University of Alberta

Characterization of PAZ/PIWI domain proteins by identification of their binding partners

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Cell Biology

Edmonton, Alberta

Fall 2004

Your file Votre référence
ISBN: 0-612-96026-9
Our file Notre référence
ISBN: 0-612-96026-9

But, I am the blossoms of sorrow
That grow on the branches of your memory

Farrokhzaad F.

To

Fateme, Louise and Mohammad

ACKNOWLEDGEMENTS

I have been very fortunate to have wonderful people around me that supported and believed in me. I would like to thank my supervisor Dr. Tom Hobman for his guidance and mentorship throughout the years of my graduate studies toward this PhD degree. The humorous, happy and friendly environment of the Hobman lab is the healthy soil in which my ideas grew and flourished. Thank you Tom for being a great human in addition to being a wonderful mentor.

I would like to thank Dr. Henry Parker for being patient and teaching me a great deal before and after I join the Hobman lab. All the members of the lab, past and present, Darren, Martin, Tracy, John Law and Jon Carmichael, Kasia, Cezar, Jason, Margaret and Ileen thank you for being the wonderful people that you are and for the gift of friendship and for your support.

I would like to thank Dr. Richard Rachubinski and Dr. Warren Gallin, the members of my supervisory committee for their constant input of critical and interesting ideas and for their support and encouragement.

I would like to thank my lovely Canadian and non-Canadian friends in Edmonton, Randy and Susan, Don and Heather, Bijan and Nina, and Marjan, that accepted me with open arms and helped me feel I am home. This achievement would be impossible without great sacrifices from my parents. My mom, whom I never met again after coming to Canada, and my dad which joined mom two years ago. Lastly but most importantly, I want to thank Louise that made the last two years the best time of my life and supported me throughout all the hardships.

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LIST OF ABBREVIATIONS

A	Ampere
Ab	antibody
ACT	Activator of CREM
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bp	base pair
cDNA	complementary deoxyribonucleic acid
Ci	Curie
CFP	cyan fluorescent protein
cpm	count per minute
CSM	complete synthetic media
CT	carboxyl terminal
°C	degrees Celsius
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DRBD	double-stranded RNA binding domain
ds	double-stranded
EGFP	enhanced GFP
FL	full-length
g	gravitational force
GD	geldanamycin
GDP	guanosine diphosphate
GFP	green fluorescent protein
GSC	germinal stem cell
GST	glutathione S-transferase
GTP	guanosine triphosphate
HEK	human embryonic kidney
IgG	immunoglobulin G
kDa	kilo Daltons
LTR	long terminal repeats

M	moles per liter
min	minutes
miRNA	micro RNA
miRNP	micro RNA-containing ribonuleoprotein
mRNA	messenger ribonucleic acid
μ	micro ($x10^{-6}$)
μg	micro gram
μl	micro liter
NP40	nonidet P40
NRK	normal rat kidney
nt	nucleotide
NT	amino terminal
ORF	open reading frame
PAZ	PIWI, Argonaute, Zwille
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pd	pull-down
PIWI	p-element induced wimpy testis
PKR	protein kinase RNA-activated protein
PNS	post-nuclear supernatant
PPD	PAZ/PIWI domain-containing proteins
PRP	proline-argenine- proline
PTGS	post-transcriptional gene silencing
PVDF	polyvinylidene difluoride
RISC	RNA-induced silencing complex
RITS	RNA-induced initiation of transcriptional gene silencing
RNA	ribonucleic acid
RNase	ribonuclease
RNAi	RNA interference
rpm	revolutions per minute
siRNA	short interfering RNA

ss	single-stranded
stRNA	small temporal RNA
TGS	transcriptional gene silencing
UTR	untranslated region
v	volts
VIGS	virus induced gene silencing
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

1-1- Introduction to RNAi

1-1-1- The discovery of RNAi

The term RNA interference (RNAi) was coined by Fire and colleagues in reference to the potent gene-silencing by homologous double-stranded RNA (dsRNA) in *Caenorhabditis elegans* (*C. elegans*) (Fire et al., 1998). This was after a surprising observation that sense RNA was as potent as antisense in inducing gene-silencing (Guo and Kemphues, 1995). Looking at the possible synergistic effect of sense and antisense RNA, Fire and colleagues discovered that dsRNA is about ten-fold more potent than either one of the sense or antisense single-stranded RNA (ssRNA) in inducing gene silencing. They also showed that dsRNA derived from promoters and introns had a minimal effect on RNAi of the corresponding genes. Furthermore, *in situ* hybridization in embryos showed that mRNA degradation was the underlying mechanism for silencing of an abundant RNA, *mex-3*. These data indicated that RNAi, as defined by Fire et al. (1998), is a post-transcriptional gene silencing (PTGS) phenomenon. RNAi related phenomena had been reported to silence expression of transgenes (Jorgensen, 1990; Pal-Bhadra et al., 1997) and viral RNA (Dougherty et al., 1994) prior to the definition of RNAi. Considering these reports, Fire and colleagues hypothesized that RNAi is a widespread physiological gene silencing phenomenon occurring via multiple mechanisms, including heterochromatin silencing (Fire et al., 1998).

1-1-2- RNAi and related phenomena

RNAi-like phenomena had been observed in transgenic plants, plants infected with viruses, and in other eukaryotes including *C. elegans*, *Drosophila melanogaster* (*D.*

melanogaster) and *Neurospora crassa* (*N. crassa*). For example, overexpression of a chalcone synthase transgene did not deepen the color of petunia petals as expected, but instead, resulted in the blocking of the pigment formation pathway (Jorgensen, 1990; Napoli et al., 1990). This phenomenon was called "co-suppression", because inactivation of a transgene was propagated to its homologous endogenous gene (Jorgensen et al., 1996). Further investigation revealed that the potency of co-suppression was dependent on promoter strength and transcript stability, suggesting an RNA-based mechanism for co-suppression (Que et al., 1997). Co-suppression, however, was not restricted to plants, as similar phenomena were observed in *C. elegans* (Dernburg et al., 2000) and *D. melanogaster* (Pal-Bhadra et al., 1997). In parallel, it was found that plants acquire immunity to RNA viruses by targeting viral RNA for destruction (Dougherty et al., 1994). Furthermore, genes encoded by viral RNA could be silenced by a process called virus-induced gene silencing (VIGS) (Kumagai et al., 1995). Silencing of endogenous genes by VIGS was deemed to be post-transcriptional, because it targeted the exons but not the introns of the endogenous gene (Ruiz et al., 1998). Finally, quelling in *N. crassa* was defined as the silencing of an endogenous gene after ectopic integration of multiple copies of a homologous transgene (Romano and Macino, 1992). Quelling was also found to be RNA-dependent (Cogoni et al., 1996).

The mechanistic common denominator of all of these gene silencing phenomena was that they were all RNA-related. Additionally, formation of dsRNA by various strategies in different organisms seemed to be required for efficient gene silencing (Bernstein et al., 2001b). For example, the dsRNA intermediates formed by many replicating RNA viruses and transposons were thought to be the basis of VIGS (Gitlin

and Andino, 2003). In *D. melanogaster*, it had been shown that inverted arrays of transgenes, which could yield stem-loop dsRNA molecules, silenced homologous genes more effectively than tandem arrays (Dorer and Henikoff, 1994). Furthermore, "aberrant RNAs" that likely contain dsRNA structures or motifs were thought to be required for the initiation of RNA-dependent silencing responses in plants (Wassenegger and Pelissier, 1998). The requirement for dsRNA in gene-silencing phenomena in different organisms suggested that RNAi is a basic part of the silencing mechanism.

1-1-3- Two steps in RNAi

The present understanding of the molecular mechanisms underlying post-transcriptional gene silencing by RNAi has largely been derived from studies in *D. melanogaster* cell cultures and cell extracts. Kennerdell and colleagues showed that injection of dsRNA into *D. melanogaster* embryos caused potent and sequence-specific silencing of several genes involved in the wingless pathway (Kennerdell and Carthew, 1998). Then, Tuschl et al. (1999) developed the first *in vitro* RNAi system using extracts from the syncitial blastoderm of *D. melanogaster* embryos. Incubation of dsRNA *in vitro* resulted in sequence-specific silencing of a luciferase reporter by degradation of its mRNA (Tuschl et al., 1999). These studies pioneered the biochemical analysis of RNAi.

The nature of the nuclease complex that destroys mRNA and the basis of its sequence specificity were the next questions to be addressed. Sense and antisense RNA molecules of approximately 25 nucleotide (~ 25 nt) that were homologous to the genes targeted by co-suppression or VIGS were soon discovered in plants (Hamilton and Baulcombe, 1999). Using dsRNA-treated *D. melanogaster* Schnider 2 (S2) cell extracts,

Hammond and colleagues partially purified a sequence-specific mRNA degradation activity along with ~25 nt RNA molecules homologous to the silenced gene. The mRNA degradation activity was associated with an approximately 500 kDa ribonucleoprotein (~500 kDa RNP) complex that was called RISC for RNA-Induced Silencing Complex. It is thought that small RNAs confer sequence-specificity to RISC (Hammond et al., 2000).

Detection of the short RNA molecules in RISC established a biochemical connection between RNAi, co-suppression and VIGS. The question of the origin of small RNA molecules was addressed by Zamore et al. (2000). They showed that the short RNA molecules could be produced by the addition of long dsRNA, but not ssRNA, to *D. melanogaster* embryo extracts in the absence of target mRNA. They proposed that RNAi consisted of two sequential steps. An "initiation step" during which dsRNA was cleaved into 21-23 nt dsRNA molecules, and a second "effector step" resulting in sequence-specific mRNA degradation guided by antisense 21-23 nt RNA molecules (Zamore et al., 2000). The challenge now was to identify the components of the nuclease activity that produced the short RNA molecules.

1-1-3-1- The initiation step of RNAi

The term short interfering RNAs (siRNAs) was coined by Elbashir and colleagues for the 21-23 nt dsRNA molecules produced from long dsRNA during the initiation step of RNAi (Elbashir et al., 2001b). RNase III enzymes, the only known enzymes that cut dsRNA, had been speculated to be involved in RNAi (Bass, 2000). Data from Elbashir and colleagues strongly supported this speculation by demonstrating that siRNAs were 5' phosphorylated, 3' hydroxylated and contained 2 nt 3' overhangs; all characteristic of

RNase III-type catalytic activity (Elbashir et al., 2001b). Furthermore, it was discovered that synthetic siRNAs (21 and 22 nt) with 2 nt 3' overhangs were sufficient for inducing potent RNAi in *D. melanogaster* embryo extracts in the absence of long dsRNA. The 5' ends of the synthetic siRNA molecules need not be phosphorylated to be functional, since phosphorylation occurs soon after addition to cell extracts (Elbashir et al., 2001b).

The *D. melanogaster* genome encodes two classes of RNase III enzymes including Drosha (Filippov et al., 2000), and two other RNase III enzymes with an additional ATPase/helicase domain called Dicer1 and Dicer2 (Bernstein et al., 2001a). Regardless of the sequence, long dsRNAs are cleaved into siRNAs by immunoprecipitates of tagged Dicer1 or endogenous Dicer, but not by Drosha immunoprecipitates. Furthermore, ATP is required for Dicer activity in S2 cell extracts. Inactivation of Dicer1 by RNAi compromised the ability of S2 cells to silence a GFP reporter gene, suggesting that Dicer1 is required for RNAi *in vivo* (Bernstein et al., 2001a). Further investigation revealed that Dicer proteins are highly conserved in a wide range of organisms from *Schizosaccharomyces pombe* (*S. pombe*) to humans (Cerutti et al., 2000).

1-1-3-2- The effector step of RNAi

During the effector step of RNAi, RISC recognizes and destroys homologous RNA. In addition to Hammond and colleagues who partially purified RISC activity in a ~500 kDa complex (Hammond et al., 2001a), RISC activity has been purified by others in smaller complexes of ~360 kDa (Nykanen et al., 2001) and ~90-160 kDa (Martinez et al., 2002). The fact that the RISC activity could still be carried out by smaller complexes,

suggests that the ~500 kDa complex may contain components that regulate localization of RISC or perhaps the level of its activity. A detailed discussion of the protein components of RISC will be presented in section 1-3-2-4.

Both sense or antisense siRNAs can be functionally incorporated into RISCs (Elbashir et al., 2001c). Schwarz and colleagues showed that the relative and absolute propensity of the 5' ends to fray determines which siRNA strand will incorporate into RISC (Schwarz et al., 2003).

A series of elegant experiments showed that mRNA is first cleaved by RISC in 21-23 nt intervals and then degraded (Zamore et al., 2000). The mRNAs were ^{32}P-radiolabeled at their 5'cap (7-methyl-guanosine) and added to the *D. melanogaster* embryo extracts along with unlabeled dsRNAs corresponding to defined mRNA sequences. SDS-PAGE and autoradiography showed that the strongest cleavage sites were 21-23 nt apart from each other. Later it was shown by similar experimental strategies that cleavage of mRNA starts approximately 10 nucleotides from the 5'-end of the region spanned by dsRNA and continues in approximately 21-23 nt intervals (Elbashir et al., 2001b). Mapping of the mRNA cleavage sites indicated that the strongest cleavage sites correspond to the middle of the synthetic siRNA sequences. Despite the observation that mRNA cleavage occurs at 21-23 nt intervals, eventually degradation of mRNA continues to completion, even when induced by only a single siRNA (Capodici et al., 2002).

1-1-4- Transcriptional and post-transcriptional gene silencing by RNAi

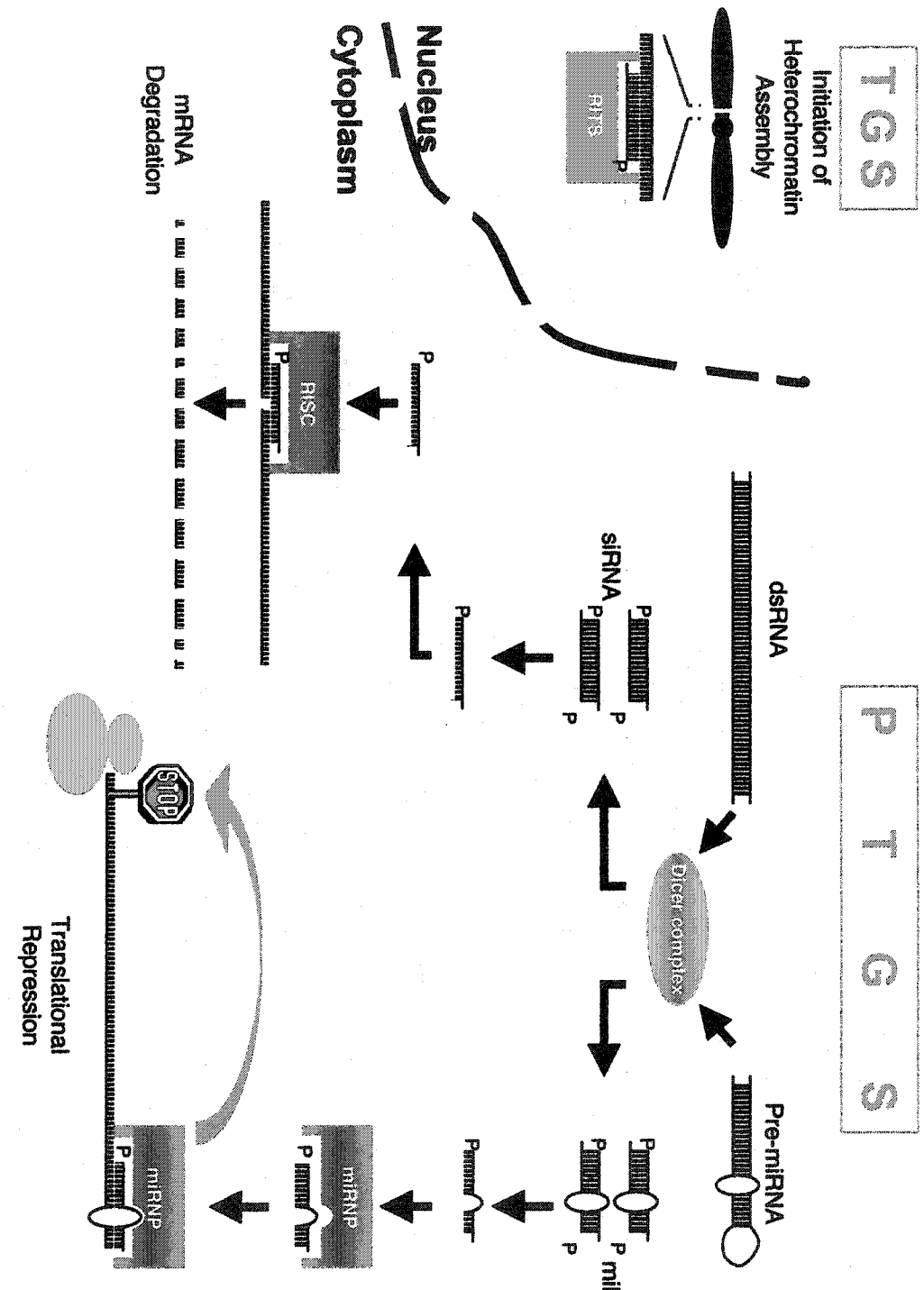
Since the original definition of RNAi by Fire and colleagues in1998, it is now clear that RNAi is not limited to gene silencing mediated by mRNA degradation.

Mechanistically related phenomena have been found to be the basis for transcriptional and post-transcriptional gene silencing (TGS and PTGS, respectively). RNAi machinery can induce TGS epigenetically without a change in DNA sequence through initiation of heterochromatin assembly (for a review, see Grewal and Moazed, 2003), or by DNA/promoter methylation (Mette et al., 2000). Alternatively, RNAi-mediated TGS may be caused by the deletion of specific DNA sequences. For example, DNA rearrangements are required for sexual reproduction in *Tetrahymena*, and this process is now known to be RNAi-dependent (Yao et al., 2003). Recently, it has been shown that heterochromatin assembly, which is initiated by RNAi (Volpe et al., 2002), takes advantage of a ribonucleoprotein complex called RITS for RNA-Induced Initiation of Transcriptional Gene Silencing Complex (Verdel et al., 2004). Incorporation of the siRNAs corresponding to the transcriptionally silenced genes into RITS, and the presence of members of PAZ/PIWI domain-containing family of proteins (PPD proteins) in RISC and RITS, suggest that RITS is the equivalent of RISC in TGS by heterochromatin assembly (Verdel et al., 2004) (Fig 1-1).

RNAi also mediates PTGS either through mRNA degradation (Elbashir et al., 2001c) or by translational repression (Grishok et al., 2001). Translational repression of *lin14* and *lin41* mRNAs by 22 nt *lin4* and *let7* small temporal RNAs (stRNA) causes developmental progression in *C. elegans* (Reinhart et al., 2000; Wightman et al., 1993). It has been shown that during the initiation step of RNAi mediated by translational repression, Dicer cleaves stRNAs from approximately 70 nt imperfect hairpin RNA precursors (Grishok et al., 2001; Ketting et al., 2001). Unlike RNAi - mediated mRNA

Figure 1-1- RNAi and regulation of gene expression.

RNAi and mechanistically related pathways are the basis of TGS and PTGS. RNAi is initiated by the cleavage of long dsRNA or approximately 70 nucleotides long imperfect hairpin RNA precursors. Cleavage of long dsRNA or hairpin RNA molecules into siRNA or miRNAs is performed by Dicer. All Dicer cleavage products contain a RNase III signature; 5' phosphorylation and a 2 nt overhang at the 3' end. During the effector step of RNAi, one of the strands of siRNA or miRNA is incorporated into RISC, or a miRNA-containing ribonucleoprotein complex (miRNP), respectively. The si/miRNA-associated RISC or miRNP complexes are competent for sequence-specific gene regulation. RISC guided by siRNA identifies a precisely homologous anti-parallel sequence in the coding region of an mRNA and degrades the target mRNA. In contrast, miRNP guided by miRNA finds an anti-parallel sequence with imperfect homology on the 3'UTR of a target mRNA and regulates its expression by translational repression. Recently, a new complex called RITS has been described in fission yeast that is required for the initiation of the heterochromatin assembly.



degradation, which requires perfect base-pairing of siRNA and its target sequence, mismatches between stRNA and their target sequences are crucial for translational repression (Doench et al., 2003; Hutvagner and Zamore, 2002). StRNAs bind to imprecisely complementary sequences at the 3'untranslated regions (3'UTR) of their target mRNAs to induce translational repression (Lee et al., 1993; Olsen and Ambros, 1999).

StRNAs now are classified together with hundreds of ~21-26 nt RNA molecules collectively known as micro RNAs (miRNAs), which are cleaved by Dicer from endogenously transcribed imperfect hairpin precursors (Carrington and Ambros, 2003; Krichevsky et al., 2003; Lau et al., 2001; Reinhart et al., 2002). During the effector step of RNAi, miRNAs are incorporated into RISC-like ribonucleoprotein complexes called miRNPs (Mourelatos et al., 2002; Schwarz and Zamore, 2002). There is ample evidence suggesting that miRNAs use stRNA-like strategies to target miRNPs for translational repression (Zeng and Cullen, 2003; Zeng et al., 2002).

1-1-5- The significance of RNAi in research and therapy

1-1-5-1- RNAi as a research tool

Functional studies of gene expression in vertebrates through site-directed mutagenesis or creation of knockouts is often complicated, time consuming and expensive (Rossant and McMahon, 1999). Compared to these conventional methods, RNAi is a much faster and cheaper way to "knockdown" expression of individual genes (Kittler and Buchholz, 2003). Initially one of the main drawbacks for the use of RNAi in research was that dsRNA longer than 30 nt triggered activation of protein-kinase RNA-activated protein

(PKR) in vertebrates. Activation of PKR results in phosphorylation of eIF2α (Levin and London, 1978), which causes a general shut down of translation and induces apoptosis through the FADD pathway (Balachandran et al., 1998). This obstacle was overcome by the direct use of siRNAs (shorter than 30 nt) for the induction of RNAi in mammalian cells (Elbashir et al., 2001a). The new technique has provided an opportunity to explore the effects of selective inhibition or downregulation of protein expression on different aspects of cellular physiology in mammalian cells. Finally, with the completion of genome projects of several multicellular organisms including human, use of RNAi will be an integral part of functional genomics (Frankish, 2003; Kamath and Ahringer, 2003; Simmer et al., 2003; Tuschl, 2003).

1-1-5-2- RNAi as a therapeutic agent

Small RNA molecules have enticing properties as therapeutic agents. They act in a sequence-specific manner providing a high level of targeting accuracy, and they have short half-lives which facilitates dosage regulation (Zamore and Aronin, 2003). In contrast to traditional drugs that target proteins (e.g., receptors or enzymes), RNAi-based drugs are expected to function at the transcriptional or post-transcriptional level to destroy defective or harmful proteins prior to their translation (Lavery and King, 2003; Shuey et al., 2002).

Many laboratories have focused their attempts on harnessing the therapeutic potential of RNAi. For example, siRNA treatment was shown to inhibit production of hepatitis B virus replicative intermediates in cell culture and in immunodeficient mice (McCaffrey et al., 2003). Moreover, siRNAs have been shown to block gene expression

and RNA synthesis of a hepatitis C subgenomic replicon in human liver cells (Wilson et al., 2003). Son et al. (2003) used siRNA-producing hairpins to target Fas, a cell surface receptor that induces apoptosis and necrosis in the liver during hepatitis. They found that siRNA treatment blocked development of disease in a mouse model of hepatitis C and improved survival (Song et al., 2003). RNAi has also been used against HIV (human immunodeficiency virus), which uses RNA intermediates at various stages of its life cycle. Investigators have also been exploring ways to target transcripts of the cellular co-factors required for HIV replication, including receptors and transcription factors (Stevenson, 2003; Surabhi and Gaynor, 2002). In oncology, RNAi has been utilized for targeting dominant or amplified oncogenes (Brummelkamp et al., 2002), or viral oncogenes (Jiang and Milner, 2002).

1-2- Physiological functions of RNAi

1-2-1- RNAi as a genome defense mechanism

RNAi in worms and plants shares common properties with the immune system of vertebrates, such as response amplification and specificity (Plasterk, 2002). Fire and colleagues noticed that injection of few copies of dsRNA is enough to trigger a general RNAi respons in worms (Fire et al., 1998). In addition, triggering RNAi by soaking *C. elegans* in dsRNA (Tabara et al., 1998), indicated that the response could not only be amplified, but it could be transferred between tissues. In plants, gene-silencing can be transferred, following grafting to a non-targeted wild-type plant and spread up to 30 cm through the stem (Palauqui et al., 1997). In addition, sequence-specificity provides RNAi with a means to recognize self from non-self. Considering that 45% of the human

genome consists of the remnants of previous transposon and virus attacks, RNAi may play a significant role in transposon silencing or protecting genomes against invaders, particularly in lower invertebrates such as *C. elegans* (Plasterk, 2002).

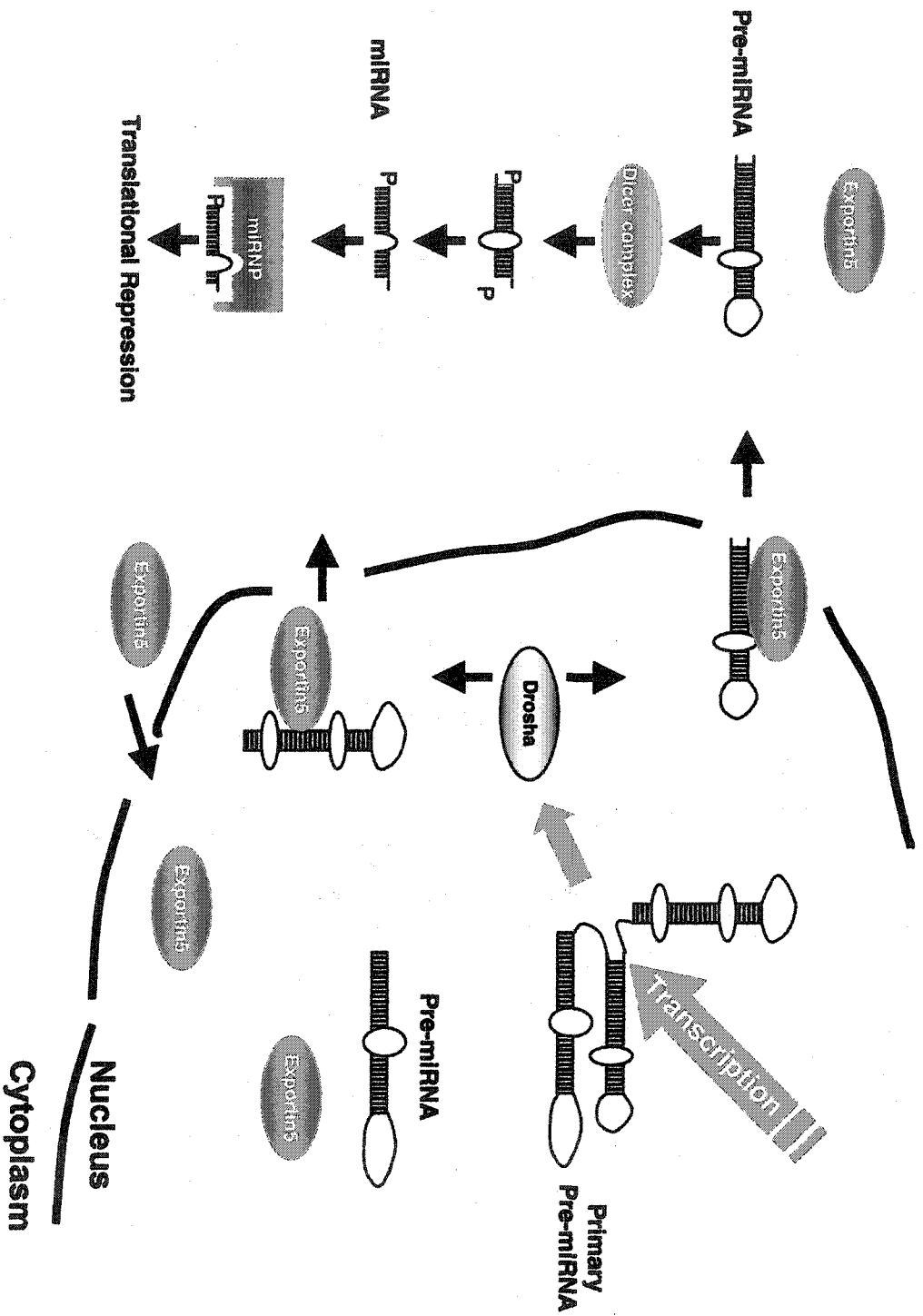
1-2-2- Regulation of gene expression by miRNAs and miRNPs

Defining an endogenous origin for dsRNA has been a challenge for scientists (Hannon, 2002). Initially, RNAi was thought of more as a defense mechanism against RNA viruses and transposable elements rather than a means to regulate gene expression (Dougherty et al., 1994; Plasterk, 2002). The discovery of miRNAs that are involved in regulation of endogenous gene expression via the RNAi machinery has permanently changed our view of how gene expression is regulated.

MiRNAs (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001) are transcribed from endogenous genes as primary pre-miRNAs in the nucleus, and are then cleaved into ~70 nt hairpin stem-loop pre-miRNAs by Drosha (Fig 1-2) (Lee et al., 2003). Pre-miRNAs are then transported to the cytoplasm by exportin-5 (Lund et al., 2003), where they are processed into miRNAs by Dicer cleavage (Hutvagner et al., 2001; Provost et al., 2002a; Zhang et al., 2002). Finally, miRNAs are incorporated into effector miRNP complexes (Dostie et al., 2003; Mourelatos et al., 2002), where an increasing body of evidence indicates that they are involved in the regulation of endogenous gene expression (Ambros, 2003; Carrington and Ambros, 2003; Finnegan and Matzke, 2003). Remarkably, miRNAs may even regulate expression of Dicer by a feed-back mechanism. Indeed, this has has been shown for *Arabidopsis thaliana* (*A. thaliana*) *miR165*, a miRNA that mediates the cleavage of *dicer-like 1* (*dcl1*) mRNA (Xie et al., 2003).

Figure 1-2- Processing of miRNAs and their incorporation into miRNPs.

Primary pre-miRNAs are transcribed from mono or poly-cistronic genes in the nucleus. Cleavage by Drosha in the nucleus results in the formation of approximately 70 nt pre-miRNA hairpins which bind to exportin5 in a Ran-GTP dependent manner and then translocate to the cytoplasm. Exportin releases miRNAs in the cytoplasm, where there is higher concentrations of Ran-GDP compared to the nucleus. Cytoplasmic processing of pre-miRNA hairpins to miRNAs is performed by Dicer. This is followed by transfer of miRNAs to miRNP complexes which regulate gene expression at the level of translation.



A large and growing number of miRNAs have been recently identified in different organisms. So far, 423 miRNAs have been isolated from the genome of *A. thaliana* (Llave et al., 2002a; Reinhart et al., 2002; Tang et al., 2003). Computational studies have estimated the presence of 200-255 miRNAs in the human genome and up to 123 miRNAs in the *C. elegans* genome (Lim et al., 2003a; Lim et al., 2003b). Approximately 30% of worm miRNAs have corresponding homologues in *D. melanogaster* and vertebrates, suggesting conserved physiological roles for these non-coding RNAs (Brennecke et al., 2003).

The presence of sequences with perfect complementarity to miRNAs in the genome of plants has facilitated identification of their candidate target genes. Interestingly, 34 of 49 predicted miRNA candidate targets in plants were transcription factors, many of them with roles in meristem development, suggesting a link between plant development and miRNAs (Rhoades et al., 2002). Furthermore, most of candidate miRNA target sequences are located in the coding region of mRNAs (Rhoades et al., 2002). Based on these observations, Rhoades et al. (2002) suggested that plant miRNAs may function as siRNAs, i.e. through mRNA degradation. This was shown to be the case for cleavage of three mRNAs encoding members of the SCARECROW family of putative transcription factors by *miR171* (Llave et al., 2002b). A later study reported that PHAVOLUTA and PHABULOSA transcripts were cleaved at the region of complementarity with *miR165/166* in wheat germ extracts (Tang et al., 2003).

Recent data indicate that miRNAs also regulate the expression of a wide variety of genes in animals. Translational repression of *lin14* and *lin41* mRNAs by *lin4* and *let7* stRNAs are the classical examples of gene expression control by miRNAs. *Lin4* and *let7*

promote transitions from early to late developmental stages and as such are referred to as heterochronic genes. *Lin4* also indirectly regulates repression of *lin28*, another heterochronic gene (Seggerson et al., 2002), and translation of the *C. elegans hbl1* (*hunchback-like*) heterochronic gene is regulated by binding of a miRNA to its 3'UTR (Abrahante et al., 2003; Lin et al., 2003). Regulation of developmental timing is but only one of many functions of miRNAs. Other examples include regulation of *D. melanogaster hid* mRNA expression, a key activator of apoptosis, by a 21 nt miRNA called *bantam*. *Bantam* was identified in a screen for mutants that exhibited growth defects, and it was subsequently determined that the *bantam* locus encoded a miRNA that repressed apoptosis and promoted cell proliferation (Brennecke et al., 2003). *MiR14* was identified in a screen for suppressors of apoptosis and probably functions through suppression of the Drice caspase. *MiR14* mutants are also stress-sensitive, have a short life span and exhibit defects in fat metabolism (Xu et al., 2003).

