Scientific Report

TRANSPARENT PROCESS

EMBO reports

The RNA surveillance complex Pelo-Hbs1 is required for transposon silencing in the *Drosophila* germline

Fu Yang[1,2], Rui Zhao[2], Xiaofeng Fang[3,4], Huanwei Huang[2], Yang Xuan[2], Yanting Ma[2], Hongyan Chen[2], Tao Cai[2], Yijun Qi[3,4] & Rongwen Xi[2,*]

Abstract

Silencing of transposable elements (TEs) in the metazoan germline is critical for genome integrity and is primarily dependent on Piwi proteins and associated RNAs, which exert their function through both transcriptional and posttranscriptional mechanisms. Here, we report that the evolutionarily conserved Pelo (Dom34)-Hbs1 mRNA surveillance complex is required for transposon silencing in the *Drosophila* germline. In *pelo* mutant gonads, mRNAs and proteins of some selective TEs are up-regulated. Pelo is not required for piRNA biogenesis, and our studies suggest that Pelo may function at the translational level to silence TEs: This function requires interaction with Hbs1, and overexpression of RpS30a partially reverts TE-silencing defects in *pelo* mutants. Interestingly, TE silencing and spermatogenesis defects in *pelo* mutants can also effectively be rescued by expressing the mammalian ortholog of Pelo. We propose that the Pelo-Hbs1 surveillance complex provides another level of defense against the expression of TEs in the germline of *Drosophila* and possibly all metazoa.

Keywords *Drosophila*; germline; Hbs1; Pelota/Dom34; transposon silencing
Subject Categories RNA Biology; Protein Biosynthesis & Quality Control
DOI 10.15252/embr.201540084 | Received 13 January 2015 | Revised 27 May 2015 | Accepted 2 June 2015 | Published online 29 June 2015
EMBO Reports (2015) 16: 965–974

Introduction

The *Drosophila* Piwi proteins and associated piRNAs defend against transposable elements (TEs) in the germline at both transcriptional and posttranscriptional levels [1]. In the cytoplasm, the transcripts are subjected to endonucleolytic cleavage by RNA-induced silencing complexes (RISCs) containing Aub or Ago3, two Piwi family proteins that generate piRNAs in the ping-pong cycle to amplify the response to the actively transcribed TEs [2,3]. In the nucleus, the Piwi-containing RISCs are guided to the chromatin via piRNAs to establish repressive chromatin state at the transposable DNA elements and other targeting loci [4–6]. Murine members of Piwi and associated piRNAs are found to be associated with ribosomes and regulate translation during spermatogenesis [7,8], but whether TEs could be regulated at the translational level is unknown.

Piwi, the founding member of the *Drosophila* piRNA pathway, was initially identified as an essential regulator of germline stem cell maintenance in the *Drosophila* ovary [9]. Later studies demonstrate that Piwi is required in both the somatic niche cells and the germline for the maintenance, proliferation, and differentiation of germline stem cells [10–12]. *Pelo* encodes a eukaryotic release factor (eRF1)-like protein, and similar to *piwi* mutants, *pelo* homozygous null females have rudimentary ovaries because of its requirement for germline stem cell maintenance [13]. Insights on the molecular function of Pelo came from yeast in which Dom34, the Pelo ortholog, is found to form a complex with a small GTPase Hbs1 to regulate endonucleolytic cleavage of mRNAs whose secondary structure causes ribosome stalling during translational elongation, a process known as no-go decay (NGD) [14]. The structure of Dom34-Hbs1 complex is similar to that of eRF1 and eRF3, but Dom34 lacks the motifs for codon recognition and peptide release [15,16]. Along with studies from biochemical analyses, it is proposed that the Dom34-Hbs1 complex binds to the ribosomal A site to promote subsequent dissociation of ribosome subunits for ribosomal recycle [17,18]. The molecular connection between Dom34-Hbs1-mediated ribosomal recycle and mRNA cleavage, and the identity of the responsible endonuclease remain unclear. In addition to the RNA quality control in the NGD pathway, Dom34-Hbs1 is also important for non-stop decay, decay of non-functional 18S rRNAs and mRNAs with premature stop codon [19–21]. Therefore, the Dom34-Hbs1 complex appears to represent a major RNA surveillance pathway in eukaryotes to detect and rescue the ribosomes stalled on mRNAs during translation.

Here, we report that the *Drosophila* Pelo is required for silencing of germline transposons in the ovary and testis. Pelo is not required for piRNA biogenesis or function, and our analyses indicate that

1 College of Life Sciences, Beijing Normal University, Beijing, China
2 National Institute of Biological Sciences, Beijing, China
3 Tsinghua-Peking Center for Life Sciences, Beijing, China
4 Center for Plant Biology, School of Life Sciences, Tsinghua University, Beijing, China
*Corresponding author. Tel: +86 10 80723241; Fax: +86 10 80723249; E-mail: xirongwen@nibs.ac.cn

Pelo might function together with Hbs1, possibly via a NGD-like mechanism, to prevent translation of transposon mRNAs.

Results and Discussion

To facilitate the study on the potential role of Pelo in transposon silencing, we generated a few hypomorphic alleles of *pelo* by imprecise excision of P-elements inserted in the *pelo* locus (Fig EV1). Based on molecular lesion, gene expression abundance, and ovary phenotype, we considered $pelo^{PB60}$ as the strongest loss-of-function allele, $pelo^{PA13}$ the weakest, and $pelo^{1}$ in-between (see Materials and Methods, and Fig EV1). By real-time PCR analysis, we observed moderate but significant up-regulation of selective germline transposons in ovaries of *pelo* homozygous or transheterozygous mutants (Fig 1A). The magnitude of TE up-regulation was correlated with the strength of *pelo* mutations (Fig 1A) and was further enhanced by ambient temperature (Fig EV2). In addition, up-regulation of multiple TE elements, such as *HeT-A*, 1731, and I-element, found in $pelo^{PB60}$ homozygous ovaries could be significantly suppressed by germline expression of a *pelo* transgene (Fig 1B). Using antibodies to I-element ORF1, we also observed protein accumulation in *pelo* ovaries in the oocyte of the stage 2 egg chambers in all ovarioles examined (23/23). As controls, accumulation of I-element protein was only observed in the oocyte of developing egg chambers in *aub* mutant ovaries, but not in wild-type ovaries (Fig 1C). Taken together, these observations suggest that *pelo* is required for transposon silencing in the *Drosophila* ovary whose mutation causes up-regulation of some germline TEs at both mRNA and protein levels.

