# University of Alberta

## Hsp90 and its co-chaperones regulate the activity of human Argonaute2 in RNA-mediated silencing pathways

by

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**Abstract**

The potent ability of small, double-stranded RNAs to silence gene expression was initially reported 1998, sparking revolutions in molecular biology and genetics. Since then, intensive research into the mechanism of RNA-mediated silencing has implicated the machinery of this pathway in the post-transcriptional regulation of the majority of mammalian genes. The major RNA effectors of silencing are miRNAs, and they target mRNAs encoding proteins involved in nearly every biological process including cell division, differentiation, development and death. These small RNAs directly associate with a member of the Argonaute family of proteins to form the RISC which functions as a component of large ribonucleoprotein complexes. Considering the extent and critical nature of the proteins regulated by the miRNA pathway, the assembly and activity of RISC is most certainly subject to extensive regulation by a variety of mechanisms.

Hsp90 is an essential, ubiquitous and highly abundant molecular chaperone that is conserved throughout evolution. Interactions with its vast array of client proteins are coordinated by a network of co-chaperones, and its activity results in conformational changes within the structure of these clients through a process known as the chaperone cycle. A generally accepted role for Hsp90 in that maturation of its clients involves the stabilization of an unfavorable structural intermediate. The stabilization of these intermediates allows for the activation of the client. A role for Hsp90 in the RNAi pathway was initially

suggested by data demonstrating a reduced interaction between the RNAi core components hAgo2 and Dicer in the absence of Hsp90 activity.

In this thesis, I have demonstrated that Hsp90 activity, together with a subset of its co-chaperones, is important for the function of the miRNA-mediated post-transcriptional gene silencing machinery.

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**Table of Contents**

## List of Tables

**List of Figures**

## List of Nomenclature and Abbreviations

| | |
|---|---|
| µ | micro (x $10^{-6}$) |
| 17-AAG | 17-N-allylamino-17-demethoxygeldanamycin |
| A | amperes |
| ADP | adenosine diphosphate |
| ATP | adenosine triphosphate |
| ATPase | adenosine triphosphatase |
| cDNA | complementary deoxyribonucleic acid |
| Ct | threshold cycle |
| DAPI | 4',6-diamidino-2-phenylindole |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | dimethyl sulphoxide |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| dNTPs | deoxyribonucleotide triphosphate |
| DOX | doxycycline |
| DsRed | *Discosoma sp.* red fluorescent protein |
| dsRNA | double-stranded ribonucleic acid |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| endo-siRNA | endogenous short interfering RNA |
| FBS | fetal bovine serum |
| FRET | fluorescence resonance energy transfer |
| FT | flow-through |
| *g* | gravitational force |
| Geld | geldanamycin |

| | |
|---|---|
| GFP | green fluorescent protein |
| GHKL | gyrase, Hsp90, histidine kinase, MutL |
| GR | glucocorticoid receptor |
| HA | hemagglutinin |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid |
| HIPP | hippuristanol |
| HSP | heat shock protein |
| IgG | immunoglobulin G |
| IP | Immunoprecipitation |
| kDa | kilo (x $10^3$) Daltons |
| m | milli (x $10^{-3}$) |
| M | moles per litre |
| MEEVD | methionine-glutamic acid-glutamic acid-valine-aspartic acid |
| miRISC | microRNA RNA-induced silencing complex |
| miRNA | microRNA |
| mRNA | messanger RNA |
| n | nano (x $10^{-9}$) |
| NGS | normal goat serum |
| NP40 | nonidet-P40 |
| nt | nucleotide |
| $^oC$ | degrees Celsius |
| P body | processing body |
| PAZ | PIWI, Argonaute, Zwille |
| PB | processing body |
| PBS | phosphate buffered saline |
| PBS-T | phosphate buffered saline, tween 20 |

| | |
|---|---|
| PCR | polymerase chain reaction |
| pH | power Hydrogenii |
| piRNA | PIWI-interacting RNA |
| pre-miRNA | precursor microRNA |
| pri-miRNA | primary microRNA |
| PTGS | post-transcriptional gene silencing |
| PVDF | polyvinyldene difluoride |
| RISC | RNA-induced silencing complex |
| RNA | ribonucleic acid |
| RNAi | RNA interference |
| RNase | ribonuclease |
| RNP | ribonucleoprotein |
| ROX | 5-carboxy-X-rhodamine |
| SDS-PAGE | sodium dodecyl-suplhate polyacrylamide gel electrophoresis |
| shRNA | short, hairpin RNA |
| siRNA | short interfereing RNA |
| TEMED | N,N,N’,N’-tetramethylenediamine |
| TPR | tetratricopeptide repeat |
| tRNA | transfer RNA |
| U | enzymatic units |
| UNG | uracil-N-glycosylase |
| V | Volts |
| v/v | volume per volume |
| w/v | weight per volume |
| WB | western blot |

# CHAPTER 1

Introduction

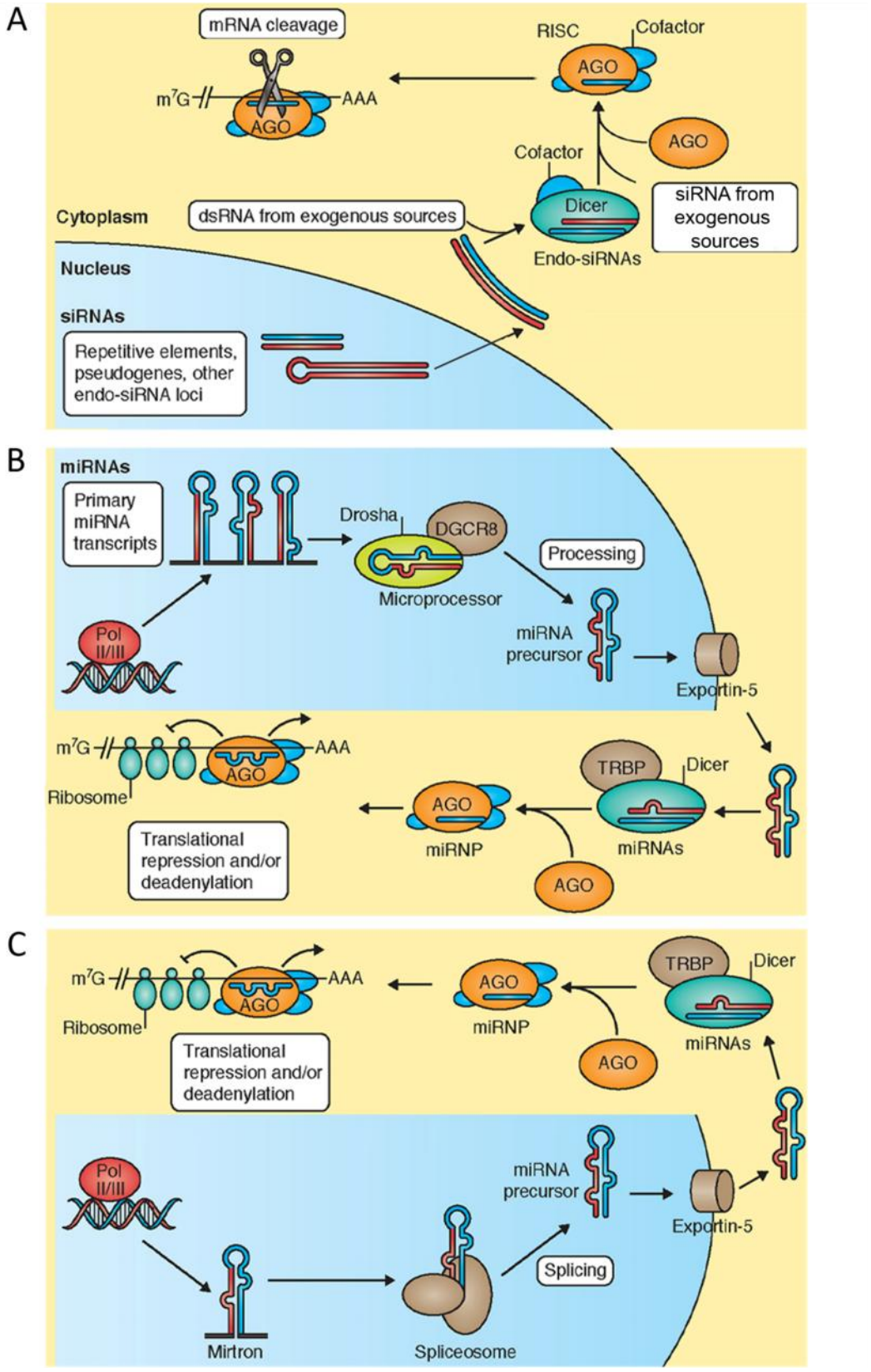
## 1.1 RNA interference

### *1.1.1 Overview*

More than a decade has passed since the potent ability of double stranded RNA (dsRNA) to interfere with protein expression at the messenger RNA (mRNA) level was discovered (Fire et al., 1998). In the intervening years, the study of RNA intereference (RNAi) has led a revolution in molecular biology, allowing researchers to specifically 'knock down' the expression of proteins using small interfering RNAs (siRNAs). The subsequent discovery of endogenous small RNA effectors of silencing, called microRNAs (miRNAs) has further reformed traditional thinking about mechanisms of translational regulation (Ambros and Horvitz, 1987; Lee et al., 1993; Olsen and Ambros, 1999; Reinhart et al., 2000; Wightman et al., 1993). The protein components of RNA-mediated silencing are evolutionarily conserved across kingdoms, are present in all metazoans and are predicted to regulate the expression of greater than half of all mammalian genes (Friedman et al., 2009). This level of conservation leaves little doubt as to the significance of RNA interference for eukaryotic biology.

Initially, the mechanism of action by which these small RNAs affect protein expression was largely unknown; however, intensive investigation has identified much of the machinery responsible for enacting the silencing effect of small RNAs. The cytoplasmic RNase Dicer, working in conjunction with TRBP (HIV transactivating response RNA-binding protein) and/or PACT (also known as PRKRA) cofactors, is responsible for cleaving dsRNA into short, ~22nt small RNAs (Haase et al., 2005; Provost et al., 2002; Zhang et al., 2002) (Figure 1.1). After this processing step, small RNAs interact with their targeted mRNAs as a component of the large ribonucleo-protein (RNP) complex known as the RNA-induced silencing complex (RISC). A member of the Argonaute family of proteins is common to all forms of RISC. These proteins associate directly with the small RNA and are responsible for the endonuclease activity within RISC.

**Figure 1.1 Pathways of small RNA biogenesis. A.** Extensively complementary RNA from endogenous or exogenous sources is processed in the cytoplasm by Dicer to yield mature, ~22 nt siRNAs. siRNAs, which can also be introduced as chemically synthesized duplexes of 22 nt, are loaded onto an Argonaute protein and mediate cleavage of complementary mRNA. **B.** Hairpin structures formed by transcripts from the nuclear genome are first processed in the nucleus by Drosha, exported to the cytoplasm and processed into mature, ~22nt duplexes by Dicer. miRNAs target Argonaute-containing miRISC complexes to mRNAs and result in translational repression and/or deadenylation of the mRNA. **C.** Drosha-independent biogenesis of miRNAs involves the spliceosome-mediated removal of an intron. The excised intron adopts a hairpin structure and serves as a substrate for Dicer. Adapted from (Ender and Meister, 2010)


A
mRNA cleavage
RISC
Cofactor
AGO
m7G
AAA
AGO
Cofactor
AGO
Dicer
siRNA from exogenous sources
Endo-siRNAs
Cytoplasm
dsRNA from exogenous sources
Nucleus
siRNAs
Repetitive elements, pseudogenes, other endo-siRNA loci
B
miRNAs
Primary miRNA transcripts
Drosha
DGCR8
Microprocessor
Processing
Pol II/III
miRNA precursor
Exportin-5
m7G
AAA
AGO
Ribosome
TRBP
Dicer
AGO
miRNP
miRNAs
Translational repression and/or deadenylation
AGO
C
m7G
AAA
AGO
Ribosome
AGO
miRNP
TRBP
Dicer
miRNAs
Translational repression and/or deadenylation
AGO
Pol II/III
miRNA precursor
Exportin-5
Splicing
Mirtron
Spliceosome

### *1.1.2 Classes of Small RNA*

#### *1.1.2.1 PIWI-interacting RNA (piRNA)*

The Argonaute family of proteins can be subdivided into two classes. The first is composed of the ubiquitously expressed Argonaute proteins that demonstrate the most similarity to Argonaute-1 of *Arabidopsis thaliana* and generally bind to dsRNA-derived small RNAs. The second subfamily of Argonaute proteins, initially identified in *Drosophila melanogaster,* is the Piwi class. This subfamily was identified, and named, due to the effects of Piwi mutants on gonadal development (Cox et al., 1998; Cox et al., 2000). Piwi proteins bind to RNA derived from a single-stranded precursor (Thomson and Lin, 2009) and evidence from multiple studies in *Drosophila* suggests that they are involved in repressing transposons (Brennecke et al., 2007; Chen et al., 2007; Pelisson et al., 2007; Savitsky et al., 2006; Shpiz et al., 2007; Vagin et al., 2004). Piwi-interacting RNAs (piRNAs) have also been identified in mammalian systems (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Watanabe et al., 2006) and are divided into two classes. Class one is defined by piRNAs expressed following the pachytene stage of meiosis. Unlike piRNAs of *Drosophila* that are enriched for sequences of transposons, more than 90% of the known members of this class of mammalian piRNAs map to unique sites within the mammalian genome (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006). The second class of mammalian piRNAs is expressed before meiotic pachytene (prepachytene piRNAs) and is derived from repetitive and transposon-rich sequences (Aravin et al., 2006; Aravin et al., 2007; Aravin et al., 2007; Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008). In both mammals and *Drosophila*, piRNA sequences are clustered within their respective genomes. In *Drosophila,* virtually all piRNA clusters are found within heterochromatin with a particular enrichment at the boundary between euchromatin and heterochromatin at the

centromeric regions of chromosomes (Aravin et al., 2003; Reinhart and Bartel, 2002).

Unlike the other classes of small RNAs discussed in this section, piRNA biogenesis is independent of Dicer activity (Houwing et al., 2007; Vagin et al., 2006), and is believed to occur via two pathways. The model of the primary pathway posits the cleavage of a long single-stranded RNA that traverses an entire piRNA cluster by an unknown nuclease. This processing step generates a free 5' end which is then loaded onto a Piwi protein. Cleavage of the loaded transcript generates its 3' end, yielding a mature piRNA. Mammalian piRNAs show a strong sequence bias for a 5' uridine residue which may serve to direct the initial cleavage of the long piRNA transcript. However, piRNAs associated with the Piwi family member AGO3 in *Drosophila* show no such 5' nucleotide bias and are thought to be loaded with piRNAs generated by a second pathway of piRNA biogenesis.

The second model for piRNA biogenesis is termed the 'ping-pong cycle' and depends on the cleavage of piRNA transcripts derived from both strands of genomic clusters of piRNA sequences (Brennecke et al., 2007; Gunawardane et al., 2007; Li et al., 2009; Liu and Paroo, 2010). It is proposed that cleavage of a long piRNA transcript is mediated by the endonuclease activity of the PIWI protein pre-loaded with a piRNA. Products of Piwi-mediated cleavage are then loaded onto Piwi proteins (*e.g.* AGO3) and direct cleavage of transcripts derived from the opposite strand of genomic DNA.

As piRNAs are a relatively recently discovered class of small RNA, there remains a great deal to be learned about their role in biological processes such as germline preservation and developmental timing.

*1.1.2.2 Small interfering RNA (siRNA)*

In 1998, Andrew Fire, Craig Mello and their colleagues published the seminal observation that gene expression can be potently and specifically inhibited by introducing dsRNAs that share perfect complementarity with the mRNA of the gene of interest (Fire et al., 1998). Upon formation of a perfect duplex between the siRNA and the targeted mRNA, the mRNA is cleaved by the endonuclease activity of the Argonaute component of RISC (Elbashir et al., 2001a; Elbashir et al., 2001b; Schwarz et al., 2002) (Figure 1.1A). The sequence-specific mediators of RNA interference were later shown to be 21- and 22-nt dsRNAs (Elbashir et al., 2001a) and since then RNA interference has become a ubiquitous technique in laboratories throughout the world.

