

RESEARCH ARTICLE

Antagonistic roles of Nibbler and Hen1 in modulating piRNA 3' ends in *Drosophila*

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ABSTRACT

In eukaryotes, aberrant expression of transposable elements (TEs) is detrimental to the host genome. Piwi-interacting RNAs (piRNAs) of ~23 to 30 nucleotides bound to PIWI clade Argonaute proteins silence transposons in a manner that is strictly dependent on their sequence complementarity. Hence, a key goal in understanding piRNA pathways is to determine mechanisms that modulate piRNA sequences. Here, we identify a protein-protein interaction between the 3'-to-5' exoribonuclease Nibbler (Nbr) and Piwi that links Nbr activity with piRNA pathways. We show that there is a delicate balance in the interplay between Nbr and Hen1, a methyltransferase involved in 2'-O-methylation at the 3' terminal nucleotides of piRNAs, thus connecting two genes with opposing activities in the biogenesis of piRNA 3' ends. With age, piRNAs become shorter and fewer in number, which is coupled with the derepression of select TEs. We demonstrate that activities of *Nbr* and *Hen1* inherently contribute to TE silencing and age-dependent profiles of piRNAs. We propose that antagonistic roles of *Nbr* and *Hen1* define a mechanism to modulate piRNA 3' ends.

KEY WORDS: *Nbr*, *Hen1*, piRNA, 3' terminal trimming, 2'-O-methylation, Transposon, Small RNA sequencing, *Drosophila*

INTRODUCTION

Transposable elements (TEs) are abundant in eukaryotes and their aberrant expression and transposition can have deleterious effects on the host genome (Levin and Moran, 2011). Piwi-interacting RNAs (piRNAs), which comprise the largest class of small non-coding RNAs in gonadal cells, are involved in silencing the expression of TEs, safeguarding genome stability (Malone and Hannon, 2009; Siomi et al., 2011). Given their central importance, the biogenesis of piRNAs and modulation of piRNA pathways are areas of broad interest.

Compared with other endogenous small non-coding RNAs, such as small interfering RNAs (siRNAs, ~21 nt) (Elbashir et al., 2001) and microRNAs (miRNAs, ~22 nt) (Tomari et al., 2007), piRNAs

are specifically germline enriched and show a much broader size range of ~23 to 30 nt, suggesting a unique biogenesis mechanism (Aravin et al., 2006). In *Drosophila*, according to the mechanistic cascades by which they are produced, piRNAs can be divided into three types: primary, secondary and tertiary (Siomi and Siomi, 2015). Primary piRNAs are produced from long precursory transcripts derived from one of 142 such piRNA clusters – discrete genomic loci comprising complex structures of transposon remnants (Brennecke et al., 2007). Zucchini (Zuc), an endonuclease, mediates cleavage to produce piRNAs that tend to begin with 5' uridine (1U) (Brennecke et al., 2007; Ipsaro et al., 2012; Nishimasu et al., 2012). Primary piRNAs are bound to Piwi or Aubergine (Aub). Aub-bound primary piRNAs cut active TE mRNAs to yield secondary piRNAs bound to Ago3, which in turn cleave original precursory piRNAs to generate new Aub-bound piRNAs, thus forming the so called ping-pong cycles that amplify piRNAs (Brennecke et al., 2007; Gunawardane et al., 2007; Li et al., 2009). Tertiary piRNAs are derived from a Zuc-dependent phased cleavage along precursor piRNAs following the initiation sites where Ago3-bound secondary piRNAs trigger the cut. Tertiary piRNAs are bound to Piwi (Han et al., 2015a; Mohn et al., 2015). Collectively, these mechanisms of biogenesis are mutually dependent and allow the production of extraordinarily abundant and diverse piRNA sequences that are ready to destroy transposon mRNAs when aberrantly expressed.

Despite recent progress, piRNA pathways are only beginning to be revealed. Previous reports have addressed mechanistic frameworks by which piRNAs can be made, but genes that modulate piRNA sequences are poorly understood. Intriguingly, most individual piRNAs are of low abundance, but one prominent testicular piRNA, AT-chX-1, has multiple isoforms with defined sequences (Nishida et al., 2007). Deep sequence analysis of piRNAs from testis reveals that AT-chX-1 all begin with 1U, followed by heterogeneous 3' ends, descending by single nucleotides. Based on established models, Zuc cleavage is tentatively involved to liberate the 5' end of AT-chX-1, given the 1U bias, but factors dictating the modulation of 3' ends have not been well specified. This raises the possibility that a putative exoribonuclease might be involved to trim piRNA from the 3' end. Using silkworm and *in vitro* biochemistry, a study predicted an active processing that determined the 3' end formation of piRNAs (Kawaoka et al., 2011). Similarly, a recent report proposed roles of Nbr, an established 3'-to-5' exoribonuclease previously described in *Drosophila* miRNA pathways, in piRNA biogenesis (Feltzin et al., 2015). The extent to which Nbr trims piRNA populations, including AT-chX-1, and whether this modulation is functionally relevant in the repression of TEs, remain uncharacterized.

A common feature of piRNA 3' ends is 2'-O-methylation, a chemical modification catalyzed by Hen1, a methyl transferase (Horwich et al., 2007; Saito et al., 2007). Studies in *Arabidopsis*

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Received 3 July 2015; Accepted 22 December 2015

have implicated a protective effect of 2'-*O*-methylation (Li et al., 2005; Yu et al., 2005). Since virtually all fly piRNAs are 2'-*O*-methylated, if that effect holds true in *Drosophila* then such a protective mechanism should have a much broader impact on piRNAs than previously estimated. However, it remains to be determined how *Hen1* impacts fly piRNAs at the genome level. In animals, the progression of normal aging is coupled with a functional deterioration in multiple systems, including fertility (Lopez-Otin et al., 2013). Provocatively, aberrant induction of TEs has been noted in aging brains, indicating a late-onset decline in the control of TEs (De Cecco et al., 2013). But whether and how piRNA pathways are modulated with age are poorly studied. Here, we interrogate *in vivo* functions of *Nbr* and *Hen1*, and dissect antagonistic roles between these two genes that profoundly impact piRNA 3' ends in *Drosophila*. We further extend our findings into the chronic modulation of piRNA pathways that relates to an age-dependent activity of *Nbr*.

RESULTS

Endogenous Nbr is ovary enriched and interacts with Piwi

To study the *in vivo* function of *Nbr*, we characterized the *Nbr* interactome based on its endogenous protein-protein interaction. We utilized the CRISPR/Cas9 method in *Drosophila* (Ren et al., 2013; Yu et al., 2013). We designed a single-stranded oligodeoxynucleotide repair template that includes a 30 nt Myc tag sequence flanked by 146 nt homology arms corresponding to the *Nbr* genomic sequence (Fig. 1A). Resulting flies expressed a Myc tag fused in frame within the N' terminus of the *Nbr* protein (Fig. 1B), hereafter termed *Nbr*^{KI-Myc}. *Nbr*^{KI-Myc} flies showed a normal miR-34 trimming pattern, suggesting that the addition of a Myc tag has no effect on native protein function (data not shown). Western blot showed that *Nbr* protein is relatively highly expressed in ovaries compared with other adult tissues (Fig. 1C).

To study the *Nbr* interactome, we dissected ovary tissues and performed *Nbr* protein immunoprecipitation using a Myc antibody pull-down followed by mass spectrometry. To ensure stringency and quantification, we ranked the result list with more than ten peptide hits, and narrowed it down to those with peptide-spectrum match (PSM) counts greater than 5-fold enrichment. Strikingly, this approach found *Piwi* among the top hits (Fig. 1D). To confirm this finding, we generated a polyclonal antibody for the *Drosophila* *Piwi* protein. We first tested the specificity of this new antibody using *piwi* mutant flies that we made via the CRISPR/Cas9 method, hereby named *piwi*^{Cas9} (Fig. S1A-C). Then, using this specific *Piwi* antibody and endogenous protein co-immunoprecipitation assay (co-IP), we detected *Piwi* when pulling down Myc-*Nbr*; reciprocally, we found *Nbr* in the *Piwi* pull-down (Fig. 1E). Together, these assays established endogenous protein-protein interaction. We next used *Drosophila* Schneider 2 cells and co-IP to determine if *Nbr* can interact with other *Piwi* family proteins. Our data showed that *Nbr* appears to strongly interact with *Piwi*, but not *Aub* or *Ago3* (Fig. S1D). Notably, it has been known that *Piwi* is a hallmark protein involved in virtually all major steps of piRNA pathways. Thus, association with *Piwi* might link *Nbr* function with piRNAs.

Nbr trims piRNAs from both germline and somatic tissues

To assess *Nbr* activity, we generated a new *Nbr* loss-of-function allele using the CRISPR/Cas9 method (Ren et al., 2013; Yu et al., 2013). We introduced two single guide RNAs (sgRNAs) that deleted 189 bp coding region for the exoribonuclease domain (Fig. 2A-C), hereafter termed *Nbr*^{Cas9}. Assessment of miR-34

isoforms confirmed *Nbr*^{Cas9} as a null mutation (Fig. 2D). We characterized *Nbr*^{Cas9} flies and identified miRNAs that were *Nbr* substrates (Fig. S2, Table S1).

To focus on piRNAs, we sequenced small RNAs from dissected germlines, comparing control with *Nbr*^{Cas9}. First, we analyzed the AT-chX-1 locus, a testis-enriched piRNA. In *Nbr* mutants, AT-chX-1 remained identical in sequence layout as in controls, but with a striking accumulation of longer forms as shown by length distribution and small RNA northern blot (Fig. 3A-C). Analysis of another well-characterized testicular piRNA derived from the *Su(Ste)* locus (Nishida et al., 2007) revealed a similar alternation at the 3' end in *Nbr*-deficient gonads (Fig. S3).

Next, we carried out analysis on piRNA populations. We used a newly developed piPipes algorithm to plot the mean length of

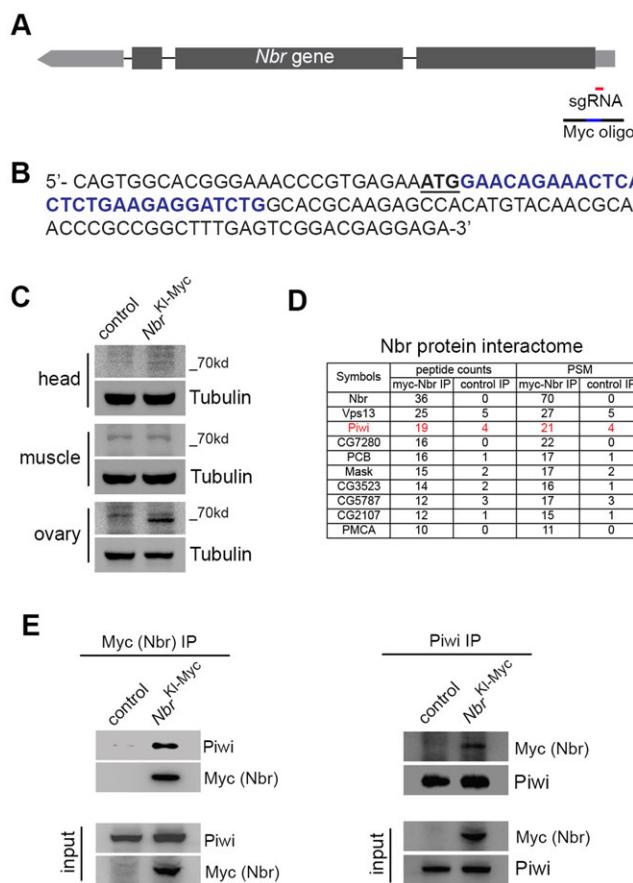
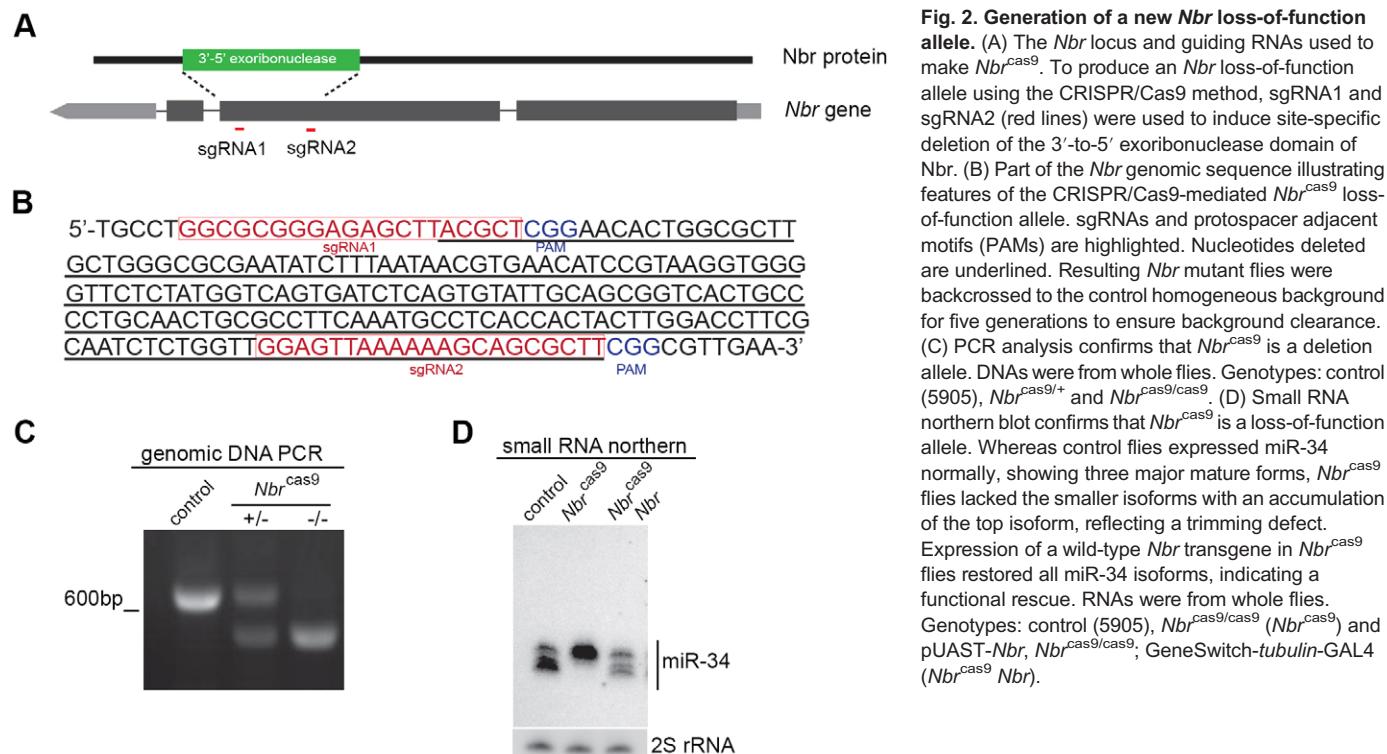


Fig. 1. Endogenous Nbr is ovary enriched and interacts with Piwi. (A) The *Drosophila* *Nbr* locus and guiding RNAs used to make *Nbr*^{KI-Myc}. To produce an *Nbr* Myc tag knock-in allele using the CRISPR/Cas9 method, sgRNA (red) was co-injected with a DNA oligonucleotide (black line, interrupted with blue representing Myc coding sequence). (B) In *Nbr*^{KI-Myc}, the *Nbr* gene contains sequence (blue) encoding the Myc tag inserted after the *Nbr* ATG start codon (underlined). Resulting *Nbr*^{KI-Myc} flies were backcrossed to the control homogeneous background for five generations to ensure background clearance. (C) *Nbr* is ovary enriched. In adult flies, a protein signal corresponding to *Nbr* protein on the western blot could be clearly seen for the ovary, but not head or muscle. Genotypes: control (5905) and *Nbr*^{KI-Myc}/*Nbr*^{KI-Myc}. (D) Endogenous protein interactome determines that *Nbr* interacts with *Piwi*. Using endogenous *Nbr* as bait for immunoprecipitation and mass spectroscopy analysis, *Piwi* (red) was captured among the top-ranked interacting proteins. Ranking was based on peptide counts. Highly stringent criteria were applied, including peptide counts greater than 10 and PSM fold change greater than 5. Proteins were from ovaries. Genotypes as in C. (E) Co-IP experiment confirming that *Piwi* interacts with *Nbr*. Proteins were from ovaries. Genotypes as in C.



piRNA reads assigned to transposons (Han et al., 2015b). The rationale of this analysis lies in the fact that mutants would accumulate long or short forms depending on the nature of defects, thus causing the mean length of piRNAs to depart from that of controls (Fig. S4). We found that loss of *Nbr* had a global impact on the length distribution of piRNAs with a clear accumulation of longer forms, regardless of their strand origins (Fig. 3D). We then characterized the impact of *Nbr* deletion on two specific piRNA clusters: 42AB piRNAs, which are bidirectional transcripts in germline cells, and *flamenco* piRNAs, which are prominently unidirectional transcripts in follicle cells of somatic tissues (Brennecke et al., 2007). The loss of *Nbr* led to a size increase in both populations of piRNAs (Fig. 3E,F), suggesting that *Nbr* is likely to regulate piRNA sequences after their transcription with minimal strand preference. To determine if the size increase in gonadal piRNAs in *Nbr*^{cas9} flies is due to a lack of nucleotide removal on either the 5' or 3' end, or both, we carried out an analysis on antisense piRNAs that have 1U bias (Brennecke et al., 2007). Comparing control with *Nbr* mutants, we found unaltered 1U bias of piRNAs (Fig. 3G), suggesting that the size increase is unlikely to be due to changes at the 5' end.

