



Etudes fonctionnelles sur les composants de la voie des piRNAs MOV10L1 et FKBP6

Jordi Xiol

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Functional studies on piRNA pathway components MOV10L1 and FKBP6

*Études fonctionelles sur les composants de la voie des
piRNA MOV10L1 et FKBP6*

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Summary

Piwi-interacting RNAs (piRNAs) associate to members of the PIWI clade of Argonaute proteins and are responsible for silencing of transposable elements in animal germ lines. In mouse, PIWI proteins mediate DNA methylation at the level of transposon promoters and are required for male fertility. piRNAs are produced through two biogenesis pathways, known as primary processing and ping-pong amplification cycle. This study focuses on the functions of two PIWI-associated proteins: the putative RNA helicase MOV10L1 and the immunophilin FKBP6. Here, the role of MOV10L1 as a primary piRNA processing factor is described. Genetic disruption of MOV10L1's RNA helicase domain results in male-specific sterility and derepression of retrotransposons due to reduction of DNA methylation. A complete loss of piRNAs is observed in the mutant, pointing to a pivotal role of MOV10L1 in piRNA biogenesis.

Tdrd1 associates with PIWI proteins and has been suggested to play a role in the piRNA pathway by recruiting some factor through its N-terminal MYND domain. We show that Tdrd1's MYND domain interacts with FKBP6, which belongs to a family of prolyl isomerases present in chaperone complexes. Biochemical studies suggest that FKBP6 is inactive and uses its prolyl isomerase domain as a structural module for binding PIWI proteins, while binding Hsp90 through its TPR domain. Analysis of an *Fkbp6* mutant mouse reveals transposon derepression and loss of DNA methylation, but little defects in primary piRNA biogenesis. These results are consistent with a role of FKBP6 downstream of Tdrd1, and we speculate that it might recruit Hsp90 to PIWI complexes to mediate ping-pong amplification cycle.

Résumé

Les *piwi-interacting RNAs* (piRNA) interagissent avec les protéines qui font partie de la branche PIWI de la famille des Argonautes. Ils participent à la répression des transposons dans la ligne germinale. Chez la souris, les protéines PIWI sont indispensables pour la fertilité des mâles et sont responsables de la méthylation de l'ADN au niveau des promoteurs des transposons. Les piRNA sont produits à partir de deux mécanismes: la biogenèse primaire et le cycle d'amplification ping-pong. Nous avons fait des études fonctionnelles sur deux protéines qui participent à la voie des piRNA: l'hélicase d'ARN MOV10L1 et l'immunophiline FKBP6. Nous avons décrit le rôle de MOV10L1 en tant que facteur de biogenèse primaire. La disruption génétique du domaine hélicase de MOV10L1 mène à une activation des transposons et à une perte de la méthylation de l'ADN. Ainsi, les piRNA ne sont pas produits chez le mutant, ce qui suggère que MOV10L1 est essentielle pour la biogenèse des piRNA.

Tdrd1 interagit avec les protéines PIWI et joue un rôle dans la voie des piRNA en recrutant quelques facteurs à partir de son domaine MYND N-terminal. Nous avons montré que le domaine MYND de Tdrd1 interagit avec FKBP6, une protéine qui appartient à une famille d'isomérases de prolines qui sont présentes dans des complexes de chaperonnes. Les études biochimiques réalisées indiquent que FKBP6 est inactive et qu'elle utilise son domaine isomérase comme un module structural qui interagit avec les protéines PIWI, alors qu'elle interagit avec Hsp90 avec son domaine TPR. L'analyse d'une souris mutante pour *Fkbp6* a révélé une dérégulation des transposons et une perte de la méthylation de l'ADN, mais peu d'effets sur la biogenèse primaire. Ces résultats sont en accord avec un rôle de FKBP6 en aval de Tdrd1, et nous pouvons penser que ce rôle pourrait être le recrutement de Hsp90 pour participer au cycle d'amplification ping-pong.

Abbreviations

Ago	Argonaute
Armi	Armitage
ATP	adenosine triphosphate
Aub	Aubergine
CDS	coding sequence
Cyp	cyclophilin
DNA	deoxyribonucleic acid
ds	double-stranded
DTT	dithiotreitol
DUF	domain of unknown function
EDTA	ethylenediaminetetraacetate
FKBP	FK506-binding protein
FL	full-length
GST	glutathione S-transferase
GTP	guanosine triphosphate
Hsp	Heat-shock protein
IPTG	isopropyl- β -D-thiogalactopyranoside
ITC	isothermal titration calorimetry
kDa	kilodalton
L	liter
L1	Line1
M	molar
miRNA	microRNA
ml	milliliter
mM	millimolar
MOV10	Moloney leukemia virus 10
MOV10L1	MOV10-like 1
mRNA	messenger RNA
MYND	myeloid, Nervi and DEAF-1
NLS	nuclear localisation signal
nm	nanometer

nM	nanomolar
nt	nucleotides
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAZ	Piwi/Argonaute/Zwille
PBS	phosphate buffered saline
PCR	polymerase chain reaction
piR-IL	piRNA intermediate-like molecule
piRNA	piwi-interacting RNA
PIWI	P-element induced wimpy testis
pNA	p-nitroanilide
PPIase	prolyl <i>cis-trans</i> isomerase
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNP	ribonucleoprotein
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecylsulfate
siRNA	small-interfering RNA
ss	single-stranded
Tdrd1	Tudor domain containing 1
TEV	Tobacco Etch Virus
TPR	tetratricopeptide repeat
UTR	untranslated region
v/v	volume (of solute) per volume (of solvent)
Zuc	Zucchini
μl	microliter
μM	micromolar

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1- INTRODUCTION

Résumé du chapitre “Introduction”

Les petits ARN non-codants jouent un rôle clé dans la régulation de l'expression génique chez les eucaryotes. Ils ont tous en commun leur association à une protéine de la famille des Argonautes. On peut distinguer trois classes de petits ARN: les *small-interfering RNA* (siRNA), les *microRNA* (miRNA) et les *piwi-interacting RNA* (piRNA). Les siRNA sont les molécules effectrices de l'ARN interférence. Ils conduisent à l'inhibition post-transcriptionnelle de leurs cibles et, dans certains cas (par exemple chez les plantes ou les levures) ils peuvent diriger la formation d'hétérochromatine. Les miRNA sont des petits ARN endogènes qui régulent l'expression génique au niveau post-transcriptionnel. Enfin, les piRNA, sur lesquels ces travaux de recherche se focalisent, s'associent avec des protéines de la branche PIWI de la famille des Argonautes et sont exprimés dans les gonades des métazoaires. Ils protègent l'intégrité du génome dans la lignée germinale en réprimant l'expression des transposons.

Les piRNA sont produits à partir de deux voies de biogenèse appelées primaire et secondaire, mais la compréhension de ces mécanismes au niveau moléculaire reste mince. Plusieurs protéines ont été décrites en tant que composants de cette voie suite à des criblages protéomiques et génétiques. Par exemple, on a montré que des protéines avec des domaines Tudor interagissent avec des arginines diméthylées des protéines PIWI et jouent un rôle dans la biogenèse des piRNA et/ou la localisation subcellulaire des protéines PIWI. L'objectif de ces travaux de recherche est de caractériser et d'analyser la fonction de deux protéines qui ont été identifiées dans notre laboratoire et qui pourraient potentiellement participer à la voie des piRNA chez la souris: la putative hélicase d'ARN MOV10L1 et la putative isomérase de prolines FKBP6.

1.1 –Small non-coding RNAs

Research over the last decades has seen an increasing importance of RNAs as regulatory molecules. The discovery of small non-coding RNAs substantially contributed to the understanding of fundamental biological processes in which they are involved, such as gene regulation, heterochromatin formation or anti-viral defence. Small RNAs are molecules of short length (~20-30 nucleotides) that associate with members of the Argonaute family of proteins and regulate RNA targets through sequence complementarity.

The first small non-coding RNA was identified in 1993 by Victor Ambros and coworkers in the nematode *Caenorhabditis elegans* (Lee *et al.* 1993). They found that *lin-4*, a negative regulator of *lin-14*, encodes for small RNAs with antisense complementarity to *lin-14*'s 3' untranslated region (UTR). At the same time, Gary Ruvkun's laboratory showed that the repression of *lin-14* by *lin-4* takes place at the post-transcriptional level, with the 3'UTR of *lin14* being necessary and sufficient for this regulation (Wightman *et al.* 1993). This was the first identification of a microRNA (miRNA) and its target, but it was thought to be a peculiarity of nematodes as no *lin-4* homologues were found in other species. Since *lin-4* mutants had defects in developmental timing, it was called a small temporal RNA (stRNA).

In 1998, Andrew Fire and Craig Mello discovered that exogenous double stranded RNA triggers specific gene silencing in *C. elegans* (Fire *et al.* 1998). They called this RNA interference (RNAi) and, shortly after, it was found to operate in a wide variety of organisms, including planaria, hydra, fungi and *Drosophila* (Kennerdell and Carthew 1998; Waterhouse *et al.* 1998; Cogoni and Macino 1999; Sanchez Alvarado and Newmark 1999). Fire and Mello's discovery could be linked to Ambros and Ruvkun's findings when RNAi was described to function through processing of the dsRNA trigger into small RNAs that mediate target mRNA cleavage (Hammond *et al.* 2000; Zamore *et al.* 2000). In parallel, a second miRNA, let-7, was discovered in *C. elegans* (Reinhart *et al.* 2000) and shown to be conserved in many metazoan lineages, including mammals (Pasquinelli *et al.* 2000). Evidence for small RNAs having a regulatory role had also been found in plants, where they were

implicated in post-transcriptional gene silencing (Hamilton and Baulcombe 1999). Altogether, these findings set the foundations for the understanding of gene regulation by small RNAs as a widespread phenomena in Eukaryotes, taking place in a very broad range of biological processes.

Small RNAs guide Argonaute proteins to their regulatory targets, and this typically results in a reduced expression of the target genes. An increasing number of small RNA classes have been described, differing in their biogenesis and functions, but they can be generally divided into three groups (reviewed in Ghildiyal and Zamore 2009): small-interfering RNAs (siRNAs), miRNAs and piwi-interacting RNAs (piRNAs). The boundaries between them are becoming very narrow but a few distinctions can still be drawn. siRNAs and miRNAs are broadly distributed and they bind members of the AGO subfamily of Argonaute proteins, while piRNAs, which are mostly restricted to animal gonads, bind members of the PIWI subfamily.

The research work of this thesis focuses on the study of piRNAs and mechanisms underlying their biogenesis and function. Among the three groups of small RNAs, piRNAs were the latest to be discovered and have been therefore studied to a more limited extent than miRNAs and siRNAs. An overview on the current knowledge about the different small RNA pathways, which have all in common the central role played by Argonaute proteins, is given below.

1.1.1 - The Argonaute Family of Proteins

Eukaryotic Argonautes can be divided into three subfamilies: AGOs, which bind miRNAs and siRNAs; PIWIs, which bind piRNAs; and WAGOs, which are only found in worms (Fig 1.1A) (Yigit *et al.* 2006; Hutvagner and Simard 2008). The degree of specialisation of Argonaute proteins for different small RNAs varies depending on the organism. As an example, fly Argonaute-1 (Ago1) and Argonaute-2 (Ago2) are specialised for miRNAs and siRNAs respectively (Forstemann *et al.* 2007), while Argonautes 1 to 4 in mammals can bind either class of small RNAs (Meister and Tuschl 2004). Argonaute proteins and small RNAs form RNA-induce

silencing complexes (RISC) (Hammond *et al.* 2000), which can contain additional proteins that extend or modify RISC's function.

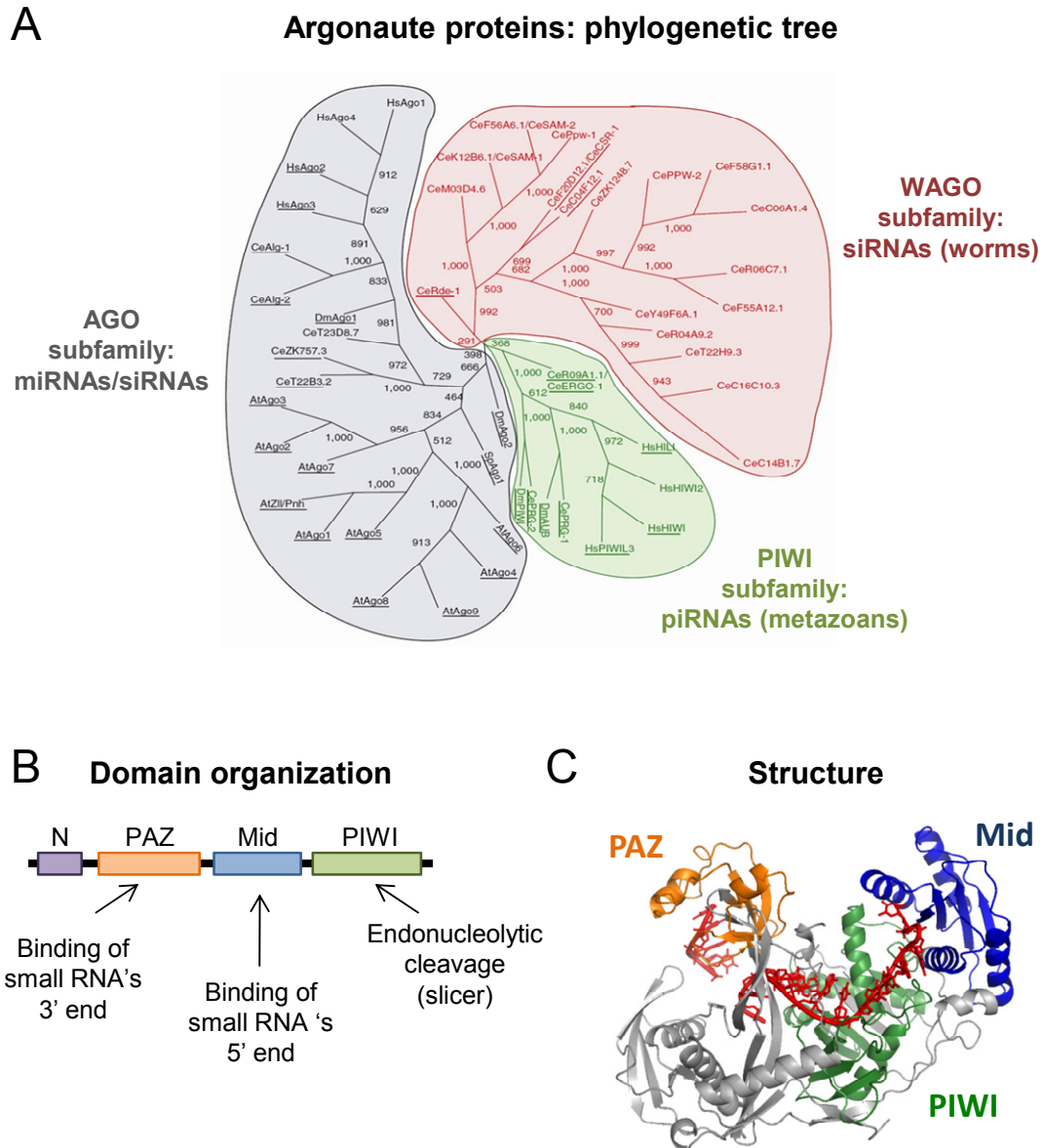


Figure 1.1: The Argonaute Family of Proteins. Shown are Argonaute proteins' phylogenetic tree (A, the three Eukaryotic subfamilies are indicated) (Sasaki *et al.* 2003), Argonaute protein's characteristic domain organisation (B) and a cartoon representation of *Thermus thermophilus* Argonaute structure, containing a DNA guide (C) (PDB Code: 3DLB, Wang *et al.* 2008b.)

Argonaute proteins have a highly conserved architecture, containing four characteristic domains: N-terminal (N), PAZ, MID and PIWI (Fig. 1.1B). The PAZ and MID domains are responsible for holding the small RNA, as they respectively

accommodate its 3' and 5' ends (Song *et al.* 2004; Ma *et al.* 2005; Parker *et al.* 2005). The PIWI domain adopts an RNase H-like fold that, in some cases, allows endonucleolytic cleavage of the target nucleic acids (Liu *et al.* 2004; Parker *et al.* 2004; Song *et al.* 2004). This activity is known as Slicer and is the first step towards target degradation, although it can also be used during small RNA biogenesis as described below. Three conserved residues in the active site (aspartate, aspartate and histidine; DDH motif) coordinate a catalytic metal ion, and mutation of any of these residues to alanine impairs Slicer activity *in vitro* and *in vivo* (Rivas *et al.* 2005). However, not all Argonaute proteins are catalytically active. For instance, it is widely accepted that, among the four human AGOs (Ago1-4), only Ago2 is able to slice (Meister *et al.* 2004).

The function of the different domains in Argonaute proteins could be uncovered thanks to structural studies. Crystal structures of archaeal and bacterial Argonautes with a nucleic acid guide and with or without the target RNA have also remarkably contributed to the understanding of small RNA pathways (Fig 1.1C) (Wang *et al.* 2008b; Wang *et al.* 2008c). The protein and small RNA establish contacts mainly through sugar-phosphate backbone interactions, explaining how Argonautes are able to take a wide variety of sequences. The small RNA's residues 2-6 are exposed and positioned in a way that can establish Watson-Crick pairing with their targets, and this has a special relevance *in vivo*, as these are the most important residues for target recognition by miRNAs (Parker *et al.* 2006; Parker *et al.* 2009).

1.1.2 - Small-interfering RNAs (siRNAs)

siRNAs are the effector molecules of RNAi. Different types exist and can be classified by different criteria; here, their origin (exogenous or endogenous) will be taken as a reference.

1.1.2.1 - Biogenesis

siRNAs arise from double-stranded precursors that are cleaved into small RNA duplexes with perfect complementarity (Fig. 1.2). Double-stranded RNA processing is

carried out by Dicer, a dsRNA-specific ribonuclease from the RNase III family (Bernstein *et al.* 2001). Some organisms, including mammals and worms, have only a single Dicer that is responsible for biogenesis of both siRNAs and miRNAs (Grishok *et al.* 2001; Hutvagner *et al.* 2001; Ketting *et al.* 2001; Knight and Bass 2001). Others express multiple Dicers (e.g. *Drosophila melanogaster* and *Arabidopsis thaliana*, which express two and four respectively), which are specialized for different pathways, as exemplified by the fruit fly where Dicer-1 processes miRNAs and Dicer-2 processes siRNAs (Lee *et al.* 2004b).

Dicer produces siRNA duplexes that have a 5' phosphate and leaves two-nucleotide overhangs at the 3' ends (Zamore *et al.* 2000; Elbashir *et al.* 2001c; Zhang *et al.* 2004). One of the two strands, called the guide, is loaded into an Argonaute protein, while the other one, called the passenger strand, is eventually degraded in most cases. Strand selection is based on thermodynamic stability of the duplex ends, with the strand having a less stably base-paired 5' end being selected as a guide (Khvorova *et al.* 2003; Schwarz *et al.* 2003). In those cases where the thermodynamic stability is the same in the two ends, both species get loaded with approximately even frequencies. In *Drosophila*, this asymmetry is sensed by R2D2/Dicer-2 heterodimer, as R2D2 positions itself in the more stable end of the duplex (Tomari *et al.* 2004). R2D2 and Dicer-2 form the RISC-loading complex (RLC), which transfers the siRNA to Ago2 (Liu *et al.* 2003). After loading, the passenger strand is sliced by Ago2 (Matranga *et al.* 2005; Miyoshi *et al.* 2005; Rand *et al.* 2005) and degraded by component 3 promoter of RISC (C3PO) (Liu *et al.* 2009). In mammals, Dicer, TRBP and Ago2 associate prior to the dsRNA trigger, forming the RISC-loading complex (Chendrimada *et al.* 2005; Gregory *et al.* 2005; Maniataki and Mourelatos 2005). This complex is capable of dicing the precursor, loading siRNA and degrading the passenger strand.

In some organisms, like flies and plants, siRNAs are modified at their 3' ends with a 2'-O-methyl group, which is added by the methyltransferase Hen1 (Yu *et al.* 2005; Horwich *et al.* 2007; Pelisson *et al.* 2007). This modification confers stability to the siRNAs and, in *Drosophila*, it prevents their tailing and trimming upon base-pairing with their extensively complementary targets (Ameres *et al.* 2010).

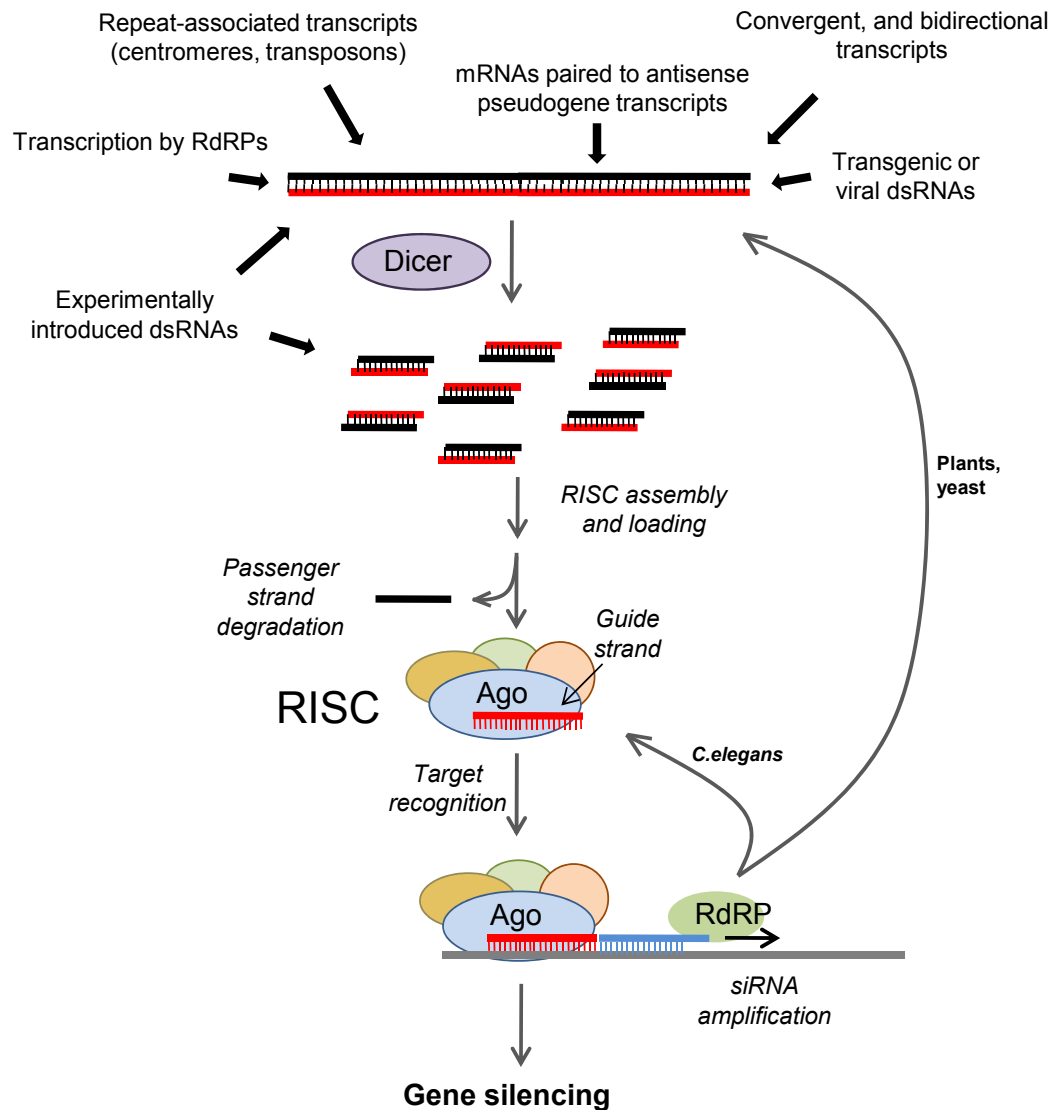


Figure 1.2: siRNA Biogenesis Pathways. Indicated are the sources of dsRNA that can trigger siRNA production through processing by Dicer enzymes and loading into Argonautes. Secondary siRNAs are direct RdRPs products in *C. elegans*, while they are further processed by Dicer in plants and yeast.

Exogenous siRNAs (exo-siRNAs)

The fact that exogenous dsRNA triggers siRNA formation has led to the usage of RNAi as a powerful technique for silencing specific genes in cell culture and *in vivo*. In vertebrates, the presence of long dsRNA activates the interferon response, but silencing can be achieved by using siRNAs instead, which induce only RNAi as they fall below the threshold for triggering an interferon response (Elbashir *et al.* 2001a).

In some organisms, like insects and plants, RNAi is used as an anti-viral defence (Voinnet 2008; Umbach and Cullen 2009). This is not the case in mammals, perhaps because they have evolved a complex protein-based immune system (Kunzi and Pitha 2005). In plants, dsRNA is not the only source for RNAi: viral ssRNAs are converted into dsRNA by RNA-dependent RNA polymerase enzymes (RdRPs), and this initiates an RNAi response through Dicer cleavage and loading into Argonautes (Qu *et al.* 2005). Similarly, highly-expressed transgenes are also targets for RdRPs (Gazzani *et al.* 2004).

In *C. elegans*, the organism where RNAi was discovered, the primary dsRNA trigger also induces synthesis of secondary siRNAs through the action of RdRPs (Pak and Fire 2007; Sijen *et al.* 2007). This takes place upon base-pairing of siRNAs to their targets. However, unlike for plants, secondary siRNAs are not generated through the action of Dicer but are direct products of RdRPs, as reflected by the fact that they are only produced from the antisense strand to the target and that they carry di- and tri-phosphate groups at their 5' ends. Secondary siRNA amplification leads to targeting of regions of the mRNA which were not targeted by the initial dsRNA trigger, and this is known as transitive RNAi (Sijen *et al.* 2001). The secondary pool of siRNAs can spread and lead to systemic silencing in the entire organism, both in *C. elegans* and plants (Baulcombe 2004).

Endogenous siRNAs (endo-siRNAs)

Endogenous siRNA production is a widespread feature in Eukaryotes, taking place in organisms such as yeast, fungi, plants and animals (Okamura and Lai 2008; Padmanabhan *et al.* 2009). Endo-siRNAs pathways are very complex and diverse in *C. elegans* (Ambros *et al.* 2003), which expresses as many as 27 Argonaute proteins. Secondary siRNAs are the most abundant ones, and, as for exogenous triggers, they are produced through the action of RdRPs. An example of this are 22G RNAs, which are 22nt long secondary siRNAs enriched in a 5' guanine. They are generated through the amplification of 26G RNAs, which are produced by Dicer but are also dependent on the generation of dsRNA by RdRPs (Gent *et al.* 2010).

In flies, endo-siRNAs have been found to be derived from transposons, heterochromatic sequences, long RNA transcripts with extensive structure and mRNAs, and they have been implicated in transposon control (Czech *et al.* 2008; Ghildiyal *et al.* 2008; Kawamura *et al.* 2008; Okamura *et al.* 2008). Similar to exo-siRNAs, they are produced through dsRNA processing by Dicer-2 and Loquacious. Endo-siRNAs have also been found in mouse oocytes, where they have very diverse genomic sources, including pseudogenes and transposons (Tam *et al.* 2008; Watanabe *et al.* 2008). They are also produced by Dicer and have been suggested to participate in transposon silencing.

Plants, like *C. elegans*, are very rich in endo-siRNAs, which can be divided in different classes. *Cis*-acting siRNAs (casiRNAs) originate from transposons, repetitive elements and tandem repeats, and require RdRPs and Dicer for their production (Xie *et al.* 2004; Herr *et al.* 2005; Pontier *et al.* 2005). Trans-acting siRNAs (tasiRNAs) are generated through the convergence of miRNA and siRNA pathways, as miRNA-directed target cleavage can trigger dsRNA formation by RdRPs and subsequent Dicer processing (Vazquez *et al.* 2004; Allen *et al.* 2005; Axtell *et al.* 2006). Some plant endo-siRNAs are produced in response to stress. Examples are natural antisense transcript-derived siRNAs (natsiRNAs), which originate from convergent transcription (Borsani *et al.* 2005), and long siRNAs (lsiRNAs), which are 30-40nt long and are produced from natural antisense transcript pairs (Katiyar-Agarwal *et al.* 2007).

1.1.2.2 - Function

In the canonical RNAi, siRNAs direct degradation of perfectly complementary mRNAs through Argonaute's Slicer activity (Fig 1.3A) (Tomari and Zamore 2005). Target cleavage occurs between the 10th and 11th nucleotide (Elbashir *et al.* 2001b), and the reaction product contains 5' phosphate and 3' hydroxyl groups. The newly generated 3' end is a substrate for oligouridylation, which promotes exonucleolytic targeting (Shen and Goodman 2004). The cleaved target dissociates from RISC and is subsequently degraded, allowing RISC recycling and cleavage of new targets (Orban and Izaurralde 2005). Some siRNAs are loaded into non-Slicer Argonautes, and some

have only partial complementarity to their targets; in these cases, siRNAs can still silence by mediating mRNA decay and/or translational repression, similar to miRNAs (Fig 1.3B) (Tomari and Zamore 2005).

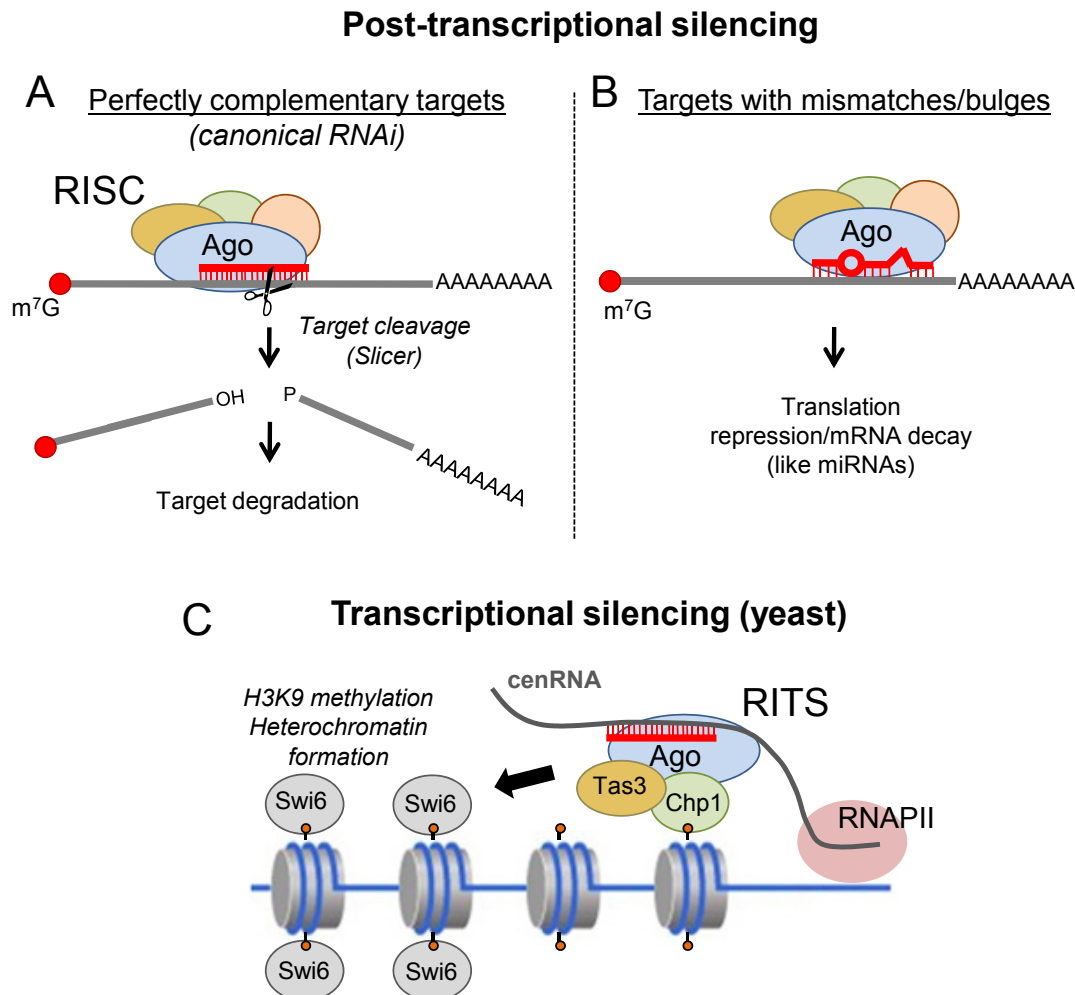


Figure 1.3: Gene Silencing by siRNAs. siRNAs can silence their targets either at the transcriptional or post-transcriptional level. In most organisms, siRNAs mediate silencing by directing target cleavage (A), provided that the targets have extensive complementarity. Otherwise, they can repress the targets in a similar way to miRNAs (B, see also Figure 1.5). Plant and yeast siRNAs can also mediate heterochromatin formation. In yeast (C), the RITS complex is tethered to centromeric transcripts (cenRNA) through base-pairing with the siRNA and binding of Chp1 to methylated H3K9 (red lollipops). This triggers siRNA amplification (not shown), H3K9 methylation by Clr4 and heterochromatin formation by Swi6.

In some organisms, siRNAs can mediate silencing at the transcriptional level (Moazed 2009). siRNA-directed heterochromatin formation is one of the hallmarks of RNAi in fission yeast (Fig 1.3C). *Schizosaccharomyces pombe*'s Argonaute-1 forms

the RNA-induced transcriptional silencing (RITS) complex, containing also the chromodomain protein Chp1 and the glycine and tryptophan(GW)-motif-containing protein Tas3 (Verdel *et al.* 2004). RITS is tethered to non-coding centromeric nascent transcripts through siRNA base-pairing and association of Chp1's chromodomain to methylated H3K9 (Motamedi *et al.* 2004; Noma *et al.* 2004; Buhler *et al.* 2006). siRNAs are amplified through the action of RdRPs and Dicer (Sugiyama *et al.* 2005), and heterochromatin formation involves recruitment of the H3K9 methyltransferase Clr4, whose catalytic activity allows binding of the heterochromatin protein Swi6 to histone tails (Iida *et al.* 2008). Transcriptional silencing by siRNAs also takes place in plants, where for instance *cas*iRNAs or virus-derived siRNAs can direct DNA methylation and formation of repressive chromatin (Chan 2008).

