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The interplay between the Argonaute proteins Piwi and Aub within Drosophila germarium is critical for oogenesis, piRNA biogenesis and TE silencing

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ABSTRACT

Transposable elements (TEs) have invaded most genomes and constitute up to 50% of the human genome. Machinery based on small non-coding piRNAs has evolved to inhibit their expression at the transcriptional and post-transcriptional levels. Surprisingly, this machinery is weakened during specific windows of time in mice, flies or plants, allowing the expression of TEs in germline cells. The function of this de-repression remains unknown. In *Drosophila*, we have previously shown that this developmental window is characterized by a reduction of Piwi expression in dividing germ cells. Here, we show that the unique knock-down of Aub in these cells leads to female sterility. It correlates with defects in piRNA amplification, an increased Piwi expression and an increased silencing of transcriptionally silenced TEs. These defects are similar to those observed when Aub is depleted in the whole germline which underlies the crucial role of this developmental window for both oogenesis and TE silencing. We further show that, with age, some fertility is recovered which is concomitant to a decrease of Piwi and TE silencing. These data pinpoint the Pilp as a tremendously important step for female fertility and genome stability. They further show that such a restricted developmental niche of germ cells may sense environmental changes, such as aging, to protect the germline all along the life.

INTRODUCTION

Transposable elements (TEs) have the capacity to transpose and invade genomes. To ensure genome integrity for the next generation, their mobilization is strictly controlled in the germline (1). Yet, TEs are major constituents of each genome suggesting that they once had the possibility to transpose in this immortal cell line. Once settled in the germline, new TE insertions are then vertically transmitted like any cellular gene.

Extensive studies performed in Drosophila and mice have identified the PIWI-interacting RNA (piRNA) pathway as the major pathway for silencing TEs in the germline and thereby inhibiting their mobilization and transmission (2–4). In Drosophila, the piRNA pathway is active in female ovaries. A pair of Drosophila ovaries composed of 15–18 ovarian subunits called ovarioles. Each ovariole is made up of an anterior functional unit called germarium and a linear string of differentiated egg chambers (5). Ovarian germline stem cells (GSC) reside in the germarium at the anterior tip of each ovariole (6). During the asymmetric division of GSC, the posterior differentiating daughter cell becomes a cystoblast which undergoes four cycles of mitotic division to form interconnected cysts of successively 2, 4, 8 and 16 germ cells. In the egg chamber, one of the final 16 germ cells becomes the oocyte and the other 15 differentiate to nurse cells, all surrounded by a monolayer of somatic follicle cells deriving from the somatic stem cells (SSCs) also present within the germarium.

In germ cells, a pool of primary piRNAs is produced from discrete genomic loci (piRNA clusters) enriched in TEs or their relics. These clusters are classified into uni-strand and dual-strand clusters both transcribed by the RNA polymerase II (RNA pol II) (3). Transcription of uni-strand clusters provides a precursor which is not different in basic RNA features from other RNA Pol II transcripts (7). A heterochromatin-dependent transcription machinery drives transcription of dual-strand piRNA clusters (8–10).

Once transcribed, piRNA precursors produced from piRNA clusters are transferred to cytoplasmic perinuclear granules called nuage where components such as Vasa (Vas), Armitage (Armi), Squash (Squ) and Zucchini (Zuc)

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are required for piRNA production (11–13). When processed into primary piRNAs, they associate with Aubergine (Aub) and target TE sense-transcripts. The slicing of the latter results in the production of a secondary pool of piRNAs bound to Ago3. In turn, secondary TE piRNAs target and induce piRNA cluster transcript processing into new piRNAs. This amplification process, which uses piRNA cluster and TE transcripts for piRNA production, has been called the ping-pong loop (3). Recent studies have reported that secondary piRNAs initiate 3′-directed slicing of target transcripts resulting in phased piRNAs (14,15). The ping-pong loop then fulfills two key roles for TE repression: amplification and phased piRNA production, and post-transcriptional silencing (PTGS) of TEs resulting from slicing. In addition, piRNAs bound to Piwi mediate transcriptional silencing (TGS) of TEs, independently of its slicer activity (16). Overall, flies with germline defects in the piRNA pathway not only display de-repression of TEs but also a dramatic increase of sterility (17).

From our current knowledge of the piRNA pathway mostly deduced from high-throughput sequencing and large-scale genetic screens, many questions remain open. Is the piRNA pathway active all along germ line development or do specific stages orchestrate TE silencing? How can the presently known actors of the piRNA pathway succeed in silencing hundreds of TE families which display distinct specificity in term of structure, distribution, evolution? In a previous study, Wang *et al*. suspected that piRNAs sequenced from whole ovaries might not fully represent the variability of piRNA levels between different stages of oogenesis (18). In support of this, weaknesses of the TE silencing machinery have been observed in the germline or its associated cells in most eukaryotes. In Arabidopsis, many genes involved in small RNA-mediated silencing and heterochromatin regulation have reduced expression in mature pollen. In the vegetative nucleus, DNMT1, required for the transcriptional silencing of TEs is absent (19). Such a temporal window, where key components of the TE transcriptional silencing are weakened, is also observed during specific stages of mammalian germline development such as the epigenetic reprogramming (20).

In a previous study, we observed that Piwi expression is reduced within a small developmental window of Drosophila oogenesis. During this stage, taking place in the dividing germline cysts of the germarium, Idefix- and P-element-based transgenes escape host control. This region has been termed the 'Piwiless pocket' or Pilp (21). Here, we show that depletion of Aub in the Pilp is sufficient to affect female fertility. The absence of Aub is accompanied by an increased Piwi expression and by defects in piRNA biogenesis. This developmental stage of oogenesis is then crucial to produce and amplify a pool of piRNAs that will ensure TE silencing.

MATERIALS AND METHODS

Drosophila stocks

All stock flies were kept at 20°C and crosses at 25°C. RNAi lines against *white* (35573), *aub* (35201), *ago3* (35232), *rhi* (35171), *hp1* (36792), *piwi* (31610) and the driver line *nos-Gal4-VP16* (chromosome 3) all came from the Bloomington stock center (line numbers in brackets). The driver line *bam-gal4* and the *NV-burdock* (*nanos-promoter-GFP::lacZ-burdock-vasa-3′UTR*) line were kind gifts from J.R. Huynh and J. Brennecke, respectively.

Sterility test

Fertilized females aged 2–5 days or 19–22 days were collected and incubated 24 hours on yeast. They were then transferred to a fresh vial and allowed to lay eggs for 24 h before being removed. Twenty four hours later, hatched and non-hatched embryos were counted. In each vial, 200 embryos were examined. Experiments were biologically replicated 6 times. Statistical relevance was assessed by Chi2 test on R software.

Immunofluorescence

Ovaries from 3- to 6-day-old flies were dissected in PBS fixed with 4% paraformaldehyde at RT for 20 min, rinsed three times with phosphate buffered saline (PBS), permeabilized 1 h in PBS–0.3% Triton, rinsed three times with PBS and incubated for 1.5 h in PBS, bovine serum albumin 1%, Triton 0,1%, prior to 4°C overnight staining with mouse anti-lamin antibody (ADL67-10, Hybridoma, 1:300 dilution), goat anti-GFP antibody (ab5450, Abcam; 1:2000 dilution), mouse anti-Aub antibody (22) (1:500 dilution), rabbit anti-Piwi antibody (sc-98264, Santa Cruz, 1:1000 dilution) and mouse anti-AGO3 antibody. Secondary antibodies coupled to Cy3, Cy-5 or Alexa-488 were used at 1/1000 dilution. Three-dimensional images were acquired on Leica SP5 and Leica SPE confocal microscopes with a 40× lens.

in situ RNA hybridization

The DNA fragment used to prepare the *412* probe for the detection of *412* transcripts was PCR amplified from the *w1118* line with primers 5′- CACTAACTCTGACGAGGAAG -3′ and 5′-ACCTAAAGGCTGTTGCGAGT-3′ and cloned into pGEM®-T Easy Vector (Promega).

Riboprobe was synthesized by digestion of pGEM®-T Easy Vector with NcoI enzyme (New England Biolabs), followed by *in vitro* transcription using T7 polymerase and digoxygenin labeled UTP (Roche), DNAse I treatment and purification.

RNA FISH was performed on ovaries from 3- to 6-day-old flies dissected in 0.2% Tween-20/PBS (PBT). Ovaries were fixed with 4% paraformaldehyde/PBT at RT for 30 min, rinsed three times with PBT, post-fixed 10 min in 4% paraformaldehyde/PBT and washed in PBT. After permeabilization 1 h in PBS-0.3% Triton, prehybridization was performed as follows: 10 min HYB- (Formamide 50%, SSC 5×, Tween 0.02%)/PBT 1:1, 10 min HYB-, 1 h HYB+ (HYB- with yeast tRNA 0.5 mg ml^{-1} (Sigma), heparin 0.25 mg ml^{-1}) at 60°C. Ovaries were hybridized overnight at 60°C with 1 μg riboprobe. They were then rinsed 20 min in HYB- and in HYB-/PBT 1:1 at 60°C, then four times in PBT at room temperature (RT) before blocking 1 h at RT in TNB 0.3% triton (Perkin-Elmer TSA kit) and immunodetection 1.30 h at RT with anti-digoxigenin-HRP (Ref: 11 207 733 910, Roche, 1:200 dilution) in TNB 0.2% Tween-20.

