of maternally deposited transcripts.

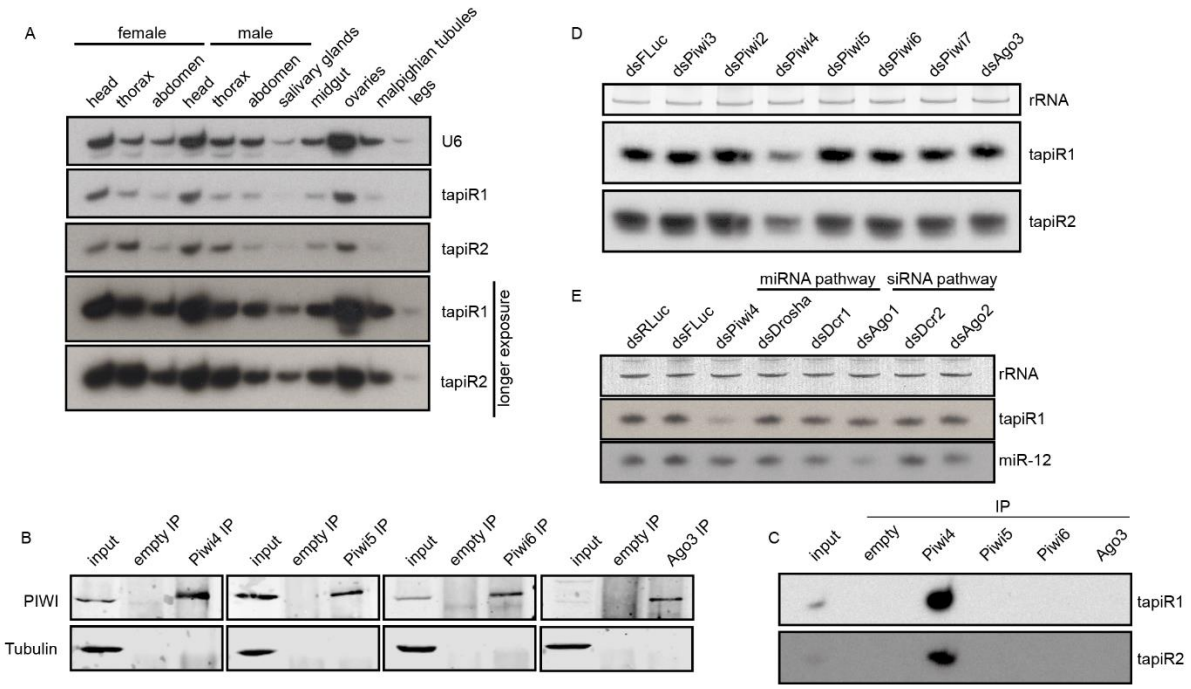
(A) Expression of tapiR1 and 2 in *Ae. aegypti* embryos as analysed by northern blot. Time indicates the age of the embryos after a 30 min egg laying period. For each time point, around 50 to 150 eggs were pooled. Ethidium bromide-stained rRNA serves as loading control.
(B) Outline of the experimental procedure for panels C-F.
(C) Percent of embryos injected with either tapiR1 or control AO that reached the indicated developmental stages 2.5 days post injection. Individual embryos were scored as either undeveloped, intermediate or fully developed, as shown in Supplementary Figure S1I. The effect on tapiR1 AO treatment on development was analysed using a X^2 test of independence: X^2 (2, N = 521) = 105.05, p<2.2e-16.
(D) Percent of embryos injected with tapiR1 or control AO that hatched four days post injection. Box-whisker plot represents mean, first and third quartile and maximum and minimum of the data. Points show the individual experiments with 20 to 60 (C), or 50 to 150 (D) embryos per group.
(E) log2 expression of genes in embryos injected with tapiR1 specific AO or control AO at 20.5 h post injection. Mean counts from five biological replicates plus a pseudo-count of one are plotted. Per replicate, 50 embryos per group were pooled. Significance was tested at an FDR of 0.01 and a log2 fold change of 0.5. Diagonal lines highlight a fold change of two.
(F) Experimental cumulative distribution of log2 fold changes of genes without or with predicted target sites for tapiR1. Target sites were grouped based on the predicted minimum free energy (mfe) of the piRNA/target duplex.
(G) Expression of tapiR1 target genes in *Ae. aegypti* embryos. RT-qPCR was performed on samples shown in (A). Abd-A is a gene not targeted by tapiR1 and serves as negative control.
(H) Fraction of genes in different classes expressed in embryos between 0 and 16 h post egg-laying. Dark blue to dark red represent changes in abundance between 0-2 h and 12-16 h that are larger than a log2 fold change of 5, 2, 0.5, between 0.5 and -0.5, or smaller than -0.5, -2, and -5, respectively. Genes were considered purely zygotic when they were covered by less than 1 read at 0-2 h.



Extended Data Figure 1: Expression of piRNAs from a satellite repeat locus.

(A) Fraction of siRNAs and piRNAs mapping on genomic features in adult *Ae. aegypti* female ovaries (germline) or carcasses (soma). Small RNAs that overlapped multiple features were assigned to only one category (see Methods). The leftmost bar depicts the fraction of each feature category in the genome.

(B) Read length distribution of tapiR1 and 2 in Aag2 cells, and adult germline and somatic tissues (β-eliminated, or untreated).