Widely used in engineered approaches to knockdown protein expression, siRNAs have also been shown to serve an important function in innate immunity against viral infection in plants and insects (Kemp and Imler, 2009; Mallory and Vaucheret, 2010). Long dsRNA molecules, generated as replication intermediates of RNA viruses, can be recognized and processed by Dicer in the same manner as engineered dsRNA used by researchers. The role for this pathway in the defense against invading RNA viruses has led to speculation that the evolutionary origin of the RNA interference pathway was a primitive immune system. This RNA-based immune system is still relied upon by plants, fungi and invertebrates (Lu et al., 2005; Morel et al., 2002; Wang et al., 2006).

The efforts of many labs working to understand the mechanism and biological significance of RNA interference has led to the discovery of a number of other classes of endogenously transcribed small RNAs.

*1.1.2.3 Endogenous siRNA (endo-siRNA)*

There is another class of small RNAs that is derived from a pathway similar to that which generates canonical siRNAs. As described above, siRNAs are

generated by processive cleavage of dsRNA precursors from exogenous sources. Alternatively, endogenous transcripts which form extended intramolecular hairpins or intermolecular duplexes give rise to endo-siRNAs. Similar to the siRNA biogenesis pathway, the biogenesis of endo-siRNAs in *Drosophila* requires Dicer (Chung et al., 2008; Czech et al., 2008; Ender and Meister, 2010; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008a; Okamura et al., 2008b). Endo-siRNAs have been described in plants, *C. elegans, Drosophila* and mice (Chapman and Carrington, 2007).

In *Drosophila,* there are two isoforms of Dicer and two members of the Argonaute subfamily and the two major small RNA pathways (miRNAs and siRNAs) utilize separate Dicer and Argonaute isoforms. siRNAs from dsRNA precursors are the products of Dicer-2-mediated cleavage and preferentially associate with *Drosophila* Ago2 to mediate the cleavage of target mRNA (Caudy et al., 2002; Forstemann et al., 2007; Lee et al., 2004). Accordingly, endo-siRNAs preferentially load onto *Drosophila* Ago2 (Czech et al., 2008; Kawamura et al., 2008) and mammalian Ago2 (Watanabe et al., 2008) to direct the cleavage of their targets. The endo-siRNA pathway in both *Drosophila* and mice appears to mainly target transposable elements, although protein-coding genes are also targeted (Czech et al., 2008; Ghildiyal et al., 2008; Okamura et al., 2008a).

*1.1.2.4 MicroRNA (miRNA)*

The *lin-4* gene of *Caenorhabditis elegans* was discovered as encoding a small RNA with complementarity to *lin-14* and it was suggested that *lin-4* regulates *lin-14* via an antisense RNA-RNA interaction (Lee et al., 1993). It is now known that *lin-4* represents a large class of small, non-coding RNAs that function to down-regulate the expression of protein-coding genes in *C. elegans* (Olsen and Ambros, 1999; Reinhart et al., 2000). These small RNAs, initially known as small temporal RNAs, are now known as microRNAs (miRNAs). Since their discovery in *C. elegans*, miRNAs have been identified in a wide range of

metazoans with described roles in nearly every aspect of biology including developmental timing, differentiation, proliferation, metabolism and even metastasis (Adams et al., 2009; Aukerman and Sakai, 2003; Brennecke et al., 2003; Chen et al., 2004; Chen, 2004; Emery et al., 2003; Johnston and Hobert, 2003; Lee et al., 2006; Li et al., 2010; Liu et al., 2004; Morita et al., 2007; Palatnik et al., 2003; Sun et al., 2011; Wang et al., 2011; Xu et al., 2003; Yin et al., 2011).

Biogenesis of miRNAs begins with transcription from the nuclear genome resulting in the production of a long single-stranded transcript. This transcript, called the primary miRNA transcript (pri-miRNA), contains partially complementary inverted repeats that lead to the formation of large hairpin structures (Kim, 2005). The pri-miRNA is cleaved by the nuclear RNaseIII-type microprocessor Drosha and its DiGeorge syndrome critical region 8 (DGCR8) cofactor, generating the pre-miRNA (Han et al., 2006). Exportin-5-dependent transport of the pre-miRNA from the nucleus to the cytoplasm allows for additional processing of the pre-miRNA by Dicer (Lund et al., 2004). Dicer-mediated cleavage of the pre-miRNA generates the mature miRNA, which is then loaded onto an Argonaute protein to form a miRNA RISC (miRISC) (Kawamata and Tomari, 2010). Once loaded onto an Argonaute protein, the miRNA duplex is unwound. Following duplex unwinding, a single stranded RNA, termed the ‘guide strand’, remains associated with an Argonaute protein as a part of the mature RISC (Figure 1.1B).

Drosha-dependent pathways are not the only mechanism for miRNA biogenesis. A second pathway, which is independent of the activity of this nuclear microprocessor, relies on spliceosome-dependent excision of short introns that mimic the structural features of a pre-miRNA. These excised introns are exported from the nucleus by Exportin-5 and are then processed by Dicer into mature miRNAs (Figure 1.1C) (Berezikov et al., 2007; Okamura et al., 2007; Ruby et al., 2007).

The mechanism by which miRNAs repress translation is poorly understood. The 3' untranslated regions of many mRNAs contain conserved sequence elements that retain a high level of complementarity to bases 2-8 of miRNA sequences, termed 'seed regions' (Lewis et al., 2003). These sites are targeted by miRNAs via base-pairing. In animals, most miRNAs have been shown to form imperfect duplexes with their binding sites on targeted mRNAs, with poor conservation outside of the seed region. Endonucleolytic cleavage of an mRNA by the Argonaute component of RISC requires extensive base-pairing, especially at positions 10-12 of the guide miRNA (Tolia and Joshua-Tor, 2007; Wang et al., 2008; Wang et al., 2008). As a result of this requirement, miRNAs in animals do not typically result in the endonucleolytic cleavage of the mRNA (Lee et al., 1993; Moss et al., 1997; Pillai et al., 2005; Wightman et al., 1993). It is generally accepted that miRNAs repress translation through one of, or potentially a combination of, two means: i) Inhibition of cap-dependent translation initiation and/or elongation (Humphreys et al., 2005; Mathonnet et al., 2007; Pillai et al., 2005; Thermann and Hentze, 2007; Wakiyama et al., 2007; Wang et al., 2006); (Chendrimada et al., 2007; Wang et al., 2008); (Kim et al., 2004; Maroney et al., 2006; Nelson et al., 2004; Nottrott et al., 2006; Vasudevan et al., 2007); (Petersen et al., 2006); (Nottrott et al., 2006); ii) Deadenylation and consequential destabilization of the target mRNA (Behm-Ansmant et al., 2006a; Eulalio et al., 2007; Eulalio et al., 2008; Eulalio et al., 2009; Fabian et al., 2009; Iwasaki et al., 2009; Takimoto et al., 2009; Till et al., 2007; Wakiyama et al., 2007). In the latter mechanism, the effect of the miRNA on the stability of the mRNA differs from the effect of an siRNA as it requires the activity of a deadenylase complex (Behm-Ansmant et al., 2006b; Eulalio et al., 2009; Fabian et al., 2009). Argonaute proteins and glycine-tryptophan (GW) repeat-containing protein of 182 kDa (GW182) are necessary for miRNA-mediated repression of translation (Eulalio et al., 2009; Eulalio et al., 2009). The proposed mechanisms

of miRNA-mediated post-transcriptional gene silencing are discussed in more detail in section 1.1.3.3.2.

### *1.1.3 Protein components of RNA-mediated silencing*

#### *1.1.3.1 RNase III enzymes*

Drosha and Dicer are double-strand (ds)-specific RNases whose cleavage products are used to provide specificity in RNA-based gene-silencing pathways (Bernstein et al., 2001; Gregory et al., 2004). These modular ~200 kDa enzymes work in conjunction with cognate-binding partners to generate small RNAs and load them onto the ribonucleoprotein complexes that effect post-transcriptional gene-silencing (Chendrimada et al., 2005; Forstemann et al., 2005; Lee et al., 2006; Liu et al., 2003).

##### *1.1.3.1.1 Drosha / DGCR8*

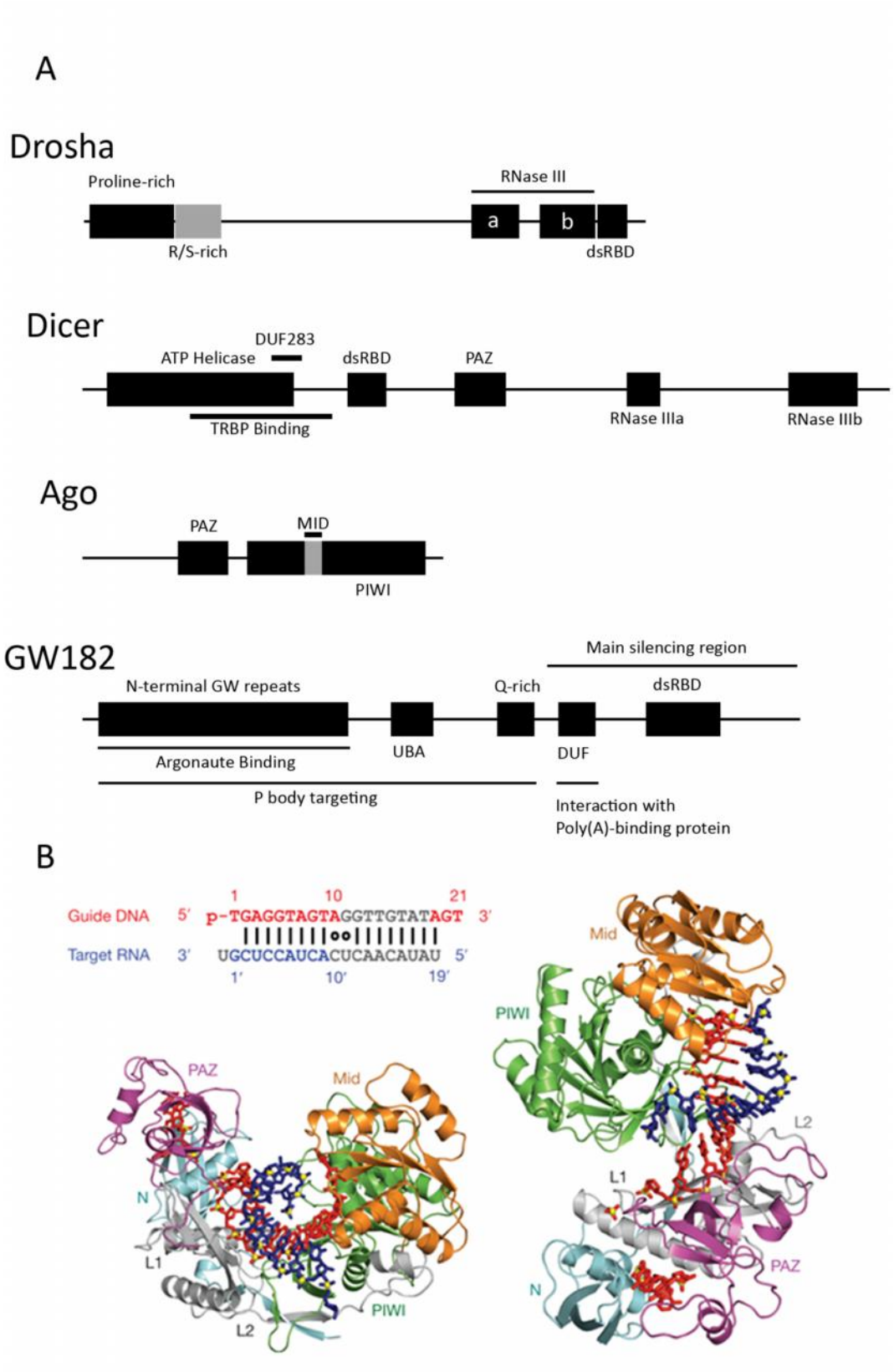
Members of the Drosha family contain two RNase III domains (a and b), a double-stranded RNA binding domain (dsRBD) and a relatively long amino-terminus (Filippov et al., 2000; Wu et al., 2000). The latter segment contains a proline-rich region and an arginine/serine-rich (RS-rich) region, both of which are thought to participate in protein-protein interactions (Fortin et al., 2002; Wu et al., 2000) (Figure 1.2A).

Drosha family members localize predominantly to the nucleus (Wu et al., 2000). In addition to their role in RNAi, Drosha proteins are important for processing pre-ribosomal RNA precursors (Wu et al., 2000). Originally termed human RNase III, inhibiting the expression of this enzyme resulted in cell death, demonstrating that Drosha is an essential enzyme (Wu et al., 2000). Work by the Kim laboratory revealed that Drosha activity is required upstream of Dicer in the microRNA (miRNA) biogenesis pathway (Lee et al., 2003). Specifically, this

enzyme is responsible for cleaving the pri-miRNAs into pre-miRNAs (Lee et al., 2003).

Drosha does not function in isolation *in vivo*, but rather is part of a multiprotein complex that has been termed Microprocessor (Denli et al., 2004). The Microprocessor complex includes the dsRNA-binding protein DGCR8 or Pasha (partner of Drosha) in *Drosophila*. Pasha is important for efficient processing of miRNA precursors as evidenced by the observations that biochemical or genetic depletion of its activity interferes with pri-miRNA processing (Denli et al., 2004; Han et al., 2004; Landthaler et al., 2004).

**Figure 1.2 The basic structure of the core components of the mammalian RNAi pathway. A.** Schematics of Drosha, Dicer, hAgo2 (Ago) and GW182 are shown depicting the locations of described domains as well as regions of interest for their role in the RNAi pathway. **B.** The crystal structure of *Thermus thermophilus* Argonaute is shown bound to a 21 nucleotide guide DNA duplexed with a 20 nucleotide target RNA. From (Wang et al., 2008).


A
Drosha
Proline-rich
RNase III
a
b
R/S-rich
dsRBD
Dicer
DUF283
ATP Helicase
dsRBD
PAZ
TRBP Binding
RNase IIIa
RNase IIIb
Ago
PAZ
MID
PIWI
GW182
Main silencing region
N-terminal GW repeats
Q-rich
dsRBD
Argonaute Binding
UBA
DUF
P body targeting
Interaction with
Poly(A)-binding protein
B
1
10
21
Guide DNA 5′ p-TGAGGTAGTAGGTTGTATAGT 3′
Target RNA 3′ UGCUCCAUCACUCAACAUAU 5′
1′
10′
19′
PAZ
Mid
N
L1
L2
PIWI
Mid
PIWI
L2
L1
PAZ
N

*1.1.3.1.2* Dicer / TRBP

Similar to Drosha, Dicer enzymes contain two RNase III domains and a dsRBD, but lack proline-rich and RS-rich domains. They are the only members of the RNase III superfamily that contain PAZ and ATP-dependent RNA helicase domains. In addition, most Dicer isoforms possess a centrally located domain (DUF283), which has been shown to have the structure of a non-canonical RNA binding fold and, at least in *Arabidopsis thaliana*, is important for protein-protein interactions (Qin et al., 2010) (Figure 1.2A).

Biochemical and candidate gene selection approaches were used to identify Dicer as the bidentate ribonuclease activity that generates 22 bp siRNAs from long dsRNA precursors (Bernstein et al., 2001). Moreover, Dicer functions downstream of Drosha to produce mature miRNAs from pre-miRNAs (Kim et al., 2009).

Dicer is a modular protein, composed of five or more different domains, listed above. The PAZ domain, located in the center of the Dicer molecule, is also present in members of the Argonaute superfamily of proteins (Cerutti et al., 2000). Structural studies have revealed that PAZ domains are oligonucleotide-binding modules (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003). By binding to the 3’ ends of dsRNA molecules, PAZ domains help to position the substrates for cleavage by the RNase III domains. PAZ domains of Dicer contain a large extended loop that is absent from Argonaute PAZ domains (Macrae et al., 2006). This difference between Dicer and Argonaute PAZ domains may be important for differential binding and transferring RNA substrates between these proteins.

The RNase III domains form the catalytic centers of Dicer enzymes and dimerization of these domains is required for RNase activity in all RNase III family members (Dunn, 1976; Robertson et al., 1968). Dimerization results in alignment of the active sites from each RNase III domain thereby creating a catalytic center.

Such an arrangement allows these enzymes to cleave two nearby phosphodiester bonds on opposite strands of the duplex. This type of cleavage gives rise to dsRNA products with 2 nt 3' protruding ends.

