

## scientific report

Maternally deposited germline piRNAs silence the *tirant* retrotransposon in somatic cellsAbdou Akkouché<sup>1†</sup>, Thomas Grentzinger<sup>2</sup>, Marie Fablet<sup>1</sup>, Claudia Armenise<sup>2</sup>, Nelly Burlet<sup>1</sup>, Virginie Braman<sup>1</sup>, Séverine Chambeyron<sup>2+</sup> & Cristina Vieira<sup>1,3++</sup><sup>1</sup>Université de Lyon, Université Lyon 1, CNRS UMR5558, Laboratoire de Biométrie et Biologie Evolutive, Villeurbanne,<sup>2</sup>Institut de Génétique Humaine, Centre National de la Recherche Scientifique, Montpellier, and <sup>3</sup>Institut Universitaire de France, Paris, France

**Transposable elements (TEs), whose propagation can result in severe damage to the host genome, are silenced in the animal gonad by Piwi-interacting RNAs (piRNAs). piRNAs produced in the ovaries are deposited in the embryonic germline and initiate TE repression in the germline progeny. Whether the maternally transmitted piRNAs play a role in the silencing of somatic TEs is however unknown. Here we show that maternally transmitted piRNAs from the *tirant* retrotransposon in *Drosophila* are required for the somatic silencing of the TE and correlate with an increase in histone H3K9 trimethylation an active *tirant* copy.**

**Keywords:** histone modifications; piRNA; somatic regulation; transposable elements

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## INTRODUCTION

Transposable elements (TEs) are major structural elements present in essentially all eukaryotic genomes [1]. Active TEs are highly mutagenic, and their silencing is necessary to ensure genetic stability [2,3]. Recent studies have shown that TE repression in *Drosophila* gonads is dependent on a small non-coding RNA pathway. This so-called piRNA pathway is on the basis of the cooperation of Argonaute proteins from the PIWI clade, which act together with PIWI-interacting RNAs (piRNAs) to target and silence active TEs [4,5]. Two distinct piRNA pathways have been identified in the *Drosophila* ovary. In the somatic tissue surrounding the germline cells, called follicle cells, piRNAs are exclusively produced by the primary biogenesis. Putative

single-stranded precursor transcripts derived from the transcription of piRNA clusters are processed randomly into 23- to 29-nucleotide primary piRNAs that are then loaded onto Piwi, the only PIWI protein expressed in somatic cells [2,6–10]. This pathway is involved in the regulation of several endogenous retroviruses, such as *gypsy*, *ZAM* and *Idefix* [5,11–14], that, when derepressed, can infect the germline [15,16]. The secondary piRNA biogenesis is on the basis of the existence of an efficient feed-forward amplification loop called the ping-pong cycle. This mechanism involves Aubergine (Aub) and Argonaute 3 (Ago3), two PIWI proteins specifically expressed in germline cells [6,17]. The initiators of the ping-pong cycle can be either primary piRNAs produced in the germline cells [18] and/or maternally transmitted piRNAs [14,18,19]. Germline piRNAs have been reported to be maternally deposited in the embryo, and such transmission has a role in the regulation of germline TEs in the offspring [20]. Here, we report that these maternally deposited piRNAs are also involved in the silencing of somatic TEs in the offspring.

## RESULTS AND DISCUSSION

To investigate the role of maternally transmitted piRNA in somatic TE silencing, we studied the regulation of the *Drosophila simulans tirant* element. We used the Chicharo strain, which is free of functional *tirant* copies, and the Makindu strain, which contains several full-length euchromatic copies of *tirant* dispersed over the chromosome arms [21,22]. These copies are expressed in both germline and somatic cells but are not translated, and no *tirant* envelope protein could be detected by immunostaining experiments using whole-mount ovaries, suggesting that *tirant* is repressed at a post-transcriptional level [21] (supplementary Fig S1 online). Similar germline repression with the nuclear accumulation of TE transcripts in the nurse cell nucleus has been previously described in the inducer strain of the I-R hybrid dysgenesis system of *Drosophila melanogaster* [23].