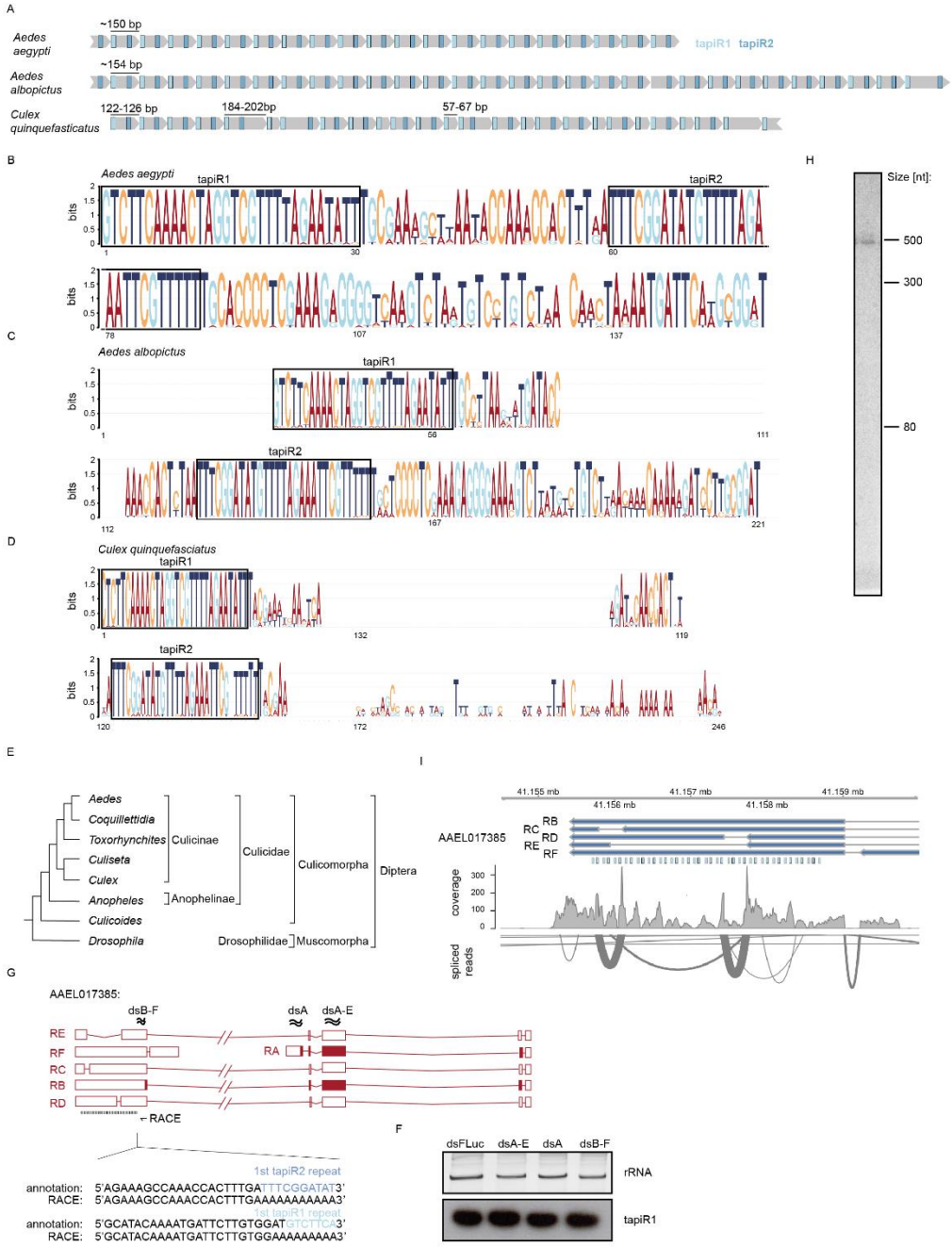


Extended Data Figure 2: tapiR1 and 2 are expressed in *Ae. aegypti* mosquitoes and associate with Piwi4.

(A, D, E) Northern blot of tapiR1 and 2 in different tissues of adult mosquitoes (A), upon dsRNA-mediated knockdown of individual PIWI genes (D), and upon knockdown of miRNA and siRNA pathway genes (E), or a control dsRNA treatment (dsFLuc and dsRLuc) in Aag2 cells. U6 snRNA or ethidium bromide-stained rRNA serves as loading controls.

(B) Western blot analysis of the indicated PIWI proteins before (input) and after immunoprecipitation (IP) used for the small RNA northern blot of panel C. An IP with empty beads serves as negative control. Tubulin was used to control for non-specific binding.

(C) Immunoprecipitation of the indicated PIWI proteins from Aag2 cells followed by northern blot analyses for tapiR1 and 2.



Extended Data Figure 3: Expression of tapiR1 is conserved in culicine mosquitoes, and is independent of AAEL017385.

(A) Schematic representation of the tapiR satellite repeat locus in *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus*. Numbers indicate repeat lengths, and, for *Cx. quinquefasciatus*, lengths of deviating repeat monomers.

(B-D) Sequence logos constructed from all individual tapiR repeat units in *Ae. aegypti* (B), *Ae. albopictus* (C), or *Cx. quinquefasciatus* (D). Gaps in the sequence logos mainly arise due to size heterogeneity in few repeat monomers.

(E) Evolutionary relationships of dipterous genera based on ref.[35]. Bar lengths are arbitrary and do not reflect evolutionary distances.

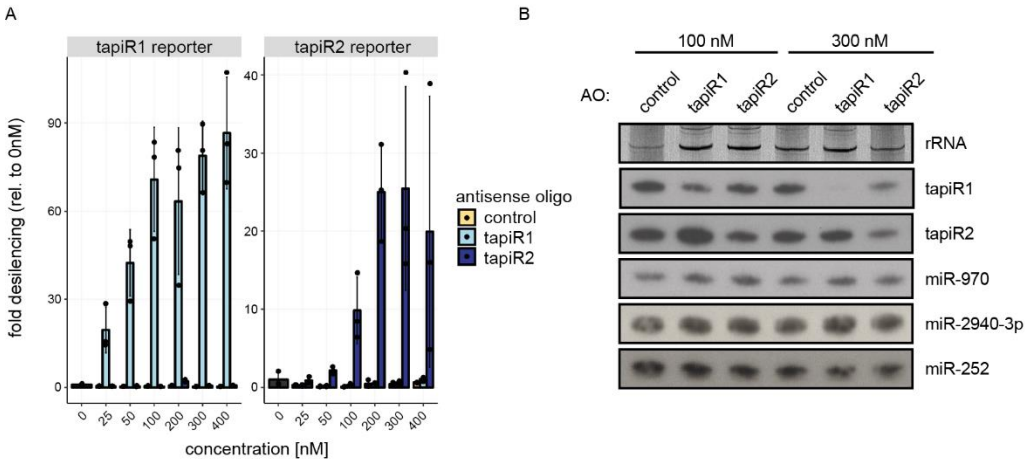
(F) Northern blot of tapiR1 in Aag2 cells treated with dsRNA targeting different transcripts of AAEL017385 (indicated in panel G), or, as control, firefly luciferase (FLuc). Ethidium bromide stained rRNA serves as loading control.

(G) Top panel: Schematic representation of the AAEL017385 locus and satellite repeat. The primer used for 3' RACE, and positions targeted by dsRNA in panel F are indicated with an arrow and wavy lines, respectively.

Bottom panel: 3' RACE analysis of AAEL017385. Indicated are sequences from the current AaegL5 genome annotation and RACE PCR products. The sequences of the 5' terminal part tapiR1 and 2 repeats are highlighted with colours.

(H) Northern blot of a potential tapiR1/2 precursor transcript.

