knockout cells, RFP–SQS was mislocalized, degraded, and aggregated, while no significant effects were observed for RFP–VAMP2.

The final evidence that EMC mediates insertion was provided by reconstitution studies combined with a protease protection assay. When the purified EMC proteins were reconstituted into liposome, SQS could be inserted with approximately 50% of native efficiency, and this could be increased to near-native levels with the help of CaM. By contrast, VAMP2 poorly inserts into EMC proteoliposomes. Thus, EMC is necessary and sufficient for membrane insertion of the TA substrate SQS.

Certainly, many interesting questions have been left unanswered. Is the Oxa1-like EMC3 subunit also responsible for the insertase activity? Can TMCO1 insert proteins on its own, and can it cooperate with the Sec translocon to promote insertion into the ER membrane? Can these homologs insert membrane proteins with different topologies other than TA proteins? Nevertheless, even at this early stage, based on the papers presented here, it is clear that Oxa1 superfamily proteins also operate in the ER.

Together, these two papers expand our understanding of the boundaries of the Oxa1/Alb3/YidC family of insertases. The new Oxa1 superfamily extends from bacteria and archaea to mitochondria, chloroplasts, and ER in eukaryotic cells. This provides an opportunity to study how the structure and function of each member has been conserved or changed during the process of evolution.

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Forum

RNA Selection by PIWI Proteins

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Gene regulation by PIWI–piRNA complexes is determined by the selection of cognate target RNAs by PIWI–piRNA. What are the mechanisms for this selection? There is a rigorous multistep control in identifying target RNAs by PIWI–piRNA structures, and RNA helicases play a potentially crucial role in this process.

Small RNA-mediated regulation has been the focus of fruitful research given its use across all domains of life. I focus here on target RNA selection by PIWI clade of the Argonaute (AGO) proteins and 24–31 nucleotide PIWI-interacting RNAs (piRNAs).

PIWI and piRNAs have been identified across animal phyla and are essential for gametogenesis. PIWI–piRNA complexes silence retrotransposons, primarily in gonads, at transcriptional and post-transcriptional levels, and are implicated in the regulation of other genes [1]. piRNAs are complementary to target RNAs and thereby guide specific PIWI proteins to these targets. Therefore, a PIWI–piRNA complex is the principal element which should recognize cognate target RNAs and avoid regulating noncognate (unrelated) RNAs. For example, to prevent transposon mobilization in the gonad, PIWI–piRNA complexes must recognize transposon RNAs (cognate targets). However, these complexes should not silence unrelated non-transposon RNAs (noncognate targets). How is this accomplished? I highlight evidence for multistep target selection performed by PIWI–piRNA structure, which has a preference for specific targets, through control by regulatory proteins and localization to specific loci. In addition, I propose that RNA helicases contribute to target selection by PIWI–piRNA.

The recent structure of a PIWI–piRNA from the silkworm *Bombyx mori* (Siwi) [2] has highlighted features contributing to complementary target recognition by PIWI–piRNA. Importantly, nucleotides 2–5 of piRNA bound to Siwi are arranged in a conformation nearly identical to the A-form helix, indicating that this short sequence performs the function of a seed region that provides the initial specificity during searching for a complementary region in potential targets (Figure 1A,B). This A-form seed sequence–target interaction is a common element in the initial target recognition by other Argonaute–guide RNA complexes, that are the members of the AGO clade distinct from PIWI, and Cas protein–crRNA–DNA target recognition in CRISPR systems [3]. Similarly to these systems, the PIWI-induced A-form helix of the seed sequence should

significantly enhance its interaction with the target by lowering the entropic cost of the interaction.