Given that *tirant* is a retrovirus-like element whose translation occurs in follicle cells [21], its repression should be exclusively dependent on the primary piRNA pathway and so be similar to that of the *gypsy* and *ZAM* elements previously described in *D. melanogaster* [6,8,11,13,14]. Therefore, the offspring from

<sup>1</sup>Université de Lyon, Université Lyon 1, CNRS UMR5558, Laboratoire de Biométrie et Biologie Evolutive, F-69622 Villeurbanne, France<sup>2</sup>Institut de Génétique Humaine, Centre National de la Recherche Scientifique, 34396 Montpellier, France<sup>3</sup>Institut Universitaire de France, Paris, France<sup>†</sup>Present address: Institut de Génétique Humaine, Centre National de la Recherche Scientifique, 34396 Montpellier, France<sup>+</sup>Corresponding author. Tel: +33 4 34 35 99 49; Fax: +33 4 34 35 99 01;

E-mail: severine.chambeyron@igh.cnrs.fr

<sup>++</sup>Corresponding author. Tel: +33 4 72 44 81 98; Fax: +33 4 72 43 13 88;

E-mail: cristina.vieira@univ-lyon1.fr

reciprocal crosses between Chicharo and Makindu populations should have the same *tirant* mRNA expression levels in the somatic cells (Fig 1A). Using quantitative reverse transcription PCR (RT-qPCR) on total RNA from F1 daughter ovaries, we observed that there were five times more *tirant* mRNAs in the F1 ovaries of Chicharo females mated with Makindu males than in the F1 ovaries of the reciprocal crosses (Fig 1B). This observation could be easily explained by the loss of the post-transcriptional silencing of the functional germline *tirant* copies in the Makindu strain when introduced into the naive Chicharo genome, as previously described for the *I element* in *D. melanogaster* [23]. Using *in situ* hybridization experiments on F1 daughter ovaries, we show that *tirant* mRNA expression occurred specifically in the follicle cells of the F1 ovaries of Chicharo females mated with Makindu males (referred to as 'Non-Regulated *Tirant*' (NRT) cross in the remainder of the manuscript), whereas *tirant* silencing was observed in the reciprocal cross (called 'Regulated *Tirant*' (RT) cross) (Fig 1A,C). We further showed that the strong follicle *tirant* mRNA expression was associated with the production of the envelope protein that localized in the somatic cells of the F1 daughters of the NRT cross (Fig 1D). In both NRT and RT crosses, the functional *tirant* copies were post-transcriptionally silenced in germline cells, where the *tirant* envelope protein was never detected (Fig 1C,D).

To determine whether the germline piRNA pathway was disrupted in NRT daughter ovaries, we assayed localization of the Ago3 and Vasa, two proteins involved in the piRNA pathway and localized to the nuage (Fig 1E). There was no difference in staining between F1 ovaries of NRT and RT crosses showing that the follicle *tirant* derepression was not a consequence of the disruption of piRNA biogenesis machinery in the germline as previously reported for P-M hybrid dysgenesis [24]. Taken together, these results indicate a maternal effect on the somatic repression of *tirant*.

To study the maternally transmitted piRNA pool following NRT and RT crosses, we performed high-throughput sequencing of small RNAs isolated from Chicharo and Makindu parental ovaries (supplementary Fig S2 online) and from the 0- to 2-h embryos laid by NRT and RT crosses (Figs 1A, 2).