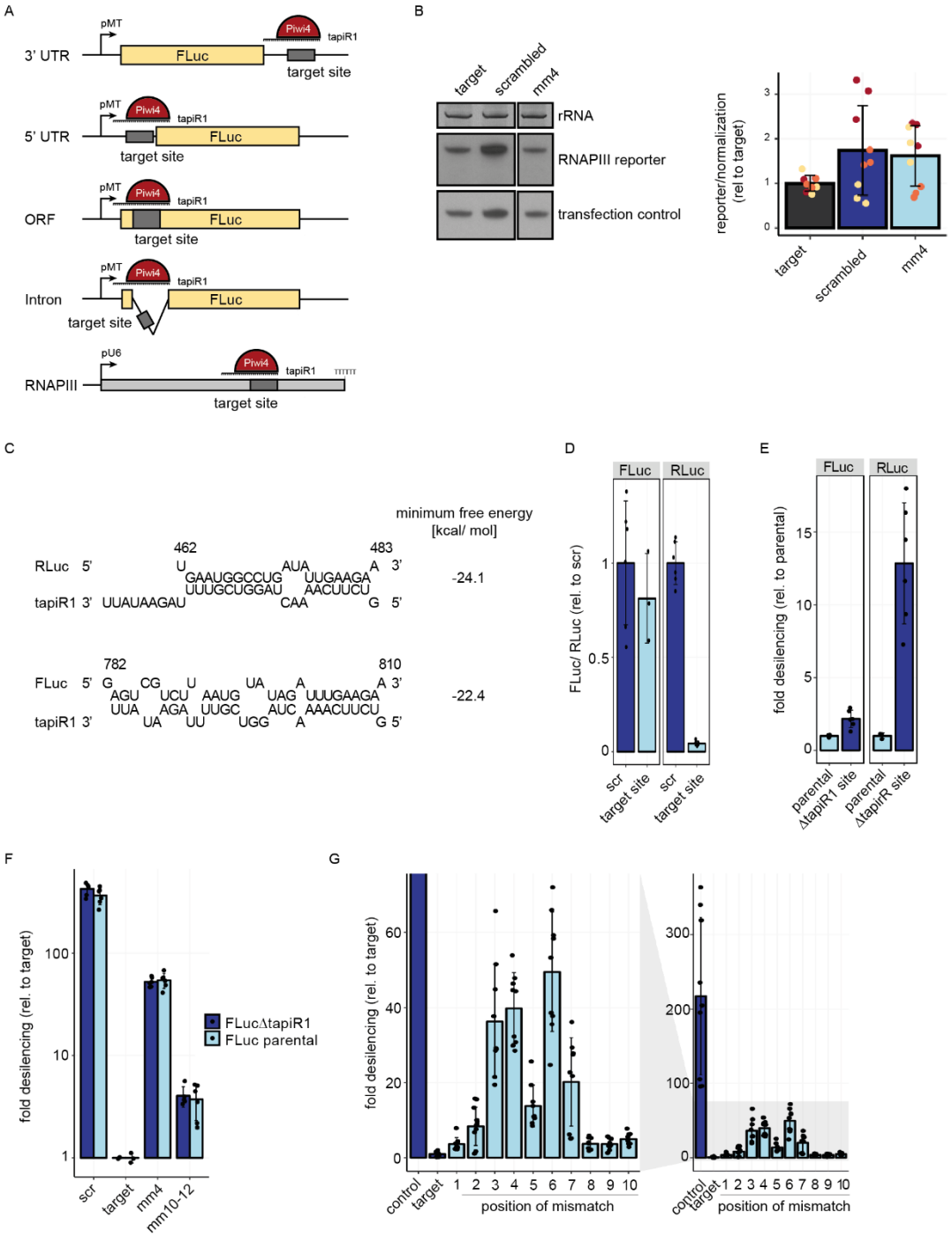
(I) RNAseq read coverage of the tapiR repeat locus and AAEL017385 (top panel), and sashimi plot indicating spliced reads (bottom panel).



Extended Data Figure 4: Antisense oligonucleotides relieve tapiR1-mediated silencing.

(A) Luciferase assay of a reporter with a fully complementary target site for tapiR1 (left panel), or tapiR2 (right panel) in the 3' UTR. Cells were co-transfected with the reporter and increasing amounts of a fully 2'*O*-methylated antisense RNA oligonucleotide (AO), or a control AO. Firefly luciferase activity was normalized to the activity of a co-transfected *Renilla* luciferase reporter. Indicated are mean, standard deviation and individual measurements from a representative experiment measured in triplicate wells.

(B) Northern blot detection of tapiR1, tapiR2, and three different miRNAs in Aag2 cells upon treatment with a the indicated concentrations of tapiR1, tapiR2, or control AO. Ethidium bromide-stained rRNA serves as loading control.

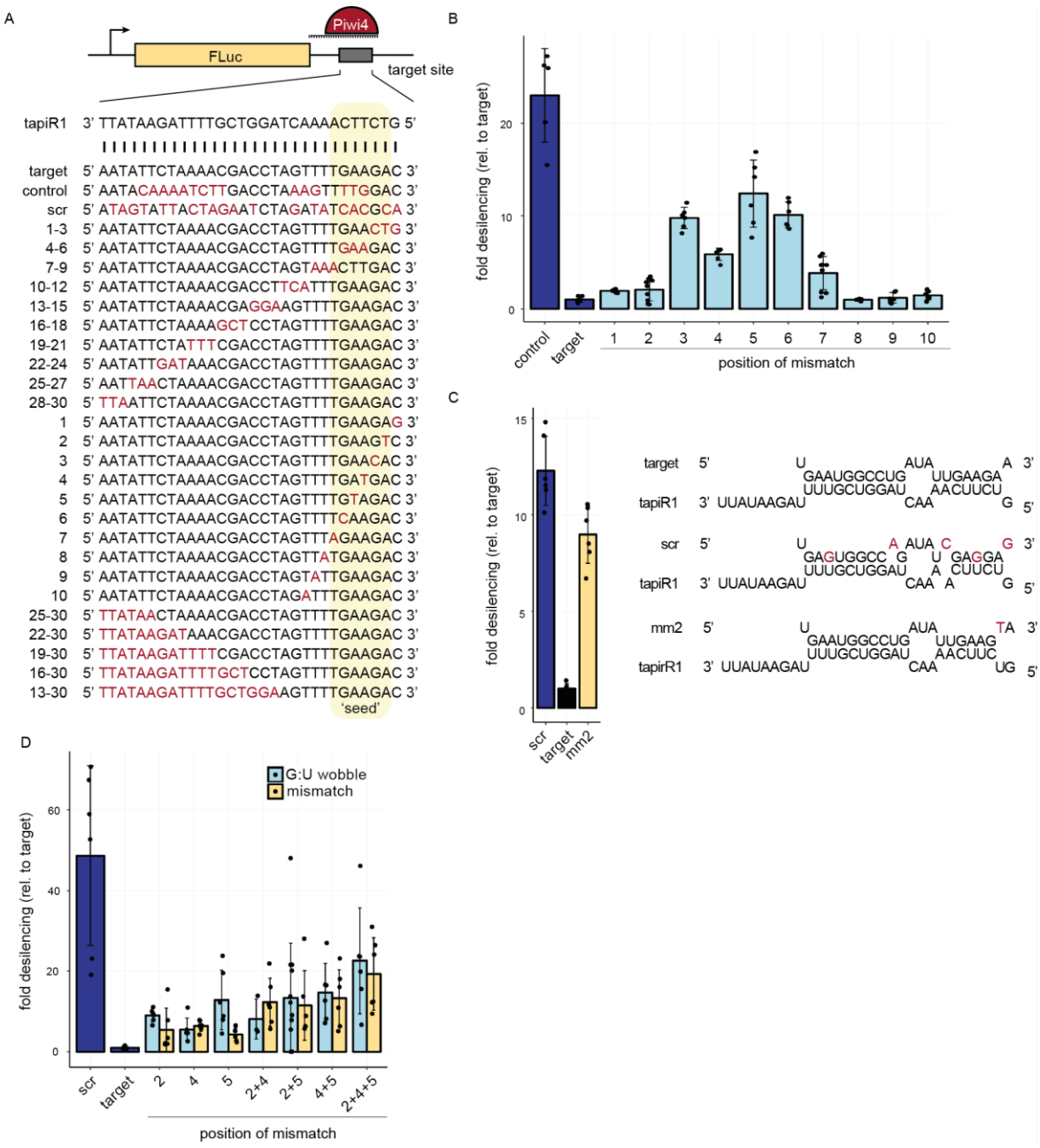


Extended Data Figure 5: *Renilla* luciferase contains a functional tapiR1 target site.

(A) Schematic representation of the different reporter constructs used in this study. pMT: metallothionein promoter; RNAPIII: RNA polymerase III reporter.

(B) Representative northern blot (right panel), and quantification of an RNAPIII reporter. Values are normalized to a non-targeted transfection control. Mean, standard deviation, and individual measures of three independent experiments (indicated with colours) quantified in triplicates are shown.