1.1.3 – microRNAs (miRNAs)

miRNAs are ~21nt-long endogenous small RNAs that regulate gene expression through different degrees of complementarity to target mRNAs (Bushati and Cohen 2007). They are found in the plant and animal branches of Eukaryota. The fact that they can target non-perfect complementary RNAs allows targeting of multiple genes by a single miRNA (Brennecke *et al.* 2005). Recent studies have estimated that ~60% of protein-coding genes could be subjected to miRNA regulation in mammals (Friedman *et al.* 2009), and the fact that single miRNAs have generally mild effects on the cell proteome (rarely exceeding 1.5-fold) has led to the designation of miRNAs as fine-tuners of gene expression (Baek *et al.* 2008; Selbach *et al.* 2008).

1.1.3.1 - Biogenesis

miRNA genes are typically transcribed by RNA polymerase II into capped and polyadenylated primary microRNAs (pri-miRNAs) (Cai *et al.* 2004; Lee *et al.* 2004a). Although there are some examples of miRNAs transcribed from separate transcription units, most miRNAs are transcribed from transcription units that give more than one product, for instance clusters of multiple miRNAs or miRNAs encoded in introns of protein-coding genes (Lee *et al.* 2002; Rodriguez *et al.* 2004).

Pri-miRNA transcripts have a characteristic stem-loop structure with flanking single-stranded regions. In animals, they are processed in the nucleus by Drosha, an enzyme of the RNase III family, to produce a ~70nt hairpin precursor miRNA (pre-miRNA) (Lee *et al.* 2003). Drosha requires a protein cofactor to perform the reaction –DiGeorge critical region 8 (DGCR8) in mammals, Pasha in flies and worms (Landthaler *et al.* 2004)- and together they form the microprocessor complex (Gregory *et al.* 2004). Some miRNAs located in introns (called mirtrons) can by-pass the requirement of the microprocessor as the intron is released as a pre-miRNA during splicing (Berezikov *et al.* 2007; Ruby *et al.* 2007).

Pre-miRNAs are exported from the nucleus by Exportin5 and the GTP-bound form of Ran (Yi *et al.* 2003). They are processed by Dicer (see Fig. 1.4 for details) (Bernstein *et al.* 2001), and strand selection is based on thermodynamic features like for siRNAs. The complementary strand of a miRNA (called miRNA*) is in some cases also loaded into Argonaute complexes and functions as a guide. A few particularities in pre-miRNA processing have been described: in mammals, Ago2 can participate in biogenesis of some miRNAs by cleaving the 3' end of pre-miRNA hairpins that have extensive complementarity. This releases an intermediate called Ago2-cleaved precursor miRNA (ac-pre-miRNA) that is further processed by Dicer (Diederichs and Haber 2007). There is even an example of a mammalian and fish pre-miRNA (pre-miR-451) that by-passes Dicer cleavage and is directly cleaved by Ago2, polyuridylated and trimmed at the 3' end by an unknown nuclease (Cheloufi *et al.* 2010; Cifuentes *et al.* 2010).

In plants, Dicer-like 1 (DCL1) processes pri-miRNAs to miRNA/miRNA* duplexes, therefore also filling the role of Drosha (Park *et al.* 2002; Reinhart *et al.* 2002). This duplex is then exported to the cytoplasm by an exportin 5 homologue, HASTY (Park *et al.* 2005). Unlike animal miRNAs, plant miRNAs are 2'-O-methylated in their 3' ends, protecting them from polyuridylation and degradation (Li *et al.* 2005; Yu *et al.* 2005).

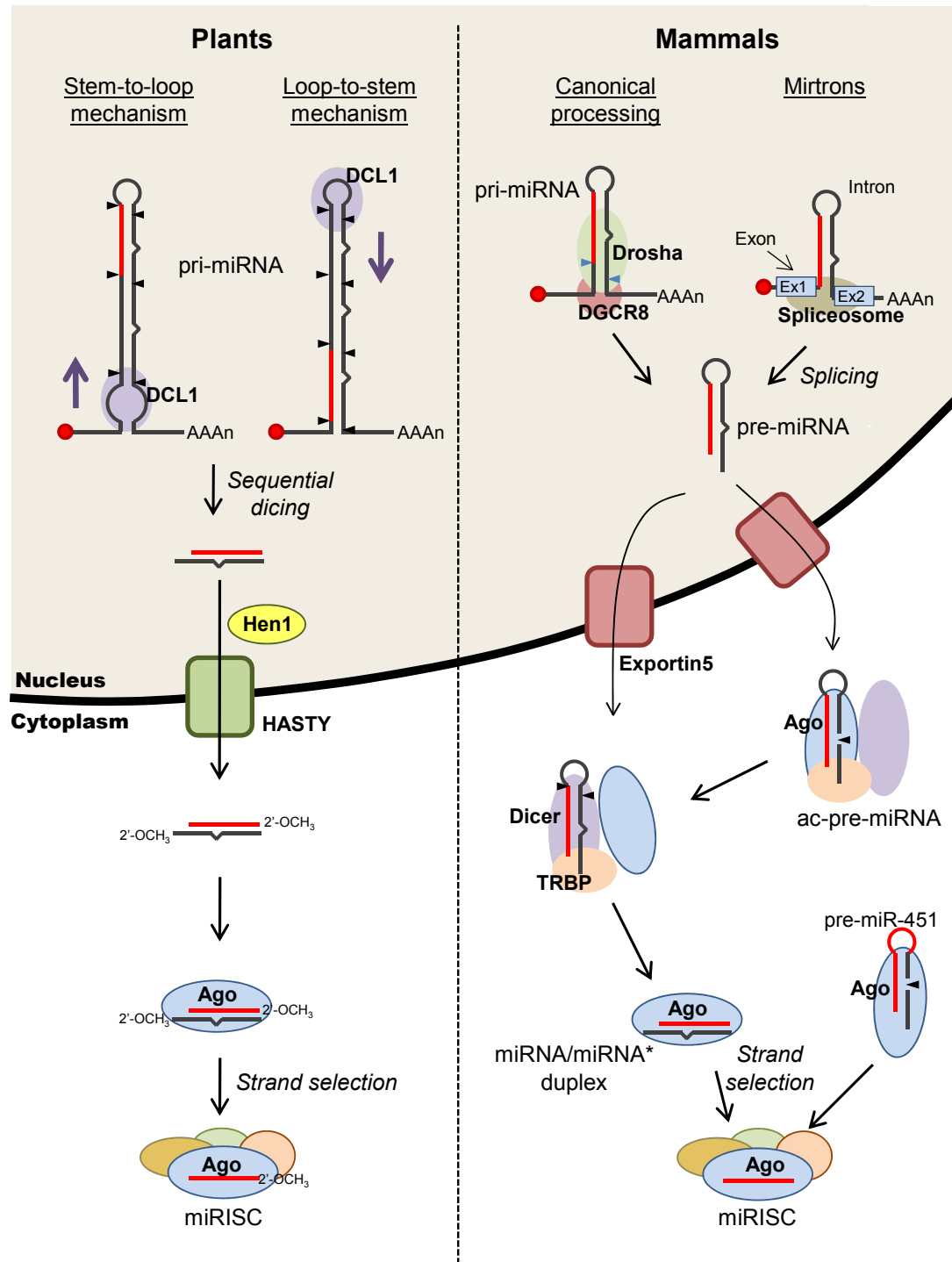


Figure 1.4: miRNA Biogenesis Pathways. Depicted are the different steps that lead to the formation of mature miRNAs loaded into Argonautes. In plants, pri-miRNAs are processed by Dicer-like 1 (DCL1) by two different mechanisms depending on the miRNA position in the hairpin. Mature miRNAs are produced after sequential rounds of Dicing, methylation by Hen1 and export to the cytoplasm (left). Mammalian miRNA biogenesis (right) is similar to that of other animals. In flies, for instance, the pathway differs from the one shown in this figure by the presence of Pasha instead of DGCR8 and by the fact that pre-miRNA cleavage is performed by Dicer-1 and Loquacious.

1.1.3.2 - Function:

miRNAs regulate the expression of complementary mRNAs. Perfect complementarity between the miRNA and the target leads to endonucleolytic cleavage of the target and subsequent degradation. While this is very common in plants (Rhoades *et al.* 2002), most animal miRNAs have discontinuous pairing to their targets, leading to translational repression and mRNA decay (Bushati and Cohen 2007). miRNA binding sites are preferentially located in mRNA's 3' UTR, but both computational and biochemical studies have identified miRNAs targeting the open reading frame (ORF) and the 5' UTR (Stark *et al.* 2007; Chi *et al.* 2009). The rules of miRNA/target interactions in animals have been defined both experimentally and with *in silico* approaches (Bartel 2009). Essentially, the most important recognition involves the miRNA's nucleotides 2-7, which is known as the seed region. miRNAs and their targets are usually mismatched at miRNA positions 10-12, preventing endonucleolytic cleavage, and the pairing is reinforced by additional matches beyond the seed region (Lewis *et al.* 2005; Grimson *et al.* 2007).

The mechanism by which most animal miRNAs regulate mRNAs has been extensively debated (Fabian *et al.* 2010). Silencing takes place at the post-transcriptional level, and there seem to be two independent events in blocking translation and promoting mRNA decay.

mRNA Decay

There is quite a big consensus on how miRNAs direct mRNA decay (Fig 1.5A). Degradation is achieved through removal of the poly(A) tail followed by decapping and 5'-3' endonucleolytic digestion (Behm-Ansmant *et al.* 2006). This is mediated by the action of GW182 proteins, which associate to the PIWI domain of Argonaute proteins through glycine-tryptophan repeats in their N-termini (Eulalio *et al.* 2009). Vertebrates express three GW182 paralogues (TNRC6A/GW182, TNRC6B and TNRC6C), while there is a single copy in *Drosophila*. GW182 interacts with poly(A)-binding protein (PABP), reducing its binding to the poly(A) tail, and recruits the CCR4-NOT1 deadenylase complex that is responsible for target mRNA deadenylation (Fabian *et al.* 2009).

A few proteins other than GW182 have been identified to participate in miRNA-mediated silencing, but their roles remain poorly understood (Hock *et al.* 2007; Landthaler *et al.* 2008). Of special relevance for this work is the putative RNA helicase Moloney leukemia virus 10 (MOV10), as it is a paralogue of one of the proteins studied during this thesis, MOV10-like 1 (MOV10L1). MOV10 interacts with human Ago1 and Ago2 and is required for miRNA-mediated silencing (Meister *et al.* 2005; Chendrimada *et al.* 2007), but mechanistic information is missing. MOV10 has also been reported to participate in transcriptional silencing by the Polycomb complex (El Messaoudi-Aubert *et al.* 2010) and to play a role during viral infections, either by helping viral transcription or inhibiting retroviral production (Haussecker *et al.* 2008; Furtak *et al.* 2010; Wang *et al.* 2010).

Translation Repression

miRNAs can also repress translation, but the mechanism by which this is achieved is a subject of great controversy (Chekulaeva and Filipowicz 2009). The biological relevance of this regulation is also unclear, as a recent study suggested that mRNA decay accounts for most of miRNA-mediated regulation *in vivo*, while they observed a very modest influence on translation (Guo *et al.* 2010). miRNAs have been proposed to inhibit translation at its initiation (Humphreys *et al.* 2005; Pillai *et al.* 2005) or elongation steps (Olsen and Ambros 1999; Nottrott *et al.* 2006; Petersen *et al.* 2006), and it has been suggested that they can use either mechanism depending on target mRNA's transcriptional history (Kong *et al.* 2008).

miRNAs have been proposed to regulate translation elongation by promoting co-translational degradation of nascent peptides (Nottrott *et al.* 2006) or ribosome drop-off from the actively translated mRNA (Fig 1.1B) (Petersen *et al.* 2006). However, most studies using cell-free based systems support that the repression takes place at the initiation stage (Fig 1.1C) (Mathonnet *et al.* 2007; Thermann and Hentze 2007). The most widely accepted model suggests that miRNAs interfere with eIF4E binding to the cap structure and therefore block small ribosomal subunit recruitment, but there is also controversy on how this is achieved. It was proposed that Ago proteins themselves can bind the cap structure through their MID domain (Kiriakidou

et al. 2007), but this was later brought into question by studies in *Drosophila* cell lines (Eulalio *et al.* 2008) and by the crystal structure of human Ago2's MID domain, as the residues proposed to be involved in cap binding are buried within the hydrophobic core of the protein (Frank *et al.* 2010). Another study suggested that miRNAs prevent assembly of 60S ribosomal subunit through recruitment of eIF6 by Ago proteins, as eIF6 inhibits association of 60S subunit to 40S subunit (Chendrimada *et al.* 2007). This is supported by the fact that 40S, but not 60S, subunits are present in miRNA-targeted mRNAs in rabbit reticulocyte lysate (Wang *et al.* 2008a), but contradicted by the fact that eIF6 knock-down has no effect on miRNA-mediated repression in *Drosophila* S2 cells (Eulalio *et al.* 2008) and *C. elegans* (Ding *et al.* 2008). It is difficult to reconcile the different models, but different mechanisms may be operating in different situations.

Others

miRNAs have been reported to have some additional functions besides post-transcriptional repression. miRNAs and Ago proteins have been found in the nuclei of human cells (Hwang *et al.* 2007), where they could have a role in transcription repression by recruitment of Polycomb complex components (Kim *et al.* 2008). miRNAs have also been shown to activate translation in particular cases, like in cells in cell cycle arrest (Vasudevan *et al.* 2007) or through 5' UTR binding sites in ribosomal proteins' mRNAs (Orom *et al.* 2008) and in the Hepatitis C virus (HCV) RNA genome (Henke *et al.* 2008). The mechanisms underlying these additional functions, however, have not been elucidated.

1.1.3.3 – Physiological Relevance:

miRNAs, like transcription factors, regulate gene expression in a very wide range of cellular pathways. The importance of miRNA-mediated regulation is reflected by the fact that mutant animals for miRNA components are nearly always lethal and show severe developmental defects (Bernstein *et al.* 2003; Wienholds *et al.* 2003).

Many miRNAs have defined roles in specific biological processes. These include regulation of embryonic development and differentiation of tissues such as

brain, the hematopoietic system or muscle among others (Stefani and Slack 2008). Some miRNAs have also housekeeping functions or participate in stress responses. They are also relevant in pathogenic processes, like cancer (Agami 2010), neurodegenerative diseases (Sonntag 2010) and viral infections (Roberts and Jopling 2010), and the control of their expression has a great therapeutic potential. miRNAs' physiological relevance goes therefore beyond the RNA-silencing field and has to be understood in the context of the specific cellular pathways in which they participate.

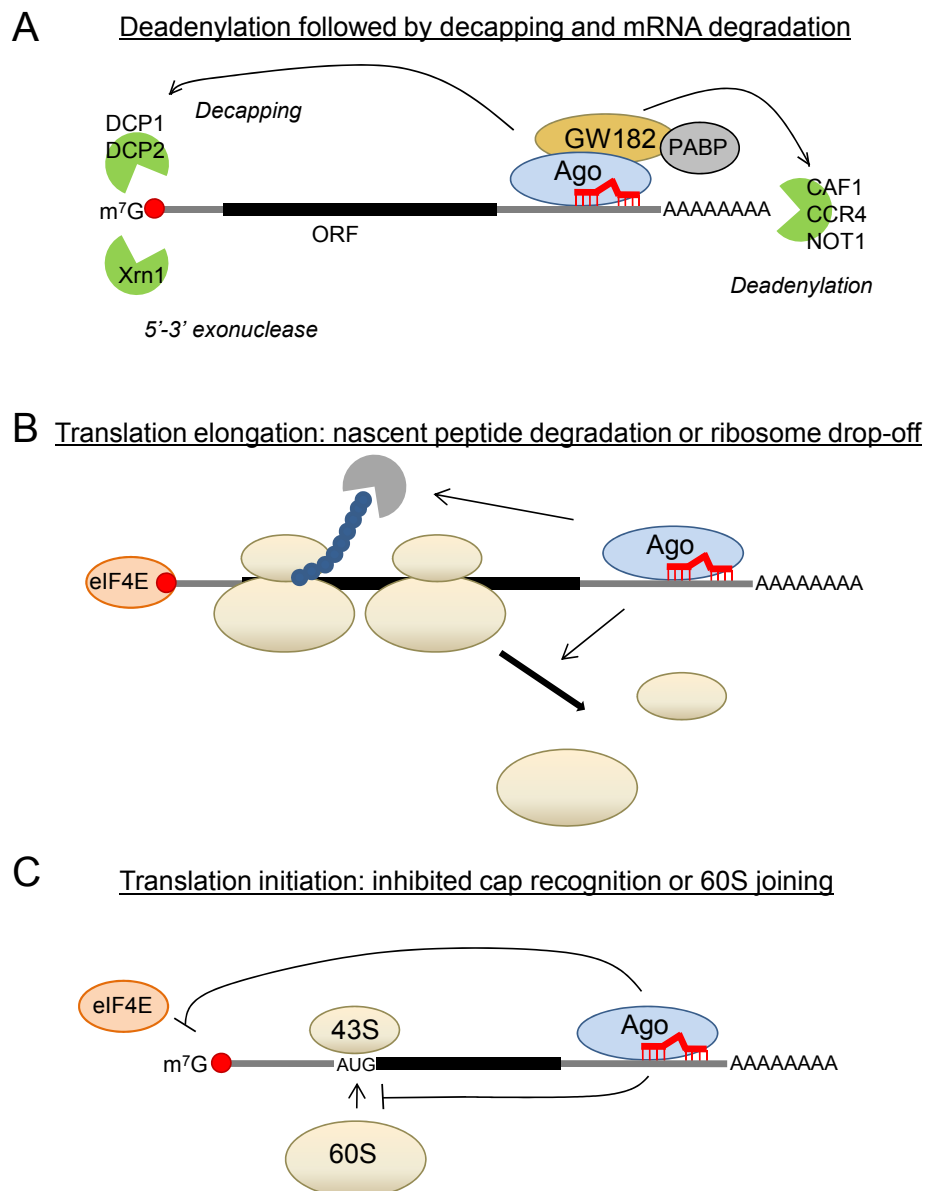


Figure 1.5: Mechanisms of Gene Regulation by miRNAs. Perfectly complementary targets are cleaved by Argonaute proteins (not shown, see Fig. 1.3). In animals, most miRNAs are partially complementary to their targets and have been proposed to direct mRNA decay (A) or to repress translation, either at the elongation (B) or initiation steps (C).

1.2 - Piwi-interacting RNAs (piRNAs)

1.2.1 - Discovery and General Features

piRNAs were discovered in *Drosophila* and were initially described as a subset of siRNAs which preferentially derived from repeat regions (Aravin *et al.* 2001). They were shown to mediate silencing of repetitive elements in the germ line and they were called repeat-associated siRNAs (rasiRNAs). Already in those early days, rasiRNAs distinguished themselves from miRNAs and siRNAs by being slightly longer in size (25-27 nt). Later they were found to be associated to members of the PIWI subfamily of Argonaute proteins and not to require Dicer for their biogenesis (Saito *et al.* 2006; Vagin *et al.* 2006), which made them essentially different from siRNAs. The finding that mouse PIWI proteins also bind a different class of small RNAs (Aravin *et al.* 2006; Girard *et al.* 2006; Grivna *et al.* 2006a) led to the designation of all PIWI-associated small RNAs, including rasiRNAs, as piRNAs.

piRNAs are generally longer (~24-30nt) than miRNAs and siRNAs and, like fly siRNAs, they are 2'*O*-methylated at their 3' ends (Kirino and Mourelatos 2007b). The methyl group is added by Hen1 (or its *Drosophila* homolog Pimet/DmHen1) (Horwich *et al.* 2007; Kirino and Mourelatos 2007a; Saito *et al.* 2007) and, in fish, this modification protects the piRNAs from being adenylated or uridylated, which would lead to their degradation (Kamminga *et al.* 2010).

piRNAs operate mostly in animal gonads, matching where PIWI proteins are expressed. As a consequence of their sequence heterogeneity they have several different functions (discussed below), but the most widespread one is that of silencing transposons. Transposons are mobile genetic elements that can move and propagate within the genome and are therefore a source of mutations (Slotkin and Martienssen 2007). By silencing them, piRNAs protect the genome integrity in the germ line, and this has been proven to be crucial as all PIWI protein mutants in mouse, fish and flies have fertility defects (Lin and Spradling 1997; Schmidt *et al.* 1999; Deng and Lin 2002; Kuramochi-Miyagawa *et al.* 2004; Carmell *et al.* 2007; Houwing *et al.* 2007; Houwing *et al.* 2008; Li *et al.* 2009). There are three PIWI proteins in *Drosophila*;

two of them, Aubergine (Aub) and Argonaute 3 (Ago3) are expressed and required both in male and female germ lines (Schmidt *et al.* 1999; Li *et al.* 2009). They are present in the apical part of testes, while in ovaries, they are expressed in nurse cells (see Fig. 1.6). The third one, Piwi, is expressed in nurse cells as well, but also in follicle cells, which are somatic cells surrounding the egg chamber (Cox *et al.* 2000). There, it participates in silencing transposons such as *gypsy*, which can pack itself into retroviral-like particles and invade the oocyte (Kim *et al.* 1994; Pelisson *et al.* 1994; Chalvet *et al.* 1999).

In mouse, piRNAs are mainly present in male gonads and are essential for their fertility as mutations for any of the three murine PIWI proteins confer male-specific sterility because of spermatogenic arrest (Deng and Lin 2002; Kuramochi-Miyagawa *et al.* 2004; Carmell *et al.* 2007). Mouse PIWI proteins are differentially expressed throughout spermatogenesis. Miwi2 is expressed only in embryonic stages (Aravin *et al.* 2008), while Mili is present in all stages but is enriched in spermatocytes and Miwi is found from meiotic pachytene stage cells to post-meiotic germ cells (Kuramochi-Miyagawa *et al.* 2001). Only pre-meiotic piRNAs are enriched in transposon sequences and participate in transposon silencing; the role of meiotic piRNAs, which derive mostly from intergenic sequences, remains elusive (Fig. 1.7). Mouse oocytes also express a limited set of piRNAs, but their functions are unclear as single PIWI mutations seem to have no effect on female fertility. It is likely that the piRNA pathway is rendered redundant due to the existence of an endo-siRNA pathway targeting transposons in oocytes (Tam *et al.* 2008; Watanabe *et al.* 2008).

A characteristic feature of piRNAs is that they have clustered genomic origins (Aravin *et al.* 2006; Girard *et al.* 2006; Brennecke *et al.* 2007). This means that they are particularly enriched in certain locations in the genome, where a lot of piRNAs are produced. Interestingly, in mammals, piRNAs do not show sequence conservation but their locations are syntenic (Aravin *et al.* 2006; Girard *et al.* 2006). In flies, piRNA clusters are preferentially found in pericentromeric and telomeric heterochromatin (Brennecke *et al.* 2007). Based on EST data, piRNA precursors are thought to be long single-stranded transcripts from these clusters (Aravin *et al.* 2006). This hypothesis is strongly supported by the fact that insertion of a *P*-element into a particular fly somatic piRNA cluster called *flamenco* abolishes piRNA production from the whole

cluster, as far as 168kb away from where the *P*-element is inserted (Brennecke *et al.* 2007).

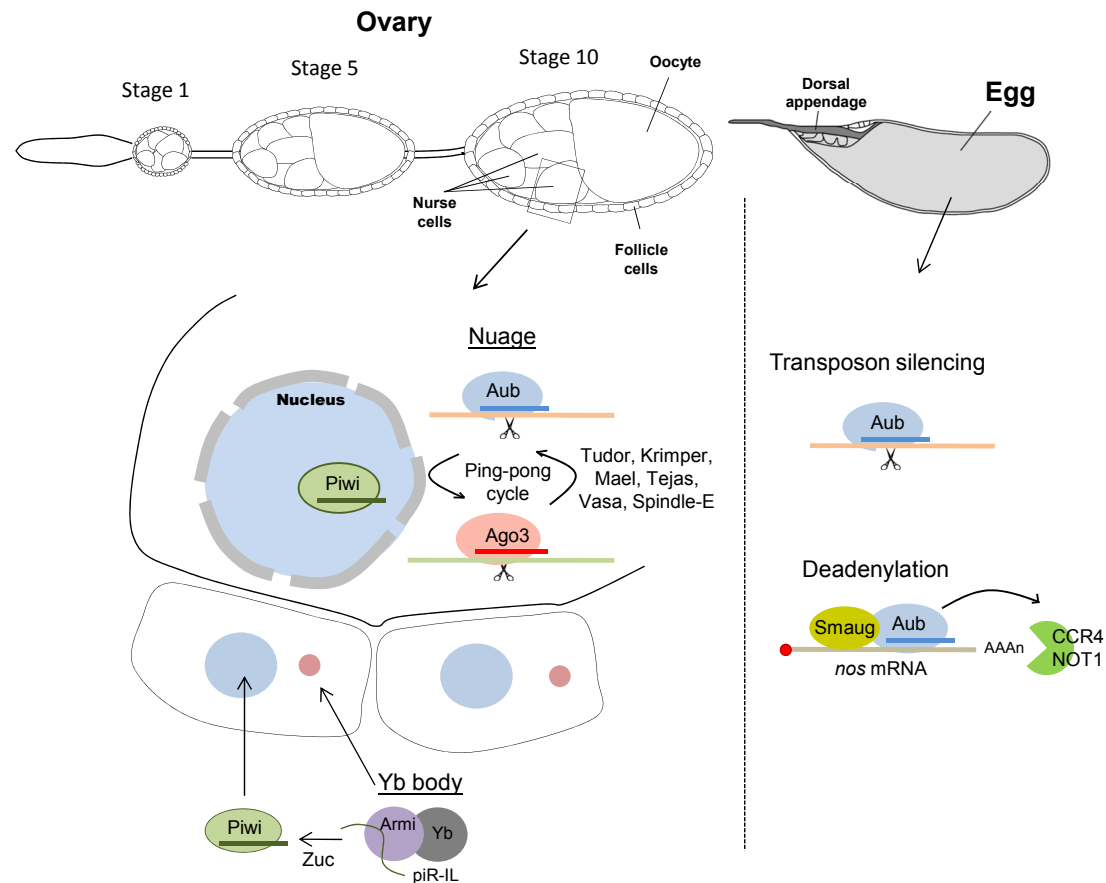


Figure 1.6: The piRNA Pathway in *Drosophila* Ovaries. Schematic representation of oogenesis in the fruit fly. Aub and Ago3 are expressed in nurse cells, where they localise in nuage and engage in the ping-pong amplification cycle (see Fig. 1.9 for details). The proteins that are implicated in nuage formation or ping-pong amplification are indicated. Piwi is nuclear and is also expressed in follicle cells, where piRNA biogenesis takes place in Yb bodies. In the embryo, piRNAs mediate transposon silencing and deadenylation of *nos* mRNA.

There are different types of clusters based on the strand within a genomic window that gives rise to piRNAs: in some of them, piRNAs are produced only from one strand; these are called uni-strand clusters and a good example is the aforementioned *flamenco*, which contains old copies from transposons that accumulated in this region. The piRNAs produced from *flamenco* have opposite

polarity to active transposons and can therefore target them (Brennecke *et al.* 2007). This cluster is conserved in different *Drosophila* species, producing only antisense piRNAs in all of them (Malone *et al.* 2009). Some clusters, such as a very prominent one in mouse chromosome 17 (conserved in humans, chromosome 6) are bidirectional, having divergent transcription from a central promoter (Aravin *et al.* 2006). Finally, there are also dual-strand clusters, which produce piRNAs from both strands, such as cluster42AB in *Drosophila* (Brennecke *et al.* 2007). One of the major questions in the field is what triggers piRNA production from the clusters. In the case of fly dual-strand clusters, it was shown that the HP1 homolog Rhino is required for transcription and piRNA production from these loci (Klattenhoff *et al.* 2009).

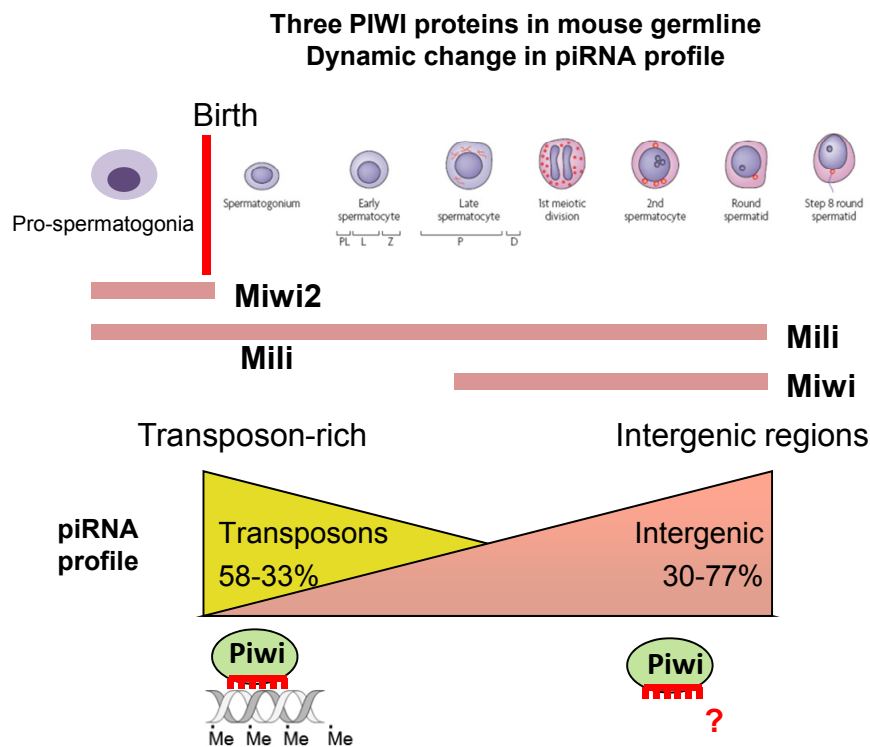


Figure 1.7: PIWI protein expression pattern and piRNA profile during spermatogenesis. Miwi2 and Mili are expressed in embryonic stages and mediate transposon silencing through DNA-methylation. Later, piRNAs are no longer transposon-rich but are mostly intergenic, and the roles of Miwi and Mili at these stages are not understood.

1.2.2 - Biogenesis

1.2.2.1 - Primary pathway

Processing of primary transcripts to small RNAs that are loaded into PIWI proteins is known as primary piRNA biogenesis, while amplification of pre-existing piRNAs is referred to as secondary piRNA biogenesis. In *Drosophila* ovarian germ cells, as well as in mammalian embryonic testicular germ cells, the two pathways coexist and it is difficult to estimate the contribution of each of them to the total piRNA pool. A distinctive feature of primary piRNAs is a strong preference for a U in their 5' residue (Aravin *et al.* 2006; Girard *et al.* 2006; Brennecke *et al.* 2007), and some of the PIWI proteins seem to preferentially load piRNAs that are produced through this mechanism, like Piwi (*Drosophila*) and Miwi (mouse). The existence of organisms and cell-types lacking secondary amplification has provided excellent systems for the study of the primary pathway. This is the case of somatic cells surrounding the germ cells in *Drosophila* ovaries (Li *et al.* 2009; Malone *et al.* 2009) and the ovarian-derived cell-lines OSS (ovarian somatic sheets) and OSC (ovarian somatic cells) (Niki *et al.* 2006; Lau *et al.* 2009), which only express the protein Piwi and have been recently used for identification and characterization of factors involved in primary biogenesis (Haase *et al.* 2010; Olivieri *et al.* 2010; Saito *et al.* 2010). Moreover, *C. elegans* offers a great and yet to be explored potential for genetic studies, given that piRNAs in this organism (also known as 21U RNAs) are exclusively produced through the primary pathway (Das *et al.* 2008).

The biogenesis of piRNAs is essentially different from that of miRNAs and siRNAs. The lack of conserved secondary structure motifs in piRNA producing regions suggested that the canonical enzymes that participate in miRNA biogenesis, Drosha and Dicer, are not required for the piRNA pathway (Girard *et al.* 2006); this could also be confirmed experimentally (Vagin *et al.* 2006; Houwing *et al.* 2007; Saito *et al.* 2010). piRNA-producing clusters are known to be transcribed into long single-stranded precursors, but there is little information as to how they are selected for entering the piRNA pathway. piRNAs originate also from mRNAs, preferentially from 3'UTRs (Robine *et al.* 2009). An example of this is the *Drosophila* gene *traffic*

jam, which is actively translated and producing piRNAs from its 3'UTR at the same time (Saito *et al.* 2009). Whether there are two mRNA subpopulations or the mRNA can be recycled for piRNA biogenesis after translation is currently not understood.

Characterization of the cellular machinery responsible for piRNA biogenesis is at its initial stages. In *Drosophila*, piRNA production was found to be defective in a mutant for a locus encoding a putative nuclease, Zucchini (Zuc), and there is solid evidence pointing to a role for Zuc in the primary pathway (Pane *et al.* 2007; Olivieri *et al.* 2010; Saito *et al.* 2010). Zuc belongs to the family of phospholipase D (PLD) phosphodiesterases, containing some members that act as nucleases, but, although Zuc's catalytic residues are conserved, such an activity has not been biochemically proven. However, a point mutation of a histidine to a tyrosine in the catalytic motif displays a similar phenotype to that of a *null* mutant, pointing to a requirement of Zuc's catalytic site in piRNA biogenesis (Pane *et al.* 2007; Haase *et al.* 2010).

Genetic and biochemical studies identified Armi and its homolog in mouse, MOV10L1, as primary pathway components (Vagin *et al.* 2006; Frost *et al.* 2010; Olivieri *et al.* 2010; Saito *et al.* 2010; Zheng *et al.* 2010). These are orthologues of *Arabidopsis* SDE-3 -which has been implicated in small RNA-mediated silencing (Dalmay *et al.* 2001)- and they contain a conserved RNA helicase domain at their C-termini. Similar to *zuc* mutants, piRNA biogenesis and transposon silencing are impaired in *armi/Mov10l1* mutants. Armi/MOV10L1 interacts directly with PIWI proteins, but its role at the molecular level has not been described yet. In *Drosophila*, Armi associates with ~25-70nt RNA species which map to piRNA-producing regions and generally overlap with mature piRNAs (Saito *et al.* 2010). They were termed piRNA intermediate-like molecules (piR-ILs) and they are the only presumable intermediates of primary biogenesis described to date. piR-ILs have a 5'-hydroxyl and a 3' cyclic 2',3'-cyclic phosphate, which in both cases is incompatible with the features of mature piRNAs, suggesting that they still have to be processed from both ends. It is tempting to speculate that piR-ILs are Zuc substrates, as they still accumulate in Armi complexes upon Zuc depletion, whereas mature piRNAs fail to be produced.

