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PERSPECTIVE

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Insights in piRNA targeting rules

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Abstract

PIWI-interacting RNAs (piRNAs) play an important role in the defense against transposons in the germline and stem cells of animals. To what extent other transcripts are also regulated by piRNAs is an ongoing topic of debate. The amount of sequence complementarity between piRNA and target that is required for effective downregulation of the targeted transcript is guiding in this discussion. Over the years, various methods have been applied to infer targeting requirements from the collections of piRNAs and potential target transcripts, and recent structural studies of the PIWI proteins have provided an additional perspective. In this review, I summarize the findings from these studies and propose a set of requirements that can be used to predict targets to the best of our current abilities.

This article is categorized under:

Regulatory RNAs/RNAi/Riboswitches > Regulatory RNAs

RNA Interactions with Proteins and Other Molecules > Protein-RNA Interactions: Functional Implications

RNA-Based Catalysis > RNA-Mediated Cleavage

KEYWORDS

non-coding RNA, piRNA, PIWI, RNA regulation, targeting

1 | INTRODUCTION

PIWI-interacting RNAs, or piRNAs, are powerful molecules in transcriptional and posttranscriptional tuning of the genomic output. They are well-known for their function in silencing transposons in the animal germline. Depending on the species and the PIWI protein, they can do this by recognizing transposon transcripts and marking them for degradation, or by inducing epigenetic modifications at the matching genomic locus. In either case they recognize their targets by sequence complementarity, and their effect is mediated by their binding partner, the PIWI protein. Many excellent reviews have been written on the biogenesis of piRNAs and the mechanisms of PIWI function (Czech et al., 2018; Luteijn & Ketting, 2013; Ozata et al., 2019), and I refer to those for further information on these aspects.

Over recent years PIWI proteins and piRNAs have also been reported in tissues outside of the germline (in fact, the somatic presence of PIWI proteins likely reflects their ancestral state (Jehn et al., 2018; Lewis et al., 2018)), and effects of piRNAs have been proposed on sequences beyond transposons (Dai et al., 2019; Galton et al., 2022; Gou et al., 2014; Jones et al., 2016; Lee et al., 2011; Nandi et al., 2016; Rajasethupathy et al., 2012; Rouget et al., 2010; Sharma et al., 2001; Shi et al., 2020; van Wolfswinkel, 2014). Further it has become clear that sequence complementarity of the piRNA to the target does not need to be perfect, extending the range of potential targets dramatically. A major question

now is to what extent detected downstream effects can be ascribed to piRNA targeting (Wu & Zamore, 2021), and to what extent sequence complementarity to a piRNA is predictive of a regulatory relationship.

In this perspective, I will summarize and interpret recent findings on the targeting rules of piRNAs.

2 | DEFINITION OF piRNAS

Before determining what the targets of piRNAs are, it is necessary to define what molecules are considered piRNAs. The name indicates that a piRNA (PIWI-interacting RNA) is an RNA that interacts with a PIWI protein, but does that mean that any RNA that binds to a PIWI protein is a piRNA, and that an RNA that does not interact with a PIWI is not?

PIWI proteins consist of an N-terminal domain, two linker domains, a PAZ domain, a MID domain, and a PIWI domain (Figure 1a). The N-terminal, MID, and PIWI domain form a central channel that is covered by the PAZ domain, and is lined with positively charged residues (Figure 1b) (Song et al., 2004). To function as a guide for PIWI protein targeting, a piRNA needs to be bound at this central channel of the PIWI protein. The optimal sequence length for binding differs somewhat between different PIWI proteins, and allows for a few bases of flexibility, but typically is somewhere between 24 and 32 nt. Therefore, a length in this range is an expected feature of a mature piRNA.

During biogenesis of piRNAs, the loading onto a PIWI protein and piRNA length determination are coupled (Figure 1c): a longer RNA fragment is initially bound by the PIWI protein and is subsequently trimmed to its mature length (Feltzin et al., 2015; Izumi et al., 2016; Tang et al., 2016). If such RNA fragments are not bound to a PIWI protein, they are rapidly degraded by exonucleases (Balaratnam et al., 2022), and once bound to a PIWI protein the release rate of a piRNA is low (Arif et al., 2022). Therefore, piRNAs are found almost exclusively bound to PIWI proteins (Gainetdinov et al., 2018), and small RNA molecules of piRNA length in the absence of a PIWI protein are more likely to be intermediates of RNA degradation or processing pathways than to be actual piRNAs.

The trimming of piRNAs to their mature length is efficient (Kawaoka et al., 2011; Tang et al., 2016), and therefore longer pre-piRNAs are found only as a minor side population of more abundant shorter mature piRNAs. After trimming, the 3′ end of the piRNA is modified by methylation, which stabilizes the piRNA. Unmodified piRNAs are targets for degradation, and this destabilization is particularly strong when the piRNA has complementarity to a target (Gainetdinov et al., 2021), which is exactly what is required for the piRNA to be functional as a guide. It is possible that some piRNAs with little complementarity to other sequences can exist in unmodified state, but presence of this 2′Ome modification strongly increases the likelihood that a small RNA is a mature piRNA that can accomplish functional targeting.

Finally, there are probabilistic expectations on the sequence content of piRNAs: piRNAs have a bias for a 5′ Uridine and/or a bias for an Adenine at position 10, and frequently are derived from specific genomic clusters (Brennecke et al., 2007; Gunawardane et al., 2007). These features however do not apply to every individual piRNA, and therefore are problematic to enforce. Most piRNAs start with a Uridine as the first nucleotide, in part due to the fact that this nucleotide fits the binding pocket of most PIWI proteins best (see below), and in part due to biases of the nuclease Zucchini that generates the 5′ ends of many pre-piRNAs (Haase et al., 2010; Ipsaro et al., 2012; Stein et al., 2019). However some PIWI proteins, such as *Drosophila* Ago3, do not have a strong preference for a specific 5′ base (Cora et al., 2014). *Drosophila* Ago3 is one of a set of PIWI proteins that bind secondary piRNAs, generated from a 5′ end that is newly created by the targeting by a primary PIWI-piRNA complex in an amplification mechanism known as the ping-pong loop (see Figure 3d). The primary piRNA-PIWI complex has a preference for an Adenine opposite the first base of its piRNA (though not due to base-pairing with the 5′U), thereby creating a class of secondary piRNAs without a strong 5′ bias but with an Adenine at position 10 (Wang et al., 2014). Thus while presence of the 5′U may strengthen confidence in a sequence as a piRNA, the absence has no predictive power. Similarly, many piRNAs derive from piRNA clusters, and thus the fact that a small RNA sequence maps to such a cluster can be considered a confirmation of its definition as a piRNA. However, piRNAs produced by ping-pong amplification could be derived from target transcripts that are distinct from these clusters. Further, some piRNAs have been found to derive from 3′UTRs of coding genes, or from structural RNA, and there is evidence that piRNAs from non-cluster locations may actually carry the majority of the functional implications (Gebert et al., 2021). Therefore, if other conditions are satisfied, the atypical source of a small RNA is not a ground to dismiss it as a piRNA as it could have downstream effects identical to those induced by piRNA sequences derived from clusters.

In conclusion, to be considered a mature piRNA, an RNA should be bound to a PIWI protein, have a length that matches the binding preference of the PIWI protein, and have 3′ methylation (Figure 1c).

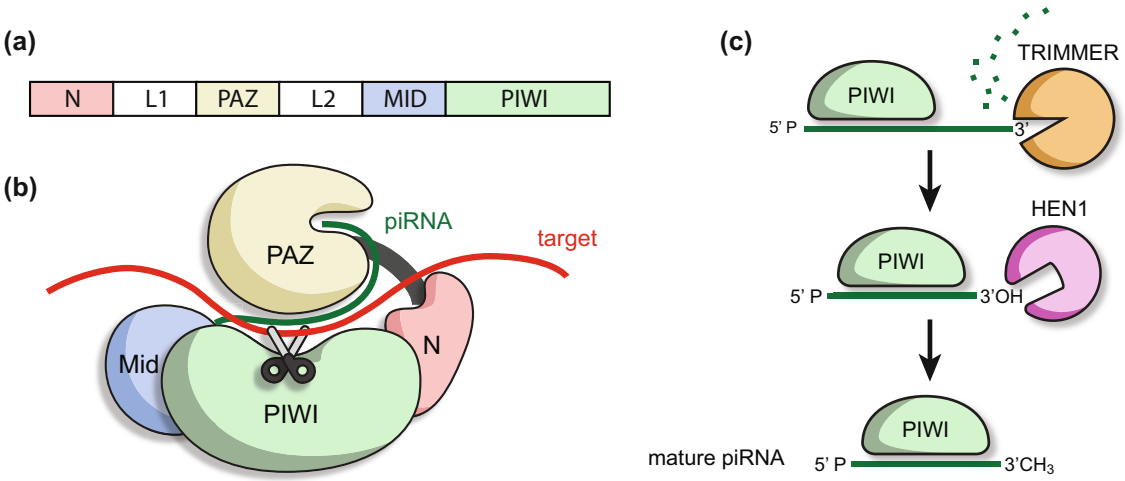


FIGURE 1 General features of PIWI proteins and piRNAs. (a). Schematic of the domains of the PIWI protein. (b). Schematic of the organization of the domains. The Mid domain binds the 5′ end of the piRNA, and the PAZ domain has a binding pocket for the 3′ end. The PIWI domain contains a catalytic site that has endonuclease activity. The PIWI-bound piRNA engages a target by sequence complementarity and both are located in the central channel of the PIWI protein. Adapted from (Song et al., 2004). (c) In the biogenesis of piRNAs, initially a longer RNA molecule with 5′ monophosphate group is bound by the PIWI protein, and this is subsequently trimmed to the correct length from the 3′ end. The trimmed piRNA is methylated by the methyltransferase HEN1, which inhibits further trimming and degradation.

3 | INSIGHTS FROM STRUCTURAL ANALYSES

Many of our expectations for piRNA targeting are based on findings regarding other regulatory small RNA mechanisms, in particular the miRNAs. miRNAs are bound by Argonaute proteins, which are closely related to the PIWI proteins (see Box 1). The structural differences between Argonautes and PIWIs therefore help tune our expectations for piRNA targeting.

Several PIWI proteins have been investigated by detailed structural analysis: the silkworm Siwi protein (Matsumoto et al., 2016), *Drosophila* PIWI (Yamaguchi et al., 2020), and an AGO-3 homolog from the sponge *Ephydatia fluviatilis* (Anzelon et al., 2021). These studies have painted a clear picture of the similarities and differences between PIWIs and Argonautes.

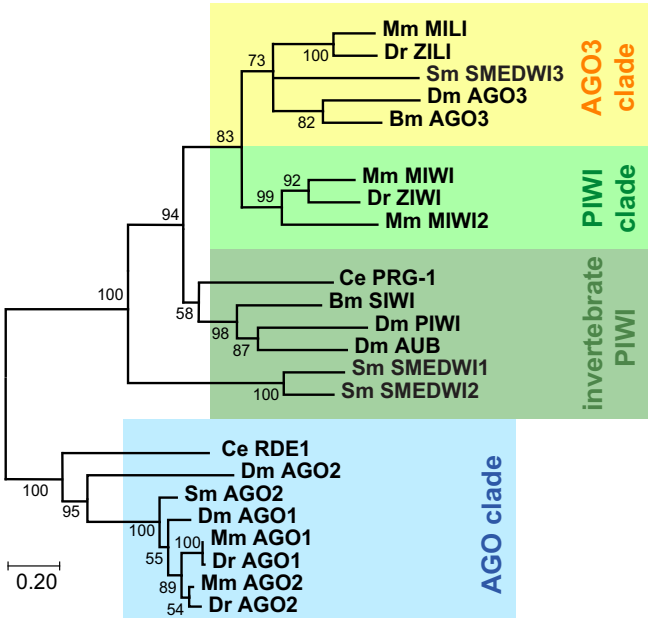
The 5′ and 3′ end of the piRNA both have dedicated binding pockets in the PIWI protein, thereby defining the optimal length of the small RNA (Matsumoto et al., 2016), similar to the situation for Argonautes (Figure 2a). The 5′ nucleotide is fitted in a binding pocket in the MID domain that recognizes a 5′ mono-phosphate. In most PIWI proteins, a loop on the MID domain, also referred to as the specificity loop, can form a hydrogen bond with a Uridine, but clashes with any of the other bases, thereby providing a structural basis for the observed 5′U bias of piRNAs (Cora et al., 2014; Frank et al., 2010; Stein et al., 2019). The binding site for the 3′ end of the piRNA in the PAZ domain has a hydrophobic pocket that fits the protective 2′Ome group that has repeatedly been detected on piRNAs (Figure 2b; Matsumoto et al., 2016).

