Antisense piRNA amplification, but not piRNA production or nuage assembly, requires the Tudor-domain protein Qin

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Qin is required for transposon silencing by the PIWI-interacting RNA (piRNA) pathway (Zhang et al., 2011; Anand & Kai, 2012). Initial descriptions of qin mutants led to conflicting explanations for the role of Qin in piRNA biogenesis. One study suggested that loss of Qin causes the accumulation of sense piRNAs instead of antisense without altering total piRNA levels or perturbing the localization of Aub and Ago3 to the perinuclear nuage (Zhang et al., 2011). A second report concluded that both piRNAs and nuage were lost from the germline in qin mutants, leading to a complete failure of the piRNA pathway (Anand & Kai, 2012). We re-analyzed the qin alleles used in the two studies: qin1, qin2 (Zhang et al., 2011) and qinkumo (Anand & Kai, 2012). These analyses corroborate our original findings that the fundamental defect in qin mutants is not a loss of piRNAs, but rather the replacement of heterotypic Aub:Ago3 with non-productive, homotypic Aub:Aub. Our data suggest that the phenotypes reported for qinkumo homozygotes are caused by a secondary mutation unlinked to qin.

Compared with genotypically matched w1118 and qinkumo/TM3 controls, homozygous qinkumo mutant ovaries are small, with few egg chambers beyond stage 10 (Supplementary Fig S1). In contrast, qin1, qin2, qinkumo in trans to a complete deletion of the qin locus (Df(3R)Excel6180; henceforth, Df), as well as qin1/qinkumo and qin2/qinkumo, all had normal ovary size and shape. qinkumo/qinkumo females laid almost no eggs (one egg per female on day 2, and none thereafter), yet qinkumo/Df females each laid approximately 50 eggs per day. Typically, the phenotype of a strong mutant allele remains the same or worsens in trans to a deficiency, but qinkumo/qinkumo was more severe than qinkumo/Df. Potential explanations include (i) qinkumo is a neomorph; (ii) the Df(3R) Excel6180 deficiency fails to uncover the entire qin gene; and (iii) qinkumo contains a secondary mutation unlinked to qin.

Our data support the idea that qinkumo is a null mutation and that Df(3R)Excel6180 removes all of qin: RNA-seq detected no qin mRNA in qinkumo/Df ovaries (Fig 1A). The qin1 allele results from a piggyBac transposon insertion and produces a truncated mRNA 4432 nt long. As anticipated, qin1/Df ovaries produced a approximately 4400 nt RNA less than half as abundant as qin mRNA in w1118 (12 versus 32 rpkm). The qin deficiency extends beyond the 5′ end of qin, disrupting the upstream gene CG7694: CG7694 mRNA abundance was 12 rpkm in w1118 but only 4.2 rpkm in qin1/Df and 3.6 rpkm in qinkumo/Df. We conclude that both qinkumo and Df(3R)Excel6180 are null alleles of qin and that a secondary mutation unlinked to qin is present on the qinkumo chromosome.

By immunofluorescence antibody staining, Ago3 and Aub were present in nuage in all genotypes tested except qinkumo/qinkumo (Fig 1B). For example, qinkumo/Df, Ago3 was correctly localized to perinuclear foci in 62 of 67 nurse cells among 12 separate egg chambers, compared with 66 of 70 in 16 separate egg chambers from qinkumo/TM3 flies and 96 of 102 in 20 separate egg chambers from w1118 ovaries; Aub was present in perinuclear puncta typical of nuage in 50 of 57 nurse cells from nine qinkumo/Df egg chambers compared to 61 of 68 nurse cells from nine qinkumo/TM3 egg chambers and 90 of 98 from 20 w1118 egg chambers. Aub localizes to the posterior end of the oocyte in late-stage egg chambers, and this localization was preserved in qin mutants (Supplementary Fig S2). In contrast, qinkumo homozygotes showed mislocalized Ago3 and Aub as previously reported (Fig 1B; Anand & Kai, 2012).