Human Dicer interacts with two closely related dsRNA-binding proteins, TRBP and PACT (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006). The roles of these proteins in miRNA biogenesis remains to be determined, as neither of them are absolutely required for processing of pre-miRNAs. Dicer and TRBP (and/or PACT) cooperate in loading the miRNA onto mammalian Argonautes (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006). The role of dsRNA-binding proteins in the miRNA pathway of *Drosophila* is better understood. Loquacious, which contains three dsRNA-binding domains, interacts with Dicer-1, and is required for miRNA biogenesis (Forstemann et al., 2005; Jiang et al., 2005; Saito et al., 2005). Moreover, the *Drosophila* protein R2D2 has a role in determining which strand of an siRNA duplex is loaded into RISC as the guide strand (Tomari et al., 2004). During siRNA biogenesis, R2D2 binds to the more thermodynamically stable end of the RNA duplex, forcing Dicer-2 to bind the opposite end of the dsRNA duplex. Thus, the siRNA is selectively oriented within the Dicer-2/R2D2 heterodimer and one strand of the duplex is preferentially loaded.

*1.1.3.2 GW182*

GW182 was originally identified and characterized as an immunoreactive antigen detected by an autoimmune serum from a human patient suffering from motor and sensory neuropathy (Eystathioy et al., 2002). There are three paralogs of GW182 in humans (TNRC6A, TNRC6B and TNRC6C), as well as a single ortholog found in *Drosophila melanogaster* and two in *Caenorhabditis elegans*. A defining feature of the GW182 family is an amino-terminal domain rich in glycine-trypophan (GW) repeats. These GW repeats have been shown to act as Argonaute-binding determinants (El-Shami et al., 2007; Eulalio et al., 2009;

Eulalio et al., 2009; Lazzaretti et al., 2009; Takimoto et al., 2009; Till et al., 2007), and are accordingly termed 'Argonaute hooks'. The interaction between GW182 and Argonaute proteins is essential for miRNA-mediated gene silencing (Baillat and Shiekhattar, 2009; Behm-Ansmant et al., 2006a; Ding and Han, 2007; Eulalio et al., 2009; Eulalio et al., 2009; Jakymiw et al., 2005; Lazzaretti et al., 2009; Lian et al., 2009; Liu et al., 2005a; Takimoto et al., 2009; Till et al., 2007; Zipprich et al., 2009). Analysis of GW182 mutants has identified sequences within the carboxyl-terminal end as being important in the repression of protein translation, independent of the ability of GW182 to bind to Argonautes (Behm-Ansmant et al., 2006a; Eulalio et al., 2009; Li et al., 2008; Lian et al., 2009) (Figure 1.2A).

Efforts to elucidate the mechanism by which GW182 affects the translation of messages targeted by miRNAs have demonstrated an interaction between GW182 and poly(A)-binding protein I (PABP-I) (Fabian et al., 2009; Zekri et al., 2009). This interaction may interfere with the circularization of the mRNA which is mediated by the interaction between eIF4G and PABP-I. Additionally, miRISC recruits the CCR4-NOT1 deadenylase complex and this requires GW182 (Behm-Ansmant et al., 2006a; Eulalio et al., 2009; Fabian et al., 2009). Despite the considerable progress made in identifying the domains of GW182 that are involved in translational silencing, the mechanism by which translation is inhibited by miRNAs is not well understood.

Components of miRISC including human Argonaute2 (hAgo2), GW182, miRNAs and targeted mRNAs are enriched in cytoplasmic RNA granules known as processing bodies (P bodies). GW182 is a key component of these granules (Jakymiw et al., 2005; Yang et al., 2004) which are also known as GW bodies. The amino-terminal domain of GW182 is critical for targeting it to P bodies (Behm-Ansmant et al., 2006a), which are believed to be involved in the storage or degradation of translationally repressed messages, and the localization of

hAgo2-associated complexes to these RNA granules may serve to enhance miRNA-mediated translational repression.

*1.1.3.3 Argonaute superfamily*

Regulation of protein expression by small RNAs requires the loading of these effector molecules onto an Argonaute protein. Named for the phenotype of an AGO-knockout in *Arabidopsis thaliana* (Bohmert et al., 1998), Argonautes are small RNA-binding proteins that are the core components of RISC and therefore are essential to the RNAi pathway. Argonaute proteins are divided into two subfamilies.

*1.1.3.3.1 Piwi subfamily*

The expression of the Piwi subfamily of Argonaute proteins is mainly restricted to the germline; where they interact with piRNAs (Thomson and Lin, 2009). Four Piwi proteins are encoded by the human genome (HIWI, HIWI2, HIWI3, HILI) and three by the mouse genome (MIWI, MILI, MIWI2). The role of the Piwi subfamily of proteins in humans is largely unknown; however, in mice they are all important for spermatogenesis (Carmell et al., 2007; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004). Consistent with their requirement for spermatogenesis, Piwi proteins silence mobile genetic elements in the germline (Aravin et al., 2007; Carmell et al., 2007). The Piwi proteins of *Drosophila* (Gunawardane et al., 2007; Saito et al., 2006) and *Rattus norvegicus* (Lau et al., 2006) have the capacity to cleave target RNAs *in vitro*, but the role of target cleavage *in vivo* is still undetermined.

Piwi subfamily members are substrates for methylation by the arginine methyltransferase PRMT5 (Kirino et al., 2009). Post-translational methylation of Piwi proteins facilitates their interaction with Tudor-domain-containing proteins (TDRDs) (Reuter et al., 2009; Vagin et al., 2009). The role of TDRDs in Piwi-mediated silencing is unclear; however, there is evidence that TDRD1 is

important for piRNA biogenesis and silencing of repetitive elements (Kojima et al., 2009; Reuter et al., 2009; Vagin et al., 2009).

*1.1.3.3.2 Argonaute subfamily*

Argonaute proteins are conserved throughout the animal, plant and fungi kingdoms, and are expressed in all multicellular and many unicellular organisms. The number of proteins of the Argonaute subfamily varies widely between species, from a single isoform in the fission yeast *Schizosaccharomyces pombe* to 10 in *Arabidopsis thaliana*. There are four members of the Argonaute subfamily in humans (Ago1, Ago2, Ago3 and Ago4). These proteins are expressed ubiquitously and bind to both miRNAs and siRNAs.

Argonaute proteins are characterized by the presence of three domains: a Piwi-Argonaute-Zwille (PAZ) domain, a PIWI domain and a MID domain (Figure 1.2A). The least conserved region of Argonaute proteins is the amino-terminal domain. As mentioned above, PAZ domains are also found in Dicer proteins and form an oligonucleotide-binding pocket which recognizes and preferentially interacts with 2 nt 3' overhangs (Lingel et al., 2003; Lingel et al., 2004; Ma et al., 2004; Song et al., 2003; Yan et al., 2003). These overhangs are characteristic of the cleavage products of RNase III family members (e.g. Drosha, Dicer). The PIWI domain of Argonaute proteins adopts an RNase H-like fold (Parker et al., 2004; Song et al., 2004; Yuan et al., 2005) and is the catalytic center of Argonaute proteins (Liu et al., 2004; Song et al., 2004). The PIWI domain is responsible for the endonucleolytic cleavage of mRNAs targeted by complete base-pairing with siRNAs. Significantly, of the four Argonaute isoforms that are expressed by the human genome, only hAgo2 contains a catalytically active PIWI domain (Liu et al., 2004; Meister et al., 2004). Accordingly, hAgo2 is the only human Argonaute capable of cleaving a targeted mRNA (Liu et al., 2004; Meister et al., 2004). The MID domain adopts a Rossman-like fold, which serves as a binding pocket for the 5' phosphate group of the small RNA (Ma et al., 2005; Parker et al., 2005). The

Patel group has crystallized *Thermus thermophilus* Argonaute in complex with single-stranded guide oligonucleotides and double-stranded duplexes (Wang et al., 2008; Wang et al., 2008; Wang et al., 2009) (Figure 1.2B). This has provided a platform for predictions about the structure of Argonautes of higher organisms; however, no complete structures of mammalian Argonautes have been solved to date.

Argonautes are guided to their target mRNA by miRNAs and siRNAs. As alluded to, evidence suggests that initial target recognition is determined by the seed region (Bartel, 2009; Filipowicz et al., 2008). The mechanism by which an Argonaute protein silences an mRNA is determined by the extent to which the guide small RNA base-pairs with the target. Complete complementarity between the guide and the target promotes endonucleolytic cleavage of the target by hAgo2. Alternatively, base-pairing mismatches in the central region of the duplex leads to inhibition of translation and/or a decrease in mRNA stability (Tolia and Joshua-Tor, 2007; Wang et al., 2008; Wang et al., 2008) (Lee et al., 1993; Moss et al., 1997; Pillai et al., 2005; Wightman et al., 1993). The vast majority of regulation of endogenous mRNAs by miRNA-mediated pathways occurs without hAgo2-mediated endonucleolytic activity; however, the mechanism of silencing is poorly understood and an area of controversy (Fabian et al., 2010).

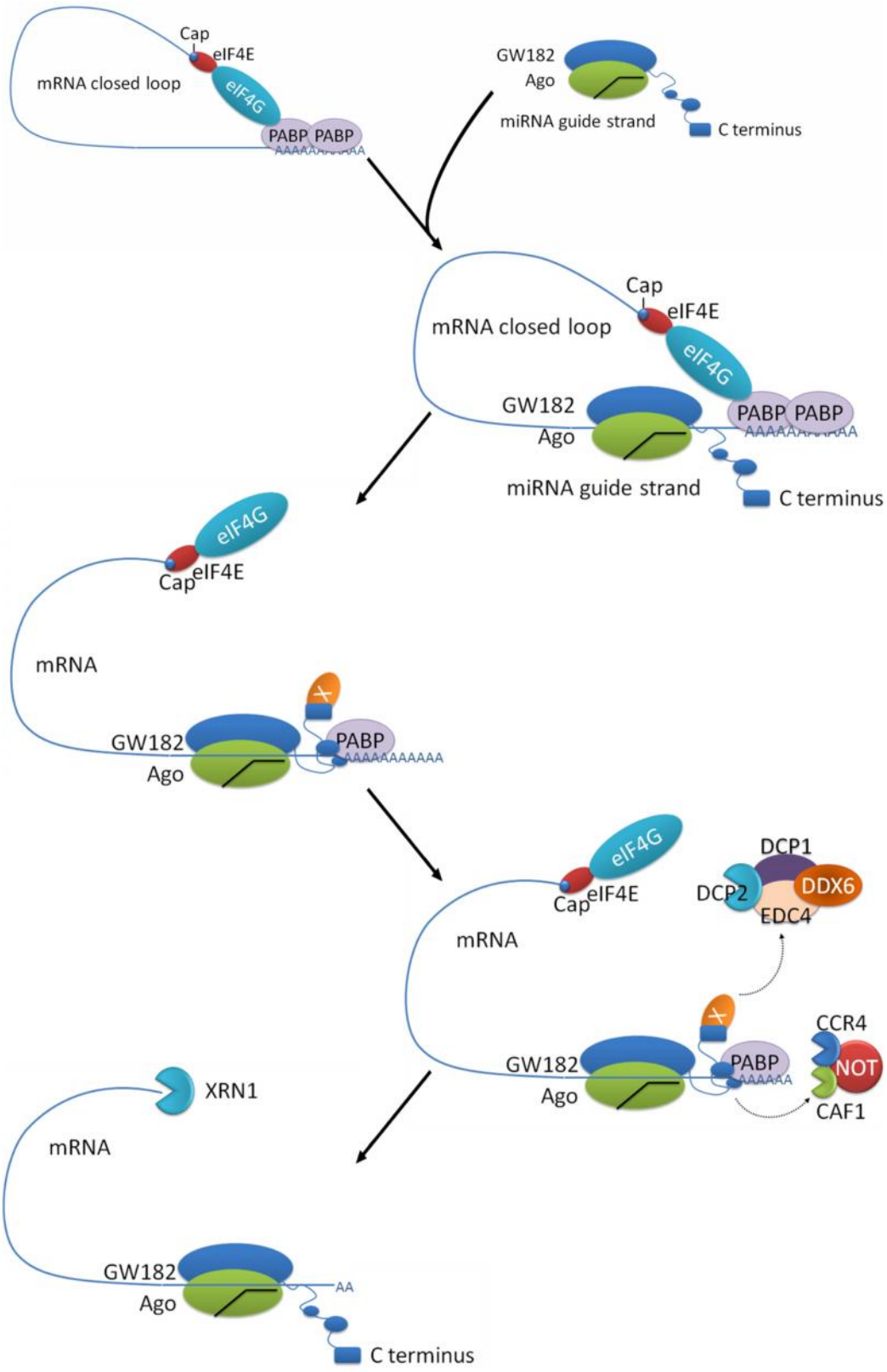
There are two models to explain miRNA-mediated silencing of protein expression. The first model contends that protein expression is decreased by inhibiting translation. The step at which this inhibition occurs is controversial. Proposed mechanisms of inhibition include; suppression of cap recognition by the EIF4E component of eIF4F and the subsequent reduction in 40S ribosomal subunit recruitment (Humphreys et al., 2005; Mathonnet et al., 2007; Pillai et al., 2005; Thermann and Hentze, 2007; Wakiyama et al., 2007; Wang et al., 2006); repression of 60S subunit joining (Chendrimada et al., 2007; Wang et al., 2008); post-initiation effects blocking elongation (Kim et al., 2004; Maroney et al., 2006;

Nelson et al., 2004; Nottrott et al., 2006; Vasudevan et al., 2007); pre-mature termination and ribosome dissociation (Petersen et al., 2006); and promoting the proteolysis of nascent polypeptides (Nottrott et al., 2006). A second model for regulation of translation, which is not necessarily mutually exclusive with the first model, predicts that Argonaute binding promotes deadenylation and destabilization of the target mRNA. This second model is based on evidence that miRISC acts to recruit the CCR4-NOT1 deadenylase complex to the target mRNA (Behm-Ansmant et al., 2006a; Eulalio et al., 2007; Eulalio et al., 2008; Eulalio et al., 2009; Fabian et al., 2009; Iwasaki et al., 2009; Takimoto et al., 2009; Till et al., 2007; Wakiyama et al., 2007). Recently, a parsimonious model of miRISC action was proposed in which repression occurs in two steps (Djuranovic et al., 2011). The authors suggest that, in all cases, translation of the mRNA is initially inhibited. The message is then deadenylated and degraded to consolidate the initial, transient, translational repression. The authors further suggest that the kinetics of the transition from the initial stage of translational inhibition to the degradation of the mRNA could be regulated by a variety of as-of-yet-unidentified factors (Figure 1.3).

Argonaute proteins function in regulating the expression of the majority of mammalian genes and affect a vast array of biological processes; therefore, it is likely that they are extensively regulated. However, comparatively little work has been done to determine how Argonaute proteins are regulated. Stability of hAgo2 is known to be influenced by hydroxylation at proline-700 (Qi et al., 2008). Additionally, there have been a number of phosphorylated residues identified in hAgo2. Phosphorylation of serine-387 was shown to impact the localization of hAgo2 to P bodies, but the functional consequence of this modification is still unknown (Zeng et al., 2008). Phosphorylation of tyrosine-529, located in the MID domain of hAgo2, prevents the binding of small RNAs (Rudel et al., 2011). As modified amino acid residues are typically found on the exposed surface of proteins, it is tempting to speculate that the association of

Argonaute proteins with other protein components of the RNAi system may be altered by post-translational modifications. The direct interaction between Argonautes and the GW182 family of proteins is potentially subject to regulation as the GW182 family plays a critical role in the silencing and degradation of miRNA-targeted mRNAs (Baillat and Shiekhattar, 2009).