In the miRNA-binding Argonaute proteins, the central cleft is narrow and stacks nucleotides 2 through 6 of the miRNA in a manner that reduces the entropic cost of base-pairing with a target RNA (Parker et al., 2009; Schirle & MacRae, 2012). Due to this stacked conformation, the pairing of these first few bases is much more rapid and energetically favorable in the context of the Argonaute protein than would be expected for the naked small RNA, and this explains the outsized weight that base-pairing of this 5′ region contributes to miRNA targeting. If bases beyond nucleotide 5 are paired, the Argonaute protein goes through a conformational change that allows base-pairing to continue up to nucleotide 8 and also exposes nucleotide 13–16 for additional pairing (Schirle et al., 2014; Sheu-Gruttadauria et al., 2019). Right after nucleotide 8 however the cleft narrows and does not allow base-pairing between miRNA and target for the next couple of nucleotides (Figure 2c) (Sheu-Gruttadauria et al., 2019). These conformational features explain the independently identified concept of miRNA targeting by base-pairing of nucleotide 2–8 of the miRNA, known as the seed region (Lewis et al., 2003), which can be complemented by additional pairing at nucleotides 13–16 (Brennecke et al., 2005; McGeary et al., 2022).

Compared to Argonaute proteins, PIWI proteins have a widened central cleft that does not narrow significantly after nucleotide 8 of the piRNA, and the initial bases of the piRNA are less organized (Figure 2c; Anzelon et al., 2021). This

BOX 1 Phylogenetic relationships between Argonautes and PIWI proteins

The family of Argonaute proteins consists of a highly conserved group of proteins that all bind small non-coding RNA (Carmell et al., 2002). All eukaryote Argonaute proteins contain the same set of domains and the same overall domain architecture, described in Figure 1. Prokaryotic Argonaute proteins are more divergent in their domain composition (Koopal et al., 2022). Based on further domain similarity, several clades of eukaryote Argonautes can be distinguished. The AGO clade (blue) contains the canonical Argonaute proteins that are conserved among animals, fungi and plants. They are typically present in every cell of the organism and bind to siRNAs (small interfering RNAs) and miRNAs (microRNAs). Animals have several more clades of related proteins that are most highly expressed in the germline and the pluripotent stem cells, and are collectively known as the PIWI proteins. Within the PIWI proteins, the AGO3-related PIWIs form a separate clade (yellow). Among the other PIWIs the vertebrate sequences cluster closely together while the invertebrate PIWIs are more divergent (green).



Mm *Mus musculus*, Dr *Danio rerio*, Dm *Drosophila melanogaster*, Bm *Bombyx mori*, Sm *Schmidtea mediterranea*, Ce *Caenorhabditis elegans*. Numbers at branches indicate bootstrap values of the Maximum Likelihood tree.

suggests that the seed region still needs to match its target to fit the cleft, but the wider cleft may allow for more lenience in the base-pairing. Further, the lack of seed stacking suggests that the weight of base-pairing in the seed region likely does not outsize the weight of other bases significantly. This implies that base-pairing at the seed region creates a weaker bond than in Argonautes, and thus that stabile binding of a target requires significant additional base-pairing outside of this region. But in contrast to the situation in Argonautes, the base-pairing can continue beyond the first 8 nucleotides and this can thus create the additional stability required.

Most Argonaute proteins have catalytic activity that is conferred by the presence of a catalytic triad of amino acids known as the DDH motif (Liu et al., 2004). This activity allows the Argonaute-guideRNA complex to cleave a target RNA opposite the bond between nucleotide 10 and 11 of the small RNA. For this activity to engage however, an energetically costly conformational change of the Argonaute protein needs to take place to correctly position the catalytic site. This requires full base-pairing around the scissile site, which puts significant strain on the Argonaute protein, and needs to be compensated by additional base-pairing. Additionally, for the target and the guideRNA to fit the central channel, the 3′ end of the small RNA needs to be released from the binding pocket, which comes at a significant

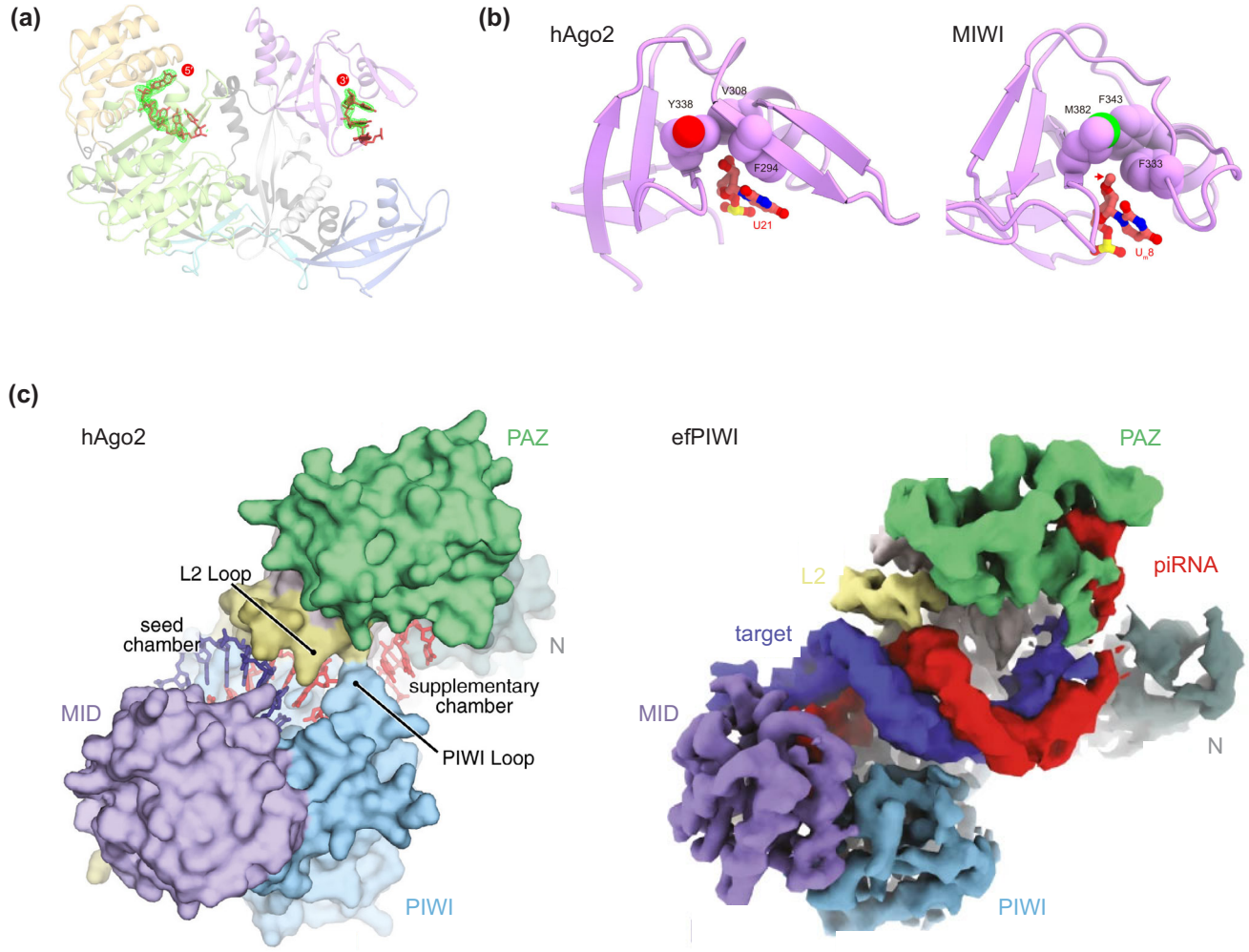


FIGURE 2 Structural comparison between Argonaute proteins and PIWI proteins. (a) Binding pockets for the 5′ and 3′ end of the piRNA in the silkworm PIWI protein SIWI MID and PAZ domain, respectively. Adapted from (Matsumoto et al., 2016). (b) Comparison of the binding pockets for the 3′ end of the small RNA between human Argonaute protein Ago2 (hAgo2) and mouse PIWI protein MIWI. Adapted from (Matsumoto et al., 2016). The PAZ domain of MIWI has more space in the binding pocket which allows for the fitting of the terminal 2′Ome (arrow). (c) Comparison of the central channel in hAgo2 (Sheu-Gruttadauria et al., 2019) and efPIWI (Anzelon et al., 2021). In hAgo2, a narrowing of the channel disrupts the base-pairing between piRNA and target at the end of the seed sequence. In efPIWI a widened channel can be formed that allows for base-pairing of piRNA and target along the whole length of the sequence. Adapted from (Sheu-Gruttadauria et al., 2019) and (Anzelon et al., 2021).

energetic cost that needs to be offset by further base-pairing between the guideRNA and the target (Tomari & Zamore, 2005). Together this means that for Argonaute catalytic activity to occur, base-pairing between the small RNA and its target needs to be (near) perfect. This typically does not happen for metazoan miRNAs, but does match what is found for siRNAs (small interfering RNAs) that function in genome defense (Elbashir et al., 2001).

The catalytic site of the PIWI protein is similar to that of the Argonaute and is positioned opposite the bond between nucleotide 10 and 11 of the piRNA. What the catalytically active conformation of the PIWI protein looks like however is not entirely known. Only recently it was found that the fully active PIWI complex requires the presence of the auxiliary Zn finger protein GTSF1 (Arif et al., 2022), and no structural studies have yet been conducted in the presence of this protein. Studies of PIWI structure in the absence of GTSF1 however found that a conformational opening of the cleft has to take place to accommodate extended sequence complementarity between the piRNA and its target and that a conformational change is required for correct positioning of the catalytic triad (Anzelon et al., 2021; Matsumoto et al., 2016). In accordance with this, activation of cleavage activity (in the absence of GTSF1) required significantly

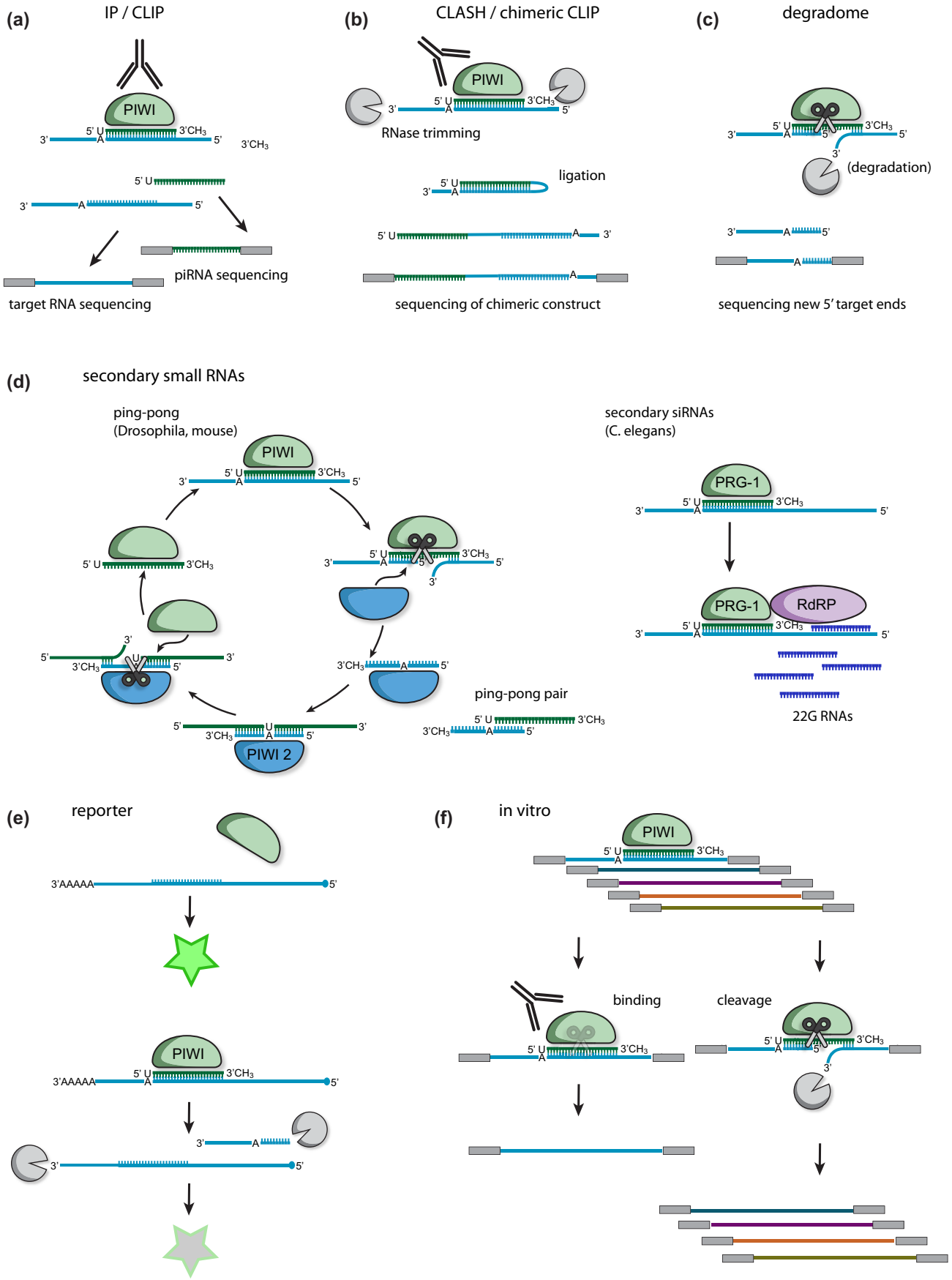


FIGURE 3 Legend on next page.

more base-pairing than just the seed region: in efPiwi, cleavage activity reached plateau only when the pairing extended to nucleotide 18 with no more than 2 mismatches (Anzelon et al., 2021).