To provide an independent test of whether qinkumo/Df disrupts nuage, we monitored the

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Figure 1. Without Qin, Ago3, Aub and Vasa still reside in nurse cell nuage.
A RNA-seq data for wild-type and qin mutant ovaries.
B Ago3 and Aub immunostaining or live EGFP-Vasa image in qin mutants. EGFP-Vasa fusion protein was expressed from a transgene using the vasa promoter.
localization of GFP-Vasa, a nuage marker, in live nurse cells (Fig 1B). We detected no disruption of the localization of GFP-Vasa in qin mutants.

We conclude that loss of Qin does not affect nuage structure in unfixed, living nurse cells.

piRNA levels in qin\(^{1}/\text{Df}\) ovaries are indistinguishable from controls (Zhang et al., 2011). We used small RNA sequencing to measure piRNA abundance in qin\(^{2}/\text{Df}\) and qin\(^{\text{numo}}/\text{Df}\) ovaries. Compared to heterozygotes, the abundance of total transposon-derived, 23–29 nt small RNAs in qin\(^{2}/\text{Df}\) and qin\(^{\text{numo}}/\text{Df}\) changed < 4% (Fig 2A; Supplementary Tables S1 and S2). Among the 93 transposon families with > 100 ppm piRNA

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Figure 2. piRNA abundance and Ping-Pong efficiency are unaltered in qin mutant ovaries.

A. piRNA length distribution. Blue, sense piRNAs; red, antisense.

B. Box plots reporting the change in abundance of all piRNAs mapping to transposons.

C. piRNA abundance (ppm) in qin\(^{1}/\text{Df}\), qin\(^{2}/\text{Df}\) and qin\(^{\text{numo}}/\text{Df}\), but not qin\(^{\text{numo}}/\text{qin}\), affect piRNA production similarly. Group 1: transposon families with piRNAs amplified by the Ping-Pong pathway and more antisense piRNAs bound to Aub and more sense piRNAs bound to Ago3. Group 2: transposon families with piRNAs amplified by the Ping-Pong pathway and more sense piRNAs bound to Aub and more antisense piRNAs bound to Ago3. Group 3: transposon families expressed in the somatic follicle cells, predominantly antisense primary piRNAs, and little Ping-Pong amplification.

D. Box plots reporting Ping-Pong Z-score by transposon family. Z-score = 1.96 corresponds to P-value = 0.05.
reads in $qin^1/TM6B$ ovaries (Zhang et al., 2011), there was no significant change in median piRNA abundance in control $w^{1118}$ ovaries relative to $w^{1118}$ controls for $qin^1/Df$ ($P = 0.57$, Wilcoxon test), $qin^2/Df$ ($P = 0.13$), or $qin^{kumo}/Df$ ($P = 0.33$; Fig 2B; for additional analyses by transposon families see http://www.umassmed.edu/uploadedFiles/zamore/Transposon_buckets.zip). However, the fraction of piRNAs with the same orientation as the corresponding transposon sense mRNA increased: the median sense fraction (i.e. sense piRNAs/all piRNAs) among 93 transposon families was 0.25 for $w^{1118}$ ovaries but 0.41 for $qin^1/Df$ mutants ($P = 5.2 \times 10^{-7}$, Wilcoxon test), 0.37 for $qin^2/Df$ ($P = 2.4 \times 10^{-4}$), and 0.38 for $qin^{kumo}/Df$ ($P = 1.1 \times 10^{-5}$; Fig 2B).

We also measured piRNA abundance in $qin^{kumo}/qinkumo$ and $qinkumo/TM3$ ovaries (Supplementary Tables S1 and S2), $qinkumo$ homozygous maternal flies, like other $qin$ loss-of-function mutations, produced amounts of piRNAs similar to their $qinkumo$ / TM3 siblings (Fig 2A). Our analysis of previously published deep sequencing data from homozygous $qinkumo$ ovaries (Anand & Kai, 2012) also led us to conclude that there was no change in total piRNA production (Fig 2A). Moreover, the effects of $qin^1/Df$ and $qin^{kumo}/Df$ on piRNA production were highly correlated ($r = 0.94$), but less well correlated with $qinkumo$ ($r = 0.85$, P-value $< 2.2 \times 10^{-16}$; Fig 2C and Supplementary Fig S3A).

