Small regulatory RNAs guide Argonaute (Ago) proteins in a sequence-specific manner to their targets and therefore have important roles in eukaryotic gene silencing. Of the three small RNA classes, microRNAs and short interfering RNAs are processed from double-stranded precursors into defined 21- to 23-mers by Dicer, an endoribonuclease with intrinsic ruler function. PIWI-interacting RNAs (piRNAs)—the 22–30-nt-long guides for PIWI-clade Ago proteins that silence transposons in animal gonads—are generated independently of Dicer from single-stranded precursors. piRNA 5′ ends are defined either by Zucchini, the Drosophila homologue of mitoPLD—a mitochondria-anchored endonuclease—and by piRNA-guided target cleavage. Formation of piRNA 3′ ends is poorly understood. Here we report that two genetically and mechanistically distinct pathways generate piRNA 3′ ends in Drosophila. The initiating nucleases are either Zucchini or the PIWI-clade proteins Aubergine (Aub) or Ago3. While Zucchini-mediated cleavages directly define mature piRNA 3′ ends, Aub/Ago3-mediated cleavages liberate pre-piRNAs that require extensive resection by the 3′-to-5′ exoribonuclease Nibbler (Drosophila homologue of Mut-7). The relative activity of these two pathways dictates the extent to which piRNAs are directed to cytoplasmic or nuclear PIWI-clade proteins and thereby sets the balance between post-transcriptional and transcriptional silencing. Notably, loss of both Zucchini and Nibbler reveals a minimal, Argonaute-driven small RNA biogenesis pathway in which piRNA 5′ and 3′ ends are directly produced by closely spaced Aub/Ago3-mediated cleavage events. Our data reveal a coherent model for piRNA biogenesis and should aid the mechanistic dissection of the processes that govern piRNA 3′-end formation.

piRNA biogenesis is initiated by endonuclease-catalyzed formation of piRNA 5′ ends (Fig. 1a). Based on the nuclease involved, this defines primary (processed by Zucchini) and secondary (processed by Aub/Ago3) piRNAs. Attributes of these two end-cleavages are a 5′ uridine signature for primary piRNAs, and a 10-nt 5′ sense/antisense offset for secondary piRNAs (ping-pong signature). Following 5′-end cleavage, piRNA intermediates are anchored with their 5′ ends in PIWI proteins before their 3′ ends are matured (we refer to these as pre-piRNAs). Zucchini also liberates 3′ ends of primary and secondary piRNAs.

In zucchini mutants, however, Aub/Ago3-bound secondary piRNAs are still abundant, indicative of alternative 3′-end formation pathways (Extended Data Fig. 1a). The 3′ ends of these piRNAs lack signs for endonuclease processing, such as a coupling signature stemming from Zucchini-mediates, phased piRNA biogenesis, or a 3′-to-5′ ping-pong signature indicative of slicer-mediated 3′-end formation (Extended Data Fig. 1b). This supports the hypothesis that Zucchini-independent piRNA 3′-end biogenesis involves exoribonuclease resection of pre-piRNAs that have been generated by Aub/Ago3 (Fig. 1a).

We identified piRNA 5′ species (piRNAs with the same 5′ end)—that besides piRNAs in the 23–29 nt range—exhibit one abnormally long isoform that extends to the cleavage position of a complementary piRNA (Fig. 1b). These isoforms are also found in libraries from immuno-purified PIWI proteins (Fig. 1b), indicating that they represent Aub/Ago3-loaded pre-piRNAs whose 3′ ends have been formed by slicing and await trimming. Consistent with this, the long isoforms lack 2′-O-methylation at their 3′ ends (Fig. 1b).

To identify the 3′ exonuclease involved, we used a piRNA biogenesis reporter that recapitulates Zucchini-independent piRNA 3′-end formation; expression of a reporter with two target sites for cellular piRNAs forces the generation of responder piRNAs in Zucchini-depleted ovaries (Fig. 1c, d). We combined this reporter with a double-shRNA expression cassette to co-deplete Zucchini and a gene of interest (Extended Data Fig. 2a–c). A strong candidate for the exonuclease is the PARN-like nuclease PNLDC1, which trims pre-piRNAs in silkworm. As PARN-family nucleases are absent in Drosophila, we tested instead the mitochondria-anchored Tudor/KH-domain protein Papi, an essential PNLDC1 co-factor in silkworm (Extended Data Fig. 2d, e). Co-depletion of Zucchini and Papi does not impair piRNA generation from two independent reporters (Fig. 1d, Extended Data Fig. 2b, f), and global piRNA levels are comparable between Zucchini- versus Zucchini and Papi co-depleted ovaries (Extended Data Fig. 2g). Notably, Pwi-bound piRNAs increase by approximately 0.5 nt in length in papi mutants (Fig. 1e, Extended Data Fig. 3a, j). As 3′ ends of Pwi-bound piRNAs are generated predominantly by Zucchini, we conclude that Papi-assisted piRNA trimming—if conserved in flies—occurs downstream of Zucchini, consistent with its role in mouse and silkworm.

We next tested the 3′-to-5′ exoribonuclease Nibbler/Mut-7, which trims some micro RNAs (miRNAs) after their loading into Ago1,11, and which has been reported to modulate piRNA lengths. Co-depletion of Zucchini and Nibbler abolates piRNA production from both reporters despite trigger piRNAs remaining abundant and silencing–competent (Fig. 1d, Extended Data Fig. 2f, h).

Consistent with Nibbler acting on slicer-generated pre-piRNAs, it is enriched in perinuclear nuage together with Aub/Ago3, while Papi co-localizes with Zucchini at mitochondria (Fig. 1f, Extended Data Fig. 2d, e). In aubergine mutants, Nibbler’s nuage localization is reduced, yet Nibbler does not enrich in Krimper foci where unloaded Ago3 accumulates (Fig. 1f). Nibbler’s co-localization with Aub/Ago3 therefore probably depends on these factors being loaded with pre-piRNAs. We did not detect robust interactions between Nibbler and Aub/Ago3 by co-immunoprecipitation (weak interactions between Nibbler and Pwi were detected, hinting at a transient interaction (Extended Data Fig. 2i, j).