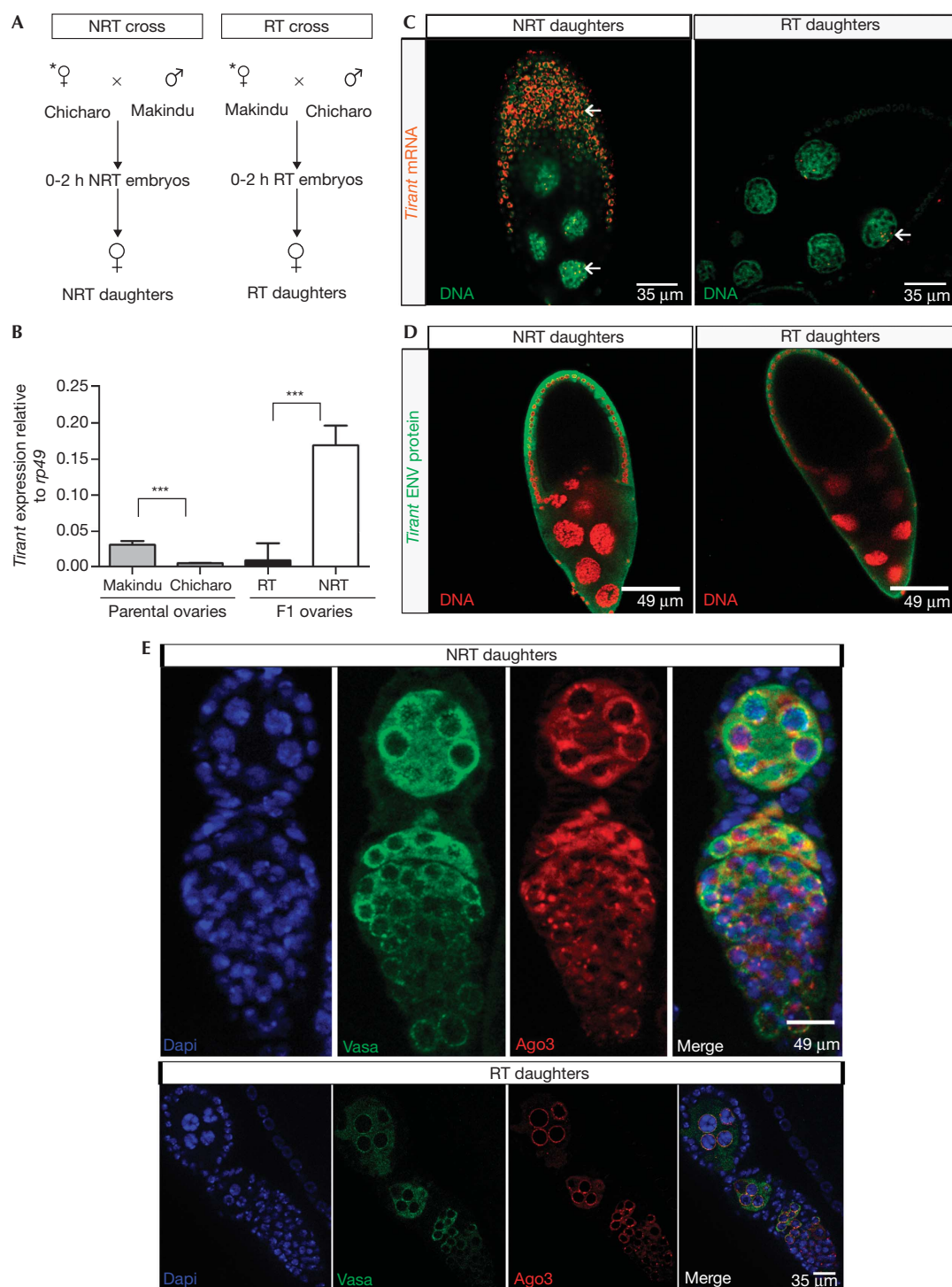
To account for differences in sequencing quality and depth, we normalized the small RNA populations to one million piRNAs as previously published [18,19] (supplementary information online). We compared the fold changes in piRNA abundances for each of the 85 major TEs between 0- and 2-h embryos laid by NRT and RT crosses using both a heatmap and a scatter plot that revealed only small differences in piRNA accumulation for the 85 studied TEs except for *tirant* piRNAs (Fig 2A,B). The embryonic levels of *tirant* piRNAs were fivefold greater in the embryos laid by RT crosses than in the embryos laid by NRT crosses, reflecting the differences in piRNA observed in the parental ovaries (Fig 2B; supplementary Fig S2 online and supplementary Table S1 online). These observations suggested that *tirant* derepression in F1 daughters of the NRT cross was specific to *tirant* element.

Embryos laid by NRT and RT crosses and both parental ovaries exhibited a strong ping-pong signature suggesting that in Chicharo strains *tirant* secondary piRNA biogenesis was efficiently achieved by defective *tirant* copies as previously described for the *I element* in the reactive strain after aging treatment [19] (Fig 2C;