**Figure 1.3 Model of miRNA-mediated post-transcriptional gene silencing.** An Argonaute-GW182 complex is targeted to the 3' end of a translating mRNA by the miRNA bound to Argonaute. Interaction between GW182 and PABP-I leads to a loss of circularization and a decrease in translation initiation. Additional interactions involving the carboxy-terminal domains in GW182 recruit the CCR4/NOT1 deadenylase complex. Ultimately, the mRNA is decapped and degraded by the XRN1 5'→3' nuclease. Adapted from (Huntzinger and Izaurralde, 2011)


Cap
eIF4E
mRNA closed loop
eIF4G
PABP PABP
AAAAAAAAAAA
GW182
Ago
miRNA guide strand
C terminus
Cap
eIF4E
mRNA closed loop
eIF4G
GW182
Ago
PABP PABP
AAAAAAAAAAA
miRNA guide strand
C terminus
eIF4G
Cap
eIF4E
mRNA
GW182
Ago
X
PABP
AAAAAAAAAA
eIF4G
Cap
eIF4E
mRNA
DCP1
DCP2
DDX6
EDC4
GW182
Ago
X
PABP
AAAAAA
CCR4
NOT
CAF1
XRN1
mRNA
GW182
Ago
AA
C terminus

## 1.2 Cytoplasmic RNA Granules & the RNAi machinery

There are a variety of mechanisms for controlling gene expression, post-transcripition, which include: modulating the initiation of translation, the stability of mRNA and altered subcellular localization of mRNAs. In eukaryotic cells, a variety of RNA granules exist with which non-translating mRNAs can associate (Bashkirov et al., 1997; Knowles et al., 1996; Nover et al., 1989; Schisa et al., 2001). The granules include P bodies, germline granules, neuronal transport granules and stress granules. Of these, P bodies and stress granules are the only granules found in the non-neuronal soma. These granules contain proteins that function in pathways involved in mRNA decay (Ingelfinger et al., 2002; van Dijk et al., 2002) and/or storage (Gallouzi et al., 2000; Stoecklin et al., 2006; Thomas et al., 2005) and are thought to consolidate translationally-repressed mRNAs (Kedersha et al., 2002). Significantly, both P bodies and stress granules contain hAgo2 (Leung et al., 2006; Liu et al., 2005b) and have therefore been implicated in small RNA-mediated post-transcriptional gene silencing pathways.

### *1.2.1 Processing bodies*

The 5'-to-3' exonuclease XRN1 was the first enzyme associated with mRNA decay reported to localize to discrete cytoplasmic foci (Bashkirov et al., 1997) which later became known as P bodies. Dcp2, Dcp1, Lsm1-7, CCR4 and other enzymes involved in the degradation of mRNA also localize to these granules (Andrei et al., 2005; Cougot et al., 2004; Sheth and Parker, 2003; Stoecklin et al., 2006; Wilczynska et al., 2005), firmly establishing a connection between mRNA degradation and these cytoplasmic granules. P bodies are highly mobile structures that move along microtubules (Aizer et al., 2008; Carmichael et al., 2006), an observation that led to speculation that bi-directional movement may be a mechanism for scanning the cytoplasm for mRNAs targeted for degradation or silencing. While P bodies are constitutively present in the

cytoplasm of most eukaryotic cells (Behm-Ansmant et al., 2006a; Ingelfinger et al., 2002; Lykke-Andersen, 2002; Sheth and Parker, 2003; Squirrell et al., 2006; van Dijk et al., 2002)(Buchan and Parker, 2009), their size, number and composition varies based on cell type and stages of the cell cycle (Moser et al., 2007; Yang et al., 2004). Considerable efforts are being made to better understand the biogenesis of P bodies. A recent publication has identified no less than 39 genes involved in the formation of P bodies in mammalian cells (Ohn et al., 2008). However, the mechanisms governing P body biogenesis are still poorly understood.

A link between P bodies and the RNAi pathway was revealed when it was shown that both hAgo2 and GW182 are critical components for P body formation in mammalian cells (Chu and Rana, 2006; Eulalio et al., 2007a; Franks and Lykke-Andersen, 2008). P bodies form as a consequence of RNAi-mediated silencing (Eulalio et al., 2007b); however, RNAi-mediated silencing of an mRNA can occur in the absence of microscopically visible P bodies (Eulalio et al., 2007b). Consequently, the role of P bodies in RNAi-mediated silencing is still a matter of controversy. Nevertheless, their persistence throughout evolution suggests that they serve an important role in RNA metabolism. Perhaps P bodies increase the efficiency of the processes of RNAi and mRNA degradation by concentrating the components of these pathways. Post-translational modifications which regulate the association of hAgo2 with P bodies have been reported (Zeng et al., 2008). It may well be that regulating the association of human Argonaute proteins with cytoplasmic P bodies serves as a mechanism for increasing the efficiency of miRNA-mediated silencing.

### *1.2.2 Stress granules*

Originally identified as concentrations of a specific subset of cellular mRNAs in the cytoplasm of heat-stressed *Lysopersicon peruvianum* (tomato) cells (Nover et al., 1989), stress granules are now known to form as a protective

measure countering temperature, translational, oxidative and metabolic stresses (Barber, 2005; Kaufman, 2004; Lu et al., 2001; Wek et al., 2006). It is believed that stress granules serve to enhance the translational inhibition of mRNAs encoding expendable proteins allowing the cell to conserve energy during stress. Consistent with this hypothesis, mRNAs that encode stress-response factors, such as heat shock proteins, are selectively excluded from stress granules (Kedersha and Anderson, 2002; Stohr et al., 2006). The exact mechanism by which this occurs is not understood.

Stress granule formation, a process which involves over 100 human genes (Ohn et al., 2008), is triggered by the stress-induced phosphorylation of eIF2α (Kedersha et al., 2005). eIF2α is a component of the eIF2 complex, which loads the initiator tRNA onto the 40S ribosomal subunit (Dever, 2002; Kimball, 2001). Phosphorylation of eIF2α inhibits initiation of protein synthesis (Krishnamoorthy et al., 2001). The second stage of stress granule assembly relies on domains which mediate protein-protein interactions (Gilks et al., 2004; Kedersha et al., 2000; Kedersha et al., 1999; Mazroui et al., 2007; Rikhvanov et al., 2007; Tourriere et al., 2003)(Buchan and Parker, 2009). Such domains are found in numerous RNA-binding proteins known to be components of stress granules. For example, TIA-1 and TIA-R are RNA-binding proteins that contain glutamine and asparagine-rich prion-like domains. These domains have the ability to self-aggregate and can therefore promote stress granule formation (Gilks et al., 2004; Kedersha et al., 2000; Kedersha et al., 1999). Stress granule formation is also dependent on an intact microtubule network. Microtubule depolymerizing drugs such as nocodazole have been shown to impair the formation of these structures (Fujimura et al., 2009; Ivanov et al., 2003; Kolobova et al., 2009; Kwon et al., 2007; Loschi et al., 2009); however, the role of microtubules is unclear.

While the composition of stress granules, and the mechanism by which they are assembled, have been the focus of intense investigation, little is known

about their function. It has been hypothesized that stress granules form as a means of repressing the translation of and/or stabilizing and storing mRNAs; however, neither the global repression of translation nor the stabilization of mRNAs explicitly requires the formation of stress granules (Buchan et al., 2008; Fujimura et al., 2009; Hilgers et al., 2006; Kwon et al., 2007; Loschi et al., 2009; Mokas et al., 2009; Ohn et al., 2008). While not strictly required for translational repression, it remains an intriguing possibility that stress granules serve to reduce translation of non-essential mRNAs to allow for optimal translation of mRNAs involved in the stress response. Emerging evidence has demonstrated that inhibition of stress granule formation leads to reduced cell survival, which suggests that these granules may play an anti-apoptotic role during stress conditions (Baguet et al., 2007; Eisinger-Mathason et al., 2008; Kwon et al., 2007). A role for stress granules in preventing or delaying the onset of apoptosis has led to speculation that they do so by sequestering pro-apoptotic factors. In the context of the stress granule, sequestration of pro-apototic factors could serve to link cell fate decisions to mRNA regulation. Certainly, a better understanding of the roles of stress granule components within the granule during the stress response will aid in elucidating the function of these highly dynamic structures.

While it has been consistently demonstrated that hAgo2 associates with stress granules (Detzer et al., 2011; Leung et al., 2006), there is little knowledge regarding the mechanism or functional consequence of this association. Existing evidence indicates only that the localization of hAgo2 to stress granules is dependent on the presence of mature miRNAs (Leung et al., 2006) and that the accumulation of hAgo2 in stress granules results in a decrease in miRNA- and siRNA-mediated post-transcriptional gene silencing (Detzer et al., 2011). As a more complete understanding of the factors that control the association of hAgo2 with both P- bodies and stress granules remains elusive, it is unclear whether the pool of hAgo2 associated with P bodies is biochemically identical to

that which is capable of being recruited to stress granules. Given the difference in the composition of P bodies and stress granules, it is tempting to speculate that hAgo2 localized to P bodies is associated with mRNAs that are in a late stage of miRNA-mediated silencing and in the process of consolidating translational repression via deadenylation and mRNA decay. Meanwhile, the hAgo2 recruited to nascent stress granules would be predicted to be associated with mRNAs which are at early stages of translational repression. Nevertheless, considering the paucity of knowledge regarding the cause and effect of hAgo2 association with stress granules, further investigation into the mechanisms regulating this aspect of Argonaute function is warranted.

### 1.3 Interaction between RISC components and the Hsp90 chaperone system

The Hobman laboratory reported that hAgo2 interacts with a protein complex containing the chaperone Hsp90 (Tahbaz et al., 2001). These results were confirmed by the Hannon laboratory, whose results further suggested that this interaction was not dependent upon additional proteins (Liu et al., 2004). Chaperones may mediate crucial protein-protein and protein-RNA interactions among components of the RNAi pathway and may therefore be important in its regulation. Indeed, inhibition of Hsp90 ATPase activity significantly reduces the association of tagged hAgo2 with a co-expressed, tagged form of human Dicer (Tahbaz et al., 2001). These findings suggest that the function of hAgo2 in the miRNA pathway may be dependent upon the activity of the Hsp90 chaperone complex.

## 1.4 The Hsp90 Chaperone System

### *1.4.1 Overview*

All organisms employ molecular chaperones to help newly synthesized proteins adopt their native conformations, as well as assemble into and disassemble from macromolecular complexes. These factors comprise a very large and structurally diverse group, but many of them are upregulated in response to heat stress and are therefore termed the heat shock proteins (HSPs). One of the most conserved HSPs, present from bacteria to humans, is Hsp90 which is highly abundant, even in unstressed cells. It is essential and, in eukaryotic cells, is found in nearly every subcellular compartment (Borkovich et al., 1989). In fact, most eukaryotic genomes encode several compartment-specific Hsp90s. Not surprisingly, due to its ubiquity and abundance, Hsp90 interacts with a wide array of 'client' proteins. In general, the clients of Hsp90 depend on the chaperone's activity to acquire their active conformation (Taipale et al., 2010).

### *1.4.2 Structure*

Each Hsp90 monomer consists of three distinct domains: amino-terminal, middle and carboxyl-terminal. Hsp90 functions as a dimer and the carboxyl-terminal domain mediates dimerization. This domain also contains a highly conserved Met-Glu-Glu-Val-Asp (MEEVD) motif that mediates interaction with many of the co-chaperones of Hsp90 (Young et al., 1998). The middle domain contains motifs that play a critical role in client recognition. The amino-terminal domain of Hsp90 shares structural homology with other members of the gyrase, Hsp90, histidine kinase and MutL (GHKL) ATPase superfamily (Bergerat et al., 1997) (Figure 1.4A). Hsp90 binds ATP via an α- and β-sandwich motif in its amino-terminal domain (Prodromou et al., 1997; Stebbins et al., 1997). The interaction of this domain with a catalytic loop in the middle domain, which contains a catalytic arginine residue, leads to ATP hydrolysis (Meyer et al., 2004;

Sato et al., 2000). The binding and hydrolysis of ATP provides the energy required by Hsp90 to elicit conformational changes in its client proteins. This maturation of clients occurs through a process known as the Hsp90 chaperone cycle (Figure 1.4B) (Chadli et al., 2000; Csermely et al., 1993; Grenert et al., 1997; Maruya et al., 1999; Prodromou et al., 2000; Sullivan et al., 1997). Significantly, the binding of ATP to Hsp90 can be inhibited with high specificity by ATP analogues (e.g. geldanamycin, 17-AAG, radicicol) (Chavany et al., 1996; Schulte et al., 1995; Schulte et al., 1998; Smith et al., 1995; Whitesell et al., 1994).

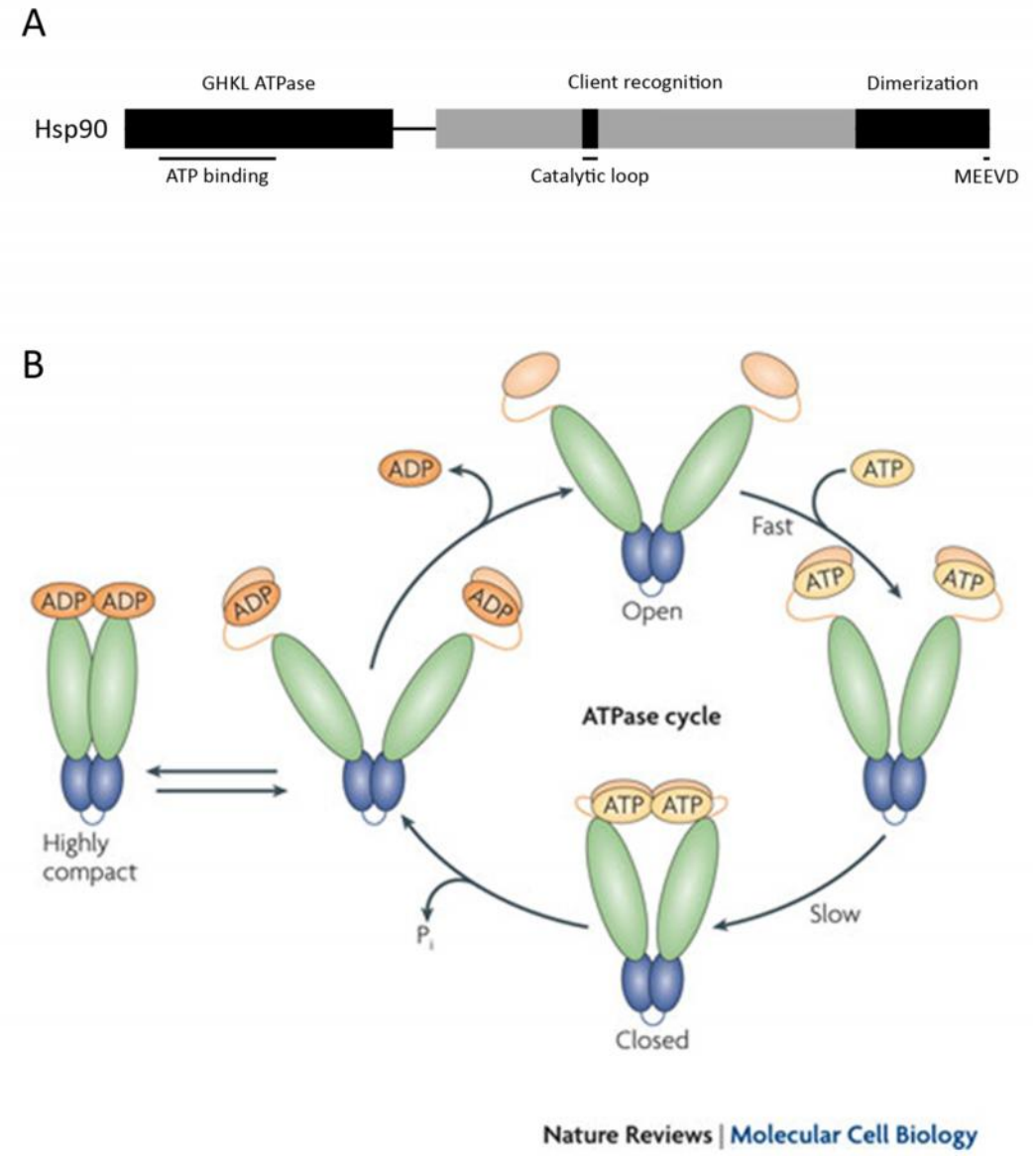



**Figure 1.4 Hsp90 domain structure and chaperone cycle. A.** The three domains of Hsp90 are the GHKL ATPase domain in the amino-terminal end, a middle domain required for client recognition and a carboxy-terminal domain important in dimerization of the chaperone. Regions important for ATP binding and catalyzing ATP hydrolysis and the MEEVD sequence required for interaction with the co-chaperones are also noted. **B.** Schematic of the proposed chaperone cycle of Hsp90. Binding of ATP results in Hsp90 adopting a more 'closed' conformation, which is predicted to modify the structure of the bound client (not shown). ATP hydrolysis and subsequent dissociation of ADP restores Hsp90 to its 'open' conformation, releasing the properly folded client and allowing additional client proteins to bind. Adapted from (Taipale et al., 2010).