To characterize Nibbler’s role in piRNA biogenesis we generated flies that express no detectable Nibbler protein (Extended Data Fig. 3b, c). As reported, nibril mutants are viable and fertile, but defective in mir-34 maturation (Extended Data Fig. 3d). As also reported, localization and abundance of PIWI proteins, overall piRNA levels, and transposon silencing are not affected (Extended Data Fig. 3e–h).
Figure 1 | The 3′-to-5′ exonuclease Nibbler matures piRNA 3′ ends from slicer-cleaved pre-piRNAs. a, Schematic illustration of piRNA 5′ and 3′ biogenesis. b, Northern blot against individual piRNA 5′ species (mature piRNAs: 23–29 nt) detects a 34 nt pre-piRNA (blue arrowhead). 3′-end methylation probed by β-elimination (miR-8 serves as control). To the right, sequencing counts of the corresponding piRNAs (normalized to 1 million miRNA reads; p.p.m.) from total small RNAs or from an Aub immunoprecipitation are shown. c, Schematic of the dual-site piRNA biogenesis reporter. UTR, untranslated region. d, Levels of small RNAs (p.p.m.) mapping to the biogenesis reporter (5′ ends only) in indicated genetic backgrounds. e, Length profiles of TE-mapping small RNAs isolated from Piwi/Aub/Ago3-immuno-precipitates (genetic background indicated; P-values calculated with two-sided t-test). f, Confocal images showing localization of GFP-Nibbler, Aub and Ago3 in w1118 or Aubergine mutant egg chambers (scale bars, 5 μm; individual channels as inverted grey scale images).

piRNA length, however, is mildly increased (Extended Data Fig. 3); our sequencing libraries span 18–40 nt, Extended Data Fig. 4). Notably, this originates primarily from Ago3-bound piRNAs, which increase >1 nt in length (Fig. 1e). This supports a specific role for Nibbler in resecting Aub/Ago3-generated pre-piRNAs. Indeed, somatic Piwi-bound piRNAs, whose 3′ ends are generated by Zucchini, show no length change in nibbler mutants, in contrast to papi mutants (Extended Data Fig. 3j)12,13. These results indicate that Nibbler does not fine-tune piRNA length as proposed12,13, but instead represents the central exonuclease of a distinct piRNA 3′-end pathway that resects slicer-generated pre-piRNAs to mature piRNAs.

If Zucchini endonuclease and Nibbler exonuclease act in separate pathways to generate piRNA populations with similar overall length, the 3′ profiles of piRNA 5′ species should differ in single-mutant ovaries. We inspected individual Aub/Ago3-bound piRNA 5′ species by northern blot analysis and sequencing (Fig. 2a, b). While piRNAs in Zucchini-depleted ovaries display a broad length profile (consistent with exonuclease resection), piRNAs in nibbler mutants display discrete length patterns with major isoforms typically being followed by uridine (downstream-U signature), a hallmark of Zucchini cleavages (Fig. 2a, b).6,9,17

To generalize these findings, we determined the downstream-U signature and the 3′-end precision index for thousands of piRNA 5′ species bound to Piwi/Aub/Ago3. This allows several conclusions. (1) In agreement with the two-pathway model, the downstream-U signature increases in nibbler mutant ovaries, yet is ablated in Zucchini-depleted ovaries (Fig. 2c; Piwi- piRNAs are lost in the absence of Zucchini). (2) In wild-type ovaries, the downstream-U signature is strong for Piwi-bound piRNAs, intermediate for Aub, and very weak for Ago3 (Fig. 2c), indicating that Zucchini acts predominantly on Piwi and Aub.

(3) The downstream-U signature correlates with the 3′-end precision index of piRNA populations (Fig. 2c). (4) In nibbler mutants, the 3′-end precision index increases for Piwi-, Aub-, and Ago3-bound piRNAs, indicating that Nibbler acts on all three PIWI proteins (Extended Data Fig. 5a). (5) Characteristics of piRNA 5′ ends do not correlate with the 3′-end precision index (Extended Data Fig. 5b), arguing that 5′-end generation does not dictate the mode of 3′-end formation.

In agreement with Nibbler and Zucchini acting in parallel pathways, the length profiles of wild-type piRNAs appear to be a composite of the two respective single-pathway profiles (Fig. 2a, b). Inspired by this observation, we computed the relative contribution of Nibbler and Zucchini for Aub/Ago3-bound piRNAs. For each of the approximately 300 analysable piRNA 5′ species, we determined the Zucchini/Nibbler contribution at which the combined length profile best mimics the wild-type profile (Fig. 2d). For both, Aub- and Ago3-bound species the wild-type profiles can be accurately modelled from the single-pathway profiles (Extended Data Fig. 5c). This results in an approximate median 70:30 dominance of Zucchini over Nibbler for Aub-bound piRNAs and an opposite ratio for Ago3-bound piRNAs (Fig. 2e), in agreement with the 3′-end characteristics of the respective piRNA populations in wild-type ovaries (Fig. 2c). Our data demonstrate that two parallel pathways with varying contributions form 3′ ends of Aub/Ago3-bound piRNAs: Zucchini generates most Aub-bound piRNAs, while Nibbler generates most Ago3-bound piRNAs.

Zucchini-mediated piRNA 3′-end formation results in processing of the downstream precursor RNA into phased piRNAs bound to Piwi (referred to as triggering)6,9,3′-end formation by Nibbler instead prevents triggering owing to degradation of the downstream precursor (Fig. 3a). As Zucchini compensates for 3′-end formation in the absence of Nibbler, triggering levels should increase in nibbler mutants. Indeed,
Zucchini-mediated downstream piRNA biogenesis that fuels nuclear consequences for piRNA loading into PIWI proteins: Nibbler limits ′ (TEs) decrease (Extended Data Fig. 6c). This indicates a competitive populations, ping-pong signatures for nearly all transposable elements (Extended Data Fig. 6a, b). As a consequence of the shifts in piRNA to Ago3 incorporating abnormally high levels of antisense piRNAs mutants (Fig. 3c). It is unclear why Aub-bound, but not Ago3-bound piRNAs increase at the expense of ping-pong piRNAs in and subsequent Nibbler-catalysed pre-piRNA resection limits the extent of triggering, especially for Ago3-bound pre-piRNAs. The occurrence of Piwi-bound piRNA 5′ ends immediately downstream of Aub/Ago3-bound piRNA 3′ ends increases in nibbler mutant compared to wild-type ovaries (Fig. 3b). As expected, this increase is more pronounced for Ago3/Piwi linkages compared to Aub/Piwi linkages. We conclude that in wild-type ovaries, downstream slicing and subsequent Nibbler-catalysed pre-piRNA resection limits the extent of triggering, especially for Ago3-bound pre-piRNAs.

In agreement with elevated triggering, the levels of Piwi-bound piRNAs increase at the expense of ping-pong piRNAs in nibbler mutants (Fig. 3c). It is unclear why Aub-bound, but not Ago3-bound piRNAs are reduced in the absence of Nibbler. Possibly this is due to Ago3 incorporating abnormally high levels of antimir piRNAs (Extended Data Fig. 6a, b). As a consequence of the shifts in piRNA populations, ping-pong signatures for nearly all transposable elements (TEs) decrease (Extended Data Fig. 6c). This indicates a competitive relationship between two piRNA 3′-end formation pathways with consequences for piRNA loading into PIWI proteins: Nibbler limits the extent at which slicer-induced piRNA biogenesis propagates into Zucchini-mediated downstream piRNA biogenesis that fuels nuclear Piwi. By contrast, Zucchini consumes piRNA precursors, reducing their participation in ping-pong during post-transcriptional regulation.