The Siwi–piRNA structure reveals an interaction between the base of piRNA nucleotide 5 and phenylalanine 436 in helix α7 that is conserved in PIWI-clade proteins (Figure 1B). This amino acid–base interaction indicates that base stacking between piRNA nucleotides 5 and 6 would be disrupted, making it difficult to associate with the target downstream of piRNA nucleotide 5 without changing the location of Phe436. Because different proteins have been shown to interact with Siwi and its homologs [4–6], it is tempting to propose that the helix α7 positioning and therefore piRNA–target interaction may be regulated by PIWI partners. Although this notion of a specific effect of a PIWI-associated regulator on piRNA–target binding awaits experimental testing, recent data indicate inhibitory effects of Tudor-domain polypeptides, SpnE and Krimp, on piRNA association with Siwi and Ago3 respectively [5–7]. SpnE and Krimp may block piRNA loading onto these PIWI proteins, and thereby target recognition until they are delivered to the special loci within ribonucleoprotein (RNP) granules. These granules are known to assemble in the germline, localize piRNA pathway components, and are referred to as germ granules [4]. This model proposes that the localization of PIWI–piRNAs to the special RNP sites prevents unintended silencing of RNAs elsewhere, thereby contributing to the specificity in selecting targets within these RNP granules.

Some PIWI proteins contribute to target selection by preferred binding to specific target nucleotides. In particular, *Drosophila* Aubergine, Siwi, and the mouse homolog Mili prefer to bind to targets which have an unpaired adenine opposite to the first 5′ nucleotide of piRNA, regardless of the identity of this piRNA base [8]. This preference for adenine in the targets was also detected for vertebrate Argonaute proteins which use miRNAs.

RNA helicases are commonly associated with PIWI–piRNA complexes and are crucial for PIWI–piRNA-dependent functions (Box 1). Accordingly, I propose that RNA helicases might enhance the selectivity of PIWI–piRNA towards specific target RNAs as follows. The helicases may constantly interfere with piRNA–target RNA basepairing and PIWI–target RNA interaction to prevent the formation of a 'cleavage-ready' noncognate ternary (PIWI–piRNA–noncognate target) complex. In other words, RNA helicases and PIWI–piRNA may be involved in a 'tug of war'