### *1.4.3 Clients of Hsp90*

Hsp90 interacts with a large number of client proteins. These clients are involved in diverse cellular functions including signal transduction, trafficking, receptor activation and the innate and adaptive immune responses (Brugge et al., 1981; Garcia-Cardena et al., 1998; Holt et al., 1999; Joab et al., 1984; Lipsich et al., 1982; Minet et al., 1999; Sanchez et al., 1985; Sato et al., 2003; Schuh et al., 1985; Sepehrnia et al., 1996; Tariq et al., 2009). Studies in yeast have demonstrated either direct or genetic interactions between Hsp90 and 1,232 unique proteins, or roughly 20% of the proteins in the yeast genome (Breitkreutz et al., 2008). Despite the abundance of Hsp90 client proteins, very little is known about the molecular basis of client recognition. Common sequences or structural motifs have not been identified and their existence seems unlikely given the diversity of proteins that rely on Hsp90 chaperone activity. More likely, co-chaperones of Hsp90 have an important role in the process of client recognition.

### *1.4.4 Roles of Hsp90 co-chaperones*

While more than 20 co-chaperones have been identified in eukaryotic cells, their individual biological functions remain largely a mystery. In general, co-chaperones modulate the biochemical activity of Hsp90 by: coordinating the interaction between Hsp90 and other chaperone systems; stimulating or (more typically) inhibiting the ATPase activity of Hsp90; and participating in the recognition of specific Hsp90 client proteins.

The largest group of Hsp90 co-chaperones contain characteristic tetratricopeptide repeat (TPR) domains which bind to the MEEVD motif of Hsp90 (Carrello et al., 1999; Chen et al., 1998; Owens-Grillo et al., 1996a; Owens-Grillo et al., 1996b; Radanyi et al., 1994; Ratajczak and Carrello, 1996; Young et al., 1998). This class of co-chaperones, in addition to a variety of other functions, is involved in the cooperative and successive action of Hsp40, Hsp70 and Hsp90. The best characterized member of this family is HOP (Hsc70 and Hsp90-

organizing protein). HOP contains multiple TPR domains that can simultaneously bind Hsp90 and Hsp70 and coordinate the transfer of a client protein between them (Taipale et al., 2010). A subfamily of the TPR domain-containing group of co-chaperones, FK506-binding proteins (FKBPs), are important positive regulators of androgen, glucocorticoid and progesterone receptor signaling pathways (Dolinski et al., 1997; Smith et al., 1993).

The Cdc37 co-chaperone links Hsp90 and its kinase clients and is also known to inhibit the ATPase activity of Hsp90. The interaction of Cdc37 with the amino-terminal domain of Hsp90 blocks ATPase activation by inhibiting the interaction of the catalytic arginine residue from the middle domain with the ATP-binding site. Cdc37 also blocks the ATP-binding pocket and holds it in an inactive conformation. This conformation of Hsp90 prevents interaction of the amino-terminal domains across the dimer, an interaction known to be important for activating Hsp90 ATPase activity (Roiniotis et al., 2005; Siligardi et al., 2002).

p23 is a co-chaperone that associates with Hsp90 late in the chaperone cycle, following ATP binding and interaction of the ATP binding site with the catalytic residue of the middle domain (Ali et al., 2006; Freeman et al., 2000; Young and Hartl, 2000). This conformation is very transient; however, p23-mediated stabilization of this conformation of Hsp90 is predicted to facilitate client maturation (McLaughlin et al., 2006).

One of the few co-chaperones known to activate the ATPase activity of Hsp90, Aha1, binds to the middle domain and facilitates the interaction of the amino-terminal ATP-binding domain with the catalytic arginine residue found in the middle domain of Hsp90 (Meyer et al., 2004). The binding of Aha1 promotes a closed conformation of Hsp90 in which the amino-terminal domains from each monomer stably associate. Stabilizing the interaction of the amino-terminal domains of the Hsp90 monomers, across the dimer, promotes efficient ATP hydrolysis (Retzlaff et al., 2010).

The range of effects that the co-chaperones have on the ATPase activity of Hsp90 likely reflects the different stages of the chaperone cycle at which they function. Generally speaking, co-chaperones that inhibit ATPase activity are more likely to be involved in client loading and/or the formation of mature Hsp90 complexes. Alternatively, co-chaperones that enhance the ATPase activity of Hsp90 are most likely involved in the stages of the chaperone cycle which elicit a conformational change in, and the subsequent release of, the mature client protein.

### *1.4.5 Maturation of client proteins*

Despite a central role in many cellular processes, many aspects of Hsp90 structure, regulation and function remain unknown. The role of Hsp90 in the maturation of kinases and steroid hormone receptors provides a great deal of insight into the molecular function of Hsp90 (Grad and Picard, 2007). The glucocorticoid receptor (GR) is a transcription factor that belongs to the superfamily of nuclear receptors. Following the binding of glucocorticoids, GRs regulate the transcription of target genes by binding specific sites in their promoters or enhancers (Mangelsdorf et al., 1995). Nascent GR is folded into a conformation with low affinity for hormone by the concerted action of Hsp40 and Hsp70 as well as several co-factors of these chaperones (Figure 1.5) (Nemoto et al., 1990). The co-chaperone HOP mediates the transfer of GR from Hsp70 to Hsp90 (Chen and Smith, 1998), and the ATP-bound Hsp90-GR complex is subsequently stabilized by p23 (Dittmar et al., 1997; Morishima et al., 2003). Hsp90 regulates the final maturation of GR and, in doing so, increases the affinity of GR for hormone by 100-fold (Nemoto et al., 1990). Structurally, the ‘open’ conformation of the hormone-binding cleft of GR is stabilized by the chaperone activity of Hsp90, allowing the receptor to bind to the hormone and acquire its active state (Figure 1.5). Stabilization of transitional, conformationally unfavorable structural intermediates to facilitate more stable, conformationally

active states is generally accepted as a major function of the Hsp90 chaperone complex in the maturation of client proteins (Grad and Picard, 2007).

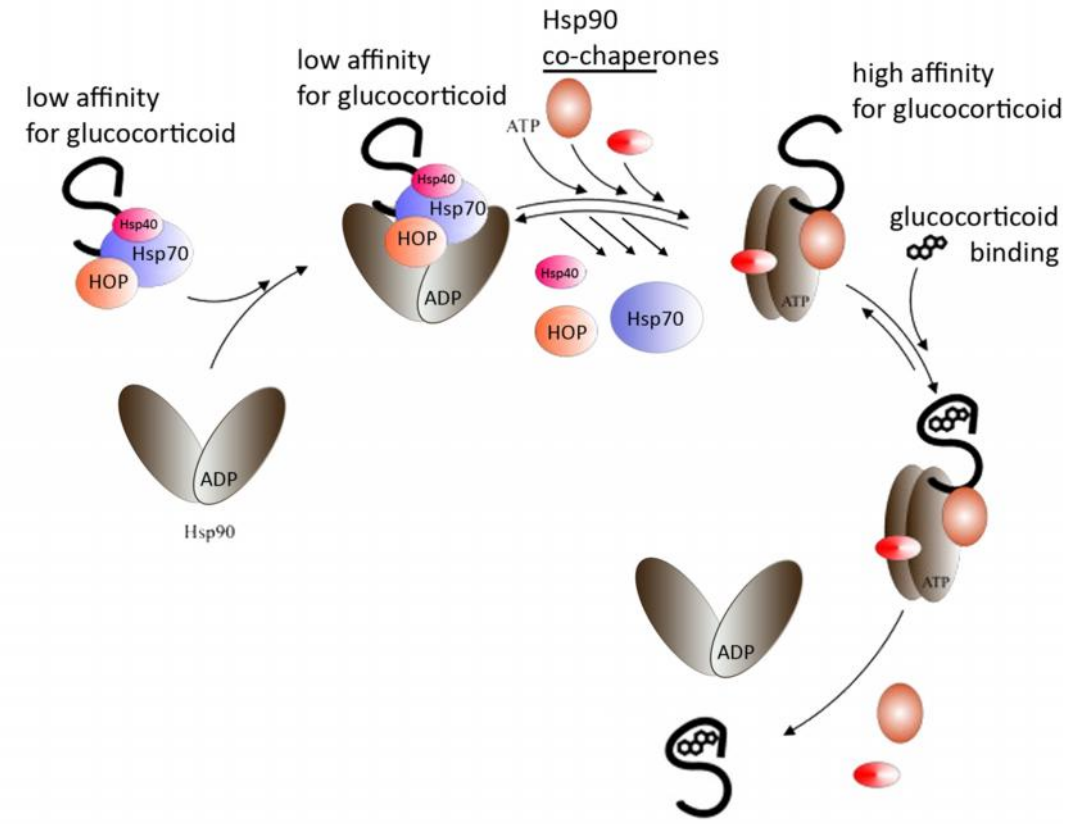



**Figure 1.5 Schematic representation of the maturation of an Hsp90 client.** Transfer of a glucocorticoid receptor (GR) from a complex containing Hsp40/Hsp70 is mediated by HOP. Hsp90 binds to ATP and its co-chaperones resulting in a conformational change in the structure of the bound client. The conformationally altered client has an increased affinity for its glucocorticoid and this high affinity state is stabilized by continued association with Hsp90. Following binding of the receptor to the glucocorticoid, ATP is hydrolyzed and the chaperone complex dissociates, releasing the GR. Adapted from (Grad and Picard, 2007).

## 1.5 Objectives

The RNAi pathway modulates the expression of greater than half of the proteins encoded by mammalian genomes (Friedman et al., 2009). Accordingly, the protein components of this system are very likely to be subject to extensive regulation. The objective of my thesis was to characterize the nature of the interaction between the molecular chaperone Hsp90 and hAgo2, the core component of the RNAi pathway in humans. The results detailed in chapter 3 demonstrate a clear requirement for the activity of the Hsp90 chaperone in the localization and function of hAgo2. Based on the results obtained in the first study, I hypothesized that Hsp90 activity is required to facilitate interaction between Argonaute, loaded with a miRNA, and the targeted messenger RNA. In chapter 4 of this thesis I present unpublished data suggesting that the interaction of RISC with a target RNA depends on Hsp90 activity. Lastly, based the role of co-chaperones in client recognition and their requirement for client maturation, I predicted specific co-chaperones would be required for the activation of Argonaute. The Hsp90 co-chaperones that co-localize with hAgo2 at stress granules served as a list of candidates for further investigation. In chapter 5, I demonstrate that knockdown of the Hsp90 co-chaperones Aha1 and Cdc37 has a significant effect on the efficiency of RNA-mediated post-transcriptional gene silencing.

# CHAPTER 2

Materials & Methods

## 2.1 Materials

### *2.1.1 Reagents*

The following reagents were used following the manufacturers' recommendations unless otherwise stated.

**Table 2.1** Commercial sources of materials, chemicals and reagents

| ***Reagent*** | ***Source*** |
|---|---|
| 40% acrylamide /bis-acrylamide solution (29:1) | Bio-Rad |
| Agar | Sigma-Aldrich |
| Agarose, ultrapure, electrophoresis grade | Invitrogen |
| Ammonium acetate | Invitrogen |
| Ammonium persulphate | Sigma-Aldrich |
| Ampicillin | Sigma-Aldrich |
| Bacto-tryptone | Becton, Dickinson & Company |
| Bacto-yeast extract | Becton, Dickinson & Company |
| Blasticidin | Invitrogen |
| Bovine serum albumin (BSA) | Sigma-Aldrich |
| 1-bromo 3-chloropropane | Sigma-Aldrich |
| Bromophenol blue | Sigma-Aldrich |
| $CO_2$-independent cell culture medium | Invitrogen |
| Casein, enzymatic hydrolysate | Sigma-Aldrich |
| Chloroform | Thermo Fisher Scientific |
| Complete™ EDTA-free protease inhibitor | Roche |
| Diatomaceous earth | Sigma-Aldrich |
| Dimethyl sulphoxide (DMSO) | Sigma-Aldrich |
| Dimethylpimelimidate | Thermo Fisher Scientific |
| Disodium hydrogen orthophosphate | Becton, Dickinson & Company |
| Dithiothreitol (DTT) | Sigma-Aldrich |
| Doxycycline | Sigma-Aldrich |
| Dulbecco's modified Eagle's medium (DMEM) High Glucose | Invitrogen |
| Dynabeads M-280 Streptavidin | Invitrogen |
| Ethanolamine | Thermo Fisher Scientific |
| Ethidium bromide solution | Sigma-Aldrich |
| Ethyl Alcohol | Commercial Alcohols |
| Ethylenediaminetetraacetic acid (EDTA) | EMD Chemicals |
| Fetal bovine serum | Invitrogen |
| Geldanamycin | LC Laboratories |
| Glycerol | Thermo Fisher Scientific |
| Glycine | Calbiochem |
| Guanidine HCl | Thermo Fisher Scientific |
| Hippuristanol | J. Pelletier (McGill University) |
| 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) | Thermo Fisher Scientific |
| Hydrochloric Acid | Thermo Fisher Scientific |

**Table 2.1** (Continued)

| ***Reagent*** | ***Source*** |
|---|---|
| Kanamycin | Sigma-Aldrich |
| Isopropanol | Commercial Alcohols |
| Isopropanol, molecular biology grade | Sigma-Aldrich |
| Lipofectamine 2000 | Invitrogen |
| Magnesium chloride hexahydrate | EMD Chemicals |
| Methanol | Thermo Fisher Scientific |
| 2-Mercaptoethanol | Thermo Fisher Scientific |
| N,N,N',N'-tetramethylenediamine (TEMED) | Sigma-Aldrich |
| Nonidet P-40 (NP-40) / Igepal CA-630 | Sigma-Aldrich |
| OptiMEM | Invitrogen |
| Paraformaldehyde | Thermo Fisher Scientific |
| Penicillin-streptomycin solution (100x) | Invitrogen |
| Phenol, buffer-saturated | Sigma-Aldrich |
| Phenol: Chloroform: Isoamyl Alcohol | Sigma-Aldrich |
| Potassium acetate | Anachemia |
| Potassium chloride | Becton, Dickinson & Company |
| Potassium hydroxide | Becton, Dickinson & Company |
| ProLong Gold with DAPI | Invitrogen |
| Protein G - sepharose Fast Flow | General Electric Healthcare |
| Puromycin | Sigma-Aldrich |
| Skim milk powder | Carnation |
| Sodium arsenite | Sigma-Aldrich |
| Sodium chloride | Thermo Fisher Scientific |
| Sodium dodecyl sulphate (SDS) | Bio-Rad |
| Sodium hydroxide | Becton, Dickinson & Company |
| Sodium molybdate | Acros Organics |
| Sodium salicylate | EM Science |
| Sucrose | EMD Chemicals |
| TRI Reagent / TRIZol | Invitrogen |
| TRIzol | Invitrogen |
| Tris base | EMD Chemicals |
| Triton X-100 | VWR International |
| 0.25% Trypsin-EDTA | Invitrogen |
| Tween-20 (polyoxyethylenesorbitan monolaureate) | Thermo Fisher Scientific |
| UltraPure distilled water | Invitrogen |
| Xylene cyanol FF | Sigma-Aldrich |
| Zeocin | Invitrogen |

**Table 2.2** Molecular size standards

| ***Standard*** | ***Source*** |
|---|---|
| GeneRuler 1 kb DNA Ladder Plus (SM1333) | Fermentas |
| PageRuler Pre-stained Protein Ladder (SM0671) | Fermentas |

**Table 2.3** DNA/RNA modifying enzymes, buffers

| *Enzyme* | *Source* |
|---|---|
| Calf intestinal alkaline phosphatase | Invitrogen |
| DNase I, amplification grade | Invitrogen |
| Restriction endonucleases | Invitrogen<br>New England Biolabs |
| RNase A | Invitrogen |
| T4 DNA ligase | Invitrogen |

**Table 2.4** Detection systems

| *System* | *Source* |
|---|---|
| Axiovert 200M with | Zeiss |
| UltraView ERS spinning disk confocal unit | Perkin Elmer Bioscience |
| Hamamatsu C9100-050-EM-CCD digital camera | |
| Volocity acquisition software | |
| FluorChem Q | Cell BioSciences |
| FluoroChem FC | Alpha Innotech Corp. |
| LSCM510 | Zeiss |
| MX3005P | Stratagene |
| Nitrocellulose membrane, Trans-Blot Transfer Medium | Bio-Rad |
| Odyssey Infrared Imaging System | LiCor |
| Polyvinyldene difluoride (PVDF) membrane (0.45 µM) | Millipore |
| Rx film | Fuji |
| SuperSignal WestPico chemiluminescent substrate | Thermo Scientific |
| Ultraviolet gel transilluminator | Thermo Fisher Scientific |
| XO-Mat Developer | Kodak |

**Table 2.5** Multi-component systems

| *System* | *Source* |
|---|---|
| Expand Long Template PCR System | Roche |
| PerfeCTa SYBR Green SuperMix, UNG, Low Rox | Quanta Biosciences |
| Platinum Taq PCR System | Invitrogen |
| QIAEX II gel extraction kit | QIAGEN |
| QIAGEN plasmid maxi kit | QIAGEN |
| QIAprep spin miniprep kit | QIAGEN |
| QIAquick PCR purification kit | QIAGEN |
| qScript Flex cDNA kit | Quanta Biosciences |

### *2.1.2 Commonly used buffers*

The following buffers were used throughout this work and their composition is detailed below in Table 2.6.