Taken together, these results demonstrated that the increase in piRNA length in *Nbr* mutants is primarily due to the accumulation of forms with extended 3' ends, indicative of a failure in 3' processing upon *Nbr* depletion. Since *Nbr* has an established 3'-to-5' exoribonuclease activity in trimming small RNAs (Han et al., 2011), these results, together with its protein-protein interaction with Piwi, demonstrate an *in vivo* biological role of *Nbr* in trimming piRNAs from germline and somatic tissues.

Genome-wide impact of *Hen1* depletion at 3' ends of piRNAs

Hen1, a methyltransferase, is known to be involved in piRNA pathways by catalyzing 2'-O-methylation at the 3' terminal nucleotide (Horwich et al., 2007; Saito et al., 2007). Yet, how *Hen1* activity impacts the piRNA profile at the genome level

remains uncharacterized. To explore its role, we generated a new *Hen1* loss-of-function allele using the CRISPR/Cas9 method (Fig. 4A-C), hereafter termed *Hen1*^{cas9}. Following the same rationale, we sequenced and analyzed piRNAs from ovaries of control and *Hen1* mutants. Interestingly, we found that the lack of *Hen1* led to a dramatic shortening of piRNA from the 3' end (Fig. 4D-G). Of note, our data provide the first insight at the genome level into the role of *Hen1*. Since depletion of *Hen1* results in a piRNA profile characterized by enhanced 3' trimming, we proposed that *Hen1*-mediated 2'-O-methylation might play a role in antagonizing trimming at piRNA 3' ends.

Antagonistic roles of *Nbr* and *Hen1* modulate piRNA 3' ends

To determine a potential interaction of *Hen1* and *Nbr* in 3' trimming of piRNAs, we sequenced and analyzed piRNAs from control, *Nbr*^{cas9}, *Hen1*^{cas9}, and *Hen1*^{cas9} *Nbr*^{cas9} double-mutant flies. Consistently, this analysis demonstrated that whereas *Nbr*^{cas9} lengthened piRNAs, *Hen1*^{cas9} shortened them (Fig. 5A,B, Table S2). Moreover, we found that flies carrying the *Hen1*^{cas9} *Nbr*^{cas9} double mutation showed a rescuing effect on the overall piRNA length profile towards that of controls (Fig. 5A,B, Table S2). Intriguingly, an oxidation and β-elimination experiment revealed that piRNAs remained 2'-O-methylated in *Nbr*^{cas9} mutants, suggesting that *Nbr*-mediated trimming is not required for methylation to take place at piRNA 3' ends (Fig. S5). None of these mutants affected the expression of core piRNA pathway factors (data not shown). piRNA abundance appeared to be at comparable levels between controls and the various mutants (Fig. S6A, Table S3). Combined, these data suggested antagonizing roles of *Nbr* and *Hen1* in modulating piRNA 3' ends.

Silencing of select TEs is disturbed upon loss of either *Nbr* or *Hen1*

Since a major function of mature piRNAs in animal gonads is for silencing aberrant TE expression (Aravin et al., 2007), we next

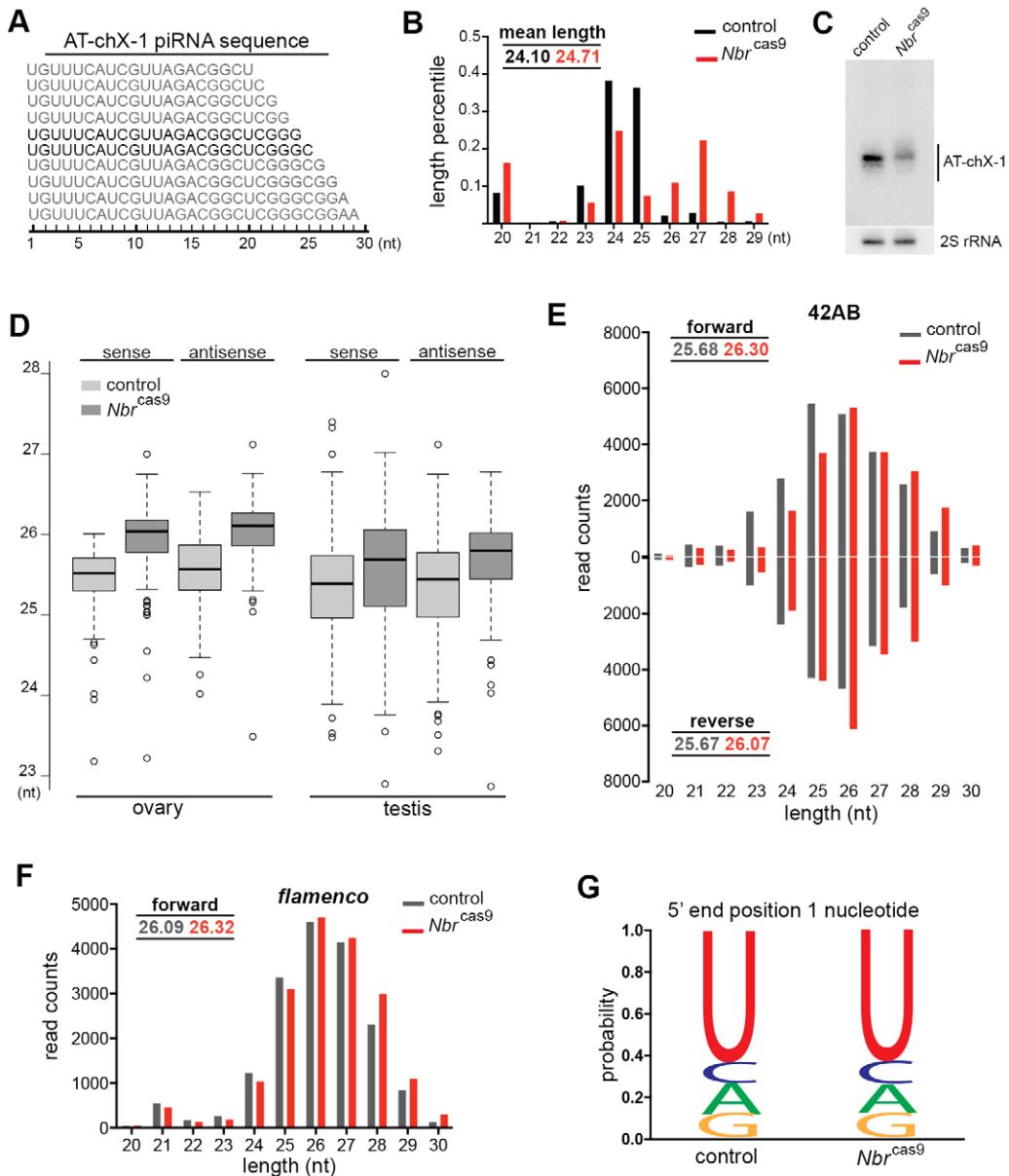


Fig. 3. *Nbr* trims piRNAs at the 3' end. (A) AT-chX-1, a piRNA enriched in the testis, displays the sequence feature of a 5' uridine and a nested series at the 3' end. The main forms are 24 and 25 nt (bold). (B) Length distribution analysis indicates that AT-chX-1 accumulates more long forms in *Nbr*^{cas9} (red) than in the control (black). RNAs were from testis. Genotypes: control (5905) and *Nbr*^{cas9/cas9} (*Nbr*^{cas9}). (C) Small RNA northern blot confirms that AT-chX-1 shows a striking size increase in length in *Nbr*^{cas9} compared with control. 2S rRNA was used as a loading control. RNAs were from testis. (D) Gonadal piRNAs become longer in *Nbr* mutants than in controls. Box plots for length distribution revealed that *Nbr*^{cas9} flies accumulated more piRNAs with long forms than in controls. Using piPipes algorithms, only piRNAs mapped to transposons were used for subsequent analysis. Each transposon is assigned a mean value based on the length of mapped piRNAs, with a total of 127 individual transposons analyzed. Control versus *Nbr*^{cas9}: ovary sense, $P<2.2\times10^{-16}$; ovary antisense, $P<2.2\times10^{-16}$; testis sense, $P=4.861\times10^{-7}$; testis antisense, $P=1.19\times10^{-10}$, Wilcoxon signed-rank test. RNAs were from ovaries and testis. (E,F) In the 42AB cluster (E), piRNAs can be derived from both forward (top) and reverse (bottom) strands, and they show accumulation of long forms regardless of their strand origin. In the *flamenco* cluster (F), piRNAs can only be generated from the forward strand (top), and they show *Nbr*-dependent length modulation. Mean lengths for piRNAs derived from the indicated clusters in control and *Nbr*^{cas9} are shown. RNAs were from ovaries. (G) Antisense piRNAs in *Nbr*^{cas9} show the same bias for 5' uridine (1U) as in the control. (C-G) Genotypes as in B.

asked if *Nbr* and *Hen1* activity might affect TE silencing. Mutations in genes that encode the core factors of piRNA pathways, such as *piwi*, *aub* and *Ago3*, lead to strong defects in fertility. Despite the shortened piRNAs, *Hen1* mutants show normal fertility, suggesting that lack of *Hen1* does not cause a strong defect in the germline (Fig. S6B). By contrast, *Nbr*^{cas9} mutants and *Hen1*^{cas9} *Nbr*^{cas9} double mutants had reduced fecundity (Fig. S6B).

To characterize the TE status in *Nbr*^{cas9} and *Hen1*^{cas9} flies, we performed qRT-PCR analysis of ovary RNAs. The selection of TEs

was based upon the existing literature that characterized representative TEs across the genome (Li et al., 2009). This analysis revealed that, among 23 TEs analyzed, eight in *Nbr*^{cas9} and six in *Hen1*^{cas9} showed loss of silencing (Fig. 5C). In *Hen1*^{cas9} *Nbr*^{cas9} double mutants, more interestingly, we observed a striking trend in that a majority of desilenced TEs (10 out of 12 upregulated in the single mutant) were now brought back to normal silenced status, with the only exceptions being *412* and *gypsy* (Fig. 5C). piRNAs mapped to *412* and *gypsy* transposons behaved as expected

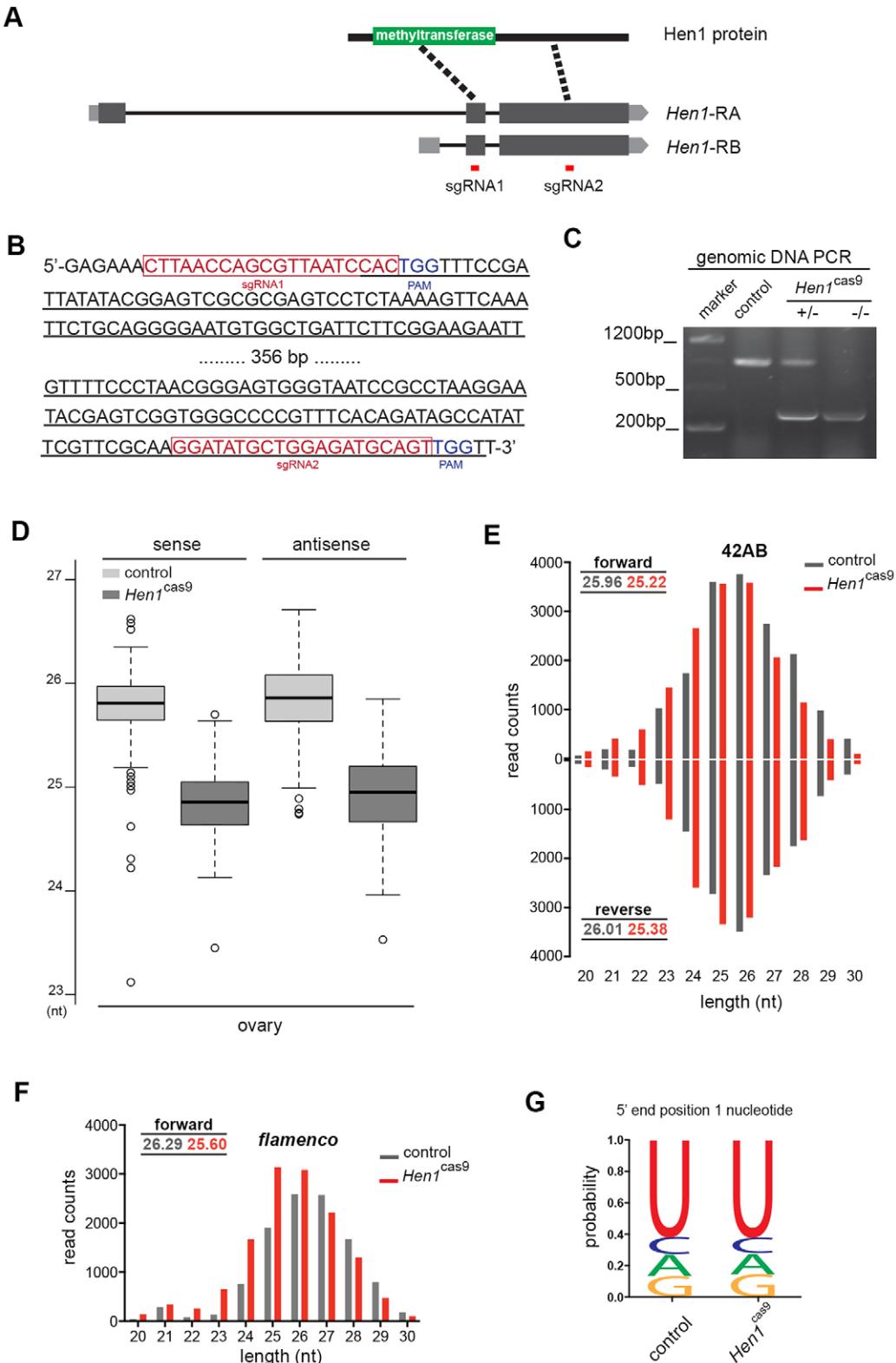


Fig. 4. Loss of *Hen1* shortens piRNAs from the 3' end. (A) The *Hen1* locus and guiding RNAs used to make *Hen1*^{cas9}. To produce the *Hen1* loss-of-function allele, sgRNA1 and sgRNA2 (red lines) were used to induce site-specific deletion. The deleted region largely removed the methyltransferase domain. (B) Part of the *Hen1* genomic sequence illustrating features of the CRISPR/Cas9-mediated *Hen1*^{cas9} loss-of-function allele. sgRNAs and PAMs are highlighted. Resulting *Hen1* mutant flies were backcrossed to the control homogeneous background for five generations to ensure background clearance. (C) PCR analysis confirms that *Hen1*^{cas9} is a deletion allele. DNAs were from whole flies. Genotypes: control (5905), *Hen1*^{cas9/+} and *Hen1*^{cas9/cas9}. (D) Box plots for length distribution reveal that *Hen1*^{cas9} flies accumulate more short form piRNAs than controls. Control versus *Hen1*^{cas9}, ovary sense, $P < 2.2 \times 10^{-16}$; ovary antisense, $P < 2.2 \times 10^{-16}$, Wilcoxon signed-rank test. RNAs were from ovaries. Genotypes: control (5905) and *Hen1*^{cas9/cas9} (*Hen1*^{cas9}). (E,F) In the 42AB cluster (E) and the *flamenco* cluster (F), piRNAs show accumulation of shorter forms. Mean lengths for piRNAs derived from the indicated clusters in control and *Hen1*^{cas9} are shown. RNAs were from ovaries. (G) Antisense piRNAs in *Hen1*^{cas9} show the same bias for 5' uridine (1U) as in control. RNAs were from ovaries. (E-G) Genotypes as in D.