They were rinsed three times in PBT, incubated 10 min with TSA-Cy3 in amplification diluent (Perkin-Elmer, 1:50 dilution), and rinsed. RNA staining was followed by incubation with mouse anti-lamin antibody (ADL67-10, Hybridoma, 1:300 dilution). Secondary antibodies coupled to Alexa-488 were used.

qRT-PCR analyses

Total RNA was isolated from 10 pairs of ovaries with Trizol (Ambion). Following DNAse I treatment, cDNA was prepared from 1 μg RNA by random priming of total RNA using Superscript IV Reverse Transcriptase (ThermoFisher Scientific). Quantitative PCR was performed with Roche FastStart SYBR Green Master and the Lightcycler® 480 Instrument. All experiments were conducted with four biological replicates and with technical triplicates. Steady-state RNA levels were calculated from the threshold cycle for amplification by the $2^{-\Delta\Delta C}{}_T$ method. *rp49* was used for normalization (Supplementary Table S1).

Small RNA sequencing

Total RNA was isolated from 40 pairs of ovaries from 3- to 6-day-old or 20- to 23-day-old flies with Trizol (Ambion). Deep sequencing of 18–30nt small RNAs was performed by Fasteris S.A. (Geneva/CH) on an Illumina Hi-Seq 2500. Illumina reads were matched to release six of the *Drosophila* genome with Bowtie2. For further analysis, 23–29nt long RNAs were selected as piRNAs. All the analyses were conducted either on piRNAs mapping to TEs allowing 0–3 mm with BWA or on genome-unique piRNAs mapping to piRNA clusters as defined by Brennecke *et al*. (3), allowing no mismatch. All libraries were normalized on the basis of the number of genome-unique piRNAs mapping the somatic cluster *flamenco*.

Ping-pong signature was assessed by counting the proportion of sense piRNAs with an overlap of 10 nucleotides (nts) with an antisense piRNAs. Proportions of overlap of 1 to 24 nts were numbered and the percentage of 10 nt overlaps defined as ping-pong signature.

Phasing signature was assessed by measuring the 3′ to 5′ distance of genome-unique reads mapping on the 42AB piRNA cluster. Mapping was performed with the small RNA mapper tool of Rozenkranz group (23) and followed by analyses of the distance between 3′ and 5′ of piRNAs using Phaser tool of the Rozenkranz group.

BioSample accessions: SAMN09010118, SAMN09010119, SAMN09010120, SAMN09010121, SAMN09010122, SAMN09010123, SAMN09010124, SAMN09010125

Quantitative Piwi fluorescence imaging

3D images acquired through the confocal microscope were automatically segmented using a Python script inside Imaris (which was only used to open and interact with the images). Nuclei from the germline cells were detected using the cytoplasmic Aub marker (Supplementary Figure S1). Aub staining is easily detected along the whole germarium in control *bam>whiteKD* ovaries where nuclei appear black. In *bam>aubKD* ovaries, Aub staining is hardly detected in the dividing cysts due to RNAi treatment but its background staining is sufficient to visualize nuclei. The mean Piwi intensity was then measured inside each nucleus, and its position along the Antero/Posterior (A/P) axis was reported. For each image, the first and last nuclei were used to define the beginning and end of the corresponding germarium, and thus determine the relative positions of the nuclei. These positions were then grouped for each 20% in R, and from this data, a mixed model analysis (with two fixed effect factors: position and genotype) was used to detect statistically significant differences between the positions for each genotype, the germarium being a random effect factor. The script is provided upon request.

RESULTS

Aubergine is required in the Pilp to ensure fertility

To elucidate the function of specific windows of time during which the piRNA machinery is weakened, we examined the Pilp function in Drosophila ovaries. As a suitable Gal4-driver to specifically lead transgene expression in the Pilp, we used the *bam-Gal4* driver. The translation repressor, bag-of-marbles (bam), is indeed only expressed in the dividing cysts (Figure 1A). On fly ovaries carrying a GFP-reporter transgene driven by *bam-gal4*, we showed through immunostaining using GFP and Piwi antibodies that GFP is expressed within the germarium where Piwi is absent as expected for a Pilp-specific driver.

With the exception of Piwi, essential proteins required for piRNA production appear to be expressed in the Pilp. This has been shown for Rhino, Aub and AGO3, HP1 which are required, respectively, for piRNA cluster transcription, the ping-pong amplification and TE transcriptional repression. Thus we wondered whether depletion of one of these proteins specifically within the Pilp could generate helpful mutants to point out functions of this developmental window. Specific RNAi-knockdown (KD) of *rhino*, *aub*, *ago3* and *HP1* was performed. When a germline driver like *nanos-gal4 (nos-Gal4)* was used to knock-down the expression from the germarium to later stages of Drosophila oogenesis, we observed a significant decrease in the fertility of *nos>ago3*KD and *>HP1*KD females aged from 3 to 6 days (d). Complete sterility was observed in *nos>aubKD* and *nos>rhi*KD females which laid eggs that did not hatch (Figure 1B). When *rhino*, *ago3*, *HP1* were knocked down in the Pilp using the *bam-gal4* driver, no sterility was observed. Surprisingly, females in which Aub was depleted thanks of the *bam* driver (*bam>aubKD* females) were completely sterile, as were *nos>aubKD* females in which Aub is depleted in the whole germline. These females lay eggs which do not hatch. These results could be explained if Aub were never recovered in the germline after its depletion within the Pilp. They could also be explained if Aub depletion affected dramatically AGO3 expression, the other crucial PIWI-clade protein required for germline piRNA biogenesis. Double immuno fluorescence (IF) experiments using antibodies addressed against Aub and AGO3 were performed on ovarioles dissected from *bam>aubKD* flies and compared to *bam>whiteKD* flies as a WT control, and *nos>aubKD* flies in which Aub is depleted in the whole germline (Figure 1C).

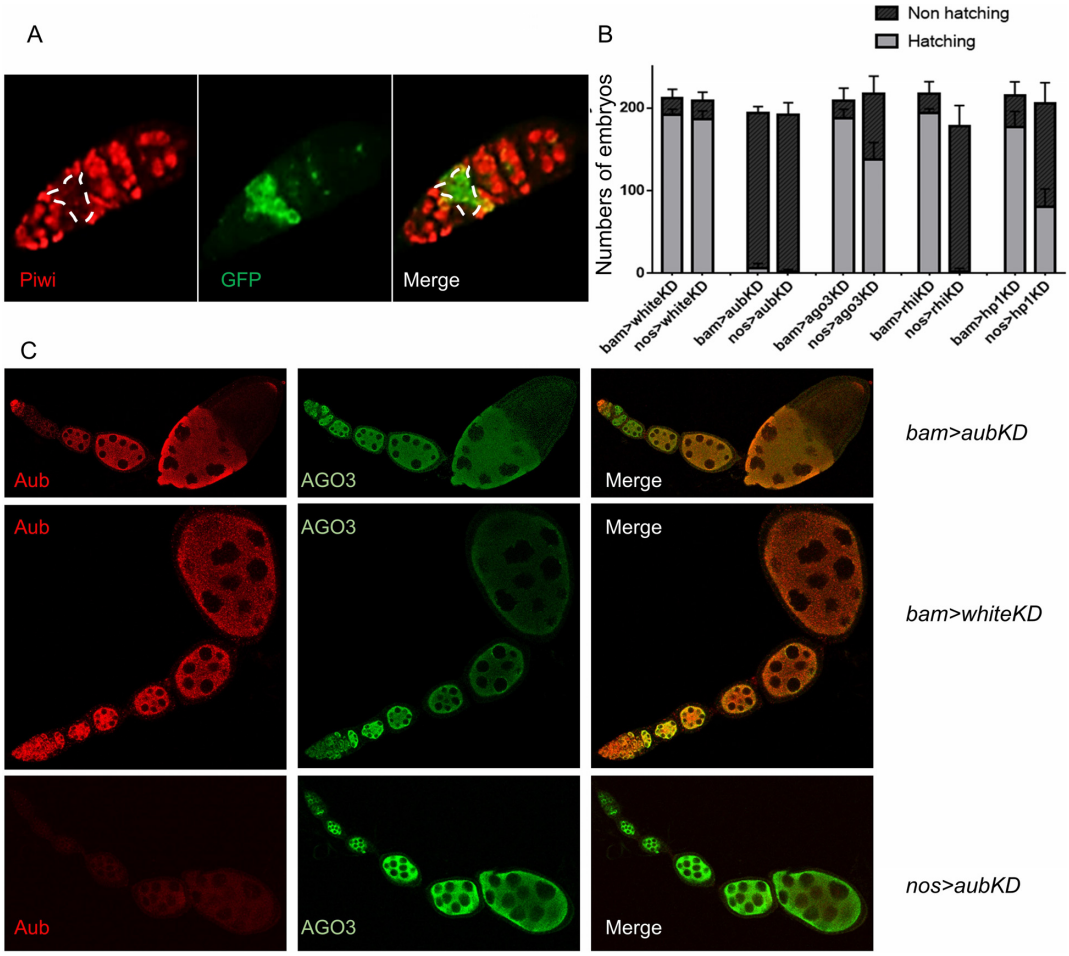

Figure 1. Different RNAi-knock-down genes driven specifically in the Pilp have different effects on fly fertility. (**A**) The *bam-gal4* driver is specifically expressed in the Pilp: Immunostaining experiments performed on ovaries of flies carrying two transgenes (*UASp-GFP* and *bam-gal4*). GFP (green) is detected in cells where Piwi staining (red) is less expressed than in the other germline cells. (**B**) Impact of aubergine (aub), ago3, rhino (rhi) or hp1 knock-down either in all the germline cells (nos) or specifically in the Pilp (bam) on the fertility. Knockdown of a *white* transgene is used as a control. Fertility of flies was measured by counting the number of hatching (gray) and non-hatching (black) eggs laid by 3–6 day old females. Sterility test was replicated 6 times. (**C**) Effect of *aub* knock-down on Aub (red) and AGO3 (green) expression and localization assessed by immunostaining of control ovaries (*bam>white*KD), of ovaries depleted for Aub in the Pilp (*bam>aub*KD) or in the whole germline (*nos>aub*KD) of 3–6 day old flies.

