論 文 の 内 容 の 要 旨

論文題目　Dynamic subcellular compartmentalization of the piRNA pathway in silkworm cells
（カイコ培養細胞におけるpiRNA経路の動的な細胞内区画化）

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In animal germ cells, PIWI proteins and Piwi-interacting RNAs (piRNAs) protect the genome from deleterious transposons by RNA silencing. piRNA-guided PIWI proteins cleave transposon RNAs using their 'slicer' activities, thereby suppress the translation of transposase as well as the activation of transposons. The cleavage products produced by the slicer are further processed into new piRNAs, therefore enable the amplification of piRNA loci. This process, known as the 'ping-pong cycle', involves a pair of PIWI proteins that cleave their target RNAs and successively and precisely hand over the cleavage products to each other, which are then further processed into mature piRNAs (**Figure 1**). It is known that the silkworm cell-line BmN4 contains a fully functional ping-pong pathway and thus serve as an excellent cell model for piRNA studies. In BmN4 cells, two PIWI proteins, BmAgo3 and Siwi are involved in this pathway, where Siwi is associated with most of the transposon-targeting piRNAs. In addition, several key factors including BmVasa (a DEAD-box RNA helicase), BmSpnE (a Tudor domain-containing RNA helicase) and BmQin (a Tudor domain-containing E3 ligase) are required for proper piRNA production, but their molecular mechanisms of action are unclear (**Figure 1**).

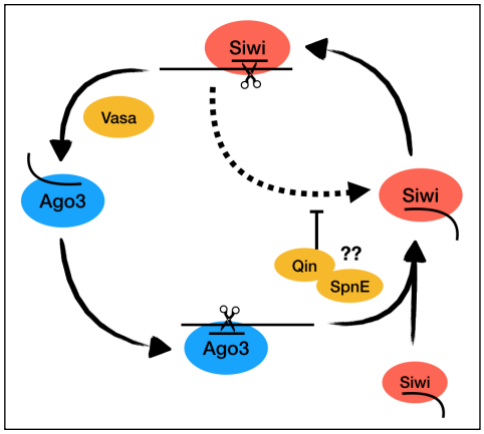

Figure 1. A simplified version of Ping-Pong amplification pathway in silkworm BmN4 cells.

The PIWI proteins and their interactors are enriched in perinuclear ribonucleoprotein (RNP) granules called 'nuage', which leads to a major opinion that nuage may function as the center for transposon silencing and piRNA biosynthesis. Previous genetic studies in mice and fruit flies have provided evidences that the proper localization of nuage proteins is functionally linked to an intact piRNA biosynthesis pathway. However, despite the extensive genetic studies on ping-pong amplification loop, there remains a lack of comprehensive understanding regarding the molecular details of these piRNA-related RNP granules. For instance, a recent study argues that in silkworms, SpnE and Qin interacts with Siwi independently of

BmVasa-containing nuage. Furthermore, studies in mice have reported the functional compartmentalization of its ping-pong cycle, where the two PIWI proteins, MILI and MIWI2, forming distinct but contacting RNP granules with their interacting proteins. Of note, MIWI2 granules are associated with processing body (P-body) proteins, which includes RNA degradation and silencing factors. Since this compartmentalization mechanism is not conserved in the well-studied fruit fly ovaries model, research efforts are greatly hindered, and the biological functions of the granules has remained largely elusive. Therefore, this study aims to elucidate the molecular details and the biological significance of piRNA-related RNP granules in silkworm ovary-derived BmN4 cells, which will form the basis of future studies on the step-wise regulation of ping-pong amplification loop and other important yet elusive piRNA sub-pathways.

To dissect the compartmentalization of piRNA biogenesis machineries, I employed the strategy to express proteins of interest with an N-terminal fluorescent tag (AcGFP or mCherry) in BmN4 cells. This also allows the expression of functional mutants for studying the relations between protein functionality and their localization. Given that the slicer activity of PIWI proteins is the driving force of the ping-pong cycle, I first examined the subcellular localization of the slicer-defective mutants of Siwi (D670A) and BmAgo3 (D697A). Unexpectedly, the single residue mutation in Siwi caused the protein to dislodge from nuage and instead re-localized to P-bodies (**Figure 2**). On the contrary, the localization pattern of BmAgo3 slicer mutant was indistinguishable from its wildtype counterpart, with most of the protein remained localizing in nuage (**Figure 2**). As Siwi-D670A mutant is capable of binding piRNA and its target RNA but not cleavage, I suspected that Siwi-D670A might have been trapped in some piRNA complexes originated from P-bodies, inspired by the study of the mammalian piRNA pathway.