**Table 2.6** Buffers and solutions

| ***Name*** | ***Composition*** |
|---|---|
| 5x protein sample buffer | 62.5 mM Tris-HCl (pH 6.8), 25% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, 5% (v/v) β-mercaptoethanol |
| 6x DNA gel loading buffer | 40% (w/v) sucrose, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF |
| Alkaline lysis buffer | 200 mM NaOH, 1% (w/v) SDS |
| Bacteria resuspension buffer | 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 µg/mL RNase A |
| Diatomaceous earth | 100 mg/mL diatomaceous earth, 6 M guanidine-HCl, 20 mM EDTA, 50 mM Tris -HCl (pH 8.0) |
| Diatomaceous earth wash buffer | 50% (v/v) isopropanol, 200 mM NaCl, 5mM EDTA, 20 mM Tris -HCl (pH7.5) |
| Hsp90 IP buffer | 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM MgOAc, 1% TritonX-100, 20 mM sodium molybdate |
| LB growth media | 1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 0.5% (w/v) NaCl, 0.1% (v/v) 1 M NaOH |
| Neutralization buffer | 3.0 M Potassium acetate (pH 5.5) |
| NP40 lysis buffer | 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% (v/v) NP-40 |
| Oligo binding buffer | 25 mM HEPES-KOH (pH 7.4), 50 mM KCl, 75 mM KOAc, 2 mM MgCl2, 1 mM DTT |
| Phosphate-buffered saline (PBS) | 137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4 (pH 7.4) |
| PBS-T | 137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4 (pH 7.4), 0.05% (v/v) Tween-20 |
| RISC wash buffer | 25 mM HEPES-KOH (pH 7.4), 50 mM KCl, 1.5 mM MgCl2, 0.5% (v/v) NP40 |
| SDS-PAGE running buffer | 250 mM glycine, 0.1% (w/v) SDS, 100 mM Tris base (pH 8.3) |
| Swelling buffer | 25 mM HEPES-KOH (pH 7.4), 50 mM KCl, 1.5 mM MgCl2, 1 mM DTT |
| TAE | 40 mM Tris-Acetate, 1 mM EDTA (pH 8.0) |
| Tris-buffered saline (TBS) | 137 mM NaCl, 2.7 mM KCl, 24 mM Tris-HCl (pH 7.4) |
| TBS-T | 137 mM NaCl, 2.7 mM KCl, 24 mM Tris-HCl (pH 7.4), 0.05% (v/v) Tween-20 |
| Western blot transfer buffer | 200 mM glycine, 25 mM Tris base (pH 8.3), 20% (v/v) methanol, 0.1% (w/v) SDS |

### *2.1.3 Oligonucleotides*

**Table 2.7** Oligonucleotides

| ***Name*** | ***Engineered sites*** | ***Sequence*** | ***Usage (Section)*** |
|---|---|---|---|
| eGFP-hAgo2-AflII-F | *Afl* II | 5'-TACTCTTAAGTCGCCACCATGGTGAGCAAGG | 2.2.2.1 |
| eGFP-hAgo2-XbaI-R | *Xba* I | 5'-ACTTCTAGATTAAGCAAAGTACATGGTGCGC | 2.2.2.1 |
| TRBP-XhoI-F | *Xho* I | 5'-GCTCGAGCCATGAGTGAAGAG | 2.2.2.3 |
| TRBP-BamHI-R | *BamH* I | 5'-GGGATCCCTTGCTGCCTGCCATG | 2.2.2.3 |
| hDcr1-XhoI-F | *Xho* I | 5'-GCCTCGAGGCATGAAAAGCCCTGCTTTGCA | 2.2.2.2 |
| hDcr1-HindIII-R | *Hind* III | 5'-GCAAGCTTGCTATTGGGAACCTGAGGTT | 2.2.2.2 |
| BoxB-MfeI-F | *Mfe* I | 5'-GTTGTTCAATTGTAAGTCCAACTACTAAACTGGGGATTC | 2.2.2.5 |
| BoxB-HpaI-R | *Hpa* I | 5'-CAACAAGTTAACGGCCGCCTCGAGATAATATC | 2.2.2.5 |
| let-7c RT primer | | Proprietary (Ambion, AM30002) | 2.2.7.2 |
| let-7c PCR primer set | | Proprietary (Ambion) | 2.2.7.3 |
| anti-cel-miR35, 2'-O-methylated RNA | | 5'-/Biotin/ACUGCUAGUUUCCACCCUGUGA | 2.2.8.2 |
| anti-hsa-miR122, 2'-O-methylated RNA | | 5'-/Biotin/CAAACACCAUUGUCACACUCCA | 2.2.8.2 |

### *2.1.4 Plasmid Vectors*

**Table 2.8** Plasmid vectors

| ***Plasmid*** | ***Source*** |
|---|---|
| pEGFP-hAgo2 | Addgene |
| pCR-BluntII-TOPO | Invitrogen |
| pcDNA 4/TO | Invitrogen |
| pcDNA 4/TO / GFP-hAgo2 | Constructed in this study |
| pDsRed-C1-Monomer | Clontech |
| pEGFP-C1 | Clontech |
| pEGFP-C1 / TIA-1 | J.F. Caceres (Western General Hospital) |
| pDEST30-HA / TRBP2 | W. Filipowicz (Friedrich Miescher Institute) |
| pcDNA 5/TO / CFP-hDicer | T.C. Hobman (University of Alberta) |
| pEGFP-C1 / hDicer | Constructed in this study |
| pWS /GFP-TNRC6A | E.K. Chan (University of Calgary) |
| pLKO.1 | Sigma-Aldrich |
| pLKO.1 / Aha1 shRNA | Sigma-Aldrich |
| pLKO.1 / Cdc37 shRNA | Sigma-Aldrich |
| pLKO.1 / FKBP52 shRNA | Sigma-Aldrich |
| pEGFP-N1 | Clontech |
| pEGFP-N1 / let7a (3' UTR) | E. Jan (University of British Columbia) |
| pEGFP-N1 / KRAS 3' UTR | E. Jan (University of British Columbia) |

**Table 2.8** (Continued)

| *Plasmid* | *Source* |
|---|---|
| phRL-TK / 5x BoxB | W. Filipowicz (Friedrich Miescher Institute) |
| pCI-Neo4 / lambda N-HA-hAgo2 | W. Filipowicz (Friedrich Miescher Institute) |
| pCI-Neo4 / HA-hAgo2 | W. Filipowicz (Friedrich Miescher Institute) |
| pCI-Neo4 / lambda N-HA-LacZ | W. Filipowicz (Friedrich Miescher Institute) |
| pEGFP-N1 / 5x BoxB | Constructed in this study |

### *2.1.5 Antibodies*

**Table 2.9** Primary antibodies

| *Antibody* | *Dilution* | *Application** | *Source* |
|---|---|---|---|
| Rabbit anti-Ago2 (2D4, PAZ) | 1:1 000 | WB | T.C. Hobman, University of Alberta |
| Human anti-GW182 | 1:1 000 | WB | M.J. Fritzler, University of Calgary |
| Mouse anti-Hsp90 (SPA-830) | 1:1 000 | WB | Assay Designs |
| Rabbit anti-FKBP52 (ab97306) | 1:1 000 | WB | Abcam |
| Mouse anti-Cdc37 (MA3-029) | 1:1 000 | WB | Thermo Fisher Scientific |
| Rabbit anti-Ahsa1 | 1:1 000 | WB | P. LaPointe, University of Alberta |
| Rabbit anti-cytochrome C | 1:1 000 | WB | L.G. Berthiaume, University of Alberta |
| Mouse anti-RAS (RAS10) | 1:1 000 | WB | Millipore |
| Rabbit anti-GFP | 1:20 000 | WB | L.G. Berthiaume, University of Alberta |
| Rabbit anti-GAPDH (ab9485) | 1:1 000 | WB | Abcam |
| Mouse anti-hnRNP U (ab10297) | 1:1 000 | WB | Abcam |
| Human anti-GW182 | 1:100 | IIF | M.J. Fritzler, University of Calgary |
| Rabbit anti-TRBP | 1:250 | IIF | A. Gatignol, McGill University |
| Rabbit anti-PACT | 1:300 | IIF | G.C. Sen, Cleveland Clinic |
| Rabbit anti-Dcp1 (ab47811) | 1:800 | IIF | Abcam |
| Goat anti-TIA (sc-1751) | 1:50 | IIF | Santa Cruz Biotechnology |
| Mouse anti-Hsp90 (SPA-830) | 1:100 | IIF | Assay Designs |
| Rabbit anti-Cdc37 (SPA-605) | 1:250 | IIF | Assay Designs |
| Rabbit anti-hAhsa1 | 1:100 | IIF | P. LaPointe, University of Alberta |
| Mouse anti-HOP (SRA-1500) | 1:250 | IIF | Assay Designs |
| Mouse anti-p23 | 1:500 | IIF | Assay Designs |
| Mouse anti-FKBP59 (SRA-1400) | 1:250 | IIF | Assay Designs |
| Goat anti-GFP | 1:2500 | IP | L.G. Berthiaume, University of Alberta |

*- IB: immunoblot; IIF: indirect immunofluorescence; IP: immunoprecipitation

**Table 2.10** Secondary antibodies

| *Antibody::Conjugate* | *Dilution* | *Application** | *Source (product ID)* |
|---|---|---|---|
| Goat anti-mouse::HRP | 1:5 000 | WB | Jackson ImmunoResearch Laboratories |
| Goat anti-rabbit::HRP | 1:5 000 | WB | Jackson ImmunoResearch Laboratories |
| Goat anti-human::HRP | 1:5 000 | WB | Jackson ImmunoResearch Laboratories |
| Goat anti-rabbit::Alexa750 | 1:10 000 | WB | Invitrogen (A21039) |
| Donkey anti-mouse::Alexa680 | 1:10 000 | WB | Invitrogen (A10038) |
| Goat anti-mouse::Alexa750 | 1:10 000 | WB | Invitrogen (A21037) |
| Donkey anti-rabbit::Alexa647 | 1: 1 000 | IIF | Invitrogen (A31573) |
| Goat anti-rabbit::Alexa594 | 1: 1 000 | IIF | Invitrogen (A11037) |
| Goat anti-mouse::Alexa647 | 1: 1 000 | IIF | Invitrogen (A21236) |
| Donkey anti-mouse::Alexa488 | 1: 1 000 | IIF | Invitrogen (A21202) |
| Donkey anti-rabbit::Alexa488 | 1: 1 000 | IIF | Invitrogen (A21206) |
| Donkey anti-human::TexasRed | 1: 1 000 | IIF | Jackson ImmunoResearch Laboratories |

*- IB: immunoblot; IIF: indirect immunofluorescence

### *2.1.6 Cell lines*

**Table 2.11** Mammalian cell culture lines

| *Cell line* | *Source* |
|---|---|
| HeLa | ATCC |
| HeLa / TREx | Invitrogen |
| HeLa / TREx / 4TO-GFP-hAgo2 | This study |
| HepG2 | D.L. Tyrrell, University of Alberta |
| Huh7.5 | D.L. Tyrrell, University of Alberta |
| MEF WT | G.C. Sen, Cleveland Clinic |
| MEF PACT -/- | G.C. Sen, Cleveland Clinic |
| MEF TRBP -/- | R.E. Braun, The Jackson Laboratory |

## 2.2 Methods

### *2.2.1 Molecular Biology*

#### *2.2.1.1 Isolation of plasmid DNA from Eschericia coli*

Small scale plasmid DNA isolation was performed using either QIAprep spin mini prep kit (Table 2.5) following manufacturer's recommendations, or using diatomaceous earth (Table 2.6). Plasmid-transformed DH5α bacteria, grown overnight in 4 mL cultures of LB (Table 2.6), were pelleted via centrifugation at 5 000 x *g* for 10 minutes. For diatomaceous earth, bacterial pellets were resuspended in 200 µL of bacteria resuspension buffer (Table 2.6). Following resuspension, 200 µL of alkaline lysis buffer (Table 2.6) was added; tubes were inverted several times and incubated at room temperature for 5 minutes to complete lysis. Lysates were neutralized with 300 µL of neutralization buffer (Table 2.6) and insoluble material was pelleted via centrifugation at > 12 000 x *g* for 10 minutes. Supernatants were transferred to a fresh 1.5 mL tube containing 750 µL of diatomaceous earth slurry. Samples were incubated for at least 10 minutes at room temperature with rocking or rotation and then applied to pre-wetted filter pipette tips on a vacuum manifold. Plasmid DNA bound to diatomaceous earth was washed in place on the filter pipette tip twice with diatomaceous earth wash buffer (Table 2.6). Following final wash, tips were left to air dry for 10 minutes and then centrifuged at 10 000 x *g* for 1 minute to remove trace isopropanol. Tips were then transferred to fresh microfuge tubes and the plasmid DNA was eluted from the diatomaceous earth with 50 µL MilliQ $H_2O$ and centrifugation at 10 000 x *g* for 1 minute.

Large scale plasmid DNA purifications from 200 – 300 mL overnight cultures were carried out using QIAGEN plasmid maxi kits (Table 2.5) following the manufacturer's recommendations.

*2.2.1.2 Restriction endonuclease digestion*

Reactions were typically carried out in a volume of 20 µL with 0.5 - 5 µg of plasmid DNA and containing 2 - 10 U of restriction endonuclease and appropriate reaction buffer (Table 2.3).

*2.2.1.3 Dephosphorylation of linearized vectors*

To reduce occurrences of vector self-ligation, vector DNA was dephosphorylated using calf intestinal alkaline phosphatase (Table 2.3) according to manufacturer's recommendations.

*2.2.1.4 Polymerase chain reaction*

DNA was amplified using Platinum *Taq* (Table 2.5) or the Expand Long Template PCR system (Table 2.5). Reactions typically contained 50 – 250 ng of plasmid DNA, linearized by restriction endonuclease digestion, 500 nM dNTPs, 200 nM of each primer and 1 – 5 U of polymerase. Reactions were performed using either a TC-312 thermocycler (Techne) or a Robocycler Gradient 40 Hot Top system (Stratagene).

*2.2.1.5 Agarose gel electrophoresis*

Electrophoresis grade agarosewas dissolved by heating in TAE (Table 2.6). Prior to pouring the gel [0.8% - 1.5% (w/v)] into the casting tray, ethidium bromide was added to a final concentration of 0.5 µg/mL. The gel was immersed in TAE and the DNA samples were mixed with 6x DNA gel loading buffer (Table 2.6) and electrophoretically resolved. Fragments were visualized using an Ultra-violet gel transilluminator (Table 2.4) or images captured using a FluoroChem FC imaging system (Table 2.4)

*2.2.1.6 Purification of DNA fragments*

A QIAquick PCR purification kit (Table 2.5) was used to purify PCR products from dephosphorylation reactions, polymerase chain reactions and restriction endonuclease digestion reactions of PCR products, which generated small (<50nt) cleavage products that were to be excluded from subsequent ligations. Restriction endonuclease digestions yielding larger products that were not compatible for exclusion by the above kit were separated by agarose gel electrophoresis and the bands of interest were excised from the gel with a clean razor blade. DNA fragments were then eluted from the agarose gel using the QIAEX II gel extraction kit (Table 2.5).

*2.2.1.7 Ligation of DNA*

DNA inserts and vectors were combined in molar ratios ranging from 2:1 – 4:1, typically using a minimum of 20 ng of vector DNA and 1 – 5 U of T4 DNA ligase (Table 2.3). Reaction volumes were kept minimal, typically not exceeding 15 uL, and performed for at least 30 minutes at room temperature (for cohesive-end ligations) or overnight at 16$^{o}$C (for blunt-end ligations).