In *bam>aubKD* flies, we detected Aub in the anterior tip of the ovariole upstream of the Pilp. It is then depleted in the dividing cysts as expected for its knock-down driven by *bam*. We observed that *aub* depletion does not perfectly coincide with stages when the *bam* driver is active, but can extend up to stage 3. At stage 4, Aub expression is recovered and similar to WT (Figure 1C upper and middle lines). It is detected within the cytoplasm of the nurse cells of developing egg chambers and enriched at the posterior oocyte pole in late egg chambers. Since the *bam* driver is only active in the Pilp (Figure 1A), a delay in the full recovery of Aub expression after its knocking-down may be explained by a delay in its translation. Alternatively, the stability of interfering RNAs could also explain this delay. However, this last hypothesis is unlikely since when *bam>rhinoKD* flies are examined, Rhino expression is recovered as soon as b*am-Gal4* is inactive (Supplementary Figure S2). RNAi-mediated depletion of Aub using the *nos-Gal4* driver (*nos>aub KD* flies) results in its nearly complete loss, Aub staining being undetectable in the germline (Figure 1C, lower line).

AGO3 expression is found unchanged in *bam>aubKD* and *nos>aubKD* compared to WT flies with a cytoplasmic accumulation along the developing egg chambers and an accumulation to nuage of nurse cell nuclei (Figure 1C). These data indicate that the absence of Aub does not affect the expression or localization of AGO3.

Thus, the transient depletion of Aub driven by *bam* is sufficient to greatly affect oogenesis and to lead to a complete sterility similar to the one observed when Aub is absent from the whole germline (Figure 1B). This argues in favor of a crucial role of Aub during this short window of germline development.

Two categories of TEs are differentially affected upon Aub knock-down in the Pilp

Since *bam>aubKD* flies are sterile, we wondered whether TE silencing could be lost in these flies. We assessed TE control by comparing TE expression from a WT control to *bam>aubKD* or *nos>aubKD* ovaries. Quantitative PCR

(qPCR) analysis revealed two classes of TEs. One category, to which *Roo*, *412, Invader1*, *RT1B* and *17.6* belong, corresponded to TEs which are over-expressed regardless of the driver used (Figure 2A). This over-expression is expected when one of the components of the piRNA pathway, here *aub*, is disrupted. Expression of the second category of TEs increased as expected when Aub was depleted in the whole germline (*nos-gal4*) but, strikingly, their steady-state RNA level significantly decreased when Aub depletion was specifically carried out with the *bam* driver. This increased silencing was observed for *Gate*, *TART*, *I*, *Het-A* and *Burdock* (Figure 2A).

TEs are usually classified according to their structure, evolutionary history or mode of transposition. However, these classifications were unable to explain the two categories observed in *bam>aubKD* flies. In 2015, Senti *et al*. defined a new repertoire of TEs (24). They defined a category of transcriptionally silenced TEs (named TGS-TEs thereafter) when loss of Aub or AGO3 leads to a RNA polymerase II (Pol-II) enrichment (more than 1.5 times) registered on their transcription start sites (Figure 2B, right). From their study, a second category of TEs can be defined as post-transcriptionally silenced TEs or PTGS-TEs when Aub or AGO3 germline depletion leads to an increased steady-state transcripts (>2-fold) of these TEs with no PolII enrichment (less than 1.5 fold) on their promoters (Figure 2B, left).

Interestingly, the two categories of TEs observed after the qPCR experiment on *bam>aubKD* ovaries perfectly coincide with this new classification of TEs. Indeed, *Gate*, *TART*, *I*, *Het-A* and *Burdock*, whose silencing is enforced, belong to the class of TGS-TEs whereas *17.6*, *RT1B*, *Invader1*, *412* and *Roo*, whose silencing is released, belong to PTGS-TEs.

qPCR gives information only on total RNAs present in the entire ovary. To know whether TGS- and PTGS-TE silencing varies during oogenesis, we used transgenic lines carrying a reporter of TGS-TE-silencing. This reporter, *nanos-promoter-GFP::lacZ-burdock-vasa-3′ UTR*, carries a fragment of the TGS-TE *Burdock* coupled to GFP and LacZ and under the control of a *nanos* promoter (*NV-burdock,* a kind gift of J. Brennecke). In WT ovaries, GFP is not detected owing to the germline silencing exerted on *NV-burdock*. If Aub depletion is achieved by the *nos* driver (*nos>aubKD* lines) *NV-burdock* silencing is lost and GFP is detected at high levels in all germ cells. Upon Aub depletion specifically in the Pilp, GFP is undetected in the whole germline including the Pilp itself (Figure 2C). Thus, this TGS-transgene is not desilenced when Aub is depleted in the Pilp. This is consistent with the decrease in the expression of the endogenous *Burdock* TE observed in qPCR upon *bam>aub*KD.

412 belongs to the PTGS-TE category. To check its expression despite the lack of a proper sensor transgene, we chose to perform a RNA-FISH experiment using a RNA-probe directed against the *412* transcript. Except few faint spots within the nuclei, no staining is observed in *whiteKD* control flies neither within the germarium (Figure 2D upper left panel) nor at later stages of oogenesis (Figure 2D lower left panel). By contrast, *412* staining is detected at a high level in germ cells upon Aub depletion from both *nos>aubKD* and *bam>aubKD* lines (Figure 2D). In the germarium, staining appears diffuse within the cytoplasm and as a bright spot within the nuclei. At later stages, a pronounced staining is detected in both the cytoplasm and nuclei. These findings confirm that PTGS-TEs are desilenced in all the cells whether Aub is depleted in the Pilp or in all the germinal cells.

Taken together, these results indicate that two categories of TEs exist that are differently affected by Aub knockdown in the Pilp. PTGS-TE silencing decreases whereas TGS-TE silencing increases. They further underline that the piRNA pathway regulation within the Pilp has a dominant impact on TE silencing which can be maintained along oogenesis.

Aub is responsible of Piwi decrease in the Pilp

The increased silencing exerted on TEs when the piRNA pathway is mutated was unexpected. Since Piwi plays a major role in the transcriptional silencing of TEs, we investigated the expression of Piwi in *bam>aubKD* flies. An immuno-fluorescence experiment was performed using Piwi and Aub antibodies on WT, *bam>aubKD* and *nos>aubKD* ovaries. In germarium from WT flies, cytoplasmic staining of Aub and nuclear staining of Piwi are observed within the germline cells with a Piwi depletion corresponding to the Pilp (Figure 3A, left). In germarium from *bam>aubKD* flies, the cytoplasmic presence of Aub is detected at the anterior tip of the germarium before its depletion from the dividing cysts (Figure 3A, middle). In *nos>aubKD*, no germline Aub staining is detected (Figure 3A, right). In both Aub-depleted flies, *bam>aubKD* and *nos>aubKD*, Piwi expression is stable in the whole germarium where no Pilp can be detected. This was further evidenced when the level of Piwi was compared between germarium depleted or not for Aub (Figure 3B). Using 3-D images of germarium from confocal microscopy, we measured fluorescence intensity inside the nuclei from the anterior tip to the 16-cells cyst. The level of Piwi was low in the first 40% of *bam>whiteKD* germarium and then increased. This reveals the position of the Pilp. In Aub-depleted flies, Piwi level is high all along the A/P axis of the germarium where no Pilp could be found (Figure 3B).

Overall, these data establish a link between the presence of Aub and the lack of Piwi in the Pilp.