In Argonautes, target cleavage leads to reduced stability of the association between the AGO-siRNA complex and each of the two cleavage products. The reduced number of base-pairs with each fragment is no longer sufficient to sustain binding, and the strain involved in base-pairing beyond the 8th nucleotide at the seed contributes to the instability, resulting in the rapid release of the target fragments after cleavage (Salomon et al., 2015; Yao et al., 2015). PIWI-piRNA complexes however retain stronger and more stable interactions with their base-paired target after cleavage. Silk moth Siwi remained bound to the cleaved target RNAs, and required the RNA helicase Vasa to release the cleavage products (Nishida et al., 2015), and in mouse both MIWI and MILI failed to release their targets after cleavage (Dowling et al., 2023), except in the presence of additional factors from testis lysate, whose identity currently remains unknown (Arif et al., 2022). *Drosophila* PIWI showed that the dynamics of this nuclear PIWI protein might be even more rigid. Modeling showed that PIWI remains tightly bound even to targets with multiple mismatches, and as *Drosophila* PIWI has lost its nuclease activity there is no obvious way to reduce the affinity (Yamaguchi et al., 2020). PIWI therefore is likely unable to release a target without the help of one or more auxiliary factors. Similarly, the release of bound targets from efPIWI in the absence of auxiliary factors was found to be comparable to the lifespan of typical mRNA molecules (Anzelon et al., 2021), suggesting that targets once bound may never be released.

Based on these studies it can be expected that effective piRNA targeting requires substantial sequence complementarity, and that mere pairing of the seed region is unlikely to suffice. On the other hand, once a target with ample base-pairing has been bound, this bond is predicted to be very stable and not easily disrupted. While these structural studies give valuable insights in the general principles of PIWI function, the structures are determined based on a very small number of piRNAs and targets, and thus more high-throughput strategies are required to determine what amount of sequence complementarity is sufficient for piRNA targeting.

4 | EXPERIMENTAL STRATEGIES TO DETERMINE piRNA TARGETS

As piRNAs find their targets by sequence complementarity, many studies have used the sequences of piRNAs to predict potential targets. Based on the structural studies it is clear that some amount of mismatching between piRNA and target is likely to be tolerated while still allowing for PIWI activation, but that does not reveal how much mismatching is found among natural targets. The study of bound target molecules thus can give further insights into the base-pairing requirements.

FIGURE 3 Methods to determine piRNA targeting. (a) Immunoprecipitation (IP) of the PIWI protein recovers both the piRNA (green, 5′U indicated for orientation) and the associated target RNA (blue). The RNAs can be separated based on length and modifications. Cloning and sequencing of the associated RNA identifies the population of small RNA as well as the population of associated target sequences. Crosslinking of the PIWI protein before IP (CLIP) to the RNA can improve the recovery and provide additional information about the most proximal residues. (b) In chimeric CLIP or CLASH the PIWI protein and RNAs are crosslinked before immunoprecipitation, and the associated RNA molecules are trimmed and ligated resulting in a chimeric molecule that combines the piRNA and the target sequence. Cloning and sequencing of these chimeric molecules thus gives direct information about the pairing between piRNA and target. (c) The endonucleolytic action of the PIWI protein results in the formation of a new monophosphorylated 5′ end at the target site, opposite nucleotide 10 of the corresponding piRNA. Cloning of these degradation fragments (degradome) and combining this with sequencing of piRNA sequences provides information about the likely combination between piRNA and target. Upon elimination of the piRNA, the reduction of the cleavage product in combination with the elevated level of the uncleaved target then confirms the regulatory interaction. (d) The downstream effect of piRNAs includes the generation of secondary small RNAs from the target RNA. In most animals, this involves the action of a second PIWI protein which through a mechanism known as the ping-pong cycle results in the generation of a second piRNA that has a 10 nucleotide overlap with the initial piRNA. This signature of ping-pong piRNAs thus indicates the effective targeting of a long RNA. In *C. elegans* the amplification mechanism involves an RNA dependent RNA polymerase (RdRP) that synthesizes novel antisense small RNAs (22G RNAs) in proximity of the targeting by the worm piRNA (21U RNA). (e) Effective piRNA targeting can be estimated by evaluating the stability of a reporter mRNA. The reporter typically encodes a fluorescent protein. Effective targeting by a piRNA will result in reduced stability of the mRNA and thus reduction of the fluorescent signal. (f) Binding and cleavage activity and kinetics of a specific PIWI-piRNA complex can be determined by in vitro approaches. Typically, a purified PIWI protein is preloaded with a specific piRNA, and exposed to a library of potential targets. To determine binding preferences, the subset of bound target molecules can be precipitated with the (catalytically inactive) PIWI protein and sequenced. To determine cleavage activity, the non-cleaved targets can be processed for sequencing, or a degradome approach can be used to clone the newly generated 5′ ends.

4.1 | *Make me a match…* piRNA targets by base-pairing and binding

A straight-forward method of experimentally identifying targets is to inspect the longer RNAs that precipitate with a PIWI protein, and determine their level of complementarity to independently sequenced piRNAs (Figure 3a). Crosslinking before immunoprecipitation (CLIP and many variants thereof) increases the stability of such interactions. Such studies have revealed that a wide range of mRNAs can associate with PIWI proteins but remarkably only a fraction could be assigned well-matching piRNAs (Barckmann et al., 2015; Gou et al., 2014; Toombs et al., 2017; Vourekas et al., 2012). In retrospect it appears that this type of IP data may be too noisy to deduce targeting principles. Co-precipitation of the longer RNA with the PIWI proteins could be mediated by pairing to a piRNA, but long RNAs could also be associated with other regions of the PIWI protein and binding could even be indirect through other interacting proteins or RNAs. Binding to any surface of the PIWI protein other than the central channel is meaningless in the context of piRNA targeting. Positional information of an added crosslink can mitigate these concerns, but it has become clear that further evidence in addition to mere binding is required to infer targeting. Further, these methods do not provide information on which piRNA matches to which target—this is only inferred from complementarity between independently sequenced reads—and therefore it is very difficult to distill targeting rules from such IP experiments.

A recent study in *C. elegans* introduced an elegant variant of target cloning called CLASH (Crosslinking, Ligation, and Sequencing of Hybrids; Kudla et al., 2011) to the piRNA field (Shen et al., 2018) in which the piRNA is ligated to the (trimmed) bound target before sequencing, thereby providing direct information about the pairing between the two (Figure 3b). This study revealed that *C. elegans* 21 U piRNAs have a 5′ seed region at (bases 2–8) in which little to no mismatches are tolerated, followed by a center region (nucleotides 9–13) in which base-pairing is more lenient, and a 3′ region (bases 14–21) in which base pairing is more stringent again (Figure 4).

Similar chimeric reads can be derived from CLIP experiments in which piRNAs and their matching long RNAs have become ligated, and these chimeras similarly can give insight in direct pairing interactions. Application of this method to *Drosophila* Aubergine (Aub) revealed limited base-pairing and detected more mismatches in the center region of the piRNA than on the 5′ seed or the 3′ end (Vourekas et al., 2016). Remarkably, this study found that among transcripts that had target sites with similar base-pairing, only a subset was bound to Aub, suggesting that other factors such as abundance and transcript length contribute to the outcome. A similar chimeric CLIP approach on S. *mediterranea* PIWI protein SMEDWI-3 led to similarly mixed results (Kim et al., 2019). While some long transcripts could be linked to matching piRNAs, many could not be assigned.

While this chimeric approach gives more insight into the pairing of piRNA and target, there still are substantial limitations. There are indications that the ligation reactions introduce biases into the analysis as there are discrepancies in the abundance of specific sequences identified by IP and those identified by chimeric methods. Further, binding does not equal functional targeting. Similar studies in miRNA targeting have shown that CLIP association is a poor predictor of regulatory effects (Chu et al., 2020). For each individual small RNA, the number of true matching target sites in the transcriptome is low, and the number of incomplete matches is orders of magnitude larger. Therefore the likelihood of catching a small RNA while it is binding to a poorly matching site—while instable—is high. The CLIP protocols cannot distinguish between the true functional pairings and the instable ones, and thus this data remains noisy. While functional targeting without detectable binding may be highly unlikely, binding without functional targeting is probably prevalent. Binding therefore is a prerequisite, but remains an inaccurate predictor for the functional effects of piRNAs.

4.2 | *As long as it works…* piRNA targets based on downstream effects

Another method for target identification is by using downstream effects as a proxy (Figure 3c–e). Cleavage by a PIWI-piRNA complex splits a target mRNA into two fragments: a capped fragment with a newly unprotected 3′ end that is rapidly degraded, and an A-tailed fragment with a newly generated uncapped 5′ end. These 5′ mono-phosphorylated fragments can be cloned for sequencing approaches and are generally referred to as the degradome (Figure 3c). If the 5′ end is positioned at the 10th nucleotide position of a matching piRNA, this strongly suggests that the matching piRNA was causal to the generation of this cleavage fragment (Goh et al., 2015; Reuter et al., 2011; Zhang et al., 2015). If the abundance of the cleavage fragment is reduced in a background that eliminates the piRNA or the PIWI protein, the case for causality is strengthened further. In such a study of MIWI, base complementarity of nucleotides 2–22 was required for cleavage in vitro, proposing the need for extensive base pairing (Reuter et al., 2011). Later in vivo analyses

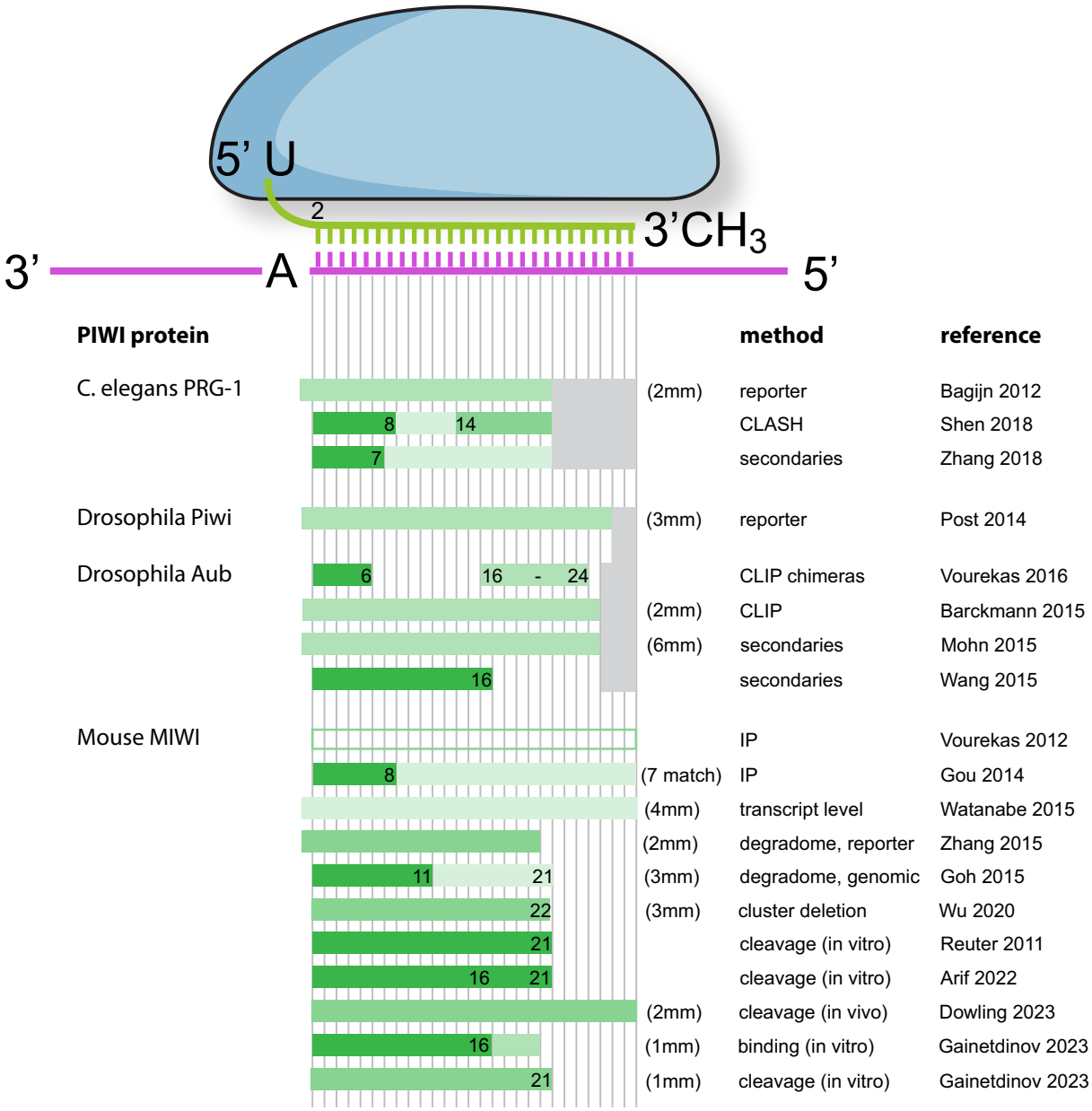


FIGURE 4 Findings on piRNA targeting. Schematic summary of the findings from various studies on the targeting rules in different PIWI proteins. Gray blocks limit the typical length of the piRNA for each PIWI protein. Indicated in green are the piRNA nucleotides that were found to match a detected target by base-pairing. The shade of green indicates the number of mismatches allowed, and where possible the maximum number of mismatches in the light green region is indicated on the right. Please note that not all detail and nuance on the identified pairing rules from these studies could be captured in the simplicity of this figure.

confirmed that extensive base-pairing is required for MIWI-mediated cleavage, although some amount of mismatches was tolerated (Dowling et al., 2023; Gainetdinov et al., 2023; Zhang et al., 2015).