*2.2.1.8 Transformation of Escherichia coli*

Three strains of *E. coli* [OneShot Chemical Competent (Invitrogen), XL10-Gold Ultracompetent cells (Stratagene) and SubCloning Efficiency DH5α competent (Invitrogen)] were used during the course of this work. In all cases, chemically competent cells were transformed and cultured following the manufacturer's recommendations.

### *2.2.2 Construction of recombinant plasmids*

All primers used for the construction of the below plasmids are documented in Table 2.7. The sources of all plasmids described below are

detailed in Table 2.8. The sequence of plasmid constructs generated by PCR was verified by DNA sequencing at the Molecular Biology Facility (Department of Biological Sciences, University of Alberta) or at The Applied Genomics Centre (TAGC, Department of Medical Genetics, University of Alberta).

*2.2.2.1 pcDNA4/TO / GFP-hAgo2*

The plasmid pEGFP-hAgo2 was linearized with *Eag* I and used as a template in a PCR with the primers eGFP-hAgo2-AflII-F and eGFP-hAgo2-XbaI-R. The resulting product (3348 base pairs) was cloned into pCR-BluntII-TOPO following the manufacturer's protocol. The GFP-hAgo2 cassette of pCR-Blunt-GFP-hAgo2 was then subcloned into pcDNA4/TO using the *Afl* II and *Xba* I restriction endonuclease sites.

*2.2.2.2 pEGFP-C1 / hDicer*

After using pcDNA 5TO / CFP-hDicer as a template in a PCR with the primers hDcr1-XhoI-F and hDcr1-HindIII-R to generate an hDicer fragment (5774 base pairs), the resulting PCR fragment was subcloned into pCR-BluntII-TOPO following the manufacturer's protocol. Due to concerns over plasmid stability as well as problems with plasmid yield, both presumably as a result of the large size of the hDicer open reading frame, the hDicer cassette was subcloned into pBluSKP using the *Xho* I and *Hind* III sites. Following propogation of pBluSKP/hDicer, the hDicer cassette was ultimately subcloned into the *Xho* I and *Hind* III sites of pEGFP.

*2.2.2.3 pEGFP-C1 and pDsRed-C1-Monomer / TRBP2*

The vector pDEST30-HA/TRBP2 was used as a template for PCR using the primers TRBP-XhoI-F and TRBP-BamHI-R. The resulting PCR product (1006 base pairs) was digested and subsequently ligated into the pDsRed-C1-Monomer and pEGFP-C1 vectors at the *Xho* I and *BamH* I sites.

*2.2.2.4 pDsRed-C1-Monomer / TIA-1*

The TIA-1-enconding sequence was subcloned from pEGFP-C1 / TIA into pDsRed-C1-Monomer using *Xho* I and *Xba* I.

*2.2.2.5 pEGFP-N1 / 5x BoxB*

After using phRL-TK/5xBoxB as a template for PCR with the primers BoxB-MfeI-F and BoxB-HpaI-R, the resulting PCR fragment (226 base pairs) was subcloned into the *Mfe* I and *Hpa* I sites of pEGFP-N1.

### *2.2.3 Culture and transfection of mammalian cell lines*

*2.2.3.1 Cell line maintenance*

HeLa, HepG2, Huh7.5, MEF WT, MEF PACT-/- and MEF TRBP-/- cells were cultured in DMEM containing 10% FBS, 20 mM HEPES (pH 7.4) and penicillin/streptomycin. HeLa / TREx cells were cultured in DMEM containing 10% FBS, 20 mM HEPES (pH 7.4), penicillin (200 U/mL), streptomycin (200 µg/mL), and blasticidin (5 µg/mL). The derivative cell line HeLa/TREx / 4TO-GFP-hAgo2 was cultured in the same media as the parent HeLa/TREx cell line with the addition of Zeocin (400 µg/mL). Cells were incubated at 37$^{o}$C in a humidified atmosphere with 5% $CO_2$.

*2.2.3.2 Transient transfection of cell lines*

HeLa and HeLa/TREx/4TO-GFP-hAgo2 (Section 2.2.3.3) cells were transiently transfected with plasmid DNA using Lipofectamine 2000. 24 hours prior to transfection, cells were seeded in 35mm dishes at a density of 1.5x10$^{5}$ cells/dish. Cells were then transfected with 1 µg of plasmid DNA and 2.5 µL of Lipofectamine 2000 in 1 mL of OptiMEM per dish. Cells were incubated with the DNA-lipocomplexes for 2 hours, after which the medium was supplemented with

an equal volume of OptiMEM containing 20% FBS and returned to a 37$^{o}$C incubator overnight. The following day the medium was replaced with normal growth media.

*2.2.3.3 Construction of HeLa/TREx/4TO-GFP-hAgo2 stable cell line*

HeLa/TREx cells were transfected, in a 35 mm dish, with pcDNA4/TO/GFP-hAgo2 (Section 2.2.2.1) using Lipofectamine 2000 following the protocol for transient transfection. 48 hours after transfection, cells were trypsinized and seeded into a 150 mm dish. Growth medium was replaced with HeLa/TREx growth medium supplemented with zeocin (described in 2.2.3.1). Medium was replaced twice weekly for approximately 2 weeks until colonies were apparent. Colonies were trypsinized using sterile cloning rings and sterilized silicone grease and re-plated into 96-well plates. Cells were grown under selection and continually passaged into increasingly larger culture dishes. Ultimately, clones were screened for inducible expression of GFP-hAgo2 by seeding cells on coverslips, inducing expression by treatment with doxycycline (1 µg/mL) for 24 hours and imaging using fluorescence microscopy. A single clone with inducible expression of GFP-hAgo2 was used in this study.

*2.2.3.4 Selection and enrichment of cells transiently transfected with pLKO.1-based vectors*

HeLa cells were cultured and transiently transfected with plasmid DNA using Lipofectamine 2000 as described in Section 2.2.3.2. Following overnight incubation in OptiMEM supplemented with FBS to a final concentration of 10%, medium was aspirated and the cells were rinsed one time with PBS (Table 2.6). Cells were subsequently grown in DMEM supplemented with 10% FBS, 20 mM HEPES (pH7.4) and puromycin (5 µg/mL) for an additional 24 to 48 hours (total of 48 to 72 hours post-transfection) as indicated.

### *2.2.4 Microscopy*

#### *2.2.4.1 Indirect Immunofluorescence*

HeLa and HeLa/TREx/4TO-GFP-hAgo2 cells were cultured on glass coverslips, treated as described and processed for indirect immunofluorescence microscopy post-transfection at times indicated. Stress granules were induced by treating the cells with either 500 µM arsenite or 1 µM hippuristanol for the indicated times prior to fixation. Cells were thrice washed with PBS and then fixed with 4% (w/v) paraformaldehyde for 15 minutes. Fixed cells were rinsed with PBS three more times prior to permeabilization with PBS containing 0.2% (v/v) TritonX-100 for 2 minutes. Following permeabilization, coverslips were again rinsed three times with PBS and then blocked for 2 hours in PBS-T containing 2% skim milk powder (Table 2.6). Primary antibody (diluted as indicated in Table 2.9) incubations were performed in PBS-T containing 2% skim milk powder for 2 hours at room temperature or overnight at $4^{o}$C. Following primary antibody incubation, coverslips were washed three times in PBS-T while rocking for a total of 2 hours. Secondary antibody (Table 2.10) incubations were performed in PBS-T for 1 hour at room temperature followed by three additional PBS-T washes, for a total of one hour. Coverslips were rinsed with PBS and then mounted in ProLong Gold antifade reagent with DAPI. Digital images of samples were captured with an Axiovert 200M (Table 2.4) or LSCM510 (Table 2.4) microscope.

#### *2.2.4.2 Live cell imaging*

HeLa cells transiently expressing fluorescent protein-tagged chimeras, or stably transfected HeLa/TREx/4TO-GFP-hAgo2 cells induced to express GFP-hAgo2 with doxycycline, were cultured in 35 mm glass-bottom dishes (MatTEK) and treated as described. Prior to imaging, the growth medium was aspirated, the cells were rinsed with PBS and the medium was replaced with $CO_2$-independent cell culture medium supplemented with 10% FBS. Cells were

imaged on a Zeiss Axiovert 200M and were kept warm by outfitting the microscope with an objective warmer (Bioptechs) and a heated stage (Zeiss).

Induction of stress granules during live cell imaging experiments was done by treating cells with either 500 µM arsenite or 1 µM hippuristanol. Untreated cells in 35 mm glass-bottom culture dishes, containing 1 mL of $CO_2$-independent media, were mounted on the heated stage. Image acquisition was started and, following the initial time point, an additional 1 mL of $CO_2$-independent media containing either 1000 µM arsenite or 2 µM hippuristanol was carefully added to the dish.

*2.2.4.3 Quantitative image analysis*

Datasets acquired with Volocity image acquisition software were exported using LIFF format and the LIFF file was opened using Imaris x64 with the MeasurementPro module (Bitplane). Stress granule number and "Absolute Intensity" were determined using a "Spots" algorithm with "Region growing" selected ("Region of interest" and "Track (over time)" were left unselected). The average diameter of nascent stress granules, typically 1 µm, was entered in the "Estimated diameter" field and a filter was added to exclude spots that were both below (reduce false positives) and above (exclude pre-existing processing bodies) a threshold "Quality". Statistics were exported in Microsoft Excel file format and analyzed.

### *2.2.5 Protein gel electrophoresis and detection*

*2.2.5.1 Sodium dodecyl-suplhate polyacrylamide gel electrophoresis (SDS-PAGE)*

Proteins were separated by discontinuous gel electrophoresis (5% stacking gel and 8%, 10% or 12% resolving gel). Stacking gels were prepared by adding acrylamide / bis-acrylamide, to final concentration of 5%, to 125 mM Tris-

HCl (pH 6.0) with 0.1% SDS, 0.1% ammonium persulphate and 0.1% TEMED. Resolving gels were prepared by combining acrylamide / bis-acrylamide, to an appropriate final concentration, in 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 0.1% ammonium persulphate and 0.1% TEMED. Protein samples were mixed with 5x protein sample buffer (Table 2.6) and denatured at 95$^{o}$C for 6 minutes. Electrophoresis was performed using the Bio-Rad mini-protean III system with SDS-PAGE running buffer (Table 2.6) at 140 – 225 Volts. After electrophoresis, the gels were processed for immunoblot analysis as described below (Section 2.2.5.2).

*2.2.5.2 Immunoblot analysis*

Following SDS-PAGE, proteins in the gels were transferred to either 0.45 µm polyvinylidene fluoride (PVDF) or nitrocellulose membranes. PVDF membranes were first activated in methanol. Membranes were then equilibrated in western blot transfer buffer (Table 2.6) for at least 10 minutes at room temperature with rocking. Protein transfer was performed using western blot transfer buffer, pre-chilled to 4$^{o}$C and a Mini Trans-Blot Electrophoresis transfer cell apparatus (Bio-Rad) at 140 V or 320 mA for at least 1 hour. Following completion of the transfer the membranes were air-dried, re-wet with methanol, and then blocked in PBS-T or TBS-T (Table 2.6) containing 5% (w/v) skim milk powder for at least 1 hour on a rocking device. PBS-T was used preferentially throughout the immunoblot procedure when blots were to be imaged using the Odyssey Infrared scanner (LiCor) {Figures 5.2, 5.3 and 5.4}; however, when blots were developed using horseradish peroxidase-conjugated secondary antibodies {Figures 3.3, 3.9, 3.10, 4.1 and 5.4 (hAgo2 and GW182)}, TBS-T was used interchangeably. Membranes were incubated with primary antibodies diluted (as described in Table 2.9) in PBS-T containing 5% (w/v) skim milk powder for 2 hours at room temperature or overnight at 4$^{o}$C. It should be noted that for the best results, when the rabbit anti-Ago2 (2D4) primary

antibody was used for immunoblot detection of endogenous hAgo2, incubations were done overnight at 4$^{o}$C. After three washes with PBS-T at room temperature for a total of 2 hours, the membranes were incubated in the secondary antibody diluted (as indicated in Table 2.10) in PBS-T containing 2% (w/v) skim milk powder for 25 minutes at room temperature. Lastly, membranes were washed three times with PBS-T for a total of 30 minutes and then detected as described below (Section 2.2.5.3 or 2.2.5.4).

*2.2.5.3 Detection of horseradish peroxidase-conjugated secondary antibodies*

Membranes were incubated in Supersignal West Pico chemiluminescent substrate (Table 2.4) for 3 minutes, after which they were either exposed to Rx film (Table 2.4) or imaged using the FluorChem Q (Table 2.4).

*2.2.5.4 Detection of fluorophore-conjugated secondary antibodies*

Membranes were rinsed with PBS and then placed, face-down, on the scanner bed of the Odyssey Infrared Imaging system (Table 2.4). The membranes were scanned at 84 µm resolution on a quality setting of "High".

### *2.2.6 Biochemical analysis of protein-protein interactions*

Immunoprecipitation of Hsp90 and detection of associated Argonaute by immunoblot analysis was performed by Paul LaPointe (University of Alberta).

*2.2.6.1 Primary Hsp90 antibody crosslinking*

Monoclonal anti-Hsp90 antibodies (Table 2.9) and protein G-sepharose fast flow beads were mixed together in PBS for 1hr at room temperature at a concentration of 3 – 5 mg of IgG per milliliter of bead volume and then washed with 10 volumes of 100 mM HEPES (pH 8.8). Following the wash, antibody was crosslinked to beads with 20 mM dimethylpimelimidate for 30 minutes at room

temperature. Samples were then washed twice with 10 volumes of 200 mM ethanolamine and incubated in 200 mM ethanolamine for an additional 2 hours at room temperature. Lastly, samples were washed three times with 10 volumes PBS and stored at 4$^{o}$C as a 1:1 slurry in PBS.

*2.2.6.2 Immunoprecipitation of Hsp90*

Four confluent 100 mm dishes of HeLa cells were treated as described and lysed in Hsp90 IP buffer (Table 2.6) supplemented with Complete$^{TM}$ Protease Inhibitor. Lysates were cleared via centrifugation at 14 000 x *g* for 10 minutes at 4$^{o}$C. Immunoprecipitation of clarified lysates was performed with 2-3 µL of protein G-sepharose beads crosslinked to anti-Hsp90 primary antibody (Section 2.2.6.1). Immunoprecipitations were carried out for 1 hour at 4$^{o}$C with rotation after which the beads were washed one time with Hsp90 IP buffer before boiling in protein sample buffer and subjected to SDS-PAGE and immunoblot analysis. Protein G-sepharose beads crosslinked to monoclonal anti-myc antibodies (following protocol in 2.2.6.1) were used as a control.

***2.2.7 RNA techniques***

*2.2.7.1 RNA isolation*

Total RNA (including small RNA species) was isolated from samples using either TRIzol reagent or TRI Reagent (Invitrogen) following the same protocol detailed below. Samples were homogenized in 20 volumes of reagent or 1 mL per 35 mm dish of cultured cells. Homogenized samples were incubated at room temperature for 5 minutes and then 1/5$^{th}$ volume of 1-bromo 3-chloropropane was added. The samples were mixed by vigorous shaking for 30 seconds prior to incubation for 5 minutes at room temperature. Aqueous and organic phases were separated via centrifugation at 12 000 x *g* for 15 minutes at 4$^{o}$C and the aqueous phase was transferred to a fresh, nuclease-free microfuge tube. An

equal volume of phenol/chloroform/isoamyl alcohol was added to the aqueous phase, samples were vortexed for 90 seconds and then re-centrifuged at 12 000 x *g* for 15 minutes at 4$^{o}$C. Again, the aqueous phase was transferred to a fresh, nuclease-free tube and an equal volume of chloroform was added. Samples were vortexed for 30 seconds and centrifuged for 10 minutes at 12 000 x *g* at 4$^{o}$C. The aqueous phase was again placed in a fresh tube and the RNA was precipitated at -20$^{o}$C for 1 hour after addition of molecular biology grade isopropanol equivalent to 50% of the original volume of TRIzol used in the lysis. Precipitated RNA was pelleted by centrifuging samples at 12 000 x *g* for 15 minutes at 4$^{o}$C. The RNA pellet was washed with 1 mL of 70% ethanol and re-pelleted via centrifugation at 8 000 x *g* for 10 minutes at room temperature. In order to remove all contaminating guanidine isothiocyanate from the purified RNA, the RNA pellet was resuspended in 135 µL of UltraPure distilled water, 5 µL of 500 mM EDTA (pH 8.0) and 15 µL of 3 M ammonium acetate. After rigorous pipetting and vortexing, to ensure resuspension of the RNA pellet, the RNA was precipitated again at -20$^{o}$C for 1 hour using 500 µL of molecular biology grade isopropanol. The precipitated RNA was repelleted and washed with 70% ethanol prior to being resuspended in UltraPure distilled water.