To date, target genes of miRNAs have not been identified in humans, but the expression of some miRNAs appears to be defective or lost in certain human diseases. For example, the steady-state levels of *miR143* and *miR145* are often reduced in the adenomatous and cancerous stages of colorectal neoplasia (Michael et al., 2003), and *miR15* and *miR16* are deleted in 68% of B-cell chronic leukemias (Krichevsky et al., 2003). Finally, Dostie and colleagues found that *miR175* is located on a candidate region for early-onset Parkinsonism and X-linked mental retardation (Dostie et al., 2003). All of these data, and the fact that many miRNAs are expressed in a tissue-specific manner (Lagos-Quintana et al., 2002), indicate that the RNAi machinery might have evolved

from a genome defense mechanism (Plasterk, 2002) to a physiological mechanism for gene regulation in higher eukaryotes.

1-3- Molecular components of RNAi

1-3-1- An overview of the RNase III family

The RNase III enzymes are the only known endoribonucleases with specificity for dsRNA. All RNase III enzymes contain one or more dsRNA-binding domains (DRBD) and RNase III domains with the signature sequence (HNERLEFLGDS) (Mian, 1997). The family has been subdivided into three classes based on domain structure: bacterial RNase III with a single RNase III domain (Nicholson, 1999), Drosha with two RNase III domains (Filippov et al., 2000), and an RNaseIII/helicase enzyme called Dicer (Bernstein et al., 2001a) (Fig 1-3.A)

1-3-1-1- The domain structure of Dicer and dsRNA cleavage

In addition to DRBD and RNase III-A and B domains, Dicer proteins often contain a central PAZ domain (section 1-3-2-1), and an N-terminal DEXH/DEAH (Asp-Glu-X/Ala-His) box-containing RNA helicase/ATPase domain (Bernstein et al., 2001a; Provost et al., 2002a; Schauer et al., 2002; Zhang et al., 2002) (Fig 1-3.A). However, no helicase activity has been demonstrated *in vitro* for Dicer. Moreover, unlike *D. melanogaster* Dicer, the function of human Dicer does not require ATP for dsRNA cleavage *in vitro*. Since the catalytic activity of the enzyme is low in typical *in vitro* assays, and RNA does not dissociate from the enzyme, it has been suggested that the

ATPase activity may be required for RNA dissociation (Provost et al., 2002a; Zhang et al., 2002).

Determination of the three dimensional structure of a bacterial RNase III (*Aquifex aeolicus* RNase or AaRNase) allowed the development of a model for the manner in which Dicer processes long dsRNA into ~21-23 nt dsRNAs (Fig 1-3. B, C, D). The cleavage of dsRNA requires antiparallel homodimerization of two RNase III domains that form a tunnel for the incoming dsRNA molecule. Two bacterial RNase III molecules form the tunnel and secure the homodimer by two "ball-and-socket" junctions on the other side of the molecule. This orientation creates two compound dsRNA-cleavage sites at the ends of the tunnel. The cleavage sites contain highly conserved residues including glutamic acid 64 and 37 (E64 and E37) in antiparallel bacterial AaRNase molecules (molecules A and B in Dimer 1 or 2, Fig 1-3.B). Promotion of the RNase III activity by binding of Mn^{2+} or Mg^{2+} results in the cleavage of long dsRNA into 13 nt long pieces with 2 nt 3' overhangs by a bacterial homodimer (Blaszczyk et al., 2001).

Formation of a dsRNA-cleavage tunnel similar to that observed in bacteria might be achieved in a single molecule of Dicer by anti-parallel dimerization of the RNase III-A and B domains. Alternatively, dimerization of two Dicer molecules could create a dsRNA processing tunnel that is twice as long as the bacterial RNase III tunnel. It has been reported that replacement of the original glutamic acid (E64 in AaRNase) with proline (P1731 in Dicer RNase III-B) destroys the carboxyl terminal dsRNA-cleavage site of the RNase III-B of Dicer (Blaszczyk et al., 2001). This was supported by experimental evidence showing that substitution of E64 with a proline in bacterial AaRNase destroyed

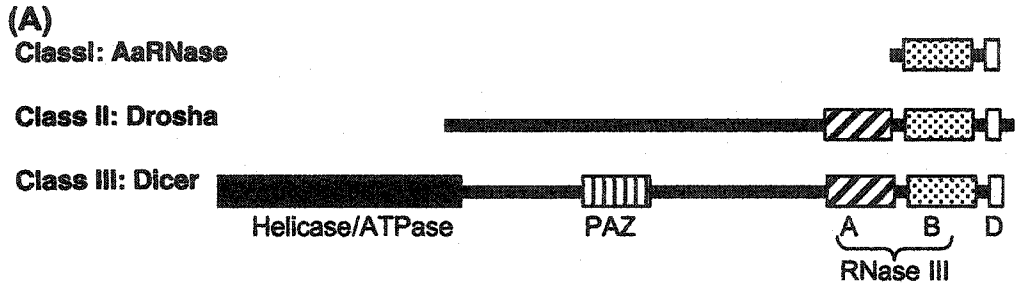
Figure 1-3- The RNase III family and processing of dsRNA by Dicer

A) Schematic representation of the domain structure of RNase III family members, including Class I: bacterial RNase III (*Aquifex aeolicus* RNase or AaRNase), Class II: *D. melanogaster* Drosha, and Class III: human Dicer. All RNase III proteins contain a double-stranded RNA-binding domain (D) and at least one RNase III domain. Drosha and Dicer contain two RNase III domains (A and B). In addition, Dicer proteins contain an ATPase/helicase domain and, in most cases, a PAZ domain.

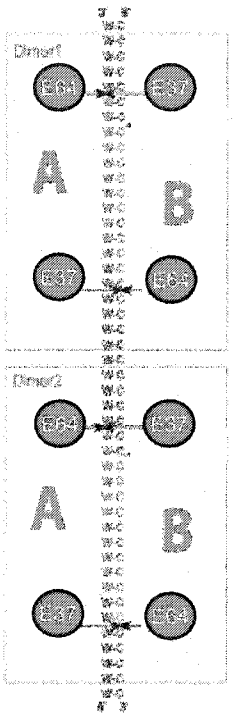
B) A model showing two independent dimeric bacterial RNase III molecules. The tunnel created by each bacterial dimer (1 or 2) cleaves dsRNA into short dsRNA molecules (distinguished with alternate colors), each containing a 9-bp dsRNA segment with a 2 base 3' overhang. The cleavage centers include conserved glutamic acid residues at positions E64 and E37 in the antiparallel members of the AaRNase dimer (Blaszczyk et. al., 2001).

C) A space filling model of (B). Protein dimers 1 and 2 are illustrated as a surface representation model; dsRNA products are shown as van der Waals spheres with alternate colors (Blaszczyk et. al., 2001).

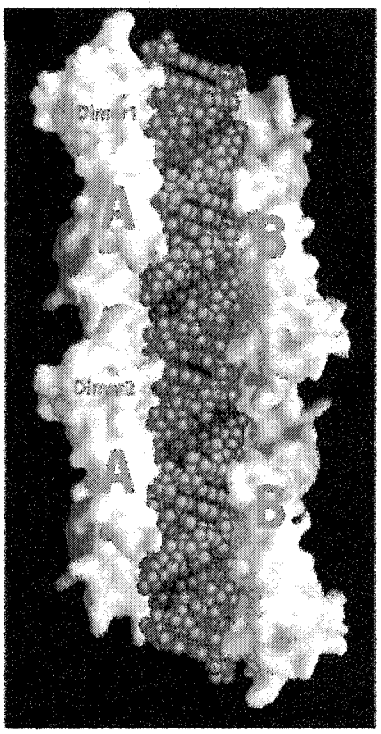
D) A schematic model showing a 22 nt dsRNA-processing tunnel formed by a Dicer dimer. Intramolecular dimerization of RNase III-A and B domains of Dicer results in the formation of 11 nt long dsRNA-processing tunnels. In the bacterial dimer the binding of Mg^{2+} to RNase III domains results in the activation of the dsRNA-cleavage centers with E37 in one molecule and E64 in the other (B). Since only cutting centers 2 and 3 remain active in Dicer dimers, the dsRNA processing by the Dicer dimer results in 22 nt dsRNA molecules.



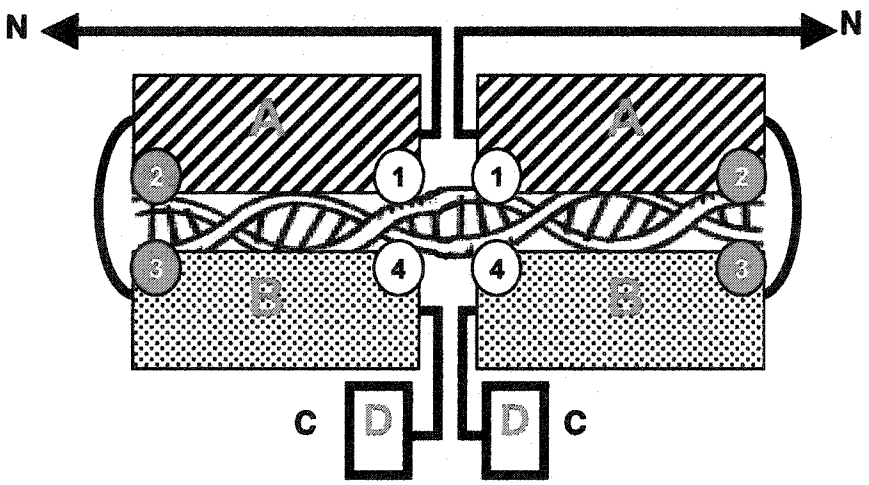
(B)



(C)



(D)



one of the active sites in a bacterial RNase III homodimer (Blaszczyk et al., 2001; Hannon, 2002). However, dimerization of two Dicer molecules would keep the inactive cutting sites in the middle of the long tunnel (inactive sites 1 and 4 in Fig 1-3.D). The Dicer dimers could then only cut dsRNA at the ends of the two tunnels. In this model, the distance between the cutting sites located at the two ends of the tunnels (~21-23 nt in a dsRNA scale) determines the length of the Dicer cleavage products.

1-3-1-2- Genetic analysis of Dicer

There are four *dicer-like* (*dcl*) genes in *A. thaliana*; however, only *dcl1* has been characterized to date (Schauer et al., 2002). Mutation of *dcl1* blocks miRNA processing but does not affect siRNA cleavage, suggesting that miRNAs and siRNAs are cleaved by different enzymes in *A. thaliana* (Finnegan et al., 2003). Null alleles of *dcl1* cause early embryonic arrest, while the hypomorphic alleles result in a variety of developmental defects (Jacobsen et al., 1999; Ray et al., 1996).

MiRNAs are detected in zebrafish *dicer1* mutants during the first week of development (Wienholds et al., 2003). However, these miRNAs disappear during the second week of the development, suggesting that they are of maternal origin. Lack of miRNAs in the second week of embryonic life of *dicer1* mutants is followed by embryonic arrest. These data suggest that the zebrafish Dicer1 is probably involved in the regulation of development through processing of miRNAs.

Dicer knockout mice do not survive past embryonic day 8.5, and *in situ* hybridization for Oct4, an embryonic stem cell marker, showed that they are depleted of

stem cells. These data suggest that Dicer is involved in regulation of stem cell maintenance and differentiation (Bernstein et al., 2003a and 2003b).

In conclusion, a link between defective miRNA processing, stem cell depletion and developmental arrest exists in Dicer mutants. However, a detailed molecular analysis of the connection between these defects is still lacking.

1-3-1-3- Dicer and its binding partners

Dicer has been found to interact with several proteins, including *C. elegans* Rde1 and Rde4 (Tabara et al., 2002), *D. melanogaster* R2d2 (Liu et al., 2003) and human PPD proteins (Doi et al., 2003; Sasaki et al., 2003; Tahbaz et al., 2004). These interactions may provide Dicer with dsRNA, mediate transfer of siRNA to RISC or miRNP, regulate Dicer activity and/or localize Dicer to specific subcellular compartments.

C. elegans RNAi-deficient mutants, *rde1* and *rde4,* are not defective in development or transposon silencing (Tabara et al., 1999). Parrish and Fire (2001) later showed that addition of siRNA could restore RNAi in *rde4*, but not in *rde1*, mutants. This observation suggested that *rde4 (+)* activity was required during the initiation step of RNAi, while the activity of *rde1(+)* was mainly required at the effector step (Parrish and Fire, 2001). Later, Tabara et al. (2002) demonstrated that the *rde4* gene encodes a dsRNA-binding protein and the interaction of Rde4 protein with long dsRNA was dependent on its binding to Rde1. Finally, they also showed that Rde4 interacted with Dcr1 enzyme (*C. elegans* Dicer), an interaction thought to be important for dsRNA transfer (Tabara et al., 2002).

Recently, a *D. melanogaster* homologue of Rde4 (~33% amino acid identity) was detected in a purified siRNA-generating activity. Since this novel protein contained two dsRNA-binding domains and interacted with Dicer2, not Dicer1, the authors called it R2d2 (Liu et al., 2003). RNAi-induced silencing of a reporter GFP construct was abolished in homozygous *r2d2* null embryos, indicating that R2d2 protein is involved in RNAi *in vivo*. A mutation that abolished dsRNA-binding of R2d2 ($R2d2^M$) did not affect R2d2 binding to Dicer2 or Dicer activity, but inhibited Dicer2 from binding to siRNA. To determine if R2d2 played a role in coupling Dicer2 and RISC activities, the authors purified RISC activity from RNAi-competent S2 cell extracts. The RISC activity initiated by the siRNA was stimulated after the addition of recombinant Dicer2/R2d2, but inhibited dose-dependently by Dicer2/$R2d2^M$. Next, Liu et al. (2003) used biotinylated siRNAs to co-purify *D. melanogaster* Ago2 (dAgo2), a known component of RISC, from siRNA-activated RISC reactions. Addition of Dicer2/R2d2, but not Dicer2/$R2d2^M$ or Dicer alone, increased association of dAgo2 with siRNAs. This observation suggested that the siRNA-binding activity of R2d2 was required for the transfer of siRNAs to RISC. Remarkably, Liu et al. (2003) did not show a direct interaction between R2d2 and dAgo2, indicating a possible role for dAgo2-Dicer2 interaction during siRNA transfer to RISC. Interaction between dAgo2 and Dicer1 had been previously reported (Hammond et al., 2001a) .

1-3-1-4- Requirement for Dicer during the effector step of RNAi

Existing evidence suggests that Dicer is required for siRNA-dependent gene silencing in mammalian cells, and thus might function during the effector step of RNAi.

Plasmids encoding firefly and *Renilla* luciferase, as well as siRNA corresponding to firefly luciferase, were transfected into different mammalian cell lines. After normalizing the silencing results to firefly/*Renilla* luciferase transfection efficiency, a five-fold sequence-specific silencing induced by siRNA was observed (Doi et al., 2003). RNAi knockdown of Dicer activity abolished sequence-specific silencing of the firefly luciferase transgene by siRNA in all mammalian cell lines tested. Knockdown of hAgo1-4 (human homologues of PPD proteins) by RNAi in parallel experiments indicated that downregulation of hAgo1 had the strongest inhibitory effect, similar to Dicer knockdown, on silencing firefly luciferase by siRNA. The siRNA-dependent silencing of an EGFP transgene was also abolished after downregulation of Dicer and hAgo1 by RNAi in mouse F9 cells. Again the results were normalized to the transfection efficiency and an unrelated siRNA did not induce any silencing of EGFP. These results suggested that in addition to the established role of Dicer in dsRNA-cleavage in the initial step, Dicer activity is required during the effector step of RNAi. The data from Doi et al. (2003) also indicate that the role of hAgo1 in the effector step of RNAi is the most significant among hAgo homologues. The authors then asked whether the effects of knockdown of hAgo1 and Dicer on siRNA-dependent silencing of firefly luciferase were additive. To detect the possible synergistic effects, lower concentrations of Dicer and hAgo1 siRNAs with minimal detectable effects on the inhibition of firefly luciferase silencing were used. The double-knockdown induced synergistic effects on siRNA-dependent silencing of firefly luciferase (Doi et al., 2003), suggesting that Dicer has hAgo1-independent activities, and thus functions other than in siRNA transfer during the effector step of RNAi.

A closer look at the literature provides more evidence to support a role for Dicer in the effector step of RNAi. Liu et al. (2003) precipitated Dicer activity partially (in 10% polyethylene glycol; PEG) or completely (in 15% PEG) from RISC activity present in the cytosolic fraction of dsRNA-activated S2 cell extracts. Addition of siRNA to reactions restored RISC activity only if the depletion of the Dicer activity was partial (Liu et al., 2003). These data are in agreement with the notion that Dicer is essential for the RISC activity.

1-3-2- An overview of the PPD family members

PAZ and PIWI domain-containing proteins are highly basic approximately 100 kDa proteins which are present in most, if not all, eukaryotes except *Saccharomyces cerevisiae* (*S. cerevisiae*) (Carmell et al., 2002). PPD family members can be divided into the Ago and Piwi subfamilies based on sequence homology as well as developmental stage and tissue-specific expression. Proteins of the Ago subfamily show high sequence similarity to *A. thaliana* Argonaute 1 (Ago1), and all members except dAgo2 contain conserved PRP motifs (proline, arginine, proline) (see section 1-3-2-1 and Chapter 5). The Ago subfamily members are ubiquitously expressed throughout development in most tissues (Carmell et al., 2002). In contrast, the Piwi subfamily members (whose sequences closely resemble *D. melanogaster* Piwi) are expressed mainly in early developmental stages and in a tissue-specific manner (Carmell et al., 2002). The PPD family also contains two orphan members: Rde1 in *C. elegans* and Qde2 in *N. crassa.*

The numbers of genes encoding PPD proteins vary in different organisms (Fig 1-4). For example, the genome of *C. elegans* contains the highest number with 24 PPD

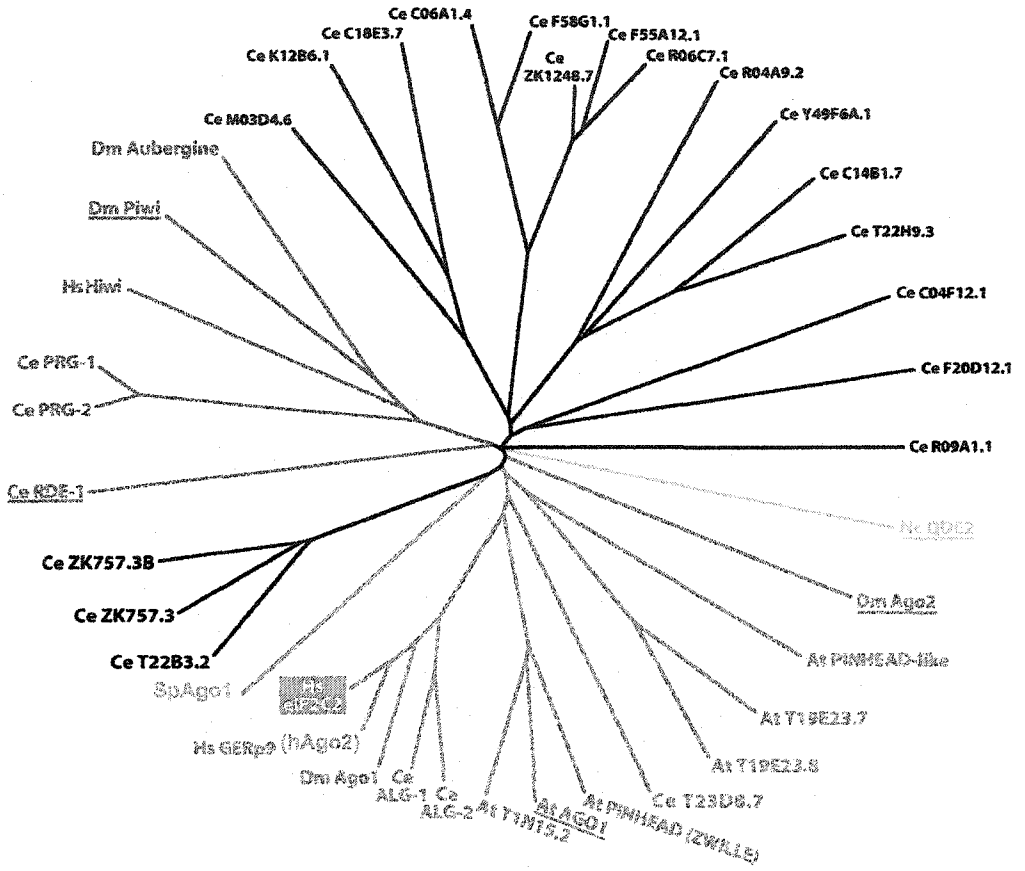


Figure 1- 4- The PPD family

(At) *A. thaliana*; (Ce) *C. elegans*; (Dm) *D. melanogaster*; (Nc) *N. crassa*; (Hs) *Homo sapiens*; (Sp) *S. pombe*. Piwi subfamily is shown in red and Ago subfamily is in green. Rde1, Qde2 and perhaps *D. melanogaster* Ago2 are the orphan members of the PPD family (adapted from Schwarz et al., 2002).

genes, while *S. pombe* has only one. Except for *A. thaliana* which has no Piwi homologues, all other organisms with multiple PPD family members encode both Ago and Piwi homologues. There are 10 PPD genes in the *A. thaliana* genome with only four of them (*ago1, zwille, ago7 and ago4*) characterized. Only five PPD genes, including *alg1* and *alg2, prg1* and *prg2* and *rde1* have been characterized in *C. elegans*. The *D. melanogaster* genome contains five PPD genes, four of which (*piwi, aubergine, dago1* and *dago2*) have been studied in detail. Finally, eight PPD genes have been identified in both genomes of mouse and human with *miwi* (*mouse piwi*), *mili* (*miwi-like*) or *hago2* (*human ago2*) and *hiwi* (*human piwi*) characterized (for a recent review, see Carmell et al., 2002).

1-3-2-1- General structure of PPD proteins

PAZ, named after three of the original members of the PPD family (Piwi, Argonaute and Zwille), is a centrally located domain comprised of 110-130 amino acid residues (Cerutti et al., 2000) (Fig 1-5). The nuclear magnetic resonance (NMR) structure of the PAZ domain of dAgo1 indicates that the core of PAZ consists of a six-stranded ß-barrel annexed with a hairpin ß-stranded appendage (Yan et al., 2003) (Fig 1-6.A). The groove between the open surface of the barrel and the hairpin can bind to short ssRNA molecules (Fig 1-6.B). Unlabeled RNA, but not DNA, was shown to effectively compete away labeled RNA binding to the dAgo1 PAZ domain in an electrophoretic mobility shift assay (Yan et al., 2003). In addition, the NMR data suggest that PAZ domains preferentially bind to RNase III cleavage products, which are phosphorylated at their 5' ends. Finally, the authors demonstrated that ssRNA binding is a general function of PAZ

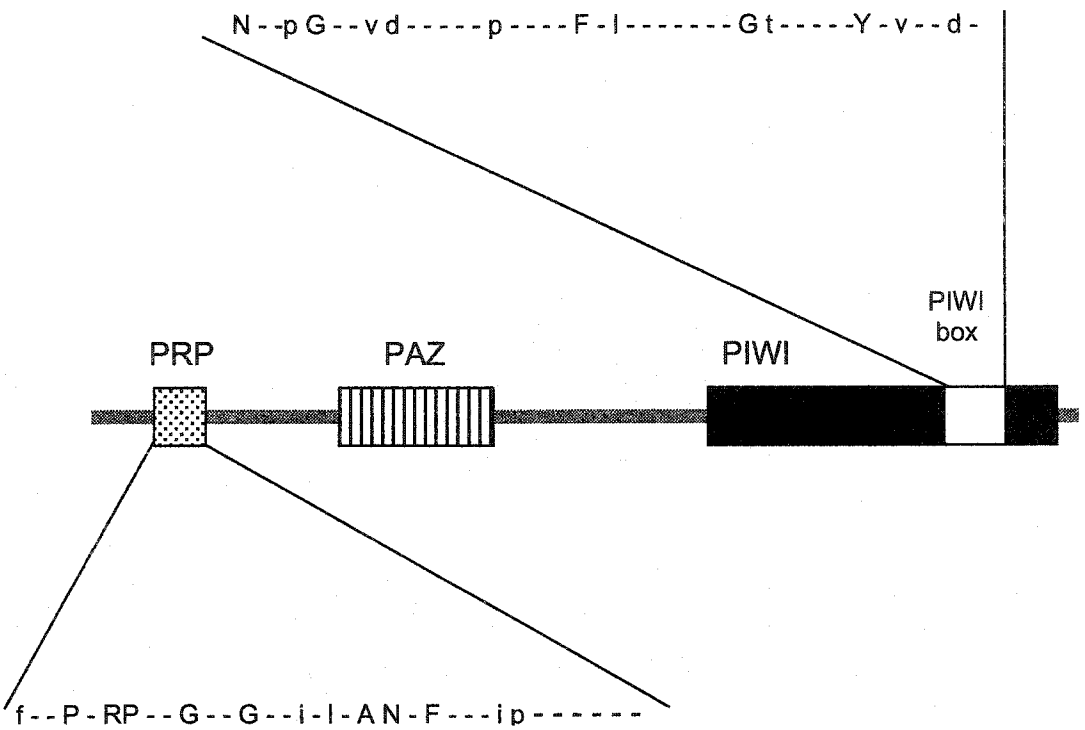


Figure 1-5- General structure of PPD proteins

PPD proteins contain PAZ and PIWI domains. PIWI-box is a highly conserved part of the PIWI domain in which the identity and position of 5 amino acids are completely conserved among all PPD proteins. PRP motif is only present in Ago subfamily members. The identity and position of 8 amino acids are completely conserved in PRP motifs. The amino acids that are completely conserved are in upper case. The less conserved amino acids are in lower case.

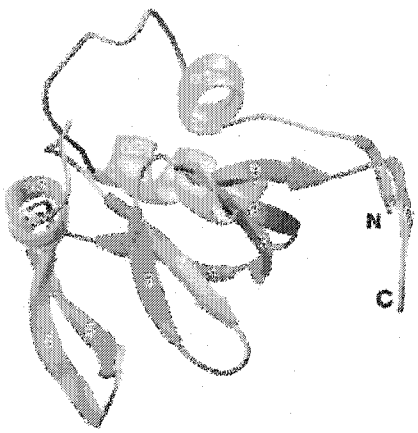
Figure 1-6- The three-dimensional structure of the dAgo1 and dAgo2 PAZ domains and their RNA-binding sites

A) Top view of the 25 superimposed NMR-derived structures of the dAgo1 PAZ domain (residues 298-430). The C-terminal unstructured residues (431-464) are omitted for clarity. The barrel formed by the β-sheets 2-9 and the appendage formed by β-sheets 5 and 6 are shown.

B) The surface electrostatic potential (blue, positive; red, negative) of the PAZ domain of dAgo1, displayed in the same orientation as A. The short ssRNA molecule binds in the cleft located between the β-sheet appendage and the barrel.

C) Molecular surface of the PAZ domain of the dAgo2 protein highlighting residues that are involved in binding to ssRNA. The orientation is the same as in A and B. The ssRNA-binding cleft is shown in green (Yan et al., 2003).

A)



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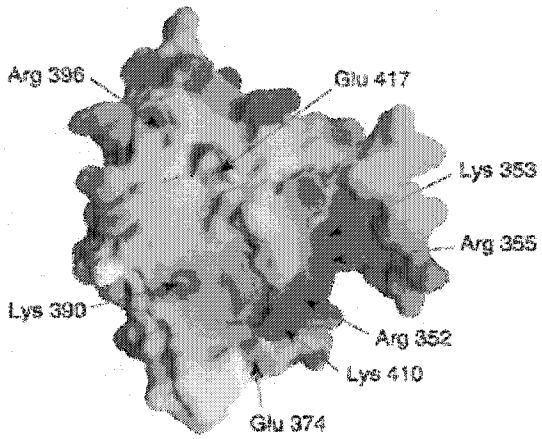

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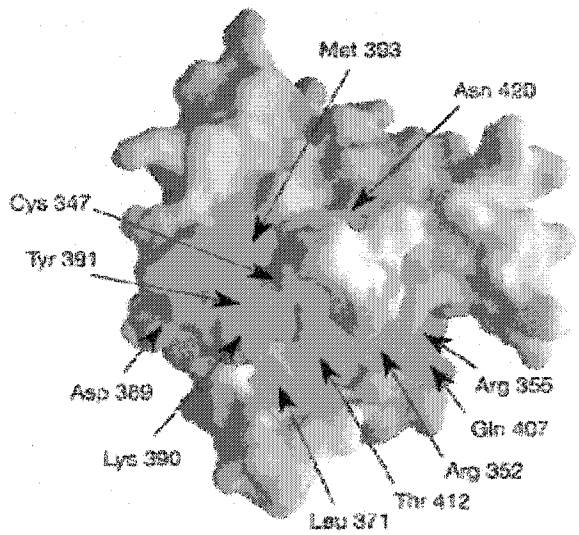


domains by showing that PAZ domains from multiple human PPD proteins (hAgo1, hAgo2 and Piwi-like 1) interact with ssRNA (Yan et al., 2003).

Resolution of the NMR structure of the PAZ domain of dAgo2 showed similar results (Lingel et al., 2003). A significant difference was that a ssDNA could compete with ssRNA for binding to dAgo2 PAZ domain, while, as mentioned, the dAgo1 PAZ domain bound only ssRNA. The observation that dAgo2 interacts with ssDNA supports the proposed role of RNAi machinery in the regulation of the transcriptional gene silencing, a process that may require RNA-DNA duplex formation (for a review, see Grewal and Moazed, 2003; Jenuwein, 2002).

The PIWI domains are highly conserved C-terminal domains approximately 300 amino acid residues in length (Cox et al., 1998) (Fig 1-5). They are present in the PPD proteins of all eukaryotes and in a limited number of prokaryotic proteins (Cerutti et al., 2000). The function of the PIWI domain is still largely unknown, but it has been shown recently that this domain is involved in interactions between PPD proteins and Dicer (Doi et al., 2003; Tahbaz et al., 2004). A highly conserved 43 amino acid motif, referred to as the PIWI-box, has been identified in PIWI domains (Cox et al., 1998). The identity and position of five amino acid residues in the PIWI-boxes are completely conserved in all known PPD proteins (Fig 1-5). The Dicer binding site on the PIWI domain of PPD proteins have been mapped to the PIWI-box (Tahbaz et al., 2004).

All members of the Ago subfamily, except *D. melanogaster* Ago2 (dAgo2), contain a novel proline-rich PRP motif, which is thought to be involved in mediating protein-protein interactions (Doi et al., 2003; Kay et al., 2000) (Fig 1-5). The PRP motif consists of 35 amino acids, with the identity and position of eight amino acids highly

conserved among Ago subfamily members from plants to mammals, except dAgo2 (Doi et al., 2003). Sequence homology searches indicate that dAgo2 may also be classified as one of the orphan PPD family members (Doi et al., 2003; Schwarz et al., 2003) (see Fig 1-4).

1-3-2-2- Genetic analysis of PPD genes

1-3-2-2-1- Heterochromatic gene silencing and cell growth: Ago1 and Ago4

Epigenetic silencing by heterochromatin assembly initiates in a nucleation site and propagates to nearby genes, transposons and tandem or inverted repeats (Avramova, 2002; Wallrath, 1998). In *S. pombe*, deacetylation of lysine 9 of histone H3 (H3K9) by Clr3 allows subsequent methylation by Clr4 and binding of Swi6, which spreads the heterochromatic state by recruitment of more Swi6 (Jenuwein and Allis, 2001; Lachner and Jenuwein, 2002). It has been shown that the RNAi machinery is required for the initiation, but not propagation, of heterochromatin assembly (Allshire, 2002; Hall et al., 2002; Schramke and Allshire, 2003; Volpe et al., 2002).

Isolation of siRNAs homologous to the silenced repeats of *S. pombe* outer centromeric heterochromatin suggested a link between heterochromatic gene silencing and RNAi (Reinhart and Bartel, 2002). Volpe et al. (2002) demonstrated that derepression of a transgene in the outermost centromeric region of *ago1*$^-$, *dcr1*$^-$ and *rdp1*$^-$ (RNA-dependent RNA polymerase) *S. pombe* mutants was accompanied by decreased methylation of H3K9. RT-PCR and nuclear run on assays indicated that both PTGS and TGS of an outermost centromeric repeat transcript were impaired in *ago1*$^-$, *dcr1*$^-$ and *rdp1*$^-$ *S. pombe* mutants. Deletion of *ago1*$^+$ had a synergistic effect on the derepression of

transcription of a heterochromatic repeat in *swi6*⁻ mutants, suggesting that the RNAi machinery may function upstream of the propagation of silencing by Swi6, i.e. at the initiation step. Subsequently, Hall et al. (2002) showed that the RNAi machinery was required for initiation of ectopic silencing by centromeric repeats from a silent mating-type locus, but was dispensable for maintenance of an established heterochromatic state at the mating-type locus. Finally, Schramke et al. (2003) demonstrated that expression of a hairpin RNA was sufficient to induce transcriptional silencing of a homologous sequence *in trans*, through the use of the RNAi machinery. Their data suggest that silencing of nearby genes by interspersed retroposon long-terminal repeats (LTRs) via RNAi might be important for regulation of gene expression during differentiation (Schramke and Allshire, 2003). Later, it was also shown that derepression of the centromeric repeats in *ago1*⁻, *dcr1*⁻ and *rdp1*⁻ strains interfered with the accurate chromosome segregation during meiosis and mitosis and disturbed telomere clustering during interphase (Hall et al., 2003; Provost et al., 2002b; Volpe et al., 2003).