A major limitation of this method is that not all action of PIWI proteins may require target cleavage, and that in some cases such as in chromatin-related effects or in the *C. elegans* piRNA pathway, association may be sufficient (Bagijn et al., 2012; Darricarrere et al., 2013; Lee et al., 2012). Therefore, several other readouts can be used instead. The effective targeting of a piRNA can also be deduced from the generation of secondary small RNAs from a matching region, suggesting that targeting by the initial piRNA was likely at the base of this amplification effect (Figure 3d). In many animals this will involve ping-pong generated piRNAs that thus require cleavage, but in the case of *C. elegans*, 22G RNAs are produced in the vicinity of piRNA targeted loci, and these can be analyzed in a similar manner. Several

studies that used this approach came to very similar conclusions, namely that to generate 22G RNAs a piRNA needs to have perfect pairing of nucleotides 2–7 (the seed) and up to 3 mismatches in nucleotides 8 through 21 (Bagijn et al., 2012; Shen et al., 2018; Zhang et al., 2018). Additionally, GU wobble bases were found to be overrepresented among the pairing interactions (Zhang et al., 2018), and whether or not such wobble bases were taken into account may explain the small differences between these studies.

Finally, downstream effects can be deduced from changes in transcript levels of targeted mRNAs, or from the intensity of fluorescent signal from reporter constructs (Figure 3e). This is typically combined with genetic manipulations that modify the piRNA repertoire or the activity of the PIWI protein to test its effect on the reporter or transcripts. Using a reporter-based approach, it was found that at perfect complementarity the silencing effects of piRNAs were weaker than the effects of miRNAs, and piRNA-mediated silencing was undetectable when matching only the seed sequence (Post et al., 2014). And a transcriptome approach to study the effect of MIWI activity on endogenous transcript levels identified a large set of altered mRNAs that contained fragments of retrotransposons, suggesting that transposon-derived piRNAs could target these transcripts for degradation (Watanabe et al., 2015). One caveat of these methods is that there is no direct connection between the proposed initiating piRNA and the downstream effect, and while a connection may be likely, it is possible that other small RNAs or indirect effects contribute to the detected outcomes. This limitation makes that this method of determining targeting has to be interpreted with caution and is likely problematic for deducing targeting rules, unless it is combined with other approaches that test the correlation between the piRNA and the effect more directly.

4.3 | piRNA targeting lessons from genomic manipulations

An interesting version of this last approach is to insert or delete an entire piRNA cluster and analyze the effects that this creates on endogenous transcripts. While there are caveats associated with such major genomic modifications, this strategy has produced some noteworthy insights.

The mouse genome contains a dozen clusters that produce the majority of piRNA molecules. Remarkably, elimination of two such prolific pachytene piRNA clusters, pi17 and pi2, from the genome had no detectable effect on murine health or fertility (Wu et al., 2020). While it is surprising that such major sources of piRNAs appear to be dispensable for adequate piRNA-mediated silencing, similar findings have been reported in *Drosophila* where the *flamenco* cluster was required for fertility, but deletion of all of the three largest piRNA clusters resulted in no detectable effect on fertility or on transposon silencing (Gebert et al., 2021).

The removal of murine piRNA clusters pi6 (Wu et al., 2020) and pi18 (Choi et al., 2021) created highly penetrant sperm maturation defects, but based on changes in transcript abundance still only few piRNA-targeted transcripts were identified in these studies. For pi18 only a single target was proposed, and for pi6 the defect was proposed to be due to direct targeting of 6 specific mRNAs. Within this limited set of targets, the matching piRNAs appeared to have no more than three mismatches in nucleotides 2–22 (although one proposed matching piRNA and target had 6 mismatches in this stretch) and the piRNA levels were estimated to be around 500 copies per cell, which was at least several-fold higher than the level of each targeted transcript (Wu et al., 2020). Together these findings indicate that the few effective piRNAs tend to be high-complementary and abundant, but that the majority of the cluster-derived piRNAs do not have functional implications for mRNA levels.

The opposite approach, insertion of a human piRNA cluster into the mouse genome, resulted in the production of abundant new piRNAs that targeted genes involved in germline development as evidenced by detection of new degradome reads at the predicted piRNA target site as well as sense ping-pong piRNAs. Based on the sequences of the newly introduced piRNAs and the affected target transcripts, the targeting requirements included perfect base-pairing at nucleotides 2–11, and no more than 4 mismatches in nucleotides 12–21 (Goh et al., 2015).

Several caveats are warranted here. First, it is uncertain which exact piRNAs cause the effects, and whether the detected effects are caused only by the piRNAs that are part of the manipulated genome fragment, or whether there are additive effects from other piRNA sources in play. Further, it was found that piRNAs can target other clusters and generate more piRNAs (Wu et al., 2020), and it thus is possible that there are indirect piRNA-mediated effects in addition to indirect transcriptome-mediated effects at play. Nevertheless, these findings suggest that highly matching piRNAs can induce cleavage of mRNAs, even though in vivo effects on steady-state transcript levels were rarely detected.

4.4 | *Under the hood…* piRNA targeting according to in vitro approaches

A more controlled way to study the binding and cleavage activity of PIWI-piRNA complexes is to isolate the PIWI protein, pre-load it with one specific piRNA sequence, and test the complex with a range of specifically designed targets in an in vitro environment (Figure 3f). This allows for direct comparison of the effect of the PIWI-piRNA complex on targets that differ in little more than the amount and positioning of their sequence complementarity. Furthermore this allows for the analysis of the kinetics of the interactions.

For example, testing the binding of synthesized and preloaded efPIWI in vitro showed that targets with only seed matching are unstably bound and rapidly dissociate (Anzelon et al., 2021). The relevance of the seed in this context was also found to be very limited: base-pairing of 9 nucleotides resulted in comparable binding stability, whether it started at the 2nd base or at a later position in the piRNA (Gainetdinov et al., 2023).

Optimal cleavage activity by MIWI loaded with a specific piRNA was found to require complementarity of nucleotides 2–22 (Reuter et al., 2011), or when supplemented with the GTSF1 reached plateau at perfect complementarity of nucleotides 2–20 (Arif et al., 2022). If complementarity was extended further, mismatches could be tolerated with limited loss of efficiency, but insertions or deletions resulted in a significant drop in efficiency (Gainetdinov et al., 2023). Remarkably, in each of three PIWI proteins tested, the position of the mismatches was found to be inconsequential for cleavage, and could include the nucleotides adjacent to the scissile bond, as long as sufficient overall base-pairing was maintained. Another interesting finding from these in vitro studies is that an extended non-pairing 3′ end of a piRNA inhibited the efficiency of the cleavage: a 16mer piRNA with perfect 2–16 complementarity was more efficient at cleavage than a 3′ extended version of the same sequence that did not add additional base-pairing (Arif et al., 2022). This suggests that the release of the 3′ end of the piRNA from the binding pocket, which is required for the active conformation of the MIWI complex with a longer piRNA, may require additional base-pairing to overcome the energetic cost of the release.

The obvious limitation of in vitro studies is that there may be additional factors or spatial considerations in the cell that alter the behaviors of the PIWI proteins, and that are not included in the in vitro models. Nevertheless, these studies provide clear and quantitative findings that form a valuable complement to the in vivo analyses.

4.5 | Conflicting findings in piRNA regulation and targeting

The different approaches each have their limitations and blind spots, leading to different findings on the interactions between piRNAs and their RNA targets, and the consequences of those interactions. The difficulties in deriving targeting rules with predictive value are nicely demonstrated by summarizing findings for the *Drosophila* PIWI protein Aubergine (Aub) and for the mouse protein MIWI.

4.5.1 | Aubergine

A CLIP study in early embryos found binding of Aub to many maternally deposited mRNAs. Bound sequences were enriched in mRNAs that had less than three mismatches to piRNAs (Barckmann et al., 2015). However, among targets with similar binding characteristics, some were bound and others were not, so this finding was not sufficient to define predictive targeting rules. Notably, due to the vast repertoire of piRNA sequences, allowing three mismatches results in potential regulation of almost the entire *Drosophila* transcriptome (Post et al., 2014), and thus becomes meaningless for predictive purposes. Further, of the Aub-bound RNAs in this study, only 31% were altered in transcript level upon loss of Aub, and only 10% of bound RNAs produced ping-pong piRNAs (Barckmann et al., 2015), suggesting that a significant fraction of the bound transcripts were not detectably regulated through cleavage. Instead, much of the effect was proposed to be mediated by de-adenylation followed by degradation of targeted transcripts in the somatic section of the embryo (Rouget et al., 2010), whereas positive regulation of adenylation stabilized transcripts in the germ plasm (Dufourt et al., 2017; Ramat et al., 2020). These effects have only been tested for an individual piRNA-target combination, and thus no further targeting rules could be deduced.

Analysis of chimeric CLIP data on Aub similarly detected binding of many transcripts, and found very limited base-pairing between piRNA and target (Vourekas et al., 2016). This study identified strong complementarity to the target only for nucleotides 2–6 of the piRNA with some additional complementarity at nucleotides 16–24, reminiscent of

miRNA binding. This low amount of complementarity allows the piRNAs to target almost any mRNA, and the study thus proposed that Aub binding to mRNAs was largely random and driven by abundance and length rather than sequence, and that it helped sequester transcripts in the germ plasm of the developing embryo.

Of two studies using the production of ping-pong piRNAs as a readout for targeting, one came to more predictive targeting rules (Mohn et al., 2015; Wang et al., 2015). This study found that nucleotides 2–16 had to match between piRNA and target, although possibly one or two mismatches could be tolerated (Wang et al., 2015). This study also found that a catalytically dead Aub phenocopied the effects of a full Aub mutant, indicating that cleavage is an essential aspect of Aub function.

4.5.2 | MIWI

Similarly, conflicting data have been reported for the mechanism of action as well as the targeting rules of the mouse PIWI protein MIWI. Several studies found that a wide range of piRNAs and mRNAs was bound to the mouse MIWI protein, and many mRNAs were altered upon loss of MIWI (Gou et al., 2014; Reuter et al., 2011; Watanabe et al., 2015; Zhang et al., 2015). These studies however differed in their conclusions on how this effect on mRNAs was brought about, and what part of this effect was mediated by piRNAs. In one study, the majority of the changes in mRNA levels could not be related to the MIWI-bound piRNA sequences, and only targeting of LINE-1 elements was found to be directly mediated by MIWI-bound piRNAs (Reuter et al., 2011). A second study identified MIWI-dependent and potentially piRNA-induced cleavage sites on ~170 mRNAs, and found that 43% of those were associated with MIWI by CLIP (Zhang et al., 2015), suggesting cleavage-directed regulation of these mRNAs. A third study focusing on late spermatocytes also found ~170 altered mRNAs, and although they were unable to detect binding of these transcripts to MIWI, they did identify an enrichment of transposon-related sequence fragments in these mRNAs as well as degradome reads from these transcripts indicating MIWI-mediated cleavage (Watanabe et al., 2015). However a fourth study, focusing on elongating spermatids, concluded that many of the bound mRNAs were de-adenylated and that this effect was independent of MIWI slicer activity (Gou et al., 2014). This finding was surprising as an earlier study had found that the sperm developmental arrest caused by a catalytically dead MIWI was largely identical to that induced by a full MIWI null mutant (Reuter et al., 2011).