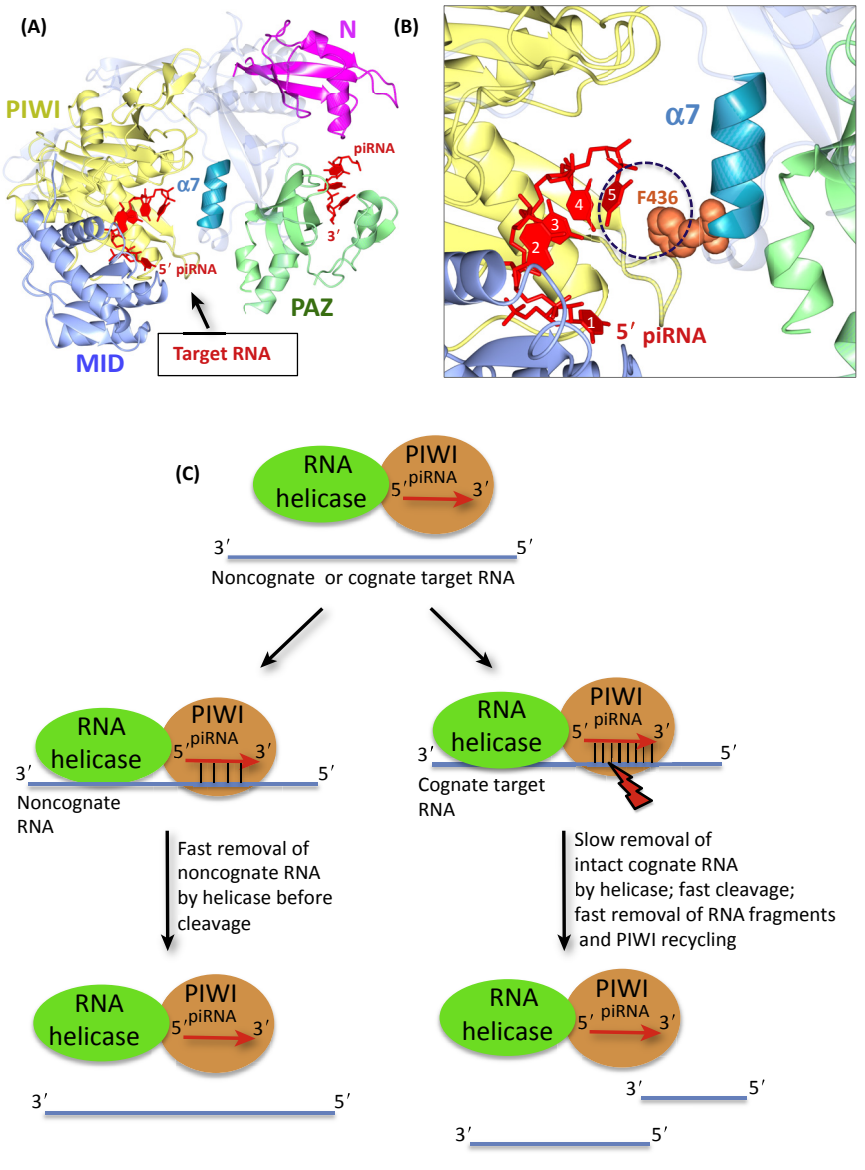

Figure 1. Selection of Target RNAs by PIWI–piRNA Complexes. (A) Siwi–piRNA structure (PDB ID: 5GUH [2]). A Siwi lobe containing PIWI and MID domains interacts with piRNA 5′ terminal nucleotides, and the

(Figure legend continued on the bottom of the next page.)

Box 1. Involvement of RNA Helicases in PIWI–piRNA Functions

RNA helicases unwind double-stranded RNA regions by hydrolyzing ATP. Therefore, they are essential components of RNP remodeling mechanisms and play a role as RNA chaperones during different stages of post-transcriptional gene regulation, from RNA splicing to translation and RNA stability [10]. Genetic analysis has implicated several RNA helicases as crucial players involved in transposon silencing, the control of piRNA loading onto PIWI proteins, and piRNA biogenesis; these include Vasa (Vas), SpnE/TDRD9, Armitage, and UAP56 [1,6,9]. Furthermore, there is strong evidence for direct interactions between Siwi and Vas [6], Aubergine and RNA helicase eIF4A [4], and mouse PIWI proteins (Mili and Miwi) and Vas homolog MVH [11]. In addition, Vas has been shown to act as a crucial RNP remodeler and assembly platform for components involved in the amplification of piRNA production, including PIWI proteins. In particular, this 'amplifier' complex has been implicated in the transfer of piRNA precursors generated from Siwi-cleaved target RNAs onto Ago3, and dissociation of these cleaved RNAs from Siwi [6,12]. Therefore, RNA helicases are central players in PIWI–piRNA-mediated functions and, in addition to their double-stranded RNA-unwinding activity (see Figure 1C in main text), they modulate the dynamic assembly of PIWI–piRNA complexes.

over target RNAs (Figure 1C). Specifically, the helicases may interfere with basepairing between antisense piRNAs and targets, and this interference may prevent cleavage of unrelated targets by PIWI. At the same time, this mechanism would also ensure rapid unwinding of piRNA–target RNA after the cleavage and the removal of cleaved RNAs from PIWI–piRNA. This model is consistent with current data and explains how the same unwinding helicase function can either prevent noncognate RNA cleavage or promote PIWI–piRNA recycling for subsequent cleavage reactions. Specifically, these two mutually exclusive events will depend on the strength of the PIWI–piRNA–target interactions. If piRNA–target RNA basepairing is not extensive and PIWI–target RNA interaction is weak, the cleavage rate will be slow and helicases will be able to move the target away from the Piwi endonucleolytic site before cleavage takes place ('helicases win the tug of war'). In this case, the RNA unwinding rate or target removal from PIWI by helicases will be faster than the cleavage rate, and therefore this mechanism will prevent RNA degradation. Alternatively, in case of strong piRNA–target basepairing and association with PIWI, the cleavage reaction occurs faster than a helicase can act ('PIWI–piRNAs win the tug of war'), and the helicase subsequently removes the remaining RNA fragments still associated with piRNA after target RNA is cut, thereby recycling the PIWI–piRNA for a new round of cognate target cleavage.

Consistent with the model, Vas helicase is required for the removal of target RNA fragments (slicer products) from Siwi–piRNA after piRNA–target extensive basepairing and cleavage by Siwi [6]. Importantly, even cognate targets were found to be ejected from the Siwi–piRNA by Vas [6] before slicing, suggesting that the helicase is engaged in the rigorous selection leading to the multiple removals of potential targets from PIWI–piRNA before its association with a cognate target results in cleavage. Recent experiments have pointed to the analogous function of mouse Vas in the release of slicer products from PIWI complexes [9].