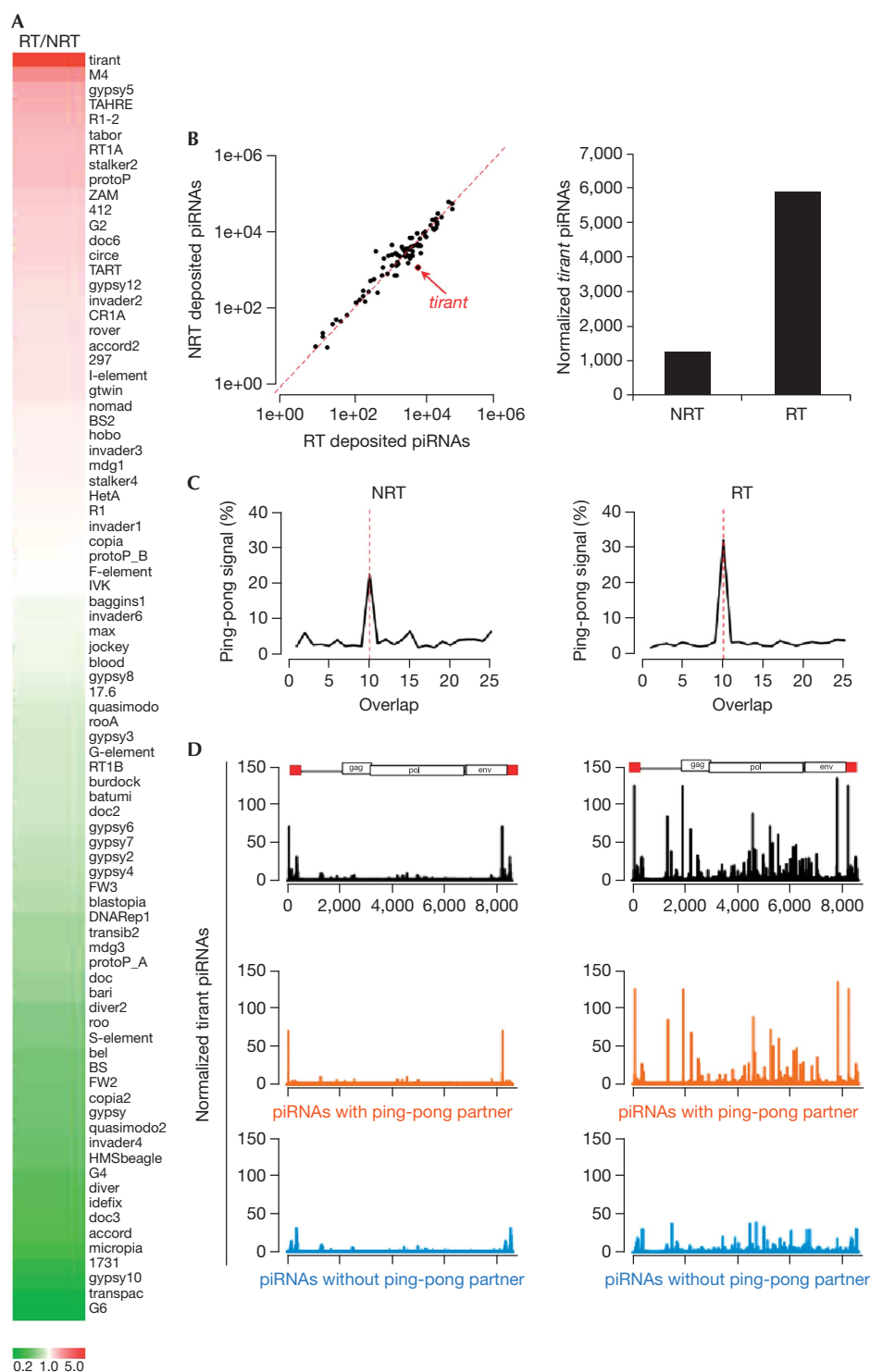
supplementary Fig S2 online). To further characterize this phenomenon, we split the *tirant* piRNA populations into piRNAs that had at least one sequenced ping-pong partner (that is, another piRNA containing a reverse complementary 5' 10-mer; supplementary information online) and all the others (Fig 2D). Interestingly, we observed that *tirant* secondary piRNAs in both Chicharo strain and embryos laid by NRT crosses mapped to long terminal repeat (LTR) sequences suggesting they were produced by short fragments or solo LTRs [25].