In *Drosophila* testis, the repeated Stellate (Ste) element is normally silenced by the piRNA pathway mediated by *Su(Ste)* piRNAs [22]. As expected, in *aub* mutant testis, where the piRNA pathway is compromised, Ste proteins accumulated and formed needle-like crystals in the spermatocyte (Fig 1D). Germline knockdown of *pelo* by nos-Gal4/UAS-pelo-RNAi also led to Ste crystal formation in the spermatocytes with 100% penetrance (49/49) though the severity varied in different testis (Fig 1D). Strong loss-of-function mutants of *pelo* ($pelo^{1/PB60}$) did not display the crystal phenotype in spermatocytes (Fig 1D), possibly because the mutant spermatocytes have not yet developed to a later stage necessary for Ste expression, as Pelo is required for the progression through meiosis I during spermatogenesis [23]. Consistent with this notion, leaky expression of a *UAS-pelo* transgene (without a GAL4 driver) in $pelo^{1/PB60}$ mutants also caused Ste crystals in the spermatocytes (Fig 1D). We generated another *pelo* transgene with a GFP tag to its C-terminal and found that this transgene is only partially functional because the transgene allowed the formation of spermatids, but failed to rescue the sterile phenotype of *pelo* mutant males (data not shown). Germline expression of the *pelo-GFP* transgene in *pelo* null testis also led to the formation of Ste crystals (Fig 1D). These data suggest that Pelo is required for Ste silencing in the developing spermatocytes.

The *Ste* elements are distributed in two clusters in the genome, the 12D euchromatic region and the distal X-heterochromatic region. Similar to previous observations, *Ste* transcripts derived from either locus was significantly up-regulated (approximately 25-fold) in $Aub^{QC42/HN2}$ mutant testes [24] (Fig 1E). By contrast, only transcripts derived from the euchromatic locus showed significant up-regulation in *pelo-RNAi* testis, and the magnitude of mRNA up-regulation was also comparatively less dramatic (approximately fourfold) (Fig 1F).

Pelo could regulate TE silencing through the piRNA pathway or other mechanisms. We first determined whether Pelo regulates the expression or function of genes in the piRNA pathway. The mRNA levels of virtually all major known genes involved in the piRNA remained unchanged in *pelo* mutant ovaries, except for Yb, whose expression was up-regulated (Fig 2A). The protein levels for several major piRNA pathway components, such as Piwi, Vasa, and Krimp, remained unaltered as well (Fig 2B). In addition, the subcellular distribution patterns of all piRNA proteins examined, including Piwi, Aub, Ago3, Vasa, and Krimp, remained unaltered in *pelo* mutant germline cysts (Fig 2C). The above data suggest that Pelo does not regulate the expression or subcellular localization of major protein components involved in the piRNA pathway.

To determine whether Pelo affects piRNA biogenesis, we profiled all small RNAs extracted from $pelo^{1/+}$ and $pelo^{1/PB60}$ ovaries by deep sequencing. After filtering out tRNA, rRNA, and degraded snoRNAs, we had about 10M reads for each sample, 60% of which mapped to the repetitive DNA regions in the genome (Fig EV3). The RNA profile in general showed that the production of endogenous siRNA (esiRNA), piRNAs, and microRNAs (miRNAs) in $pelo^{1/PB60}$ ovaries was largely similar to that in the control ovaries. We specifically analyzed small repetitive RNAs uniquely mapped to the two major piRNA clusters, the 240-kb *42AB* cluster that exclusively processes germline piRNAs, and the 179-kb *flamenco* cluster that predominantly produces somatic piRNAs. Both the abundance and distribution of small RNAs derived from these two clusters were virtually unchanged in $pelo^{1/PB60}$ ovaries (Fig 3A). We further examined the small RNAs mapped to specific transposable DNA elements. *HeT-A* is a germline-specific TE, and *ZAM* is soma-specific. The abundance of small RNAs derived from both TEs also remained unaltered in $pelo^{1/PB60}$ ovaries (Fig 3B). In parallel, we examined the production levels of several piRNAs, including *roo*, *HeT-A*, and *AT-chX-1*, by polyacrylamide gel electrophoresis and Northern blotting analysis. As a positive control, piRNAs were significantly reduced in $Aub^{QC42/HN2}$ ovaries (Fig 3C). We found that the production of these piRNAs remained unchanged in $pelo^{1/PB60}$ ovaries, and the production of *miR-8* was unaltered in either $Aub^{QC42/HN2}$ or $pelo^{1/PB60}$ ovaries (Fig 3D). Finally, Northern blot analysis revealed that *Su(Ste)-4* piRNA production in testis was significantly compromised in *Aub* mutants but remained at comparable level to the control in *pelo-RNAi* mutants (Fig 3E). Taken together, these data agree with the global RNA profiling analysis and suggest that Pelo does not regulate the biogenesis of small RNAs, including piRNAs and miRNAs. In addition, Pelo does not seem to generally regulate the levels of mRNAs. As shown previously, the mRNA expression of many germline-specific genes was largely unaltered in *pelo* mutants (Fig 2A). We also examined a set of housekeeping genes (polymerase genes), and their mRNA expression was virtually unaltered or within twofold of up-regulation in *pelo* mutants (Fig EV3).

In yeast, Dom34/Pelo complexes with Hbs1 in the NGD process to regulate endonucleolytic cleavage of mRNAs in stalled ribosomes during translational elongation [14]. We found that in *Drosophila*, Pelo displayed abilities to interact with the *Drosophila* Hbs1 (CG1898) in the yeast two-hybrid assay (Fig 4A). Furthermore, Pelo