Ago4-dependent epigenetic silencing has been linked to the initiation and effector step of RNAi in *A. thaliana*. Ago4 is required for siRNA formation and TGS via methylation of both DNA and H3K9 (Zilberman et al., 2003). Interestingly, the function of Ago4 at the initiation step of RNAi is reported to be gene-specific, suggesting a role for Ago4 in targeting specific transcripts for Dicer cleavage.

The *ago1*⁻ and *dcr1*⁻ mutants also exhibit defects in the regulation of cytokinesis, cell cycle progression and mating in *S. pombe* (Carmichael et al., 2004). *Ago1*⁺ and *dcr1*⁺, but not *rdp1*⁺ genes, are required for hyper-phosphorylation of Cdc2, a cyclin-dependent kinase that regulats the S-M checkpoint. Interestingly, overexpression of

ago1^{+} suppressed cytokinesis and S-M checkpoint defects of *dcr1*$^{-}$, but not the chromosome segregation defect. These results suggest that regulation of Cdc2-dependent cell cycle events by *ago1*$^{+}$ and *dcr1*$^{+}$ is independent from *rdp1*$^{+}$ function and perhaps from the classical RNAi pathway (Carmichael et al., 2004).

1-3-2-2-2- Stem cell maintenance: Piwi, Prg1, Prg2, Ago1, Zll/Pnh and Hiwi

Mutations in *D. melanogaster piwi* are recessive and often result in depletion of germinal stem cells (GSC) in both female and male flies (Lin and Spradling, 1997). In normal ovariol, the functional unit of ovaries, GSCs undergo asymmetric cell divisions that serve to renew the GSC pool, as well as to produce cystoblasts that differentiate into oocytes (for a recent review, see Lin, 2002). Mutations in *piwi* result in the impairment of GSC asymmetric cell division, and the differentiation of GSCs into cysts without renewal thereby causing GSC depletion (Cox et al., 1998). *In situ* hybridization showed that Piwi is expressed in both germinal stem cells and somatic cells of the *D. melanogaster* ovary. Analysis of mosaic flies indicated that expression of Piwi in adjacent somatic cells is required for asymmetric cell division of GSCs (Cox et al., 1998). Moreover, overexpression of Piwi in somatic cells increases GSC asymmetric cell division in a dose-dependent manner, suggesting that Piwi functions via an intercellular signaling mechanism. In contrast, *piwi*$^{-}$ GSC clones exhibit decreased rates of cell division, suggesting that Piwi also acts autonomously to increase GSC division (Cox et al., 2000).

TGS and PTGS of transgenes are impaired in *piwi* mutants (Pal-Bhadra et al., 2002). TGS by Piwi is dependent on the Polycomb group of chromatin-repressive genes and is thought to occur at the level of initiation of chromatin assembly. Defects in siRNA

formation in *piwi* mutants seem to be the reason that PTGS mediated by mRNA degradation is impaired, suggesting that Piwi may be required for the activity of Dicer (Pal-Bhadra et al., 2002). Two of the known *piwi* suppressor genes, *serendipity-δ* and *similar*, encode transcription factors, which interact with *piwi* in a negative and dosage-dependent manner to regulate GSC division (Smulders-Srinivasan and Lin, 2003). It is hypothesized that transcriptional or post-transcriptional downregulation of these transcription factors by Piwi results in the regulation of the expression of genes required for asymmetric cell division. However, direct evidence showing that the RNAi machinery regulates expression of endogenous Piwi-target genes is lacking.

Cox et al. (1998) also demonstrated that the RNAi-mediated knockdown of *piwi* homologues *prg1* and *prg2* (*piwi-related genes 1* and *2*) in *C. elegans* resulted in infertile worms and depletion of their GSCs. Similarity between the *piwi* and *prg1* and *prg2* mutant phenotypes suggests that GSC maintenance is one of the conserved functions of Piwi subfamily members.

A. thaliana Ago1 and Zwille/Pinhead (Zll/Pnh) proteins are involved in the regulation of development and appear to have partially redundant functions. Homozygous *ago1* mutants are sterile, exhibit pleiotropic developmental defects in the general plant architecture and have filamentous structures that resemble small squids, hence the name argonaute (Ago) (Bohmert et al., 1998). The apical shoot meristem terminates prematurely, and the axilary meristem is inactive in *ago1* mutants, indicating that Ago1 is required for maintenance of meristerm cells (plant stem cells) in an undifferentiated state (Bohmert et al., 1998; Lynn et al., 1999). In contrast to Ago1, which is expressed ubiquitously, Zll/Pnh is expressed during embryonic life and in a few adult organs.

Zll/Pnh was shown to play a critical role in the maintenance of undifferentiated cells in the apical shoot meristem and axilary meristem (Moussian et al., 1998). Ectopic expression of Zll/Pnh results in a loss of determination for differentiation in the lateral organs (Newman et al., 2002), and overexpression of Ago1 disrupts development and causes formation of cupped leaves (Bohmert et al., 1998). Development of the double-mutant *ago1/zll* is arrested early in embryogenesis, suggesting that Ago1 and Zll/Pnh have redundant functions (Lynn et al., 1999). In summary, it seems that both Ago1 and Zll/Pnh are involved in regulation of meristem cell fate determination.

The *A. thaliana ago1* mutants, but not *zll/pnh*, are also defective in PTGS (via mRNA degradation) and show decreased viral resistance (Fagard et al., 2000). Interestingly, hypomorphic fertile *ago1* mutants with impaired PTGS have been isolated (Morel et al., 2002). Uncoupling of PTGS mediated by mRNA degradation and developmental defects in *ago1* mutants suggests that these defects are caused by two independent pathways, but does not exclude possible connections between RNAi and development in *ago1* mutants. For instance, *ago1* mRNA contains a predicted miRNA target site for *miR168*, suggesting that the expression of Ago1 during plant development may be regulated in an RNAi-dependent manner (Rhoades et al., 2002). Finally, developmental defects of *ago1* or *zll/pnh* mutants may be related to defects in TGS or translational repression by RNAi, instead of mRNA degradation.

Hiwi, the human homologue of Piwi, is expressed in undifferentiated $CD34^+$ hematopoietic stem cells, but not in more differentiated $CD34^-$ counterparts (Sharma et al., 2001). $CD34^+$ cells lose Hiwi expression in culture conditions that support differentiation, suggesting a possible role for Hiwi in the maintenance of hematopoietic

stem cells in an undifferentiated state. Qiao et al. (2002) have localized the *hiwi* gene to chromosome 12, band 12q24.33, a region with genetic linkage to testicular germ cell tumors of adults and adolescents. Overexpression of the *hiwi* gene is common in seminomas (undifferentiated germinal cell tumors), suggesting a role for Hiwi in maintenance of cells in an undifferentiated state, a situation reminiscent of Piwi's role in preventing GSCs from differentiating (Qiao et al., 2002).

1-3-2-2-3- Developmental Timing: Alg1, Alg2 (Ago-like genes 1 and 2) and Ago7

Homozygous *alg2* mutant worms show subtle defects in development and fertility (Grishok et al., 2001). However, RNAi-mediated knockdown of *alg1* gene expression results in a burst vulva phenotype (Grishok et al., 2001). RNAi-mediated double-knockdown of Alg1 and Alg2 or *alg1/alg2* double-mutants results in embryonic lethality, indicating that Alg1 and Alg2 may have overlapping functions. The coding regions of genes encoding these two proteins are 80% identical. However, neither of the genes was necessary for PTGS mediated by mRNA degradation in *C. elegans* (Grishok et al., 2000; and 2001). Knockdown of Alg1 by injection of full-length *alg1* dsRNA appeared to partially inhibit Alg2 and produce viable animals, referred to "*alg1/alg2*" RNAi worms, with defects in development and fertility, as well as the burst vulva phenotype (Grishok et al., 2001). Interestingly, mutation of the *dcr1* gene (*C. elegans* Dicer) caused burst vulva and similar developmental and fertility defects, except that *dcr1* mutants were also RNAi defective. Grishok et al. (2001) showed that *alg1/alg2* and *dcr1* also cause heterochronic defects, which phenocopy *lin4* and *let7*mutations. The *lin4* and *let7* 22 nt stRNAs are cleaved out of ~70 nt hairpin precursors by Dicer, and regulate translation of

lin14 and *lin41* mRNAs, respectively (Grishok et al., 2001; Lee et al., 1993). Grishok et al. (2001) also showed that the heterochronic defects of *alg1/alg2* and *dcr1* are suppressed by *lin14* and *lin41* mutations. Furthermore, reporter genes regulated by *lin14* and *lin41* 3'UTRs are upregulated in *alg1/alg2* and *dcr1* mutants, as well as in *lin4* and *let7* mutants. These results indicate that the heterochronic defects of *alg1/alg2* and *dcr1* are mediated by interruption of *lin4* and *let7* functions. Northern blot analysis confirmed this conclusion by revealing that processing of *lin4* and *let7* stRNAs are defective in *alg1/alg2* and *dcr1* mutants.

Grishok et al. (2001) established a link between RNAi machinery and regulation of developmental timing. According to their model, Dicer activity is required for both forms of sequence-specific PTGS: mRNA degradation and translational repression. In contrast, Alg1 and Alg2 are required downstream of Dicer for translational repression of *lin14* and *lin41* mRNAs. Additionally, Alg1 and Alg2 are required upstream of Dicer for stability and perhaps processing of *lin4* and *let7* precursors.

Loss of function *ago7* mutations in *A. thaliana* also result in developmental timing defects, but similar to *zll/pnh* mutants, no defect in PTGS was observed (Hunter et al., 2003). The observation that PTGS mediated by mRNA degradation is not defective in *zll/pnh* and *ago7* does not exclude defects in other forms of RNAi in these mutants.

1-3-2-2-4- Fertility: Aub, Miwi and Mili

The *D. melanogaster* PPD protein Aubergine (Aub) also known as Sting (Sti) is required for RNAi-induced silencing of the X-linked *stellate* (*ste*) tandem repeat by its paralogous Y-linked tandem repeat sequence, *suppressor of stellate* (*su(ste)*) (Aravin et

al., 2001). Derepression of *ste* repeats in *aub* mutant flies with intact *su(ste)* repeats, results in infertility, possibly due to aggregation of Ste protein and formation of protein crystals (Stings) in male germinal cells (Bozzetti et al., 1995; Schmidt et al., 1999). Remarkably, *aub* mutations do not affect transposon silencing (Aravin et al., 2001; Gvozdev et al., 2003). This observation supports the notion that PPD proteins are involved in targeting RNAi machinery to specific downstream pathways.

Aub is involved in RNAi through mRNA degradation and translational regulation of gene expression in *D. melanogaster* oocytes (Kennerdell and Carthew, 1998). Maternal mRNAs are translationally inactive in arrested oocytes, where mRNA degradation is also inactive. Maturation of oocytes after fertilization is followed by translation of some maternal mRNAs, which become sensitive to RNAi-mediated mRNA degradation, while quiescent mRNAs remain insensitive to RNAi. Mutations in the *aub* gene perturb RNAi mediated by mRNA degradation, as well as translational silencing of mRNAs during oogenesis, indicating that they may be regulated via similar molecular mechanisms (Kennerdell and Carthew, 1998).

Links between Aub and regulation of gene expression extend to the embryonic and adult life of *D. melanogaster*. *Oskar* is involved in regulation of germline formation and abdominal body patterning in the embryo. It is thought that Aub upregulates translation of *oskar* mRNA by inhibition of its translational repressors in a 3'UTR-dependent manner (Harris and Macdonald, 2001). Finally, Aub is required for localization of Maelstrom to Nuage granules; RNP granules present in the germinal cells of *D. melanogaster* during adulthood. Interestingly, *D. melanogaster* Ago2 and Dicer are

mislocalized in *maelstrom* mutants implicating a connection between Aub, dAgo2 and Dicer (Findley et al., 2003).

Miwi expression can be detected 14 days post-partum, which corresponds to the onset of spermiogenesis. Differentiation of germinal cells is arrested at the round spermatid stage in *miwi* mutants and seminiferous tubules are devoid of sperm. Miwi is hypothesized to be a major regulator of spermiogenesis, a process that converts round spermatids into elongated sperm (Deng and Lin, 2002). Interestingly, Miwi binds to the activator of CREM (ACT) mRNAs and to CREM target mRNAs, CREM being a major regulator of spermiogenesis. The ACT and CREM target mRNAs are downregulated in *miwi* mutants, indicating that Miwi may be required for their stability (Deng and Lin, 2002). Altogether, these data suggest that the role of Miwi in differentiation of germinal cells is mediated through regulation of stability and/or translation of ACT and CREM target mRNAs, perhaps through an RNAi-dependent pathway.

Spermatogenesis is impaired in *mili* mutants and *mili*-null mice are sterile (Kuramochi-Miyagawa et al., 2004). Development of germinal cells in *mili*-null mice is blocked at the early stages of meiosis, a phenotype similar to *mvh*-null mice. The *mvh* or *mouse vasa homologue* encodes a DEAD-box helicase that is only expressed in the germinal lineage up to round spermatid, and is required for the development of the germ cells (Tanaka et al., 2000). The Mvh protein interacts with both Mili and Miwi, although its affinity is higher for interaction with Miwi. Finally, both Mili and Miwi colocalize with Mvh in the cytoplasm and might be required for mRNA stability during spermatogenesis (Kuramochi-Miyagawa et al., 2004).

1-3-2-3- PPD Proteins and human disease

The human genome contains eight *PPD* genes with *eif2c* (*eukaryotic initiation factor 2c*) *1-4/hago1-4* encoding proteins of the Ago subfamily and *piwiL1/hiwi*, *piwiL2/hili*, *piwiL3* and *piwiL4/hiwi2* encoding proteins of the Piwi subfamily (Sasaki et al., 2003). All human PPD proteins have been shown to bind human Dicer (Doi et al., 2003; Sasaki et al., 2003; Tahbaz et al., 2004). HAgo1, hAgo3 and hAgo4 are encoded by genes tandemly arranged on human chromosome 1, band 1p34-35, a region which is lost in Wilm's tumors (Koesters et al., 1999). Loss of hAgo1, hAgo3 and hAgo4 in Wilm's tumors is consistent with a role for these genes in cellular differentiation. Interestingly, Hiwi is required for the persistence of hematopoietic stem cells in an undifferentiated state (Sharma et al., 2001). Furthermore, Hiwi overexpression is common in seminomas, and loss of Hiwi expression is correlated with hypogonadism (Qiao et al., 2002). Finally, an isoform of hAgo2, Eif2c2, binds to 40 different miRNAs (Mourelatos et al., 2002). The role of miRNAs in human disease is just beginning to be explored (Dostie et al., 2003).

1-3-2-4- Components of the RISC and miRNP complexes

The known protein components of the *D. melanogaster* RISC and miRNP complexes include: dAgo2 (Bernstein et al., 2001a; Hammond et al., 2001a), Vig (Vasa intronic gene) or Dmp68, dFxr (*D. melanogaster* fragile-X related), dAgo1 (Caudy et al., 2002; Ishizuka et al., 2002, and reviewed in Carthew, 2002) and Tudor-SN (Tsn) (Caudy et al., 2003). In addition, hAgo2 (Mourelatos et al., 2002) Gemin3 (Charroux et al., 1999) and Gemin4 (Charroux et al., 2000) have been identified in human miRNP complexes.

Generally, RISC or miRNP complexes contain a PPD protein (dAgo1, dAgo2, hAgo2), an Asp-Glu-Ala-Asp (DEAD) box-containing RNA helicase (Vig, Gemin3), a nuclease (Tsn) and RNA-binding proteins (dFxr and Gemin4). Here, I will discuss the components of RISC and then will continue by introducing the components of miRNP.

Hammond et al. (2001) showed that dAgo2 is a bona fide RISC component, which also interacts with Dicer. Although dAgo2 was the first RISC component to be identified, no dAgo2 mutants have been isolated to date. Vig, also known as Dmp68, is a DEAD-box RNA helicase which binds to and unwinds short dsRNAs (Huang and Liu, 2002). DFxr is the *D. melanogaster* homologue of human Fmrp or fragile-X mental retardation protein. Loss of Fmrp expression as a result of promoter methylation is the underlying cause of fragile-X mental retardation syndrome (Bardoni and Mandel, 2002). Fmrp is a RNA-binding protein and is involved in translational inhibition. It has been shown that Fmrp associates with polyribosomes, as well as with large RNP complexes at synapses (Brown et al., 2001; Ceman et al., 1999; Feng et al., 1997). Interestingly, polyribosome assembly is defective in patients with fragile-X syndrome (Feng et al., 1997). It has been shown that association between dAgo2, Vig and dFxr is not dependent upon RNA (Caudy et al., 2002; Ishizuka et al., 2002). Remarkably, although Caudy et al. (2002) demonstrated that anti-dFxr1 antibodies could immunopurify RISC activity from S2 cell lysates, they did not observe a strong impact on RNAi following knockdown of dFxr1 expression by RNAi. Similarly, Ishizuka et al. (2002) observed no effect on RNAi after inhibition of dFxr1. Both groups reported that dFxr1 copurified on sucrose gradients with the miRNAs and not siRNAs. Finally, it has been reported recently that the human homologues of dFxr interact with pre-miRNAs and miRNAs, as well as with Dicer and

hAgo2 (Jin et al., 2004). In general, these data indicate that although fragile-X mental retardation proteins associate with RISC and miRNPs, they may not be required for RNAi via mRNA degradation.

The *D. melanogaster* orthologue of hAgo2, dAgo1, does not seem to be required for RNAi in S2 cell extracts (Caudy et al., 2002). However, genetic studies showed that dAgo1 is required for efficient RNAi in the embryo downstream of siRNA formation (Williams and Rubin, 2002). Unlike dFxr, dAgo2 and Vig, which cofractionate in a ~500 kDa complex, dAgo1 cofractionates with miRNAs at ~250 kDa and does not seem to interact with dFxr or Vig (Caudy et al., 2002). These data suggest that dAgo1 is part of either the RISC or miRNP complex during different stages of *D. melanogaster* life.

Tsn is a 103 kDa protein with four complete and one incomplete amino-terminal Staphylococcal/micrococcal nuclease repeats fused to a Tudor repeat (Caudy et al., 2003). Tudor repeats are known to bind to methylated amino acids (Maurer-Stroh et al., 2003). *D. melanogaster* Tsn cofractionates with dFxr and dAgo2 in a complex of ~500 kDa that exhibited RISC activity. Tsn associates with dFxr, dAgo2 and Vig, as well as both miRNAs and siRNAs in *D. melanogaster* cells. The data for human and *C. elegans* Tsn homologues show some discrepancies with *D. melanogaster* Tsn. For instance, in mammalian cells, interaction of hAgo2 and Fmrp with Tsn requires siRNA transfection, suggesting that Tsn is not stably associated with miRNP components (Caudy et al., 2003). Moreover, Tsn1 (*C. elegans* Tsn) associates with *let7* and Vig1 (*C. elegans* Vig), and cofractionates with Vig1 in a ~250 kDa complex (Caudy et al., 2003), suggesting that Tsn1 is a component of miRNP complexes. These data suggest that Tsn homologues are componets of RISC and/or miRNP complexes in various organisms.

RNAi knockdown of Vig1 and Tsn1 results in loss of translational repression by *let7*, as measured by a *let7*-responsive reporter gene (Caudy et al., 2003). However, this was only a partial loss of *let7* function since phenotypes normally associated with loss of *let7* function (e.g. burst vulva) were not detected in Vig1 and Tsn1 knockdowns. In addition, knockdown of Vig1 and Tsn1 did not show a significant effect on RNAi through mRNA degradation.

Reports from several laboratories indicate that mRNA cleavage by RISC includes a sequence-specific step during which the putative RISC "slicer" makes the first cut in the middle of a siRNA-mRNA hybrid, followed by a non-specific RNA degradation step (Elbashir et al., 2001c; Hutvagner and Zamore, 2002; Llave et al., 2002b; Tang et al., 2003). Tsn is an unspecific RNase/DNase that could support RISC activity during mRNA degradation, but not during mRNA cleavage, by the RISC "slicer" (Caudy et al., 2003).

Eif2c2, an isoform of hAgo2, resides in cytoplasmic 15S miRNP complexes, along with Gemin3 and Gemin4 and 40 different miRNAs (Mourelatos et al., 2002). Eif2c2 has also been found in a ~500 kDa RISC-like complex and binds *let7* (Caudy et al., 2003). Hutvagner and Zamore (2002) showed that a *let7*-programmed RISC activity could be immunoprecipitated from HeLa cells using an antibody against hAgo2, indicating that miRNP complexes can be reprogrammed to function as RISC (Hutvagner and Zamore, 2002). Altogether, it seems that RISCs and miRNPs are structurally and functionally similar complexes containing PPD proteins.

1-4- Objectives

The goal of this thesis project was to gain insight into the functions of PPD proteins by identification of their binding partners. I found that PPD proteins interact with Hsp90, an interaction which is required for the stability of PPD proteins and their association with membranes. Interaction with Hsp90 also seems to be required for the interaction of PPD proteins with their protein partners, as shown in the case of Dicer. Finally, I mapped the interaction sites on PPD proteins and Dicer, and together with our collaborators, we have demonstrated that the interaction of Dicer with PPD proteins downregulates Dicer activity *in vitro*. This thesis project provides insight into the functions of PPD proteins based on a biochemical analysis of their interactions with other proteins, specifically Hsp90 and Dicer.

CHAPTER 2

MATERIALS AND METHODS

2-1- Reagents and commonly used buffers

Reagents were obtained from the following sources:

TABLE 2-1: Commonly used reagents

REAGENT	*SOURCE*
40 % Acrylamide/Bis solution, 29:1	Bio-Rad
agar	Difco
Agarose A, electophoresis grade	Rose Scientific LTD.
ammonium persulphate	BDH
aprotinin	Roche
ampicilin	Sigma Chemical Co.
bovine serum albumin	Sigma Chemical Co.
bromophenol blue	BDH
activated charcoal	BDH
Complete™ EDTA-free protease inhibitor	Roche
coomassie Brilliant Blue	ICN Biomedicals
CSM-leu-his or 2DO	BIO 101
CSM-leu-his-trp-ade or 4DO	BIO 101
D-(+)-glucose	Sigma Chemical Co.
dimethyl sulphoxide (DMSO)	Sigma Chemical Co.
dithiothreitol (DTT)	ICN Biomedicals
Dulbecco's modified Eagle's medium high glucose	Invitrogen
ethanol	Commercial Alchohols
ethylenediaminetetraacetic acid (EDTA)	Sigma Chemical Co.
fetal bovine serum	Invitrogen
fibronectin	Sigma Chemical Co.
formamide	BDH
formaldehyde, 37% (v/v)	BDH
geldanamycin	Sigma Chemical Co.
glass beads (G-9268)	Sigma Chemical Co.
glutathione conjugated to Sepharose 4B	Amersham Pharmacia Biotech.
glycerol	BDH
glycine	EM SCIENCE

TABLE 2-1 (continued)

guanidine HCl	ICN Biomedicals
hydrochloric acid	Fisher
isopropanol	Fisher
methanol	Fisher
***Minimum* essential medium lacking cysteine/methionine**	ICN Biomedicals
N,N,N',N'-tetramethylenediamine (TEMED)	Invitrogen
peptone	Difco
phenol, buffer-saturated	Invitrogen
phenylmethylsulphonylfluoride (PMSF)	Roche
polyuridylic acid-Agarose	Sigma Chemical Co.
Polyadenilyc acid-Agarose	Sigma Chemical Co.
ponceau S	Sigma Chemical Co.
potassium chloride	BDH
potassium phosphate, monobasic (KH_2PO_4)	Merck
potassium phosphate, dibasic (K_2HPO_4)	Merck
Protein A and G sepharose	Amersham Pharmacia Biotech.
radicicol	Sigma Chemical Co.
Restore™ Western Blot Stripping Buffer	PIERCE
RNasin	Promega
salmon sperm DNA, sonicated	Sigma Chemical Co.
sephadex G50	Amersham Pharmacia Biotech.
sodium acetate	BDH
sodium chloride	Merck
sodium citrate	BDH
sodium dodecyl sulfate (SDS)	Bio-Rad
sodium hydroxide	BDH
sodium phosphate, dibasic (Na_2HPO4)	BDH
sodium salicylate	EM SCIENCE
sorbitol	BDH
Tris Base	Roche
Triton X-100	BDH
Tryptone	Difco
Tween 20 (polyoxyethylenesorbitan monolaureate)	CALEDON
VECTASHIELD Mounting Medium for IF H-1000	VECTOR Labs
yeast extract	Difco
yeast nitrogen base w/out amino acids and ammonium sulfate	Difco

TABLE 2-2: Commonly used buffered solutions

BUFFER	*COMPOSITION*
1 X PBS (IF)	137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4
1 x PBS	137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4
20 x SSC	3 M NaCl, 0.3 M sodium citrate, pH 7.0
2 x SDS sample b.	200mM dithiotheritol, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 100 mM Tris HCl, pH 6.8
elution b.	10mM reduced glutathione in 50 mM Tris HCl pH 8.0
FSB	10 mM KOAc pH 7.5, 45 mM MnCl2, 10 mM CaCl2, 10 mM KCl, 3 mM hexaminecobalt chloride, 10% glycerol
hypotonic b.	10 mM KCl, 5 mM $MgCl_2$, 10 mM Tris HCl pH 7.5
lower gel b.	0.1% SDS, 375 mM Tris HCl, pH 8.8
upper gel b.	0.1% SDS and 250 mM Tris HCl, pH 6.8
low salt b.	25 mM KCl, 5 mM $MgCl_2$, 50 mM Tris HCl pH 7.5
NP40 Lysis B.	150 mM NaCl, 2 mM EDTA, NP40 1%, Tris HCl pH 7.4
PBSCM	0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.05% Azide in 1x PBS
RIPA b.	150 mM NaCl, sodium deoxycholate 0.5%, SDS 0.1%, NP40 1%, 50 mM Tris HCl, pH 8.0
SDS-PAGE run. b.	250 mM glycine, 0.1% SDS and 100mM Tris Base
sonication lysis b.	200 mM NaCl, 2.5 mM $MgCl_2$, NP40 0.05%, 20 mM Tris HCl pH 7.4
TAE	40 mM Tris-Acetate, 1 mM EDTA, pH 8.0
TBS	137 mM NaCl, 2.7 mM KCl, 24 mM Tris HCl pH 7.4
TBST	TBS + 0.05% Tween 20
TCA 100%	100 g of TCA in 45.4 ml ddH_2O
TE	1mM EDTA, 10 mM Tris HCl pH 7.5
transfer b.	200mM Glycine, 25 mM Tris Base

2-2- Materials

2-2-1- Multicomponent systems

FuGENE™ 6 Transfection Reagent	Roche
Pwo polymerase	Roche
Platinum *Pfx* DNA polymerase	Invitrogen
Perfectin transfection reagent	Gene Therapy Systmes Inc.
TNT™-coupled reticulocyte lysate system	Promega
Yeast two-hybrid system (MATCHMAKER III)	CLONTECH
GeneTrapper cDNA Positive Selection System	Invitrogen
T-Rex™ 293 cell line and reagents	Invitrogen

BCA Protein Assay Reagent	PIERCE
Random Primed DNA Labeling Kit	Roche

2-2-2- DNA modifying enzymes

Restriction endonucleases	NEB, Promega, Invitrogen
T4 DNA ligase	NEB
T4 DNA polymerase	NEB

2-2-3- Radiochemicals and detection systems

Promix [^{35}S]methionine/cysteine (1000 Ci/Mm)	Amersham Pharmacia
translation grade [^{35}S]methionine (1000 Ci/Mm)	Amersham Pharmacia
X-ray film (BioMaxMR, X-Omat AR and X-Omat XK-1)	Kodak
Immobilon™-P (PVDF) membranes	MILLIPORE
Hybond™ membranes	AmershamPharmacia
RX film	Fuji
Supersignal Westpico Chemiluminescent Substrate	PIERCE

2-2-4- Molecular size standards

^{14}C-labeled protein standard	Amersham Pharmacia
prestained protein marker, broad range	NEB
Kaleidoscope prestained standards	Bio-Rad
Precision plus prestained™ standards	Bio-Rad
10 kD unstained protein marker	NEB
1 kb DNA ladder	Invitrogen

2-2-5- Plasmid vectors

pBluescript SK and KS	Stratagene
pENTR1a, pENTR3C pcDNA4/TO, pcDNA5/TO, pCMV5, pCMVSPORT2	Invitrogen
pEBG	Barker lab, McGill U

2-2-6- Mammalian cell lines

COS, HEK293T, NRK52E and NRK52F cells were from American Type Culture Collection.

2-2-7- Antibodies

2-2-7-1- Primary antibodies

2-2-7-1-1-Mouse monoclonal antibodies

anti-P23 (JJ3), anti-Hsp90 (3G3), anti-P50^{Cdc37} (C1)	Affinity Bioreagents
anti-Fkbp52, anti-Fkbp54, anti-Hsp90, anti-Hsp70, anti-Hop (P60)	Stressgen
anti-Ago2 (9g2), and anti-HA	Hobman lab
anti-Cmyc	ATCC
anti-Eif2c2 (8C7)	Dreyfuss lab, U of Pennsylvania

2-2-7-1-2- Rabbit polyclonal antibodies

anti-Ago2 (7C6)	Hobman lab
anti-Mannosidase II (Man II)	Farquhar lab, U of San Diego
anti-P50^{Cdc37}, anti-calnexin, anti-Bip	Stressgen

2-2-7-2- Secondary antibodies

Horseradish peroxidase-conjugated goat anti-mouse IgG	Bio-Rad
Horseradish peroxidase-conjugated anti-rabbit IgG	Bio-Rad

Texas Red-conjugated anti-mouse IgG and Fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (double labeling grade) were from Jackson ImmunoResearch Laboratories.

2-3- Methods

2-3-1- Analysis of DNA

2-3-1-1- Polymerase chain reactions

DNA sequences were amplified by PCR using primers containing engineered restriction sites (for subcloning). *Pwo* polymerase or *Pfx* DNA polymerase were used and reactions were performed according to the manufacturer's instructions. Primers were designed as 18-21 nt long oligonucleotides with predicted Tm of 58-64°C. Reactions normally contained 100 ng of plasmid DNA, 15 pM of each primer, 10 μM of each dNTP (dATP, dTTP, dCTP and dGTP), 2 units of the polymerase and 5 μl of 10 x reaction

buffer in a total volume of 50 μl. Most reactions were carried out for 30 cycles in 0.6 ml PCR tubes in a DeltaCycler II™ System (ERICOMP) with a hot top attachment.

2-3-1-2- Restriction endonuclease digestion

DNA digestions were performed following the manufacturer's instructions, usually in 20 μl of total volume, containing 5 units of the enzyme, restriction digestion buffer and 1-2 μg of DNA.

2-3-1-3- Ethanol precipitation of DNA

DNA precipitation was performed by the addition of sodium acetate (from a 3 M, pH 5.2, stock) to the DNA solution in a 1.5 ml microtube to a final concentration of 0.3 M. Absolute or 95% ethanol was then added (2-2.5 volumes) to the DNA-containing sample, followed by gentle mixing and incubation at –20°C for 30-60 min (Sambrook et al., 1989). Precipitated DNA was collected by centrifugation at 14,000 x *g* for 30 min at 4°C and rinsed by adding 1 ml of cold 70% ethanol followed by centrifugation at 14,000 x *g* for 5 min at 4°C. DNA then was dried in a rotary vacuum desiccator (Eppendorf Concentrator 5301) or on the bench for 10-20 min and dissolved in 10 mM Tris HCl, pH 7.5-8, or deionized RNase-free water.

2-3-1-4- Agarose gel electrophoresis

Following PCR or after treatment with restriction endonucleases, DNA samples were mixed with 6x DNA sample dye (40% sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol FF), and DNA fragments were separated by electrophoresis in agarose gels

(1% or 2% agarose in TAE) containing 0.5 μg ethidium bromide/ml (Maniatis et al., 1982). Electrophoresis was conducted at 10 V/cm in TAE, and DNA was visualized on an ultraviolet transilluminator (FisherBiotech Electrophoresis Systems).

2-3-1-5- DNA purification from agarose gels

DNA fragments of interest were excised with a clean razor blade and purified using QIAEX II Gel Extraction Kit (150) (Qiagen), according to the manufacturer's instructions.

