Doctoral Dissertation (Censored)

博士論文（要約）

Elucidation of a novel mechanism underlying piRNA-mediated transcriptional transposon silencing

(piRNA によるトランスポゾン転写の新規抑制機構の解明)

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Contents

1. Abstract

1. Abstract

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

2. Abbreviations

Ago1	Argonaute1
Ago2	Argonaute2
Ago3	Argonaute3
apiRNA	Artificial piRNA
Armi	Armitage
ATP	Adenosine triphosphate
Aub	Aubergine
BAP	Brm-associated protein
Brm	Brahma
BSA	Bovine serum albumin
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
co-IP	Co-immunoprecipitation
Ctrl	Control
DMP	Dimethyl pimelimidate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein

Egg	Eggless
FBS	Fetal bovine serum
FPKM	Fragments Per Kilobase of exon per Million mapped fragments
Gasz	Germ cell specific protein with four Ankyrin repeats, a Sterile alpha motif, and a putative leucine Zipper
GFP	Green fluorescent protein
Gnf1	Germ line transcription factor 1
GO	Gene ontology
Gtsf1	Gametocyte-specific factor 1
H1	Linker histone H1
H3K9me3	Histone 3 lysine 9 trimethylation
HEPES	4-(2-Hydroxyethyl)-1 -piperazineethanesulfonic acid
HMG	High mobility group
HMT	Histone methyltransferases
HP1a	Heterochromatin protein 1 a
HRP	Horseradish peroxidase
Hsp70	Heat shock protein 70
IgG	Immunoglobulin G
kb	Kilobase pairs

KD	Knockdown
kDa	Kilodaltone
l(1)G0020	Lethal (1) G0020
LRR	Leucine-rich repeat
LTR	Long terminal repeats
Luc	Luciferase
Mael	Maelstrom
MDa	Megadaltone
miRNA	Micro RNA
mRNA	Messenger RNA
MS	Mass Spectrometry
n.i.	Non-immune immunoglobulin
NP40	Nonyl phenoxypolyethoxylethanol
Nt	Nucleotide
Nxf2	Nuclear RNA export factor 2
Nxt1	NTF2-related export protein 1
OSC	Ovarian somatic cell
Panx	Panoramix
PB	Polybromo
PBAP	Polybromo-containing BAP
PBS	Phosphor buffered saline
PcG	Polycomb group

PCR	Polymerase chain reaction
piRISC	piRNA-induced silencing complex
piRNA	PIWI-interacting RNA
PNP	Panx-Nxf2-p15
Pol	Polymerase
qRT-PCR	Quantitative reverse transcription PCR
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RNAi	RNA interference
RNase	Ribonuclease
RT	Room temperature
SDS	Sodium dodecyl sulfate
Sfmbt	Scm-related gene containing four mbt domains
siRNA	Small interfering RNA
Snr1	Snf5-related 1
SoYb	Sister of Yb
SWI/SNF	Switch/ Sucrose Non-Fermentable
TE	Transposable element
T-PBS	0.1% Tween 20 in PBS
Tj	Traffic jam

Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride buffer
TSS	Transcriptional start sites
UTR	Untranslated region
Vret	Vreteno
Wde	Windei
WT	Wildtype
Yb	Female sterile (1) Yb
Zuc	Zucchini

3. Introduction

3. Introduction

3.1. Transposon and piRNA

Transposons are DNA sequences which are capable of transposing themselves randomly within their host genomes. While the transpositions of transposons have functioned as evolutional drivers, they cause mutations in the host genomes and threatens individual survival. Especially, in germ cells, which have function to transmit genetic information correctly to the offsprings, the mechanisms to repress transposons are conserved among most of the eukaryotes.

Gonad-specific 24-31 nt small non-coding RNAs, PIWI-interacting RNAs (piRNAs), protect germline genome from invasion of transposons by repressing their expression (*1-4*). Whereas miRNAs and siRNAs are expressed ubiquitously and form complexes with AGO subfamily proteins of Argonaute proteins, Ago1 and Ago2 respectively, piRNAs form complexes with PIWI subfamily proteins and interact with transposon RNAs which are complement to the sequences of piRNAs (Fig.3.1.). The complexes are referred to as piRNA-induced silencing complexes (piRISCs) and repress expression of transposons in various ways (1-4). piRNAs are discovered initially in *Drosophila melanogaster*,

and many of milestone findings have been made in this model organism. piRNAs also have been found in mammals, such as mice (1-3). Loss of piRISC desilences transposons, leading to DNA damage in the germline genome, malfunction of gonads and infertility (5, 6).

3.2. PIWI proteins in *Drosophila*

Drosophila has three PIWI genes, *piwi*, *aub* and *ago3*, all of which are essential in gonadal development (7, 8) (Fig. 3.1.). Ovarian germ cells express all these members, whereas ovarian follicle cells express Piwi, but not others (9).

