Function of The Mouse PIWI Proteins and Biogenesis of Their piRNAs in The Male Germline

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Cell Biology in the Graduate School
of Duke University

2009

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ABSTRACT

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Abstract

PIWI proteins belong to an evolutionary conserved protein family as the sister sub-family of ARGONAUTE (AGO) proteins. While AGO proteins are functionally well-characterized and shown to mediate small RNA-guided gene regulation, the function of PIWI proteins remain elusive. Here we pursued functional characterization of PIWI proteins by studying MILI and MIWI, two PIWI proteins in the mouse.

We first show that both MIWI and MILI co-immunoprecipitate with a novel class of non-coding small RNAs from the post-natal mouse testis extract, which are named Piwi-interacting RNAs (piRNAs). Our cloning efforts identified thousands of different piRNA sequences, mostly derived from intergenic regions. Interestingly, both MILI and MIWI piRNAs correspond to the same regions on the genome and differ primarily in length. We propose piRNAs in the adult testis are produced by the processing of long, single stranded RNA precursors, based on the observation that they originate in clusters from a number of sites on the genome in a head-to-tail homology. In support, we bioinformatically predicted their putative promoters, and yeast-one-hybrid analysis on two such regions found out that they interact with Krueppel C2H2 type zinc finger transcription factors. We did not observe the features of the "ping-pong" mechanism in their biogenesis: Both MILI and MIWI piRNAs are biased for 5` Uracil without an

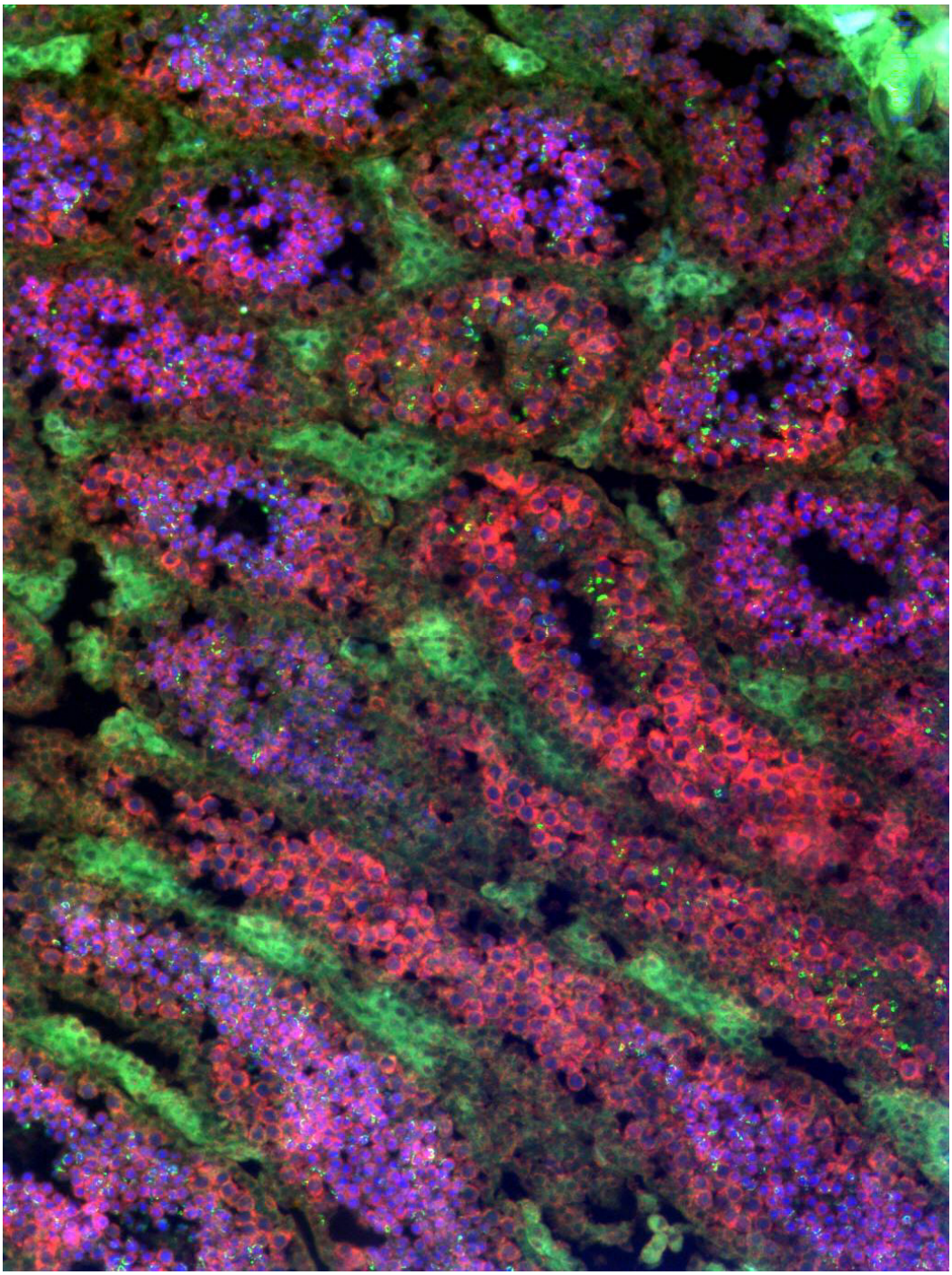
Adenine bias on the 10th nucleotide position, and do not significantly consist of sequences complementary to each other along their first 10nt. Moreover, MILI piRNAs are not down-regulated in *Miwi*$^{-/-}$ testis. These results indicate that the adult testicular piRNAs are produced independent of the "ping-pong" mechanism.

Although piRNAs are highly complex, PAGE and *in situ* analyses showed that they are germ cell-specific with a predominant expression in spermatocytes and round spermatids, suggestive of a meiotic function. Correspondingly, we found that *Miwi*$^{-/-}$; *Mili*$^{-/-}$ mice undergo only male infertility with terminal spermatogenic arrest during meiosis. piRNAs show a nucleo-cytoplasmic distribution, with enrichment in the "chromatoid" and "dense" bodies, two male germ cell-specific structures. The "dense body" has been implicated in synapsis and in the heterochromatinization of the sex chromosomes during male meiosis, a process known as meiotic sex chromosome inactivation (MSCI). Our histological analysis on *Miwi*$^{-/-}$; *Mili*$^{-/-}$ testes showed that, while the overall synapsis is not affected, the sex chromosomes retain the euchromatin marker acetyl-H4K16 and lacks the heterochromatin marker H3K9-dimethyl. These observations indicate that murine PIWI proteins are necessary for MSCI. Moreover, we identified piRNA production from the X chromosome before MSCI, and propose PIWI proteins utilize piRNAs to target and silence unpaired chromosomal regions during meiosis.

Dedicated to the loving memory of

my grandfather Nurettin Orhan.

May he rest in peace. . .



Cover Figure: *Adult mouse testis cross-section immunostained for round spermatid marker CREM-I shown in blue, spermatid marker TRA-54 in punctate green, and a MILI-associated piRNA in red.*

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List of Abbreviations

dpp : days post partum

dpc : days post coitum

nt : nucleotide

GSC : Germline Stem Cell

PGC : Primordial Germ Cell

Kb : Kilobase

KDa : Kilodalton

piRNA: Piwi-interacting RNA

rapiRNA: repeat-associated piRNA

miRNA: microRNA

siRNA : small interfering RNA

snRNA: small nuclear RNA

MSCI : Meiotic Sex Chromosome Inactivation

MSUD : Meiotic Silencing of Unpaired DNA

MSUC : Meiotic Silencing of Unpaired Chromosome

AUB : AUBERGINE

AGO : ARGONAUTE

ZNF : Zinc Finger

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Presented here is a journey I have taken in the past several years. With the hope that the destination reached will contribute at least a drop to the ocean of "knowledge", I would like to thank those who helped me start this journey and those who joined me along the way.

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1. General Introduction

Reproduction is the ability of a life form to produce similar individuals of its own, whereby it can preserve and further spread its existence. However, the continuity of a life form not only depends on its ability to reproduce, but also to adapt the changes in its environment through the formation of a genetic variety. Sexual reproduction has emerged during evolution as a means of increasing the genetic variety by combining the genetic materials of two individuals for the formation of a progeny with a new genetic arrangement. At the center of this process are two mechanisms: reductional division and conjugation.

In sexually reproducing animals, specialized cells called "germline" undergo reductional division during gametogenesis to decrease their genomic content by half. During this process, the genetic material is rearranged by means of independent assortment and recombination, and modified with the rest of the cell to be competent for the subsequent conjugation. The resultant cell type, gamete, conjugates with the gamete of the other sex during fertilization, fulfilling the appropriate amount of genomic content and forming a new individual with a genetic composition different than each parent. Each step in these events has to be under strict regulation since any error directly affects the next generation. What regulates the integrity, modification, and movements of the genetic material during gametogenesis? What are the autonomous

and non-autonomous mechanisms regulating gametogenesis? What is the gene regulatory network in this process and how are these genes regulated? In mammalian males, gametogenesis is maintained by a stem cell population throughout the reproductive life span. What is the molecular machinery that regulates the maintenance and differentiation of these stem cells?

PIWI proteins have been shown to be necessary for gametogenesis, some being at the stem cell level. They belong to the same protein family as ARGONAUTE proteins, which have been shown to mediate small RNA-guided gene regulation. Therefore, PIWI proteins lie at the intersection of stem cells, gametogenesis, small RNAs and gene regulation, and thus their functional characterization is crucial in elucidating the link between these mechanisms. In this dissertation, I have studied MILI and MIWI to shed light on the function of PIWI proteins in the mouse. In this chapter, I will present a general knowledge regarding spermatogenesis, small RNA-induced gene regulation, and PIWI/ARGONAUTE proteins forming the context of this dissertation.

1.1 Spermatogenesis in the Mouse

Mammalian spermatogenesis is a stem cell-driven process and occurs in the seminiferous tubules of testis in a peripheral-to-luminal fashion (***Figure 1A***) (Lin 1997)**.** The stem cells are a sub-population of spermatogonia that form the basal layer of germ

cells adjacent to the basal lamina, which is an epithelial extracellular matrix separating outside of the tubule from the inside. Differentiation of germ cells from this stem cell and progenitor population occurs towards the lumen, forming layers of increasingly more differentiated germ cells. Spermatozoa are the ultimately differentiated cell type of this lineage and are located at the center of the lumen. Hence, germ cells can be discriminated based on their relative position to the basal lamina in a transverse section of a seminiferous tubule, as well as their morphological characters and cell-specific markers. In addition to the germ cells, seminiferous tubules encase a somatic cell type, called Sertoli. These large cells are always in contact with the germ cells and function in nurturing them.

Spermatogenesis in the mouse is a highly temporally regulated process and occurs during the reproductive life-span of males in cycles by virtue of the stem cell population present (***Figure 1B***) (Bellve, Millette et al. 1977; Goetz, Chandley et al. 1984). The first wave of spermatogenesis starts at 5-6dpp with the stem cells A_1 spermatogonia mitotically dividing to produce other spermatogonia types. After multiple rounds of mitotic amplification, type B Spermatogonia form, which then divide once more to produce the primary spermatocytes by 9dpp. Primary spermatocytes are distinguished as the cell population which has lost their contact with the basal lamina. The switch from mitosis to meiosis occurs as the primary spermatocytes enter the first meiotic

division around 10dpp. By 12dpp, pachytene spermatocytes start emerging. Round, haploid spermatids form around 20dpp; which then have to undergo a multi-step differentiation process called spermiogenesis to yield the spermatozoa of this first cycle of spermatogenesis around 35dpp. This temporally defined progression of spermatogenesis offers a unique opportunity for developmental and biochemical characterization of different stages of spermatogenesis. Depending on the developmental time point of the testis samples, one can know what types of cells are under investigation.

Figure 1: Spermatogenesis in the mouse

A) A wedged cartoon cross-section of a seminiferious tubule. Germ cells can be discriminated based on their morphology and relative location to the basal lamina in addition to cell-specific markers.

B) A schematic diagram depicting the highly temporally regulated spermatogenesis in the mouse. dpp: days post partum.

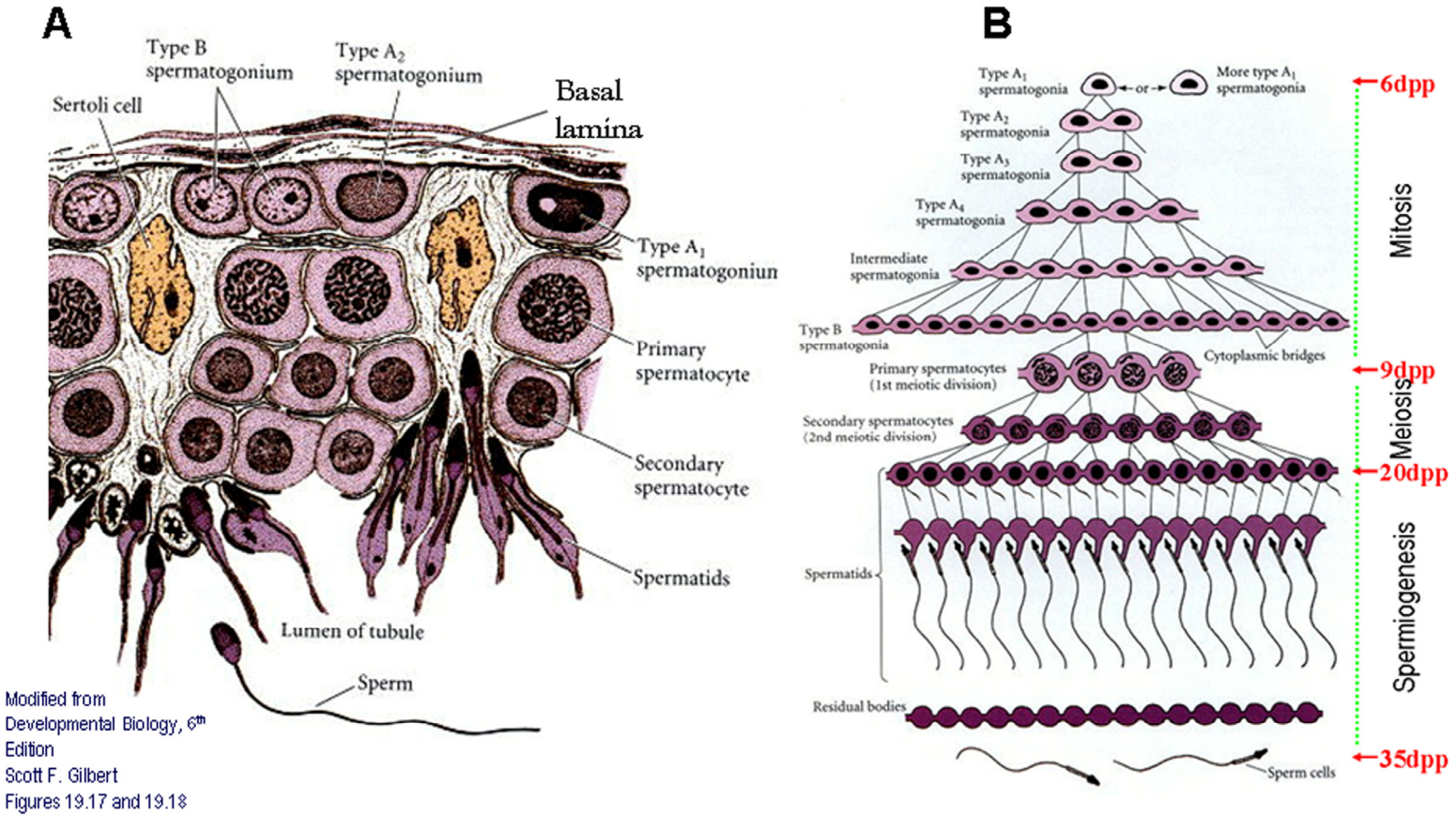

Figure 1: Spermatogenesis in the mouse

1.2 Small RNA-Mediated Gene Silencing

Small non-coding RNAs of 18-32 **n**ucleo**t**ides (**nt**) in length have emerged as potent regulators of gene expression in the last decade (Fire, Xu et al. 1998; Matzke and Birchler 2005). Studies on the function of small RNAs demonstrated their pivotal roles in the regulation of developmental timing (Lee, Feinbaum et al. 1993), cell proliferation and apoptosis (Brennecke, Hipfner et al. 2003; Johnson, Grosshans et al. 2005), morphogenesis (Johnston and Hobert 2003; Palatnik, Allen et al. 2003; Chang, Johnston et al. 2004; Giraldez, Cinalli et al. 2005), transposon silencing (Sijen and Plasterk 2003), genomic DNA elimination (Mochizuki, Fine et al. 2002; Mochizuki and Gorovsky 2004) and viral defense (McManus 2004). These diverse functions are achieved via an evolutionarily conserved cellular mechanism, called **RNA**-**i**nduced gene silencing (**RNAi**) (Napoli, Lemieux et al. 1990; Lindbo and Dougherty 1992; Fire, Xu et al. 1998). First shown to exert its function at the post-transcriptional level by mRNA degradation or translational repression, now it is known that, RNAi is also involved in transcriptional gene regulation by altering the epigenetic state of the targeted chromatin loci (Mette, Aufsatz et al. 2000; Volpe, Kidner et al. 2002; Stevenson and Jarvis 2003; Kawasaki and Taira 2004; Motamedi, Verdel et al. 2004; Pal-Bhadra, Leibovitch et al. 2004; Verdel, Jia et al. 2004; Zilberman, Cao et al. 2004; Matzke and Birchler 2005).

The first finding related to RNAi was the observation of gene silencing in plants, fungi and nematodes in the early 1990s. In *Petunia* (Napoli, Lemieux et al. 1990) and *Neurospora crassa* (Romano and Macino 1992), introduction of a transgene unexpectedly led to the reduction of the RNA levels of the endogenous genes that shared homology with the transgene. This then so-called "co-suppression" or "quelling" effect was later shown to be the consequence of the RNA product itself of the transgene as the same effect was also achieved via the direct introduction of the RNA alone (Lindbo and Dougherty 1992). However, the properties of this RNA trigger was not unraveled until the studies in *Caenorhabditis elegans*, where it was shown that double stranded RNAs were the causing agents of this transgene-induced gene silencing (Fire, Xu et al. 1998). Further mechanistic studies led to the identification of small RNAs as the products of these double stranded RNA triggers. With the discovery of endogenous small regulatory RNAs (Lee, Feinbaum et al. 1993; Reinhart, Slack et al. 2000; Lagos-Quintana, Rauhut et al. 2001) and RNAi in protozoans (Djikeng, Shi et al. 2001; Mochizuki, Fine et al. 2002; Mochizuki and Gorovsky 2004; Mochizuki and Gorovsky 2004) as well as metazoans, what was first thought to be an artifact of gene manipulation turned out to be an evolutionary conserved gene regulation mechanism.

One such endogenous small regulatory RNA was identified in 1993 during a study on the genes controlling developmental timing in *C. elegans* (Lee, Feinbaum et al.

1993). These so-called "heterochronic" genes regulate the timely events of post-embryonic development in *C. elegans* (Ambros 1989), which consists of four larval cycles, classified from L1 to L4. The perturbation of their functions results in precocious or retarded development (Ambros and Horvitz 1984). For instance, LIN-14 protein level is high during the L1 stage but down-regulated afterwards (Ambros and Horvitz 1987). While the loss-of-function mutations skip the L1 stage and execute the later stages prematurely, gain-of-function mutations lead to a retarded phenotype. Interestingly, *lin-14* is negatively regulated by another heterochronic gene, *lin-4*, whose perturbation results in a similar phenotype as *lin-14* gain-of-function. Mapping the *lin-14* gain-of-function mutations (Wightman, Burglin et al. 1991) followed by gene fusion experiments (Wightman, Ha et al. 1993) showed that the 3` **u**n**t**ranslated **r**egion (**UTR**) of *lin-14* is necessary for the antagonistic effect of *lin-4* on *lin-14*. Moreover, *lin-14* RNA level remains constant during all the larval stages (Wightman, Ha et al. 1993) despite the fact that the protein level decreases after the L1 stage (Ruvkun and Giusto 1989). These observations led to the conclusion of translational-control of *lin-14* by *lin-4*. However, the nature of this interaction and the molecular identity of *lin-4* were not revealed until the cloning and functional characterization efforts (Lee, Feinbaum et al. 1993). Lee *et al* (1993) showed that merely a particular small portion of the *lin-4* locus was able to rescue the *lin-4* null mutant and it corresponded to an intron of another gene; and thus, it was not competent for a protein product. Additionally, mutagenesis of the predicted open

reading frames in *lin-4* did not diminish its rescuing ability. The *lin-4* orthologs from 3 other *Caenorhabditis* species were able to rescue the null mutant of *C. elegans* as well, however did not comprise any conserved open reading frames. Concluding that *lin-4* did not code for a protein, Lee *et al* searched for any putative RNA product. They were able to identify two small transcripts of 22 and 61 nucleotides in length, the larger one having a hairpin secondary structure. The comparison of their sequences indicated the small species to be produced from the stem of the larger transcript. Most interestingly, alignment of *lin-14* RNA with this small transcript showed that the 3` UTR of *lin-14* RNA contained seven regions that were partially complementary to the small transcript. This observation suggested that the negative regulation of *lin-14* by *lin-4* resulted from an antisense RNA-RNA interaction. Subsequent analyses in animals and plants showed the existence and evolutionary conservation of the same type of 21-23nt long small RNAs, whereby the macro world of **mi**croRNAs (**mi**RNAs) was born (Lagos-Quintana, Rauhut et al. 2001; Reinhart, Weinstein et al. 2002).

Mechanistic studies on the biogenesis of miRNAs proved the large transcript, first observed by Lee *et al*, to be the precursor processed by a cytosolic RNaseIII type of enzyme, **DICER** (**DCR**), to form the mature miRNA (Bernstein, Caudy et al. 2001; Grishok, Pasquinelli et al. 2001; Hutvagner, McLachlan et al. 2001), while, another RNaseIII enzyme, DROSHA, processes the primary transcripts to form the precursors in

the nucleus (Lee, Ahn et al. 2003; Denli, Tops et al. 2004). Functional studies demonstrated that mature miRNA is loaded onto the cellular gene targeting machinery **R**NA-**I**nduced **S**ilencing **C**omplex (**RISC**) (Hammond, Bernstein et al. 2000; Pham, Pellino et al. 2004). The resultant miRISC utilizes the incorporated miRNA as a guide to trace mRNAs with complementary sequences to the miRNA. In animals, miRNAs mostly show imperfect complementarity with their targeted mRNAs. This partial complementarity leads to translational repression of the target with a yet-to-be identified mechanism (***Figure 2***) (Tang 2005).

Another group of small non-coding RNAs, siRNAs, are generated from long double stranded RNA precursors that are not uniform in size. Such double stranded RNA fragments can be administered to the cell exogenously or produced endogenously from repeat-associated regions in the genome via bi-directional transcription. As with miRNA precursors, these double stranded RNAs are processed by DCR to produce double stranded small RNAs. One, and occasionally each, of the small RNA strands is incorporated into RISC. The resultant siRISC targets and endonucleolytically cleaves the mRNAs that are fully complementary to this "guide" strand (***Figure 2***) (Tang 2005).

In addition to the biogenetic and functional similarities, miRNAs and siRNAs are structurally similar to each other as well. As DCR products, both of them are 21-23nt

long and carry the characteristic features of RNaseIII processing: The unwound double stranded product has 5` phosphates and 3` hydroxyl groups with 2nt overhangs at its 3` ends. These signature motifs are necessary for the small RNAs to enter the RNAi pathway, distinguishing them from fragments of degraded RNA (Tomari, Matranga et al. 2004). Interestingly most of mi/siRNAs start with Uracil, due to the bias of DCR activity towards Uracils.

Although miRNAs and siRNAs are preferentially generated by two different DCR homologs in the fly, there is only one DCR protein responsible for the biogenesis of both in mammals. Interestingly, miRNAs that are fully complementary to their targets can act like siRNAs and result in target cleavage (Llave, Xie et al. 2002). Likewise, siRNAs with partial complementarity cause translational repression of the targets, similar to miRNAs (Doench, Petersen et al. 2003). These observations indicate that the miRNA and siRNA pathways are interchangeable, with the degree of complementarity of the targeted mRNA being the major underlying factor affecting its fate. Therefore, separate classification of siRNAs and miRNAs is mostly a reference to the subtle differences in their precursors.

Besides their control at the post-transcriptional level, the involvement of small RNAs in gene silencing at the transcriptional level has been well documented (Mette,

Aufsatz et al. 2000; Volpe, Kidner et al. 2002; Stevenson and Jarvis 2003; Kawasaki and Taira 2004; Motamedi, Verdel et al. 2004; Pal-Bhadra, Leibovitch et al. 2004; Verdel, Jia et al. 2004; Matzke and Birchler 2005). For instance, in the fission yeast, DCR-dependent small RNAs guide the effector "**R**NA-**i**nduced **t**ranscriptional **s**ilencing" (**RITS**) complex to the centromeric repeats. Interaction of the RITS complex with the epigenetic machinery results in the modification of the histones in the targeted loci, and ultimately leads to their heterochromatinization (Verdel, Jia et al. 2004). Similarly, double-stranded RNAs with cognate sequences to promoter regions can induce the transcriptional inactivation of the corresponding genes in plants, after being endogenously processed into ~23nt small RNAs (Mette, Aufsatz et al. 2000). This silencing is correlated with the DNA methylation of the targeted promoter regions. Interestingly, *Tetrahymena thermophilia* utilizes ~28nt small RNAs, termed **sca**n**n**RNAs (**scn**RNAs) to target and remove the complementary genomic sequences in its newly formed vegetative macronucleus following sexual reproduction. Hence, this protozoon adopted the ultimate approach to silence redundant genomic sequences simply by eliminating them with the guidance of small RNAs (Mochizuki, Fine et al. 2002; Mochizuki and Gorovsky 2004).

Figure 2: Schematic diagram of RNA-induced post-transcriptional gene silencing

Double stranded RNAs are processed by DICER to smaller fragments of approximately 21nt, which carry 5` phosphates and 3` hydroxyl groups, and comprise 2nt overhangs on their 3` ends. Hairpin precursors of miRNAs are incised by DICER at specific scissile bonds, producing miRNAs with particular sequences; whereas long double stranded RNAs are processed quasi-randomly. After unwinding, one of the strands is incorporated into RISC to guide it in targeting mRNAs. While partially annealed guide RNAs cause translational repression, full complementarity leads to target cleavage. In animals, miRNAs usually anneal partially to their targets.

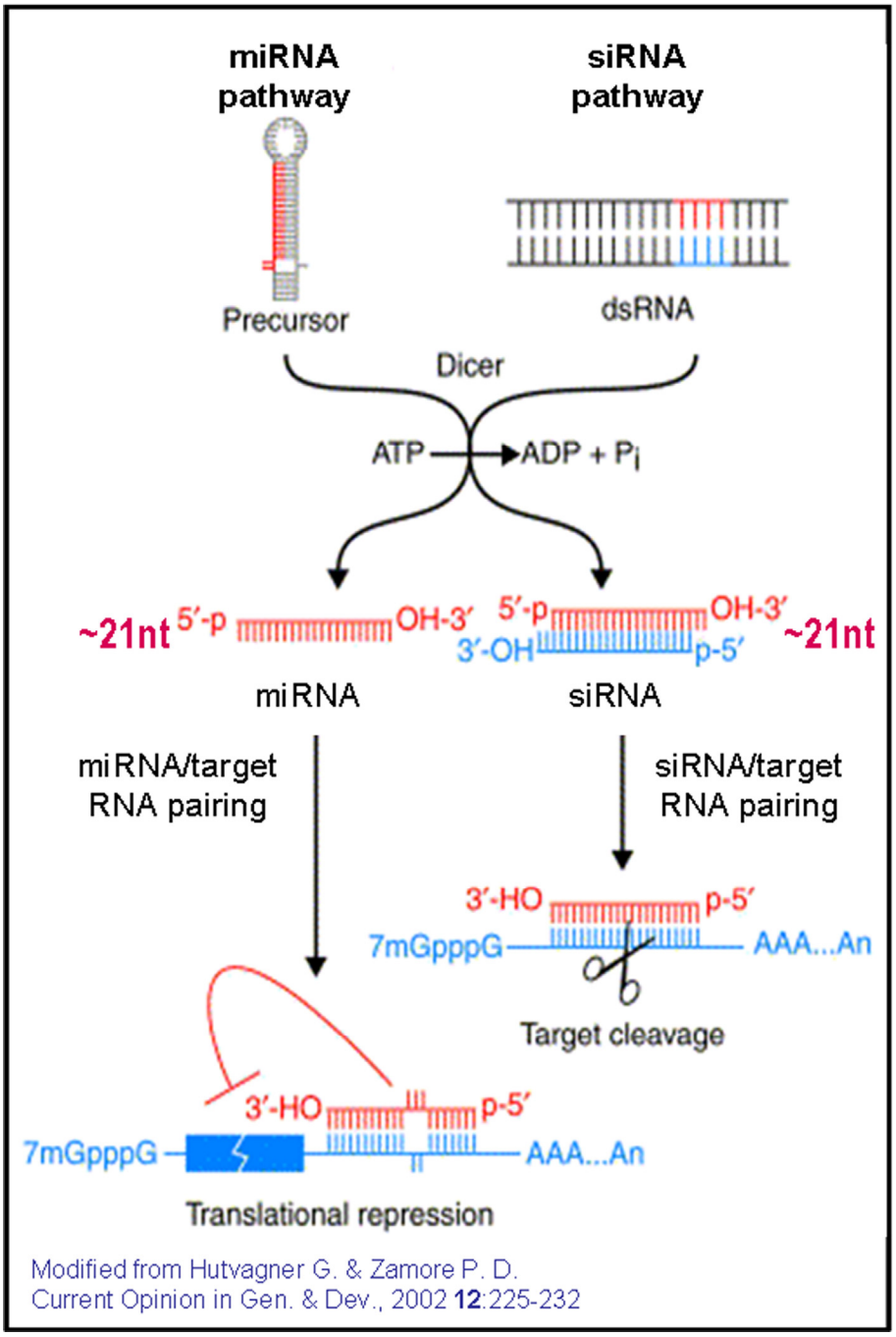

Figure 2: Schematic diagram of RNA-induced post-transcriptional gene silencing

1.3 PPD Family of Proteins

1.3.1 ARGONAUTE Proteins

Genetic and biochemical analyses have revealed **A**R**GO**NAUTE (**AGO**) sub-family proteins of the **P**iwi-**P**az **D**omain (**PPD**) family (a.k.a Argonaute/Piwi protein family) to be the core enzymes of the RNAi effector complexes, RISCs (Hammond, Boettcher et al. 2001; Liu, Carmell et al. 2004; Meister, Landthaler et al. 2004; Pillai, Artus et al. 2004; Peters and Meister 2007). PPD family is a highly conserved group of proteins present in prokaryotes and eukaryotes (Hall 2005; Cerutti and Casas-Mollano 2006). It was first discovered with the identification of *piwi* in Drosophila during a mutational screen for the genes affecting germline stem cell maintenance (Lin and Spradling 1997; Cox, Chao et al. 1998). Phylogenetic analysis of this protein family deciphers the divergence of two sub-families in eukaryotes: Ago and Piwi (***Figure 3A***). In the mouse, there are 5 members from the Ago and 3 members from the Piwi subfamily (Carmell, Xuan et al. 2002).

PPD members comprise two signature motifs termed **PAZ** (**P**iwi **A**rgonaute **Z**wille) and PIWI domains in addition to the less conserved N-Terminal and **Mid**dle (**Mid**) domains (***Figure 3B***) (Carmell, Xuan et al. 2002). Structural studies demonstrated that PAZ domain comprises an **O**ligonucleotide/**O**ligosaccharide-**b**inding (**OB**) fold and interacts with the 3` end of small RNAs, while PIWI domain resembles RNase H

enzymes (Lingel, Simon et al. 2003; Song, Liu et al. 2003; Yan, Yan et al. 2003; Ma, Ye et al. 2004; Parker, Roe et al. 2004; Song, Smith et al. 2004; Lingel and Sattler 2005). Mutating the cleft in PAZ domain, where the 3` end of the small RNA binds, highly reduces but does not abolish the small RNA interaction (Parker, Roe et al. 2004). It was later shown that the 5` end of the small RNA binds to a groove in between PIWI and Middle domains, called "PIWI fold" (Parker, Roe et al. 2004; Ma, Yuan et al. 2005; Parker, Roe et al. 2005). These structural analyses implied two aspartate and one glutamate residues in PIWI domain to form the catalytic site of AGO proteins (Parker, Roe et al. 2004; Song, Smith et al. 2004; Miyoshi, Tsukumo et al. 2005). Similarly, RNase H enzymes have three carboxylate groups in their active sites contributed by aspartate or glutamate residues (Chapados, Chai et al. 2001). Homology comparison of AGO proteins demonstrated that these three residues, termed the "catalytic triad", are also conserved among most AGO proteins (Parker, Roe et al. 2004; Song, Smith et al. 2004). Mutating these residues abolishes the endonucleolytic (a.k.a "slicer") activity of RISC, while retains its small RNA binding capacity (Liu, Carmell et al. 2004). On the other hand, some AGO proteins are incapable of cleaving targeted mRNAs despite having these three residues. These observations indicate that the catalytic triad is necessary but not sufficient for the cleavage. For example, human AGO2 can cleave targeted mRNAs *in vivo* provided that the targeting occurs via a fully complementary small RNA guide; while the other human AGO homologs, which also possess the catalytic triad, do not

(Liu, Carmell et al. 2004; Meister, Landthaler et al. 2004). Such non-cleaving AGO proteins have been proposed to cause translational repression of the targets instead. Therefore in addition to the degree of complementarity between the small RNA and the target mRNA, the type of the AGO protein utilized and possibly the protein partners involved seem to determine the fate of the targeted mRNA. The effect in either case is negative, ultimately leading to a decreased gene expression, and hence the name "silencing".

Figure 3: PPD Family of Proteins

A) The phylogenetic tree of PPD family of proteins deciphers the divergence of two sub-families: Ago, resembling the ***A****rabidopsis* ***t****haliana* (**At**) ARGONAUTE 1 and Piwi, resembling the ***D****rosophila* ***m****elanogaster* (***Dm***) Piwi. PIWI proteins are not found in fungi and plants; and all organisms expressing PIWI proteins show sexual reproduction.

B) Schematic representation of the RNA interactions of AGO proteins is shown. 3` end of the guiding small RNA binds to a pocket in the PAZ domain, while the 5` end associates with the groove in between the Middle and Piwi domains. Target mRNA positions in the longitudinal groove between the PAZ and Piwi domains. RNA endonucleolytic (Slicer) residues reside in the Piwi domain.

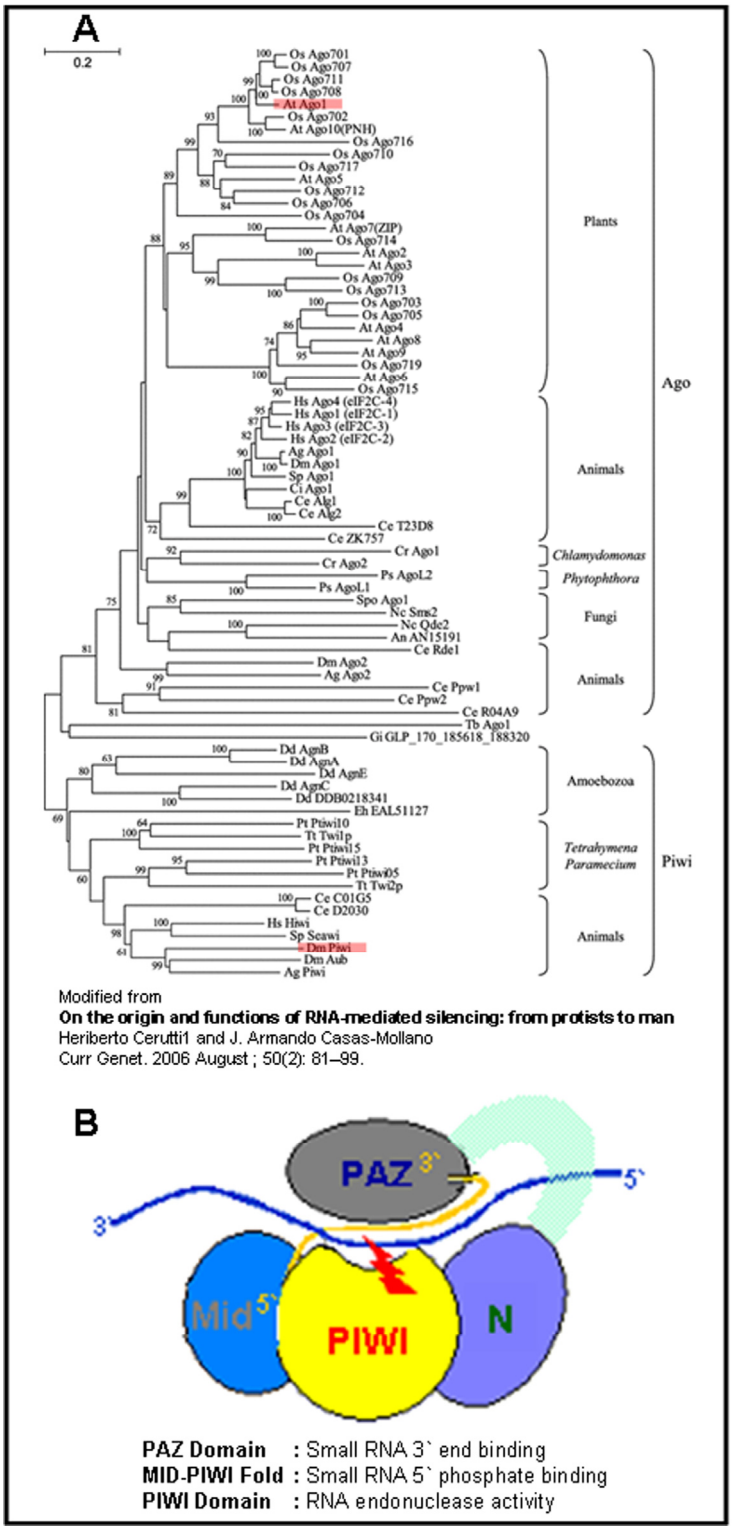

Figure 3: PPD Family of Proteins

The content of the Section 1.3.2 has been published in
Piwi-interacting RNAs (piRNAs), by Ergin Beyret and Haifan Lin
MicroRNAs: From Basic Science to Disease Biology, edited by Krishnarao Appasani
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1.3.2 PIWI Proteins

1.3.2.1 Biological Functions of PIWI Proteins in *Drosophila*

Drosophila genome contains three *piwi* homologs: *piwi*, *aubergine (aub)* and *argonaute3 (ago3)*. Although named as "Argonaute", *Drosophila ago3* belongs to the *Piwi* sub-family of the PPD family due to its closer homology to *Drosophila piwi* rather than *Arabidopsis AGO1* (Seto, Kingston et al. 2007), and thus its nomenclature is misleading.

PIWI subfamily proteins are predominantly detected in gonads and largely associated with stem cell maintenance. As anticipated, they are necessary for germline development and the progression of gametogenesis, with their loss of function leading to infertility. For instance, *piwi* has been shown to be necessary for the maintenance of the male and female **g**ermline **s**tem **c**ells (**GSC**) in *Drosophila* (Lin and Spradling 1997; Cox, Chao et al. 1998). In the ovary of *piwi* mutant flies, GSCs enter oogenesis without self-renewal (Cox, Chao et al. 1998), ultimately leading to flies devoid of germ cells. Additionally, oogenesis in the mutants is often aberrant (Lin and Spradling 1997), implicating a post-GSC function of *piwi* in oogenesis as well. Likewise *ago3* is necessary

for the maintenance of male germline stem cells in the fly, and *ago3* mutant females lay fewer eggs (Li, Vagin et al. 2009).

Cytological analyses showed that Piwi is present in the nuclei of germ cells, including GSCs, and also in the somatic niche cells of the adult ovary in addition to the **p**rimordial **g**erm **c**ells (**PGC**s) (Cox, Chao et al. 2000). Interestingly, the function of *piwi* for GSC maintenance in the ovary resides in the somatic niche cells: removing *piwi* from the germline does not affect GSC self-renewal (Cox, Chao et al. 1998), yet specific expression of *piwi* in the somatic niche cells of *piwi* mutants rescues the GSC phenotype (Szakmary, Cox et al. 2005). Furthermore, overexpressing *piwi* in the somatic niche cells expends the GSC niche and leads to a significant increase in the number of the GSCs (Cox, Chao et al. 2000). On the other hand, germline expression of *piwi* promotes the GSC division (Cox, Chao et al. 2000), ensures normal progression of subsequent steps of oogenesis (Lin and Spradling 1997), and supplies maternal PIWI for future embryogenesis (Cox, Chao et al. 1998). Subsequently, this maternal supply becomes highly enriched in the germplasm in early *Drosophila* embryos, as a component of a germline-specific organelle called "polar body" (Megosh, Cox et al. 2006). Depleting maternal Piwi leads to failure in establishing PGCs, yet doubling and tripling the dosage of *piwi* proportionally increases the number of PGCs (Megosh, Cox et al. 2006). These results indicate that *piwi* is necessary for germline determination and maintenance, and

promotes its division rate during gametogenesis. Likewise, although no abnormality in gametogenesis has been reported for *aub* mutants, maternal supply of Aub has been shown to be necessary for the formation of the PGCs (Harris and Macdonald 2001).

In addition to these well-characterized germline functions, Piwi and Ago3 may also have crucial functions for the soma. For instance, embryos without the maternal supply of Piwi or Ago3 are lethal with full penetrance and display various mitotic and morphogenetic defects (Cox, Chao et al. 1998; Li, Vagin et al. 2009). However, currently it is not clear if these embryonic phenotypes are merely a manifestation of impaired oogenesis that produces defective eggs in the absence of these PIWI proteins.

1.3.2.2 Biological Functions of PIWI Proteins in the Mouse

The mouse genome has three members of the *Piwi* sub-family: ***Miwi*** (**M**ouse P**iwi**), ***Mili*** (**M**ouse P**i**wi-**li**ke), and *Miwi2* (Peters and Meister 2007). They all are predominantly expressed in the male germline. Among these, only *Mili* expression is detected in the female germline as well (Kuramochi-Miyagawa, Kimura et al. 2001; Aravin, Sachidanandam et al. 2008; Watanabe, Totoki et al. 2008). However, loss of MILI, MIWI or MIWI2 causes only spermatogenic arrest with no oogenic or maternally derived defects (Deng and Lin 2002; Kuramochi-Miyagawa, Kimura et al. 2004; Carmell, Girard et al. 2007). The spermatogenic arrest in either case is followed by extensive

apoptosis of the established germline, including the GSCs. While knocking out *Miwi* causes post-meiotic arrest of the germline, *Mili*$^{-/-}$ or *Miwi2*$^{-/-}$ mice show spermatogenic arrest during early prophase I of meiosis. Additionally, the maintenance and differentiation of the GSCs have been proposed to be defective in *Mili*$^{-/-}$ (Unhavaithaya, Hao et al. 2008) and *Miwi2*$^{-/-}$ mice (Carmell, Girard et al. 2007). Despite these severe phenotypes in the homozygous knock-outs, the heterozygotes have the full complement of germ cells and phenotypically act like wild-types.

The spermatogenic arrests described above appear to be autonomous defects unlike the *piwi* mutant in the fly, since the murine PIWI homologs are specifically detected in the germline. Expression of MILI starts in the primordial gonad at 12,5dpc which correlates with the PGC immigration (Kuramochi-Miyagawa, Kimura et al. 2001). The expression increases in the adult male gonad and localizes predominantly to the cytoplasm of spermatogonia and spermatocytes. In addition to this homogeneous cytoplasmic distribution, MILI localizes to the "nuage" of spermatocytes (Unhavaithaya, Hao et al. 2008) and round spermatids (Aravin, Sachidanandam et al. 2008; Wang, Saxe et al. 2009), therein called the "chromatoid body"- a functionally elusive, germline-specific perinuclear structure. Interestingly, expression of *Mili* overlaps with that of its close paralog *Miwi* following entry of the germline into meiosis (***Figure 4***) (Kuramochi-Miyagawa, Kimura et al. 2001; Deng and Lin 2002). Like *Mili*, *Miwi* is expressed in

spermatocytes and round spermatids, and predominantly found in the cytoplasm with enrichment in the nuage/chromatoid body. The expression of the third homolog, *Miwi2*, starts at 15.5dpc in the PGCs and becomes undetectable at 4dpp (Aravin, Sachidanandam et al. 2008; Kuramochi-Miyagawa, Watanabe et al. 2008). Although it was initially thought to be expressed in Sertoli cells (Carmell, Girard et al. 2007), subsequent studies showed this not to be the case (Aravin, Sachidanandam et al. 2008). Furthermore, *Miwi2*$^{-/-}$ testes transplanted with wild-type spermatogonia can fully undergo spermatogenesis (Carmell, Girard et al. 2007), further indicating that the germline effect of MIWI2 is cell autonomous. The germline autonomy of the murine PIWI proteins suggests that there is no true ortholog of the fly *piwi* in the mouse, pointing to functional differences between the two organisms.

Figure 4: Expression patterns, and homology of *Mili* and *Miwi*

A) *Mili* is expressed in the germline from the germline stem cell stage until the formation of round haploid spermatids in the adult testis. *Miwi* expression starts during pachynema, overlapping with that of *Mili*.

B) *Mili* has a moderate homology with its mouse paralog *Miwi*.