(C) Schematic representation of predicted tapiR1 target sites and minimum free energy of the indicated structures in the coding sequences of *Renilla* luciferase (RLuc) or firefly luciferase (FLuc). Numbers indicate the position of the targets relative to the first nucleotide in the ORFs.
(D) Luciferase assay of Aag2 cells transfected with reporters carrying either a scrambled (scr) site or the predicted target site from firefly luciferase (left panel), or from *Renilla* luciferase (right panel) from panel (C) in the 3'UTR of FLuc.
(E) Luciferase activity of FLuc or RLuc constructs with synonymous mutations introduced into the predicted tapiR1 target site (Δtapir1 site) and the parental clones.
(F) Luciferase reporter assay of reporters carrying target sites for tapiR1 as indicated in panel C in the 3'UTR of either the parental firefly luciferase, or the ΔtapiR1 firefly luciferase version.
(G) Reporter assay with luciferase carrying tapiR1 target sites with single mismatches in the 3' UTR as used in Extended Data Fig 6B, using RLuc with a mutated tapiR1 target site (ΔtapiR1 site) for normalization. Left panel is a zoom to the x-axis of the right panel. Shown are mean, standard deviation and individual measurements from representative experiments performed with at least two different clones per construct, and each measured in triplicate wells.



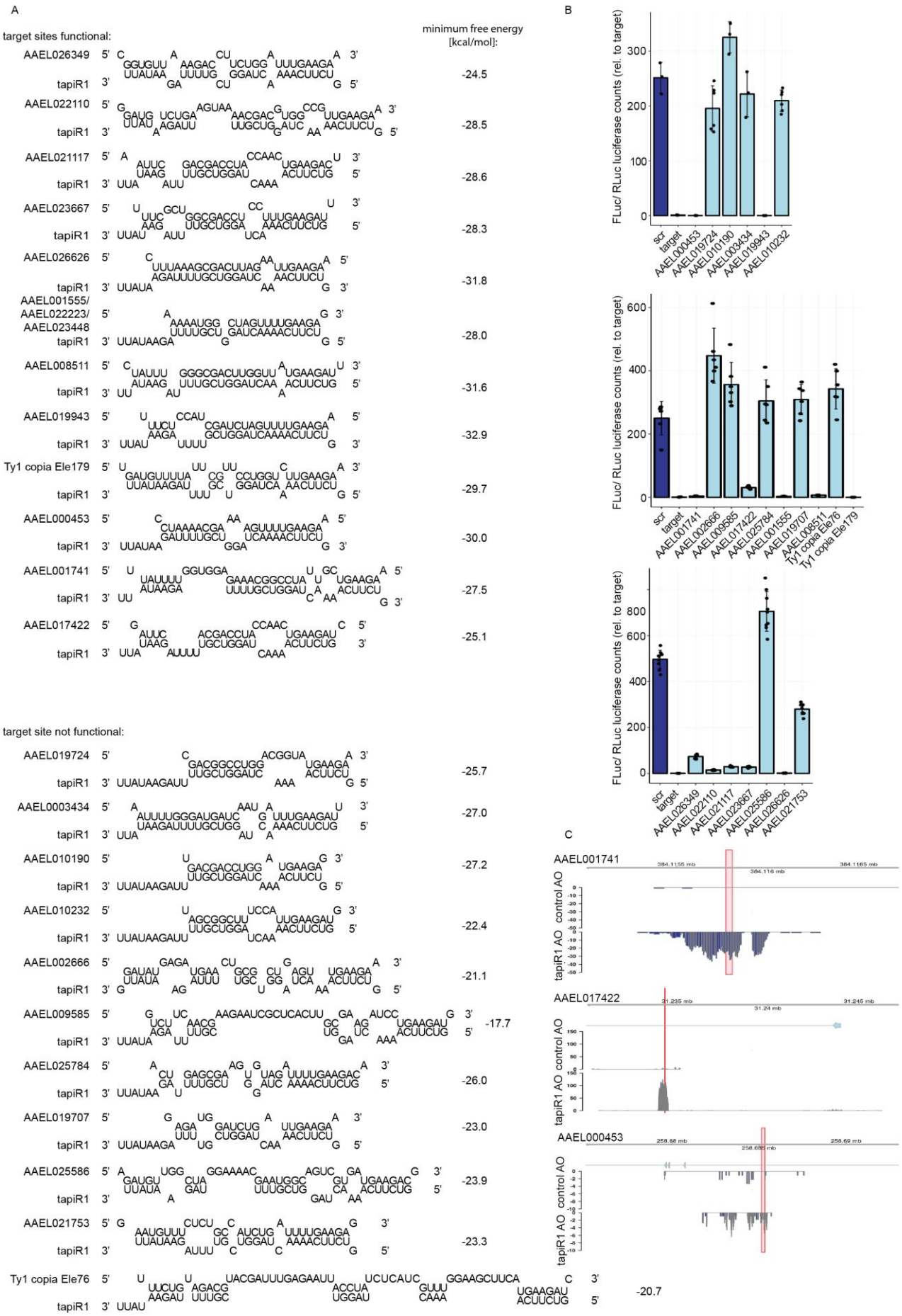
Extended Data Figure 6: tapiR1 uses a G:U wobble sensitive seed sequence for target recognition. (A) Schematic representation of the reporter constructs used in panel B and Figure 2. Numbers indicate the position of the mismatch relative to the 5' end of the piRNA.

(B) Luciferase assay of reporters carrying a tapiR1 target site with single mismatches in the 3' UTR.

(C) Luciferase activity of reporters with the tapiR1 target site from RLuc and indicated mismatches in the 3' UTR of FLuc (left panel). The tapiR1-target duplexes and mutants are presented in the right panel.

(D) Luciferase activity of tapiR1 reporters carrying mismatches or G:U wobble basepairs at the indicated positions. Firefly luciferase activity was normalized to the activity of a co-transfected

Renilla luciferase reporter to control for differences in transfection efficiencies. Data represent mean, standard deviation and individual measurements of representative experiments with two independent clones per construct and measured in triplicates wells.