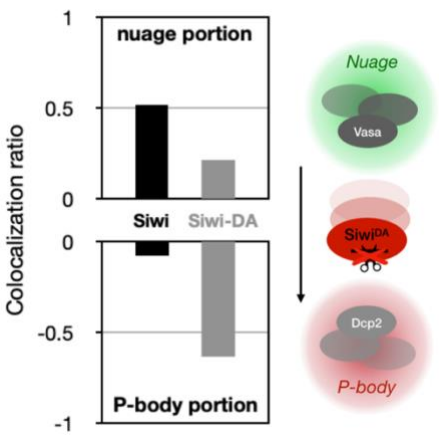

Figure 2. Slicer-defective Siwi is mislocalized to P-body. Quantification of co-localization ratio of Siwi-D670A mutant between nuage marker BmVasa or P-body marker BmDcp2 shows that Siwi-D670A slicer mutant mislocalized to P-body.

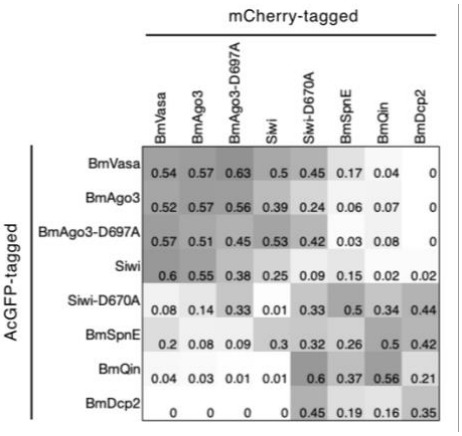

Figure 3. Subcellular compartmentalization of piRNA pathway. Heatmap showing co-localization ratio of piRNAs revealed the presence of two distinct clusters of piRNA granules, representing nuage (top-left) and piP-body (bottom-right).

Indeed, in a localization analysis of piRNA factors which involves A total of 28 combinations from 5 core piRNA factors and BmDcp2 (a well-known P-body protein), I found that in addition to Siwi-D670A, BmSpnE and BmQin exclusively localized in BmDcp2-containing cytoplasmic P-bodies **(Figure 3)**. In contrast, wildtype Siwi, BmAgo3,

BmAgo3-D679A and BmVasa extensively colocalized in nuage but not in P-bodies **(Figure 3)**. Interestingly, I also found that the P-body localization of BmSpnE and BmQin increased when Siwi-D670A was expressed, pointing to an extensive interaction between the slicer-defective Siwi mutant and its P-body interactors. These results suggested that in silkworm cells, piRNA pathway is compartmentalized into nuage and piP-body in a way similar to mouse but not fruit fly.

Since these experiments were performed by over-expressing protein mutants, mis-localization could be simply caused by aberrant aggregation of a large quantity of epitope-tagged protein. To eliminate the effect of this possible overexpression artifact, I cloned the tagged proteins into Tet-On plasmids, where the expression rate of the proteins can be induced by applying Doxycycline to cell cultures. With the new constructs, I successfully validated the key co-localization pairs between Siwi-D670A and P-body markers, while reducing the expression level of the tagged-proteins down to 0.08 folds of their endogenous counterpart. This excluded the possibility that piP-body localization of piRNA factors is merely an artifact caused by overexpression.

Given that Siwi-D670A led to an increased localization of BmSpnE and BmQin in piP-bodies, I suspected that Siwi-D670A and its interactors are trapped on long P-body mRNAs as solid aggregates. This hypothesis was proved correct by FRAP (Fluorescence-recovery after photobleaching) experiments, where the slower recovery of Siwi-D670A foci after a photobleaching event suggests decreased molecular exchange between the foci and the cytoplasm. Furthermore, by utilizing an aliphatic alcohol named 1,6-hexanediol which has been used in studies to dissolve liquid-like condensate, I found that Siwi-D670A foci were not affected by the chemical, while wildtype Siwi-foci were completely dissolved. These together suggest that Siwi-D670A along with its interacting proteins, resembles a solid-like RNP aggregates in piP-body. In other words, the slicer activity of Siwi plays an important role in maintaining the molecular dynamics of itself and its protein partners at steady state.