2-3-1-6- Ligation and transformation

PCR products were digested with restriction enzymes and purified as described in the previous section. The DNA fragments were then ligated into pre-digested vectors using 1-5 units of T4 DNA ligase in the buffer supplied by the manufacturer. Ligations were performed in a total volume of 20 μl, at the molar ratio of 3 to 1 vector to insert. After incubation at room temperature for 30-60 min, *E. coli* DH5α strain was transformed with ligation products either chemically or by electroporation.

2-3-1-6-1- Electroporation of *E. coli*

Electrocompetent DH5α cells were prepared as described (Dower et al., 1988). Briefly, 5 ml of LB medium was inoculated with a single colony of DH5α and incubated overnight in a 37°C rotary shaker at 200-250 rpm. The next day, 500 ml of LB was inoculated, and cultures were grown to OD_{600}= 0.5-0.8 before cells were harvested by centrifugation at 4,000 x *g* for 15 min at 4°C. Cells were washed twice in 0.5 liter of ice-

cold water, once with 10 ml of ice-cold 10% (v/v) glycerol and then resuspended in 1 ml of 10% glycerol. Cells were aliquoted into 50 μl batches, frozen in a dry ice/ethanol bath and stored at –80°C. For electroporation, 40 μl aliquots of electro-competent cells were mixed with 1-5 μl of DNA (100-500 ng) on ice, transferred between the electrodes (0.2 cm apart) of an ice-cold Bio-Rad Gene Pulser Cuvette, incubated on ice for 1 min and submitted to an electrical pulse in a Bio-Rad MicroPulser (BioRad). Cells were immediately transferred into 1 ml of LB culture, incubated in a 37°C rotary shaker at 150-200 rpm for 1 hour, plated onto LB agar plates (containing antibiotics), and incubated overnight at 37°C.

2-3-1-6-2- Chemical transformation of *E. coli*

One ml of an overnight culture of DH5α in 2xYT (1.6% bacto-tryptone, 1.6% bacto-yeast extract, 0.5% NaCl) was used to inoculate 30 ml of 2xYT and was grown to OD_{600}=0.3-0.5. Cells were washed in 5 ml of ice-cold FSB (TABLE 2-2) and divided into three 1 ml aliquots. DMSO (35 μl) was added and mixed with pellets from each aliquot on ice and incubation was continued for 15 min. Aliquots were washed in 1 ml ice-cold FSB twice and divided into smaller aliquots (200 μl each), frozen in a dry ice-ethanol bath and stored at -80°C.

Chemical transformations were performed according to Sambrook et al. (1989). Briefly, 100-500 ng DNA was added to 40 μl aliquots of *E. coli* strain DH5α chemical competent cells thawed on ice. Cells were mixed with the DNA on ice, and incubation was continued for 30 min followed by a 45 second heat-shock at 42°C. Transformed cells were then recovered by growth at 37°C for 1 hour in a rotary shaker at 150-200 rpm,

plated onto LB agar plates containing appropriate antibiotic, and incubated overnight at 37°C.

2-3-1-7- Automated sequencing

Dideoxynucleotide sequencing using fluorescently labeled DNA was performed using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) according to the manufacturer's instructions. Briefly, fluorescent dideoxy terminators are incorporated randomly during elongation of DNA sequences with a modified version of *Taq* DNA polymerase. Capillary electrophoresis was used to separate reaction products, and fluorescence was detected and recorded by an ABI 310 Genetic Analyzer (PE Applied Biosystems).

2-3-1-8- Plasmid DNA isolation from *E. coli*

2-3-1-8-1- Minipreps

DNA minipreps were often prepared using a laboratory modified diatomaceous earth protocol. Two ml cultures were inoculated and grown overnight in LB. The cells were subjected to centrifugation at 5000 x *g* in microfuge tubes and resuspended in 100 μl of solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris HCl, pH 8.0, and 100μg RNase A /ml). Two hundred μl of freshly made solution II (0.2 M NaOH, 1% SDS) was added and gently mixed with solution I followed by a 5 min incubation at room temperature. Proteins were precipitated after lysis with solution II by the addition and gentle mixing of 150 μl of solution III (5 M potassium acetate, pH 4.8, in 11.5% glacial acetic acid (v/v)). The tubes were subjected to centrifugation at 14,000 x*g* for 10 min to pellet the proteins, and the supernatants were transferred to new tubes containing 600 μl

of diatomaceous earth slurry. After 1-2 min incubation at room temperature, the DNA was absorbed to diatomaceous earth, washed 2 x (200 mM NaCl, 5 mM EDTA, 50% ethanol, 20 mM Tris HCl, pH 7.5), and eluted in TE or deionized water heated to 70°C.

Prior to use in DNA isolation, diatomaceous earth was prepared as follows: Diatomaceous earth (2.5-3 grams) was washed on a filter paper with 300 ml deionized water, dryed in a dessicator and mixed by gentle heating with 300 ml of guanidine-HCl and merlin III (7% glacial acetic acid (v/v), 12% potassium acetate in deionized water pH: 5.5), filtered and aliquoted.

In some cases, mini-preps were also prepared using WizardPlus Miniprep kit (Promega) according to the manufacturer's instructions.

2-3-1-8-2- Maxi and Mega-preps

Qiagene kits were used according to the manufacturer's protocol and then DNA was ethanol precipitated and adjusted to 1μg/μl in 10 mM Tris HCl (pH 7.5-8).

2-3-1-9- Construction of plasmids

Pwo polymerase was used to amplify rat Ago2 (rAgo2) constructs. All human constructs were prepared by PCR using platinum P*fx* DNA polymerase. The Dicer mammalian cell expression constructs were prepared by our collaborators in Dr. Filipowicz's lab (Basel, Switzerland). Sequences of primers used for subclonings are shown in TABLE 2-3.

2-3-1-9-1- Constructs used in the TNT™-coupled reticulocyte lysate system

The Hdj2 cDNA was subcloned into the *Bam*HI and *Xho*I sites of the pGADT7 (Clontech) to create HA-tagged Hdj2 for *in vitro* protein synthesis. HAgo2 cDNAs (G4, D9, E10, F9) were originally cloned in pCMVSPORT2 and expressed from the SP6 promoter during *in vitro* protein synthesis.

2-3-1-9-2- Constructs used in yeast two-hybrid assays

The hDicer RNase III subdomains including RNase III-A (aa 1270-1582), RNase III-B (aa 1699-1831) and DRBD (aa 1848-1922), were fused to Gal-4 activation domain in the vector pGADT7. Forward and reverse primers included *Cla*I and *Xho*I sites (underlined), respectively (primers 1-6). Sequences encoding PAZ (aa 227-380) and PIWI (aa 508-829) domains of hAgo2, PAZ (aa 271-425) and PIWI (aa 543-861) domains of Hiwi and PIWI-boxes of hAgo2 (aa 723-780) and Hiwi (aa 751-807) were amplified using primers 7-18, and subcloned into pGBKT7 in frame with the Gal-4 DNA-binding domain. CDNAs encoding amino terminal (aa 1-323) or carboxy terminal (aa 309-863) regions of rAgo2 were amplified using primers 43-46 and subcloned into pGADT7. The forward primers contained *Eco*RI and the reverse primers contained *Bam*HI sites (underlined).

2-3-1-9-3- Mammalian cell expression constructs

GST fusion constructs of PPD proteins and their subdomains: PPD cDNAs were subcloned into the pEBG vector such that they were in-frame with GST. Regions encoding the PIWI (aa 505-831) and PAZ (aa 215-354) domains of hAgo2 and the PIWI

(aa 550-855) and PAZ (aa 271-396) domains of Hiwi (Qiao et al., 2002) were amplified by PCR using primers 19-26 and subcloned into pEBG (Mizushima and Nagata, 1990) vector. Full-length hAgo2 and Hiwi cDNAs were amplified by PCR using primers 27-28 and 29-30 respectively, and subcloned into pEBG. Finally full-length rAgo2 cDNA or its amino (aa 1-323) and carboxy (aa 545-863) terminal coding regions were amplified by PCR using primers 39-40, 39-41 and 42-40 respectively, and subcloned into pEBG. Forward and reverse primers used for subcloning into pEBG included *Bam*HI and *Not*I sites (underlined), respectively.

GST and CFP fusion constructs of Dicer and its subdomains: A region encoding the Dicer PAZ domain (aa 612-1078) was amplified by PCR with primers 33-34 using pBS-Dicer (Zhang et al., 2002) as a template. The PCR fragment was digested with *Eco*RI and *Not*I and ligated into pENTR3C (Invitrogen), yielding pENTR-PAZ. Fragments corresponding to the Dicer RNase III (aa 1271-1922) and Helicase/PAZ (aa 1-1078) were similarly amplified, using primers 35-36, and 37-38 respectively. The resulting PCR fragments were digested with *Sal*I and *Not*I and ligated into pENTR1A, yielding pENTR-RNase III and pENTR-Helicase/PAZ. The Dicer cDNA inserts were transferred from the entry vectors into pDEST27 by LR clonase reaction (Invitrogen), yielding pGST-PAZ, pGST-RNase III, and pGST-Helicase/PAZ. To obtain the mammalian expression plasmids encoding full-length hDicer fused to GST, the Dicer cDNA in pENTR-Dicer (Zhang et al., 2002) was recombined using LR clonase into the pDEST 27 vector (Invitrogen). pCFP-Dicer, encoding the cyan fluorescent protein fused to human Dicer has been described (Billy et al., 2001). All Dicer constructs were prepared in Dr. Filipowicz's lab.

Other mammalian constructs: The cDNA of hAgo2 was amplified by PCR using primers 31-32, digested with *Eco*RI and *Bam*HI respectively, and subcloned into pcDNA4/TO. CDNA of HA-tagged Hdj2 was excised from pGADT7 and subcloned into pCMV5 using *Bgl*II and *Pst*I restriction sites.

TABLE 2-3: List of primers

INSERT	*VECTOR*	*PRIMER*
RNaseIII-A **Dicer**	pGADT7	Forward # 1: CAAGTATCGATTTCAAGTGCTCAAGGGCAGG Reverse # 2: GAACTCTCGAGTCAAAAAGCTGAGCAGCCCTCTC
RNaseIII-B **Dicer**	pGADT7	Forward # 3: CAAGTATCGATTCACTGATTGTTACCAGCGCT Reverse # 4: GAACTCTCGAGTCAGACTGTCTCCAGTGACATCC
DRBD **Dicer**	pGADT7	Forward # 5: CAAGTATCGATCTGCAAATGTACCCCGTTCC Reverse # 6: GAACTCTCGAGTCAGCTATTGGGAACCTGAGC
PIWI **hAgo2**	pGBKT7	Forward # 7: TGATCGAATTCCACCTGAAGAACACGTATGCG Reverse # 8: TAACTGGATCCTTAGCTTCCTTCAGCACTGTCATG
PIWI-box **hAgo2**	pGBKT7	Forward # 9: TGATCGAATTCGAGCGGGTTGGGAAAAGTGG Reverse # 10: TAACTGGATCCTTACAGCTCATCAGAGGAGAAACG
PAZ **hAgo2**	pGBKT7	Forward # 11: TGATCGAATTCAAGGCACAGCCAGTAATCGAG Reverse # 12: TAACTGGATCCTTAAATCTCTTCTTGCCGATCGGG
PIWI **Hiwi**	pGBKT7	Forward # 13:TCGACGAATTCAGAGTCTTACAGCAAAAGGTC Reverse # 14: TCAGCGGATCCCTAGAGGTAGTAAAGGCGGT
PIWI-box **Hiwi**	pGBKT7	Forward # 15: TCGACGAATTCCAGTCTGGAGGAAGACTTCAG Reverse # 16: TCAGCGGATCCCTATGGTCTGGCTTCAGGCCGCT
PAZ **Hiwi**	pGBKT7	Forward # 17: TCGACGAATTCCATAAAGTCCTTCGAAGTGAGA Reverse # 18: TCAGCGGATCCCTAAATGAGTCGTCCCACTTCAC
PIWI **hAgo2**	pEBG	Forward # 19: ATCGAGGATCCATGTTCCGGCACCTGAAGAAC Reverse # 20: AGCATGCGGCCGCTTAGGTATGGCTTCCTTCAGCA
PAZ **hAgo2**	pEBG	Forward # 21: GACTCGGATCCATGCTGAATATTGATGTGTCA Reverse # 22: GACTAGCGGCCGCTTAAATACATCTTTGTCCTGCC
PIWI **Hiwi**	pEBG	Forward # 23: GTGTGGATCCACAGCAGACACCCAGATAG Reverse # 24: GATGCGGCCGCTTATTGACAGTGACAGATTTGGC
PAZ **Hiwi**	pEBG	Forward # 25: GTGTGGATCCCATAAAGTCCTTCGAAGTG Reverse # 26: GATGCGGCCGCTTACATTTTATCAGTTAGACCTG
hAgo2	pEBG	Forward # 27: TGATCGGATCCATGCACCCATTCCAGTGGTGT Reverse #28: TGATCGCGGCCGCTTAAGCAAAGTACATGGTGCGCAG
Hiwi	pEBG	Forward # 29: TGATCGGATCCATGACTGGGAGAGCCCGAG Reverse # 30: TGATCGCGGCCGCTTAGAGGTAGTAAAGGCGGT

TABLE 2-3 (continued)

hAgo2	PcDNA 4/TO	Forward # 31: TCAAGGAATTCATGCACCCATTCCAGTGGTGT Reverse # 32: GATCAGGATCCAGCAAAGTACATGGTGCGCAG
PAZ **Dicer**	pENTR3C	Forward # 33: CGGAATTCATGCCACCATATGTGTTGAGGC Reverse #34 : ATAAGAATGCGGCCGCTTAAGCATCGCTGGCAGTCTG
RNaseIII **Dicer**	pENTR1 A	Forward # 35: ACGCGTCGACATGCTCAAGGGCAGGATGG Reverse # 36: ATAAGAATGCGGCCGCTCAGCTATTGGGAACCTGAG GTTG
Hel/PAZ **Dicer**	pENTR1 A	Forward # 37: ACGCGTCGACATGAAAAGCCCTGCTTTGC Reverse # 38: ATAAGAATGCGGCCGCTTAAGCATCGCTGGCAGTCTG
rAgo2	pEBG	Forward # 39: GTGTGGATCCATGTTACCCATGTACTCGG Reverse # 40: GCTAGCGGCCGCAACATGTCAAGCAAAGTACA
N-term **rAgo2**	pEBG	Forward # 39: GTGTGGATCCATGTTACCCATGTACTCGG Reverse # 41: GCTAGCGGCCGCAACATGTCAAGCAAAGTACA
C-term **rAgo2**	pEBG	Forward # 42: CGATCGGATCCAACGTGCAGAGGACGACGCC Reverse # 40: GCTAGCGGCCGCAACATGTCAAGCAAAGTACA
N-term **rAgo2**	pGBKT7	Forward # 43: GTGTGAATTCATGTTACCCATGTACTCGG Reverse # 44: GCTAGGATCCAACATGTCAAGCAAAGTACA
C-term **rAgo2**	pGBKT7	Forward # 45: CGATCGAATTCAACGTGCAGAGGACGACGCC Reverse # 46: GCTAGGATCCAACATGTCAAGCAAAGTACA

2-3-2- Analysis of proteins

2-3-2-1- Mammalian cell culture and transfections

HEK293T and COS cells were cultured in DMEM containing 10% fetal bovine serum, 2 mM gluthamine, 10 mM HEPES and antibiotics (penicilin and streptomycin, 100 u/ml). DMEM with 5% fetal bovine serum was used to culture NRK52E cells. For transient transfections, HEK293T ($1x10^6$) or COS ($5x10^5$) cells were transfected in 60 mm diameter (p60) plates using 21 μl of PerFectin and a total of 8 μg of plasmid DNA according to the manufacturer's instructions. FuGENE™ 6 was also used to transfect 100 mm diameter (p100) plates of COS cells, in which case 20 μl of FuGENE™ 6 and a total

of 5 μg DNA was used. The transfected cells were used for experiments 40-48 hr post-transfection.

HEK293T cells were plated in fibronectin-coated p60 plates. P60s were coated by addition of two ml of 10 μg/ml fibronectin in PBS, followed by incubation at 37°C for 20 min and 3 washes with PBS.

To create stable cell lines expressing hAgo2 (TRex293+hAgo2), TRex™ 293 cells were transfected with 6-8 μg of pcDNA-5TO-hAgo2 followed by selection in 100 μg/ml hygromycin B and 5 μg/ml blasticidin-S. The surviving colonies were isolated and screened for regulated expression of hAgo2 by immunoblotting and indirect immunofluorescence. For some Dicer-PPD binding experiments, the stable cell lines overexpressing hAgo2 were transiently transfected with 4-6 μg of a plasmid encoding the GST-hDicer fusion protein.

2-3-2-2- SDS-PAGE

Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described by Sambrook et al. (1989). Equal volumes of 2 x concentrated sample buffer (TABLE 2-2) were added to the protein samples, which were then denatured in a 95°C water bath for 5 min, and separated by discontinuous gel electrophoresis. Stacking gel consisted of 4% acrylamide (40% acrylamide/Bis 29:1 ratio stock; Bio-Rad) in the upper gel buffer (TABLE 2-2). The resolving gel consisted of 6, 8 or 10% acrylamide in lower gel buffer (TABLE 2-2). 0.1% (v/v) TEMED and 0.1% (w/v) ammonium persulfate were added just before pouring the gels. Electrophoresis was

conducted in SDS-PAGE running buffer (TABLE 2-2) at 80-150 V using Bio-Rad Mini-PROTEAN III or Mini-PROTEAN II vertical gel system.

2-3-2-3- Fluorography

Protein samples labeled with [^{35}S]methionine *in vitro* or [^{35}S]methionine/cysteine in cell cultures, were processed for fluorography as described (Cikaluk et. al., 1999). After the electrophoresis, gels were fixed in 25% isopropanol and 10% acetic acid for 30 min, rinsed in deionized water and incubated for a further 30 min in 100 mM sodium salycilate solution containing 0.01% (v/v) ß-mercaptoethanol. The gels were then dried at 80°C for 2 hours on a Bio-Rad gel dryer Model 583 and exposed to X-Omat AR film (Kodak) at –80°C.

2-3-2-4- Immunoblotting

SDS-PAGE gels were equilibrated for 5 min in transfer buffer (TABLE 2-2), before transfer to PVDF membranes. PVDF membranes were rinsed in methanol (30-60 sec) and deionized water, and equilibrated in transfer buffer for 5 min. Transfer was performed at 4°C using a Hoefer™ TE22 mini-gel transfer apparatus (Amersham Pharmacia Biotech.), at a 300-350 mAmp current for 2 hours (6% gel) or 3 hours (8-10% gel). Following transfer, membranes were rinsed in TBST (TABLE 2-2), and blocked in TBST containing 5% skim milk for 1 hour. Incubations with primary antibodies or horseradish peroxidase-conjugated secondary antibody (1/3000 dilution) were performed in the blocking solution for 30-60 min. Membranes were washed 3 x (5-10 min each) at room temperature in TBST, between the first and second antibody incubations and prior

to signal detection. Membranes were incubated with Supersignal Westpico Chemiluminescent Substrate reagents (PIERCE) and then exposed to Fuji RX film.

2-3-2-5- Silver staining

Silver staining was performed following a procedure adapted from Nesterenko et al. 1994. First gels were fixed for 30 min in 50% (v/v) methanol and 10% (v/v) acetic acid and sensitized in 0.02% (w/v) sodium thiosulfate. Next, gels were impregnated in 0.1% (w/v) silver nitrate solution at 4°C for 25 min, and finally gels were developed in 2% (w/v) anhydrous sodium carbonate solution containing 0.02% (v/v) formaldehyde for 2-5 min. A 1.4% (w/v) EDTA sodium salt solution was used to stop the staining reactions (Nesterenko et al., 1994).

2-3-2-6- Metabolic labeling and radioimmunoprecipitation

Confluent dishes of NRK52E and COS cells were washed once with PBS and incubated in minimum essential medium minus cysteine and methionine, containing 5% dialyzed fetal bovine serum, for 15 min at 37°C. Cells were labeled for various time periods with 150-500 μCi of Promix [^{35}S]methionine/cysteine/ml of the same labeling medium. Where indicated, cells were treated with geldanamycin (5 μM) before, during and after the labeling period. Radiolabeled cells were washed 3 x with ice-cold PBS and lysed on ice in NP40 lysis buffer (TABLE 2-2) containing Complete™ EDTA-free protease inhibitors. The lysates were subjected to centrifugation at 14,000 x*g* for five min at 4°C and the resulting supernatants were subjected to immunoprecipitation with various antibodies and protein A- or G-sepharose beads. Immune complexes were washed 3 x

with ice-cold PBS containing 1% Triton X-100 or NP40 and once with water. When using the rabbit anti-Ago2 antibody in pulse-chase experiments, the samples were washed 3 x with RIPA buffer (TABLE 2-2), and once with water. Samples were then heated at 95°C in SDS gel sample buffer for 5 min to denature the proteins before SDS-PAGE.

2-3-2-7- TNT™-coupled rabbit reticulocyte lysate system

[^{35}S]methionine labeled proteins were synthesized *in vitro* using a transcription/translation-coupled rabbit reticulocyte lysate kit according to the manufacturer's instructions. When canine pancreatic microsomal membranes (Cikaluk et al., 1999) were added to the reactions, half the normal amount of TNT reaction buffer was used and the amount of [^{35}S]methionine used in the reaction mixture was doubled. Samples were diluted with 100 volumes of TBS containing TX-100 1%, followed by radioimmunoprecipitation with antibodies as described above. Canine pancreatic microsomal membranes were a kind gift from Dr. Christopher V. Nicchitta (Duke University).

2-3-2-8- *In vitro* binding assay

In vitro binding assays were performed as described (Beatch and Hobman, 2000) to confirm results obtained by yeast two-hybrid screening (section 2-3-3-1). The cDNAs identified through yeast two-hybrid screening were subcloned into pGADT7 in-frame with an HA-tag epitope. HA-fused positive "preys" and their corresponding Cmyc-fused (in pGBKT7) "baits" were translated *in vitro* in the same or separate TNT™-coupled reticulocyte lysate reactions according to the manufacturer's instructions. Five μl of

translation products were pooled in a tube and subsequently diluted with 100 volumes of TBS+TX-100 1%. Incubation at 4°C and rotation was continued for 1 hour, and followed by reciprocal immunoprecipitation. Briefly, either anti-Cmyc or anti-HA monoclonal antibodies (9E10 or 12CA5, respectively) were added to the mixture of the labeled proteins and incubation was continued at 4°C for 3 hours. Immune complexes were isolated by binding to protein G-sepharose and collected by 5 min centrifugation at 500 x*g*, washed 3 x with incubation buffer, and resolved on SDS-PAGE followed by fluorography.

2-3-2-9- Protease protection assay

These assays were performed as previously described (Cikaluk et al., 1999). Briefly, rat or human Ago2 and VSV G were synthesized *in vitro* with or without canine pancreatic microsomes. After translation samples were adjusted to 10 mM $CaCl_2$ and kept on ice for 10 min to stabilize microsomes. Samples were then incubated on ice for 60 min with or without trypsin/chymotrypsin (2-20 μg) in the presence or absence of Triton-X100. Proteolysis was terminated by the addition of aprotinin, and samples were then analyzed by SDS-PAGE and fluorography.

2-3-2-10- TCA precipitation of proteins

Proteins were precipitated by the addition of 100% TCA (trichloroacetic acid) to a final concentration of 20%, followed by mixing and incubation on ice for 30 min. Protein samples were pelleted by centrifugation at 14,000 x*g* for 10 min. Pellets were washed by addition of 80% acetone (in deionized water) for 5 min on ice, followed by a 5 min

centrifugation at 14,000 x*g* and 4°C. The samples were resuspended in sample buffer, heated to 95°C for 5 min and subjected to analysis by SDS-PAGE and immunoblotting.

2-3-2-11- GST pull-down assay

Forty hours post-transfection, cells were washed with ice-cold PBS and then lysed with or without sonication. In the first case, cells from confluent p60 dishes were lysed in sonication lysis buffer (TABLE 2-2) containing protease inhibitors, followed by brief sonication on ice using a Branson Sonifier 250, and centrifugation at 21,000 x*g* for 18 min at 4°C (Mourelatos et al., 2002). In cases where sonication was not used, cells were lysed in NP40 lysis buffer containing protease inhibitors. The resulting supernatants were incubated with 400 μl of PBS glutathione-sepharose 4B bead slurry (10% in PBS) for a minimum of 3 hours at 4°C on a rotating device. The beads were collected by centrifugation at 500 x*g* for 5 min and then washed 3 x with ice-cold lysis buffer. Proteins were eluted by two sequential treatments with freshly prepared elution buffer (TABLE 2-2) for 10 min at room temperature. Eluted proteins were TCA precipitated and then analyzed by SDS-PAGE and silver staining or immunoblotting. For some experiments, beads were heated to 95°C for 5 min in SDS sample buffer (instead of elution) and analysed by SDS-PAGE. Where indicated, GD was added (5 μM), 2-8 hours prior to harvesting cells. Control samples were treated with equal volumes of DMSO.

To determine if RNA was required for complex stability, the glutathione-sepharose 4B beads and their associated proteins were treated with 0.5 U/μl micrococcal nuclease (total volume of 50 μl sonication buffer plus 1 mM $CaCl_2$) on ice for 1 hour. Activity of the nuclease was verified by parallel digestion of 2-5 μg DNA in 10-60 min

time periods. The reactions were stopped by the addition of 5 mM EDTA. Samples were washed two more times in the sonication buffer prior to SDS-PAGE and immunoblotting with anti-GFP and anti-Dicer to detect endogenous and CFP-Dicer.

2-3-2-12- Membrane flotation assay

Assays were performed according to a previously published protocol (Matsumoto et al., 1997). Confluent p60 dishes of HEK293T, NRK52E or COS cells were washed 3 x in ice-cold PBS, followed by addition of 1 ml of hypotonic buffer (TABLE 2-2), plus complete EDTA-free protease inhibitors for 10 min on ice. Cells were scraped from dishes and homogenized by 15 passages through a 26.5 gauge needle in the cold-room. Homogenates were subjected to centrifugation at 1,000 x*g* for 5 min at 4°C to prepare post-nuclear supernatants (PNS). PNS (446 μl) was mixed with 1.78 ml of 72% (w/v) sucrose solution prepared in low salt buffer (TABLE 2-2) and loaded at the bottom of SW50.1 centrifugation tubes. The sucrose-adjusted PNS was overlaid with 2.23 ml of 55% (w/v) and topped with 0.54 ml of 10% (w/v) sucrose prepared in low salt buffer. The samples were subjected to centrifugation at 140,000 x*g* for 12 hours at 4°C. One ml fractions were collected from top to the bottom of the gradients, diluted in low salt buffer. Proteins were precipitated with TCA from diluted fractions, followed by SDS-PAGE analysis and immunoblotting.

For some experiments, gradient fractions were used for GST pull-down assays. Samples from top and bottom fractions of multiple parallel membrane flotation assays were collected and concentrated prior to GST pull-down assays.

2-3-2-13- Immunofluorescence microscopy

Cells grown on 12-mm glass coverslips, were processed for indirect immunofluorescence microscopy as described (Hobman et al., 1992; Kuismanen and Saraste, 1989). Briefly, coverslips were rinsed 3 x in PBSCM (TABLE 2-2), fixed and permeabilized with methanol at –20°C for 6 min, followed by 3 washes (5 min each) in PBSCM. After blocking in PBSCM+1% BSA for 15 min, coverslips were incubated with primary antibodies in blocking solution for 1 hour and washed 3 x with PBSCM+BSA 0.1%. Next, dilutions of secondary antibodies (1/200) were added to the coverslips and incubated for 30-60 min in the dark. Finally, the coverslips were rinsed 3 x with PBSCM+BSA 0.1% and mounted in a drop of VECTASHIELD mounting medium (TABLE 2-1) on slides. Where indicated, cells were treated with the Hsp90 inhibitors, geldanamycin (5-10 μM) or radicicol (10 μM), 6-12 hours before processing for immunofluorescence microscopy.

2-3-2-14- Dicer activity assay

DsRNA processing assays were performed by our collaborators in Dr. Filipowicz's lab (Basel, Switzerland) as previously described (Zhang et al., 2002), and contained 129-bp dsRNA uniformly labelled with [^{32}P]UTP, 20 ng of recombinant human Dicer-HisC protein, and various amounts of GST-Hiwi, GST-hAgo2, or GST.

2-3-3- Screening methods

2-3-3-1- Yeast two-hybrid screen for rAgo2-binding proteins

All reagents, yeast strains and plasmids were obtained from MATCHMAKER SYSTEM III. MATCHMAKER SYSTEM II yeast two-hybrid human cDNA libraries (in pACT2) were used for screening.

The sequences encoding the amino terminal (aa 1-323) and carboxy terminal (aa 554-863) regions of rAgo2, were subcloned into pGBKT7 and used as "bait" in yeast two-hybrid screen. The "bait" plasmids were co-transformed along with the yeast two-hybrid human prostate or testis cDNA libraries into *S. cerevisiae* strain AH109. The transformants were plated on complete synthetic media lacking leucine, tryptophan, histidine and adenine (CSM-leu-trp-his-ade) also referred to as quadruple drop-out plates (4DO). Putative positive colonies were picked from 4DO plates after 7-10 days, cultured in 5 ml liquid 4DO medium and subjected to "smash and grab" miniprep (next section), to recover the "prey" plasmids. Plasmids were retransformed into the AH109 strain, along with their corresponding "bait" or negative controls (pGBKT7+p53 or no insert). The putative positives that grew on 4DO only in the presence of their corresponding "bait", were re-isolated from yeast by "smash and grab" and sequenced. The identities of the sequences were determined after "Blast" searches to compare them with the non-redundant human database at NCBI. Further confirmation of the interactions with rAgo2 was obtained by *in vitro* binding assays, as described in section 2-3-2-8.

To test for the direct interactions and to map interaction sites, selected regions of the cDNAs of human Dicer or PPD proteins (hAgo2 and Hiwi) were fused in-frame to the Gal-4 activation domain of pGADT7 or the Gal-4 DNA-binding domain of pGBKT7,

respectively (TABLE 4-1). The resulting plasmid constructs were transformed into AH109 and plated on CSM-leu-trp (2DO) for 2-3 days at 30°C followed by streaking onto 4DO. Combinations of "bait" and "prey" that grew in fewer than 7 days, were deemed as positive interactions, whereas lack of growth after 14 days was deemed as negative.

2-3-3-1-1- Yeast smash & grab DNA miniprep

DNA was isolated from yeast by "smash and grab" DNA miniprep technique as described (Rose MD et al, 1990). Single yeast colonies were grown in 5 ml cultures at 30°C on a wheel at 50 rpm for 20-24 hours. Yeast was harvested by centrifugation at 1000 x*g* for 5 min in microfuges. After addition of 0.3 g of glass beads (425-600 microns), 0.2 ml of the lysis buffer (100 mM NaCl, 1mM EDTA, 1% SDS, 2% Triton X-100 and 10 mM Tris HCl, pH 8.0), and equal volume of 1:1 mix of phenol-chloroform, the microfuge tubes were vortexed vigorously for 2 min and then subjected to centrifugation at 14000 x*g* for 5 min at room temperature. The aqueous supernatants were transferred to new tubes and DNA was precipitated by addition of 2 volumes of 100% ethanol. DNA was recovered by 2-3 min centrifugation at 14,000 x*g* at room temperature, rinsed in 70% ethanol, air dried and dissolved in TE.

The isolated DNAs that contained both "prey" (pGADT7; ampicilin resistant) and "bait" (pGBKT7; kanamycin resistant) plasmids, were transformed into *E. coli*, and colonies containing the "prey" plasmids were recovered on ampicilin plates.

2-3-3-2- GeneTrapper cDNA positive selection system

Full-length cDNAs encoding isoforms of a human orthologue of rAgo2 were recovered from a human leukocyte cDNA library in collaboration with Invitrogen. Briefly, target cDNAs were enriched by GeneTrapper cDNA Positive Selection System, identified by hybridization to ^{32}P-labeled probes and cloned.

