

**Doctoral Dissertation (Censored)**

博士論文（要約）

**Siwi cooperates with Par-1 kinase to resolve the autoinhibitory effect of Papi  
for Siwi-piRISC biogenesis**

（Siwi-piRISC 生合成において Siwi はリン酸化酵素 Par-1 と協調して  
Papi の自己抑制効果を解除する）

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## **1. Abstract**

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本章については、5年以内に雑誌等で刊行予定のため、非公開。









## **2. Abbreviations**

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aDMA	asymmetrical dimethylarginine
Ago3	Argonaute 3
CLIP	UV-crosslinking and immunoprecipitation
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
GFP	Green fluorescent protein
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
IDR	Intrinsically disordered region
KD	Knockdown
kDa	Kilodalton
KH domain	K homology domain
miRNA	Micro RNA
mRNA	Messenger RNA
n.i.	Non-immune immunoglobulin
NP40	Nonyl phenoxypolyethoxylethanol
nt	Nucleotide

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
piRISC	piRNA-induced silencing complex
piRNA	PIWI-interacting RNA
PTGS	Post-transcriptional gene silencing
qRT-PCR	Quantitative real-time PCR
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
RNP	Ribonucleoprotein
sDMA	symmetrical dimethylarginine
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
TGS	Transcriptional gene silencing
T-PBS	0.1% Tween 20 in PBS
Trim	Trimmer
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride buffer
Triton X-100	2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol
WT	Wildtype
Zuc	Zucchini

### **3. Introduction**

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#### **3.1. RNA silencing**

RNA silencing, also known as RNA interference (RNAi), is a sequence-specific gene regulatory mechanism conserved in eukaryotes (Aravin and Tuschl, 2005; Ghildiyal and Zamore, 2009; Hutvagner and Simard, 2008; Plasterk, 2002). The main players of the system are 20-35 nucleotide (nt)-long small noncoding RNAs and Argonaute family proteins. To exert their functions in RNA silencing, small RNAs and Argonaute proteins associate with each other to form RNA-induced silencing complexes (RISCs) (Fig. 3.1.A).

Argonaute proteins are divided into AGO and PIWI subfamilies depending on the degree of peptide sequence similarity (Fig. 3.1.B). AGO and PIWI proteins also show different properties: While AGO proteins are expressed ubiquitously, PIWI proteins are expressed mainly in germline tissues (i.e., ovaries and testes). Small RNAs are classified into three categories; small interfering RNA (siRNA), micro RNA (miRNA), and PIWI-interacting RNA (piRNA) (Fig. 3.2) (Chu and Rana, 2007; Ghildiyal and Zamore, 2009; Hutvagner and Simard, 2008; Ipsaro and Joshua-Tor, 2015). While piRNAs are germline-specific and are specifically loaded onto PIWI proteins (see section 3.2 for the details), siRNAs and miRNAs are expressed ubiquitously and assemble RISCs with AGO proteins.

siRNAs are normally 21-25 nt long and are produced from long double-stranded RNA precursors by Dicer, a member of the RNase III family (Fig. 3.2) (Chu

and Rana, 2007; Ghildiyal and Zamore, 2009). miRNAs are normally 22-30 nt long and are produced from hairpin-shaped miRNA precursors in a stepwise manner, firstly by Drosha, another member of the RNase III family, and then by Dicer (Fig. 3.2). miRNAs regulate protein-coding genes differently in different cell types, contributing to the assignment of cell identity (Iwakawa and Tomari, 2015; Krol et al., 2010). siRNAs can also regulate protein-coding genes and other genes that show high complementarity to them when siRNAs are artificially designed to achieve silencing of the targets (Mocellin and Provenzano, 2004). Endogenous siRNAs (endo-siRNAs), particularly in nematode and *Drosophila*, are known to silence transposons (Britton et al., 2020; Ghildiyal and Zamore, 2009; Hoogstrate et al., 2014; Kawamura et al., 2008; Watanabe et al., 2008). It is also known that when nematode, *Drosophila*, and even plants are infected, the viral genes produce siRNAs, which the hosts use as a self-defense system to protect themselves from viral invasion (Agius et al., 2012; Huang et al., 2013; Swevers et al., 2018).