Piwi increase might well result in an increased TGS-TE repression as observed in Figure 2A. This prompted us to test the steady state RNA levels of TEs when both Aub and Piwi are depleted within the Pilp ([*bam>aubKD; >piwiKD] flies*) (Figure 3C). The TGS-TEs *Gate*, *I, Blood* and *Het-A* were strongly over-expressed in these double mutant ovaries (more than 6 fold for the I-element). This result contrasts dramatically with the silencing observed in single depletions of Aub or Piwi where TGS-TE transcript levels were lowered or unchanged, respectively. By contrast, PTGS-TEs *17-6*, *RT1B* and *Roo* seemed not to be sensitive to Piwi depletion and had similar steady state RNA levels in *bam>aubKD* flies and in double mutants [*bam>aub; >piwiKD]*. These data support that the increased TGS silencing observed in Aub depleted ovaries compared to WT is due to the increased Piwi expression in the Pilp. This experiment highlights another relevant new aspect of TE con-

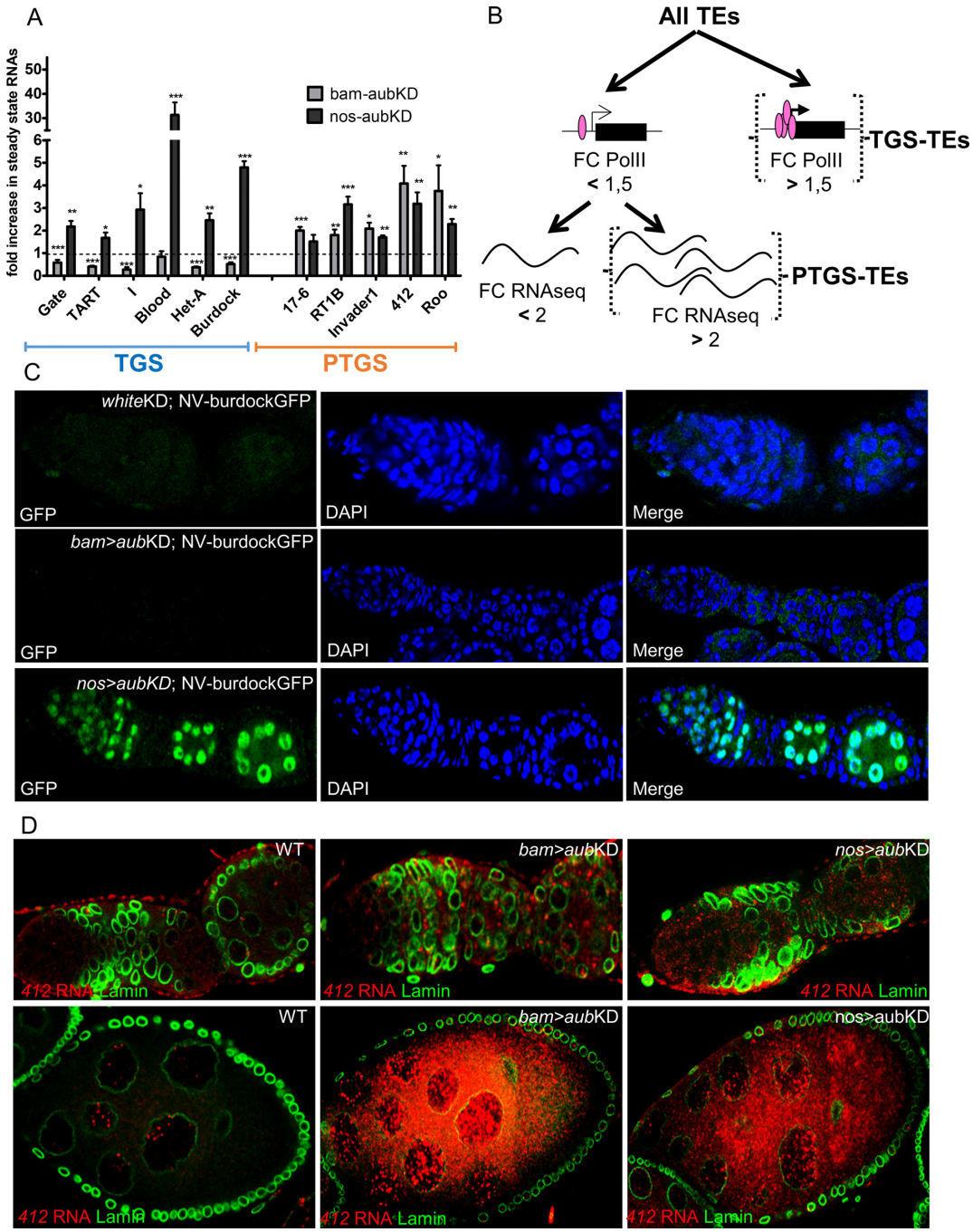

Figure 2. Depletion of Aub in the Pilp affects differently the silencing of two categories of TEs. (**A**) Fold change in steady state RNA level of TEs in nos>Aub-KD and bam>aub-KD flies. Tested TEs are indicated above the graph (the positions of primers are indicated in Supplementary Table S1). RNA levels are normalized to white-KD. The dotted line corresponds to a fold change of 1 and indicates no change in the RNA level. Statistical tests were made using *t*-test function on R software. In Aub-depleted Pilp, the silencing of one category of TEs (six TEs on the left) increases whereas it decreases for the second category (5 TEs on the right). At least 5 biological replicates was used. * corresponds to a *P* value <0.05, ** to a *P* value <0.01 and *** to a *P* value <0.001. (**B**) The cartoon shows two modes of TE silencing by the piRNA pathway in the germ line (24). If loss of Aub or AGO3 leads to a pronounced Pol II peak (>1.5) that coincides with the transcription start site of the element (arrow), the TE is classified as TGS-TE for transcriptionally silenced TE. If an increase of transcription is observed with no PolII peak, the TE is classified as PTGS-TE for post-transcriptionally silenced TE. (**C**) Silencing of the *nanos-promoter-GFP::lacZ-burdock-vasa-3′UTR* -sensor transgene (*NV-burdockGFP*). Repression of *NV-BurdockGFP* used an example of TGS-TE silencing is assessed in the control line (*whiteKD*), *bam>aubKD* and *nos>aubKD* flies (first, second and third lines, respectively). GFP (green) is visualized by immunofluorescence using and anti-Piwi antibody. Nuclei are labelled using DAPI. (**D**) PTGS-TE silencing is examined through *in situ* RNA hybridization of the PTGS-TE *412* transcripts. In situ hybridization are performed on ovaries of the control line (*whiteKD*), and on *bam>aubKD* and *nos>aubKD* flies. *412* transcripts (red) are presented within germarium (upper line) and at later stages (lower line). Nuclear membranes are labelled using an anti-lamin antibody.

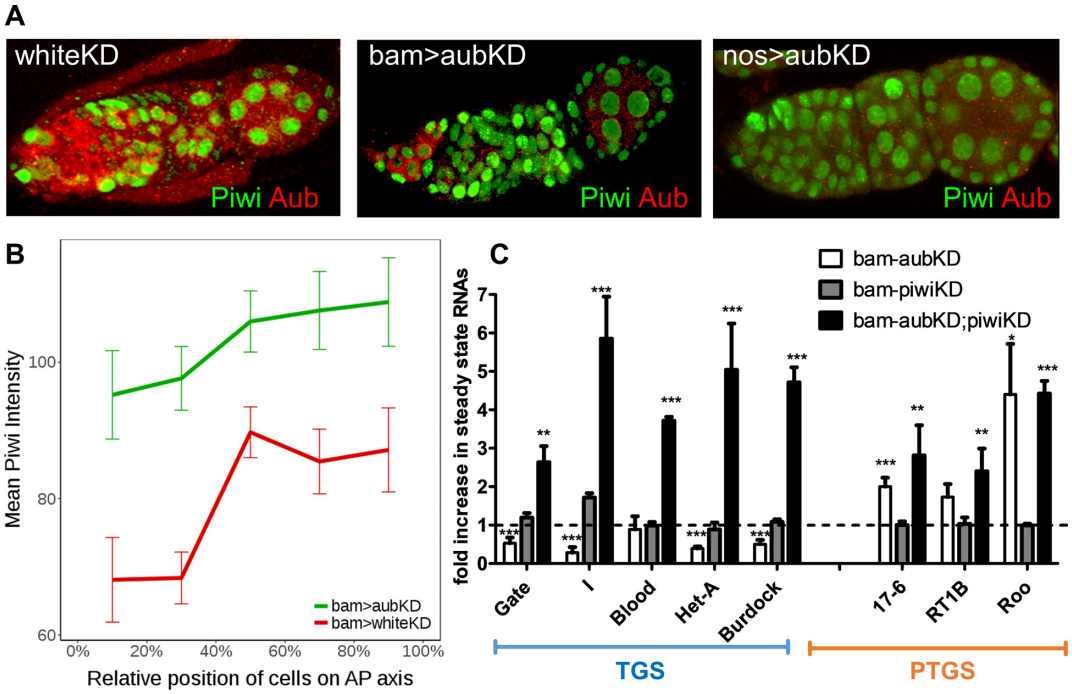

Figure 3. Aub depletion leads to Piwi stabilization in the Pilp. (**A**) Immunostaining of Aub (red) and Piwi (green) in the control line (*whiteKD*) and in flies depleted for Aub in the whole germline (*nos>aubKD*) or specifically in the Pilp (*bam>aubKD*). (**B**) The graph represents the mean intensity of Piwi inside the nuclei along the A/P axis of the germarium. A mixed model analysis reveals that the mean Piwi intensity is significantly different in the nuclei located in the first 40% of the germarium and those located in the last 60% (P-value < 0.0001) from *bam>whiteKD* (red line). The low level of Piwi expression corresponds to the Pilp position. Comparatively, no statistically significant difference can be detected along *bam>aubKD* germarium (green line) where Piwi staining remains high and no Pilp can be detected (P-value $= 0.06342$). (**C**) Impact of the double knock-down of aub and piwi in the Pilp (*bam-aubKD;-piwiKD*) on TEs silencing. Knockdown of aub (*bam-aubKD*) or piwi (*bam-piwiKD*) individually are presented. Fold change in steady-state RNA levels was quantified by q-RT-PCR using specific primers recognizing the indicated TEs, TGS-TEs are underlined in blue and PTGS-TEs in orange. Results were normalized using white-KD flies as a control, dotted lines indicate no change in RNA levels corresponding to a fold change of 1. Statistical tests were made using t-test function on R software. At least 4 biological replicates are represented. ** correspond to a P value <0.01 and *** to a P value <0.001.

trol in the Pilp: the silencing of TGS-TEs may be exerted by Piwi and Aub. By contrast, it depends solely on Aub for PTGS-TEs.