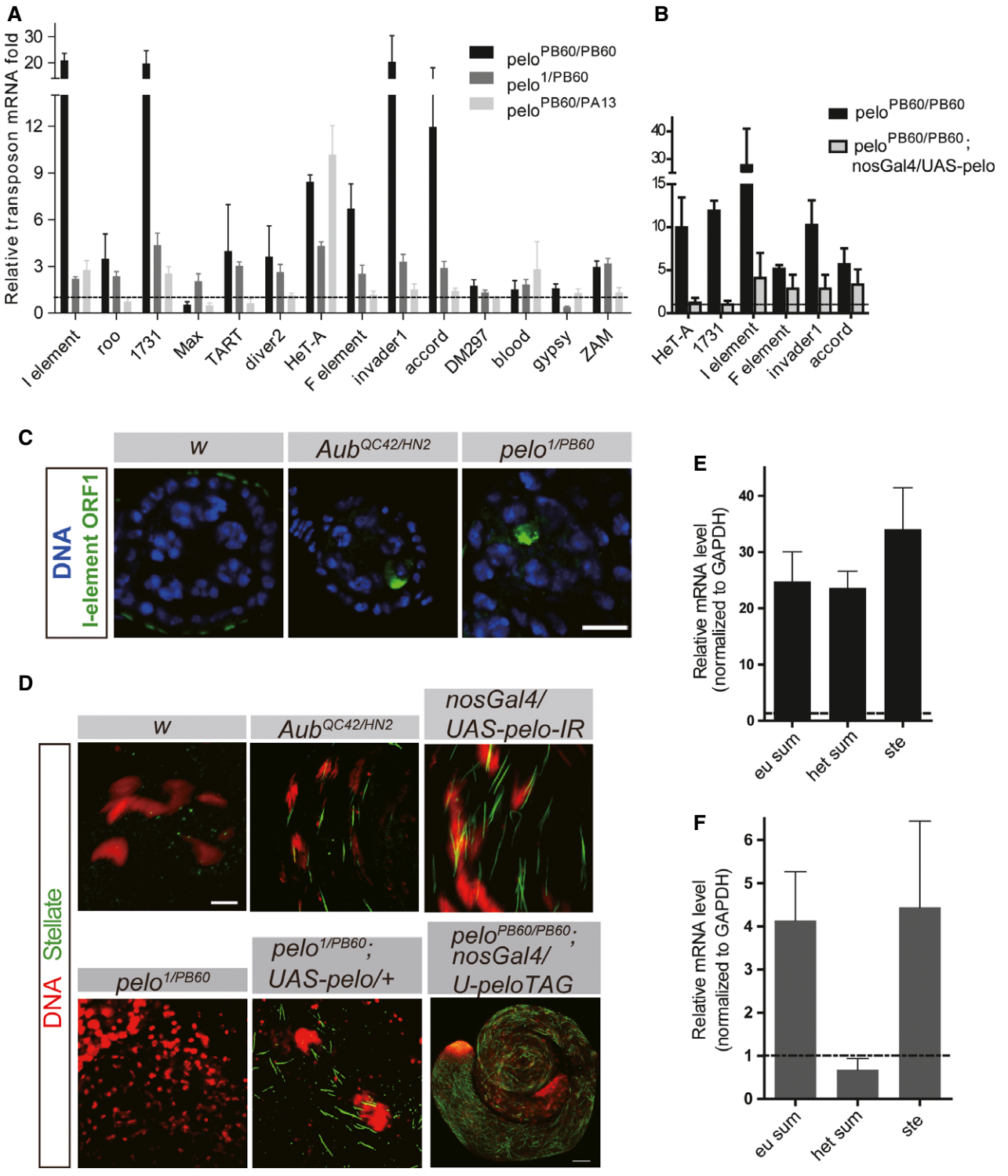

Figure 1. Pelo is required for transposon silencing in the *Drosophila* germline.

A, B Quantitative RT–PCR analysis of retro-transposon transcripts in ovaries of the indicated genotypes. The expression levels were relative to that in $pelo^{PB60/+}$ ovaries. Germline expression of *Pelo* transgene suppressed TE up-regulation in $pelo^{PB60}$ mutant ovaries (B). Values are means ± SEM, n = 3–4.

C Egg chambers immunostained with anti-I-element ORF1. I-element ORF1p was detected in newly formed egg chambers in $Aub^{QC42/HN2}$ and $pelo^{1/PB60}$ ovaries but is absent in wild-type ovaries (63× magnification); scale bar represents 10 μm.

D Testes with the indicated genotypes were immunostained with anti-Stellate (Ste, green). The needle-like Ste crystals were found in *aub* mutant and *pelo* weak mutant testes. DAPI staining is shown in red. Scale bars represent 20 μm except in the last image, which is 100 μm.

E, F Quantitative RT–PCR analysis of stellate transcript in $Aub^{QC42/HN2}$ (D) and *pelo*-RNAi (E) testes. Values are means ± SEM, n = 4.

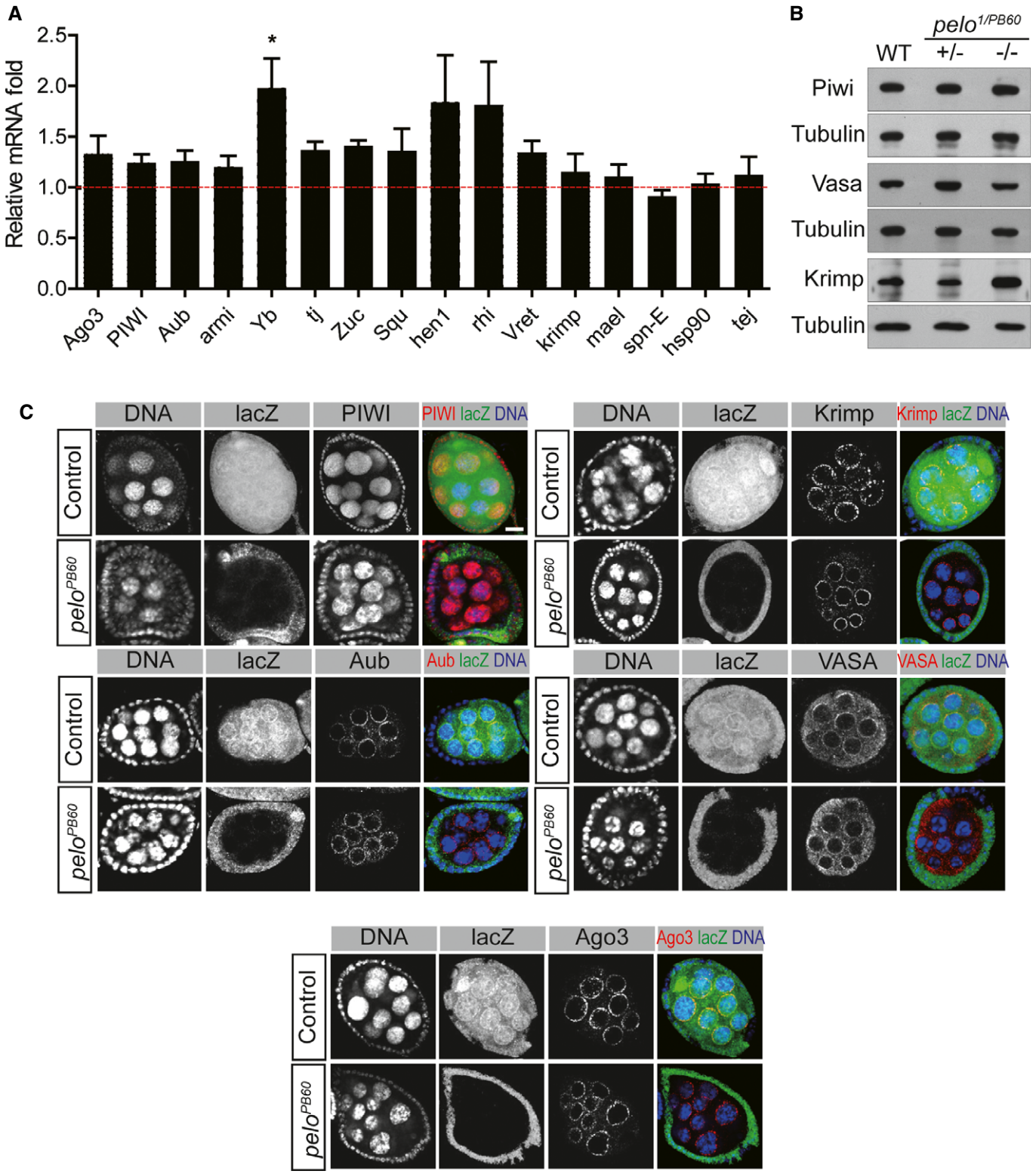

Figure 2. Pelo does not affect the expression, stability or localization of core protein components in the piRNA pathway.