It is known that Aub-piRISC and Ago3-piRISC function in the cytoplasm of ovarian germ cells. Aub forms complex with piRNA derived from piRNA precursor via initial Aub-piRNA production pathway, called 'primery pathway', and cleaves mRNAs of transposons using its slicer activity (9, 10). Ago3 forms complex with piRNA derived from the remnant of transposon mRNA after Aub-piRISC cleavage, to accelerate the Aub-piRNA-driven production by cleaving piRNA precursor (9, 10). The pathway is called 'ping-pong amplification pathway' (9, 10) (Fig. 3.2.).

On the other hand, in both ovarian germ and somatic cells, Piwi-piRISC does not cleave mRNAs of transposons in the cytoplasm, but is imported into the nucleus and repress transcription of transposon (11). Therefore, it is suggested that the transposon repression in the nucleus is not post-transcriptional repression, but transcriptional repression (12-15).

3.3. Overview of Piwi-piRNA pathway

First, Piwi-piRNA precursors are transcribed from a genomic locus enriched with transposon fragments, which is called *flamenco* piRNA cluster (1-3). The Piwi-piRNA precursors are exported to the cytoplasm, processed into piRNA intermediates and captured in Yb body, which is a cytoplasmic granule containing Yb protein and other piRNA precursors (Fig.3.3.). Yb body is thought to have a function to promote pre-Piwi-piRISC formation by accumulating piRNA intermediates and Piwi proteins (16). Then, Piwi-piRNA intermediates translocate with Piwi to mitochondria and are processed into 24-31 nucleotide-long mature piRNAs by mitochondrial endonuclease Zucchini (Zuc) (17). Finally, Piwi-piRISC is imported to the nucleus, and represses

transcription of transposons. At present, the relationship between this repression mechanism and heterochromatin formation has been strongly suggested (12-15) (Fig.3.3.).

3.4. piRNA factors

For elucidation of molecular mechanism of piRNA pathway, in previous studies, RNAi screening were performed to identify essential factors for transposon silencing in *Drosophila* ovary (18, 19). These factors are called 'piRNA factors.' Especially, piRNA factors in ovarian follicle cells are involved in Piwi-piRNA pathway. piRNA factors in ovarian follicle cells could be categorized in two types: the factors for Piwi-piRNA biogenesis and the factors for Piwi-mediated silencing. Actually, by using OSC (Ovarian Somatic Cell), which is a cultured cell line derived from ovarian follicle cells, both can be classified (18, 19). When piRNA factors for biogenesis are depleted in OSCs by RNAi, both reduction of Piwi-piRNA and increase of transposon mRNA can be observed, but when piRNA factors for silencing are depleted, while Piwi-piRNA can be produced, transcription of transposon is up-regulated (18).

Examples of piRNA factors for Piwi-piRNA biogenesis include the following: Yb, Armitage, Sister of Yb, Vreteno for

capturing piRNA intermediates, Gasz and Zuc for processing piRNA intermediates into mature size of piRNAs (16, 17, 21). piRNA factors for silencing include the following: Gtsf1/Asterix (Gtsf1), Panoramix/Silencio (Panx), Mael, Nxf2, p15/Nxt1, Eggless (Egg), Windei (Wde), Heterochromatin protein 1 a (HP1a) and linker histone H1 (H1) (12, 22- 32).

3.5. piRNA factors for transcriptional silencing

As piRNA factors for silencing include histone methyltransferase and heterochromatin proteins, it was presumed that Piwi-mediated silencing of transposon transcription involves heterochromatin formation. Actually, the concept of small RNA-mediated heterochromatin formation has already been proposed in *Saccharomyces pombe* (33). In *Saccharomyces pombe*, siRNA and Ago1 recognize a nascent transcript of centromeric repeats and induce H3K9 methylation via a H3K9-specific histone methyltransferase and heterochromatin formation (33) (Fig. 3.4.). Based on this model, it has been presumed that piRNA and Piwi in *Drosophila* repress transposon transcription in a similar manner and several laboratories have begun to provide evidence to support the assumption.

As mentioned, Piwi-mediated transposon silencing requires multiple co-factors, including Gtsf1, Panx, Nxf2, p15, Mael, Egg, Wde, H1, and HP1a, (12, 22-32). Previous studies have mainly performed knockdown (KD) analyses and the depletion phenotypes of these factors in OSC were reported. Depletion of these factors impacts on transposon silencing, but the effects are different in each case.

Here, I have further classified these factors from phenotypes of their depletion into three groups: (I) Factors whose depletion phenotype are similar to Piwi depletion (Gtsf1, Panx, Nxf2 and p15), (Ⅱ) Heterochromatin factors (HP1a, H1, Egg and Wde) and (Ⅲ) Factor whose depletion phenotype is partly similar to Piwi (Mael). The followings are summaries of what are known about the factors in each group.