In contrast to PIWI–piRNA complexes, helicases might not similarly contribute to target selection by AGO proteins loaded with siRNAs or miRNAs, and it is likely that these non-PIWI AGO proteins reject noncognate targets much more efficiently than PIWI. Consistent with this idea, Ago2–siRNA itself effectively ejected the target RNA slicer products [6].

Similarly to the proposed role of RNA helicases in target selection by PIWI–piRNAs that slice the targets, the helicases may be involved in PIWI–piRNA selection

piRNA 3′ end is extended to the lobe composed of N and PAZ domains. The piRNA middle segment was not defined. Helix α7 shown at the center of the structure is likely to play an important role in the regulation of piRNA–target RNA interaction because it needs to move away from the RNA binding surface to allow efficient RNA duplex formation. (B) A detailed view of piRNA 5′ segment–Siwi interaction. Bases 2–5 at the 5′ end of piRNA constitute a seed sequence which adopts an A-form helix-like conformation when bound to Siwi. Phenylalanine 436 (F436) in the α7 helix interacts with the base of nucleotide 5 of the piRNA. This interaction should prevent stacking between bases 5 and 6 of the piRNA, and therefore the α7 helix should change its location to allow piRNA–target RNA duplex formation. In human Argonaute2, which is a representative of the AGO clade and binds to miRNAs, helix α7 changes its location to allow the guide-target RNA basepairing interaction and, together with the PIWI domain, α7 even stabilizes this interaction by associating with the minor groove of the RNA–RNA double helix [3]. It is thus likely that helix α7 in PIWI proteins may be an important element required for the stabilization of the initial complementary interaction between the piRNA seed sequence and the target. Importantly, different cellular polymerases also interrogate the proper geometry of nucleotide base-pairs by interacting with the minor groove of cognate base pairs including DNA and RNA polymerases (interact with template–new nucleotide base pair) and the ribosomes (associate with cognate codon–anticodon pairs) [13]. Therefore, the mechanisms involving interactions of cellular machines, which synthesize macromolecules, with the minor grooves of transient base pairs seem to be universal and contribute to the fidelity of DNA, RNA, and protein synthesis. These mechanisms may also enhance the accuracy of gene regulation by Argonaute proteins. The figure was generated using the program CCP4mg. (C) 'Tug of war' between RNA helicase and PIWI–piRNA may contribute to fidelity during selection and silencing of cognate target RNAs, and rejection of noncognate RNAs. RNA helicase and PIWI–piRNA are engaged in two mutually exclusive reactions: (i) RNA helicase helps to dissociate targets from PIWI–piRNA, and (ii) PIWI–piRNA binds to and subsequently cleaves the targets. Therefore, there is a dynamic equilibrium between these two reactions, and helicase-enhanced dissociation of noncognate RNA from PIWI–piRNA is carried out faster than RNA cleavage by PIWI owing to limited interactions between PIWI–piRNA and noncognate RNA. By contrast, there is a higher affinity of cognate target RNA for PIWI–piRNA and a lower dissociation rate owing to helicase activity, which results in cognate target cleavage. The accuracy of target selection will be directly proportional to the activity of RNA helicase which accelerates the dissociation of potential targets from PIWI–piRNA.

of targets that are silenced without cleavage during PIWI–piRNA-dependent recruitment of the deadenylation complex to the targets or transcriptional silencing which involves the recognition of nascent cognate transposon RNA by PIWI–piRNA followed by the formation of repressive chromatin. Two helicases involved in transposon silencing, UAP56 and TDRD9, are potential candidates to contribute to nascent RNA selection in the nucleus by piRNA-guided *Drosophila* Piwi and mouse Miwi2, respectively.

In summary, rigorous target selection is likely to be accomplished at the level of PIWI–piRNA structure through PIWI–piRNA interactions with regulatory proteins, localization to specific loci, and the involvement of RNA helicases. Future research should provide mechanistic insights into the effects of PIWI partners on PIWI–piRNA structure and target recognition.

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