### **3.2. piRNA, PIWI protein, and piRISC**

piRNAs are normally 24-31 nt long and are produced from single-stranded piRNA precursors (Fig. 3.2). Unlike siRNA and miRNA biogenesis, piRNA biogenesis does not depend on Dicer. As briefly mentioned above, piRNAs are rich in the germline, where they associate with PIWI proteins to form piRISCs. The piRISCs repress transposons to maintain the stability of the germline genome (see section 3.3 for the details) (Czech and Hannon, 2016; Ghildiyal and Zamore, 2009; Hiraoka and Siomi,

2019; Klattenhoff and Theurkauf, 2008; Yamashiro and Siomi, 2018). Otherwise, the organisms become infertile, disrupting the inheritance of the species.

The piRNA system is highly conserved in organisms with sexual reproduction (except plants). Nonetheless, the piRNA study has mainly been conducted using *Drosophila melanogaster* and mice (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Kim, 2006; Lau et al., 2006; Saito et al., 2006; Vagin et al., 2006; Watanabe et al., 2011). Both *Drosophila* and mice express three PIWI proteins; Piwi, Aubergine (Aub), and Ago3 in *Drosophila* and MIWI, MIWI2, and MILI in mice. All these PIWI members become piRISCs with piRNAs in the cytoplasm. Upon this, Aub-piRISC and Ago3-piRISC in *Drosophila* and MIWI-piRISC and MILI-piRISC in mice remain in the cytoplasm and repress transposons post-transcriptionally by cleaving target gene mRNAs using the slicer activity. On the other hand, Piwi in flies and MIWI2 in mice are transported into the nucleus upon piRISC formation and repress transposons transcriptionally by depositing repressive histone marks or inducing DNA methylation at the target loci, facilitating heterochromatinization (Chuma and Nakano, 2013; Ernst et al., 2017; Onishi et al., 2021; Ozata et al., 2019). Interestingly, the piRNA pathway in mice is male-specific, while the pathway in *Drosophila* can be observed in both sexes (Quénerch' du et al., 2016; Saint-Leandre et al., 2020; Taborska et al., 2019). Studies showed that mice depend on endo-siRNAs to repress transposons in the ovaries (Stein et al., 2015).



### 3.3. piRISC-mediated repression of transposons

Transposons move around within the genome, from one location to another, via either ‘copy and paste’ or ‘cut and paste’ method, thus are also known as “jumping genes” (Bourque et al., 2018; Dubin et al., 2018; Feschotte and Pritham, 2007; Muñoz-López and García-Pérez, 2010). The translocation of transposons creates a variety of mutations in the genome; therefore, transposons can be a driving force of evolution. However, the selfish movement of transposons also causes severe damages to the genome. To avoid this situation in the germline, piRNAs repress transposons (Grimson et al., 2008; Khurana and Theurkauf, 2010; Vagin et al., 2006). Endo-siRNAs in *Drosophila* silence transposons in regular, non-germline-type somatic cells (Ghildiyal and Zamore, 2009; Kawamura et al., 2008; Watanabe et al., 2008). Endo-siRNA-dependent transposon silencing may also happen in the germline. However, it seems that the contribution of endo-siRNAs to repress transposons in the tissues may be much lower than that of piRNAs.

### 3.4. piRNA biogenesis in silkworm cultured ovarian germ cells, BmN4 cells

Germline tissues consist of two types of cells, germ cells and somatic cells surrounding the germ cells. To analyze separately the molecular mechanisms occurring in the two types of cells, cultured cells consisting of only one type of cells are desired. Cultured ovarian somatic cells have already been established from *Drosophila* and are called OSCs (Saito et al., 2006). In contrast, cultured ovarian germ cells have not been established in *Drosophila* (and in mice, too). Thus, in our

laboratory, other members and I have used BmN4 cells to understand the mechanism underlying piRNA biogenesis in ovarian germ cells. BmN4 cells are derived from silkworm ovaries and consist of only ovarian germ cells, so are a powerful tool to analyze the piRNA biogenesis pathway biochemically.