These findings prompted us to re-examine the long-standing question of why secondary piRNA populations from some TEs remain abundant in Zucchini-depleted ovaries (’robust TEs’, for example, Doc), while others collapse (’sensitive TEs’, for example, I-element; Extended Data Fig. 6d)16. We reasoned that only TEs with a minimal abundance of Nibbler substrates (reflected by abundant ping-pong piRNAs) could maintain piRNA biogenesis in the absence of Zucchini. That is because Zucchini generates piRNA 3′ ends independent of additional precursor cleavages, while Nibbler-mediated 3′-end formation requires a second piRNA-guided cleavage event close by (Fig. 3a). Indeed, ping-pong piRNA levels are substantially higher for robust compared to sensitive TEs in wild-type ovaries (Extended Data Fig. 6d). TEs with ping-pong piRNAs below a threshold level therefore cannot compensate for Zucchini loss, as the production of Nibbler substrates is too inefficient. As ping-pong is a feed-forward loop, this results in the collapse of piRNA biogenesis.

As the most direct test for two separate piRNA 3′-end pathways, we co-depleted Zucchini and Nibbler. As expected from the piRNA biogenesis reporter experiments (Fig. 1d), this results in piRNA loss for some TEs, which generate abundant piRNAs in Zucchini-depleted ovaries (Extended Data Figs 1a and 7a). Surprisingly, however, piRNAs that map to several other TEs are only mildly affected (Extended Data Fig. 7a, b), total germline piRNA levels are reduced less than twofold compared to Zucchini-depleted ovaries (Extended Data Fig. 7c), and TE derepression is similar to Zucchini-depleted ovaries (Extended Data

Figure 2 | Two genetically independent pathways generate piRNA 3′ ends. a, b, Northern blot analysis (loading control: miR-8) and sequencing counts for Ago3- (a) or Aub- (b) enriched piRNAs in indicated genetic backgrounds. Bar charts display fraction of reads with indicated length. Respective RNA sequences are shown. c, Sequence logos display nucleotide bias around dominant 3′ ends of Piwi/Aub/Ago3-bound piRNAs isolated from ovaries of indicated genetic background. Bar plots display the nucleotide composition at the position following dominant 3′ ends (piRNAs are split into ten equally sized bins sorted for their precision index). d, Shown is how the Zucchini/Nibbler contribution to 3′-end formation of individual piRNA 5′ species was determined. e, Shown are the Zucchini/Nibbler contributions for 3′-end formation for Aub- and Ago3-bound piRNAs (left, as stacked bar diagram; right, as box plot; Tukey definition; ***P < 0.001 after Wilcoxon rank-sum test).
A closer look at their mappings provides an explanation of how their 3’ ends are generated (Fig. 4a). In double-depleted ovaries, novel ping-pong pairs emerge between two distantly spaced ping-pong pairs, thereby reducing the cleavage intervals to around 20–30 nt. Also, piRNA 3’ ends change from a bell-shaped profile (consistent with Nibbler-mediated exo-resection) to discrete profiles where a single dominating 3’ end precedes the 5’ end of a flanking piRNA by 1 nt (Fig. 4a). This suggests that two slicer events, spaced by one piRNA length, directly generate 5’ and 3’ ends of piRNAs. To test this prediction we turned to the piRNA biogenesis reporter with two cleavage sites spaced by 52 nt that is incompatible with piRNA biogenesis in Zucchini and Nibbler co-depleted ovaries (Fig. 4b). Introducing a third central target site re-installs biogenesis for two responder piRNAs, whose 3’ ends map precisely to the downstream slicer sites (Fig. 4b).

We systematically analysed Zucchini/Nibbler-independent piRNAs for two characteristic signatures, namely 3’/5’ coupling (nucleotide-precision phasing), and 3’/5’ ping-pong (presence of complementary piRNA 5’ ends 10 nt downstream of piRNA 3’ ends). Both signatures—while absent in Zucchini-depleted ovaries—are pronounced in double-depleted ovaries, indicative of tightly spaced ping-pong pairs (Fig. 4c). When piRNAs are grouped into length cohorts, coupling of flanking piRNAs is apparent for all size classes (Fig. 4d). No piRNA coupling is observed in Zucchini-depleted ovaries, as here Nibbler allows ping-pong pairs to be spaced in a larger window (Fig. 4e, Extended Data Fig. 8d).

Zucchini/Nibbler-independent piRNAs bound to Aub or Ago3 retain their respective nucleotide bias of 1U and 10A (Extended Data Fig. 8e). Given the precise piRNA coupling, this explains why slicer/slicer-generated piRNAs display downstream-1U and downstream-10A signatures (Extended Data Fig. 8e). As uridine residues are not spaced in pre-fixed patterns, this requires flexibility on the Argonaute-side to accommodate piRNAs with different lengths. Indeed, while approximately 80% of piRNA species in Zucchini-depleted ovaries are approximately 80% of piRNA species in Zucchini-depleted ovaries are 23–27 nt, this length accounts for only approximately 50% in Zucchini and Nibbler co-depleted ovaries (Fig. 4d, e, Extended Data Fig. 8b). Nucleotide-resolution northern blots confirm the existence of piRNAs as short as 21 nt and as long as 32 nt (Fig. 4f, Extended Data Fig. 9a). While these piRNAs are similarly abundant as the corresponding
species in wild-type ovaries, their length is restricted to essentially a single isoform. When examining slicer/slicer-generated piRNAs for their 2′-O-methylation status, a hallmark of mature piRNA 3′ ends, even 32-nt-long piRNAs are methylated (Fig. 4f). This extends to all piRNA sizes (Extended Data Fig. 9b), indicating that 2′-O-methylation can occur independently of Zucchini- and Nibbler-mediated 3′-end formation. As pre-piRNAs with similar length are not methylated in wild-type ovaries (Fig. 1b), Nibbler probably acts faster than Hen1.

Together with recent findings, our data provide a blueprint for piRNA 3′-end formation. Two separate exonucleolytic pathways—initiated by endonucleolytic cleavages—are dedicated to pre-piRNA trimming: the Papi/PNLDC1 pathway, and the Nibbler/Mut-7 pathway. This is probably an ancient pathway architecture as—similar to ping-pong—all involved nucleases (Zucchini, PNLDC1, and Nibbler/Mut-7) are conserved from sponges to mammals (Extended Data Fig. 10). There are, however, interesting exceptions. (1) Nematodes have lost Zucchini and an entirely different small RNA biogenesis system fuels their PIWI proteins. In C. elegans, PARN trims PIWI-bound small RNAs, while Nibbler/Mut-7 is required for 22G siRNA biogenesis. (2) While PARN or PNLDC1 have been sporadically lost in several lineages (for example, fish), only flies have lost both enzymes. We postulate that this central pre-piRNA exonuclease is dispensable in flies as here Zucchini directly forms mature piRNA 3′ ends. (3) Only two groups have lost Nibbler: all Anopheles species, and several mammals ping-ponging in these species is currently unclear.