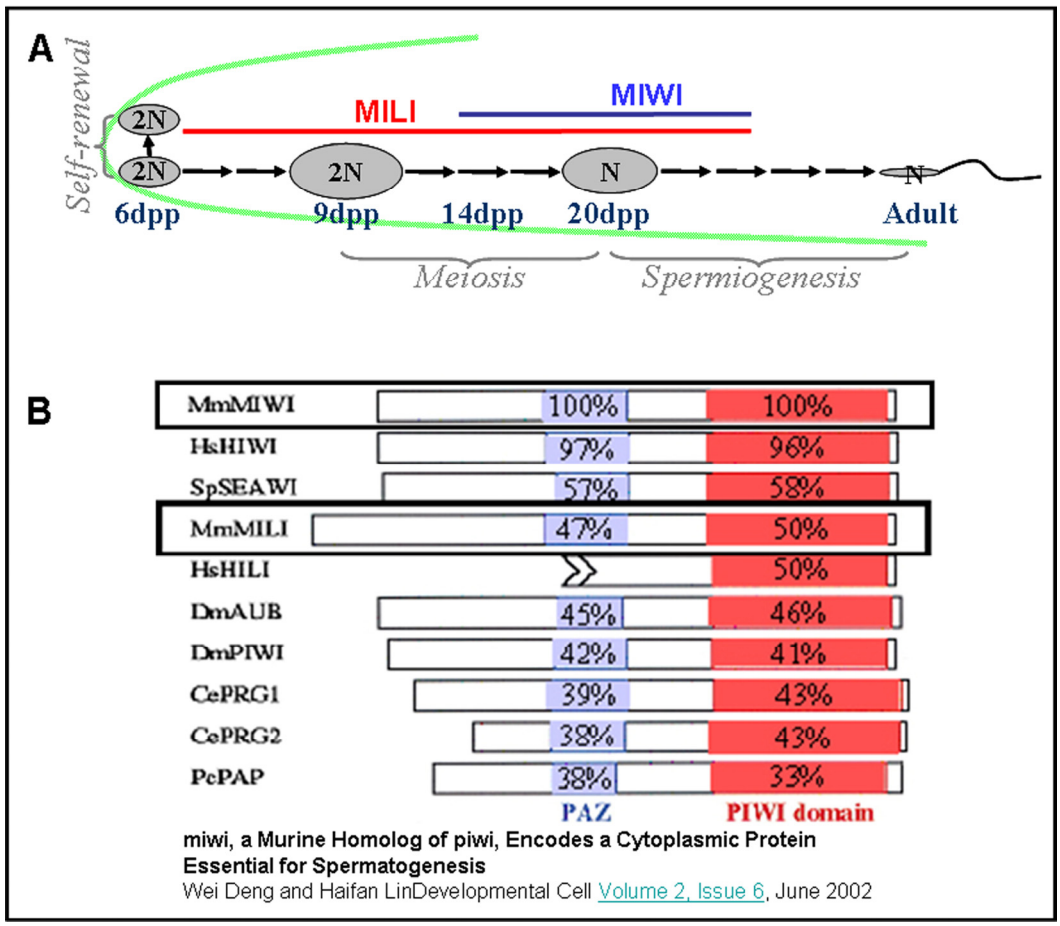

Figure 4: Expression patterns and homology of *Mili* and *Miwi*

1.3.2.3 Biological Functions of PIWI Proteins in Other Organisms

The functions of PIWI proteins in other organisms have mostly been characterized in the germline as well. In *C. elegans*, RNAi depletion of two *piwi* homologs, *prg-1* and *prg-2* results in reduced germline mitotic proliferation zone, which is the *C. elegans* equivalent of GSCs (Cox, Chao et al. 1998). Studies on *Piwi* homologs in other animals such as jellyfish, zebrafish, and frogs likewise reflect their involvement in an evolutionary conserved germline function, some potentially to be at the stem cell level (Tan, Lee et al. 2002; Seipel, Yanze et al. 2004; Houwing, Berezikov et al. 2008; Wilczynska, Minshall et al. 2009). In human, there is no report of loss-of-function phenotypes for the PIWI proteins. However, in regard of the dosage effect, the human homologs *HIWI* (Qiao, Zeeman et al. 2002) and *HILI* (Lee, Schutte et al. 2006) have been shown to be correlated with testicular seminomas, although it is not clear whether the elevated levels of these proteins is the cause or the result of the oncogenesis.

Somatic function of PIWI proteins in higher organisms have been proposed by expression analyses in several somatic tissues (Sharma, Nelson et al. 2001; Lee, Schutte et al. 2006) and the correlation between *HIWI* overexpression and gastric malignancies (Liu, Sun et al. 2006). Little is known beyond these studies.

1.3.2.4 Biochemical Functions of PIWI Proteins

The biochemical function(s) of PIWI proteins have recently started unraveling. Genes that are near heterochromatin are usually inactivated due to heterochromatin spreading. This so-called "positional effect variegation" is suppressed in the absence of Piwi in the fly. This deficiency in silencing is correlated with the lack of heterochromatinization of the genes as assessed with decreased levels of heterochromatic modifications of histones and epigenetic repressor proteins such as **H**eterochromatin **P**rotein 1 (**HP**1). Furthermore, *Drosophila* Piwi associates with chromatin and directly interacts with HP1 (Brower-Toland, Findley et al. 2007). These observations indicate that the fly Piwi is involved in epigenetic gene silencing. In addition to the transcriptional level, PIWI proteins might be involved in gene silencing at the post-transcriptional level since *Drosophila* Piwi (Saito, Nishida et al. 2006) and its rat homologue RIWI (Lau, Seto et al. 2006) have the "slicer" activity *in vitro*. Moreover, mouse PIWI homologues, MIWI (Grivna, Pyhtila et al. 2006) and MILI (Unhavaithaya, Hao et al. 2008) have been proposed to interact with the translational machinery and mRNAs. As they have been shown to be necessary for the progression of spermatogenesis, this interaction may be necessary for the translational regulation of spermatogenesis-related genes.

1.3.3 ARGONAUTE and PIWI Sub-families Differ From Each Other

In addition to the phylogenetic difference, AGO and PIWI proteins differ in various aspects. While AGO proteins are found in diverse eukaryotes including plants and fungi, PIWI proteins seem to have been lost from these kingdoms during evolution. They have been detected only in animals and some protists thus far (Cerutti and Casas-Mollano 2006). The common feature of the PIWI-expressing organims is that they all show sexual reproduction. In agreement with this observation, PIWI proteins are detected predominantly in the gonads or during the sexual reproductive cycle of these organisms. On the other hand, AGO proteins overall are ubiquitously and constitutively expressed. Furthermore, echoing their expression pattern, mutations in animal PIWI proteins all result in infertility due to defects in germline determination and/or gametogenesis (Harris and Macdonald 2001; Megosh, Cox et al. 2006; Klattenhoff and Theurkauf 2008), while *ago* phenotypes are more correlated with somatic functions and can be as severe as lethality (Liu, Carmell et al. 2004; Alisch, Jin et al. 2007).

The observations reviewed above suggest that the function of PIWI and AGO proteins differ from each other. However, a functional difference between ARGONAUTE proteins and PIWI proteins has not been shown at the molecular level yet. Therefore, functional characterization of MILI and MIWI is not only crucial to comprehend PIWI sub-family proteins but also to unravel the functional differences

between PIWI and AGO sub-families. Moreoever, our studies on these proteins should provide a significant insight about the role of PIWI proteins in germline functions. In pursuit of these aims, in the following chapters, I will present the identification of a novel class of small RNAs, which specifically associate with PIWI proteins and appear to guide them to identify the unpaired regions during meiosis. Additionally, I will provide detailed information about the biogenesis and expression pattern of these small RNAs.

2. Discovery of piRNAs

*The main content of this chapter has been published as a research paper by Shane T. *Grivna, *Ergin Beyret, Zhong Wang and $^{\Phi}$Haifan Lin in Genes & Development 20:1709-14 (2006)*

**Co-first authors*

$^{\Phi}$Primary Investigator

2.1 Author Contribution

Shane T. Grivna performed the electrofractionation of total RNA from 24dpp *Miwi*$^{+/-}$ and *Miwi*$^{-/-}$ testes, cloned piRNAs presented here, co-immunoprecipited piRNAs with MIWI, and performed small RNA Northern blotting on the testicular polysome fraction.

Ergin Beyret performed the nuclease treatments on piRNAs, evaluated their expression pattern during post-natal testis development in *Mili*$^{+/-}$ and *Mili*$^{-/-}$ mice, analyzed different tissues for piRNA expression, compared the expression pattern and size of piRNAs with miRNAs, and performed electrofractionation of total RNA from the testicular polysome fraction.

Zhong Wang performed the bioinformatics analyses of the cloned piRNAs: mapped piRNAs on the genome; analyzed their genomic regions, sequences and size distribution.

2.2 Summary

Small noncoding RNAs, including siRNAs and miRNAs of ~21 nucleotides in length, regulate gene expression at both transcriptional and post-transcriptional levels in diverse organisms. Here we report the identification of a novel class of small RNAs termed Piwi-interacting RNAs (piRNAs). piRNAs are ~30 nucleotides in length and specifically detected in the mouse male germline following entry into meiosis. They associate with MIWI, and depend on it for their biogenesis and/or stability. Furthermore, a minor subpopulation of piRNAs co-sediments with ribosomes, but does not seem to interact with them suggesting that piRNAs are not involved in translational regulation.

2.3 Introduction

The importance of small noncoding RNAs as regulators of transcription, RNA stability, and translation is becoming increasingly evident (Storz, Altuvia et al. 2005; Plasterk 2006). Although hundreds of small RNAs have been identified from mammalian somatic tissues, relatively little is known about small RNAs in germ cells.

To date, only 43 miRNAs have been cloned from the mouse testis (Lagos-Quintana, Rauhut et al. 2002; Yu, Raabe et al. 2005). One such miRNA, miR-122a, targets a reporter mRNA containing sequences from the 3'-UTR of the Transition Protein 2 (Tnp2) mRNA. Tnp2 is a small basic protein that initially replaces the histones during the condensation of haploid chromosomes in spermatids as they undergo morphogenetic changes to become sperm, a process known as spermiogenesis. Because transcription ceases as chromosomes condense, all genes involved in spermiogenesis, such as Tnp2, must be transcribed in early spermatids but translationally repressed for up to seven days until later stages of spermiogenesis (Kleene 2003).

Here we report the identification and preliminary characterization of a novel class of small RNAs. These small RNAs are ~30 nucleotides in length, longer and much more abundant than the known small RNAs, and detectable only in testis following entry into meiosis. We show that they interact with the mouse PIWI protein, MIWI, and this interaction is necessary for their cellular accumulation. Because of these properties, these small RNAs are termed piRNAs (*p*iwi-*i*nteracting RNAs). piRNAs map to intergenic, RNA-coding, and repetitive sites in the mouse genome. A subpopulation of them co-fractionates with polysomes, suggesting a potential role in translational regulation.

2.4 Materials and Methods

2.4.1 Total RNA Isolation and Separation

Total and polysomal RNA was isolated using Trizol (Invitrogen) according to manufacturer's protocol. Concentrations were determined by UV spectrometry and gel quantification.

2.4.2 Cloning of Testicular piRNAs

Small RNAs of 29-34nt from the testis were purified from 500 μg of total testicular RNA by excision of corresponding gel slices from ethidium-bromide-stained 15% denaturing polyacrylmide gels. Small RNAs were recovered from the excised gel slices and cloned according to the protocol of (Lau, Lim et al. 2001). A total of 46 clones were isolated. Sequence analysis revealed that 5 are cloning artifacts; the remaining 41 are genuine clones, which represent 40 independent sequences.

2.4.3 Polyacrylamide Gel Electrophoresis (PAGE)

For total RNA analysis by ethidium bromide staining, 40 μg of total RNA from 16 and 24dpp *Miwi*$^{+/-}$ and *Miwi*$^{-/-}$ testes was electrofractionated by 6M Urea/15% PAGE. For total RNA analysis by ^{32}P end-labeling, 1 μg of total RNA samples isolated from 9, 14, 22dpp and adult (~ 3 months old) *Mili*$^{+/-}$ and *Mili*$^{-/-}$ testes were 5'-end labeled with ^{32}P by T4 polynucleotide kinase (New England Biolabs) following dephosphorylation with

calf intestinal phosphatase (New England Biolabs). One ng of 5'-end labeled DNA 45-mer was added to each sample as indicated in Figure 5B. Labeled RNA or DNA samples were purified using Sephadex G25 spin columns (Roche). Nuclease digestion, where indicated, was performed with 100 μg RNase A (Sigma), and 7.5 units RQ1 DNase (Promega) for 30 minutes at 37°C. For polysomal RNA analysis, one adult testis-equivalent (160 mg testis) of polysomal RNA was used. 10nt DNA markers and 10nt RNA markers were purchased from Invitrogen and Ambion, respectively. Typhoon 9400 phosphoimager was used for radioactive imaging analysis.

2.4.4 Northern Blot Analysis of piRNAs

For Northern blot analysis, electrofractionated RNA was transferred onto Hybond-N+ membrane (Amersham Biosciences). For polysomal analysis, one-tenth of one testis-equivalent of 24dpp *Miwi*$^{+/-}$ total testicular RNA and one testis-equivalent of polysomal RNA were used. Hybridizations were performed at 42°C in hybridization buffer [1X SSC, 7% SDS, 20 mM Na_2HPO_4 (pH 7.2), 1X Denhardt's solution, 0.1 mg/mL salmon sperm DNA]. Blots were probed with radiolabeled DNA oligos (IDT DNA) complementary to mature pi- or miRNA sequences. Probes were labeled on their 5` ends with kinase reaction, using [^{32}P-γ]-ATP and T4 PNK (NEB). Blots were washed twice in 1X SSC, 0.1% SDS at 42°C for 10 min. and analyzed with phosphorimager.

2.4.5 RNA Isolation From Polysomes

Two testes from 24 day-old *Miwi*$^{+/-}$ and *Miwi*$^{-/-}$ mice were subjected to continuous sucrose density gradient fractionation as in (Grivna, Pyhtila et al. 2006). Fractions containing polysomes were combined and total RNA was isolated as above. For the polysome profiling of the adult testes, one testis (Figure 10B) or two testes (Figure11) from the indicated genetic backgrounds were likewise fractionated. In Figure 10B, fractions containing 40S and 60S subunits were combined with the free RNP fractions, and total RNA therein was isolated as above. The monosomal (80S) and polysomal RNAs were likewise extracted. In Figure 11, the 40S and 60S fractions were combined to give the "subunit" fraction, and the free RNP fraction was analyzed separately than the subunit fraction. The monosomal and polysomal fractions were combined to give the "ribosomal" fraction. The total RNA in the free RNP, subunit and ribosomal fractions were isolated as above.

2.4.6 Co-immunoprecipitation of piRNAs

MIWI and its associated RNA was immunoprecipitated from 2 mg of post-nuclear extract from 24dpp *Miwi*$^{+/-}$ testes by a rabbit polyclonal antibody R133 against amino acid residues 239-524 of MIWI (Deng and Lin 2002). The extract was combined with an equal volume of NT2 buffer [50mM Tris (pH7.4), 150mM NaCl, 1 mM $MgCl_2$,

0.05% NP-40] and incubated with Protein A-Sepharose beads coated with either anti-MIWI or pre-immune serum. To recover co-precipitated RNA, 200 μL of TE/1% SDS was added to the precipitate and heated to 95°C for three minutes, followed by phenol/chloroform extraction and isopropanol precipitation. Precipiated RNA was separated by 15% denaturing PAGE and visualized with ethidium bromide.

2.4.7 piRNA Sequence Analyses

piDNA sequence logos were drawn by WebLogo (http://weblogo.berkeley.edu/logo.cgi). piRNA (plus 50nt upstream and 50nt downstream) secondary structure predictions were carried out with RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). In order to annotate piRNAs, BLAT searches were conducted to map them onto individual chromosomes (http://genome.ucsc.edu/cgi-bin/hgBlat). For individual piRNAs with multiple hits, only the highest scoring hits with a sequence identity of more than 96% were kept. Exonic, intronic, intergenic and repeat annotations were retrieved based on mouse genome assembly 34, by an in-house developed computer program.

The 77.6% frequency of 5'U in piRNAs refers to the percentage of sequences that have a 5'-U in a combined dataset of the Imai Set (367 sequences) (Watanabe, Takeda et al. 2006) and our set (40 sequences). The frequency of 5'U in our set is 30%, in contrast to

the Imai Set (82.8%). To determine whether it is likely that our set is a subset of the Imai set, we randomly sub-sampled 40 sequences from the Imai's 367 sequences to form 10,000 sets of 40 sequences and calculated the number of sequences that have 5'-Us. Among these random sets, there are 22-40 sequences per set that have a 5'U (mean=33.15 and standard deviation=2.25). Therefore, the probability of observing 30% 5'U-containing piRNAs from our set as a fluctuation of the Imai set is less than 0.0001. This result suggests that our sequences likely represent a distinct population of small RNAs than Imai's piRNAs.

2.5 Results and Discussion

2.5.1 The Mouse Testis Comprises a Novel Class of Small RNAs, Which Depends on MIWI for Expression

PIWI and PAZ domains of ARGONAUTE proteins have been shown to bind to mi/siRNAs. As the members of PIWI/ARGONAUTE protein family (a.k.a PIWI-PAZ Domain family), PIWI proteins also possess these domains. Additionally, scan RNAs in *Tetrahymena thermophila* depend on their PIWI protein partner, TWI1P for their cellular accumulation. In order to assess if mouse PIWI proteins likewise interact with small RNAs, we first evaluated the small RNA content of the testis, where mouse PIWI proteins are abundantly expressed. When we resolved the small RNAs of 24dpp *Miwi*$^{+/-}$ and *Miwi*$^{-/-}$ testes by polyacrylamide gel eletrophoresis (PAGE), we identified an abundant species of RNA migrating at 31-35 nt relative to a DNA ladder marker, but

significantly reduced in 24dpp *Miwi*$^{-/-}$ testes (***Figure 5A***). We used 24dpp testes, because the tissue composition of *Miwi*$^{+/-}$ testis afterward differs than *Miwi*$^{-/-}$ testis as spermatogenesis is arrested during this stage in the knock-out. Because DNA migrates 10% faster than RNA in our denaturing PAGE, the observed small RNAs correspond to 28-32nt in length (also see ***Figure 5B*** with an RNA ladder marker). In order to ensure that this 28-32nt species is indeed composed of RNA, we treated the ^{32}P-end-labeled testicular total RNA samples with RNase or DNase, after adding a 45-nt single-stranded DNA oligomer to the samples as an internal control. The RNase did not have any contaminating DNase activity as the DNA control was unaffected, yet effectively degraded the entire RNA sample, including the 28-32nt species (***Figure 5B***). In contrast, the DNase treatment eliminated the 45nt internal DNA control without affecting the 28-32nt species, confirming that this species is composed of RNA (***Figure 5B***).

Figure 5: Testis comprises a novel RNA species which depends on MIWI for expression
(Panel A by Shane T. Grivna/Haifan Lin Lab)

A) PAGE analysis of equal amounts of total RNA from 24dpp *Miwi*$^{+/-}$ (+/-) and *Miwi*$^{-/-}$ (-/-) testes shows an abundant quantity of small RNAs of ~30nt in length exists in *Miwi*$^{+/-}$ but not in *Miwi*$^{-/-}$ testis.

The electrofractionated samples were visualized with ethidium bromide staining.

B) PAGE analysis of equal amounts of total RNA from adult *Mili*$^{+/-}$ (+/-) and *Mili*$^{-/-}$ (-/-) testes, treated with or without RNase or DNase, shows the ~30nt species is composed of RNA.

The electrofractionated samples were visualized with autoradiography by means of ^{32}P-end-labeling with kinase reaction.

M: 10nt DNA (**A**) or RNA (**B**) molecular weight ladder marker. O: 45-nt DNA oligomer only. Closed arrowhead: the novel small RNA species, open arrowhead: DNA oligomer.

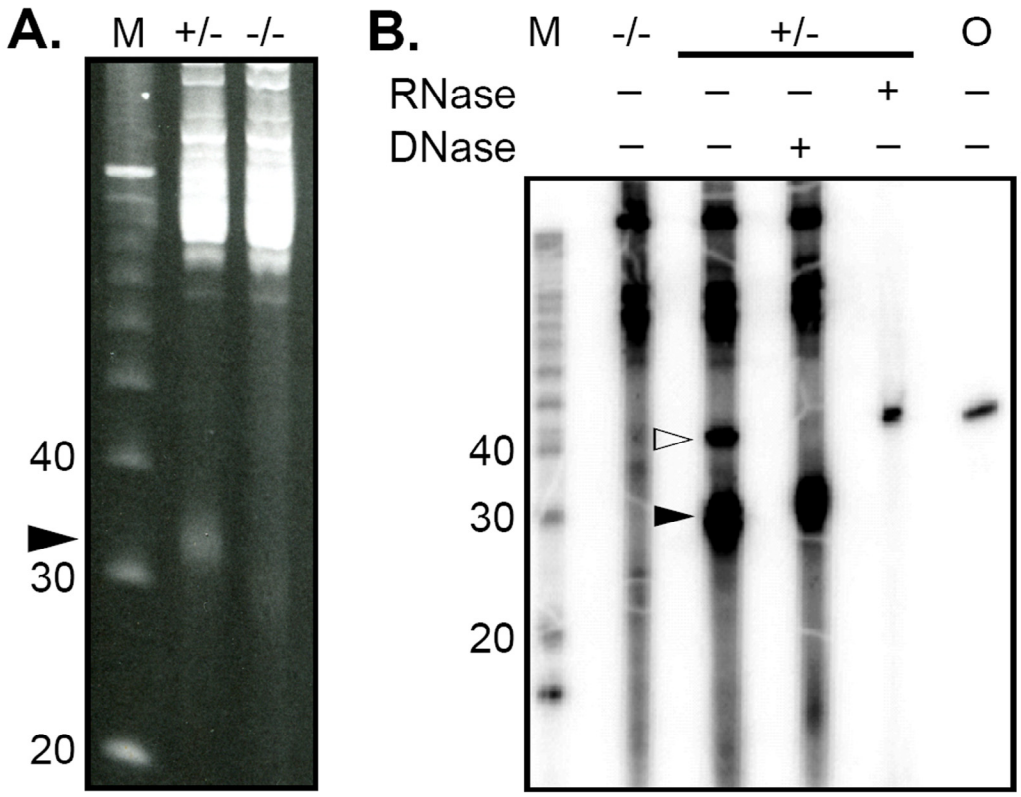

Figure 5: Testis comprises a novel RNA species which depends on MIWI for expression

(Panel A by Shane T. Grivna/ Haifan Lin Lab)

2.5.2 The Novel RNA Species is Detectable Only in the Testis Following Entry Into Meiosis

To further characterize these RNAs, we have analyzed their temporal and spatial expression pattern, and compared with the expression patterns of 8 representative miRNAs. Our PAGE analyses indicate that this RNA species is distinct from miRNAs in terms of length, abundance and expression pattern: First, while miRNAs are 20-23nt in size, these RNAs are approximately 30nt (***Figure 6***). Second, they are much more abundant than miRNAs that they can be visualized even with ethidium bromide staining or ^{32}P-end-labeling of the total cellular RNA, unlike any known small RNAs (***Figure 5 and 6***). Third, they become highly detectable during 14 to 22dpp of the testicular development, corresponding to the period of meiosis and coinciding with the expression of MIWI, while the miRNAs tested do not overall show such trend (***Figure 6***). Lastly, among the tissues tested, they appear to be restricted to the testis, agreeing with the expression pattern of the mouse PIWI proteins (***Figure 7A and B***). Notably, they are not detected in the caudal epididymus, suggesting that they are not loaded into mature sperm and thus do not function as paternally contributed factors. These observations cumulatively indicate that the observed species is a novel class of RNA, which depends on MIWI for its biogenesis and/or stability.

Figure 6: The novel RNAs are uniformly upregulated following entry into meiosis unlike miRNAs

The novel RNA species is upregulated between 14dpp and 22dpp, corresponding to the period of meiosis. The tested miRNAs do not show such trend overall. (**A-E**) PAGE analysis of equal amounts of total RNA from 8dpp, 9dpp, 14dpp, 22dpp and 2 months old-adult (Ad) *Mili*$^{+/-}$ (HET) and *Mili*$^{-/-}$ (KO) testes. (**A-D**) Samples were visualized with ethidium bromide staining and northern blotted for microRNAs Mirn100, Mirn15b (**A**); Mirn101, Mirn16 (**B**); Mirn465, Mirn34b (**C**); Mirn466, Mirn449a (**D**). As an external positive control and size marker, the last lane of each gel was loaded with 5 fmol reverse complementary oligo DNA of the indicated miRNA to be probed for (c-MiDNA). U6snRNA was used as the loading control. One of the cloned novel RNA species (piRNA-T4) was probed for on the same samples in (**A**). (**E**) Equal amounts of samples were 5` end-labeled and visualized with autoradiography.

M_D & M: 10nt DNA & RNA molecular weight ladders. The sizes of the small RNAs probed for are indicated on their right. The external controls migrate faster than the endogenous miRNAs since DNA migrates 10% faster than RNA under our denaturing (Urea)-PAGE conditions.

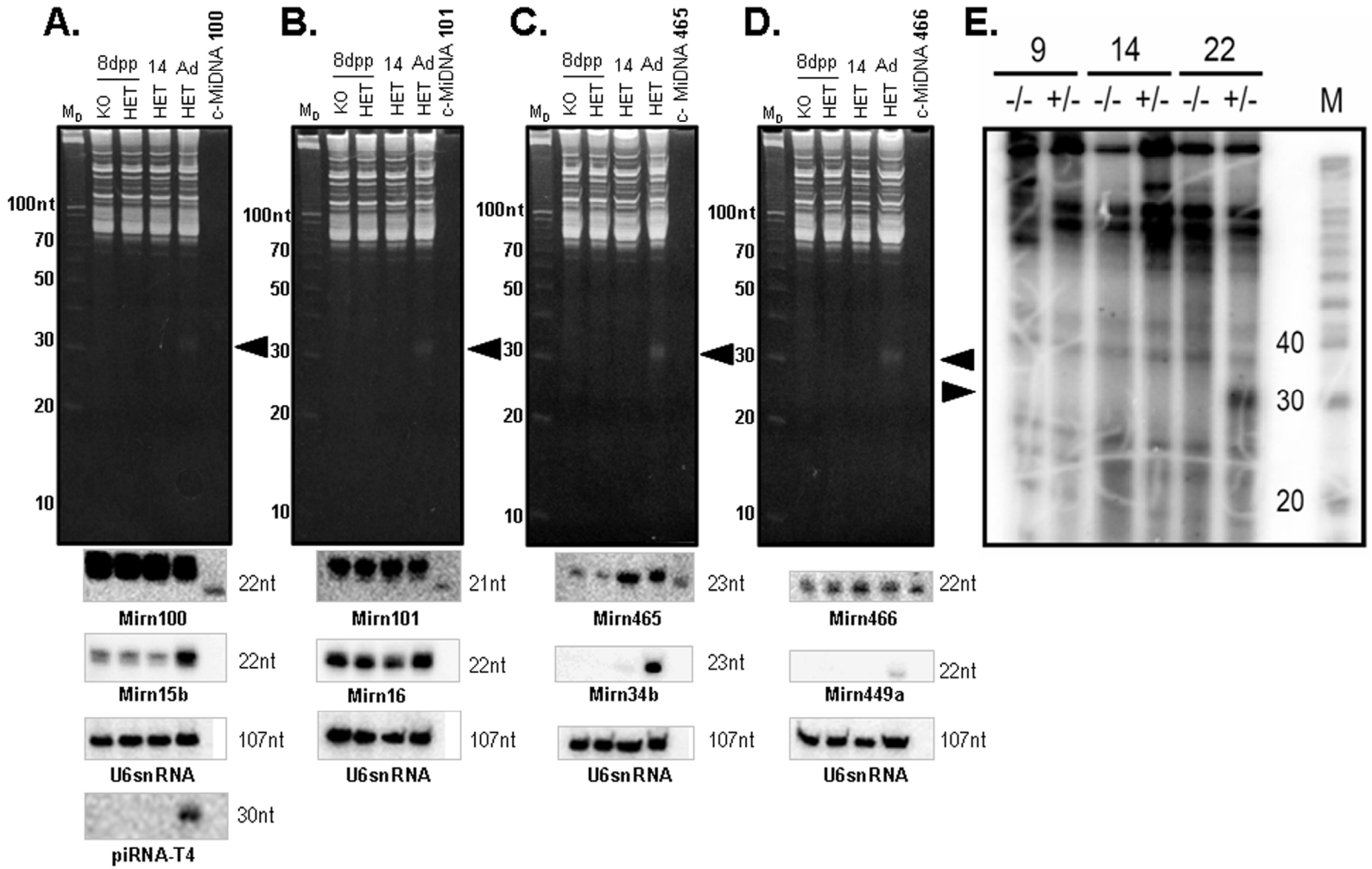

Figure 6: The novel RNAs are uniformly upregulated following entry into meiosis unlike miRNAs

2.5.3 MIWI Interacts With the Novel RNAs, *piRNAs*

Since the expression of this novel RNA species requires and coincides with the expression of MIWI, we asked whether the two interact. For this purpose, we immunoprecipitated MIWI from the 24 dpp *Miwi*$^{+/-}$ testicular extract and analyzed the total RNA content of the immunoprecipitates with Urea-PAGE. We observed that the novel RNAs co-immunoprecipite with MIWI (**Figure 7C**). Due to this interaction, we named them ***P***iwi-***i***nteracting RNAs (***pi***RNAs).

Figure 7: The novel RNA species is detectable only in the testis and interacts with MIWI (Panel C by Shane T. Grivna/Haifan Lin Lab)

A) PAGE analysis of equal amount of total RNA from the liver (Li), brain (Br), kidney (Ki), testis (Te), ovary (Ov), spleen (Sp), embryonic stem cells (E), intestine (In), caudal epididymus (Ep), lung (Lu), heart (H), and stomach (St), shows that the novel RNAs are only detected in the testis with ethidium bromide staining.

B) Northern blot analysis of RNA from (A), showing one of the cloned novel RNAs (piRNA-T4) is detectable only in the testis.

C) PAGE analysis of 10 μg testicular RNA input (I), and RNA co-immunoprecipitated with MIWI antibody (αMW) or its preimmune sera (Pre) shows that the novel RNA species co-immunoprecipitates with MIWI. Shown is the negative image of the ethidium-bromide stained gel.

M: 10nt DNA ladder.

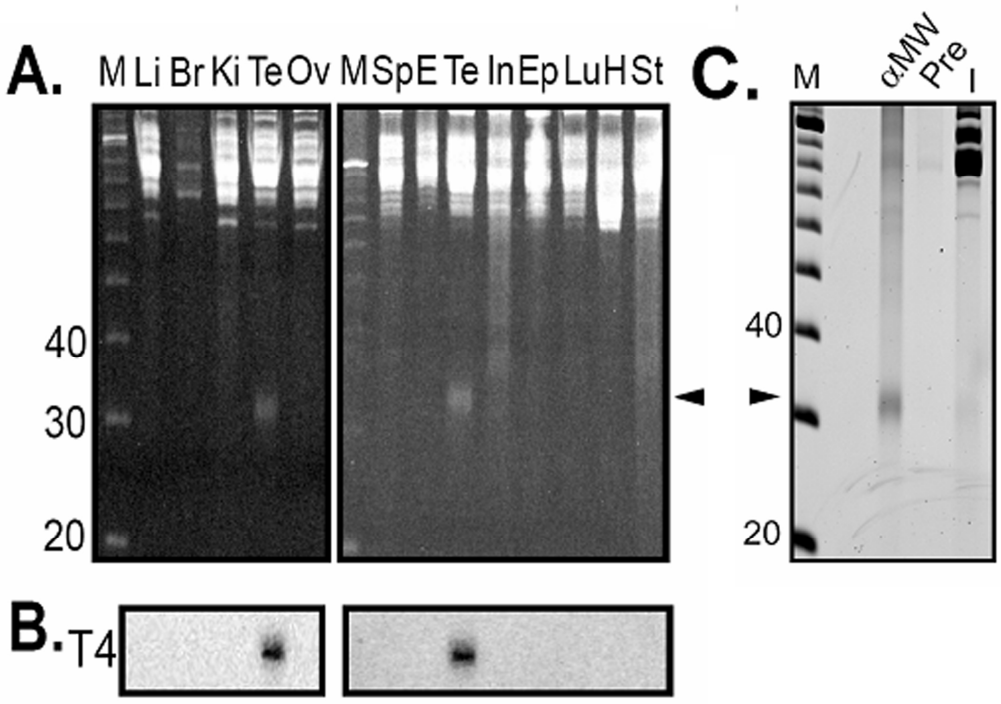

Figure 7: The novel RNA species is detectable only in the testis and interacts with MIWI

(Panel C by Shane T. Grivna/Haifan Lin Lab)

2.5.4 piRNAs are 29-30nt in Median Length and Mostly Start With Uracil

In order to identify the sequences of individual piRNAs, we cloned them from the corresponding band in electrofractionated total testicular RNA using the method of Lau *et al* (Lau, Lim et al. 2001). This method takes advantage of the characteristic 5'-phosphate and 3'-hydroxyl moieties present on siRNAs and miRNAs, allowing effective selection against typical breakdown products that contain 5'-hydroxyl and 3'-phosphate groups. As a result, 40 independent novel RNA species were cloned (***Table 1***). Subjecting the cloned sequences to BLAT searches against the current mouse genomic databases revealed that 35 of the clones each corresponded to at least one region in the genome. The length of these small RNAs ranges from 25 to 35nt, with the majority between 29-31nt (***Figure 8A***), confirming our original observations (***Figure 5***). Interestingly, two of the 40 RNAs have also been identified by Watanabe *et al.* of the Imai lab (Watanabe, Takeda et al. 2006) in their cloning of 367 germline-specific small RNAs from the mouse (gsRNAs, GeneBank accession No. AB250971-AB251337, herein called the Imai set). Given that the length distributions of these two independent sets of clones are very similar (***Figure 8A and B***) and that they are both from the mouse germline, they likely represent two subsets of the same class of germline specific small RNAs. The small overlap between these two sets of small RNAs suggests that the total number of piRNAs is much larger than these two sets combined.

Table 1: The piRNA-T* sequences (by Shane T. Grivna)

Small RNAs of 29-34nt were cloned from the corresponding gel slice of the electrofractionated testicular total RNA according to the protocol of (Lau, Lim et al. 2001).

Note that cloned sequences were named from piRNA-T1 to piRNA-T49; shown are the 40 genuine piRNA sequences identified.

Table 1: The piRNA-T* sequences

(by Shane T. Grivna/ Haifan Lin Lab)

Name	Sequence
piRNA-T1	gaaugaacuuggcaguacccucuagauga
piRNA-T2	gaagaucauaaccauccucaugucuacaa
piRNA-T3	gccuuaaagcucagaaacaauaaggccca
piRNA-T4	uagacagcuuaggacacugaaaauugucua
piRNA-T5	uucugcacaguacucuuaugcucugug
piRNA-T10	agucaggaggaggguucagcaugaaagccugac
piRNA-T11	cgagugcgaugcuuuguuuuaauuacaca
piRNA-T12	uaagucagaaucccgcccaggcggaac
piRNA-T13	gauuccgugggugguggugcauggucguucuu
piRNA-T14	cagaaagccugcccuagcauggauggugagc
piRNA-T15	caauauagauggauacgaauaaggcgu
piRNA-T16	auaauaagcugauggaugcuugaucguca
piRNA-T17	uaggaacaugacugcuuuguaaagggcca
piRNA-T18	uugggucauauugaacuuuacuugaaugug
piRNA-T19	uccugagaagugugugacaguugguuacc
piRNA-T20	uaugguucucaguuuagcuguguuuuaau
piRNA-T22	auggacuuucugucagauuuugagaugauguua
piRNA-T23	auuagaguguaaaauauuuccuggguuguuug
piRNA-T24	auucguugucauauaaauucgcaaaaa
piRNA-T25	aacuaaaauuuuugcggcugucuga
piRNA-T26	aaaguggacaaaacuccugggaagg
piRNA-T29	ucaugaucuugagaaagucauuuccuuc
piRNA-T30	cagaaucagcggggaaagaagacccuguuga
piRNA-T31	aauauagagaaauuuucucuauuccagucu
piRNA-T33	aagaacgaaagucggagguucgaagacgauc
piRNA-T34	aucagaccccagaaaagguguugguugauau
piRNA-T35	uaccgauuggaugguuuagugaggcccucgg
piRNA-T36	acuuuuccuagaguccaucaagcga
piRNA-T37	aaagcaucugggauaaaaaaaaaaaaaaacccaga
piRNA-T38	uuauauguuggguuacugaaguacuuucagu
piRNA-T39	ugcagauacauguaccuaaacacucaugua
piRNA-T40	augugauuaggauguugggugauuuaaaa
piRNA-T41	aauuccauuagauagaugggaaaagcugcggc
piRNA-T42	uaucagugagacuugagaacccacaaaggg
piRNA-T43	auagucaacaaguaccguaagggaaaguugaa
piRNA-T44	ccgcagccaagggaacgggcuuggcggaa
piRNA-T46	aaaaaaacuagcgguuaggauuccugguu
piRNA-T47	agcguauauuaaaguugcugcaguuaaaaa
piRNA-T48	aauauaauguuagaaucuggauguuucc
piRNA-T49	cagaacaggaacucaaacagggcgguaac
* T stands for testis.	

Figure 8: piRNAs are 29-30nt in median length

(by Zhong Wang/Haifan Lin Lab)

Length distributions of piRNAs from this study (**A**) and from the Imai set (**B**) show that piRNAs cloned have a median length of 29-30nt.

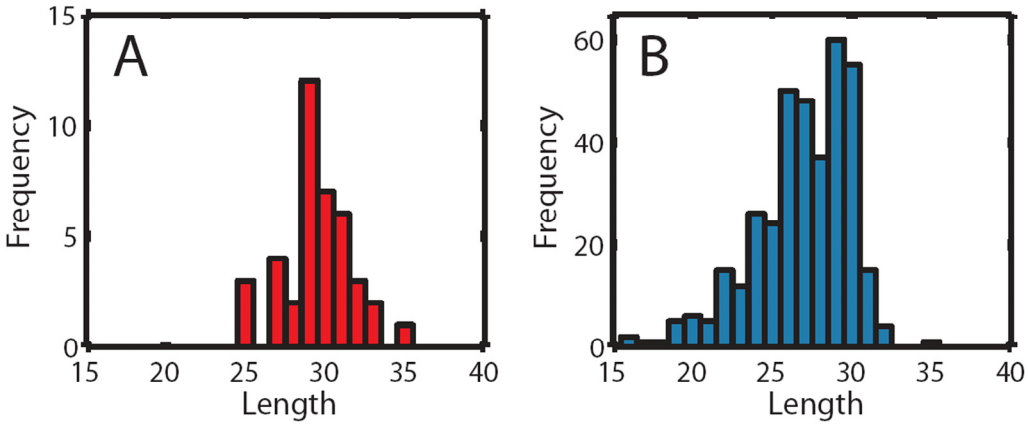

Figure 8: piRNAs are 29-30nt in median length

(by Zhong Wang/Haifan Lin Lab)

It has been reported that DICER products, including miRNAs and siRNAs have a strong preference for pyrimidine residues, especially Uridine, at their 5'-most position (Aravin, Lagos-Quintana et al. 2003). piRNAs overall show a very strong sequence bias towards U (77.6%), (***Table 2 and Figure 14B for a more comprehensive analysis***), suggesting that they may also be generated by a DICER-like cleavage.

Among DICER-generated small RNAs, miRNAs are known to be generated from precursors that have a unique fold-back hairpin secondary structure (Bartel 2004). To test whether piRNAs share similar structures, we randomly selected eight piRNAs, added 50 bases of flanking genomic sequence from both ends of each piRNA coding sequence, and predicted their secondary structures. None of the eight structures resembles those of miRNA precursors, nor do these structures resemble each other. Therefore, it remains unclear whether piRNAs are derived from precursors with unique secondary structures.

2.5.5 piRNAs Mostly Originate in Clusters and From Intergenic Regions

To further characterize piRNAs, we determined the corresponding genomic regions of our piRNAs and the Imai set, totaling 407 piRNAs. Mapping them on the mouse genome ("Material and Methods", ***Figure 9*** and ***Table 2***) resulted in the successful

annotation of 75.4% of the 407 piRNAs. The remaining 24.6% (37) sequences did not match to any genomic sequences. Several striking features are revealed through this analysis:

First, piRNA-coding sequences display highly uneven distribution among chromosomes (***Table 2***). For example, Chromosome (Chr) 17, representing only 3.1% of the genome, encodes 17.6% of the piRNAs. Other piRNAs are enriched on Chr 5 (11.6%), Chr 4 (10.7%) and Chr 2 (10.2%), but are under-represented on Chr 1, 3, 16, 19 and X. For example, the X chromosome, representing 5.5% of the genome, contains only two piRNA sequences (0.4%), 14-fold lower than the expected value for random distribution. The Y chromosome is devoid of known piRNAs.

Table 2: piRNAs are biased for 5` Uracil and map unevenly on the genome (by Zhong Wang/Haifan Lin Lab)

Mapping the piRNAs on the genome shows that they are unevenly distributed among the chromosomes, with the chromosome 17 comprising the highest number of the piRNAs. None of the piRNAs correspond to the Y chromosome.

Note that a total of 407 piRNAs from our set and the Imai set combined were mapped to 450 sites on the genome due to some piRNAs mapping to more than one locus.

Nucleotide composition of the piRNAs shows that they are biased for Uracil on their 5` ends. See Figure 14B for a more comprehensive analysis.

Table 2: piRNAs are biased for 5` Uracil and map unevenly on the genome

(by Zhong Wang/Haifan Lin Lab)

Blat Mapping				**Chromosomal distribution**		
Total piRNAs	407			Chrom	Count	Percentage
Mapped	370			1	5	1.1%
Not Mapped	37			2	46	10.2%
				3	3	0.7%
Percent Mapped	75.43%			4	48	10.7%
				5	52	11.6%
First base composition		**Percent**		6	34	7.6%
A	40	9.83%		7	39	8.7%
G	26	6.39%		8	11	2.4%
C	25	6.14%		9	16	3.6%
U	316	77.64%		10	21	4.7%
Total piRNAs	407			11	10	2.2%
				12	25	5.6%
				13	8	1.8%
				14	5	1.1%
				15	34	7.6%
				16	1	0.2%
				17	79	17.6%
				18	9	2.0%
				19	1	0.2%
				X	2	0.4%
				Y	0	0.0%
				Un_random	1	0.2%
				Total	450	

Second, piRNA-coding sequences show a strong tendency to cluster (***Figure 9, Table 3 and Figure 17A for a more comprehensive analysis***). 89% of the piRNAs form 35 clusters containing 2-62 piRNAs, with an average of 10.3 piRNAs per cluster. Each cluster spans 0.4-73.5kb (median 21.8kb). The largest cluster is located on Chr17 and contains 62 piRNAs in a 73.5kb region. Among the 35 clusters, five may be created by intra-chromosomal segmental duplications, with one cluster on Chr 4 containing 17 duplicated 152bp sequences defined by two piRNAs. However, the other 30 clusters do not appear to be caused by duplications. In either case, this highly clustered organization raises the possibility that the piRNAs within each cluster are coordinately expressed and might share related functions.

Figure 9: piRNAs map on the genome mostly in clusters (by Zhong Wang)

Mapping the piRNAs on the genome shows that they are frequently produced in clusters. The chromosomes were drawn in scale and aligned by their centromere positions. piRNAs from this study and from the Imai set are indicated in red and black respectively. The number of piRNAs in a particular cluster is indicated by the number next to the cluster.

See Figure 17A for a more comprehensive analysis.

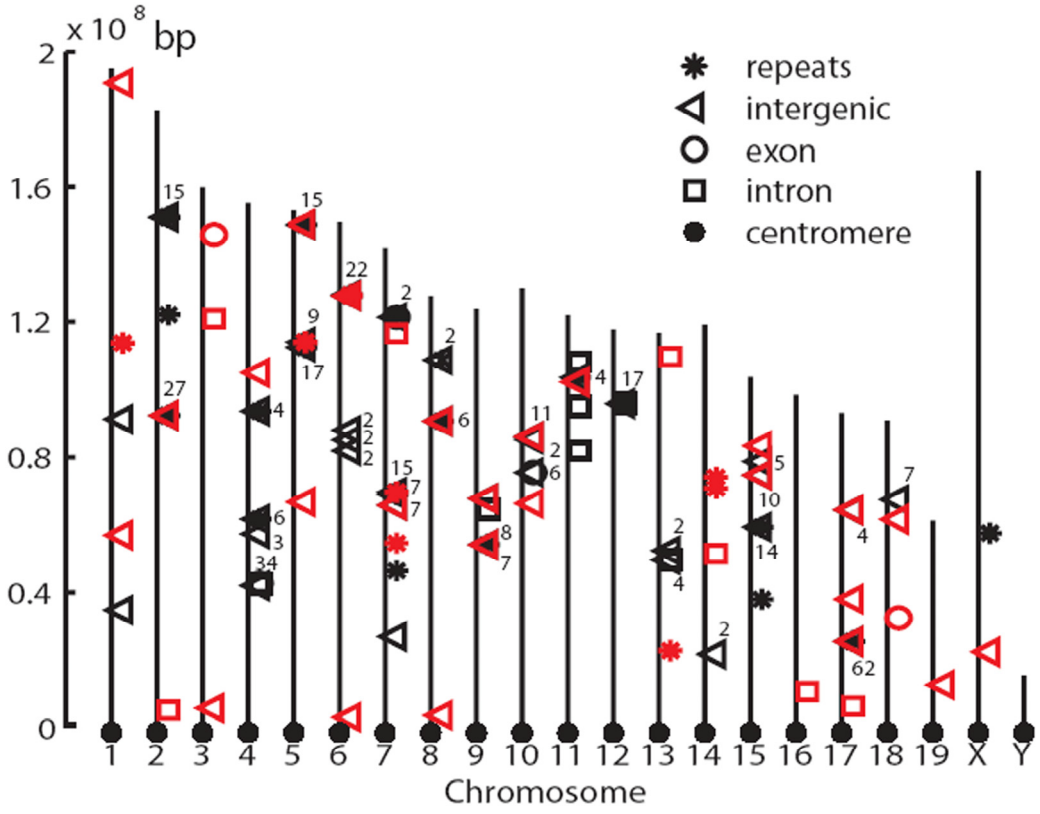

Figure 9: piRNAs map on the genome mostly in clusters

(by Zhong Wang/Haifan Lin Lab)

Table 3: The distribution of piRNA clusters in the genome (by Zhong Wang)

Mapping the piRNAs on the genome shows that approximately 90% of them clusters into a number of regions, with the biggest cluster observed on the chromosome 17. 62 out of 405 piRNA sequences mapped correspond to this cluster, which spans approximately 74Kb. piRNAs were considered to cluster if they were at most 1.5Kb apart from each other.

Table 3: The distribution of piRNA clusters in the genome

(by Zhong Wang/ Haifan Lin Lab)

Cluster	Chrom	Start	End	Span	Size
1	2	92392091	92449353	57262	27
2	2	150891762	151150195	258433	15
3	4	41948209	42806145	857936	34
4	4	57402727	57405532	2805	3
5	4	61655436	61671483	16047	6
6	4	93701967	93721992	20025	4
7	5	112515135	112543698	28563	17
8	5	114039621	114073693	34072	9
9	5	148731425	148759710	28285	15
10	6	3153642	3154056	414	2
11	6	81914441	81928791	14350	2
12	6	85141128	85141659	531	2
13	6	127807835	128213734	405899	22
14	7	65761455	65788317	26862	7
15	7	68964011	68975080	11069	7
16	7	69632947	69703015	70068	15
17	7	121425571	121427163	1592	2
18	8	90783243	90788781	5538	6
19	8	108728061	108729327	1266	2
20	9	54244207	54280957	36750	7
21	9	67739352	67760451	21099	8
22	10	75493650	75515410	21760	6
23	10	85308838	85317282	8444	2
24	10	86129518	86180840	51322	11
25	11	103545028	103547907	2879	4
26	12	95882531	95941138	58607	17
27	13	49691732	49775258	83526	4
28	13	52437439	52437923	484	2
29	14	21772334	21772885	551	2
30	15	59317491	59354889	37398	14
31	15	74721171	74727020	5849	10
32	15	78732535	78748711	16176	5
33	17	25476281	25549803	73522	62
34	17	64370851	64432848	61997	4
35	18	67432484	67457171	24687	7
			Average	67030.51429	10.34286
			Median	21760	7
			Total		362
			percent		89.38%
		Total Hits (excluding some overlap ones)			405

Third, piRNAs are diversely distributed among exonic, intronic, intergenic, and repeat sequences (***Table 4 and Figure 13C for a comprehensive analysis***). The majority of piRNAs (90%) are found within intergenic regions, 9.3% within introns of known genes, and 1.3% in exons. The exonic piRNAs can be distinguished from the degradation products of mRNAs by Northern blot analysis where piRNAs are detected as discrete bands of ~30nt (*data not shown*). This distribution is not significantly different from the genome-wide averages of 68.1% intergenic, 29.9% intronic, and 2.1% exonic sequences. Interestingly, 17% of piRNAs map to repetitive sequences, noticeably lower than the presence of such sequences in 42.3% of the genome. The relatively even distribution of piRNAs among all types of genomic sequences suggests their diverse roles.