A Quantitative RT–PCR analysis to detect changes in the expression of indicated genes in *pelo*$^{1/PB60}$ ovaries compared to the heterozygous controls. Values are means ± SEM, $n = 3$. *$P < 0.05$, *t*-test.

B Western blot analysis of indicated proteins in wild-type, *pelo*$^{1/+}$, and *pelo*$^{1/PB60}$ ovaries. Tubulin was used as a loading control.

C Immunostaining to detect subcellular localization of the indicated proteins (red) in *pelo*^{PB60} and control egg chambers. *pelo* mutant clones are visualized by the absence of LacZ expression (green). Scale bar represents 10 μm.

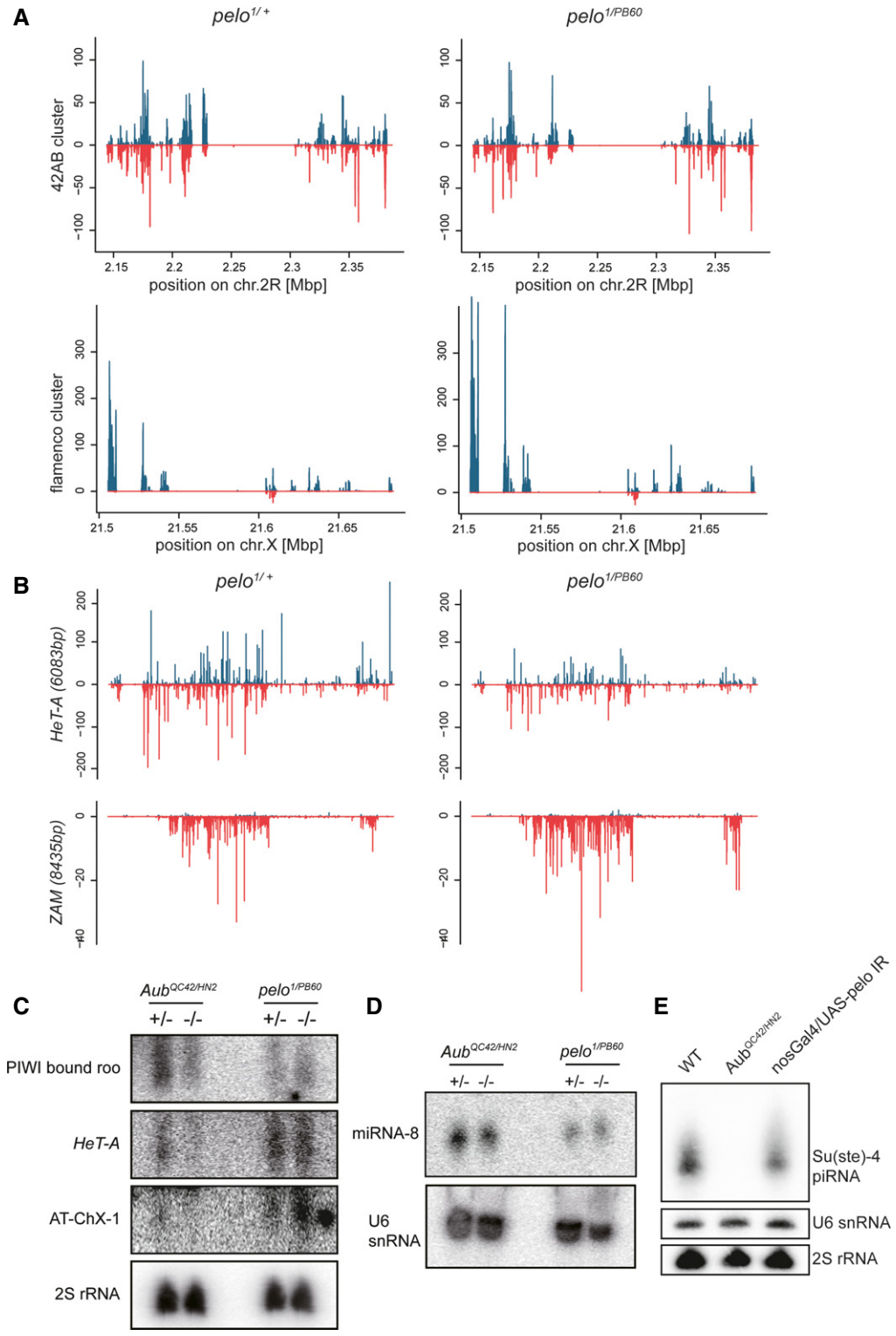

Figure 3. Small RNA biogenesis is not disrupted in *pelo* mutant ovaries.

A Normalized and calibrated piRNA density profiles mapped to *42AB* and *flamenco* cluster in $pelo^{1/PB60}$ and $pelo^{1/+}$ ovaries. Plus and minus strands are shown in blue and red, respectively.

B piRNA density profiles mapped to *HeT-A* and *ZAM* transposon loci. Plus and minus strands are shown in blue and red, respectively.

C–E Small RNAs extracted from ovaries (C, D) or testes (E) of the indicated genotypes were hybridized with probes complimentary to *roo*, *HeT-A*, *AT-ChX-1* piRNAs (C), *miRNA-8* (D), and *Su(Ste)-4* piRNA (E). 2S rRNA and U6 snoRNA were used as loading controls.

co-immunoprecipitated with Hbs1 in ovarian extracts (Fig 4B). To determine whether Pelo functions in a complex with Hbs1 in regulating TE silencing in the *Drosophila* germline, we generated a *Hbs1* null *Drosophila* strain. Ovaries from *Hbs1* mutants showed a moderate but significant increase in TE expression levels compared to the wild-type controls (Fig 4C). In addition, mutation of *Hbs1* significantly enhanced the up-regulation of *HeT-A* element expression caused by *pelo* mutation, although it did not further increase 1731 or *ZAM* expression (Fig 4D). In yeast NGD pathway, mutational analysis suggests that Hbs1 has a much minor role in the process compared to the requirement for Dom34, possibly due to the redundant functions by other eRF3-like proteins [14]. Structural and biochemical data have shown that the conserved PGF motif in the central domain of Pelo/Dom34 interacts with a conserved RDF motif in the GTPase domain of Hbs1. The PGF motif is also required for efficient NGD activity as a P to A mutation in this motif, which does not completely abolish the interaction [16], reduces NGD activity [25,26]. We therefore generated a transgene for Pelo carrying P210 to A (P210A) mutation in the PGF motif and examined the ability of the mutant Pelo in rescuing the observed defects in $pelo^{1/PB60}$ ovaries. Indeed, the P210A mutant Pelo had significantly reduced ability to repress TE levels (Fig 4E). These observations suggest that Pelo might form a complex with Hbs1 (and possibly other eRF3-like proteins) in the *Drosophila* germline to regulate transposon silencing.