The balance between the two 3′-end generating pathways defines the extent to which precursors are processed into PIWI-bound versus Aub/Ago3-bound piRNAs. Ultimately, this determines the ratio between transcriptional (PIwi) and post-transcriptional silencing (Aub/Ago3). Aub-bound piRNAs, which are antisense-biased, are more abundant than Ago3-bound sense piRNAs. PIWI-bound piRNAs, which are generated in response to Aub/Ago3-initiated triggering, are, however, antisense-biased. Ago3-bound cleavage intermediates must therefore be transferred more efficiently to mitochondria for Zucchini-mediated 3′-end formation than Aub-generated intermediates. Also, Nibbler matures Ago3-bound pre-piRNAs probably more frequently compared to Aub-bound pre-piRNAs, because Aub-bound piRNAs are more abundant than Ago3-bound piRNAs. Consequently, two closely spaced cleavages will be more frequent downstream of Aub than of Ago3.

In the absence of Zucchini and Nibbler, PIWI proteins accommodate two 2′-O-methylated piRNAs ranging in length from 20–34 nt. We speculate that the slicer-only pathway potentially represents an ancient small RNA-generating unit, onto which dedicated endo- and exonucleases are added during evolution in order to efficiently generate piRNAs of optimal length.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 23 June; accepted 18 October 2016.

Published online 16 November 2016.

1. Ghildyal, M. & Zamore, P. D. Small silencing RNAs: an expanding universe. Nat. Rev. Genet. 10, 94–108 (2009).
2. Maione, C. D. & Hannon, G. J. Small RNAs as guardians of the genome. Cell 136, 656–668 (2009).
3. Iwasaki, Y. W., Siomi, M. C. & Siomi, H. PIWI-interacting RNA: its biogenesis and functions. Annu. Rev. Biochem. 84, 405–433 (2015).
4. Nishimatsu, H. et al. Structure and function of Zucchini endoribonuclease in piRNA biogenesis. Nature 491, 284–287 (2012).
5. Ippso, J. R., Haase, A. D., Knott, S. R., Joshua-Tor, L. & Hannon, G. J. The structural biochemistry of Zucchini implicates it as a nuclease in piRNA biogenesis. Nature 491, 279–283 (2012).
6. Brennecke, J. et al. Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila. Cell 128, 1089–1103 (2007).
7. Gunawardane, L. S. et al. A slicer-mediated mechanism for repeat-associated siRNA 5′ end formation in Drosophila. Science 315, 1587–1590 (2007).
8. Han, B. W., Wang, W., Li, C. Z., Weng, Z. & Zamore, P. D. Noncoding RNA-guided transposon cleavage initiates Zucchini-dependent, phased piRNA production. Science 348, 817–821 (2015).
9. Mohn, F., Handler, D. & Brennecke, J. Noncoding RNA piRNA-guided slicing specifies transcripts for Zucchini-dependent, phased piRNA biogenesis. Science 348, 812–817 (2015).
10. Han, B. W., Hung, J. H., Weng, Z., Zamore, P. D. & Amers, S. L. The 3′-to-5′ exonuclease domain of slicer nucleases shapes the 3′ ends of microRNAs bound to Drosophila Argonaute1. Curr. Biol. 21, 1878–1887 (2011).
11. Liu, N. et al. The exoribonuclease slicer controls 3′ end processing of microRNAs. Cell 120, 1888–1893 (2011).
12. Feltz, V. L. et al. The exoribonuclease slicer regulates age-associated traits and modulates piRNA length in Drosophila. Aging Cell 14, 443–452 (2015).
13. Wang, H. et al. Antagonistic roles of Nibbler and Hen1 in modulating piRNA 3′ ends in Drosophila. Development 143, 530–539 (2016).
14. Vourekas, A. et al. Mi1 and Miwi target RNA repertoire reveals piRNA biogenesis and function of Miwi in spermiogenesis. Nat. Struct. Mol. Biol. 19, 773–781 (2012).
15. Kawaoka, S., Iwam, N., Katsuma, S. & Tomari, Y. 3′ end formation of PIWI-interacting RNAs in vitro. Mol. Cell 43, 1015–1022 (2011).
16. Olliver, D., Senti, K. A., Subramanian, S., Sachidanandan, R. & Brennecke, J. The cohesin shordenuplo determines a group of siRNA biogenesis factors essential for all piRNA populations in Drosophila. Mol. Cell 47, 954–969 (2012).
17. Izumi, N. et al. Identification and functional analysis of the Pre-3′-end interor in silkworms. Cell 164, 952–973 (2016).
18. Saxe, J. E., Chen, M., Zhao, H. & Lin, H. TDn1 is essential for spermatogenesis and participates in primary piRNA biogenesis in the germline. EMBO J. 32, 1869–1885 (2013).
19. Sato, K. et al. Klimper modifies an antisense bias on piRNA pools by bindingAGO3 in the Drosophila germline. Mol. Cell 59, 553–563 (2015).
20. Webster, A. et al. Aub and Ago3 are recruited to Nuage through two mechanisms to form a ping-pong complex assembled by Klimper. Mol. Cell 59, 564–575 (2015).
21. Horwich, M. D. et al. The Drosophila RNA methyltransferase, Dom1, modifies mature piRNA sequences and single-stranded piRNAs in RISC. Curr. Biol. 17, 1265–1272 (2007).
22. Sato, K. et al. Fmrt, the Drosophila homolog of HEN1, mediates 2′-O-methylation of PIWI-interacting RNAs at their 3′ ends. Genes Dev. 21, 1603–1608 (2007).
23. Grimson, A. et al. Early origins and evolution of microRNAs and PIWI-interacting RNAs in animals. Nature 455, 1193–1197 (2008).
24. Sarkies, P. et al. Ancient and novel small RNA pathways compensate for the loss of piRNAs in multiple independent nematode lineages. PLoS Biol. 13, e1002061 (2015).
25. Tang, W., Tu, S., Lee, H. C., Weng, Z. & Mello, C. C. The RNAse PARN-1 trims piRNA 3′ ends to promote transcriptome surveillance in C. elegans. Cell 164, 974–984 (2016).
26. Gu, W. et al. Distinct argonaute-mediated 22G-RNA pathways direct genome surveillance in the C. elegans germline. Mol. Cell 63, 231–244 (2009).
27. Scott, K. A., Jurczak, D., Sajic, J. & Brennecke, J. piRNA-guided slicing of transposon transcripts enforces their transcriptional silencing via the nuclear piRNA repertoire. Genes Dev. 29, 1747–1762 (2015).
28. Wang, W. et al. Slicing and binding by Ago3 or Aub trigger PIWI-bound piRNA production by distinct mechanisms. Mol. Cell 59, 819–830 (2015).
METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