Table 4: piRNAs are mostly derived from intergenic regions (by Zhong Wang)

Annotation of the piRNA loci shows that they mostly reside in the intergenic regions that are depleted for repetitive sequences.

See Figure 13C for a comprehensive analysis.

Table 4: piRNAs are mostly derived from intergenic regions

(by Zhong Wang/Haifan Lin Lab)

Sequence type	Count	Percentage of piRNAs	Sequence type/Genome*
Exon	6	1.3%	2.06%
Intron	42	9.3%	29.88%
intergenic	402	89.3%	68.06%
repeats	77	17.1%	42.29%
* Percentage based on calculation of mm34			

2.5.6 A Minor Amount of piRNAs Co-sediments With the Translational Machinery but Does not Interact With It

Since MIWI is implicated in translational regulation (Grivna, Pyhtila et al. 2006), the association of piRNAs with MIWI suggests a possible role for piRNAs in MIWI-mediated translational regulation. To test this potential role, we first examined whether piRNAs co-sediment with polysomes through a continuous sucrose gradient (a representative testicular polysome profile is shown in ***Figure 10A***). We fractionated testicular extracts from adult *Miwi*$^{+/-}$ mice (***Figure 10B***) as well as 24dpp (***Figure 10C***) *Miwi*$^{+/-}$ and *Miwi*$^{-/-}$ mice with sucrose gradient sedimentation (see "Materials and Methods"). The RNA content was isolated from the pooled free RNP, monosomal, and polysomal fractions of adult *Miwi*$^{+/-}$ mice, end-labled with ^{32}P, and separated with denaturing PAGE (***Figure 10B***). We observed that piRNAs are mostly present in the RNP fraction; but a minor amount of them co-sediments with monosomes, and polysomes. Northern blotting for three individual piRNAs confirmed their presence in the polysomal fraction (***Figure 10C***). As expected, piRNAs are absent from the polysomal fraction of *Miwi*$^{-/-}$ testes.

Figure 10: A minor amount of piRNAs co-sediments with polysomes.

(Panel C by Shane T. Grivna/Haifan Lin Lab)

A) A representative A_{260} profile of adult wild-type testicular extract separated by continous 15-50% sucrose density gradient fractionation, with RNP, 40S, 60S, monosomal, and polysomal fractions indicated. Each tick indicates a collected sample fraction

B) PAGE analysis of RNA from pooled RNP, monosome (80S), and polysome fractions of adult *Miwi*$^{+/-}$ testicular extract end-labeled with ^{32}P. 40S and 60S fractions were included in the RNP fraction. piRNAs are indicated by the arrowhead.

C) Northern blots of total RNA and polysome-associated RNA from 24dpp *Miwi*$^{+/-}$ and *Miwi*$^{-/-}$ testes probed for three individual piRNAs. MicroRNA miR-16 was used as a loading control.

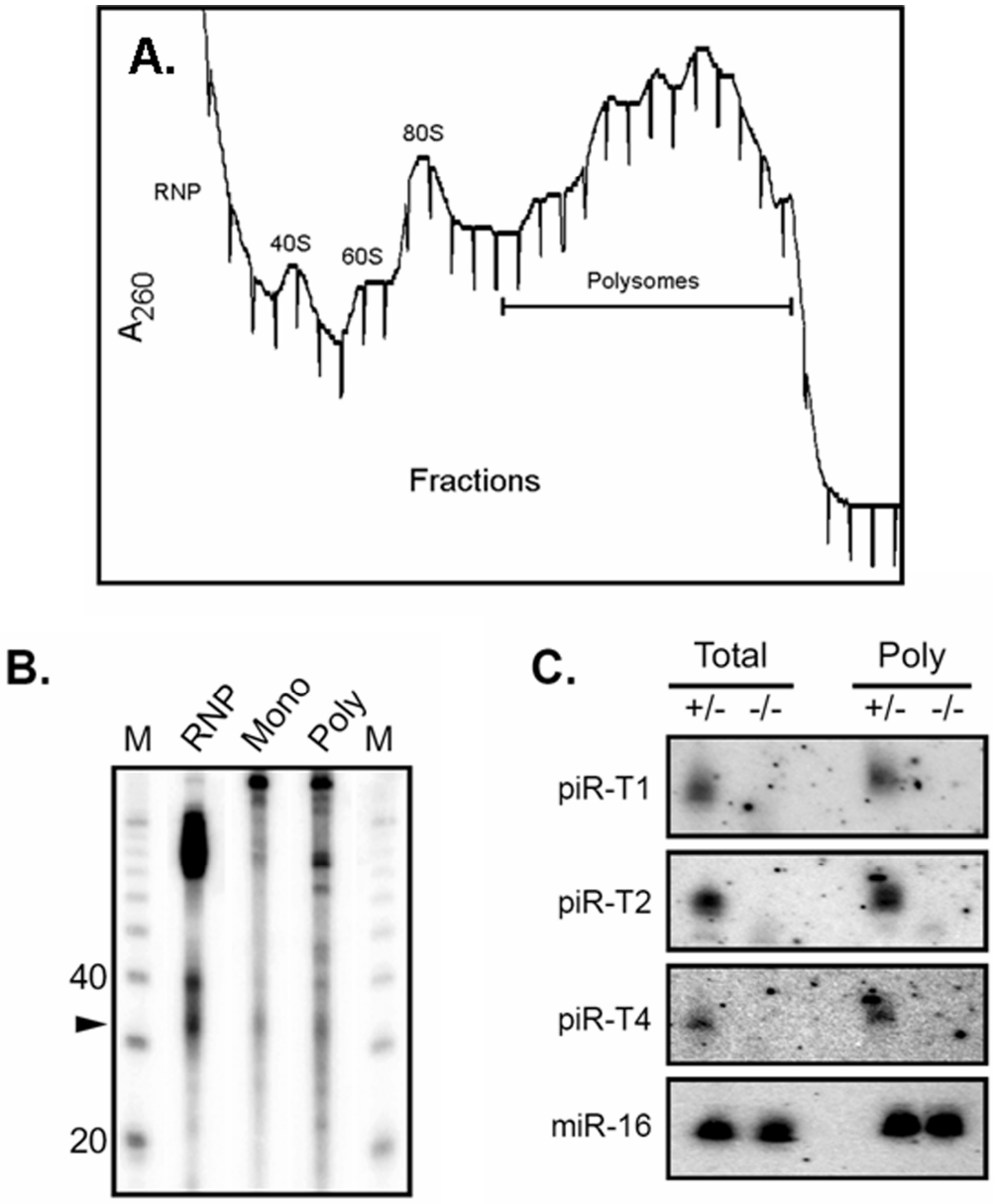

Figure 10: A minor amount of piRNAs co-sediments with polysomes

(Panel C by Shane T. Grivna/Haifan Lin Lab)

Next, we evaluated if the piRNAs in the monosomal and polysomal fractions interacted with ribosomes. For this reason, we analyzed the piRNA sedimentation profile of EDTA-treated testicular extracts. EDTA disrupts the quaternary structure of ribosomes, and thus dissociates them into their subunits. If piRNAs interact with ribosomes, EDTA treatment should decrease the amount of piRNAs in the monosomal and polysomal fractions, concurrently with the amount of ribosomes. However, the piRNA distribution seemed largely unaffected upon EDTA treatment, which disrupted the quaternary structure of almost all the ribosomes, shifting the ribosomal peaks toward the 40S and 60S subunits as evidenced by OD_{260nm} read-out (***Figure 11***). Therefore, the minor piRNA population that co-fractionates with the translational machinery does not seem to interact with it. It is possible that a small amount of piRNAs reside in a molecular complex that has an equal sedimentation velocity as ribosomes or this observation is due to a "leak-through" of piRNAs during the sedimentation process.

Figure 11: piRNAs do not interact with ribosomes

Disintegration of the ribosomes in the testicular lysate with EDTA treatment shows that co-sedimentation of piRNAs with ribosomes is independent of the quaternary structure of ribosomes. 2 testes of an adult wildtype mouse was lysed either in the presence (**A**) or absence (**B**) of $MgCl_2$, supplemented with (**B**) or without (**A**) the indicated amount of EDTA. The lysates were fractionated with sedimentation centrifugation through continuous 15-50% sucrose gradient as in Unhavaithaya *et al* (2008) followed by real-time OD_{260nm} analysis of the fractions (**A-B**). Each tick indicates a collected sample fraction. FreeRNP, 40S (small ribosomal subunit), 60S (large ribosomal subunit), 80S (monosome) and polysome peaks are labeled. The numbers inside the square brackets indicate the collected sample fractions within each peak that were pooled into one. The sample fractions shown in shaded areas at the junction of two neighboring peaks were excluded from either pool in order to decrease cross-contamination.

Subunit: 40S + 60S fractions, Ribosomal: 80S (monosome) + polysome fractions.

Equal volumes from each sample, except the "Free RNP" sample, were analyzed with 15% Urea-PAGE and cyber gold staining (**C**). For the "Free RNP" sample, 1/5th of the volume analyzed for other samples was used in order to prevent over-

saturation of the image. Green asterisks on the right of the samples indicate the piRNA populations co-sedimenting with ribosomes. M_D: 10nt DNA ladder marker

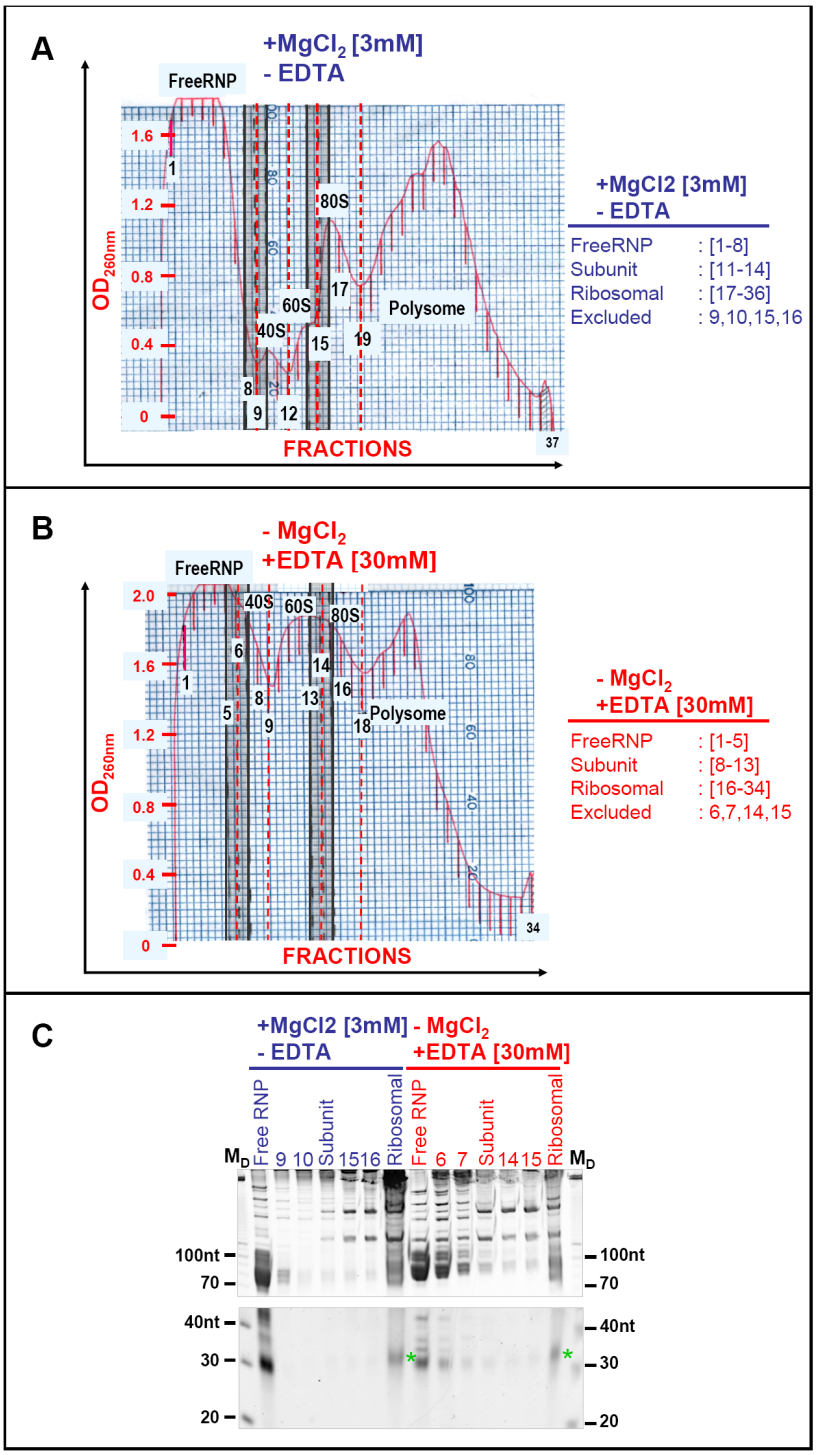

Figure 11: piRNAs do not interact with ribosomes

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2.6 Conclusions

We have previously shown that PIWI sub-family of the PIWI/ARGONAUTE (a.k.a Piwi Paz Domain) protein family is necessary for gametogenesis, including maintenance of germline stem cells by some of its members, (Cox, Chao et al. 1998; Cox, Chao et al. 2000; Deng and Lin 2002; Kuramochi-Miyagawa, Kimura et al. 2004; Unhavaithaya, Hao et al. 2008) as well as for the establishment of the fly germline (Megosh, Cox et al. 2006). Although PIWI proteins have been genetically well characterized, their function at the molecular level remained elusive. In pursuit of the biochemical characterization of PIWI proteins, here we identified a novel class of small RNAs, which we named **p**iwi-**i**nteracting RNAs (**pi**RNAs) due to their interaction with the mouse PIWI protein, MIWI. Moreover, we found that MIWI is necessary for their biogenesis/stability. Different than the small RNA partners of AGO proteins in various aspects, this new class of small RNAs forms the first molecular distinction between PIWI and AGO sub-families and is the key in elucidating the biochemical function of PIWI proteins.

Concurrently with our studies (Grivna, Beyret et al. 2006), four other groups independently identified piRNAs in the mouse, rat and human (Aravin, Gaidatzis et al. 2006; Girard, Sachidanandam et al. 2006; Lau, Seto et al. 2006; Watanabe, Takeda et al. 2006). These studies showed that in addition to MIWI, its mouse paralog MILI and rat ortholog RIWI are likewise associated specifically with piRNAs but not miRNAs, indicating that piRNAs are universal small RNA-partners of PIWI proteins. While piRNAs of MIWI display a median length of 30nt, MILI piRNAs are mostly 26nt long. Expectedly, two equivalent populations of piRNAs are also present in human testis (Aravin, Gaidatzis et al. 2006; Girard, Sachidanandam et al. 2006), implicating their likely interaction with the orthologs of MIWI and MILI in humans, HIWI and HILI, respectively.

Additional works identified piRNAs in various, and only in PIWI-expressing organisms including protists (Mochizuki, Fine et al. 2002; Mochizuki and Gorovsky 2004), insects (Brennecke, Aravin et al. 2007; Gunawardane, Saito et al. 2007; Yin and Lin 2007), worms (Batista, Ruby et al. 2008; Palakodeti, Smielewska et al. 2008; Wang and Reinke 2008), fishes (Houwing, Kamminga et al. 2007; Houwing, Berezikov et al. 2008) and amphibians (Houwing, Kamminga et al. 2007; Houwing, Berezikov et al. 2008; Wilczynska, Minshall et al. 2009), indicating their conservation during evolution, and

thus their biological significance. In *Drosophila*, each of the three PIWI proteins, PIWI, AGO3 and AUB, interacts with a distinct population of piRNAs, as MIWI and MILI. Approximately 75% of the *Drosophila* piRNAs are derived from the highly repetitive sequences in the genome; thus a small number of them were initially identified as **r**epeat-**a**ssociated **si**RNAs (**rasi**RNAs) (Vagin et al., 2006). This is in contrast to the fact that only ~15% of mammalian piRNAs are derived from the repetitive sequences despite approximately 40% of the mammalian genome is annotated as repeats.

piRNAs differ from miRNAs not only in size and abundance but also in expression pattern and complexity. They are only detectable in the testis, especially become highly abundant after the pachytene stage of spermatogenesis (Aravin, Gaidatzis et al. 2006; Girard, Sachidanandam et al. 2006; Grivna, Beyret et al. 2006; Watanabe, Takeda et al. 2006). At present, at least a total of 55,000 individual piRNAs have been identifed to be associated with MIWI and MILI. The total number of piRNAs is expected to significantly exceed the known number, because many piRNAs have been identified by a single hit during the sequencing efforts.

Genomic mapping of the piRNA sequences showed that they are largely derived in clusters from the genome. Although individual piRNA sequences do not seem to be

evolutionary conserved unlike miRNAs, the piRNA clusters themselves show synteny among mouse, rat and human genomes (Aravin, Gaidatzis et al. 2006; Girard, Sachidanandam et al. 2006). Remarkably, most piRNAs correspond to intergenic regions previously thought to be untranscribed and thus referred as "junk" DNA. These peculiar features of piRNAs raised several questions: How are they produced and regulated? Are these piRNAs merely "junk" RNAs or do they have any functions? If so, what are their functions? In the following chapters, I am going to address these questions.

3. Biogenesis of piRNAs

3.1 Author Contribution

Ergin Beyret co-immuno-precipitated piRNAs with MILI and MIWI from the wild-type testes, cloned the MILI piRNAs and MIWI piRNAs presented here, performed β-elimination reaction and small RNA northern blotting on 24dpp *Miwi*$^{+/-}$ and *Miwi*$^{-/-}$ testicular RNA, co-immuno-precipitated MILI piRNAs from the *Miwi*$^{-/-}$ testis, performed the yeast-one-hybrid analyses.

Hang Yin performed the bioinformatics analyses for the questions raised by Ergin Beyret: extracted the piRNA sequences and mapped them on the genome; analyzed their genomic regions, sequences and size distribution; compared the homology of MILI piRNAs to MIWI piRNAs; predicted the putative piRNA promoter regions; performed the pairwise alignment of piRNAs within a cluster.

3.2 Summary

piRNAs have been recently discovered as a novel class of small non-coding RNAs with specific affinity for PIWI proteins in various organisms. Although their biogenesis is largely unknown, a sub-population of them rich in repeat-associated sequences has been shown to be produced and/or amplified via the interplay between

two PIWI proteins. Termed "ping-pong", this mechanism has also been shown to operate between MILI and MIWI2 in the primordial testis of the mouse. However the major piRNA expression occurs in the adult testis, when only MILI and MIWI are detectable among the three PIWI proteins. Here we characterized piRNAs in the post-natal mouse testis for their biogenesis, with a focus on the adult. For this purpose, we have comprehensively identified testicular MIWI piRNAs for the first time, and MILI piRNAs with a higher coverage than before. These piRNAs in the adult testis appear to be produced from transposon-poor, intergenic regions through long, single stranded precursors instead of the "ping-pong" mechanism. Additionally we provide evidence for the transcription of these precursors from certain regions on the genome under the control of Krueppel C2H2 type zinc finger transcription factors.

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3.3 Introduction

The biogenesis of piRNAs appears to be distinct from that of siRNAs and miRNAs**.** Similar to miRNAs and siRNAs, piRNAs show a high bias for Uracil at their 5` ends. This echoes the products of the RNase III enzymes, such as DROSHA and

DICER. However, computational prediction for piRNA precursors so far has not revealed any configuration similar to miRNA precursors. No common secondary structure motif among piRNA clusters has been found, either. These bioinformatic results have been augmented with the experimental observation that *Drosophila dicer1* or *dicer2* loss-of-function mutants do not show any reduction in rasiRNA/piRNA levels (Vagin, Sigova et al. 2006). Although this does not exclude the possibility of redundancy between the two *Dicer* paralogs, a more convincing line of evidence came from zebrafish. Houwing *et al* (2007) observed that zygotic *Dicer* is not necessary for piRNA biogenesis in the zebrafish. However, in this case, the maternally loaded DICER may be involved in piRNA production. Intriguingly, we (Grivna, Beyret et al. 2006) and Aravin *et al* (2007) showed that MIWI and MILI piRNAs are highly down-regulated in the MIWI and MILI-deficient mice, respectively, while miRNAs are unaffected. The same has been observed for at least one piRNA partner of ZIWI, the ortholog of MIWI in the zebrafish (Houwing, Kamminga et al. 2007). Given that PIWI proteins possess the "slicer" activities (Saito, Nishida et al. 2006; Gunawardane, Saito et al. 2007) it is possible that PIWI proteins are the enzymes involved in processing yet-to-be-identified precursors into piRNAs. Additionally, they may bind to mature piRNAs to form an effector complex. This interaction in turn may be necessary for the stabilization of piRNAs,

which might otherwise be rapidly degraded by cellular nucleases in the absence of their partner proteins.

Although biogenesis of piRNAs is largely elusive, one major mechanism, named "ping-pong model" has been proposed for the amplification of primary, pre-existing piRNAs and production of secondary, new piRNAs of transposonic origin in the fly, fish and mouse (Aravin, Sachidanandam et al. 2007; Brennecke, Aravin et al. 2007; Gunawardane, Saito et al. 2007; Aravin, Sachidanandam et al. 2008; Houwing, Berezikov et al. 2008; Klattenhoff and Theurkauf 2008). This model was first derived from the bioinformatic analyses of the fly piRNAs. Among the three fly PIWI proteins, AUB and PIWI associate with piRNAs rich in sequences corresponding to the anti-sense strands of transposons or their derivatives. AGO3, on the other hand, associates mostly with sense piRNAs of transposonic origin. Although AUB and PIWI piRNAs show the 5` Uracil bias, AGO3 piRNAs do not display such propensity. Instead, they frequently have Adenine on their 10th nucleotide position. Particularly, AUB piRNAs and AGO3 piRNAs that are derived from the same clusters are fully complementary to each other for the first 10 nucleotides. This observation raised the possibility of their production in a "slicer"-based mechanism, since the "slicer" activity corresponds to the tenth nucleotide position of the small RNA in cleaving RISCs (***Figure 12***). In this model,

AGO3 and AUB reciprocally act on the production of each other's piRNA partners. For instance, an AUB piRNA guides its partner protein AUB to transcripts with complementary sequences. The "slicer" activity of AUB incises the precursor, forming the 5` end of the corresponding AGO3 piRNA. Upon the formation of the 3` end by a yet-unknown enzyme, the resulting new piRNA is loaded onto AGO3, which in turn targets the AUB piRNA precursor and produces more AUB piRNAs (***Figure 12A***). In support of this amplification model, matching piRNAs are indeed present at a higher abundance in the *Drosophila* ovarian piRNA library and this profile is highly abolished in the absence of AUB (Malone, Brennecke et al. 2009). Likewise, loss of AGO3 not only destabilizes the AGO3 piRNAs but also AUB piRNAs (Li, Vagin et al. 2009). Currently it is not clear whether PIWI also engages in "ping-pong".

The "ping-pong" mechanism is a secondary processing mechanism, requiring complementary transcripts to a pre-existing pool of piRNAs. Since piRNAs are enriched in intergenic regions, any fully complementary transcript mainly comes from transposons. Indeed, matching piRNAs are rich in transposonic sequences. Hence, the "ping-pong" mechanism mostly concerns rasiRNAs, the repeat-associated sub-population of piRNAs, and does not elucidate the production of piRNAs that do not

display the 10nt match. On the other hand, how the primary processing occurs is not known.

The "ping-pong" mechanism accounts for the formation of the 5` ends of piRNAs but does not identify the enzymes responsible for the 3` cleavage. The ultimate size of a piRNA might be established by means of the interaction between an individual PIWI protein and the RNase responsible for the 3′ cleavage. ZUCCHINI and SQUASH are two such candidate RNases in *Drosophila* (***Figure 12A***) , as implied by the study of Pane *et al* (Pane, Wehr et al. 2007). ZUCCHINI belongs to the phospholipase-D/nuclease family, and SQUASH has a homology with *Agrobacterium tumefaciens* RNase HII. They both interact with AUB and are necessary for the production of rasiRNAs/piRNAs in *Drosophila.*

A peculiar feature of piRNAs is that they are 2`-O methylated at their 3` ends by HEN1, unlike animal miRNAs but similar to siRNAs and plant miRNAs (Kirino and Mourelatos 2007; Ohara, Sakaguchi et al. 2007). The mechanism and functional significance of this methylation is not known, yet. Nevertheless, it has been shown to confer stability to plant miRNAs (Yu, Yang et al. 2005; Yang, Ebright et al. 2006) and scanRNAs of *Tetrahymena thermophilia*, which are the counterpart of piRNAs in this

organism (Kurth and Mochizuki 2009). Such modification for piRNAs in animals may serve the same function. Alternatively, it might be necessary to recruit protein partners or may simply act as a protection mechanism against RNA editing (Li, Yang et al. 2005).

MILI and MIWI2 have been proposed to engage in "ping pong" for the biogenesis of rasiRNAs/piRNAs in pre-meiotic germ cells of the mouse testis (***Figure 12B***) (Aravin, Sachidanandam et al. 2008). Indeed, MIWI2 piRNAs are not detectable in the absence of MILI. However, although MIWI2 expression is not detected beyond 4dpp (Kuramochi-Miyagawa, Watanabe et al. 2008), the major boost of piRNA expression occurs after 14dpp, during meiosis (Aravin, Gaidatzis et al. 2006; Girard, Sachidanandam et al. 2006; Grivna, Beyret et al. 2006; Lau, Seto et al. 2006; Watanabe, Takeda et al. 2006). This period corresponds to the onset of MIWI expression, overlapping with MILI expression. Here we analyzed the biogenesis of piRNAs in the post-natal testis, and asked if MILI and MIWI engages in "ping-pong".

Figure 12: The "ping-pong" mechanism for piRNA biogenesis and amplification

A) "Ping-pong" between AUB and AGO3 in the fly. piRNAs produced by the "primary processing" start predominantly with Uracil (U). A primary piRNA guides AUB to a transcript that has a complementary sequence. The slicer activity of AUB cleaves this transcript and generates the 5` end of a new piRNA with a complementary sequence to the primary piRNA along the first 10 nucleotides, causing the Adenine (A) bias on the 10$^{th}$ nucleotide. ZUCCHINI (Zuc) and SQUASH (Squ) are two candidate RNases for the formation of the 3` ends in the fly. The generated piRNA is loaded onto AGO3 and re-starts another loop. Most AUB piRNAs start with U and display such complementarity with some AGO3 piRNAs.

B) "Ping-pong" in the mouse. Matching piRNAs have been observed among the piRNAs of both MILI and MIWI2 in the pre-pachytene testis with the most prominent being between MILI and MIWI2. Most MILI piRNAs start with U, while MIWI2 piRNAs are relatively enriched for A on the 10th nt position.

Hypothetical sequences are shown.

Figure 12A has been published in

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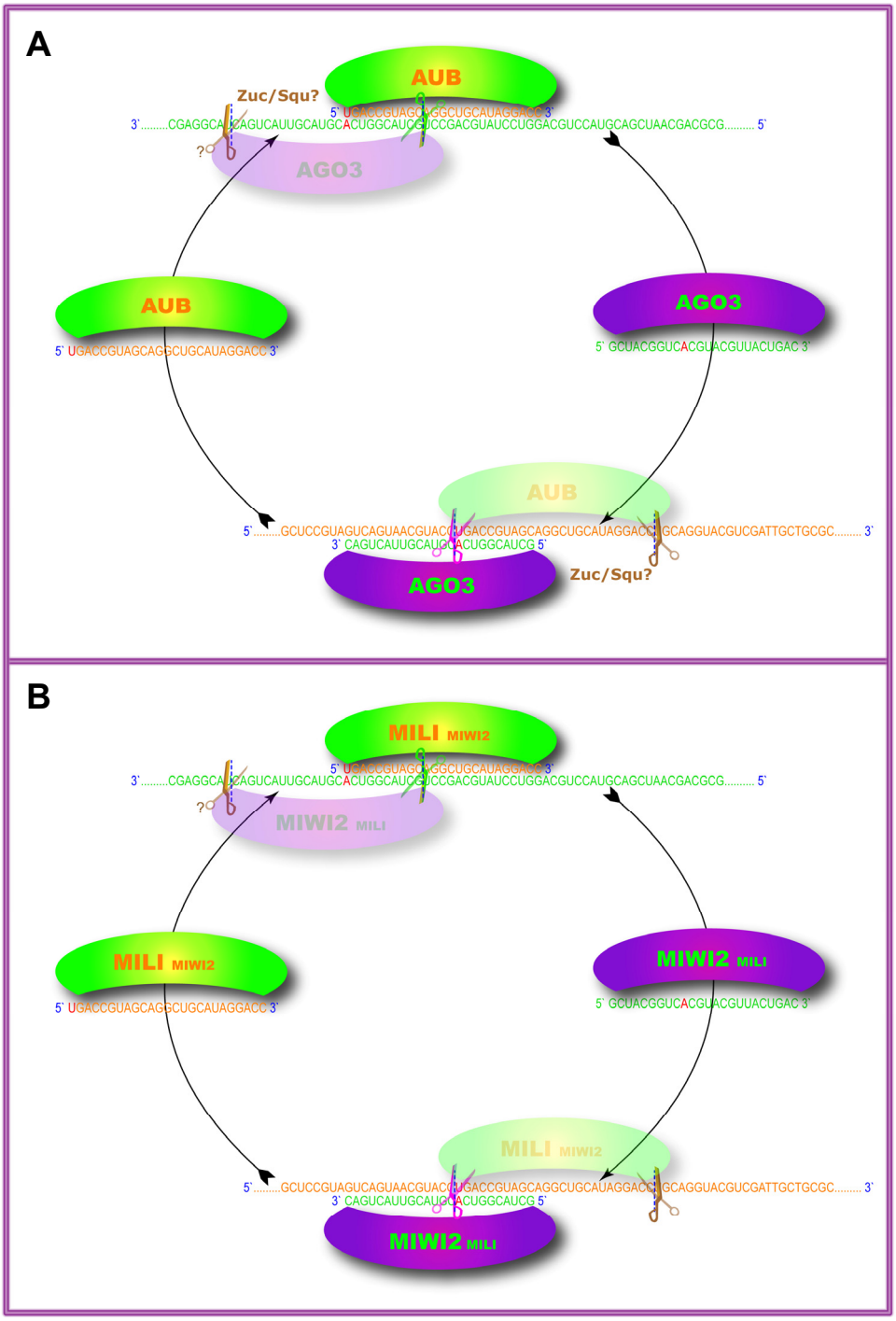

Figure 12: The "ping-pong" mechanism for piRNA biogenesis and amplification

3.4 Materials and Methods

3.4.1 Animals

The generation and genetic backgrounds of *Mili*$^{-/-}$ and *Miwi*$^{-/-}$ mice are described in (Deng and Lin 2002; Kuramochi-Miyagawa, Kimura et al. 2004) respectively. The homozygous mutants were obtained by crossing heterozygous males to homozygous mutant females. CD1 strain mice were used as the wild type model and in the identification of the piRNAs associated with MILI and MIWI.

3.4.2 Antibodies

MIWI and MILI complexes were immunoprecipitated with the R133 anti-MIWI serum (Deng and Lin 2002), and affinity purified MILI peptide antibody (Unhavaithaya, Hao et al. 2008) respectively.

3.4.3 Immuno-precipitation of the PIWI/piRNA Complexes

Immuno-precipitation was performed as described in (Unhavaithaya, Hao et al. 2008) from the crude lysates of mouse testes with the following modifications: Briefly, mice of the indicated ages were euthanized with cervical dislocation or asphyxiation with CO_2. Testes were dissected out, flash frozen in liquid nitrogen and stored at -80C^{o} until needed. After removing tunica albuginea, they were homogenized in [50mM

HEPES pH7.4, 150mM KOAc, 0.1% Triton X-100, 0.1% NP-40, 2mM MgOAc, 10% glycerol, 1mM DTT, 80u/ml RNaseOUT (Invitrogen), complete mini EDTA-free Protease inhibitor cocktail tablet (Roche), 200μM Cycloheximide] with a dounce homogenizer for at least 20 strokes under RNase-free conditions. Immunoprecipitations were performed overnight at 4C^{o} using protein A-Sepharose beads conjugated with affinity purified MILI peptide antibody or R133 anti MIWI serum. As the negative controls, antibodies blocked with the antigen peptide as in (Unhavaithaya, Hao et al. 2008) and antibody-free protein A-Sepharose beads were used for the MILI and MIWI immunoprecipitations, respectively. For the identification of the piRNAs, co-immunoprecipitated RNA was extracted from the immunoprecipitates with TRIZOL Reagent (Invitrogen), and 5` end-labeled with [$^{32}$P-γ] ATP by T4 Polynucleotide Kinase (New England Biolabs). Following the labeling, the sample was saturated with non-radioactive ATP to ensure all the 5` ends are phosphorylated, which is required to ligate the 5` adapter during the cloning. The samples were purified off un-incorporated nucleotides with Sephadex G-25 columns (Amersham Biosciences) and resolved with 10% Urea-PAGE alongside end-labeled 10 nucleotide DNA marker (Invitrogen) to distinguish the co-IPed piRNA population. piRNAs were extracted from the gel and precipitated with NaOAce/EtOH. Cloning was performed as in (Lau, Lim et al. 2001). The adapters were obtained from Integrated DNA Technologies (IDT). cDNAs of the piRNAs were amplified with PCR

and the resulting pi-DNA libraries were identified with high throughput pyro-sequencing by 454 (Roche).

3.4.4 Denaturing PAGE Analysis of piRNAs

Indicated amount of RNA samples were denatured in 50% formamide at 55C° for 15 minutes. The samples were resolved in polyacrylamide gel containing 6M Urea with 1X TBE. For the analysis of the radiolabeled samples, the gels were exposed to X-ray films (Kodak) or phosphorimager screen.

3.4.5 Small RNA Northern Blotting

Following denaturing PAGE, the gel was stained with 1µg/ml ethidium bromide in 1X TBE for ~15 minutes to assess the global piRNA content and integrity of the samples. Afterwards, the gel was de-stained in 1X TBE for ~10 minutes and transferred onto Hybond-N nylon membrane (Amersham Biosciences) in 1X TBE for 30 minutes at 350miliAmp using Hoefer TE 22 tank transfer unit at 4C°. The membrane was dried at 75C° for 5-10 minutes. The samples were cross-linked to the membrane with UV light of 120mJ/cm^2 followed by baking at 75C° for one hour. The membrane was stored at -80C° until needed or used immediately for probing. Hybridizations were performed overnight at 42C° in [5X SSC, 20mM Na_2HPO_4 at pH 7.2, 7% SDS, 1X Denhardt`s

Solution, 0.1mg/ml boiled salmon sperm DNA] following pre-hybridization in the same buffer composition without Denhardt's Solution for at least 30 minutes at 42C°. Probes were prepared as follows: DNA oligonucleotides with reverse complementary sequences for individual small RNAs were obtained from IDT and radiolabeled on their 5` ends with kinase reaction. For piRNAs, oligos were LNA modified to yield a Tm value of 75C° (+/-3C°). Labeled probes were boiled for 1 minute before adding into the hybridization buffer. Following hybridization, blots were washed twice in 1×SSC and 0.1% SDS at 42°C for 10 minutes and analyzed by PhosphorImager. If necessary, blots were stripped by boiling in 0.1% SDS for 10 minutes.

3.4.6 Analysis of the 3` Ends of the piRNAs in the *Miwi*$^{-/-}$ Testis

Modification status of the piRNA 3` termini was determined with β-elimination reaction as in (Horwich, Li et al. 2007). 40μg total RNA from a group of 23-24dpp *Miwi*$^{-/-}$ testes alongside 23-24dpp *Miwi*$^{+/-}$ testes was subjected to periodate oxidation and β-elimination. Samples were resolved with 20% urea-PAGE and analyzed with northern blotting. Controls with no β-elimination were performed alongside experimental samples by substituting H_2O for $NaIO_4$, borax-boric acid buffer and NaOH. miRNA-16, which is not modified on its 3` end, was used as a positive control for the β-elimination reaction.

3.4.7 Probes Used for Norhern Blotting

The nucleotides with the "+" sign on their left are LNA-modified.

mir-16 : CGC CAA TAT TTA CGT GCT GCT A

U6 snRNA : TGT GCT GCC GAA GCG AGC AC

piRNA probes:

piRNA T4 is a MIWI piRNA, the rest are MILI-associated.

Transposonic 2 : GGA C+CG +GT+C T+GC AG+C TGC TGA GTC GTA

Anti-sense Exonic : GGG ACA CA+C T+CA +GC+A C+TC +CT+T TGC A

Sense Intronic 1 : GGG CAG GTG +AG+A G+GA +TCC ATG GCC CA

Repeat-associated 1 : TA+T CA+T A+GT +CA+T CA+T CA+T C+AT +CG+T CA

piRNA T4 : TAG ACA ATT TTC AGT GTC CTA AGC TGT CTA

BZLES : GA+C AA+C GGG A+CA G+GA +TA+T T+TG AA+A T+CA

EI893 : CAG GA+C CA+C AAA GT+C A+CT +CT+T T+CC A

ETCZG : GGG A+CG GGC A+CA TGT GCA AGA +CTG CA

CMBG0 : GGG G+CA +TC+C T+CC ATT GAG ATG TA+T C+CC A

3.4.8 Bioinformatic Analysis of piRNAs

Bioinformatics analysis of the piRNAs cloned was performed by Dr. Hang Yin as below: Sequence extraction and genomic mapping was performed by using an in-

house small RNA analysis pipeline. Briefly, all extracted unique sequences were mapped to the *Mus musculus* genome released in July, 2007 (mm9) unless otherwise stated. If no matches were found, single mismatches were allowed in order to circumvent technical sequencing errors and polymorphisms between the experimental strain and that of the genomic map. If allowing single mismatches was not adequate, a final round of scanning was performed with double mismatches allowed. Any sequence that did not pass this stepwise mapping was termed "not defined". Mapped candidates were further aligned with mouse noncoding RNA sequences to exclude known microRNAs, snoRNAs, snRNA, rRNAs, etc. The remaining small RNAs are considered *bona fide* piRNAs.

In order to evaluate the involvement of the "ping-pong" machinery in the biogenesis of adult testicular piRNAs, piRNA libraries were pairwise analyzed for the presence of matching piRNAs that are complementary along their first 10 nucleotides. As a negative control, position specific score matrices were determined for the piRNA libraries of MILI and MIWI, and random piRNAs were generated based on these matrices. For both libraries, random piRNAs were generated in the same number as the sequenced piRNAs. The percentages of the matching piRNAs shared between these randomly generated libraries were likewise calculated.

3.4.9 Yeast-one-hybrid Analysis

Yeast strains used:

YM4271: *MATa*, *ura3-52*, *his*3-*200, lys2-801, ade2-101, ade5,* ***trp1-901***, *leu2-3, 112, tyr1-501,gal4*Δ, *gal80*Δ, *ade5 : : hisG*

Y187: *MAT*α*, ura3-52*, *his*3-*200, ade 2-101*, *trp 1-901, leu 2-3*, *112*, *gal4*Δ, *met–*, *gal80*Δ, *URA3 : : GAL1*UAS-*GAL1*TATA-*lacZ, MEL1*

AH109: *MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4*Δ, *gal80*Δ, *LYS2 : : GAL1UAS-GAL1TATA-HIS3, MEL1 GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1TATA-lacZ*

Yeast-one-hybrid analysis was performed as in (Bart Deplancke 2006). Briefly, 27052634-27052942 region of the chromosome 17 and 54047985-54048243 of the chromosome 9 according to the UCSC mm8 database were PCR amplified from the genomic tail DNA of CD1 strain mouse, and cloned into the pMW#2-HIS3 reporter destination vector- (Addgene plasmid 13349) and pMW#3-lacZ reporter destination vector- (Addgene plasmid 13350) (Bart Deplancke 2006; Deplancke, Mukhopadhyay et al. 2006). The resultant reporter constructs were integrated respectively into the HIS3

and Ura loci of the YM4271 yeast strain. Self activation of the lacZ and HIS3 reporters of the transformants were respectively assessed based on the color development with β-galactosidase assay, and their growth level on increasing concentrations of 3-**A**mino-1,2,4-**t**riazole (3-**AT**). Colonies that do not grow on the lowest 3-AT concentration and develop the minimum level blue color with β-galactosidase assay were selected. Library screening was performed by mating these "bait" strains with the Y187 yeast strain that had been previously transformed with testicular total cDNA library, which carries Leu marker (Clontech 638872). Colonies with the activated reporters were selected based on their growth level on –His, -Ura, -Leu plates with the lowest 3-AT concentration that prevents the growth of the "bait" strains alone (50mM for Chr17-1 and 20mM for Chr9-1), and screened for blue color development with β-galactosidase assay. In order to assess the background level growth and color development, "bait" strains were mated with Y187 strain that carries T antigen in fusion to GAL4-AD (Clontech/pGADT7-T). As a positive control, yeast strain AH109 that carries a p53-GAL4-BD fusion construct (Clontech/pGBKT7-53) was mated with Y187 that has the T antigen-GAL4-AD fusion construct.

3.5 Results

3.5.1 Post-natal Testicular piRNAs in the Mouse are Largely Derived From Non-transposonic, Intergenic regions

In order to gain an insight about the biogenesis of piRNAs in the post-natal testis, we first bioinformatically addressed testicular piRNAs of 7-8dpp, 13dpp and 2 months old adult mice. The testicular germline consists of only spermatogonia at 8dpp. At 13dpp, it has reached meiosis-I and contains early primary spermatocytes up to pachytene stage in addition to spermatogonia. At 2 months, the full complement of spermatogenic cells is present. Since MIWI piRNAs have not been comprehensively identified before and the coverage of MILI piRNAs identified (Aravin, Gaidatzis et al. 2006; Aravin, Sachidanandam et al. 2007; Aravin, Sachidanandam et al. 2008) is far from saturation (Betel, Sheridan et al. 2007), we first cloned the corresponding piRNAs from the testicular immunoprecipitates of MILI and MIWI (***Figure 13A***), and identified with high throughput sequencing. Approximately 32000 and 14000 unique sequences were cloned from adult testicular MILI and MIWI complexes respectively (***Figure 14A***). Despite the overall high number of piRNAs identified, most of them were hit only once during the sequencing in both populations, indicating that the sequencing effort is still unsaturated for both.

In the "ping-pong" mechanism, transcripts with fully complementary sequences to the available pool of piRNAs are necessary for the production of the secondary piRNAs. Hence, piRNAs involved in this mechanism are enriched for the sequences of genic and transposonic RNAs. For this reason, we evaluated the genomic regions from which the testicular piRNAs are derived. Mapping of the piRNAs on the genome revealed that majority of them is derived from single sites, and repeat-associated sequences are under-represented considering that such sequences form approximately 40% of the mouse genome (***Figure 13B***). However we also noticed that relative to the adult testis, pre-meiotic testis is somewhat enriched for piRNAs that have more than one mapping site. Evaluation of the annotation of the corresponding sequences indicated that our testicular piRNA libraries at 7-8dpp and 13dpp, as well as at adult, are almost equally poor in transposonic sequences. Instead all these piRNA libraries are mostly derived from non-transposonic, intergenic regions implying their biogenesis is mostly, if not completely, independent of the "ping-pong" machinery (***Figure 13C***).

We noticed that MILI piRNAs of pachytene stage at 13dpp are relatively rich in sequences derived from coding genes. This stage corresponds to an overall increase in transcriptional activity. It is possible that the resultant transcripts might enter the piRNA biogenesis pathway to be processed into the observed genic piRNAs.

Supporting this view, most of these piRNAs correspond to the sense strand of the genes (*Data not shown*).

Figure 13: piRNAs are largely derived from non-transposonic, intergenic regions during post-natal testis development (Bioinformatics in Panels B and C by Hang Yin/Haifan Lin Lab)

A) MIWI and MILI associate with piRNAs in the testis. piRNAs isolated from the immunoprecipitates of MILI and MIWI were analyzed with 5` end-labeling and 15% Urea-PAGE. Notice the size difference between MILI piRNAs and MIWI piRNAs.

B) Pie charts showing the number of the genomic hits of sequenced piRNAs from the MILI and MIWI complexes. MILI piRNAs predominantly match to single (unique-mapping) sites on the genome through-out spermatogenesis as is the case for adult testicular MIWI piRNAs. Repeat-associated piRNAs form a bigger fraction by 13dpp relative to the adult testis.

C) Pie charts showing the genomic annotations of sequenced piRNAs from the MILI and MIWI complexes. MILI piRNAs are largely derived from intergenic regions and poorly from transposonic regions through-out spermatogenesis, as adult testicular MIWI piRNAs. 13dpp pachytene stage testis is rich in piRNAs that correspond to coding genes relative to the other stages examined. These piRNAs are mostly sense to the corresponding genes.