In yeast, RpS30a, a ribosomal protein, has been identified as a high-copy suppressor of the growth defect caused by Dom34 ablation [27]. Interestingly, overexpression of RpS30a is able to complement the NGD defects and allow some cleavage of NGD substrates in a *Dom34* strain [25]. The underlying mechanism is unclear, but it has been speculated that extra RpS30a might extend the elongation pausing allowed for mRNA cleavage [25]. We generated a transgenic fly expressing the *Drosophila* RpS30a and found that RpS30a overexpression in the germline significantly reduced the TE levels in $pelo^{-/-}$ ovaries, an effect that is largely similar to overexpression of Pelo (Fig 4F). By contrast, overexpression of RpS30a failed to reduce the TE levels in $Aub^{QC42/HN2}$ ovaries (Fig 4G). Germline overexpression of RpS30a also improved the egg production (Fig 4H) and the hatching rate of *pelo* females, similar to the germline expression of Pelo (Fig 4I). Taken together, these data indicate that Pelo might regulate transposon silencing at the translational level, possibly through the NGD pathway to cleave TE transcripts that have been loaded with ribosomes, thereby preventing translation.

Studies with artificially expressed mRNAs have shown that the Dom34-Hbs1 complex can detect and dissociate stalled ribosomes with no-stop mRNAs [14]. But the *in vivo* targets are largely unknown. A recent study also suggests that Dom34 does not generally dissociate ribosomes on coding sequence but on 3′ UTRs [28]. Because TE transcripts loaded on the ribosomes could still be potentially recognized by complementary piRNAs, one possibility is that the binding of piRNAs to the transcript may produce no-go mRNAs, followed by endonucleolytic cleavage via NGD. If this is true, Pelo-mediated TE silencing must rely on proper piRNA biogenesis. In Ago3- and Aub-depleted ovaries, where the piRNA biogenesis is reduced, we found that mutation in *pelo* was able to further enhance *Het-A* and 1731 up-regulation (Fig EV4). In addition, depletion of Pelo could still further enhance TE up-regulation by additionally depleting Dcr2 (Fig EV4), which generates esiRNAs that are implicated in TE silencing in somatic cells [29,30]. Because disrupting the ping-pong cycle may reduce, but not eliminate the piRNA production, it remains undetermined whether Pelo-mediated TE silencing is dependent or independent of piRNAs.

RNA surveillance mediated by Dom34-Hbs1 appears to be an evolutionarily conserved mechanism from yeast to human [14,18]. We found that expression of the mouse *Pelo* (*mPelo*) gene in *Drosophila* was able to rescue the observed defects found in $pelo^{1/PB60}$ mutants, including TE depression (Fig 5A) and defects in spermatogenesis (Fig 5B and C). Ubiquitous expression but not germline-restricted expression of *mPelo* effectively rescued the sterility of $pelo^{1/PB60}$ mutants (Fig 5D), suggesting that the function of Pelo in transposon silencing could be conserved in mammals. On another note, insufficient rescue by the germline-restricted expression of Pelo indicates a role for Pelo in somatic cells as well.

Our studies here suggest an interesting hypothesis that in addition to the transcriptional and posttranscriptional levels of TE silencing by Piwi proteins and piRNAs, the mRNA surveillance complex Pelo-Hbs1 may provide another level of defense against TE transcripts that have escaped from the piRNA pathway-mediated endonucleolytic cleavage. One plausible mechanism would be that these escaped transcripts are loaded with ribosomes for translation, but the NGD machinery is able to recognize them, followed by degradation. How the loaded TE mRNAs are recognized by the NGD machinery is unclear, but the involvement of piRNAs remains to be a possibility worthy of future investigation. Given that TEs are particularly active in the germline, the involvement of multiple levels of defense against TEs in the germline ensures the integrity of genetic information to be passed to the next generation. We propose that the Pelo-Hbs1 RNA surveillance complex as another gatekeeper against the expression of transposons could be a common feature in the metazoan germline.

Figure 4. Pelo possibly functions at the level of translation in transposon silencing.

A Pelo and Hbs1 interaction was detected by the yeast two-hybrid assay.

B Co-immunoprecipitation of Pelo with Hbs1 in ovarian extracts.

C–G Quantitative RT–PCR to detect the relative amount of transposon mRNAs from ovaries of the indicated genotypes. Fold changes were compared to heterozygous controls. (C) Transposon mRNAs were mildly increased in $Hbs1^{-/-}$ ovaries at 29°C. (D) *Hbs1* mutation further enhanced *HeT-A* up-regulation in *pelo* mutant ovaries. (E) Transgene expression of Pelo (P210A) could not prevent *HeT-A* and *ZAM* up-regulation in *pelo* mutant ovaries. (F, G) Transgene expression of RpS30a reduced TE levels in $pelo^{1/PB60}$ but not $Aub^{QC42/HN2}$ (G) ovaries. Values are means ± SEM, n = 3–5. n.s., not significant; *$P < 0.05$, **$P < 0.01$, t-test.

H, I Germline expression of *RpS30a* partially restored egg production (H) and hatching rate (I) of $pelo^{1/PB60}$ females, similar to the effect caused by germline expression of *pelo*. Values are means ± SEM, n = 16–40 females.