Primary piRNA biogenesis in *Drosophila* somatic cells takes place in cytoplasmic foci that are known as Yb bodies (Fig. 1.6) (Olivieri *et al.* 2010; Saito *et al.* 2010; Qi *et al.* 2011), which are termed after the localization pattern of the protein Yb (Szakmary *et al.* 2009). Yb contains a helicase and a Tudor domain and it interacts and co-localizes with Armi and most probably with piR-ILs, although *in situ* hybridization data is lacking. Yb and Armi are dependent on each other for their localization in Yb bodies (Olivieri *et al.* 2010; Saito *et al.* 2010), and depletion of Yb impairs piRNA biogenesis and association of Armi to piR-ILs. Piwi, which is normally nuclear, is found in the cytoplasm in any situation where it cannot be loaded; this includes Armi and Yb depletions and internal mutations that abolish the binding to small RNAs. Interestingly, in the particular case of Zuc depletion, Piwi is found in cytoplasmic bodies together with Armi. It is therefore reasonable to think that piRNAs are loaded into Piwi in Yb/Armi bodies and some signal allows Piwi to go to the nucleus when biogenesis is complete. This might be achieved by regulating the accessibility of nuclear import factors to the nuclear localization signal (NLS) that is present in Piwi N-terminus. Yb bodies are located in the close vicinity of mitochondria, where Zuc is found. Surprisingly, unlike Armi and Zuc, Yb is not required in *Drosophila* germ cells, where primary piRNA biogenesis also takes place (Olivieri *et al.* 2010). However, two paralogues of Yb exist in the *Drosophila* genome, so it is likely that one of them might be performing similar functions to Yb in the germ line. In mouse, MOV10L1 is the only biogenesis factor that has been identified (Fig. 1.8). Tdrd12 (the murine Yb orthologue) and Zuc are also conserved and display germ line specific expression, suggesting that similar mechanisms to *Drosophila* are operating. In *C. elegans*, in contrast, BLAST searches for these components do not give any significant hits, so it is difficult to speculate about the conservation of the pathway in this organism.

Overall, primary piRNA biogenesis only begins to be understood. The first intermediates of primary piRNA biogenesis (piR-ILs) have been recently described (Saito *et al.* 2010), but it has yet to be confirmed that these are bona fide precursors. How processing of single-stranded primary transcripts – as long as several kilobases in some cases- into piR-ILs (~25-70nt) takes place is currently a black box. On the other hand, three factors have been identified - Zuc, Armi (MOV10L1 in mouse) and Yb - that are likely to participate in late stages of biogenesis, but their exact roles

remain elusive. Armi and Yb might define a subcellular localization for piRNA biogenesis, where Armi carries the precursors and would hypothetically hand them over to Piwi, while Zuc is possibly the nuclease trimming the piR-IL down to the size of a mature piRNA. Zuc could either be processing both ends of the precursor or there might be a need for another nuclease. In any case, the activities of these proteins have to be reconstituted *in vitro* in order to confirm these hypotheses.

PIWI proteins themselves might also play a role in biogenesis. This is unlikely to be due to their catalytic activity, as this has been shown not to be required for primary biogenesis (Saito *et al.* 2009), but rather for their structural features. The fact that there is such a strong bias towards a U in the 5' position of piRNAs suggests that there is high specificity for this residue either from the nuclease that generates the 5' end or from the 5' binding pocket of the PIWI protein, which lies in its MID domain. The structure of the MID domain of human Ago2 revealed the presence of a rigid loop that establishes contacts with the 5' nucleotide, giving Ago2 a strong preference for A or U residues (Frank *et al.* 2010). The amino-acids forming this loop are not conserved in PIWI proteins, but it seems realistic to imagine that a similar loop could be present, explaining the specificity for a 5' U. In contrast, piRNA 3' ends seem quite variable when analyzing deep-sequencing data. For example, in mouse, Mili and Miwi piRNAs in adult stages originate from the same genomic clusters, and the profiles look essentially the same (Aravin *et al.* 2006 and Pillai lab, unpublished data). Mili binds shorter RNAs (~26nt) than Miwi (~30nt) and, interestingly, this 4 nucleotide difference lies on the 3' end, while the 5' ends perfectly overlap (more than 90% of Mili and Miwi reads have identical 5' ends). It is therefore easy to draw a scenario where the PIWI protein binds first to the 5' end of the precursor and its footprint determines where the 3' end processing ends.

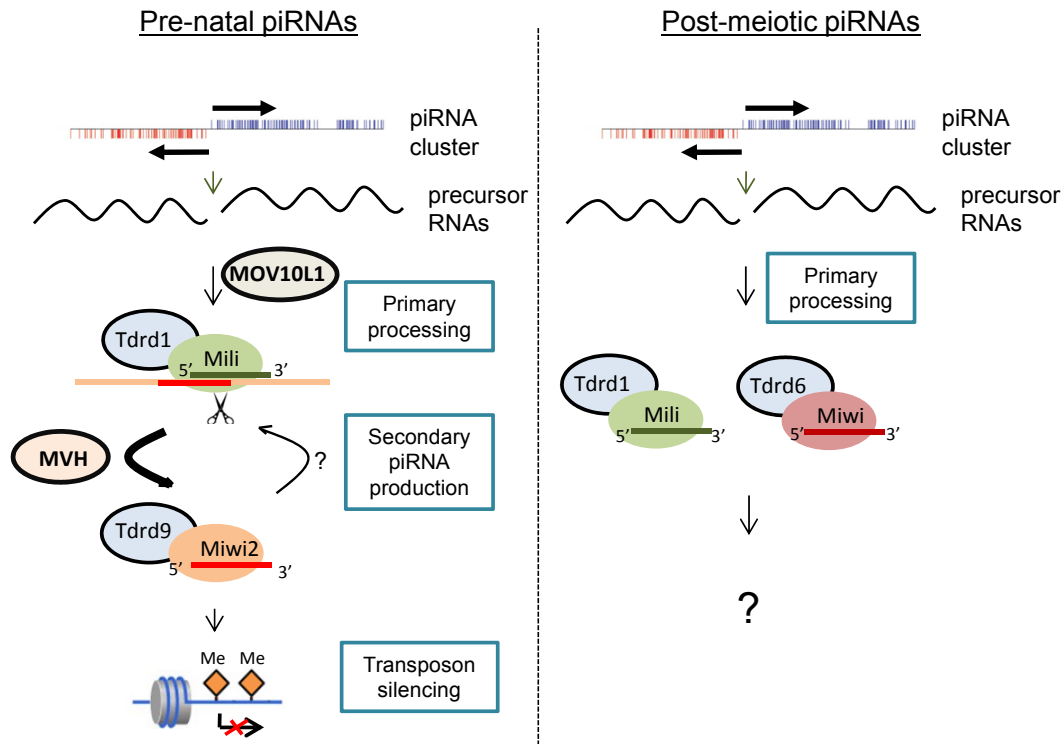


Figure 1.8: Biogenesis pathways in mouse. piRNA precursors are thought to be long single-stranded transcripts from piRNA clusters. Primary piRNA biogenesis is dependent on MOV10L1, while secondary biogenesis requires MVH. PIWI proteins associate with Tudor proteins, which have an impact both in biogenesis and localisation of piRNA pathway components. The factors that participate in post-meiotic stages have been studied to a more limited extent given that most mouse mutants have spermatogenesis arrested before these stages.

1.2.2.2 - Secondary piRNA Biogenesis

piRNA amplification through the so-called ping-pong cycle was first described in *Drosophila* after the observation that piRNAs bound to different PIWI proteins are asymmetrically distributed on the two strands of a piRNA-producing cluster (Brennecke *et al.* 2007; Gunawardane *et al.* 2007). In particular, Aub and Piwi preferentially bind piRNAs that arise from the antisense strand of transposon loci, while Ago3 is biased for small RNAs mapping to the sense strand. In a few clusters this trend is inversed, but even in these cases the asymmetry between the different proteins is maintained. The small RNAs are not randomly distributed through the clusters, but there is a correlation by which piRNAs from opposite strands have an overlap of 10nt at their 5' ends. Fly PIWI proteins, similar to some members of the Argonaute family called Slicers, have the ability to cleave perfectly complementary targets between the 10th and the 11th position counting from the 5' end of the small

RNA (Saito *et al.* 2006; Gunawardane *et al.* 2007). It was suggested that this cleavage defines the 5' end of a new piRNA, explaining the overlap of exactly 10nt between piRNAs from opposite strands. An unknown nuclease would then trim the 3' end to the size of a mature piRNA. Generally, primary piRNAs are bound to Aub and Piwi and have a 5'U. As a consequence, piRNAs originated by a slicing event and bound to Ago3 tend to have an A at the 10th position, and this is the main feature of secondary piRNAs. Upon base-pairing of secondary piRNAs to their targets, another slicing and 3' trimming events generate piRNAs that have primary features, closing an amplification loop that is known as ping-pong cycle (Fig. 1.9) (Brennecke *et al.* 2007; Gunawardane *et al.* 2007).

In *Drosophila* ovaries, ping-pong occurs only in germ cells, where all three PIWI proteins are expressed, although it is mostly Aub and Ago3 that participate in the cycle, while Piwi, like in somatic cells, is restricted to primary biogenesis (Li *et al.* 2009; Malone *et al.* 2009). The ping-pong cycle is conserved and seems to have existed since the origin of metazoans, as ping-pong signatures are already found in very primitive animals such as cnidarians and sponges (Grimson *et al.* 2008). In zebrafish, Ziwi and Zili engage in a ping-pong cycle that resembles very much to that in *Drosophila*, with each of the proteins binding piRNAs from opposite polarity within the same clusters (Houwing *et al.* 2008). Ziwi, similar to Aub, provides the primary pool, as it binds RNAs with a strong bias for a 5'U, while Zili, similar to Ago3, has a preference for an A at the 10th position. In mouse, in contrast, most of the piRNAs bound to all three PIWI proteins display primary features.

Murine piRNA populations dynamically change over the different stages of spermatogenesis, and they are generally classified in three groups: embryonic, pre-pachytene and pachytene (Aravin *et al.* 2008). Ping-pong pairs (piRNAs from opposite strands with an overlap of 10nt) can be found in all stages, but in pachytene stage they represent a very minor proportion of the total pool (Berninger *et al.* 2011). In pre-pachytene population in post-natal germ cells, only Mili is expressed and Mili-Mili pairs are found but only for transposon-derived piRNAs, which at this stage constitute 35% of the total pool (Aravin *et al.* 2007). Secondary biogenesis is most obvious in embryonic stages, where, in the absence of Mili, Miwi2 fails to get loaded with piRNAs and fails to localize in the nucleus (Aravin *et al.* 2008). It is however

unlikely that this is due only to ping-pong, as, although there are abundant Mili-Miwi2 ping-pong pairs, most of Miwi2 piRNAs have primary signatures. In a *Miwi2* knock-out, in contrast, Mili is normally loaded with piRNAs, suggesting that either the pathway is linear or the contribution of Miwi2 to amplification of Mili-bound piRNAs is very small (Kuramochi-Miyagawa *et al.* 2010).

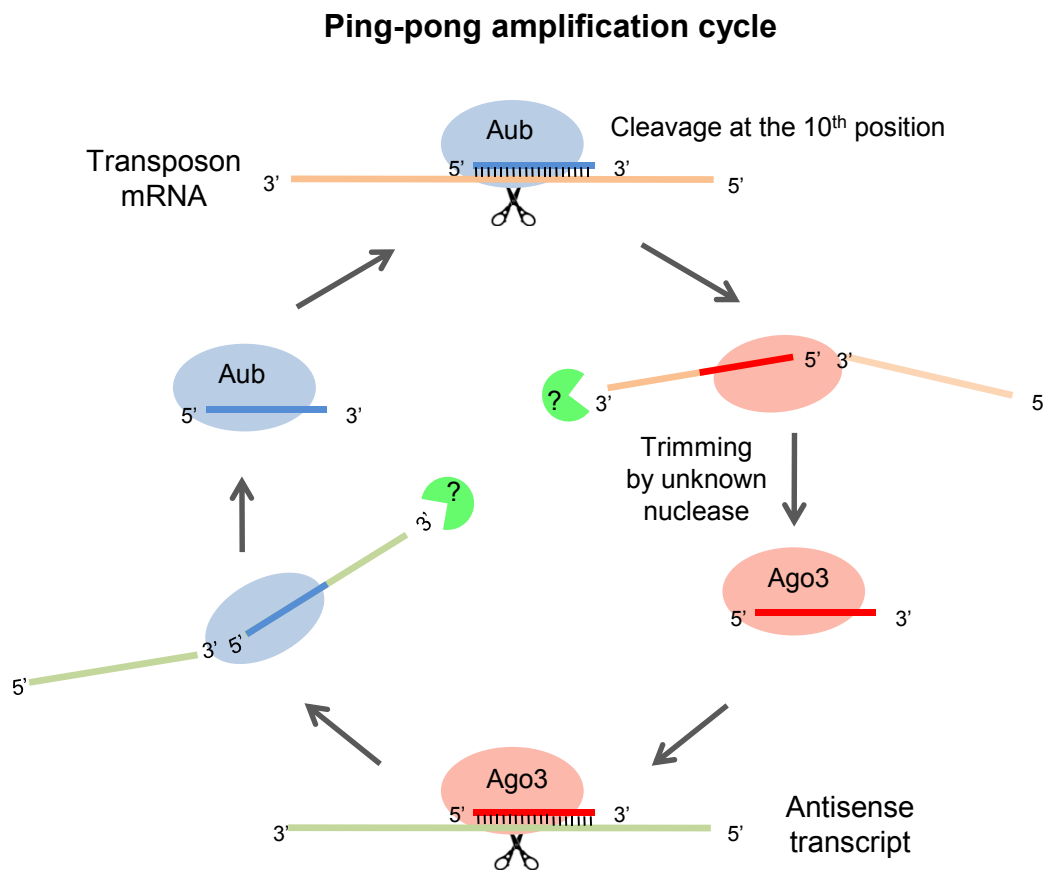


Figure 1.9: The ping-pong amplification cycle for piRNA biogenesis. The ping-pong cycle functions through PIWI proteins Slicer activity, where target cleavage defines the 5' end of a new piRNA. It allows amplification of piRNAs generated through the primary pathway, as well as maternally inherited piRNAs. Mechanistic information, such as the identity of the nuclease that trims the 3' end to the size of a mature piRNA, is still missing.

The ping-pong model was built on the grounds of bioinformatic analysis and there is genetic and biochemical evidence supporting it, but molecular understanding is lacking. It is unclear how target slicing by a PIWI protein recruits another PIWI protein to bind the newly generated 5' end, as well as how the 3' end is trimmed.

Some factors which specifically operate in secondary biogenesis have been described. These include two Tudor domain containing proteins -Spindle-E and Krimper- and Vasa, an RNA helicase that has been widely used as a germ cell marker. Mutations of these genes in *Drosophila* impair ping-pong amplification but do not affect primary biogenesis (Malone *et al.* 2009). The mouse orthologue of Vasa, Mouse Vasa Homolog (MVH), is also required for secondary biogenesis, as a *Mvh* knock-out has a very similar phenotype to a *Mili* knock-out, being Miwi2 unloaded and mislocalized (Kuramochi-Miyagawa *et al.* 2010). The helicase activity and RNA-binding capacity of Vasa have been long known to be required for its function (Liang *et al.* 1994), but the molecular details of these requirements remain elusive. Deeper analysis will be needed in order to understand mechanistic aspects of the ping-pong cycle and the role of the factors described above.

1.2.2.3 - Compartmentalization of Biogenesis Pathways

The precise steps of piRNA biogenesis have not been clearly defined yet, nor has their subcellular localization. The final step, where piRNAs are loaded into PIWI proteins, is believed to happen in defined cytoplasmic structures. Even if some of the PIWI proteins are nuclear -Piwi in *Drosophila*, Miwi2 in mouse, Zili in zebrafish (Cox *et al.* 2000; Aravin *et al.* 2008; Houwing *et al.* 2008)- , there is good evidence indicating that they are loaded in the cytoplasm prior to their movement to the nucleus (Aravin *et al.* 2008; Olivieri *et al.* 2010; Saito *et al.* 2010). In the germ line, cytoplasmic PIWI proteins and piRNA biogenesis factors colocalize in a perinuclear electron-dense structure called nuage (Brennecke *et al.* 2007; Houwing *et al.* 2007; Lim and Kai 2007). Nuage, which means “cloud” in French, also contains components of the miRNA and mRNA degradation pathways, similar to P-bodies in somatic cells (Lin *et al.* 2008; Lim *et al.* 2009). Nuage is one of the hallmarks of animal germlines. In *C. elegans*, for instance, it is present in the form of perinuclear granules that are known as P granules (Updike and Strome 2010), while in *Drosophila* it is found in nurse cells in ovaries. In early stages of spermatogenesis in mammals, nuage is also called intermitochondrial cement due to its tight association to mitochondria. This could be related to the fact that an important primary piRNA biogenesis factor, Zuc (MitoPLD in mammals), is located in the outer mitochondrial membrane (Choi *et al.* 2006; Saito *et al.* 2010). Later in spermatogenesis, mammalian nuage is condensed into one

compact filamentous perinuclear granule that conserves similar contents (including piRNA pathway and P-body components) and that is known as chromatoid body (reviewed in Kotaja *et al.* 2006).

Localization of piRNA pathway components is orchestrated through interaction of PIWI proteins with Tudor domain containing proteins. RG/GR repeats in the N-termini of PIWI proteins constitute a substrate for arginine symmetrical dimethylation by Prmt5; this modification is used as a binding platform for Tudor domains, which contain a characteristic aromatic cage that recognizes symmetrically dimethylated arginines (sDMAs) (Kirino *et al.* 2009; Nishida *et al.* 2009; Reuter *et al.* 2009; Vagin *et al.* 2009; Kirino *et al.* 2010b). A number of Tudor domain containing proteins have been described to participate in the piRNA pathway, most of them playing a role in piRNA biogenesis and/or in compartmentalization of biogenesis pathways. Mutation of the methyltransferase Prmt5 in flies, for instance, leads to general defects in PIWI protein localization, with Aub spreading in the cytoplasm instead of concentrating in nuage (Nishida *et al.* 2009). Similar observations have been made in mutants for other proteins containing Tudor domains, such as Tudor (the protein that gave name to the family), Tejas, Spindle-E or Krimper, with piRNA biogenesis being affected at different levels in the different mutants (Lim and Kai 2007; Nishida *et al.* 2009; Patil and Kai 2010).

In late stages *Drosophila* oocytes, Aub localizes to the posterior pole together with Vasa, Tudor and several other proteins and RNAs (Hay *et al.* 1988b; Kim-Ha *et al.* 1991; Bardsley *et al.* 1993; Harris and Macdonald 2001). After fertilization, the posterior pole defines where pole cells, the progenitors of the germ line, will form in the developing embryo (Strome and Lehmann 2007). Mislocalization of any of the components to the posterior pole in oocytes leads to sterility defects in the progeny due to failure of germ cell formation, and genes that are responsible for this are known as grand-childless genes. *Prmt5* and its cofactor, *valois*, as well as *tudor*, belong to this group of genes (Boswell and Mahowald 1985; Schupbach and Wieschaus 1986; Gonsalvez *et al.* 2006) and are required for Aub localization in the posterior pole (Kirino *et al.* 2009; Nishida *et al.* 2009). However, the phenotypes of *Prmt5* and *valois* mutants are somewhat less severe than that of some Tudor domain containing proteins and PIWI proteins themselves, which indicates that Prmt5-

dependent methylation cannot account for the function of all Tudors in the piRNA pathway. For instance, Yb, which contains one Tudor domain, is required for primary biogenesis in somatic cells while Prmt5 and Valois are not, suggesting that other methylases, perhaps mediating asymmetric dimethylation, might be involved in the pathway (Olivieri *et al.* 2010).

In mouse embryos, proteins from the Tudor family define specific granules within nuage for Mili and Miwi2. Tdrd9 is required for cytoplasmic Miwi2 localization in piP-bodies (Shoji *et al.* 2009), which also contain the canonical P-body components and are in the vicinity of pi-bodies, where Mili accumulates in a Tdrd1-dependent manner (Aravin *et al.* 2009). It is unclear how Mili and Miwi2 participate together in the ping-pong cycle while being localized in distinct granules. In post-meiotic cells, Miwi and Mili colocalize with Tdrd1, Tdrd6, Tdrd7 and Tdrd9 in the chromatoid body (Chuma *et al.* 2003; Hosokawa *et al.* 2007; Vasileva *et al.* 2009), and the architecture of this structure and Miwi localization have been reported to be severely impaired in Tdrd6 mutants (Vasileva *et al.* 2009).

Formation of germ cell granules in different organisms, as well as localization to the posterior pole in flies, is a hierarchical process in which Tudor domain proteins are upstream of PIWI proteins. On top of the hierarchy there is Vasa, which is present in fly and mammalian nuage at all stages (Hay *et al.* 1988a; Toyooka *et al.* 2000), but not in somatic Yb bodies as it is a germ cell specific component. Vasa also contains RG repeats where the arginines are symmetrically and asymmetrically dimethylated and are likely to recruit Tudor proteins to the specific granules (Kirino *et al.* 2010a). Direct interaction with Oskar anchors Vasa in the posterior pole (Breitwieser *et al.* 1996), but how Vasa defines nuage is currently not understood. Some proteins other than Vasa and Tudors have also been reported to be essential for nuage formation and correct localization of PIWI proteins. This is the case for Mael, which both in mammals and flies is required for transposon silencing and correct assembly of P-body components and PIWI proteins in nuage (Lim and Kai 2007; Soper *et al.* 2008; Aravin *et al.* 2009). In mouse, GASZ is required for correct localization of Mili and Tdrd1 in the intermitochondrial cement, and piRNA biogenesis is also affected in *gasz* mutants (Ma *et al.* 2009).

Assembly of germ cell granules is achieved through a complex network of interactions and recruitment of components in which arginine methylation and Tudor domain binding play a key role. The specificity of the different methylated arginines for the different Tudor domains is unclear, but the fact that there are proteins that contain more than one Tudor domain (e.g. Tudor, which has eleven, or Tdrd1, which has four) might indicate that they act as a scaffold for different proteins or different molecules of the same protein. It is also possible that some of them have redundant functions, given that the defects observed in some of the mutants are only partial (Kirino *et al.* 2009; Nishida *et al.* 2009). The impact of correct localization of PIWI proteins on piRNA biogenesis is variable; while formation of Yb bodies in *Drosophila* somatic ovarian cells is essential for the primary piRNA pathway (Olivieri *et al.* 2010; Saito *et al.* 2010), mislocalized PIWI proteins in the murine and fly germ lines have been reported to be loaded with piRNAs in different Tudor protein mutant backgrounds (Nishida *et al.* 2009; Reuter *et al.* 2009). However, the piRNA profile is altered in these mutants, suggesting that Tudors and localization to nuage might play a role in quality control of piRNA biogenesis.

1.2.3 - Function

1.2.3.1 - Transposon Silencing

piRNAs, as other classes of small RNAs, guide Argonaute proteins (in this case from the PIWI clade), to their complementary targets, leading in most cases to their silencing. piRNAs are enriched for transposon sequences, and mutants for most PIWI proteins and piRNA pathway components display transposon activation in their germ lines (Sarot *et al.* 2004; Vagin *et al.* 2006; Aravin *et al.* 2007; Carmell *et al.* 2007; Li *et al.* 2009). This leads to the generation of double-stranded DNA breaks and results in meiotic blockade or developmental defects in the offspring due to the activation of a DNA-damage checkpoint (Klattenhoff *et al.* 2007).

There are different mechanisms by which piRNAs silence transposons. The three *Drosophila* PIWI proteins have been shown to be Slicers (Saito *et al.* 2006; Gunawardane *et al.* 2007), and transposon repression has been found to take place at

the post-transcriptional level and to be dependent on mRNA degradation enzymes (Lim *et al.* 2009). These data strongly suggest that piRNAs can silence their targets in a very similar manner to siRNAs during RNAi. On the other hand, Piwi is localised in the nucleus and has been found to interact with HP1a (Brower-Toland *et al.* 2007) and to participate in heterochromatin formation during position effect variegation (Pal-Bhadra *et al.* 2004). Although no link has been established with transposon silencing and *piwi* mutation has been reported not to affect HP1 recruitment to piRNA clusters (Moshkovich and Lei 2010), this data suggest that Piwi could have an epigenetic function.

In *Drosophila*, maternally deposited PIWI proteins and piRNAs defend the developing embryo against transposon activation (Brennecke *et al.* 2008). Crosses between males that carry active *P*- or *I*-elements with females that do not have these transposons result in sterile offspring due to transposon activation, while the progeny from the reciprocal cross are fertile. This phenomenon is known as hybrid dysgenesis (Kidwell *et al.* 1977) and is explained by the fact that the protection is conferred by the piRNA pathway, which is transmitted by the mothers (Brennecke *et al.* 2008).

In mouse, transposon silencing is thought to take place both at the transcriptional and post-transcriptional level. Slicer activity is not demonstrated for the mammalian PIWI proteins which are implicated in transposon silencing, but this is expected as piRNAs display signatures of ping-pong amplification, which implies Slicer activity (Aravin *et al.* 2007). The only mammalian PIWI protein which has been shown to be a Slicer is rat Riwi (Lau *et al.* 2006), but its functions are unknown. Therefore, transposon post-transcriptional silencing is expected but has not been proven. In contrast, there is a link with transcriptional silencing. Primordial germ cells (PGCs) undergo a wave of DNA demethylation during early stages of embryonic development, soon after they settle in the gonadal compartment (Hajkova *et al.* 2002). In males, DNA methyl marks are re-established approximately between 14.5 days post-coitum (dpc) to 16.5 dpc (Kato *et al.* 2007). During this stage, the piRNA pathway directs selective *de novo* methylation of transposon sequences (Aravin *et al.* 2008; Kuramochi-Miyagawa *et al.* 2008). This is mediated by Miwi2, which is nuclear. Loss of Mili, Miwi2 and other piRNA pathway components leads to a failure in establishing methyl marks at the level of transposon promoters, which leads to their

derepression (Aravin *et al.* 2007; Carmell *et al.* 2007). The methylation is carried out by Dnmt3a in collaboration with its enzymatically inactive regulatory factor Dnmt3L (Bourc'his and Bestor 2004), but the biochemical link between the methyltransferase complex and Miwi2 has not been shown. It is possible that Miwi2 gains access to target genomic regions by tethering to nascent transcripts like the RITS complex in fission yeast, but there is no evidence proving this hypothesis.

1.2.3.2 - Protein-Coding Gene Silencing

piRNAs have also been shown to silence protein-coding genes other than transposons. The first example was *Stellate*, which forms crystals in *Drosophila* male gonads if it is not silenced. Genetic analyses identified a locus that is responsible for *Stellate* silencing, which was called *Suppressor of Stellate* [*Su(Ste)*] (Tritto *et al.* 2003). This locus was found to code for piRNAs which are antisense to *Stellate* mRNA (Aravin *et al.* 2001) and are loaded into Aub and Ago3 (Vagin *et al.* 2006; Nagao *et al.* 2010). Mutations in *aub* and *ago3*, as well as in other piRNA pathway components, such as *armi*, *zuc*, or *spindle-E*, lead to formation of *Stellate* crystals (Aravin *et al.* 2001; Vagin *et al.* 2006; Pane *et al.* 2007; Nagao *et al.* 2010). Silencing is likely to take place at the post-transcriptional level, given that an RNA with *Stellate* sequence was shown to be sliced by Aub immunoprecipitates (Nishida *et al.* 2007). Examples of other protein-coding genes which are down-regulated by PIWI proteins are *vasa*, which is also targeted by piRNAs in *Drosophila* testis (Nishida *et al.* 2007), and *Fasciclin III* (*FasIII*), for which antisense piRNAs are produced from *traffic jam*'s 3' UTR in *Drosophila* ovarian somatic cells (Saito *et al.* 2009).

In addition to this, piRNAs have been recently reported to function in a similar way to animal miRNAs in *Drosophila* embryos, as maternally deposited piRNAs trigger deadenylation of the mRNA of an embryonic posterior morphogen, Nanos (Nos) (Rouget *et al.* 2010). This is achieved through partial complementarity of piRNAs to this mRNA and the action of the RNA-binding protein Smaug and the CCR4-NOT deadenylation complex.

1.2.3.3 - Others

piRNAs have some additional functions beyond transposon silencing and gene regulation. First, the role of meiotic piRNAs in mammals, derived mostly from intergenic sequences, is still unclear. Male knock-out mice for *Miwi* are sterile because of a block in spermatogenesis (Deng and Lin 2002), but there is no transposon derepression in the mutants. It remains therefore a mystery what these piRNAs are targeting. Mili has been proposed to upregulate translation (Unhavaithaya *et al.* 2009) and Miwi associates with the translation machinery (Grivna *et al.* 2006b), but the mechanism by which they do so and the link with the piRNAs they carry have not been described.

In *Drosophila*, a subset of shorter piRNAs (~19-22nt) was recently shown to participate in the assembly of the telomere protection complex (Khurana *et al.* 2010). On the other hand, Piwi and one of its associated piRNAs were also reported to mediate epigenetic activation by promoting the formation of euchromatin (Yin and Lin 2007). However, the mechanisms by which piRNAs perform these additional roles remain elusive.

Aim of the thesis

One of the approaches taken in the Pillai group to further understand piRNA biogenesis and function has been to find and characterise protein factors that participate in the pathway besides PIWI proteins themselves. Such an approach led to the identification of Tdrd1 as an essential component for transposon silencing by the piRNA pathway (Reuter *et al.* 2009). This study focuses on two additional factors identified in the Pillai group: MOV10L1, which was found in a PIWI protein proteomic screen, and FKBP6, following a yeast two-hybrid screen to search for Tdrd1 interactors. The aim of this PhD project was to:

- (1) Investigate whether MOV10L1 and FKBP6 are piRNA pathway components by testing and mapping their interaction with PIWI proteins and, in the case of FKBP6, Tdrd1.
- (2) Analyse *Mov10l1* and *Fkbp6* mutant mice to examine whether these proteins are required for efficient transposon silencing in the germ line. Determine at which step of the pathway they act by analysing, among others, piRNA profile and subcellular localisation of the different components.
- (3) Perform biochemical analyses to gain mechanistic information about MOV10L1 and FKBP6 in the piRNA pathway. Determine whether their enzymatic activity is required or, alternatively, which additional roles they could play.

Overall, the goal was to understand the function of FKBP6 and MOV10L1, if possible at the molecular level, to shed light on how piRNAs accomplish the essential task of silencing transposons in the germ line.

2-MATERIALS AND METHODS

2.1 - Clones

2.1.1 - Plasmids and cDNAs

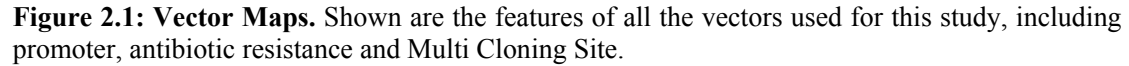
The following plasmids were used for recombinant protein expression in *E. coli*: pETM-11 (EMBL) for His-tagged proteins and pETM-30 (EMBL) for GST-tagged proteins. pFastBac-HTa (Invitrogen) was used for expression in Sf21 cells. pcDNA3 (Invitrogen) containing a 3xMyc or FLAG tag and pCiNeo (Promega) containing an NHA tag were used for mammalian cell expression, while pCR2.1 was used for cloning blunt PCR products. The maps of all the vectors used are shown in Fig. 2.1.

Most of the cDNAs used for this study were purchased from commercial cDNA libraries. Some of them were obtained by RT-PCR using Superscript one-step RT-PCR with Platinum Taq Kit (Invitrogen). A list of all the cDNAs used, as well as the way they were obtained and their accession number is shown in Table 2.1. cDNAs were amplified by PCR with DNA primers containing flanking restriction sites and cloned into the target vector multi-cloning site by standard methods. Pwo polymerase (Peqlabs) was used for the amplification and T4 DNA ligase (Fermentas) for the ligation. Plasmids were transformed and amplified in chemocompetent TOP10 cells (Invitrogen). Cloning strategies were designed using VectorNTI software (Invitrogen) and positive clones were sequenced by MacroGen Korea.

For gene mutation, site-directed mutagenesis was performed using the overlapping extension strategy (Ho *et al.* 1989).

2.1.2 - Constructs

The constructs used in Chapter 3 are described within the chapter. A summary table of the constructs used in Chapter 4 is shown in Table 2.2.



HEK293 cells were grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (v/v), penicillin, streptomycin and L-

glutamine. Cells were transfected with Lipofectamine Plus (Invitrogen) in 6cm² dishes and harvested 48h after transfection. The amount of DNA used ranged from 500ng to 2µg depending on the levels of expression.

Protein	Organism	Accession number	Obtained from
Ago2	<i>Homo sapiens</i>	NM_012154.3	Pillai <i>et al.</i> 2005
BmAgo3	<i>Bombyx mori</i>	NM_001104597.2	Kawaoka <i>et al.</i> 2009
FKBP12	<i>Homo sapiens</i>	BC005147 IMAGE:3504715	Geneservice Ltd.
FKBP3	<i>Mus musculus</i>	BC002122 IMAGE:3492535	Geneservice Ltd.
FKBP5	<i>Homo sapiens</i>	BC042605 IMAGE:4539929	Geneservice Ltd.
FKBP6	<i>Homo sapiens</i>	NM_003602.3	RT-PCR from human testes RNA
FKBP6	<i>Mus musculus</i>	NM_033571.2	RT-PCR from mouse testes RNA
FKBP8	<i>Mus musculus</i>	BC027808 IMAGE:5324380	Geneservice Ltd.
Mili	<i>Mus musculus</i>	NM_021308	Reuter <i>et al.</i> 2009
Miwi	<i>Mus musculus</i>	NM_021311.3	Reuter <i>et al.</i> 2009
Miwi2	<i>Mus musculus</i>	NM_177905.3	Reuter <i>et al.</i> 2009
MOV10	<i>Homo sapiens</i>	BC002548.1 IMAGE:3138543	Geneservice Ltd.
MOV10	<i>Mus musculus</i>	BC053743 IMAGE:6510959	Geneservice Ltd.
MOV10-like	<i>Homo sapiens</i>	NM_018995 IMAGE:100000082	Geneservice Ltd.
MOV10-like	<i>Mus musculus</i>	NM_031260.2 IMAGE:100068158	ImaGenes
Mov10-like (complete N-terminus)	<i>Mus musculus</i>	JF319145	RT-PCR from mouse testes RNA
Shut-down	<i>Bombyx mori</i>	Not annotated	RT-PCR from BmN4 RNA
Siwi	<i>Bombyx mori</i>	NM_001104596.2	Kawaoka <i>et al.</i> 2009
Tdrd1	<i>Mus musculus</i>	NM_031387.3	Reuter <i>et al.</i> 2009

Table 2.1: List of cDNAs Used for this Study. Shown are species, accession number (including IMAGE clone number if applicable) and the way they were obtained.