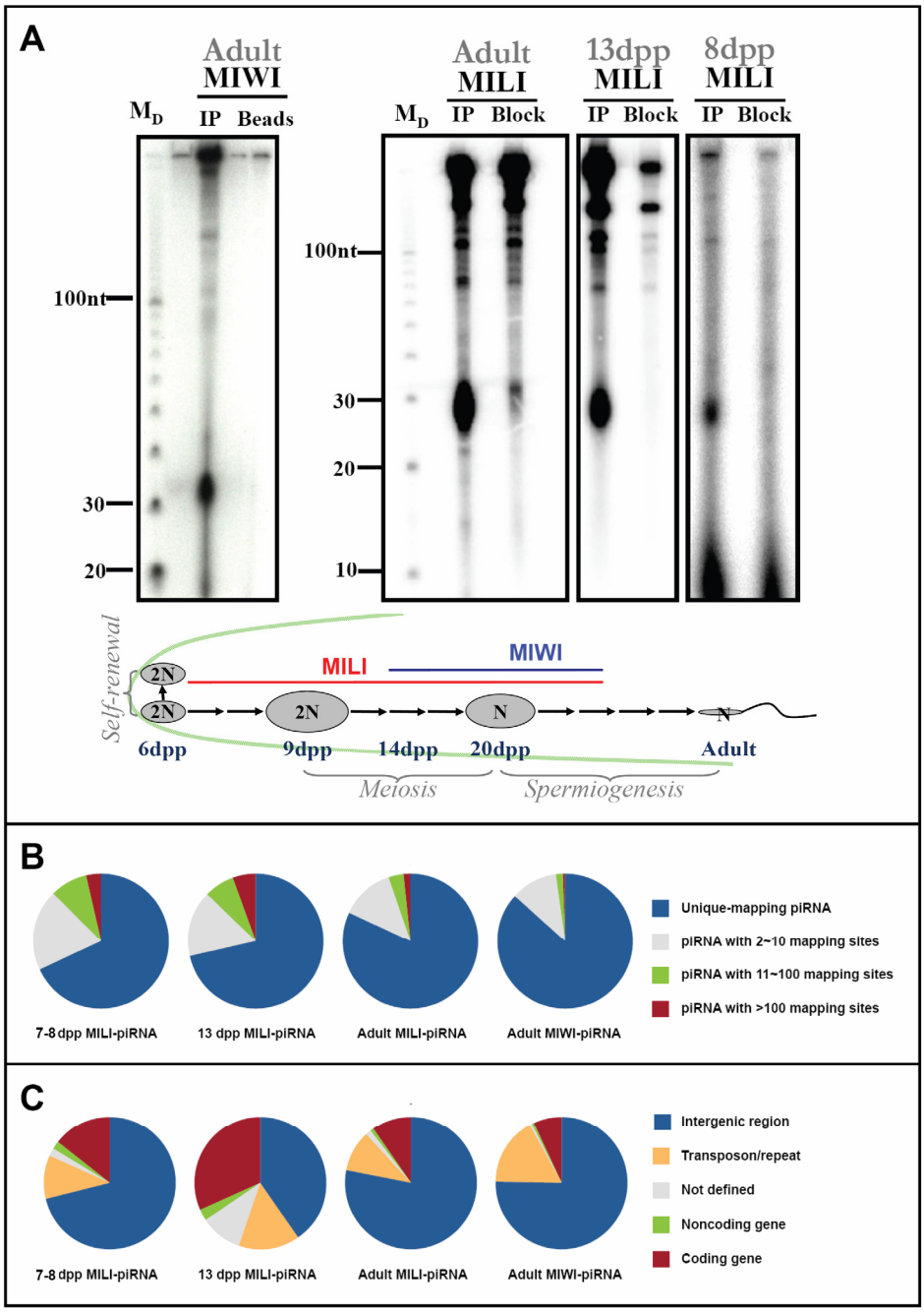

Figure 13: piRNAs are largely derived from non-transposonic, intergenic regions during post-natal testis development

(Bioinformatics in Panels B and C by Hang Yin/Haifan Lin Lab)

3.5.2 MILI piRNAs and MIWI piRNAs in the Adult Testis are Highly Homologous to Each Other and Differ Mainly in Length

We asked if MILI and MIWI piRNA populations in the adult testis carry a sequence signature. piRNAs generated by the "ping-pong" machinery show complementary sequences along their first 10 nucleotides due to the position of the "slicer" activity. Since primary piRNAs are enriched for 5` U, "ping-pong" generates secondary piRNAs with A bias on the 10$^{th}$ nucleotide position. Comparison of the sequences showed that both populations are biased for Uracil on their 5` ends and neither displays any nucleotide bias on the 10th nucleotide position. Further analysis showed that MILI piRNAs and somewhat MIWI piRNAs are biased for Guanine on their second nucleotide position (***Figure 14B***). Additionally, while the last two nucleotides of the MILI piRNAs tend to be Cytosine, such tendency is not observed for the MIWI piRNA population. As shown before (Aravin, Gaidatzis et al. 2006; Girard, Sachidanandam et al. 2006; Grivna, Beyret et al. 2006), MILI interacts with piRNAs ranging from 22nt-31nt with a median lenght of 26nt and MIWI interacts with piRNAs of 26nt-33nt in size, preferentially with 30nt (***Figure 14C***). Despite these differences, piRNA populations of MILI and MIWI in the adult are highly homologous to each other, and thus unlikely to display the 10nt complementarity (***Figure 14D***).

Figure 14: piRNA populations of MILI and MIWI are highly homologous to each other and differ mainly in length (Bioinformatics by Hang Yin/Haifan Lin Lab)

A) Mapping of the adult testicular piRNAs shows they are complex in sequence and mostly originate from single sites on the genome. 32000 and 14000 different piRNA sequences were identified from the immunoprecipitates of MILI and MIWI respectively.

B) Homology comparison of the piRNAs within each population shows MILI piRNAs are biased for U on the 5` end, somewhat for G on the second-most position and C on the last nucleotide. MIWI piRNAs are biased for 5` U only.

C) Size distribution of the MIWI piRNAs and MILI piRNAs shows that the two populations differ in length.

D) Homology comparison of the two piRNA populations by a home-made algorithm allowing a maximum of two mismatch shows they are highly homologous to each other.

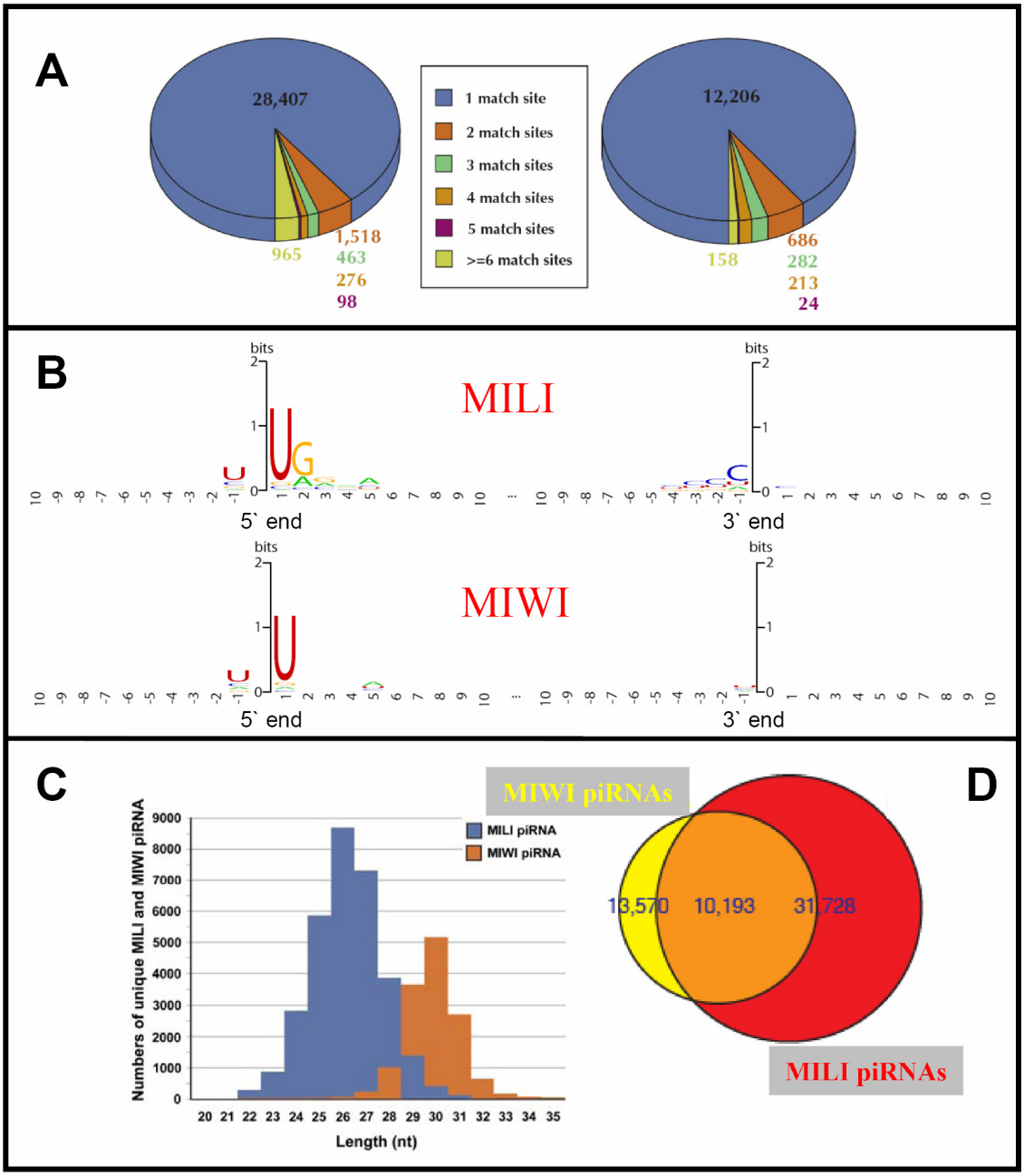

Figure 14: piRNA populations of MILI and MIWI are highly homologous to each other, display 5` Uracil bias only, and differ mainly in length

(Bioinformatics by Hang Yin/Haifan Lin Lab)

3.5.3 piRNAs in the Adult Testis are not Produced by the "Ping-Pong" Mechanism

Next, we directly analyzed the adult testis for the presence of matching piRNAs that show the 10nt complementarity. However, we found that such propensity does not significantly exist, even among the piRNAs with transposonic sequences (***Figure 15***).

Figure 15: Adult testis does not comprise matching piRNAs with complementary sequences along their first 10 nucleotides (Bioinformatics by Hang Yin/Haifan Lin Lab)

Shown are the charts that indicate the degree to which complementary 5′ 10-mers ("ping-pong" signature) are found in pairwise library comparisons. The chart on the left is based on all sequenced piRNAs in the MILI or MIWI complexes, whereas the one on the right is based on those with transposonic sequences only. The values belong to the libraries on the row.

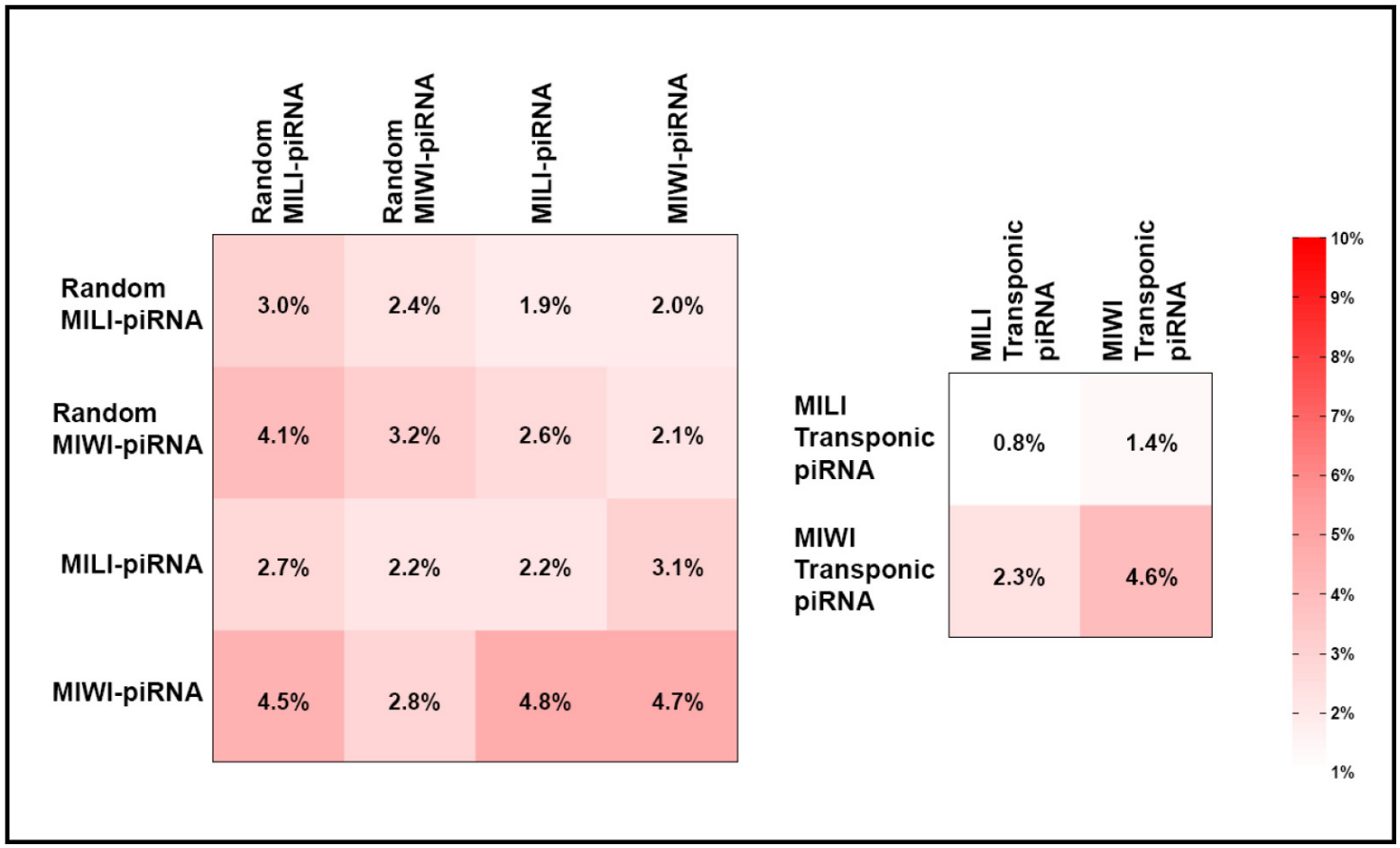

Figure 15: Adult testis does not comprise matching piRNAs with complementary sequences along their first 10 nucleotides

(Bioinformatics by Hang Yin/Haifan Lin Lab)

Next, we sought to experimentally address the involvement of the "ping-pong" machinery among the adult testicular piRNAs. Since the "ping-pong" mechanism is a feedback loop, requiring the cooperative physical interaction of two PIWI complexes carrying primary and secondary piRNAs, we asked if MILI and MIWI complexes are inter-dependent and/or interact with each other. For this purpose, we first consecutively immuno-precipitated MILI and MIWI complexes from the same testicular extract to see if they interacted. Analysis of their piRNA content with kinase labeling showed that MILI and MIWI interacted with distinct populations of piRNAs in the same extract (***Figure 16A***). Likewise immunoblotting sub-samples from these immunoprecipitates for MILI and MIWI did not show any signs of interaction *(Data not shown).*

Then, we used northern blotting for representative piRNAs to test if MILI piRNAs are affected in the absence of MIWI. We have previously reported that MIWI piRNAs are down-regulated in *Miwi*$^{-/-}$ testis (Grivna, Beyret et al. 2006) with Mili expression unaffected (Kuramochi-Miyagawa, Kimura et al. 2004). If a positive feed-back loop operates between the two PIWI complexes in the production of each other's piRNAs, then both piRNA populations are expected to be down-regulated in the absence of one of the complexes. Since piRNAs of MILI and MIWI are highly homologous to each other and differ only in size, northern blotting for one piRNA

typically yields two major bands corresponding to the MIWI piRNA and that of MILI. Our northern blotting analyses show that, while MIWI piRNAs are undetectable in *Miwi*$^{-/-}$ testes at 24dpp, MILI piRNAs are not down-regulated (***Figure 16B***).

The PIWI proteins in the fly interact with the piRNA methyl transferase and this interaction may be necessary for the methylation of piRNAs (Saito, Sakaguchi et al. 2007). Therefore, in order to ascertain the small RNAs we observe in *Miwi*$^{-/-}$ are indeed MILI piRNAs but not de-stabilized MIWI piRNAs, we tested if they are still modified. Noticeably, β-elimination reaction on them did not result a shift in migration unlike the internal miRNA control, indicating they retain their modification (***Figure 16C***). Additionaly, this signal is depleted upon immuno-precipitation of MILI, confirming it as the MILI piRNAs (***Figure 16D right panel***).

It is possible that MILI piRNAs are overall down-regulated while the particular ones we tested are not. Therefore, we analyzed MILI piRNAs at the global level by comparing the piRNA content of the MILI immunoprecipitates from 24dpp *Miwi*$^{-/-}$ versus *Miwi*$^{+/-}$ testicular extract with end labeling. Confirming the northern analyses, we did not detect any obvious difference at the global level, either (***Figure 16D***).

Figure 16: MILI piRNAs are not down-regulated in the absence of MIWI

A) MILI and MIWI interact with independent populations of piRNAs. MILI and subsequently MIWI complexes were immuno-precipitated from the same adult testicular extract. Associated piRNAs were isolated and analyzed as in Figure 13.

B) Northern blotting for representative piRNAs reveals MILI piRNAs are not down-regulated in the absence of MIWI. 20μg total testicular RNA samples from 24dpp *Miwi*$^{+/-}$ and *Miwi*$^{-/-}$ were resolved with 15% Urea-PAGE and northern blots were probed for individual piRNAs. U6snRNA (U6) was used as an internal loading control. Ethidium bromide staining was used to assess the global level of piRNAs.

C) Terminal nucleotides of MILI piRNAs retain their 2`-O-methyl group in the absence of MIWI. 40μg total testicular RNA samples from 23-24dpp *Miwi*$^{+/-}$ and *Miwi*$^{-/-}$ were either subjected to β-elimination reaction or not, resolved with 20% Urea-PAGE. miRNA mir-16 was used as an internal loading control and positive control for the β-elimination reaction. Ethidium bromide staining and northern blotting were used to assess the piRNAs at the global and individual levels respectively.

D) Immuno-precipitation of MILI from *Miwi*$^{-/-}$ testes shows that MILI piRNAs overall are not down-regulated in the absence of MIWI.

Left panel: Immuno-precipitates (IP). Associated piRNAs were isolated and analyzed as in Figure 13. Right panel: Negative image of ethidium bromide stained control samples.

I: Input, SN: Supernatant, T: 70μg Total testicular RNA, Adlt: Adult. Adult *Mili*$^{-/-}$ testis was used as the negative control for MIWI piRNAs as well as MILI piRNAs since the spermatogenic arrest occurs before MIWI expression starts.

Tp2: Transposonic 2, A-exo: Anti-sense exonic, Rapi1: Repeat-associated 1, S-Intro: Sense intronic, T4: piRNA-T4. The annotations indicate the genomic regions piRNAs are derived from (e.g; "anti-sense exonic" is a piRNA that corresponds to the anti-sense strand of an exon). Long and short arrows denote piRNAs of MIWI and MILI respectively. Blank arrow indicates the location of mir-16.

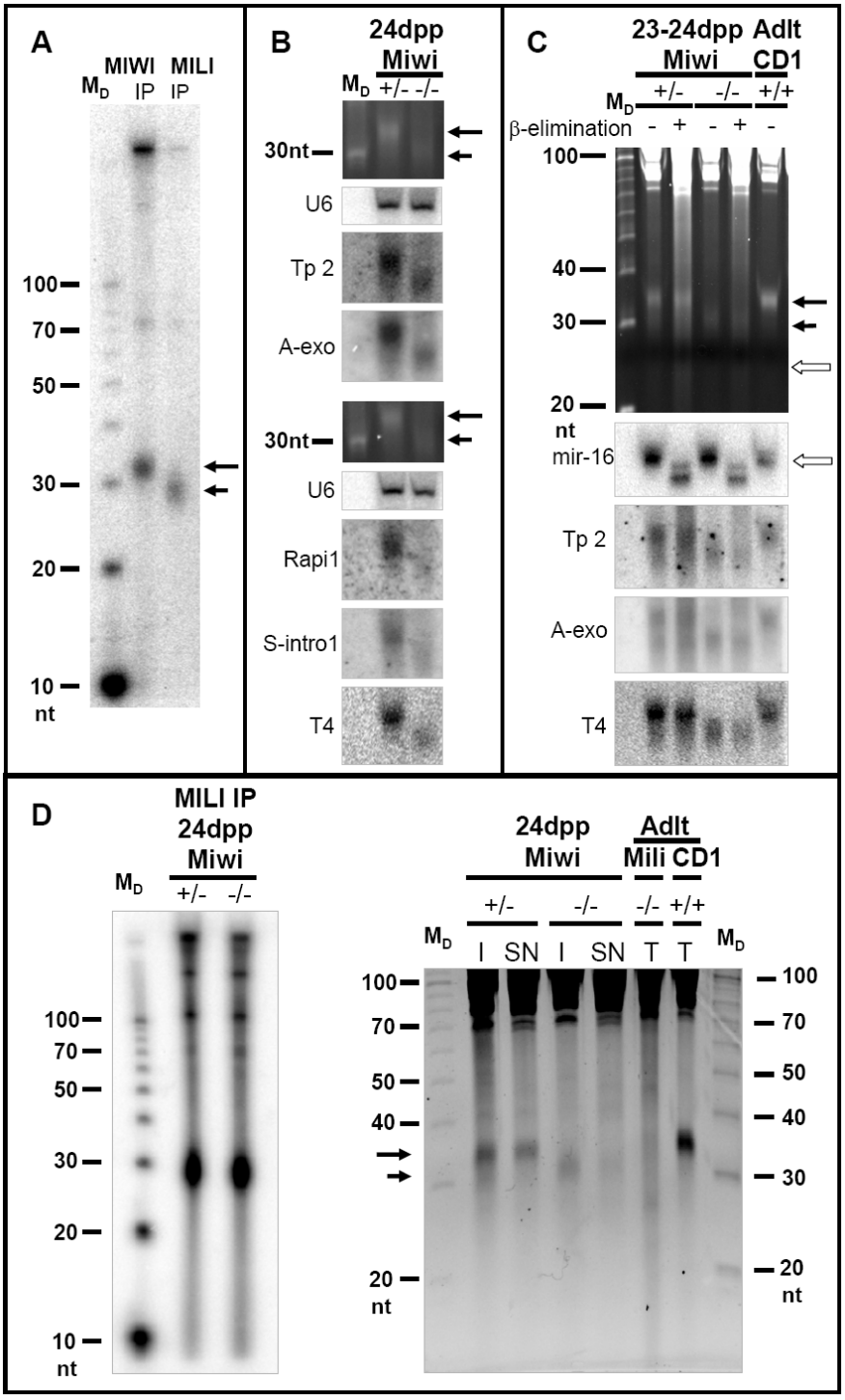

Figure 16: MILI piRNAs are not down-regulated in the absence of MIWI

3.5.4 piRNAs in the Adult Testis are Produced by the Primary Processing Mechanism

Upon concluding that piRNAs in the adult mouse testis are not produced by the "ping-pong" machinery, we asked if we could deduce their mode of biogenesis based on the genomic regions they are derived from. Plotting the piRNAs on the genome showed that, most of them originate in clusters. Interestingly, we found that both piRNA populations of MILI and MIWI are derived from the same genomic regions and strand, which elucidates their close homology (***Figure 17A and 17C***).

We observed that piRNAs within a cluster are derived from one genomic strand and in most of the clusters, this strand origin of synthesis is abruptly switched to the other strand at a certain position, resulting in a bi-directional profile (***Figure 17B***). These piRNA-free positions (***Figure 17C***) seem to be the promoter regions directing transcription of long, single stranded precursors of the piRNAs in each direction based on several observations: First, mouse testes exceptionally contain long ESTs with unknown functions and some of the piRNA clusters seem to be their origin, indicating that these ESTs might be the precursors of the corresponding piRNAs (***Figure 17C***). Moreover, such ESTs also switch direction at approximately the same positions as the piRNAs do. Second, clustered piRNAs show head-to-tail homology as if they are processed from the same type of precursors (***Figure 17D***). Third, the edges of each

cluster are less dense in piRNA production, which may be due to the processivity of the RNA polymerase driving the transcription of the precursor (***Figure 17B***). Interestingly, the strand switch of both MILI piRNAs and MIWI piRNAs occurs at the same piRNA-free region indicating that the parameters of their biogenesis are the same (***Figure 17C***).

Figure 17: MILI piRNAs and MIWI piRNAs in the adult testis are processed from single stranded precursors derived from the same genomic strands and loci (Bioinformatics by Hang Yin/Haifan Lin Lab)

A) Mapping of the MILI piRNAs and MIWI piRNAs reveals they are both derived from the same regions and usually in clusters. Cluster locations are indicated with proportionally sized triangles according to the number of piRNAs in each cluster. Chromosome 5 has 3 major clusters, two of which are in close proximity, which are below the resolution of this graph.

B) piRNAs are derived from one of the genomic strands only, and while piRNAs in a few clusters are derived from the same genomic strand along the entire cluster locus, in most, they abruptly switch to the opposing strand at the center resulting in a bi-directional profile. Only MILI piRNAs are shown.

C) Higher resolution of a bi-directional cluster on Chromosome 17 shows that MILI and MIWI piRNAs are derived from the same genomic strands and switch origin of synthesis to the other strand at a same, piRNA-free region.

D) Pairwise alignment of clustered piRNAs shows they have overlapping sequences.

Each bar in B and C indicates one piRNA sequence. Bar height or length represents the size of the piRNA. piRNAs in blue or green are derived from the Watson strand; orange and red is for the Crick strand and ESTs are in black. The asterisks on

Chromosomes 9 and 17 show the piRNA clusters whose putative promoter regions were used for yeast-one-hybrid analyses in this work.

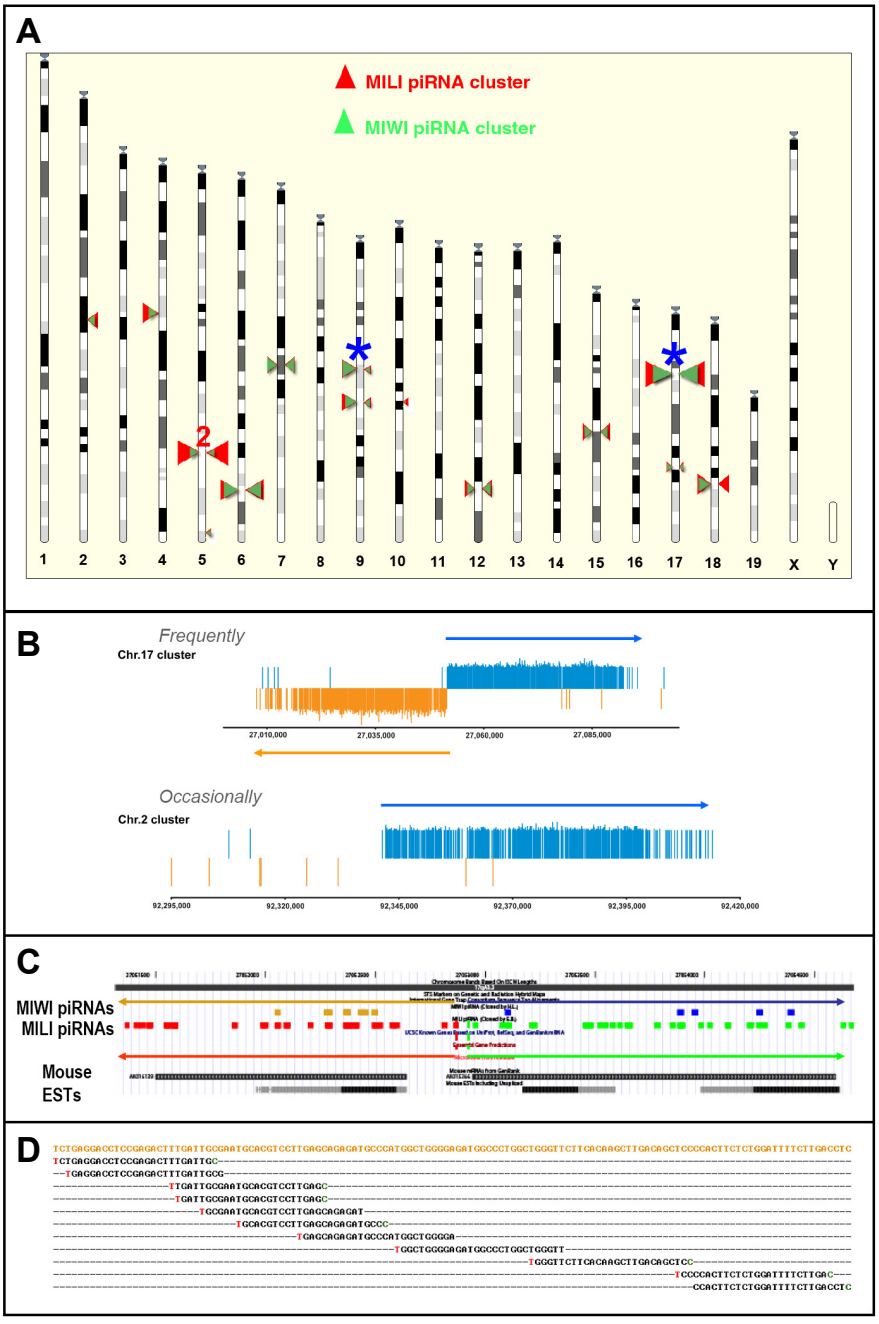

Figure 17: MILI piRNAs and MIWI piRNAs in the adult testis are processed from single stranded precursors derived from the same genomic strands and loci

(Bioinformatics by Hang Yin/Haifan Lin Lab)

Next, we sought to experimentally address if the regions of the strand switch we identified in the bi-directional clusters are indeed promoter sites for the precursors of the piRNAs therein. For this purpose, we first looked for any consensus sequence or motif among them; however we were unable to identify any. Therefore, we decided to focus on a few representative clusters and address each of them individually. We selected the first piRNA cluster on Chromosome 17 (26936426-27108585 mm8) (***Figure 17 A* asterisk on Chromosome 17**) since it is the most populated bi-directional cluster with more than 2500 MILI-associated single-mapping piRNAs. Moreover, its piRNA-free region at the center of the stand switch (27052881-27052942 mm8) is only 63bp long, which is the shortest among all the clusters. However, deletion of this piRNA-free region did not lead to any phenotype or any change in the levels of the piRNAs from either direction indicating it is not necessary for their biogenesis (***Figure 18***).

Figure 18: The minimum piRNA-free region at the junction of the strand switch in the Chr17-1 piRNA cluster is not necessary for the piRNA biogenesis from this cluster

A) PCR genotyping confirms the targeted region is successfully replaced with the targeting construct. Each lane corresponds to a different individual mouse. Shown is the negative image of the ethidium bromide stained gel.

B) Northern blotting for representative piRNAs uniquely mapping to the Chr17-1 piRNA cluster indicated that piRNA biogenesis is not impaired in the absence of the minimum piRNA-free region at the junctional area of this cluster.

17-1 +/+ vs. -/- : Wild type vs. knock-out of the (27052881-27052942 mm8) region on the chromosome 17. Total RNA from adult *Mili*$^{-/-}$ testis (Mili -/-), was used as a negative control for global piRNA expression. The inscriptions beneath the piRNA numbers indicate the sequencing IDs of the individual piRNAs. Uppermost panels are the negative images of the ethidium bromide stained gels showing total piRNAs, while the middle panels correspond to the individual piRNA northern blots. U6snRNA northern blots were used as loading controls.

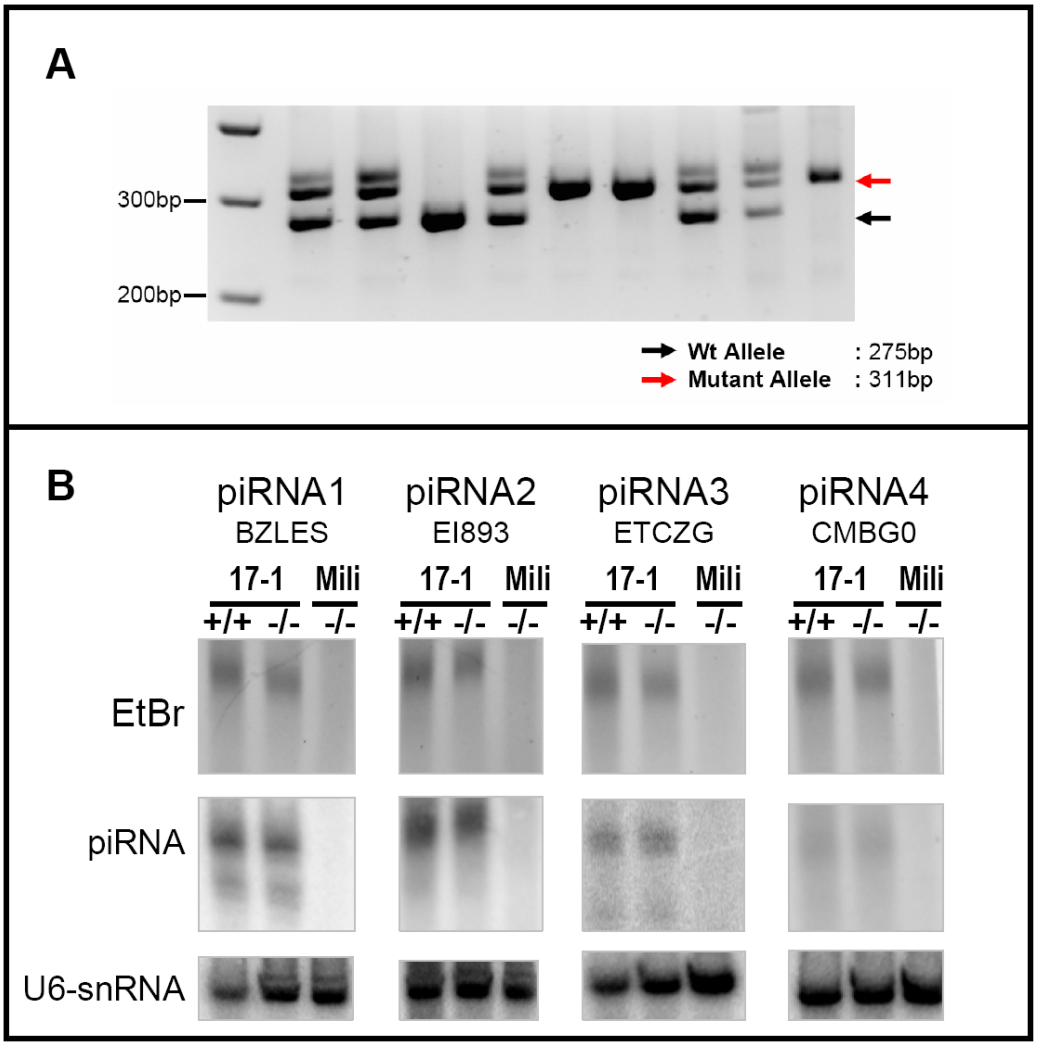

Figure 18: The minimum piRNA-free region at the junction of the strand switch in the Chr17-1 piRNA cluster is not necessary for the piRNA biogenesis from this cluster

Next, assuming that our bioinformatic identification of the promoter region was not accurate enough, we further zoomed out of this region on the UCSC genome browser until we were able to detect two long ESTs running in each direction. Considering that these ESTs might be the precursors of the piRNAs, we used yeast-one-hybrid (Y1H) analysis to identify the testicular proteins that interact with the corresponding 309 bp long region (27052634-27052942 mm8) in between their start sites ("Chr17-1 promoter" herein), which also covers the 63bp long piRNA-free region. In order to decrease the false positive rates, we used two reporter constructs carrying *HIS3* or *lacZ*, and integrated both into the genome of the YM4271 yeast strain (***Figure 19A***). We detected 58 clones that strongly activated both of the reporters. We were not able to identify 3 of these due to technical reasons. Out of the 55 clones we identified, 33 of them (60%) were **K**rueppel-**l**ike **f**actor 4 (**KLF** 4) and 9 of them (16%) were related transcription factors (KLF15 and 17, Transcription factor SP1 and 3) (***Figure 19B*** and ***Table 5***). Moreover, the KLF4 clones corresponded to 9 different cDNA fragments, indicating that KLF4 is likely an authentic interactor of Chr17-1 promoter. Likewise, we performed the same analysis for the 259bp long piRNA-free region (54047985-54048243 mm8) in between the strand switch of the first cluster on the Chromosome 9 (Chr9-1 herein) (***Figure 17 A* asterisk on Chromosome 9**) as another example. Out of the 192 clones that strongly activated both of the reporters, we were able to successfully identify

184 of them. 181 (98%) of these double positives corresponded to 5 different cDNA clones of **Zi**n**c** **f**inger protein 821 (**ZNF**821) (***Figure 19C*** and ***Table 5***), which is a Krueppel C2H2 type zinc finger transcription factor as KLF4.

Figure 19: Krueppel C2H2 type zinc finger transcription factors interact with the putative promoters of piRNA precursors

A) Identification of the protein interactors of a DNA sequence via yeast-one-hybrid analysis. The DNA of interest is cloned upstream of a reporter, which displays a minimum expression. In parallel, a cDNA library is fused with the **a**ctivation **d**omain (**AD**) of GAL4. The respectively resultant "bait" and "prey" constructs are introduced into a host yeast. Subsequent interaction between the library protein and the DNA of interest brings the AD domain of GAL4 close to the reporter promoter whereby upregulates reporter activity.

HIS3 and lacZ reporters were used in this study for each piRNA promoter to be analyzed. Mouse testis cDNA library was used as the "prey".

B) The chromosome 17-1 promoter interacts with KLF4. 60% of the "prey"s identified as the strong activators of both HIS3 (left panel) and lacZ (right panel) reporters corresponds to KLF4 (pie chart). 16% corresponds to related transcription factors, KLF15 and 17, Sp1 and 3. Nine different cDNAs of KLF4 (1-9) and two different cDNAs of Sp3 (Sp3-1, Sp3-2) were identified.

K: KLF, 2+: a cDNA clone of KLF4 that is 17nt longer than the KLF clone 2 on the 5` end.

C) The chromosome 9-1 promoter interacts with ZNF821. 98% of the "prey"s identified as in **B** corresponds to ZNF821 (pie chart). Five different cDNAs of ZNF821 were identified.

Library screening was performed by mating the "bait" strains with the "prey" strain. The background (Bcgr) and positive (+) controls were used as a reference during the screen (see "Materials and Methods").

Bcgr: "Bait" strain mated with Y187 strain that carries T antigen in fusion to GAL4-AD.

+: AH109 strain that carries p53 in fusion to GAL4-BD mated with Y187 that has the T antigen-GAL4-AD fusion construct.

-: AH109 strain that carries Lamina in fusion to GAL4-BD mated with Y187 that has the T antigen-GAL4-AD fusion construct

Colonies were grown on –His, -Ura, -Leu plates with the indicated amounts of 3-**A**mino-1,2,4-**t**riazole (3-**AT**).

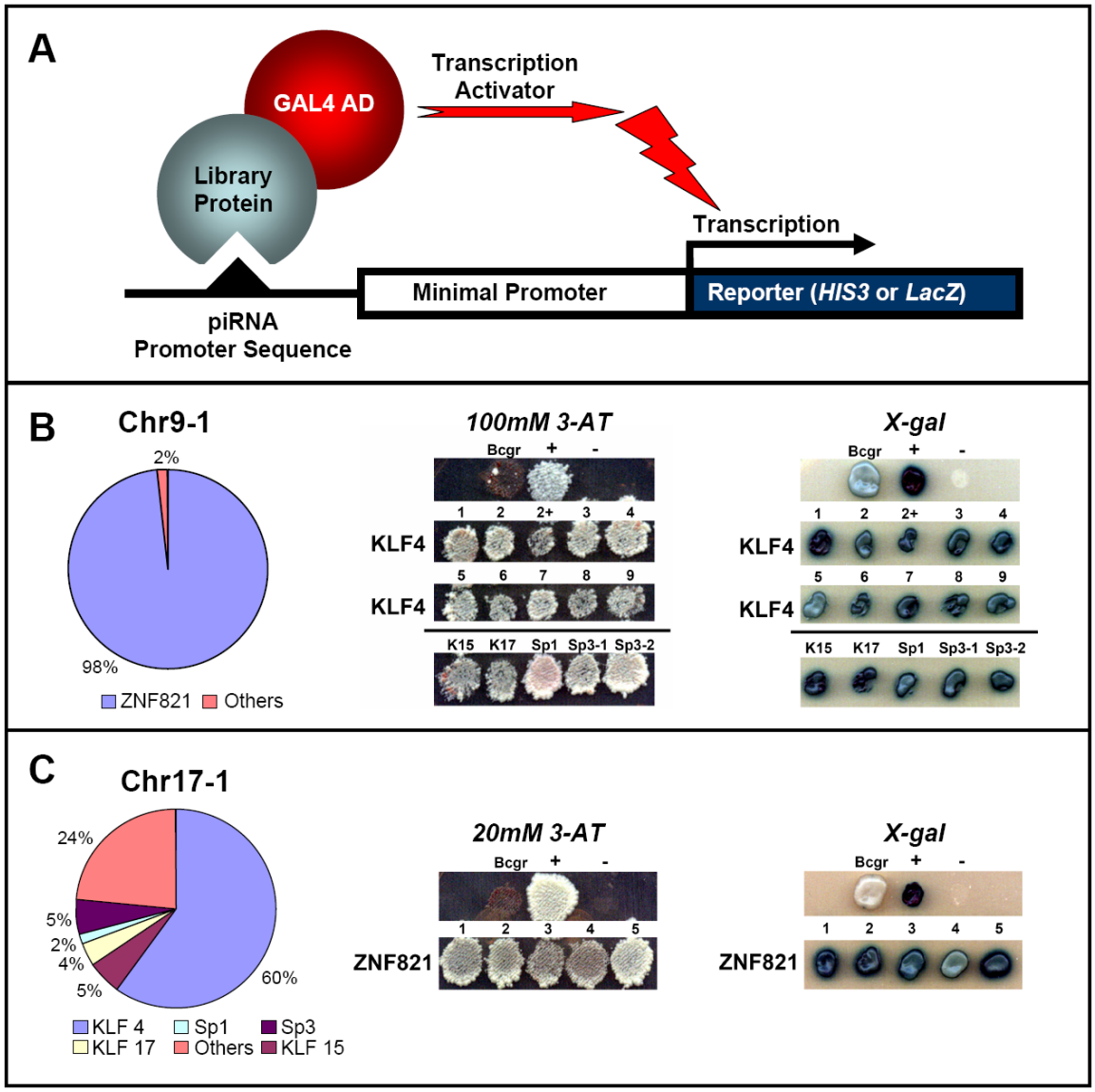

Figure 19: Krueppel C2H2 type zinc finger transcription factors interact with the putative promoters of piRNA precursors

Table 5: Identified protein interactors of the putative promoters of piRNA precursors

Screening with yeast-one-hybrid analysis identified KLF4 in 33 colonies out of 55 as the protein interactor of the putative promoter region in the Chromosome 17-1 cluster, and ZNF821 in 181 colonies out of 185 for the Chromosome 9-1 cluster.

Table 5: Identified protein interactors of the putative promoters of piRNA precursors

CHROMOSOME 17-1 FISHOUTS	
Genes identified	**Hits**
Kruppel-like factor (KLF) 4	33
Kruppel-like factor (KLF) 15	3
Kruppel-like factor (KLF) 17	2
Transcription factor SP3	3
Transcription factor SP1	1
G protein pathway suppressor 2 (partial match)	4
Glucose-6-phosphate isomerase	1
A disintegrin and metallopeptidase domain 34	1
Leucine-rich repeat-containing protein 18	1
Coiled-coil domain-containing protein 86	1
Calmodulin 3	1
HRAS-like suppressor 5	1
PI3-kinase p85 subunit gamma	1
Chromosome 17 genomic contig	1
CKLF-like MARVEL transmembrane domain-containing protein 2A	1
CHROMOSOME 9-1 FISHOUTS	
Genes identified	**Hits**
Zinc Finger Protein 821 (ZNF821)	181
Chromosome 1 genomic contig	1
protein tyrosine phosphatase-like	1
phenylalanine-tRNA synthetase 2 (mitochondrial) Gene	1

3.6 Discussions

Here we asked whether the "ping-pong" mechanism operates in the adult mouse testis, where the major piRNA expression occurs (***Figure 6***). Although the "ping-pong" mechanism operates between MILI and MIWI2 in the primordial testis, MIWI2 is not detectable in the adult, raising the question whether it operates between MILI and MIWI instead. However, we did not detect any physical interaction or a positive feed-back between MILI and MIWI for the biogenesis of their piRNAs. On the contrary, there seems to be competition more than cooperation between the two since some of the MILI piRNAs analyzed with northern blotting appear to be even upregulated in the absence of MIWI. Given that the two populations of piRNAs are derived from the same genomic regions, they may be processed from precursors in common. Thus, in the absence of one of the complexes, the equilibrium of piRNA biogenesis is shifted towards the other. These observations indicate that the two complexes do not interact but share a common piRNA biogenesis pathway.

Furthermore, we looked for the signs of the "ping pong" model bioinformatically. Our observations indicate that such mechanism does not exist for the production of the majority of piRNAs in the adult testis: First, adult testis is poor in piRNAs with transposonic sequences, which is the major piRNA population involved in

the "ping-pong" mechanism. Second, piRNA populations of MIWI and MILI originate from the same genomic loci and correspond to the same genomic strands, and thus not likely to be complementary to each other. Instead, they are highly homologous to each other. Third, although the reciprocal piRNAs involved in the "ping-pong" are complementary to each other along their first 10nt due to the "slicer" activity of PIWI proteins, we did not observe such matching partners among the piRNA populations of MILI and MIWI. Fourth, both populations are biased for Uracil on the 5` end and not biased for Adenine on the 10th position. These observations cumulatively indicate that most, if not all, of the piRNAs in the adult testis are produced independent of the "ping-pong" mechanism.

What is the mechanism responsible for the biogenesis of piRNAs in the adult mouse testis? Most of adult testicular piRNAs originate in clusters in a head-to-tail homology indicating they are processed from long single stranded precursors. Currently we do not know the molecular machinery behind this processing. However, Houwing *et al* (2007) and Vagin *et al* showed that DICER is not necessary for the production of piRNAs in the zebrafish and fly respectively. We also observed that most of the piRNA clusters have a small piRNA-free region at approximately their centers, where the genomic strand origin of the piRNAs switched to the other strand generating

a bi-directional outline. Additionally, testis specific long ESTs with a similar bi-directional outline have been identified around some of these regions, suggesting such ESTs might be the corresponding piRNA precursors and the region in between them is a promoter for a bi-directional transcription. Indeed, most of the protein interactors identified for both of such regions are transcription factors, supporting the notion of these regions being promoters. We identified Krueppel-like and SP transcription factors for the Chr17-1 promoter and ZNF 821 for the Chr9-1 promoter. Although, we did not detect any common protein interactor for both of the regions, all these transcription factors belong to Krueppel C2H2 type zinc finger protein family. These transcription factors have been shown to regulate the RNA Polymerase II-based transcription of target genes via interaction with other cofactors like CRSP, p300/CBP, TAFII 130.

Our observations collectively indicate that the "ping-pong" machinery is not the major mechanism underlying the biogenesis of the piRNAs in the adult testis. Instead, they seem to be processed from single stranded precursors that are mainly bi-directionally transcribed from certain regions by RNA polymerase II under the control of the Krueppel-like C2H2 type transcription factors (***Figure 20***).

Figure 20: Biogenesis of the adult testicular piRNAs

The majority of piRNAs in the adult mouse testis are derived in clusters, most of which seem to be under the control of a bi-directional promoter region. Individual piRNAs within a cluster are derived from only one of the DNA strands in the same direction with a head-to-tail homology indicating long, single stranded precursors produced from these regions are processed to form the individual piRNAs. RNA Polymerase II is highly likely to be responsible for the transcription of these precursors based on the observation that Krueppel-like transcription factors interact with these regions.