3.5.1. Gtsf1, Panx, Nxf2 and p15

In the first place, loss of Piwi leads to transposon derepression and reduction in the level of H3K9me3 at target loci in OSCs (12). Similar phenotypes can be observed in loss of Gtsf1, Panx, Nxf2 and p15, so it can be assumed that these factors are indispensable for Piwi-mediated transcriptional silencing of transposon (22-25, 28-31) (Fig.3.4 A and B). Particularly, Panx,

Nxf2 and p15, form complex called PNP, and are thought to be important factors. In previous studies, not only KD analyses but also artificial tethering assays using lambda N fusion proteins and boxB integrated luciferase reporter have been performed (22, 23, 28-31) (Fig. 3.4. C). In this assay, it is possible to tether an arbitrary protein on nascent RNA by fusing lambda N protein, which binds to stem-structured RNA derived from boxB sequence, and to observe its silencing effect. Several laboratories have been reported that tethering of Panx, Nxf2 or p15, efficiently silence ubiquitin-promoter or ß-tubulin-promoter-driven luciferase reporter and induces H3K9me3 (22,23, 28-31). This finding indicates that PNP itself have function to induce transcriptional silencing. Interestingly, tethering of Piwi could not silence transcription of the reporter (22, 23, 29). This confusing result indicates the possibility that Piwi requires recognition of target RNA to induce silencing. In fact, it has been shown that LRR domain of Nxf2 interact with target transposon mRNA and is necessary for silencing of endogenous transposons (28-31). Therefore, it has been proposed that, after Piwi recognized target transposon, PNP is recruited by Piwi and reinforces the association of Piwi and target transposon mRNA by binding to both Piwi and target RNAs (29, 30). From these reasons, PNP is

thought to be a core complex for tethering repression factors to nascent RNA and inducing H3K9me3 and transcriptional silencing of transposons.

3.5.2. HP1a, H1, Egg and Wde

Generally, heterochromatin factors also involved are in transposon silencing. HP1a is a core component of heterochromatin formation. HP1a mediates gene silencing via associating with K9-trimethylated histone H3 tail. Histone H1 is also involved in stabilization of heterochromatin by interacting with linker DNA and histones. Depletion of HP1a or H1 causes strong derepression of transposons and collapses heterochromatin (26, 27). However, the destruction of heterochromatin can be observed not only Piwi-dependent transposons, but also Piwi-independent transposons (26, 27). Certainly, HP1a and H1 play an important role for transposon silencing, but it is inferred that the specificity of the target loci is determined by Piwi and PNP.

Egg and Wde are also involved in that repression pathway. *Drosophila melanogaster* has three H3K9-specific histone methyltransferases, Su(var)3-9, G9a and Egg. Previous study showed that only Egg is involved in transposon silencing but the

others are not (23). Depletion of Egg results in transcriptional activation of transposon and H3K9 demethylation (23) (Fig. 3.4. C). In addition, it has been also reported that depletion of Egg cancels reporter silencing, which is induced by artificially tethered PNP (22, 23). Recently, our laboratory reported that Wde interacts with Egg and support its function by maintaining Egg on nucleosomes (32). For these reasons, Egg-dependent H3K9me3 induction has been considered as a most essential event for transcriptional silencing of transposons. However, there is a room for reconsideration. It is true that depletion of Egg causes transcriptional activation of Piwi-dependent transposons, but the activation rate of endogenous transposon is significantly low compared to depletion of Piwi (23) (Fig. 3.4. D). Therefore, Piwi may silence transposons by unknown mechanism parallel to H3K9me3 induction.

3.5.3. Mael

Mael was originally identified as a factor that is involved in anterior-posterior axis formation of early oocyte (34). Recently, Mael is also known to play a crucial role in Piwi-mediated transposon silencing (12). Depletion of Mael in OSC resulted in accumulation of RNA Pol II at the target loci and derepression of

transposons (Fig.3.5. A and B). Mael loss in OSC actually led to an increase of chromatin accessibility, but the levels of H3K9me3 at the loci were fairly maintained (12, 26) (Fig3.5. B). Recently, Mael further has been reported to be able to repress canonical transcription by RNA Pol II both inside and outside dual-stranded piRNA clusters which contain transposon remnants capable of providing promoter activity (35). These studies suggest that Mael may finally achieve the establishment of nucleosome-dense structure which effectively prevents RNA Pol II recruitment to maintain the Piwi-mediated transposon silencing downstream of, or in parallel to, the Egg-dependent H3K9me3 establishment.

3.6. Recent model of Piwi-mediated transcriptional silencing of transposons

The model currently proposed is as follows. Evidences have so far shown that Piwi-mediated transposon silencing is divided into four consecutive steps; (1) searching of target RNAs by Piwi that occurs in a relatively randomized fashion, (2) Piwi-target RNA binding through RNA-RNA base-pairings, (3) recruitment of two piRNA factors, PNP and Gtsf1, and (4) induction of local heterochromatinization in concert with histone

methyltransferases Egg, H1 and HP1. Upon this, RNA Pol II no longer has access to the Piwi-target loci, resulting in transcriptional silencing (22-25, 28-32) (Fig. 3.6.).