The post-transcriptional germline regulation of *tirant* in both NRT and RT daughters (Fig 1C,D) suggests either that the amount of maternally deposited piRNAs in embryos laid by Chicharo females is sufficient to initiate the amplification loop in the germline [19] or that the initiation of this amplification loop is dependent on primary piRNAs only [18]. However, the deficit in maternally deposited *tirant* piRNAs in embryos laid by Chicharo females was associated with the activation of this TE in the somatic follicle cells of F1 NRT crosses. We further investigated whether the *tirant* derepression was already noticeable in the NRT embryos. We therefore measured the amount of *tirant* mRNAs in 12- to 16-h embryos from RT and NRT crosses (Fig 3A). We found that embryonic expression of *tirant* was significantly lower in embryos derived from RT crosses than those derived from NRT crosses ( $P < 0.05$ ). We then compared the *tirant* expression profile by *in situ* hybridization on whole-mount embryos from Makindu, RT and NRT crosses (Fig 3B). We observed that, in NRT embryos, *tirant* mRNA was specifically expressed in several tissues and particularly in gonadal cells, while no *tirant* transcript was detected in RT embryos (Fig 3B). Moreover, *tirant* mRNAs accumulate in follicle cell precursors as its expression profile corresponds to *Traffic jam* profile that is a somatic-specific marker surrounding Vasa germline-specific marker (Fig 3C,D). These data support the hypothesis that maternally deposited *tirant* piRNA-dependent regulation takes place in follicle precursor cells in early *Drosophila* development.

Small RNAs associated with Argonaute proteins can guide sequence-specific chromatin modification and thus contribute to the establishment and maintenance of distinct chromatin domains [26]. In *Drosophila*, the role of the PIWI protein in heterochromatin formation, as well as a direct interaction between PIWI and the HP1 protein, have been reported [26–30]. Recently, Piwi has been described to be involved in TE silencing at the transcriptional level [31], confirming previous results showing that PIWI TE regulation is independent of its slicing activity [32,33]. To determine whether *tirant* repression occurred at a transcriptional level in somatic cells, we studied the chromatin state of a functional full-length *tirant* insertion located in an intron of the *tkv* gene (chromosome 2L) (Fig 4A) [25]. ChIP experiments using RT and NRT daughter ovaries revealed a significant increase in H3K9me3 in RT samples compared with NRT, both in the 5'- and 3'-flanking regions of the euchromatic copy of *tirant*, consistent with the silent state of *tirant* in the offspring of the RT crosses. By contrast, in the offspring of the NRT crosses, in which maternally deposited *tirant* piRNA level was low, the level of H3K9me3 enrichment remained low, and this low level of H3K9me3 was associated with the expression of *tirant* (Fig 4B). To determine whether this chromatin change was specifically correlated with the amount of Piwi-associated *tirant* piRNAs, we compared by RT-qPCR the level of two *tirant* piRNAs in total RNA extraction from ovaries