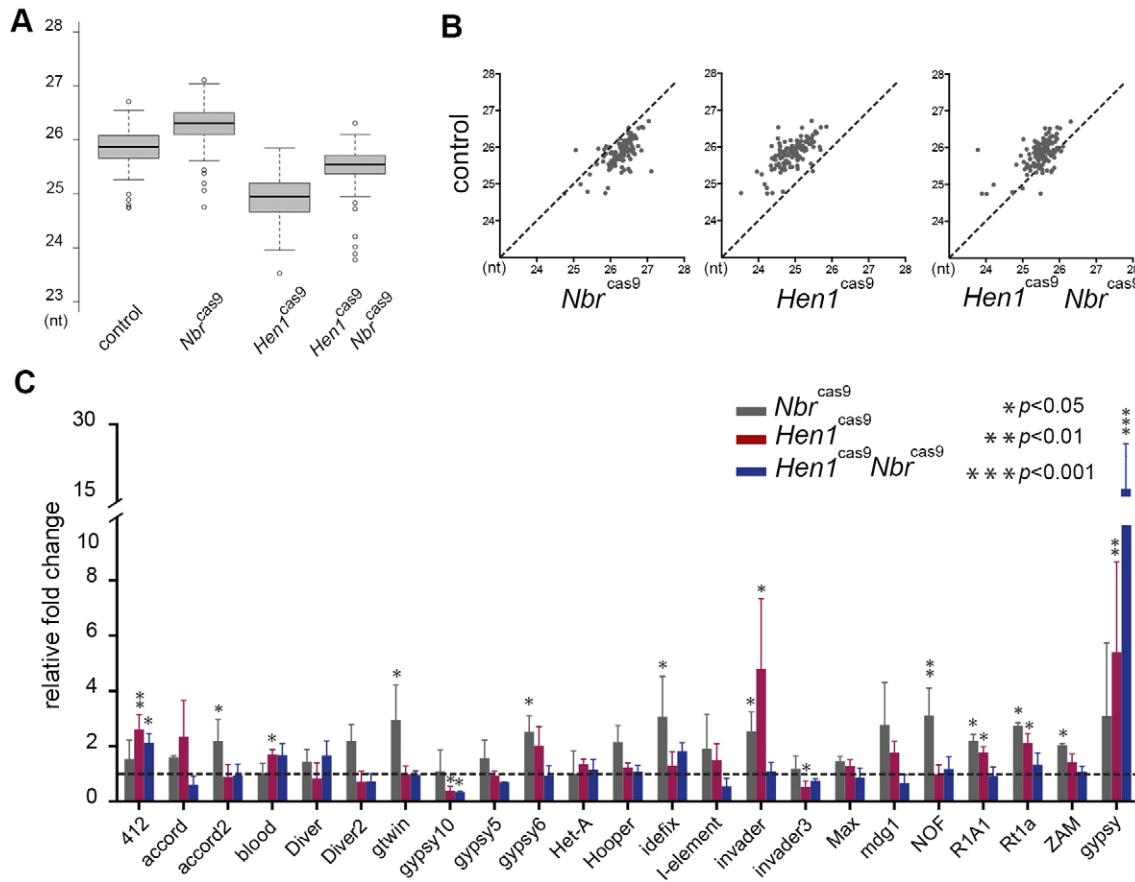


Fig. 5. Antagonistic roles of *Nbr* and *Hen1* in piRNA pathways. (A,B) Box plots (A) and scatter plots (B) showing that *Nbr* and *Hen1* modulate piRNA length. Whereas lack of *Nbr* lengthened piRNAs, lack of *Hen1* shortened them. Flies lacking both *Nbr* and *Hen1*, however, showed restoration of the piRNA profile towards that of controls. *Nbr*^{cas9} versus control (5905), $P<2.2\times10^{-16}$; *Hen1*^{cas9} versus control, $P<2.2\times10^{-16}$; *Hen1*^{cas9} *Nbr*^{cas9} versus control, $P<2.2\times10^{-16}$, Wilcoxon signed-rank test. piRNA sequencing and analysis of RNA from ovaries of 3-day-old animals of the indicated genotypes. Genotypes: *Nbr*^{cas9}/*Nbr*^{cas9} (*Nbr*^{cas9}), *Hen1*^{cas9}/*Hen1*^{cas9} (*Hen1*^{cas9}) and double mutant. (C) *Nbr* and *Hen1* are functionally relevant in repressing TEs. qRT-PCR analysis was used to determine the expression status of TEs among different genotypes. Whereas lack of either *Nbr* or *Hen1* caused upregulation of select transposons, flies mutant for both *Nbr* and *Hen1* exhibited a clear reversion in that the majority of the desilenced TEs became repressed, the only exceptions being 412 and *gypsy*. Mean±s.d., n=3 independent experiments; Student's t-test. RNAs were from ovaries. Genotypes as in A.

in control and the various mutant backgrounds (Tables S2 and S3), so modulation of 412 and *gypsy* cannot be attributed to alteration of the piRNA profile per se, but rather influenced by other effects in *Hen1*^{cas9} *Nbr*^{cas9} double mutants. Since *Nbr* is involved in both miRNA and piRNA pathways, the biological consequence of double mutants might in part be contributed by defective trimming of select miRNAs. Nevertheless, our data indicated that the proper control of TE silencing could be disturbed upon the loss of either *Nbr* or *Hen1*. Importantly, rescue of TE silencing in the double mutant was accompanied by a restoration of piRNA length, suggesting a regulatory mode that is sensitive to piRNA length. Since *Nbr* and *Hen1* have opposing effects on piRNA length, our data seemed to suggest that the integrity of piRNAs, including their length, is essential to maintain the normal repression status of most TEs.

piRNAs are age modulated

A fertility test revealed that flies demonstrate an age-associated decline in fecundity (Fig. S7A); yet, few studies have addressed whether late-onset fertility decline is coupled with an age modulation of TE status and piRNAs. To approach these issues, we first characterized the expression profile of TEs during aging. We found that aged flies demonstrated significant derepression of

select TEs compared with young animals (Fig. 6A). Next, we asked if the piRNA profile was also modulated during aging. We sequenced piRNAs from ovaries of control flies at 3 (newly eclosed adult), 15 (young), 30 (mid-age) and 45 (old) days of age (Fig. 6B). Since our control isogenic flies had a median life span of ~60-70 days, we avoided testing animals at 60 days to uncouple potential defects with lethality. Interestingly, during normal aging, piRNAs appeared to undergo a progressive shortening from the 3' end (Fig. 6B, Fig. S7B, Table S4). Coupled with the shortening, piRNA abundance also showed a slight decline with age (Fig. 6C, Table S5). Although the change in abundance is very minor, it is statistically significant. Together, these data provided a first glimpse into the piRNA profile of the adult life cycle and revealed a potential link between the age-modulated piRNA profile and a deterioration of TE silencing status with age.

Nbr contributes to the age-modulated piRNA profile

Age modulation of piRNAs showed a uniform pattern of change, suggesting a controlled event rather than dysregulated activities of *Nbr* or *Hen1* upon aging. Using qRT-PCR, we found that the steady-state levels of both *Nbr* and *Hen1* increased in aging ovaries (Fig. S8A). In *Nbr*^{KI-Myc} flies, *Nbr* protein levels remained roughly the same between 3 and 30 days (Fig. S8B). To test the possibility

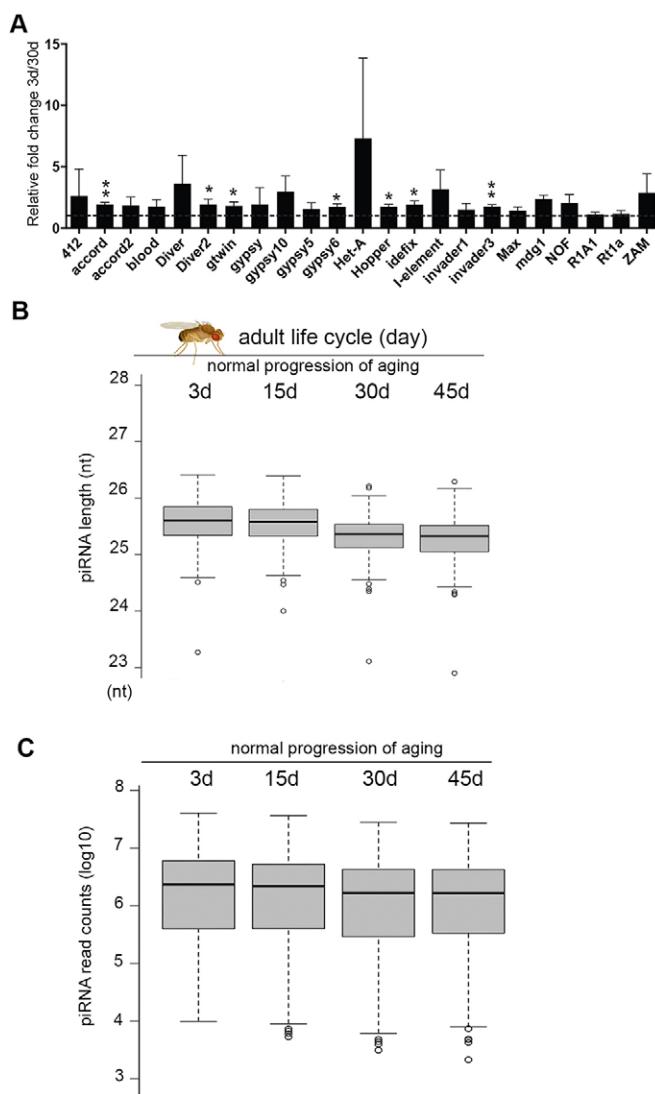


Fig. 6. piRNAs are age modulated. (A) Select transposons are upregulated in aged ovaries. qRT-PCR analysis was used to determine the expression status of TEs in ovaries at 30 days versus 3 days. Mean \pm s.d., n=3 independent experiments; *P<0.05, **P<0.01, Student's t-test. (B) The length of piRNAs is age modulated. RNA from ovaries of control flies at 3 (newly eclosed adult), 15 (young), 30 (mid-age) and 45 (old) days of age were used to generate age-modulated piRNA profiles. Box plots show that with age piRNAs become progressively shortened from the 3' end. Fifteen versus 3 days, P=0.001371; 30 versus 3 days, P<2.2 \times 10 $^{-16}$; 45 versus 3 days, P<2.2 \times 10 $^{-16}$, Wilcoxon signed-rank test. (C) The abundance of piRNAs in ovaries becomes slightly decreased with age. Box plots for normalized read counts are shown as log₁₀ value. Fifteen versus 3 days, P=3.182 \times 10 $^{-1}$; 30 versus 3 days, P<2.2 \times 10 $^{-16}$; 45 versus 3 days, P<2.2 \times 10 $^{-16}$, Wilcoxon signed-rank test. (A-C) Flies were of genotype 5905.

that the reduction in piRNA length at the 3' end during aging was due to enhanced trimming by Nbr, we compared the profile of known miRNA substrates of Nbr in young and old flies. Interestingly, the levels of miR-34-5p shorter isoforms, which are established Nbr trimming products, were markedly increased in aged ovaries (Fig. 7A). Similar changes were also found for miR-275-3p and miR-317-3p, with their Nbr-dependent isoforms being elevated with age (Fig. 7A), which was consistent with the increased activity of Nbr with age.

To further determine if Nbr is involved, we sequenced piRNAs from control, *Nbr*^{cas9} and *Hen1*^{cas9} animals of 3 or 20 days of age.

Day 20 was chosen to uncouple the onset of age-associated phenotypes in *Nbr* (current study) or *Hen1* (Abe et al., 2014) mutants. Strikingly, our data revealed that whereas piRNAs displayed age-associated shortening in control and *Hen1*^{cas9}, piRNAs in *Nbr*^{cas9} did not (Fig. 7B, Fig. S8C). The abundance of piRNAs also showed a slight but statistically significant decline between 20 days and 3 days in control and *Hen1*^{cas9}, but not in *Nbr*^{cas9} (Fig. 7C, Fig. S8D). Together, these data implicated an age-associated activity of Nbr leading to enhanced trimming, which in turn contributes to the age-modulated piRNA profile as characterized by a gradual shortening as well as a decline in abundance.

DISCUSSION

A long-standing question in piRNA pathways is to understand the biogenesis and modulation of mature piRNAs. To date, a spectrum of protein factors has been implicated in piRNA biogenesis pathways; by contrast, few genes have been carefully studied in the modulation of mature piRNAs. Here, we provide evidence to suggest a model for the modulation of piRNA 3' ends (Fig. 8). First, piRNA substrates longer than the mature length are loaded onto the Piwi protein. Nbr, an established 3'-to-5' exoribonuclease, mediates trimming until Hen1 catalyzes the 2'-O-methylation on mature piRNAs of proper length. Yet, our data implicate that trimming and methylation are not coupled events. Mature piRNAs of proper length and 3' end modification can be readily enrolled into piRNA pathways. Within this model, there is a delicate balance in the interplay of Nbr and Hen1: genetic mutation abolishing either component overwhelmingly alters the balance, diminishing the efficacy of piRNA pathways; on the other hand, enhancement of one player over the other, such as an increased activity of Nbr with age, gradually tilts the balance, altering piRNAs in response to the progression of natural aging. Intriguingly, flies with loss of both *Nbr* and *Hen1* show shorter piRNAs than with loss of *Nbr* alone (Fig. S9), implicating a possible effect of a second, as yet unknown, 'trimmer'. As such, Nbr and Hen1 might represent examples of a group of emerging factors crucial for the modulation of piRNA sequences.

Nbr and Hen1 might define one key aspect shared by additional factors in shaping piRNA 3' ends. Previous studies identified Papi, a Tudor domain protein that can influence piRNA length (Han et al., 2015a; Honda et al., 2013). Yet, analysis of the Nbr interactome based on an endogenous protein bait and a rigorous filter only captured Piwi, a signature protein of piRNA pathways, but not Hen1 and Papi. These data might suggest that these factors do not necessarily form a protein complex but instead mediate parallel processes in the modulation of piRNA 3' ends. Although we have shown protein interactions between Nbr and Piwi, these results do not exclude the possibility that Nbr may function together with Aub or Ago3 through transient or dynamic interactions. Alternatively, rather than being direct contact, adapter proteins might be necessary to bridge Nbr with other PIWI family members. Importantly, our data clearly show an effect of Nbr in trimming of AT-chX-1, a testicular piRNA bound to Aub but not Piwi protein (Nishida et al., 2007). Therefore, a detailed mechanism remains to be elucidated by which Nbr can differentially trim piRNAs bound to distinct PIWI family proteins. Furthermore, it is worth noting that Piwi protein can shuttle between the nucleus and cytoplasm, whereas Nbr is primarily cytoplasmic (Feltzin et al., 2015). Thus, Nbr activity can only partially overlap with that of Piwi.

Fertility is an adult-onset trait, which can progressively decline in old age. Interestingly, strong mutations that abolish piRNA

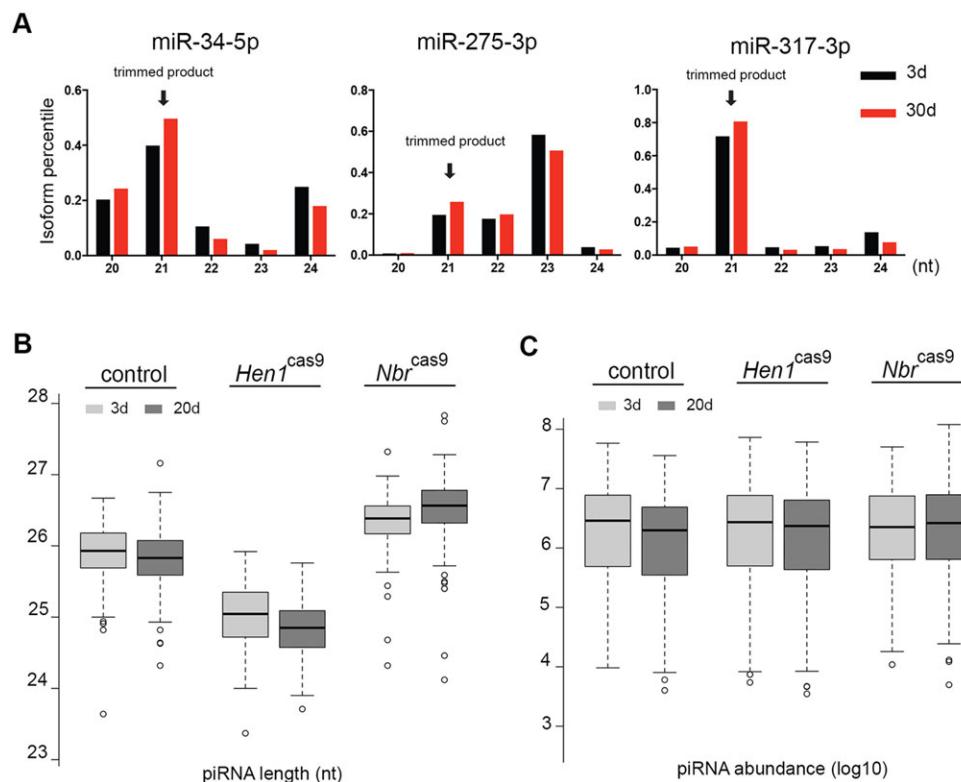


Fig. 7. The age modulation of piRNAs is dependent on Nbr activity. (A) Nbr trimming activity increases with age. Small RNA deep sequence datasets were used. Known Nbr-trimmed isoforms (arrows) of miR-34-5p, miR-275-3p and miR-317-3p show increased levels at 30 days compared with 3 days. RNAs were from ovaries of genotype 5905. (B) Nbr contributes to age modulation of piRNA length. Box plots show that aging piRNAs become shortened in control and *Hen1*^{cas9}, but not in *Nbr*^{cas9}. Twenty versus 3 days: control (5905), $P=2.948 \times 10^{-10}$; *Hen1*^{cas9}, $P=2.634 \times 10^{-15}$; *Nbr*^{cas9}, $P=7.098 \times 10^{-8}$; Wilcoxon signed rank test. *Nbr*^{cas9/cas9} (*Nbr*^{cas9}), *Hen1*^{cas9/cas9} (*Hen1*^{cas9}). (C) Nbr modulates piRNA abundance with age. Box plots showed a trend in that with aging piRNA levels were decreased in control and *Hen1*^{cas9}, but not in *Nbr*^{cas9}. Twenty versus 3 days: control (5905), $P<2.2 \times 10^{-16}$; *Hen1*^{cas9}, $P=8.454 \times 10^{-11}$; *Nbr*^{cas9}, $P=0.1271$; Wilcoxon signed-rank test. Genotypes as in B.

pathways are always coupled with a severe fertility defect, indicating an underlying connection between the integrity of piRNAs and the maintenance of adult fertility. Here, we provide evidence that in the adult life cycle piRNAs undergo a progressive shortening from the 3' end and a decline in abundance, striking age-related features that have not been previously demonstrated. Furthermore, our data support the activity of Nbr in the promotion of the piRNA aging profile, indicating an active mode of trimming that proceeds along with the progression of aging. Nevertheless, aging is a highly complex biological event that converges effects from multiple intrinsic drivers as well as the environment (Kirkwood, 2005). Our data that link Nbr trimming with the chronic modulation of piRNAs present a novel facet in the

understanding of age-associated events. Future studies may address how this link might impact the long-term maintenance of TEs and fertility in the adult life cycle.