Fly husbandry and strains. Flies were kept at 25 °C. Short hairpin RNA (shRNA) fly lines were crossed to the maternal triple driver (MTD)-GAL4 line (#3777; Bloomington stock centre) to drive expression of the shRNA in the germ line. shRNA constructs for double depletion of Zucchini-Nibbler and Zucchini-Papi were generated by PCR with Recombinant TripGLO10111 line was used (#35227; Bloomington) for depleting Zucchini alone. GFP reporter constructs and GFP-tagged RAC rescue constructs were inserted into the attP40 landing site. A nibbler and a papi allele with frame-shift mutations were generated by CRISPR/Cas9 using guide RNAs TGAGCCGCCCTTGGACGCAA and CGACGCCGCTTTAAAGCCGATC, respectively, as previously described. N-terminally Flag-tagged nibbler allele was generated using a guide RNA CACGGGAAAACCCCAGTGAG. The resulting allele has an insertion of IDYKDHDGDYKDHDIDYKDDD after the start codon.

w1118 strain was used as a wild-type control throughout the study except the analysis of TE expression from ovaries using RNA sequencing where an shRNA line against white crossed to MTD-GAL4 was used as a control. Flies were aged for 6 days and kept on apple juice agar plates supplemented with yeast paste to ensure consistent ovarian morphology.

Construction of shRNA expression vectors. To achieve simultaneous knockdown of two genes with a single shRNA construct, we modified the Valium20 vector and inserted two tandem shRNA sequences. The two hairpins are separated by the sequence that spans the miR-6-3 and miR-6-2 hairpins in the genome in order to maximize efficient processing (referred to as miR-6 backbone in Extended Data Fig. 2a). NheI/EcoRI and AgeI/SphI sites were used to clone the two shRNA oligos. The modified shRNA expression cassette (restriction sites used for cloning of shRNA oligos are underlined and the miR-6 spacer sequence is in bold).

TTCCAGCCGATATCTGGAACGTGACCTTTGAATATAACTATTAGGAT
GGATATGTCGAACTA

Zucchini shRNA reverse:
AACATCGGCAATCAGGTAGTGAATAGCAAGGTGATGGTTGAATAG

The shRNA oligo sequences are:

Zucchini shRNA forward (guide miR sequence is underlined):
CTAGCAGTCATGTGGAACGTGACCTTTGAATATAACTATTAGGAT
GGATATGTCGAACTA

Nibbler shRNA forward (guide miR sequence is underlined):
CCGGTGATGATGTCTGATGCTGATGCTGATGCTGATGCTGATGCTGAT

Nibbler shRNA reverse:
CCGGTGATGATGTCTGATGCTGATGCTGATGCTGATGCTGATGCTGAT

Papi shRNA forward (guide miR sequence is underlined):
CCGGTGATGATGTCTGATGCTGATGCTGATGCTGATGCTGATGCTGAT

Papi shRNA reverse:
GCACGGCAGGTAGTGAATAGCAAGGTGATGGTTGAATAG

RNAi depletion from total RNA. For the depletion of 2S rRNA from 10 μl of total ovarian RNA, 100 μl slurry of Myone Streptavidin C1 beads (Invitrogen) were used. The beads were washed twice with 500 μl 0.5 × SSC. After washing, the beads were re-suspended in 300 μl 0.5 × SSC and 1 μl of 100 μM s-RNA-as-oligo (Bio-AGTCTTACAACCCTCAACCATATGTAGTCCAAGCAGCACT) were added and mixed. This mixture was incubated on ice for 30 min, followed by© 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
a wash with 500 μl of 0.5 × SSC to remove unbound 2′-RNA as-oligo. After 
resuspension of the beads in 100 μl, they were incubated at 65 °C for 5 min. The 
RNA was denatured for 5 min at 80 °C for 5 min and mixed with the beads, 
followed by incubation at 50 °C for 1 h. Fraction not bound to the beads was 
collected and the RNA was ethanol precipitated and served as input for the small 
RNA cloning procedure.

**Small RNA cloning and sequencing.** Small RNA libraries were prepared as 
described previously. In brief, total RNA from respective ovaries was isolated 
using TriR (Thermo Scientific). The transcript was subjected to 2′-dephosphorylation. Oxidation was done by 
incubating 2′-dephosphorylated total RNA in borate buffer (pH 8.6) containing 25 mM 
NaOAc, at room temperature for 30 min. RNA samples from total RNA and from 
IP experiments were resolved on a denaturing polyacrylamide gel and small RNAs 
ranging from 18–40 nt were excised and recovered. These were subsequently 
ligated to 3′ and 5′ adapters containing four random nucleotides at both ends to 
reduce ligation biases. Ligated RNA was reverse-transcribed, PCR amplified and 
the libraries were sequenced on an Illumina HiSeq 2500 machine in single-read 
50 bp mode. IP libraries of the Zucchini/Nibbler-depleted flc were sequenced in 
single-read 100 mode.

**RNA sequencing.** PolyA+ RNA seq was performed as described in Mohn et al.26 
using NEBNext Ultra directional RNA library prep kit for Illumina (NEB) and 
libraries were sequenced on Illumina HiSeq 2500 in paired-end 125 bp mode. Six 
bases at each end of the reads were trimmed and the remaining part was split into 
three reads (37, 38, 38 nt). Reads were mapped against TE consensus sequences 
using Bowtie (0. 12. 9) allowing up to three mismatches. Filtered small RNA reads were 
subsequently mapped to TE consensus sequences allowing three mismatches. Second, 5′ and 3′ ends of mapped reads were counted at each 
nucleotide position on both strands. For each linkage analysis, the statistical 
significance of observing a certain linkage was calculated as follows.

1) For example, the calculation of canonical ping-pong, piRNA 5′ ends were 
randomly resampled for a fixed number of times from antisense and sense 
mapped piRNAs. We sampled for the number of a 5′ of TE length (bp). For 
instance, if the size of a TE is 5,000 bp, we sampled 5′ ends 1,000 times. A 
fixed number of samplings allows for a fair comparison between libraries of 
different sequencing depths. The shuffle function in bash 4.2.37(1) was used to 
obtain random numbers.

2) We counted the number of 5′ ends of antisense piRNAs that find at least one 
5′ end of sense piRNA at 10-nt off-set (N).

3) We computationally repeated (1) and (2) for 500 times and calculated the 
mean of Nmean.