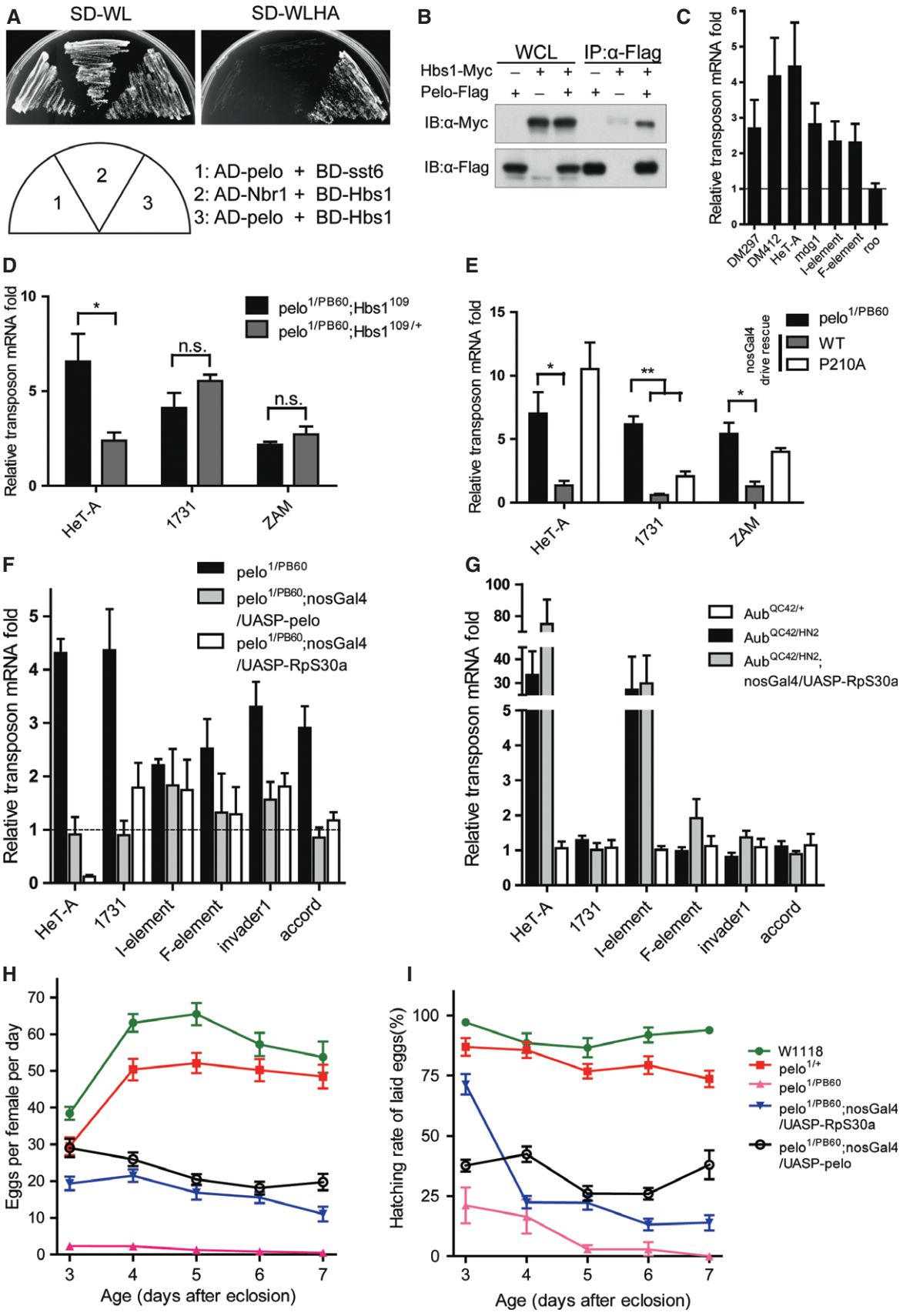

Figure 4.

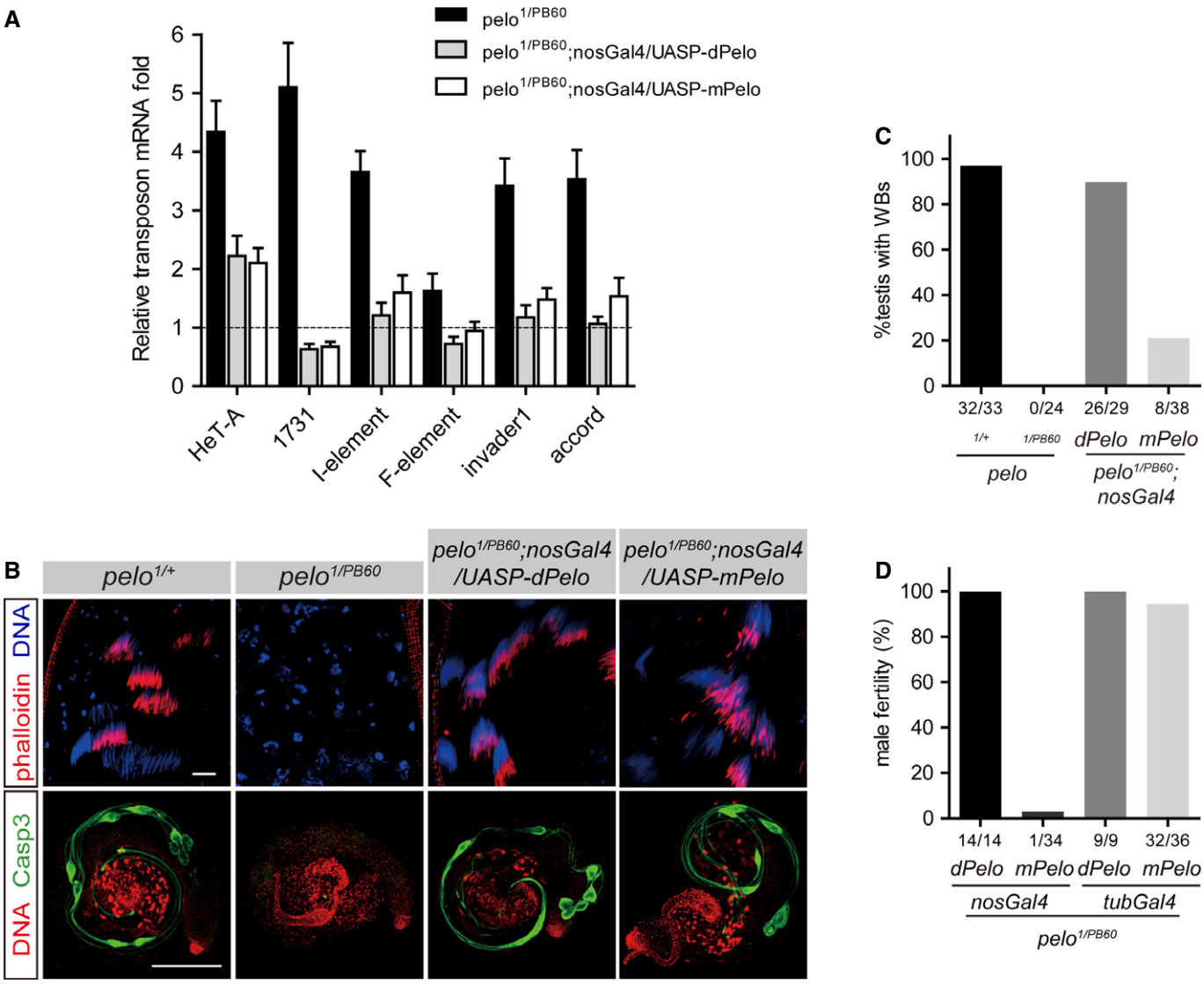

Figure 5. Rescue of *pelo* mutant phenotypes by the mouse *Pelo* (*mPelo*) transgene.

A Quantitative RT–PCR results of transposon mRNAs from ovaries of the indicated genotypes. Expression of mouse *Pelo* (*mPelo*) significantly reduced TE up-regulation in $pelo^{1/PB60}$ ovaries. Values are means ± SEM, $n = 5$.

B Rescuing spermatogenesis by transgene expression of mPelo in $pelo^{1/PB60}$ testes. Immunostaining of individualization complex (IC) in testes with indicated genotypes (upper panels). IC was visualized by phalloidin staining (red). Immunostaining of anti-caspase-3 (green) in testes with indicated genotypes (lower panels). Normally, individual IC travels along the axonemes (visualized by anti-caspase-3) to remove cytoplasmic content, which accumulates in the waste bags (WB, green) at the apical end of testes.

C Quantitative data of WB formation in testes of the indicated genotypes.

D Fertility test of male flies with the indicated genotypes.

Materials and Methods

Additional methods are described in the Appendix file.