3.7. SWI/SNF in *Drosophila melanogaster*

SWI/SNF is known to be an approximately 2 MDa active chromatin remodeling complex consists of ATP-dependent DNA helicase and several factors. SWI/SNF contributes to tissue-specific development and differentiation by promoting nucleosome accessibility to support recruitment of transcriptional factors and RNA Pol Ⅱ (35) (Fig. 3.7. A). In *Drosophila*, Brahma (Brm) is an ATP-dependent DNA helicase of the Brm (SWI/SNF) chromatin remodeling complex (37-43). The Brm chromatin remodeling complex is known to act as a positive regulator of homeotic genes and other genes including those related to oogenesis (39,44). The Brm complex consists of seven core proteins and accessory factors and is divided further into the BAP and PBAP complexes depending on the accessory proteins. The BAP complex contains Osa absent in the PBAP complex, while the PBAP complex contains Polybromo and some other accessory proteins absent in the BAP complex (37-43) (Fig. 3.7. B). The specificity of the two complexes to their target loci are speculated

to be determined by the accessory proteins. The two complexes have been suggested to function in different biological processes, but the differences are not yet fully understood.

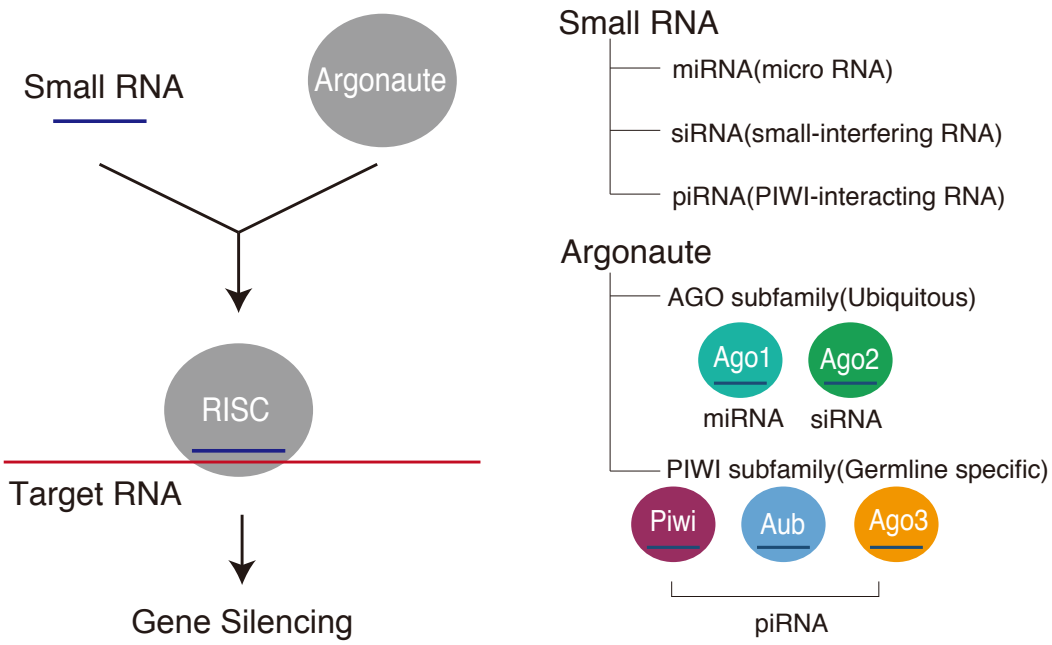


Figure 3.1. RNA silencing

Schematics show mechanism of small RNA-mediated gene silencing and classification of Argonaute family proteins and small RNAs in *Drosophila melanogaster*.