Overall, these findings show that in WT flies, Aub is involved in the lack of Piwi in the Pilp and can operate a post-transcriptional silencing of all TEs.

The Pilp is a crucial developmental stage for ping-pong amplification

Relaxed transcriptional repression in the Pilp could be exploited to boost the generation of piRNAs that match active TEs. To test this hypothesis, we investigated piRNA production and sequenced small RNAs from 3- to 6-day-old ovaries, depleted or not for *Aub*. The *whiteKD* line was used as a control. Small RNAs of 23–29 nucleotides, the characteristic length of piRNAs, were taken into consideration in this study and mapped onto TGS-TE and PTGS-TE sequences allowing 0–3 mismatches (mm). Normalization by the number of unique piRNAs mapping on the somatic cluster *flamenco*, unaffected by germinal knockdown, enabled us to compare piRNA production in different genotypes. Moreover, piRNAs have been sequenced from whole ovary extracts, germinal and somatic cells included. Hence specific patterns of piRNAs produced by the germline could be masked by somatic reads. In this analysis, we only considered piRNAs mapping to TEs described to produce more germinal than somatic piRNAs (24). Upon depletion of Aub in the germline thanks to the *nos-gal4* driver, a significant decrease of piRNAs matching both categories of TEs with 0–3 mm was observed (Figure 4A, Supplementary Figure S3A). Interestingly, this decrease was also observed upon depletion of Aub thanks to the *bam* driver (Figure 4A, Supplementary Figure S3A). We then checked the production of piRNAs targeting individual TEs (Figure 4B and Supplementary Figure S3B and C). Scatterplots indicated that piRNAs mapping to most TGS- and PTGS-TEs decrease in *bam>aubKD* ovaries compared to the control. This was particularly evident for TGS-TEs like Het-A, TART or TAHRE, which are repressed in *bam>aubKD* ovaries but whose piRNAs decreased to 66.7%, 63.6% and 63.4% of the control level, respectively (Supplementary Figure S3B). To go deeper in the analysis of germline piRNAs, we examined sense and anti-senses piRNAs produced from *bam>aubKD* and *nos>aubKD* ovaries. To this end, we firstly removed piRNAs mapping to *flamenco*, the principal source of somatic piRNAs which could introduce a strand bias due to the large majority of them being antisense. This analysis revealed that loss of Aub both in the Pilp and in the whole germ line results in a reduction of anti-sense piRNAs mapping to PTGS-TEs, the fold change ratio sense/antisense piRNAs being around 2 (Supplementary Figure S4C and D). A more severe bias between sense

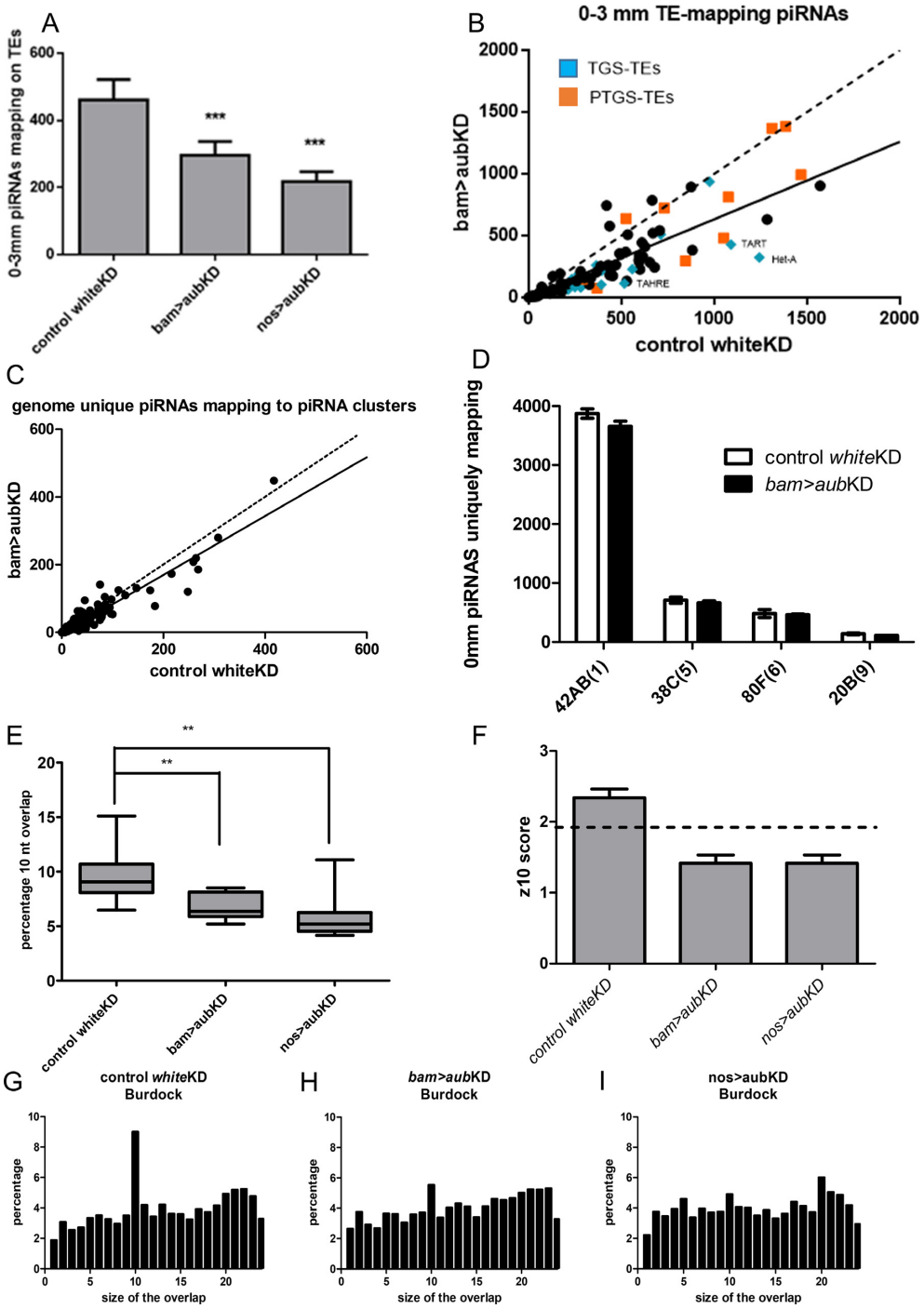

Figure 4. Depleting Aub in the Pilp decreases piRNA in the ovaries of 3- to 6-day-old females. (**A**) Percentage of 0–3 mismatches (mm) piRNAs matching on RepBase TEs. piRNAs found in ovaries of control *whiteKD*, *bam>aubKD*, *nos>aubKD* are presented. Significance was assessed using ANOVA. *** correspond to a *P* value < 0.001. (**B**) Scatterplot showing 0–3 mm piRNAs mapping to RepBase TEs. piRNAs from *bam>aubKD* and *whiteKD* ovaries are reported on y- and x-axes respectively. TGS-TEs are represented as blue diamond and PTGS-TEs as orange squares. piRNAs were normalized by the number of unique piRNAs mapping to the somatic cluster *flamenco*. Dotted line represent the standard regression expected if no changes occurred between the conditions and plain black line represents the standard regression observed. (**C**) Scatterplot showing normalized counts of piRNAs mapping to piRNA clusters with 0mm: piRNAs from *bam>aubKD* and *whiteKD* ovaries are reported on y- and x-axes respectively. piRNAs were normalized by the number of unique piRNAs mapping to the somatic cluster *flamenco*. (**D**) Histograms showing the number of 0mm piRNAs mapping uniquely to the more important germinal piRNAs clusters (x-axis). piRNAs were normalized by the number of unique piRNAs mapping to the somatic cluster *flamenco*. (**E**) Boxplot representing the ping-pong signature (defined by the percentage of piRNA sense and antisense pairs presenting an overlap of 10nts) from ovaries of control *whiteKD*, *bam>aubKD*, *nos>aubKD*. Statistical significance was assessed using ANOVA. ** correspond to a *P* value <0.01. (**F**) Histograms showing the z-score of piRNAs with a 10-nt overlap signature from ovaries of *whiteKD, bam>aubKD* and *nos>aubKD* flies. (**G**–**I**) Histograms showing the percentage of piRNA pairs with a size specific overlap (comprised between 1 and 24nt) in a control line (G) or upon depletion of Aub specifically in the Pilp (H) or in the whole germline (I). Light gray histograms represent the z-score of the 10nt overlap.

and anti-sense piRNAs was observed for piRNAs mapping to TGS-TEs in *nos>bamKD* ovaries where the fold change ratio sense/antisense is >3 as shown for Blood and Burdock (Supplementary Figure S4A and B). By contrast, loss of Aub in the Pilp results in a higher reduction of sense than anti-sense piRNAs mapping to TGS-TEs, the fold change ratio sense/anti-sense being lower than 1 (0.4 and 0.7 for Blood and Burdock respectively, Supplementary Figure S4A,B).