4) Suppose two sets of random 5′ ends, the probability of an antisense 5′ end 
to find a ping-pong partner p = 1 − (1 − L/L)^3 where L is the length of TE. The 
expected number of N from a random set (Nspecific) is L/5 × p.

5) Z-score was calculated as (Nmean − Nspecific/σ) where the standard deviation 
σ is (L/5 × p × (1 − p)^3)^0.5.

We used the following sets of sequencing reads for calculation. Canonical ping-
pong: 5′ ends of antisense and sense mapped reads from total libraries (10-nt off-
set, Extended Data Figs 1b and 8c). 3′/5′ coupling: 3′ ends of antisense piRNA 
mapped reads from total libraries (1-nt off-set, Extended Data Figs 1b and 4c). 3′/5′ 
ping-pong: 3′ ends of antisense mapped reads and 5′ ends of sense mapped reads 
(10-nt off-set, Extended Data Figs 1b and Fig. 4c).

**Mapping small RNA reads to reporter constructs.** Responder and trigger 
piRNA reads were determined by mapping the total small RNA reads including 
genome-unmapped reads to reporter construct sequences using bowtie allowing 
zero mismatches. To determine trigger piRNA levels, only the first 20 nt of 
mapped reads were used in order to account for 3′ heterogeneity. Mapped reads were 
normalized to 1 million miRNA reads.

**Measure the definition of 3′ ends.** The analysis was adapted from Mohn et al. In 
brief, piRNA 5′ end positions where the downstream 20–35 nt window had equal 
number of mappings in the reference genome were selected as unambiguous 5′ end 
positions. We only included the reads whose 5′ ends are defined as unambiguous 
(29 analyses). These analysable reads were collapsed on 5′ ends yielding the 
counts of each length per given 5′ end. 5′ ends that have more than 20 raw counts 
as well as more than 2 counts normalized to one million TE-mapping reads (p.p.m.) 
were included in the analysis. These cut-offs allowed the comparison of different 
sequencing depths as well as to have the confident assessment of 3′ end variants. Finally, 
the fraction of the counts representing the dominant length per 5′ end was calculated as 
a percentage (definition). For the analysis of somatic piRNAs, piRNAs that mapped to 
soma-enriched 1 kb tiles were used with a cut-off of soma-index greater than 8 δ.

**Nucleotide analysis.** Only piRNA reads that passed the requirements for 3′-end 
analysis (see above) were used for the analysis. The entire analysis is sequence-
based, not read-based (cloning frequency is therefore not considered). Nucleotide 
windows surrounding the respective positions were extracted using get-fasta 
from the fastX-toolkit (Hannon laboratory). For each 5′ end, the dominant 
3′ end position was used to extract the surrounding sequences. When indicated, 
the 5′ ends were binned into ten groups containing an equal number of 5′ ends 
according to the definition of their 3′ ends. The nucleotide signatures were 
generated using weblogo 3.4 and Prism 6 was used for visualization.

**Contribution analysis for Nibbler and Zucchini.** The relative contribution of 
Nibbler and Zucchini in forming the 3′ ends of Aub- or Ago3-bound piRNAs was 
modelled as follows:
Analysable piRNA 5' ends (see above) with at least 20 p.p.m. in the respective IP libraries of all three genotypes (u1118, nibbler−/−, and Zucchini depletion) were included in the analysis. First, the 3'-end composition of individual 5' ends in a window of 20–30 nt of length was determined. The 3'-end profile of piRNAs from nibbler−/− and Zucchini-depletion libraries were summed in different ratios (0–100% and the reciprocal values) to generate a combined profile. Second, the combined profile was compared to the w−/− profile using a linear regression model. The R² value was used to assess the best fit. Finally, the ratios of Nibbler versus Zucchini contribution were extracted at the maximal R² value.

Heat maps for 3'-to-5' coupling. Heat maps were generated as previously described9. In brief, filtered reads (see above) from total small RNA libraries were mapped to TE consensus sequences allowing up to three mismatches. We selected pairs of complementary reads with 10 nt 5' overlap (ping-pong piRNA pairs). Ping-pong piRNAs that were cloned greater than 10 p.p.m. and more than 2% of the most abundant piRNA per individual TEs were selected for the analysis. We extracted the counts of 5' and 3' ends mapped in the window of ±100 nt for each ping-pong piRNA (two instances per pair) using annotatePeaks (Homer v.4.5) and transformed the counts at each position to a percentage value (read count table). The read count tables per ping-pong piRNA were sorted by the length of the dominant ping-pong responder piRNA and by the relative abundance of the ping-pong responder piRNA in the window, and were assembled into heat maps. The distribution of 5'-to-5' distance of flanking ping-pong piRNAs was calculated by summing the read count table of all ping-pong piRNAs in the respective genotypes (Extended Data Fig. 8d).

Orthologue search. Orthologues for Zucchini/MitoPLD, PARN, PNLDC1, and Nibbler/Mut7 were first searched using orthoDB v.9 against metazoan species (http://www.orthodb.org/)39. Orthologue groups (EOG091G04CM: PARN/PNLDC1, EOG991G04KJ: Nibbler/Mut7) were analysed. Protein entries that were not annotated with the following Pfam domains were excluded: Zucchini/MitoPLD: PLDc_2 (PF13091), PARN/PNLDC1: CAFI (PF04876), Nibbler/Mut7: DNA_pol_A_exo1 (PF01612). Pfamscan (http://www.ebi.ac.uk/Tools/pfa/pfamscan/) was used to search for the domains (pfam-A, E-value: 0.1). We defined PARN as proteins that have RNA_bind (PF08675) and/or RRM_7 (PF16367) and PNLDC1 as proteins that do not have these annotations. Species that did not have orthologues in orthoDB were further searched for orthologues first by BLASTp (NCBI) against non-redundant protein sequences and second by tBLASTn (NCBI) against whole genome shotgun sequences using the default cut-offs. Hits from BLASTp and tBLASTn were reciprocally surveyed for predicting the query proteins as the most confident homologue. All hits were combined and displayed in a phylogenetic tree (either presence or absence) using iTOL (http://itol.embl.de)40.

Code availability. The code that underlies the computational analyses in this manuscript is available at https://gitlab.com/groups/Genetic_and_mechanistic_diversity_of_piRNA_3prime_end_formation.

Data availability statement. All sequence data that support the findings of this study (see SI) have been deposited in NCBI GEO with the accession code GSE83698. All fly lines used in this study are available from VDRC (http://stockcenter.vdrc.at/control/main). Source data for all gel images are provided with the paper (see Supplementary Information).