Construct	Organism	Residues	Expression	Vector
FKBP12	<i>Homo sapiens</i>	Full-length	Bacteria	pETM-11
FKBP3	<i>Mus musculus</i>	Full-length	Bacteria	pETM-11
FKBP5	<i>Homo sapiens</i>	Full-length	Bacteria	pETM-30
FKBP6	<i>Mus musculus</i>	19-301	Bacteria	pETM-11 and pETM-30
FKBP6	<i>Mus musculus</i>	Full-length	HEK293 cells	pCI-neo
FKBP6 FK domain	<i>Mus musculus</i>	19-145	Bacteria	pETM-11 and pETM-30
FKBP6 FK domain	<i>Mus musculus</i>	1-145	HEK293 cells	pCI-neo
FKBP6 TPR domain	<i>Mus musculus</i>	145-301	Bacteria	pETM-30
FKBP6 TPR domain	<i>Mus musculus</i>	145-327	HEK293 cells	pCI-neo
FKBP8	<i>Mus musculus</i>	Full-length	Bacteria	pETM-30
Mili	<i>Mus musculus</i>	Full-length	Reticulocyte lysate	pCI-neo
Mili ΔN-terminus	<i>Mus musculus</i>	384-971	Reticulocyte lysate	pCI-neo
Mili N-terminus	<i>Mus musculus</i>	1-383	Reticulocyte lysate	pCI-neo
Miwi	<i>Mus musculus</i>	Full-length	Reticulocyte lysate	pCI-neo
Miwi2	<i>Mus musculus</i>	Full-length	Reticulocyte lysate	pCI-neo
Tdrd1	<i>Mus musculus</i>	Full-length	Reticulocyte lysate	pCI-neo

Table 2.2: List of Constructs Used for this Study. Shown are the species, the residues that the constructs comprise, the system used for expression and the vectors in which they were cloned.

2.3 - *In Vivo* Studies

2.3.1 - Mouse Strains and Genotyping

Testes from mice of the indicated age were harvested and extracts were normalized by protein estimation using the Bradford method. FKBP6 mutant mouse is published (Crackower *et al.* 2003) and genotyping was performed as described with small modifications: mouse tails were digested with Proteinase K in a buffer containing 50mM Tris pH 8, 100mM NaCl and 1% SDS. After overnight incubation at 55°C, the samples were cleared by centrifugation and the DNA was precipitated with isopropanol. The resulting pellet was resuspended in water and 100ng of DNA were

used per PCR reaction. The wild-type and mutant alleles were amplified separately using specific primers for each of them, and the PCR products were analysed with a 1% agarose gel.

2.3.2 - Antibodies and Western Blot

Antisera against MOV10L1 were raised against three different regions of the protein, comprising residues 1-200, 690-805 and 878-933 respectively. These three fragments were expressed in *E.coli* and purified from inclusion bodies due to their insolubility. For that purpose, bacterial pellets were resuspended and sonicated in PBS containing 1mM PMSF. Lysates were centrifuged at high speed and the supernatant discarded. Pellets were then washed with a buffer containing 0.1M Tris-HCl pH 7 and 20mM EDTA, and subsequently washed again with a buffer containing 60mM EDTA, 6% Triton X-100 and 1.5M NaCl at pH 7. In order to recover the protein, pellets were resuspended in 8M urea and agitated at 4°C for at least two hours. The resulting mixture was cleared by centrifugation and the supernatant was used for rabbit immunization. Two rabbits were immunized for each MOV10L1 construct. Only the antibody produced with the antigen comprising residues 1-200 gave a specific signal for MOV10L1 and was used for the experiments shown.

For antibody purification, the protein used for immunization was loaded on an SDS-PAGE without molecular weight marker or well separation and transferred to a nitrocellulose membrane. Ponceau staining allowed identification of the strip of membrane where the protein was present, which was then cut out and washed with water to remove the staining. Blocking was performed in 5% milk prior to hybridisation with the serum. Generally, 3ml of serum per strip of membrane were used. Hybridisation was performed in 5% milk at 4°C and left overnight in agitation. The membrane was then washed three times with PBS containing 0.1% Tween (PBS-T). For the elution, the strip was incubated with 500µl of a buffer containing 0.1M glycine and 150mM NaCl at pH 3. After 3 minutes of incubation, the eluate was immediately neutralized with 100µL of a buffer containing 0.5M Tris and 150mM NaCl at pH 8. This step was performed twice. Purified antibodies were aliquoted and stored at -20°C after addition of 10% glycerol and 0.02% NaN₃.

Other antibodies used were Mili, Miwi (Reuter *et al.* 2009), Miwi2 (J. Martínez and Greg Hannon), FKBP6 (Crackower *et al.* 2003), Hsp90 (Santa Cruz Biotechnology), tubulin (Abcam), HA (Santa Cruz Biotechnology), Myc (EMBLCF) and FLAG (Sigma).

For Western blot, 10-15% SDS-PAGE were transferred into a nitrocellulose membrane using a semidry blotting equipment (Bio-rad). The membrane was blocked with 5% milk in PBS-T for approximately 30min and incubated for 2 hours with the primary antibody in 5% milk PBS-T. Dilutions of the antibody ranged from 1/150 to 1/2000 depending on the signal obtained. For Western blots against MOV10L1, blocking and incubation with the primary antibody was performed in 5% BSA PBS-T. After extensive washes with PBS-T, the membrane was incubated with a 1/10.000 dilution of anti-mouse or anti-rabbit antibodies coupled to HorseRadish Peroxidase (HRP, GE Healthcare), followed by three washes in PBS-T. Membranes were developed using ECL+ (GE Healthcare) and chemiluminiscence films (GE Healthcare).

2.3.3 - Immunoprecipitation (IP)

HEK293 were lysed in a buffer containing 50mM Tris pH8, 150mM NaCl, 5mM MgCl₂, 1mM DTT, 10% glycerol, 0.5% Triton X-100 and Complete –EDTA free– protease inhibitor cocktail (Roche). For testis extracts, the same buffer was used adding 0.5% sodium-deoxycholate and increasing the concentration of Triton X-100 to 1%. In the case that small RNAs had to be extracted after the IP, the buffer contained also 2mM Vanadyl-RC (Sigma) and 50µg/mL tRNA to prevent their degradation. Cells/testes were homogeneized in a douncer and the lysate was cleared by centrifugation.

HA-tagged proteins were immunoprecipitated using Anti-HA affinity matrix (Roche), while Anti-FLAG M2 affinity gel (Sigma) was used for FLAG-tagged proteins. Other antibodies were coupled to protein G-sepharose beads (GE Healthcare) following the manufacturer's instructions. Extracts were incubated with the beads for 2-4h at 4°C

and washed extensively with a buffer containing 10mM Tris pH 8, 150mM NaCl, 1mM MgCl₂ and 0.01% NP-40. Immunoprecipitates were resolved by SDS-PAGE and analysed by Western blotting.

When required, antibodies were chemically cross-linked to the beads using dimethylpimelidate (Sigma) in order to reduce the background in Western blot. In these cases, immunoprecipitated proteins were eluted from the beads using 0.1M glycine pH 3 and precipitated with methanol and chloroform prior to SDS-PAGE.

For small RNA extraction, 300µL of a buffer containing 10mM Tris pH 7.5, 0.5% SDS, 5mM EDTA and 30µg/mL Proteinase K were added to the washed beads and incubated at 55°C during 30 minutes. The RNA was recovered by phenol-chloroform extraction and precipitated with ethanol at -20°C. After dephosphorylation with shrimp alkaline phosphatase (Roche), the RNA was 5' labelled using T4 polynucleotide kinase (Fermentas) in the presence of [γ -³²P]ATP (Perkin Elmer). Small RNAs were resolved in a denaturing 15% urea-PAGE, exposed to a PhosphorImager screen (GE Healthcare) after gel drying and visualised with a Typhoon Scanner (GE Healthcare).

2.3.4 - RT-PCR and Northern Blot

Analysis of *Mov101l* expression in different stages was performed by RT-PCR. Total RNA from mouse testes was extracted using TriReagent (Molecular Research Center, Inc.) and treated with DNase I (Roche). After phenol-chloroform extraction, 1µg of total RNA was used for reverse transcription with random primers and Superscript III reverse transcriptase (Invitrogen). The resulting cDNA was amplified by PCR using Taq polymerase (EMBLCF), using *Mov101l*-specific primers and actin as a control. The results were analysed on a 2% agarose gel stained with ethidium bromide.

The level of expression of the transposons Line1 and IAP in the germ lines of *Mov101l* and *Fkbp6* mutant mice was analysed by quantitative RT-PCR and Northern blot. In both cases actin was used as a reference.

Quantitative RT-PCR analysis for Line1 and IAP transposons was performed as described (Lane *et al.* 2003; Aravin *et al.* 2007). cDNA obtained by reverse transcription as described above was used for real-time PCR using the SYBR Green method and a Stratagene real-time PCR machine. Reactions were performed in triplicates and at least two biological replicates were used per quantification.

For Northern blotting, 10µg of total RNA were resolved in a 1% agarose gel containing 6.7% formaldehyde (v/v). The quality of the migration was assessed by ethidium bromide staining and the RNA was transferred by capillarity to a Nylon membrane (Hybond N+, Amersham). After the transfer, the RNA was UV cross-linked to the membrane. Pre-hybridization was performed for 2h in Church buffer (0.25M NaPO₄ pH 7.2, 1mM EDTA, 1% BSA, 7% SDS). Probes were labelled with [α -³²P]dCTP (Perkin Elmer) using the Random Primed DNA Labeling Kit (Roche), denatured for 5min at 95°C and used for overnight hybridization at 65°C in Church Buffer. After washing, the membrane was exposed to a PhosphorImager screen and visualized with a Typhoon Scanner.

2.3.5 - Southern Blot and Bisulfite Sequencing

Analysis of CpG methylation at the level of Line1 transposon promoters in different genetic backgrounds was performed with two different techniques, methylation-sensitive Southern blot and bisulfite sequencing. Probes and primers for these techniques were used as described (Carmell *et al.* 2007).

DNA was extracted from the resulting pellets after preparation of testes extracts for immunoprecipitation. First, proteinase K treatment was performed overnight at 55°C in a buffer containing 20mM Tris pH 8, 100mM EDTA, 100mM NaCl, 1% SDS and 0.5mg/mL proteinase K. The samples were then homogenized by pipetting and the DNA was isolated by three consecutive phenol-chloroform extractions and subsequently precipitated with ethanol.

For methylation-sensitive Southern blot, 5µg of DNA were digested with HpaII and MspI restriction enzymes in different reactions. Both enzymes recognize the same

DNA sequence for cleavage, but MspI is insensitive to CpG methylation while HpaII cannot cut if the CpGs are methylated. Therefore, HpaII will give different digestion patterns for a given sequence in case the samples have a different methylation status. Digestion was carried out overnight at 37°C and the samples were resolved in a 1% agarose gel. After the run, the gel was incubated in 0.25M HCl for 15 minutes, followed by two incubations in a buffer containing 0.5M NaOH and 1.5M NaCl (10 and 45 minutes respectively) and finally neutralized in 1M Tris pH 8 and 1.5M NaCl for 20 minutes. The DNA was transferred by capillarity to a Nylon membrane and the following steps were performed as described for Northern blotting.

Conversion of unmethylated cytosine residues to uracil for bisulfite sequencing was performed with the EZ DNA Methylation-Gold Kit (Zymo Research). 50ng of DNA were used per reaction, and 40% of the eluate was used for PCR amplification. PCR products were purified in a 1% agarose gel and cloned using the TA-Cloning Kit (Invitrogen). Ninety-six clones were inoculated for each analysed condition (e.g. wild-type mouse, P10) and mini-preps and sequencing were performed at the EMBL Gene Core Facility, Heidelberg. Two scripts were coded in Visual Basic for analysis of sequencing results. The first one allowed removing the sequence from the vector backbone and filtering out the clones that had no insert due to self-ligation of the vector. Next, a sequence alignment of all the positive clones for a given condition was performed using VectorNTI software (Invitrogen). The second script processed the alignment file, looking for positions where cytosines are subject to methylation and checking whether they had been changed for a thymine (indicating lack of methylation at that CpG) or they had been kept as cytosines (indicating methylation of that CpG). The output is a graphic representation of the clones analysed, where methylated CpGs are shown as filled dots and unmethylated CpGs are shown as empty dots (see Fig. 3.3).

2.3.6 - Small RNA Library Preparation for High-Throughput Sequencing

Small RNA libraries were prepared using the Digital Gene Expression for Small RNA kit (Illumina), which allows ligating RNA adaptors at both ends of the RNA of

interest in order to perform RT-PCR and obtain a cDNA library that can be analysed by deep-sequencing. For a schematic representation of the procedure followed, see Fig. 2.2A.

Starting material was RNA extracted from an IP or 10µg of total RNA. The first step was isolation of the RNAs of the desired size by purification from a 15% urea-PAGE. For that purpose, one tenth of an IP was radiolabelled as described above. For preparation of total small RNA libraries (~18-33nt) no radiolabelling was performed to prevent cloning of degradation fragments, as they contain a 5'-hydroxyl and phosphorylation would facilitate ligation to the 5' adaptor in a later step. The gel was run with two different RNA markers and exposed wet to a PhosphorImager in the presence of radioactive letters that would serve as a visual reference (see Fig. 2.2B for an example). The region of interest -which for IPs was visible and for total RNA had to be determined with the help of the markers- was excised from the gel and the acrylamide was crushed with a round pipette tip. Next, 400µl of 0.3M NaCl were added and the mixture was left overnight in agitation at 25°C. The pieces of acrylamide were removed by filtration and the RNA was extracted with phenol-chloroform and precipitated with ethanol.

All the RNA recovered from the previous step was used for ligation of the 3' adaptor with T4 RNA ligase (Fermentas). After incubation at 20°C for 6h, the reaction was stopped by addition of RNA loading dye and the samples loaded in a 15% urea-PAGE. The same procedure as described above was followed for gel-purification and subsequent ligation of the 5' adaptor. A last step of purification in 15% urea-PAGE was carried out before proceeding to reverse transcription. Superscript III Reverse Transcriptase (Invitrogen) and the RT Primer provided in the Illumina kit were used for this purpose. PCR amplification of the resulting cDNA library was performed using Phusion polymerase (NEB) and primers matching the adaptors. Annealing temperature was 60°C and the number of cycles was only 15 to reduce the bias associated to amplification. PCR products were migrated in 3.5% low-melting agarose in the cold room and bands of the correct size (90-100nt) excised using Gel-extraction kit (Qiagen).

The libraries were deep-sequenced (Solexa, Illumina) at EMBL Gene Core Facility. Bioinformatic analysis was performed in collaboration with Alexander Stark (IMP, Vienna) and Ravi Sachidanandam (MSSM, NY, USA).

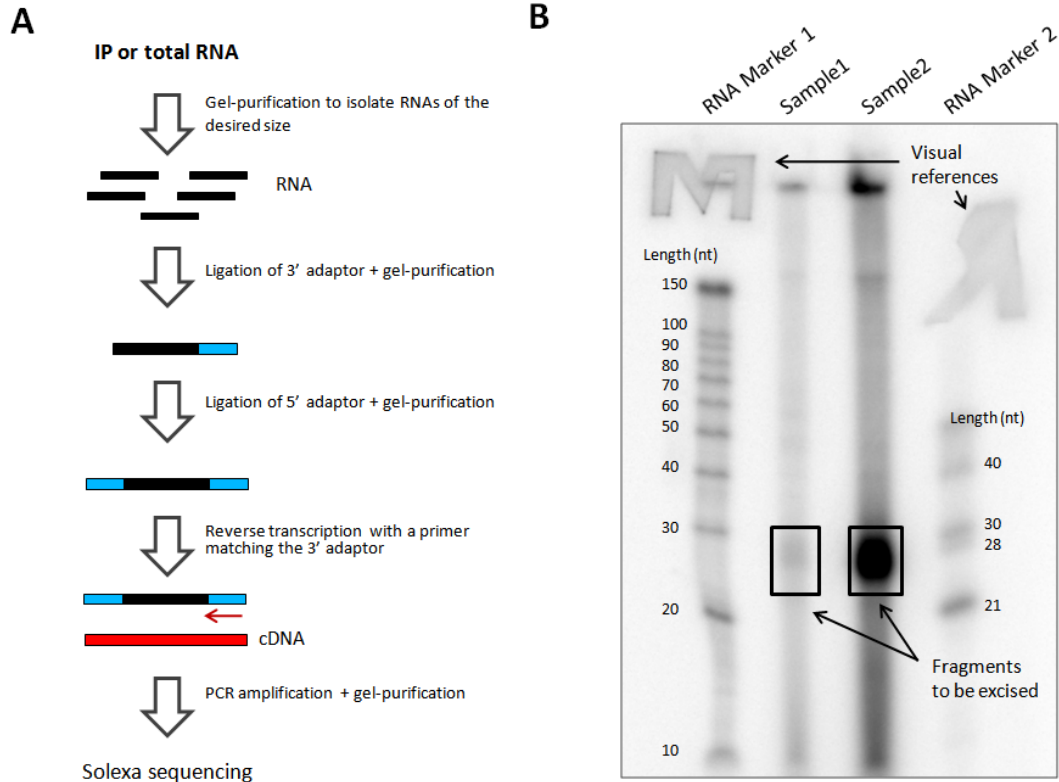


Figure 2.2: Library Preparation for High-Throughput Sequencing. (A) Workflow for preparing a small RNA library. (B) Example of a gel picture, with the visual references, for the gel-elution steps required during small RNA library preparation.

2.4 - Protein Expression and Purification

2.4.1 - Bacteria

Expression in *E. coli* was carried out in Rosetta strain (Novagen), which is optimized for usage of rare Arg, Ile, Gly, Leu and Pro codons. All the vectors used are inducible with isopropyl-beta-thio-galactoside (IPTG, Euromedex), and simultaneous usage of two vectors was possible by making use of different antibiotic resistances. Bacteria were grown in agitation at 37°C, using Luria-Bertani (LB) media and the

corresponding antibiotics. When the cell density reached an OD₆₀₀ of 0.6-0.9, expression was induced with IPTG at a final concentration of 1mM, and the cells were left shaking overnight at 20°C.

Cells were harvested by centrifugation and resuspended in 25ml of purification buffer per liter of culture. The buffer generally contained 20mM Tris pH 8, 150mM NaCl, 2mM β-mercaptoethanol and Complete –EDTA free- protease inhibitor, but some modifications were introduced depending on the protein that was purified. The concentration of NaCl was increased to 500mM for FKBP6 purifications, while Phosphate Buffered Saline (PBS) was used for GST-fusions of MOV10L1. For His-tagged versions of MOV10L1 the pH of the Tris buffer was reduced to 7.5. Resuspended bacteria were sonicated during 1min10s per liter of culture and the lysate was cleared by centrifugation.

His-tagged proteins were purified by immobilized metal ion affinity chromatography (IMAC) using Ni-NTA beads (Qiagen) or Chelating Sepharose Fast Flow Resin (GE Healthcare) charged with Ni²⁺ and equilibrated with purification buffer, which contained additional 20mM imidazole. The amount of resin used depended on the expected amount of protein, taking 5mg of protein per ml of bed volume as a reference. Lysates were run through the resin by gravity flow, and the beads were washed with 10 column volumes of purification buffer containing 50mM imidazole. The concentration of imidazole was increased to 500mM for the elution, which was monitored by Bradford Protein Assay (Biorad). The eluted fractions, as well as the input, the wash and the flow-through were analysed by 10-15% SDS-PAGE and Coomassie staining.

When indicated, the N-terminal His-tag was removed using TEV protease (common name for the 27 kDa domain of the Nuclear Inclusion a protein encoded by the tobacco etch virus, TEV). TEV protease was added in a ratio of 1mg per 100mg of eluted protein and the sample dialyzed overnight in a 6-8kDa cut-off Spectra/por dialysis bag (Spectrum Laboratories) against 2L of purification buffer to reduce the imidazole concentration. The cleaved protein was run through a second nickel column and was collected in the flow-through, as in the absence of a His-tag it no longer bound the resin.

GST-fusion proteins were purified using glutathione affinity resin (Macherey-Nagel). Bacterial lysates were incubated with equilibrated beads during 2h in agitation. The resin was collected by centrifugation and washed twice with purification buffer. Elution was performed in a column using purification buffer with 10mM glutathione. Purified proteins were dialyzed against 2L of purification buffer as described for His-tagged proteins.

2.4.2 - Insect Cells

Insect cell expression using baculovirus was performed as described (Fitzgerald *et al.* 2006). For bacmid preparation, constructs cloned in pFastBac vector were transformed into DH10 MultiBac cells, which contain a YFP gene that allows monitoring of protein expression. Transformants were plated on LB-agar containing selective antibiotics, IPTG and X-Gal for blue/white selection. After growth, white colonies were picked for isolating the bacmid, which was transfected into Sf21 cells using FuGene (Roche). Viral particles in the media were collected after two days and amplified by infecting increasing volumes of Sf21 cells grown in suspension. Protein expression was performed in 100ml cultures as a starting volume and different viral concentrations were tried for optimization. A small sample was collected every day to measure YFP fluorescence and the cells were harvested at the peak of YFP expression. For solubility tests, cell pellets were resuspended in PBS, sonicated and the soluble and insoluble fractions loaded in a 10% SDS-PAGE. Purification trials of His-tagged proteins were performed as described for bacterial expression.

2.4.3 - Mammalian Cells

HEK293T cells were grown in roller bottles for expression of FLAG-tagged versions of MOV10L1. Transfection was performed using 1mg polyethylenimine (PEI, Sigma) and 0.5mg DNA per roller bottle. Cells were harvested four days after transfection and collected by centrifugation in 50ml Falcon tubes. Pellets were washed in PBS and resuspended in a buffer containing 50mM Tris pH 8, 150mM NaCl and protease inhibitors. Cells were lysed by sonication and the protein was

purified using anti-FLAG M2 Affinity Gel (Sigma) following the manufacturer's instructions. Elution was carried out with 3X FLAG Peptide (Sigma), which competes with the tagged protein for binding to the resin.

2.5 - Biochemical Methods

2.5.1 - Pull-down Assays

Interaction mappings between recombinant and *in vitro* translated proteins were performed using pull-down assays. *In vitro* translation was carried out with TnT Coupled Reticulocyte Lysate System (Promega), which allows coupling of transcription and translation in the same reaction and protein labelling with ³⁵S-Methionine (Perkin-Elmer). 100µg of recombinant protein (expressed in *E.coli* with either GST or His-tag and purified as described above) were incubated for 1h with one fifth of an *in vitro* translation reaction in a buffer containing 20mM Tris pH 8, 300mM NaCl, 0.01% NP-40 and 20mM imidazole. The same buffer was used for washing and equilibrating 25µl of Ni-NTA resin (even for GST-fusion proteins, as they also contain a His-tag in their N-terminus). The beads were blocked with 1% BSA for 30min and incubated with the previously mixed proteins for 2 hours. After thorough washing, elution was performed in 500mM imidazole, followed by 10% SDS-PAGE. The gel was fixed in 45% methanol and 10% acetic acid, dried and exposed to a PhosphorImager screen to visualize the ³⁵S-labelled proteins.

2.5.2 - ATPase and Unwinding Assays

ATP hydrolysis reactions were performed in 20µl a buffer containing 50mM Tris pH 7.5, 100mM KCl, 5mM MgCl₂, 0,01% NP-40, 1mM DTT, 0.5mM cold ATP and 20nM [γ -³²P]ATP. Generally, the purified protein was added to a concentration of 5µM, and the mixture was incubated at 37°C for 30min or the indicated time. The reaction was stopped by addition of 4µl formic acid and 2µl were spotted on a thin layer chromatography (TLC) plate (cellulose fibers on PET foils, Fluka) and migrated in 0.5M LiCl and 0.5M formic acid in a migration chamber for 45min. Free phosphate

can be distinguished from ATP because it migrates faster in a TLC; quantification of the radioactive signals was performed using ImageQuant software (Molecular Dynamics), and the percentage of hydrolysed ATP was calculated using the following formula: % hydrolysis = intensity Pi / (intensity Pi + intensity ATP).

For unwinding assays, 10pmol of a 21nt long RNA were 5'-labelled with [γ - 32 P]ATP and pre-hybridized with 20pmol of a complementary 40nt long cold RNA in a buffer containing 10mM Tris pH 8, 100mM KCl and 2mM MgCl₂. To form the hybrid, the mixture was heated to 95°C and cooled to 37°C. Unwinding assays were performed in the presence of a 5-fold excess of a DNA oligo having the same sequence as the 32 P-labelled RNA to prevent reannealing after unwinding. Different protein concentrations were tested, ranging from 20nM to 10 μ M, and the reactions were performed at 37°C for 1h in the presence or absence of 1mM ATP. Samples were quenched on ice and stopped by adding a final concentration of 0.3% SDS, 10mM EDTA and 5% glycerol, prior to loading to a native 15% polyacrylamide gel. The radioactive bands were visualized by phosphorimaging and quantified using the ImageQuant software.

2.5.3 - *In Vitro* Methylation Assay

In vitro methylation analysis of Mili was performed using an assay that was developed for Sm proteins (Meister *et al.* 2001) with small modifications. HEK293 cells from one 6cm² plate were lysed in 100 μ l of a buffer containing 10mM Tris pH8, 20mM KCl, 1.5mM MgCl₂, 0.5mM DTT and protease inhibitors. Methylation reactions were performed in 50mM Tris pH 7.5 and 10mM EDTA, using 100 μ g of purified GST or the GST-fusion of Mili N-terminus, 4 μ l of the HEK293 cleared extract and 4 μ l of [14 C]-S-adenosyl methionine (SAM, 0.02mCi/ml, Perkin Elmer) per reaction. The reaction volume was 12 μ l and either FKBP6 or GST was added with the indicated ratios. The samples were incubated for one hour at 37°C and subsequently resolved by 10% SDS-PAGE. Visualization of the radioactive bands was performed by phosphorimaging.

2.5.4 - Prolyl Isomerisation Assay

Prolyl *cis-trans* isomerase (PPIase) activity was determined with a protease-coupled assay that measures the *cis* to *trans* isomerisation of the proline-leucine bond in the peptide N-succinyl-Ala-Leu-Pro-Phe-p-nitroanilide (pNA). The assay was performed as described (Siekierka *et al.* 1989; Shan *et al.* 1994), with small modifications. Reactions (1ml) contained 50µg of α -chymotrypsin, 50mM Tris pH 8, 100mM NaCl and 20µg of the indicated FKBP or GST. The samples were chilled on ice, and 10µl of 5mM peptide (Bachem), dissolved in trifluoroethanol containing 470mM LiCl, were added to initiate the reaction. After mixing, the increase of the absorbance at 390nm was measured at 0.5s intervals during 5 minutes. The log of the difference between absorbance at steady state and absorbance at time t was plotted against time. The first order rate constant, k (s^{-1}), is calculated from the slope of the resulting straight line.

2.5.5 - Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) experiments were carried out using a Microcalorimeter ITC200 (GE Healthcare). Proteins were dialyzed against the titration buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl). FK506 solution at 10µM (the maximum solubility in aqueous buffer) in the calorimetric cell was titrated with 100 µM FKBP12 or FKBP6. Titrations were carried out by constant volume injections (21 injections of 1.5 µl). The evolved heat after each protein injection was obtained from the integral of the calorimetric signal. The resulting binding isotherms were analyzed by non-linear least squares fitting of the experimental data to a model corresponding to a single set of sites. Analysis of the data was performed using Microcal Origin (OriginLab Corporation).

3- MOUSE MOV10L1 IS A PRIMARY piRNA BIOGENESIS FACTOR

Résumé du chapitre “*Mouse MOV10L1 Is a Primary piRNA Biogenesis Factor*”

L'étude que nous avons effectuée nous a permis de caractériser MOV10L1 en tant que premier facteur de biogenèse primaire des piRNA chez la souris. MOV10L1 avait été identifiée dans des complexes de protéines PIWI par trois groupes différents, y compris le notre (Chen *et al.* 2009; Reuter *et al.* 2009; Vagin *et al.* 2009). Nous avons montré que MOV10L1 interagit avec les protéines PIWI et les piRNA *in vivo*, et des techniques de séquençage haut-débit ont révélé que les piRNA associés à MOV10L1 couvrent tout le spectre des piRNAs murins.

Nous avons observé que la disruption génétique de MOV10L1, comme celle des protéines PIWI, cause un arrêt de la spermatogenèse qui a pour conséquences la stérilité des mâles mutants. Les piRNA ne sont pas produits dans les souris *Mov10l1*^{-/-} et Miwi2 (une des protéines PIWI) ne parvient plus à entrer dans le noyau. Par conséquent il y a une perte de la méthylation d'ADN au niveau des promoteurs de certains transposons, ce qui mène à leur activation. Ces résultats indiquent que MOV10L1 est un facteur indispensable pour la biogenèse des piRNA. Nous avons essayé de réaliser des essais biochimiques *in vitro* pour prouver que MOV10L1 est une hélicase d'ARN et pour déterminer quel type d'ARN elle lie, mais nous n'avons pas réussi à produire de la protéine recombinante à cause de problèmes de solubilité. Des études postérieures à la notre ont montré que la fonction de MOV10L1 est conservée chez la *Drosophila* (Olivieri *et al.* 2010; Saito *et al.* 2010), où l'orthologue de MOV10L1, Armi, s'associe à des molécules qui pourraient être des précurseurs des piRNA. Nous pensons que la fonction de MOV10L1 pourrait être de tenir les précurseurs pour faciliter leur maturation par des nucléases et peut-être aussi leur chargement dans les protéines PIWI.

3.1-Article: Mouse MOV10L1 associates with Piwi proteins and is an essential component of the Piwi-interacting RNA (piRNA) pathway

3.1.1 - Contribution to the published article

The following article was a collaboration between the Pillai and Wang laboratories in which the Pillai lab contributed with its expertise in the small RNA field while the Wang lab participated with mouse genetics. The aim of this note is to point out the experiments that were performed by us and can therefore be considered as part of the thesis. These include antibody production, IP, small RNA sequencing and interaction experiments in HEK293 cells (Fig. 1, Fig. S2). *Mov10l1* mutant mouse was generated in the Wang's lab and we performed transposon activation analysis by Northern and Southern blot (Fig. 3), as well as Mili and Miwi2 IPs from P0, P10 and P14, and total small RNA sequencing (Fig. 4). We also carried out some additional experiments that are described in the section "Additional results" (page 79).

Mouse MOV10L1 associates with Piwi proteins and is an essential component of the Piwi-interacting RNA (piRNA) pathway

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Piwi-interacting RNAs (piRNAs) are essential for silencing of transposable elements in the germline, but their biogenesis is poorly understood. Here we demonstrate that MOV10L1, a germ cell-specific putative RNA helicase, is associated with Piwi proteins. Genetic disruption of the MOV10L1 RNA helicase domain in mice renders both MILI and MIWI2 devoid of piRNAs. Absence of a functional piRNA pathway in *Mov10l1* mutant testes causes loss of DNA methylation and subsequent derepression of retrotransposons in germ cells. The *Mov10l1* mutant males are sterile owing to complete meiotic arrest. This mouse mutant expresses Piwi proteins but lacks piRNAs, suggesting that MOV10L1 is required for piRNA biogenesis and/or loading to Piwi proteins.

meiosis | RNA helicase | transposon silencing

The Piwi clade of Argonaute proteins associates with a class of 26–31-nt germline-specific small RNAs called “piRNAs”. Together they participate in suppression of transposable elements in all animals studied (1–4). In mice, the Piwi clade contains three members: *Miwi2*, *Mili*, and *Miwi*. These three Piwi members exhibit distinct developmental expression patterns. *Miwi2* is expressed in perinatal male germ cells (5), whereas *Mili* is more broadly expressed from embryonic germ cells to postnatal round spermatids (6). *Miwi* expression begins in pachytene spermatocytes and persists in haploid round spermatids (7). The overlapping temporal expression of *Mili* with *Miwi* and *Miwi2* points to the pivotal role of MILI in the piRNA pathway, as further supported by the fact that MILI is associated with developmental stage-dependent pools of piRNAs: prenatal, prepachytene, and pachytene piRNAs (5, 8, 9).

The mechanisms of piRNA biogenesis are largely unclear (1–4). One feature of piRNAs in all species is their highly clustered genomic origins. Several of these clusters produce piRNAs only from one strand. This leads to a hypothesized primary processing pathway whereby an unknown nuclease cleaves off mature piRNAs from a long single-stranded precursor transcript. On the other hand, some piRNAs in prenatal and prepachytene pools display signatures indicative of a proposed RNA-mediated amplification loop that uses primary piRNAs to generate secondary piRNAs from precursor transcripts (ping-pong mechanism) (10, 11). Apart from the Piwi proteins themselves, factors directly impacting piRNA production are unknown.

We previously identified *Mov10l1* as a gene specifically expressed in mouse germ cells, which encodes a putative RNA helicase of unknown function (12). Whereas the N-terminal half of MOV10L1 is not homologous to any other mouse proteins, its C-terminal RNA helicase domain exhibits low homology (45% amino acid identity) with MOV10, the vertebrate homolog of *Drosophila* *Armi*, is ubiquitously expressed. In mammalian cells, MOV10 is associated with Argonaute proteins in the RNA-induced silencing complex (RISC) and is functionally re-

quired for RNA interference (13, 14). Here we demonstrate that MOV10L1 is an essential factor in the piRNA pathway.