Drosophila strains

Flies were cultured on standard food media with yeast paste added to the food surface. The culture temperature was 25°C unless otherwise noted. Strains used in this study were as follows: $Ago3^{T2}$, $Ago3^{T3}$, Aub^{QC42}, and Aub^{HN2} (gifts from Phillip Zamore); $pelo^{1}$ is a P-element insertional allele [23], and RT–PCR analysis suggests that this allele produces a C-terminal truncated product. $pelo^{PB60}$ and $pelo^{PA13}$ are insertion and deletion alleles, respectively, generated by imprecise excision of P-element insertions: $pelo^{PB60}$ has a 107-base-pair deletion in the 2nd exon which creates an early stop codon and can be considered as a genetic null allele; $pelo^{PA13}$ has a 558-base-pair insertion in the 5′ UTR, and RT–PCR analysis suggests that the mutation causes approximately 80% reduction of gene product (see Fig EV1); $Hbs1^{109}$ (a null allele, details will be described elsewhere); nos-Gal4VP16 [31]; *UAS-pelo-RNAi* (VDRC #34770, #34771); and tub-Gal4 (BDSC #5138).

Generation of transgenic flies

To make a *UASp-RpS30a* construct, the *RpS30a* cDNA was cloned from a w^{1118} ovarian cDNA library and then subcloned into a pUASP vector with *Kpn*I and *Xba*I. Mouse *pelo* cDNA from C57BL/6 mice (The Jackson Laboratory) was cloned into a pUASp vector with *Kpn*I and *Xba*I. The Gateway cloning technology (Invitrogen) was used to generate *UASp-pelo-GFP*, *UASp-pelo-Flag*, and *UASp-Myc-Hbs1* constructs. The P210A point mutant was generated by QuickChange Site-Directed Mutagenesis Kit (Stratagene). All the plasmids were verified by DNA sequencing. The plasmid DNA was introduced to w^{1118} embryo by a standard procedure to generate transgenic flies.

Immunostaining and microscopy

Drosophila ovaries were dissected and immunostained as described previously [32]. The following primary antibodies were used: anti-Piwi, anti-Ago3, anti-Aub (1:1,000, gifts from Gregory J. Hannon), anti-Krimp (1:10,000, a gift from Toshie Kai), anti-Vasa (DSHB, 1:100), anti-Stellate (1:1,000, a gift from William E. Theurkauf), anti-I-element ORF1p (1:50, a gift from David Finnegan), anti-β-gal (DSHB, 1:50), and anti-cleaved caspase-3 (Cell Signaling, 1:300). For IC staining, fixed testes were incubated with rhodamine-conjugated phalloidin (Molecular Probes, 1:10) at 37°C for 1 h. Secondary antibodies, including goat anti-rabbit and goat anti-mouse IgGs, conjugated to Alexa (488 or 568) (Molecular Probes) were used at a dilution of 1:300 (DAPI (4′,6-diamidino-2-phenylindole), Sigma; 0.1 mg/ml, 5 min incubation). Images were collected by either a Zeiss Meta 510 confocal microscope system or a Zeiss Imager Z1 equipped with an ApoTome. All acquired images were processed in Adobe Photoshop and Illustrator.

RNA isolation and qPCR assays

Total RNA from 10-20 ovaries was extracted by TRIzol reagent (Invitrogen). After DNase treatment, complementary DNA (cDNA) was synthesized using an oligo dT primer and High-Fidelity cDNA Synthesis Kit (Roche). RT–qPCR was performed in three duplicates using SYBR Premix Ex Taq RT-PCR Kit (Takara) on an ABI PRISM 7500 fast Real-time PCR System (Applied Biosystems). Endogenous *Actin5c* mRNA levels were measured for normalization. Fold changes for mRNA were calculated using $\Delta\Delta C_t$ method [33]. The primers used are listed in Table EV1.

Small RNA isolation and Northern blot

Small RNA was enriched by PEG8000 precipitation from total ovaries RNA, and Northern blot was performed according to Qi *et al* [34]. ^{32}P-end-labeled oligonucleotides complimentary to small RNA sequence were used as probes which are listed in Table EV1.

Fertility test

To test female fertility, two virgins were collected and mated with two 5- to 7-day-old w^{1118} males in a small cage with 35-mm apple juice agar plate with yeast paste as described [35]. After 2 days, the plate was replaced by a new one every day. The number of eggs per female per day was counted every 24 h, and the hatching rate was scored 48 h after the plate was changed. At least eight plates were scored in total for each genotype at each time point. To test male fertility, each 2- to 5-day-old male was collected and mated with three 3- to 7-day-old w^{1118} virgins. Fertility was determined by examining the appearance of larvae 5 days after mating.

Small RNA cloning and sequencing

Ten micrograms enriched small RNA were separated on a 15% denaturing polyacrylamide gel.

18- to 30-nt RNAs were purified according to RNA oligo markers and ligated to adapters to generate the libraries for subsequent sequencing on the Illumina GA II instrument. The small RNA-seq library was prepared by following the manufacturer's instructions.

The GEO accession code for the RNA-seq data is GSE69468.

Statistical analysis

Data are presented as mean ± SEM. *P*-values were calculated using one-way ANOVA or unpaired two-tailed Student's *t*-test by GraphPad Prism 5 (GraphPad Software Inc.).

Expanded View for this article is available online:
http://embor.embopress.org

Acknowledgements
We thank Phillip Zamore, Greg Hannon, David Finnegan, Toshi Kai, William Theurkauf, the Bloomington Drosophila Stock Center, and Vienna Drosophila Resource Center for providing fly stocks and reagents; Xijuan Li and Anying Kang for technical assistance; and members of the Xi Laboratory for helpful comments and discussion. This work was supported by National Basic Science 973 grants (2011CB812700 and 2014CB849700) from the Chinese Ministry of Science and Technology.

Author contributions
FY, RZ and RX conceived and designed the experiments, FY and RX analyzed the data and wrote the manuscript. FY, RZ, XF, HH, YX, YM, HC, TC, and YQ performed the experiments and analysis.

Conflict of interest
The authors declare that they have no conflict of interest.