It is considered that the absolute number of Piwi-bound piRNAs antisense to the nascent target transcript at the promoter region defines Piwi transcriptional silencing (25). We examined whether such anti-sense piRNAs might be detected at TGS-TE promoters in *bam>aubKD* and *nos>aubKD* ovaries in which Aub-bound piRNAs are depleted and only Piwi-bound piRNAs remain (24). This analysis was restricted to TEs for which our data indicated that there is a differential Aub-dependent regulation of the two categories of TEs (Figure 2). We found that anti-sense piRNAs are detected at the TGS-TE promoters of Blood, Burdock or Gate whether flies are depleted or not for Aub. However, not all TGS-TEs conserve piRNAs anti-sense to their nascent transcript as observed for I, HeT-A or TART (Supplementary Figures S4A and S5, left). If, as reported, PTGS-TEs are post-transcriptionally silenced, anti-sense piRNAs detected at their promoters should be Aub-bound piRNAs and lost when Aub is depleted. This is indeed what we observed for RT1b, Opus/Nomad and Invader 1 but not for Roo and 17.6 (Supplementary Figures S4B and S5, right). Therefore, half of the TEs examined confirm the central role of Piwi-piRNAs and Aub-piRNAs in orchestrating the transcriptional control of TGS-TEs and the post-transcriptional control of PTGS-TEs, respectively.

Overall, these results indicate that Aub expression within the Pilp is required for germline piRNA production. If depleted at this stage of oogenesis, the piRNA population is then characterized by a severe decrease of sense piRNAs mapping TGS-TEs consistent with an increased transcriptional repression of these TEs likely due to Piwi-piRNAs.

With regard to piRNA production, Aub is required both for phasing initiation, which leads to primary piRNAs, and for ping-pong loop amplification. To decipher which of these roles might be affected in the Aub-depleted Pilp or whether both of them are, we first considered piRNAs that map solely to piRNA clusters. We found no significant overall decrease in these piRNAs (Figure 4C). When we focused on the genomic clusters that produce most of the germline piRNAs (42AB, 38C, 80F and 20B), we observed no decrease in the amount of piRNAs in *bam>aubKD* ovaries (Figure 4D). To evaluate if the phased production of piRNAs is affected, we measured the distance between the 3′ end of a piRNA and the 5′ end of the next one. We concentrated the analysis on genome unique piRNAs that map to the piRNA cluster 42AB. Upon phased production of piRNAs, the most frequent distance between the 3′ and the 5′ of the next piRNAs was 1 nt as expected from published data (Supplementary Figure S6A). Thus, mutation of Aub in the Pilp does not affect the production of phased piRNAs.

We then considered whether depletion of Aub in the Pilp affects ping-pong amplification. This can only be determined if the variation is high enough to be significantly detected in whole ovary extracts. We checked for 10-nt 5′-overlaps between sense and antisense piRNAs and the median z-score for 10nt overlap as hallmarks of ping-pong amplification (Figure 4E and F, Supplementary Figure S6B and C). In whole ovaries, this ping-pong signature was significantly reduced in *bam>aubKD* to the same extent as when Aub is depleted in all the germline (*nos>aubKD* flies). This was observed for both TGS- and PTGS-TEs (Supplementary Figure S7A and B). This means that Aub depletion in the Pilp alone or in the whole germline has a similar impact on ping-pong and shows the importance of this developmental window for ping-pong amplification. Three individual TEs taken from both categories were also examined (Figure 4G–I and Supplementary Figure S7C). As expected from the results above, ping-pong signatures of individual TGS-TE and PTGS-TEs are significantly reduced when Aub is depleted within the Pilp or in all the germline.

The amount of ovarian piRNAs and ping-pong signatures decreases therefore upon Aub depletion in the Pilp but this depletion is nonetheless associated with a greater silencing exerted on the specific category of TGS-TE. This might be due to the increased Piwi expression and consequently the inability of transcriptionally silenced TEs to produce mRNAs engaged in the ping-pong loop for piRNA amplification.

Female sterility associated with aub knock-down in the Pilp reverts with aging

In the course of crosses leading to *bam>aub-Gal4* flies, we found that young (aged 3–6 days) and old *Aub*KD females (aged 20–23 days) lay the same number of eggs as *whiteKD* control females. However, when young females were totally sterile, 52% of the eggs deposited by old females hatched and gave rise to progeny (Figure 5A).

We checked whether TE repression is recovered with age of the mother. TE transcripts were examined by qRT-PCR experiments performed on young and old female ovaries dissected from *bam>aubKD* and *whiteKD* flies. Differences were observed with age and between TGS- and PTGS-TEs. In old *bam>aubKD* females, the level of both TGS- and PTGS-TE expression tends to reach that of WT expression observed in control flies. The level of TGS-TE transcripts increases with age while those of PTGS-TEs significantly decreases. This result suggests that the over-repression of TGS-TEs and the de-silencing of PTGS-TEs observed in flies depleted for Aub within the Pilp are lost with age (Figure 5B).

Recovering a WT control of TEs could be due to a decreased Aub depletion within the Pilp as females age. Using Aub-ab and AGO3-ab, double immuno-fluorescence experiments were performed to verify expression of the PIWI proteins in old females. We found that *aub* depletion in young and old *bam>aubKD* females is similar. Depletion is observed in the germarium as expected for a depletion driven by *bam*. Expression is recovered around stage 4 (Figure 5C, upper lines). It is then similar to WT with an Aub enrichment at the posterior oocyte pole in late egg chambers (Figure 5C, top and middle lines). In *nos>aubKD* ovaries of old females, Aub is depleted within the whole germline and never recovered (Figure 5C, lower line). In young and old