Results

MOV10L1 Is Associated with Piwi Proteins. To identify potential interaction partners, we isolated MOV10L1-containing protein complexes from testicular extracts by immunoprecipitation. Mass spectrometry analyses of three specific protein bands in the MOV10L1 complex revealed that they corresponded to MOV10L1/TDRD1, MILI, and MIWI (Fig. S1). We and others have also found MOV10L1 in immunoprecipitated MILI, MIWI, and MIWI2 complexes by mass spectrometry (15, 16). Consistent with the mass spectrometry data, coimmunoprecipitations followed by Western blot analysis showed abundant association of MOV10L1 with MILI but less with TDRD1 and MIWI (Fig. 1*A* and *B*). Further coimmunoprecipitation experiments done with cotransfected human 293T cells strongly suggested that MOV10L1 binds to MILI, MIWI, and MIWI2 (Fig. 1*C*), because mammalian somatic cells lack piRNA pathway components.

To determine whether MOV10L1 is associated with piRNAs, we immunoprecipitated MOV10L1, MILI, and MIWI from adult mouse testes and assessed the presence of any associated small RNAs (Fig. 1*D*). As expected, MILI and MIWI were associated with ~26-nt and ~30-nt small RNAs, respectively. Consistent with its interaction with Piwi proteins, the MOV10L1 purification revealed the presence of small RNAs in the 26–30-nt size range, with a majority migrating similar to MILI-associated piRNAs (Fig. 1*D*). It is very likely that MOV10L1 is associated with piRNAs indirectly through its interaction with Piwi proteins.

We then performed Solexa deep sequencing of a small RNA library from MOV10L1 complexes isolated from adult testes. A total of ~2.2 million reads perfectly mapped to the genome and peaked around 26 to 27 nt (Fig. 1*E*), similar to those in small RNA libraries prepared from MILI or its interacting partner, TDRD1 (17). A notable fraction (~8%) of MOV10L1 reads was 29–32 nt in length, a size similar to MIWI-associated piRNAs (18, 19). The MOV10L1 reads display a strong preference (~80%) for a uridine at position one (U1-bias). Genome annotation of the MOV10L1

Author contributions: K.Z., J.X., R.S.P., and P.J.W. designed research; K.Z., J.X., M.R., S.E., N.A.L., and K.J.M. performed research; A.S. and R.S. analyzed data; and R.S.P. and P.J.W. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: Solexa deep-sequencing data have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE21763).

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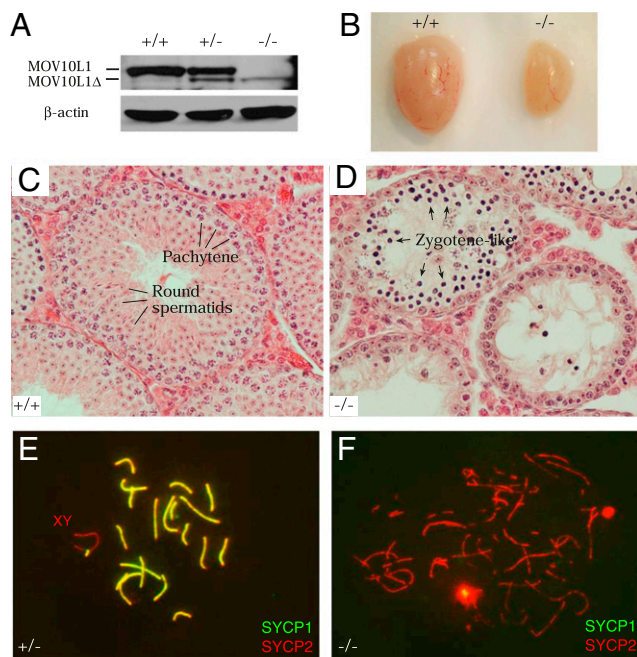


Fig. 2. *Mov10l1* is essential for spermatogenesis and chromosomal synapsis. (A) Western blot analysis of adult wild-type, *Mov10l1*^{+/−}, and *Mov10l1*^{−/−} testes. The mutant MOV10L1 protein (1,061 aa; MOV10L1Δ) is indicated. (B) Dramatic size reduction in 7-wk-old *Mov10l1*^{−/−} testes. (C and D) In contrast to wild-type tubules with full spermatogenesis (C), *Mov10l1*^{−/−} tubules from adult mice exhibited early meiotic arrest (D). (E) Normal pachytene spermatocyte with 19 pairs of fully synapsed autosomes and the partially synapsed sex chromosomes. (F) Zygotene-like spermatocytes from *Mov10l1*^{−/−} adult testes.

Mov10l1^{−/−} females displayed normal fertility, but *Mov10l1*^{−/−} males were sterile. Disruption of *Mov10l1* caused a sharp reduction in testis size (Fig. 2B). The weight of *Mov10l1*^{−/−} testes (48.3 ± 9.1 mg per pair) from 5- to 7-wk-old mice was <37% that of *Mov10l1*^{+/−} testes (131.1 ± 6.9 mg per pair) (Student's *t* test, $P < 0.0001$). In contrast to wild-type seminiferous tubules (Fig. 2C), tubules from adult *Mov10l1*^{−/−} testes contained only early spermatogenic cells, with a complete lack of postmeiotic germ cells (Fig. 2D). The most advanced germ cells in the mutant were zygotene-like spermatocytes, indicating an early meiotic arrest. The stage of meiotic arrest in *Mov10l1*^{−/−} mice is similar to that in *Mili* and *Miw2* mutant mice (6, 26). Thus, MOV10L1 is required for male meiosis and is essential for male fertility.

To further define the meiotic defects in *Mov10l1*^{−/−} testes, we analyzed the assembly of the synaptonemal complex by immunostaining central and axial elements with anti-SYCP1 and anti-SYCP2 antibodies. In *Mov10l1*^{+/−} spermatocytes, all chromosomes were fully synapsed except the XY chromosomes, which were only synapsed at the pseudoautosomal region (Fig. 2E). In *Mov10l1*^{−/−} spermatocytes, synapsis failed to occur, evident from the absence of SYCP1 (Fig. 2F). Thus, disruption of *Mov10l1* causes meiotic blockade before the pachytene stage.

MILI and TDRD1 Are Lost in *Mov10l1*^{−/−} Spermatocytes but Retained in Spermatogonia. We next investigated the consequence of loss of MOV10L1 function on the expression of piRNA pathway components. Normally, MILI and TDRD1 are expressed in both spermatogonia and spermatocytes (Fig. S5). However, both MILI and TDRD1 were detectable in spermatogonia but not in spermatocytes in *Mov10l1*^{−/−} testis (Fig. S5B and D). In contrast, MVH, MAEL, and GASZ were still expressed in both spermatogonia and spermatocytes in *Mov10l1*^{−/−} testis (Fig. S5F) (22, 27). The expression pattern of MILI and TDRD1 in P14 *Mov10l1*^{−/−} testes was the same as in the adult (P60) mutant testes. Thus, disruption

of MOV10L1 impacted the abundance of MILI and TDRD1 most dramatically. Decreased abundance of nuage proteins has also been observed in *Gasz* mouse mutant and a number of *Drosophila* nuage mutants (22, 28, 29).

Binary Derepression of LINE1 and IAP Retrotransposons in Postnatal *Mov10l1*^{−/−} Testes. piRNAs are required for silencing of transposable elements in the germline in various species (1, 2, 30). We examined the expression of LINE1 and IAP retrotransposons in *Mov10l1*^{−/−} testes. P10 testes contain predominantly spermatogonia and also preleptotene and leptotene spermatocytes. P14 testes also contain more advanced spermatocytes, such as zygotene and pachytene cells. Quantitative RT-PCR analyses showed that the abundance of both LINE1 and IAP transcripts increased sharply in *Mov10l1*^{−/−} testes (Fig. 3A). We confirmed these findings by Northern blot analyses (Fig. 3B). Western blot analyses showed that LINE1 ORF1p abundance increased significantly in P10 *Mov10l1*^{−/−} testes and more dramatically in P14 mutant testes (Fig. 3C). IAP protein abundance increased modestly in P10 and P14 *Mov10l1*^{−/−} testes (Fig. 3C). Thus, disruption of *Mov10l1* results in derepression of LINE1 and IAP retrotransposons.

We next identified the cell types that express retrotransposon-encoded proteins by immunofluorescence analyses on adult testis sections (P60), in which spermatogonia and spermatocytes can be unequivocally identified. In wild-type tubules, LINE1 and IAP were barely detectable (Fig. 3D). *Mov10l1*^{−/−} testes, however, exhibited highly abundant expression of LINE1 in spermatocytes but not spermatogonia (Fig. 3E). In contrast, IAP protein was readily visualized in spermatogonia but not in spermatocytes of mutant testes (Fig. 3F). This binary derepression pattern of LINE1 and IAP was also observed in P14 *Mov10l1*^{−/−} testes. These data suggest that these two classes of retrotransposons (LINE1 and IAP) are silenced in a MOV10L1-dependent manner but are regulated differently in spermatogonia and spermatocytes.

Cytosine DNA methylation of retrotransposon regulatory regions in mouse causes transcriptional silencing (31). Methylation-sensitive Southern blotting showed that demethylation of LINE1 elements was discernible in *Mov10l1*^{−/−} testes at P10 and clearly detectable in P14 mutant testes (Fig. 3G). Quantification of LINE1 demethylation by bisulfite sequencing showed methylation of 84% and 85% of CpGs in P10 and P14 *Mov10l1*^{+/−} testes, respectively. Consistent with our Southern result, 72% of LINE1 CpGs were methylated in P10 *Mov10l1*^{−/−} testes, but only 54% were methylated in P14 mutant testes. These data showed that disruption of *Mov10l1* results in loss of DNA methylation and thus derepression of LINE1 and IAP retrotransposons.

MILI Is Depleted of piRNAs in Postnatal *Mov10l1*^{−/−} Testes. Given the association of MOV10L1 with the MILI piRNA ribonucleoprotein particles (piRNPs), we examined the impact of loss of *Mov10l1* on piRNA populations. In testes from P10 and P14 mice, MILI binds to prepachytene and pachytene piRNAs, respectively (8, 20). Although the abundance of MILI was reduced in *Mov10l1*^{−/−} testes, a substantial amount of MILI was immunoprecipitated from P10 or P14 mutant testes but was found to be devoid of piRNAs in *Mov10l1*^{−/−} testes (Fig. 4A). To rule out the possibility that the observed loss of MILI-bound piRNAs is due to the detection limit of immunoprecipitation and 5'-end radiolabeling assay, we repeated the experiment and, in parallel, performed immunoprecipitations with serial dilutions of P10 wild-type testis extract. MILI-associated piRNAs were readily detected in wild-type controls, even when MILI protein was not detectable by Western blotting (Fig. 4B, lanes 5 and 6), demonstrating the high sensitivity of this method. In conclusion, MILI is unloaded in the P10 and P14 *Mov10l1*^{−/−} testes.

It is possible that, in *Mov10l1* mutant testes, piRNAs are produced but fail to get incorporated into MILI. To test this, we prepared and sequenced 18–32-nt total small RNA libraries from

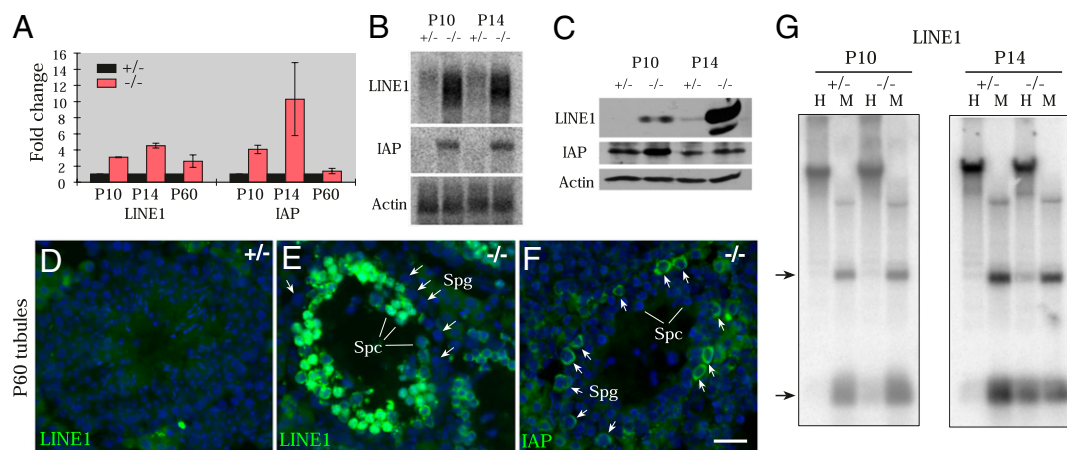


Fig. 3. Binary derepression of LINE1 and IAP retrotransposons in mitotic vs. meiotic germ cells. (A) Quantitative RT-PCR analysis. (B) Northern blot analysis. (C) Western blotting analysis. (D and E) Immunofluorescence analysis of LINE1 ORF1p in 2-mo-old (P60) *Mov10l1*^{+/+} and *Mov10l1*^{-/-} testes. (F) Immunofluorescence analysis of IAP in *Mov10l1*^{-/-} testes. Spc, spermatocytes; Spg, spermatogonia. (G) Methylation-sensitive Southern blot analysis of testis genomic DNA. Genomic DNA was digested with methylation sensitive (HpaII, H) and methylation insensitive (MspI, M) enzymes. Arrows indicate the position of methylation-sensitive (H) restriction products in the *Mov10l1*^{-/-} testes. (Scale bar in F, 25 μ m for D–F.)

testes of P10 *Mov10l1*^{+/+} and *Mov10l1*^{-/-} mice. Analysis of read-lengths (Fig. 4C) revealed a loss of \approx 26–28-nt sequences (presumably piRNAs) in *Mov10l1*^{-/-} testes. The \approx 21-nt reads, the bulk of which are annotated miRNAs, are still present in the *Mov10l1*^{-/-} testes (Fig. 4C). These data demonstrate that MOV10L1 is required for biogenesis of piRNAs but not miRNAs.

Both MILI and MIWI2 Are Depleted of piRNAs in *Mov10l1*^{-/-} Perinatal Germ Cells. Given that MOV10L1 is expressed in perinatal gonocytes (Fig. 1F), we wondered whether the piRNA pathway is affected in the early germ cells in *Mov10l1* mutant. We first examined the expression of MILI and MIWI2 in perinatal *Mov10l1*^{-/-} germ cells. MIWI2 localized predominantly to the nucleus in P0 *Mov10l1*^{+/+} gonocytes, but it was excluded from the nuclei and accumulated in the cytoplasm of *Mov10l1*^{-/-} gonocytes (Fig. 4D). A nuclear-to-cytoplasmic redistribution of MIWI2 was also observed in both *Mili* and *Tdrd1* mouse mutants (5, 16, 17). The cytoplasmic localization of MILI was maintained in *Mov10l1*^{-/-} gonocytes (Fig. 4D). Furthermore, LINE1 elements were strongly activated, and IAP was moderately derepressed in *Mov10l1*^{-/-} gonocytes (Fig. S6). These data strongly indicate that the piRNA pathway is defective in *Mov10l1*^{-/-} perinatal gonocytes.

In embryonic germ cells, MILI and MIWI2 are associated with repeat-rich prenatal piRNAs (5, 9). To ascertain the piRNA association status of MILI and MIWI2 in *Mov10l1*^{-/-} perinatal germ cells, we immunoprecipitated both proteins from P0 testes. In testes from *Mov10l1*^{+/+} pups, MILI and MIWI2 were loaded with their respective \approx 26- and \approx 28-nt small RNAs. In contrast, both MILI and MIWI2 were completely devoid of piRNAs in *Mov10l1*^{-/-} testes (Fig. 4E). Taken together with our findings in P10 and P14 mutant testes, we conclude that MOV10L1 is required for biogenesis and/or stability of both perinatal (MILI- or MIWI2-bound) and prepachytene (MILI-bound) piRNAs.

Discussion

Here we report a mouse mutant in which MILI-associated piRNAs are absent, whereas the MILI protein is still detectable. Our studies point to a central role for MOV10L1 in the biogenesis and/or stability of MILI-, MIWI2-, and possibly MIWI-bound piRNAs. First, we have demonstrated an essential role for MOV10L1 in the biogenesis of MILI-bound piRNAs in both perinatal (P0) and prepachytene (P10) stages. Second, MOV10L1 may also be directly required for the loading of presumably secondary piRNAs onto MIWI2 in perinatal gonocytes, because these two proteins bind to

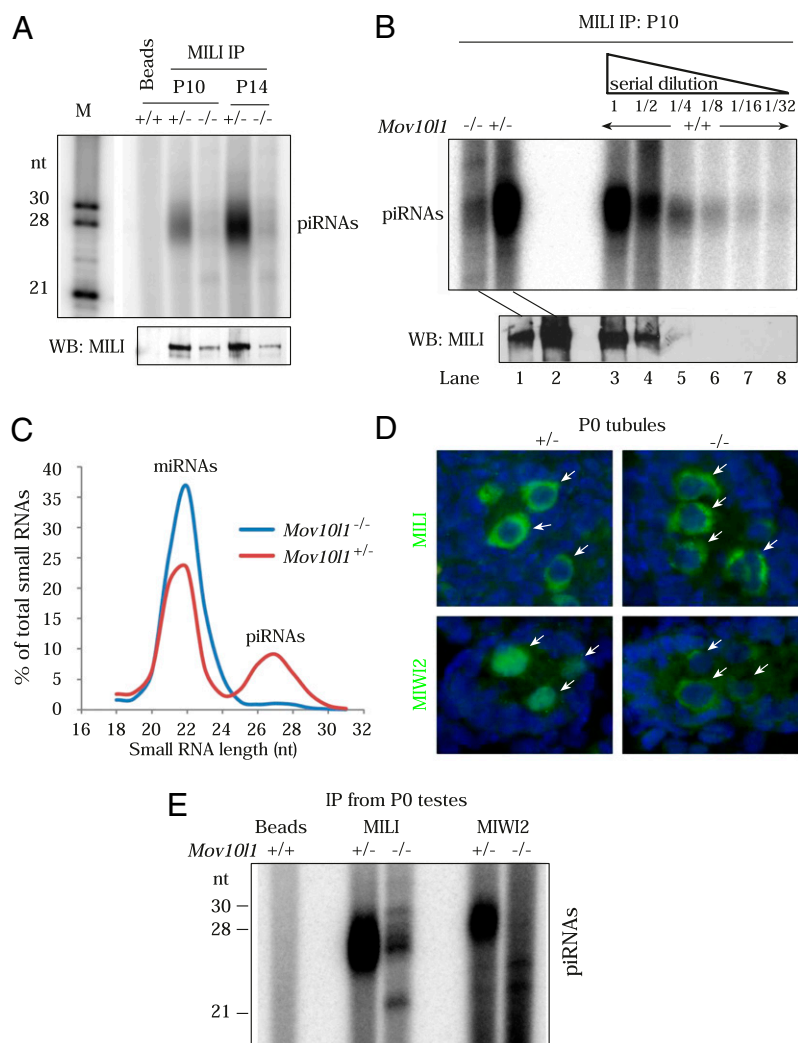
each other and MIWI2 is unloaded in the *Mov10l1* mutant. Alternatively, in the *Mov10l1* mutant, the “empty” MILI (lack of bound primary piRNAs) may fail to guide the biogenesis of secondary piRNAs that would bind to MIWI2. Such a possibility is further supported by the unloaded status of MIWI2 in the *Mili* mutant (5). Finally, the fact that MOV10L1 is associated with both MILI and MIWI in adult mouse testes also implicates it in the pachytene piRNA biogenesis. However, this is not readily testable, because disruption of *Mov10l1* causes meiotic block before the pachytene stage.

MOV10L1 exhibits limited homology with MOV10, which is the vertebrate homolog of *Drosophila* Armi. Armi is a sequence homolog of SDE3, which is required for posttranscriptional gene silencing in *Arabidopsis* (32). Armi is required for RISC maturation and *oskar* mRNA silencing (33, 34). Armi is also important for piRNA biogenesis and silencing of the *Stellate* repeat locus and retrotransposons (28, 35). Therefore, Armi functions in both RNAi and piRNA pathways in *Drosophila*. Our data demonstrate that the vertebrate-specific MOV10L1 is specialized in the piRNA pathway.

Members of the RNA helicase superfamily are required for all biological processes involving RNA molecules, such as ribosome biogenesis, splicing, translation, and RNA interference (36). MOV10L1 is a putative RNA helicase that contains all of the conserved helicase motifs, including ATPase and unwindase domains. Our findings that the truncated mutant protein lacks only the RNA helicase domain and is defective in piRNA biogenesis demonstrate that this domain is required for MOV10L1 functions. Biochemical studies of *Drosophila* Armi suggest that Armi facilitates ATP-dependent incorporation of single-stranded siRNA into RISC (34). Analogous to the role of Armi in RISC maturation, MOV10L1 may facilitate loading of piRNAs into piRNP complexes. One explanation for the loss of 25–32-nt small RNAs (piRNAs) in the total small RNA library from P10 *Mov10l1* mutant testes is that piRNAs are generated but fail to incorporate into MILI and thus are degraded. Alternatively, MOV10L1 might be required for the primary processing in the biogenesis of piRNAs.

Materials and Methods

Antibodies, Western Blot Analyses, and DNA Constructs. Two GST-MOV10L1 (amino acids 1–101 and 53–253) fusion proteins were expressed in *Escherichia coli*. Purified recombinant proteins were used to immunize rabbits. Other antibodies used were MILI (Abcam), MIWI (Abcam), MIWI2 (J. Martinez and G. Hannon), TDRD1, RNF17, MVH (T. Noce), LINE1 ORF1p (S. L. Martin), IAP



analyses: MOV10L1 (≈ 2.2 million), *Mov10l1*^{-/-} ($\approx 500,000$), and *Mov10l1*^{+/-} ($\approx 125,000$). Small RNA sequencing data used in this study are deposited in the GEO database under the accession number GSE21763.

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Houston) for GASZ antibody, T. Noce (Shiga University of Medical Science, Shiga, Japan) for MVH antibody, and C. Höög (Karolinska Institutet, Stockholm) for SYCP1 antibody; we also thank C. Yuan for mass spectrometry and Stephanie Eckhardt for imaging analysis; the European Molecular Biology Laboratory (EMBL) Protein Expression and Gene Core facilities for antibody production and Solexa sequencing; and D. O'Carroll, F. Yang, A. Verdel, and members of the Pillai group for comments on the manuscript. This work was supported by EMBL (R.S.P.), Agence National de la Recherche Jeune Chercheur program (piRmachines) (R.S.P.), and National Institutes of Health/National Institute of General Medical Sciences Grant RO1GM076327 (to P.J.W.).

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Supporting Information

Zheng et al. 10.1073/pnas.1003953107

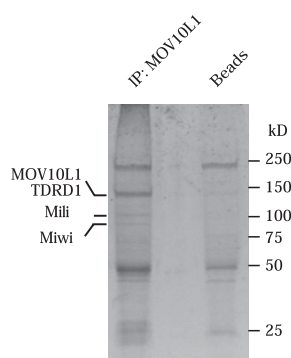


Fig. S1. Purification and characterization of MOV10L1-associated proteins from 18- to 20-d-old testes by immunoprecipitation (IP) and mass spectrometry. Protein identity for each band is indicated. In brief, to isolate MOV10L1-associated proteins, eight pairs of 18- to 20-d testes (≈ 300 mg) were homogenized in 3 mL radioimmunoprecipitation assay buffer (RIPA) in the presence of proteinase inhibitor mixture. Protein lysate was centrifuged twice. Supernatants were precleared with protein A agarose beads. Precleared lysate incubated with affinity-purified anti-MOV10L1 antibody, followed by binding to protein A agarose beads. Immunoprecipitated proteins were extensively washed, run on a 4–15% gradient SDS/PAGE gel, and stained with SYPRO Ruby (Bio-Rad).

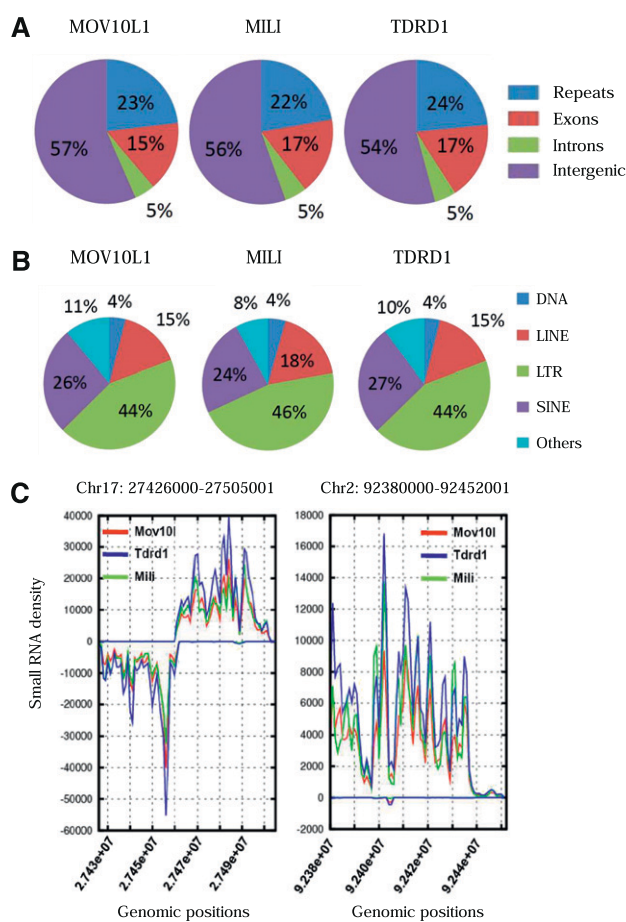


Fig. S2. Profiles of small RNA reads from MOV10L1, MILI, and TDRD1 libraries. (A) Annotation profile of MOV10L1 reads resembles those in MILI and TDRD1 libraries. (B) Pie charts showing representation of repeat piRNAs belonging to different transposon classes in indicated libraries. (C) Common genomic origins of MOV10L1-, MILI-, and TDRD1-associated piRNAs. All three libraries have similar read-depths (≈ 2 million). The Piwi-interacting RNA (piRNA) density for the top two pachytene clusters was plotted. The chromosome 17 (Chr17) cluster is ≈ 79 kb in length and bidirectional, with piRNAs arising from both strands in a nonoverlapping manner, whereas the Chr2 cluster is ≈ 72 kb and unidirectional, with most piRNAs deriving from the top strand.

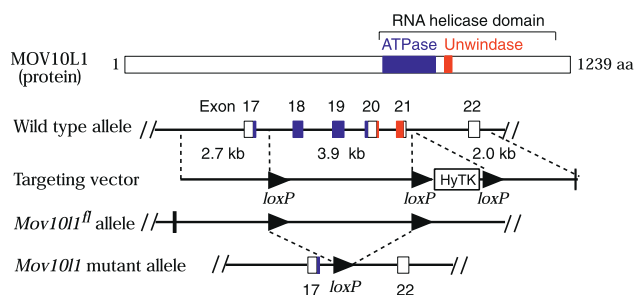


Fig. S3. Targeted inactivation of the *Mov10l1* gene. *Top*: Illustration of the predicted RNA helicase domain of mouse MOV10L1 protein. The mouse *Mov10l1* gene consists of 27 exons and spans a >72-kb genomic region. This deletion removes the ATPase and unwindase domains, which are highly conserved among RNA helicases.

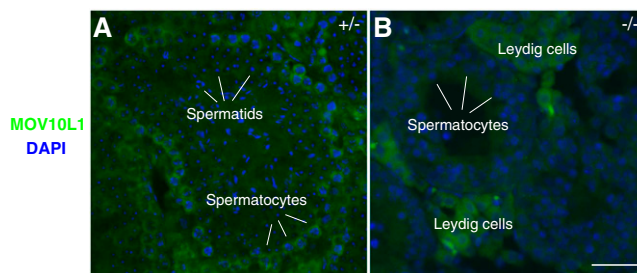


Fig. S4. Immunofluorescence analyses of MOV10L1 in testes from adult *Mov10l1*^{+/-} (A) and *Mov10l1*^{-/-} (B) mice. Leydig cells give strong autofluorescence. In *Mov10l1*^{-/-} spermatocytes (B) the internally deleted mutant MOV10L1 protein was barely detectable by immunofluorescence analysis, owing to its sharply reduced abundance. (Scale bar, 25 μ m.)

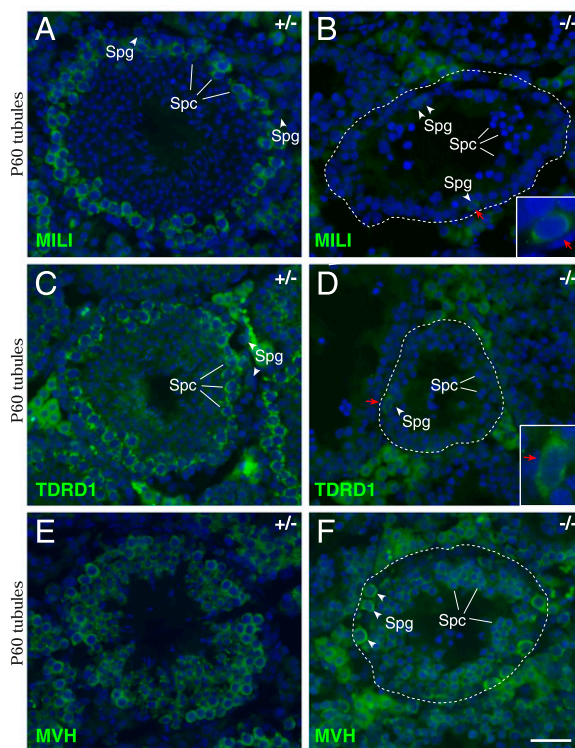


Fig. S5. MILI and TDRD1 persist in spermatogonia but not in spermatocytes in *Mov10l*^{+/-} testes. Frozen testis sections from 2-mo-old (P60) *Mov10l*^{+/-} (A, C, and E) and *Mov10l*^{-/-} (B, D, and F) were immunostained with anti-MILI, anti-TDRD1, and anti-MVH antibodies. Chromatin was stained with DAPI. *Mov10l*^{-/-} seminiferous tubules are demarcated with dash lines. *Insets* (B and D): Enlarged view of indicated spermatogonia. Spg, spermatogonia; Spc, spermatocytes. (Scale bar, 25 μ m.)

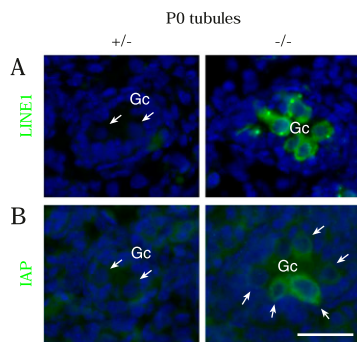


Fig. S6. Derepression of LINE1 and IAP retrotransposons in *Mov10l1^{-/-}* perinatal gonocytes. Newborn (P0) testis sections were immunostained with anti-L1 ORF1p and anti-IAP antibodies. Gonocytes (Gc) are indicated by arrows. (Scale bar, 25 μ m.)

3.1.4 - Additional Results

This chapter will include data that is complementary to the results published in PNAS (Zheng, Xiol *et al.* 2010), but that was not included in the final manuscript mostly due to lack of space.

Mouse *Mov10l1* Gene Annotation

MOV10L1 orthologues in different vertebrates were identified by BLAST search, and sequence alignment revealed a high level of homology (Fig. 3.1A). Surprisingly, the N-terminal part of the murine protein was shorter than that of its counterparts in other species, including rat and human. Approximately 50 aminoacids were lacking in the mouse protein, some of them being conserved among all the other vertebrates (see Fig 3.1A). In order to investigate this, we browsed the genomic location of *Mov10l1* looking for a possible open reading frame (ORF) of around 150bp upstream of the predicted start codon. A fragment coding for 52 aminoacids was found starting in a region annotated as an intron and going over the first exon, which was annotated as 5'UTR (Fig. 3.1B). The sequence coded for this region was almost identical to that of the rat protein (96% identity), suggesting this fragment might have been misannotated in the public databases. To test this, we designed primers to amplify this fragment by RT-PCR, anchoring the 3' primer in the annotated CDS. Cloning and sequencing of the resulting PCR product confirmed that this was a part of the ORF present in the mRNA, and the sequence is shown in Fig. 3.1C in a new alignment with its orthologues. The new sequence has been submitted to NCBI and the accession number is JF319145.