A slightly modified version of Figure 20 has been published in
Piwi-interacting RNAs (piRNAs), by Ergin Beyret and Haifan Lin
MicroRNAs: From Basic Science to Disease Biology, edited by Krishnarao
Appasani
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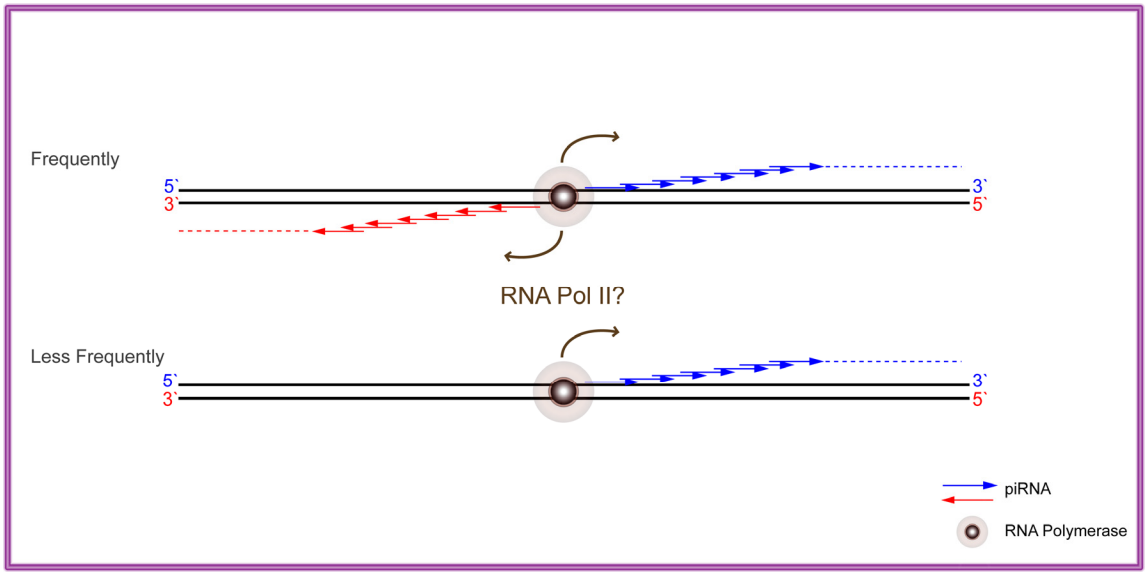

Figure 20: Biogenesis of the adult testicular piRNAs

3.7 Conclusions

Elucidation of the biogenesis of piRNAs is important in understanding the formation of the PIWI/piRNA complexes. piRNAs derived from repeat-associated regions on the genome (a.k.a rasiRNAs) can be produced and/or amplified via the interaction of two PIWI proteins in the "ping-pong" mechanism. Although this mechanism has been shown to operate in the fly, fish and prenatal mouse gonads, it only relates to this sub-population of piRNAs. In this study, we characterized piRNAs in the mouse testis for their biogenesis, and focused on the adult testis, where the major piRNA expression is observed. In pursuit of this purpose, we have identified testicular MIWI piRNAs for the first time and new MILI piRNAs with a higher coverage than before. We found that piRNAs in the adult testis appear to be produced from transposon-poor, intergenic regions through long, single stranded precursors and provided evidence that RNA Polymerase II might be responsible for the transcription of these precursors from certain regions on the genome. Although currently we do not know how these precursors are processed into piRNAs, we found no evidence for the involvement of the "ping-pong" machinery.

3.8 Acknowledgements

We thank Mrs. Hua Li for her assistance in preparing the MIWI piRNA library.

We are grateful to Dr. Na Liu for her re-analyses and confirmation of the bioinformatics.

4. Function of PIWI proteins and piRNAs

4.1 Author Contribution

Ergin Beyret performed all the experiments presented here.

Na Liu performed bioinformatics analyses for the questions raised by Ergin Beyret: evaluated the distribution of the piRNAs on the X and Y chromosomes.

4.2 Summary

PIWI proteins and piRNAs have been linked to transposon silencing in the primordial mouse testis, but their function in the adult testis remains elusive. Here we report the cytological characterization of piRNAs in the adult mouse testis and the phenotypic analysis of *Miwi*$^{-/-}$; *Mili*$^{-/-}$ mice. We show that piRNAs are specifically present in germ cells, especially abundant in spermatocytes and early round spermatids, regardless of the type of the genomic sequences to which they correspond. piRNAs and PIWI proteins are present in both the cytoplasm and nucleus. In the cytoplasm, they are enriched in the nuage/"chromatoid body"; whereas in the nucleus they are enriched in the "dense body". The "dense body" is a male-specific organelle associated with synapsis and the formation of the "sex body", which is an epigenetically silenced form of the sex chromosomes during meiosis in the males. While *Miwi*$^{-/-}$; *Mili*$^{-/-}$ mice are

impaired only in spermatogenesis due to the meiotic arrest of the germ cells with no obvious defect in synapsis, a euchromatic mark, acetyl-H4K16, fails to disappear on the sex chromosomes. Correspondingly, H3K9 on the sex chromosomes fails to be hyper di-methylated to mark epigenetic silencing. These observations implicate PIWI-piRNA complexes function in meiotic sex chromosome inactivation during spermatogenesis.

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4.3 Introduction

All sexually reproducing organisms undergo meiosis to decrease their genomic content by half in order to reproduce. Lying at the center of meiosis is the pairing of the homologous chromosomes, which has to be under strict regulation since any mis-pairing can lead to aneuploidy in the progeny. However, in most organisms, sex determination is regulated by means of unequal sex chromosomes, and the presence of such heterogeneity causes unpaired regions on the sex chromosomes (Namekawa and Lee 2009). Such unpairing on the sex chromosomes trigger their transcriptional silencing, a phenomenon termed "**m**eiotic **s**ex **c**hromosome **i**nactivation" (**MSCI**) (Turner 2007).

MSCI is a natural outcome of a general mechanism termed "**m**eiotic **s**ilencing of **u**npaired **c**hromosomes" (**MSUC**) (Baarends, Wassenaar et al. 2005; Turner, Mahadevaiah et al. 2005; Turner, Mahadevaiah et al. 2006; Turner 2007), and conserved

among organisms that show partial sex chromosome synapsis (Turner, Aprelikova et al. 2004; Maine, Hauth et al. 2005; Hense, Baines et al. 2007; Turner 2007; Namekawa and Lee 2009; Schoenmakers, Wassenaar et al. 2009). Although its biological purpose is elusive, this mechanism is similar to the RNAi-related **m**eiotic **s**ilencing of **u**npaired **D**NA (**MSUD**) in *neurospora crassa*, which is thought to be a defense mechanism against transposons (Lee, Pratt et al. 2003; Kelly and Aramayo 2007). Under normal circumstances, unpaired regions naturally occur between the heteromorphic sex chromosomes of the male mice, since the sex chromosomes and autosomes in the female can fully synapse. If they are not recognized and sequestered as the heterochromatinized structure "sex body", this anomaly causes the cells to be arrested during pachytene stage presumably due to a meiotic check-point.

The "sex body" is the condensed state of the sex chromosomes in spermatocytes (Hoyer-Fender 2003). It is formed en route to the transcriptional inactivation of the sex chromosomes during prophase I of meiosis. Cytologically, the "sex body" is discerned during pachynema and diplonema as a darkly stained globular chromatin structure, and marked by the phosphorylation of the H2A variant H2AX (γH2AX) (Hoyer-Fender 2003). In addition to marking double stranded breaks (Rogakou, Pilch et al. 1998; Mahadevaiah, Turner et al. 2001), γH2AX also marks any unpaired region during

meiosis (Mahadevaiah, Turner et al. 2001; Turner 2007). It coats the sex chromosomes of the late zygotene spermatocytes in a tadpole-like shape during the zygonema-pachynema transition and takes the globular form of the "sex body" during pachynema (Mahadevaiah, Turner et al. 2001; Blanco-Rodriguez 2009).

A likely molecular activity of PIWI/piRNA complexes is transposon silencing as most *piwi* mutations in various organisms show de-regulation of some transposon types (Sarot, Payen-Groschene et al. 2004; Vagin, Sigova et al. 2006; Aravin, Sachidanandam et al. 2007; Carmell, Girard et al. 2007; Chambeyron, Popkova et al. 2008; Das, Bagijn et al. 2008; Desset, Buchon et al. 2008; Houwing, Berezikov et al. 2008). Moreover, most piRNA sequences in *Drosophila* match transposons (Brennecke, Aravin et al. 2007; Yin and Lin 2007) and their downregulation is correlated with deregulation of the corresponding transposon types (Brennecke, Aravin et al. 2007). Similarly, in the primordial mouse testis, MILI and MIWI2 associate with piRNAs rich in transposonic sequences relative to piRNAs in the adult testis (Aravin, Sachidanandam et al. 2008; Kuramochi-Miyagawa, Watanabe et al. 2008). Therefore, it has been proposed that PIWI proteins use piRNAs to target and silence transposons in the germline.

Although the mode of this PIWI/piRNA mediated silencing still remains elusive, the "ping-pong" model proposed for the piRNA biogenesis in the fly suits for post-transcriptional silencing of transposons as well (**Figures 12 and 21**). piRNAs that are antisense to transposons may engage in the "ping-pong" loop, generating secondary piRNAs with sense transposonic sequences, while disintegrating the transposon intermediates. In addition to post-transcriptional silencing of transposons, piRNAs may be involved in transcriptional silencing (***Figure 21***). Since the epigenetic involvement of PIWI is well documented (Pal-Bhadra, Leibovitch et al. 2004; Brower-Toland, Findley et al. 2007; Yin and Lin 2007), it is possible that piRNAs guide PIWI proteins to the genomic loci being targeted. In support, *Line1* and *IAP* type transposons are hypomethylated in the absence of MILI or MIWI2 specifically in the germline (Aravin, Sachidanandam et al. 2007; Carmell, Girard et al. 2007; Kuramochi-Miyagawa, Watanabe et al. 2008). Even though MILI is a predominantly cytoplasmic protein, it has been suggested that its effect on transcriptional regulation of transposons may be via its interaction with MIWI2, which is predominantly nuclear.

Although the primordial mouse testis comprises abundant amount of piRNAs with transposonic sequences, adult testicular piRNAs are mostly derived from non-transposonic regions (***Figure 13C***) (Aravin, Gaidatzis et al. 2006; Girard, Sachidanandam

et al. 2006; Aravin, Sachidanandam et al. 2007). Therefore, the majority of piRNAs in the adult testis seems to have a function irrespective of transposon regulation. To elucidate this function, here we report the phenotypic and cytological characterization of PIWI proteins and piRNAs in the adult mouse testis. We show that both PIWI proteins and piRNAs are specifically present in germ cells, where they exhibit a nucleo/cytoplasmic distribution with localization to the male germ cell-specific structures "chromatoid body" and "dense body". Moreover, piRNAs are highly up-regulated in the meiotic cells regardless of the type of the genomic regions they correspond to. In the absence of a functional PIWI/piRNA complex, spermatogenesis is arrested during meiosis and the sex chromosomes fail to acquire a heterochromatic nature, implicating a role for the PIWI/piRNA complexes in meiotic sex chromosome inactivation in the male.

Figure 21: piRNA-mediated transposon silencing in the germline

piRNAs with complementary sequences to retrotransposons can guide Piwi proteins to target the corresponding elements and lead to their silencing in the germline. This surveillance can be achieved at the transcriptional or post-transcriptional level via the epigenetic and "slicer" activities of PIWI proteins respectively.

Figure 21 has been published in

Piwi-interacting RNAs (piRNAs), by Ergin Beyret and Haifan Lin

MicroRNAs: From Basic Science to Disease Biology, edited by Krishnarao Appasani

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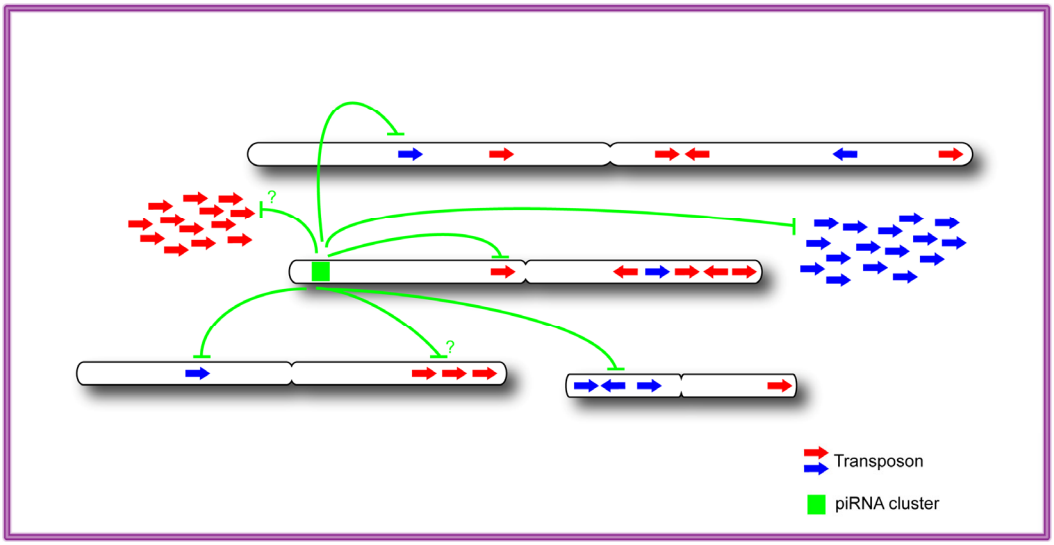

Figure 21: piRNA-mediated transposon silencing in the germline

4.4 Materials and Methods

4.4.1 Animals

Same as in Chapter 3

4.4.2 Denaturing PAGE Analysis of piRNAs

Same as in Chapter 3

4.4.3 Small RNA Northern Blotting

Same as in Chapter 3

4.4.4 Antibodies

The following antibodies were used for indirect immunofluorescence at the indicated dilution: R133 anti-MIWI 1:200 (Deng and Lin 2002), GP15 anti-MILI 1:200 (Unhavaithaya, Hao et al. 2008), anti-SCP1 1:25 (Santa Cruz), anti-SCP3 1:50 (Novus Biologicals) anti-EE2 1:200, anti-CyclinD3 1:100, anti-CREM-I (X-12) 1:200 (Santa Cruz Biotechnology), anti-H3K9me2 1:400 (Cell Signaling), anti-TRA54 1:400, anti-Tsx 1:1000, anti-Fibrillarin 1:300 (Novus Biologicals), anti-γH2AX 2-5μg/ml (Millipore), anti-acetyl-Histone H4 (Lys16) 1:200 (Millipore). Fluorophore-conjugated secondary antibodies obtained from Jackson Immuno Research Laboratories were used between

1:100-1:1000. Dilution factors for immuno-blotting: anti-GAPDH 1:4000 (Sigma), anti-Pol II (CTD4H8) 1:1000 (Santa Cruz Biotechnology), R133 anti-MIWI 1:1000, GP15 anti-MILI 1:1500, MILI peptide antibody 1:1500 (Unhavaithaya, Hao et al. 2008). HRP-conjugated secondary antibodies obtained from Jackson Immuno Research Laboratories were used between 1:1000-1:10000.

4.4.5 Spermatocyte Spread Preparation

Mice of the indicated ages were euthanized with cervical dislocation or asphyxiation with CO_2. The testicular cells were mechanically isolated as previously described (Aravindan, Pineau et al. 1996) from flash-frozen testis with the following modifications: The 1X PBS solution used until the fixation of the samples was kept ice-cold and supplemented with [complete mini EDTA-free Protease inhibitor cocktail tablet (Roche), 1mM EDTA, 1mM DTT]. The cell suspension was filtered sequentially through 70μm and 20μm nylon mesh, and lastly through glass wool, that were equilibrated with 1X PBS pre and post-filtration. The cells were precipitated at 700g for 10 minutes at $4C^{o}$ and resuspended in 100μl of the hypotonic extraction buffer of (Peters, Plug et al. 1997) per testicular sample. Nuclei were spreaded as in (Peters, Plug et al. 1997) with the following modifications: The samples were precipitated at 700g for 10 minutes at $4C^{o}$ after the incubation in the hypotonic solution. The pellet was resuspended in 50μl of the

1X PBS solution and supplemented with 50µl 100mM sucrose (pH8.2) per testicular sample. 20µl of the suspension was spread per slide. Spread nuclei were dried in a humidifying chamber at 55C° for 2 hours before washing with the Photoflo (Kodak) solution. The slides were dried at room temperature and kept at -80C° until needed.

4.4.6 Immuno-fluorescence Analyses

Unless otherwise indicated, 8-10µm cyrosections were used. Freshly dissected whole testes were fixed in [4% paraformaldehyde-1X PBS] solution overnight at 4C°. The next day, they were dehydrated by sequential incubation in 10-15-20-30% sucrose solutions in 1X PBS at room temperature for 30-45-60-60 minutes respectively, followed by [30% sucrose-1X PBS] : OCT (1:1) overnight at 4C° and [30% sucrose-1X PBS] : OCT (1:3) for 30 to 60 minutes at room temperature. The samples were cryopreserved in OCT at -80C° until sectioning.

For immuno-flourescent detection of proteins on cryosections, samples were first re-hydrated with 1X PBS and then incubated in the blocking solution of 10% serum in 1X PBT [1X PBS, 0.1% BSA, 0.1% Tween-20] for at least one hour at room temperature. Afterwards, samples were sequentially treated with the primary and secondary antibodies diluted in the same blocking solution. Incubations with the antibodies were

performed at room temperature for 2 hours or at 4C° overnight. Following the secondary antibody treatment, the samples were stained with DAPI in 1X PBT for 6 minutes at room temperature to visualize the DNA. The samples were rinsed with 1X PBT for three times 10 minutes each, following the primary antibody incubation, and once again after DAPI staining. Slides were mounted with VectaShield mounting medium (Vector Labs).

Immuno-fluorescence on spermatocyte spreads was performed as above except that the samples were first incubated in [0.5% Triton X-100, 1X PBS] for 5 minutes at room temperature, followed by washing with 1X PBS three times for 2 minutes each. Incubations with the blocking solution, primary and secondary antibodies were performed respectively for 1-1.5 hour, overnight and 1-2 hours at 37C°.

4.4.7 *In situ* Hybridization of piRNAs

LNA-modified DNA oligonucleotides with reverse complementary sequences of individual piRNAs were labeled with the DIG Oligonucleotide Tailing Kit (Roche) according to the manufacturer's instructions and purified with Sephadex G25 Columns. Labeling efficiency was estimated with dot blotting based on the reference set provided by the kit. The hybridization buffer is composed of [50% (v:v) deionised formamide, 0.3M NaCl, 20mM Tris HCl pH8.0, 5mM EDTA, 10mM $NaPO_4$ buffer pH8.0, 10% (w:v)

Dextran Sulfate, 1X Denhardt's Solution, 100μg/ml PolyA oligo DNA of 30nt length, 0.1 mg/ml Heparin and freshly boiled 0.5 mg/ml yeast tRNA]. Before the hybridization, the sections were acetylated with freshly made [0.25% acetic anhydride, 1.165% triethanolamine] for 10 minutes followed by 1X PBS wash for three times five minutes each. Probes were incubated in the hybridization buffer at 65C^{o} for 5 minutes and quickly chilled on ice to denature secondary structures. Hybridization was performed overnight at 46C^{o} in a humidifying chamber of [1XSSC, 50% formamide]. Slides were sequentially washed once in 5X SSC at room temperature for 15-20minutes, twice in [50% formamide, 0.1% Tween-20, 1X SSC] for 15 minutes, once in 0.5X SSC and once in 1X PBS. Antibody detection was performed as in (Heller, Sheane et al. 1998) except the concentration of the alkaline-phosphatase (AP) conjugated F_{ab} fragments (Boehringer Mannheim) used was 1:750, and PBS in the last two washes were replaced with NTL buffer [150mM NaCl, 100mM Tris-HCl pH8.1, 0.5mg/ml Levamisole]. The slides were incubated in the dark with 1-STEP NBT/BCIP plus Suppressor Solution (Pierce) until the desired color intensity was reached. Color development was terminated with [1X PBS pH 5.5, 1mM EDTA].

Combined RNA and protein detection was performed similar to RNA detection alone with the following modifications in the antibody detection: Primary antibodies for

the counter staining were combined with the AP conjugated F_{ab} fragments. Following the incubation, the slides were washed three times at room temperature in 1X PBT for 15 minutes each. The samples were incubated with a combination of the secondary antibodies and AP-conjugated F_{ab} fragments in the same blocking buffer for 2 hours at room temperature or overnight at $4C^{o}$. Washes were performed as in RNA detection alone. The slides were additionally washed with [0.1M Tris-HCl pH 8.1, 0.5mg/ml Levamisole] for 15 minutes then incubated in the dark with FastRed solution (Roche). [1X PBS pH 5.5, 1mM EDTA] was used to terminate the color development.

4.4.8 Probes Used for *in situ* Hybridization and Northern Blotting

The nucleotides with the "+" sign on their left are LNA-modified.

mir-34b : CAA +TC+A G+CT +AA+T TA+C A+CT +GC+C TA

mir-100 : C+AA +GTT +CG+G AT+C TA+C G+GG +TT

mir-465 : T+CA +CA+T C+AG +TG+C C+AT +TC+T AAA +TA

mir-16 : CGC CAA TAT TTA CGT GCT GCT A

Negative Oligo : AA+C GA+C TCG CAG TA+C GTC A+CG T+CT A+TG G

U6 snRNA : TGT GCT GCC GAA GCG AGC AC

piRNA probes:

"Sense Intronic" and piRNA T4 are MIWI piRNAs, the rest are MILI-associated.

Transposonic 1 : G+AG +CC+G C+CC +TCA CAT TCG CTG TTG CA

Transposonic 2 : GGA C+CG +GT+C T+GC AG+C TGC TGA GTC GTA

Sense Exonic : GGG CTC TGT GGT +GG+C T+TT +TCG TCG TGC CA

Anti-sense Exonic : GGG ACA CA+C T+CA +GC+A C+TC +CT+T TGC A

Sense Intronic 1 : GGG CAG GTG +AG+A G+GA +TCC ATG GCC CA

Sense Intronic 2 : TAG G+CC +CTT +CAT +CA+C G+GA +TG+G ATT ATT GAG

Anti-sense Intronic : GAA CCA GTT C+CA +CG+A G+TG +TT+G C+CC A

Repeat-associated 1: TA+T CA+T A+GT +CA+T CA+T CA+T C+AT +CG+T CA

Repeat-associated 2: G+TA +GGT +CT+C CAG CA+T CA+C A+TC T+TT G+TA

piRNA T4 : TAG ACA ATT TTC AGT GTC CTA AGC TGT CTA

4.4.9 Chromosome Painting

X and Y chromosomes were painted on spermatocyte spreads with the probes obtained from Cambio (StarFISH Mouse Chromosome Specific Probes) according to the manufacturer's recommendations except: Spermatocyte spreads were washed at room temperature once in 0.5% TritonX-100 for 5 minutes and three times in 1X PBS for 2 minutes each before incubating in methanol:acetic acid (3:1) for 10 minutes. Denaturation of the probes and the slides were performed at 70C^{o} for 2 minutes. The probes and the prepared slides were introduced at 65C^{o} and the temperature was

gradually decreased to 37C°, at which it was kept overnight for the hybridization. γH2AX staining was performed as above in between the hybridization and the detection steps following the post-hybridization washes. The slides were mounted with VectaShield mounting medium (Vector Labs) after counter-staining with DAPI. Images were analyzed with Axioimager (Carl Zeiss Incorporation). 3D images were captured using Apotome deconvolution microscopy.

4.4.10 Separation of the Nuclear and Cytoplasmic Fractions of the Testicular Lysate

A pair of 2 months old adult testes, which had been flash-frozen and stored at -80C°, was homogenized with a tight (B) pestle (clearance: 0.0010-0.0030 inch, Kontes Glass Company) in 1ml of the same lysis buffer used for immunoprecipitation. The lysate was spun at 1300g for 10 minutes at 4C° to precipitate the nuclei and intact cells. The supernatant was spun one more time to remove residual nuclei and kept as the "cytoplasmic fraction". The pellet was washed with 500μl lysis buffer, grinded again to eliminate contaminating unbroken cells and re-spun. After one more round of cleaning, the pellet was lastly resuspended in 1 ml lysis buffer and kept as the "nuclear fraction". The supernatants of the washes were pooled and kept as the "wash fraction". For the unfractionated control, a pair of testes was likewise homogenized in 1 ml buffer without any further separation. Total RNAs were extracted from equal volumes of the samples,

fractionated with 15% Urea-PAGE and northern blotted to assess their piRNA content. 25 μg kidney total RNA was used as a negative control for piRNAs. The blot was stripped and re-probed with a 30mer LNA-modified oligo DNA with no matching sequence on the genome as a negative control for hybridization (*Data not shown*). Likewise, equal volumes were analyzed with western blotting onto nitrocellulose membranes (Bio-Rad Laboratories) to assess their MILI-MIWI content and the degree of cross contamination.

4.4.11 piRNA Distribution Along the X and Y Chromosomes

piRNAs cloned in Chapter 3 were extracted from the adapter sequences, and those that map to the X and Y chromosomes were identified. The corresponding piRNA sequences were plotted across the X and Y chromosomes to calculate their piRNA content with the following criteria: We considered the sequencing read number for each piRNA as the representation of its abundance in the sample. Therefore, if the same piRNA was sequenced from "n" different clones, we counted its abundance as "n". If a piRNA corresponded to more than one region on the genome, we divided "n" with the number of the corresponding genomic regions, assuming that each region has the same likelihood of producing the piRNA. The resultant values for piRNAs within each non-overlapping 50Kb window were summed to yield the total number of piRNA clones in

each window. To reduce any discrepancy between the sequencing coverages, the results were normalized according to the total sequencing read number of the MILI piRNAs in the adult testis (MILI adult) in the following way: The number of total piRNA clones within each window was multiplied with the total read number of the "MILI adult" sample, and the result was divided with the total read number of the sample being normalized (e.g; MILI piRNAs in 13dpp testis).

4.5 Results

4.5.1 *Miwi*$^{-/-}$*; Mili*$^{-/-}$ Mice Phenocopy the *Mili*$^{-/-}$ Defect

While MIWI, MIWI2 and their piRNAs have been detected only in the male germline, it is possible that they have escaped detection and show redundancy with MILI in the female germline. In order to assess if a redundancy exists between MIWI and MILI, we generated *Miwi*$^{-/-}$*; Mili*$^{-/-}$ mice by intercrossing *Miwi*$^{+/-}$*; Mili*$^{+/-}$ animals.

We were able to obtain *Miwi*$^{-/-}$*; Mili*$^{-/-}$ males and females with the expected Mendelian ratios, indicating that loss-of-function of *Miwi* and *Mili* together is not lethal. While the males are infertile as expected, females still proved to be unaffected. We did not notice any obvious defects other than male-only infertility. We also did not detect any abnormalities in the F2 generation of the *Miwi*$^{-/-}$*; Mili*$^{-/-}$ females. Therefore, even

though MILI/piRNA complex is present in the female germline and seems to be maternally delivered to the early embryo, loss of MIWI and MILI together does not seem to lead to a zygotic or maternal phenotype in the females.

Collectively these results point to an essential function of mouse PIWI proteins only in the male germline. In order to evaluate what this function might be, we examined *Miwi*$^{-/-}$; *Mili*$^{-/-}$ testes. Histological analysis showed that only spermatogonia and early spermatocytes are visible in the germline without any defect in the testicular soma (***Figure 22***). This observation indicates that *Miwi*$^{-/-}$; *Mili*$^{-/-}$ testis phenocopies *Mili*$^{-/-}$ testis, which is spermatogenically arrested during mid-pachynema (Kuramochi-Miyagawa, Kimura et al. 2004).

Figure 22: Spermatogenesis is arrested during meiosis in *Miwi*$^{-/-}$; *Mili*$^{-/-}$ mice

A) *Miwi*$^{+/-}$; *Mili*$^{+/-}$ testes show the full complement of germ cells.

B) *Miwi*$^{-/-}$; *Mili*$^{-/-}$ testes do not consist of germ cells beyond primary spermatocyte stage.

Germ cell types are assessed based on DAPI staining and the spermatogonia marker EE2. The histological analysis was performed on testis cryosections of 14 weeks old mice.

R.Spt: Round spermatid, E.Spt: Elongating spermatid, Spc: Primary spermatocyte. Shown in parentheses are the markers used.

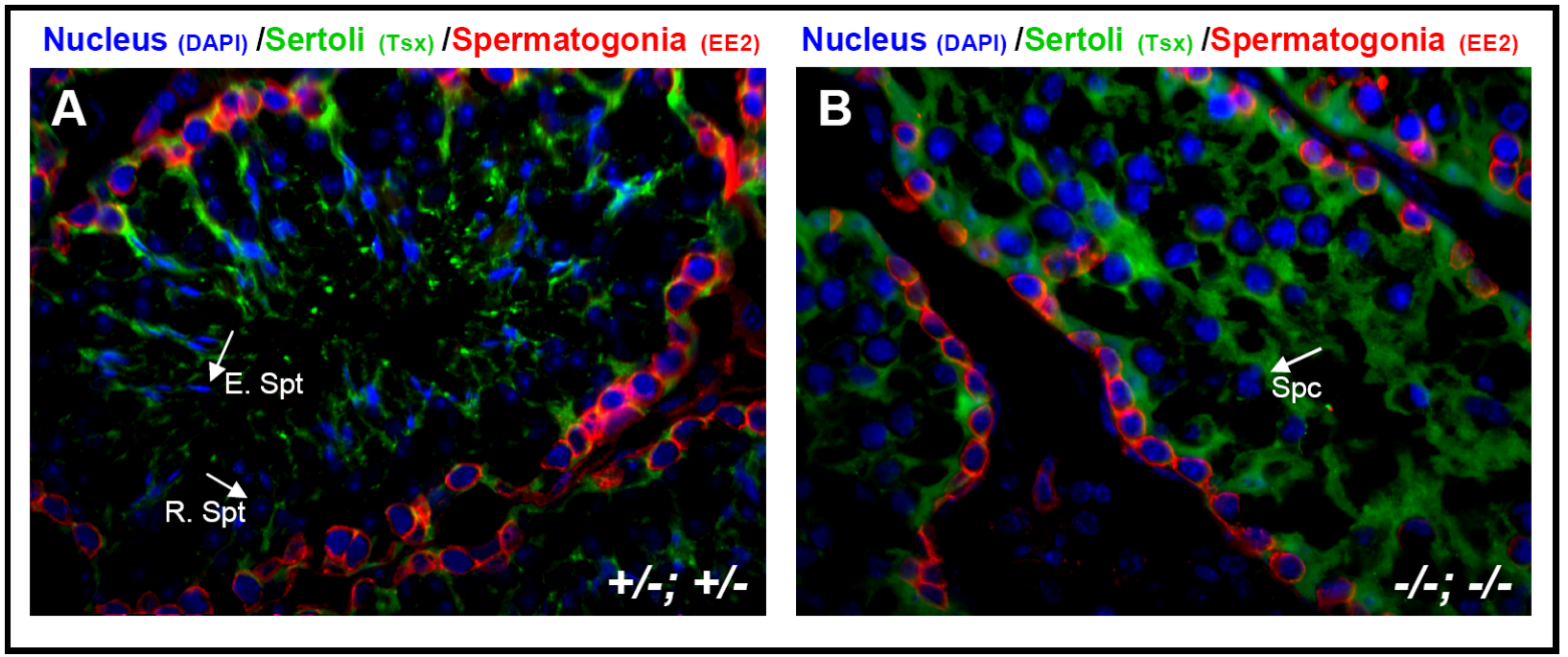

Figure 22: Spermatogenesis is arrested during meiosis in *Miwi*$^{-/-}$*; Mili*$^{-/-}$ mice

Since MILI is necessary for the assembly and localization of the MIWI2/piRNA complex in the testis (Aravin, Sachidanandam et al. 2008), the *Miwi*$^{-/-}$; *Mili*$^{-/-}$ mice are expected to be defective in the function of MIWI2 as well, and thus represent the loss-of-function of all of the three PIWI proteins in the mouse. Therefore our results indicate that, in the absence of a functional PIWI/piRNA complex, spermatogonial stem cells can still form and differentiate until the onset of meiosis, but cannot progress further in the mouse, pointing to an essential function of the mammalian PIWI proteins for male meiosis.

4.5.2 Testicular piRNAs are Germ Cell-Specific and Highly Abundant in the Meiotic Cells

We and others have previously shown that piRNAs overall are highly upregulated by 22dpp, when spermiogenesis is initiated, but are not detectable in the adult epididymide, where mature sperm are stored (Aravin, Gaidatzis et al. 2006; Girard, Sachidanandam et al. 2006; Grivna, Beyret et al. 2006; Watanabe, Takeda et al. 2006). These observations indicate that piRNAs have a significant function during spermatogenesis and are not paternally loaded to the embryo. To further explore this function, we analyzed the expression profiles of individual piRNAs during postnatal testis development with Northern blotting. We chose representative piRNAs derived from different types of sequences in the genome, including repeat-associated and

transposonic regions, to test if their expression patterns differed. A total of 4 MILI-associated piRNAs were tested. Irrespective of their genomic annotation, all of the tested piRNAs become highly abundant by 22dpp, almost reaching to the plateau level of expression, and are not detectable before 14dpp by northern blotting (***Figure 23***). This developmental window corresponds to the first arrival of meiosis. Thus, piRNAs, just like their protein partners MILI and MIWI, are upregulated during meiosis, implying a significant function of PIWI/piRNA complexes during meiosis irrespective of their genomic origins.

Figure 23: piRNAs become abundant during meiosis by the onset of spermiogenesis

Northern blotting for individual piRNAs shows that piRNAs are upregulated during meiosis irrespective of their annotation. Each panel corresponds to an individual piRNA with a different annotation (e.g; sense intronic: a piRNA derived from the sense strand of an intron)

20μg total RNA from 8dpp, 14dpp, 22dpp and 2 months old adult wild type testes was analyzed. 20μg liver total RNA was used as a negative control for piRNAs. U6 snRNA (U6) was used as an internal loading control.

M_D: 10nt DNA marker, Tp2: Transposonic 2, A-exo: Anti-sense exonic, Rapi1: Repeat-associated 1, S-Intro1: Sense intronic 1, T4: piRNA-T4.

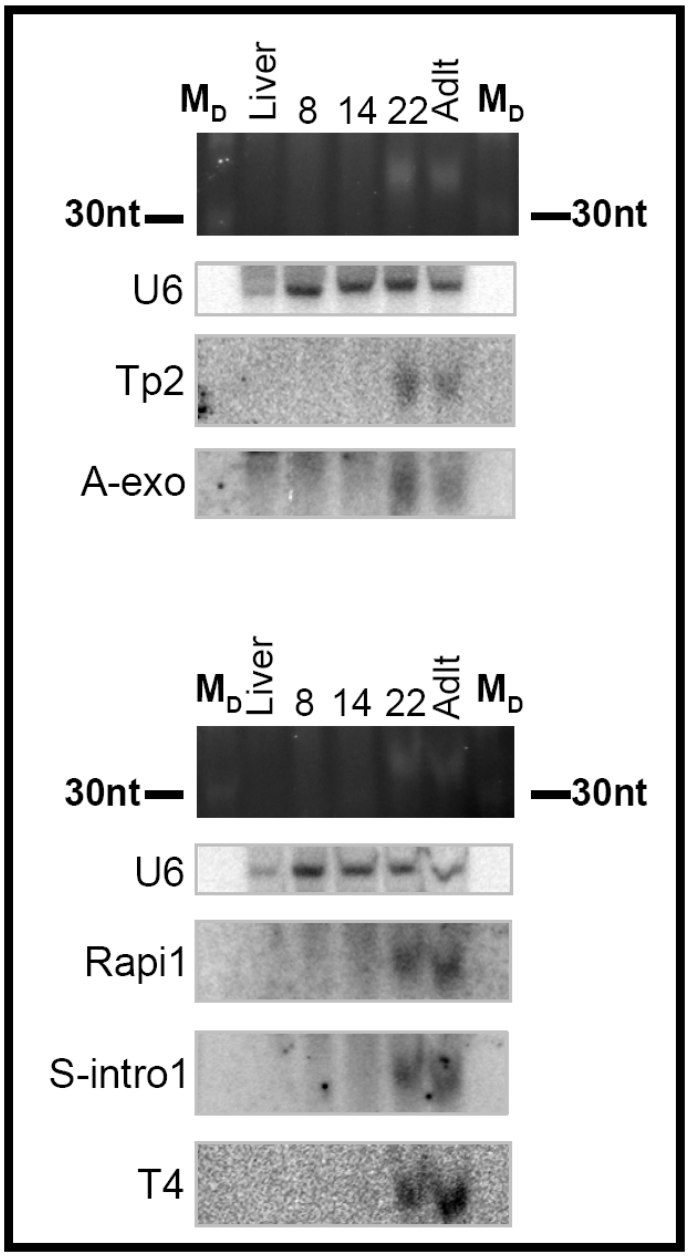

Figure 23: piRNAs become abundant during meiosis by the onset of spermiogenesis

To further characterize piRNA expression during meiosis, we performed *in situ* hybridization of representative piRNAs on the adult testis. We first assessed if our technique is reliable in the detection of small RNAs by comparing the Northern and *in situ* expression profiles of several micro RNAs (miRNAs) during spermatogenesis. As expected, a miRNA with a decreasing Northern expression profile during spermatogenesis was enriched in the early spermatogenetic cells in our *in situ* analysis; whereas those with an increasing expression profile were enriched accordingly later in the germ cells (***Figure 24***). These data validate our small RNA *in situ* analysis technique.

Figure 24: Expression patterns of small RNAs can be reliably detected with *in situ* hybridization

A) Expression patterns of miRNAs during post-natal testis development as evaluated with Northern Blotting are shown. mir-100 expression decreases as the development progresses; whereas mir-34 shows elevated expression in the adult with no detectable expression at 8dpp. mir-465 can be detected at 8 dpp and reaches to the peak value around 14dpp. Northern blotting for an individual piRNA (piRNA-T4) and ethidium bromide staining shows the abundant expression of piRNAs in the adult. Each indicated developmental stage of heterozygous (HET) in addition to 8dpp knock-out (KO) *Mili* testis was analyzed as in Figure 23. U6 snRNA was used as an internal loading control. Mirn 100 and Mirn 465 are 5 fmols of the sense mir-100 and mir-465 DNA oligomers respectively.

B) *In situ* hybridization of the miRNAs in the testis reflects their expression patterns during post-natal testis development. miRNAs mir-34b and mir-465 increase in the late germ cells; whereas mir-100 decreases. mir-465 can be detected in the spermatogonia layer while mir-34b expression therein is close to the background level.

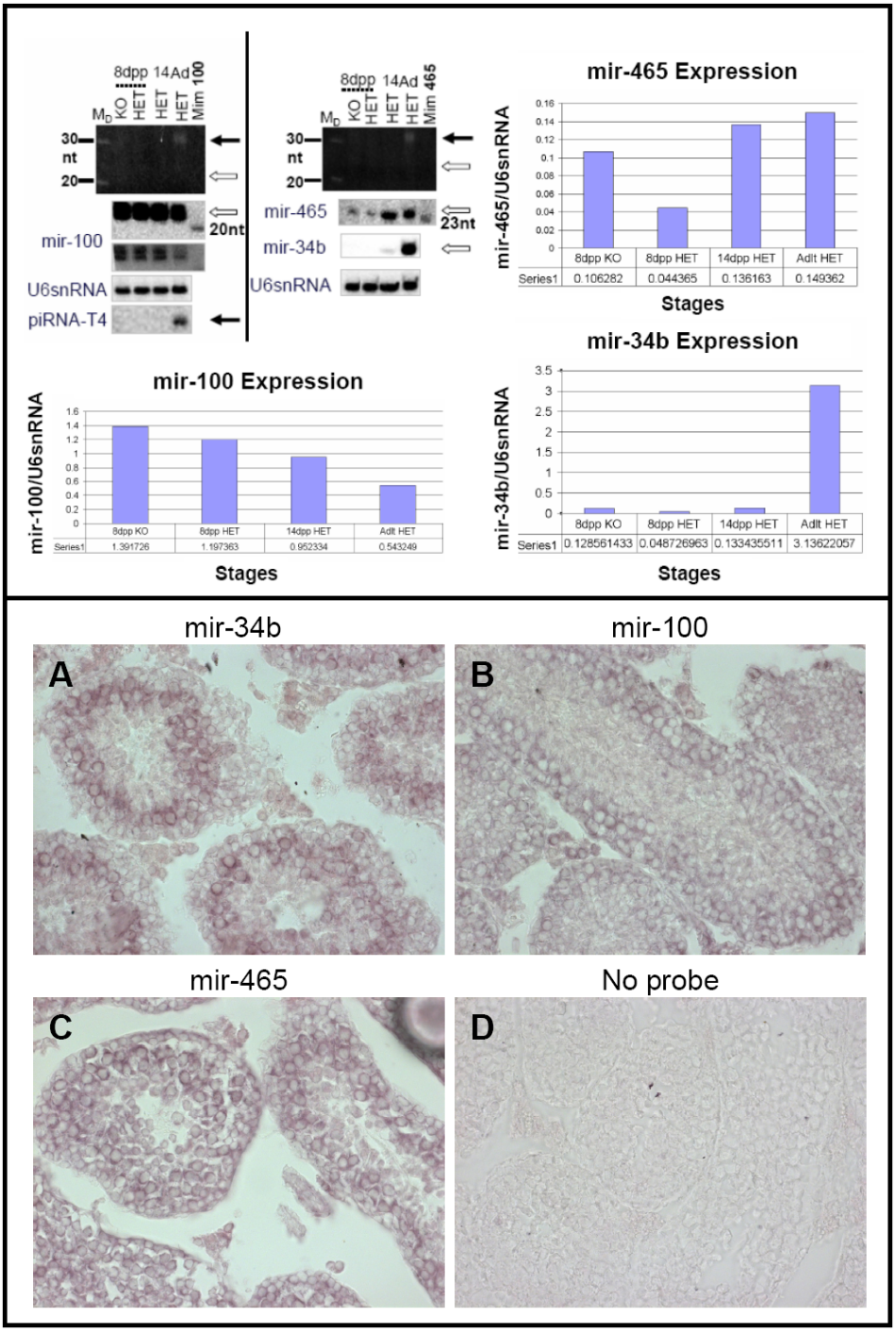

Figure 24: Expression patterns of small RNAs can be reliably detected with *in situ* hybridization

We then conducted *in situ* hybridization for a total of 17 piRNAs, four of which are MIWI-associated and the rest are MILI-associated. We chose these piRNAs based on their corresponding genomic regions, including repeat-associated, transposonic, exonic and intronic regions. All of these piRNAs are upregulated during the mid-stages of spermatogenesis, agreeing with the Northern data (***Figure 25***). Particularly, the piRNA-expressing cells are spermatoyctes and round spermatids, based on their morphology and location in the tubules. Some probes also yielded signal in the basal layer of the tubule where spermatogonia reside. This expression pattern is in synchrony with the expression patterns of MILI and MIWI (Deng and Lin 2002; Kuramochi-Miyagawa, Kimura et al. 2004; Grivna, Pyhtila et al. 2006; Aravin, Sachidanandam et al. 2008; Unhavaithaya, Hao et al. 2008; Wang, Saxe et al. 2009).

Even though several piRNAs with similar sequences may associate with MILI and/or MIWI, we noticed the same pattern of staining in *Miwi*$^{-/-}$ testis as well (*Data not shown*), where MILI piRNAs, but not MIWI piRNAs, are detected (***Figure 16***). These results indicate MILI piRNAs exist both in spermatocytes and round spermatids in addition to spermatogonia (***Figure 13A***) and primordial germ cells (Aravin, Sachidanandam et al. 2008; Kuramochi-Miyagawa, Watanabe et al. 2008). Unfortunately

we cannot test the same for MIWI piRNAs since the germline does not progress beyond mid-pachynema in *Mili*$^{-/-}$ testis.

Figure 25: piRNAs are abundantly expressed in spermatocytes and round spermatids

A-I) *In situ* hybridization analysis on the adult testis shows that piRNAs are abundant in the cytoplasm of spermatocytes and round spermatids. In addition to the homogenous cytoplasmic staining, piRNAs localize to peri-nuclear puncta that resemble chromatoid bodies. Early spermatocytes show a nuclear punctum staining as well. Cell type assessment was based on cellular morphology and relative location to the lumen periphery.

Yellow arrow head: "chromatoid body"-like staining; black arrow head: nuclear punctum staining. Insets are the magnified views of the regions indicated with the arrow heads. Each panel corresponds to an individual piRNA staining, described by the region it is derived from (e.g; anti-sense exonic: a piRNA derived from the anti-sense strand of an exon). Negative oligo: a 28mer oligonucleotide without a match on the genome was used as the negative control to assess the background level. "Sense-intronic 2" is a MIWI piRNA, the rest is MILI associated.

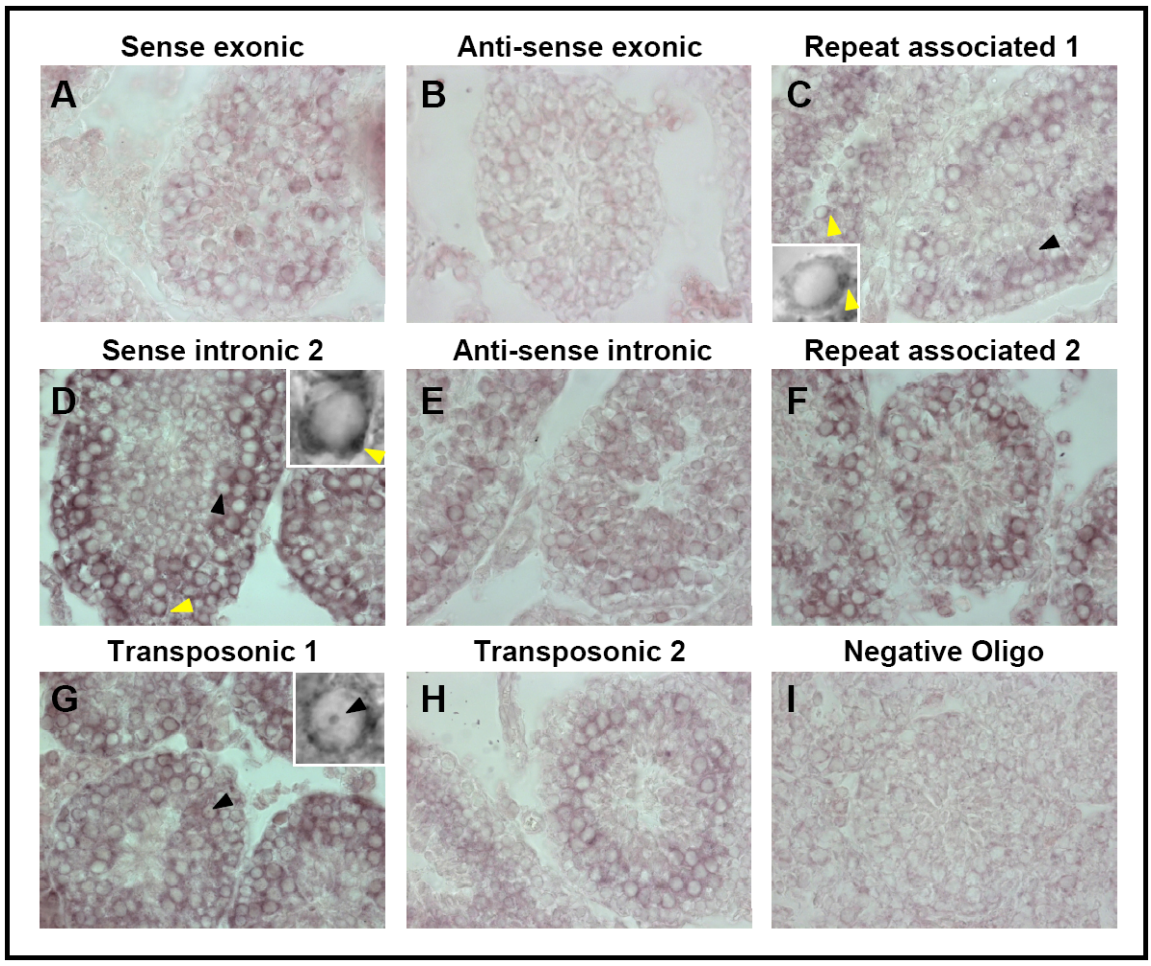

Figure 25: piRNAs are abundantly expressed in spermatocytes and round spermatids

In order to more precisely identify the expression window of piRNAs during spermatogenesis, we co-stained adult testis for piRNAs and cell-specific markers. This analysis showed that piRNA expression is close to the background level in spermatogonia, highly elevated in spermatocytes, moderate in round spermatids and already decreases to an undetectable level by the time elongating spermatids are formed (***Figure 26 A-C and A`-C`***).