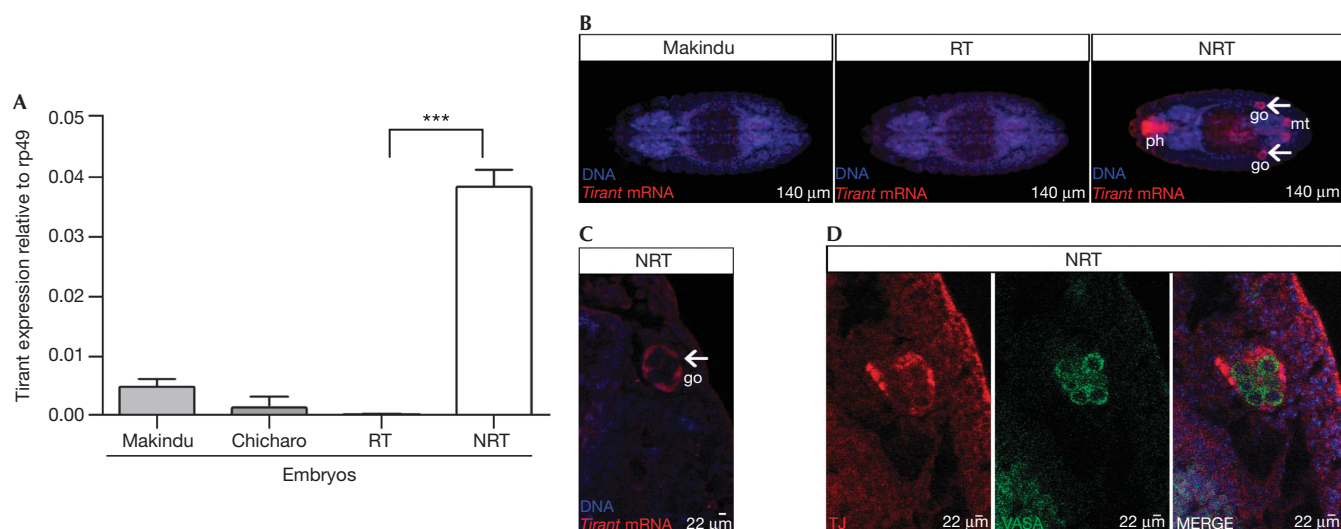


**Fig 1** | Progenies of crosses between two wild-type strains of *D. simulans* show different *tirant* expression. (A) Schematic representation of the NRT (non-regulated *tirant*) and RT (regulated *tirant*) crosses. (B) Quantitative real-time PCR of randomly reverse-transcribed total RNA from the ovaries of the parental strains (grey bars), NRT (white bar) and RT (black bar) daughters. *tirant* transcript levels are expressed relative to an internal *rp49* mRNA control ( $n = 3$ ; error bars mean  $\pm$  s.d., two-tailed Student's *t*-test, \*\*\* $P < 0.0001$ ). (C) RNA FISH on whole-mount ovaries from NRT (left panel) and RT (right panel) daughters. *tirant* transcripts are in (red) and DNA is labelled in green (Sytox Green). White arrows indicate *tirant* transcripts localization. (D) Immunostaining of *tirant* envelope protein (green) in ovaries from NRT (left panel) and RT (right panel) daughters. DNA is stained in red (propidium iodide). (E) No disruption of sub-cellular organization of the germline transposon silencing machinery in both NRT (upper panel) and RT (lower panel) daughters. Immunostaining of Vasa (green) and Ago3 (red) proteins. DNA is labelled with 4,6-diamidino-2-phenylindole (DAPI; blue).

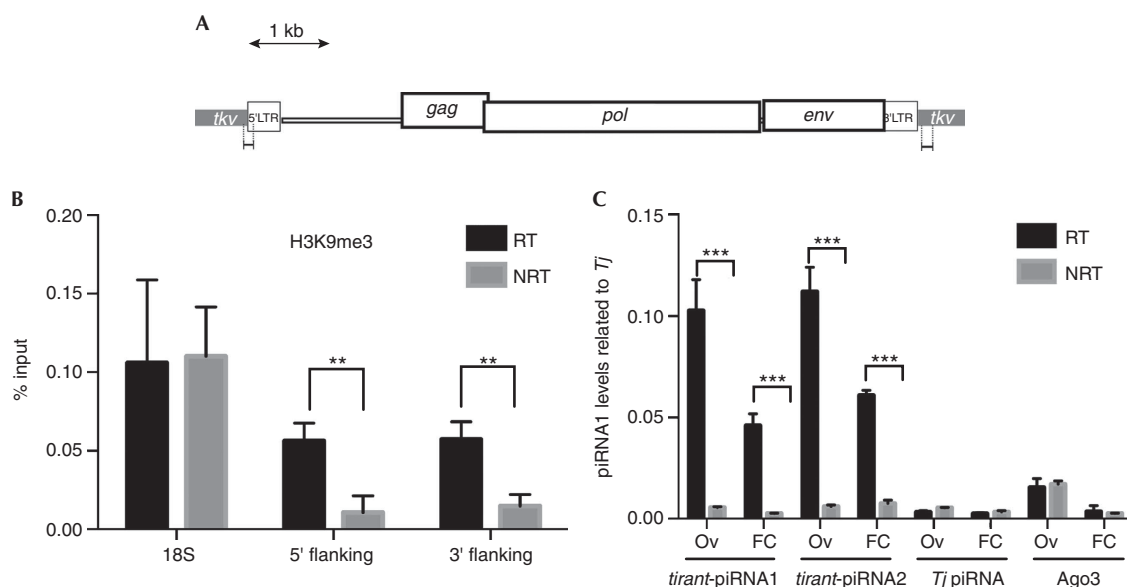


**Fig 2** | Differences in *tirant* Piwi-interacting RNA (piRNA) populations between regulated *tirant* (RT) and non-regulated *tirant* (NRT) embryos reflect a difference in maternal piRNA populations. (A) Transposon piRNA ratios between RT and NRT embryos are shown as a heatmap for each of the 85 most highly targeted *Drosophila* transposable elements (TEs) (up to four mismatches allowed between reads and RepBase sequences). (B) (Left) Pairwise comparison of the TE piRNA levels in NRT and RT embryos. A scatter plot displays correlation between normalized piRNA abundances for each of the 85 major TEs. (Right) Normalized counts of *tirant* piRNAs in NRT and RT embryos. (C) Both maternally deposited *tirant* piRNA populations displayed a strong ping-pong signature. (D) Density profile of piRNAs matching *tirant* sequence (up to four mismatches) split into piRNAs with ping-pong partners (orange) and other piRNAs (blue) (see supplementary information online for ping-pong partner bioinformatic identification). *Tirant* structure with *gag*, *pol* and *env* open-reading frames is reported (red rectangles represent long terminal repeats).