BmN4 cells express two PIWI proteins, Siwi (*Drosophila* Aub homolog) and Ago3 (Kawaoka et al., 2008). In BmN4 cells, piRNAs are produced through primary pathway and ping-pong pathway (see below in this section) (Fig. 3.3) (Nishida et al., 2015; Sakakibara and Siomi, 2018). In the primary pathway, piRNAs are derived from piRNA clusters, intergenic regions enriched with transposon sequences. *Flamenco* is a representative of piRNA clusters in *Drosophila* and has been extensively analyzed (Kofler, 2020; Yamanaka et al., 2014). Although piRNA clusters are considered to exist in the BmN4 genome, the actual status remains unclear.

piRNA precursors transcribed from piRNA clusters are processed into mature piRNAs in a step-wise manner in the cytoplasm and are loaded onto Siwi to produce Siwi-piRISCs (Fig. 3.3) (Izumi et al., 2016, 2020; Nishida et al., 2015, 2018). piRNAs loaded onto Siwi are mostly antisense to transposon mRNAs. Thus, Siwi-piRISC cleaves transposon mRNAs upon binding to them through piRNAs. DEAD-box helicase Vasa then transfers the cleavage products to Ago3 (Nishida et al., 2015; Xiol et al., 2014). The RNA fragments bound to Ago3 are then processed to piRNAs, producing Ago3-piRISC. Because of this, piRNAs bound to Ago3 are mostly sense piRNAs (because they are derived from transposon mRNAs). Next, Ago3 cleaves

transposon transcripts and piRNA precursors in antisense orientation. Then, another DEAD-box helicase DDX43 transfers the RNA cleavage products to unloaded Siwi (Murakami et al., 2021).

These reactions depending on the slicer activity of Siwi and Ago3 are repetitively continued, and through this, Siwi-piRISC and Ago3-piRISC are generated abundantly, which efficiently repress transposons post-transcriptionally (Sakakibara and Siomi, 2018). This system is known as the ping-pong pathway. The ping-pong pathway is conserved in species, including *Drosophila*, but *Drosophila* OSC cells do not possess the pathway (Parhad and Theurkauf, 2019; Zamore, 2010). Thus, OSC cells cannot be used to analyze the mechanism of the ping-pong cycle.

### **3.5. Papi, a Tudor domain-containing piRNA factor**

Papi was originally identified in *Drosophila* ovaries as a factor interacting with PIWI proteins (Liu et al., 2011). In *Drosophila* ovaries, Papi interacts with Ago3 in its symmetrical dimethylarginine (sDMA) modification-dependent manner (Saigusa et al., 2011) (Fig. 3.4.A). Papi and Ago3 colocalize to nuage, germline-specific cytoplasmic granules (Liu et al., 2011). These traits suggest that Papi is involved in piRNA biogenesis in *Drosophila*. However, the expression level of piRNAs was hardly changed by the loss of Papi. Furthermore, the expression levels of transposons were hardly altered in the ovaries of Papi knockout flies. Based on these observations, it was claimed that Papi is not necessary for piRNA biogenesis in *Drosophila* (Han et al., 2015; Handler et al., 2011).

On the other hand, mouse Papi (Tdrkh) is necessary for piRNA biogenesis and repression of LINE-1 retrotransposon in mice testes (Saxe et al., 2013). *Bombyx* Papi is also essential for piRNA biogenesis: the previous study showed that siRNA-based depletion of Papi in BmN4 cells causes a severe reduction in the level of piRNAs (Nishida et al., 2018). Papi is anchored on the mitochondria surface via its mitochondrial localization signal (MLS) located at the N-terminus. Besides, Papi has three functional domains; two K homology (KH) domains, one Tudor domain, and an auxiliary domain (Honda et al., 2013) (Fig. 3.4.B and C). Generally, KH domain is a well-conserved domain, which binds to single-stranded nucleic acids (Nicastro et al., 2015; Siomi et al., 1994). Tudor domain binds dimethylated lysine and arginine residues to mediate protein–protein interactions (Chen et al., 2011; Pek et al., 2012). The KH domain of Papi binds to piRNA precursors and the Tudor domain interacts with Siwi and Ago3 in an sDMA modification-dependent manner (Honda et al., 2013; Izumi et al., 2016; Nishida et al., 2018).

In BmN4 cells, not only Papi but also Siwi, endonuclease Zucchini (Zuc), and exonuclease Trimmer (Trim) are on the surface of mitochondria, where Papi co-functions with them (Izumi et al., 2016; Nishida et al., 2018) (Fig.3.4.D). Firstly, unbound Siwi is modified with sDMA by Protein Arginine Methyltransferase 5 (PRMT5) (Anne et al., 2007; Gonsalvez et al., 2006; Honda et al., 2013; Kirino et al., 2009). Next, Siwi interacts with Papi on mitochondria depending on the sDMA modification, and the Papi-Siwi complex binds to a piRNA precursor. The 3'-end of

piRNA precursor is cleaved by Zuc and further trimmed by Trimmer (Trim), giving rise to mature Siwi-piRISC (Izumi et al., 2016; Nishida et al., 2018).