The MIWI studies also reported a range of conclusions regarding the targeting rules. In vitro studies found that binding requires at least 15 continuous nucleotides of base-pairing, and that cleavage reaches plateau efficiency if this stretch is extended to 19 nucleotides, while limited mismatches are tolerated in such longer stretches (Arif et al., 2022; Gainetdinov et al., 2023; Reuter et al., 2011). However in vivo, Gou et al., who had reported a miRNA-like mechanisms for transcript regulation, found that many spermatogenic mRNAs were targeted by MIWI-bound piRNAs through very lenient base-pairing that had no mismatches in nucleotides 2–8, but could include up to 6 mismatches in nucleotides 9–18 (Gou et al., 2014). This targeting was verified using reporter constructs, but only for a few very highly expressed piRNAs. In contrast, two studies that focused on degradome readouts found that nucleotides 2–21 can contain no more than three mismatches in order to achieve cleavage (Goh et al., 2015; Zhang et al., 2015). Differentiating by expression levels of individual piRNA sequences revealed that more flexibility in base-pairing is tolerated for highly abundant piRNAs, and that for those piRNAs base-pairing can also start well after the 2nd nucleotide but that significant base-pairing over a stretch of 15–20 nucleotides has to be maintained (Gainetdinov et al., 2023). Remarkably, both in vitro and in vivo studies found that the positions of the mismatches that are tolerated by MIWI without significant effect on activity include the nucleotides next to the scissile site (nucleotides 10 and 11, which in Argonaute proteins need to be perfectly paired for cleavage to occur) (Dowling et al., 2023; Gainetdinov et al., 2023). An intriguing possibility is that GTSF1 helps to arrange MIWI into a catalytically active conformation and thereby has reduced the importance of individual base-pairing positions in PIWI proteins.

4.5.3 | Combining observations

It is clear that studies not always agreed on the proposed piRNA targeting rules for Aub and MIWI. There are several confounding factors that may explain these discrepancies between the results. Different tissues or developmental phases may play by different rules, in vitro approaches may lack certain accessory factors, and the expression levels of the piRNAs may affect the targeting rules that they follow (see below). One recurring observation however is that

the downstream effect that is scored affects the stringency of the detected targeting rules. Focusing on degradome and cleavage approaches to MIWI targeting, the findings match remarkably well between studies, and found that significant complementarity of bases 2–21 of the piRNA to the target is detected, and no more than 2 or 3 mismatches in these 20 bases can be tolerated (Arif et al., 2022; Dowling et al., 2023; Gainetdinov et al., 2023; Goh et al., 2015; Reuter et al., 2011; Zhang et al., 2015). When instead scoring mRNA reduction (which could include effects by cleavage, de-adenylation, or potential indirect effects) or binding, the targeting rules identified are much more lenient (Gou et al., 2014; Vourekas et al., 2012). A similar observation can be made from the *Drosophila* data.

One possible explanation for this discrepancy in rules based on the readout is that effects on mRNA level may involve additional factors and considerations in addition to mere piRNA cleavage, and that this obfuscates the rules for piRNA targeting. Further, it is likely that methods involving changes in RNA levels or CLIP did not always allow for the definitive identification of the correct functional pairings between piRNA and target, as the identification of the piRNA that causes the effect is more ambiguous, and this could lead to more difficulty in distilling the underlying rules. Another real possibility is that some of the effects for which no clear base-pairing rules could be identified, are in fact sequence-independent. Argonaute proteins have been proposed to have sequence-independent affinity for single-stranded RNA (Ameres et al., 2007; Salomon et al., 2015), and a similar affinity could explain how large sets of abundant mRNAs could associate with PIWI proteins, especially during developmental transitions that involve major reorganization of the transcriptome. PIWI proteins could well have functional roles in the formation of large protein-RNA aggregates involved in such transitions and thereby confer effects on large groups of transcripts. Finally, it is possible that piRNAs are able to function through different mechanisms, and that while stringent base-pairing is required for transcript regulation by cleavage, less accurate base-pairing may still lead to regulation by de-adenylation. However, less complete base-pairing also results in a less stable interaction, and in a higher expected number of competing interactions in the transcriptome that have similar or better binding affinity. An important question to consider then is how much effect an individual piRNA sequence can reasonably have, given the sequence space of the transcripts that it can regulate, relative to its number of copies in the cell.

5 | ROLE OF STOICHIOMETRY AND KINETICS IN piRNA TARGETING

The above findings indicate that the currently uncovered pairing rules can identify potential targets of piRNAs, but only a subset of those transcripts are detectably regulated. This phenomenon is well known in miRNA targeting, and it is clear that both cooperation between target sites and the stoichiometry of the miRNA and the target play a role in the regulatory outcome (Bartel, 2009). There therefore are three quantitative aspects to consider: the cooperation between target sites, the molecular ratio between piRNAs and targets, and the turnover of PIWI-piRNA-target complexes.

For miRNAs, the presence of a matching seed sequence in the transcriptome is a frequent occurrence, especially when mismatches are allowed. Almost all mRNAs contain multiple potential miRNA seed matches, but when two sites occur in close proximity, cooperation between them can stabilize the binding of miRNA-loaded Argonautes (Krek et al., 2005; Saetrom et al., 2007). The biochemical basis for this cooperation lies in the binding of TNRC6 which bridges adjacent AGO-miRNA complexes, leading to cooperative interactions (Briskin et al., 2020; Broderick et al., 2011; Elkayam et al., 2017).

In the case of piRNAs no such bridging proteins are known, and there thus is no biochemical basis for cooperation between binding sites. Nevertheless, there are functional indications that some form of cooperation likely does apply. In *C. elegans*, most functional targets were targeted by multiple piRNAs, and deletion of individual piRNAs resulted in only partial effects on the piRNA-regulated endogenous transcripts (Shen et al., 2018), suggesting that multiple piRNAs cooperate to accomplish silencing. A reporter study in *Drosophila* cell culture found that large numbers of diverse piRNAs were required to establish robust silencing (Post et al., 2014), and that loci that were stably silenced by PIWI typically had several thousands of RPM of piRNAs matching to them. And an analysis of functional target sites of MIWI in mouse testes found that for 75% of the targets more than 1 matching piRNA sequence could be found (Zhang et al., 2015).

For miRNAs, perfect seed matches are more stable, but much rarer than imperfect ones, and thus miRNA-loaded Argonautes spend a large fraction of their time bound to imperfect sites that have little to no functional implications. If the number of copies of a given miRNA is small, the chance that a perfectly functional target site is ever bound by any of these copies is slim, and thus targeting rules that are adequate for highly abundant miRNAs may be insufficient to accomplish significant regulatory effects for rare ones (Bartel, 2009; Brancati & Grosshans, 2018).

piRNAs require more sequence complementarity to engage, and thus the expected frequency of functional matches is even lower than for miRNAs. At any given time, a large fraction of piRNA-loaded PIWI protein will be bound to insufficiently matching sites with variable levels of stability. The numerous binding sites with insufficient sequence complementarity are unable to position the target in a conformation required for catalytic activity and thus are unlikely to have functional implications (although cleavage-independent functions cannot be excluded). The number of PIWI-piRNA complexes compared to the number of potential target sites determines the equilibrium of these interactions. A recent in vitro study determined that if there is more target than matching piRNA-loaded PIWI, reliable target cleavage required the full 20 nucleotides of base-pairing. However if the piRNA-loaded PIWI was in large excess then base-pairing by only 15 nucleotides could be sufficient (Arif et al., 2022). This means that some of the highly abundant piRNAs may be able to achieve targeting with more limited complementarity, as was also found in in vivo MIWI targeting analyses (Gainetdinov et al., 2023; Gou et al., 2014). In fact, Gainetdinov et al. found by regression analysis that the two most predictive features for piRNA function in vivo are their molar abundance and their base-pairing stability with the target, and that to some extent these two factors can compensate for each other. Most piRNAs however exist in low copy numbers (Genzor et al., 2021), and for such rare piRNA sequences to have an effect, the base-pairing has to be high in order for the piRNA to reliably find that target.

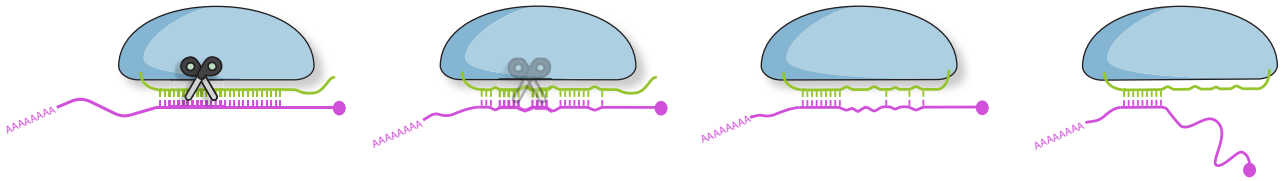
The last quantitative consideration involves the turnover of the PIWI complex. A recent study of MIWI activity suggested that without the presence of the auxiliary protein GTSF the nuclease activity of PIWI proteins is orders of magnitude slower than that of Argonaute proteins, and when matched by less than 20 nucleotides of base-pairing, the cleavage rate of the PIWI-piRNA complex was even further reduced (Arif et al., 2022). Furthermore, Argonaute proteins can rapidly disengage from their target upon the completion of target cleavage, and a single Argonaute-miRNA complex can thus target many transcripts sequentially. The structural studies of PIWI proteins however have suggested that these proteins remain bound to their target even when cleavage has already taken place, and that they require a helper helicase such as Vasa to release (Arif et al., 2022; Nishida et al., 2015). Even in vivo, in the presence of all required auxiliary proteins, cleavage fragments were found to be retained on the PIWI protein, likely blocking its ability to target another transcript (Dowling et al., 2023). Together this implies that the multiple-turnover of PIWI proteins is likely low, and thus that rare piRNAs, even if they have significant sequence complementarity to a target, are unlikely to make much of an impact if not in collaboration with other piRNAs.

The only remarkable exception to the slow dynamics of PIWI proteins may be the use of short perfectly complementary small RNAs. In vitro studies of MIWI found that perfectly matching 16mers actually display relatively rapid target finding, and allow for the release of the cleaved target without assistance by Vasa (Arif et al., 2022). These molecules would not typically be considered piRNAs as they do not match the typical piRNA length. It is however possible that such molecules exist in certain cell types or cell states (Ishino et al., 2021; Perera et al., 2019; Yang et al., 2019), and that they may actually mediate a more rapid and dynamic PIWI mechanism, as long as they have (almost) perfect complementarity to their target. In this context it is intriguing that piRNAs associated with human and hamster Piwil3 were found to be very short (Ishino et al., 2021; Yang et al., 2019). Regulatory effects of Piwil3 and these short small RNAs may well have been overlooked as these short RNAs have likely been excluded from most piRNA analyses.

6 | FINAL THOUGHTS ON FUNCTION

While not everything in biology has a logical explanation, thinking about the function of piRNAs does give the targeting rules some perspective. One of the major functions of the piRNAs is to defend the genome against invasive sequences. Such sequences can rapidly amplify and evolve, and thus some degree of mismatching must be tolerated in order to recognize novel variants of these elements. On the other hand, the targeting must be restrictive enough to reliably distinguish these invasive sequences from essential "self" RNAs. The long nucleotide sequence of the piRNAs combined with substantial base-pairing while allowing scattered mismatches are a way to accommodate both these requirements (Gainetdinov et al., 2023).