Interaction with PIWI Proteins

The interaction of MOV10L1 with Mili and Miwi in mouse testes extracts, tested by the Wang laboratory, has been shown in the above manuscript (Fig. 1A). Complementary studies had also been performed for this thesis and will be presented in the current section. In fact, MOV10L1 was first identified in our lab in a Mili proteomic screen (Reuter *et al.* 2009), which was the basis for the study of the protein

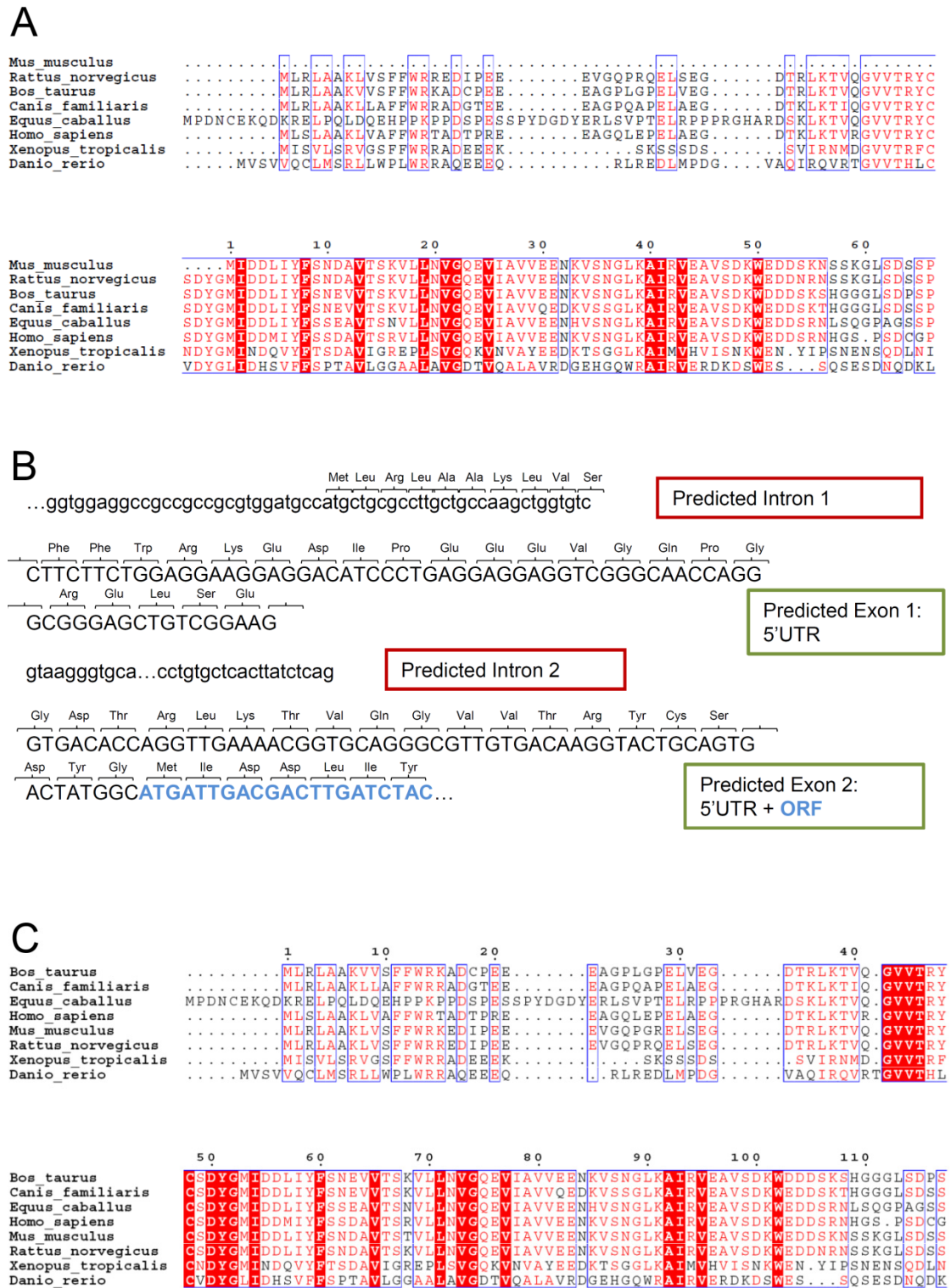


Figure 3.1: Annotation of MOV10L1 N-terminus. (A) Sequence alignment of the N-terminal part of MOV10L1 and its orthologues in other vertebrates. (B) Genomic annotation of *Mov10l1* upstream of the start codon; the amino acid sequence of the new ORF found starting in intron 1 is displayed. (C) New alignment with the additional 52 amino acids cloned for mouse MOV10L1.

in our group. Co-immunoprecipitation experiments were used to analyse the interaction between PIWI proteins and MOV10L1 in mouse testes. MOV10L1 could be pulled down by Mili and, to a lower extent, by Miwi (Fig 3.2A). Given that Miwi2 expression is restricted to embryonic testis (Aravin *et al.* 2008), it was not possible to obtain sufficient material for testing its interaction with MOV10L1. However, *Mov10l1* could be shown to be already expressed at embryonic stages by RT-PCR (Fig. 3.2B), and the expression continued into adult stages as confirmed by Western Blot (Fig. 3.2C). This expression pattern and the fact that in the *Mov10l1* mutant piRNA biogenesis is impaired as early as in the embryonic stage suggest MOV10L1 acts as a primary biogenesis factor in different stages of spermatogenesis.

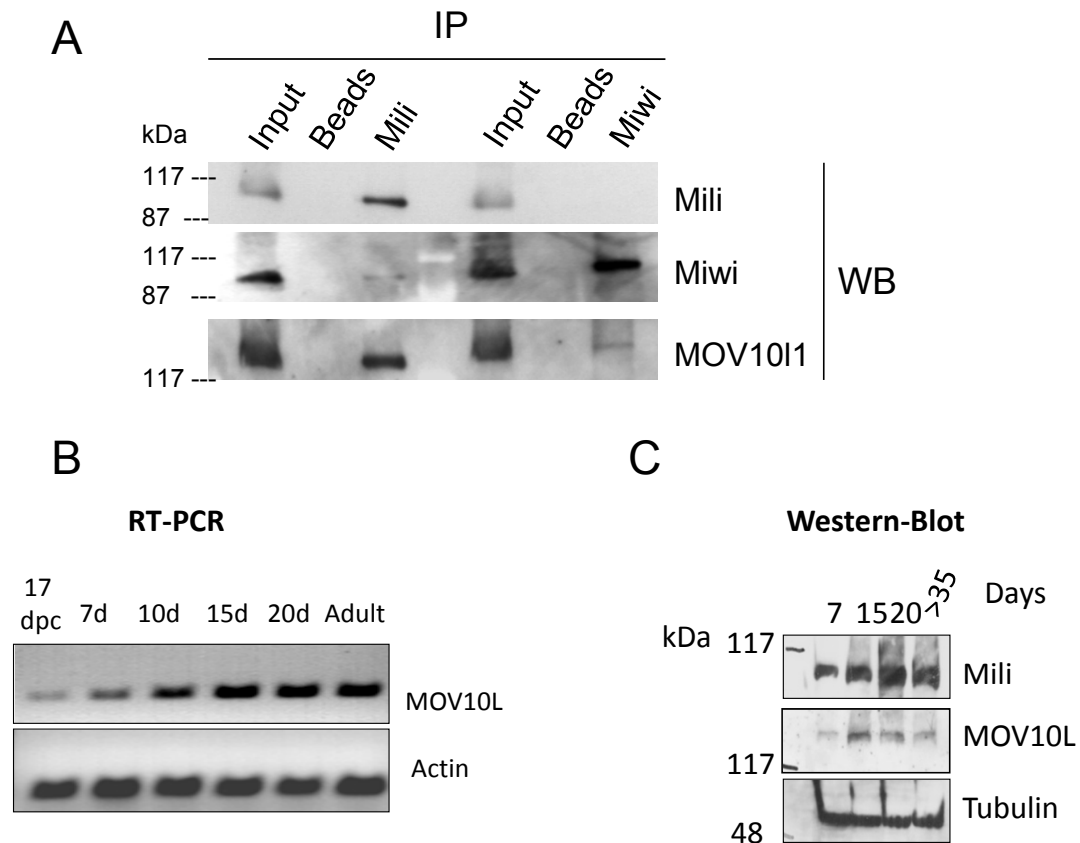


Figure 3.2: MOV10L1 Interaction with PIWI Proteins and Expression Pattern *In Vivo*. (A) MOV10L1 co-immunoprecipitates with Mili and Miwi in adult mouse testes. Empty beads were used as a control. (B) MOV10L1 is expressed at all stages as shown by RT-PCR and Western blot (C).

Bisulfite Sequencing

DNA methylation at the level of Line1 promoter in *Mov10l1*^{-/-} mice was analysed by Southern blot (see Fig. 3) and quantified by bisulfite sequencing. A reduction of methylated CpGs was observed in homozygous mutants compared to their heterozygous siblings, both at age 10 and 14 (Fig. 3.3). The loss of DNA methylation in germ cells is likely to be bigger than the percentages shown, as the sample was prepared from total testis DNA and contains therefore somatic cells, where most probably Line1 promoters remain methylated in *Mov10l1* mutants.

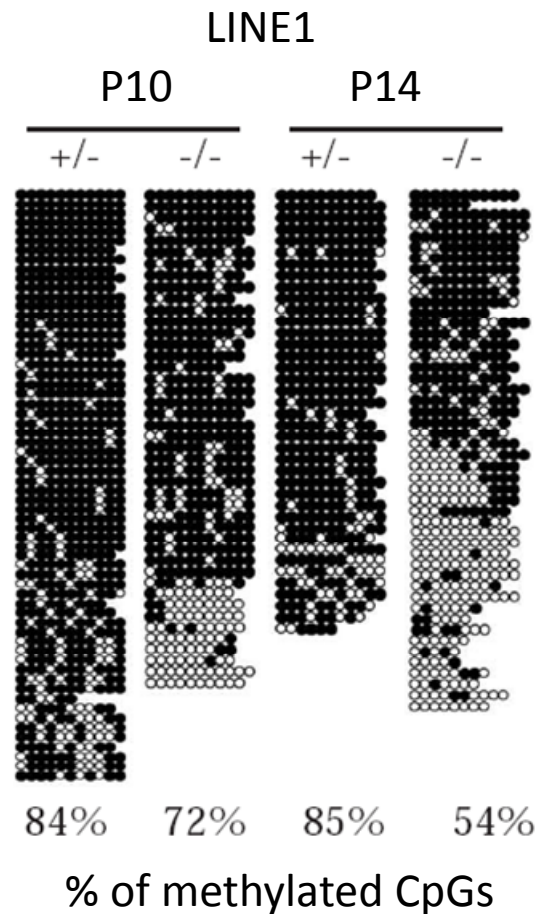


Figure 3.3: Bisulfite Sequencing of Line1 Promoters. DNA methylation is lost at the promoters of Line1 transposons in age 10 and 14 *Mov10l1* mutant mice. Methylated CpGs are represented as filled circles and unmethylated CpGs are represented as empty circles. Each of the lanes represents a sequenced clone.

3.2 - *In vitro* Studies

In order to gain mechanistic information on MOV10L1's role in the piRNA pathway, we aimed to perform a detailed biochemical study on the protein. None of the MOV10L1 orthologues (SDE3 in plants, Armi in flies) or paralogues (MOV10) has been shown to be an active RNA helicase, and the requirements of this activity for their function *in vivo* have not been described. We took an *in vitro* approach as an attempt to characterize MOV10L1 as an enzyme; such an approach would also allow to determine its preferences regarding its RNA substrates, as well as to test different hypotheses on MOV10L1's function in piRNA biogenesis at the molecular level. For instance, while Ago2 expressed in mammalian cells can be easily loaded with small RNAs *in vitro* (Rivas *et al.* 2005), several attempts to load PIWI proteins the same way have failed (Pillai lab, unpublished data). This could indicate that a PIWI-specific factor might be required for piRNA loading, and the phenotype of *Mov10l1* mutant mouse, where PIWI proteins are devoid of piRNAs, would fit with such a requirement. Recombinant MOV10L1 could therefore be tried in piRNA-loading assays, and individual domains could be tested for interaction with PIWI proteins *in vitro*.

MOV10L1's homologues in different organisms were identified by BLAST search and aligned using Clustalw. For the alignment, the different proteins were divided into four groups, corresponding to Armi, MOV10L1, MOV10 and SDE3 orthologues respectively. Based on conservation, the protein could be divided into three blocks, each of them separated by a low homology region (Fig. 3.4). The C-terminal block corresponds to the RNA helicase domain, including the DEAGQ/H motif for ATP hydrolysis, and it is highly conserved among the four groups. The other two blocks lie on the N-terminus and they were provisionally defined as DUF1 (Domain of Unknown Function 1) and DUF2. Interestingly, while DUF2 is also highly conserved among the four groups, DUF1 is only conserved within each group. All the proteins aligned are associated to Argonautes but they have different preferences for the two subfamilies: SDE3 and MOV10 for Agos (Dalmay *et al.* 2001; Meister *et al.* 2005; Chendrimada *et al.* 2007); MOV10L1 and Armi for Piwis (Frost *et al.* 2010; Olivieri *et al.* 2010; Saito *et al.* 2010; Zheng, Xiol *et al.* 2010). We

therefore speculate that DUF2 might be involved in Argonaute interaction while DUF1 might provide specificity for each of the pathways.

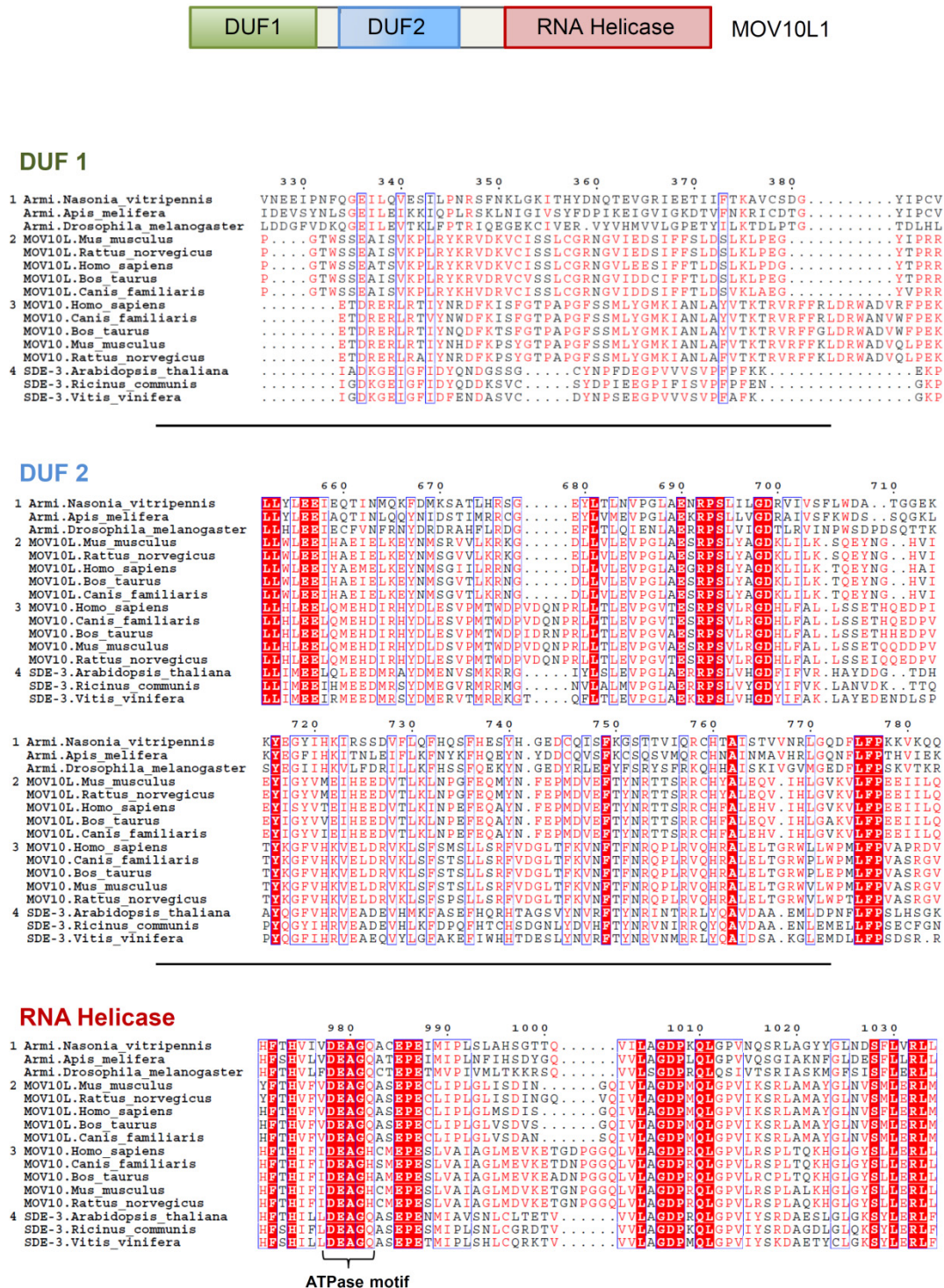


Figure 3.4: MOV10L1 Domain Organization. MOV10L1 has a C-terminal RNA helicase domain and two N-terminal domains of unknown function (DUFs) that were defined upon sequence conservation (top). Shown is a fragment of an alignment for each of the regions.

E. coli expression: GST and His-tagged versions			Insect cell expression: His-tagged versions		
Construct	Aminoacids	Region	Construct	Aminoacids	Region
1	1-106	DUF1	17	1-1239	Full-length
2	1-148	DUF1	18	1-325	DUF1
3	1-325	DUF1	19	408-672	DUF2
4	118-325	DUF1	20	408-1175	DUF2 + RNA Helicase
5	130-312	DUF1	21	566-1149	DUF2 + RNA Helicase
6	322-1155	DUF2 + RNA Helicase	Mammalian cell expression: FLAG-tagged versions		
7	404-1155	DUF2 + RNA Helicase	Construct	Aminoacids	Region
8	408-1175	DUF2 + RNA Helicase	22	1-1239	Full-length
9	408-672	DUF2	23	1-325	DUF1
10	566-1149	DUF2 + RNA Helicase	24	566-1149	DUF2 + RNA Helicase
11	566-1164	DUF2 + RNA Helicase			
12	709-1175	RNA Helicase			
13	727-1109	RNA Helicase			
14	738-1109	RNA Helicase			
15	744-1109	RNA Helicase			
16	824-1109	RNA Helicase			

Table 3.1: Constructs for Expression of Recombinant MOV10L1. Shown are the boundaries of all the constructs used in purification trials in *E.coli*, insect cells and mammalian cells.

A few constructs comprising the different MOV10L1 domains were designed for expression in *E. coli*. The boundaries were decided based on conservation and secondary structure predictions, and a summary is shown in Table 3.1. Each of the constructs was expressed both His-tagged and as a GST fusion, but overall the solubility was very low and the recombinant protein accumulated in inclusion bodies. Increasing the salt concentration or changing the buffer conditions did not improve the solubility. Only construct number 10, comprising DUF2 and the RNA helicase domain, could be purified when expressed as a GST fusion, with a yield of ~50µg/liter of culture. However, it eluted in the void volume of an S200 column in size-exclusion chromatography, indicating that it was forming soluble aggregates of at least 200kDa. Besides, a contaminant of approximately 50kDa copurified with MOV10L1 as observed by SDS-PAGE (Fig. 3.5A), and mass-spectrometry analysis of this band identified the bacterial protein chaperonin. To score for ATP hydrolysis activity, the protein was incubated with [γ -³²P]ATP followed by thin layer chromatography to

separate free phosphate, the end product of the reaction. ATPase activity was detected both in the absence and presence of RNA (Fig 3.5B). This came as a surprise because closely related helicases like UPF1 are only active in the presence of RNA (Czaplinski *et al.* 1995). To rule out the possibility that the observed activity was due to the contaminating chaperonin, a catalytically inactive mutant where the DEAGQ motif was substituted for GGAAG was expressed, but the mutation rendered the protein insoluble. Besides, we failed to show any unwinding activity of two RNAs in a native gel-based assay (Fig 3.5C). Altogether, we cannot conclude that the purified protein is active, perhaps due to the fact that it forms soluble aggregates.

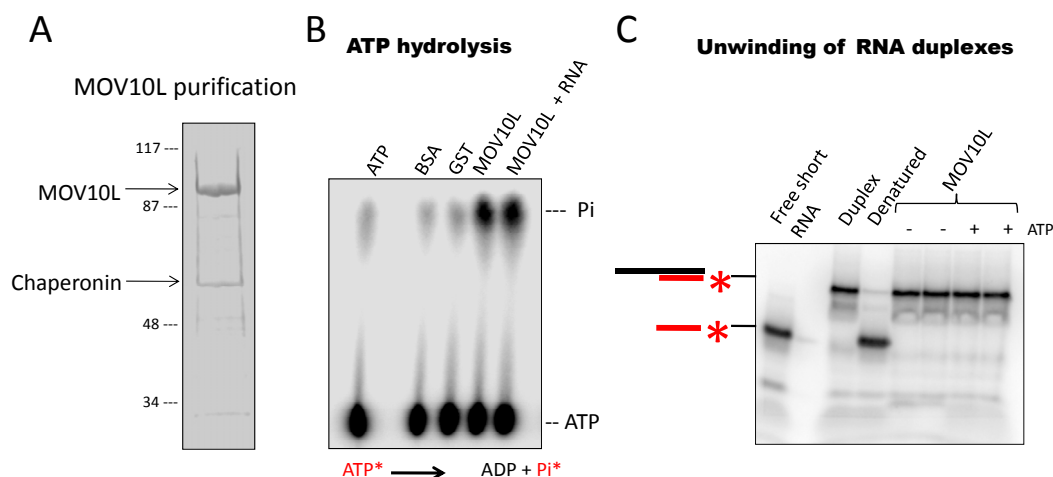


Figure 3.5: MOV10L1 Enzymatic Assays. Recombinant MOV10L1 purified from bacteria contained chaperonin as a contaminant (A). This preparation had ATPase activity irrespective of the presence of RNA (B), as shown by the increased amount of free phosphate in the lanes containing MOV10L1, but failed to unwind an RNA duplex (C). Unwinding was determined using a native gel, where a radioactively labelled RNA migrates faster when it is single-stranded than when it is hybridised with a longer RNA. The duplex was denatured at 95°C as a control that it remains single-stranded after unwinding.

The poor results obtained with expression in *E. coli* led us to move to a eukaryotic system. Full-length MOV10L1 and deletions comprising the different domains were expressed in insect cells using the baculovirus system (Table 3.1). However, none of the constructs tried yielded soluble protein, even if the protein was expressed at high levels. Different lysis methods were tried, some of them avoiding nuclear disruption and therefore preventing a hypothetical association to DNA, but none of them were successful, and nor was changing the purification conditions. We

therefore moved to a mammalian system and full-length MOV10L1 as well as the deletions shown in Table 3.1 were expressed in HEK293 cells with a FLAG tag. The same construct that had given soluble protein in bacteria (construct number 22, in this case), as well as the corresponding catalytic mutant, could be purified as detected by Western blot (Fig 3.6A). Both constructs were tested in ATPase and unwinding assays; while no unwinding activity was observed (data not shown), ATP hydrolysis quantified in different time-points was substantially higher for the wild-type protein than for the catalytic mutant (Fig. 3.6B). However, the fact that the mutant retained some residual activity could suggest that some contaminating NTPase could have been copurified with MOV10L1 and prompts us to be cautious with this result.

Despite the great potential that an *in vitro* system could offer for mechanistic studies, especially for a germ cell specific pathway that cannot be readily studied in any mammalian cell line, we unfortunately could not obtain good quality recombinant MOV10L1 for these purposes.

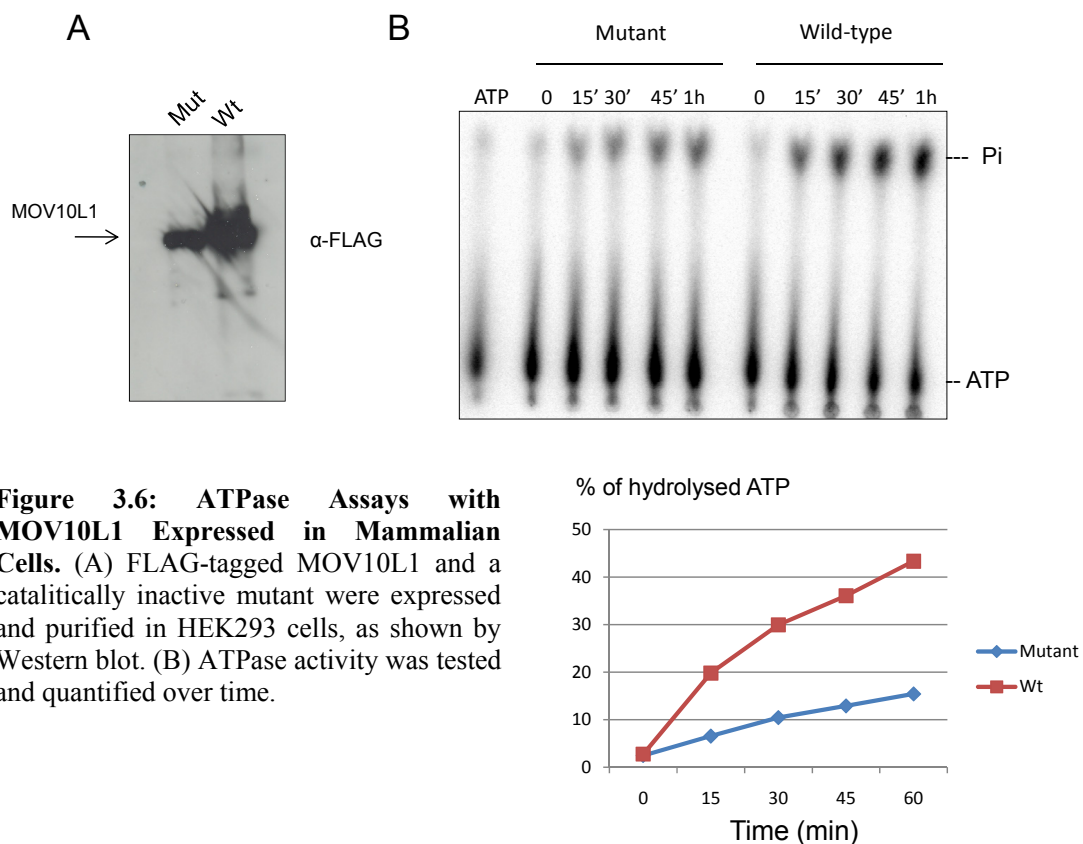


Figure 3.6: ATPase Assays with MOV10L1 Expressed in Mammalian Cells. (A) FLAG-tagged MOV10L1 and a catalitically inactive mutant were expressed and purified in HEK293 cells, as shown by Western blot. (B) ATPase activity was tested and quantified over time.

3.3 Discussion and Future Perspectives

Early studies on the piRNA pathway determined that piRNA biogenesis is independent of Dicer, the canonical enzyme involved in miRNA and siRNA production (Vagin *et al.* 2006; Houwing *et al.* 2007). Since then, extensive efforts have been made to identify the factors that participate in primary and secondary biogenesis pathways (Pane *et al.* 2007; Chen *et al.* 2009; Reuter *et al.* 2009; Vagin *et al.* 2009). This study, together with another one published at the same time (Frost *et al.* 2010), describes MOV10L1 as the first piRNA biogenesis factor in mouse. The data presented above, showing that the PIWI proteins Mili and Miwi2 are devoid of piRNAs in the male germ line of a *Mov10l1* mutant mouse, allow us to place MOV10L1 as a primary processing factor. We could also show derepression of Line1 and IAP transposons in the absence of a functional piRNA pathway, as well as a loss of DNA methylation at the level of Line1 promoters, leading to meiotic arrest and male-specific sterility.

We speculate that MOV10L1 might be required either for processing of precursors for primary biogenesis or loading of mature piRNAs into PIWI proteins. The fact that no piRNAs were found in the total small RNA pool of *Mov10l1* mutant testes would argue against the second hypothesis, but it is also possible that piRNAs are rapidly degraded when they cannot be loaded. Meiotic block in *Mov10l1* mutants takes place prior to the expression of the third murine PIWI protein, Miwi; the possibility that MOV10L1 is also required for biogenesis of Miwi-associated piRNAs could therefore not be tested, but is supported by the fact that MOV10L1 was found to interact with Miwi *in vivo*. Moreover, Mili and Miwi display almost identical small RNA profiles in pachytene stage (Aravin *et al.* 2006), so the processing pathways are likely to also be very similar. A good way of testing this would be generating a conditional mutant in which the expression of MOV10L1 is disrupted after birth, thus allowing progression of meiosis until the stages where Miwi is expressed.

The role of MOV10L1's orthologue in *Drosophila*, Armi, was unclear before this study was performed. Armi had been described to be involved in RNAi, mediating an ATP-dependent step of RISC assembly (Tomari *et al.* 2004), and to be

required for biogenesis of some repeat-associated siRNAs (rasiRNAs, the name piRNAs were given before they were found to be associated to PIWI proteins) (Vagin *et al.* 2006). A more extensive study revealed that piRNA biogenesis was partially affected in *armi* mutants, being piRNAs lost from some specific clusters -e.g. cluster 42AB- but still produced from the main primary biogenesis cluster -i.e. the *flamenco* cluster (Malone *et al.* 2009)-. Ping-pong signatures could also still be detected. The fact that RISC assembly was found to be impaired in *armi* mutants could perhaps be explained by the later finding that a DNA-damage response is activated in piRNA pathway mutants due to transposon activation (Klattenhoff *et al.* 2007), and this may in turn inactivate the RNAi pathway. It was not until the present study had already been published that the Brennecke and Siomi labs could show, making use of an *in vivo* RNAi-based approach and an ovary derived cell-line (OSC cells), that Armi, like MOV10L1, is indeed a primary piRNA biogenesis factor (Olivieri *et al.* 2010; Saito *et al.* 2010). The mild defects in piRNA biogenesis described before in *armi* mutants were due to the presence of a second somatic-specific promoter that was still active in the mutant, leading to the expression of the protein in follicle cells (Olivieri *et al.* 2010).

Studies of Armi in *Drosophila* led to some interesting findings that might also be relevant for MOV10L1 in mouse. In somatic cells, Armi interacts with Yb and they colocalize in Yb bodies (Olivieri *et al.* 2010; Saito *et al.* 2010). It would be worth determining if MOV10L1 associates with Tdrd12, the murine orthologue of Yb, which is expressed both in the male and female germ line. Armi associates with RNA species of 25-70nt length whose sequences overlap with those of piRNAs. They were called piRNA intermediate-like molecules (piR-ILs) and they are likely to be piRNA precursors (Saito *et al.* 2010). In contrast, we found MOV10L1 to be associated with mature piRNAs that cover the whole Mili-associated piRNA spectrum, suggesting MOV10L1 is a stable component of piRNPs, but no longer RNA species were detected. This surprising difference could be due to the fact that, in *Drosophila*, Piwi travels to the nucleus after being loaded with piRNAs and Armi stays in Yb bodies (Olivieri *et al.* 2010; Saito *et al.* 2010), while both Mili and MOV10L1 remain cytoplasmic in mouse. Hence, a hypothetical association of MOV10L1 with piR-ILs could perhaps be masked by the rapid processing of these into piRNAs given the abundant presence of Mili in the cytoplasm, while in flies, translocation of Piwi into

the nucleus once biogenesis is complete could leave Yb bodies and Armi as a *reservoir* of piRNA precursors waiting for new Piwi molecules to arrive. A possibility to prove this hypothesis would be to immunoprecipitate MOV10L1 in a *Mili* mutant background and check whether piR-ILs accumulate.

Even though all studies, including ours, have placed MOV10L1 and Armi as primary piRNA biogenesis factors, we cannot exclude that it also participates in secondary biogenesis. The primary pathway constitutes a source for secondary amplification, so the direct effect of *Mov10l1* mutation on secondary biogenesis could not be analysed in the absence of a functional primary pathway. In *Drosophila*, ping-pong signatures are still present in *armi* mutants (Malone *et al.* 2009); this could either be due to amplification of maternally deposited piRNAs, ruling out an involvement of Armi in ping-pong amplification, or to the fact that the analysed *armi* allele (Cook *et al.* 2004) is in fact a hypomorph. Uncoupling primary and secondary pathways is a challenging task which could perhaps be achieved by establishing an *in vitro* system that recapitulates piRNA biogenesis. Regardless of whether the effect of MOV10L1 on Miwi2 piRNA biogenesis is direct or indirect, Miwi2 failed to go to the nucleus in *Mov10l1* mutants. The same observation had already been made in a *Mili* mutant, where Miwi2 is also not loaded with piRNAs (Aravin *et al.* 2008). Interestingly, *Drosophila* Piwi also remained cytoplasmic in an Armi knock-down or any other situation where it failed to be loaded (Olivieri *et al.* 2010; Saito *et al.* 2010). Piwi contains an NLS at its N-terminus and localizes in the nucleus when expressed in S2 cells, which do not contain piRNA pathway components (Saito *et al.* 2009; Saito *et al.* 2010). A possible explanation is that the NLS is masked *in vivo*, retaining Piwi in the cytoplasm, and loading of the protein with piRNAs leads to some conformational change that leaves the NLS exposed and allows Piwi to translocate to the nucleus. It would be interesting to see if a similar mechanism operates in mouse, for instance by checking whether Miwi2 is nuclear when expressed in HeLa or HEK293 cells.

A question that remains unanswered is whether MOV10L1 is also required for piRNA biogenesis in mouse oocytes. piRNAs are also produced in oocytes (Tam *et al.* 2008), but this pathway has been studied very little given that mutation of piRNA

pathway components has no effect on female fertility. MOV10L1 is also expressed in ovaries (Baillet *et al.* 2008), so it is likely that it plays a similar role as in testes.

We attempted to gain further mechanistic and biochemical information by characterizing MOV10L1 *in vitro*. Unfortunately, we had very little success in producing and purifying recombinant MOV10L1 in good amounts using different expression systems (bacteria, insect cells and mammalian cells). It is possible that MOV10L1 requires some interaction partner to be correctly folded. In fact, MOV10L1 expressed in bacteria was copurified with a chaperonin (Fig. 3.5A) and MOV10L1 produced in mammalian cells was found to associate with the chaperone Hsp70-2 when incubated with testes extracts (Frost *et al.* 2010); these observations could be an indication that MOV10L1 is misfolded when expressed in a heterologous system. Armi was shown to form oligomeric complexes *in vivo* (Olivieri *et al.* 2010); this could therefore also be the case for MOV10L1, making it a difficult target for recombinant expression. In any case, a deletion fragment of MOV10L1 containing the RNA helicase domain could be purified at low yields from bacteria and mammalian cells and used for enzymatic assays. However, unwinding activity could never be shown and the results from ATP hydrolysis assays have to be taken with caution given the presence of contaminating ATPases in the purification.

Our study points to the requirement of the helicase activity of MOV10L1 for piRNA biogenesis, given that the mutant mouse expresses a part of the protein that lacks the conserved motifs for RNA binding and unwinding. However, the truncation affects the stability of the protein *in vivo* as detected by Western blot, so whether the protein is well folded in the mutant is not certain. *In vitro*, mutation of the conserved ATPase motif from DEAGQ to GGAAG had a negative effect on the recombinant protein solubility, preventing the enzymatic tests from being conclusive. Once the problems in expressing MOV10L1 can be overcome and the protein can be confidently shown to be active, efforts can be made to find a point mutant that is fully inactive but that is equally stable for testing the activity requirements *in vivo*.