Papi was detected as multiple bands on western blots, suggesting that Papi is modified post-translationally (Nishida et al., 2018). Dephosphorylation assay suggested that Papi was phosphorylated. Thus, the band shift on western blots was claimed to be caused by phosphorylation of Papi (Nishida et al., 2018). UV-crosslinking and immunoprecipitation (CLIP) assays showed that the top band of Papi on the western blots showed stronger RNA-binding activity compared with other bands (Nishida et al., 2018). This suggested that phosphorylation of Papi might control the RNA-binding activity of Papi (Nishida et al., 2018). However, its regulatory mechanism remains elusive.

### **3.6. Post-translational modification of piRNA factors**

Post-translational modifications are known to modify protein functions and dynamically coordinate their signaling networks (Conibear, 2020; Lin and Carroll, 2018; Ramazi and Zahiri, 2021; Xu et al., 2018). The modifications include phosphorylation, ubiquitylation, SUMOylation, methylation, and so on. Post-translational modifications of piRNA factors known so far include sDMA and asymmetrical dimethylarginine (aDMA) modifications of Vasa (Kirino et al., 2010), and sDMA modification of Siwi and Ago3 (Honda et al., 2013; Nishida et al., 2018). The sDMA and aDMA modifications of Vasa are conserved among animals, such as mouse, *Xenopus*, and *Drosophila* (Kirino et al., 2010; Xiol et al., 2014). Siwi and

Ago3 interact with other piRNA factors that have Tudor domains in an sDNA-dependent manner, and many of such protein–protein interactions via sDMA modification are necessary for piRNA biogenesis in BmN4 cells (Honda et al., 2013; Nishida et al., 2018).

To the best of my knowledge, no studies have been done to show that the function of piRNA factors is regulated by phosphorylation, although an earlier study revealed that the RNA-binding activity of human Ago2 (Golden et al., 2017) is regulated by phosphorylation and that this influence the efficiency of RNAi in the organism.

### **3.7. The aim of this study**

本節については、5 年以内に雑誌等で刊行予定のため、非公開。

### **3.8. Summary of this study**

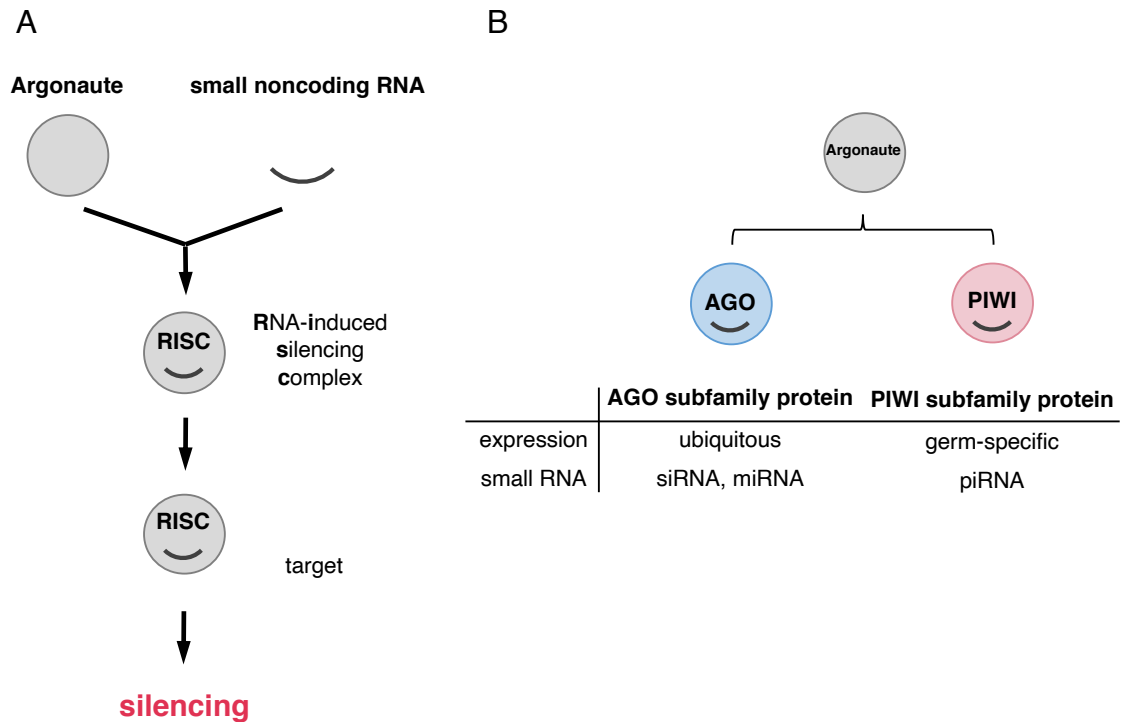
In this study, I revealed:

- 1) Ser547 phosphorylation in Papi is necessary for facilitating Siwi-piRISC biogenesis.
- 2) Par-1, a conserved serine/threonine kinase, is responsible for Ser547 phosphorylation in Papi.
- 3) Siwi interacts with Par-1 in the cytoplasm and targets Par-1 to mitochondrial Papi to promote Ser547 phosphorylation.
- 4) Dephosphorylation of Papi-pSer547 is unnecessary for Papi to be free from RNAs for second round piRISC production reaction.
- 5) The interaction of Papi with Siwi via the Tudor domain and the additional negative charge within the auxiliary domain of Papi by Par-1 are required for Papi to be bound with Siwi-binding piRNA precursor.

In conclusion, this study reveals the unique mechanism by which Siwi cooperates with Par-1 to control the role of Papi in Siwi-piRISC biogenesis. The RNA-binding activity of Papi is necessary for two steps: the interaction with Siwi

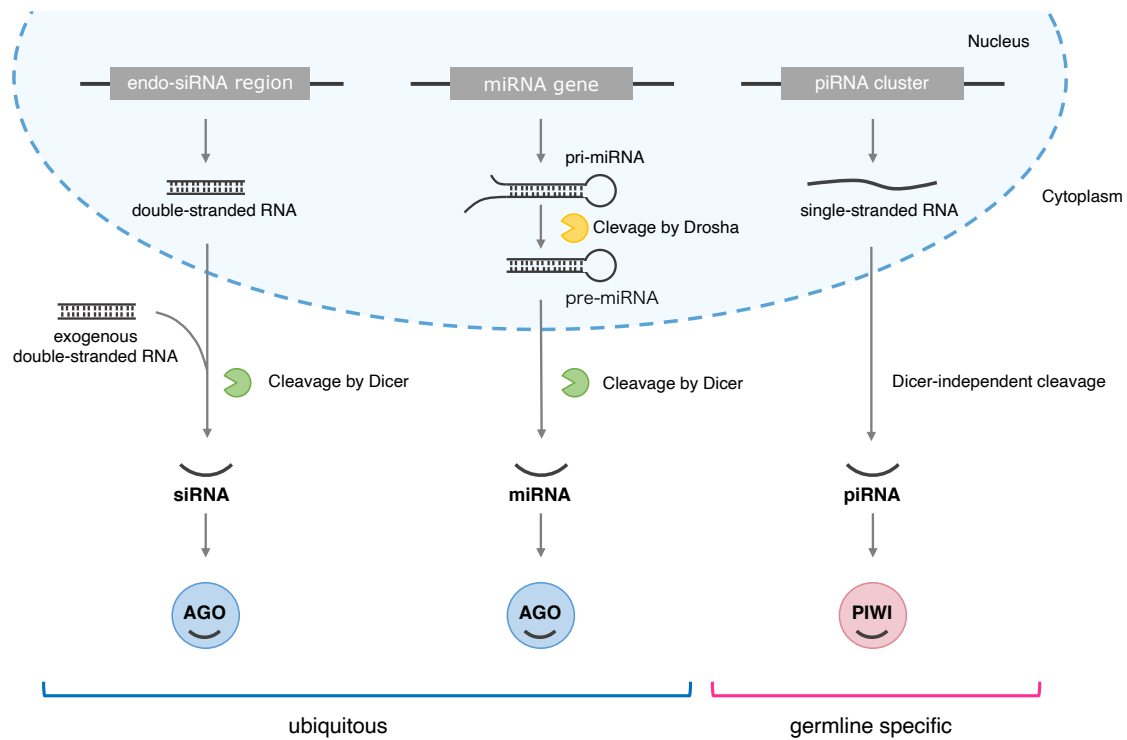
and the negative charge addition to Papi by Par-1. The way of how Par-1 mechanistically regulates the RNA-binding activity of Papi necessary for Siwi-piRISC biogenesis in BmN4 cells has now emerged.