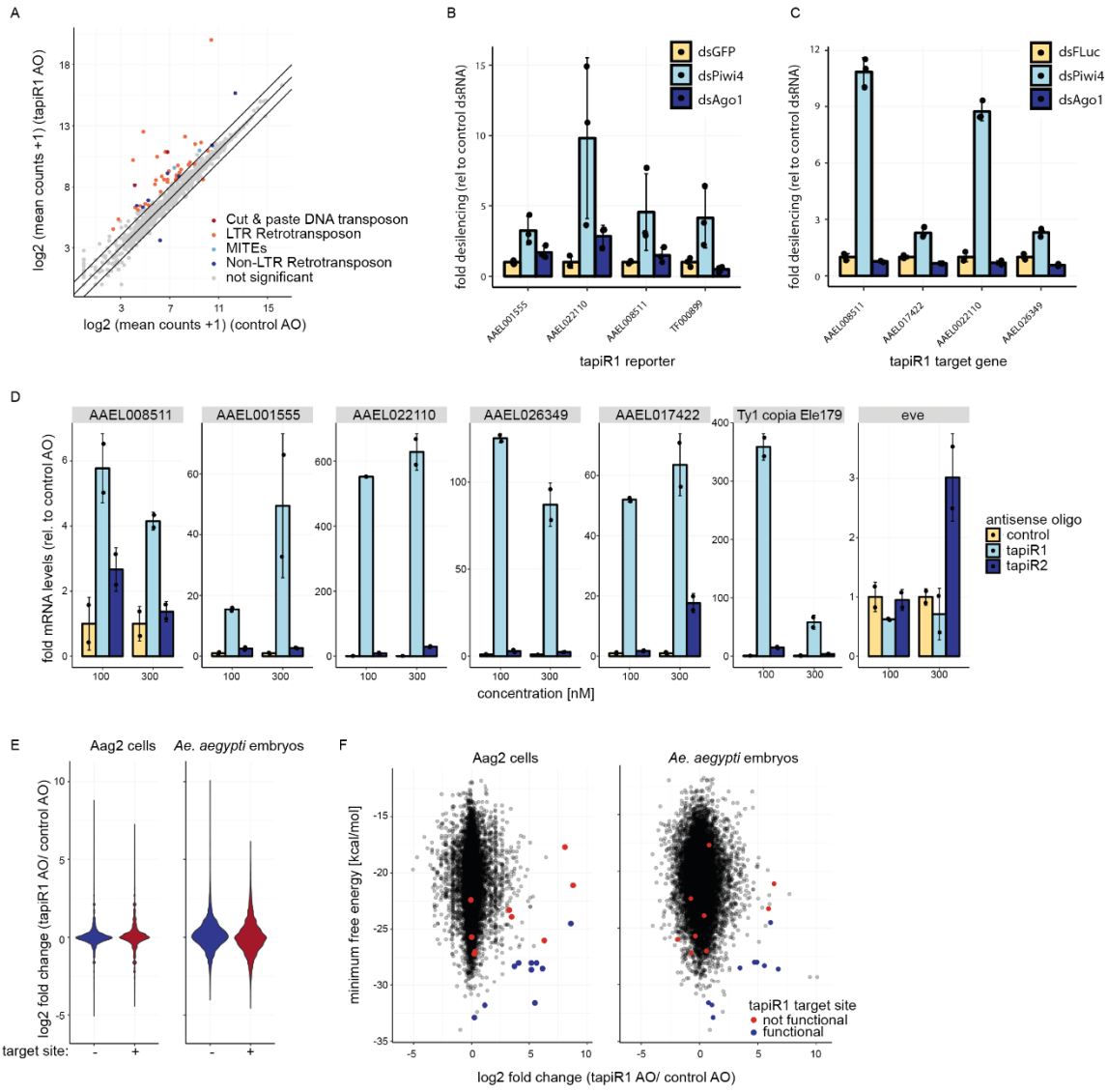

A
target sites functional:
minimum free energy [kcal/mol]:
AAEL026349 5' C A CU A A 3'
GGUGUU AAGAC UCUGG UUUGAAGA
UUAUAA UUUUG GGAUC AAACUUCU
tapiR1 3' GA CU A G 5'
-24.5
AAEL022110 5' G AGUAA AACGAC G CCG UUGAAGA A 3'
GAUG UCUGA UGG
UUAU AGAUU UUGCUG AUC AACUUCU G 5'
tapiR1 3' A G AA
-28.5
AAEL021117 5' A CCAAC U 3'
AUUC GACGACCUA UGAAGAC
UAAG UUGCUGGAU ACUUCUG 5'
tapiR1 3' UUA AUU CAAA
-28.6
AAEL023667 5' U GCU CC U 3'
UUC GGCGACCU UUUGAAGAU
AAG UUGCUGGA AAACUUCUG 5'
tapiR1 3' UUAU AUU UCA
-28.3
AAEL026626 5' C AA A 5'
UUUAAAGCGACUUAG UUGAAGA
AGAUUUUGCUGGAUC AACUUCU
tapiR1 3' UUAUA AA G 3'
-31.8
AAEL001555/
AAEL022223/ 5' A G 3'
AAEL023448 AAAAUGG CUAGUUUUGAAGA
UUUUGCU GAUCAAAACUUCU
tapiR1 3' UUAUAAGA G G 5'
-28.0
AAEL008511 5' C A U 3'
UAUUU GGGCGACUUGGUU UGAAGAU
AUAAG UUUGCUGGAUCAA ACUUCUG 5'
tapiR1 3' UU AU A
-31.6
AAEL019943 5' U CCAU A 5'
UUCU CGAUCUAGUUUUGAAGA
AAGA GCUGGAUCAAAACUUCU
tapiR1 3' UUAU UUUU G 3'
-32.9
Ty1 copia Ele179 5' U UU UU C A 3'
GAUGUUUUA CG CCUGGU UUGAAGA
UUAUAAGAU GC GGAUCA AACUUCU
tapiR1 3' UUU U A G 5'
-29.7
AAEL000453 5' C AA A 5'
CUAAAACGA AGUUUUGAAGA
GAUUUUGCU UCAAAACUUCU
tapiR1 3' UUAUAA GGA G 3'
-30.0
AAEL001741 5' U GGUGGA U GC A 5'
UAUUUU GAAACGGCCUA U UGAAGA
AUAAGA UUUUGCUGGAU A ACUUCU
tapiR1 3' UU C AA G 3'
-27.5
AAEL017422 5' G CCAAC C 5'
AUUC ACGACCUA UGAAGAU
UAAG UGCUGGAU ACUUCUG 3'
tapiR1 3' UUA AUUUU CAAA
-25.1
target site not functional:
AAEL019724 5' C ACGGUA A 3'
GACGGCCUGG UGAAGA
UUGCUGGAUC ACUUCU
tapiR1 3' UUAUAAGAUU AAA G 5'
-25.7
AAEL0003434 5' A AAU A U 3'
AUUUUGGGAUGAUC G UUUGAAGAU
UAAGAUUUUGCUGG C AAACUUCUG 5'
tapiR1 3' UUA AU A
-27.0
AAEL010190 5' U A G 3'
GACGACCUGG UGAAGA
UUGCUGGAUC ACUUCU
tapiR1 3' UUAUAAGAUU AAA G 5'
-27.2
AAEL010232 5' U UCCA G 3'
AGCGGCUU UUGAAGAU
UUGCUGGA AACUUCUG 5'
tapiR1 3' UUAUAAGAUU UCAA
-22.4
AAEL002666 5' GAGA CU G A 3'
GAUAU UGAA GCG CU AGU UGAAGA
UUAUA AUUU UGC GG UCA ACUUCU
tapiR1 3' G AG U A AA G 5'
-21.1
AAEL009585 5' G UC AAGAAUCGCUCACUU GA AUCC G 3'
UCU AACG GC AG UGAAGAU
AGA UUGC UG UC ACUUCUG 5'
tapiR1 3' UUAUA UU GA AAA
-17.7
AAEL025784 5' A AG G A A 3'
CU GAGCGA U UAG UUUUGAAGAC
GA UUUGCU G AUC AAAACUUCUG 5'
tapiR1 3' UUAUAA U G
-26.0
AAEL019707 5' G UG A A 3'
AGA GAUCUG UUGAAGA
UUU CUGGAU AACUUCU
tapiR1 3' UUAUAAGA UG CAA G 5'
-23.0
AAEL025586 5' A UGG GGAAAAC AGUC GA G 3'
GAUGU CUA GAAUGGC GU UGAAGAC
UUAUA GAU UUUGCUG CA ACUUCUG 5'
tapiR1 3' A GAU AA
-23.9
AAEL021753 5' G CUCU C A G 3'
AAUGUUU GC AUCUG UUUUGAAGA
UUAUAAG UG UGGAU AAAACUUCU
tapiR1 3' AUUU C C G 5'
-23.3
Ty1 copia Ele76 5' U U UACGAUUUGAGAAUU UCUCAUC GGAAGCUUCA C 3'
UUCUG AGACG ACCUA GUUU UGAAGAU
AAGAU UUUGC UGGAU CAAA ACUUCUG 5'
tapiR1 3' UUAU
-20.7
B
FLuc/ RLuc luciferase counts (rel. to target)
scr
target
AAEL000453
AAEL019724
AAEL010190
AAEL003434
AAEL019943
AAEL010232
AAEL001741
AAEL002666
AAEL009585
AAEL017422
AAEL025784
AAEL001555
AAEL019707
AAEL008511
Ty1 copia Ele76
Ty1 copia Ele179
AAEL026349
AAEL022110
AAEL021117
AAEL023667
AAEL025586
AAEL026626
AAEL021753
C
AAEL001741
AAEL017422
AAEL000453
control AO
tapiR1 AO

Extended Data Figure 7: Validation of tapiR1 target genes.