MATERIALS AND METHODS

Fly genetics

Flies were grown at 25°C. To make *Nbr*^{cas9} flies, we followed the method developed by Yu et al. (2013). To make the *Hen1*^{cas9} and *piwi*^{cas9} mutants, we used the method developed by Ren et al. (2013). To make *Nbr*^{KI-Myc} flies, we first recombined the *Lig4*^{-/-} mutant with flies expressing nano-Cas9 (TH00788, N), followed by microinjection of sgRNA and the oligonucleotide template with the Myc tag sequence. Fly microinjection was conducted by the *Drosophila* Core Facility, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. To induce *Nbr* transgene expression in adults, we recombined *Nbr* pUAST transgenic flies with the *Tubulin-GeneSwitch-GAL4* line, and fed adult flies with standard media containing 100 µg/ml Mifepristone (Sigma). Further details of strain construction are provided in the supplementary Materials and Methods.

Fly wing posture phenotype was scored at days 3 and 10. For each genotype at each time point, 40 flies were scored. Three independent experiments for each genotype and time point were performed for statistics. To determine lifespan, newly eclosed males were maintained at 15 flies per vial, transferred to fresh vials every 2 days while being scored for survival. A total of 150–200 flies were used per genotype per lifespan. The spreadsheet for the lifespan was first generated in Excel (Microsoft) and then analyzed by Prism software (GraphPad) for survival curves and statistics. To determine female fecundity, we crossed three healthy wild-type males with one virgin female of defined genotype or age. The crosses lasted for 48 h, and then adult flies were removed. After an additional 10 days, emerged flies of the next generation were counted. For each genotype, ten independent virgin females were scored.

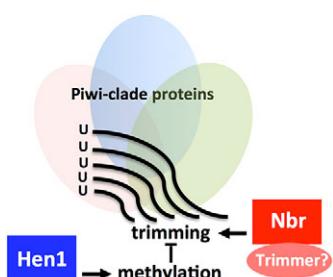


Fig. 8. Model depicting antagonistic roles of Nbr and Hen1 in the modulation of piRNA 3' ends. There is a delicate balance in the mechanism by which Nbr and Hen1 modulate piRNA 3' ends. Mature piRNAs of ~23–30 nt are bound to PIWI clade Argonaute proteins (ovals), including Piwi, a nodal protein that is crucial in piRNA pathways. We provide evidence that Piwi can interact with Nbr. Our data reveal interplay between Nbr-mediated exoribonuclytic trimming and Hen1-dependent methylation that antagonizes trimming, thus linking two genes with opposing activity in shaping piRNA 3' ends in the biogenesis of piRNAs. Nbr might represent just one of a large cohort of additional, as yet unknown, factors with roles in trimming piRNA 3' ends.

Protein immunoprecipitation and mass spectrometry

Adult virgin female flies of control and *Nbr*^{KI-Myc} were collected and aged to 3 days. 400 ovaries for each genotype were dissected and homogenized in 1 ml lysis buffer [50 mM Tris-HCl pH 7.5, 5% glycerol, 0.4% NP40,

1.5 mM MgCl₂, 125 mM NaCl, 25 mM NaF, 1 mM Na₃VO₄, 1 mM DTT, 1 mM EDTA and Complete Protease Inhibitor (Roche)] using a pre-cooled Dounce tissue homogenizer. The homogenates were centrifuged at 12,000 g for 5 min at 4°C. The supernatants were passed through a 100 µm cell strainer followed by 0.45 µm PVDF membrane. 100 µl anti-c-Myc agarose beads (Sigma) were washed with 1 ml TBS buffer (50 mM Tris HCl, 150 mM NaCl, pH 7.4) and then mixed with protein extracts. After a 2 h incubation at 4°C, beads were washed three times with 1 ml TBS buffer. After the final wash, beads were dissolved in 60 µl 8 M urea, 100 mM Tris pH 8.5, and then reduced by 5 mM Tris (2-carboxyethyl) phosphine for 20 min and alkylated by 10 mM iodoacetamide for 15 min in the dark. The solution was then diluted 1:4 with 100 mM Tris pH 8.5, and digested with 1 µg trypsin at 37°C overnight. The digestion was terminated by adding 2% formic acid, and subjected to C18 desalting tips. The resulting peptides were dried in a vacuum concentrator. The peptides were analyzed by online nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS). Briefly, the peptides were first separated on a nano column (100 µm×15 cm, C18, 1.9 µm, 120 Å) using an EASY-nLC 1000 system (Thermo Scientific). The separated peptides were analyzed using an Orbitrap Fusion mass spectrometer (Thermo Scientific). A cycle of one full-scan mass spectrum (300–1800 m/z) at a resolution of 120,000 followed by higher-energy collisional dissociation-ion trap (HCD-IT) MS/MS spectra at 32% normalized collision energy was repeated continuously in top-speed mode. The mass spectrometry data were analyzed by Proteome Discoverer (Thermo Scientific, ver. 1.4). The tandem mass spectra were searched against the UniProt *Drosophila melanogaster* protein database (release date: 27/05/2015) using the Sequest HT search engine (Eng et al., 1994). Carbamidomethyl of cysteine was set as a static modification, and dynamic modification of methionine oxidation was used. The peptide false discovery rate was controlled at 1% by Percolator (Kall et al., 2007). Peptide-spectrum match (PSM) numbers of corresponding proteins were used to assess differences in protein abundance.

Small RNA sequencing

Fly tissues were dissected in ice-cold 1× phosphate buffered saline solution (Sangon Biotech), and total RNA was isolated using Trizol according to the manufacturer's instructions (Life Technologies). DNA was removed using the Ambion TURBO DNA-free Kit (Life Technologies). RNA was fractionated using 15% TBE-urea pre-cast PAGE gels (Life Technologies). Small RNA of ~20–29 nt was sliced from the gel and recovered using the ZR Small-RNA PAGE Recovery Kit (Zymo Research). To determine library quality, qRT-PCR was used to quantify the library concentration. The normalized libraries were denatured with 0.1 M NaOH solution (Sigma). Pooled libraries with different barcodes were sequenced on the Illumina MiSeq platform.

Sequence analysis

Adapter sequence was removed using FASTX (http://hannonlab.cshl.edu/fastx_toolkit/). Reads without adapter, shorter than 18 nt or mapped to rRNAs were filtered out. For annotating miRNAs, we used miRDeep2 (Friedlander et al., 2008). *Drosophila* genome release 6 from FlyBase was used as reference, and the miRNA precursor and mature miRNAs were from miRBase. miRNA isoform statistics were carried out according to miRDeep2 output, and reads were restricted with perfect match. For annotating piRNAs, small RNA reads were mapped to transposon reference sequence downloaded from FlyBase using Bowtie2 (Langmead and Salzberg, 2012) with two mismatches allowed. Probability for 5' nucleotide was calculated by FASTX. Length distribution and graphic analysis of piRNAs derived from different transposons were performed using the piPipes algorithm (Han et al., 2015b). Scatter plots were produced using piPipes. Box plots were produced using R language, according to piPipes output. To calculate piRNA abundance, reads was first normalized to siRNAs to attain normalized counts. Dm3 was chosen as genomic reference and two mismatches were allowed for mappings.

Small RNA northern

For small RNA northern, total RNA was isolated from fly head, whole flies or fly ovaries using Trizol reagent as above. For each lane, 3 µg RNA was

fractionated on 15% TBE-urea pre-cast PAGE gels (Life Technologies), and then transferred onto a Hybond nylon membrane (GE Healthcare). Prior to hybridization, RNA blots were prehybridized with Ambion Oligohyb (Life Technologies), and then incubated with radioactively labeled RNA probes made using the Ambion MaxiScript-T7 *In Vitro* Transcription Kit (Life Technologies), supplemented with ³²P-labeled UTP. Oligonucleotide templates were prepared by annealing two single-stranded DNA oligonucleotides into a duplex (99°C for 5 min and then allowed to cool to room temperature). Probes are listed in the supplementary Materials and Methods.

Molecular biology

To clone full-length cDNA, RT-PCR was conducted using RNAs from ovaries. Primers are listed in the supplementary Materials and Methods.

To carry out *Drosophila* Schneider 2 cell-based co-IP experiments, 8×10⁶ cells were seeded onto a 10 cm plate, and plasmid DNAs were transfected using Effectene (Qiagen). Cells were collected 36 h after transfection and lysed in lysis buffer (as above) for 30 min on ice. Lysates were centrifuged 12,000 g at 4°C for 5 min, and the supernatants were transferred to new tubes. 10% supernatants were used as input. Protein A/G PLUS-agarose (Santa Cruz) was added for preclear at 4°C for 1 h. The anti-Flag antibody (1:1000; Sigma, F1894) was added and incubated at 4°C for 2 h. Then protein A/G PLUS-agarose was added. Flag-tagged proteins were immunoprecipitated overnight at 4°C. Beads were washed three times with RIP buffer (150 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5% NP40). For western blots, 15 adult male heads, five adult muscles, or five adult ovaries per sample were homogenized in 50 µl Laemmli buffer (Bio-Rad) supplemented with 5% 2-mercaptoethanol, heated to 95°C for 5 min and 10 µl was loaded onto 10% bis-Tris gels (Life Technologies), then transferred to nitrocellulose membrane (Bi-Rad) and blotted following standard protocols. Primary antibodies used were anti-Flag (1:1000; Sigma, F1894), anti-HA (1:2000; Roche, 12013819001), anti-Tubulin (1:10,000; MBL, PM054) and anti-Myc (1:2000; Santa Cruz Biotechnology, sc-40). Secondary antibodies were goat anti-rabbit (1:5000; Sigma, A9169) and goat anti-mouse (1:5000; Sigma, A4416) conjugated to HRP, then developed by chemiluminescence (ECL, Thermo Scientific). The final image was obtained by Fuji scanner (GE Healthcare). To make the Piwi polyclonal antibody, 200 amino acids of the N-terminus of the Piwi protein were used as antigen and injected into rabbit according to established methods (GenScript).

To determine transposon expression status, we performed qRT-PCR using RNA from fly ovaries. The analysis was performed using the QuantStudio 6 Flex real-time PCR system (Life Technologies).

Dataset access

All sequence datasets have been uploaded into the Sequence Read Archive (SRA, <http://trace.ncbi.nlm.nih.gov/Traces/>) with access ID SRP062894.

Acknowledgements

We thank Renjie Jiao and Luping Liu for technical help with the *Drosophila* CRISPR/Cas9 system; Ying Li, Muofang Liu, Junhao Hu, Dangsheng Li and Yikong Rong for helpful discussion; Junying Yuan, Yingchuan Qi and Feng Liu for critical comments on the manuscript; Wei Wu and the *Drosophila* Core Facility, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai, China for fly stocks and fly microinjections.

Competing interests

The authors declare no competing or financial interests.

Author contributions

H.W. and N.L. designed the experiments. H.W. together with Z.M., Y.X., K.N., X.W. and C.P. performed the experiments. H.W., Y. Zhao, K.W., Y. Zhang and N.L. analyzed the data. H.W. and N.L. wrote the manuscript with input from all authors.

Funding

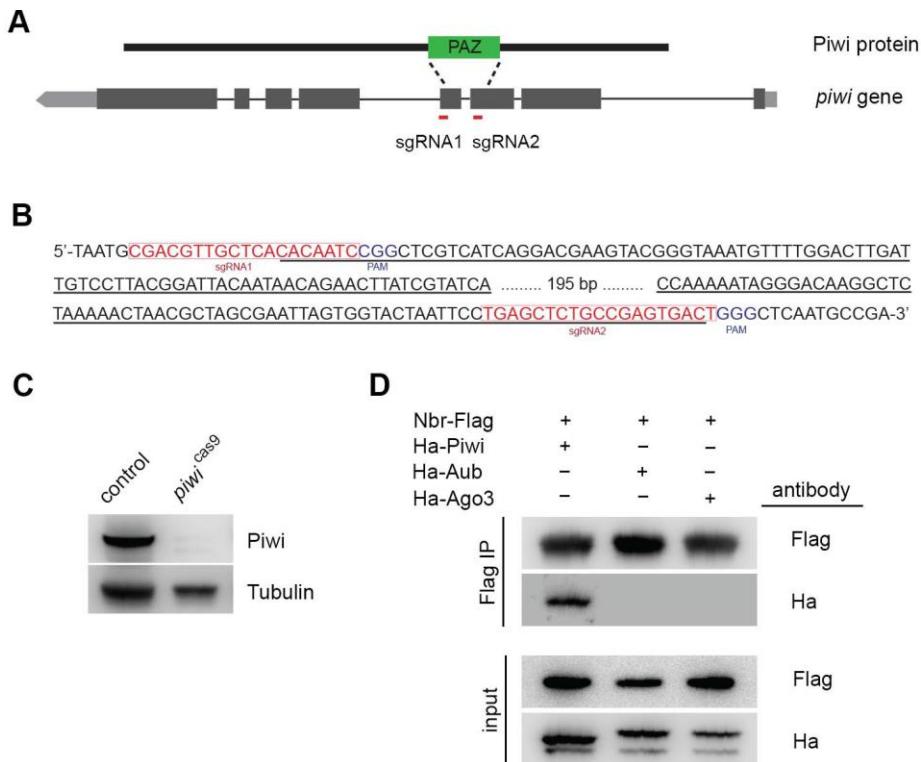
N.L. is a Junior Scholar of the 1000 Plan of China. This work was supported by the startup fund of the 1000 Plan of China and grants from the National Natural Science Foundation of China to N.L. [31371326] and Y. Zhao [31371492]; and a National Institutes of Health (NIH) grant [HG006465] to K.W. Deposited in PMC for immediate release.