Following a search in the human EST database, a 5' sense oligonucleotide corresponding to the nucleotides 305-364 in the ORF of rAgo2 (5'-GGAGAGTTAACA-GGGAAATCGTGG-3') and a 3' sense oligonucleotide corresponding to the nucleotides 2565-2590 in the ORF of rAgo2 (5'-CAGTGTACCACCTGGTGGATAAGG-3'), were chosen for hybridization to and isolation of hAgo2. In the first step, the oligos were synthesized by Invitrogen and purified on a 12% (19:1 w/w) acrylamide/bisacrylamide, 8 M urea and 1x TBE buffer gel. Oligonucleotides (3μg) were biotinylated by the addition to a reaction containing Biotin-14-dCTP and TdT in 1 x TdT buffer, followed by brief vortexing and incubation for 1 hour at 30°C. The biotinylated oligos then were ethanol-precipitated, dried and redissolved in TE. In the second step, a human leukocyte cDNA library (demonstrated by PCR to contain full-length hAgo2) was treated with Gene II in 1 x Gene II buffer and Exo III to produce an anti-sense single stranded cDNA library. Gene II binds to the f1 origin of replication in phagemid vectors (in this case pCMVSPORT2), and nicks the viral strand of the supercoiled DNA. After nicking, Exo III was added and incubated at 37°C for 1 hour to digest the sense strand of pCMVSPORT2, leaving a single-stranded (ss) cDNA library with the opposite polarity to the biotinylated oligonucleotides. Next, ss cDNA libraries were hybridized to the denatured biotinylated oligonucleotides by incubation at 37°C for 1 hour, and paramagnetic streptavidin beads

were used to capture the biotinylated complexes. Captured ss cDNAs then were eluted from the beads and ethanol precipitated. The ss cDNAs were converted to ds cDNAs in a DNA synthesis reaction using non-biotinylated form of the same oligos that were used to captured them, followed by ethanol precipitation of DNA. The captured dsDNAs were then electroporated into high efficiency ELECTROMAX DH10B *E. coli* cells ($10^9/\mu l$) and plated on agar plates containing 100 μg/ml ampicilin. Bacterial colonies were transferred to nylon Hybond™ membranes. A 935 bp 5'-oligonucleotide was prepared from rAgo2 cDNA by PCR using p10 (forward): 5'-GCTAGTTTGGGGAAGATTAG CC-3' and p9 (reverse): 5'-GCTTTATCTCCACCTTTAGACC-3' primers. The 935 bp partial rAgo2 cDNA was labeled with [α^{32}P]-dCTP using Random Primed DNA Labeling Kit and hybridized with the membranes. The membranes were blotted dry, exposed to X-Omat AR film (Kodak) at –80°C. Positive colonies were picked from duplicate agar plates, and plasmids were isolated by miniprep and sequenced.

CHAPTER 3

RESULTS

A version of this chapter has been published in "**Tahbaz, N.**, Carmichael, J.B. and Hobman, T.C. (2001) GERp95 belongs to a family of signal-transducing proteins and requires Hsp90 activity for stability and Golgi localization. *J Biol Chem*, 276, 43294-43299" and in "Cikaluk, D.E., **Tahbaz, N.**, Hendricks, L.C., DiMattia, G.E., Hansen, D., Pilgrim, D. and Hobman, T.C. (1999) GERp95, a membrane-associated protein that belongs to a family of proteins involved in stem cell differentiation. *Mol Biol Cell*, 10, 3357-3372". Reproduced with permission.

Overview

Golgi and endoplasmic reticulum-associated 95 kDa protein (GERp95) was identified in a monoclonal antibody screen for novel membrane-associated proteins (Cikaluk et al., 1999). Non-redundant data base searches indicated that GERp95 is a PPD protein, and it was renamed as rat Ago2 (rAgo2). Our data suggested that a pool of rAgo2 exists as a peripheral membrane protein, that is part of a protease-resistant complex (Cikaluk et al., 1999). A search for rAgo2-binding proteins was undertaken to gain insight into the intermolecular interactions of rAgo2.

Yeast two-hybrid screening and affinity purifications identified members of the Hsp90 heterocomplex as rAgo2-binding proteins. Association between rAgo2 and an Hsp90 heterocomplex was confirmed in transcription/translation-coupled rabbit reticulocyte lysate system (TNT). In addition, pulse-chase experiments and immunoblotting revealed that biogenesis and stability of rAgo2 required functional Hsp90. However, no colocalization between rAgo2 and Hsp90 was detected, suggesting that their interaction is transient. Furthermore, in the absence of Hsp90 activity, translocation of rAgo2 to the membranes was inhibited (Tahbaz et al., 2001), suggesting that Hsp90 is required for incorporation of rAgo2 into membrane-associated complexes.

3-1- RAgo2 is present in a protease-resistant complex

Our data indicated that rAgo2 is a cytosolically exposed peripheral membrane protein, that associates with intracellular membranes (Cikaluk et al., 1999). Unexpectedly, protease protection assays of microsomes followed by immunoblotting

revealed that rAgo2 is partially resistant to proteolytic digestion. Our data showed that rAgo2 was degraded only in the presence of detergent and high concentration of proteases. In contrast, ß-Cop, another cytosolically exposed peripheral membrane protein on ER/Golgi membranes (Duden et al., 1991), was sensitive to protease digestion in the absence of detergent. The Glucosidase II ß-subunit, an intraluminal ER protein, was used as a positive control for integrity of the microsomes. This 80 kDa protein was degraded only after the detergent was included in the assay (Cikaluk et al., 1999).

We asked whether rAgo2 was inherently protease-resistant or if it was protected from proteases due to its presence in a complex (Cikaluk et al., 1999). *In vitro* synthesized ^{35}S-labeled rAgo2 was highly sensitive to protease degradation, indicating that rAgo2 is not inherently protease-resistant (Fig 3-1; upper panel, lanes 3, 6, 9). When canine pancreatic microsomal membranes (a kind gift from Dr. Chris Nicchitta, Duke University) were included in the *in vitro* synthesis reactions, a portion of the newly synthesized rAgo2 became protease-resistant. This protease-resistant form of rAgo2 was degraded at a similar rate in the presence (- TX-100) or absence (+TX-100) of intact membranes (Fig 3-1; upper panel, lanes 4,5,7,8,10,11). The vesicular stomatitis virus glycoprotein (VSV G) was used as a positive control for translocational activity of the microsomes. VSV G is an integral membrane protein of which all but 29 amino acids are translocated into microsomes during translation (Katz et al., 1977). VSV G was synthesized in the presence or absence of microsomal membranes *in vitro*, and the reactions were then subjected to the protease-protection assay with or without detergent. The intraluminal part of the glycoprotein was protease-sensitive only when the detergent was included during incubation with the protease, indicating that membranes remained

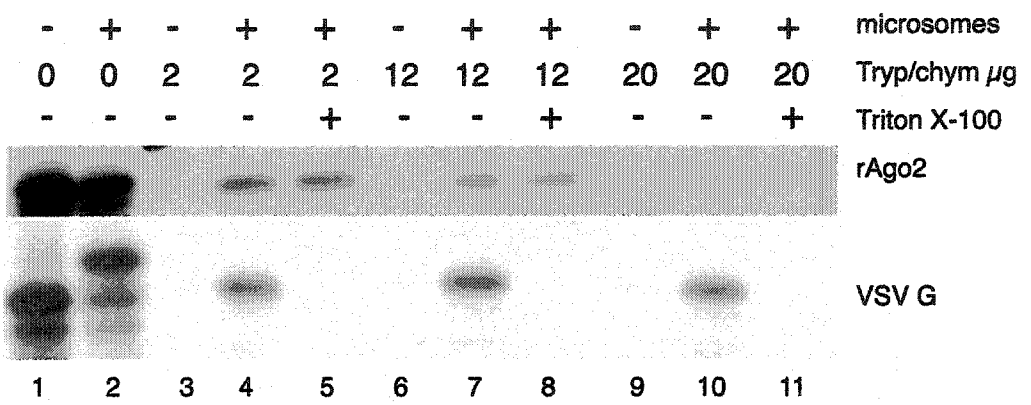


Figure 3-1- A pool of rAgo2 is incorporated into a protease-resistant complex

^{35}S-labeled rAgo2 (upper panel) was synthesized *in vitro* in the presence or absence of canine pancreatic microsomes. Samples were subjected to digestion with varying amounts of trypsin/chymotrypsin with or without Triton X-100 before SDS-PAGE and autoradiography. In reactions that contain membranes, a portion of rAgo2 became protease-resistant (lanes 4, 7 and 10). Protease-resistance of rAgo2 persisted even after solubilization of membranes by detergent (lanes 5, 8 and 11). VSV G protein (lower panel) was used as a positive control to show translocational activity of the microsomes. The intraluminal portion of VSV G is protected from protease digestion when microsomes are present.

intact during the experiment (Fig 3-1; lower panel, lanes 5, 8, 11).

Our data indicated that rAgo2 is incorporated into a protease-resistant complex in the presence of membranes. Once formed, the stability of the protease-resistant complex is not dependent on the integrity of the membranes. These results are consistent with the simultaneous existence of both membrane-bound and cytosolic forms of the rAgo2-containing complexes *in vivo*. The fact that only a portion of rAgo2 enters the complex *in vitro* could mean that the components required for the assembly of the complex are limiting in the rabbit reticulocyte lysates and/or in the microsomal preparations.

3-2- Yeast two-hybrid screen for rAgo2-binding proteins

Yeast two-hybrid screening has been used extensively to detect interactions between proteins (Fields and Song, 1989). Using a full-length rAgo2 cDNA fused to the DNA-binding domain of Gal-4 as "bait", a number of potential rAgo2-binding proteins, including heat-shock proteins, had previously been identified in our laboratory (our unpublished data). Next, we were interested in identifying rAgo2-binding proteins that associated with the divergent amino terminus or highly conserved carboxy terminus of the protein.

RAgo2 is a highly basic protein containing 863 amino acid residues. The cDNAs encoding the amino terminal (aa 1-323) and carboxyl terminal (aa 545-863) regions of rAgo2 were subcloned into the MATCHMAKER SYSTEM III "bait" plasmid, pGBKT7, in-frame with the C-Myc-tag and the DNA-binding domain of Gal4. The plasmid constructs were named pGBKT7-NT and pGBKT7-CT, respectively. Since Northern blot analysis revealed the highest levels of rAgo2 transcripts were in testis (Cikaluk et al.,

1999), testis and prostate yeast two-hybrid libraries (cloned in pACT2) were used in the screens. However, rat testis and prostate yeast two-hybrid libraries were not available, and therefore we used human cDNA libraries. Human Ago2 is 97.8 % identical to rAgo2; the minor differences are at the amino terminal regions of the two proteins. Accordingly, we expected to find the human orthologues of rAgo2-binding proteins in our screen.

PGBKT7-NT or CT "bait" plasmids were co-transformed along with prostate or testis yeast two-hybrid libraries into *S. cerevisiae* strain AH109. Approximately $1x10^6$ transformants were screened. The "prey" plasmids were isolated by "smash and grab" DNA miniprep from colonies that grew on 4DO plates. The inserts were sequenced and then compared with non-redundant cDNA databases to identify the encoded proteins.

3-2-1- Clones encoding potential rAgo2-binding proteins

The putative positives that were found in the yeast two-hybrid screen using the amino terminus of rAgo2 as a "bait" included Hdj2, Filamin, FHL2, Caldesmon and several novel proteins of unknown function. When the carboxyl terminus of rAgo2 was used as "bait", Filamin, Initiation Factor3/subunit 5, Proteosome (PSMB4), Sorbitol Dehydrogenase, MHC class I (HLA B51 mRNA) and several novel proteins were identified as potential binding proteins.

Filamin, FHL2 and Caldesmon were deemed to be false positives since the "prey" plasmids encoding them grew on 4DO plates in the presence of "bait" plasmids with no insert. In contrast, Hdj2, Initiaton Factor3/subunit 5, Proteosome (PSMB4), Sorbitol Dehydrogenase and MHC class I (HLA B51 mRNA) together with an unknown human hypothetical protein (GI: 39645249) appeared to be true rAgo2-interacting proteins

(TABLE 2, Appendix). However, our *in vitro* binding assays were limited to the study of rAgo2-Hdj2 interactions.

3-2-2- Interaction between Hdj2 and rAgo2 *in vitro*

Hdj2 is the human homologue of bacterial Dna-J and is one of the members of the Hsp40 family of heat-shock proteins (Leng et al., 1998). Hdj2 is both a weak chaperone and a co-chaperone. As a co-chaperone, Hdj2 interacts with Hsp70 and enhances its chaperone activity (Wittung-Stafshede et al., 2003). In addition, Hdj2 and Hsp70 bind to substrates and present them to Hsp90 (Minami and Minami, 1999). To confirm the interactions obtained by yeast two-hybrid screening, glutathione *S*-transferase (GST) alone or rAgo2 fused to GST was transiently overexpressed in COS cells, with or without human Hdj2. Transfected COS cells were labeled with [^{35}S]methionine/cysteine before lysis in NP40 lysis buffer (TABLE 2-2). GST, GST-rAgo2 and associated proteins were captured on glutathione-sepharose 4B beads, washed and eluted from the beads, resolved on SDS-PAGE and visualized by fluorography. The data obtained by GST pull-downs confirmed the results of the yeast two-hybrid screening, indicating that human Hdj2 binds rAgo2 (Fig 3-2).

3-3- Interaction between rAgo2 and Hsp90

3-3-1- The amino terminus of rAgo2 binds Hsp90

Affinity purification of proteins by GST pull-downs has been widely used to identify novel binding interactions (Zhan et al., 2001). Constructs encoding full-length rAgo2 (GST-FL), plus the amino terminal (aa 1-323) (GST-NT) and carboxyl terminal (aa 554-

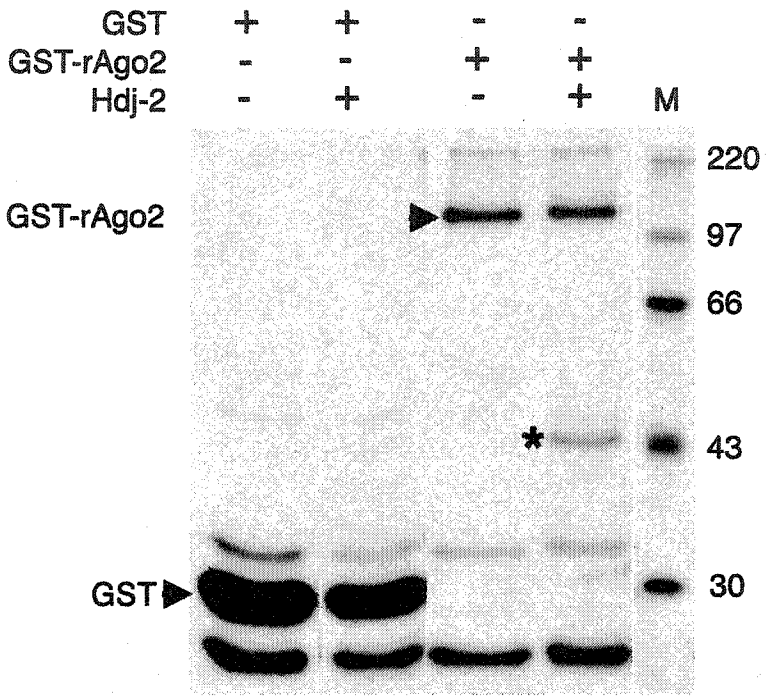


Figure 3-2- Confirmation of the binding between rAgo2 and Hdj2 using GST pull-downs

GST-rAgo2 or GST alone were transiently expressed in COS cells with or without Hdj2, and labeled overnight with Promix [^{35}S]methionine/cysteine. Lysates were affinity purified on glutathione-sepharose 4B beads, and eluted proteins were resolved on SDS-PAGE and visualized by fluorography. GST-rAgo2 and GST alone are indicated by arrow heads. Hdj2 is marked by an asterisk. The ^{14}C-labeled protein markers (M) are shown on the right side of the gel.

863) (GST-CT) regions fused to GST and GST alone were expressed transiently in COS cells (Chen et al., 2002; Sanchez et al., 1994). The GST-fusion proteins (Fig 3-3 A) were affinity purified together with their binding proteins on glutathione-sepharose 4B beads. The proteins were eluted from the beads, resolved by SDS-PAGE and visualized by silver staining. As expected, the rAgo2-GST fusion proteins bound a number of proteins that did not bind to GST alone (Fig 3-3 B). We were especially interested in proteins that bound to the GST-NT or GST-CT fusion proteins and to GST-FL rAgo2. Silver staining revealed a prominent approximately 90 kDa protein that co-purified only with the GST-NT and GST-FL rAgo2. Based on its apparent mass and our data indicating that Hdj2 interacts with rAgo2, we tested whether the 90 kDa protein was Hsp90 by immunoblotting. Indeed this turned out to be the case (Fig 3-3 C). Moreover, endogenous Hsp90 was coimmunoprecipitated by anti-Ago2, but not by preimmune serum from rat liver microsomal preparations (Fig 3-3 D). Finally, it is important to mention that Hsp90 was also identified as a hAgo2 (human orthologue of rAgo2) binding protein by mass spectrometry (Parker and Hobman, unpublished data).

A second protein of approximately 70 kDa was also affinity purified by GST-NT, GST-CT and GST-FL rAgo2, but not by GST alone (Fig 3-3 B). This protein was identified by immunoblotting as Hsp70 (discussed below). Hsp70 was also identified by mass spectrometry among hAgo2-binding proteins affinity purified from cultured human cell lines (Parker and Hobman, unpublished data).

3-3-2- Members of Hsp90 heterocomplexes associate with rAgo2

Hsp90 is a highly conserved essential heat-shock protein which is expressed in all

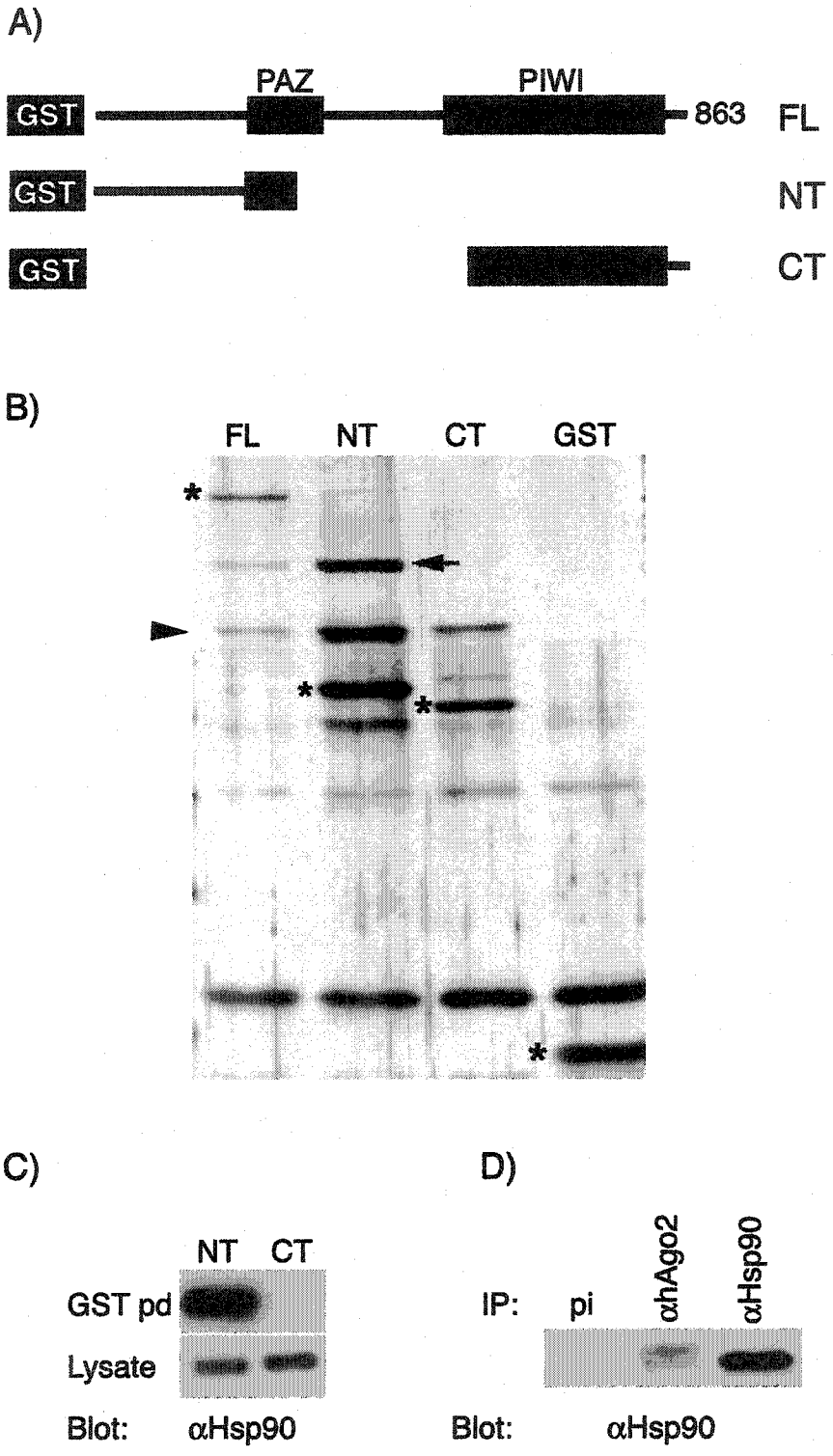
Figure 3-3- RAgo2 binds to Hsp90

A) The coding regions of full-length (FL), amino terminal (NT) (1-323 aa), and carboxyl terminal (CT) (554-863 aa) regions of rAgo2 were fused to GST.

B) GST alone and GST fused to FL, NT, or CT of rAgo2 were transiently expressed in COS cells and then immobilized on glutathione-sepharose 4B beads. Proteins were eluted with reduced glutathione, separated by SDS-PAGE, and silver-stained. The 90-kDa protein (arrow) that binds to FL and NT of rAgo2 was shown to be Hsp90 by immunoblotting. Arrow head points to a 70 kDa protein which was shown to be Hsp70 by immunoblotting. GST fusion proteins are marked by asterisks.

C) Anti-Hsp90 was used to immunoblot GST pull-downs (pd) and lysates from COS cells transiently expressing GST-rAgo2 constructs.

D) Aliquots from rat liver microsomal preparations were immunoprecipitated with anti-Ago2 or preimmune serum (Pi), or with anti-Hsp90 as a positive control. The immunoprecipitates were resolved on SDS-PAGE, transferred to PVDF membranes and blotted with an antibody to Hsp90.



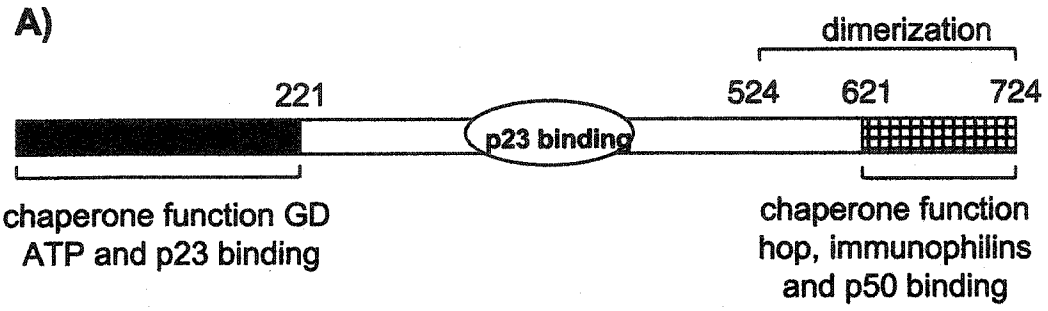
eukaryotes. Unlike Hsp70, Hsp90 is not normally involved in folding of nascent proteins, but instead associates with a limited number of substrates (Young et al., 2001). There are two major forms of Hsp90 heterocomplexes that associate with kinases or steroid hormone receptor complexes (Pratt et al., 1999). The Hsp90-kinase complex contains $P50^{Cdc37}$, whereas the Hsp90-steroid hormone receptor complexes contain immunophilins, such as Fkbp52 or Cyp40. $P50^{Cdc37}$ is the mammalian homologue of the yeast cell cycle regulatory protein Cdc37 that binds to protein kinases such as pp60(src) and v-Raf, as well as to Hsp90. The $P50^{Cdc37}$ and immunophilin binding-sites overlap on Hsp90 and consequently the two forms of the Hsp90 heterocomplexes are distinct (Silverstein et al., 1998). In addition, both types of Hsp90 heterocomplexes contain a core set of chaperones and co-chaperones including Hsp70, Hdj2, P23 and Hop (also called P60). Hsp90 hydrolyzes ATP and induces a conformational change in bound substrates that generally results in substrate activation (Grenert et al., 1999). Consequently, activated steroid hormone receptors are able to bind steroid hormones and translocate them to the nucleus, whereas activated kinases are able to phosphorylate their substrates after interaction with Hsp90 (Czar et al., 1997; Whitesell et al., 1994). The function of Hsp90 is specifically inhibited by ansamycin antibiotics including geldanamycin (GD) and radicicol (Rad), both of which compete with ATP for binding to Hsp90 (Roe et al., 1999) (Fig 3-4 A).

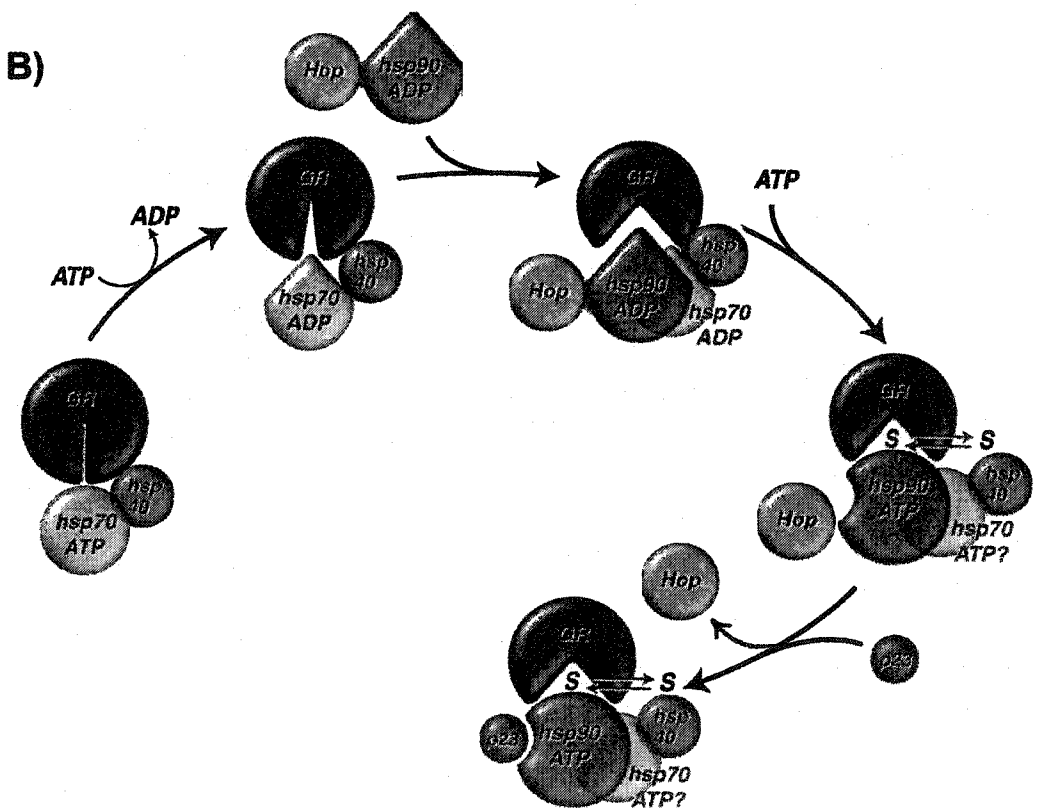
Association between Hsp90 heterocomplexes and their substrates involves several sequential steps (Morishima et al., 2000). First, Hsp70 and Hdj2 bind to substrate in the presence of ATP and transfer them to Hsp90-Hop complexes. This is followed by the interaction of Hsp90 with ATP and P23, a process that results in dissociation of Hop and and stabilization of the ATP-associated form of the Hsp90-substrate complex

Figure 3-4- Structure of Hsp90 and the Hsp90 chaperone cycle

A) Hsp90 is composed of 724 amino acid residues, with the amino and carboxyl termini required for chaperoning function. The amino terminus of Hsp90 has an ATPase domain which is the binding site for ATP, geldanamycin and P23. P23 also interacts with a region in the middle of Hsp90. The carboxyl terminus of Hsp90 is the binding site for Hop, immunophilins and $P50^{Cdc37}$, and is also required for dimerization of Hsp90 (adapted from Pratt and Toft, 1998; 2003).

B) The mechanism of activation for steroid hormone receptor substrates (Glucocorticoid Hormone Receptor (GR)), by Hsp90 heterocomplexes is well characterized. The ATP-bound form of Hsp70 and Hsp40 (Hdj2) binds to GR, and the hydrolysis of ATP to ADP results in the slight opening of the hydrophobic glucocorticoid-binding cleft of GR. Next, the Hsp70-Hsp40 complex transfers GR to the ADP-bound Hsp90-Hop complex. Maturation of the heterocomplex is induced by the ADP to ATP exchange and addition of P23 to the complex, which is followed by the dissociation of Hop. Finally, activated GR interacts with its substrate (S), (Glucocorticoid Hormone) and translocates to the nucleus (adapted from Pratt and Toft, 2003).





(Sullivan et al., 1997). Finally, hydrolysis of ATP to ADP by the ATPase activityof Hsp90 causes a conformational change in the substrate and releases the activated protein (Young and Hartl, 2000) (Fig 3-4 B).

We investigated the association of rAgo2 with Hsp90 heterocomplexes using GST pull-down assays. GST-FL, GST-NT or GST-CT of rAgo2 or GST alone were expressed transiently in COS cells and lysates were prepared in NP40 lysis buffer (TABLE 2-2). Lysates were then subjected to affinity purification with glutathione-sepharose 4B beads, SDS-PAGE, and immunoblotting with antibodies to Hsp90, P23, Hop, Hdj2 and Hsp70 (TABLE 2, Appendix). The results demonstrated that the amino terminus of rAgo2 interacts directly or indirectly with all five chaperone/co-chaperones (Fig 3-5). However, the data are slightly different for full-length rAgo2, in that the full-length protein did not bind well to P23 and Hdj2. Furthermore, the binding of Hop to full-length rAgo2 was not detected. The carboxyl terminus of rAgo2 bound weakly to Hsp70 and Hdj2 but not to Hsp90, P23 or Hop. Hsp70 is a general chaperone that together with Hdj2 (Landry, 2003; Wittung-Stafshede et al., 2003) binds to substrates and delivers them to the Hsp90-Hop complex. Thus, the observed binding of Hsp70 and Hdj2 to different regions of rAgo2 is not unexpected. Neither Fkbp52 nor $P50^{Cdc37}$ were present in detectable amounts in any of our GST-rAgo2 affinity purifications (data not shown). Our data suggest that Hsp90-rAgo2 may represent a novel type of Hsp90 complex that is different from kinase and steroid hormone receptor complexes.

Binding to Hsp90 heterocomplex may induce a conformational change in rAgo2 that is important for the interaction of rAgo2 with other proteins, possibly through the more conserved carboxyl terminus. The stable association of GST-NT with Hsp90 and its

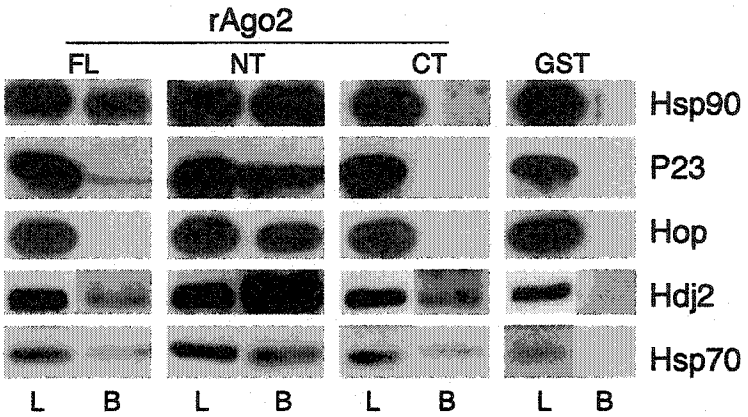


Figure 3-5- RAgo2 associates with members of Hsp90 heterocomplex

COS cells were transiently transfected with GST-rAgo2, lysed in NP40 lysis buffer (TABLE 2-2) and affinity purified on glutathione-sepharose 4B beads. GST-rAgo2 and its associated proteins were eluted from the beads in reduced glutathione. Lysates (L) and GST fusion protein bound fractions (B) were subjected to SDS-PAGE and immunoblotted with antibodies to Hsp90, P23, Hop, Hdj2, and Hsp70. Twice (percentage of total) as much of the bound fractions were loaded in order to detect weaker interactions.

co-chaperones may indicate that the isolated amino terminus of rAgo2 adopts a conformation that traps the Hsp90 heterocomplex and inhibits its release.

3-3-3- Hsp90 and its co-chaperones associate with rAgo2 in rabbit reticulocyte lysates

Rabbit reticulocyte lysate-mediated transcription/translation has been routinely used to study interactions between Hsp90 heterocomplexes and their substrates (Hutchison et al., 1992; Scherrer et al., 1992; Smith et al., 1993). This system contains adequate levels of Hsp90 and its co-chaperones to form functional complexes which regulate the activities of steroid hormone receptors and kinases (Nimmesgern and Hartl, 1993; Schumacher et al., 1994). To ascertain that the interaction between rAgo2 and Hsp90 heterocomplex was not an aberration resulting from rAgo2 overexpression in mammalian cells, we verified this interaction in a TNT-coupled rabbit reticulocyte lysate system.

Full-length rAgo2 or GFP (negative control) were synthesized *in vitro* in the presence of [^{35}S]methionine. The translation products were immunoprecipitated with antibodies against Ago2, GFP, or Hsp90, Hop, P23, Hdj2 and Hsp70, followed by SDS-PAGE and fluorography. Radiolabeled rAgo2 was efficiently coimmunoprecipitated with antibodies to Hsp90, Hop, P23, Hdj2 and Hsp70 (Fig 3-6, top panel). In contrast, no interactions were detected between rAgo2 and Fkbp52 or p50^{Cdc37}. As expected GFP did not interact with Hsp90 or any other co-chaperones (Fig 3-6, lower panel). Identical results were obtained later, when hAgo2 was used instead of rAgo2 in this experiment (section 4-1, and see TABLE 2, Appendix).