29. Markstein, M., Pitsouli, C., Villalta, C., Celniker, S. E. & Perrimon, N. Exploiting position effects and the gypsy retrovirus insulator to engineer precisely expressed transgenes. Nat. Genet. 40, 476–483 (2008).
30. Gokezade, J., Sienski, G. & Duchek, P. Efficient CRISPR/Cas9 plasmids for rapid and versatile genome editing in Drosophila. G3 (Bethesda) 4, 2279–2282 (2014).
31. Ni, J. Q. et al. A genome-scale siRNA resource for transgenic RNAi in Drosophila. Nat. Methods 8, 405–407 (2011).
32. Venken, K. J. et al. Versatile P[acman] BAC libraries for transgenesis studies in Drosophila melanogaster. Nat. Methods 6, 431–434 (2009).
33. Ejsmont, R. K. et al. Recombination-mediated genetic engineering of large genomic DNA transgenes. Methods Mol. Biol. 772, 445–458 (2011).
34. Jayaprakash, A. D., Jabado, O., Brown, B. D. & Sachidanandam, R. Identification and remediation of biases in the activity of RNA ligases in small-RNA deep sequencing. Nucleic Acids Res. 39, e141 (2011).
35. Seitz, H., Ghiidiyal, M. & Zamore, P. D. Argonaute loading improves the 5’ precision of both MicroRNAs and their miRNA* strand in flies. Curr. Biol. 18, 147–151 (2008).
36. Mohn, F., Sienski, G., Handler, D. & Brennecke, J. The rhino-deadlock-cut-off complex licenses noncanonical transcription of dual-strand piRNA clusters in Drosophila. Cell 157, 1364–1377 (2014).
37. Fall, G. S., Codony-Servat, C., Byrne, J., Ritchie, L. & Hamilton, A. Carbodiimide-mediated cross-linking of RNA to nylon membranes improves the detection of siRNA, miRNA and piRNA by northern blot. Nucleic Acids Res. 35, e60 (2007).
38. Kvitentseva, E. V. et al. OrthoDB v8: update of the hierarchical catalog of orthologs and the underlying free software. Nucleic Acids Res. 43, D250–D256 (2015).
39. Letunic, I. & Bork, P. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics 23, 127–128 (2007).
Extended Data Figure 1 | 3′ ends of Zucchini-independent ping-pong piRNAs are formed by an exonuclease. a, Scatter plot showing the fold change in piRNA levels (for 63 germline-dominant TEs) in Zucchini-depleted compared to control ovaries (calculated as sum of normalized Piwi/Aub/Ago3-bound piRNAs). TEs were grouped into robust (red) and sensitive (blue) on the basis of piRNA loss (threshold = 5× loss). b, Box plots displaying the Z-scores of canonical 5′/5′ ping-pong, 3′/5′ coupling, and 3′/3′ ping-pong for piRNAs isolated from ovaries of indicated genotype (for the 19 robust germline-enriched TEs that maintain piRNA production in Zucchini-depleted ovaries; defined in panel a). Midline indicates the median value, box ranges from the first to the third quartile, whiskers are 1.5× the interquartile range.
Extended Data Figure 2 | See next page for caption.
Extended Data Figure 2 | Nibbler, but not Papi, is required for the generation of piRNAs in the absence of Zucchini. a, Design of the short hairpin expression cassette that allows the simultaneous RNAi-mediated knockdown of two genes in a tissue specific manner. shRNAs are separated by the miR-6 backbone and can be cloned via indicated restriction sites. b, c, Confocal sections of egg chambers (scale bars, 10 μm) of indicated genotype expressing GFP-tagged Zucchini (b, c) and GFP-tagged Papi (b), showing the efficient shRNA-mediated knockdown of Zucchini and Papi (b), or Zucchini and Nibbler (c) in the germ line. Nibbler was detected using a monoclonal antibody. d, Confocal images showing the localization of GFP-tagged Papi and Nibbler in Drosophila egg-chambers (scale bars, 10 μm). Functionality of GFP–Nibbler is demonstrated in Extended Data Fig. 3d. e, Confocal sections through single nurse cell nuclei of egg chambers expressing GFP-tagged Zucchini (left) or Papi (right) and stained for mitochondria (immuno-staining of ATP synthase). f, Shown are mappings of piRNAs (5′ ends only; red, sense; black, antisense) from ovaries of indicated genotype to a second reporter construct (as in Fig. 1d). Values are normalized to 1 million sequenced miRNA reads. g, Scatter plot displaying miRNA-normalized piRNA levels mapping to 63 germline-dominant TEs in Zucchini-depleted versus Zucchini/Papi-depleted ovaries. h, Shown are confocal sections of egg chambers expressing indicated piRNA biogenesis reporters (GFP-fluorescence; DNA stained with DAPI) in wild-type (top) or Zucchini and Nibbler-depleted ovaries (middle and bottom). Top, reporter with no target site; middle, reporter used in Fig. 1d; bottom, reporter used in Extended Data Fig. 2f. i, Ovary lysates expressing N-terminally Flag-tagged wild-type Nibbler was immunoprecipitated (IPed) using M2 magnetic beads. Wild-type ovary lysates were used as a control. Red colour in the blot indicates a saturated signal. ATP synthase 5A (ATP syn) serves as loading control. Ovary volume indicates the amount of loaded lysate/IP fraction. j, Eluates from IP were blotted with indicated antibodies. Piwi is slightly enriched in the Flag–Nibbler IP fraction, while there is no detectable enrichment of Aub or Ago3 in the IP fraction.
Extended Data Figure 3 | Molecular characterization of the piRNA pathway in nibbler and papi mutant flies. a, papi gene locus indicating the position of the Cas9-induced frameshift allele. b, nibbler gene locus indicating the position of the Cas9-induced frameshift allele. c, Western blot analysis showing the loss of detectable Nibbler protein in nibbler−/− ovaries. ATP-synthase 5A antibody is used as loading control. Loaded amounts of ovary lysates are indicated. d, Northern blot analysis of the Nibbler-substrate miR-34 comparing small RNAs obtained from ovaries of w1118 or nibbler−/− flies. The GFP-Nibbler rescue transgene (used in Fig. 1f) restores miR-34 processing. e, f, Immunostainings (e) and western blot analysis (f) of Piwi, Aub and Ago3 in w1118 or in nibbler−/− ovaries, showing that localization and expression of the three PIWI-clade proteins are unperturbed (arrow heads; scale bars, 10 μm). ATP synthase 5A (ATP syn) served as loading control. g, Scatter plot displaying the steady-state RNA level of TEs in indicated genetic background (only TEs with RPKM >1 in either background; n = 40). h, Bar chart displaying TE mapping piRNA levels in w1118 or in nibbler−/− ovaries (values normalized to 1 million sequenced miRNA reads). i, j, Length profiles of TE mapping small RNA reads obtained from ovaries of indicated genotypes. Shown are all ovarian small RNAs (i) or Piwi-bound piRNAs defined as soma-enriched (j; see Methods). Displayed are fractions of reads of indicated length as a percentage (mean lengths are indicated below).
Extended Data Figure 4 | A small RNA library cloning approach that allows the recovery of longer piRNA species. *Drosophila* total RNA contains large amounts of the 30-nt long 2S rRNA. Previous cloning approaches therefore typically restrict small RNA cloning to the 18–29 nt window by cutting these small RNA populations from a gel. We used a previously published 2S rRNA depletion method\(^3\), followed by extracting small RNAs ranging from 18–40 nt in length for library preparation. Shown are size distributions of TE mapping small RNAs (obtained from *w*\(^{1118}\) ovaries) comparing the standard small RNA cloning protocol (left) and the protocol using total RNA depleted of 2S rRNA (middle; see Methods). An overlay of the longer reads (>27 nt) is displayed to the right.
Extended Data Figure 5 | Zucchini and Nibbler generate Aub- and Ago3-bound piRNA 3′ ends independently of piRNA 5′-end formation. 