Supplementary information

Supplementary information available online at
<http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.128116/-DC1>

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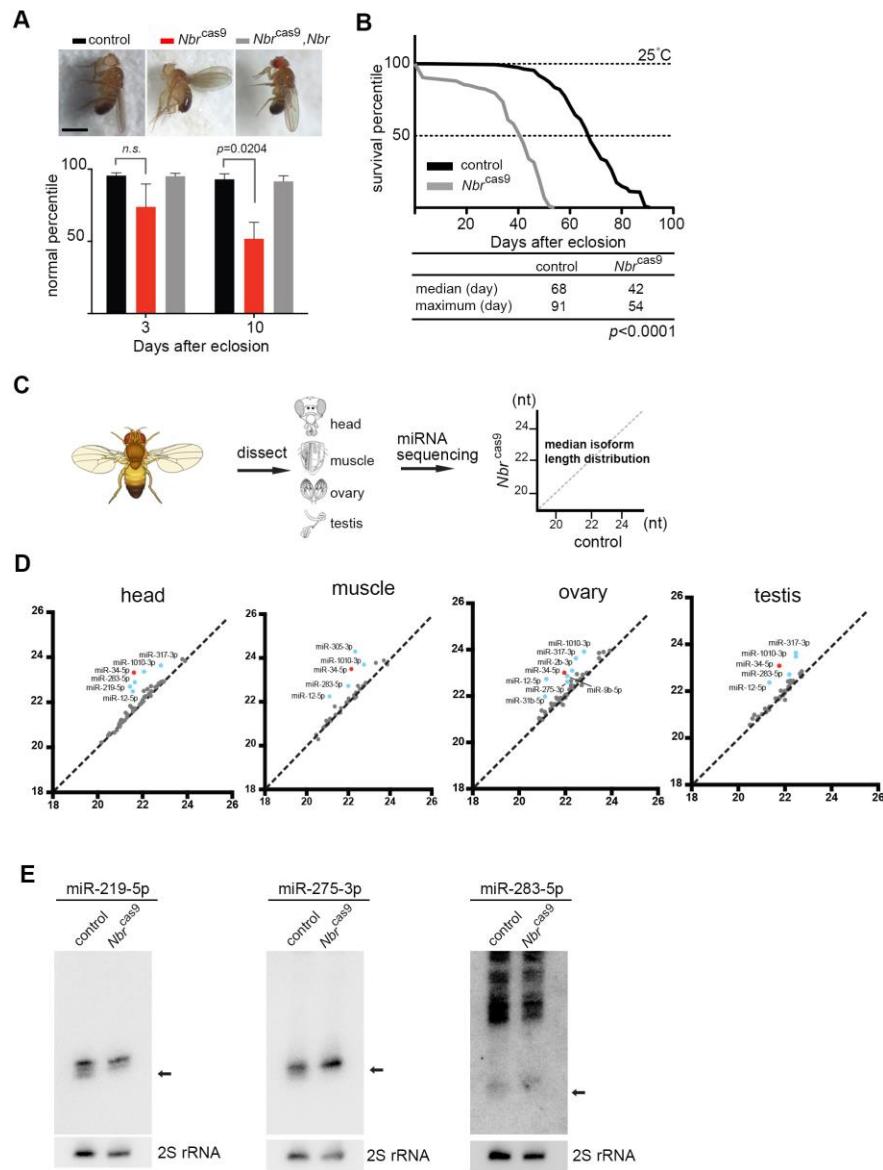
Supplementary Figure 1. Nbr interacts with Piwi at the protein level.

A. Map of the *piwi* locus and guiding RNAs used to make *piwi*^{cas9}. To make *piwi* loss-of-function allele based on the CRISPR/Cas9 method, sgRNA 1 and sgRNA 2, as illustrated in red lines, were used to induce site-specific deletion. The deleted region corresponds to the PAZ domain of Piwi.

B. *piwi*^{cas9} carries a genomic deletion on the *piwi* gene. Part of *piwi* genomic sequence was shown to illustrate design features of CRISPR/Cas9-mediated *piwi* loss-of-function allele. sgRNAs and PAMs were highlighted at a nucleotide resolution. Nucleotides removed were underlined. Resulted *piwi* mutant flies were backcrossed to the control homogeneous background for five generations, to ensure background clearance.

C. Western blot confirms the specificity of Piwi antibody. Flies of indicated genotypes were assayed by western analysis using the polyclonal antibody for Piwi. Proteins were from ovaries. Genotypes: control (5905). *piwi*^{cas9} (*piwi*^{cas9/cas9}).

D. Nbr interacts with Piwi. In *Drosophila* S2 cells, co-IP experiments were conducted to determine if Nbr could interact with Piwi, Aub, and Ago3. Proteins were from S2 cells.



Supplementary Figure 2. Loss of *Nbr* induces adult-onset, age-associated deficits, and miRNA trimming defects.

A. Lack of *Nbr* triggers an adult-onset, progressive deficit. *Nbr*^{cas9} flies exhibited expected adult emergence and gross morphology, suggesting normal development. Examination of adult animals revealed that, whereas controls had flat wing posture (top left panel), some *Nbr*^{cas9} flies showed wing held-up (Top middle panel), a phenotype commonly linked with stress conditions. Upon slight age, the defect of wing postures became significantly enhanced, suggesting progressive deterioration. Flies were raised at 25°C. Wing held up phenotype: Mean \pm SD, n \geq 40 flies

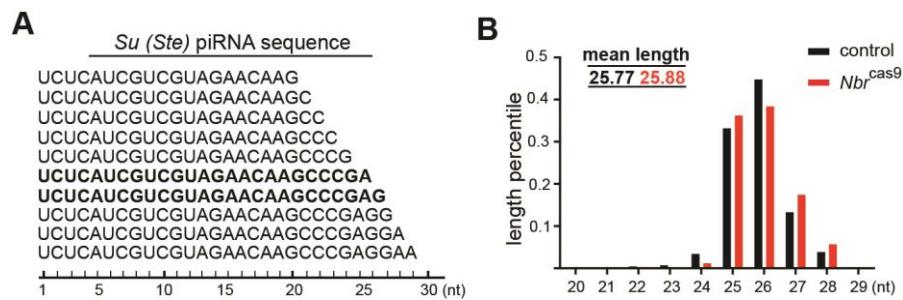
(student *t*-test). Genotypes: control (5905), *Nbr*^{cas9} (*Nbr*^{cas9/cas9}), *Nbr*^{cas9} *Nbr* (pUAST-*Nbr*, *Nbr*^{cas9/cas9}; GeneSwitch-*tubulin-GAL4*).

B. *Nbr*^{cas9} flies (grey) had a sharp decline in adult viability compared to controls (black). Flies were raised at 25°C. Mean ± SE, n ≥ 200 flies for curve (*p*<0.0001, log-rank test). Genotypes: 5905 and *Nbr*^{cas9/cas9}.

C. A scheme to reveal miRNAs trimmed by Nbr. Using dissected adult tissues, we compared length for all miRNA reads between control and mutant. Our rationale lies at the fact that *Nbr* loss would accumulate higher isoforms due to defective trimming, thus tweaking the ratio among isoforms.

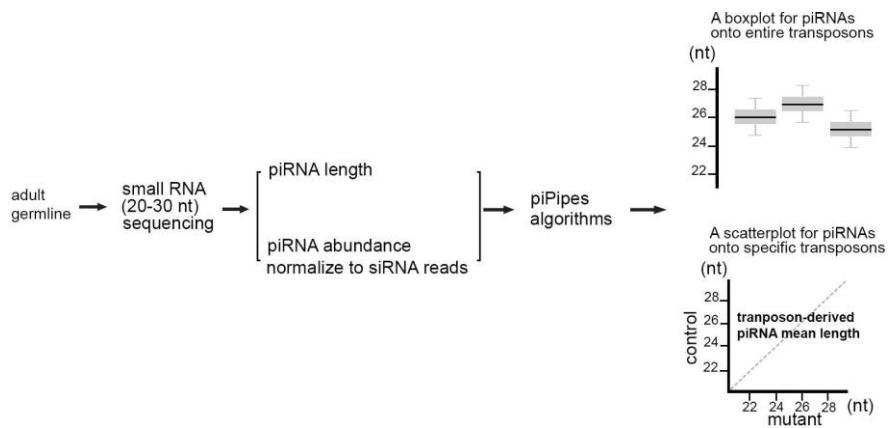
D. miRNAs with altered length in *Nbr*^{cas9}. Scatterplots for indicated adult tissues were made based on miRNA length alterations between control and *Nbr*^{cas9}. To highlight significantly lengthened miRNAs, we chose a cutoff of 0.5 nt, a mean length difference for particular miRNAs between control and mutant. In the graph, miR-34-5p was highlighted in red, while other miRNAs with a significantly lengthened size in *Nbr*^{cas9} were shown in blue. Genotypes: 5905 (x-axis) and *Nbr*^{cas9/cas9} (y-axis).

E. Nbr-substrate miRNAs show abolished lower isoforms in *Nbr*^{cas9}. Small RNA northern was using RNA from heads (miR-219-5p, miR-283-5p) and ovaries (miR-275-3p). Arrow indicated trimmed isoforms in control, which became disappeared upon *Nbr* loss. 2S rRNA was used as a loading control. Genotypes as in B.



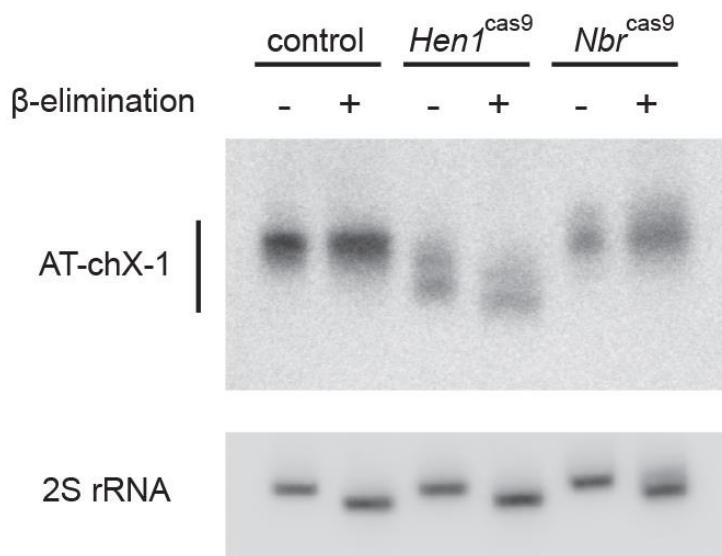
Supplementary Figure 3. *Nbr* loss impacts sequence-specific piRNA

- A. In the deep sequencing result, a testicular piRNA, *Su(Ste)*, has a defined sequence, starting with a 5' uridine, with heterogeneous 3' ends of nested series. Main forms of 25 and 26 nt were shown in bold.
- B. Length distribution graph indicates that *Su(Ste)* piRNAs accumulate more long forms in *Nbr*^{cas9} than in control. RNAs were from testis. Genotypes: 5905 and *Nbr*^{cas9/cas9}.



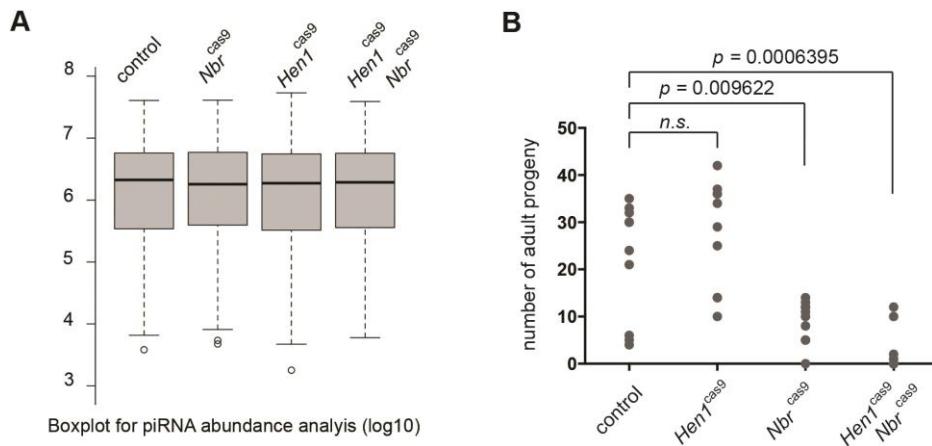
Supplementary Figure 4. Computational pipeline to analyze piRNAs

A dedicated algorithm called piPipes was used for piRNA analysis. Through piPipes, piRNAs mapped to 127 major *Drosophila* transposons were used. A mean value was calculated based on the length of piRNAs mapped to specific transposons. In boxplots, the horizontal bar (black) inside the box represented the median length of all 127 transposons, while the area of box (grey) represented the range of 25%-75% in the mean length of all 127 transposons. The whiskers indicated variability outside the upper and lower quartiles. Outliers beyond the whiskers were plotted as points. The use of boxplot allowed side-by-side comparison across multiple genotypes/samples. In scatterplots, individual transposons were shown as single dots (black), allowing comparison for individual transposons between two genotypes/samples.



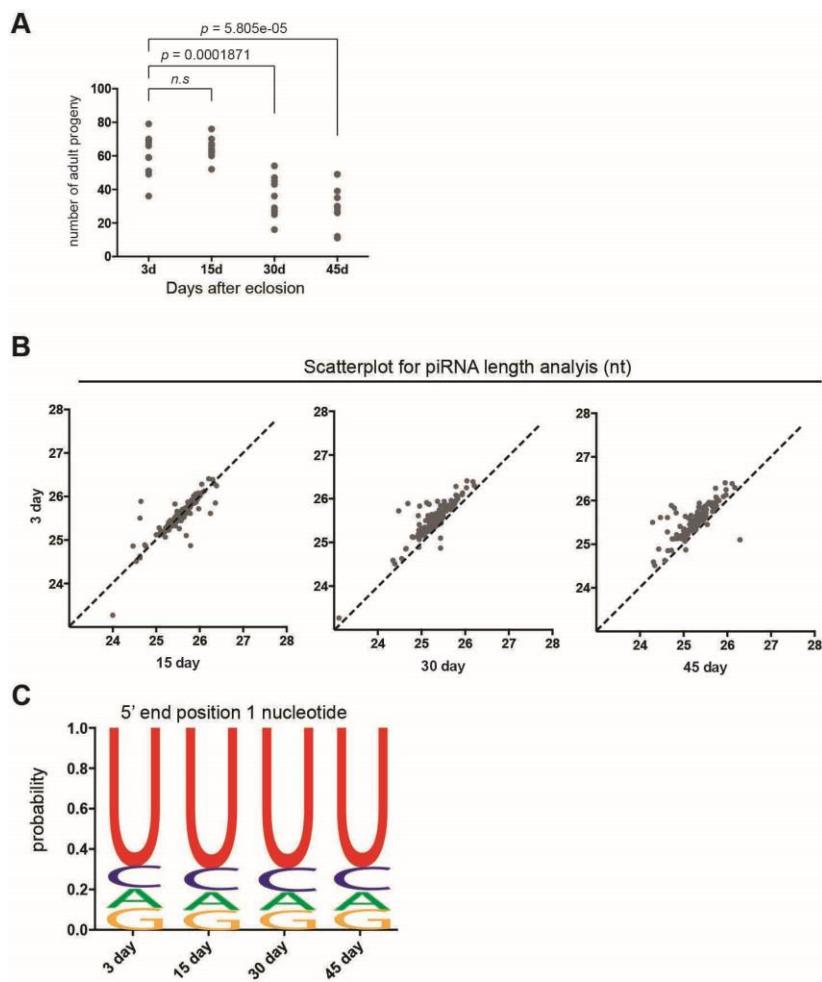
Supplementary Figure 5. piRNAs remain 3' methylated in *Nbr* mutants.

Small RNA northern blot revealed that, AT-chX-1 piRNAs were resistant to beta-elimination, in control and *Nbr*^{cas9} mutants, but not in *Hen1*^{cas9} mutants, suggesting that these piRNAs were 2'-*O*-methylated at 3' ends. Note that AT-chX-1 became shorter in *Hen1*^{cas9} mutants compared to controls, reflecting enhanced trimming upon loss of *Hen1*. RNAs were from testis. Genotypes: control (5905), *Hen1*^{cas9} (*Hen1*^{cas9/cas9}), *Nbr*^{cas9} (*Nbr*^{cas9/cas9}).



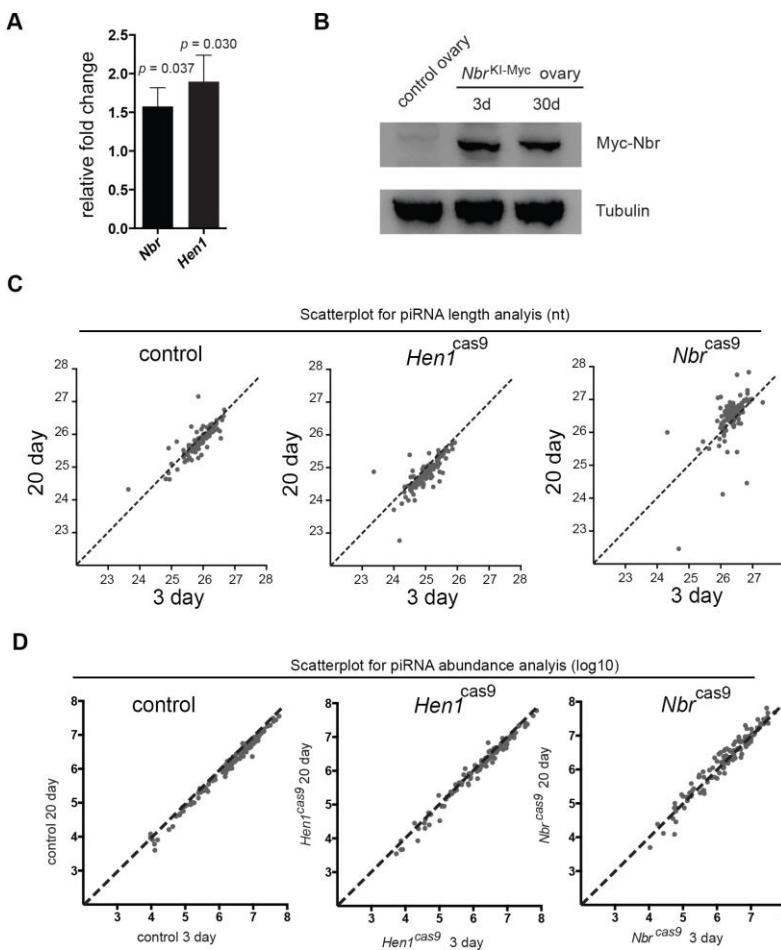
Supplementary Figure 6. Characteristics of mutants.