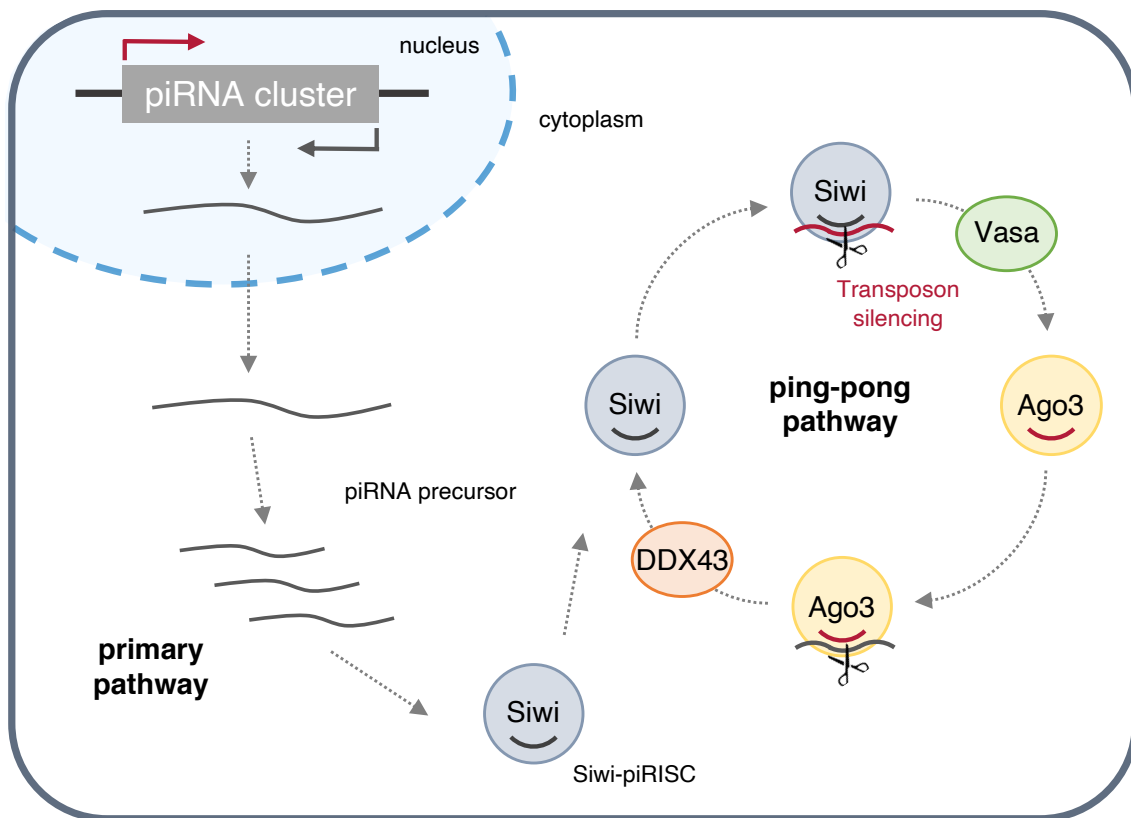
**Figure 3.1. RNA silencing**

(A) The schematic drawing shows an overview of RNA silencing. Other factors may also involve silencing of target genes. (B) Argonaute proteins are divided into two subfamilies; AGO subfamily and PIWI subfamily. Their expression patterns and small RNAs bound to them are different each type.