a, Box plots (**P < 0.001 by two-sided t-test) showing the 3′-end definition (see Methods) of Ago3-, Aub-, and Piwi-bound piRNAs isolated from ovaries of the indicated genotypes. Soma-enriched Piwi-bound piRNAs (see Methods) are shown in boxes with diagonal lines. 
b, Stacked bar plots displaying the nucleotide composition at the 5′ end or position 10 of piRNAs bound to Aub-, Ago3-, or Piwi (isolated from ovaries of indicated genotypes). The plots show the composition within ten equally sized bins, sorted for their 3′ end precision index (see Methods). c, Box plots (**P < 0.001 by Wilcoxon rank-sum test) showing the $R^2$ values of the comparison between the 3′-end profiles of Ago3- or Aub-bound piRNAs from w1118 ovaries and those of nibbler−/− ovaries (Zucchini only), those of ovaries depleted of Zucchini (Nibbler only), and those of the calculated composite that provides the highest $R^2$ (best fit). Midlines in a and c indicate the median value, box ranges from the first to the third quartile, whiskers are 1.5× the interquartile range.
Extended Data Figure 6 | Ago3 incorporates more TE antisense piRNAs in nibbler mutant flies. a, b, Scatter plots displaying the strand bias (antisense divided by sense) of piRNA populations in w1118 versus nibbler−/− ovaries. a, piRNAs bound to Piwi, Aub, or Ago3. b, piRNAs from total ovarian RNA. c, Z-scores of 5′/5′ ping-pong levels per TE (63 germline dominant TEs) in w1118 versus nibbler−/− ovaries. d, Grouping of TEs (63 germline dominant TEs) based on fold change in piRNA levels between Zucchini-depleted and control ovaries (left). Box plot indicates Aub/Ago3-bound piRNA levels in wild-type ovaries for defined TE groups (midline indicates the median value, box the first and third quartiles, whiskers are 1.5× the interquartile range; ***P < 0.001 after Wilcoxon rank-sum test).
Extended Data Figure 7 | piRNAs are abundantly produced in Zucchini/Nibbler double-depleted ovaries. 

**a**, Heat map displaying fold changes in antisense piRNA levels per TE ($n = 63$) in indicated genotypes versus control (six biological replicates). Dots mark TEs, which lose piRNAs only in Zucchini/Nibbler-depleted ovaries. 

**b**, Mappings (5′ ends only; red, sense; black, antisense) of piRNAs onto the GATE and Doc TE consensus sequences. The plots at the top are from a piRNA library obtained from Zucchini-depleted ovaries, and the ones at the bottom are from a piRNA library obtained from Zucchini/Nibbler double-depleted ovaries. The ~100-bp window of Doc that is detailed in Fig. 4a is depicted by brackets.

**c**, Size distributions of TE-mapping small RNAs obtained from total small RNA libraries from Zucchini-depleted (top) or Zucchini/Nibbler-depleted (bottom) ovaries. Shown are the average values from six biological replicates (reads were normalized to 1 million sequenced miRNA reads).

**d**, Scatter plots displaying the steady-state RNA level of TEs in indicated genetic background (only TEs with RPKM $> 1$ in either background are shown; $n = 74$).
Extended Data Figure 8 | Precise coupling of neighbouring ping-pong piRNAs in Zucchini/Nibbler-depleted ovaries. a, Immunofluorescence images (confocal sections) of egg chambers of indicated genotype stained for endogenous Piwi protein (scale bars, 10 μm). Note that the shRNA-mediated knockdown is specific for germline cells. Somatic follicle cells that also express Piwi therefore serve as control. b, Length profiles (fractions of reads per indicated length as a percentage) of TE-mapping piRNAs bound to Aub/Ago3 in indicated genotypes. c, Box plot (midline indicates the median value, box ranges from the first to the third quartile, whiskers are 1.5× the interquartile range) displaying the distribution of Z-scores for canonical 5′/5′′ ping-pong of piRNA populations isolated from ovaries of indicated genotypes. The analysis is restricted to the 11 germline enriched TEs that maintain piRNA production in Zucchini/Nibbler-depleted ovaries (see Methods). Midline indicates the median value, box ranges from the first to the third quartile, whiskers are 1.5× the interquartile range. d, Histogram showing the frequencies of a cloned ping-pong piRNA 5′ end downstream of a responder piRNA 5′ end at a certain distance on the same strand. e, Sequence logos displaying the nucleotide composition within and downstream of Aub- and Ago3-bound piRNAs cloned from ovaries of indicated genotypes. 5′ end position and position 10 are measured from the piRNA 5′ end. 3′ end position is the dominant 3′ end of a certain piRNA 5′ species. Downstream positions are anchored by the dominant 3′-end position. Nucleotide signatures with respect to the position of sense and antisense slicer piRNAs are depicted in the cartoons below.
Extended Data Figure 9 | piRNAs generated by slicer/slicer are methylated. a, Shown are the mappings of those piRNAs (5' ends in black; 3' ends in grey) that were probed by northern blots in Fig. 4f. The 5' ends of antisense piRNAs (red) map precisely 10 nt downstream of the predicted slicer sites. Mappings were normalized to 1 million sequenced miRNA reads. b, An oxidized library control of the heat map shown in Fig. 4d, showing the precise coupling of piRNA 5' ends (blue) and 3' ends (red) in Zucchini/Nibbler-depleted ovaries.
Extended Data Figure 10 | Conservation of Zucchini/MitoPLD, PARN, PNLDCl, and Nibbler/Mut-7 across metazoa. 328 metazoan species are displayed in a phylogenetic tree (generated using iTOL). The presence of indicated orthologues in each species is marked (black, Zucchini/MitoPLD; green, PARN; blue, PNLDCl; red, Nibbler/Mut-7). Taxonomic groups mentioned in the text are highlighted (for details see Methods).