- A. Analysis of piRNA abundance. RNAs were from ovaries of 3d old animals with indicated genotypes. Boxplots for normalized read counts were shown as log10 value. (*Nbr*^{cas9} vs. 5905: $p = 0.05525$; *Hen1*^{cas9} vs. 5905: $p = 0.0003845$; *Nbr*^{cas9}, *Hen1*^{cas9} vs. 5905: $p = 0.233$; Wilcoxon signed-rank test). Genotypes: control (5905), *Hen1*^{cas9} (*Hen1*^{cas9/cas9}), *Nbr*^{cas9} (*Nbr*^{cas9/cas9}) and double mutant.
- B. Flies without *Nbr* show reduced female fecundity. Fertility test revealed that whereas *Hen1*^{cas9} mutants had normal fertility, *Nbr*^{cas9} mutants and *Hen1*^{cas9} *Nbr*^{cas9} double mutants demonstrated a significant decrease in female fecundity. n=10 independent tests, (student *t*-test with Welch correction). Genotypes: control (5905), *Hen1*^{cas9} (*Hen1*^{cas9/cas9}), *Nbr*^{cas9} (*Nbr*^{cas9/cas9}) and double mutant.



Supplementary Figure 7. With age, piRNAs become progressively shortened from 3' ends.

- A. Female fecundity shows age-associated decline. n=10 independent tests, (student *t*-test with Welch correction). Genotypes: control (5905).
- B. piRNAs become progressively shortened with age. Scatterplots for piRNA length analysis revealed age-dependent length changes. (15d vs. 3d: $p = 0.001371$; 30d vs. 3d: $p < 2.2 \times 10^{-16}$; 45d vs. 3d: $p < 2.2 \times 10^{-16}$; Wilcoxon signed-rank test). RNAs were from ovaries of indicated ages. Genotype as in A.
- C. piRNAs in adult flies with indicated ages show the same bias for 5' uridine (1U). Genotype as in A.



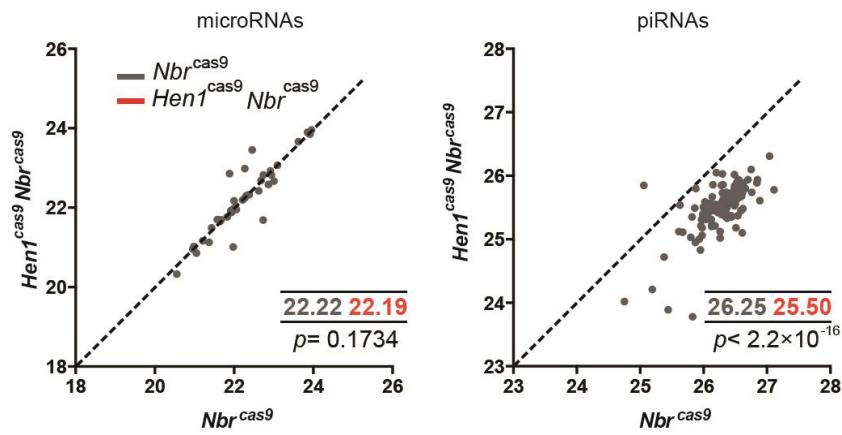
Supplementary Figure 8. *Nbr* contributes to the age-modulated piRNA profile.

A. *Nbr* and *Hen1* show increased expression level with age. qRT-PCR was used. RNAs were from ovaries at 3d or 30d. Mean \pm SD, n = 3 independent experiments (student *t*-test). Genotype (5905).

B. *Nbr* proteins show unchanged levels with age. Proteins were from ovaries of indicated age. Genotypes: control (5905), *Nbr*^{KI-Myc} (*Nbr*^{KI-Myc/KI-Myc}).

C. *Nbr* contributes to age-modulation of piRNAs. Scatterplots showed that, with slight aging, piRNAs became shortened in control (left panel) and *Hen1*^{cas9} (middle panel), but not in *Nbr*^{cas9} (right panel). RNAs were from ovaries of indicated genotypes. (5905: 20d vs. 3d: $p = 2.948 \times 10^{-10}$; *Hen1*^{cas9}: 20d vs. 3d: $p = 2.634 \times 10^{-15}$; *Nbr*^{cas9}: 20d vs. 3d: $p = 7.098 \times 10^{-8}$; Wilcoxon signed rank test). Genotypes: control (5905), *Hen1*^{cas9} (*Hen1*^{cas9/cas9}), *Nbr*^{cas9} (*Nbr*^{cas9/cas9}).

D. *Nbr* modulates piRNA abundance with age. Scatterplots showed a trend in that, with slight aging, piRNA levels became decreased in control (left panel) and *Henl^{cas9}* (middle panel), but not in *Nbr^{cas9}* (right panel). RNAs were from ovaries of indicated genotypes. (5905: 20d vs. 3d: $p < 2.2 \times 10^{-16}$; *Henl^{cas9}*: 20d vs. 3d: $p = 8.454 \times 10^{-11}$; *Nbr^{cas9}*: 20d vs. 3d: $p = 0.1271$; Wilcoxon signed-rank test).



Supplementary Figure 9. piRNAs may be trimmed by an unknown “trimmer”.

Sequence analysis and comparison revealed piRNAs, but not miRNAs, became further shortened in $Hen1^{cas9} Nbr^{cas9}$ double mutants compared to Nbr^{cas9} single mutants. This data may implicate the effect of a second “unknown” trimmer that modulates piRNA sequence. RNAs were from ovaries of indicated genotypes. Wilcoxon rank sum test.

Supplementary Table 1. miRNAs trimmed by Nbr

This table summarizes a list of miRNAs that are trimmed by Nbr at a tissue resolution. For miRNAs with relatively high expression, small RNA northern was used for validation, together with small RNA sequencing data.

	tissue type	validation
miR-2b-3p	ovary	sequencing
miR-3-3p	whole body	sequencing; small RNA northern
miR-7-5p	whole body	sequencing; small RNA northern
miR-9b-5p	ovary	sequencing
miR-10-3p	whole body	sequencing; small RNA northern
miR-11-3p	whole body	sequencing
miR-12-3p	head;muscle;testis;ovary	sequencing; small RNA northern
miR-31b-5p	ovary	sequencing
miR-34-5p	head;muscle;testis;ovary	sequencing; small RNA northern
miR-100-5p	whole body	sequencing
miR-190-5p	whole body	sequencing; small RNA northern
miR-219-5p	head	sequencing; small RNA northern
miR-263a-5p	whole body	sequencing
miR-263b-5p	whole body	sequencing
miR-275-3p	ovary	sequencing; small RNA northern
miR-281-1/2-5p	whole body	sequencing
miR-283-5p	head;muscle;testis;ovary	sequencing; small RNA northern
miR-305-5p	muscle	sequencing; small RNA northern
miR-317-3p	head;ovary;testis	sequencing; small RNA northern
miR-1010-3p	head;muscle;ovary;testis	sequencing

Supplementary Table 2. Mean length for piRNA lengths in different mutants

Summary for major transposons in the fly genome was listed. Transposons further analyzed by qRT-PCR were in red.

	control	<i>Nbr</i> ^{cas9}	<i>Hen1</i> ^{cas9}	<i>Hen1</i> ^{cas9} <i>Nbr</i> ^{cas9}
<i>frogger</i>	25.34	27.11	25.48	25.78
<i>aurora_element</i>	25.33	26.54	23.96	25.37
<i>R1_2</i>	25.38	26.5	25.35	25.56
<i>jockey2</i>	24.75	25.86	24.38	25.49
<i>X_element</i>	25.41	26.49	24.44	25.53
<i>jockey</i>	24.89	25.93	24.23	25
<i>G6</i>	25.31	26.3	24.6	25.54
<i>GATE</i>	25.38	26.37	24.73	25.66
<i>Tc1_2</i>	25.91	26.84	24.92	25.89
<i>transib1</i>	25.45	26.31	24.87	25.86
<i>R1A1-element</i>	25.68	26.54	24.41	25.65
<i>Rt1a</i>	25.81	26.66	24.7	25.8
<i>baggins</i>	25.86	26.6	25.04	25.86
<i>Max-element</i>	25.87	26.6	24.67	25.68
<i>stellateHet</i>	25.76	26.49	24.98	25.61
<i>G2</i>	26.03	26.75	24.92	26.1
<i>BS3</i>	25.9	26.61	24.92	25.1
<i>Rt1b</i>	25.65	26.35	24.46	25.59
17	25.64	26.33	25.07	25.71
<i>stellate</i>	25.95	26.62	24.91	25.48
<i>HeT-A</i>	25.49	26.13	24.34	25.22
<i>gypsy8</i>	25.92	26.55	25.67	25.94
<i>gypsy7</i>	25.97	26.59	24.58	25.45
<i>suffix</i>	24.79	25.38	24.67	24.72
<i>Rt1c</i>	25.78	26.37	24.97	25.5

<i>opus</i>	25.88	26.47	24.99	25.75
<i>Doc</i>	25.38	25.96	24.36	25.21
<i>Fw3</i>	25.41	25.99	24.59	25.34
<i>suste</i>	25.44	26.01	24.59	25.46
<i>mdg3</i>	25.5	26.07	24.53	25.39
<i>Osvaldo</i>	26.02	26.59	24.36	25.45
<i>diver2</i>	25.56	26.12	24.84	25.31
<i>G3</i>	25.67	26.23	25.06	25.42
<i>roo</i>	25.94	26.5	25.18	25.69
<i>looper1</i>	25.49	26.02	25.06	25.33
<i>INE_1</i>	25.58	26.11	24.82	25.3
<i>S_element</i>	25.75	26.28	24.94	25.85
<i>1731</i>	25.77	26.3	24.81	25.45
<i>Fw2</i>	25.46	25.98	25.07	25.06
<i>Doc3_eleme</i>	25.56	26.08	24.75	25.43
<i>G_element</i>	25.84	26.36	24.65	25.74
<i>accord</i>	25.47	25.98	24.71	25.18
<i>TAHRE</i>	25.69	26.2	24.74	26.05
<i>BS</i>	25.77	26.27	24.97	25.57
<i>invader3</i>	25.79	26.29	24.97	25.5
<i>G4</i>	25.79	26.29	25.01	25.43
<i>R2_element</i>	25.89	26.39	25.25	25.62
<i>1360</i>	25.93	26.43	24.89	25.6
<i>Porto1</i>	25.67	26.16	25.21	25.45
<i>pogo</i>	25.7	26.18	25.12	25.21
<i>F_element</i>	25.51	25.99	24.49	25.39
<i>lvk</i>	25.83	26.3	24.97	25.73
<i>Tirant</i>	25.96	26.43	25.29	25.37

<i>TART_C_TAR</i>	25.72	26.18	24.65	25.55
<i>accord2</i>	25.93	26.39	25.18	25.62
<i>transib2</i>	26.24	26.7	25.45	25.94
<i>I-element</i>	25.78	26.24	24.72	25.47
<i>Doc2_eleme</i>	25.72	26.17	24.92	25.48
<i>copia</i>	26.1	26.55	24.57	25.71
<i>HMS_Beagle</i>	26.01	26.44	25.11	25.59
<i>mst40</i>	25.73	26.15	25.13	25.86
<i>Tc1</i>	25.54	25.95	25.43	24.83
<i>3S18</i>	25.69	26.1	24.6	25.57
<i>gypsy12</i>	25.94	26.35	25.03	25.58
<i>rooA</i>	26.03	26.44	25.16	25.67
<i>invader2</i>	26.12	26.53	25.12	25.81
<i>diver</i>	25.63	26.02	24.76	25.59
<i>McClintock</i>	25.91	26.3	25.3	26.03
<i>gypsy3</i>	25.95	26.34	24.85	25.36
<i>HMS_Beagle</i>	26.13	26.51	24.74	25.18
<i>Stalker</i>	26.23	26.61	25.38	25.73
<i>invader4</i>	26.53	26.89	24.54	25.61
<i>G5A</i>	25.78	26.13	24.37	25.2
<i>Cr1a</i>	26.17	26.52	25.05	25.89
<i>Stalker4</i>	26.21	26.56	25.35	25.66
<i>NOF</i>	25.77	26.11	25.08	25.48
<i>hobo</i>	26.14	26.48	25.28	25.77
<i>gypsy4</i>	26.15	26.49	25.14	25.67
<i>Stalker2</i>	26.32	26.66	25.48	25.8
<i>invader1</i>	25.96	26.29	24.77	25.51
<i>Dm88</i>	26.3	26.63	25.16	25.89

<i>Q_element</i>	26.71	27.04	25.45	26.31
<i>springer</i>	25.98	26.3	24.96	25.39
<i>gtwin</i>	26.55	26.85	25.85	25.94
<i>blood</i>	26.06	26.35	25.05	25.43
<i>Helena</i>	26.21	26.5	25.37	25.67
297	26.29	26.58	25.31	25.75
<i>TARTA</i>	25.86	26.14	25.32	25.67
<i>gypsy2</i>	26.14	26.42	25.51	25.72
<i>Idefix</i>	26.15	26.43	25.29	25.57
<i>mdg1</i>	26.16	26.44	25.17	25.35
<i>Circe</i>	26.03	26.31	25.13	25.63
<i>gypsy10</i>	26.26	26.53	25.57	25.64
<i>gypsy9</i>	26	26.26	24.09	25.12
<i>ZAM</i>	26.03	26.28	25.21	25.39
<i>Tabor</i>	26.19	26.44	25.44	25.58
<i>micropia</i>	26.52	26.76	25.19	25.74
412	26.08	26.31	25.42	25.58
<i>gypsy</i>	26.08	26.3	25.39	25.58
<i>gypsy6</i>	24.99	25.19	24.17	24.21
<i>rover</i>	25.61	25.8	24.74	25.03
<i>flea</i>	25.84	26.03	24.9	25.46
<i>G5</i>	25.86	26.05	24.7	25.37
<i>Transpac</i>	26.18	26.37	24.88	25.33
<i>hopper2</i>	25.26	25.44	23.53	23.89
<i>Stalker3</i>	26.38	26.56	25.59	25.93
<i>S2</i>	25.86	26.03	24.86	25.31
<i>Burdock</i>	25.84	26	24.6	25.51
<i>Quasimodo</i>	26.1	26.23	25.11	25.47

<i>gypsy11</i>	26.37	26.49	25.28	25.84
<i>mariner2</i>	25.54	25.63	24.55	25.54
<i>hopper</i>	25.75	25.82	24.46	25.35
<i>gypsy5</i>	26.42	26.49	25.55	25.71
<i>Bari1</i>	25.55	25.61	24.68	25.12
<i>Doc4_element</i>	25.82	25.87	24.55	24.95
<i>transib3</i>	26.23	26.26	24.53	25.02
<i>FB</i>	24.74	24.75	24.24	24.02
<i>HB</i>	26	25.97	25.5	25.31
<i>Juan</i>	26.53	26.49	25.7	26.02
<i>G7</i>	26.02	25.88	24.66	25.8
<i>invader5</i>	25.96	25.67	25.14	25.11
<i>Tom1</i>	25.92	25.06	25.69	25.85
<i>transib4</i>	26.14	22.75	24.72	25.4

Supplementary Table 3. piRNA abundance in different mutants

Transposons further analyzed by qRT-PCR were in red.