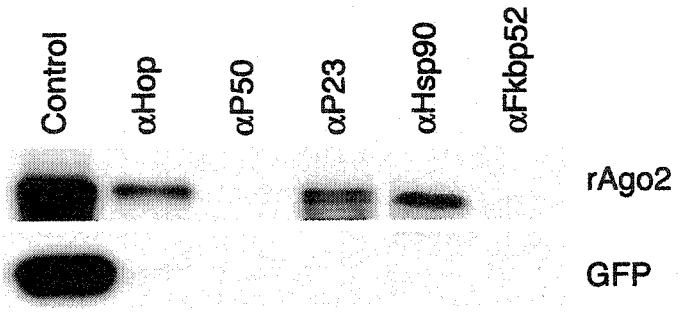


Figure 3-6- RAgo2 binds to Hsp90 and a subset of co-chaperones in the rabbit reticulocyte lysate system

^{35}S-Labeled rAgo2 and GFP were synthesized in the TNT™-coupled rabbit reticulocyte lysate system. Reactions were subjected to radioimmunoprecipitation with antibodies to, Hop, P50^{Cdc37}, P23, Hsp90 and Fkbp52. Immune complexes were analyzed by SDS-PAGE and fluorography. Immunoprecipitations with rabbit anti-Ago2 and rabbit anti-GFP were used as positive controls.

The interacting partners of full-length rAgo2 were slightly different in rabbit reticulocyte lysate system when compared to COS cells. Specifically, full-length rAgo2 interacted with Hsp90, Hop, P23, Hsp70 and Hdj2 *in vitro*, whereas interaction with P23 in COS cells was not strong. Moreover, detectable amounts of Hop were not present in pull-downs from GST-rAgo2 overexpressing COS cells. As mentioned earlier, interaction with P23 stabilizes the ATP-bound Hsp90-substrate complex and releases Hop. The presence of Hop among the Hsp90 heterocomplex members that interact with rAgo2 *in vitro* suggests that maturation of the rAgo2-containing Hsp90 heterocomplex is inefficient in reticulocyte lysates.

3-4- Characterization of the interaction between rAgo2 and Hsp90

3-4-1- Hsp90 activity is required for the stability of nascent rAgo2

Hsp90 activity is necessary for the stability of many of its substrates (Goes and Martin, 2001). To determine if Hsp90 activity was required for the biogenesis of rAgo2, we performed biosynthetic labeling experiments and radio-immunoprecipitations in the presence or absence of Hsp90 activity. Since the endogenous rAgo2 expression level is low in most cell types (including COS), rAgo2 was transiently expressed in COS cells. Forty hours later, COS cells were pulse-labeled with [^{35}S]methionine/cysteine, chased fordifferent time points and radio-immunoprecipitated with anti-Ago2. Samples were resolved by SDS-PAGE followed by fluorography. When Hsp90 activity was inhibited (+GD), newly synthesized rAgo2 turned over more rapidly than in control conditions (-GD) (Fig 3-7 A). On average only 7% of the newly synthesized rAgo2 was detected after two hours when Hsp90 activity was inhibited (+GD), compared to more than 20% under

control conditions (-GD) (Fig 3-7 B). These results indicate that Hsp90 activity is required for the stability of nascent rAgo2.

Steady state levels of rAgo2 were also determined in the presence or absence of active Hsp90. As shown in Fig 3-7 C, rAgo2 levels were reduced by 50% when Hsp90 activity was inhibited (+GD). This is not a general effect of Hsp90 activity inhibition, since the endogenous pools of endoplasmic reticulum-localized proteins calnexin and Bip were largely unaffected by GD (Fig 3-7 C and data not shown). These results indicate that the effect of inhibition of Hsp90 activity is limited to a subset of proteins including rAgo2. Similar results have been reported for a variety of Hsp90 substrates such as Mik1, Pim1, Wee1 and Swe1 (Goes and Martin, 2001; Miyata et al., 2001; Mizuno et al., 2001).

3-4-2- Hsp90 does not colocalize with rAgo2 on intracellular membranes

Our previous biochemical data demonstrated that a pool of rAgo2 localizes to intracellular membranes. Specifically, we showed by immunofluorescence microscopy that a large portion of endogenous rAgo2 colocalizes with Golgi-resident proteins in certain epithelial cell types (Cikaluk et al., 1999). In light of these findings, we investigated whether a pool of Hsp90 colocalizes with rAgo2 at the Golgi complex in NRK52E cells. NRK52E cells were co-stained for Mannosidase II (Man II), an integral membrane protein of the Golgi apparatus, and endogenous rAgo2 or Hsp90. Unlike rAgo2, Hsp90 did not colocalize with Mann II in NRK52E and other cultured cell types studied (Fig 3-8 A). These data suggest that Hsp90 and rAgo2 interact in the cytosol or that Hsp90 interaction with membranes is transient.

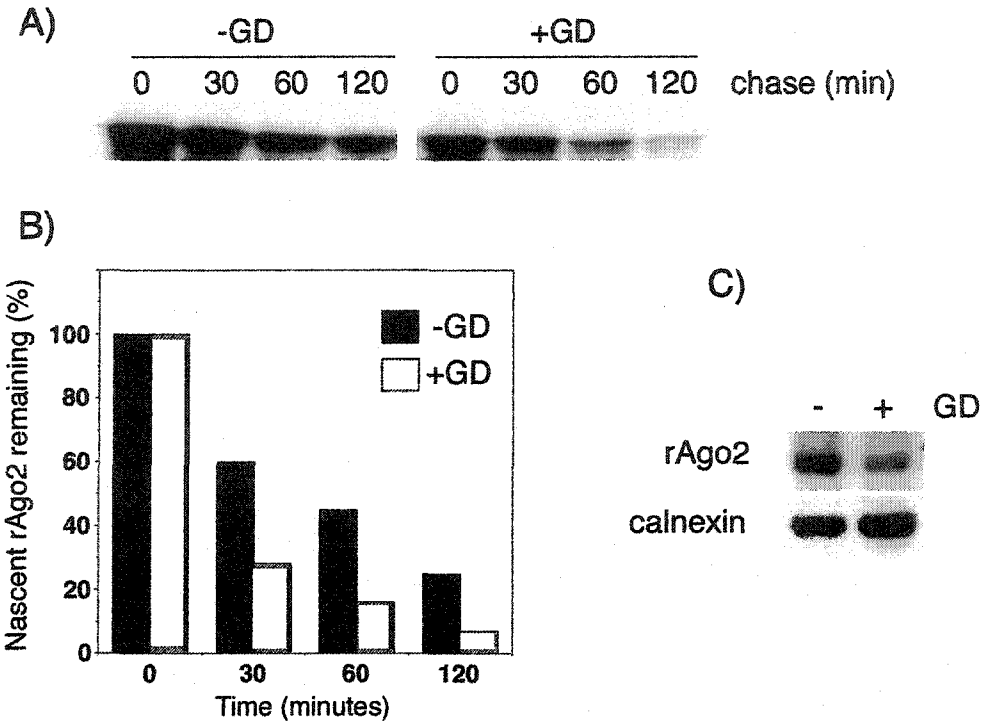


Figure 3-7- Inhibition of Hsp90 results in degradation of rAgo2

A) COS cells were transiently transfected with rAgo2. Forty hours post-transfection, cells were labeled for 15 min with Promix [^{35}S]methionine/cysteine and then chased for the indicated time periods in the absence of radioactivity. During the pulse and chase periods cells were also treated with 5 μM (+) or without (-) GD. Radioimmunoprecipitates using anti-Ago2 were subjected to SDS-PAGE and fluorography.

B) Quantitation of radiolabeled rAgo2 was performed using a PhosphorImager. The percentage of remaining nascent rAgo2 is shown in the diagram. Results are representative of two independent experiments.

C) Twenty μg of lysates from NRK cells treated for 16 hours with (+) or without (-) GD were subjected to SDS-PAGE and immunoblotting with anti-Ago2 and anti-calnexin. The total amount of rAgo2 but not calnexin is decreased in the presence of GD.

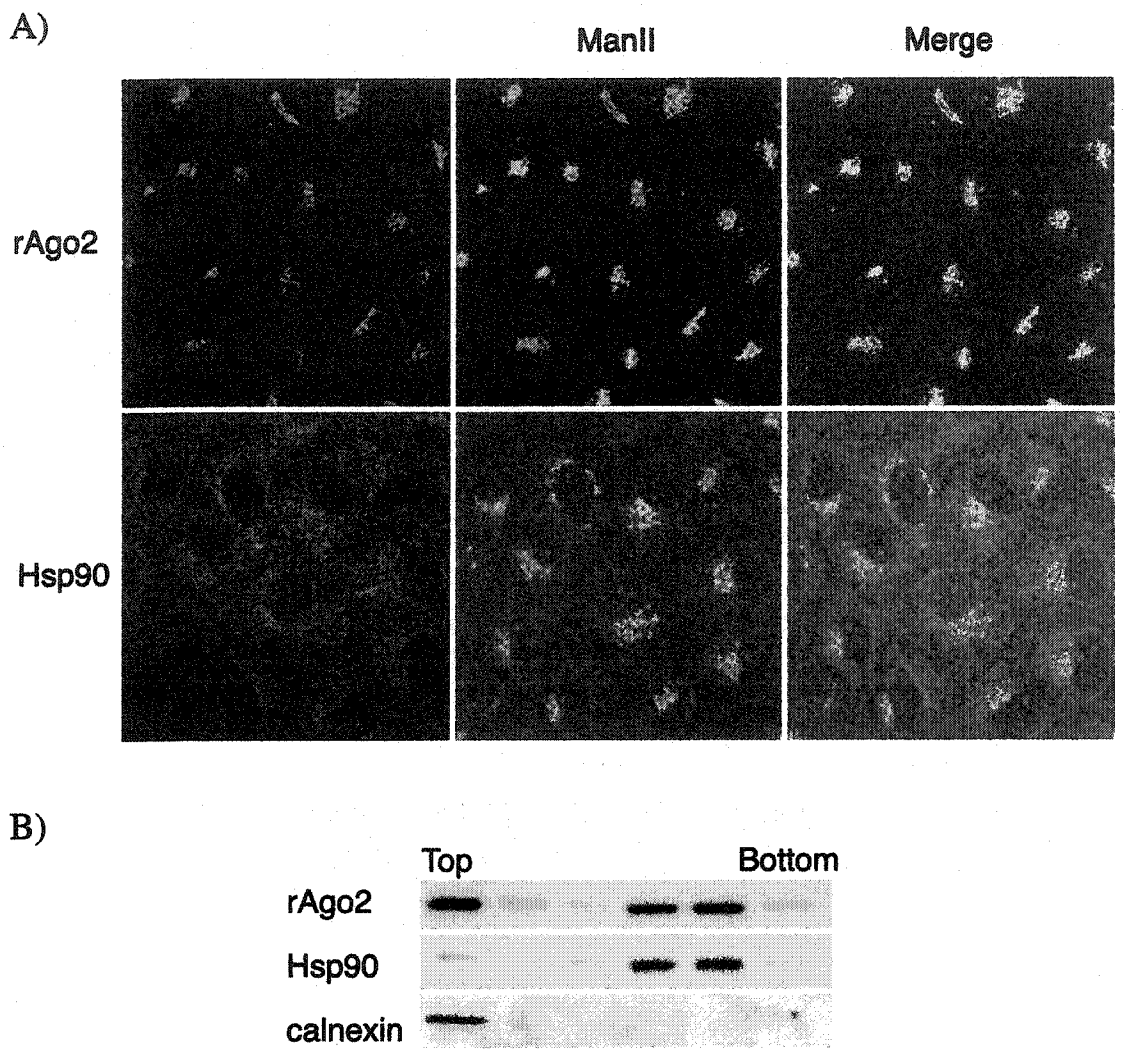


Figure 3-8- Membrane-associated rAgo2 does not bind Hsp90

A) NRK cells were co-stained with monoclonal mouse antibodies to Ago2 or Hsp90 and rabbit antibodies to the resident Golgi membrane protein Man II.

B) PNS was prepared from NRK cells and subjected to membrane flotation assays. Fractions were resolved on SDS-PAGE and proteins were visualized by immunoblotting with antibodies to Ago2 and Hsp90. The membrane-associated pool of rAgo2 floated to the top of the gradient, whereas the cytosolic fraction remained near the bottom with Hsp90. Calnexin, a transmembrane protein of the endoplasmic reticulum, was only present in the top membrane-enriched fraction.

3-4-3- Interaction between Hsp90 and rAgo2 occurs in the cytosol and is transient

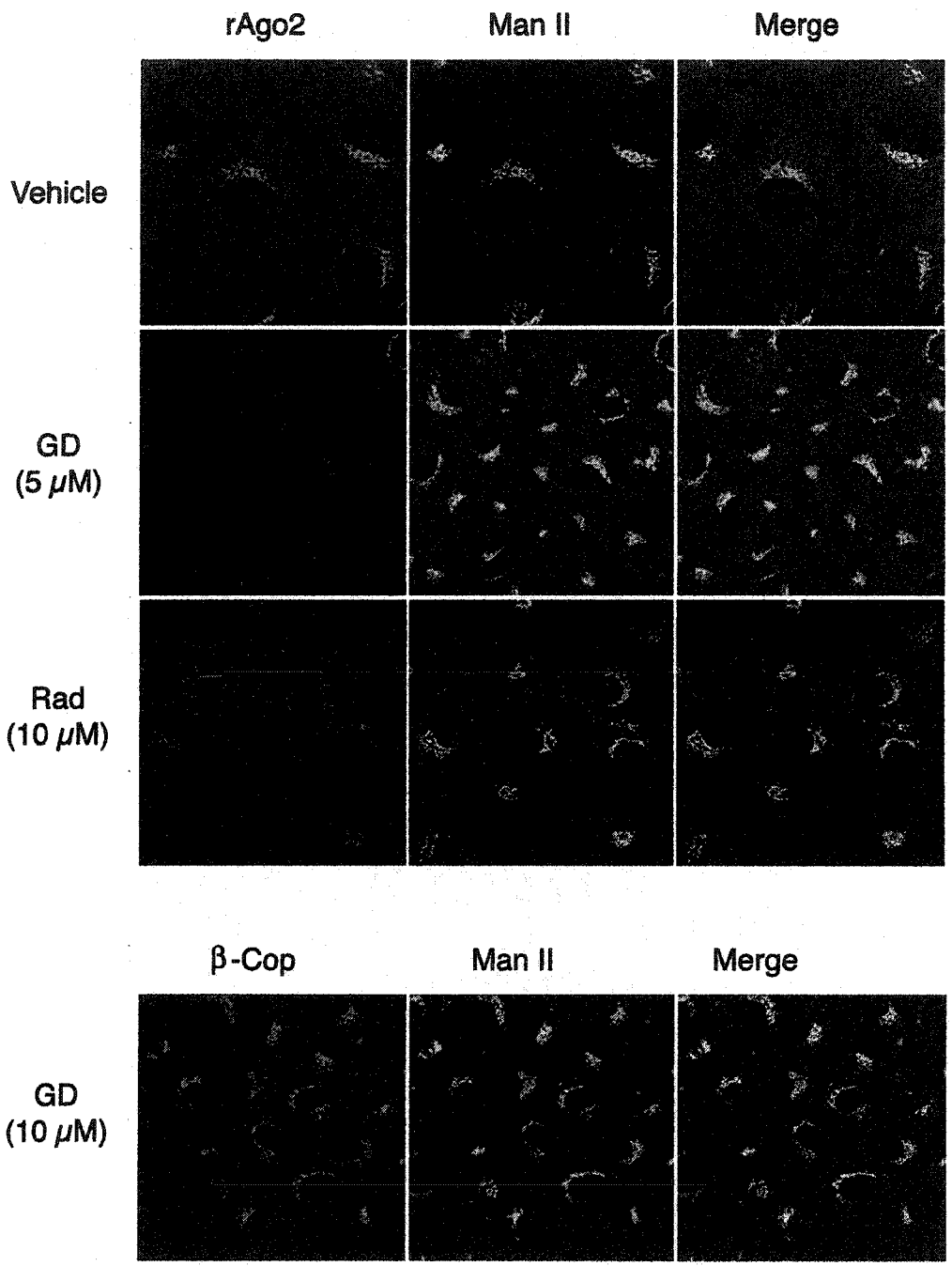
The lack of colocalization between Hsp90 and rAgo2 on the Golgi complex of NRK cells was an unexpected result. Therefore we asked whether Hsp90 was associated with any membranous pool that contained rAgo2 in NRK cells. Post-nuclear supernatants (PNS) were prepared from NRK cells and fractionated on discontinuous sucrose gradients to separate membranes (top) from the cytosol (bottom fractions). Western blot analysis indicated that a significant amount of rAgo2 cofractionated with membranes at the tops of the gradients (Fig 3-8 B). Calnexin, an integral membrane protein of the ER, was used as a positive control for the membrane fraction. The membrane-enriched fractions were essentially devoid of Hsp90. Moreover, fractionation experiments using other cell types (HEK293T) later indicated that Hsp90 was not detected in the membrane fractions, but was confined to the cytosolic fractions. These data are consistent with a scenario in which interactions between Hsp90 and rAgo2 occur in the cytosol, and are supported by the fact that we did not observe colocalization between Hsp90 and rAgo2 in the cytoplasm of NRK and COS cells by confocal immunoflourescent microscopy. The lack of colocalization between rAgo2 and Hsp90 could be explained by the transient nature of their interaction in the cytosol and/or on membranes.

3-4-4- The membrane pool of rAgo2 is depleted in the absence of Hsp90 activity

We next asked whether Hsp90 activity was required for association of rAgo2 with membranes. Immunofluorescence microscopy was used to investigate changes in the association of rAgo2 with Golgi membranes in the presence or absence of Hsp90 activity. After 12-14 hours of incubation with Hsp90 inhibitors (GD or Rad), the amount of rAgo2

Figure 3-9- Hsp90 inhibitors block association of rAgo2 with the Golgi complex

NRK cells were incubated for 14 hours with 5 μM GD, 10 μM Rad, or vehicle (DMSO or ethanol) and then processed for indirect immunofluorescence microscopy with mouse anti-Ago2 and rabbit antibodies to the Golgi marker Man II. Samples were analyzed using a Zeiss 510 confocal microscope. To show that inhibition of Hsp90 activity did not result in dissociation of all peripheral membrane proteins from the Golgi, cells treated with GD (10 μM) were also stained for ß-Cop, a peripheral membrane protein of the Golgi complex. Neither GD nor RD (not shown) affected localization of ß-Cop, indicating that the effect is specific for rAgo2.



associated with the Golgi membranes was drastically reduced (Fig 3-9). In contrast, the association of ß–Cop (a peripheral membrane protein) with Golgi membranes was not affected by GD or Rad. Interestingly, the effects of GD or Rad on rAgo2 localization were not immediate, but rather occurred after a minimum treatment of 4-6 hours. This observation suggests that the loss of rAgo2 from Golgi membranes is not simply a result of its redistribution to the cytosol. Instead, we favor the hypothesis that the reduction of rAgo2 on Golgi membranes in the presence of GD is due to the depletion of nascent rAgo2 in the cytosol coupled with turnover of membrane-bound rAgo2.

3-5- Conclusion: Sequential incorporation of rAgo2 into protease-sensitive and protease-resistant complexes

Our data are consistent with a scenario where formation of the protease-resistant and perhaps functional rAgo2 complex occurs in two steps. During the first step, rAgo2 associates with Hsp90, forming an Hsp90-rAgo2 complex which is protease-sensitive. The progress to the second step is dependent on the availability of membranes. In the presence of membranes, rAgo2 incorporates into a protease-resistant and presumably functional complex. Formation of the second rAgo2-containing complex probably requires maturation of the Hsp90-rAgo2 complex, a process that includes replacement of P23 with Hop. In the absence of membranes, the rAgo2-Hsp90 complex probably does not mature as indicated by the presence of Hop in Hsp90 heterocomplex associated with rAgo2 (reticulocyte lysate data). By analogy to other Hsp90 substrates, ATP hydrolysis by Hsp90 may induce conformational changes in rAgo2 exposing binding sites, perhaps at the conserved carboxyl terminal region, that interact with other proteins. Solubilization

of the membranes *in vitro* does not eliminate the protease-resistant rAgo2 complex, suggesting that, after formation, the complex could exist both on the membranes and in the cytosol (Fig 3-10).

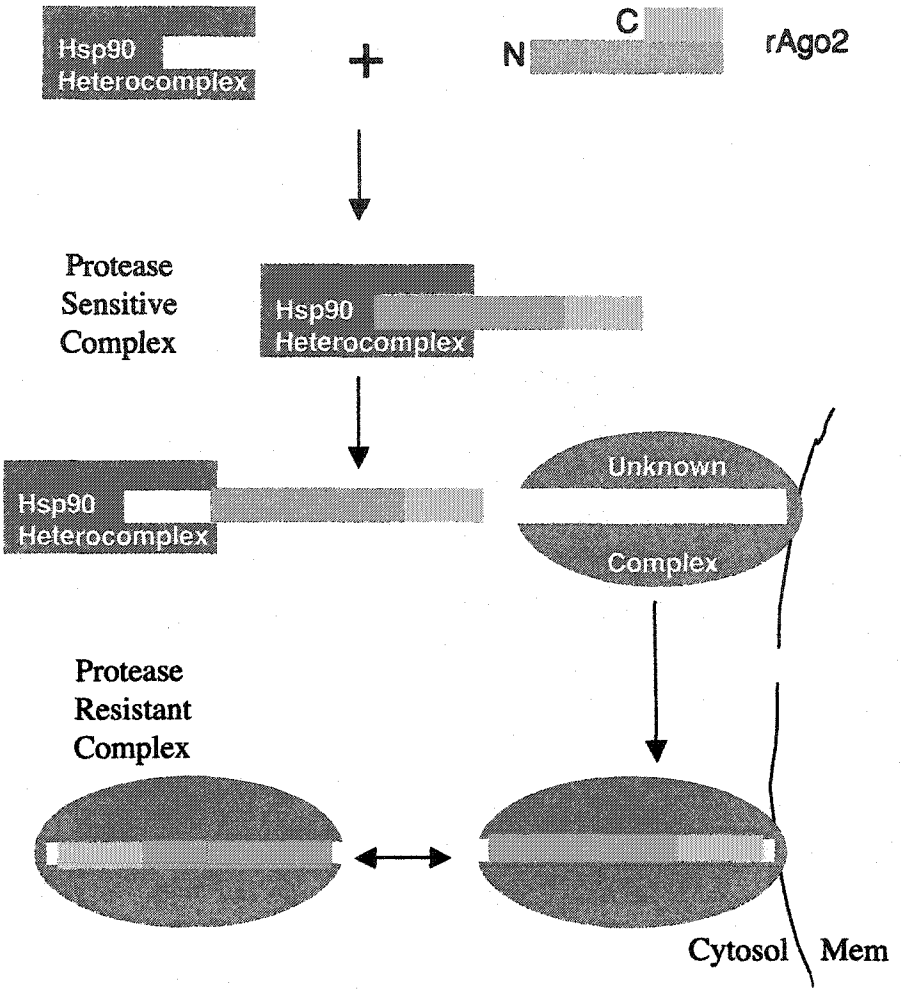


Figure 3-10- A model for sequential incorporation of rAgo2 into protease-sensitive and protease-resistant complexes

RAgo2 associates with the Hsp90 heterocomplex, to form a protease-sensitive complex. Interaction of the amino terminal region of rAgo2 with the Hsp90 heterocomplex may induce conformational changes that are required for the interaction of rAgo2 with other proteins. For instance, availability of the carboxy terminal region of rAgo2 may be required for its incorporation into a protease-resistant membrane-associated complex. After formation, the protease-resistant complex can exist in membrane-bound or soluble pools.

CHAPTER 4

RESULTS

A version of this chapter has been published. "**Tahbaz, N.**, Kolb, F.A., Zhang, H., Jaronczyk, K., Filipowicz, W. and Hobman, T.C. (2004) Characterization of the interactions between mammalian PAZ PIWI domain proteins and Dicer. *EMBO Rep*, 5, 189-194. Reproduced with permission.

Overview

Interactions between PPD proteins and Dicer have been reported previously (Doi et al., 2003; Hammond et al., 2001a; Sasaki et al., 2003). Heterotypic interactions between PAZ domains were originally thought to mediate binding between these two classes of proteins (Baulcombe, 2001). However, no evidence existed to support this theory. Here, I have characterized in detail the interactions between PPD proteins and Dicer. My research demonstrates that the PAZ domains are not required for the interaction between PPD proteins and Dicer. Rather, a sub-region of the PIWI domain in PPD proteins, the PIWI-box (Cox et al., 1998), binds to the Dicer RNase III-A domain. The interaction between PPD proteins and Dicer is direct and is not dependent on RNA. However, stable binding between PPD proteins and Dicer requires Hsp90 activity. In addition, we report that binding of PPD proteins to Dicer inhibits the RNase activity of Dicer *in vitro*. Finally, our data indicate that PPD-Dicer complexes are present in soluble and membrane-associated fractions.

4-1- Cloning of the human Ago2 by the GeneTrapper™ system

Affinity purifications followed by silver nitrate staining revealed several proteins that interact with rAgo2. Identification of these proteins by mass spectrometry proved to be unsuccessful due to the lack of a large and inclusive rat genome data-base. Since most of the human genome had been sequenced at the time, we elected to clone hAgo2, the human orthologue of rAgo2, and continue our search for PPD-binding proteins using a proteomics approach.

The "GeneTrapper cDNA Positive Selection System" facilitates rapid isolation of full-length cDNAs from libraries. A complementary oligonucleotide is biotinylated and hybridized to its targets in a single-stranded (ss) cDNA library. Then the hybrids of oligonucleotide and ss plasmids containing target cDNAs are affinity-purified on streptavidin paramagnetic beads. The isolated single-stranded plasmids are then converted into double-stranded plasmids during a DNA synthesis reaction. Finally, *E. coli* is transformed with the plasmids, and colonies are screened by hybridization to labeled probes to identify plasmids with target cDNAs.

A non-redundant human EST (Expressed Sequence Tag) database was searched using the rAgo2 sequence to identify homologous EST sequences. The 5'- and 3'-oligonucleotides were chosen (section 2-3-3-2), synthesized, biotinylated and used to isolate hAgo2 cDNAs. Three cDNAs were retrieved from a human leukocyte cDNA library (Invitrogen). The sequences of cDNAs deviated from each other at their 5'-end indicating that they did not represent truncations of the same cDNAs. Sense and antisense sequencing indicated that all of these cDNAs encode proteins that are identical throughout their lengths except at their predicted amino termini (Fig 4-1 A). The cDNAs were used for TNT reactions *in vitro* and supported synthesis of proteins with the expected sizes (95-97 kDa) (Fig 4-1 B). The proteins were immunoprecipitated with the polyclonal anti-Ago2 antibody but not the preimmune serum (Fig 4-1 C). *In vitro* synthesized hAgo2 isoforms were also coimmunoprecipitated with antibodies against Hop, P23 (Fig 4-1 C), Hdj2, Hsp70 and Hsp90 (data not shown), indicating that hAgo2 associated with the Hsp90 heterocomplex *in vitro*. The predicted protein product encoded by G4 cDNA is 861 amino acid residues long with a mass of 97 kDa. This correlates to

Figure 4-1- Human homologues of rAgo2 interact with the members of the Hsp90 heterocomplex

A) "GeneTrapper cDNA positive selection system" was used to clone full-length cDNAs that encode hAgo2. Three full-length cDNAs were identified (G4, D9 and F9), and sequenced in forward and reverse directions. The amino acid sequences of the N-terminal regions of the predicted proteins are shown. The predicted sequences of G4p and D9p only deviate from each other at the extreme amino-terminal regions (red). The F9 cDNA, deviates from G4 and D9 at the extreme 5'-end. Translation of F9p presumably starts at the second methionine present in both G4p and D9p (MDIPK). The putative start sites are shown in bold (**M**). The PRP motif is underlined.

B) ^{35}S-labeled hAgo2 isoforms were synthesized in a TNT^{TM}-coupled rabbit reticulocyte lysate system, resolved on SDS-PAGE and visualized by fluorography. G4, F9 and D9 cDNAs supported production of proteins with the expected sizes (95-97 kDa).

C) ^{35}S-labeled G4p, F9p and D9p were synthesized *in vitro*, and coimmunoprecipitated with the antibodies to P23 or Hop. Immunoprecipitations with anti-Ago2 or its preimmunue serum (Pi) were used as positive and negative controls, respectively. The smallest band among hAgo2-D9 protein synthesis products may have resulted from translational initiation at a down stream methionine. Alternatively, it may have resulted from partial degradation of the larger protein synthesis products.