(A) Predicted structures and minimum free energy of tapiR1/target duplexes analysed in panel B.

(B) Luciferase assay of reporters carrying the predicted target sites from panel A in the 3’ UTR of firefly luciferase. Firefly luciferase activity was normalized to the activity of a co-transfected *Renilla* luciferase reporter to control for differences in transfection efficiencies. Indicated are mean, standard deviation and individual measurements from representative experiments performed with two to three independent clones per construct and measured in triplicate wells.

(C) AAEL017422, AAEL001741, and AAEL000453 were annotated in the previous AaegL3 gene set, but not in the current AaegL5 gene set. Read coverage in tapiR1 AO and control AO treated Aag2 cells at these genomic regions suggests that these regions are actively transcribed, but repressed by tapiR1. Red boxes indicate the positions of tapiR1 target sites.



Extended Data Figure 8: tapiR/Piwi4 silence gene expression in Aag2 cells.

(A) log2 mRNA expression of transposable elements in Aag2 cells treated with a tapiR1 specific antisense oligonucleotide (AO) or control AO. Depicted are the means of three biological replicates. A pseudo-count of one was added to all values in order to plot values of zero. Diagonal lines represent a fold change of two. Significance was tested at an FDR of 0.01 and a log2 fold change of 0.5.

(B) Luciferase assay of reporters harbouring different tapiR1 target sites (from Extended Data Fig 7) in the 3' UTR of firefly luciferase. Firefly luciferase activity was normalized to the activity of a co-transfected *Renilla* luciferase reporter to control for differences in transfection efficiencies. Data represent mean, standard deviation and individual measurements of representative experiments measured in triplicate wells.

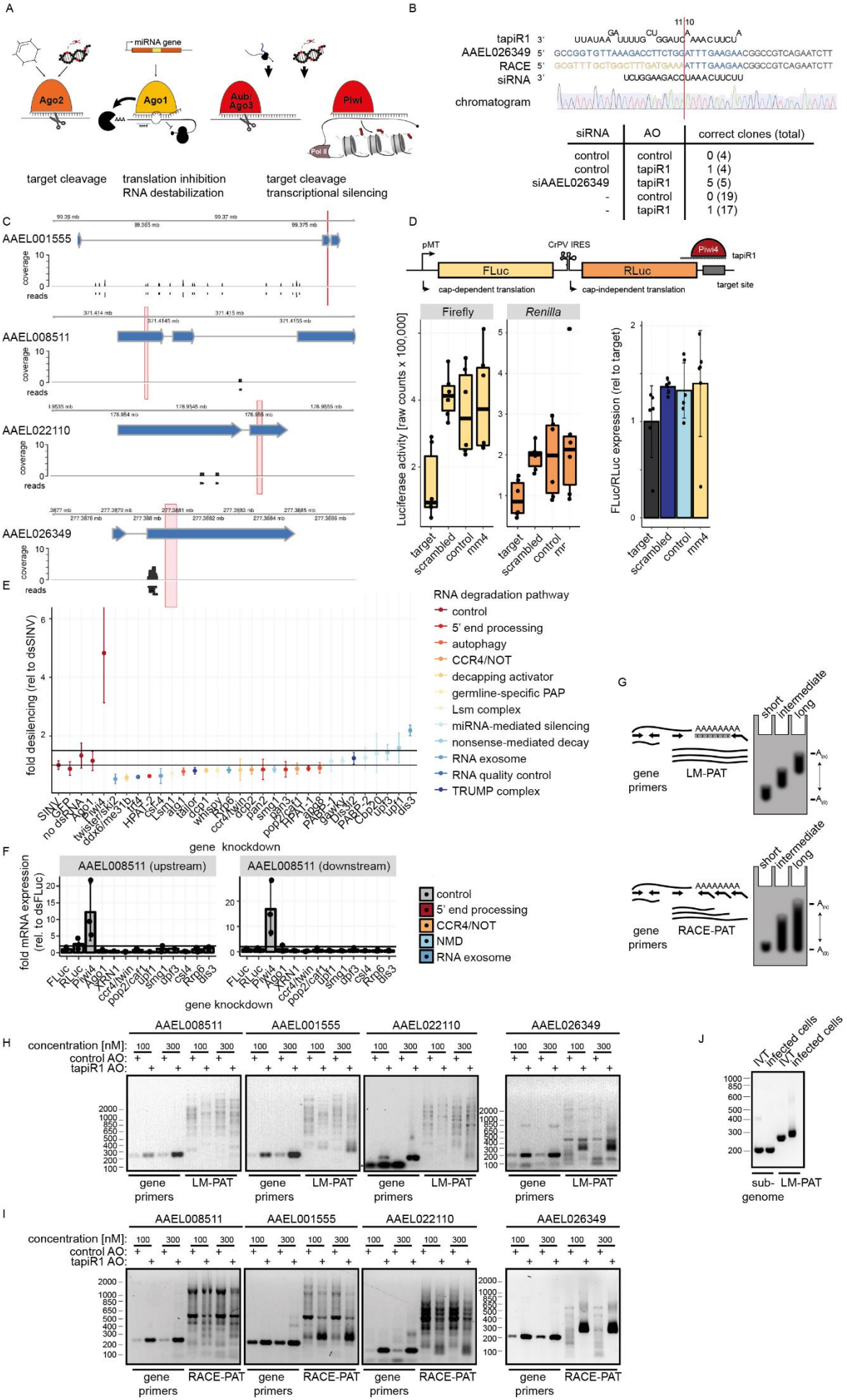
(C, D) RT-qPCR of tapiR1 target genes upon dsRNA-mediated knockdown of FLuc (control), Piwi4, or Ago1 in Aag2 cells (C), or after treatment with different concentrations of control, tapiR1,

or tapiR2 AO (D). Depicted are mean, standard deviation, and individual measurements of a representative experiment as measured from triplicate wells (C), or from duplicate wells (D). Even skipped (eve) does not harbor a tapiR1 target site, and serves as control.

(E) Experimental cumulative distribution of log2 fold changes of genes without or with predicted target sites for tapiR1. Target sites were grouped based on the predicted minimum free energy of the piRNA/target duplex.

(F) log2 fold changes of genes upon treatment with tapiR1 or control AO in Aag2 cells (left) and mosquito embryos (right) plotted against the minimum free energy of predicted tapiR1-target duplexes. Blue dots indicate target sites that were confirmed to be functional, and red dots indicate target sites that were not functional in luciferase reporter assays (see Extended Data Fig 7).

(G) Violin plot of log2 fold changes of all genes in Aag2 cells (left) and mosquito embryos (right), either with or without predicted tapiR1 target site.