	control	<i>Nbr</i> ^{cas9}	<i>Hen1</i> ^{cas9}	<i>Hen1</i> ^{cas9} <i>Nbr</i> ^{cas9}
<i>R1A1-element</i>	40555792	40918873	53738491	38956250
<i>roo</i>	34546733	35677729	28894838	35213717
<i>Rt1b</i>	29893067	25404192	24329941	23540594
<i>GATE</i>	29765871	35781378	37764822	36524184
<i>Max-element</i>	23878570	25915874	24995514	25651025
<i>gtwin</i>	18375221	18757781	15419291	14571126
<i>F_element</i>	15829868	19596311	13607558	21286131
<i>412</i>	13198049	15899266	15254488	17610607
<i>Doc</i>	12467788	14848681	9749282	12410250
<i>X_element</i>	12327023	10212972	10113076	12670588
<i>Stalker</i>	11054786	13311987	10426252	11011733
<i>Rt1a</i>	10994816	16169664	11452185	14926210
<i>Stalker4</i>	10673738	13019675	10454206	11022726
<i>Doc3_element</i>	9375582	10887232	6993792	7804955
<i>invader4</i>	9235976	5773847	7579986	6632277
<i>HeT-A</i>	9183285	12781578	9122531	8472331
<i>gypsy12</i>	9083661	10678551	11400864	10570090
297	8993479	28827042	27198270	34197061
<i>baggins</i>	8694890	7710230	6498647	6779597
<i>G_element</i>	8311368	8700602	6584400	6133818
<i>mdg1</i>	8243544	9424532	9163660	9354462
<i>opus</i>	7499437	5915539	9016755	9486698
<i>gypsy4</i>	7489508	7631847	5406112	6786272
<i>blood</i>	7362489	6970538	6238376	7016394
<i>gypsy6</i>	7139684	7690015	6041633	6593523
<i>G2</i>	7063689	5572263	5550461	5677810
<i>Stalker2</i>	6868471	8201728	6108261	6372059

<i>rooA</i>	6690092	9158656	5677760	8500340
<i>Cr1a</i>	6662661	8449936	8062565	8541705
<i>Circe</i>	6311811	6415745	6729623	5672003
<i>TART_C_TAR</i>	5917997	5977014	6808865	6027270
<i>gypsy</i>	5749546	5892751	4519596	4736007
<i>springer</i>	5716495	5417446	4028415	4104576
<i>TAHRE</i>	5466748	7620330	5754409	4856857
<i>gypsy2</i>	5430234	5767966	5318034	5257971
<i>flea</i>	5287676	1147693	7972798	1225206
1731	5230114	3279110	2961506	3748954
<i>gypsy10</i>	5122992	5046472	3982439	4931326
<i>Tabor</i>	4764209	6048619	4705033	5737882
<i>HMS_Beagle</i>	4541105	3387774	2720143	2966920
<i>Quasimodo</i>	4414961	4078264	3124211	3514222
<i>BS</i>	4390388	4191312	2563274	4923770
<i>ldefix</i>	4228648	4258130	3982744	4772878
<i>HMS_Beagle</i>	4224771	2270861	2103363	2171112
3S18	4188719	5760739	3948456	5686902
<i>aurora_element</i>	4021801	3685493	3357721	3857174
<i>rover</i>	3913934	4439335	3740341	4413802
17	3757103	4108886	5071916	5560644
<i>gypsy3</i>	3682778	3554386	2830184	2884272
<i>gypsy8</i>	3655179	3728606	2722929	3411123
<i>micropia</i>	3559118	2417278	2013311	2119921
<i>jockey</i>	3376139	3297674	3546945	4146091
<i>mdg3</i>	3296156	1235716	2524741	1345156
1360	3285512	5156759	5335734	6568159
<i>jockey2</i>	2981839	1342190	1868498	1301785
<i>NOF</i>	2918775	2971627	3143341	4836216
<i>Doc2_element</i>	2688086	2651555	2132027	2253330

<i>invader3</i>	2584627	2078870	1284719	2739027
G6	2576079	627074	945758	379915
<i>invader1</i>	2458468	1693207	1281924	1931929
<i>diver</i>	2372031	1987955	1590040	1618401
<i>R2_element</i>	2260035	2483339	1794447	1517019
<i>gypsy5</i>	2111953	2479300	2062569	2389746
<i>accord</i>	2106928	1801875	3248478	2882497
<i>hobo</i>	1954811	1809029	1713479	2094122
<i>Osvaldo</i>	1877233	1627265	932764	1151843
<i>lvk</i>	1845080	1550842	1819617	1687646
<i>I-element</i>	1718477	1794646	2068711	1382814
<i>diver2</i>	1550859	1815087	1296286	1787576
<i>mst40</i>	1530674	1362018	1409514	1731263
<i>accord2</i>	1527798	2096587	1512048	2010345
<i>invader2</i>	1523485	1374037	1423788	1649748
<i>suste</i>	1482810	1785831	1558074	1835264
<i>ZAM</i>	1476973	1666397	1097342	1276684
<i>S_element</i>	1444778	1610151	2033213	2184220
<i>transib2</i>	1366037	1032032	2351520	1815126
G5	1315973	1064191	274110	232997
<i>Bari1</i>	1314823	1157956	874114	1132476
<i>BS3</i>	1295669	1514826	1189674	1432758
<i>Juan</i>	1283974	1530649	1264812	1231537
<i>Dm88</i>	1261245	1345772	696032	1065214
<i>TARTA</i>	1230226	1386330	2538630	1668275
<i>Burdock</i>	1182124	1236358	1616024	1696223
<i>copia</i>	1168058	1237437	919496	2176140
<i>McClintock</i>	1130508	1836263	1800464	2155603
<i>Q_element</i>	1007549	1362920	876787	1298171
<i>Rt1c</i>	921877	979467	516378	460132

<i>Transpac</i>	664480	842077	1398779	919364
<i>G4</i>	655365	668500	515109	503493
<i>R1_2</i>	599844	565842	538270	427914
<i>pogo</i>	550207	20837	64731	89295
<i>INE_1</i>	536650	537523	411091	342295
<i>Porto1</i>	444522	490967	325868	381115
<i>G5A</i>	343242	364904	118977	103700
<i>Fw2</i>	322497	326305	540390	437726
<i>gypsy7</i>	317098	433764	258502	359194
<i>gypsy11</i>	308232	318691	322509	308570
<i>Helena</i>	269447	393630	418463	418829
<i>Stalker3</i>	245588	282495	217954	282695
<i>Tc1_2</i>	230163	308591	156323	336822
<i>suffix</i>	225365	382428	109094	282544
<i>looper1</i>	204936	131436	157977	113107
<i>hopper</i>	199395	217477	232908	283920
<i>Doc4_element</i>	185309	169848	95216	85550
<i>hopper2</i>	183781	255913	464215	500264
<i>G3</i>	149500	157859	85833	113329
<i>stellateHet</i>	127860	156267	178952	145553
<i>Tirant</i>	115183	106368	173196	90752
<i>stellate</i>	93590	118223	122122	85177
<i>transib3</i>	90213	95001	14480	30864
<i>FB</i>	82216	96167	90481	108577
<i>gypsy9</i>	74008	50655	29568	58405
<i>Fw3</i>	64433	72945	29862	48315
<i>Tc1</i>	57170	88076	27440	44741
<i>transib1</i>	50297	54878	48608	35706
<i>HB</i>	44986	43360	26373	29927
<i>frogger</i>	37533	48780	35868	19516

<i>S2</i>	30233	65358	31936	38142
<i>mariner2</i>	17963	13494	7214	10729
<i>Tc3</i>	8331	8130	4704	5988
<i>Tom1</i>	8029	21705	20794	27279
<i>G7</i>	7580	9506	5654	6052
<i>Bari2</i>	7498	4743	1789	5988
<i>transib4</i>	6560	5420	12446	16633
<i>invader5</i>	3827	8130	7513	7386

Supplementary Table 4. Mean length for piRNA lengths in adult life cycle

Transposons further analyzed by qRT-PCR were in red.

	3 day	15 day	30 day	45 day	30 day compared to 3 day
<i>HB</i>	25.72	25.98	24.48	24.83	-1.24
<i>transib4</i>	25.89	24.65	24.69	24.72	-1.2
<i>gypsy9</i>	25.83	25.43	24.96	24.79	-0.87
<i>S2</i>	25.91	25.91	25.12	25.67	-0.79
<i>invader5</i>	25.88	25.92	25.16	25.75	-0.72
<i>Tc1_2</i>	25.87	25.73	25.18	25.49	-0.69
<i>mariner2</i>	25.61	26.25	24.93	24.47	-0.68
<i>Tc1</i>	25.94	25.95	25.37	25.36	-0.57
<i>invader4</i>	26.28	26.05	25.79	25.73	-0.49
<i>I_element</i>	25.41	25.29	24.95	24.92	-0.46
<i>G3</i>	25.61	25.59	25.15	24.63	-0.46
<i>gypsy7</i>	25.94	25.87	25.52	25.62	-0.42
<i>transib1</i>	25.64	25.75	25.24	25.04	-0.4
<i>Tirant</i>	25.84	25.76	25.44	25.72	-0.4
<i>Bari2</i>	25.5	24.63	25.1	24.29	-0.4
<i>NOF</i>	25.51	25.42	25.14	25.21	-0.37
<i>Q_element</i>	26.41	26.21	26.04	25.95	-0.37
<i>1731</i>	25.62	25.49	25.26	25.3	-0.36
<i>mdg3</i>	25.13	25.07	24.79	24.85	-0.34
<i>Porto1</i>	25.28	25.07	24.94	24.63	-0.34
<i>frogger</i>	25.85	26.36	25.51	25.47	-0.34
<i>accord2</i>	25.66	25.48	25.33	25.17	-0.33
<i>R2_element</i>	25.55	25.5	25.22	25.16	-0.33
<i>Osvaldo</i>	25.74	25.67	25.41	25.32	-0.33
<i>X_element</i>	25.12	25	24.8	24.78	-0.32
<i>Quasimodo</i>	25.85	25.77	25.54	25.35	-0.31
<i>Bari1</i>	25.32	25.34	25.01	25	-0.31
<i>aurora_element</i>	25.12	25.1	24.82	24.74	-0.3
<i>gypsy11</i>	26.08	25.97	25.79	25.74	-0.29
<i>gypsy5</i>	25.96	25.88	25.68	25.58	-0.28
<i>ZAM</i>	25.83	25.74	25.55	25.41	-0.28
<i>rover</i>	25.35	25.27	25.08	25.01	-0.27
<i>Circe</i>	25.71	25.63	25.44	25.33	-0.27
<i>Doc3_element</i>	25.45	25.33	25.18	25.14	-0.27
<i>BS3</i>	25.71	25.68	25.44	25.47	-0.27
<i>stellate</i>	25.65	25.59	25.38	25.29	-0.27
<i>invader3</i>	25.67	25.58	25.41	25.36	-0.26
<i>copia</i>	25.6	25.5	25.34	25.11	-0.26
<i>F_element</i>	25.19	25.2	24.93	24.96	-0.26
<i>lvk</i>	25.51	25.46	25.25	25.19	-0.26
<i>Rt1c</i>	25.6	25.65	25.34	25.39	-0.26
<i>pogo</i>	25.33	25.44	25.07	25.12	-0.26

<i>G_element</i>	25.65	25.55	25.4	25.35	-0.25
<i>GATE</i>	25.19	25.12	24.94	24.94	-0.25
<i>rooA</i>	25.79	25.74	25.54	25.52	-0.25
<i>G5</i>	25.54	25.47	25.29	25.25	-0.25
<i>G2</i>	25.83	25.76	25.58	25.5	-0.25
<i>Fw2</i>	25.28	25.22	25.04	25.04	-0.24
<i>opus</i>	25.63	25.62	25.39	25.36	-0.24
<i>Dm88</i>	26.06	25.91	25.82	25.76	-0.24
<i>gypsy8</i>	25.7	25.65	25.46	25.42	-0.24
<i>suffix</i>	24.59	24.63	24.35	24.31	-0.24
<i>Idefix</i>	25.92	25.82	25.69	25.55	-0.23
<i>Rt1a</i>	25.59	25.56	25.36	25.39	-0.23
<i>Helena</i>	25.85	25.89	25.62	25.35	-0.23
<i>transib2</i>	25.72	25.66	25.49	25.48	-0.23
<i>gypsy3</i>	25.7	25.6	25.47	25.42	-0.23
<i>Doc2_element</i>	25.46	25.44	25.23	25.22	-0.23
<i>R1_2</i>	25.23	25.29	25	25.1	-0.23
<i>suste</i>	25.22	25.1	24.99	24.84	-0.23
<i>Doc</i>	25.13	25.1	24.91	24.94	-0.22
<i>HMS_Beagle</i>	25.75	25.75	25.53	25.45	-0.22
<i>gypsy4</i>	25.93	25.85	25.71	25.58	-0.22
<i>baggins</i>	25.59	25.52	25.37	25.31	-0.22
<i>Stalker3</i>	26.25	26.39	26.03	25.97	-0.22
<i>gypsy12</i>	25.61	25.57	25.39	25.29	-0.22
<i>gtwin</i>	26.39	26.3	26.18	26.11	-0.21
<i>gypsy6</i>	24.85	24.76	24.64	24.64	-0.21
<i>BS</i>	25.59	25.57	25.38	25.4	-0.21
<i>hopper2</i>	24.86	24.47	24.65	24.72	-0.21
<i>HMS_Beagle</i>	25.93	25.88	25.72	25.64	-0.21
<i>1360</i>	25.63	25.62	25.42	25.51	-0.21
<i>TART_C_TAR</i>	25.35	25.27	25.15	25.2	-0.2
<i>Cr1a</i>	25.79	25.74	25.59	25.5	-0.2
<i>TAHRE</i>	25.43	25.36	25.23	25.16	-0.2
<i>412</i>	25.91	25.81	25.72	25.63	-0.19
<i>gypsy10</i>	26.01	25.86	25.82	25.76	-0.19
<i>roo</i>	25.71	25.62	25.52	25.51	-0.19
<i>hobo</i>	26.01	26.01	25.82	25.78	-0.19
<i>Transpac</i>	25.59	25.67	25.4	25.39	-0.19
<i>Juan</i>	26.13	26.11	25.94	25.97	-0.19
<i>R1A1_element</i>	25.39	25.37	25.21	25.17	-0.18
<i>3S18</i>	25.49	25.51	25.31	25.27	-0.18
<i>McClintock</i>	25.57	25.47	25.39	25.26	-0.18
<i>Stalker</i>	26	25.98	25.83	25.81	-0.17

<i>Stalker4</i>	25.96	25.94	25.79	25.74	-0.17
<i>accord</i>	25.23	25.24	25.07	25.07	-0.16
<i>gypsy</i>	25.87	25.84	25.71	25.65	-0.16
<i>invader1</i>	25.52	25.64	25.36	25.37	-0.16
17	25.31	25.3	25.15	25.2	-0.16
<i>FB</i>	23.27	24	23.11	22.9	-0.16
<i>S_element</i>	25.51	25.52	25.35	25.36	-0.16
<i>Tabor</i>	25.95	25.94	25.79	25.66	-0.16
<i>Stalker2</i>	26.09	26.06	25.93	25.91	-0.16
<i>G6</i>	25.15	25.11	24.99	24.94	-0.16
<i>TARTA</i>	25.35	25.3	25.19	25.28	-0.16
<i>blood</i>	25.72	25.73	25.57	25.5	-0.15
<i>diver2</i>	25.31	25.32	25.16	25.19	-0.15
<i>G4</i>	25.59	25.66	25.44	25.37	-0.15
<i>diver</i>	25.41	25.33	25.27	25.25	-0.14
<i>Max_element</i>	25.55	25.57	25.42	25.46	-0.13
<i>hopper</i>	25.07	25.21	24.95	25.03	-0.12
<i>jockey2</i>	24.51	24.54	24.39	24.34	-0.12
<i>gypsy2</i>	25.85	25.82	25.73	25.68	-0.12
<i>HeT_A</i>	25.24	25.24	25.12	25.05	-0.12
<i>mdg1</i>	25.88	25.85	25.76	25.65	-0.12
<i>invader2</i>	25.61	25.78	25.5	25.4	-0.11
<i>mst40</i>	25.37	25.38	25.26	25.23	-0.11
<i>G5A</i>	25.26	25.3	25.16	25.01	-0.1
<i>springer</i>	25.81	25.81	25.71	25.7	-0.1
<i>micropia</i>	26.3	26.33	26.21	26.17	-0.09
<i>transib3</i>	25.45	25.58	25.36	25.54	-0.09
<i>jockey</i>	24.63	24.63	24.55	24.57	-0.08
<i>Burdock</i>	25.33	25.39	25.25	25.28	-0.08
<i>Rt1b</i>	25.43	25.48	25.35	25.33	-0.08
<i>Doc4_element</i>	25.15	25.14	25.07	25.05	-0.08
297	25.75	25.78	25.68	25.66	-0.07
<i>flea</i>	25.53	25.64	25.47	25.37	-0.06
<i>Fw3</i>	25.26	25.53	25.2	25.09	-0.06
<i>INE_1</i>	25.37	25.4	25.33	25.26	-0.04
<i>stellateHet</i>	25.44	25.57	25.45	25.3	0.01
<i>looper1</i>	24.89	24.74	24.94	24.43	0.05
<i>Tom1</i>	25.1	25.69	25.44	26.29	0.34
<i>G7</i>	24.87	25.79	25.44	25.05	0.57

Supplementary Table 5. piRNA abundance in adult life cycle

Transposons further analyzed by qRT-PCR were in red.