All $qin$ allelic combinations showed significant ($Z > 46$; P-value $< 2.2 \times 10^{-16}$) Ping-Pong amplification as measured by comparing piRNA pairs overlapping by 10 bp to other lengths of overlap (Fig 2D and Supplementary Fig S3B). Finally, we reached these same conclusions when normalizing the data by two alternative strategies—microRNA abundance and non-coding RNA abundance (Supplementary Figs S4, S5 and S6). We conclude that Qin is not required to maintain overall piRNA levels or for Ping-Pong amplification.

We used RNA-seq to measure transcript abundance in $w^{1118}$, $qin^1/Df$, and $qin^{kumo}/Df$ ovaries. Without Qin, RNA sequences mapping uniquely to the 42AB cluster, which is the longest piRNA cluster in flies and produces approximately 30% of all ovary piRNAs (Brennecke et al., 2007), increased from 1.5 rpkm in $w^{1118}$ flies to 2.0 rpkm in $qinkumo/Df$ and 2.5 in $qin^1/Df$ flies (Supplementary Fig S7A). We note that our result disagrees with the finding that transcripts from the 42AB cluster declined in $qin^{kumo}$ homozygous ovaries as measured by qRT-PCR (Anand & Kai, 2012). Among the 142 previously defined piRNA clusters (Brennecke et al., 2007), the steady-state abundance of transcripts from six clusters increased significantly in $qin^1/Df$ ovaries (>5-fold; $q < 0.05$); just one decreased significantly (>2-fold; $q < 0.05$; Supplementary Fig S7B). In $qinkumo/Df$ ovaries, the transcript abundance for 11 clusters increased significantly (>5-fold; $q < 0.05$), none decreased significantly (Supplementary Fig S7B).

Both $qin^1/Df$ and $qin^{kumo}/Df$ ovaries suffered increased transposon expression, as measured by RNA-seq (Supplementary Fig S7B). Of the 93 transposon families we examined, the steady-state RNA abundance of 13 families increased significantly (>6-fold; $q < 0.05$) in $qin^1/Df$, compared with $w^{1118}$. Similarly, in $qinkumo/Df$ ovaries the steady-state RNA abundance of 12 transposon families increased significantly (>4-fold; $q < 0.05$). Expression of ten transposon families increased significantly in both $qin^1/Df$ and $qinkumo/Df$ ovaries ($q < 0.05$), including eight of the 11 transposon families whose abundance was reported to increase significantly when measured using both whole-genome tiling microarrays and qRT-PCR (Zhang et al., 2011). Transposon expression in $qin^1/Df$ and $qinkumo/Df$ were highly correlated ($r = 0.95$; P-value $< 2.2 \times 10^{-16}$).

We conclude that loss of $qin$ in the fly ovary does not affect nuage assembly or overall piRNA abundance. Instead, loss of Qin leads to an increase in sense piRNAs and a decrease in antisense piRNAs. The result presented here, together with those reported previously (Zhang et al., 2011) are consistent with the loss of heterotypic Aub: Ago3 Ping-Pong in $qin$ mutants. Without Qin, piRNA cluster transcripts accumulate, rather than decline. Thus, when Aub:Aub Ping-Pong predominates (Zhang et al., 2011), Ping-Pong amplification appears to consume cluster transcripts less efficiently, consistent with a role for Qin in piRNA precursor processing. Understanding how Qin couples Aub with Ago3 to efficiently generate piRNAs and silence transposons remains a challenge for future studies.

Accession numbers

Sequence data generated in this study are available via the NCBI trace archives (http://www.ncbi.nlm.nih.gov/Traces/) using accession number SRP024291.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

ZZ, WET and PDZ conceived the experiments. BSK performed the immuno-staining, ZZ conducted the experiments. JW mapped the sequence reads. ZZ analyzed the sequencing data with guidance from JW and ZW. CT helped with fly fertility test. ZZ and PDZ wrote the manuscript in consultation with all authors.

Conflict of interest

The authors declare that they have no conflicts of interest.

References