A)

```
                                                                                                  50
G4p: MHPFQWCNALAPPAPPPPIQGYAFKPPPRPDFGTSGRTIKLQANFFEMDI
F9p:  ----------------------------------------------------------------------------------------MDI
D9p: MYSGAGP - ALAPPAPPPPIQGYAFKPPPRPDFGTSGRTIKLQANFFEMDI

                                                                                                 100
G4p: PKIDIYHYELDIKPEKCPRRVNREIVEHMVQHFKTQIFGRKPVFDGRKNL
F9p: PKIDIYHYELDIKPEKCPRRVNREIVEHMVQHFKTQIFGRKPVFDGRKNL
D9p: PKIDIYHYELDIKPEKCPRRVNREIVEHMVQHFKTQIFGRKPVFDGRKNL
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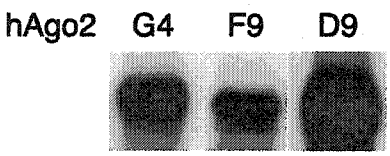
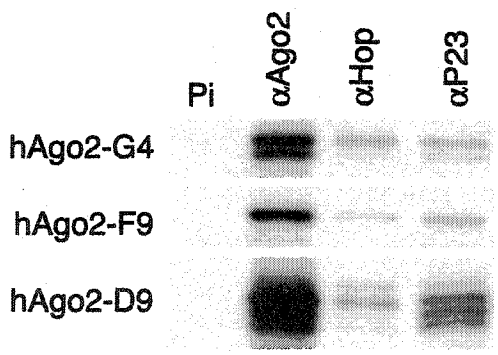
B)



C)



the larger band in hAgo2-G4 *in vitro* TNT reactions (Fig 4-1 C). We used G4 cDNA for all subsequent experiments because the apparent molecular mass of the G4 translation products corresponded well to the size of hAgo2 bands detected by immunoblotting in cultured cells (compare Fig 4-1 C and Fig 4-9 A).

4-2- Interaction between PPD proteins and Dicer

Previous reports indicated that dAgo2 and dAgo1 interact with Dicer in *D. melanogaster* cells (Hammond et al., 2001a; Ishizuka et al., 2002). To determine whether human Dicer interacts with hAgo2, we immunoprecipitated Dicer using anti-Dicer antibody (Zhang et al., 2002) from HEK293 cells stably overexpressing hAgo2 (G4 isoform). The immunoprecipitation samples were resolved on SDS-PAGE, transferred to membranes and immunoblotted with anti-Ago2. Our results revealed that Dicer coimmunoprecipitates hAgo2 (Fig 4-2).

The nature of the interactions between PPD proteins and Dicer and their effects on Dicer activity have not been thoroughly investigated. As a first step toward understanding this process, we mapped the regions within PPD proteins and Dicer that mediate their interaction. Two distantly related human PPD proteins, hAgo2 and Hiwi (Qiao et al., 2002; Sharma et al., 2001) were chosen for these studies. HAgo2 and Hiwi belong to the Ago and Piwi subfamilies of PPD proteins, respectively, and the level of identity between them is approximately 22.7% overall (Fig 1-4 A). Theoretically, selection of two distantly related PPD proteins to study the interactions with Dicer would enable us to extrapolate our findings to other PPD family members.

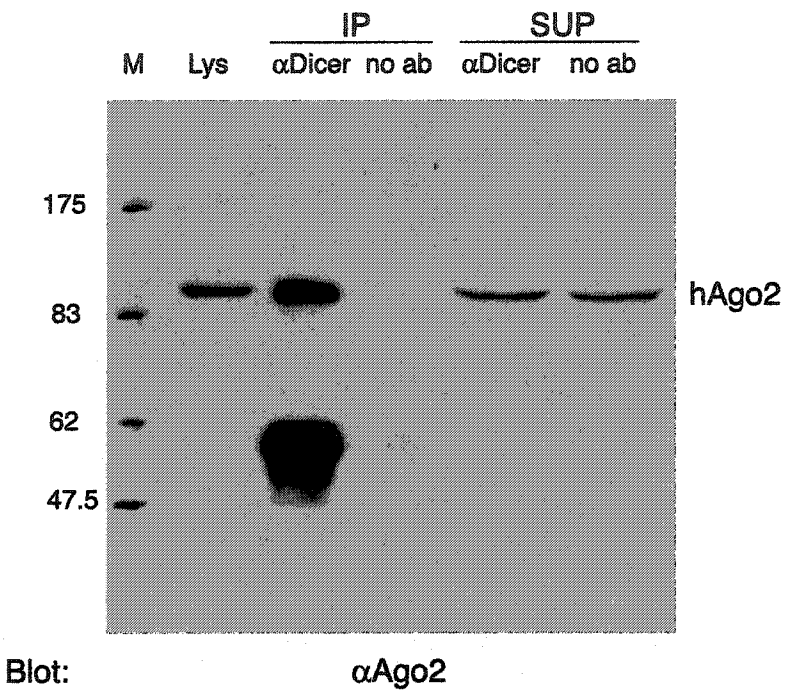


Figure 4-2- Anti-Dicer coimmunoprecipitates hAgo2

Polyclonal antibodies to Dicer were used for immunoprecipitation (IP) from lysates (Lys) of HEK293 cells stably overexpressing hAgo2. Dicer and its associated proteins were resolved on SDS-PAGE, transferred to the PVDF membranes and immunoblotted with anti-Ago2. A band corresponding to the IgG heavy chain (about 55-60 kDa) is only present in anti-Dicer immunoprecipitations.

4-2-1- The PIWI domains of PPD proteins interact with Dicer

To establish if either one of the two signature domains of PPD proteins was sufficient for binding to Dicer, the PAZ and the PIWI domains of hAgo2 and Hiwi were fused to GST and expressed in HEK293T cells together with CFP-Dicer (Fig 4-3 A). The GST-PAZ and GST-PIWI fusion proteins and their associated proteins were affinity purified on glutathione-sepharose 4B beads. After washing, proteins were eluted from the beads by boiling and samples were subjected to SDS-PAGE followed by immunoblotting with antibodies to GST or GFP. Stable interactions between the PAZ domains of hAgo2 or Hiwi and CFP-Dicer were not detected, but strong binding between CFP-Dicer and the PIWI domains of both hAgo2 and Hiwi was evident (Fig. 4-3 B). No interaction was observed between GST (negative control) and Dicer. Our data indicate that the PAZ domains of PPD proteins are not involved in the interaction between PPD proteins and Dicer. However, PIWI domains of PPD proteins are necessary and sufficient for their interaction with Dicer.

4-2-2- The RNase III domain of Dicer is required for interaction with PPD proteins

The region of Dicer involved in the interaction with PPD proteins had not been previously identified. To determine which domain of Dicer was responsible for this interaction, GST alone and GST fused to full-length Dicer or its ATPase/helicase/PAZ, PAZ or RNase III regions (Fig. 4-4 A), were expressed in HEK293T cells stably overexpressing hAgo2. Supernatants from sonicated cell lysates prepared in non-ionic detergents (Mourelatos et al., 2002) were subjected to affinity purification with glutathione-sepharose 4B beads. GST-fusion proteins and their associated proteins were

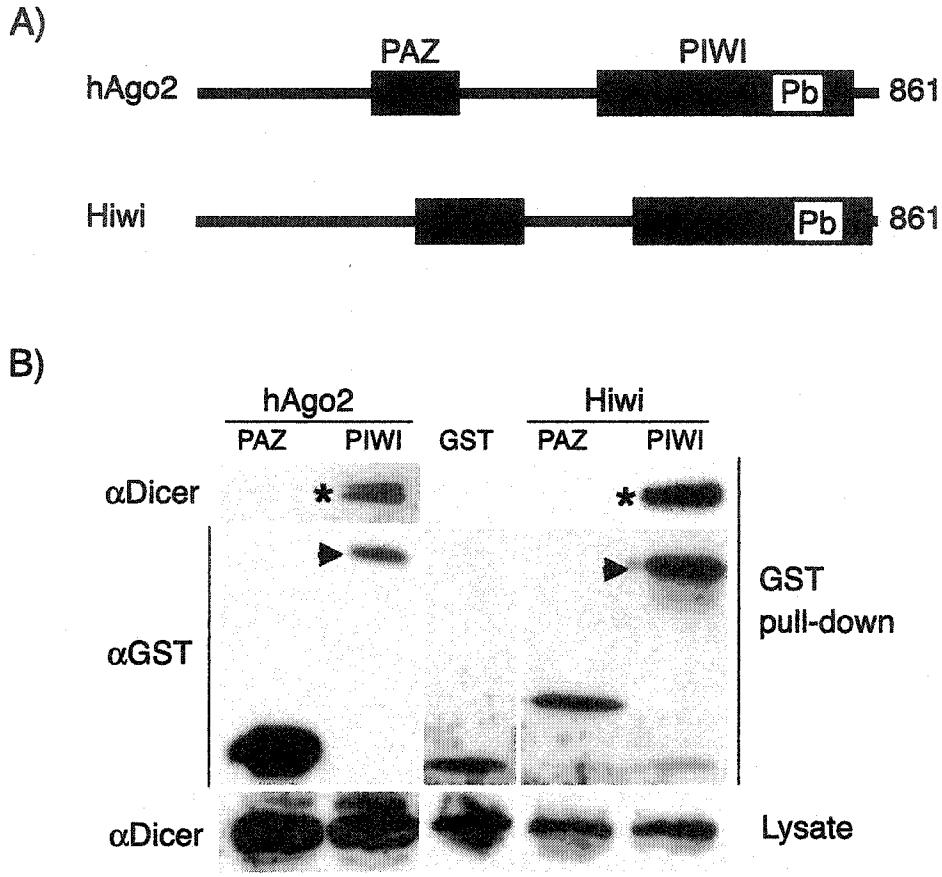


Figure 4-3- PIWI domains of hAgo2 and Hiwi interact with Dicer

A) Schematic representation of hAgo2 and Hiwi proteins used for binding studies. The PIWI domains, PIWI box (Pb) and PAZ domains are indicated. The 5' ends of the hAgo2 and Hiwi cDNAs encoding PAZ and PIWI domains were fused in-frame to the GST sequence in the context of the pEBG mammalian expression vector.

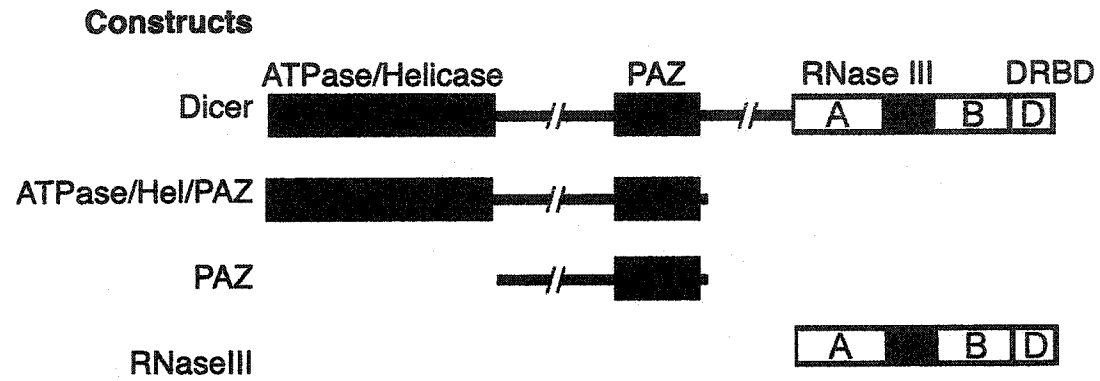
B) GST and GST-PPD fusion proteins were co-expressed with CFP-Dicer in HEK293T cells. Proteins were purified on glutathione-sepharose 4B beads, resolved by SDS-PAGE, and visualized by immunoblotting with antibodies to Dicer and GST. The GST-PIWI domain constructs (arrow heads) of hAgo2 and Hiwi interact with Dicer (asterisks). The PAZ domains fused to GST or the GST negative control, do not interact with Dicer.

Figure 4-4- The RNase III region of Dicer is required for binding to hAgo2

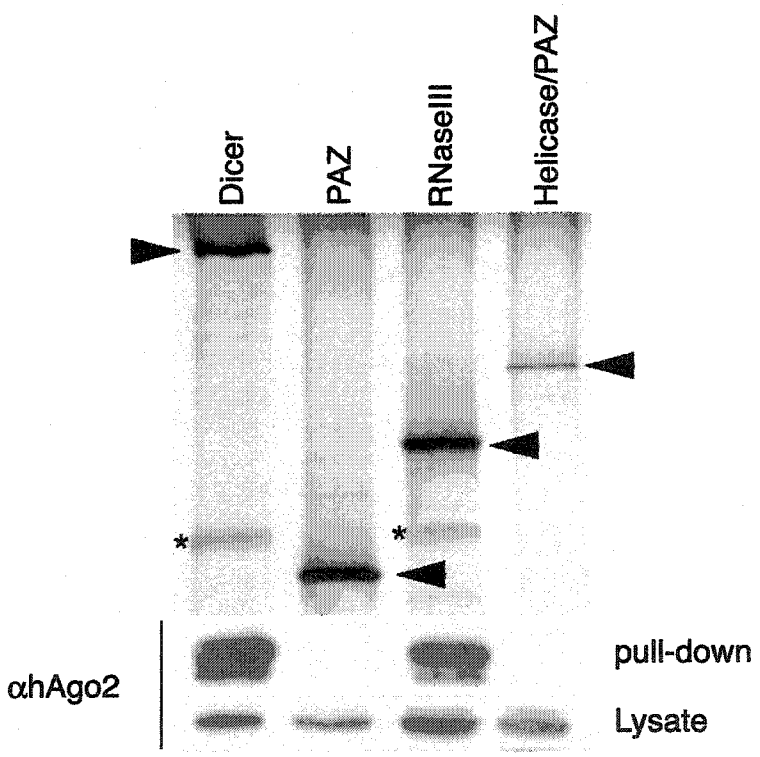
A) Schematic representation of Dicer and GST-fusion proteins used for binding studies. The three structural regions of Dicer are indicated ATPase/Helicase/PAZ, PAZ and RNase III domains. The RNase III domain is further subdivided to indicate the presence of two RNase III domains, A and B, and a dsRNA-binding domain (DRBD) (D). Plasmids encoding GST fused to the amino terminus of Dicer, ATPase/Helicase/PAZ, PAZ and RNase III domains were constructed.

B) GST fusion proteins were transiently expressed in a HEK293T cell line over-expressing hAgo2. Following GST pull-downs, proteins were visualized by silver staining (top panel) and immunoblotting (bottom panels). Arrowheads point to the silver-stained GST-fusion proteins, whereas hAgo2 is indicated by asterisks. Both Dicer and its carboxyl terminal RNase III region including RNase III-A, B and DRBD interact with hAgo2.

A)



B)



separated by SDS-PAGE, visualized by silver staining or by immunoblotting with a mouse monoclonal anti-Ago2 and anti-GST. These experiments demonstrated that the PAZ domain of Dicer is not required for binding to hAgo2. Instead, hAgo2 binds only to full-length Dicer or its carboxyl terminal region. The carboxyl terminal region of Dicer lacks the PAZ and helicase domains but does contain the dsRNA binding domain (DRBD) and RNase III-A and B domains (Fig 4-4 A). Together, these data indicated that the interaction between PPD proteins and Dicer is mediated by their PIWI domains and RNase III regions, respectively. Moreover, PAZ domains are not involved in the interaction between PPD and Dicer proteins.

4-2-3- The Dicer-PPD interaction is direct

In collaboration with Dr. Filipowicz's laboratory (Basel, Switzerland), the interactions between the purified Dicer and PPD proteins were further examined to determine if their association is direct. Dicer that was carboxyl terminally tagged with six histidine residues was expressed in Sf9 insect cells and purified as described (Zhang et al., 2002). GST-hAgo2 and GST-Hiwi fusion proteins were expressed in COS1 cells and purified on glutathione-sepharose 4B beads (Tahbaz et al., 2001). In the experiment shown in figure 4-5 A, protein A-sepharose beads coated with the anti-Dicer antibody (Billy et al., 2001) were used for immunoprecipitation of complexes of Dicer with either GST-hAgo2, GST-Hiwi or GST alone. Protein A-sepharose beads coated with an antibody against an unrelated human protein GAR1 (Dragon et al., 2000) were used as control. The proteins that coimmunoprecipitated with Dicer were visualized by immunoblotting using anti-GST antibody. The data showed that the GST-hAgo2 and

GST-Hiwi were retained by anti-Dicer beads but not by the control anti-GAR1 beads (Fig 4-5 A, upper and middle panels). Neither anti-Dicer nor anti-GAR1 beads retained GST alone (Fig 4-5 A, lower panel). These data suggest that the interactions between Dicer and PPD proteins are direct.

4-2-4- Dicer-PPD interactions are not affected by nuclease digestion

Since both PPD proteins and Dicer have been shown to bind RNA (Deng and Lin, 2002; Kataoka et al., 2001; Kuramochi-Miyagawa et al., 2001; Provost et al., 2002a; Zhang et al., 2002), we investigated if nuclease digestion would affect Dicer-PPD interaction. GST-hAgo2 and CFP-Dicer were expressed transiently in HEK293T cells. Cell lysates were subjected to GST pull-down with glutathione-sepharose 4B beads to immunopurify GST fusion proteins and their associated proteins. The beads and their associated proteins were treated with micrococcal nuclease, followed by washes to remove unbound proteins. Proteins associated with GST-hAgo2 were resolved on SDS-PAGE, transferred and immunoblotted using anti-GST, anti-GFP and anti-Dicer (Fig 4-5 B). Our data showed that interaction between GST-hAgo2 and Dicer is not affected by nuclease digestion. Similar results were obtained with purified Hiwi and Dicer proteins (Dr. Fillipowicz, personal communication).

4-2-5- The PIWI-boxes of PPD proteins interact with the RNase III-A domain of Dicer

We next investigated the interactions between Dicer, hAgo2 and Hiwi using the yeast two-hybrid assay. Our goal was to further map the interaction sites on PPD proteins

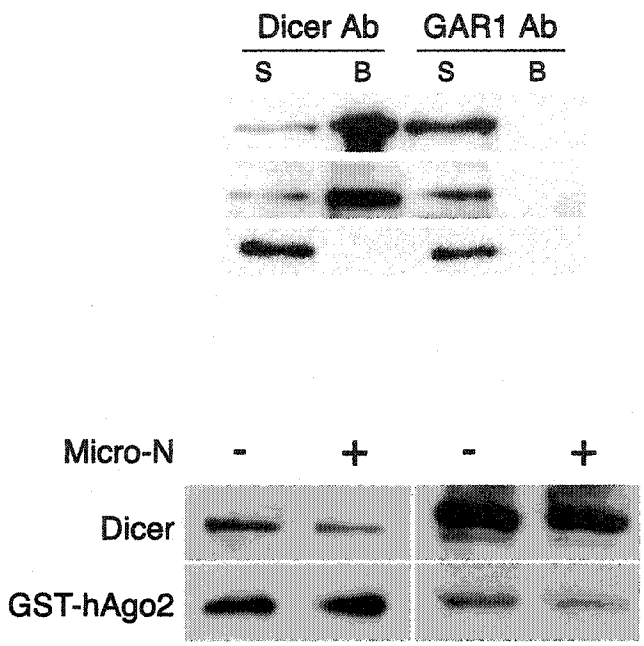


Figure 4-5- Dicer-PPD interaction is direct and is not affected by nuclease digestion.
A) Protein A-sepharose beads coated with affinity purified-antibodies to Dicer or the control protein GAR1 were incubated with mixtures of purified Dicer and GST-hAgo2 (upper panel), GST-Hiwi (middle panel) or GST alone (lower panel). Bead-associated (B) and unbound (S) material was subjected to SDS-PAGE and immunoblotting with anti-GST antibodies (courtesy of Dr. Filipowicz's laboratory).
B) GST-hAgo2 and CFP-hDicer were transiently expressed in HEK293T cells. GST pull-downs were performed and beads were treated with or without micrococcal nuclease (Micro-N) and washed to remove unbound proteins. The proteins were resolved on SDS-PAGE followed by blotting with antibodies to GST and Dicer.

and Dicer and to eliminate the possibility that proteins co-purifying with either one of Dicer-Hisx6 or GST-PPD preparations are involved in binding. The budding yeast *S. cerevisiae* does not encode PPD or Dicer homologues, and therefore, if interactions between PPD proteins and Dicer are detected in this system, it is likely that they result from direct binding. Plasmids encoding PAZ, PIWI or PIWI-box of hAgo2 or Hiwi (see Fig 4-3 A) fused to the DNA-binding domain of Gal-4 were constructed. Similarly, plasmids encoding the domains of Dicer RNase III region including DRBD, RNase III-A or RNase III-B (see Fig 4-4 A) fused to the activation domain of Gal-4 were also constructed. Different combinations of plasmids encoding PPD or Dicer constructs as shown in TABLE 4-1 were transformed into *S. cerevisiae* strain AH109. Transformants were plated on complete synthetic medium minus leucine and tryptophan, and positives were identified by streaking the transformed colonies onto 4DO plates. Using this method, no interactions between the PAZ domains of hAgo2 or Hiwi and Dicer domains were evident (see TABLE 4-1). In contrast, interactions between the PIWI domains of hAgo2 and Hiwi and the RNase III-A domain of Dicer were detected, although these interactions were not particularly strong. The reason for this is unclear, but one possibility is that the PIWI domains are not stable and/or efficiently translocated into the nuclei of yeast. The strongest interactions occurred between the RNase III-A domain of Dicer and the PIWI- boxes of hAgo2 and Hiwi (Fig 4-6 and TABLE 4-1). Relatively weak interactions were observed between the PIWI-boxes and RNase III-B domains. No interactions were detected between PAZ or PIWI domains or PIWI-boxes of the PPD proteins and the DRBD of Dicer (Fig 4-6, TABLE 4-1). Together, these data indicate that the PIWI-boxes of PPD proteins interact directly with the RNase III-A domain of Dicer.

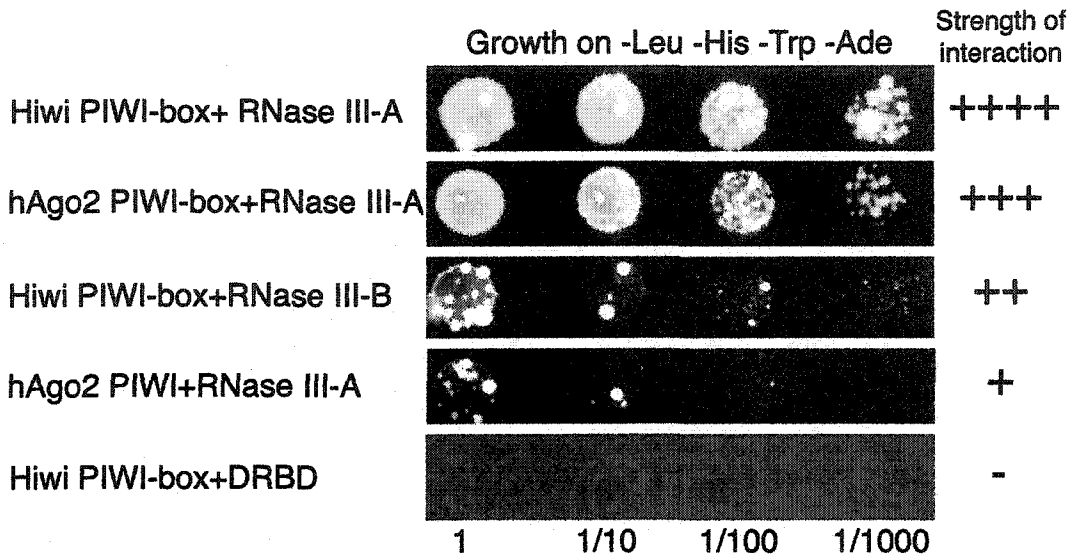


Figure 4-6- The PIWI-boxes of the PPD proteins interact with the RNase III-A domain of Dicer

Different combinations of pGBKT7 (hAgo2 or Hiwi subdomains) and pGADT7 (Dicer subdomains) constructs were transformed into the AH109 strain of *S. cerevisiae* and tested for interaction by growth on selective media. Four serial dilutions were spotted for each combination. Scoring ranged from – to ++++ depending upon how well the strains grew on media lacking leucine, tryptophan, adenine and histidine. Graded examples of some PPD-Dicer interactions are shown. A complete listing of all of the tested interactions is shown in TABLE 4-1.

TABLE 4-1- List of yeast two-hybrid interactions Tested

pGBKT7 with cDNA for PPD proteins	*pGADT7 with cDNA for Dicer*	*Evidence of Interaction (growth on 4DO)*
hAgo2 PIWI-box	RNase III-A	+++
hAgo2 PIWI-box	RNase III-B	+
hAgo2 PIWI-box	DRBD	-
hAgo2 PIWI-box	No insert	-
hAgo2 PIWI domain	RNase III-A	+
hAgo2 PAZ domain	RNase III-A	-
No insert	RNase III-A	-
No insert	DRBD	-
No insert	RNase III-B	-
Hiwi PIWI-box	RNase III-A	++++
Hiwi PIWI-box	RNase III-B	++
Hiwi PIWI-box	DRBD	-
Hiwi PIWI-box	No insert	-
Hiwi PIWI domain	RNase III-A	+
Hiwi PAZ domain	RNase III-A	-

4-3- Characterization of the interaction between Dicer and PPD proteins

4-3-1- Hsp90 activity is required for stable interactions between PPD proteins and Dicer

Previously, we reported that Hsp90 activity is required for the stability of nascent rAgo2 and its association with membranes (Tahbaz et al., 2001). Here we examined the effect of GD on the interactions between Dicer and PPD proteins. Plasmids encoding CFP-Dicer and either GST-hAgo2 or GST-Hiwi were transfected into HEK293T cells.

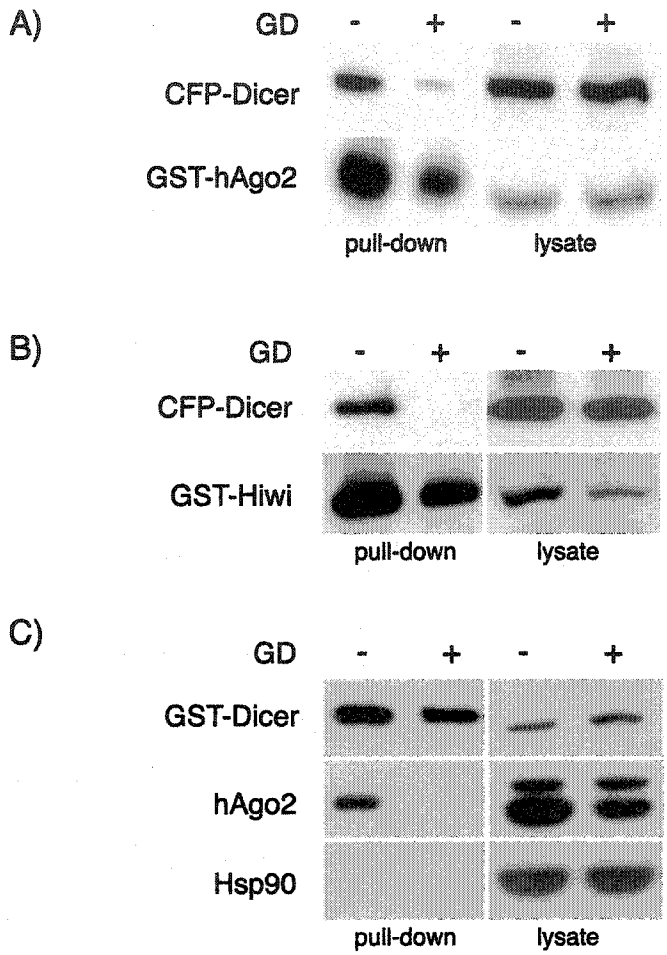


Figure 4-7- Hsp90 activity is required for stable interactions between PPD proteins and Dicer

GST-hAgo2 (**A**) or GST-Hiwi (**B**) were transiently co-expressed with CFP-Dicer in HEK293T cells. Cells were incubated with 5 μM (+) or without (-) GD for 2 hours prior to lysis. Proteins were recovered on glutathione-sepharose 4B beads and visualised by immunoblotting. CFP-Dicer was detected using a polyclonal anti-GFP antibody, whereas GST-hAgo2 and GST-Hiwi were detected using anti-GST antibodies.

C) GST-Dicer was expressed in a HEK293T cell line stably expressing hAgo2. Forty hours post-transfection, cells were treated with 5μM (+) or without (-) GD and were lysed 8 hours later. Lysates were subjected to affinity purification on glutathione-sepharose 4B beads, and proteins were resolved by SDS-PAGE and visualised by immunoblotting with anti-GST, anti-Ago2 and anti-Hsp90.

Two hours prior to lysis, cells were treated with (5μM) GD or with vehicle alone. GST fusion proteins and their associated proteins were affinity purified on glutathione-sepharose 4B beads, resolved by SDS-PAGE and immunoblotted with antibodies to GST and GFP. The associations of CFP-Dicer with GST-hAgo2 (Fig 4-7 A), and GST-Hiwi (Fig 4-7 B) were greatly inhibited in the presence of GD, suggesting that Hsp90 plays a general role in regulating the binding of PPD proteins to Dicer. The GD-dependent inhibition of Dicer-hAgo2 interactions was confirmed by reciprocal pull-down assays using GST-Dicer expressed transiently in stable cell lines overexpressing hAgo2 (Fig 4-7 C). It should be noted that GD treatment caused some degradation of PPD proteins. However during the short incubation times used here, the extent of GST-hAgo2 and GST-Hiwi degradation was not sufficient to account for the nearly complete GD-dependent block of Dicer binding. Immunoblotting with anti-Hsp90 antibody indicated that Hsp90 is not present in hAgo2-Dicer complex, as expected (4-7 C).

4-3-2- Dicer RNase activity is inhibited by binding to PPD proteins *in vitro*

Dicer cleavage products (siRNAs/miRNAs) are incorporated into PPD protein-containing RISC or miRNPs that serve as the effector complexes in RNAi. Interactions between PPD proteins and Dicer are thought to be required for the transfer of siRNAs or miRNAs from Dicer to the effector complexes of RNAi (Baulcombe, 2001; Denli and Hannon, 2003; Sasaki et al., 2003). Indeed, previous studies have documented the interaction between PPD proteins and Dicer in *D. melanogaster* and human cell extracts (Doi et al., 2003; Hammond et al., 2001b), but the effects of these interactions on Dicer activity have not been investigated. Given that PPD proteins bind to the RNase III domain of Dicer, we reasoned that this interaction could affect Dicer activity. To test this

possibility, the activity of purified Dicer was assayed in the presence of hAgo2 or Hiwi (in collaboration with Dr. Filipowicz's laboratory, Basel, Switzerland). Full-length hAgo2 or Hiwi fused to GST or GST alone were purified on glutathione-sepharose 4B beads from COS1 cell extracts. The affinity purified proteins were added together with a 129 bp [^{32}P]-labeled substrate dsRNA to purified Dicer *in vitro* (Zhang et al., 2002). A dose-dependent decrease in Dicer activity was observed in the presence of GST-hAgo2 and GST-Hiwi but not with GST alone (Fig 4-8 A and B). Similar results were obtained when Dicer and hAgo2 were preincubated together prior to the addition of the dsRNA substrate or when pre-let7 RNA hairpin was used as a processing substrate instead of dsRNA (data not shown).

4-3-3- PPD proteins and Dicer are present in membrane-associated and soluble pools

Our previous work showed that rAgo2 is present in membrane-associated and cytosolic pools (Cikaluk et al., 1999; Tahbaz et al., 2001). Moreover, recent studies have shown that Dicer also localizes to the cytoplasm (Billy et al., 2001; Findley et al., 2003; Provost et al., 2002a), but quantitative evidence of membrane-association has not been reported. Importantly, the intracellular site(s) of Dicer-PPD interactions have not been investigated, and therefore we compared the intracellular distributions of PPD proteins and Dicer in membrane fractionation assays. PNS was prepared from HEK293T cells transfected with GST-Hiwi and fractionated on a discontinuous sucrose gradient to separate membranes (top fraction) from cytosol (bottom fractions). This approach was necessary because anti-Hiwi antibodies are not available. Similar to hAgo2, a large pool

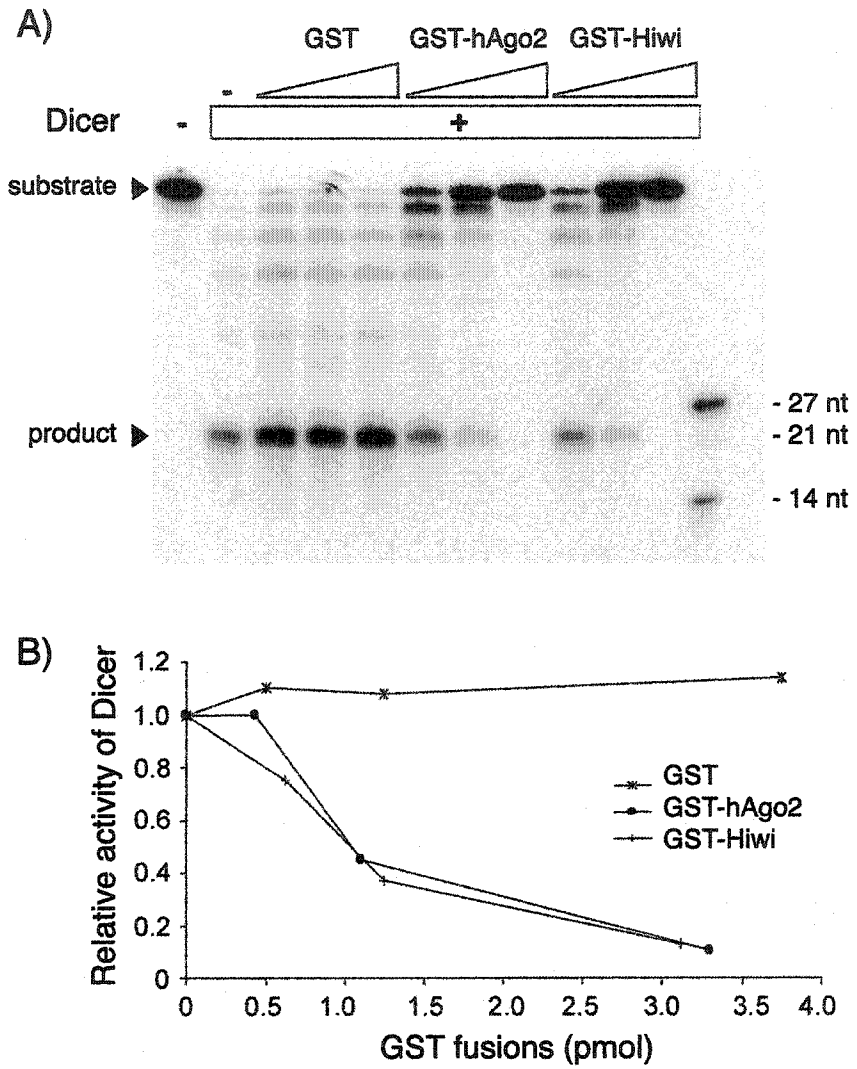


Figure 4-8- The RNase activity of Dicer is inhibited by interaction with PPD proteins *in vitro* (courtesy of Dr. Filipowicz's laboratory)

A) Effect of Hiwi and hAgo2 on Dicer-mediated cleavage of dsRNA. Increasing amounts of GST-Hiwi, GST-hAgo2 or GST alone were added to Dicer *in vitro* cleavage reactions containing a 129-bp ^{32}P-labelled dsRNA substrate. Reaction products were analysed by 8M urea PAGE. The positions of 14-, 21- and 27-nt size markers are indicated (21-nt marker was only visible upon longer exposure). The addition of GST-hAgo2 or GST-Hiwi, but not GST, inhibited Dicer activity in a dose-dependent manner.

B) PhosphorImager quantitation of the cleavage reactions from a typical experiment.

of endogenous Dicer was present in soluble fractions, whereas a smaller but significant cohort of Dicer co-purified with membranes (Fig 4-9 A). Calnexin, an integral membrane protein of the ER, was used as a marker for the membrane fraction. Hsp90 was only present in the soluble fractions, as previously reported (Tahbaz et al., 2001). Since antibodies to Hiwi are not available, the distribution of GST-Hiwi transiently expressed in HEK293T cells was examined. As with hAgo2 and Dicer, GST-Hiwi was present in both soluble and membrane-associated fractions (Fig 4-9 A). The data presented here are consistent with the existence of Dicer-PPD complexes in cytosolic and membranous fractions.

4-4-4- A portion of Dicer-PPD complexes associate with membranes

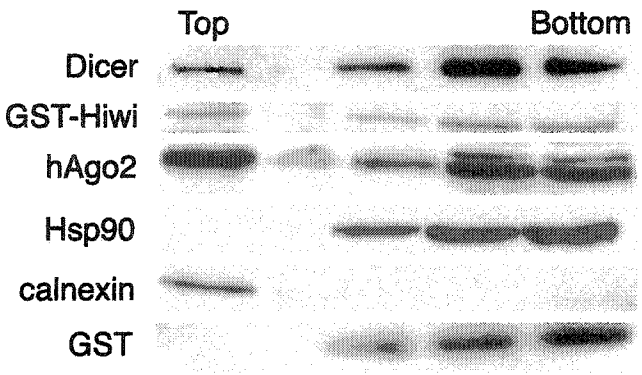
The association of PPD proteins and Dicer with cytosolic and membranous fractions is consistent with the existence of cytosolic and membranous Dicer-PPD complexes. To obtain biochemical evidence about the localization of the Dicer-PPD complexes, GST-Dicer was transiently expressed in HEK293T cells stably overexpressing hAgo2. PNS was prepared and fractionated on discontinuous sucrose gradients to separate membranes (top fraction) from cytosol (bottom fractions). The top fraction and bottom fractions were subjected to GST pull-down on glutathione-sepharose 4B beads. Proteins that associated with GST-Dicer were resolved on SDS-PAGE and immunoblotted with anti-hAgo2, anti-Dicer, anti-GST and anti-Hsp90. Our results indicate that pools of hAgo2-Dicer complexes are present in the cytosol and on membranes (Fig 4-9 B). As expected Hsp90 is present only in the cytosolic fractions, not in the membrane-enriched fraction.

Figure 4-9- A portion of the Dicer-hAgo2 complex associates with the membranes

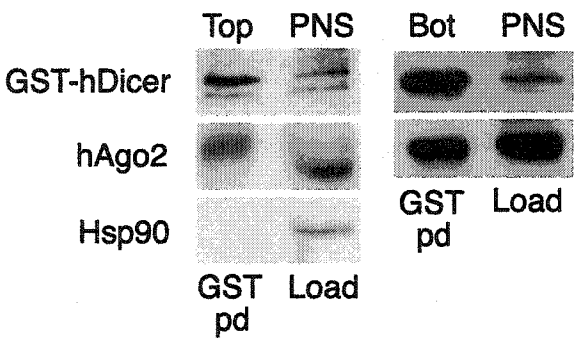
A) hAgo2, Hiwi and Dicer are present in soluble and membrane-associated pools. Homogenates of HEK293T cells transiently expressing GST-Hiwi were subjected to membrane flotation assays on discontinuous sucrose gradients. Fractions were collected from the tops of gradients, TCA precipitated, resolved by SDS-PAGE and subjected to immunoblotting with antibodies specific for hAgo2, GST, Dicer, calnexin and Hsp90. Membrane-associated material is found at the top of the gradient, whereas soluble proteins remain in the three bottom fractions. Calnexin and Hsp90 are the positive controls for the membranous and cytosolic fractions,respectively.

B) GST-Dicer was transiently expressed in a HEK293T cell line stably overexpressing hAgo2. PNS was prepared and subjected to membrane flotation assay. One ml fractions were collected from top to bottom of six gradients prepared in parallel experiments. The top and bottom fractions were concentrated and then subjected to affinity purification on glutathione-sepharose 4B beads. Proteins were resolved on SDS-PAGE, transferred to PVDF membranes and blotted with antibodies to Dicer, Ago2 and Hsp90. PNS: post-nuclear supernatant, Bot: bottom fraction.

A)



B)



CHAPTER 5

DISCUSSION

The RNP complexes involved in the effector step of RNAi have been extensively studied. It has been shown that there is functional and structural overlap between RISC and miRNP complexes. For example, endogenous *let7* st/miRNA guided a hAgo2-containing miRNP complex to cleave a target RNA that contained a perfectly complementary *let7* sequence (Hutvagner et al., 2001). Moreover, it has been shown that siRNAs can function as miRNAs by binding to pre-designed imprecisely complementary 3'UTRs of target mRNAs to enact translational repression (Doench et al., 2003). In addition, RISC and miRNP complexes share protein components. For instance, dAgo1 seems to be a component of RISC in *D. melanogaster* embryos (Williams and Rubin, 2002) and part of miRNP complexes during adulthood (Caudy et al., 2002). Furthermore, it has also been shown that Fmrp and hAgo2, which are components of miRNP complexes (Ishizuka et al., 2002; Mourelatos et al., 2002), can be recruited to RISC following siRNA transfection (Caudy et al., 2003).