	3 day	15 day	30 day	45 day
<i>R1A1_element</i>	40149494	36291700	25497365	26969138
<i>GATE</i>	36200017	31304570	27711696	26748727
<i>roo</i>	35675764	30643595	19848730	19264499
<i>Rt1b</i>	34953281	26327271	22733196	22880241
<i>Max_element</i>	27013660	19962756	15565090	15456889
<i>gtwin</i>	20638963	24749484	17198903	18627402
<i>X_element</i>	18882137	16030127	12404895	12860296
<i>F_element</i>	17841155	13882554	10670632	10532304
<i>412</i>	14199186	14886229	10912423	10447799
<i>Rt1a</i>	12342207	9037441	7017629	7211254
<i>Stalker</i>	11815536	11149429	8209559	8095500
297	11733421	9899430	6068894	6503649
<i>Stalker4</i>	11359090	10775434	7849227	7737890
<i>Doc</i>	11155458	8622955	6514117	6600559
<i>HeT_A</i>	10866145	8044428	5371141	5636340
<i>gypsy12</i>	9858453	7255424	6741561	6355131
<i>baggins</i>	9479222	9181496	6010960	5739030
<i>Doc3_eleme</i>	9233522	6726210	4901132	4895609
<i>G_element</i>	9038177	8347814	6189835	7051197
<i>G2</i>	8977548	8157545	6108594	7401294
<i>invader4</i>	8688627	3194886	1776162	1926899
<i>mdg1</i>	8629514	8354851	5872604	5587464
<i>opus</i>	8509769	6055708	4776948	4665800
<i>blood</i>	8216045	7447303	5678949	5567485
<i>gypsy4</i>	8079424	7515554	5153740	5221108
<i>Stalker2</i>	7968694	7702292	5928923	5765623
<i>gypsy6</i>	7833386	7263663	5403867	5837567
<i>Cr1a</i>	7643300	5730179	4289993	3950680
<i>rooA</i>	6664184	4620345	3477339	3000546
<i>TART_C_TAR</i>	6565761	4234011	3270942	3897139
<i>TAHRE</i>	6547458	5278724	3796539	3643353
<i>springer</i>	6034446	5960137	4562510	4531777
<i>gypsy</i>	5926067	5853801	4275651	4720695
<i>gypsy2</i>	5804611	6312498	4713480	5169306
<i>flea</i>	5804114	5333677	3398308	4003042
1731	5335138	4799662	4028331	4385128
<i>Circe</i>	5285349	4785171	4338762	4258726
<i>jockey2</i>	5137398	4772287	4844968	5538225
<i>jockey</i>	5116156	2888586	2899912	2867890
<i>aurora_element</i>	5039573	4201405	3254655	3301386
<i>gypsy10</i>	4919490	5155103	3379989	3502320
17	4842873	3911056	3204167	2638982

Tabor	4818395	4853131	3187811	3236161
3S18	4810801	4261124	2765247	2995868
rover	4688795	4510215	3111821	3063232
BS	4509666	4416808	3736840	3688545
1360	4482970	4077240	2744281	3432990
mdg3	4268598	4140842	2536220	2827198
<i>Idefix</i>	4184704	3741405	2450034	2175237
HMS_Beagle	4047172	3546749	2837921	2808631
gypsy8	3985241	3041061	2038114	2048662
Quasimodo	3761814	3697928	2655263	2786049
gypsy3	3704236	3328920	2459980	2531901
Doc2_element	3276300	2463778	1897414	2188494
HMS_Beagle	3088080	2841300	2384560	2617595
G6	3050094	3265730	2347513	3420069
<i>NOF</i>	3011242	2451218	2009031	1914479
<i>diver</i>	2665323	2183802	1675724	2014422
<i>lvk</i>	2641988	2253030	1864675	1844343
<i>accord</i>	2620613	2343948	2083276	1670584
<i>micropia</i>	2519827	2668472	2125307	2413575
<i>hobo</i>	2492375	2353232	1447496	1641215
<i>I_element</i>	2344597	1798880	1418673	1480244
<i>R2_element</i>	2312717	2154713	1865020	1678777
<i>invader3</i>	2118410	2351199	1768962	2523771
<i>gypsy5</i>	2117436	2076401	1508718	1309772
BS3	2076707	1500980	1145342	1227964
<i>invader1</i>	1996632	1510904	1419024	1642850
S_element	1983889	1749371	1314173	1384391
<i>accord2</i>	1837048	1339040	1018886	1049342
Osvaldo	1800284	1566766	956962	1081023
suste	1769987	1427051	1164533	1274612
<i>diver2</i>	1654800	1222300	1046871	975509
Bari1	1617589	1696346	1393285	1651368
<i>ZAM</i>	1497284	1476513	913261	1016746
<i>invader2</i>	1494532	1013052	752028	703943
G5	1488462	1549040	1310582	1323043
TARTA	1448778	819524	624228	741940
transib2	1446683	1409786	962421	1171414
Juan	1412748	869102	699604	761760
McClintock	1382263	1186945	840793	920253
mst40	1312908	1090813	828324	901922
Burdock	1170489	854586	610722	749044
Dm88	1054366	781726	546047	589883
Rt1c	1042622	936303	707642	689381

<i>Q_element</i>	1022428	849717	591619	533779
<i>copia</i>	862532	817383	582655	695158
<i>G4</i>	697355	734439	529479	499899
<i>INE_1</i>	652425	520421	372078	335836
<i>Transpac</i>	636517	549715	332734	388516
<i>R1_2</i>	603864	547275	414498	483696
<i>Porto1</i>	584686	496188	398384	381970
<i>pogo</i>	576576	583754	451750	496305
<i>G5A</i>	399482	401396	284512	335612
<i>gypsy7</i>	380036	239860	176674	180249
<i>Helena</i>	374873	353301	292051	296274
<i>Fw2</i>	359787	222923	181493	185351
<i>hopper2</i>	317303	221750	160005	177218
<i>gypsy11</i>	285920	300027	231100	197246
<i>Tc1_2</i>	267403	223833	146688	154295
<i>suffix</i>	244809	186652	163388	131056
<i>looper1</i>	237418	318165	197189	290165
<i>hopper</i>	229359	177266	124378	122331
<i>Stalker3</i>	227080	234181	170115	184483
<i>Doc4_eleme</i>	210917	170387	158771	160762
<i>stellateHet</i>	183820	126164	80324	77346
<i>stellate</i>	126277	91664	66487	64054
<i>G3</i>	125407	94663	99224	62350
<i>Fw3</i>	106822	92368	67660	91478
<i>Tirant</i>	100437	114981	78011	87151
<i>Tc1</i>	90072	93740	76416	77920
<i>gypsy9</i>	86561	64218	50454	54897
<i>FB</i>	72769	61616	51875	43906
<i>transib3</i>	67171	54944	41652	55638
<i>frogger</i>	60667	58249	23945	39532
<i>transib1</i>	60025	43633	40111	63124
<i>HB</i>	45792	24157	19818	17715
<i>S2</i>	39590	32801	26868	22301
<i>mariner2</i>	15780	11236	3130	7380
<i>G7</i>	14206	6097	4821	4739
<i>Tom1</i>	12933	8989	3969	2138
<i>invader5</i>	12239	6742	7095	4887
<i>transib4</i>	11757	9551	14054	10996
<i>Bari2</i>	9901	5337	4392	4276

Supplementary Materials and Methods

Fly genetics

To make *Nbr*^{cas9} flies, we co-injected two sgRNAs (100ng/μl; target sequences: 5'-GGCGCGGGAGAGCTTACGCT-3' and 5'-GGAGTTAAAAAAGCAGCGCTT-3') with Cas9 mRNA (1μg/μl) into fly embryo. To make *Hen1*^{cas9} flies, we co-injected two U6a-sgRNA-short plasmids (250ng/μl; targets sequence: 5'-CTTAACCAGCGTTAACCCAC-3'; 5'-GGATATGCTGGAGATGCAGT-3') into fly embryo expressing *nano*-Cas9 (TH00788. N). To make *piwi*^{cas9} flies, we co-injected two U6a-sgRNA-short plasmids (250ng/μl; targets sequence: 5'-CGACGTTGCTCACACAATC-3'; 5'-TGAGCTCTGCCGAGTGACT-3') into fly embryo expressing *nano*-Cas9 (TH00788. N). To make *Nbr*^{KI-Myc} flies, we injected one sgRNA (250ng/μl; target sequences: 5'-GGCTCTTGCCTGCCATTCTCA-3') and an oligo template with Myc tag sequence (5'-GGTTCTCCATGTTCTCCTCGTCCGACTCAAAGCCGGCGGTATTGCGTTGTACATGTG GCTCTTGCCTGCCAGATCCTCTTCAGAGATGAGTTCTGTTCCATTCTCACGGGTT CCCGTGCCACTGCAGAGCAAGTGCAATTGCCAATTGCAAATGCAATTGACGG TAA-3'). To screen flies carrying mutant alleles, we used single fly PCR assay. Single fly was homogenized in 50μl squashing buffer (10 mM Tris buffer [pH 8.0], 25 mM NaCl, 1 mM EDTA, 200 μg/ml Proteinase K), then incubated at 37°C for 30 min followed by inactivation at 85°C for 10 min. For PCR amplification, 1 μl of DNA extract was used as template. PCR primers were as follows: *Nbr*^{cas9} (Forward: 5'-CCTGGATTCTGAATGGATGC-3', Reverse: 5'-TCACTTAACATGGGCACCCCG-3'), *Hen1*^{cas9} (Forward: 5'-GTGGACATCGACAAGTCCTTGCTC-3', Reverse: 5'-GCAATAGGCATATTACAAGTGTCC-3'), *piwi*^{cas9} (Forward: 5'-CGTTGGATTCATATCGTGTGCTGAG-3', Reverse: 5'-CAGGTCAAGAACATCGGACGGACTAGC-3'), and *Nbr*^{KI-Myc} (Forward: 5'-GAACAGAAACTCATCTCTGAAGAGG-3', Reverse: 5'-TAAAGGCTATCATGCTGGTG-3'). To generate *Nbr* pUAST constructs, RT-PCR amplification was conducted using RNA from whole flies, with primers (Forward: 5'-ATG TATCCAGTTGGACAACAG-3', Reverse: 5'-TCACTTATCGTCGTCATCCTTGTAAACATGGGCACCCCG-3').

Small RNA northern

Oligo probes were used to make probes. Oligos used for miR-34-5p (5'-GATAATACGACTCACTATAGGGAGA-3'/5'-AAAAAAATGGCAGTGTGGTTAGCTGGTTGTCTCCCTATAGTGAGTCGTATTATC-3'), miR-219-5p (5'-GATAATACGACTCACTATAGGGAGA-3'/5'-AAAAAAATGATTGTCCAAACGCAATTCTCTCCCTATAGTGAGTCGTATTATC-3'), miR-275-3p (5'-GATAATACGACTCACTATAGGGAGA-3'/5'-AAAAAAATCAGGTACCTGAAGTAGCGCGCGTCTCCCTATAGTGAGTCGTATTATC-3'), miR-283-5p (5'-GATAATACGACTCACTATAGGGAGA-3'/5'-AAAAAAAATATCAGCTGGTAATTCTGGTCTCCCTATAGTGAGTCGTATTATC-3'), AT-chX-1 (5'-GATAATACGACTCACTATAGGGAGA-3'/5'-AAAAAAATGTTCATCGTTAGACGGCTCGGGCGTCTCCCTATAGTGAGTCGTATTATC-3'), and 2S rRNA (5'-GATAATACGACTCACTATAGGGAGA-3'/5'-TGCTTGGACTACATATGGTTGAGGGTTGTATCTCCCTATAGTGAGTCGTATTATC-3').

Molecular biology

To clone full-length cDNA, RT-PCR primers were as follows: *Nbr*-Flag (Forward: 5'-ATGGCACGCAAGAGCCACATG-3', Reverse: 5'-TCACTTATCGTCGTACCTTGTAATCCTAACATGGGCACCCG-3'), *Ha-piwi* (Forward: 5'-ATGTACCCATACGATGTTCCAGATTACGCTGCTGATGATCAGGGACGT-3', Reverse: 5'-TTATAGATAATAAAACTTCTTTC-3'), *Ha-aub* (Forward: 5'-ATGTACCCATACGATGTTCCAGATTACGCTAACATTACCACCAAACCCCTG-3', Reverse: 5'-TTACAAAAAGTACAATTGATT-3'), *Ha-ago3* (Forward: 5'-ATGTACCCATACGATGTTCCAGATTACGCTCTGGAAGAGGAAATTG-3', Reverse: 5'-TTAAAGATAAAAATAGTTTTCAG-3'). qRT-PCR primers were as follows: *rp49* (5'-CCGCTTCAAGGGACAGTATCTG-3'/5'-ATCTGCCCGAGTAAACGC-3'), *piwi* (5'-CGTCCACTTAACGAAGATGATT-3'/5'-CCTCGAGAGCTCTCTCTCT-3'), *aub* (5'-GTCCCTCGATAGAGAAATCCAGT-3'/5'-CGTGAATACACCAAATCCGTTAT-3'), *Ago3* (5'-AAATTGATCGCAAGCAGTTGA-3'/5'-AACCGTCGCAGCATAGCATTAAA-3'), *Nbr* (5'-GAAGACGTGCAGCTACTTGCATT-3'/5'-CTTCAGAATGAGCTCCAGCAGAG-3'), *Hen1* (5'-TAATCGAGCACGTTACGACGAT-3'/5'-GTGACCACTCGAACATTGTGATCC-3'), *412* (5'-AACAGCTCATCACCACCAGACA-3'/5'-AGATTGGGTCGTGTTGAAGCAGT-3'), *accord* (5'-ACAATCCACCAACAGCAACA-3'/5'-AAAAGCCAAATGTCGGTTG-3'), *accord2* (5'-GAGGTCGTTGAATAGACTGCCG-3'/5'-GGTCGCCGATGCCCTATTACAAT-3'), *blood* (5'-AGAGGGGAGGTGTAGTATGTCA-3'/5'-GCTTATGCCATATATGCCAGC-3'), *diver* (5'-GGCACCAACATAGACACATCG-3'/5'-GTGGTTGCATAGCCAGGAT-3'), *diver2* (5'-TGCAGTTGATTCTGGCAGAGAC-3'/5'-GGCTCCAACAGAGTCCTCAAGG-3'), *gtwin* (5'-TTCGCACAAGCGATGATAAG-3'/5'-GATTGTTGACGGCGACCTT-3'), *gypsy* (5'-GGGTACAGAGTTGCAGGTGGAA-3'/5'-TCTCCAGGCCACATACTCGTCTT-3'), *gypsy10* (5'-GCATACCCAGATTCCACTCAGCC-3'/5'-CTGGGTGACGATTGGTGTTC-3'), *gypsy5* (5'-TGCAGCTTATCAGACGTTAGGG-3'/5'-GGATAGGCAAATGTCCTGAGGGG-3'), *gypsy6* (5'-GACAAGGGCATAACCGATACTGTGGA-3'/5'-AATGATTCTTCCGGACTTCCGTCT-3'), *het-A* (5'-CGCGCGGAACCCATCTTCAGA-3'/5'-CGCCGCAGTCGTTGGTGAGT-3'), *hopper* (5'-TAAGTATGGCTGCAAGATCCCCTG-3'/5'-ATTGAGTGGCCAAGACAGCAA-3'), *idefix* (5'-ATTCACCGCGTTCATACCG-3'/5'-TCTGACTCTCGCGTGTCTT-3'), *I-element* (5'-GTCGTGCCTCTCAGTCTAAAGCC-3'/5'-GAGCCCGATTAGCGGTATTGTT-3'), *invader1* (5'-GTACCGTTTTGAGCCCCTA-3'/5'-AACTACGTTGCCATTCTGG-3'), *invader3* (5'-CAGCTATCGTTGGATGCAGA-3'/5'-GGTTCCGTTGCTGTTCTCG-3'), *Max* (5'-TCTAGCCAGTCGAGGCCTAT-3'/5'-TGGAAAGAGTGTGCGTTGTG-3'), *mdg1* (5'-AACAGAAACGCCAGCAACAGC-3'/5'-CGTTCCCATGTCCGTTGTGAT-3'), *NOF* (5'-GTGAAACCCGATGAACAATGCGG-3'/5'-CTTCCTCCACAATGCAGCT-3'), *RIAI* (5'-AATTCCCAGCTGTGCTAGA-3'/5'-GTCTCAAGGCACCTTCAGC-3'), *rt1a* (5'-CCACACAGACTGAGGCAGAA-3'/5'-ACGCATAACTTCCGGTTG-3'), *ZAM* (5'-ACTTGACCTGGATACACTCACAAC-3'/5'-GAGTATTACGGCGACTAGGGATAC-3').