The solid aggregation nucleated by Siwi-D670A raises a new question: why there is a reduction but not an increase of Siwi-D670A signals in nuage, when the protein is capable to trap and 'freeze' piRNA complexes? I speculated that BmVasa, a nuage DEAD-box helicase that has a putative function as releasing cleaved RNAs from Siwi, is responsible for maintaining the integrity of nuage by actively expelling Siwi-D670A with its helicase activity. Surprisingly, co-expression of a BmVasa ATPase mutant (E339Q, previously characterized in Xiol et al, 2014) and the Siwi-D670A prompted an enormous aggregation between the proteins, while wildtype Siwi co-localized with BmVasa-E339Q in smaller loci **(Figure 4)**.

This suggested that the ATPase activity of BmVasa actively remodels Siwi and Siwi-D670A RNP complexes, thus result in the segregation of Siwi-D670A foci from nuage. When both the BmVasa and Siwi activities are mutated, nuage and piP-body partition is impaired and their once separated piRNA complexes aggregates into larger condensates. To conclude, I found that Siwi slicer and BmVasa ATPase play important roles in maintaining nuage/piP-bodies partition, as well as the molecular dynamics of piRNA complexes.

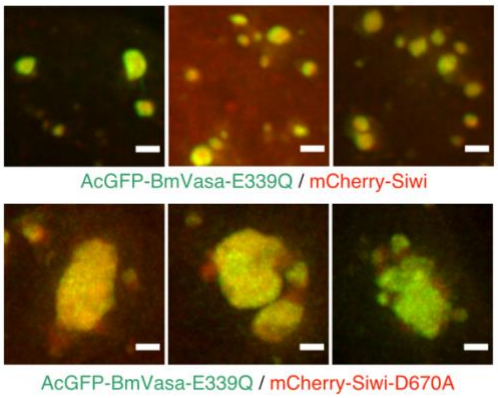

Figure 4. BmVasa ATPase mutant traps Siwi and Siwi-D670A. Representative microscopic images of BmVasa-E339Q co-aggregating with Siwi or Siwi-D670A. The aggregation size was significantly larger in

To further extend the scope of the research, I sequenced the total small RNA from Siwi-D670A-expressing cells to probe for the effect of a disrupted nuage/piP-body partition on piRNA biosynthesis. While most of the piRNA originated from transposon RNAs were not affected, I found that in Siwi-D670A- but not Siwi-expressing cells, non-transposon piRNAs increased for about 4-folds **(Figure 5)**. These non-transposon piRNAs were mapped to UTRs and CDS but not the introns of the protein-coding mRNAs, including those who have essential functions like ribosomal RNAs and translation initiation factors. I also confirmed that these non-transposon piRNAs was of the same length to normal piRNAs and carried similar 5′ nucleotide bias. Non-transposon piRNAs were almost exclusively mapped to the sense strand of mRNAs, suggesting a cytoplasmic, mature mRNA origin. Given that a battery of piRNAs were being trapped in the newly found piP-bodies, I proposed that presence of Siwi-D670A may have trapped some unknown piRNA fidelity determining factors in piP-bodies, causing the endogenous piRNA pathways to start processing cytoplasmic mRNAs. These results support a role of nuage/piP-body subcellular compartmentalization in regulating the fidelity of piRNA biosynthesis in silkworm BmN4 cells. A manuscript was prepared with the abovementioned discovery and has been accepted in *EMBO Reports* (Chung *et al*, 2021).

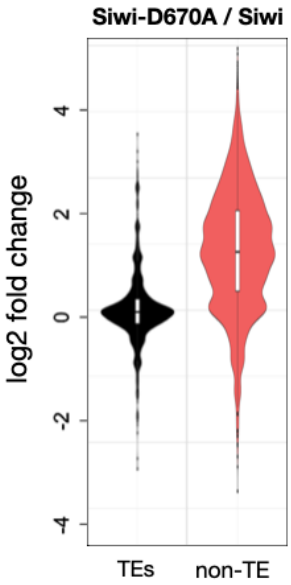

Figure 5. Siwi-D670A causes promiscuous production of non-transposon piRNAs. Small RNA-seq revealed that Siwi-D670A increases non-transposon piRNAs (non-TE) for 2 to 4 folds, compared to Siwi-expressing cells. Transposon piRNAs (TEs) were not affected.