A
miRNA gene
Ago2
Ago1
Aub/
Ago3
Piwi
target cleavage
translation inhibition
RNA destabilization
target cleavage
transcriptional silencing
B
tapiR1
AAEL026349
RACE
siRNA
chromatogram
siRNA
AO
correct clones (total)
control
control
0 (4)
control
tapiR1
1 (4)
siAAEL026349
tapiR1
5 (5)
-
control
0 (19)
-
tapiR1
1 (17)
C
AAEL001555
AAEL008511
AAEL022110
AAEL026349
coverage
reads
D
pMT
FLuc
CrPV IRES
RLuc
Piwi4
tapiR1
cap-dependent translation
cap-independent translation
target site
Firefly
Renilla
Luciferase activity [raw counts x 100.000]
FLuc/RLuc expression (rel to target)
target
scrambled
control
mm4
E
fold desilencing (rel to dsSINV)
gene knockdown
RNA degradation pathway
control
5' end processing
autophagy
CCR4/NOT
decapping activator
germline-specific PAP
Lsm complex
miRNA-mediated silencing
nonsense-mediated decay
RNA exosome
RNA quality control
TRUMP complex
F
AAEL008511 (upstream)
AAEL008511 (downstream)
fold mRNA expression (rel. to dsFLuc)
gene knockdown
control
5' end processing
CCR4/NOT
NMD
RNA exosome
G
short
intermediate
long
gene primers
LM-PAT
RACE-PAT
H
AAEL008511
AAEL001555
AAEL022110
AAEL026349
concentration [nM]:
control AO:
tapiR1 AO:
gene primers
LM-PAT
I
RACE-PAT
J
IVT
infected cells
sub-genome

Extended Data Figure 9: tapiR1 regulates gene expression on a post-transcriptional level.

(A) Schematic representation of different modes of silencing of all three small RNA silencing pathways in Drosophila.

(B) Top panel: Sequences of tapiR1, target gene AAEL026349, an siRNA targeting the gene at the same position, and Sanger sequencing results of the 5' RACE of Aag2 cells treated with tapiR1 AO and siRNAs. The tapiR1 target site is indicated in blue, RACE sequencing adapter in yellow, gene sequence in dark grey. The predicted slice site between nucleotides 10 and 11 is marked with a red vertical line. Bottom panel: Summary of the 5' RACE in the indicated conditions. Numbers refer to the number of sequenced clones with the 5'RACE adapter ligated to the predicted slice site, and the total number of sequenced clones between brackets.

(C) Small RNA coverage in Aag2 cells (unnormalized) and individual reads (direction of the arrow indicates the strand) on tapiR1 target genes. Red boxes indicate positions of the tapiR1 target site on the mRNA.

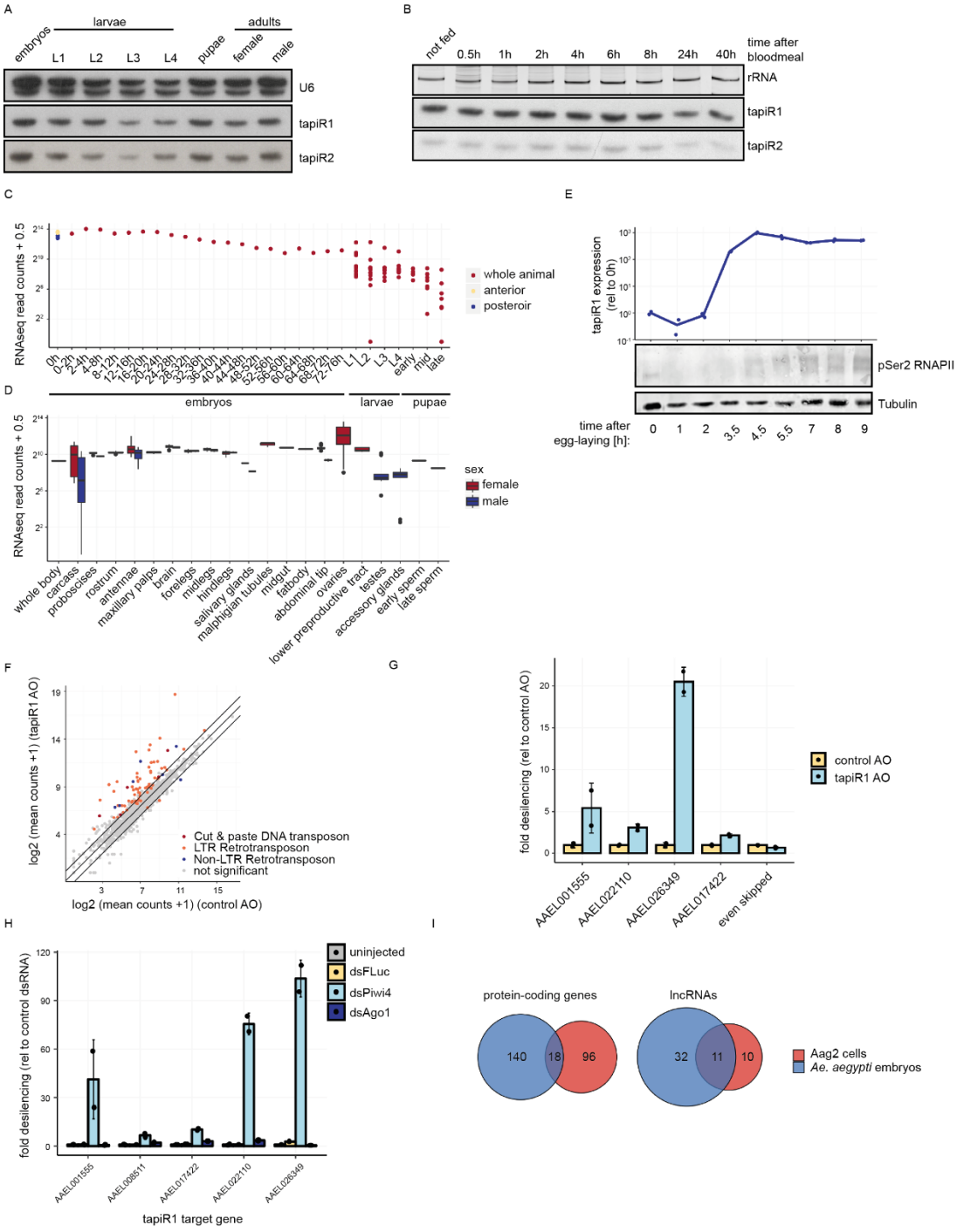
(D) Schematic representation (top panel) and luciferase expression (bottom panels) of IRES-containing reporter constructs. Depicted are mean, standard deviation, and individual measurements of a representative experiments performed in triplicate wells with two different reporter clones. The bottom left panel illustrates firefly luciferase expression normalized to *Renilla* luciferase, while bottom right panels show raw luciferase counts from the same experiment.

(E) Luciferase activity of a reporter harbouring the tapiR1 target site of AAEL001555 in the 3' UTR of FLuc upon dsRNA-mediated knockdown of the indicated genes. FLuc expression was normalized to RLuc expression to control for differences in transfection efficiencies. Depicted are mean and standard deviation of one experiment performed in triplicates.

(F) AAEL008511 target gene expression as measured by RT-qPCR upon knockdown of the indicated genes. Mean, standard deviation, and individual measurements of one out of three experiments performed in triplicate are shown. The other two experiments are presented in Supplementary Fig. S1F,G. Bars are color-coded according to the indicated RNA decay pathways.