References

1. Guzzardo PM, Muerdter F, Hannon GJ (2013) The piRNA pathway in flies: highlights and future directions. *Curr Opin Genet Dev* 23: 44 – 52
2. Brennecke J, Aravin AA, Stark A, Dus M, Kellis M, Sachidanandam R, Hannon GJ (2007) Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. *Cell* 128: 1089 – 1103
3. Gunawardane LS, Saito K, Nishida KM, Miyoshi K, Kawamura Y, Nagami T, Siomi H, Siomi MC (2007) A slicer-mediated mechanism for repeat-associated siRNA 5′ end formation in *Drosophila*. *Science* 315: 1587 – 1590

4. Le Thomas A, Rogers AK, Webster A, Marinov GK, Liao SE, Perkins EM, Hur JK, Aravin AA, Toth KF (2013) Piwi induces piRNA-guided transcriptional silencing and establishment of a repressive chromatin state. *Genes Dev* 27: 390 – 399
5. Rozhkov NV, Hammell M, Hannon GJ (2013) Multiple roles for Piwi in silencing *Drosophila* transposons. *Genes Dev* 27: 400 – 412
6. Huang XA, Yin H, Sweeney S, Raha D, Snyder M, Lin H (2013) A major epigenetic programming mechanism guided by piRNAs. *Dev Cell* 24: 502 – 516
7. Grivna ST, Pyhtila B, Lin H (2006) MIWI associates with translational machinery and PIWI-interacting RNAs (piRNAs) in regulating spermatogenesis. *Proc Natl Acad Sci USA* 103: 13415 – 13420
8. Unhavaithaya Y, Hao Y, Beyret E, Yin H, Kuramochi-Miyagawa S, Nakano T, Lin H (2009) MILI, a PIWI-interacting RNA-binding protein, is required for germ line stem cell self-renewal and appears to positively regulate translation. *J Biol Chem* 284: 6507 – 6519
9. Cox DN, Chao A, Baker J, Chang L, Qiao D, Lin H (1998) A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. *Genes Dev* 12: 3715 – 3727
10. Cox DN, Chao A, Lin H (2000) piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development* 127: 503 – 514
11. Jin Z, Flynt AS, Lai EC (2013) *Drosophila* piwi mutants exhibit germline stem cell tumors that are sustained by elevated Dpp signaling. *Curr Biol* 23: 1442 – 1448
12. Ma X, Wang S, Do T, Song X, Inaba M, Nishimoto Y, Liu LP, Gao Y, Mao Y, Li H *et al* (2014) Piwi is required in multiple cell types to control germline stem cell lineage development in the *Drosophila ovary*. *PLoS ONE* 9: e90267
13. Xi R, Doan C, Liu D, Xie T (2005) Pelota controls self-renewal of germline stem cells by repressing a Bam-independent differentiation pathway. *Development* 132: 5365 – 5374
14. Doma MK, Parker R (2006) Endonucleolytic cleavage of eukaryotic mRNAs with stalls in translation elongation. *Nature* 440: 561 – 564
15. Becker T, Armache JP, Jarasch A, Anger AM, Villa E, Sieber H, Motaal BA, Mielke T, Berninghausen O, Beckmann R (2011) Structure of the no-go mRNA decay complex Dom34-Hbs1 bound to a stalled 80S ribosome. *Nat Struct Mol Biol* 18: 715 – 720
16. Chen L, Muhlrad D, Hauryliuk V, Cheng Z, Lim MK, Shyp V, Parker R, Song H (2010) Structure of the Dom34-Hbs1 complex and implications for no-go decay. *Nat Struct Mol Biol* 17: 1233 – 1240
17. Shoemaker CJ, Eyler DE, Green R (2010) Dom34:Hbs1 promotes subunit dissociation and peptidyl-tRNA drop-off to initiate no-go decay. *Science* 330: 369 – 372
18. Pisareva VP, Skabkin MA, Hellen CU, Pestova TV, Pisarev AV (2011) Dissociation by Pelota, Hbs1 and ABCE1 of mammalian vacant 80S ribosomes and stalled elongation complexes. *EMBO J* 30: 1804 – 1817
19. Cole SE, LaRiviere FJ, Merrikh CN, Moore MJ (2009) A convergence of rRNA and mRNA quality control pathways revealed by mechanistic analysis of nonfunctional rRNA decay. *Mol Cell* 34: 440 – 450
20. Tsuboi T, Kuroha K, Kudo K, Makino S, Inoue E, Kashima I, Inada T (2012) Dom34:hbs1 plays a general role in quality-control systems by dissociation of a stalled ribosome at the 3′ end of aberrant mRNA. *Mol Cell* 46: 518 – 529
21. Saito S, Hosoda N, Hoshino S (2013) The Hbs1-Dom34 protein complex functions in non-stop mRNA decay in mammalian cells. *J Biol Chem* 288: 17832 – 17843
22. Vagin VV, Sigova A, Li C, Seitz H, Gvozdev V, Zamore PD (2006) A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* 313: 320 – 324
23. Eberhart CG, Wasserman SA (1995) The pelota locus encodes a protein required for meiotic cell division: an analysis of G2/M arrest in *Drosophila* spermatogenesis. *Development* 121: 3477 – 3486
24. Kotelnikov RN, Klenov MS, Rozovsky YM, Olenina LV, Kibanov MV, Gvozdev VA (2009) Peculiarities of piRNA-mediated post-transcriptional silencing of Stellate repeats in testes of *Drosophila melanogaster*. *Nucleic Acids Res* 37: 3254 – 3263
25. Passos DO, Doma MK, Shoemaker CJ, Muhlrad D, Green R, Weissman J, Hollien J, Parker R (2009) Analysis of Dom34 and its function in no-go decay. *Mol Biol Cell* 20: 3025 – 3032
26. van den Elzen AM, Henri J, Lazar N, Gas ME, Durand D, Lacroute F, Nicaise M, van Tilbeurgh H, Seraphin B, Graille M (2010) Dissection of Dom34-Hbs1 reveals independent functions in two RNA quality control pathways. *Nat Struct Mol Biol* 17: 1446 – 1452
27. Davis L, Engebrecht J (1998) Yeast dom34 mutants are defective in multiple developmental pathways and exhibit decreased levels of polyribosomes. *Genetics* 149: 45 – 56
28. Guydosh NR, Green R (2014) Dom34 rescues ribosomes in 3′ untranslated regions. *Cell* 156: 950 – 962
29. Ghildiyal M, Seitz H, Horwich MD, Li C, Du T, Lee S, Xu J, Kittler EL, Zapp ML, Weng Z *et al* (2008) Endogenous siRNAs derived from transposons and mRNAs in *Drosophila* somatic cells. *Science* 320: 1077 – 1081
30. Czech B, Malone CD, Zhou R, Stark A, Schlingeheyde C, Dus M, Perrimon N, Kellis M, Wohlschlegel JA, Sachidanandam R *et al* (2008) An endogenous small interfering RNA pathway in *Drosophila*. *Nature* 453: 798 – 802
31. Van Doren M, Williamson AL, Lehmann R (1998) Regulation of zygotic gene expression in *Drosophila* primordial germ cells. *Curr Biol* 8: 243 – 246
32. Li X, Han Y, Xi R (2010) Polycomb group genes Psc and Su(z)2 restrict follicle stem cell self-renewal and extrusion by controlling canonical and noncanonical Wnt signaling. *Genes Dev* 24: 933 – 946
33. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402 – 408
34. Qi Y, Denli AM, Hannon GJ (2005) Biochemical specialization within Arabidopsis RNA silencing pathways. *Mol Cell* 19: 421 – 428
35. Zhao R, Xuan Y, Li X, Xi R (2008) Age-related changes of germline stem cell activity, niche signaling activity and egg production in *Drosophila*. *Aging Cell* 7: 344 – 354