We also analyzed if piRNA expression in the mouse testis is germline-specific, since this is the case for PIWI proteins (Kuramochi-Miyagawa, Kimura et al. 2001; Deng and Lin 2002; Kuramochi-Miyagawa, Kimura et al. 2004; Aravin, Sachidanandam et al. 2008; Unhavaithaya, Hao et al. 2008). The mouse testis consists of three types of resident somatic cells: Sertoli cells are the only somatic cell types inside the seminiferous tubules, Myeoid and Leydig cells reside in the interstitial space. We noticed that the piRNAs tested are not detectable in these cell types (***Figure 26***). Therefore, piRNAs in the mouse testis appear to be germ cell-specific as their partners PIWI proteins.

Figure 26: piRNAs are germ cell-specific

Co-staining for cell type-specific markers confirms the expression of the piRNA tested is highly abundant in spermatocytes (**A-C**, **A`-C`**) and round spermatids (**B**, **B`**) but close to the background level in spermatogonia (**A**, **A`**) and elongating spermatids (**A**, **A`-C**, **C`**). It is not detectable in the interstitial soma Leydig and Myeoid cells (**B**, **B`**), and luminal soma Sertoli cells (**D**-**F**). Shown are wildtype adult testis sections.

Spg: Spermatogonia, Spt: Spermatid, R.Spt: Round spermatid, E.Spt: Elongating spermatid, Spc: Spermatocyte, Sert: Sertoli Cell, Ldg: Leydig cell, Myd: Myeoid cell, rapiRNA1: repeat-associated piRNA1.

Shown in parentheses are the markers used. Middle panels correspond to the nuclear staining of the uppermost panels with DAPI in blue.

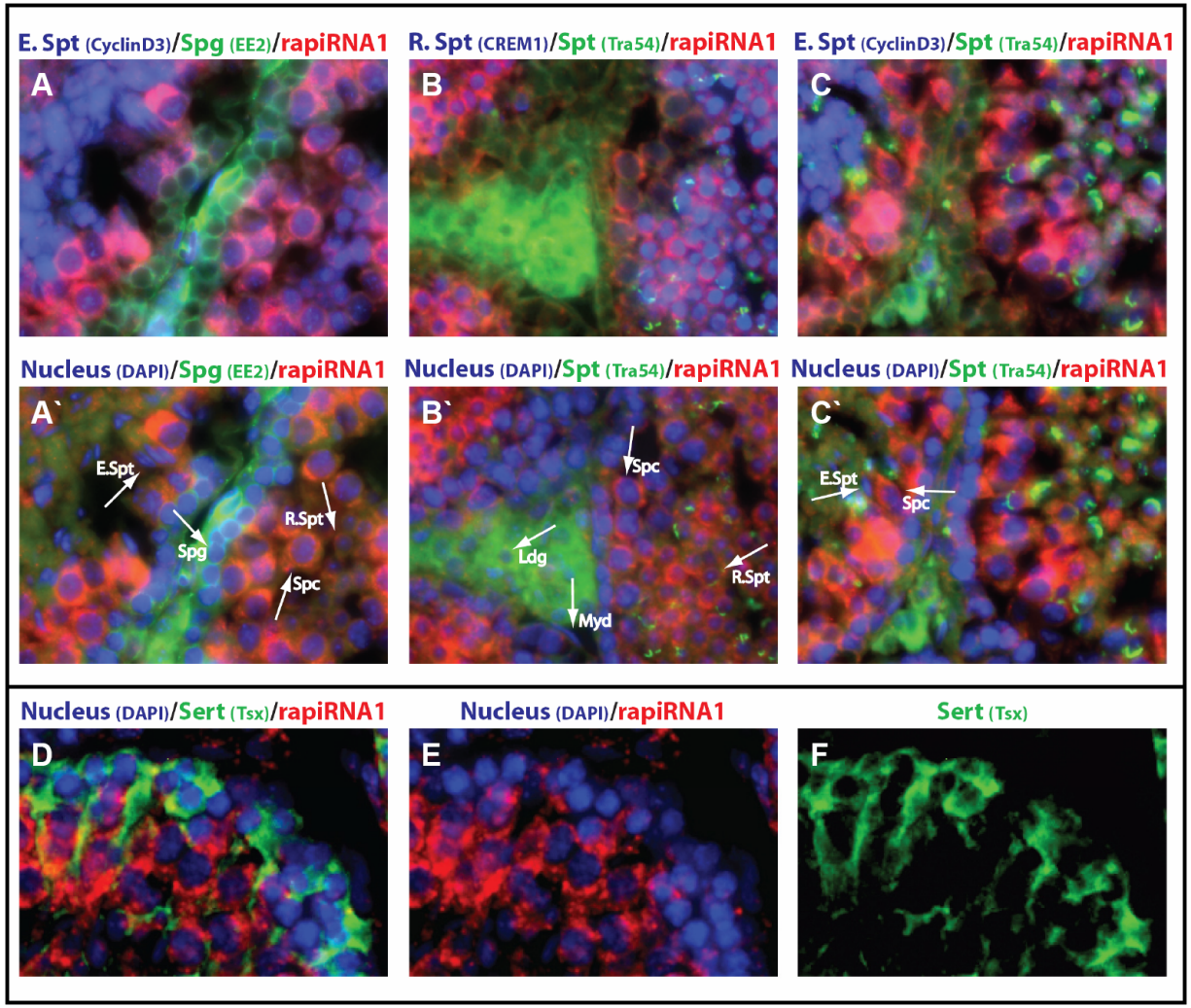

Figure 26: piRNAs are germ cell-specific

4.5.3 piRNAs Localize to Discrete Structures in Both the Cytoplasm and Nucleus of Spermatogenic Cells

We examined the subcellular localization of piRNAs to further characterize their spatial expression pattern. piRNAs mainly localize to the cytoplasm of the germ cells, including perinuclear granules that are likely nuage/chromatoid body, where PIWI proteins have also been shown to localize (***Figure 25 C-D***) (Grivna, Pyhtila et al. 2006; Kotaja, Bhattacharyya et al. 2006; Kotaja, Lin et al. 2006; Houwing, Kamminga et al. 2007; Aravin, Sachidanandam et al. 2008; Batista, Ruby et al. 2008; Unhavaithaya, Hao et al. 2008; Li, Vagin et al. 2009; Malone, Brennecke et al. 2009; Wang, Saxe et al. 2009). This highly dynamic germ cell-specific structure has been proposed to act as a warehouse and a processing center for RNAs produced during early spermatogenesis to be used later (Soderstrom and Parvinen 1976; Parvinen 2005; Kotaja, Bhattacharyya et al. 2006; Kotaja and Sassone-Corsi 2007), and as a surveillance checkpoint to monitor the trafficking of transposon intermediates through nuclear pores via the piRNA pathway (Aravin, Sachidanandam et al. 2008; Klattenhoff and Theurkauf 2008).

In addition, piRNAs are detected in the nuclei of early spermatocytes, where they localize to a punctum of approximately 1-2 micrometer (***Figure 25 G***). To explore the potential function of piRNAs in the nucleus, we sought to characterize this nuclear structure. MIWI and MILI largely co-localize with piRNAs in spermatocytes, including

at this punctum (***Figure 27 A-G***). This punctum does not correspond to the piRNA encoding genomic sequence, because it is devoid of DNA (***Figure 27***). It is not nucleolus or Cajal body either, as indicated by the lack of Fibrillarin, a common marker for these structures (***Figure 27 H-K***). These properties are consistent with those of the "dense body", a male-specific electro-dense structure of 1-2μm diameter found only in early spermatocyte nuclei.

Figure 27: piRNAs, MIWI and MILI localize to the "dense body" and "nuage/chromatoid body"

A-G) MILI, MIWI and the piRNA tested localize to a DAPI-negative nuclear punctum present only in primary spermatocytes. Shown in B-G is the magnified view of the spermatocyte boxed in A.

H-K) Co-staining for a nucleolus/Cajal body marker (Fibrillarin in red) shows that the nuclear punctum is not part of nucleolus or Cajal body. Cyan arrow head: nucleolus; orange arrow: "nuage/chromatoid body"-like structure; red arrow: "dense body"-like structure. Images are 3μm thick 3D views.

The immunofluorescence was performed on wildtype adult testis cryosections.

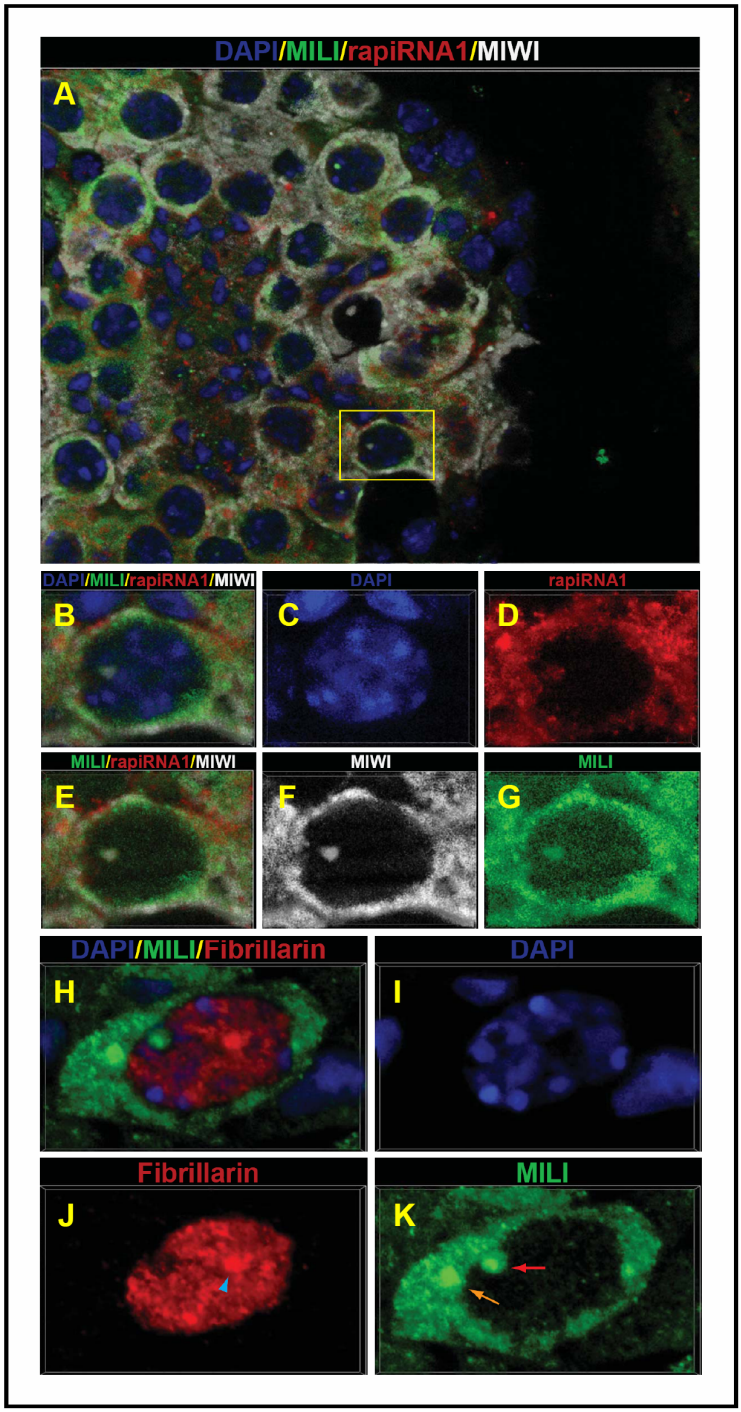

Figure 27: piRNAs, MIWI and MILI localize to the "dense body" and "nuage/chromatoid body"

We next asked if the nuclear and cytosolic staining in our *in situ* analyses indeed represent piRNAs rather than a precursor or complementary transcript. For this purpose, we separated adult testicular extract into nuclear and cytoplasmic fractions and analyzed for their piRNA content with ethidium bromide staining and Northern blotting. This analysis revealed that, irrespective of their annotation, a substantial amount of piRNAs as well as MIWI and MILI does exist in the nucleus in addition to the cytoplasm (***Figure 28***).

Figure 28: A substantial amount of MILI, MIWI and piRNAs exists in the nucleus as well as cytoplasm

A) Western blots of equal volumes of the testicular fractions, showing the sub-cellular distribution of MIWI and MILI, and the degree of cross-contamination as assessed by the cytoplasmic marker GAPDH and the nuclear marker phosphoserine 5 RNA Polymerase II (Pol-II).

Samples from the same fractions have been analyzed in two different occasions, stripped and re-probed as required. Those belonging to each gel are separated by the horizontal bar.

B) Urea-PAGE of equal volumes of fractionated RNA, showing the sub-cellular distribution of piRNAs. Ethidium bromide staining and northern blotting show the global and individual piRNA distribution respectively.

25 μg total RNA from kidney was used as a negative control, where piRNAs are not detected but microRNA miR-16 is.

Total: Unfractionated testicular extract; cyto: Cytoplasmic fraction; Nclr: Nuclear fraction. MD: 10nt DNA marker, S-exo: Sense exonic, Tp2: Transposonic 2, Rapi1: Repeat-associated 1. Blank arrow indicates the location of mir-16.

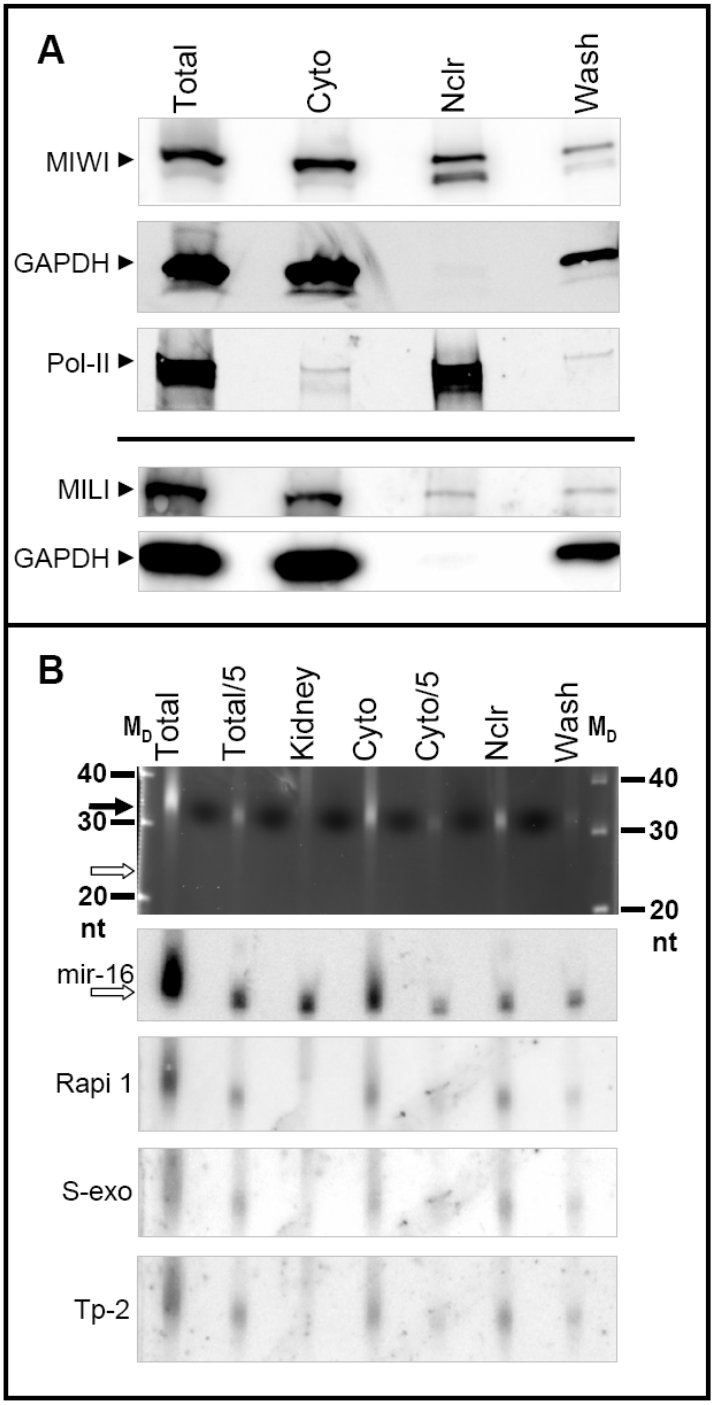

Figure 28: A substantial amount of MILI, MIWI and piRNAs exists in the nucleus as well as cytoplasm

4.5.4 Sex Chromosomes in Spermatocytes are not Heterochromatinized in the Absence of a Functional PIWI/piRNA Complex

Since a component of the "dense body" has been shown to be necessary for the proper synapsis and the formation of the "sex body" (Crackower, Kolas et al. 2003; Kolas, Marcon et al. 2005), we analyzed if any of these events is impaired in the absence of a functional PIWI/piRNA complex by conducting chromosome painting on *Miwi*$^{-/-}$; *Mili*$^{-/-}$ spermatocyte spreads. We observde that X and Y chromosomes are in the vicinity of each other and covered with globular γH2AX staining indicating that homolog recognition as well as formation of the sex body is not impaired (***Figure 29 A-D***). Co-staining for the axial/lateral element SCP3 and the transverse element SCP1 of the synaptonemal complex did not indicate any overall obvious defect in synapsis although we noticed that SCP1 staining was somewhat faint in *Miwi*$^{-/-}$; *Mili*$^{-/-}$ spermatocytes (***Figure 29 E-J***). These results indicate that PIWI proteins are not required for the pairing of the homologous chromosomes or in sequestering the sex chromosomes for the formation of the sex body.

Figure 29: *Miwi*$^{-/-}$; *Mili*$^{-/-}$ spermatocytes are not defective in synapsis of homologous chromosomes

A-D) Co-staining spermatocyte spreads from 6 weeks old *Miwi*$^{-/-}$;*Mili*$^{-/-}$ (-/-; -/-) mice for nucleus (DAPI), X and Y chromosomes and the "sex-body" (γH2AX) shows that the sex chromosomes of *Miwi*$^{-/-}$; *Mili*$^{-/-}$ spermatocytes are able to recognize each other during homologous chromosome pairing in meiosis, and can aggregate into a compact "sex body".

Shown is a 2μm 3D image of a pachytene spermatocyte identified by the globular γH2AX staining over the sex chromosomes.

E-J) Co-staining spermatocyte spreads from 6 weeks old *Miwi*$^{+/-}$; *Mili*$^{+/-}$ (+/-; +/-) mice (E-G) and *Miwi*$^{-/-}$; *Mili*$^{-/-}$ mice (-/-; -/-) (H-J) for the transverse (SCP1) and lateral (SCP3) elements of the synaptonemal complex shows that synapsis in *Miwi*$^{-/-}$; *Mili*$^{-/-}$ testis is not overtly impaired. DAPI and γH2AX were used to stain the nucleus and the "sex body", respectively. White arrowheads point the synapsed pseudo-autosomal regions of the sex chromosomes.

Shown are 5-7μm 3D images of pachytene stage spermatocytes. Insets are the 2D images of the sex body in a different *Miwi*$^{-/-}$; *Mili*$^{-/-}$ spermatocyte sample.

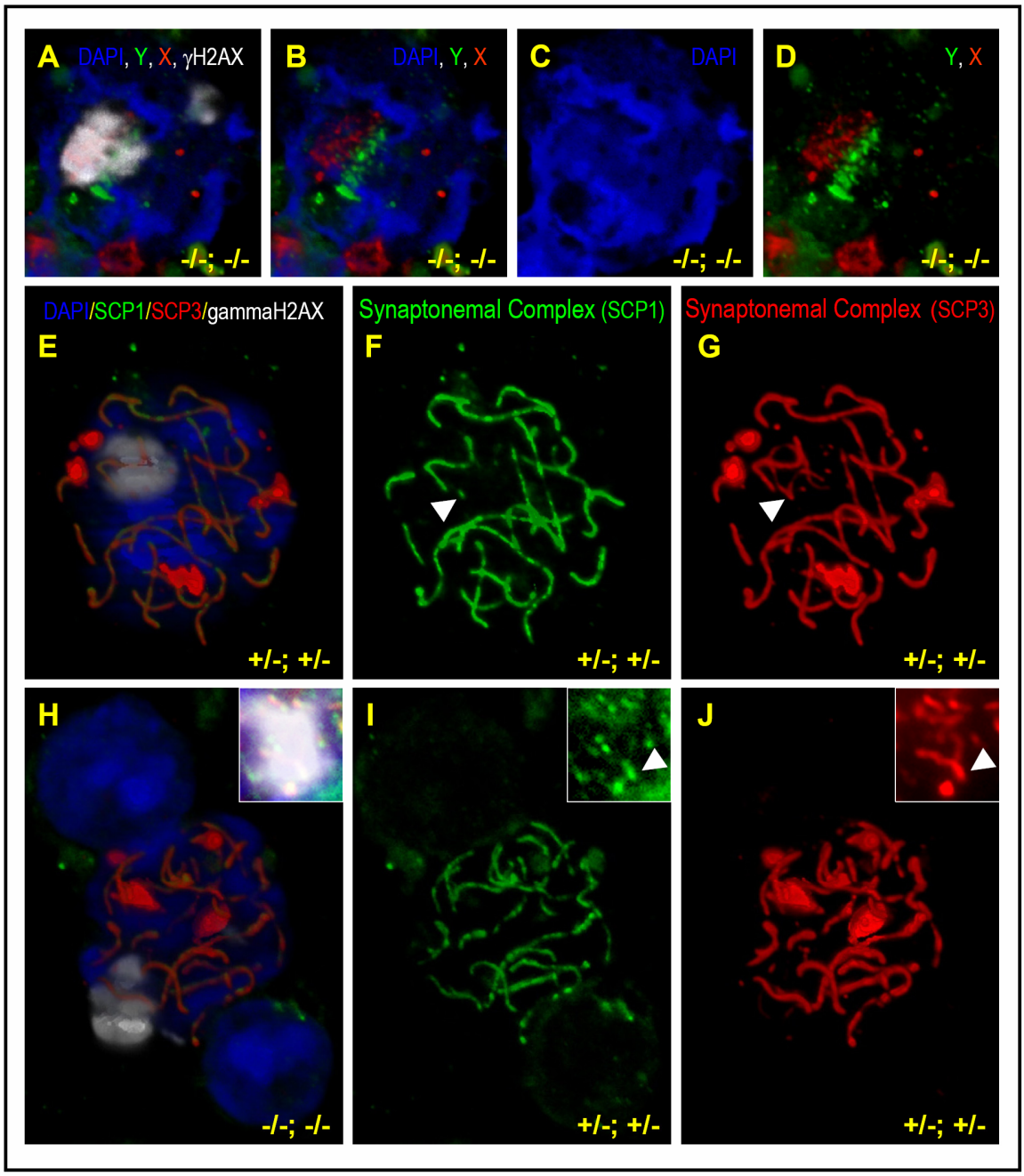

Figure 29: *Miwi*$^{-/-}$; *Mili*$^{-/-}$ spermatocytes are not defective in synapsis of homologous chromosomes

Next, we examined the epigenetic status of the sex body in *Miwi*$^{-/-}$; *Mili*$^{-/-}$ spermatocytes. Due to its highly heterochromatinized nature, the sex body is normally rich in heterochromatin markers and lacks euchromatin markers. For instance, the heterochromatin marker H3K9-me2 highly accumulates on the sex body between early and late pachynema (Khalil, Boyar et al. 2004; van der Heijden, Derijck et al. 2007). However, we observed that sex chromosomes in *Miwi*$^{-/-}$; *Mili*$^{-/-}$ spermatocytes all remain hypomethylated for H3K9me2, even though they are coated with globular γH2AX aggregates (***Figure 30***).

Figure 30: The sex body in *Miwi*$^{-/-}$; *Mili*$^{-/-}$ spermatocytes is not heterochromatic

Co-staining 14 weeks old *Miwi*$^{+/-}$; *Mili*$^{+/-}$ (**A-D**) and *Miwi*$^{-/-}$; *Mili*$^{-/-}$ (**E-H**) testis cryosections for nucleus (DAPI) (**A, E**), the heterochromatin marker H3K9me2, and γH2AX (**B-D, F-H**) shows that the sex body in *Miwi*$^{-/-}$; *Mili*$^{-/-}$ spermatocytes is not heterochromatinized.

White and blue arrows indicate examples of the sex bodies (globular γH2AX) with under or hyper-dimethylated H3K9, respectively. White arrowhead and insets point to a late zygotene spermatocyte, as identified by the tadpole-like γH2AX staining. Insets correspond to another image.

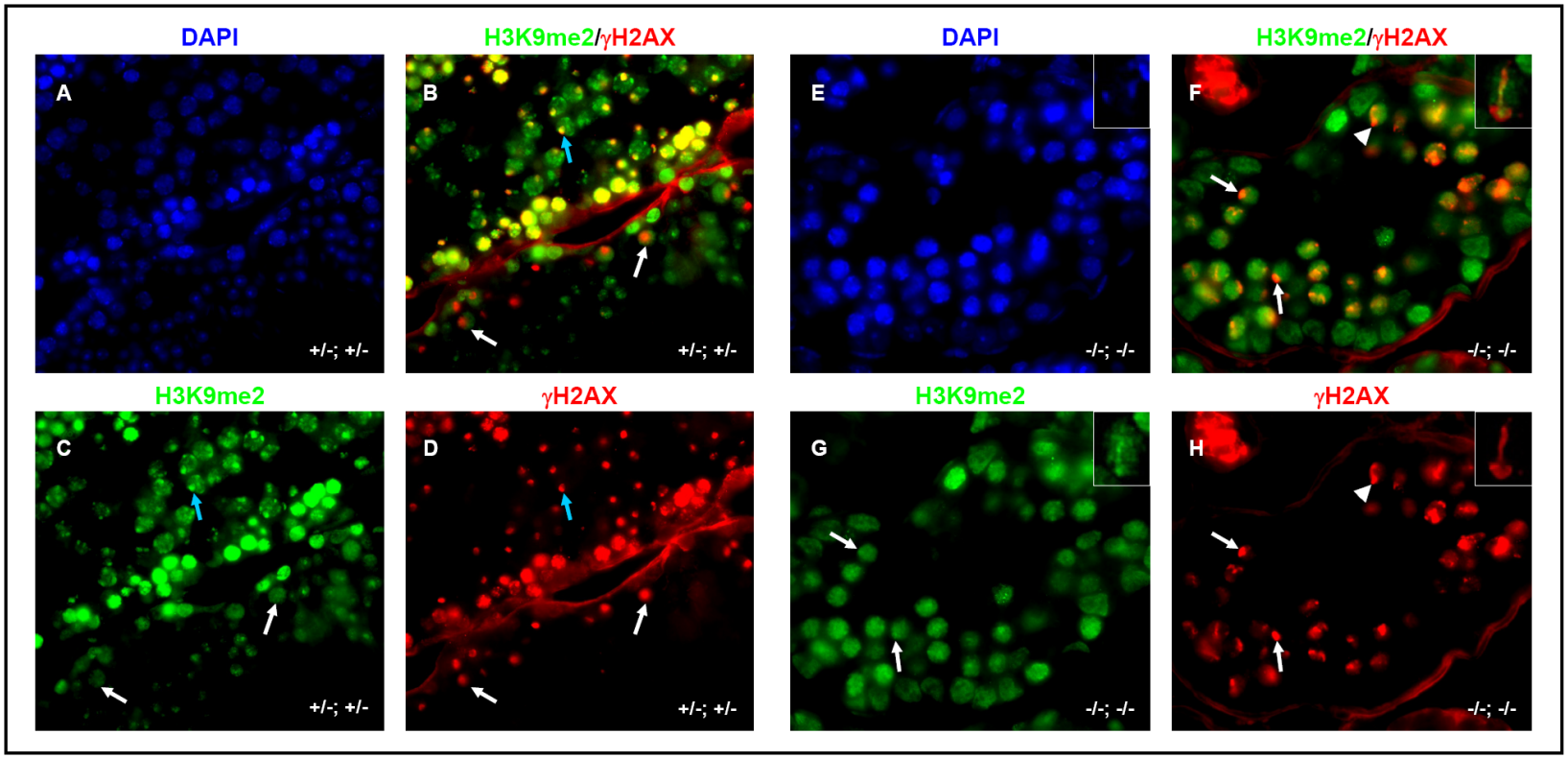

Figure 30: The sex body in *Miwi*$^{-/-}$; *Mili*$^{-/-}$ spermatocytes is not heterochromatic

It is possible that lack of H3K9 hyper-dimethylation on the *Miwi*$^{-/-}$; *Mili*$^{-/-}$ sex bodies is due to the spermatogenic arrest occurring before the methylation. To test this possibility, we examined for an earlier epigenetic mark, acetyl-H4K16, which marks euchromatin and disappears from the sex chromosomes upon formation of the "sex body" during early pachynema (Khalil, Boyar et al. 2004). While all the sex bodies in *Miwi*$^{+/-}$; *Mili*$^{+/-}$ spermatocytes lack acetyl-H4K16, *Miwi*$^{-/-}$; *Mili*$^{-/-}$ sex bodies are still covered with the mark (***Figure 31***). These results indicate that the silencing of the sex chromosomes is impaired in *Miwi*$^{-/-}$; *Mili*$^{-/-}$ testes.

Figure 31: The sex body in *Miwi*$^{-/-}$; *Mili*$^{-/-}$ spermatocytes is euchromatic

Co-staining 14 weeks old *Miwi*$^{+/-}$; *Mili*$^{+/-}$ (**A-D**) and *Miwi*$^{-/-}$; *Mili*$^{-/-}$ (**E-H**) testis cryosections for nucleus (DAPI) (**A, E**), the euchromatin marker acetyl-H4K16 (A-H4K16), and γH2AX (**B-D, F-H**) shows that the sex body in *Miwi*$^{-/-}$; *Mili*$^{-/-}$ spermatocytes has a euchromatic nature.

White arrows and insets point to pachytene spermatocytes, as identified by the globular γH2AX staining. Insets are 5μm thick 3D views from another image.

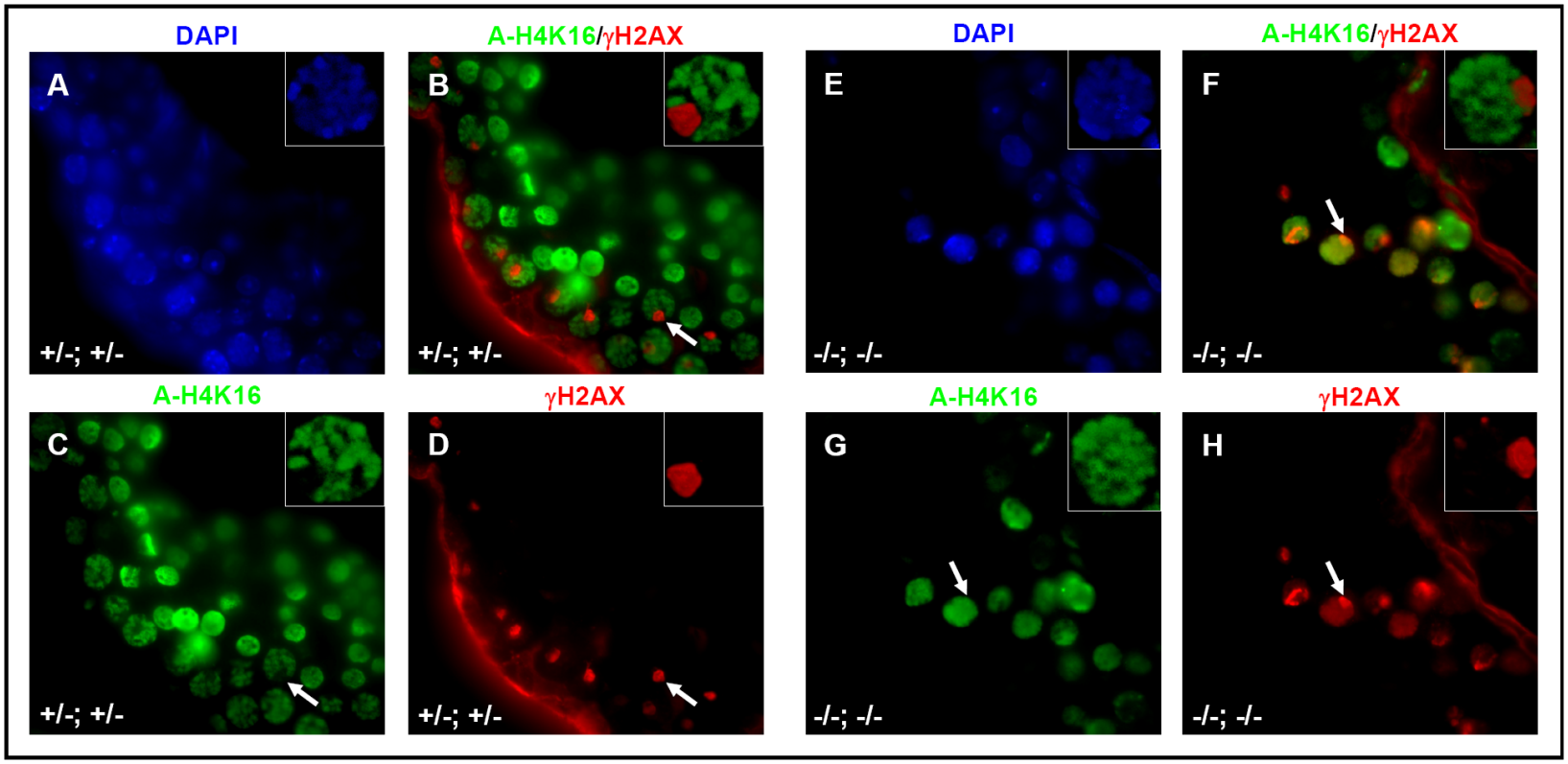

Figure 31: The sex body in *Miwi*$^{-/-}$; *Mili*$^{-/-}$ spermatocytes is euchromatic

Since piRNAs are thought to guide PIWI proteins to target genomic regions or transcripts, we asked if there are piRNAs produced from the sex chromosomes in the testis. Although the adult testis is highly depleted for piRNA sequences corresponding to the sex chromosomes (***Table 2***), there might be piRNA production at the earlier stages, before MSCI occurs. For this reason we compared 7-8dpp and 13dpp testes with the adult testis for the presence of piRNAs that match to the sex chromosomes. Indeed, X-linked piRNAs are relatively enriched in the testis at 7-8dpp, and their abundance decreases in the adult, pointing to a correlation between their production and the subsequent silencing of the regions they are derived from (***Figure 32***).

Figure 32: piRNAs are produced from the X chromosome in the testis before MSCI

A-D) Comparison of the piRNA profiles of 7-8dpp (**A**), 13dpp (**B**) and adult (**C-D**) testes shows that piRNAs are produced from the X chromosome before MSCI. Shown are the normalized number of piRNA clones (y axis) plotted along their corresponding region on the X chromosome (x axis) (see "Materials and Methods"). Each bar denotes a cluster of piRNAs within 50Kb window, with the bar height indicating the normalized number of piRNA clones within each window. piRNAs derived from the Watson strand are indicated with blue, those corresponding to the Crick strand are in red. piRNAs were cloned from the immuno-precipitates of MILI and MIWI in Chapter 3.

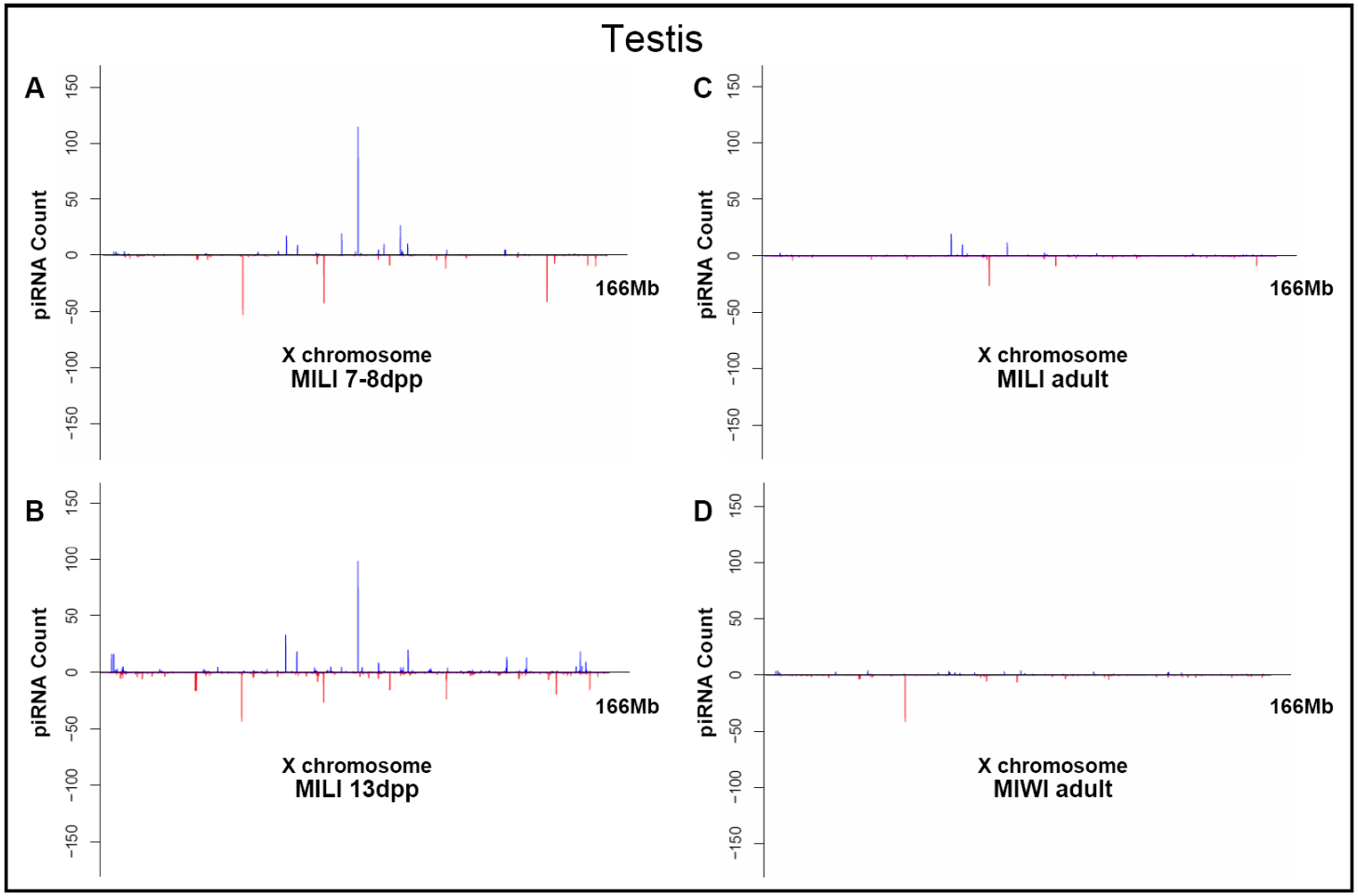

Figure 32: piRNAs are produced from the X chromosome in the testis before MSCI

(Bioinformatics by Na Liu/Haifan Lin Lab)

4.6 Discussions

Here we characterized the function of PIWI proteins and piRNAs in the adult mouse testis by phenotypic and cytological analyses. We show that PIWI proteins and piRNAs in the mouse are enriched in the "dense body", a male-specific sub-nuclear structure found exclusively in spermatocytes during prophase I of meiosis. Interestingly, in the absence of a functional PIWI/piRNA complex, spermatogenesis is terminally arrested during this period. Moreover, meiotic silencing of the sex chromosomes, an event specific to male meiosis, is impaired as assessed by the epigenetic marks H3K9-dimethyl and acetyl-H4K16. Our results indicate that PIWI proteins and piRNAs are necessary for the silencing of the sex chromosomes during spermatogenesis, and thus provide the first insight about the cause of the male-specific infertility phenotype of murine PIWI proteins.

Our phenotypic analyses indicate that *Miwi*$^{-/-}$; *Mili*$^{-/-}$ mice phenocopy *Mili*$^{-/-}$ mice with complete spermatogenic arrest during meiosis (Kuramochi-Miyagawa, Kimura et al. 2004). We did not observe any other phenotype, including embryonic, somatic, oogenic or maternally derived defects, indicating that murine PIWI/piRNA complexes are indispensable only for the progression of spermatogenesis and especially during meiosis. Indeed, our cytological analysis shows that piRNAs are

germ cell-specific and highly upregulated during meiosis, similar to PIWI proteins. Although maintenance and division of the spermatogonial stem cells and their progenitors are impaired in the absence of *Mili* (Unhavaithaya, Hao et al. 2008), currently it is not clear whether these phenotypes represent an independent stem cell function of MILI or whether they are merely an indirect effect of the spermatogenic arrest during meiosis.

What can this male-specific meiotic function be? Although PIWI proteins and piRNAs have been implicated in the silencing of the transposons in the pre-meiotic germline (Aravin, Sachidanandam et al. 2008; Kuramochi-Miyagawa, Watanabe et al. 2008), piRNAs with transposonic sequences (a.k.a rasiRNAs) constitute only a minor fraction of the adult testicular piRNAs (***Chapter 3***), which is enriched with meiotic piRNAs. This observation indicates that at least the majority of piRNAs in the adult can not function in targeting transposons. Indeed, we did not observed any difference in the expression patterns of the piRNAs that differed in their genomic annotation. Interestingly, we observed that PIWI proteins localize to two male-specific structures: the "chromatoid body" and the "dense body".

The "chromatoid body" is believed to be the form of "nuage" in spermatocytes and spermatids. Although nuage is a fibrous material surrounding the nucleus and specific to germ cells in general, the chromatoid body is a peri-nuclear conspicuous sphere observed in only spermatocytes and round spermatids. It is thought to be an RNA processing and storage center (Soderstrom and Parvinen 1976; Parvinen 2005; Kotaja, Bhattacharyya et al. 2006; Kotaja and Sassone-Corsi 2007), and also an intra and inter-cellular carrier vessel (Ventela, Toppari et al. 2003). Thus, the chromatoid body may be the site of piRNA production from the precursors and/or functions in shuttling piRNAs to their destinations.

The function of the "dense body" remains elusive as well. It has been described in the Chinese hamster spermatocytes (Moses 1977; Dresser and Moses 1980; Takanari, Pathak et al. 1982) as well as in the mouse (Fletcher 1979; Goetz, Chandley et al. 1984) as a dynamic structure during prophase I of meiosis. In the mouse, it is detectable from pachynema until diplonema and can be found away from the "sex body" before mid-pachynema but associates with the distal unpaired portion of the X chromosome during mid-to-late pachynema (Moses 1977; Fletcher 1979; Dresser and Moses 1980). Its appearance and subsequent association with the sex chromosomes during the period they undergo silencing is suggestive of its involvement in the meiotic sex chromosome

inactivation. It is possible that the dense body detects the γH2AX signal on the unpaired regions and carries the machinery needed for their silencing.

Several observations indicate that PIWI/piRNA complexes in the mouse may be the underlying components of MSCI. First, PIWI proteins have been shown to be involved in epigenetic silencing. Second, murine PIWI/piRNA complexes are detected predominantly in the germline and abundantly during meiosis. Third, mutations in the PIWI proteins are correlated with transposon de-regulation specifically in the germline. Fourth, PIWI/piRNA complexes are found in the nucleus of germ cells and localize to the male-specific "dense body", which associates with the "sex body" and whose components have been shown to be necessary for the proper formation of heterochromatinized sex chromosomes (Kolas, Marcon et al. 2005). Fifth, MILI and MIWI interact with MAELSTROM (MAEL), which has been shown to localize to the "sex body" as well as nuage/chromatoid body (Findley, Tamanaha et al. 2003; Costa, Speed et al. 2006). In addition to the PIWI proteins, MAEL associates with the chromatin remodeler SNF5 and the chromatin-associated protein SIN3B. And lastly, the lack of a functional PIWI/piRNA complex leads to male-only infertility due to meiotic arrest at pachytene stage of meiosis, corresponding to the period of MSUC. This sexually

dimorphic phenotype can be attributed to the presence of heteromorphic chromosome homologs in the males only.

Indeed, we observed that although the sex chromosomes in *Miwi*$^{-/-}$; *Mili*$^{-/-}$ spermatocytes are covered with γH2AX, they lack the heterochromatin mark H3K9me2, and remain rich in the euchromatin mark acetyl-H4K16. Enrichment of the sex chromosomes with H3K9me2 corresponds to the stage of the spermatogenic arrest observed in *Miwi*$^{-/-}$; *Mili*$^{-/-}$ mice. Thus, although it is possible that the lack of H3K9me2 is due to the lack of the germ cells, an earlier epigenetic mark, acetyl-H4K16, is also affected in the double mutants. This observation indicates that the defect on the epigenetic status of the sex chromosomes is not a secondary effect resulting from the disintegrity of the tissue composition.

We further show that piRNAs are produced from the X chromosome before it is silenced during spermatogenesis. Although it is currently possible that piRNA production from the X chromosome before but not after meiosis is the result rather than the cause of the silencing, the X chromosomes in the ovary do not produce any piRNA despite being transcriptionally active (Watanabe, Totoki et al. 2008). Therefore we think the production of piRNAs specifically from the X chromosome of the male germline is

the driving force of its epigenetic silencing during meiosis. Interestingly we did not observe a significant level of piRNA production from the Y chromosome even before it is silenced (*Data not shown*). We propose that the association of MILI with the X-linked piRNAs before and/or during meiosis inititates the piRNA-guided targeting and subsequent silencing of the corresponding regions on the X chromosome, which may spread along the unpaired regions of both homologs (***Figure 33***).

Turner *et al* (2005 and 2006) showed that MSUC is not specific to the sex chromosomes in males but occurs on any unpaired chromosome regions as a direct consequence of asynapsis. This mechanism is also present in the female germline since the only X chromosome of mice with Turner Syndrome, and the X and Y chromosomes of the XY^{tdym1} oocytes are silenced. Interestingly the MILI/piRNA complex has been detected in the female germline as well. It would be interesting to analyze if MSUC still operates in the female germline of the Turner mice and the XY^{tdym1} mice in the absence of PIWI proteins.

Figure 33: Function of the PIWI proteins and piRNAs in the adult testis

piRNAs are produced from the X chromosome before its meiotic inactivation, possibly through the processing of the precursors in the nuage/chromatoid body. PIWI proteins might be loaded with the piRNAs in the nuage/chromatoid body, and target the corresponding genomic regions, resulting in their silencing during meiosis. Silencing might be spreaded along the entire homologs to cover the Y chromosome. Such piRNA mediated targeting occurs only on the chromosomes that show asynapsis, since synapsis probably makes the genomic regions inaccessible for the targeting.