The identification of the putative RNA helicase MOV10L1 as a primary pathway component constitutes one of the first steps towards the understanding of primary piRNA biogenesis. Later studies confirmed that this function is conserved in

Drosophila, where Armi has also been described to associate to piRNA precursors. We have shown that the interaction of MOV10L1 with PIWI proteins is very likely to be direct, and this interaction was shown to be largely dependent on RNA in *Drosophila* (Olivieri *et al.* 2010). It is possible that MOV10L1 is carrying the precursors and handing them over to PIWI proteins, perhaps participating also in the loading of piRNAs. RNA helicases have traditionally been seen as RNP remodelers (Linder 2006), but an increasing number of them have been shown to play slightly different roles. For instance, eIF4AIII holds mRNAs in its active site and is used as an anchor for the Exon Junction Complex (EJC) (Bono *et al.* 2006). MOV10L1 could play a similar role in the piRNA pathway, holding precursors for their processing by nucleases prior to loading to PIWI proteins.

4- UNCOVERING A ROLE FOR FKBP6 IN THE piRNA PATHWAY

Résumé du chapitre “*Uncovering a Role for Fkbp6 in the piRNA Pathway*”

L'étude de FKBP6 a démarré avec l'objectif d'identifier la protéine qui est recrutée par le domaine MYND de Tdrd1 et qui pourrait expliquer l'effet de Tdrd1 sur la biogenèse secondaire des piRNA et l'inhibition des transposons. Suite à un criblage double-hybride et à des études d'interaction *in vitro* on a déterminé que le domaine MYND de Tdrd1 interagit avec FKBP6. De plus, FKBP6 interagit avec les protéines PIWI en utilisant un domaine isomérase de proline. Des alignements avec d'autres FKBP montrent que les résidus catalytiques de ce domaine ne sont pas conservés, et des essais biochimiques ont prouvé que ce domaine n'est pas actif. Nous pensons que ce domaine est devenu un module structural permettant de lier des protéines PIWI. FKBP6 interagit avec la partie N-terminale de Mili, une région qui contient des arginines diméthylées. Nous avons déterminé que FKBP6 inhibe la méthylation de Mili *in vitro*, probablement parce que l'interaction avec FKBP6 empêche la méthyltransférase d'accéder à son substrat, mais nous ignorons si cela est pertinent *in vivo*.

FKBP6 est indispensable pour la fertilité des mâles chez la souris, comme toutes les protéines qui participent à la voie des piRNA. Nous avons prouvé que les souris *Fkbp6*^{-/-} subissent une activation des transposons et une perte de la méthylation de l'ADN dans leur promoteur, ce qui est en accord avec un rôle de FKBP6 dans la voie des piRNA. Nous croyons que la fonction de FKBP6 pourrait être liée à la chaperonne Hsp90, étant donné que nous avons montré que ces deux protéines interagissent par le biais du domaine TPR de FKBP6. Hsp90 participe à la biogenèse des miRNA et des siRNA et a aussi été impliquée dans la voie des piRNA. Nous proposons que Hsp90 est recrutée par Tdrd1 vers les complexes de protéines PIWI à travers la co-chaperonne FKBP6 pour faciliter la biogenèse secondaire des piRNA. Nos données sont en accord avec ce modèle mais de futures investigations seront nécessaires pour le prouver.

4.1 Introduction

The basis for the research work presented in this chapter lies on the identification of FK506-binding proteins (FKBPs) as potential components of the piRNA pathway. Identification and characterization of new factors have been one of the leading edges in piRNA research and have substantially contributed to the understanding of piRNA biogenesis and function. Two main approaches have been taken for factor discovery: identification of PIWI-associated components by proteomics (Chen *et al.* 2009; Reuter *et al.* 2009; Vagin *et al.* 2009), and genetic screens in flies, looking for factors that mediate transposon silencing in the germ line (Lim and Kai 2007; Pane *et al.* 2007; Vagin *et al.* 2009). The study on FKBPs that is presented below has been built both on proteomic and genetic evidence.

4.1.1 – Search for a Tdrd1-Interacting Protein via a Yeast Two-Hybrid Screen

Previous work in the lab had been carried out concerning Tudor domain containing protein 1 (Tdrd1), which was shown to participate in transposon silencing by piRNAs (Reuter *et al.* 2009). Tdrd1 has an impact both in PIWI protein localization and piRNA biogenesis. The piRNA profile is altered in a *tdrd1* mutant mouse, with an increased amount of exon-derived piRNAs in pachytene stages. Interestingly, ping-pong signatures are lost in embryonic stages, leading to a depletion of secondary piRNAs targeting Line1 sequences and subsequent derepression of Line1 elements (Vagin *et al.* 2009). The amount of nuclear Miwi2 is reduced in the mutant, while in the cytoplasm it fails to localise in the specific granules where it is found in wild-type conditions. Tdrd1 is a 130kDa protein that has an N-terminal MYND (myeloid, Nervy and DEAF-1) domain followed by four tandem Tudor domains (Fig. 4.1A). Tdrd1 uses its Tudor domains to interact with symmetrically dimethylated arginines in PIWI proteins (Reuter *et al.* 2009; Vagin *et al.* 2009). The MYND domain is a zinc-finger binding domain that, in other proteins, participates in protein-protein interactions (Matthews *et al.* 2009). Tudor proteins are usually scaffolds for assembly of macromolecular complexes and it was hypothesized that the MYND domain recruits an additional factor to piRNPs (Reuter *et al.* 2009). Identification of this factor might give further insights into Tdrd1's function, and may as well deepen the understanding

of transposon silencing by piRNAs at the molecular level. For this reason, a yeast two-hybrid screen was performed in the Pillai group using the N-terminal part of Tdrd1 (comprising the MYND domain) as a bait with a cDNA library from adult mouse testis as a prey. The prolyl *cis-trans* isomerase FKBP8 was obtained as the main hit. In parallel, an independent proteomics study identified FKBP3, FKBP5 and FKBP6 in endogenous Mili and Miwi2 complexes (Vagin *et al.* 2009). The aim of this research project is to determine whether FKBP6 are involved in the piRNA pathway and to understand their role at the molecular level.

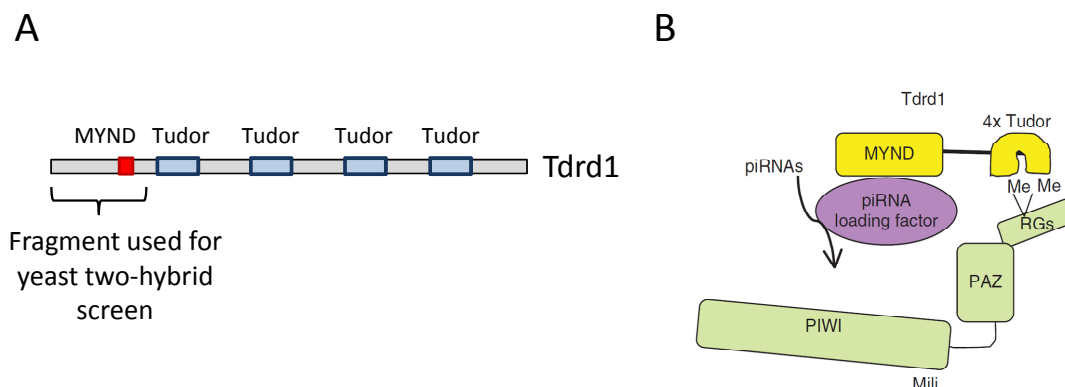


Figure 4.1: Tdrd1 in the piRNA Pathway. (A) Domain representation of mouse Tdrd1. The fragment that was used as a bait in the yeast two-hybrid screen is indicated. (B) Model for Tdrd1 function in the piRNA pathway, where the MYND domain is suggested to recruit a biogenesis/loading factor that would facilitate proper piRNA assembly (Reuter *et al.* 2009).

4.1.2 - Functions of FKBP6

Proline is the only aminoacid that can exist in both *cis* and *trans* conformations. Interconversion between the two states (Fig. 4.2A) is a rather slow process that can be accelerated by enzymes known as prolyl *cis-trans* isomerases (PPIases) or rotamases (reviewed in Lu *et al.* 2007). FKBP6 are among the proteins that can catalyze this reaction, together with three other structurally unrelated families: cyclophilins, parvulins and the protein phosphatase 2A (PP2A) phosphatase activator (PTPA). FKBP6 and cyclophilins have been extensively studied for being targets of immunosuppressive drugs that are commonly used to prevent rejection in organ

transplantation (Fischer *et al.* 1989; Harding *et al.* 1989; Siekierka *et al.* 1989; Takahashi *et al.* 1989). FK506 (tacrolimus) and rapamycin (sirolimus) bind FKBP12, which generates an interaction surface for calcineurin and mammalian target of rapamycin (mTOR) respectively, and thus promote formation of a ternary complex that inhibits calcineurin phosphatase or mTOR kinase (Liu *et al.* 1991; Schreiber and Crabtree 1992; Cardenas *et al.* 1995). This blocks IL-2 response and prevents T cell activation. Cyclosporin A (also used as an immunosuppressant) binds another PPIase, cyclophilin, and together they exhibit the same behaviour as the FKBP12/FK506 complex (Liu *et al.* 1991). Therefore, even if these drugs inhibit FKBP and cyclophilin's PPIase activity, their physiological action does not involve the catalytic activity *per se*.

Prolyl *cis-trans* isomerization events are relevant in a very broad range of cellular pathways, given that they provide a backbone switch that can be used for protein folding and regulation. This isomerization can mediate conformational changes either at a local level, for instance by causing a change in the accessibility of a given residue for post-translational modification, or at a more global level, by modifying the spatial orientation of an entire domain. Examples of local changes are prolyl isomerization by the parvulin Pin1, which regulates dephosphorylation of tau and Cdc25C and is required for cell division in yeast (Zhou *et al.* 2000), or by the FKBP Fpr4, which prevents methylation of H3K36 (Fig. 4.2B) (Nelson *et al.* 2006). On the other hand, displacement of the transcription factor MLL1's Bromo domain as a consequence of prolyl isomerisation by the cyclophilin Cyp33 is a good example of a global conformational change mediated by this catalytic activity (Fig. 4.2C) (Wang *et al.* 2010).

FKBPs have been reported to have some functions that are independent of their catalytic activity. FKBP12, the first identified member in the family, regulates palmitoylation of h-Ras through prolyl *cis-trans* isomerization (Ahearn *et al.* 2011), but does not require this activity for binding ryanodine receptor (Ryr) calcium release channel and helping to maintain it in a closed conformation (Timerman *et al.* 1995; Timerman *et al.* 1996). A subgroup of FKBPs contain a tetratricopeptide repeat (TPR) domain at their C-termini that is used for binding the C-terminal peptide MEEVD

from Hsp90 (Young *et al.* 1998). These FKBP6s act therefore as co-chaperones, but there are few demonstrations that the isomerase activity is required in this context: for example, while glucocorticoid receptor modulation by FKBP52 is dependent on both its catalytic activity and Hsp90 binding capacity (Riggs *et al.* 2003), aryl hydrocarbon receptor regulation by the FKBP XAP2/AIP requires Hsp90 but not prolyl *cis-trans* isomerization (Ma and Whitlock 1997; Meyer *et al.* 1998). The defined roles of FKBP6s as co-chaperones are currently poorly understood, and they might differ for each of the cases.

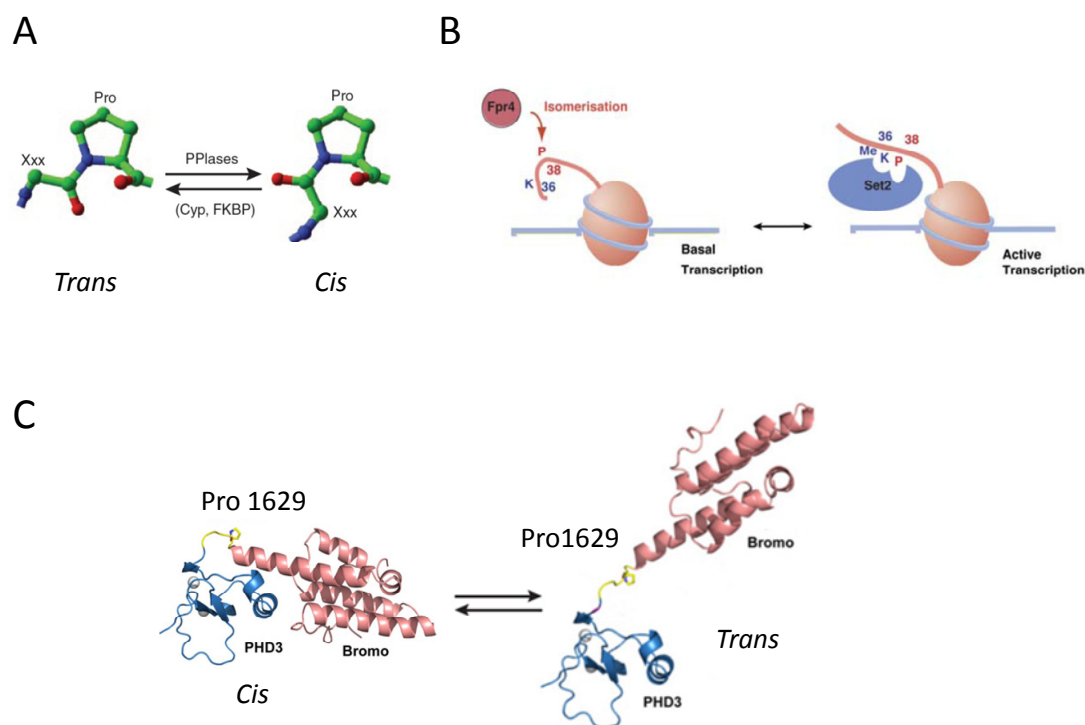


Figure 4.2: Prolyl *Cis-Trans* Isomerization as a Molecular Switch. (A) Graphic representation of proline in *cis* and *trans* conformations (Lu *et al.* 2007) (B) Prolyl isomerization by the FKBP Fpr4 in yeast inhibits H3K36 methylation by Set2 and regulates transcription (Nelson *et al.* 2006). (C) Binding and spatial orientation of MLL1's PHD3 and Bromo domains is regulated by *cis-trans* isomerization, carried out by the cyclophilin Cyp33. Adapted from Wang *et al.* 2010.

4.1.3 – Germ Line Functions of FKBP6/Shut-down

Yeast two-hybrid screen from the Pillai lab identified FKBP8 as a Tdrd1 MYND domain interactor, while FKBP6, FKBP3 and FKBP5, but not FKBP8, were found in an endogenous proteomic screen for PIWI proteins (Vagin *et al.* 2006). Among the three, FKBP6 was the one identified with a bigger coverage, with up to 44% in a Miwi2 screen, while the others were all below 20%. These results might be complementary rather than contradictory, given that FKBP6 and FKBP8 share the same domain organization –an N-terminal FK domain and a C-terminal TPR domain - and have a reasonably high sequence identity (22%). It would therefore be possible that an artificial system like yeast two-hybrid does not identify the actual *in vivo* interactor, but a very similar one from the structural point of view. In fact, FKBP8 has been shown to play a role in neural tube formation. Two *Fkbp8* mouse mutant alleles have been characterized, one of them being embryonically lethal due to defects in neural tube formation (Bulgakov *et al.* 2004), and the second one being viable but giving mice with spina bifida (Wong *et al.* 2008). FKBP8 prevents apoptosis by protecting Bcl-2 from caspase-mediated degradation (Shirane and Nakayama 2003; Choi *et al.* 2010), and this is required for neurone survival in the developing neural tube. Given that the piRNA pathway is restricted to the germ line, FKBP8 does not stand as a good candidate for a piRNA component, but the possibility that it plays different roles in different tissues cannot be ruled out. In contrast, FKBP6 is a germ cell specific component with some features that would very much fit with those of a protein that participates in the piRNA pathway. For example, *Fkbp6* mutant mice display male-specific sterility due to spermatogenic arrest (Crackower *et al.* 2003), similar to PIWI protein or other piRNA pathway component mutants (e.g. MOV10L1, Fig. 4.3A). Moreover, there are striking similarities in the expression pattern of FKBP6 orthologue in *Drosophila*, Shut-down (Munn and Steward 2000), and the PIWI protein Aub (Schmidt *et al.* 1999), suggesting participation in a shared pathway (Fig. 4.3B, left). Indeed, examination of fly mutants for Shut-down and Aub reveals compromised fertility in both males and females, with the few eggs that are laid displaying axis specification defects (Fig. 4.3B, right) (Wilson *et al.* 1996; Munn and Steward 2000). Altogether, genetic data point to a germ line role of FKBP6 rather than FKBP8, but both will be considered for this study.

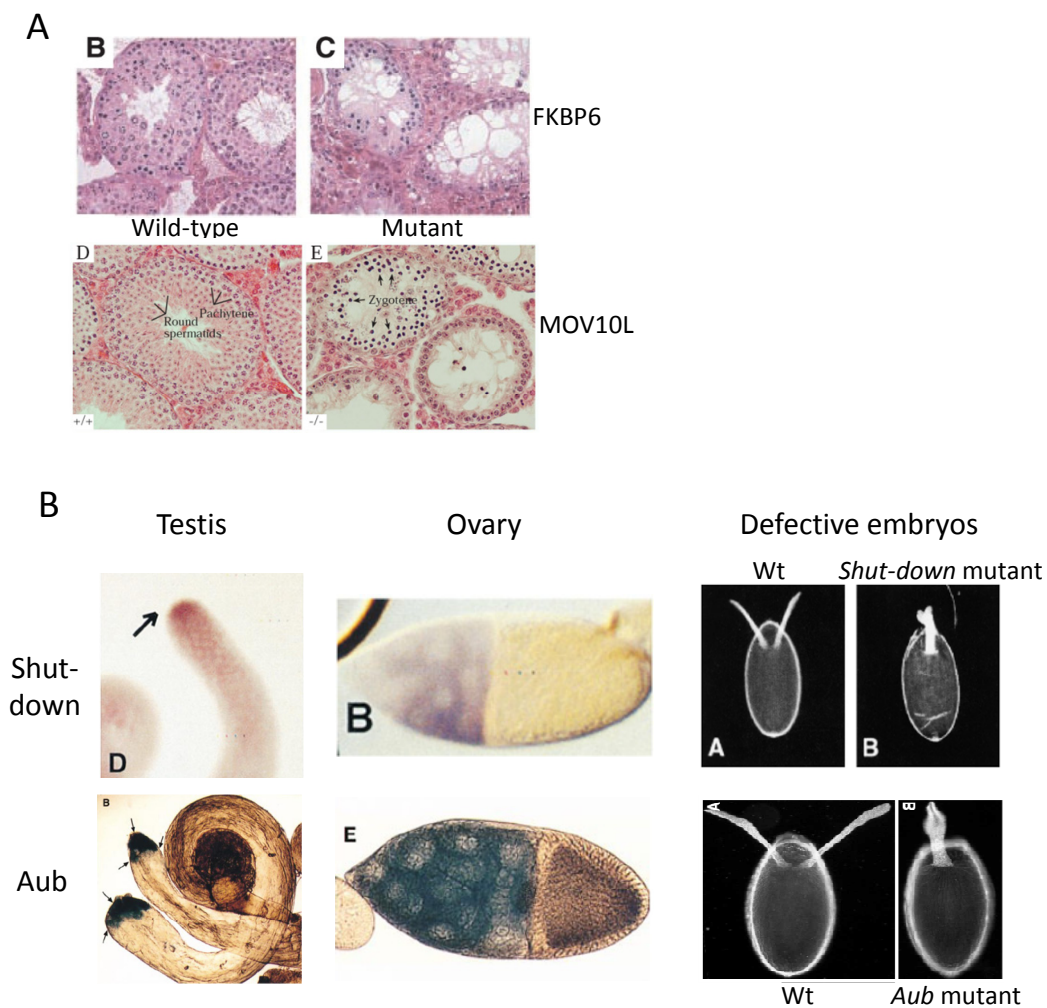


Figure 4.3: Germ Line Function of FKBP6/Shut-down. (A) Meiotic arrest in *Fkbp6*^{-/-} (Crackower *et al.* 2003) and *Mov10l1*^{-/-} (Zheng *et al.* 2010) tubules (right), in contrast to wild-type tubules with full spermatogenesis (left). (B) Expression pattern of Shut-down (Munn and Steward 2000) and Aub (Schmidt *et al.* 1999) in testis and ovaries, shown by *in situ* hybridisation and β-galactosidase of an Aub-lacZ transgenic line, respectively (left). Dorsal surfaces of eggshells from wild-type or the indicated mutant mothers (right). The dorsal appendages are fused in eggs from both *aub*⁻ (Wilson *et al.* 1996) and *shut-down*⁻ (Munn and Steward 2000) mothers.

4.1.4 - Hsp90 in Argonaute/PIWI Complexes

FKBP6 and FKBP8 belong to the subgroup of FKBP6s containing a C-terminal TPR domain which could potentially be used for interaction with the chaperone Hsp90. It should be noted that Hsp90 has already been shown to play a role in small RNA pathways, both in PIWI and Ago complexes. Three independent studies showed that Hsp90, together with Hsc70, mediates loading of small RNA duplexes into Ago

proteins in an ATP-dependent fashion in plants, humans and *Drosophila* (Iki *et al.* 2010; Iwasaki *et al.* 2010; Miyoshi *et al.* 2010). Loading of single-stranded small RNAs and target cleavage do not require Hsp90 or ATP. Hsp90 is believed to have an additional role in protecting Argonaute proteins from degradation by the proteasome, as human Ago2 is destabilized and mislocalized from cytoplasmic foci upon Hsp90 inhibition (Pare *et al.* 2009; Suzuki *et al.* 2009; Johnston *et al.* 2010).

Interestingly, there are also links between Hsp90 and the piRNA pathway. Stellate silencing in *Drosophila*'s male germ line, which is carried out by the piRNA pathway, is defective in an *Hsp83* mutant (*Drosophila*'s Hsp90). Transposons are also derepressed, both in testes and ovaries, and at least two piRNAs fail to be produced (Specchia *et al.* 2010). Moreover, Hsp90 and the co-chaperone Hsp70/Hsp90 Organizing Protein (HOP) interact with Piwi *in vivo* and influence Piwi's phosphorylation status (Gangaraju *et al.* 2011). Hsp90 and piRNAs have been proposed to participate together in canalization, a means to buffer environmental changes to reduce phenotypic variation. Altogether, there are increasing hints pointing to a role of Hsp90 in the piRNA pathway, although it has currently not been defined as for the miRNA and siRNA pathways.

4.2 - Results

4.2.1 - FKBP6 Is a piRNA Pathway Component

A yeast two-hybrid screen for the N-terminal part of Tdrd1, containing the MYND domain, identified a fragment of FKBP8 that included its TPR domain (Pillai lab, unpublished data). On the other hand, proteomic analyses revealed the presence of FKBP3, FKBP5 and FKBP6 in Mili and Miwi2 complexes (Vagin *et al.* 2009). In order to clarify which of these proteins interact with Tdrd1, recombinant versions of the four identified FKBP6s were expressed and purified from bacteria and tested in pull-down assays. Only FKBP6 and FKBP8 showed interaction with *in vitro* translated Tdrd1 (Fig. 4.4A). This result is consistent both with the two-hybrid screen, where FKBP8 was pulled-down, and the proteomics data, given that FKBP6 was identified with a largely bigger coverage than FKBP3 and FKBP5. FKBP6, like Tdrd1, is required for progression of meiosis in mouse males (Crackower *et al.* 2003; Chuma *et al.* 2006), while FKBP8 participates in neural tube development (Wong *et al.* 2008). This suggests that, even if both FKBP6 and FKBP8 can interact with Tdrd1 *in vitro*, only FKBP6 might be relevant *in vivo*. Tdrd1 participates in silencing of Line1 elements by the piRNA pathway (Reuter *et al.* 2009; Vagin *et al.* 2009), so we aimed to determine whether this is also the case for FKBP6. Line1 expression was indeed derepressed in *Fkbp6* mutant mice as shown by Northern blot (Fig. 4.4B). Silencing of these elements by the piRNA pathway is achieved through DNA methylation at the level of their promoters (Aravin *et al.* 2007; Carmell *et al.* 2007; Kuramochi-Miyagawa *et al.* 2008). Consistently, methylation-sensitive Southern blot analysis revealed a loss of DNA methylation in Line1 promoters in the *Fkbp6* mutant (Fig. 4.4C). Together with the interaction data, this strongly suggests that FKBP6 participates in the piRNA pathway.

We then asked whether FKBP6 and FKBP8 can also interact with PIWI proteins. Recombinant FKBP6, but not FKBP8 or GST, pulled-down *in vitro* translated Mili, Miwi and Miwi2 (Fig. 4.5A). This further strengthens the point that FKBP6, and not FKBP8, is a piRNA pathway component. Next, we mapped the

interaction between Mili and FKBP6. The N-terminal part of FKBP6, containing the catalytic FK domain, interacts with the N-terminal part of Mili, and these two fragments are sufficient for the interaction (Fig 4.5B).

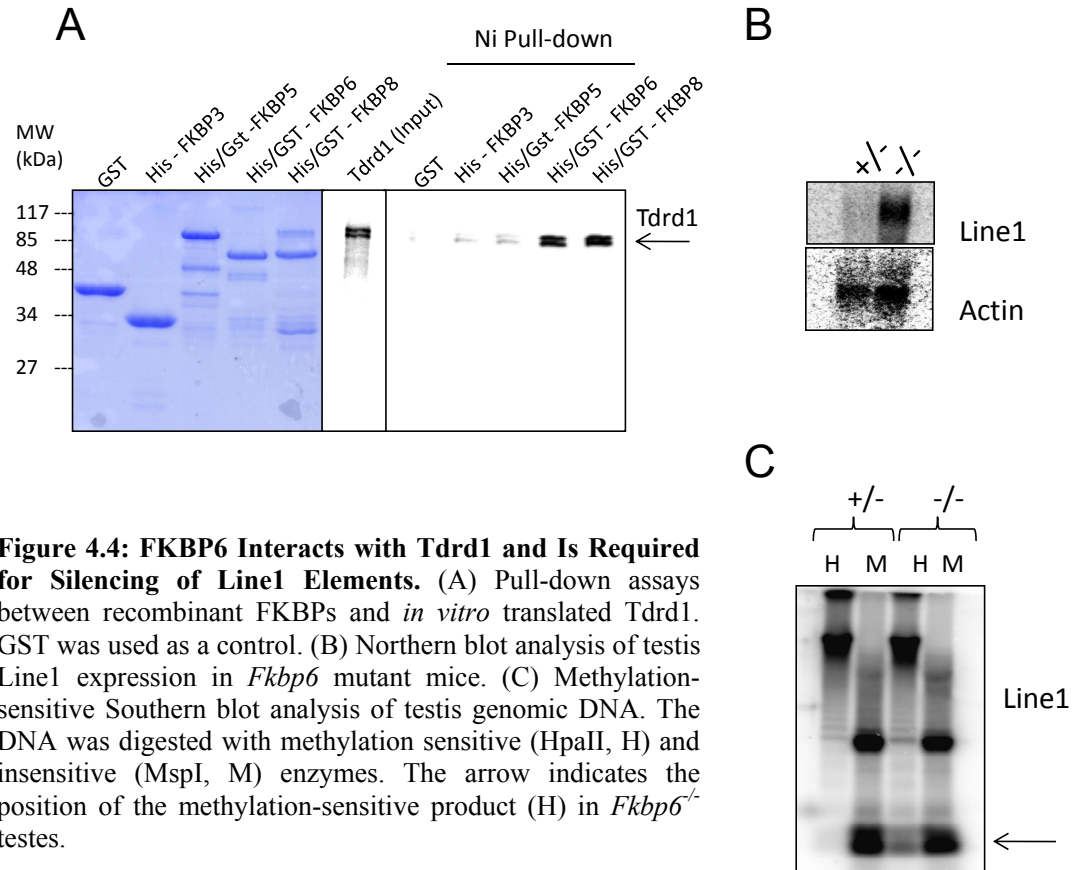


Figure 4.4: FKBP6 Interacts with Tdrd1 and Is Required for Silencing of Line1 Elements. (A) Pull-down assays between recombinant FKBP6 and *in vitro* translated Tdrd1. GST was used as a control. (B) Northern blot analysis of testis Line1 expression in *Fkbp6* mutant mice. (C) Methylation-sensitive Southern blot analysis of testis genomic DNA. The DNA was digested with methylation sensitive (HpaII, H) and insensitive (MspI, M) enzymes. The arrow indicates the position of the methylation-sensitive product (H) in *Fkbp6*^{-/-} testes.

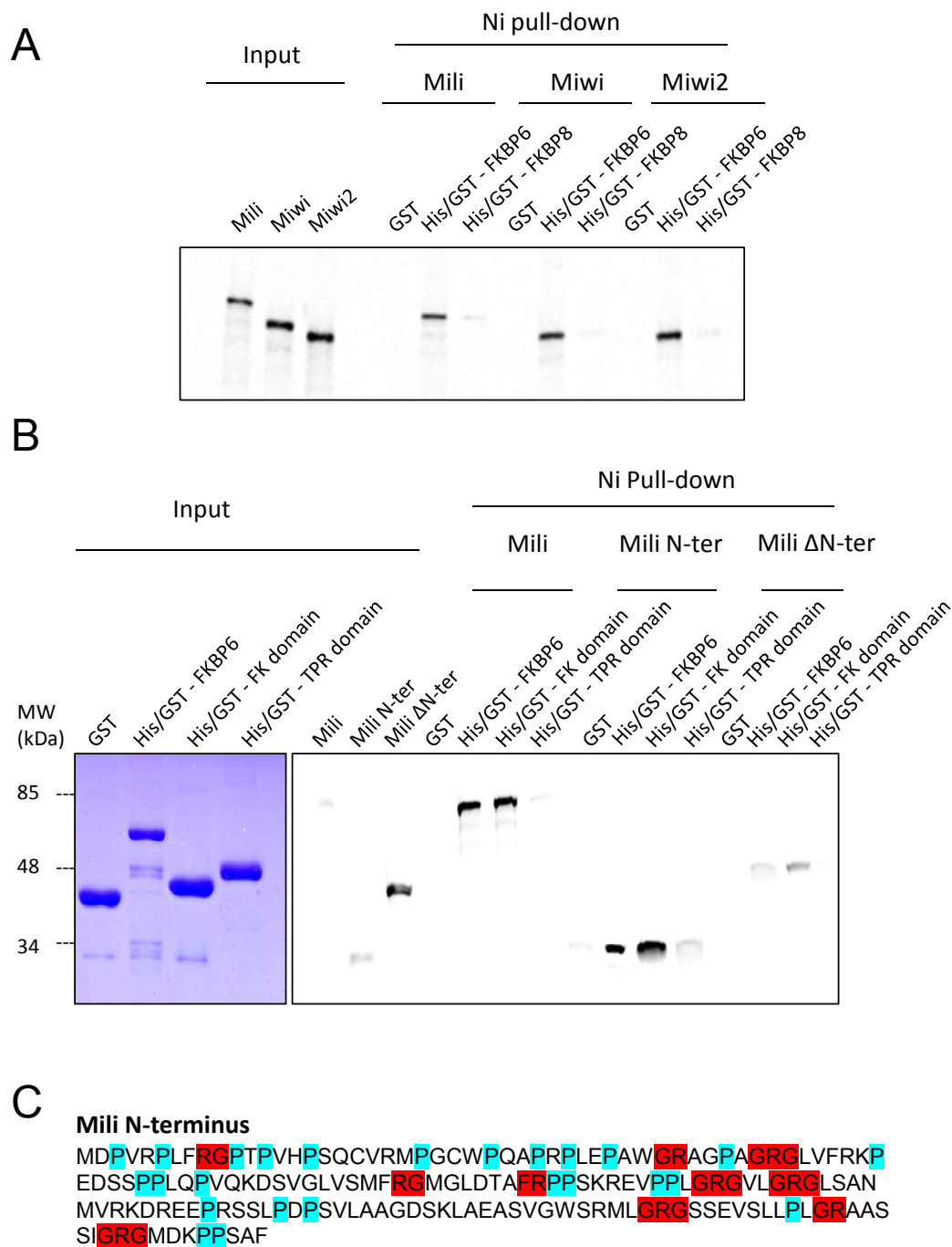


Figure 4.5: The FK Domain of FKBP6 Interacts with the N-terminus of Mili. (A) Pull-down assays between recombinant FKBP6 or FKBP8 and PIWI proteins. (B) The interaction between Mili and FKBP6 was mapped in pull-down assays, using full-length and deletion versions of recombinant FKBP6 and *in vitro* translated Mili. (C) Aminoacid sequence of Mili N-terminus. RG/GR repeats, potential methylation sites, are highlighted in red, while all the prolines are highlighted in blue.

4.2.2 - FKBP6's FK Domain Has Evolved into a Structural Module

The N-terminus of Mili contains RG/GR repeats that are modified by symmetrical dimethylation on arginines (Kirino *et al.* 2009). Interestingly, we noted the presence of abundant prolines interspersed between these arginines in this region (Fig. 4.5C). Post-translational modifications, such as lysine methylation, have already been shown to be regulated by isomerisation of neighbouring prolines (Nelson *et al.* 2006). Hence, a reasonable possibility would be that Mili methylation is regulated by prolyl isomerisation, given that FKBP6's catalytic domain interacts with the N-terminus of Mili.

In order to test this hypothesis, we aimed to analyse FKBP6's catalytic activity. Prolyl *cis-trans* isomerisation can be tested using a protease-coupled assay that has been widely used for FKBP6s, cyclophilins and parvulins (Fischer *et al.* 1984; Gothel and Marahiel 1999). A small peptide with the sequence Ala-Leu-Pro-Phe is coupled to the chromogen p-nitroanilide (pNA) and treated with chymotrypsin, which can cut (releasing pNA) only if the proline-leucine bond is in the *trans* conformation. Kinetics of chymotrypsin cleavage are faster than those of prolyl isomerisation, so the rate of *cis-trans* interconversion can be calculated as the rate of pNA release, which is measured by absorbance at 390nm. Surprisingly, neither full-length FKBP6 nor its FK domain alone showed any PPIase activity, while as many as three positive controls (FKBP3, 5 and 12) that were purified and tested in the same conditions were highly active, with first order rate constants in the same range as the ones reported in the literature (Fig 4.6A) (Siekierka *et al.* 1989; Jin *et al.* 1992; Wiederrecht *et al.* 1992).

All known active FKBP6s are inhibited by FK506, an immunosuppressive drug that binds the catalytic pocket (Van Duyne *et al.* 1991; Kang *et al.* 2008). The residues that are used for prolyl *cis-trans* isomerisation are the same that bind FK506, and a few FK domains that have lost their catalytic activity due to mutations in the active site have also been reported to lose their ability to bind FK506 (Kamphausen *et al.* 2002; Denny *et al.* 2005). Using isothermal titration calorimetry (ITC), we tested whether FKBP6 can bind FK506. While FKBP12, used as a positive control, proved

to have a very high affinity for FK506 (a K_d of 5,2nM), no binding at all was observed between this drug and the FK domain of FKBP6 (Fig. 4.6B).