Different species may have filled in the details in different ways. In *Drosophila*, the majority of the piRNAs match transposon sequences, and they can thus be considered somewhat of a transposon library. *C. elegans* piRNAs on the other hand are not enriched in transposons and rather appear to span the sequence space (McEnany et al., 2022), making them much more likely to target "self" sequences. Additionally, *C. elegans* piRNAs are much shorter than those of most other animals, and appear to use a seed-matching strategy, increasing the risk of "self" targeting further. It has been proposed that to compensate for that, *C. elegans* has a dedicated mechanism in place to counteract the targeting of



base-pairing to piRNA nucleotides	2-21	2-21 with 3 mismatches	2-8 (”seed”) plus 6 matches	2-8 (”seed”)
expected frequency in transcriptome by chance	1/1x10^12	1/1x10^9	1/1x10^6	1/1.6x10^4
number of competing target sites expected	none	0 or 1	~40	~600
PIWI endonuclease activity	yes	probably (slower)	no	no
probability of piRNA-mediated effect on the target	very high	likely, especially if target matches with multiple piRNAs or highly abundant piRNAs	possible, but only if the piRNA is at very high copy number or otherwise locally concentrated	unlikely

FIGURE 5 Targeting predictions for piRNAs. Schematic spanning the range of proposed base-pairing for piRNAs to their targets. Perfect base-pairing of nucleotides 2–21 of the piRNA (left) is sufficient to activate cleavage activity and in all assays has been predicted to result in regulation of the transcript. The finding of such a match therefore is highly predictive of regulatory activity. On the other end of the spectrum, base-pairing of just the seed (right) results in instable binding and is not sufficient to activate cleavage activity. In addition, matches of a 7mer are expected to occur several hundreds of times in a 10 Mb transcriptome. This makes it very unlikely that an individual target with this amount of base-pairing is actually regulated by the piRNA. Up to 3 mismatches in the nucleotides 2–21 of the piRNA still give significant evidence for functional targeting in the various experimental approaches, and is expected to be sufficient to activate the cleavage activity of the PIWI protein. Based on in vitro studies this activity is expected to be slower than that of piRNA-PIWI complexes with full base-pairing in the 2–21 nt region, and therefore the molecular ratio between the piRNA and the mRNA starts to become important. Targets with less base-pairing may on occasion still be regulated by the piRNA, but these effects depend on external factors, and therefore the predictive value of such target matches is very low.

germline RNAs in the form of the nematode-specific Argonaute protein CSR-1 which can temper the piRNA-mediated silencing response to “self” transcripts (Seth et al., 2013; Wedeles et al., 2013). Challenging that view however, CSR-1 is conserved in more clades of nematodes than the piRNA pathway, and appears to target similar transcripts in worms without any PIWIs, indicating that CSR-1 likely at least has additional roles independent of piRNAs (Sarkies et al., 2015; Zagoskin et al., 2022).

It is entirely possible that the context of a match also matters. Several studies found that target sites with similar complementarity can have different functional outcomes. In *Xenopus*, levels of several coding mRNAs remained unaffected even in the presence of multiple perfectly matching piRNAs, whereas several non-coding RNAs with similar matching piRNAs were degraded (Toombs et al., 2017). Similarly, in mouse down-regulation of transposon transcripts is prevalent, but a perfectly matching piRNA to the 3′UTR of a coding gene resulted in cleavage without accomplishing noticeable changes to the mRNA level (Dowling et al., 2023). Such a distinction could be caused by a difference in developmental context or a difference in quantitative aspects of the piRNA-target interaction. Alternatively, there may be specific features of the target transcripts that contribute to the regulatory outcome. *C. elegans* germline genes, which are most at risk of getting erroneously targeted, contain specific sequence elements that may make them less sensitive to piRNAs (Frokjaer-Jensen et al., 2016; Zhang et al., 2018). And more than 70% of the functional MIWI target sites was located in the 3’UTRs of transcripts (Zhang et al., 2015) (though this finding was not confirmed in a more recent study (Gainetdinov et al., 2023)), suggesting that a match in this region may be more likely to lead to functional consequences. Finally, it is possible that the targeting rules of transcriptional gene silencing (TGS) effects are distinct from those for post-transcriptional gene silencing (PTGS). Most of the studies so far have focused on the posttranscriptional effects, and thus it will be interesting to find whether the same rules hold up inside the nucleus.

Stoichiometry and kinetics need to be part of the considerations regarding targeting. Apart from the fact that the huge diversity in piRNA sequences makes it impossible for all piRNAs to be included in every individual cell (Genzor et al., 2021), the slow dynamics of PIWI proteins make it unlikely that rare piRNAs can make much of an impact on the transcriptome unless in collaboration with large numbers of other piRNAs that are targeting the same transcript.

For poorly matching piRNAs the stability of the binding is reduced, and while it is possible they are still able to contribute to gene regulation, they most likely require much higher copy numbers and even more collaborating piRNAs to accomplish an effect.

Does this mean that piRNAs only function in bulk? The data suggests that this must be the case for the majority of the regulatory events—and this again makes sense when considering the typical piRNA targets. A legitimately targeted repetitive element may well change some of its sequence, but is unlikely to change much of it at once. The collaborative action of multiple piRNAs therefore is likely to retain silencing capability independent of the rapid evolution of transposons, while still minimizing the chances that a "self" mRNA gets caught up in the action. But of course there can always be exceptions. A compelling example of regulation by an individual piRNA sequence has been reported in the silkworm *Bombyx mori*. Here a piRNA that is encoded on the female-specific sex chromosome plays a vital role in sex determination by inducing cleavage of a cellular mRNA involved in masculinization of the embryos (Kiuchi et al., 2014). This single piRNA accumulates to uncommonly high levels (>1000 RPM) in the silk worm ovaries, and this thus appears to be the exception rather than the rule. Interestingly a similar phenomenon was found in *C. elegans*, where the single most abundant piRNA is encoded on the sex chromosome, and was found to target a male-specific regulator (Tang et al., 2018), resulting in suppression of male differentiation. These two remarkably similar findings in distant species indicate that this phenomenon of targeting by a single piRNA sequence may be specific to the highly dose-sensitive sex determination process.

7 | CONCLUSION

In summary, much has become clear about piRNA targeting from a combination of structural, biochemical, functional, and genetic studies. The emerging picture is that targeting resulting in cleavage and potential amplification of the piRNA response is stringent, and requires extensive base-pairing between piRNA nucleotides 2 and 21 with no more than 2 or 3 mismatches (Figure 5), and typically involves the collective action of many piRNAs—either many copies of the same sequence, or many distinct piRNA sequences that match the same target. Interactions based on more limited base-pairing are energetically instable and as the number of potential targets for each piRNA increases, the number of piRNA molecules required for a substantial effect on an individual target becomes extremely high. In addition, such effects likely require contributions from other non-piRNA related factors as only a subset of these sparse matches have detectable effects, making them impossible to predict based on piRNA sequence alone. It is entirely possible that PIWI proteins have additional functions that are independent of piRNA-mediated targeting. Such interactions may still be functional, and may explain some of the observed PIWI-related phenomena; however they are unlikely to be sequence-specific.

When predicting regulatory effects based on piRNA sequences we thus currently can only reasonably consider the cleavage type effects, and only target sequences that obey the high-stringency rules listed above can be reasonably proposed to be regulated. Future discoveries may allow us to also predict weaker interactions with high confidence, and the many ongoing investigations into the mechanisms of piRNA function will further clarify the regulatory implications of both the transient and the stable interactions to provide an ever more complete picture of the regulatory complexities of the piRNAs.

AUTHOR CONTRIBUTIONS

Josien C. van Wolfswinkel: Conceptualization (lead); funding acquisition (lead); visualization (lead); writing – original draft (lead); writing – review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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REFERENCES

Ameres, S. L., Martinez, J., & Schroeder, R. (2007). Molecular basis for target RNA recognition and cleavage by human RISC. *Cell*, *130*(1), 101–112. https://doi.org/10.1016/j.cell.2007.04.037

Anzelon, T. A., Chowdhury, S., Hughes, S. M., Xiao, Y., Lander, G. C., & MacRae, I. J. (2021). Structural basis for piRNA targeting. *Nature*, *597*(7875), 285–289. https://doi.org/10.1038/s41586-021-03856-x

Arif, A., Bailey, S., Izumi, N., Anzelon, T. A., Ozata, D. M., Andersson, C., Gainetdinov, I., MacRae, I. J., Tomari, Y., & Zamore, P. D. (2022). GTSF1 accelerates target RNA cleavage by PIWI-clade Argonaute proteins. *Nature*, *608*(7923), 618–625. https://doi.org/10.1038/s41586-022-05009-0

Bagijn, M. P., Goldstein, L. D., Sapetschnig, A., Weick, E. M., Bouasker, S., Lehrbach, N. J., Simard, M. J., & Miska, E. A. (2012). Function, targets, and evolution of *Caenorhabditis elegans* piRNAs. *Science*, *337*(6094), 574–578. https://doi.org/10.1126/science.1220952

Balaratnam, S., Hoque, M. E., West, N., & Basu, S. (2022). Decay of Piwi-interacting RNAs in human cells is primarily mediated by 5′ to 3′ exoribonucleases. *ACS Chemical Biology*, *17*(7), 1723–1732. https://doi.org/10.1021/acschembio.2c00007

Barckmann, B., Pierson, S., Dufourt, J., Papin, C., Armenise, C., Port, F., Grentzinger, T., Chambeyron, S., Baronian, G., Desvignes, J. P., Curk, T., & Simonelig, M. (2015). Aubergine iCLIP reveals piRNA-dependent decay of mRNAs involved in germ cell development in the early embryo. *Cell Reports*, *12*(7), 1205–1216. https://doi.org/10.1016/j.celrep.2015.07.030

Bartel, D. P. (2009). MicroRNAs: Target recognition and regulatory functions. *Cell*, *136*(2), 215–233. https://doi.org/10.1016/j.cell.2009.01.002

Brancati, G., & Grosshans, H. (2018). An interplay of miRNA abundance and target site architecture determines miRNA activity and specificity. *Nucleic Acids Research*, *46*(7), 3259–3269. https://doi.org/10.1093/nar/gky201

Brennecke, J., Aravin, A. A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R., & Hannon, G. J. (2007). Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila. *Cell*, *128*(6), 1089–1103. https://doi.org/10.1016/j.cell.2007.01.043

Brennecke, J., Stark, A., Russell, R. B., & Cohen, S. M. (2005). Principles of microRNA-target recognition. *PLoS Biology*, *3*(3), e85. https://doi.org/10.1371/journal.pbio.0030085

Briskin, D., Wang, P. Y., & Bartel, D. P. (2020). The biochemical basis for the cooperative action of microRNAs. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(30), 17764–17774. https://doi.org/10.1073/pnas.1920404117

Broderick, J. A., Salomon, W. E., Ryder, S. P., Aronin, N., & Zamore, P. D. (2011). Argonaute protein identity and pairing geometry determine cooperativity in mammalian RNA silencing. *RNA*, *17*(10), 1858–1869. https://doi.org/10.1261/rna.2778911

Carmell, M. A., Xuan, Z., Zhang, M. Q., & Hannon, G. J. (2002). The argonaute family: Tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. *Genes & Development*, *16*(21), 2733–2742. https://doi.org/10.1101/gad.1026102

Choi, H., Wang, Z., & Dean, J. (2021). Sperm acrosome overgrowth and infertility in mice lacking chromosome 18 pachytene piRNA. *PLoS Genetics*, *17*(4), e1009485. https://doi.org/10.1371/journal.pgen.1009485

Chu, Y., Kilikevicius, A., Liu, J., Johnson, K. C., Yokota, S., & Corey, D. R. (2020). Argonaute binding within 3′-untranslated regions poorly predicts gene repression. *Nucleic Acids Research*, *48*(13), 7439–7453. https://doi.org/10.1093/nar/gkaa478

Cora, E., Pandey, R. R., Xiol, J., Taylor, J., Sachidanandam, R., McCarthy, A. A., & Pillai, R. S. (2014). The MID-PIWI module of Piwi proteins specifies nucleotide- and strand-biases of piRNAs. *RNA*, *20*(6), 773–781. https://doi.org/10.1261/rna.044701.114

Czech, B., Munafo, M., Ciabrelli, F., Eastwood, E. L., Fabry, M. H., Kneuss, E., & Hannon, G. J. (2018). piRNA-guided genome defense: From biogenesis to silencing. *Annual Review of Genetics*, *52*, 131–157. https://doi.org/10.1146/annurev-genet-120417-031441

Dai, P., Wang, X., Gou, L. T., Li, Z. T., Wen, Z., Chen, Z. G., Hua, M. M., Zhong, A., Wang, L., Su, H., Wan, H., Qian, K., Liao, L., Li, J., Tian, B., Li, D., Fu, X. D., Shi, H. J., Zhou, Y., & Liu, M. F. (2019). A translation-activating function of MIWI/piRNA during mouse spermiogenesis. *Cell*, *179*(7), 1566–1581. https://doi.org/10.1016/j.cell.2019.11.022

Darricarrere, N., Liu, N., Watanabe, T., & Lin, H. (2013). Function of Piwi, a nuclear Piwi/Argonaute protein, is independent of its slicer activity. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(4), 1297–1302. https://doi.org/10.1073/pnas.1213283110

Dowling, M., Homolka, D., Raad, N., Gos, P., Pandey, R. R., & Pillai, R. S. (2023). In vivo PIWI slicing in mouse testes deviates from rules established in vitro. *RNA*, *29*(3), 308–316. https://doi.org/10.1261/rna.079349.122

Dufourt, J., Bontonou, G., Chartier, A., Jahan, C., Meunier, A. C., Pierson, S., Harrison, P. F., Papin, C., Beilharz, T. H., & Simonelig, M. (2017). piRNAs and Aubergine cooperate with wispy poly(A) polymerase to stabilize mRNAs in the germ plasm. *Nature Communications*, *8*(1), 1305. https://doi.org/10.1038/s41467-017-01431-5

Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W., & Tuschl, T. (2001). Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. *The EMBO Journal*, *20*(23), 6877–6888. https://doi.org/10.1093/emboj/20.23.6877

Elkayam, E., Faehnle, C. R., Morales, M., Sun, J., Li, H., & Joshua-Tor, L. (2017). Multivalent recruitment of human argonaute by GW182. *Molecular Cell*, *67*(4), 646–658. https://doi.org/10.1016/j.molcel.2017.07.007

Feltzin, V. L., Khaladkar, M., Abe, M., Parisi, M., Hendriks, G. J., Kim, J., & Bonini, N. M. (2015). The exonuclease Nibbler regulates age-associated traits and modulates piRNA length in Drosophila. *Aging Cell*, *14*(3), 443–452. https://doi.org/10.1111/acel.12323

Frank, F., Sonenberg, N., & Nagar, B. (2010). Structural basis for 5′-nucleotide base-specific recognition of guide RNA by human AGO2. *Nature*, *465*(7299), 818–822. https://doi.org/10.1038/nature09039

Frokjaer-Jensen, C., Jain, N., Hansen, L., Davis, M. W., Li, Y., Zhao, D., Rebora, K., Millet, J. R. M., Liu, X., Kim, S. K., Dupuy, D., Jorgensen, E. M., & Fire, A. Z. (2016). An abundant class of non-coding DNA can prevent stochastic gene silencing in the *C. elegans* germline. *Cell*, *166*(2), 343–357. https://doi.org/10.1016/j.cell.2016.05.072

Gainetdinov, I., Colpan, C., Arif, A., Cecchini, K., & Zamore, P. D. (2018). A single mechanism of biogenesis, initiated and directed by PIWI proteins, explains piRNA production in Most animals. *Molecular Cell*, *71*(5), 775–790. https://doi.org/10.1016/j.molcel.2018.08.007

Gainetdinov, I., Colpan, C., Cecchini, K., Arif, A., Jouravleva, K., Albosta, P., Vega-Badillo, J., Lee, Y., Ozata, D. M., & Zamore, P. D. (2021). Terminal modification, sequence, length, and PIWI-protein identity determine piRNA stability. *Molecular Cell*, *81*(23), 4826–4842. https://doi.org/10.1016/j.molcel.2021.09.012

Gainetdinov, I., Vega-Badillo, J., Cecchini, K., Bagci, A., Colpan, C., De, D., Bailey, S., Arif, A., Wu, P.-H., MacRae, I. J., & Zamore, P. D. (2023). Relaxed targeting rules help PIWI proteins silence transposons. *Nature*, *2023*, 394–402. https://doi.org/10.1038/s41586-023-06257-4

Galton, R., Fejes-Toth, K., & Bronner, M. E. (2022). Co-option of the piRNA pathway to regulate neural crest specification. *Science Advances*, *8*(32), eabn1441. https://doi.org/10.1126/sciadv.abn1441

Gebert, D., Neubert, L. K., Lloyd, C., Gui, J., Lehmann, R., & Teixeira, F. K. (2021). Large Drosophila germline piRNA clusters are evolutionarily labile and dispensable for transposon regulation. *Molecular Cell*, *81*(19), 3965–3978. https://doi.org/10.1016/j.molcel.2021.07.011

Genzor, P., Konstantinidou, P., Stoyko, D., Manzourolajdad, A., Marlin Andrews, C., Elchert, A. R., Stathopoulos, C., & Haase, A. D. (2021). Cellular abundance shapes function in piRNA-guided genome defense. *Genome Research*, *31*(11), 2058–2068. https://doi.org/10.1101/gr.275478.121

Goh, W. S., Falciatori, I., Tam, O. H., Burgess, R., Meikar, O., Kotaja, N., Hammell, M., & Hannon, G. J. (2015). piRNA-directed cleavage of meiotic transcripts regulates spermatogenesis. *Genes & Development*, *29*(10), 1032–1044. https://doi.org/10.1101/gad.260455.115

Gou, L. T., Dai, P., Yang, J. H., Xue, Y., Hu, Y. P., Zhou, Y., Kang, J. Y., Wang, X., Li, H., Hua, M. M., Zhao, S., Hu, S. D., Wu, L. G., Shi, H. J., Li, Y., Fu, X. D., Qu, L. H., Wang, E. D., & Liu, M. F. (2014). Pachytene piRNAs instruct massive mRNA elimination during late spermiogenesis. *Cell Research*, *24*(6), 680–700. https://doi.org/10.1038/cr.2014.41

Gunawardane, L. S., Saito, K., Nishida, K. M., Miyoshi, K., Kawamura, Y., Nagami, T., Siomi, H., & Siomi, M. C. (2007). A slicer-mediated mechanism for repeat-associated siRNA 5′ end formation in Drosophila. *Science*, *315*(5818), 1587–1590. https://doi.org/10.1126/science.1140494

Haase, A. D., Fenoglio, S., Muerdter, F., Guzzardo, P. M., Czech, B., Pappin, D. J., Chen, C., Gordon, A., & Hannon, G. J. (2010). Probing the initiation and effector phases of the somatic piRNA pathway in Drosophila. *Genes & Development*, *24*(22), 2499–2504. https://doi.org/10.1101/gad.1968110

Ipsaro, J. J., Haase, A. D., Knott, S. R., Joshua-Tor, L., & Hannon, G. J. (2012). The structural biochemistry of Zucchini implicates it as a nuclease in piRNA biogenesis. *Nature*, *491*(7423), 279–283. https://doi.org/10.1038/nature11502

Ishino, K., Hasuwa, H., Yoshimura, J., Iwasaki, Y. W., Nishihara, H., Seki, N. M., Hirano, T., Tsuchiya, M., Ishizaki, H., Masuda, H., Kuramoto, T., Saito, K., Sakakibara, Y., Toyoda, A., Itoh, T., Siomi, M. C., Morishita, S., & Siomi, H. (2021). Hamster PIWI proteins bind to piRNAs with stage-specific size variations during oocyte maturation. *Nucleic Acids Research*, *49*(5), 2700–2720. https://doi.org/10.1093/nar/gkab059

Izumi, N., Shoji, K., Sakaguchi, Y., Honda, S., Kirino, Y., Suzuki, T., Katsuma, S., & Tomari, Y. (2016). Identification and functional analysis of the pre-piRNA 3′ trimmer in silkworms. *Cell*, *164*(5), 962–973. https://doi.org/10.1016/j.cell.2016.01.008

Jehn, J., Gebert, D., Pipilescu, F., Stern, S., Kiefer, J. S. T., Hewel, C., & Rosenkranz, D. (2018). PIWI genes and piRNAs are ubiquitously expressed in mollusks and show patterns of lineage-specific adaptation. *Communications Biology*, *1*, 137. https://doi.org/10.1038/s42003-018-0141-4

Jones, B. C., Wood, J. G., Chang, C., Tam, A. D., Franklin, M. J., Siegel, E. R., & Helfand, S. L. (2016). A somatic piRNA pathway in the Drosophila fat body ensures metabolic homeostasis and normal lifespan. *Nature Communications*, *7*, 13856. https://doi.org/10.1038/ncomms13856

Kawaoka, S., Izumi, N., Katsuma, S., & Tomari, Y. (2011). 3′ end formation of PIWI-interacting RNAs in vitro. *Molecular Cell*, *43*(6), 1015–1022. https://doi.org/10.1016/j.molcel.2011.07.029

Kim, I. V., Duncan, E. M., Ross, E. J., Gorbovytska, V., Nowotarski, S. H., Elliott, S. A., Sanchez Alvarado, A., & Kuhn, C. D. (2019). Planarians recruit piRNAs for mRNA turnover in adult stem cells. *Genes & Development*, *33*(21–22), 1575–1590. https://doi.org/10.1101/gad.322776.118

Kiuchi, T., Koga, H., Kawamoto, M., Shoji, K., Sakai, H., Arai, Y., Ishihara, G., Kawaoka, S., Sugano, S., Shimada, T., Suzuki, Y., Suzuki, M. G., & Katsuma, S. (2014). A single female-specific piRNA is the primary determiner of sex in the silkworm. *Nature*, *509*(7502), 633–636. https://doi.org/10.1038/nature13315

Koopal, B., Mutte, S. K., & Swarts, D. C. (2022). A long look at short prokaryotic argonautes. *Trends in Cell Biology*, *33*, 605–618. https://doi.org/10.1016/j.tcb.2022.10.005

Krek, A., Grun, D., Poy, M. N., Wolf, R., Rosenberg, L., Epstein, E. J., MacMenamin, P., da Piedade, I., Gunsalus, K. C., Stoffel, M., & Rajewsky, N. (2005). Combinatorial microRNA target predictions. *Nature Genetics*, *37*(5), 495–500. https://doi.org/10.1038/ng1536

Kudla, G., Granneman, S., Hahn, D., Beggs, J. D., & Tollervey, D. (2011). Cross-linking, ligation, and sequencing of hybrids reveals RNA-RNA interactions in yeast. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(24), 10010–10015. https://doi.org/10.1073/pnas.1017386108

Lee, E. J., Banerjee, S., Zhou, H., Jammalamadaka, A., Arcila, M., Manjunath, B. S., & Kosik, K. S. (2011). Identification of piRNAs in the central nervous system. *RNA*, *17*(6), 1090–1099. https://doi.org/10.1261/rna.2565011

Lee, H. C., Gu, W., Shirayama, M., Youngman, E., Conte, D., Jr., & Mello, C. C. (2012). *C. elegans* piRNAs mediate the genome-wide surveillance of germline transcripts. *Cell*, *150*(1), 78–87. https://doi.org/10.1016/j.cell.2012.06.016

Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P., & Burge, C. B. (2003). Prediction of mammalian microRNA targets. *Cell*, *115*(7), 787–798. https://doi.org/10.1016/s0092-8674(03)01018-3

Lewis, S. H., Quarles, K. A., Yang, Y., Tanguy, M., Frezal, L., Smith, S. A., Sharma, P. P., Cordaux, R., Gilbert, C., Giraud, I., Collins, D. H., Zamore, P. D., Miska, E. A., Sarkies, P., & Jiggins, F. M. (2018). Pan-arthropod analysis reveals somatic piRNAs as an ancestral defence against transposable elements. *Nature Ecology & Evolution*, *2*(1), 174–181. https://doi.org/10.1038/s41559-017-0403-4

Liu, J., Carmell, M. A., Rivas, F. V., Marsden, C. G., Thomson, J. M., Song, J. J., Hammond, S. M., Joshua-Tor, L., & Hannon, G. J. (2004). Argonaute2 is the catalytic engine of mammalian RNAi. *Science*, *305*(5689), 1437–1441. https://doi.org/10.1126/science.1102513

Luteijn, M. J., & Ketting, R. F. (2013). PIWI-interacting RNAs: From generation to transgenerational epigenetics. *Nature Reviews. Genetics*, *14*(8), 523–534. https://doi.org/10.1038/nrg3495

Matsumoto, N., Nishimasu, H., Sakakibara, K., Nishida, K. M., Hirano, T., Ishitani, R., Siomi, H., Siomi, M. C., & Nureki, O. (2016). Crystal structure of silkworm PIWI-clade argonaute Siwi bound to piRNA. *Cell*, *167*(2), 484–497. https://doi.org/10.1016/j.cell.2016.09.002

McEnany, J., Meir, Y., & Wingreen, N. S. (2022). piRNAs of Caenorhabditis elegans broadly silence nonself sequences through functionally random targeting. *Nucleic Acids Research*, *50*(3), 1416–1429. https://doi.org/10.1093/nar/gkab1290

McGeary, S. E., Bisaria, N., Pham, T. M., Wang, P. Y., & Bartel, D. P. (2022). MicroRNA 3′-compensatory pairing occurs through two binding modes, with affinity shaped by nucleotide identity and position. *Elife*, *11*(e69803), 1–37. https://doi.org/10.7554/eLife.69803

Mohn, F., Handler, D., & Brennecke, J. (2015). Noncoding RNA. piRNA-guided slicing specifies transcripts for Zucchini-dependent, phased piRNA biogenesis. *Science*, *348*(6236), 812–817. https://doi.org/10.1126/science.aaa1039

Nandi, S., Chandramohan, D., Fioriti, L., Melnick, A. M., Hebert, J. M., Mason, C. E., Rajasethupathy, P., & Kandel, E. R. (2016). Roles for small noncoding RNAs in silencing of retrotransposons in the mammalian brain. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(45), 12697–12702. https://doi.org/10.1073/pnas.1609287113

Nishida, K. M., Iwasaki, Y. W., Murota, Y., Nagao, A., Mannen, T., Kato, Y., Siomi, H., & Siomi, M. C. (2015). Respective functions of two distinct Siwi complexes assembled during PIWI-interacting RNA biogenesis in Bombyx germ cells. *Cell Reports*, *10*(2), 193–203. https://doi.org/10.1016/j.celrep.2014.12.013