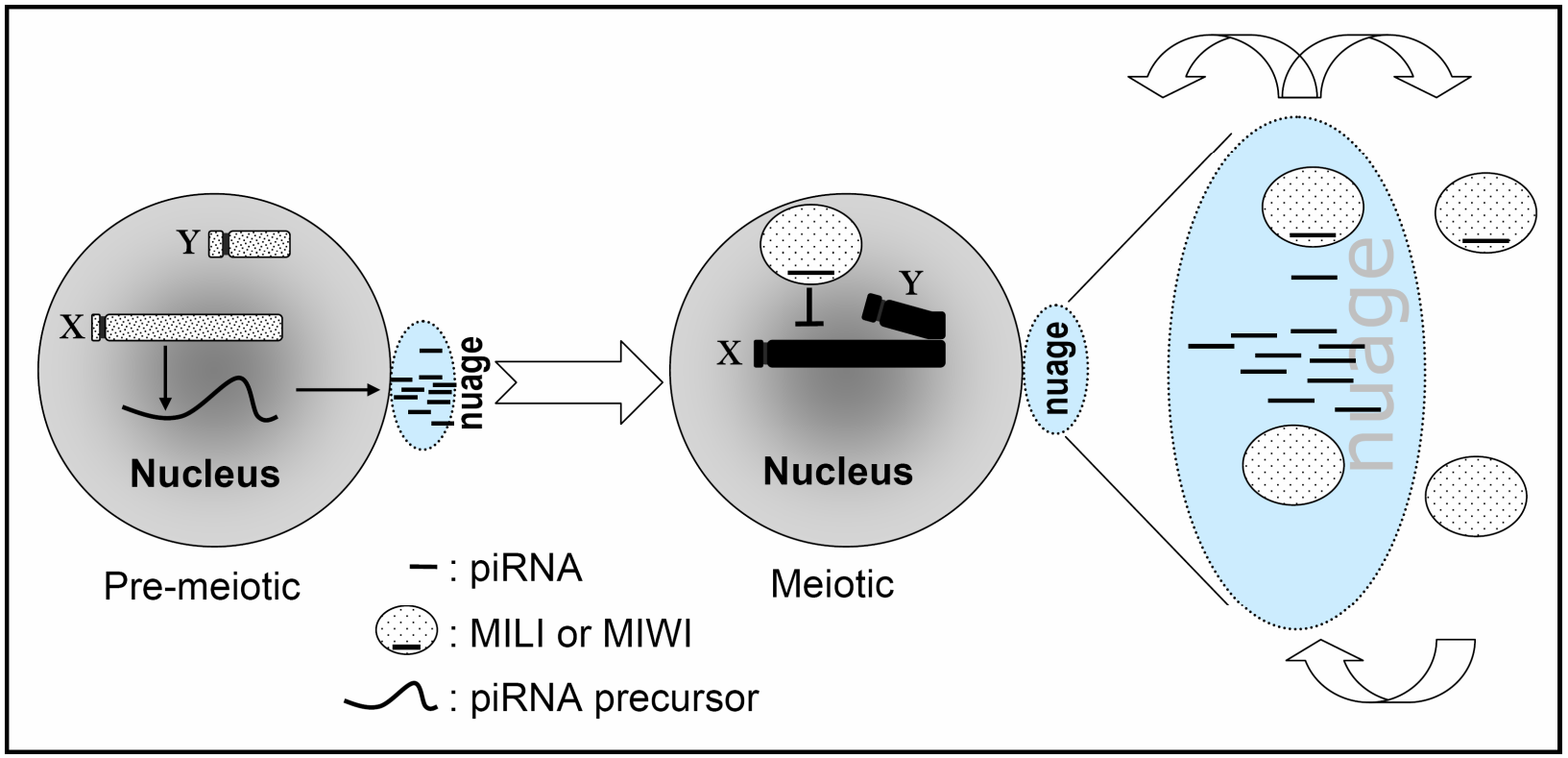

Figure 33: Function of the PIWI proteins and piRNAs in the adult testis

4.7 Conclusions

Our results elucidate the sexually dimorphic phenotype of the murine piwi mutants for the first time. We show that PIWI/piRNA complexes are concentrated in the male germ cell-specific structures "chromatoid body" and "dense body". Although the functions of these structures are elusive, the dense body has been implicated in synapsis and formation of the sex body during spermatogenesis. While synapsis is not impaired in *Miwi*$^{-/-}$;*Mili*$^{-/-}$ spermatocytes, sex chromosomes fail to acquire a heterochromatic nature indicating that murine PIWI/piRNA complexes are necessary for "meiotic sex chromosome inactivation" (MSCI). Naturally occurring only in the males due to the heteromorphic sex chromosomes, MSCI is necessary for the progression of spermatogenesis. Thus, in the absence of a functional PIWI/piRNA complex, only spermatogenesis is affected.

4.8 Acknowledgements

We thank Dr. Yoshitake Nishimune for graciously providing us with the EE2 and TRA54 antibodies, Dr. Debra J. Wolgemuth for the Cyclin D3 antibody and Dr. Philip Avner for the Tsx antibody.

5. General Discussion

5.1 Conclusions and Significance of the Work

The goal of the research in this dissertation has been to elucidate the function of PIWI proteins by studying MILI and MIWI, two PIWI proteins in the mouse. Although PIWI proteins have been genetically shown to be necessary for the establishment and/or physiology of the germline, their function at the molecular level remained elusive. Biochemical analyses of the PIWI proteins described here led to the discovery of piRNAs, and thus defined a clear molecular distinction between PIWI proteins and ARGONAUTE proteins. Characterization of this novel class of non-coding small RNAs not only helped elucidating the biochemical function of PIWI proteins, but also brought a new dimension to the biogenesis and function of small RNAs. Unlike miRNAs or siRNAs, piRNAs are specifically detected in the germ cells. In the adult testis, they appear to be processed from single stranded precursors in a manner independent of the "ping-pong" mechanism. Quasi-random processing of these long transcripts yields thousands of individual piRNAs, much more complex than any known type of small RNAs. Their extensive biogenesis from the intergenic regions on the genome raises the re-examination of the concept of "junk" regions, which were previously assumed to be untranscribed and have no function. It is unlikely that piRNAs themselves are "junk" since the germ cells not only invest energy in producing a tremendous amount of them,

but also in stabilizing them by the 2`-O-methylation on their 3` ends. Current progress, including the research presented here, suggests that piRNAs function in guiding PIWI proteins to genomic regions and/or transcripts (Brennecke, Aravin et al. 2007; Gunawardane, Saito et al. 2007; Yin and Lin 2007). In particular, our results indicate that piRNAs might guide PIWI proteins in scanning the genome to identify the unpaired regions during meiosis in the mouse. Since such unpaired regions normally occur between the heteromorphic sex chromosomes in the male mice, the absence of a functional PIWI/piRNA complex is manifested only in the males, and particularly on the sex chromosomes. Therefore, our results provide the first explanation for the sexually dimorphic phenotype observed in the murine *piwi* mutants. Furthermore, the results presented here also elucidate the molecular mechanism of MSUC for the first time, illuminating one of the major black boxes of meiosis. Last but not the least, our sub-cellular analyses define PIWI proteins and piRNAs as the first components of the dense body after FKBP6. Therefore, PIWI proteins form an excellent platform for the genetic dissection of MSUC, and for the functional elucidation of the dense body. The results leading to these conclusions were discussed in depth in the preceding chapters. In the following sections of this chapter, I will discuss about the biological role of MSCI followed by future directions for expanding our knowledge of PIWI proteins and piRNAs, and their involvement in MSUC.

5.2 Biological Role of Meiotic Sex Chromosome Inactivation

Several roles have been proposed for the occurrence of such phenomenon among heterogametic animals (Turner 2007). The recent observations indicating that similar inactivation occurs on any unpaired chromosomal regions suggested MSCI is merely a manifestation of MSUC on the heteromorphic sex chromosomes (Baarends, Wassenaar et al. 2005; Turner, Mahadevaiah et al. 2005). Then what is the biological purpose of MSUC?

A plausible explanation may lie beneath the risks of meiosis, which involves a series of unique events, including the shuffling of the genomic material, their recombination and reduction by half. Any error in these processes directly affects the next generation and thus the fitness of the species. It is possible that MSUC is a cellular surveillance mechanism, which protects the next generation from aneuploidy by identifying and eliminating the germ cells that display erroneous pairing (Turner 2007). The silencing of any unpaired chromosomal region makes the germ cell null with respect to the genes on the silenced region. If any of these regions include genes that are necessary for the survival of the cell or its progression through meiosis, the silencing is expected to lead to its meiotic arrest, and thus the erroneous cell is eliminated from the

germline pool. For instance, the only X chromosome in the XO female is meiotically silenced and most of the germline undergoes meiotic arrest unless the X chromosome self-synapses and escapes silencing (Turner, Mahadevaiah et al. 2005). While the silencing of the X and Y chromosomes in the male has no effect, this has been attributed to the male-specific expression of the retroposed copies in the autosomes (Wang 2004). These genes are specifically expressed at the onset of MSCI, and thus compensates for the inactivation of the sex chromosomes. However, mammalian spermatogenesis is a syncytial event, and currently it is not clear why silencing of a critical gene in one cell should have such an impact on the cell despite the gene product can be obtained from the other cells in the syncytium.

Another role of MSUC has been suggested by meiotic silencing of unpaired DNA (MSUD) observed in *Neurospora crassa* (Lee, Pratt et al. 2003; Kelly and Aramayo 2007). When this fungus undergoes meiosis, any hemizygous region is silenced and further invokes silencing of the similar sequences in the genome in *trans.* This mechanism is thought to be a surveillance mechanism against transposable elements, which are expected to be hemizygous upon integration into the host genome. It is possible that upper eukaryotes conserved this mechanism while evolving the heteromorphic sex chromosomes, which is manifested as MSCI.

Evolution of the heteromorphic sex chromosomes caused the issue of dosage compensation (Namekawa and Lee 2009). The heterogametic sex has half dose of the genes that reside in the unpaired segment compared to the homogametic sex. Therefore one of the sex chromosomes of the homogametic sex undergoes silencing during embryogenesis to compensate for the dosage difference. In mammals, sex is determined by the XY system, and in eutharians, two types of dosage compensation occur: While the X chromosome in the true embryo is randomly inactivated, the extraembryonic lineage always inactivates the paternally derived X chromosome. The random X-inactivation has been shown to be mediated by a mechanism involving a non-coding RNA, called *Xist*, which has been shown to be dispensable for MSCI (Namekawa, Park et al. 2006). Although the onset of this event has been thought to be the blastocyst stage, recent reports indicate it can occur as early as at the two cell stage (Huynh and Lee 2003). This observation raised the possibility that the paternal X chromosome might be delivered to the early embryo in an already inactive state. Indeed, the sex chromosomes in the males largely retain their inactive state even after meiosis (Namekawa, Park et al. 2006; Turner, Mahadevaiah et al. 2006). Moreover, marsupials do not have an *Xist* gene and the dosage compensation is maintained by the inactivation of only the paternal X

chromosome. Therefore it is possible that MSCI may function in imprinting the paternal X chromosome for its inactivation in the future embryo.

The future research on PIWI proteins and piRNAs will likely shed light on these potential biological roles of MSCI. In the rest of this chapter, I will discuss about the directions to pursue with the aim of laying out a roadmap for the related future research.

5.3 Future Directions

5.3.1 Evaluation of the Transcriptional Activity of the Sex Chromosomes During Spermatogenesis in the Absence of PIWI Proteins

The phenotypic analyses of *Miwi*$^{-/-}$; *Mili*$^{-/-}$ mice showed that sex chromosomes fail to become heterochromatinized during spermatogenesis in the absence of a functional PIWI/piRNA complex. Although epigenetic marks represent the transcriptional state of the chromatin, a more direct analysis will derive from the staining of *Miwi*$^{-/-}$;*Mili*$^{-/-}$ spermatocytes for Cot 1 RNA and RNA Polymerase II, which were previously shown to be excluded from the sex chromosomes during male meiosis in the wild-type mice (Khalil, Boyar et al. 2004; Baarends, Wassenaar et al. 2005; Turner, Mahadevaiah et al. 2005). Cot 1 is a repetitive sequence found frequently in the introns of genes throughout

the genome and thus, *in situ* RNA analysis for Cot 1 marks the primary transcripts. Our analyses suggest that the sex chromosomes in the male germline will display RNA Polymerase II and Cot 1 staining in *Miwi*$^{-/-}$; *Mili*$^{-/-}$ background unlike in the wildtype.

Interestingly while *Mili*$^{-/-}$ and *Miwi2*$^{-/-}$ mice undergo spermatogenic arrest during meiosis, *Miwi*$^{-/-}$ mice successfully complete meiosis. In the wild-type males, the silenced state of the sex chromosomes is maintained post-meiotically (Namekawa, Park et al. 2006; Turner, Mahadevaiah et al. 2006). Therefore, it is possible that while MILI and its associated piRNAs initiate this silencing, the MIWI/piRNA complex maintains it post-meiotically. To explore this possibility, I propose to examine the transcriptional state of the sex chromosomes in the male germline of *Miwi*$^{-/-}$ mice with the above mentioned strategies. The spermatocytes of *Miwi*$^{-/-}$ mice are unlikely to be impaired since they are able to complete spermatogenesis, most likely due to the presence of the MILI/piRNA complex. The round spermatids on the other hand are more likely to be defective, since they do not complete spermiogenesis. Similar analyses can be done on *Miwi2*$^{-/-}$ spermatocytes to explore if their meiotic arrest is also correlated with a defect in MSCI. Likewise, although *Miwi*$^{-/-}$;*Mili*$^{-/-}$ mice are expected to phenotypically represent the loss of function of all the PIWI/piRNA complexes in the mouse, a more conclusive answer for

the function of the PIWI/piRNA complexes will be derived from the analysis of the *Miwi*$^{-/-}$; *Miwi2*$^{-/-}$; *Mili*$^{-/-}$ mice.

5.3.2 Identification of the Genomic Regions That are Modulated by the PIWI/piRNA Complexes

Since our results indicate that PIWI proteins utilize piRNAs to target the unpaired regions during meiosis, I propose to examine the mouse spermatocytes for the localization of PIWI proteins and piRNAs on the chromosomes. This evaluation can be achieved via immuno-fluoresence on spermatocyte spreads, which provides a higher resolution than cryosections. Likewise genome-wide **ch**romatin **i**mmuno-**p**recipitation (**ChIP**) on isolated spermatocytes by using the antibodies that recognize mouse PIWI proteins is expected to unravel the targeted chromosome regions. The epigenetic status of the identified regions can be elucidated by comparing with the genome-wide H3K9-me2 and acetyl-H4K16 ChIPs. In order to test the involvement of piRNAs, one can perform the above mentioned experiments in the presence of RNase A. Additionally, the ChIP results can be compared with the piRNA profile of the pre-meiotic testis to assess if a correlation between the piRNA loci and the PIWI-targeted loci exist. If such a correlation is observed, RNase H and RNase III treatments can be used to assess whether piRNA targeting of the genomic regions occurs through the interaction with the

genomic DNA or with a nascent RNA. These RNases degrade the RNA component of the DNA-RNA hybrids and double stranded RNA respectively.

5.3.3 Extending the Function of the PIWI/piRNA Complexes to the Autosomes and the Female Germline

Mice with translocated chromosomes display synaptic failure during meiosis with unpaired regions undergoing silencing (Mahadevaiah, Turner et al. 2001; Turner, Mahadevaiah et al. 2005; Turner, Mahadevaiah et al. 2006). Hence, meiotic silencing is not specific to the sex chromosomes but occurs on any unpaired regions. An interesting question is whether the silencing of the unpaired regions in these models will be affected in the absence of PIWI proteins. In parallel, the piRNA profile of these animals pre and post-silencing can be analyzed to evaluate if a correlation exists between the piRNA loci and the silenced regions. These analyses will extend the function of the PIWI/piRNA complexes from the sex chromosomes to the whole genome.

Additionally, this mechanism operates in the female germline as the only X chromosome of the XO female mice (Turner mice) is silenced during meiosis unless self-synapsed (Turner, Mahadevaiah et al. 2005). Only approximately 30% of the oocytes escape the silencing due to self-synapsing. Those that undergo silencing die because the X chromosome contains genes necessary for oogenesis. Interestingly, MILI and its

associated piRNAs have also been identified in the female germline (Kuramochi-Miyagawa, Kimura et al. 2001; Aravin, Sachidanandam et al. 2008; Watanabe, Totoki et al. 2008). To address whether they function in the silencing of the unpaired regions during oogenesis as well, I propose to examine the XO oocytes that lack a PIWI protein. This assessment will extend the function of the PIWI/piRNA complexes to the female germline. If PIWI/piRNA complexes indeed function for the MSUC in the female germline, the X chromosome will not be silenced at all. This effect will likely cause an increase in the survival rate of the oocytes. Therefore *piwi* mutations are expected to suppress the fertility defect of the Turner Syndrome albeit possibly at the expense of an increased risk in aneuploidy.

5.3.4 Extending the Function of the PIWI/piRNA Complexes From the Mouse to other Systems

Interestingly, PIWI proteins and piRNAs have not been reported in birds and reptiles yet. The sex is determined with the ZW system in birds, in which the hetorogametic sex is the female. The heteromorphic sex chromosomes of the females are silenced during oogenesis (Schoenmakers, Wassenaar et al. 2009). Since our observations in the mouse indicate that PIWI proteins and piRNAs are the underlying factors of this silencing, the germline of birds is expected to contain PIWI/piRNA complexes. However, their loss-of-function is expected to be manifested in the females

rather than the males due to the presence of heteromorphic chromosomes only in the females.

Reptiles on the other hand are more elusive. While in some species, the sex is determined by heteromorphic sex chromosomes, in others it is determined by the temperature at which the embryo develops (Modi and Crews 2005). For instance, the house gecko lizard and crocodiles fall into the first and second category respectively. In any case, reptiles are also expected to have PIWI proteins and piRNAs since they also undergo meiosis, and thus require surveillance for the detection of any unpaired region. However, the lack of a naturally occurring set of heteromorphic chromosomes brings about the problem of readout in studying the role of PIWI/piRNA complexes in MSUC in these animals.

Of interest would be the examination of the sexually reproducing animals that show haploid parthenogenesis. For instance, in the honey bee *Apis mellifera*, the males always develop from unfertilized eggs, whereas fertilized eggs develop into females. Therefore, only the female germline undergoes both meiosis I and meiosis II, while the male germline skips directly to meiosis II. PIWI proteins and piRNAs have been found in insects (Yin and Lin 2007; Zhou, Liao et al. 2007; Kawaoka, Hayashi et al. 2008), and at

least one PIWI homolog has been predicted in the genome of the honey bee (Zhou, Liao et al. 2007) . Since our results indicate that PIWI proteins and piRNAs function during meiosis I, the female germline of the honey bee is expected to be enriched for PIWI proteins and piRNAs relative to the male germline.

5.3.5 Dissecting the Biogenesis of piRNAs

Our bioinformatic analyses indicate that piRNAs in the adult mouse testis are produced through the processing of long, single stranded precursors. How these precursors are produced and processed is not known. Our yeast-one-hybrid analyses showed that ZNF821 and KLF4 interact with the putative promoters of the piRNA clusters on Chr 9 and Chr 17, respectively. These results indicate that Krueppel C2H2 type zinc finger transcription factors may modulate the transcription of the precursors. As the next step, I propose to evaluate these interactions *in vivo* with ChIP from the adult testis. In parallel, we would like to test the involvement of KLF4 in piRNA production by analyzing a germline-specific KLF4 knock-out mouse model.

Our observation that PIWI/piRNA complexes localize to the chromatoid body may indicate that precursors of piRNAs are exported to nuage/chromatoid body to be processed into mature piRNAs, which in turn load onto the PIWI proteins present.

Subsequently, PIWI/piRNA complexes might be delivered to the nucleus and cytoplasm from the chromatoid body. This hypothesis can be tested by pulse-chase experiments. Although similar experiments previously showed that chromatoid body stores transcripts synthesized early in spermatogenesis, the fate of these transcripts was not analyzed. Following the chase, the chromatoid body can be isolated by biochemical means and its RNA content can be analyzed to assess if the labeled transcripts are processed into piRNAs. In parallel, co-immunoprecipitation with the mouse PIWI antibodies can show if the processed transcripts are loaded onto the PIWI complexes. Indeed, the chromatoid body moves around the nuclear envelope and makes transient contacts with nuclear pores as if being loaded with components from nucleus or delivering those from cytoplasm (Fawcett, Eddy et al. 1970; Soderstrom and Parvinen 1976; Parvinen and Parvinen 1979; Parvinen 2005). In support of this view, electron micrographs show material continuities between the chromatoid body and the nucleus through nuclear pores at these contact sites. Additionally, it has been shown that the chromatoid body travels between the syncytial spermatids along the cytoplasmic bridges (Ventela, Toppari et al. 2003). This observation raises the possibility that the chromatoid body acts as a carrier vessel. Therefore, it is possible that PIWI/piRNA complexes are loaded onto this structure to be distributed among the germ cells.

The complex involved in the processing of the precursors can be purified by subjecting the testicular extract to sequential column chromatography. The enzymatic activity can be monitored by tracing the formation of the piRNAs and concurrent disappearance of the precursors. The components of the purest fraction that displays the enzymatic activity can be identified with mass spectrometry and a candidate approach can be used to identify the enzyme among the components that has RNA endonuclease activity.

In conclusion, the research presented in this dissertation has opened up a new era in the biogenesis and function of small RNAs, and provided new prospects for related future research, some of which was discussed at length in this chapter. It is no doubt that the near future is bound to provide answers to at least some of these questions, extending our view of the big world of small RNAs.

6. Appendix I

6.1 Potential Involvement of MILI and piRNAs in Post-Transcriptional Regulation

The well-established catalytic role of ARGONAUTE proteins in translational regulation and mRNA stability control suggested their close relatives, PIWI proteins, might have the same functions in the gonads. For instance, both MILI and MIWI are predominantly cytoplasmic PIWI proteins in the mouse, and thus may potentially act in similar post-transcriptional regulation mechanisms.

In support of the MILI-mediated translational regulation, Unhavaithaya *et al* (2008) were able to pull down general translation initiation factors, eukaryotic cap complex and polyA tailed RNAs from testicular extracts by using anti-MILI. They additionally observed that a minor sub-population of MILI co-sedimented with poly-ribosomes (polysomes), and this profile was sensitive to EDTA or monococcal nuclease treatments, which interfere with the integrity of the polysomes. EDTA disrupts the quaternary structure of ribosomes, dissociating them into their subunits, while monococcal nuclease digests the mRNA component of the polysomes and reduces them into mono-ribosomes (monosomes). Similar results were observed for MIWI as well (Grivna, Pyhtila et al. 2006).

In support of the mRNA stability control, (Deng and Lin 2002) observed that mRNAs of the spermiogenesis-related CREM target genes co-immunoprecipitate with MIWI and this interaction is necessary for their stability, as they are not detected in the *Miwi*$^{-/-}$ testes.

However, my studies summarized in this appendix have not supported a role for MILI or piRNAs in the translational regulation or stability of genic mRNAs, in contrast to the previously proposed roles of MIWI and MILI in these aspects.

6.1.1 MILI Does not Interact With the Translational Machinery of Rough Endoplasmic Reticulum

The cellular translational machinery comprises two pools of ribosomes: membrane-bound ribosomes on rough ***E***ndoplasmic ***R***eticulum (***ER***), and free cytosolic ribosomes. Therefore, I first assessed the potential interaction between MILI and ER-bound translational machinery. Co-staining for the rough ER markers GRP94 (***Figure 34***) or Trapα (***Figure 35***) and MILI showed that MILI mostly, if not completely, do not localize to ER. Characterization of the MILI and MIWI antibodies used throughout this work is shown in (***Figure 36***).

Figure 34: MILI does not co-localize with the rough ER marker GRP94

Co-staining for the ER lumen marker GRP94 shows that MILI does not localize to ER.

Shown are the confocal images of the adult seminiferous tubules. GP15-MILI antibody was used to detect MILI. Staining was performed on testis cryosections as in Chapter 4. anti-GRP94 was a gift from Chris Nicchitta Lab.

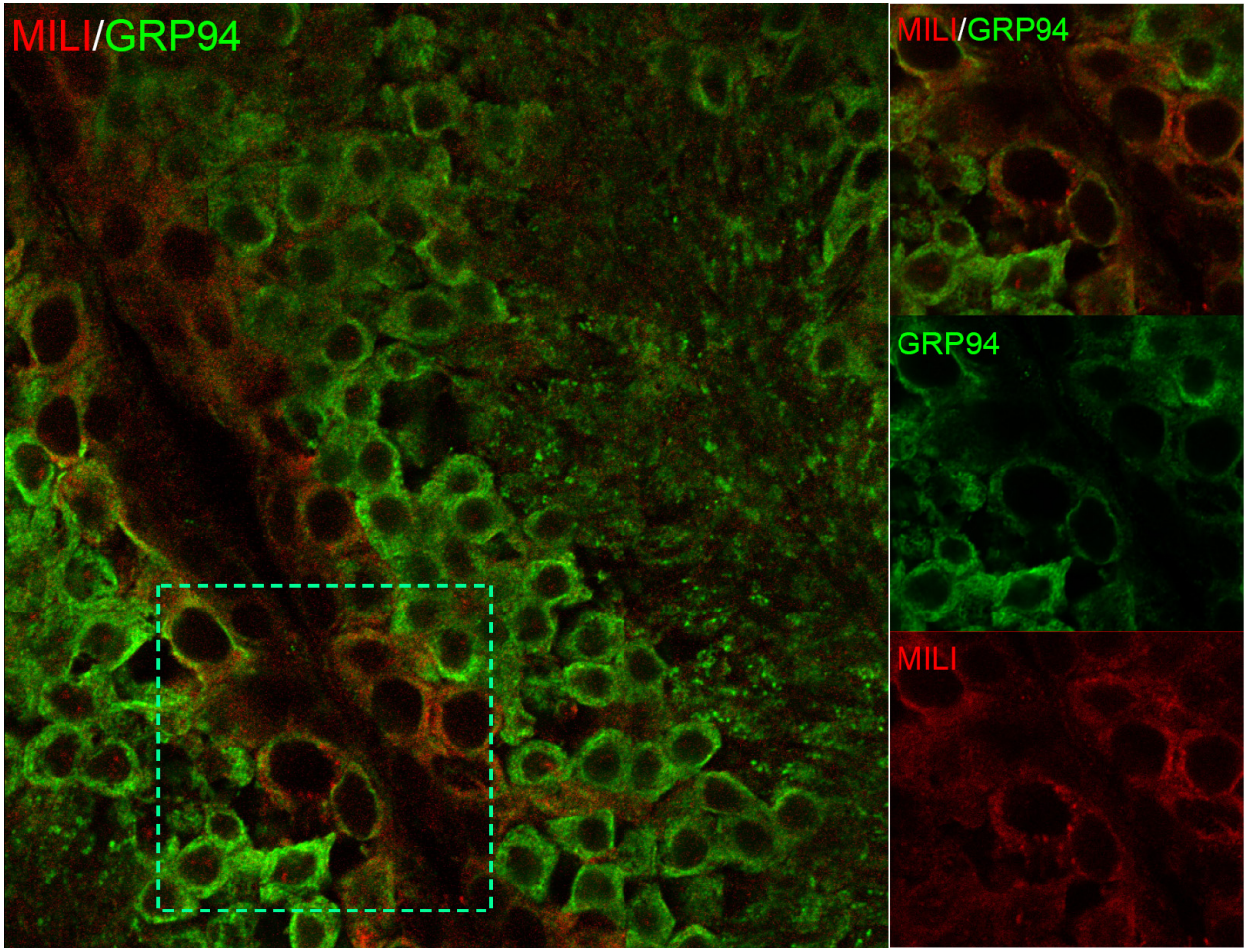

Figure 34: MILI does not co-localize with the rough ER marker GRP94

Figure 35: MILI does not co-localize with the rough ER marker TRAPα

Co-staining for the ER membrane marker TRAPα shows that MILI does not localize to ER.

Shown are the confocal images of the adult seminiferous tubules. GP15-MILI antibody was used to detect MILI. Staining was performed on testis cryosections as in Chapter 4. anti-TRAPα was a gift from Chris Nicchitta Lab.

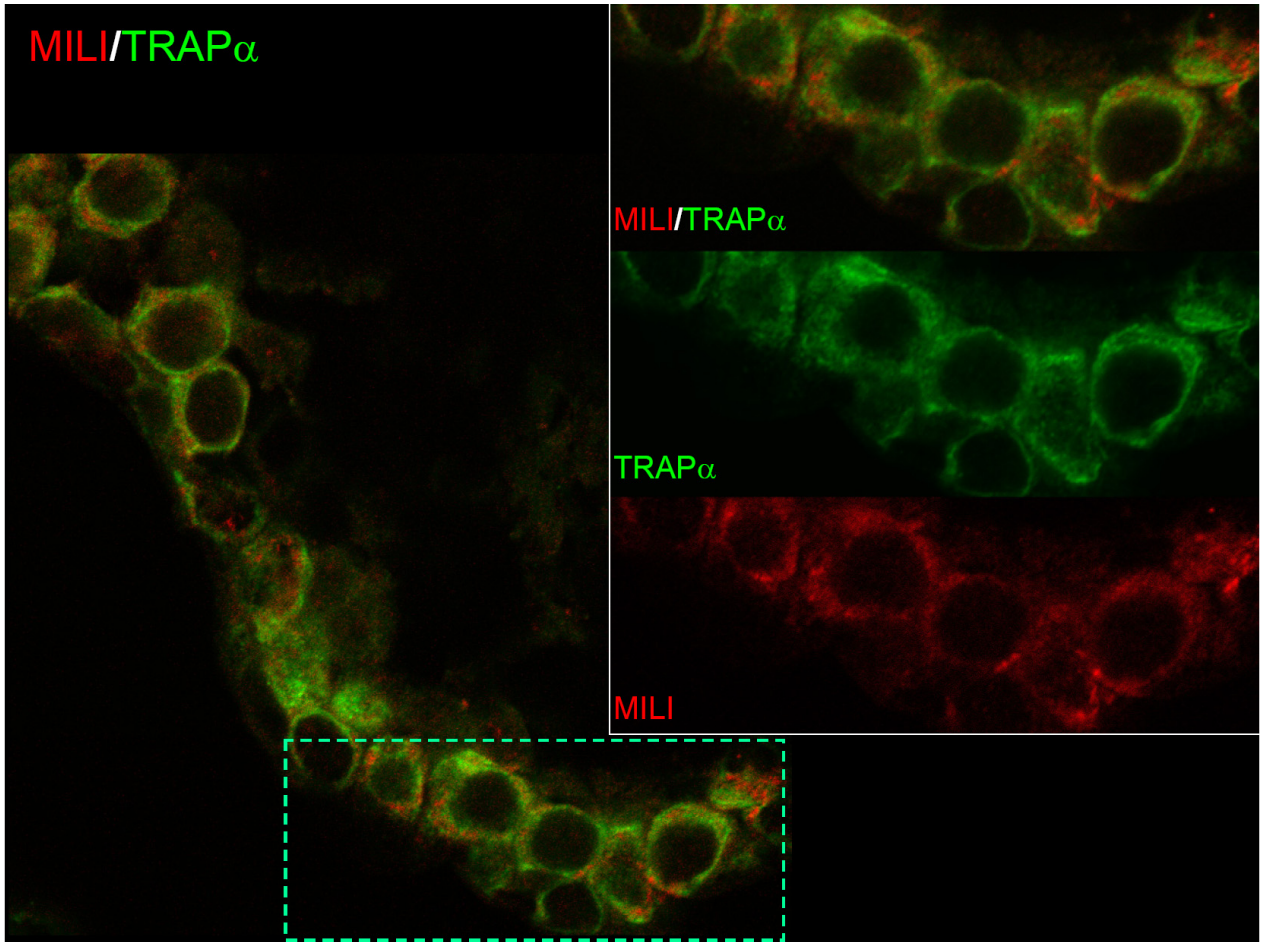

Figure 35: MILI does not co-localize with the rough ER marker TRAPα

Figure 36: MILI and MIWI antibodies utilized are specific for the antigens

A-C) MILI peptide antibody (A2847) largely co-localizes with the GP15 anti-MILI.

D) The signal is abolished largely in the *Miwi*$^{-/-}$; *Mili*$^{-/-}$ background.

E) The signal is completely abolished in the absence of the primary antibodies.

F) MILI peptide antibody and GP15 anti-MILI can efficiently recognize MILI. Immunoprecipitation was performed with the peptide antibody followed by immunoblotting with the GP15 antibody.

G) anti-MIWI can efficiently recognize MIWI. Whole extracts of *Miwi*$^{-/-}$ (-/-) and *Miwi*$^{+/+}$ (+/+) testes were immunoblotted with the MIWI antibody.

Shown in (**A-E**) are the confocal images of the adult seminiferous tubules, and in (**F-G**) are the western blots of the testicular samples.

Staining was performed on testis cryosections as in Chapter 4.

For the western blotting, protein samples were resolved with 7.5% SDS-PAGE.

Sup: Supernatant. Block: negative control, where the antibody was blocked with the peptide antigen prior to the immunoprecipitation. Exp.: Experimental sample. IP: Immuno-precipitates.

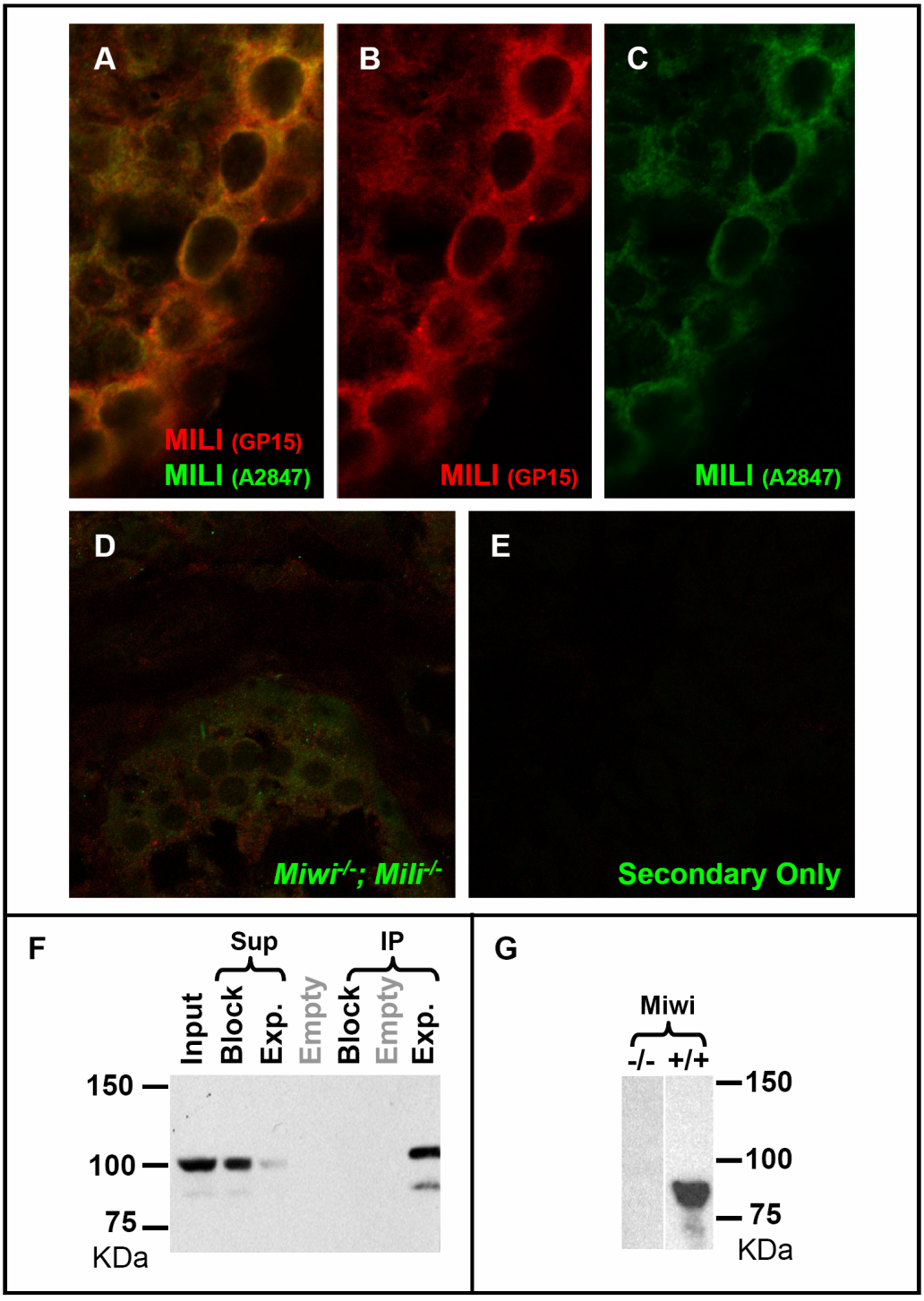

Figure 36: MILI and MIWI antibodies utilized are specific for the antigens

In parallel to this cytological strategy, I utilized membrane flotation analysis, whereby I separated membranous structures in the testicular extract, and assessed the distribution of MILI and the rough ER membrane marker TRAPα. This biochemical assay also showed that MILI and ER localize to separate fractions (***Figure 37***).

Figure 37: MILI does not co-fractionate with the rough ER membrane

Membrane flotation analysis of the testicular lysate from a 2 months old wild-type CD1 strain mouse shows MILI and ER localize to different fractions.

The analysis was performed on discontinuous sucrose gradient as in (Grivna, Pyhtila et al. 2006) using one testis. Fractions were assessed with 8% SDS-PAGE and western blotting for MILI and the ER membrane marker TRAPα. anti-TRAPα was a gift from Chris Nicchitta Lab.

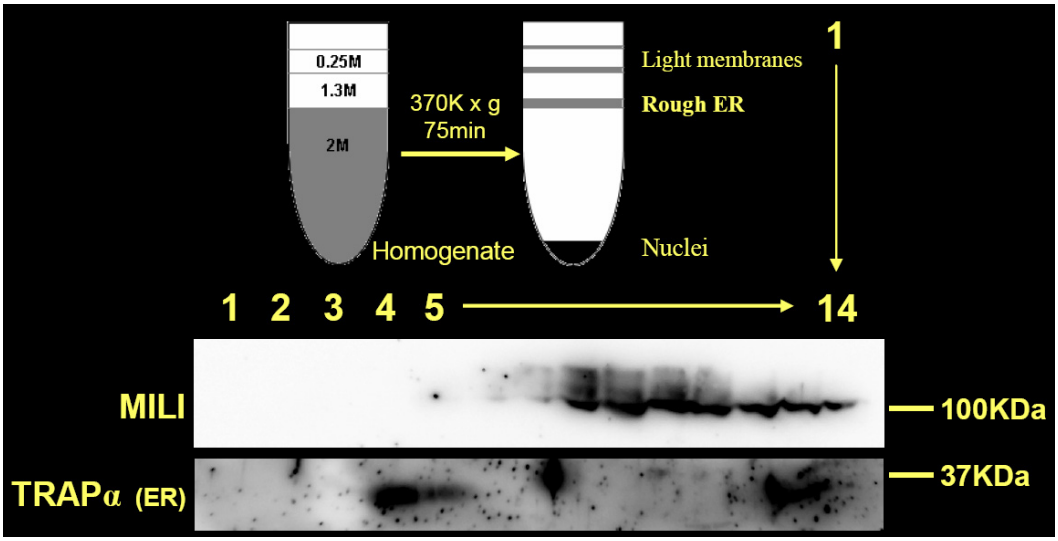

Figure 37: MILI does not co-fractionate with the rough ER membrane

These observations collectively rule out the possibility of MILI's interaction with the ER-bound translational machinery. Same was concluded for MIWI as well (Grivna, Pyhtila et al. 2006). Hence only the possibility of interaction with the free cytosolic translational machinery remains under question.

6.1.2 MILI Does not Interact With the Cellular Translational Machinery

Although (Unhavaithaya, Hao et al. 2008) observed the amount of MILI in the polysomal fractions decreased upon EDTA treatment concomitantly with the amount of ribosomes, I have not observed the same (***Figure 38***).

Fractionating the testicular lysate as in Unhavaithaya *et al* showed that MILI mostly co-sediments in the ribosome-free fractions ("FreeRNP") (***Figure 38D***). The minor population that co-sediments with ribosomes ("80S" and "polysomes") did not decrease with EDTA treatment (***Figure 38D***), which almost completely dissociated ribosomes to their subunits as evidenced by the OD_{260nm} reading (***Figure 38A*** and ***B***) and the analysis of the ribosomal RNA content of the fractions (***Figure 38C***).

Figure 38: MILI does not interact with ribosomes

Disintegration of the ribosomes in the testicular lysate with EDTA treatment shows that co-sedimentation of MILI with ribosomes is independent of their quaternary structure.

Each testis of an adult wild-type CD1 strain mouse was lysed either in the presence (**A**) or absence (**B**) of $MgCl_2$, supplemented with (**B**) or without (**A**) the indicated amount of EDTA. The lysates were analyzed as in Figure 11. FreeRNP, 40S (small ribosomal subunit), 60S (large ribosomal subunit), 80S (monosome) and polysome peaks are labeled. Numbers inside the square brackets indicate the collected sample fractions within each peak that were pooled into one.

Equal volumes from each pool were analyzed on 1% denaturing agarose gel (**C**) for their ribosomal RNA content to evaluate the cross-contamination between the pools, and by 7% SDS-PAGE for their content of MILI and ***M***ouse ***V***asa ***H***omolog (***MVH***) (**D**). The slight increase in the amount of MILI in the "40S" pool of the EDTA treated sample relative to the non-treated sample is due to contamination from the "Free" fractions during pooling as evidenced by the western blot of MVH. M_R: RNA ladder marker

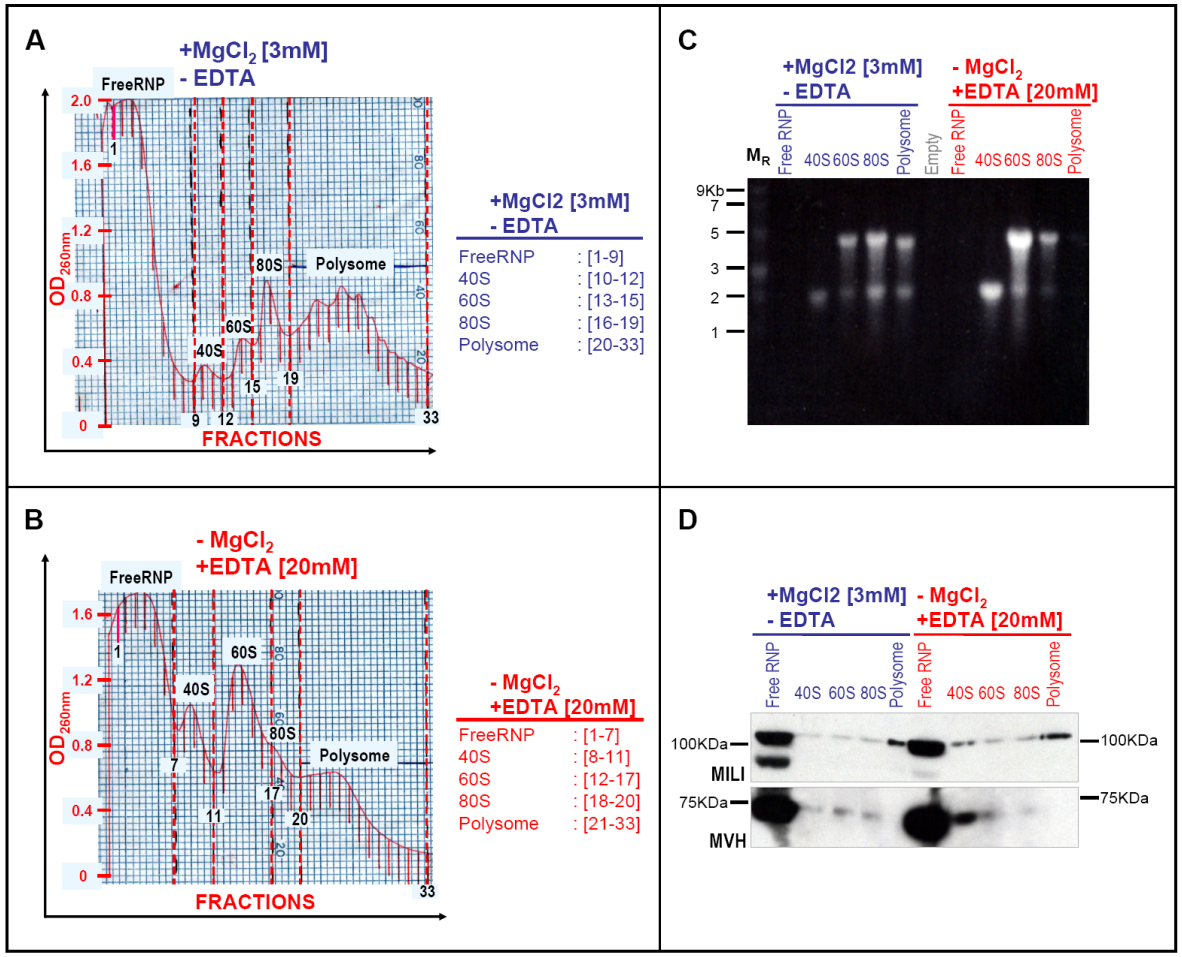

Figure 38: MILI does not interact with ribosomes

Treatment of the testicular extracts with translational inhibitors cycloheximide as in (Unhavaithaya, Hao et al. 2008) or GMP-PNP did not cause any difference on the polysomal profiles indicating these *in vitro* treatments are not actually effective under the conditions used (*Data not shown*).

These results indicate the MILI population that co-fractionates with the translational machinery does not interact with it and echoes the same observation obtained for piRNAs (***Figure 11***). However, it is also possible that the inconsistency between the observations presented here and those presented by Unhavaithaya *et al* might be due to an experimental error on my side despite I followed the same methodology of Unhavaithaya *et al* (2008) where applicable.

6.1.3 Adult Testicular piRNAs are Unlikely to Be Involved in Post-transcriptional Regulation of Genes

Although MILI and MIWI have been proposed to interact with the general translation initiation factors (Grivna, Pyhtila et al. 2006; Unhavaithaya, Hao et al. 2008), the overall polysome profile does not change in the MIWI knockout indicating that at least MIWI does not govern translation at the global level. Hence, it has been hypothesized that specificity might be acquired via piRNAs that are complementary to

mRNAs as in the case of miRNAs. However, my observations indicate that piRNAs are not involved in targeting genic mRNAs.

First, piRNAs mostly originate from intergenic regions, and thus unlikely to comprise fully complementary sequences for genic mRNAs (***Chapter 3***), and those that originate from genic regions mostly correspond to the sense strand (*Data not shown*). Therefore if piRNAs are involved in target mRNA recognition, this targeting can occur only through extensive mismatches in base pairing, which implies a very degenerate gene targeting.

Second, the piRNA distribution on the polysomes does not seem to change upon EDTA treatment indicating that piRNAs do not interact with ribosomes and thus they are unlikely to be involved in translational regulation.

As an alternative approach, we sought to characterize the piRNA population that co-fractionates with ribosomes. We speculated if indeed a sub-population of piRNAs is involved in translational regulation, the piRNAs in the ribosomal fraction may have a different profile than the rest. For instance, those with complementary sequences to genic mRNAs might be enriched in the ribosomal fractions. For this purpose, I co-

immunoprecipitated and identified the piRNAs of MILI and MIWI from the "Free RNP", "subunit" (40S and 60S) and "ribosomal" (80S and polysome) fractions with high-throughput pyrosequencing. However, there were no significant differences between the piRNAs in the three fractions in terms of their genomic origins or annotation (i.e; sense/anti-sense, exonic, intronic, repeat associated, transposonic etc.) (***Figure 39***).

Figure 39: piRNAs that co-fractionate with ribosomes are not distinctive (Bioinformatics by Hang Yin/Haifan Lin Lab)

Bioinformatics analysis of the piRNAs co-sedimenting with the "Ribosome" fractions does not reveal any distinctive feature.

piRNAs were co-immunoprecipitated and identified as in Chapter 3 from the "Free RNP", "Ribosomal Subunit" and "Ribosome" fractions by using a mixture of anti-MILI and anti-MIWI conjugated Protein-A beads.