(G) Schematic illustration of the two poly(A) tail length (PAT) assays and expected results of genes with increasing poly(A) tail lengths. LM-PAT, ligation mediated-PAT.

(H-J) Electrophoretic analysis with ethidium bromide stained agarose gels of a LM-PAT assay (H), or RACE-PAT assay (I) of different tapiR1 target genes upon treatment with two concentrations of tapiR1 or control AO. As positive control, poly(A) tail length was measured from Sindbis virus (SINV) RNA *in vitro* transcribed from a plasmid, or in infected Aag2 cells where the poly(A) tail is elongated during viral replication[61] (J).

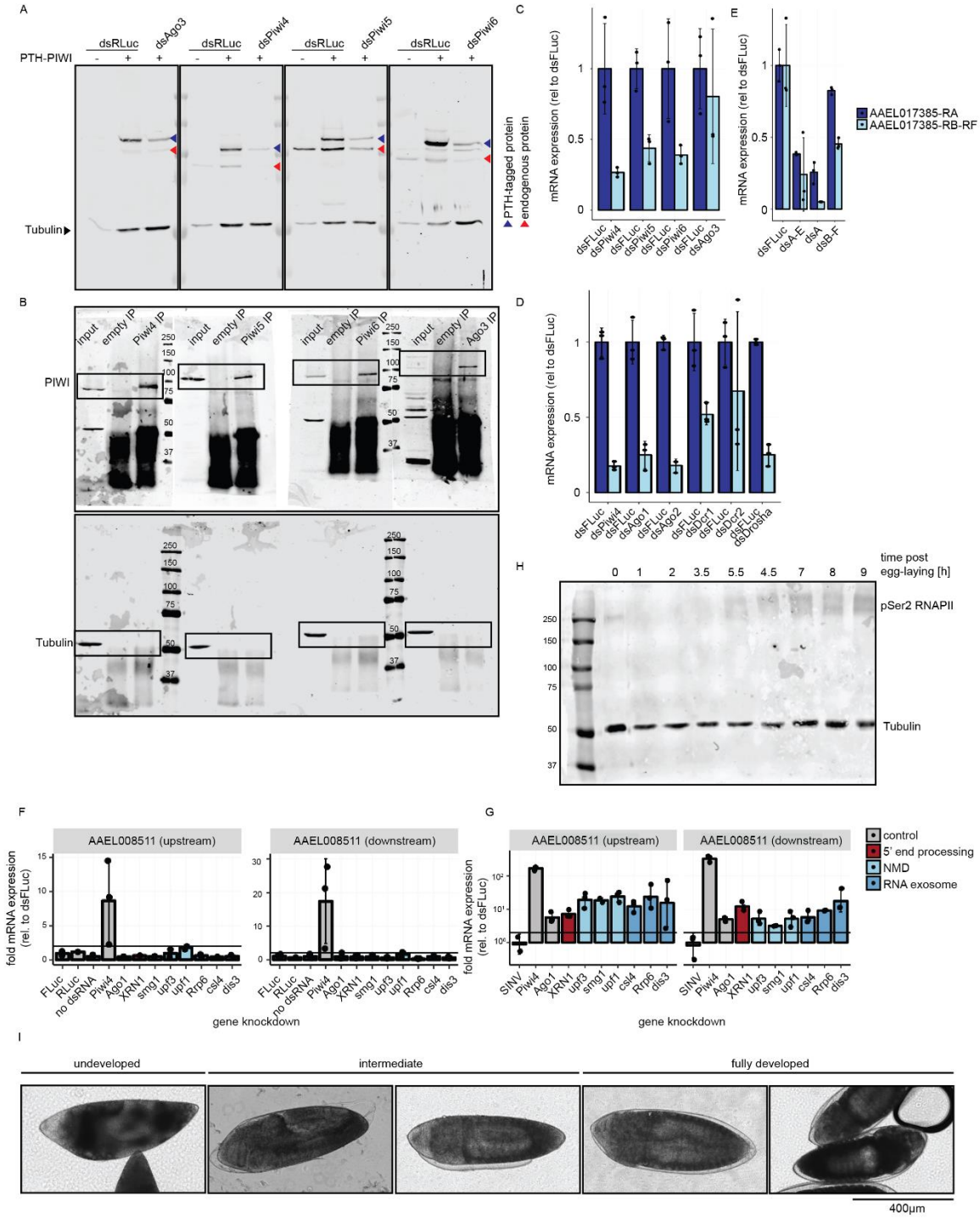


Extended Data Figure 10: tapiR1 regulates gene expression in mosquito embryos.

(A, B) Northern blot analysis of tapiR1 and 2 in developmental stages of *Ae. aegypti* mosquitoes (A), or at different time points after blood feeding (B). U6 snRNA (A) or ethidium bromide-stained rRNA (B) were analysed to verify equal loading.

(C, D) RNAseq read coverage of Piw4 in the indicated adult *Ae. aegypti* tissues (C), or developmental stages (D). Libraries used are listed in Supplementary Table S6.

(E) Western blot analysis of phosphorylated polymerase II (Ser2) in embryos at the indicated timepoints after egg-laying (bottom panel), and corresponding stem-loop RT-qPCR of tapiR1 measured in technical duplicates.
(F) log2 mRNA expression of transposable elements in embryos injected with tapiR1-specific or control AO. Mean counts of five biological replicates are shown. Significance was tested at an FDR of 0.01 and a log2 fold change of 0.5. Diagonal lines indicate a fold change of two.
(G, H) RT-qPCR of the indicated tapiR1 target genes 9h after injection of control or tapiR1-specific AOs (G), or dsRNA-mediated knockdown of FLuc (control), Piwi4, or Ago1 (H) in embryos. Mean, standard deviation and individual measurements of a representative experiment are presented. Even skipped (eve) is not a tapiR1 target gene and serves as negative control.
(H) Overlap of upregulated tapiR1 target genes (log2 fold change ≥1, with a predicted target site with mfe ≤ 24) in Aag2 cells and *Ae. aegypti* embryos.



Supplementary Data Figure S1: Antibody validation, uncropped Western blot images, knockdown efficiencies, knockdown of RNA degradation pathway components, and scoring scheme for the development of *Ae. aegypti* embryos.

(A) Validation of *Ae. aegypti* PIWI antibodies. Specificity was confirmed by detection of an additional band in PTH (Protein A, TEV cleavage site, 6x His)-tagged PIWI-expressing Aag2 cells, and loss of signal upon dsRNA-mediated knockdown. Knockdown with dsRNA targeting RLuc (dsRLuc) serves as negative control.

(B) Uncropped Western blot images corresponding to Extended Data Fig 2B.

(C-E) Knockdown efficiencies of PIWI genes shown in Extended Data Fig 2D (C), siRNA and miRNA pathway genes shown in Extended Data Fig 2E (D), and AAEL017385 isoforms in the experiment shown in Extended Data Fig 3F (E).

(F,G) RT-qPCR of the tapiR1 target gene AAEL008511 upon knockdown of the indicated genes. Presented are two independent repeats of the experiment shown in Extended Data Fig 9G.

(H) Uncropped Western blot images corresponding to Extended Data Fig 10E.

(I) Representative images of embryos scored as either undeveloped, intermediate or fully developed at 2.5 days post injection with antisense RNA oligonucleotides.