**Fig 3** | *tirant* expression in the somatic gonadal cells. (A) Quantitative real-time PCR of randomly reverse-transcribed total RNA from 12- to 16-h embryos of Makindu and Chicharo parental strains (grey bars) and of regulated *tirant* (RT; black bar) and non-regulated *tirant* (NRT; white bar) crosses. *tirant* transcript levels are expressed relative to an internal *rp49* mRNA control ( $n=3$ ; error bars mean  $\pm$  s.d., two-tailed Student's *t*-test,  $***P<0.0001$ ). (B,C) RNA FISH on whole-mount 12- to 16-h embryos from Makindu strain (left panel), from RT (middle panel) and NRT (right panel) crosses. *tirant* transcripts were labelled in red. Anterior is to the left in all panels. DNA is labelled with 4,6-diamidino-2-phenylindole (DAPI; blue). White arrows indicate gonadal cells; go, gonad; ph, pharynx; mt, Malpighian tubules. (C) Higher magnification of gonadal cells of NRT embryos expressing *tirant* transcripts (scale bar, 22  $\mu$ m). (D) Double immunostaining on 12- to 16-h embryos using anti-Traffic Jam (TJ) antibody (red) and anti-Vasa antibody (green) labelled the somatic and the germline gonadal cells, respectively. DNA is labelled with DAPI (blue).



**Fig 4** | *tirant* repression correlates with the accumulation of both H3K9me3 on its active sequence and PIWI-associated *tirant* piRNAs in follicle cells. (A) Structure of *tirant* with gag, pol and env domains, inserted in the *tkv* gene. The positions of the amplicons obtained in ChIP experiments are indicated by the bottom lines. (B) Chromatin immunoprecipitation assay using chromatin extracts from the ovaries of the offspring of regulated *tirant* (RT; black bars) and non-regulated *tirant* (NRT; grey bars) crosses. H3K9me3-relative enrichment was studied in three regions: the 5'- and 3'- flanking regions of the full-length *tirant* insertion in the *tkv* gene and in the *18S* gene. Enrichment is computed relative to the input ( $n=3$ ; error bars mean  $\pm$  s.d., two-tailed Student's *t*-test,  $**P<0.001$ ). (C) Quantitative real-time PCR of reverse-transcribed total RNA from ovaries (Ov) and purified follicle cells (FC) of the offspring of RT (black bars) and NRT (grey bars) crosses. piRNA levels and Ago3 mRNA are expressed relative to *Traffic jam* mRNA ( $n=3$ ; error bars mean  $\pm$  s.d., two-tailed Student's *t*-test,  $***P<0.0001$ ).

and from purified follicle cells of RT and NRT daughters (Fig 4C). The level of *tirant* piRNAs was significantly higher in both total ovaries and purified follicle cells of RT crosses compared with NRT crosses. The loss of Ago3 germinal-specific mRNA in isolated follicle cells is around three times more important than the loss of *tirant* piRNAs. These observations indicated that PIWI-associated *tirant* piRNAs detected in the follicle cells correlates with somatic *tirant* repression in progeny of RT crosses.

Our results support the hypothesis that in *D. simulans*, maternally inherited piRNA populations promote effective responses against TE invasion in progeny, as previously reported for *D. melanogaster* [19,20] and *D. virilis* [34]. Unexpectedly, our study revealed a critical role for maternally deposited *tirant* piRNAs in the silencing of the *tirant* retrovirus-like element in somatic cells that correlates with changes in the chromatin of *tirant*-active copy. These results raise new questions about the role of maternally deposited piRNAs in somatic regulation of TEs across generations and, more particularly, the mechanism with which maternal piRNAs can epigenetically regulate TE expression in the somatic cells remains to be elucidated.