Thirteen residues have been described to be important for PPIase activity, and they are highly conserved among active FKBP6s (Kay 1996). We performed sequence alignment of different FKBP6 orthologues (including *Drosophila* Shut-down) to both active and inactive FK domains and analysed the conservation of the residues in the active site. Consistent with the biochemical data, some of the key residues for isomerisation are not conserved in FKBP6, similar to other reported inactive FK domains (Fig. 4.6C). For instance, methionine 84 in mouse and human FKBP6 is occupied by an aromatic residue in all active FK domains (preferentially a tryptophan), while it has been mutated in all the inactive FKBP6s. In fact, mutation of this single residue in FKBP12 to methionine, leucine or isoleucine (the residues that are found in inactive FK domains in this position) was shown to decrease its activity to 20-30% compared to that of the wild-type protein (Ikura and Ito 2007). Crystal structures of the FK domains of human FKBP12 and FKBP6 have been solved (Szep *et al.* 2009 and PDB ID: 3B7X, unpublished), and it is not surprising to find that this tryptophan falls exactly at the bottom of the catalytic pocket (Fig. 4.6D, Trp60; see also the corresponding Met95 in FKBP6), suggesting that it has a key role in catalysis. However, the overall shape of the pocket remains quite similar, with an abundant presence of aromatic residues, perhaps suggesting that FKBP6 is still able to fit a proline in the active site. Some of the residues that are not conserved in FKBP6 (i.e. Arg82 and Met84), however, cannot be seen in the structure because they are disordered, while the corresponding phenylalanines in FKBP12 are visible. We speculate that FKBP6's FK domain has evolved into a structural module -similar to what has been suggested for FKBP5's inactive FK domain, which mediates interaction with progesterone receptor (Sinars *et al.* 2003)- to bind the proline-rich N-termini of PIWI proteins.

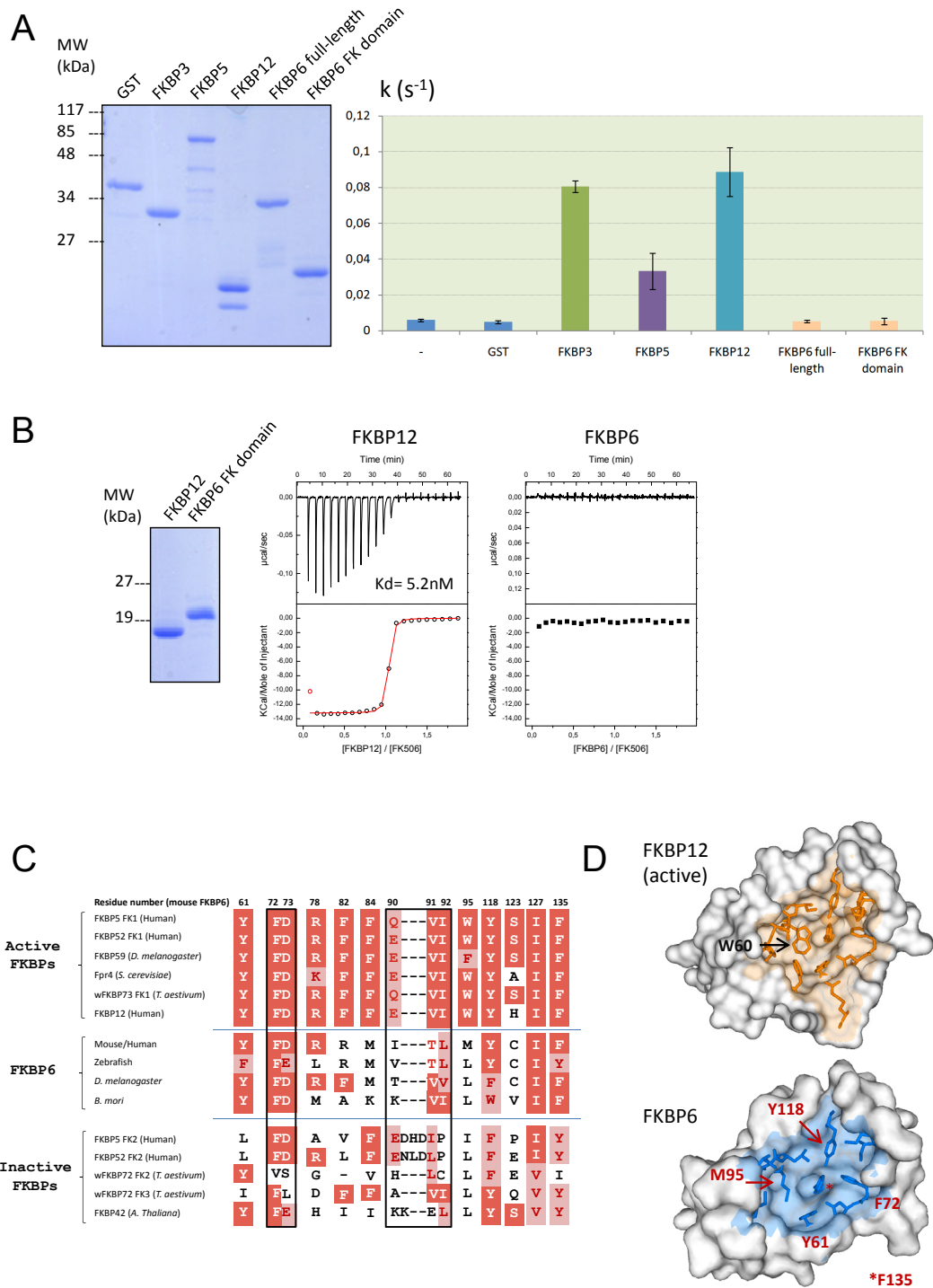


Figure 4.6: FKBP6 Has No PPIase Activity. (A) Prolyl isomerization assays using recombinant FKBP6s (left panel) and a succ-Ala-Leu-Pro-Phe-pNA peptide. First order rate constants (right panel) were calculated as the average of three independent experiments, and the standard error is shown. (B) ITC measurements of FKBP12 and FKBP6 binding to FK506. 1mM FKBP was added to 10μM of FK506. In the lower panels, the circles represent experimental data and the continuous line corresponds to the best fit to a model with one binding site. (C) Comparison of residues in the active site of active FKBP6s, FKBP6 and inactive FKBP6s. Human and mouse FKBP6 are shown in the same lane because they have the same sequence. (D) Surface representation of the FK domain of human FKBP12 (PDB ID: 2PPN) and human FKBP6 (PDB ID: 3B7X). Indicated are the aromatic residues in FKBP6's catalytic pocket, as well as the central methionine 95 and the corresponding tryptophane 60 in FKBP12.

4.2.3 - Methylation of Mili Is Blocked by FKBP6 *In Vitro*

FKBP6 could potentially interfere with Mili methylation, given that it binds the region that contains symmetrically dimethylated arginines. In order to test this, we set an assay that allows analysis of Mili methylation *in vitro*. The first 70 aminoacids of Mili –containing three potential methylation sites- were expressed in bacteria fused to GST and separated by a TEV cleavage site. The purification yielded a mixture of different species (Fig. 4.7A, left panel), which we reason might be degradation fragments as this region of Mili is intrinsically unstructured and is therefore probably accessible to proteases. The GST-fusion protein was incubated with HEK293 cell extracts, which contain the ubiquitously expressed methyltransferase Prmt5 that is responsible for PIWI protein methylation (Vagin *et al.* 2009), and ^{14}C -labelled S-adenosyl methionine (SAM, the methyl donor in the reaction). The radioactive bands detected after SDS-PAGE could be proven to be specific to Mili because their molecular weight decreases after cleavage with TEV, which separates them from GST (Fig 4.7A, right panel). Increasing amounts of FKBP6 were added to the reaction and a reduction of Mili methylation to almost background levels was observed when the ratio was 1 to 1, while no effect was observed upon addition of GST (Fig. 4.7B). This suggests that binding of FKBP6 to the N-terminus of Mili blocks methylation by Prmt5, probably by masking the methylation site. We take this result as an additional

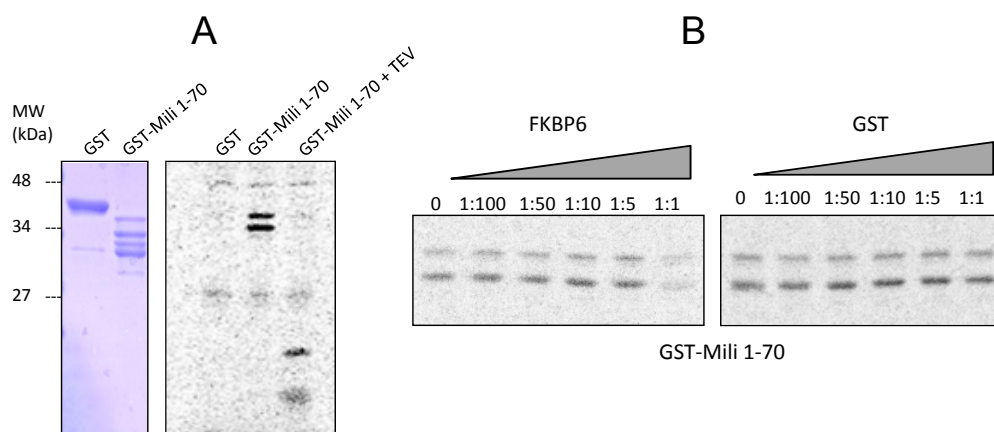


Figure 4.7: FKBP6 Blocks Mili Methylation *In Vitro*. (A) A GST fusion of the first 70 aminoacids of Mili was purified from bacteria. Note that GST alone is bigger because it is fused to an unrelated sequence from the expression vector. *In vitro* methylation using HEK293 cell extract and a radioactive substrate gives two specific bands that can be released by TEV cleavage. (B) Analysis of methylation of Mili upon addition of increasing amounts of FKBP6 or GST. The molar ratio is indicated.

evidence that FKBP6 interacts with the N-terminal region of Mili, but it is difficult to determine whether blocking of methylation also takes place *in vivo*.

4.2.4 - *Fkbp6* Mutation Alters the piRNA Profile

As described in the introduction, piRNAs are generally classified into two categories, primary and secondary, based on their proposed mode of biogenesis. In post-natal germ cells, Mili and Miwi incorporate primary piRNAs, while secondary piRNAs are loaded into Mili and Miwi2 in the embryonic mouse germ line and are generated by the ping-pong amplification cycle (Aravin *et al.* 2008). *Tdrd1* is not required for primary piRNA biogenesis. However, the primary piRNA profile is altered and secondary ping-pong signatures are reduced in *Tdrd1*^{-/-} mice (Reuter *et al.* 2009; Vagin *et al.* 2009). If *Tdrd1*'s role was to recruit FKBP6 to PIWI complexes, we would expect to see a similar situation in an *Fkbp6* mutant.

To examine any impact on piRNA biogenesis, we performed immunoprecipitation experiments using testis extracts from P20 mice (20 days after birth). At this stage during germ cell development, Mili and Miwi are expressed (Aravin *et al.* 2008); immunoprecipitation with their respective antibodies from *Fkbp6*^{+/-} animals revealed presence of distinctly sized small RNAs identifying them as Mili- and Miwi- associated piRNAs. Y12, an antibody against sDMAs, co-immunoprecipitates both these species indicating the methylated status of Mili and Miwi. Primary piRNA biogenesis is unaffected in the *Fkbp6* mutant as indicated by the presence of piRNAs in Mili complexes (Fig 4.8A). The absence of Miwi-associated piRNAs is explained by the block in germ cell development, which takes place at leptotene/zygotene stage, before the onset of Miwi expression in pachytene stage. Interestingly, piRNAs could also be immunoprecipitated using Y12 antibody, indicating that Mili is still methylated, at least at a few sites, in the *Fkbp6* mutant.

To examine the piRNA profile in the *Fkbp6* mutant, we prepared small RNA libraries for Solexa deep sequencing from the Mili and Y12 immunoprecipitates that are shown in Fig. 4.8A. A total of 0.4-1.7 million reads from the individual libraries were perfectly matched to the mouse genome and were used for the analysis. The size

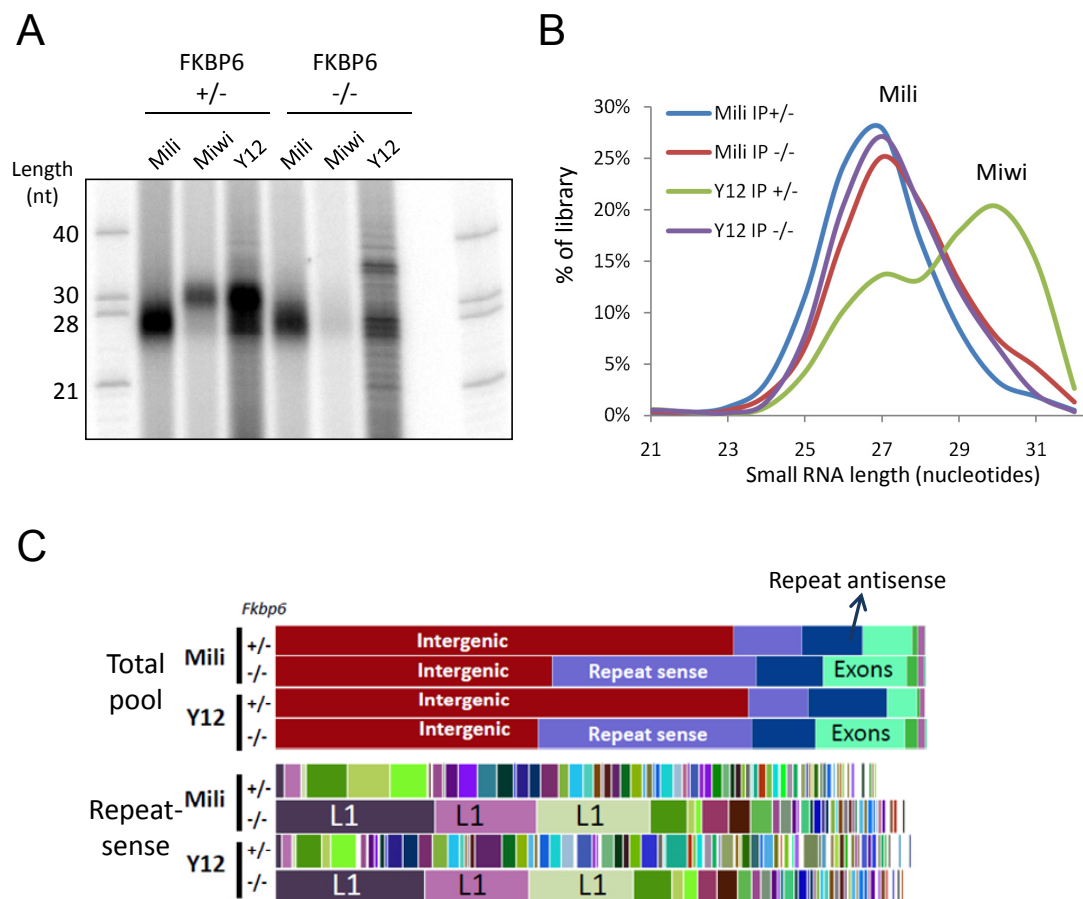


Figure 4.8: piRNA Profile Analysis in *Fkbp6*^{-/-} Mice. (A) Immunoprecipitations with the indicated antibodies were performed from *Fkbp6*^{+/-} and *Fkbp6*^{-/-} P20 testis, and the small RNAs were extracted, labelled and resolved in 15% urea-PAGE. (B) Length distribution of reads from the indicated libraries. (C) Genomic annotation of small RNAs from Mili and Y12 libraries (top). Repeat-derived sense reads were classified according to their origins (bottom). Each color represents a class of repeat, but only Line1 (L1) is indicated as the amount is significantly increased in *Fkbp6*^{-/-} libraries.

distribution of all libraries peaked at 27nt, corresponding to Mili-associated piRNAs, except for the Y12 library from heterozygous mice that showed a second peak at 30nt corresponding to Miwi piRNAs (Fig 4.8B). Genome annotation showed an expansion of repeat-derived sense piRNAs in *Fkbp6*^{-/-} Mili and Y12 libraries (Fig 4.8C, top panel). A substantial increase of Line1-derived sequences in the mutant libraries was observed when plotting only piRNAs arising from the sense strand of repeats (Fig 4.8C, bottom panel). piRNA populations are dynamic during spermatogenesis, and the amount of repeat-derived piRNAs is substantially higher in pre-pachytene than in pachytene stages. Given that spermatogenic arrest in *Fkbp6* mutant mice takes place

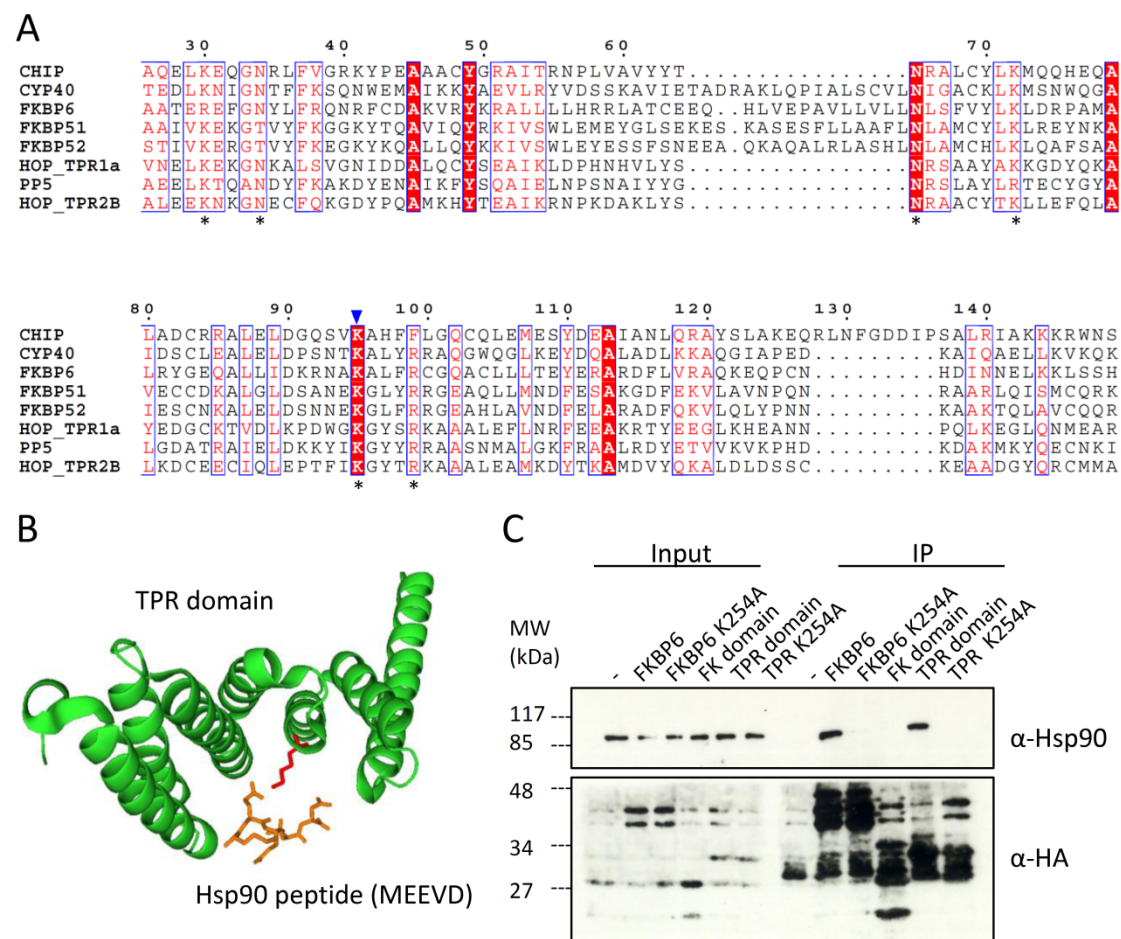
before the pachytene stage, the libraries from *Fkbp6*^{-/-} testis correspond to a pre-pachytene profile. In contrast, the *Fkbp6*^{+/-} libraries have a pachytene profile because meiosis has already reached this stage at the age at which the samples were collected (P20). Hence, these two sets of libraries cannot readily be compared as the stage difference accounts for most of the variations observed. However, comparison of the FKBP6 mutant libraries to a published wild-type pre-pachytene Mili library still shows an expansion of Line1 elements: in the library from Aravin et al. (2007), 15% of the repeat-class piRNAs are derived from Line1 elements, while this ratio is increased to 60% in the *Fkbp6*^{-/-} library.

Taken together, these results indicate that primary piRNA biogenesis is still functional in *Fkbp6* mutants, although the profile seems to be altered as a consequence of Line1 activation. A similar scenario was described in a *Tdrd9* mutant (Shoji *et al.* 2009), and probably reflects a responsive system to the activation of this transposon. However, a more detailed analysis, using younger mice for collecting the samples, is required to compare piRNA profiles from the same stages.

4.2.5 - FKBP6 Is a Co-chaperone

Considering that *Fkbp6*^{-/-} phenotype fits with a role downstream of *Tdrd1*, and that its FK domain is not catalytic but rather structural, the function of FKBP6 in the piRNA pathway could be related to Hsp90 recruitment through its TPR domain. An alignment with other TPR domains shows that the residues that mediate interaction with Hsp90 (indicated with an asterisk) are conserved in FKBP6 (Fig. 4.9A). Mutation of a single lysine to an alanine (pointed with a blue triangle, corresponding to lysine 254 in FKBP6), was shown to be sufficient to abolish interaction between FKBP52 and Hsp90 (Riggs *et al.* 2003). As seen in the crystal structure of the TPR domain of FKBP5 together with the MEEVD peptide from Hsp90 (Wu *et al.* 2004), this lysine establishes direct contact with the peptide (Fig. 4.9B). In order to determine whether FKBP6 interacts with Hsp90, hemagglutinin (HA)-tagged deletion versions of FKBP6, as well as the corresponding K254A mutant, were expressed in HEK293 cells. Anti-HA immunoprecipitation and Western blot analysis with antibodies against endogenous Hsp90 revealed specific association of FKBP6's TPR domain to Hsp90,

which is abolished upon mutation of lysine 254 (Fig. 4.9C). This confirms that FKBP6 is a co-chaperone and, based on interaction data, it could be bridging PIWI complexes to Hsp90.



4.3 – Discussion and Future Perspectives

Tudor domain-containing proteins bind methylated arginines in the N-termini of PIWI proteins (Nishida *et al.* 2009; Reuter *et al.* 2009; Vagin *et al.* 2009; Kirino *et al.* 2010). A good number of Tudor proteins have been identified in the piRNA pathway, but their exact functions are poorly understood. In particular, Tdrd1 was shown to play a role in production of secondary piRNAs from Line1 sequences, which is required for silencing of these elements by Miwi2-mediated DNA methylation (Reuter *et al.* 2009; Vagin *et al.* 2009). Here we have shown that Tdrd1's MYND domain recruits FKBP6, which additionally interacts with PIWI proteins through an inactive prolyl *cis-trans* isomerase domain. Interestingly, an uncharacterized *Drosophila* Tudor protein containing a MYND domain (CG9925) was reported to interact with a putative PPIase from the cyclophilin family (CG3511) in a genome-wide two-hybrid screen (Giot *et al.* 2003). Although no link to the piRNA pathway has been proven for this case, this might reflect a conserved role for Tudor/MYND proteins in recruiting immunophilins.

We also confirm that FKBP6 interacts with Hsp90 through its TPR domain. Consistent with a role of FKBP6 in the piRNA pathway, Line1 elements are derepressed and demethylated in the male germ line of an *Fkbp6* mutant mouse, leading to an arrest in spermatogenesis. FKBP6 has been implicated in glyceraldehyde-3-phosphate dehydrogenase (GAPDH) inhibition (Jarczowski *et al.* 2009), disassembly of clathrin vesicles (Jarczowski *et al.* 2008) and homologous chromosome pairing during meiosis (Crackower *et al.* 2003). It is possible that, as a co-chaperone, FKBP6 has multiple functions, but its role in transposon silencing by the piRNA pathway seems the most plausible explanation to the fertility defects observed in the mutant, given the similarity of the phenotype with that of other piRNA pathway components.

We show that FKBP6 catalytic domain interacts with a proline-rich region in the N-terminus of Mili, which also contains the arginines that are substrates for symmetrical dimethylation. The residues in FKBP6's catalytic site are not conserved with active FKBP6s, and we could not detect any PPIase activity *in vitro*. A further

proof for FKBP6 being inactive is its inability to bind FK506, an immunosuppressive drug that has been reported to inhibit all active FKBP6s. Jarczowski *et al.* (2008) show that FKBP6 recovers residual activity upon mutation of arginine 82 to leucine in the active site. They speculate that, *in vivo*, some unknown post-translational modifications or activators could compensate for the lack of conservation of the catalytic residues. It is possible that, *in vitro*, mutation of all catalytic residues back to the ones in active FKBP6s allows FKBP6 to gain full activity, given that the overall shape of the catalytic pocket is conserved. However, there is evidence arguing against a requirement for FKBP6 being active *in vivo*: while patients treated with rapamycin display sterility problems, probably due to inhibition of mTOR pathway by FKBP12 (Deutsch *et al.* 2007; Zuber *et al.* 2008), treatment with FK506, which would inhibit FKBP6, has little or no reported effect on male fertility (Huyghe *et al.* 2007; Skrzypek and Krause 2007). Moreover, studies in rats have shown that progression of meiosis is not affected by FK506 treatment (Tai *et al.* 1994; Hisatomi *et al.* 1996). Altogether, these data suggest that the role of FKBP6 in spermatogenesis is unrelated to prolyl *cis-trans* isomerization. Instead, we propose that the FK domain has evolved into a structural module to bind the proline-rich N-terminus of Mili.

We have attempted to examine whether FKBP6 binding to Mili interferes with arginine symmetrical dimethylation. Making use of an assay that was originally developed for analysing sDMA modification of Sm proteins (Meister *et al.* 2001), we could show that FKBP6 highly reduces methylation of Mili *in vitro*, probably because the steric hindrance posed by a bulky protein prevents the methyltransferase Prmt5 from reaching the substrate arginines. This result further proves that FKBP6 interacts with the N-terminus of Mili, but it is difficult to estimate whether this has any relevance *in vivo*, as FKBP6 might bind Mili when it is already methylated. However, this assay could be useful to analyse how other factors influence PIWI protein methylation.

Our data suggest that FKBP6 acts as a bridge between piRNPs and Hsp90. We have shown that Hsp90 and FKBP6 interact, confirming that FKBP6 is a co-chaperone. Co-chaperones modulate Hsp90 function in different ways, by coordinating interplay between Hsp90 and other chaperone systems, enhancing or

inhibiting Hsp90's ATPase activity or recruiting Hsp90 to client proteins (Taipale *et al.* 2010). We speculate that FKBP6 can target Hsp90 to piRNPs upon recruitment by Tdrd1's MYND domain. Hsp90 participates in miRNA and siRNA pathways, mediating an ATP-dependent conformational change that opens Ago proteins so that they can take bulky small RNA duplexes (Iki *et al.* 2010; Iwasaki *et al.* 2010; Miyoshi *et al.* 2010). Hsp90 could potentially play a similar role in the piRNA pathway, but piRNA precursors are thought to be single-stranded (Aravin *et al.* 2006; Brennecke *et al.* 2007). Accordingly, primary piRNA biogenesis is not affected in *Tdrd1* and *Fkbp6* mutants. In contrast, it is possible that ping-pong amplification requires an ATP-dependent step mediated by the chaperone machinery. Secondary piRNAs are drastically reduced in *Tdrd1* mutant (Vagin *et al.* 2009), perhaps indicating a failure in recruiting Hsp90. Besides, some piRNAs produced through this mechanism are absent in *Drosophila Hsp83* mutant (Specchia *et al.* 2010). Such a model would predict that ping-pong signatures are lost in an *Fkbp6* mutant. Profiling of embryonic piRNA populations will be required to confirm this hypothesis. A similar requirement of co-chaperone might also be true for Ago proteins, given that addition of recombinant Hsc70-4 and Hsp83 is not sufficient to reconstitute RISC-loading activity of small RNA duplexes (Iwasaki *et al.* 2010). It would be interesting to determine which co-chaperone specifically recruits Hsp90 to Ago complexes.

Hsp90 has an additional function in Ago proteins stabilization and localization to P-bodies (Johnston *et al.* 2010). This activity could be uncoupled from that of loading small RNA duplexes thank to the use of an *in vitro* system to test RISC formation. Mouse PIWI proteins also localize in granules (Aravin *et al.* 2009) and this might as well be dependent on the chaperone machinery. The fact that there is no *in vitro* system that recapitulates piRNA biogenesis might hinder the study of these two separate functions. However, a recently characterized cell line (BmN4) derived from *Bombyx mori* ovaries offers a great potential for the study of FKBP6 and Hsp90, given that it is the first cell line where the ping-pong cycle has been shown to be operating (Kawaoka *et al.* 2009). This will allow using drug treatment to block Hsp90, as well as point mutants of FKBP6 that abolish binding to Hsp90. *In vitro*, the use of ATP analogues allowed determining the step at which ATP hydrolysis is required for RISC loading (Iki *et al.* 2010). In a cell line, an Hsp90 mutant that can

bind ATP but is defective in hydrolysis could be used as a similar tool, trying to trap Hsp90 on PIWI complexes to uncover its function in biogenesis. Loss of PIWI protein localization to granules in BmN4 cells has already been observed upon abolishing binding to Tudor proteins (Pillai lab, unpublished data), and it would be interesting to determine if a similar effect is observed upon blocking chaperone function.

Taking together our data and published evidence, we propose a very speculative model for the role of FKBP6 and Hsp90 in the piRNA pathway. One of the questions concerning secondary biogenesis is how a PIWI protein is able to recruit another PIWI protein after target slicing. We have seen that Tdrd1 recruits FKBP6 and probably Hsp90. The fact that Hsp90 functions as a dimer, with each of the monomers being able to bind a co-chaperone (Taipale *et al.* 2010), might indicate that it bridges assembly of the two PIWI proteins and uses its ATPase activity to allow loading of the newly generated 5' end. However, Hsp90 interacts with *Drosophila* Piwi (Gangaraju *et al.* 2011), which does not participate in ping-pong amplification (Li *et al.* 2009; Malone *et al.* 2009), arguing against this hypothesis. On the other hand, Hsp90 facilitates maturation of a very wide range of proteins, so it could play different roles in each of the cases. Alternatively, the chaperone machinery could have a common function in mediating PIWI complex assembly or remodelling, given the complexity of such RNPs.

To conclude, this study has characterized FKBP6 as the factor that is recruited by Tdrd1's MYND domain. Analysis of an *Fkbp6* mutant mouse supports the idea of FKBP6 acting downstream of Tdrd1, but more detailed experimentation is required to nail down the impact of FKBP6 on the piRNA pathway. There is evidence pointing to chaperone recruitment as the function of the Tdrd1-FKBP6 complex, and we have proposed a very hypothetical model to explain the role of Hsp90 in piRNA biogenesis. Although there are interesting hints supporting it, further investigation will be needed to prove this model and to understand the molecular details of FKBP6's function.

5 - Concluding Remarks

piRNAs participate in transposon silencing in animal gonads and are essential for protecting the genome integrity in the germ line. Similar to miRNAs and siRNAs, piRNAs associate with members of the Argonaute family of proteins, in this case with members of the PIWI clade. However, fundamental questions regarding their biogenesis and function remain unsolved, as the molecular mechanisms underlying these pathways have not been elucidated. From a historical perspective, identification of factors other than Argonaute proteins in small RNA pathways has greatly contributed to their understanding. Examples of this are the discovery of Dicer and Drosha, which explained small RNA biogenesis (Bernstein *et al.* 2001; Lee *et al.* 2003), or GW182, which allowed understanding of how miRNAs mediate target mRNA decay (Ding *et al.* 2005; Fabian *et al.* 2009). Therefore, the approach taken by our research group and for this study in particular has been to try to identify and characterise novel factors that participate in the piRNA pathway.

Following the identification of the putative RNA helicase MOV10L1 in murine PIWI complexes, we have found that MOV10L1 associates with PIWI proteins and piRNAs and is essential for male spermatogenesis and transposon silencing by DNA methylation. We have characterised the first mouse mutant (other than the mutants for PIWI proteins themselves) that is completely devoid of piRNAs, and this allowed us to place MOV10L1 as a primary biogenesis factor. Given that no such factors had been identified before in mouse, we believe that this finding sets a starting point for the understanding of primary piRNA biogenesis.

A major advance in recent piRNA research has been the discovery that Tudor proteins participate in the piRNA pathway through interaction with methylated arginines in the N-termini of PIWI proteins (reviewed in Siomi *et al.* 2010). Tudor protein mutants have been analysed, both in mouse and *Drosophila*, and impacts on localization and piRNA biogenesis have been found, but the exact role of each of these proteins remains elusive. Here we have shown that Tdrd1 uses its MYND domain to recruit FKBP6 to PIWI complexes. We have confirmed that FKBP6 is required for

transposon silencing and DNA methylation by the piRNA pathway. Besides, FKBP6 contains an inactive prolyl *cis-trans* isomerase domain that is used as a structural module for binding the N-terminus of PIWI proteins. FKBP6 is a co-chaperone, as its TPR domain is used for Hsp90 interaction. Given the role of Hsp90 in other small RNA biogenesis pathways and the phenotypes of *Tdrd1* and *Fkbp6* mutants, we speculate that recruitment of FKBP6 and Hsp90 by *Tdrd1* might be required for piRNA secondary amplification.

This work has characterised two novel factors in the piRNA pathway. The discovery of MOV10L1 has been followed by studies in flies that determined that its function is conserved and gave some further insights into its role, such as the interaction with Yb or the association to putative piRNA precursors (Olivieri *et al.* 2010; Saito *et al.* 2010). On the other hand, we have shown that FKBP6 is a piRNA pathway component and we have built a model that implicates Hsp90. We have performed the identification and genetic analysis of these components, and we believe that biochemical studies will uncover the mechanism by which these factors participate in the piRNA pathway.

6 - References

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