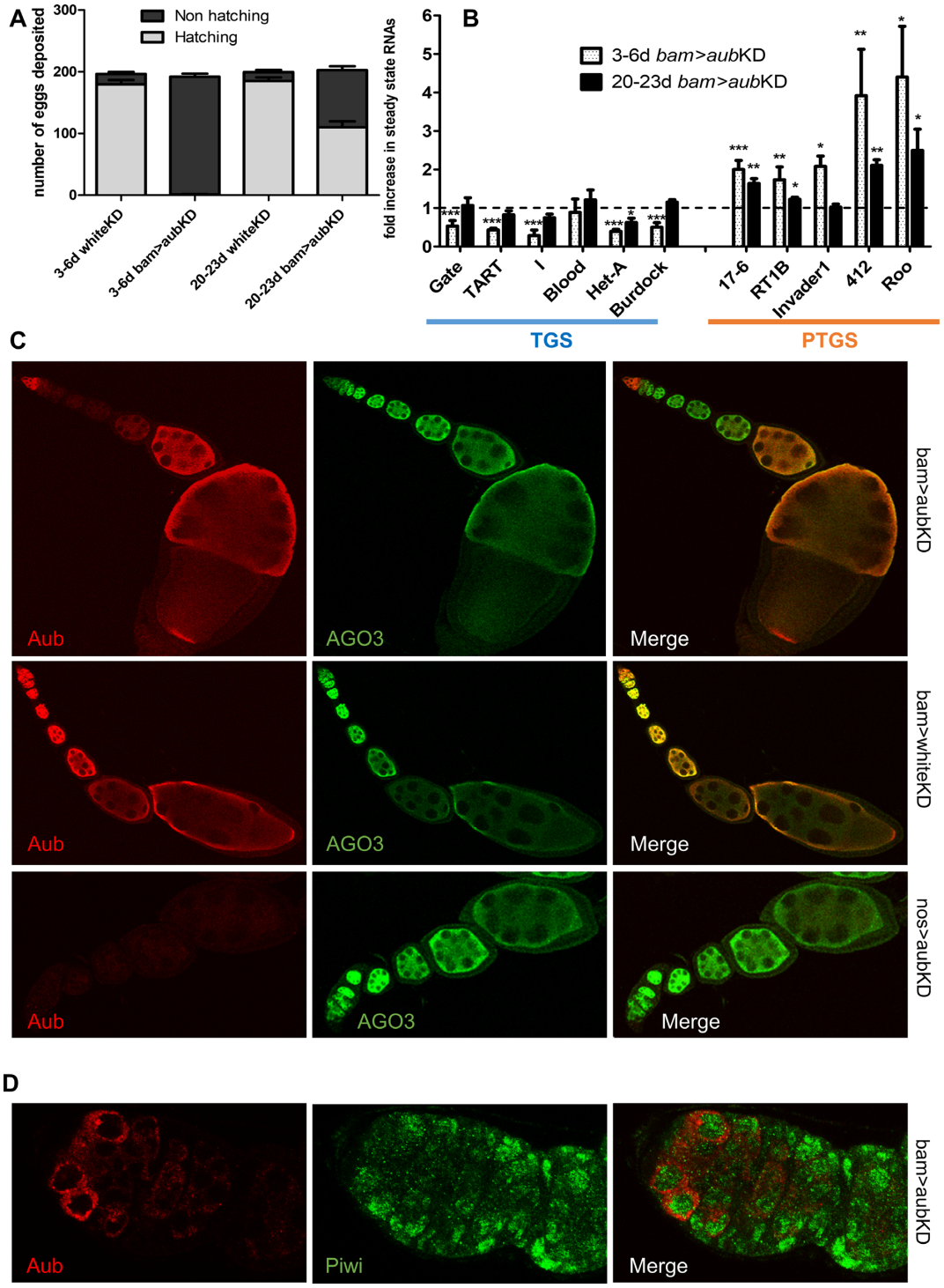

Figure 5. Effect of Aub-depleted Pilp on the fertility of young and old females. (**A**) Fertility of flies depleted for Aub in Pilp was measured in young 3–6 day old females (3–6 days *bam>aubKD*) and in old 20–23 day old females (20–23 days *bam>aubKD*) by counting the number of hatched (grey) and non-hatched (black) 24 h old embryos. (**B**) Fold change in steady state RNA level of TEs from *bam>aubKD* young and old flies. Tested TEs are indicated above the graph (the positions of primers are indicated in Supplementary Table S1). RNA levels are normalized to *bam>whiteKD*. The dotted line corresponds to a fold change of 1 and indicates no change in the RNA level. Statistical tests were made using *t*-test function on R software. * correspond to a *P* value <0.05, ** to a *P* value <0.01 and *** to a *P* value <0.001. (**C**) Effect of *aub* knock-down on Aub (red) and AGO3 (green) expression and localization assessed by immunostaining of control ovaries (*bam>white*KD), of ovaries depleted for Aub in the Pilp (*bam>aub*KD) or in the whole germline (*nos>aub*KD) of 20–23 day old flies. (**D**) Immunostaining of the germarium of a 20–23 day old *bam>aubKD* fly using antibodies against Aub (in red) and Piwi (in green).

females, AGO3 is expressed as in WT (Figure 5C). When Aub and Piwi antibodies were used, contrasting with young females, Piwi expression was found very low within the germarium in germ cells up to stage 16-cell cysts (Figure 5D).

We then considered whether these changes could be correlated with changes in the piRNA profile. We sequenced small RNAs from old *bam>aubKD* ovaries and examined piRNAs. The defects observed in young and old Aub-depleted females were similar but tended to fade with aging (Figure 6A and B). Young and old *bam>aubKD* ovaries both had decreased piRNAs mapping to TEs (67% and 82%, respectively) and ping-pong signature (the median *z*-score for 10nt overlap was 1.72 and 1.95, respectively, for 95% confidence interval). When the two populations of TEs were examined separately, we found that the rate of piRNAs homologous to TGS-TEs did not change with age. By contrast, piRNAs targeting PTGS-TEs increased to reach a WT amount (Figure 6C and D).

When piRNAs mapping to piRNA clusters were examined, the level of piRNAs produced from old *bam>aubKD* flies was similar to that of WT flies (Figure 6E) as reported above for young *bam>aubKD* flies (Figure 4D). Thus, *aub* depletion in the Pilp does not affect piRNA production from major piRNA clusters active in the germline whatever the age.

On the one hand, the decreased TGS-TE silencing with age can be explained by the decrease of Piwi within the germarium. On the other hand, the decrease of PTGS-TE transcription correlates with an increased amount of piRNAs targeting PTGS-TEs and reaching the level of WT flies. Aub/AGO3-bound piRNAs trigger primary piRNA biogenesis (14,15). When Aub is depleted, predominantly AGO3-loaded piRNAs could bring an alternative route for primary piRNA biogenesis.

Since each PIWI-clade protein binds a specific piRNA, we further characterized piRNAs produced in old and young *bam>aubKD* flies, and in old ovaries from both *bam>auKD* and *bam>whiteKD* flies. The 1U and 10A bias was revealed similar in all the populations of piRNAs (Supplementary Figure S8A).

Remarkably, their size distribution varied (Figure 6F). The major 26 nt piRNA size remains stable but a clear increase of 23–25 nts long piRNAs was observed from young to old *bam>aub KD* females. This increase is not detected in old WT females. This indicates that 23–25 nt long piRNAs might be the input required to keep the post-transcriptional capacity of the pathway in aged ovaries. These 23–25 nt long piRNAs are mostly sense piRNAs (Supplementary Figure S8B) in young (66.1%) and old females (64.3%) suggesting that they could be mostly Ago3 bound piRNAs. However, the 10A signature of these 23–25 nt long piRNAs does not change upon aging in *bam>aub*KD flies (Supplementary Figure S7C).

DISCUSSION

Transposable elements (TEs) can mobilize and trigger genetic instability of eukaryotic genomes and disease associated mutations (26,27). Protection mechanisms have evolved that limit TE expression in animal gonads. Any decrease in the silencing capacity of germ cells could be seen as a weakness allowing TE mobilization and genome invasion. It is then surprising to find that in several species, the transcriptional TE silencing machinery is weakened during specific stages of the germline development. Performing a detailed analysis of such a restricted niche of germline development in *Drosophila melanogaster* ovaries, we show that actually, such decrease of TE silencing ensures efficient piRNA biogenesis to limit TE expression in the whole germline.

The relationship between Piwi and Aub in the Pilp plays a pivotal role in piRNA production

In *Drosophila*, we have reported that Piwi expression is lowered in a spatially and developmentally restricted niche of germ cells located in the dividing cysts during Drosophila oogenesis. This niche has been called the Pilp. Our present study brings to light how the relationships between PIWI Argonaute proteins in the Pilp help to produce piRNAs. We show that the presence of Aub is required to decrease Piwi expression in the Pilp. This decrease hampers transcriptional repression exerted on TEs. The resulting mRNAs can then be wired into the ping-pong cycle and give rise to an increased amount of piRNAs due to the presence of Aub and AGO3. Consistently, if Aub is removed from the Pilp, Piwi expression increases together with an increased transcriptional repression of TEs and a decrease of homologous piRNA amplification. An increased amount of Piwi-loaded piRNAs might be processed from piRNA cluster precursors but not engaged in a ping-pong loop for amplification due to the absence of Aub. This hypothesis is supported by the analysis of sense and anti-sense piRNAs when only germline TEs are examined in *bam>aubKD* ovaries. A decrease of TGS-TE sense-piRNAs is detected as expected if the loss of Aub in the Pilp results in an increased transcriptional repression of these TEs triggered by Piwi-loaded anti-sense piRNAs. Interestingly, half of the TGS-TEs examined display anti-sense piRNAs associated to their promoters in both WT and Aub-depleted Pilp. These data reinforce the fact that Piwi-piRNAs indeed engage Piwi-transcriptional silencing within the Pilp. They also suggest that another level of regulation exists which is probably specific to the element itself through its enhancers, the number of active copies or the sites of insertion. If Piwi in addition to Aub is depleted in the Pilp, the absence of both leads to a high release of TGS-TE silencing, much higher than upon the depletion of Piwi or Aub individually. The absence of both Aub and Piwi may compromise the post-transcriptional control, including slicing and piRNA processing, of the produced mRNAs. However, all TEs are not submitted to the Piwi transcriptional control. A few years ago, Senti *et al*. defined a population of TEs which are transcriptionally silenced (referred here to TGS-TEs) (24). Interestingly, TEs which are over-repressed in an Aub-depleted Pilp, belong to this category. A second category, which we called PTGS-TEs (Post-transcriptionally silenced TEs), as opposed to the TGS-TEs, does not require Piwi for its repression. Upon Aub-depletion, PTGS-TEs display a default of silencing evidenced by increased transcription and a severe decrease of their homologous piRNA pool. The presence of Aub in the