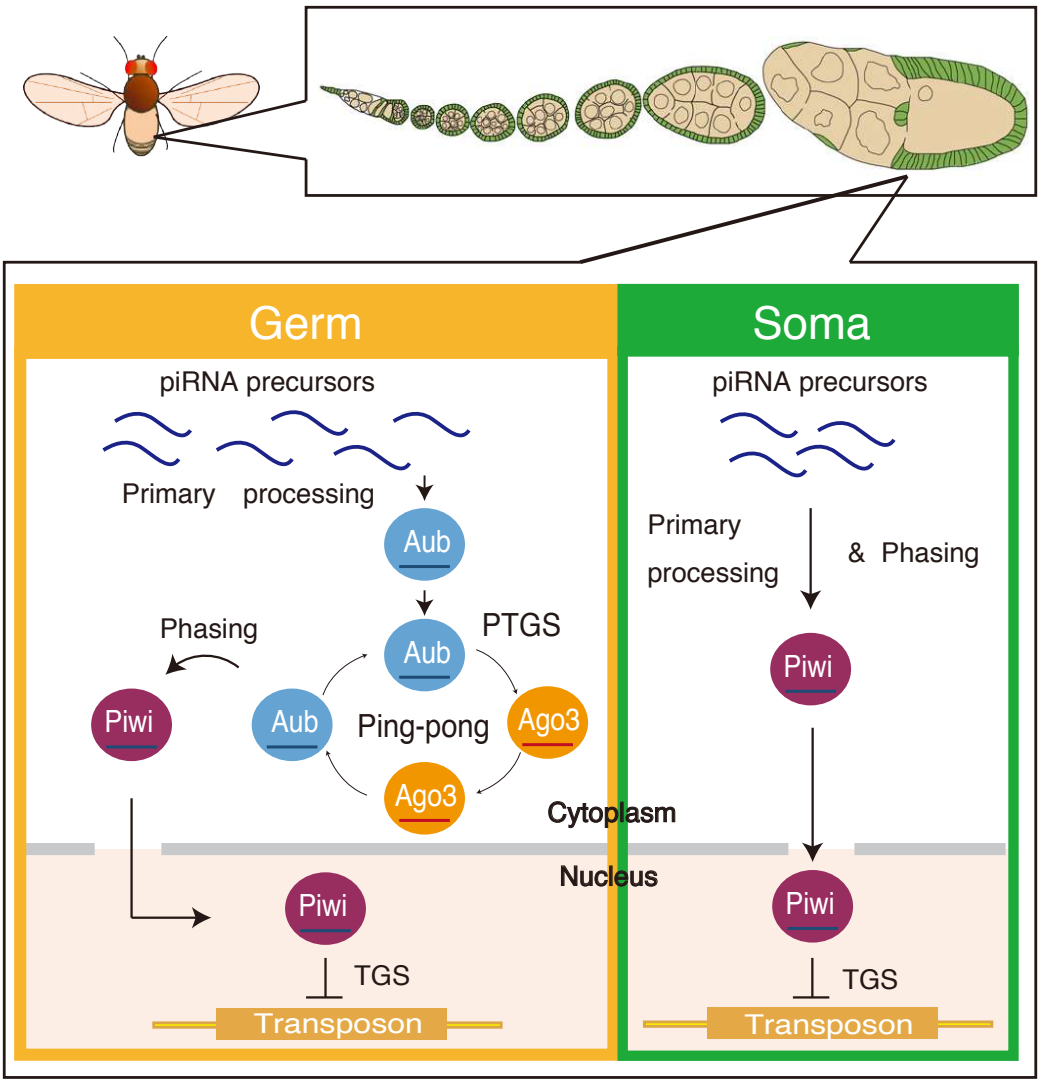


Figure 3.2. piRNA-mediated transposon silencing in *Drosophila melanogaster*

In germ cells of *Drosophila* ovary, all PIWI proteins are expressed. Aub and Ago3 functions in the cytoplasm, But Piwi in the nucleus. Piwi is also expressed in somatic cells surrounding the germ cells and represses transcription of transposons. Drawing of ovary is modified from Handler et al., 2011(44).