Ozata, D. M., Gainetdinov, I., Zoch, A., O'Carroll, D., & Zamore, P. D. (2019). PIWI-interacting RNAs: Small RNAs with big functions. *Nature Reviews. Genetics*, *20*(2), 89–108. https://doi.org/10.1038/s41576-018-0073-3

Parker, J. S., Parizotto, E. A., Wang, M., Roe, S. M., & Barford, D. (2009). Enhancement of the seed-target recognition step in RNA silencing by a PIWI/MID domain protein. *Molecular Cell*, *33*(2), 204–214. https://doi.org/10.1016/j.molcel.2008.12.012

Perera, B. P. U., Tsai, Z. T., Colwell, M. L., Jones, T. R., Goodrich, J. M., Wang, K., Sartor, M. A., Faulk, C., & Dolinoy, D. C. (2019). Somatic expression of piRNA and associated machinery in the mouse identifies short, tissue-specific piRNA. *Epigenetics*, *14*(5), 504–521. https://doi.org/10.1080/15592294.2019.1600389

Post, C., Clark, J. P., Sytnikova, Y. A., Chirn, G. W., & Lau, N. C. (2014). The capacity of target silencing by Drosophila PIWI and piRNAs. *RNA*, *20*(12), 1977–1986. https://doi.org/10.1261/rna.046300.114

Rajasethupathy, P., Antonov, I., Sheridan, R., Frey, S., Sander, C., Tuschl, T., & Kandel, E. R. (2012). A role for neuronal piRNAs in the epigenetic control of memory-related synaptic plasticity. *Cell*, *149*(3), 693–707. https://doi.org/10.1016/j.cell.2012.02.057

Ramat, A., Garcia-Silva, M. R., Jahan, C., Nait-Saidi, R., Dufourt, J., Garret, C., Chartier, A., Cremaschi, J., Patel, V., Decourcelle, M., Bastide, A., Juge, F., & Simonelig, M. (2020). The PIWI protein Aubergine recruits eIF3 to activate translation in the germ plasm. *Cell Research*, *30*(5), 421–435. https://doi.org/10.1038/s41422-020-0294-9

Reuter, M., Berninger, P., Chuma, S., Shah, H., Hosokawa, M., Funaya, C., Antony, C., Sachidanandam, R., & Pillai, R. S. (2011). Miwi catalysis is required for piRNA amplification-independent LINE1 transposon silencing. *Nature*, *480*(7376), 264–267. https://doi.org/10.1038/nature10672

Rouget, C., Papin, C., Boureux, A., Meunier, A. C., Franco, B., Robine, N., Lai, E. C., Pelisson, A., & Simonelig, M. (2010). Maternal mRNA deadenylation and decay by the piRNA pathway in the early Drosophila embryo. *Nature*, *467*(7319), 1128–1132. https://doi.org/10.1038/nature09465

Saetrom, P., Heale, B. S., Snove, O., Jr., Aagaard, L., Alluin, J., & Rossi, J. J. (2007). Distance constraints between microRNA target sites dictate efficacy and cooperativity. *Nucleic Acids Research*, *35*(7), 2333–2342. https://doi.org/10.1093/nar/gkm133

Salomon, W. E., Jolly, S. M., Moore, M. J., Zamore, P. D., & Serebrov, V. (2015). Single-molecule imaging reveals that Argonaute reshapes the binding properties of its nucleic acid guides. *Cell*, *162*(1), 84–95. https://doi.org/10.1016/j.cell.2015.06.029

Sarkies, P., Selkirk, M. E., Jones, J. T., Blok, V., Boothby, T., Goldstein, B., Hanelt, B., Ardila-Garcia, A., Fast, N. M., Schiffer, P. M., Kraus, C., Taylor, M. J., Koutsovoulos, G., Blaxter, M. L., & Miska, E. A. (2015). Ancient and novel small RNA pathways compensate for the loss of piRNAs in multiple independent nematode lineages. *PLoS Biology*, *13*(2), e1002061. https://doi.org/10.1371/journal.pbio.1002061

Schirle, N. T., & MacRae, I. J. (2012). The crystal structure of human Argonaute2. *Science*, *336*(6084), 1037–1040. https://doi.org/10.1126/science.1221551

Schirle, N. T., Sheu-Gruttadauria, J., & MacRae, I. J. (2014). Structural basis for microRNA targeting. *Science*, *346*(6209), 608–613. https://doi.org/10.1126/science.1258040

Seth, M., Shirayama, M., Gu, W., Ishidate, T., Conte, D., Jr., & Mello, C. C. (2013). The *C. elegans* CSR-1 argonaute pathway counteracts epigenetic silencing to promote germline gene expression. *Developmental Cell*, *27*(6), 656–663. https://doi.org/10.1016/j.devcel.2013.11.014

Sharma, A. K., Nelson, M. C., Brandt, J. E., Wessman, M., Mahmud, N., Weller, K. P., & Hoffman, R. (2001). Human CD34(+) stem cells express the hiwi gene, a human homologue of the Drosophila gene piwi. *Blood*, *97*(2), 426–434. https://doi.org/10.1182/blood.v97.2.426

Shen, E. Z., Chen, H., Ozturk, A. R., Tu, S., Shirayama, M., Tang, W., Ding, Y. H., Dai, S. Y., Weng, Z., & Mello, C. C. (2018). Identification of piRNA binding sites reveals the Argonaute regulatory landscape of the *C. elegans* germline. *Cell*, *172*(5), 937–951. https://doi.org/10.1016/j.cell.2018.02.002

Sheu-Gruttadauria, J., Xiao, Y., Gebert, L. F., & MacRae, I. J. (2019). Beyond the seed: Structural basis for supplementary microRNA targeting by human Argonaute2. *The EMBO Journal*, *38*(13), e101153. https://doi.org/10.15252/embj.2018101153

Shi, S., Yang, Z. Z., Liu, S., Yang, F., & Lin, H. (2020). PIWIL1 promotes gastric cancer via a piRNA-independent mechanism. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(36), 22390–22401. https://doi.org/10.1073/pnas.2008724117

Song, J. J., Smith, S. K., Hannon, G. J., & Joshua-Tor, L. (2004). Crystal structure of Argonaute and its implications for RISC slicer activity. *Science*, *305*(5689), 1434–1437. https://doi.org/10.1126/science.1102514

Stein, C. B., Genzor, P., Mitra, S., Elchert, A. R., Ipsaro, J. J., Benner, L., Sobti, S., Su, Y., Hammell, M., Joshua-Tor, L., & Haase, A. D. (2019). Decoding the 5′ nucleotide bias of PIWI-interacting RNAs. *Nature Communications*, *10*(1), 828. https://doi.org/10.1038/s41467-019-08803-z

Tang, W., Seth, M., Tu, S., Shen, E. Z., Li, Q., Shirayama, M., Weng, Z., & Mello, C. C. (2018). A sex chromosome piRNA promotes robust dosage compensation and sex determination in *C. elegans*. *Developmental Cell*, *44*(6), 762–770. https://doi.org/10.1016/j.devcel.2018.01.025

Tang, W., Tu, S., Lee, H. C., Weng, Z., & Mello, C. C. (2016). The RNase PARN-1 trims piRNA 3′ ends to promote transcriptome surveillance in *C. elegans*. *Cell*, *164*(5), 974–984. https://doi.org/10.1016/j.cell.2016.02.008

Tomari, Y., & Zamore, P. D. (2005). Perspective: Machines for RNAi. *Genes & Development*, *19*(5), 517–529. https://doi.org/10.1101/gad.1284105

Toombs, J. A., Sytnikova, Y. A., Chirn, G. W., Ang, I., Lau, N. C., & Blower, M. D. (2017). Xenopus Piwi proteins interact with a broad proportion of the oocyte transcriptome. *RNA*, *23*(4), 504–520. https://doi.org/10.1261/rna.058859.116

van Wolfswinkel, J. C. (2014). Piwi and potency: PIWI proteins in animal stem cells and regeneration. *Integrative and Comparative Biology*, *54*(4), 700–713. https://doi.org/10.1093/icb/icu084

Vourekas, A., Alexiou, P., Vrettos, N., Maragkakis, M., & Mourelatos, Z. (2016). Sequence-dependent but not sequence-specific piRNA adhesion traps mRNAs to the germ plasm. *Nature*, *531*(7594), 390–394. https://doi.org/10.1038/nature17150

Vourekas, A., Zheng, Q., Alexiou, P., Maragkakis, M., Kirino, Y., Gregory, B. D., & Mourelatos, Z. (2012). Mili and Miwi target RNA repertoire reveals piRNA biogenesis and function of Miwi in spermiogenesis. *Nature Structural & Molecular Biology*, *19*(8), 773–781. https://doi.org/10.1038/nsmb.2347

Wang, W., Han, B. W., Tipping, C., Ge, D. T., Zhang, Z., Weng, Z., & Zamore, P. D. (2015). Slicing and binding by Ago3 or Aub trigger Piwi-bound piRNA production by distinct mechanisms. *Molecular Cell*, *59*(5), 819–830. https://doi.org/10.1016/j.molcel.2015.08.007

Wang, W., Yoshikawa, M., Han, B. W., Izumi, N., Tomari, Y., Weng, Z., & Zamore, P. D. (2014). The initial uridine of primary piRNAs does not create the tenth adenine that is the hallmark of secondary piRNAs. *Molecular Cell*, *56*(5), 708–716. https://doi.org/10.1016/j.molcel.2014.10.016

Watanabe, T., Cheng, E. C., Zhong, M., & Lin, H. (2015). Retrotransposons and pseudogenes regulate mRNAs and lncRNAs via the piRNA pathway in the germline. *Genome Research*, *25*(3), 368–380. https://doi.org/10.1101/gr.180802.114

Wedeles, C. J., Wu, M. Z., & Claycomb, J. M. (2013). Protection of germline gene expression by the *C. elegans* Argonaute CSR-1. *Developmental Cell*, *27*(6), 664–671. https://doi.org/10.1016/j.devcel.2013.11.016

Wu, P. H., Fu, Y., Cecchini, K., Ozata, D. M., Arif, A., Yu, T., Colpan, C., Gainetdinov, I., Weng, Z., & Zamore, P. D. (2020). The evolutionarily conserved piRNA-producing locus pi6 is required for male mouse fertility. *Nature Genetics*, *52*(7), 728–739. https://doi.org/10.1038/s41588-020-0657-7

Wu, P. H., & Zamore, P. D. (2021). Defining the functions of PIWI-interacting RNAs. *Nature Reviews. Molecular Cell Biology*, *22*(4), 239–240. https://doi.org/10.1038/s41580-021-00336-y

Yamaguchi, S., Oe, A., Nishida, K. M., Yamashita, K., Kajiya, A., Hirano, S., Matsumoto, N., Dohmae, N., Ishitani, R., Saito, K., Siomi, H., Nishimasu, H., Siomi, M. C., & Nureki, O. (2020). Crystal structure of Drosophila Piwi. *Nature Communications*, *11*(1), 858. https://doi.org/10.1038/s41467-020-14687-1

Yang, Q., Li, R., Lyu, Q., Hou, L., Liu, Z., Sun, Q., Liu, M., Shi, H., Xu, B., Yin, M., Yan, Z., Huang, Y., Liu, M., Li, Y., & Wu, L. (2019). Single-cell CAS-seq reveals a class of short PIWI-interacting RNAs in human oocytes. *Nature Communications*, *10*(1), 3389. https://doi.org/10.1038/s41467-019-11312-8

Yao, C., Sasaki, H. M., Ueda, T., Tomari, Y., & Tadakuma, H. (2015). Single-molecule analysis of the target cleavage reaction by the drosophila RNAi enzyme complex. *Molecular Cell*, *59*(1), 125–132. https://doi.org/10.1016/j.molcel.2015.05.015

Zagoskin, M. V., Wang, J., Neff, A. T., Veronezi, G. M. B., & Davis, R. E. (2022). Small RNA pathways in the nematode Ascaris in the absence of piRNAs. *Nature Communications*, *13*(1), 837. https://doi.org/10.1038/s41467-022-28482-7

Zhang, D., Tu, S., Stubna, M., Wu, W. S., Huang, W. C., Weng, Z., & Lee, H. C. (2018). The piRNA targeting rules and the resistance to piRNA silencing in endogenous genes. *Science*, *359*(6375), 587–592. https://doi.org/10.1126/science.aao2840

Zhang, P., Kang, J. Y., Gou, L. T., Wang, J., Xue, Y., Skogerboe, G., Dai, P., Huang, D. W., Chen, R., Fu, X. D., Liu, M. F., & He, S. (2015). MIWI and piRNA-mediated cleavage of messenger RNAs in mouse testes. *Cell Research*, *25*(2), 193–207. https://doi.org/10.1038/cr.2015.4

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