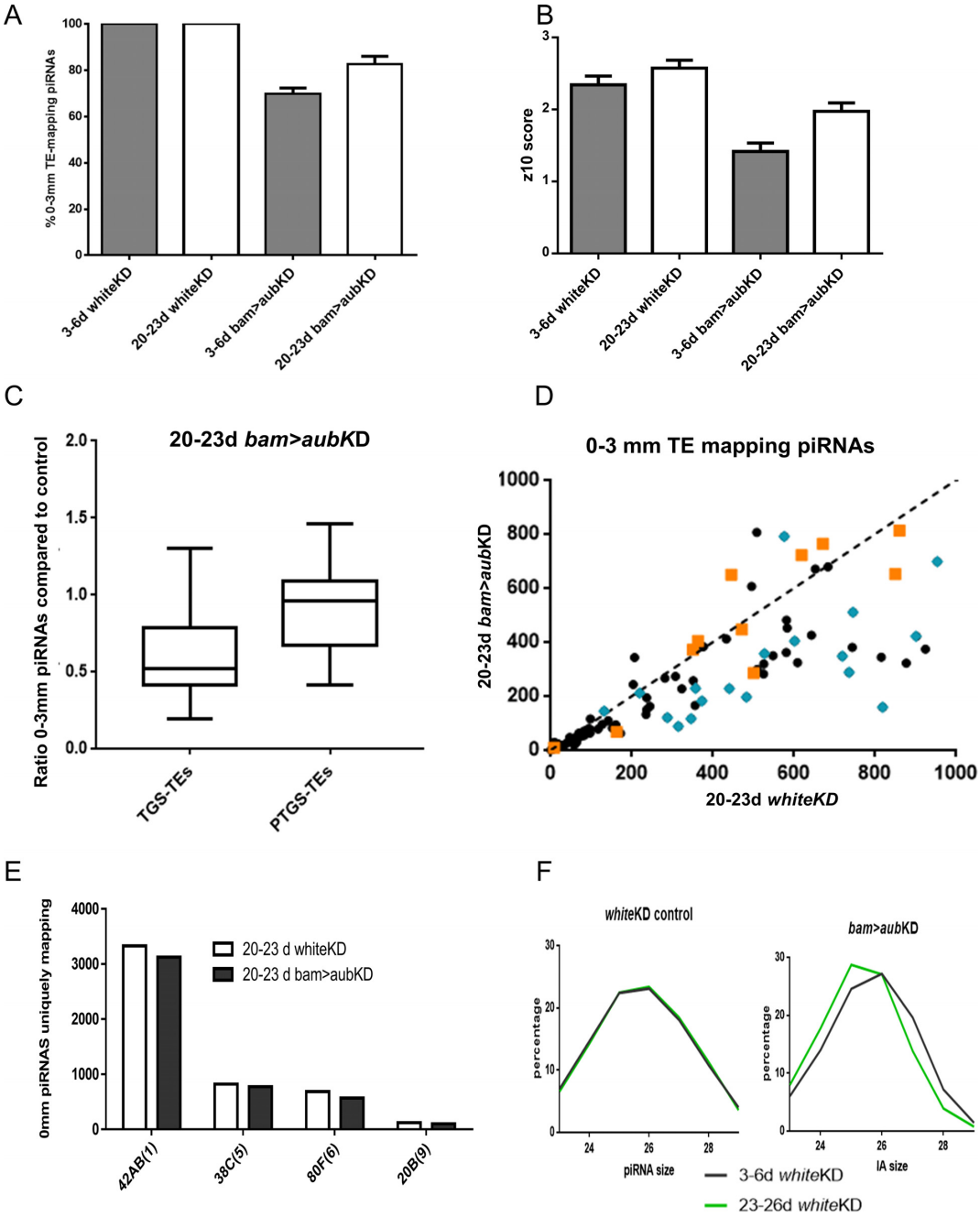

Figure 6. Impact of Aub depletion on piRNA machinery in young and old females. (**A**) Histograms showing the percentage of piRNAs matching on RepBase TEs with 0–3 mismatch (mm) from ovaries of control *whiteKD*, *bam>aubKD* lines in young and old females. (**B**) Histograms showing the z-score of piRNAs with a 10-nt overlap signature from ovaries of *whiteKD*, *bam>aubKD*, *nos>aubKD* old females (20–23 day old). (**C**) Boxplot showing the fold change of the number of 0–3mm piRNA mapping either to TFS-TEs or PTGS-TEs in 20–23 day old *bam>aubKD* compared to *whiteKD* control. (**D**) Scatterplot showing 0–3mm piRNAs mapping to RepBase TEs. piRNAs from 20–23 day old *bam>aubKD* and *whiteKD* ovaries are reported on y- and x-axes respectively. TGS-TEs are represented as blue diamond and PTGS-TEs as orange squares. piRNAs were normalized by the number of unique piRNAs mapping to the somatic cluster *flamenco*. Dotted line represent the standard regression expected if no changes occurred between the conditions and plain black line represents the standard regression observed. TGS-TEs are in blue, PTGS-TEs in orange. (**E**) Histograms showing the number of piRNAs mapping uniquely to the more important germinal piRNAs clusters with 0mm (x-axis) and sequenced from ovaries of *whiteKD*, *bam>aubKD*, *nos>aubKD* old females (20–23 day old). piRNAs were normalized by the number of unique piRNAs mapping to the somatic cluster flamenco. (**F**) Distribution of piRNA sizes from 23 to 29 nt long (X-axis) in young (3–6 days, grey line) or old (20–23 days, green line) in *whiteKD* control (left panel) or *bam>aubKD* (right panel).

Pilp is then required to establish the pool of piRNAs which silence both TGS- and PTGS-TEs in WT flies.

Interestingly the increased silencing exerted on TGS-TEs upon Aub depletion indicates that Piwi silencing is the dominant input for their control. Our data bring no clue to know why Aub leads to Piwi decrease only in the Pilp but suggest that another factor is required to provide this cell specificity. It has been reported that the failure to load Piwi with a piRNA prevents Piwi nuclear accumulation and leads to its cytoplasmic accumulation and ultimately degradation (1112). In a complementary experiment, we assessed the role of piRNAs in the Pilp and their translocation into the nucleus of WT flies. Transgenic flies expressing a point mutant of Piwi, referenced as Piwi-YK, fused to GFP were used. The transgenic *GFP-Piwi-YK* does not associate with piRNAs due to a substitution of two conserved amino acid residues (Y551L and K555E) and is found in the cytoplasm (28). Staining due to GFP-Piwi-YK and endogenous Piwi were found similar (Supplementary Figure S9). They were both observed within all the germline either in the cytoplasm or in the nucleus respectively, with the exception of the Pilp where they were undetectable. Thus the piRNA population present within the Pilp is not involved in the destabilization of Piwi in the Pilp. Further analysis is then needed to evaluate which additional factors are required to establish the link between Aub and Piwi within the Pilp.

Aub depletion in the Pilp leads to female sterility, which decreases with aging

Depleting Aub specifically in the Pilp leads to female sterility. These latter lay the same number of eggs as the WT control but the eggs do not hatch. Interestingly, they partially recover fertility after 20 days. This is reminiscent of the sterility observed in the I-R system of hybrid dysgenesis (29). The I-R system involves two categories of *D. melanogaster* flies. One category comprises 'inducer' strains that contain genomic copies of the non-LTR retrotransposons called *I-factor*. The second category, called 'reactive' strains, is devoid of functional *I-elements*. Sterility is observed in the female progeny (SF females) of crosses performed between reactive females and inducer males. This sterility is associated with a high transposition rate of the *I-factor* and an increased mutation rate. Like females depleted for Aub in the Pilp, SF females lay eggs which do not hatch but the hatching percentage increases regularly as the females age and can eventually reach control values (30). Grentzinger *et al.* reported that aged ovaries produce more *I-factor* piRNAs (31). They proposed two hypotheses to explain this increased production of piRNAs. First, cellular aging can affect relationships between mitochondria and the piRNA machinery leading to improved piRNA production in the whole germline. Second, the successive divisions of this immortal cell line might lead to an increased amount of piRNAs accumulating with age in GSC and transmitted to the cystoblasts. Interestingly, the depletion of Aub in the Pilp not only recapitulates the sterile phenotype of SF females but also major molecular changes observed with aging. Not restricted to the *I-factor*, the general percent of piRNAs observed in aged compared to young ovaries increases. This percent mostly concerns piRNAs homologous to PTGS-TEs and likely explains why PTGS-TE repression is recovered and reaches levels found in WT flies. Interestingly, the piRNA length varies with an increase of piRNAs from 23 to 25 nt long while 26 nt remain stable. PIWI clade Argonaute proteins are known to bind different lengths of small RNAs with 24, 25 and 26 nt long piRNAs preferentially bound onto AGO3, Aub and Piwi, respectively (3). It has been reported that AGO3 can compensate to some extent the loss of Aub (24). With age, the increase of 23–25 nt piRNAs could reflect that AGO3 may compensate the absence of Aub to trigger piRNA biogenesis. The control exerted on TGS-TEs whose over-repression appears to be lost and replaced by a repression similar to WT remains more obscure. Although a low rate of piRNAs compared to WT is still observed, the decreased Piwi expression in old germarium suggests that TE transcriptional repression is lost. The specific increase of 23–25 nt long piRNAs homologous to TGS-TEs might then be sufficient to exert a post-transcriptional repression reaching a control value similar to WT. At this stage of our study, we cannot attempt to explain why the absence of Aub in old ovaries does not correlate with a stable Piwi expression as in young females. Is there a factor increasing with age that acts to decrease Piwi expression independently of Aub? In the contrary, is there a factor preventing Aub to interfere with Piwi expression. Additional data are needed at this stage to understand Piwi regulation in the Pilp and its variation with aging.

Overall our data point the fact that weaknesses in the piRNA pathway in the germ line are useful to wire TE RNAs for piRNA cleavage and ping-pong amplification. They might also be primary steps at the crossroad of the molecular mechanisms that sense environmental changes, such as aging, to ultimately protect the germline all along the life. One can anticipate that the relationships established between Piwi and Aub during early stages of germ line development which assure both TE silencing and piRNA amplification will be discovered in other organisms.

DATA AVAILABILITY

Data has been deposited to the Sequence Read Archive under accession number SRP144288 (https://www.ncbi.nlm.nih.gov/sra/SRP144288).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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