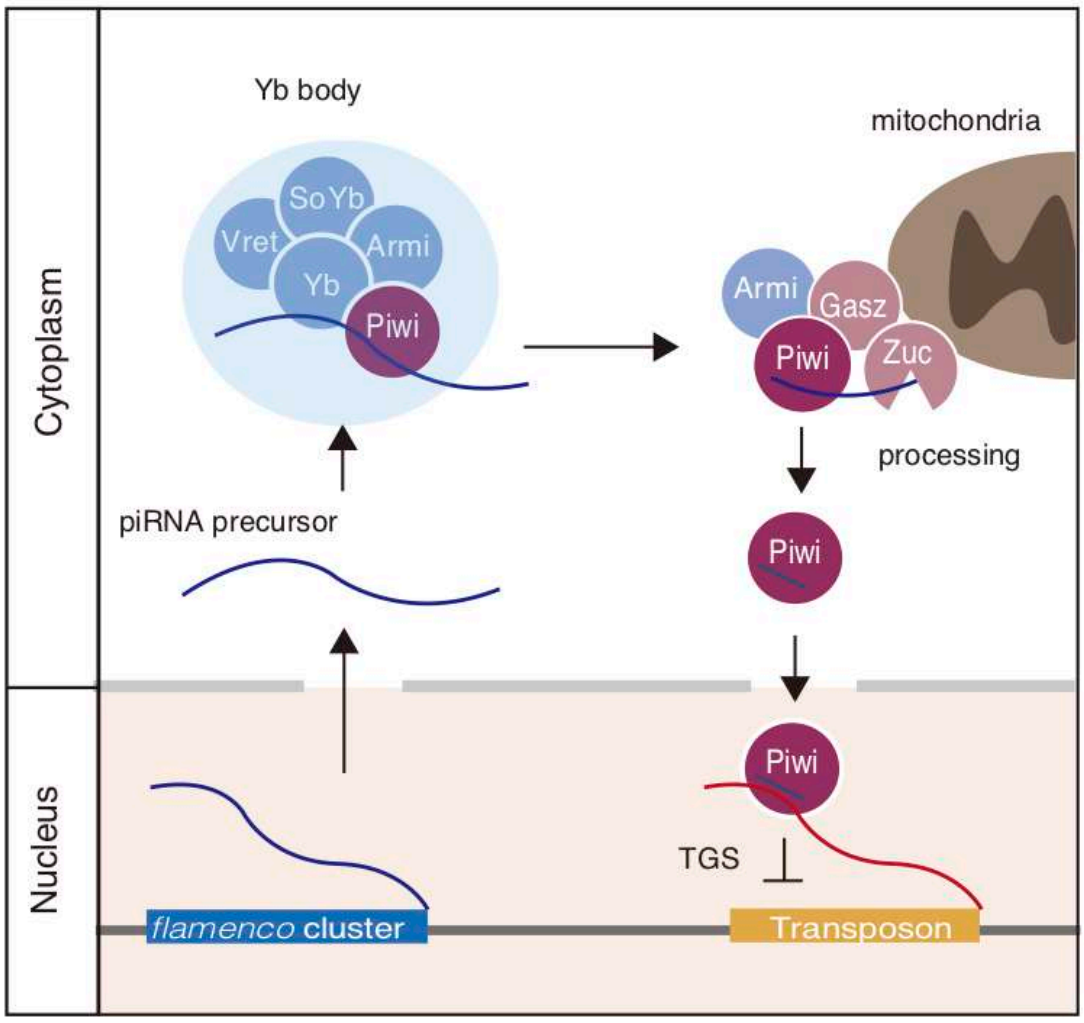


Figure 3.3. Overview of Piwi-piRNA pathway in OSC

Schematic shows overview of Piwi-piRNA pathway. piRNA precursor is transcribed from *flamenco* cluster and exported to the cytoplasm. In the cytoplasm, it is captured in Yb body and binds to Piwi proteins and finally processed on mitochondria. Piwi-piRISC is imported to the nucleus, and repress transcription of transposons.

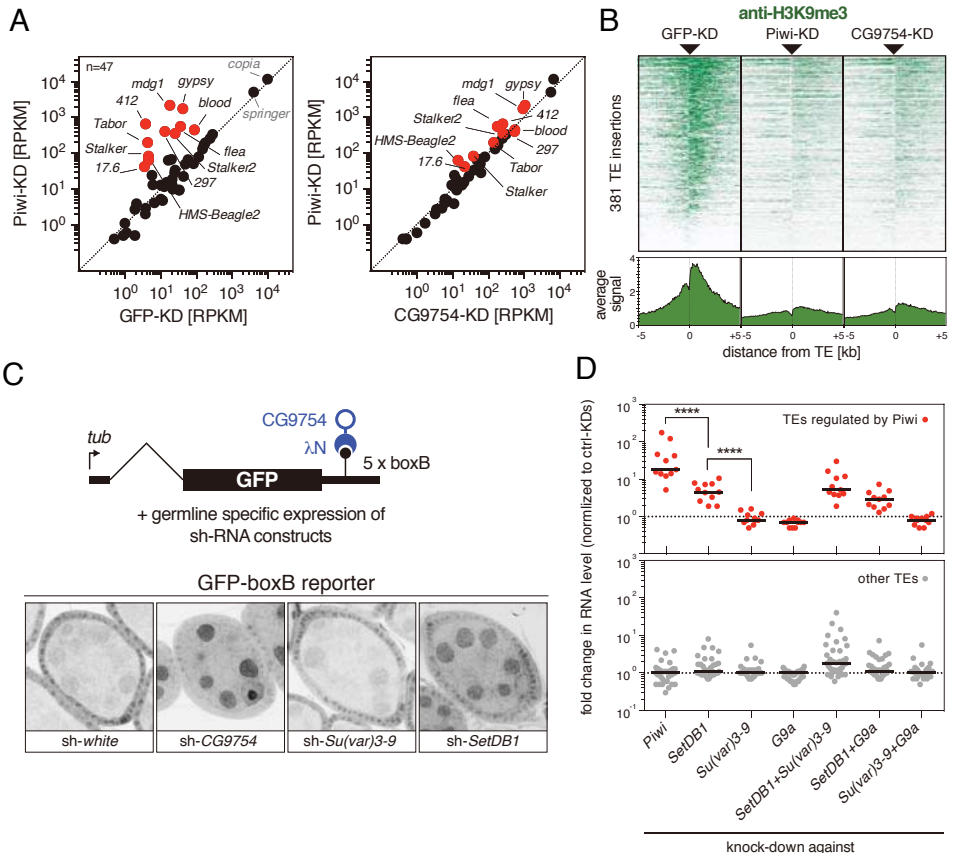


Figure 3.4. Panx induces Egg-dependent transcriptional repression

These figures are modified from Sienski, 2015 (23). CG9754 represents Panx. (A) Scatter plots showing the similarity of transposon types desilenced by Piwi KD and Panx KD. (B) Piwi KD and Panx KD induce demethylation of H3K9me3 at transposon loci. (C) Artificial tethering of Panx repress reporter gene, and the repression is Egg-dependent. SetDB1 represents Egg. (D) Egg KD causes derepression of transposons targeted by Piwi, but the rate of the derepression is relatively low compared to Piwi KD.