Compared to the research focused on the identification of RISC components, for a long period of time very little was reported on Dicer-binding proteins. Perhaps the main reason is that it was possible to study Dicer activity in the absence of other proteins. Indeed, *in vitro* purified Dicer is sufficient to support the cleavage of dsRNA or pre-miRNA molecules to siRNA or miRNA, respectively (Provost et al., 2002a; Zhang et al., 2002). Remarkably, the first Dicer-binding protein to be identified was dAgo2, which was also the first identified component of RISC (Hammond et al., 2001a). It has been suggested that interaction between dAgo2 and Dicer is transient and is required for the transfer of siRNA from Dicer to RISC (Hannon, 2002). The discovery of *D. melanogaster* R2D2, which was shown to load siRNA from Dicer onto dAgo2, revealed

that the transfer of siRNA to RISC may not be performed by Dicer *per se* (Liu et al., 2003).

In this chapter, I will discuss the role of Hsp90 in Dicer-PPD interactions and propose various possible links between Hsp90 activity and RNAi. In addition, through analyses of our results and data from other labsoratories, I have developed a model for the role of Dicer-PPD complexes in RNAi. This model suggests that Dicer-PPD complexes are stable, and their activity extends beyond the initiation step. Indeed, Dicer might be the putative "slicer", the endonuclease that mediates the siRNA-directed cleavage of target mRNA.

5-1- The interactions between PPD proteins and Hsp90

Members of the Hsp90 heterocomplex were the first PPD-binding proteins that we identified in this study. Our data revealed that an Hsp90 heterocomplex interacts with the amino terminus of PPD proteins. By analogy with other Hsp90 substrates such as steroid hormone receptors and kinases (Pratt and Toft, 2003; Young et al., 2001), I hypothesize that binding to Hsp90 causes conformational changes in PPD proteins, thus increasing their ability to interact with other proteins in downstream pathways. This may indeed be the case for PPD interaction with Dicer. In addition, binding of Dicer to the carboxyl terminus of a PPD protein may promote dissociation of Hsp90 from the amino terminus of PPD proteins. This is consistent with our data showing that Dicer-PPD complexes do not contain Hsp90, and that in the absence of the carboxyl terminal region of rAgo2, interaction with Hsp90 is increased (Tahbaz et al., 2001).

Interaction with Hsp90 heterocomplexes might also be required for the delivery of PPD proteins to destinations in the cytoplasm where their function in RNAi or signal transduction pathways is required (Pratt et al., 1999). Immuno-electron microscopy indicates that rAgo2 associates with unknown cytoplasmic filaments (our unpublished data). Furthermore, the sea urchin homologue of Piwi, also known as Seawi, was immunopurified together with microtubules (Rodriguez, et al., 2001). It is known that the interaction between a Hsp90 heterocomplex and microtubule-associated dynein is mediated through immunophilins (Galigniana et al., 2001; and 2002). However, we did not detect Fkbp52, a steroid hormone receptor-associated immunophilin, among rAgo2-associated Hsp90 heterocomplex members by immunoblot analysis. It should be mentioned that our analysis does not exclude presence of other immunophilins such as Fkbp51, Fkbp59 or Cyp40 in Hsp90-rAgo2 complex. In addition, since weak interactions mediate associations among hsp90 heterocomplex members (Murphy et al., 2001; Pratt and Toft, 1997; Radanyi et al., 1994), immunophilins might have been dissociated during the pull-down experiments.

The role of Hsp90 as a capacitor of evolution (Rutherford and Lindquist, 1998) has been linked to the epigenetic regulation of gene expression (Sollars et al., 2003). Pharmacological impairment or mutations in Hsp83 (*D. melanogaster* homologue of Hsp90) caused phenotypic variation of nearly all adult structures. Genetic selection of *hsp83* mutants showed that morphological variants exist in nature, but are silent due to the buffering activity of Hsp90. Interestingly, mutations in the *hsp83* gene or either one of the genes of the *Trithorax Group* (*TrxG*) induced an abnormal outgrowth in the eyes of flies with a dominant mutation of *kruppel* gene ($Kr^{If\text{-}1}$), a gene that encodes a zinc-finger

transcription factor (Sollars et al., 2003). TrxG proteins maintain the "active" highly acetylated chromatin state required for gene expression in *D. melanogaster*. Inhibition of the function of *hsp83* in $Kr^{If\text{-}1}$ flies, as a consequence of feeding them GD, resulted in increased incidence of the abnormal eye phenotype in the progeny. In contrast, feeding them histone deacetylase inhibitors reversed the effect of GD, suggesting that the function of Hsp83 as a capacitor of evolution might be carried out by an epigenetic mechanism involving TrxG proteins. Involvement of heat-shock proteins in the regulation of the chromatin assembly machinery is not unprecedented. For example, it was previously reported that the *heat-shock cognate 4* (*hsc4*) gene is an enhancer of *PcG* (*Polycomb Group*) genes (Mollaaghababa et al., 2001). *PcG* genes are antagonists of *TrxG* genes, and together they regulate silencing and activation of gene expression in *D. melanogaster*, respectively (Orlando, 2003). However, it is not known how PcG and TrxG proteins associate with chromatin to convey their epigenetic effects.

The role of Hsp90 as a capacitor of evolution may be mediated through its interaction with PPD proteins and/or other proteins that are involved in epigenetic regulation of gene expression. Evidence suggests that PPD proteins might be involved in the recruitment of PcG and TrxG-like complexes to chromatin in a locus and sequence-specific manner. For instance, Piwi is required in PcG-dependent transcriptional gene silencing (Pal-Bhadra et al., 2002). In addition, *A. thaliana* Ago4 is involved in locus and sequence-specific methylation of DNA and histones, perhaps through recruitment of PcG and TrxG-like complexes (Zilberman et al., 2003).

A requirement for Hsp90 during RNAi-mediated mRNA degradation has been reported recently in plants (Gossele et. al. , 2004). Indeed, assaying the

requirement for Hsp90 in RNAi-mediated mRNA degradation in mammalian cells may not be a simple task. First, Hsp90 is required for the activity of many kinases that may be required for transcription and translation. Thus, inhibiting the activity of this chaperone may be expected to lead to pleiotropic effects. Second, although my recent data suggest otherwise, Hsp90 function may not be critical for activity of all PPD proteins that are involved in mRNA degradation. All multicellular organisms express multiple PPD proteins, many of which have overlapping functions. Consequently, determining the precise role of Hsp90 activity in RNAi awaits further analysis.

5-2- Interaction between Dicer and PPD proteins

We have characterized the interaction between Dicer and PPD proteins. Mapping of the interaction sites indicated that the PIWI domains of PPD proteins and the RNase III domain of Dicer are involved in complex formation. Moreover, we showed that strongest interactions occurred between the RNase III-A domain of Dicer and the PIWI-box of PPD proteins. Further deletion analysis or alanine scanning analysis in the PIWI-box or RNase III-A domain could be used to determine the residues involved in the interaction between the two classes of proteins. The candidate amino acids for further analyses include the five amino acids that are absolutely conserved in PIWI-box (Fig 1-5), and the amino acids of the RNase III signature (Mian, 1997). A detailed understanding of the interaction between PPD proteins and Dicer may help us to understand the biochemical basis of the downregulation of Dicer activity by PPD proteins *in vitro*.

My data showed that Dicer and PPD proteins are present in both membrane-enriched and cytosolic fractions. Furthermore, hAgo2 was detected in GST-Dicer pull-downs from both membranous and cytosolic fractions suggesting that Dicer-PPD complexes are present on membranes (Fig 4-9 B) and in the cytosol (Fig 4-9 C). I suggest that the discrete pools of the Dicer-PPD complexes may be involved in processing specifically localized membranous and cytosolic pools of mRNA. Furthermore, Dicer association with membranes may be a result of its association with membrane-bound ribosomes. DFmr1, which binds to the 50S large ribosomal subunit, may be the link between Dicer, dAgo2 and ribosomes. Both dFmr1 and dAgo2 coimmunoprecipitate Dicer independent of RNAi activation, suggesting that Dicer exists in a stable complex with these proteins (Ishizuka et al., 2002). In addition, a cytosolic pool of Dicer-PPD complex may serve as a reservoir for the membranous pool, an activity which is consistent with our observation that depletion of rAgo2 from membranes in the absence of functional Hsp90 occurs after 12-14 hours (Tahbaz et al., 2001).

Destruction of target mRNAs by RISC has been suggested to occur in two steps. During the first step an endonuclease, termed "slicer", cleaves mRNA in the area spanned by dsRNA in 21-23 nt intervals (Elbashir et al., 2001b). This is followed by the degradation of mRNA cleavage products by a nuclease that completely degrades mRNA. To date, efforts to isolate "slicer" from RISC have been unsuccessful. Schwarz et al. (2004) recently demonstrated that RISC contains a Mg^{2+}-dependent endonuclease activity that leaves 5'-phosphate and 3'-hydroxyl groups at the ends of its cleavage products. Accordingly, RISC "slicer" has Dicer-like properties, although it should be noted that Dicer has never been isolated as a component of RISC. In contrast, Tsn which was

isolated as a RISC component, seems to be a good candidate for the enzymatic activity that degrades mRNA during the second step of RISC activity (Caudy et al., 2003). Interestingly, specific inhibitors of Staphylococcal nucleases do not inhibit mRNA cleavage by RISC "slicer" (Schwarz et al., 2004), reiterating that enzymatic activities that cleave and degrade mRNA during the effector step of RNAi are independent. Indeed, Staphylococcal nucleases hydrolyse DNA and RNA into 3'-phosphomononucleotides and dinucleotides in a Ca^{2+}-dependent manner (Cunningham et al., 1956; Reddi 1958;1960).

It is tempting to speculate that Dicer is the "slicer", the enzyme that starts mRNA cleavage by RISC. In addition to the data from Schwarz et al. (2004), the following evidence (presented in detail in section 1-3-1-4) supports this hypothesis. First, Doi et al. (2003) showed that RNAi knockdown of Dicer reduced siRNA-dependent mRNA degradation five-fold, suggesting that the function of Dicer is required during the effector step of RNAi through mRNA degradation. Second, data from Liu et al. (2003) showed that following complete depletion of Dicer, mRNA degradation during the effector step of RNAi was abolished. Finally, Drosha and Dicer are the only known RNase III enzymes in eukaryotes. Since Drosha is a nuclear protein, Dicer is a better candidate for the RISC endonuclease that cleaves siRNA-mRNA hybrids.

Cleavage of mRNA in 21-23 nt intervals prior to its degradation during the effector step of RNAi is reminiscent of processing of long dsRNA or pre-miRNA by Dicer. However, cleavage in 21-23 nt intervals may not be a property of the "slicer" but rather the result of cleavage in the middle of adjacent siRNAs. Indeed, it has been shown that a single siRNA could program cleavage of mRNA by RISC. If Dicer is the "slicer",

Figure 5-1- A model for the role of Dicer-PPD complexes during the effector step of RNAi

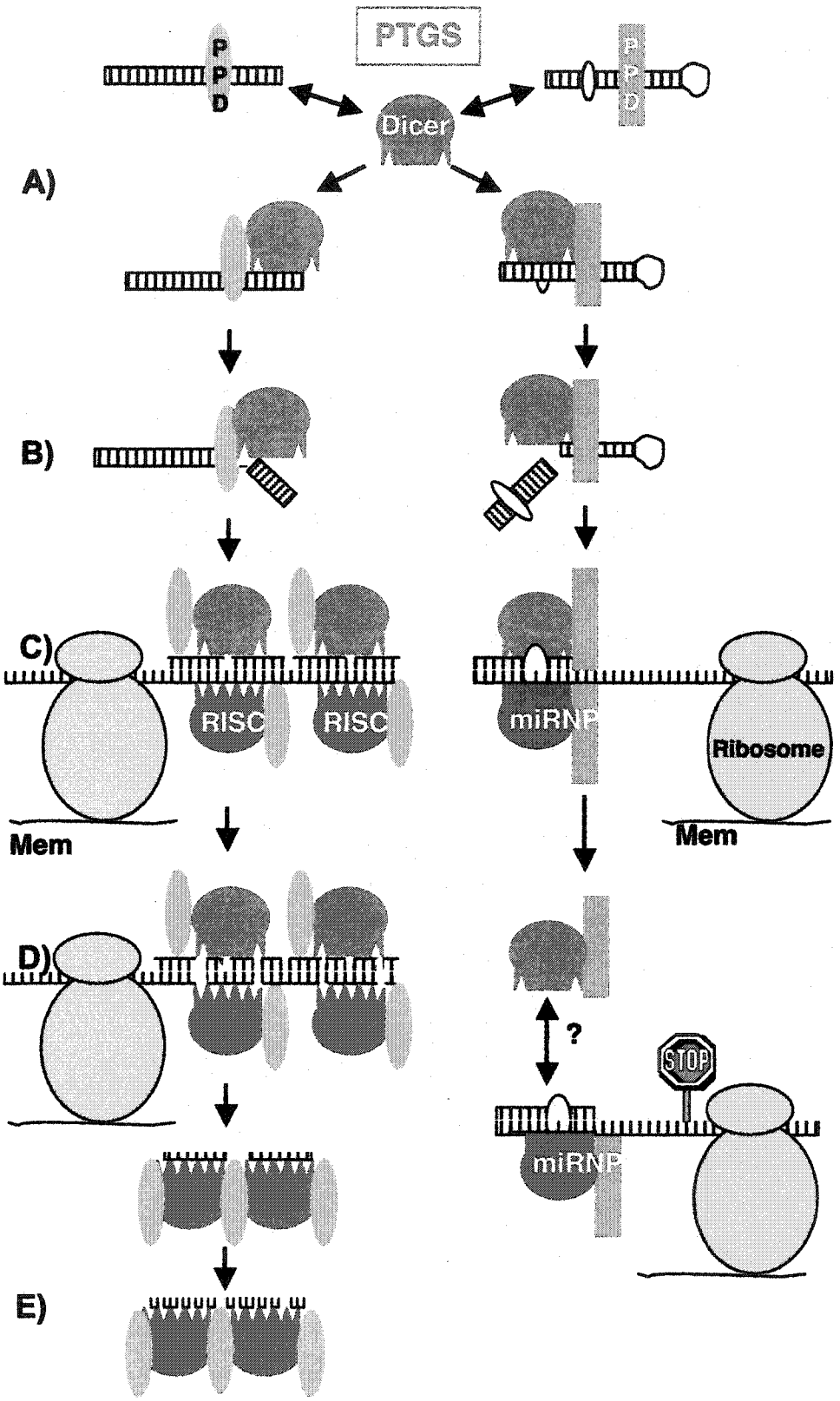
A) Long dsRNAs or pre-miRNA hairpins are introduced to Dicer by PPD proteins and Rde4 homologues (in *C. elegans* and *D. melanogaster*).

B) Long dsRNAs or pre-miRNAs are cleaved by Dicer-PPD complexes.

C) Dicer-PPD complexes containing siRNA/miRNAs then associate with ribosomes through binding to dFxr1 homologues. RISC or miRNP complexes also bind to the surface of the large subunit of ribosomes by the homologues of dFxr1. The siRNA/miRNA then recruits the Dicer-PPD and RISC/miRNP complexes to the specific sequences on mRNA.

D) Dicer cleaves the siRNA-mRNA hybrids in the middle of the siRNA. The Dicer-PPD complex may be only involved in the transfer of miRNA to the miRNPs during the effector step of translational repression.

E) Finally, exonucleases such as Tsn degrade the mRNA.



one would predict that Dicer monomers should be active dsRNA cutters, as has been suggested for the Dicer dimers (Blaszczyk et al., 2001).

In conclusion, I suggest a model in which recruitment of Dicer-PPD complexes to ribosomes, which may or may not be membrane-associated, is required to prime RISC activity, perhaps by cleavage of siRNA-mRNA hybrids or by presentation of siRNA to RISC or both (Fig 5-1). Dicer-PPD complexes may also be involved in the transfer of miRNA to miRNP complexes during translational repression.

Despite the supporting data mentioned, the model presented here is still controversial. Dicer, together with PPD proteins, might only be required for transfer of si/miRNA to RISC or miRNP complexes, and not for the cleavage of hybrids of siRNA and mRNA. In fact, RISC activity has been reported in a 90-160 kDa complex containing Eif2c2/hAgo2 (Martinez et al., 2002). Since PPD proteins do not show enzymatic activity, the endonuclease activity of 90-160 kDa RISC might have been provided by nearly undetectable amounts of a highly catalytic unknown nuclease. However, a final evaluation of the model presented here awaits isolation of "slicer" from RISC.

5-3- PPD proteins are multifunctional

The *D. melanogaster* Aubergine (Aub) is a good example of the multifunctionality of PPD proteins. Aub is involved in embryonic body patterning via indirect regulation of *oskar* mRNA translation (Harris and Macdonald, 2001). It is also required for the correct localization of Dicer and dAgo2 in the germline and somatic cells of adult ovarioles via Maelstrom (Findley et al., 2003). In addition, Aub is essential for PTGS and translational repression of maternal transcripts in the oocyte (Kennerdell et al.,

2002), and for suppression of *ste* repeats by the *su(ste)* repeats during adulthood (Aravin et al., 2001).

Association of Eif2c2, an isoform of hAgo2, with more than 40 different miRNAs (Mourelatos et al., 2002) indicates that this protein is probably involved in regulation of expression of a wide variety of genes. Analysis of cDNA sequences encoding hAgo2 isoforms suggests that the *hago2* mRNA is alternatively spliced, perhaps as a way to generate different hAgo2 isoforms with different activities. Comparison of the amino acid sequences encoded by the hAgo2 cDNAs cloned during this thesis project shows that they are more than 96% identical, differentiating only at their extreme amino termini (Fig 4-1). The amino acid sequences of D9p and G4p deviate upstream of the first alanine in the amino acid sequence ALAPPAPPP (Fig 4-1). Analysis of the genomic sequences encoding hAgo2 isoforms, indicates that the nucleotide sequences upstream of the regions encoding ALAPPAPPP are provided by different exons (Fig 5-2). The *hago2* gene includes 19 exons (Sasaki et al., 2003) with exons 3-19 common among all hAgo2 isoforms and located in successive order on chromosome 8. The first exons incorporated into *D9* and *eif2c2* mRNAs are derived from 8q24.3, the same region of chromosome 8 that provides exons 3-19 to all *hago2* mRNAs. In contrast, the first exon of *G4* mRNA is derived from 321 contiguous nucleotides found on the short arm of chromosome 8 (8p12). This could have been caused by a trans-splicing event that might have transferred exon 1 to *G4* mRNA (Garcia-Blanco, 2003). Alternatively, given that the hAgo2 cDNAs were isolated as intact fragments, the presence of exon 1 of G4 in 8p12 might have resulted from an artifact in the assembly of the corresponding genomic region. Finally, even if exon 1 of the *G4* mRNA is a cloning artifact, the results presented in this thesis do

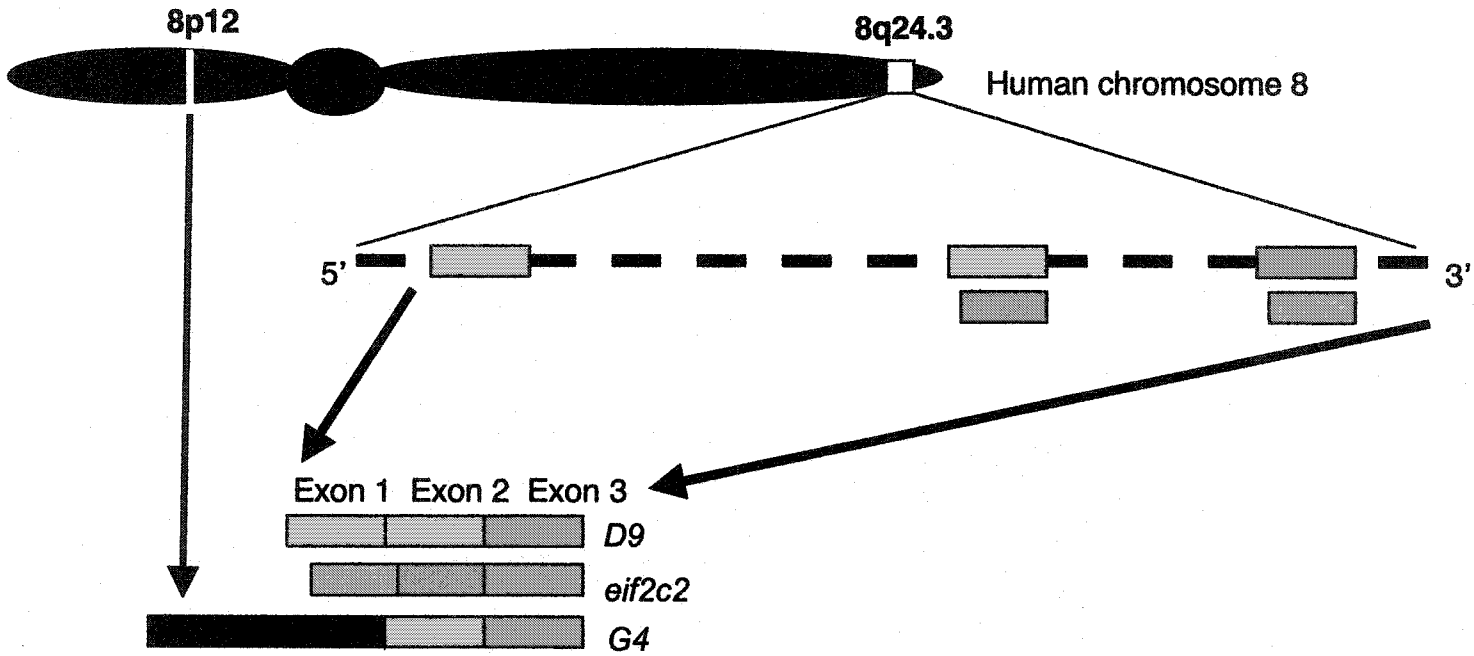


Figure 5-2- The *hago2* mRNA is alternatively spliced.

The genomic origin of the first three exons of hAgo2 mRNAs are shown. The first exons of hAgo2-encoding mRNAs (*D9*, *eif2c2* and *G4*) are different, indicating that these mRNAs are alternatively spliced. The first exons of *D9* (green) and *eif2c2* (dark purple) mRNAs are derived from 8q24.3. In contrast, the first exon of *G4* mRNA (black box) is transcribed from the 8p12 region. The second exons of the *D9*, *G4* (bright blue) and *eif2c2* (dark blue) mRNAs are identical, except that the 5' end (encoding PRP motif) is missing from eif2c2 mRNA. Interestingly, the first exon of *eif2c2* (dark purple) is a shorter form of exon 3 (bright purple) missing its 5'end (TABLE 1, Appendix). Exons 3-19 encoding all hAgo2 isoforms are identical and transcribed in a successive order from 8q24.3. Exons are not shown past exon 3.

not change significantly, since exon 1 only contributes eight amino acids to the beginning of G4p (Fig 4-1).

An interesting difference among the hAgo2 cDNAs is that the Eif2c2 cDNA lacks the coding region for PRP motif. In contrast, both G4 and D9 isoforms encode this motif (Fig 1-5). Analysis of our data provides clues to the activity of PRP motif. Our immunoblot analysis detected two species of endogenous hAgo2 in cell lysates. Only the high molecular weight form associates with the membranes (Fig 4-9 A) and is present in the membrane-associated Dicer-hAgo2 complex (4-9 B). *In vitro* TNT reactions using hAgo2 or rAgo2 encoding cDNAs also resulted in the synthesis of two forms for both proteins (Fig 4-1 C and our unpublished data). Formation of two species of hAgo2 (G4p) or rAgo2 might be caused by the post-translational modifications or alternative initiation of translation. However, we have shown that rAgo2 does not contain asparagine-linked glycans, nor does it appear to be stably phosphorylated (Cikaluk et al., 1999). Given the high level of sequence conservation between rAgo2 and hAgo2 (>97.8%), we believe the same holds true for the human orthologue. Therefore, differential initiation of translation may account for the two hAgo2 species *in vivo*. This is confirmed by the comparison of *in vitro* protein synthesis products of G4 and F9 cDNAs (Fig 4-1 C). The two most upstream methionines encoded by the G4 cDNA are in the context of MHPF and then MDIPK. The ATG codons for both of these methionines are surrounded by partial Kozak sequences (A or G at -3 and G at +4) suggesting that they are translational initiation sites (Kozak, 1999). The existence of two upstream methionines potentially allows for the synthesis of two hAgo2 species. Interestingly, *in vitro* protein synthesis with the F9 cDNA that encodes only one upstream methionine (MDIPK) produced one protein with

the equivalent size to the lower band of the *in vitro* protein synthesis products of G4 (Fig 4-1). Since our data indicates that only the larger protein translocates to membranes, the region between MHPF and MDIPK, which contains most of the PRP motif, may be required for membrane localization. However, it should be mentioned that the notion that the PRP motif is required for membrane localization of Ago subfamily members does not exclude the presence of other membrane localization determinants in members of Piwi subfamily, e.g. Hiwi lacks the PRP motif but perhaps is associated with membranes.

The PRP motif may also be involved in the interaction with Dicer or may be required for the regulation of Dicer activity. Doi et al. (2003) showed that the amino terminus of human hAgo1, containing PRP and PAZ, interacts weakly with Dicer. In contrast, our data revealed that the PAZ domain does not mediate Dicer-PPD interaction, suggesting that PRP motif may be a second site for Dicer-PPD interaction. Unpublished data from our collaborators (Dr. Witold Filipowicz, Basel, Switzerland) showed that addition of PIWI or PAZ domains to an *in vitro* dsRNA-cleavage assay did not affect Dicer activity. It would be of interest to determine the effect of the addition of the purified PRP motif on the rate of dsRNA cleavage by Dicer.

Finally, D9p or G4p that are 94.5% identical to Eif2c2 may also be components of miRNP complexes. The existence of different isoforms of hAgo2 in miRNP complexes, may be part of a mechanism of targeting miRNP complexes to specific downstream pathways.

5-4- Concluding remarks

The scientific understanding of RNAi has come a long way since it was defined in 1998 by Fire and colleagues. The discovery that cleavage of dsRNA and pre-miRNA by Dicer is independent of mRNA degradation revealed that RNAi is comprised of initiation and effector steps (Zamore et al., 2000). However, the classical view that the two steps of RNAi are independent has been challenged by new data. Dicer seems to be required for siRNA-dependent RNAi (Dio et al., 2003; Liu et al., 2003), and the RISC endonuclease activity shows Dicer-like properties (Schwarz et al., 2004). The quest for the identification of RISC endonuclease awaits the results of intensive investigations by several laboratories.

The complexity of different aspects of the role of PPD proteins in RNAi is starting to emerge. Once thought of as scaffolds for RNP complexes involved in transfer of RNA between initiation and effector steps (Baulcombe, 2001), PPD proteins now seem to be involved in specification of RNAi and channeling it to different downstream pathways. This view was first suggested by Grishok et al. (2002) who showed Alg1 and Alg2 are required for both introduction of pre-miRNA to Dicer and targeting to miRNAs to different downstream pathways.

Our data that PPD proteins stably interact with Dicer are consistent with previously unrecognized roles for both Dicer and PPD proteins in the regulation of RNAi. Based on our data and data from other laboratories, I proposed a model suggesting that Dicer might be the "slicer". Confirmation of this model requires biochemical data showing an actual association between RISC and Dicer. Interestingly, this model also predicts that the demonstration of an association between Dicer-PPD complexes and

RISC would be a difficult task. The reason is first the large size of the putative "RISC-PPD-Dicer" complex, and second that the RISC and Dicer complexes may only be indirectly associated with each other, for instance via dFxr1 or siRNA-mRNA hybrids. In addition, the association between Dicer-PPD complexes and RISC may be transient and only required to prime RISC activity rather than being required for complete mRNA degradation.

We are only a few years removed from the discovery of RNAi. Yet, RNAi not only is revolutionizing our views on the regulation of gene expression and thus cell physiology, it is also changing our strategies of drug discovery and thus our approaches to curing disease. Accordingly, acquiring a better understanding of RNAi has become the goal of many academic and industrial laboratories. Our work on the characterization of PPD proteins and their interactions with Dicer and Hsp90 provides insight into the details of the molecular mechanism of RNAi.

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APPENDIX:

TABLE 1: The genomic structure of hAgo2 isoforms

	EXON 1	*EXON 2*	*EXON 3*
D9 mRNA	6170708-6170654	6120481-6120287	6108104-6107979
eif2c2 mRNA	6108085-6107979	6120364-6120287	6108104-6107979
G4 mRNA	3745308-3474491	6120481-6120287	6108104-6107979

TABLE 2: Ago2 partners identified in this thesis work

	YEAST TWO-HYBRID	*GST PULL-DOWN*	*RETICULOCYTE LYSATE*
Hsp90		+ (r)	+ (r & h)
Hsp70		+ (r)	
Hdj2	+ (NT-r)	+ (r)	
P23		+ (r)	+ (r & h)
Hop			+ (r & h)
P50			- (r)
Fkbp52			- (r)
Initiation factor 3 Subunit 5	+ (CT-r)		
Proteosome (PSMB4)	+ (CT-r)		
Sorbitol Dehydrogenase	+ (CT-r)		
MHC class I	+ (CT-r)		
Hypothetical protein (GI: 39645249)	+ (CT-r)		
Dicer		+ (h)	

NT: amino terminal region
CT: carboxyl terminal region
r: rat Ago2
h: human Ago2