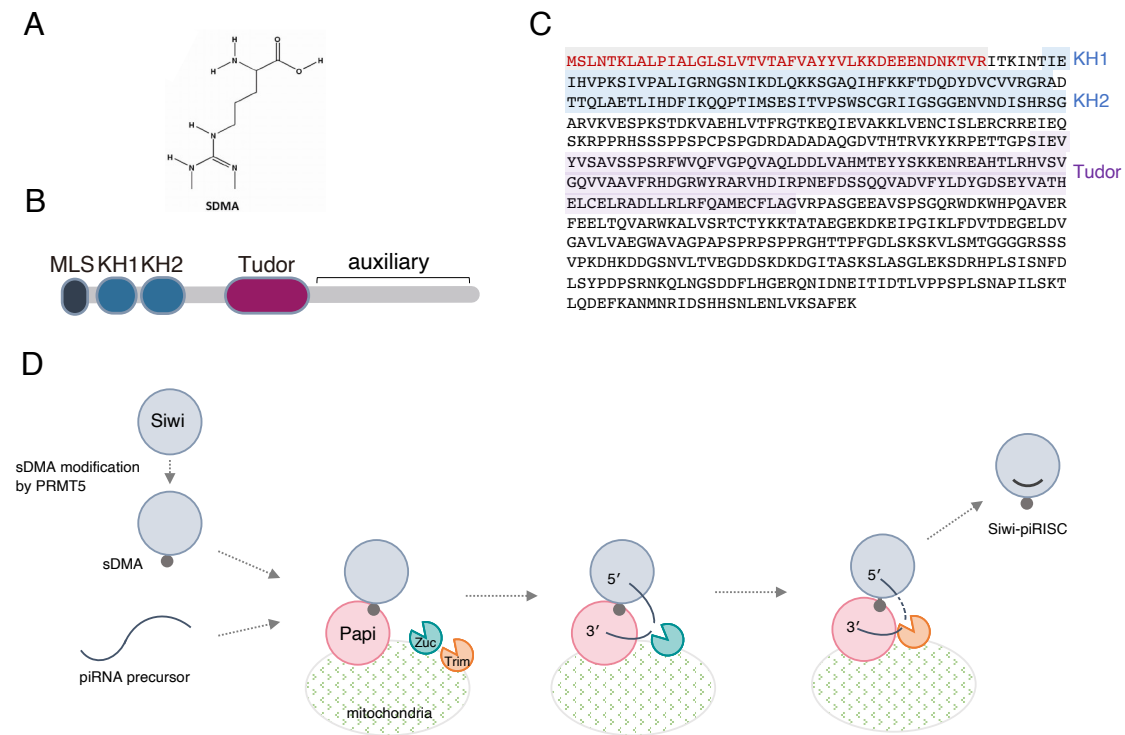
**Figure 3.2. Classification of Argonaute proteins and small RNAs in animals**

The schematic drawing shows an overview of three RNA silencing pathway in animals. siRNAs are produced from endogenous and exogenous double-stranded RNAs and loaded onto AGO subfamily protein. miRNAs are produced from double-stranded hairpin RNAs derived from miRNA genes and loaded onto AGO subfamily protein. piRNAs are produced from single-stranded RNA derived from piRNA clusters and loaded onto PIWI subfamily protein.



**Figure 3.3. piRNA pathway in BmN4 cells**

The schematic drawing shows the current model of piRNA biogenesis pathway in BmN4 cells. In BmN4 cells, piRNAs are produced by primary and ping-pong pathway. In primary pathway, single-stranded piRNA precursors are processed stepwisely, and matured piRNAs are loaded onto Siwi. In ping-pong pathway, Siwi and Ago3 cleave piRNA precursors using their slicing activity and efficiently produce piRNAs. The black lines indicate antisense piRNAs. The red lines indicate sense piRNAs.



**Figure 3.4. Papi functions as a scaffold of piRNA maturation mechanism in BmN4 cells**

(A) The chemical structure of sDMA. This figure is modified from Saigusa et al., 2011. (B) The schematic drawing shows the domain structure of Papi. (C) Amino acid sequence of Papi. The mitochondria localization signal (MLS) is shown by red letters; the KH domains are boxed in blue; the Tudor domain is boxed in purple. (D) The schematic drawing shows the current model of piRNA maturation mechanism in BmN4 cell. Firstly, Papi interacts with Siwi modified with sDMA by PRMT5 and Papi-Siwi complex binds to a piRNA precursor. Siwi binds to the 5' end of piRNA precursor and Papi binds to the 3' end of piRNA precursor. The piRNA precursor is processed by Zuc and Trim, and Siwi-piRISC is produced.

## **4. Materials and methods**

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## **5. Results**

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## **6. Discussion**

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## **7. Conclusion**

## **7. Conclusions**

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## 9. Acknowledgements

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