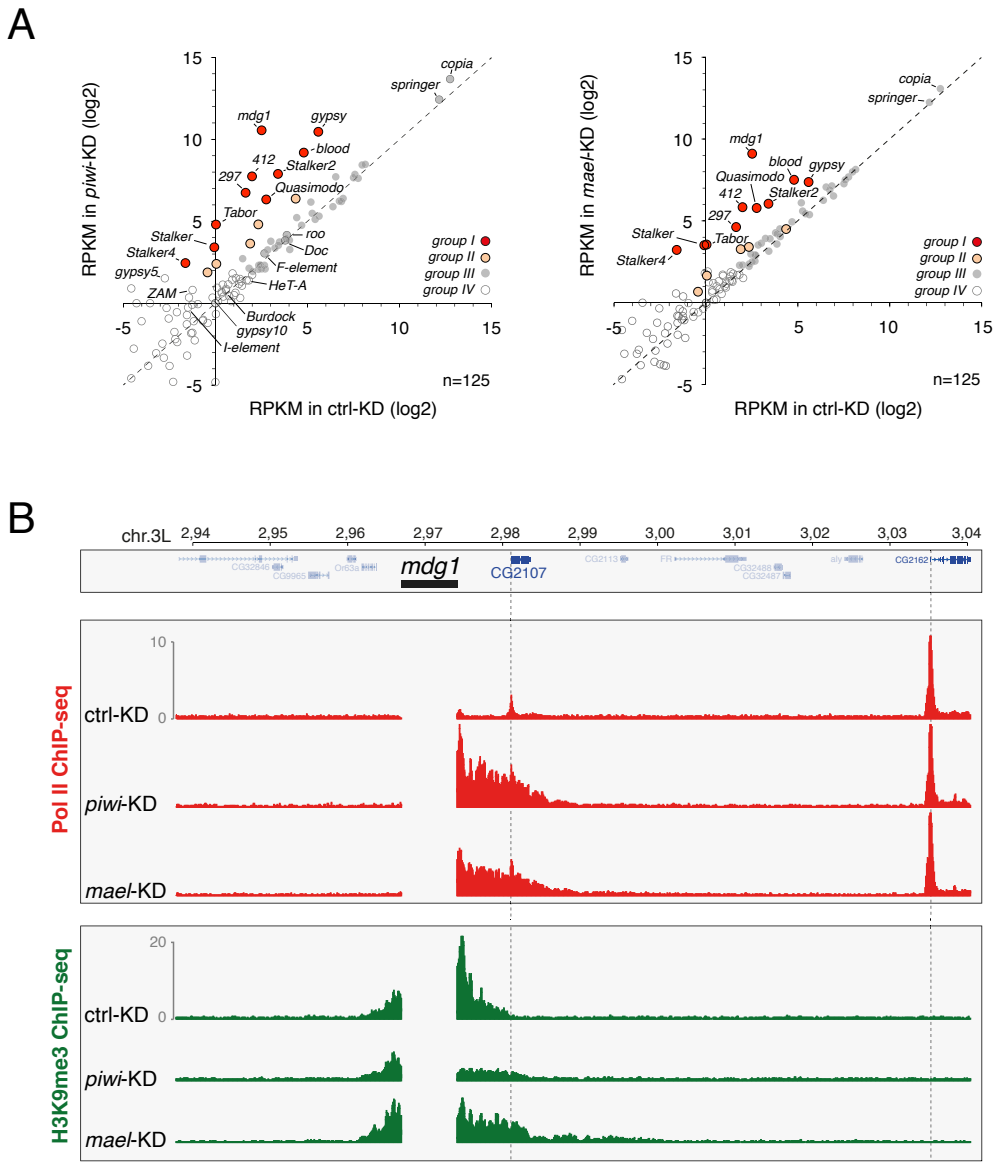


Figure 3.5. Mael KD causes activation of transposons, but little effect on H3K9me3 level. These figures are modified from Sienski, 2012 (12). (A) Scatter plots showing the similarity of transposon types desilenced by Piwi KD and Mael KD. (B) Density plot showing RNA Pol ll levels and H3K9me3 levels in indicated KD OSCs.