Cluster/Non-cluster: Clustered/Non-clustered piRNAs, S./A.S.: Sense/ Anti-sense, CDS: Coding Sequence, UTR: Un-translated region

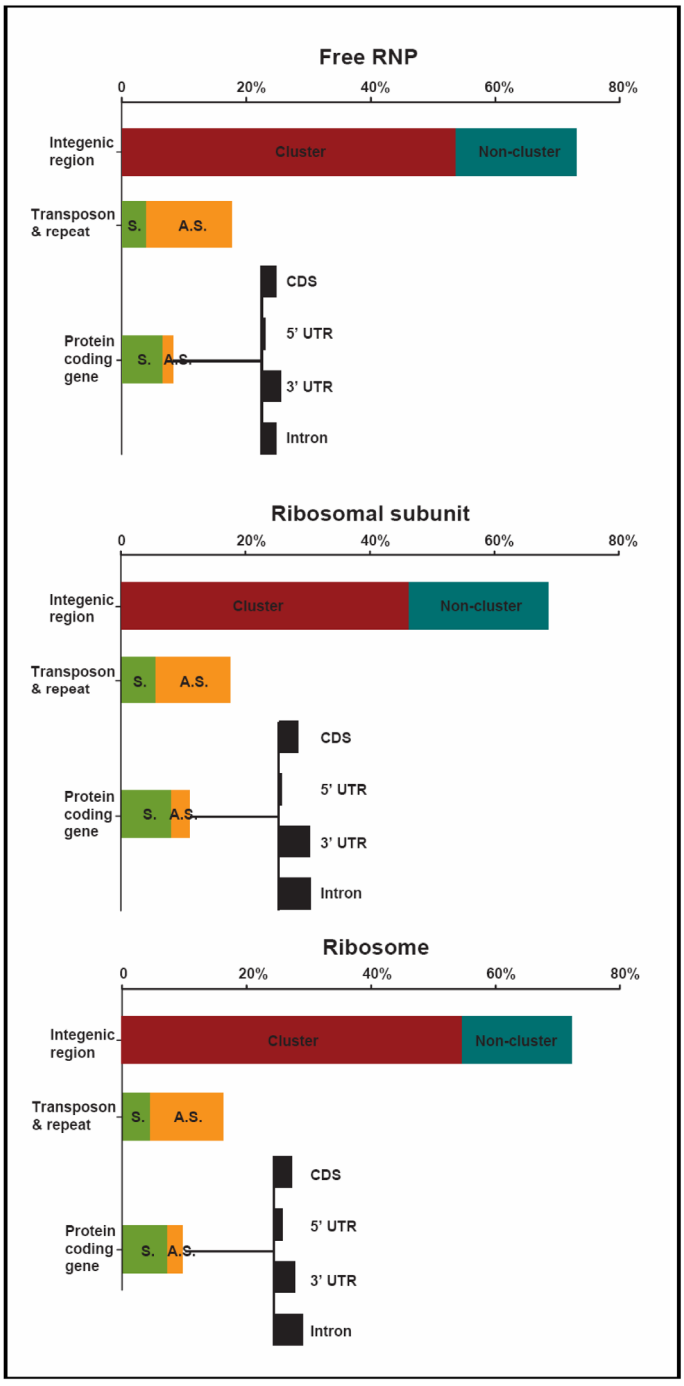

Figure 39: piRNAs that co-fractionate with ribosomes are not distinctive

(Bioinformatics by Hang Yin/Haifan Lin Lab)

6.1.4 MILI Does not Strongly and Specifically Interact With miRNAs and mRNAs

It is still possible that PIWI proteins may translationally regulate genes either via circulating piRNAs rather than a distinct pool or independent of piRNAs, maybe via miRNAs. Although cloning the small RNAs from the immunoprecipitates of MIWI and MILI, and subsequent bioinformatics analyses did not indicate a general association between PIWI proteins and miRNAs, we might have missed the detection amidst the overwhelming number of piRNAs. PIWI and PAZ domains of ARGONAUTE proteins have been shown to be necessary for the association with miRNAs and PIWI proteins also comprise these domains. Moreover, a minority of piRNAs identified from the immunoprecipitates, especially those of MILI, are within the size-range of miRNAs. Therefore I considered the possibility of MILI utilizing miRNAs for translational regulation. In order to test this possibility, I specifically addressed if MILI interacted with miRNAs by analyzing the MILI immunoprecipitates from adult testicular lysates with miRNA microarray. However, relative to the negative controls used, I did not consistently detect any miRNA in the precipitates (***Figure 40***).

Figure 40: MILI does not associate with miRNAs (Heatmaps by Summer Goodson/Scott Hammond Lab)

A representative miRNA microarray analysis of the total RNA isolated from a MILI immuno-precipitate (shown in black font) does not indicate any interaction between MILI and miRNAs relative to the negative controls (shown in blue font). Immuno-precipitations were performed with the MILI peptide antibody as in Chapter 3 from the adult testicular lysates of the indicated genotypes. Extracts contained 30mg total protein according to the Bradford Assay. Sub-samples were analyzed by western blotting to ensure the efficiency of the experimental immuno-precipitation and the negative controls. Approximately 50% of MILI was immuno-precipitated.

Experimental Sample: anti-MILI precipitates from *Mili*$^{+/-}$ (+/-) testicular extract

Negative Controls: peptide antigen blocked anti-MILI precipitates (Block), *Mili*$^{-/-}$ (-/-) testicular extract, pre-immune precipitates (Pre-I)

Yellow and blue colors on the heat map indicate "high" and "low" amount, respectively.

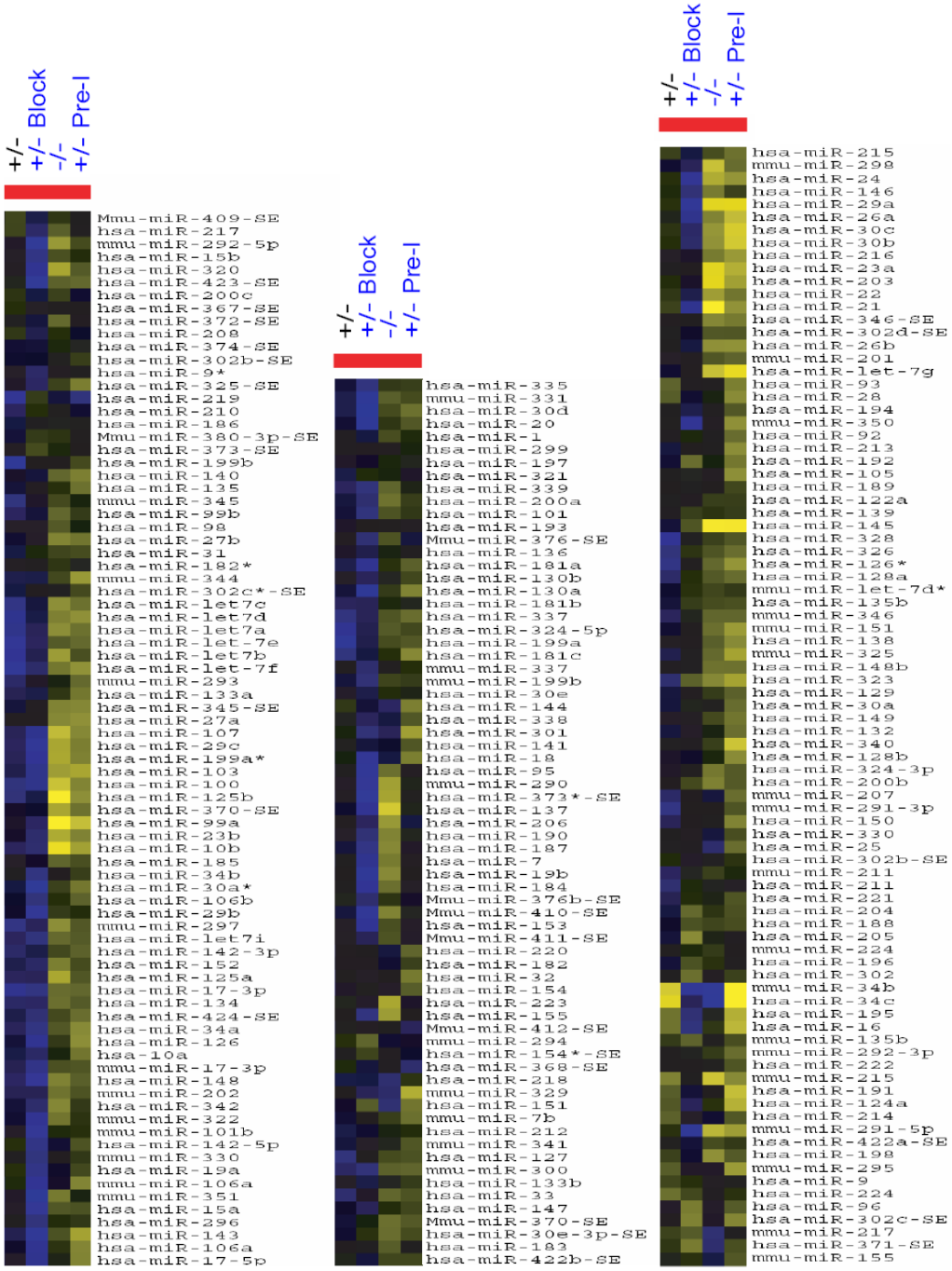

Figure 40: MILI does not associate with miRNAs

(Heatmaps by Summer Goodson/Scott Hammond Lab)

It is possible that the material obtained from the immuno-precipitates was not adequate for the detection of any putative MILI-associated miRNA by the microarray analyses. Therefore, I compared the total RNA samples from 9dpp *Mili*$^{-/-}$ vs *Mili*$^{+/-}$ testes to evaluate the effect of the loss-of MILI on the overall miRNA profile considering that miRNAs are highly de-stabilized in the absence of their partner ARGONAUTE proteins. However, I did not detect an overall difference in the miRNA levels concluding that MILI is not necessary for the biogenesis or stability of miRNAs in general (***Figure 41***). Collectively these results indicate that MILI does not associate with miRNAs and thus, rule out the possibility of miRNA-guided translational regulation by MILI.

Figure 41: Loss-of MILI has no significant effect on the overall miRNA profile (Heatmaps by Summer Goodson/Scott Hammond Lab)

A representative miRNA microarray analysis of total RNA from *Mili*$^{-/-}$ (-/-) testes relative to *Mili*$^{+/-}$ (+/-) or *Mili*$^{+/+}$ (+/+) testes shows that loss-of MILI does not significantly affect the overall miRNA profile of the testis. 5µg total RNA from each sample was analyzed as in Figure 40. Differences between the samples from the adults highly likely result from the difference in the tissue composition as *Mili*$^{-/-}$ testes do not comprise germ cells beyond mid-pachytene stage, and the established germ cells undergo extensive apoptosis. The tissue composition at 9dpp, before the onset of meiosis, is similar between *Mili*$^{-/-}$ and *Mili*$^{+/-}$ testes, and thus allows a direct comparison.

Yellow and blue colors on the heat map indicate "high" and "low" amount respectively.

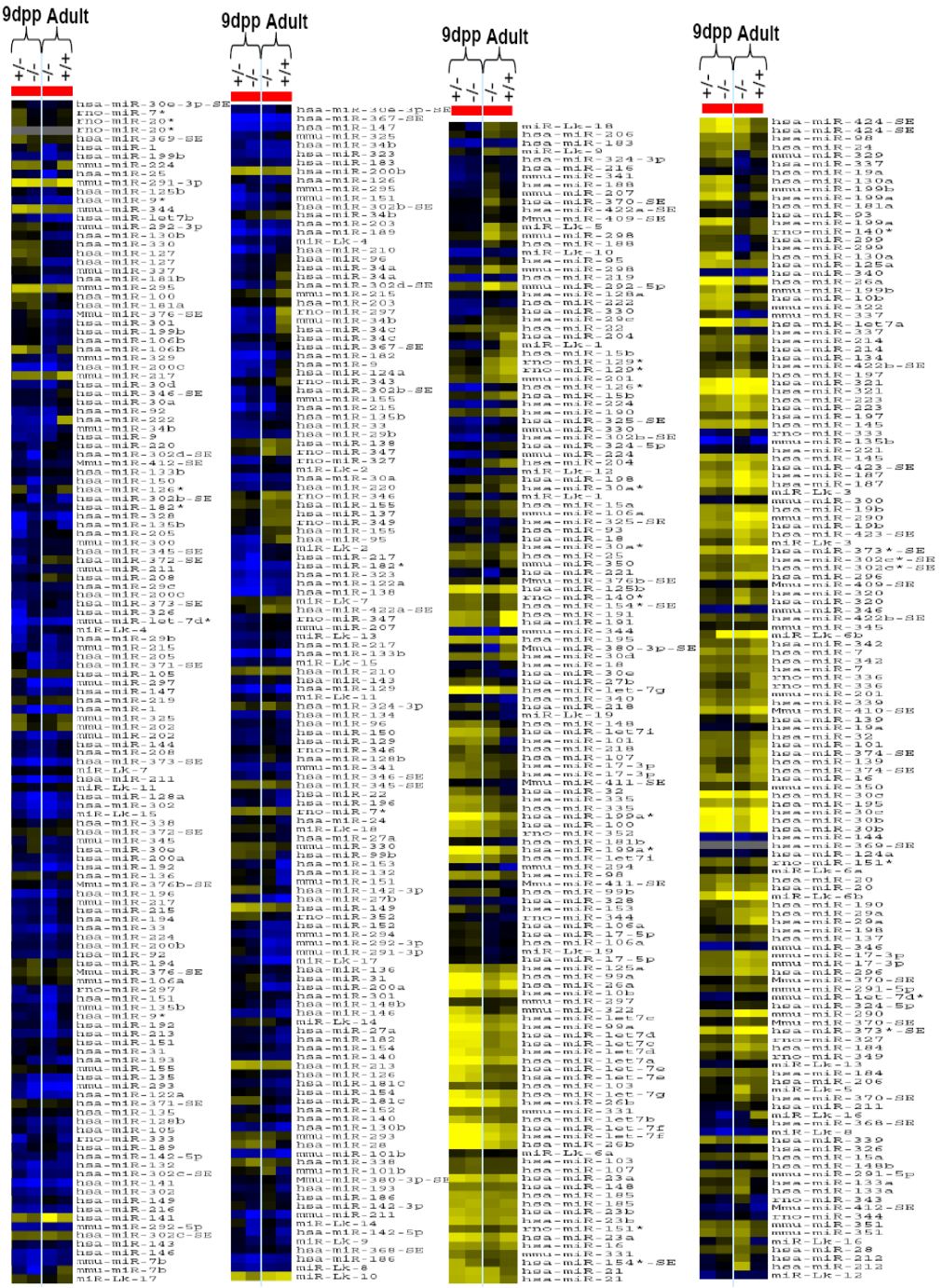

Figure 41: Loss-of MILI has no significant effect on the overall miRNA profile

(Heatmaps by Summer Goodson/Scott Hammond Lab)

Next, I addressed if MILI may be involved in translational regulation independent of any type of small RNAs. Therefore, I tested if MILI associated with mRNAs by analyzing the MILI immunoprecipitates from adult testicular lysates with cDNA microarray. Indeed, I was able to see approximately 2 fold enrichment of some mRNAs in the precipitates relative to the negative control. This result was confirmed with quantitative RT-PCR from the precipitates. However, it does not seem to be specific since a house-keeping gene GAPDH also yielded the same enrichment despite it was not identified as a MILI-associated mRNA in the microarray analysis (***Figure 42***).

Figure 42: MILI associates weakly and non-specifically with mRNAs

A) A representative example of quantitative RT-PCR from the MILI immuno-precipitates for one of the putative "target" mRNAs (Frg1: FSHD region gene 1) show that MILI associates with mRNAs, albeit weakly. Only approximately 2 fold enrichment was detected in the MILI immunoprecipitates (Exp IP) compared to the negative control, peptide antigen blocked anti-MILI precipitates (Block IP).

B) The quantitative RT-PCR for the housekeeping gene, GAPDH, which is not among the putative "targets", also yielded the same enrichment indicating MILI associates with mRNAs non-specifically.

Putative "targets" were identified by cDNA microarray of the MILI immunoprecipitates, which was normalized to the immunoprecipitates of the peptide antigen blocked anti-MILI. The efficiency of the peptide-blocking was confirmed with immuno-blotting of the immuno-precipitates for MILI.

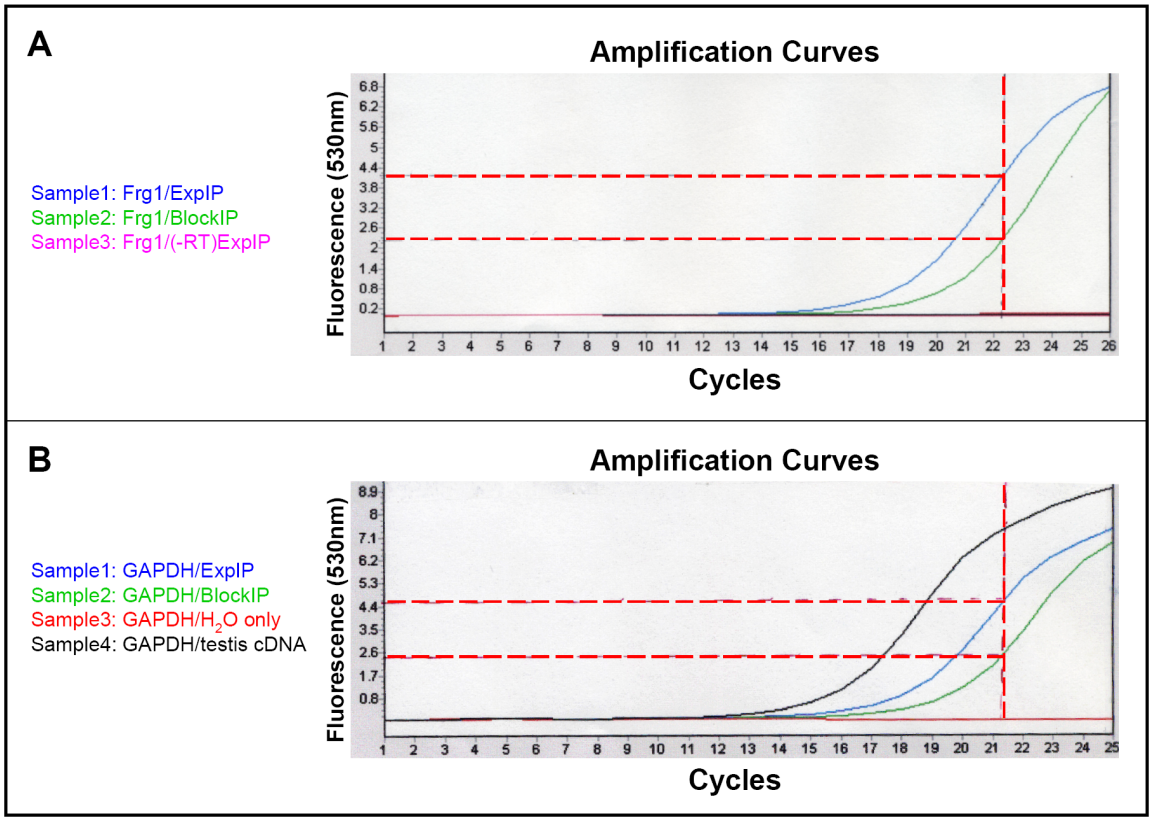

Figure 42: MILI associates weakly and non-specifically with mRNAs

Collectively, my results do not support a role for MILI and piRNAs in translational regulation. My observations indicate that the ribosomal pool of MILI and piRNAs in the polysome profile is either a technical "leak-through" during sedimentation centrifugation or part of a translational machinery-independent complex with similar sedimentation profiles to ribosomes.

6.1.5 MILI and piRNAs do not Regulate the Stability of Genic mRNAs but Potentially Some Transposonic mRNAs

All the three PIWI proteins in the mouse possess the "catalytic triad" necessary for the "slicer" activity in the ARGONAUTE proteins (Peters and Meister 2007) and MIWI has been proposed to regulate the stability as well as translation of some mRNAs (Deng and Lin 2002; Grivna, Pyhtila et al. 2006). Therefore I addressed if MILI and piRNAs are involved in the control of mRNA stability. For this purpose, I compared the individual mRNA levels in the 9dpp *Mili*$^{-/-}$ and *Mili*$^{+/-}$ testes with cDNA microarray. The tissue compositions of the *Mili*$^{-/-}$ and *Mili*$^{+/-}$ testes are similar at 9dpp. Intriguingly, we did not observe a significant difference in the mRNA levels, including those that are enriched ~2 fold in the MILI immuno-precipitates (**Figure 43**). Same was concluded independently by another group (Kuramochi-Miyagawa, Watanabe et al. 2008).

Figure 43: MILI does not overall regulate the stability of mRNAs

(Bioinformatics by Hang Yin)

cDNA microarray analyses of the 9dpp *Mili*$^{+/-}$ (horizontal axis) and *Mili*$^{-/-}$ (vertical axis) testicular total RNA show that mRNA levels do not significantly change in the absence of *Mili*. Shown in red are the mRNAs of 802 genes, which were enriched overall 2 fold in the MILI immunoprecipitates relative to the negative control, and in gray are the mRNAs of the remaining 14,845 genes in the mouse genic transcriptome represented in the array. All values are in $\log_2$ scale. The red dashed lines indicate the borders for 2-fold difference. Mouse AROS V3.0 oligo array (Operon), which represents the genic but not the transposonic transcriptome of the mouse, was used as the cDNA array. The results are derived from five independent replicas for total RNAs from *Mili*$^{+/-}$ and *Mili*$^{-/-}$ testes, and three replicas for the immunoprecipitates. Samples isolated from the immunoprecipitates were subjected to one round amplification with the MessageAmp II RNA amplification kit (Ambion Inc.) prior to the microarray analysis. Amplification and microarray analysis were performed by Duke Institute of Genome Sciences and Policy.

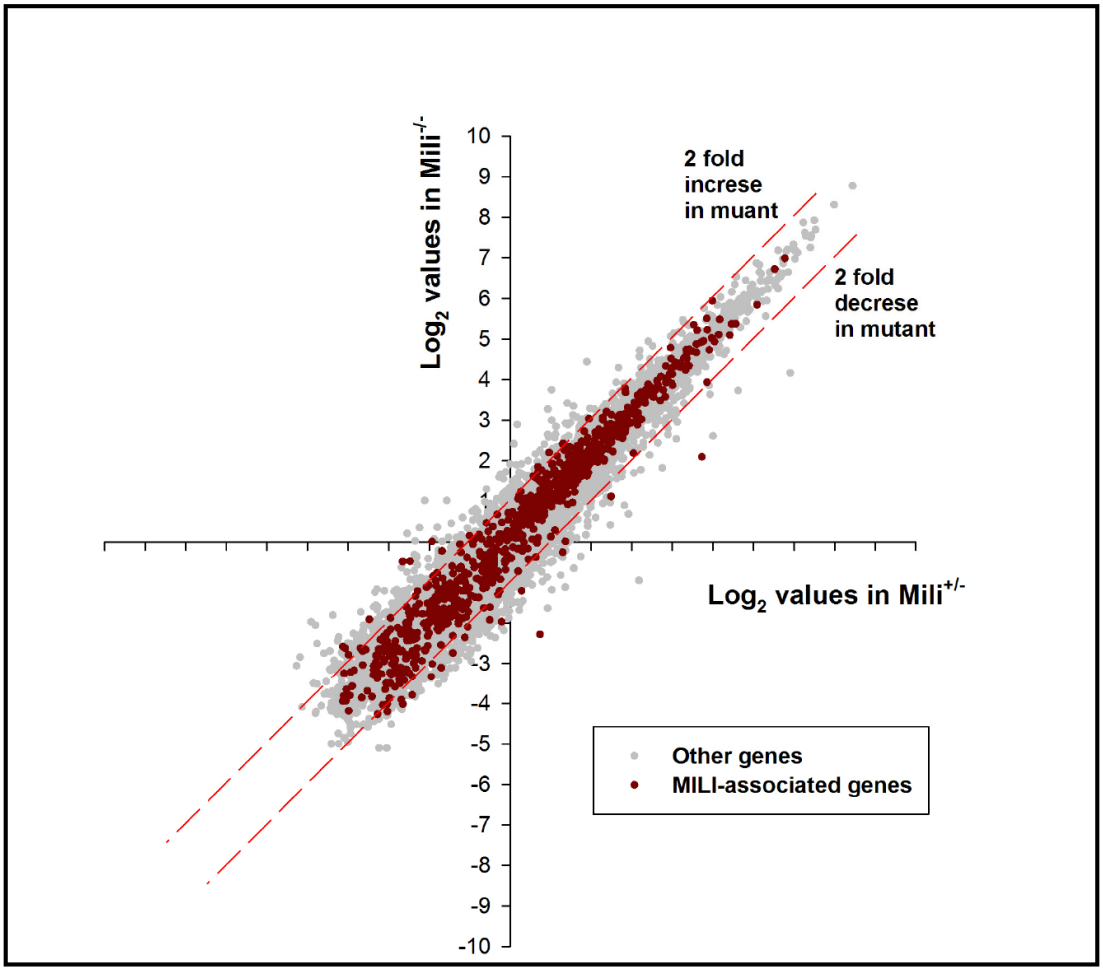

Figure 43: MILI does not overall regulate the stability of mRNAs

(Bioinformatics by Hang Yin)

In addition to the observation above, our bioinformatics analyses showed that only a minor population of piRNAs comprises genic sequences (***Chapter 3***) and they mostly correspond to the sense sequences (*Data not shown*), suggesting that a very small fraction of piRNAs are expected to be fully complementary to genic mRNAs.

These results indicate that MILI and piRNAs do not seem to be involved in mRNA stability either. Instead others observed that while genic mRNAs are unaffected, some transposonic RNA intermediates are upregulated in the *Mili*$^{-/-}$ or *Miwi2*$^{-/-}$ testes (Aravin, Sachidanandam et al. 2007; Kuramochi-Miyagawa, Watanabe et al. 2008), as well as some *Piwi* homologs in other model systems (Chambeyron, Popkova et al. 2008; Houwing, Berezikov et al. 2008), implicating them in transposon destabilization. This observation was correlated with an increase in the methylation status of the transposonic loci. Therefore the relative contribution of post-transcriptional silencing of transposons over transcriptional silencing is not clear. It is also elusive if this observation represents a direct function of PIWI proteins and piRNAs, since only a minor fraction of adult mammalian testicular piRNAs comprise complementary sequences to transposons.

7. Appendix II

7.1 Testicular miRNA Expression Profile During the Post-natal Development

In pursuit of the evaluation of the potential association between MILI and miRNAs, I analyzed the miRNA expression profile of the testis during the post-natal development as a preliminary approach. Since the post-natal testicular development mirrors the stages of the spermatogenesis, our rationale was to determine the miRNAs that were enriched in the juvenile testes, which are enriched for the MILI-expressing germ cells, and afterwards pursue these miRNAs as the candidate partners of MILI. As I showed in the preceding chapters, my subsequent works did not indicate any association of MILI with miRNAs but specifically with a novel class of small RNAs, which we named piRNAs. However, this preliminary study provided us comprehensive information regarding the miRNA expression profile in the post-natal testis (***Figure 44***). Given that miRNA biogenesis is necessary for spermatogenesis (Hayashi, Chuva de Sousa Lopes et al. 2008), our work may shed light onto the role of miRNAs in testis physiology, and more specifically, in spermatogenesis.

Figure 44: miRNA expression profile of the developing testis reveals miRNA expression pattern during spermatogenesis
(Heatmaps by Summer Goodson/Scott Hammond Lab)

miRNA microarray analyses of total testicular RNA isolated from the *Mili*$^{+/-}$ mice of the indicated ages demonstrates a dynamic expression pattern of miRNAs during post-natal testicular development. 5μg total RNA from each sample was analyzed as in Figure 40.

The germline comprises only spermatogonia at 8dpp, and it is at meiosis I at 14dpp, with spermatogonia and primary spermatocytes up to mid-pachynema present. At 22dpp, it has completed meiosis and consists of early round spermatids in addition to spermatogonia, primary and secondary spermatocytes. Adult testis has the full complement of germ cells in its germline, with the inclusion of the more differentiated round spermatids, elongating/elongated spermatids and spermatozoa to the germline at 22dpp.

MILI is expressed in the spermatogonia, spermatocytes and early round spermatids.

Representative miRNAs with different expression patterns are indicated with the colored boxes. Red box shows examples of miRNAs with the highest expression at 8dpp, decreasing onwards, indicating these miRNAs are highly abundant in spermatogonia. Pink box shows examples with highest expression during mid-spermatogenesis and no detectable expression at 8dpp, indicating their expression starts in early spermatocytes and decreases beyond round spermatids. Green box

shows examples with detectable expression starting by 22dpp and maintained in the adult, indicating their expression starts in the late spermatocytes/early spermatids and maintained in the germline onwards. Gray box shows examples with the highest expression in the adult, and with detectable expression at 22dpp, indicating their expression start with the formation of the round spermatids and increase in the germline onwards. The miRNA northern blotting results in Figure 6 and the *in situ* analyses in Figure 24 largely agree with the array results.

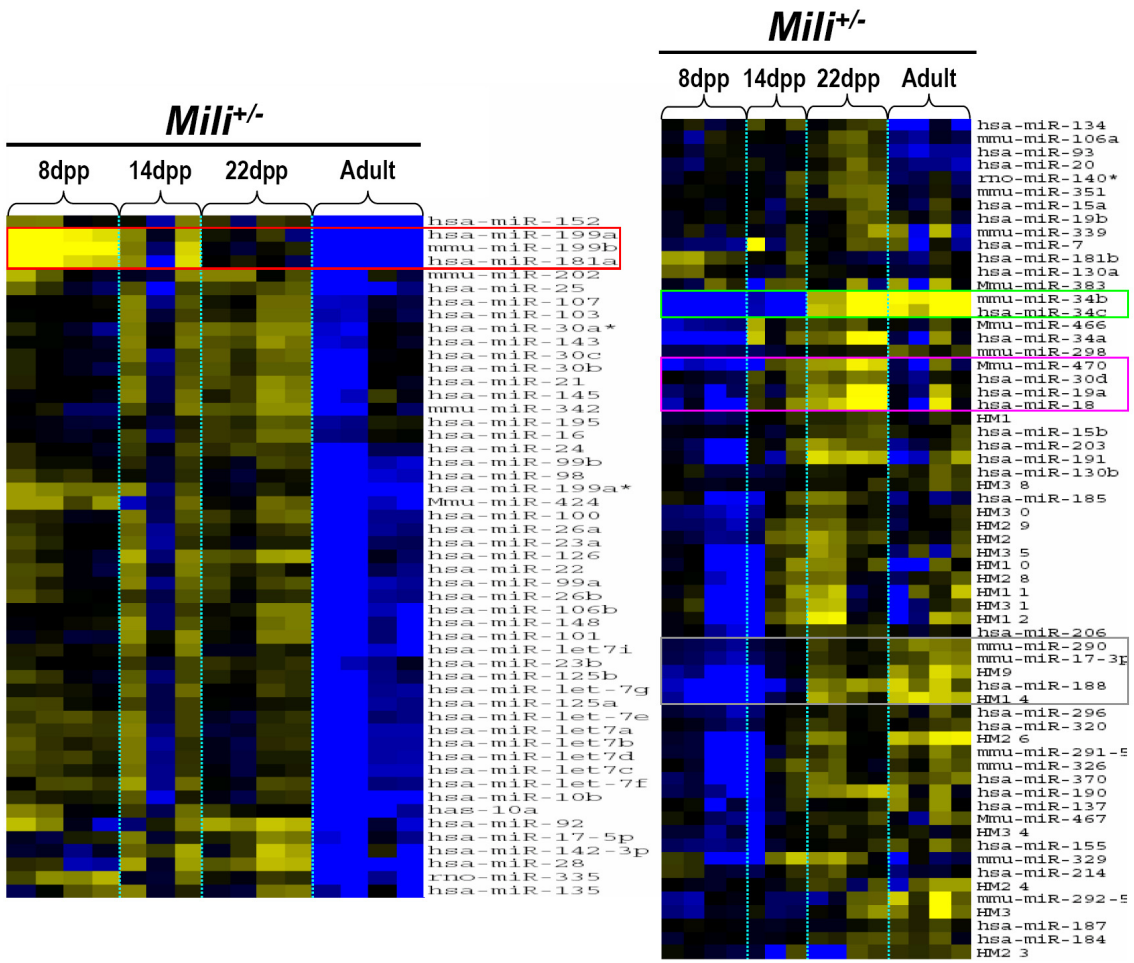

Figure 44: miRNA expression profile of the developing testis reveals miRNA expression pattern during spermatogenesis

(Heatmaps by Summer Goodson/Scott Hammond Lab)

8. Appendix III

8.1 Hematopoietic capacity of Miwi$^{-/-}$; Mili$^{-/-}$ mice

Hiwi is the orthologue of *Miwi* in the human, and human hematopoietic stem cells have been reported to express *Hiwi* mRNA (Sharma, Nelson et al. 2001). Therefore we wondered if mammalian PIWI proteins may function in hematopoiesis. We did not observe an embryonic lethality phenotype in *Miwi*$^{-/-}$ and *Mili*$^{-/-}$ single knock-outs, or *Miwi*$^{-/-}$; *Mili*$^{-/-}$ double knock-outs. We did not observe any obvious decrease in their life-expectancy, either. Therefore, at least MIWI and MILI, do not seem to be necessary for development or homeostasis, including hematopoiesis. In order to quantitatively assess the hematopoietic capacity of the mouse hematopoietic stem cells in the absence of *Miwi* and *Mili* we transplanted the bone marrow of *Miwi*$^{-/-}$; *Mili*$^{-/-}$ mice to irradiated wild type mice. However, quantitative comparison of the hematopoietic cell types between the recipient animals transplanted with the bone marrow from the *Miwi*$^{-/-}$; *Mili*$^{-/-}$ vs *Miwi*$^{+/-}$; *Mili*$^{+/-}$ siblings did not reveal an overt difference even after 6 months. Although, we observed a slight deficiency in the number of single positive thymocytes (CD4-CD8+ or CD4+CD8-) derived from the *Miwi*$^{-/-}$; *Mili*$^{-/-}$ donors, these cell types were produced efficiently, even somewhat more, in the recipients of *Miwi*$^{-/-}$; *Mili*$^{-/-}$ bone marrow. Similarly, we observed a slight deficiency in regulatory T cells in the recipients of *Miwi*$^{-/-}$; *Mili*$^{-/-}$ bone marrow; yet, the *Miwi*$^{-/-}$; *Mili*$^{-/-}$ donor did not display such deficiency.

Therefore, although these deficiencies are slightly statistically significant, they are probably false positives due to the very low absolute numbers. These results indicate that *Miwi*$^{-/-}$; *Mili*$^{-/-}$ hematopoietic stem cells are capable of homing to their niche and reconstituting hematopoeisis almost as well as *Miwi*$^{+/-}$; *Mili*$^{+/-}$ stem cells (***Table 6***).

Table 6: *Miwi*$^{-/-}$; *Mili*$^{-/-}$ mice are not defective in hematopoeisis

(Reconstitution analysis by Qi Jiang/Lishan Su Lab)

Hematopoeitic reconstitution analysis indicates that *Miwi*$^{-/-}$; *Mili*$^{-/-}$ HSCs are not impaired in homing to their niche and in their clonogenic capacity.

Bone marrow samples derived from adult *Miwi*$^{-/-}$; *Mili*$^{-/-}$ mice vs. their *Miwi*$^{+/-}$; *Mili*$^{+/-}$ siblings were used to reconstitute irradiated wild-type mice. Cell sorting from the recipients was performed 6 months after the transplantation.

Table 6: *Miwi*$^{-/-}$; *Mili*$^{-/-}$ mice are not defective in hematopoeisis

(Reconstitution analysis by Qi Jiang/Lishan Su Lab)

		Lymphocyte gate	Donor cells gate			Recipient cells gate			granlu gate
***Miwi*$^{+/-}$;*Mili*$^{+/-}$**		% Donor cells	% CD4+8-	% CD4-8+	% T_{reg}	% CD4+8-	% CD4-8+	% T_{reg}	% Donor cells
1	LE	92.3	25.8	17.1	2.8	18	37.4	5.2	87.7
2	RE	94.8	21.4	20.2	2.6	19.3	31	4.4	89.7
3	DE	89.7	20.1	14.7	4	23.5	39.2	5.8	87.5
4	NE	89.2	30.2	13.3	3.4	25.9	42	6.2	86.9
5	LE	86.8	24.7	12.9	3.1	21.8	37.9	4.9	75.6
6	RE	86.1	27	13.8	3.2	18.7	43.5	5.3	77.5
7	DE	87.1	26.4	12.7	3.5	19	30.3	6.9	79.2
average		89.4	25.1	15.0	3.2	20.9	37.3	5.5	83.4
SD		3.2	3.4	2.8	0.5	2.9	5.1	0.8	5.8
***Miwi*$^{-/-}$;*Mili*$^{-/-}$**									
8	NE	88.9	19.7	7.8	2.4	19.3	46.8	1.9	91.3
9	LE	91.9	21.6	8.8	3.8	19.3	45.3	3.2	88.8
10	RE	92.7	17.2	8.6	3.5	12.1	60.1	4	92.9
11	NE	91.7	15.8	7.7	2.7	27.5	49.6	3.9	92.2
12	LE	84.9	20.1	10	3	38.9	36.1	3	81.6
13	RE	90.9	22	11.2	2.8	24	47.1	3.4	85.4
14	DE	92.1	23.4	11.8	2.4	25.9	46.5	2.4	84.1
average		90.4	20.0	9.4	2.9	23.9	47.4	3.1	88.0
SD		2.7	2.7	1.6	0.5	8.4	7.1	0.8	4.4
TTEST		0.534	0.009	0.001	0.307	0.395	0.010	0.0001	0.120

9. Appendix IV

9.1 Evaluation of the Available Testicular Cell Lines for Endogenous piRNA Expression

The importance of *ex vivo* systems in cell biology is evident. In order to establish a tissue culture system for the analysis of the mouse PIWI/piRNA complexes, we sought to explore the available testicular cell lines for the presence of piRNAs. However, we did not detect an abundant piRNA expression in the cell lines tested with electrophoretic fractionation of their total RNAs (***Figure 45***). This result indicates that their intrinsic cellular features have changed during the immortalization process and/or their testicular niche is necessary for the expression of piRNAs. Nevertheless, we should note that electrophoretic fractionation of total RNAs does not allow the detection of the piRNA populations that have low levels of expression. For instance, although this technique showed the expression of MIWI piRNAs (***Figure 6 and 7***), detection of MILI piRNAs (***Chapter 3***) and MIWI2 piRNAs (Aravin, Sachidanandam et al. 2008; Kuramochi-Miyagawa, Watanabe et al. 2008) required enrichment through immuno-precipitation of their protein partners.

Figure 45: Testicular cell lines analyzed do not show endogenous piRNA expression

Electrophoretic fractionation of the total RNAs isolated from several available cell lines does not show a detectable level of endogenous piRNA expression.

Total RNA samples isolated with Trizol Reagent (Invitrogen) were provided by the Richard Freiman lab, labeled with ^{32}P on their 5` ends with kinase reaction as in Chapter 2 and fractionated with 15% Urea-PAGE.

TM3 and TM4 are testicular somatic cell lines derived from 11-13 day old mice, and believed to have originated from Leydig and Sertoli cells, respectively (Mather 1980). GC1 and GC2 are testicular germ cell lines. GC1 cell line shows the characteristics of the spermatogonia between the stages of type B spermatogonia and primary spermatocytes (Hofmann, Narisawa et al. 1992). GC2 cell line is a temperature-sensitive cell line and can undergo meiosis under the permissive temperature indicating its pre-spermatocyte origin (Hofmann, Hess et al. 1994). NIH 3T3 is a mouse embryonic fibroblast cell line. Total testicular RNA samples from an adult wild-type mouse (+ Ctrl WT) and an adult *Mili*$^{-/-}$ mouse (- Ctrl KO) were used as the positive and negative controls, respectively. Marker: 10nt DNA ladder marker. Red arrow indicates the location of the testicular piRNAs detectable with this analysis.

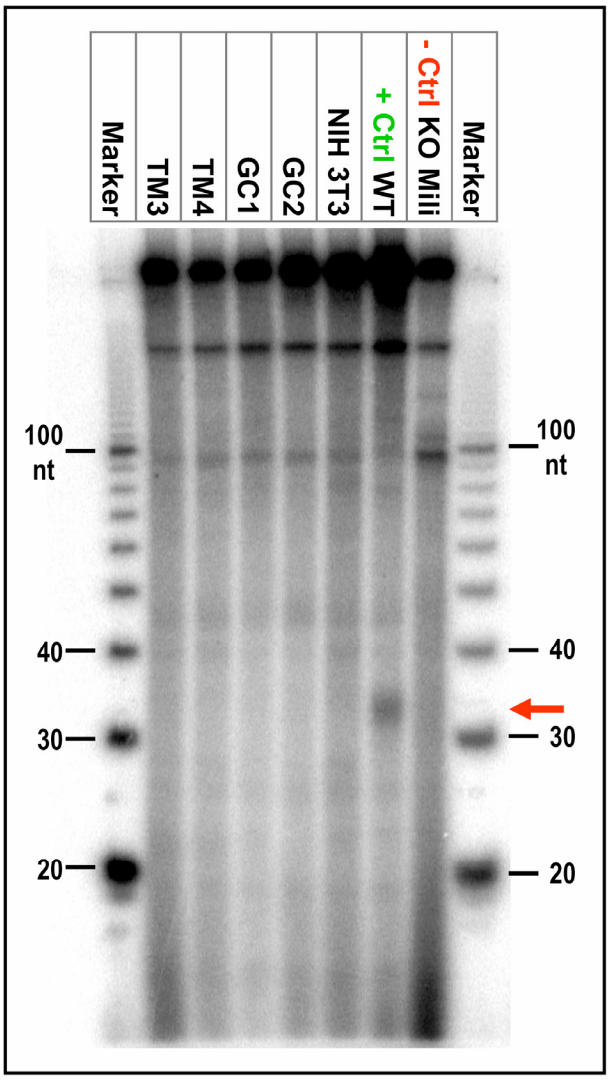

Figure 45: Testicular cell lines analyzed do not show endogenous piRNA expression

10. Appendix V

10.1 Evaluation of Taf4b$^{-/-}$ Testis for piRNA Expression

TAF4b is a general transcription factor highly enriched in the gonads. In the post-natal testis, it is detected in spermatids, spermatogonia, and the precursors of spermatogonia, called gonocytes. It forms one of the subunits of the TFIID complex, which functions in the recognition of the promoter regions to recruit RNA polymerase II for transcriptional activation. Interestingly, ovarian follicle development and maintenance of spermatogenesis require TAF4b, indicating that it regulates gonadal gene expression, and thus may be involved in the biogenesis of piRNAs. The spermatogenic defect in the *Taf4b*$^{-/-}$ mutants is evident as early as 2dpp. Although the number of gonocytes in the mutants is the same as in the wildtypes at 1dpp, their amount is significantly lower by 2dpp. This difference between the wildtype and mutant germ cells progressively increases as the wildtype gonocytes form the spermatogonia, which then undergo mitotic proliferation. Additionally, although the first wave of spermatogenesis occurs in *Taf4b*$^{-/-}$ testis, seminiferious tubules are progressively depleted of germ cells afterwards, indicating the progression of spermatogenesis is not affected but its maintenance is impaired. These results indicate that TAF4b is not necessary for the establishment, but for the proliferation and maintenance of the germline stem cells in the testis.

We asked if TAF4b is necessary for the biogenesis of piRNAs. We observed that *Taf4b*$^{-/-}$ testes comprise significantly less piRNAs than *Taf4b*$^{+/-}$ or *Taf4b*$^{+/+}$ testes. However, this observation seems to be due to lack of germ cells in the mutants rather than impaired piRNA biogenesis since the piRNA level in *Taf4b*$^{-/-}$ testis decreases progressively as the animals age and lose their germ cells (***Figure 46***).

Figure 46: TAF4b is not necessary for piRNA biogenesis

Electrophoretic fractionation of total *Taf4b*$^{-/-}$ testicular RNA samples shows that the global piRNA level decreases in *Taf4b*$^{-/-}$ testes as the animals age and lose their germ cells, indicating that the piRNA deficiency is due to the lack of germ cells rather than impaired piRNA biogenesis.

Total RNA samples isolated with Trizol Reagent (Invitrogen) were provided by the Richard Freiman lab, labeled with ^{32}P on their 5` ends with kinase reaction as in Chapter 2 and fractionated by 15% Urea-PAGE.

KO / HET / WT: *Taf4b*$^{-/-}$ / *Taf4b*$^{+/-}$ / *Taf4b*$^{+/+}$ testicular RNA. Numbers in front of the genotypes are the ages of the experimental animals in weeks. Total testicular RNA samples from an adult wild-type mouse (+ Ctrl WT CD1) and an adult *Mili*$^{-/-}$ mouse (-Ctrl KO Mili) were used as the positive and negative controls, respectively.

Marker: 10nt DNA ladder marker. Red arrow indicates the location of the piRNAs detectable with this analysis.

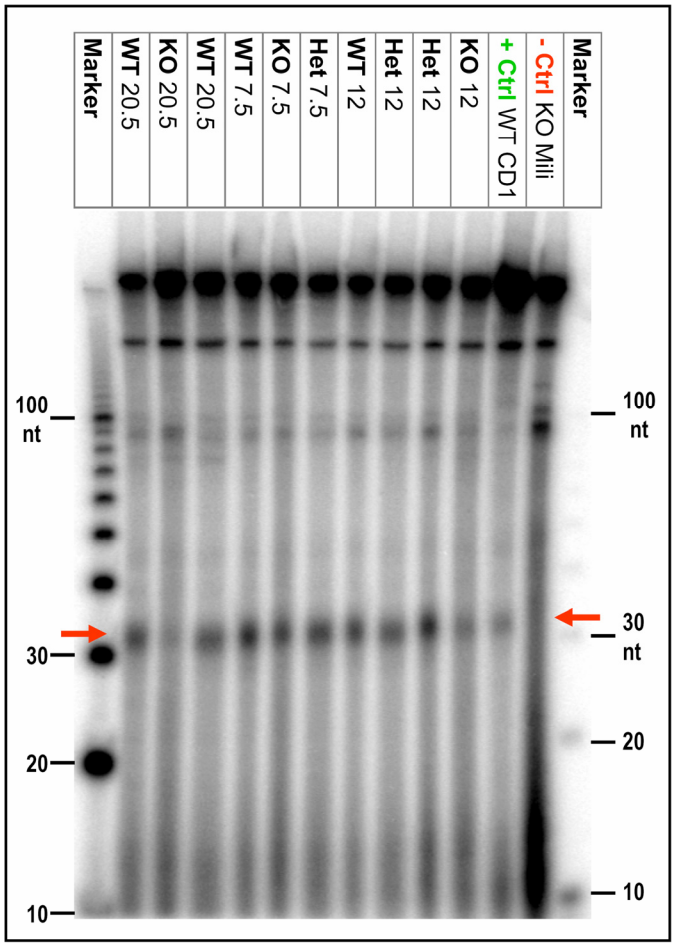

Figure 46: TAF4b is not necessary for piRNA biogenesis

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