## METHODS

**Drosophila strains.** We used samples from two wild-type strains of *D. simulans*: Chicharo (Portugal) and Makindu (Kenya). These strains were maintained in the laboratory at 24 °C as isofemale lines. We previously fully characterized the *tirant* copies, insertion sites [25] and expression patterns [21] in these strains.

**RNA fluorescence in situ hybridization (FISH) and immunostaining.** RNA FISH on ovaries was carried out according to a previously described procedure [21]. More details are available in supplementary information online.

**Preparation of follicle cells.** Follicle cells were isolated as described in Bryant *et al* [35]. Briefly, 50–100 pairs of ovaries were dissected in Schneider's medium 10% FBS (Gibco) and washed three times in 1 × PBS. After incubation in 0.5% trypsin in PBS for 15 min at RT with discontinuous vigorous shaking, the supernatant was removed, filtered through a 40-µm nylon mesh into tubes containing Schneider's medium, pelleted at 1,000 g for 7 min and resuspended in 1 ml of Schneider's medium 10% FBS, washed two times and stored on ice before Trizol RNA extraction.

**Isolation of total RNAs, synthesis of complementary DNA and quantitative RT-PCR analysis.** Total RNAs of 100 or 50 ovaries pairs were extracted by Trizol for quantification of *tirant* mRNA and *tirant* piRNAs, respectively. cDNA synthesis for *tirant* mRNA quantification was performed as previously described in Akkouché *et al* [21].

cDNA synthesis for *tirant* piRNA quantification was carried out according to Balcells *et al* [36] with the following modifications: 100 ng of total RNA was reverse transcribed in a 10-µl reaction volume containing 10 × poly(A) buffer, 0.1 mM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP), 100 units of Superscript II (Invitrogen), 0.1 mM of ATP, 1 µM of RT-primer (5'-CAGGTCCAGTTTTTTTTTTTTTTVN; V is A, C and G; N is A, C, G and T; the primer was purchased from TAG Copenhagen (Denmark)) and 1 unit of poly(A) Polymerase (BioLabs), incubated for 37 °C for 10 min, followed by incubation of 95 °C for 50 min, and 70 °C for 15 min.

For piRNA quantification, PCR was performed in 10 µl with 0.2 µl of cDNA, using LightCycler 480 SYBR Green I Master

(Roche). Cycling conditions (95 °C for 8 min, 45 cycles (95 °C for 10 s) and 60 °C for 30 s)). Each experiment was performed in biological triplicates with technical triplicates. RNA levels were calculated relative to rp49 or to Traffic jam-positive controls for *tirant* mRNA and *tirant* piRNAs, respectively. Primers for quantitative RT-PCR are listed in supplementary Table S2 online. Data were analyzed with LightCycler software (Roche).

**Chromatin immunoprecipitation and qPCR analysis.** ChIP assay was performed as previously described [37]. The sheared chromatin was incubated overnight at 4 °C with antibodies against H3K9me3 (ab8898, Abcam) and rabbit IgG (Sigma-Aldrich I5006). Real-time PCR was performed as above. Reactions were performed in triplicate on three independent biological samples, and standard curves were calculated from serial dilutions of the input. The relative enrichment of the 18S gene and *tirant* insertion-flanking sites with H3K9me3 was calculated as a ratio to the input gene enrichment. Primers are listed in supplementary Table S2 online.

**Small RNA purification.** Small RNA purification for RNA-seq was performed as previously described in Grentzinger *et al* [19]. More details are available in supplementary information online.

**Statistical analyses.** All statistical analyses were performed using R software [38]. *P*-values were calculated using two-tailed Student's *t*-test after testing normal distribution (Shapiro–Wilk test) and variance homogeneity (Levene's test).

**Bioinformatic analysis of small RNA libraries.** Our pipeline was on the basis of the analytical strategy, which has been previously described (Grentzinger *et al* [19] and supplementary information online). Heatmaps were drawn using MultiExperimentViewer application [39,40].

**Data access.** Small RNA libraries generated in this study are available through the NCBI Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>) under accession no. GSE40592, data sets GSM997551 to GSM997554 to GSM850354.

**Supplementary information** is available at EMBO reports online (<http://www.emboreports.org>).

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**Author contributions:** A.A. performed, designed experiments, analyzed data, wrote paper; T.G., N.B. and V.B. performed experiments; M.F. analyzed data; C.A. performed all bioinformatic analyses; S.C. analyzed data, designed experiments, wrote paper; C.V. analyzed data, designed experiments and wrote the paper.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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