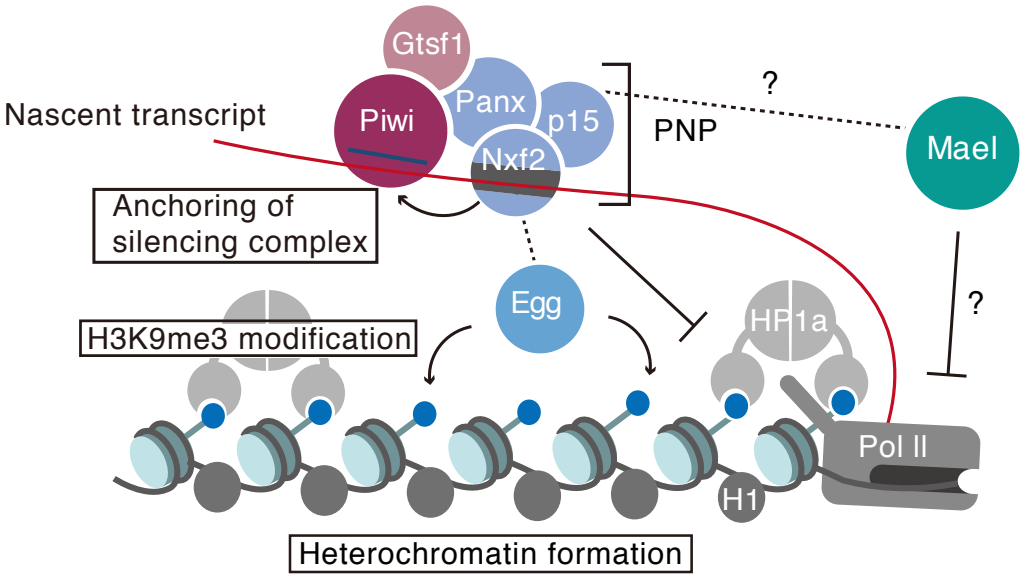
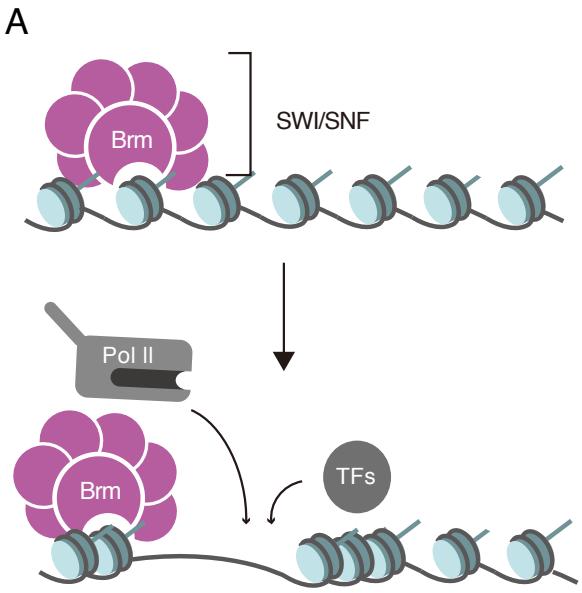


Figure 3.6. Recent model of Piwi-piRISC-mediated transposon silencing

Considering the previous studies, this model can be established. At first, Piwi-piRISC recognizes nascent transcript of transposon. Then, it recruits several factors, such as PNP, and induce heterochromatin formation and transcriptional repression. However, function of Mael is difficult to be interpreted from this model.



B

	Specific Factors	Core Subunit
BAP	Osa	Brm Bap111 Moira Snr1 Bap55 Bap60 Actin
PBAP	PB Bap170 SAYP	

Figure 3.7 SWI/SNF in *Drosophila melanogaster*

(A) Schematic showing mechanism of transcriptional activation by SWI/SNF. (B) Protein components of BAP and PBAP.

4. The aim of this study

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piRNA-mediated transposon silencing is a conserved mechanism that protects genome of reproductive cells. Especially, the silencing mechanism in the nucleus is important because it fundamentally prevents transposon expression before transcription. Therefore, elucidation of the mechanism is an urgent task.

In addition, although Mael is an essential factor for piRNA-mediated silencing and conserved among species, the function is largely unknown. Therefore, revealing the function of Mael in *Drosophila melanogaster* can be an important clue to clarify the function of Mael function in other species.

The model of Piwi-mediated silencing has been established based on KD analyses of Piwi and other cofactors. As depletion of these factors cause transcriptional activation of transposons and H3K9me3 demethylation, it has been concluded that Piwi and cofactors might recruit H3K9me3-specific histone methyltransferase and induce heterochromatin formation. Moreover, previous study shows that tethering of the cofactors enables inducing H3K9me3 and heterochromatin formation, so it is certain that the establishment of H3K9me3 plays an important

role in the Piwi-mediated transposon silencing (12, 22-25, 28-31*)*. However, it is controversial whether induction of H3K9me3 is sufficient for transcriptional silencing, because it is difficult in a conventional model to interpret the Mael depletion phenotype, which shows transcriptional activation of transposon but no decrease in H3K9me3 level (12).

For these motivations, in this study, I aim to clarify a role of Mael in Piwi-mediated transcriptional transposon silencing and to propose a novel model that is consistent with previous studies.

5. Materials and methods

5. Materials and methods

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。

6. Results

6. Results

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。

7. Discussion

7. Discussion

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。

8. Conclusion

8. Conclusion

Most of previous studies have been performed analyses on the factors whose depletion cause derepression of Piwi-dependent transposons, based on the idea that Piwi-piRISCs catalyze 'induction of repression machinery'. Consequently, little has been clarified about what the molecular entity of transcriptional machinery of the transposons that is the target of Piwi-piRISCs is. In this study, I revealed that most of Piwi-dependent transposons are regulated by Brm, SWI/SNF component. This is the first to show the character of Piwi-dependent transposon transcription.

Furthermore, analyses using artificial piRNA system elucidated Piwi silences Brm-dependent transcription by decreasing the binding of Brm to the target loci. Interestingly, the silencing was independent of Egg and occurred before H3K9me3 accumulation. In addition, tethering analyses using lambda N-boxB system showed that Mael has capability of downregulateing Brm-dependent transcription. From these results, I propose a model that Piwi-piRISC repress binding of SWI/SNF to the target transposon loci and repress its transcription via Mael, before H3K9me3 induction. The model is consistent to the other previous studies and provides a novel insight that Piwi-piRISCs

regulate transposons, not only by ‘induction of repression machinery’ but also by ‘inhibition of activation machinery’.

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