*2.2.7.2 First strand synthesis of miRNA*

Total RNA samples were isolatedfrom HeLa cells as described in 2.2.6.1. Prior to first-strand synthesis, samples were treated with amplification grade DNase I (Table 2.3) as per the manufacturer's recommendations. First strand synthesis reactions were set up using 2 µg of total RNA, the qScript Flex cDNA synthesis kit (Table 2.5) and let-7c RT primer (Table 2.7) as per the manufacturer's recommendations.

*2.2.7.3 qPCR analysis of miRNA level*

Quantitative PCR reactions were conducted on an MX3005P thermocycler (Table 2.4) using PerfeCTa SYBR Green SuperMix, UNG, Low Rox (Table 2.5).

Reactions were performed as technical triplicates in a final volume of 25 µL containing 2 µL of cDNA generated in Section 2.2.7.2 and 0.5 µL of each primer from the let-7c primer set (Table 2.7). The thermal profile of the qPCR reaction consisted of an initial denaturation step of 10 minutes at 95$^{o}$C followed by 40 cycles of 95$^{o}$C for 30 seconds, 1 minute at 55$^{o}$C and 1 minute at 72$^{o}$C. Fluorescence was quantified at the end of the 55$^{o}$C step. A dissociation curve was used to verify that a single product was amplified, and the temperature range used was 55$^{o}$C to 95$^{o}$C.

Analysis of miRNA levels bound to GFP-hAgo2 was complicated by the lack of an unrelated small RNA that could be used to normalize the samples. Therefore, the method used to determine the relative levels of let-7c miRNA between samples was solely based on an efficiency of amplication of 100%. The equation used in the calculation of relative levels of miRNA was [2^(Ct{variable}-Ct{control}], where the product of the equation is the fold decrease in the amount of miRNA associated with the variable sample relative to the control.

*2.2.7.4 Analysis of RNA integrity for microarray profiling*

Total RNA samples isolated as in 2.2.6.1 were analyzed on a NanoDrop 2000c spectrophotometer (Thermo Scientific). Samples with A260/A280 and A260/A230 ratios greater than 1.8 were sent to the Alberta Transplant Applied Genomics Center (ATAGC, University of Alberta) for analysis using a BioAnalyzer 2100 (Agilent). Samples with an RNA Integrity Number (automatically determined by the software of the BioAnalyzer 2100) of greater than 8.0 were used in downstream labeling for microarray analysis.

*2.2.7.5 Microarray profiling of miRNA expression*

Samples were submitted to the ATAGC (University of Alberta) and 1 µg of total RNA was labeled following their protocol for sample preparation for miRNA microarray analysis. Labeled RNA was analyzed and hybridized (by ATAGC) to a

GeneChip miRNA 2.0 microarray chip (Affymetrix) following the manufacturer's recommendations.

### *2.2.8 Biochemical analysis of protein-RNA interactions*

#### *2.2.8.1 Immunoprecipitation of GFP-hAgo2 for detection of associated miRNA*

Stably transfected HeLa/TREx / 4TO-GFP-hAgo2 cells (Section 2.2.3.3) were seeded into 100 mm dishes and grown to approximately 80% confluency. The expression of GFP-hAgo2 was induced by supplementation of the growth medium with doxycycline to a final concentration of 1 µg/mL. Six hours following the addition of doxycycline, the cells were treated with geldanamycin at a final concentration of 1 µg/mL, or DMSO as a control, for an additional 12 hours. Subsequent to GFP-hAgo2 induction and treatment with geldanamycin, cells were lysed with NP40 lysis buffer (Table 2.6) supplemented with Complete™ Protease Inhibitors. Lysates were clarified via centrifugation at 10 000 x *g* for 10 minutes at 4°C. Immunoprecipitation of clarified lysates was performed with 0.5 – 2.0 µg of polyclonal goat anti-GFP (Table 2.9) for 2 hour at 4°C with rotation prior to recovery on protein G-sepharose beads. Protein G-sepharose beads were prepared by washing 3 times in 10 volumes of PBS followed by washing with 10 volumes of NP40 lysis buffer. The beads were then blocked in NP40 lysis buffer supplemented with 1% (w/v) casein for 1 hour at 4°C with rotation. Following blocking, the beads were collected via centrifugation at 500 x *g* for 3 minutes, and resuspended in NP40 lysis buffer containing 1% (w/v) casein to create a 1:1 slurry. Twenty microliters of beads were then added to the antibody-containing lysates and the samples were rotated for an additional hour at 4°C. Beads were collected via centrifugation at 500 x *g* for 3 minutes at 4°C and washed once in NP40 lysis buffer. Following wash step, the beads were resuspended in 200 µL of NP40 lysis buffer and then aliquoted into fractions and harvested separately. Samples were harvested in either 400 µL of TRIzol reagent

for RNA extraction or protein sample buffer for SDS-PAGE and immunoblot analysis.

*2.2.8.2 Pulldown of RISC using 2'-O-methylated oligonucleotides*

Huh7.5 cells were grown to confluency in order to obtain $2.0x10^7$ cells per sample.

For each sample, 15 µL of Dynabeads M-280 streptavidin beads were prepared by washing three times with 1 mL of oligo binding buffer (Table 2.6). Following the third wash, the bead suspension was divided into two 1.5 mL centrifuge tubes and the excess wash buffer was aspirated. For every 15 µL of streptavidin beads prepared above, 30 µL of the 2'-O-methylated oligonucleotides anti-cel-miR35 or anti-has-miR122 (Table 2.7) were diluted from 60 µM stock into oligo binding buffer supplemented with 1 M NaCl and 2 mM DTT to a final concentration of 1 µM. Beads were adsorbed with anti-sense oligonucleotides for 120 minutes at $4^oC$ with rotation. Following the adsorbtion, the beads were washed three times with oligo binding buffer and then resuspended using 200 µL of oligo binding buffer for each 15 µL of prepared beads. The resuspended beads were aliquoted into fresh 1.5 mL centrifuge tubes and the buffer was aspirated.

Typically, greater than $3.0x10^8$ cells were harvested by trypsinization from culture dishes, combined and collected by centrifugation at 1 000 x *g* for 5 minutes. The pellet was resuspended in 25 volumes of PBS. A haemocytometer was used to determine the total number of cells harvested. Cells were then re-pelleted by centrifugation at 1 000 x *g* for 5 minutes after which they were resuspended in 4 mL of ice-cold swelling buffer (Table 2.6) supplemented with Complete™ Protease Inhibitor. The cells were allowed to swell on ice for 20 minutes before they were homogenized with 40 strokes using a 7 mL glass dounce homogenizer. Cellular debris, nuclei and unbroken cells were pelleted via centrifugation of the homogenate at 12 500 x *g* for 15 minutes. The supernatant

was collected and diluted with additional swelling buffer so that the final concentration of the homogenate was equivalent to $2.0x10^7$ cells/mL. Cell homogenate was divided in half and 100 μM geldanamycin was added to one aliquot while an equal volume of DMSO was added as a control to the other. The oligonucleotide-adsorbed beads prepared as above were then combined with 1 mL of cellular homogenate and incubated for 60 minutes at $30^oC$, with rotation. Following the incubation, the beads were collected, resuspended in 1 mL of RISC wash buffer (Table 2.6) and transferred to a new microfuge tube. Following the wash step, the beads were collected and boiled in 100 μL of protein sample buffer. Protein samples were then subjected to SDS-PAGE and immunoblot analysis.

# CHAPTER 3

Hsp90 regulates the function of Argonaute 2 and its recruitment to stress granules and P bodies

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### 3.1 Rationale

Previous studies by the Hobman laboratory and others have revealed that Argonaute proteins bind to the molecular chaperone Hsp90 (Hock et al., 2007; Liu et al., 2004; Maniataki and Mourelatos, 2005; Tahbaz et al., 2001; Tahbaz et al., 2004). Given the robust nature of the physical interaction between Hsp90 and hAgo2 (Liu et al., 2004) and previous data showing that the interaction between hAgo2 and Dicer was negatively affected by inhibition of Hsp90 (Tahbaz et al., 2004), I predicted that hAgo2 is a *bona fide* client of Hsp90. The specific requirement for this chaperone in Argonaute function had not been examined. Unlike most other chaperones that assist in folding of nascent polypeptides, Hsp90 has a restricted but diverse set of client proteins (Grad and Picard, 2007). The precise mechanisms by which Hsp90 recognizes, binds to and "matures" client proteins are not known, but it is generally believed that its clients require the chaperone to acquire their enzymatically active conformations.

In the cytoplasm, Argonaute-dependent post-transcriptional gene-silencing (PTGS) has been linked to discrete cytoplasmic puncta called GW- or P bodies (Jakymiw et al., 2005; Liu et al., 2005b; Sen and Blau, 2005). In addition to P bodies, Argonautes rapidly associate with stress granules when cells encounter translational stress. Microscopically visible stress granules are not present in "unstressed" cells; however, treatment with arsenite or the translational repressor hippuristanol results in rapid formation of stress granules (Bordeleau et al., 2006; Leung et al., 2006). Moreover, targeting of hAgo2 to P bodies and stress granules appears to require that hAgo2 be actively involved in post-transcriptional gene silencing (Leung et al., 2006; Liu et al., 2005b; Rudel et al., 2011).

If my hypothesis is correct and hAgo2 is a true client of Hsp90, then inhibition of the Hsp90 activity would result in reduced hAgo2 function and a

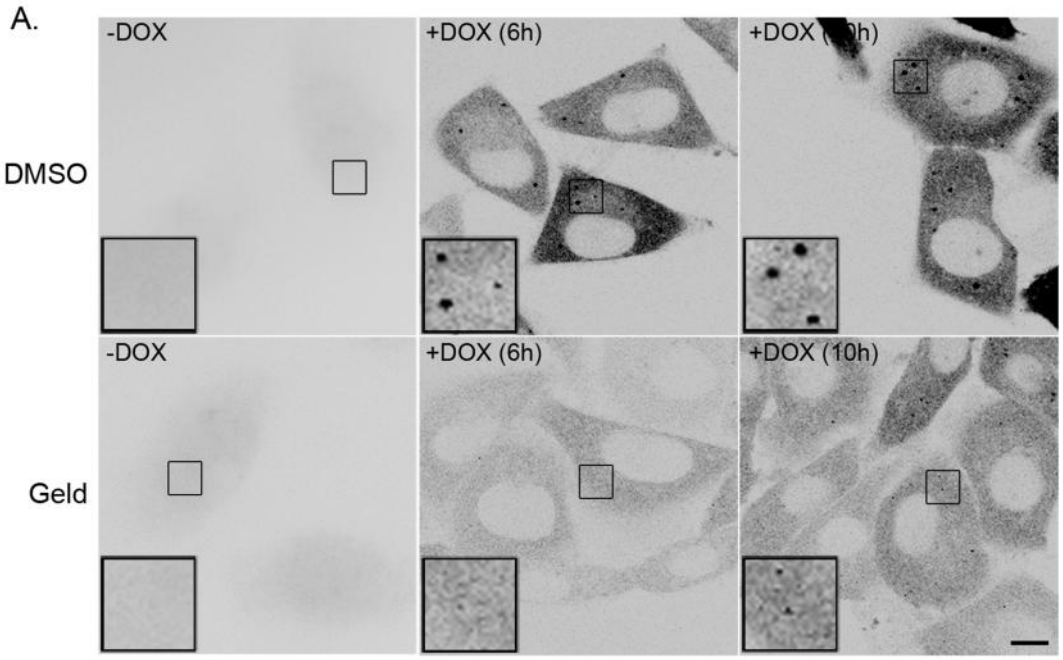
consequential decrease in miRNA-mediated and siRNA-mediated post-transcriptional gene silencing. In addition, I predicted that a reduction in the miRNA- and siRNA-mediated PTGS activity of hAgo2 would result in a reduced recruitment of hAgo2 to both processing bodies and stress granules.

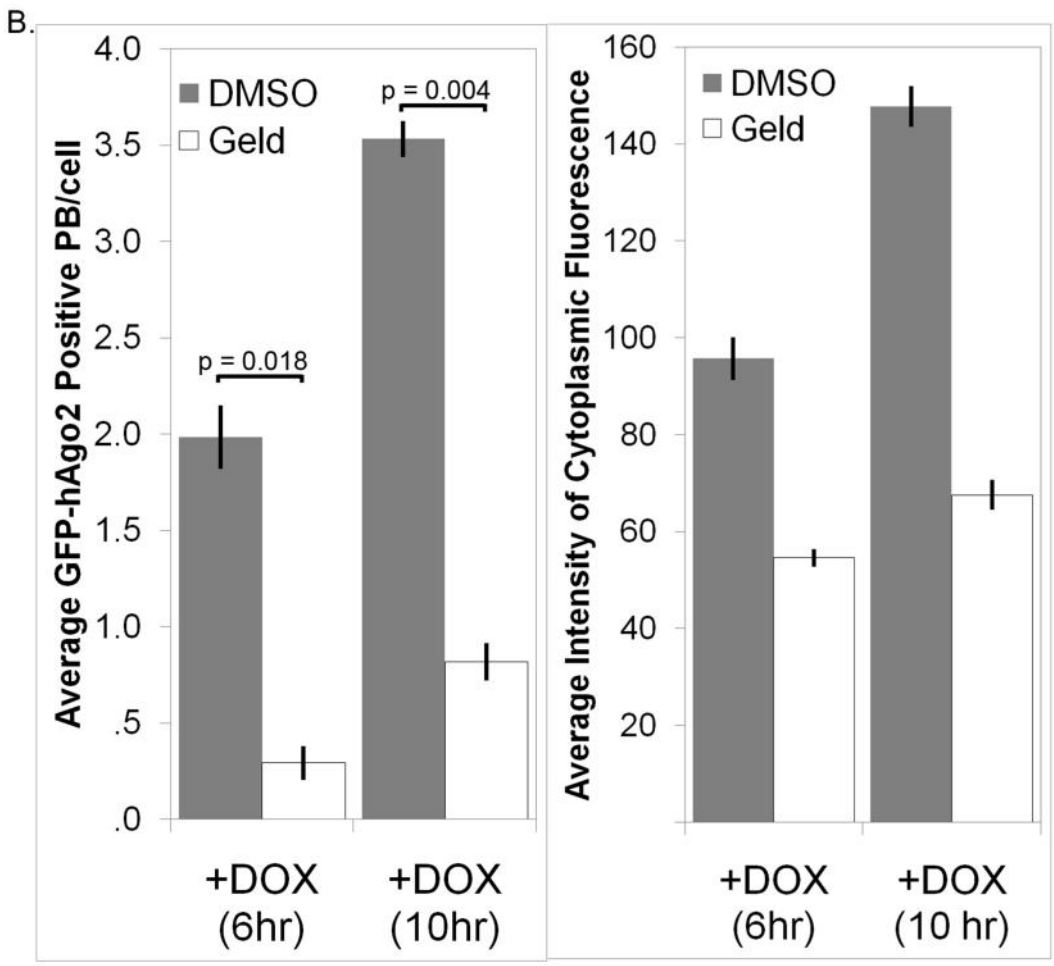
## 3.2 Results

### *3.2.1 Hsp90 activity is required for the recruitment of nascent GFP-hAgo2 to P bodies*

The first experiment of this study was designed to determine the effect of inhibition of Hsp90 on the ability of newly synthesized green fluorescent protein-(GFP-) tagged hAgo2 (GFP-hAgo2) to be recruited to P bodies in the absence of Hsp90 activity. First, I determined the kinetics of GFP-hAgo2 induction and recruitment to P bodies in a HeLa/TREx/4TO-GFP-hAgo2 stable cell line. HeLa/TREx/4TO-GFP-hAgo2 cells were cultured in glass-bottom 35 mm dishes and imaged prior to, as well as every hour following, the addition of doxycycline (1 µg/mL) to the media. Using this method, it was determined that nascent GFP-hAgo2 was sufficiently accumulated in P bodies as to be microscopically visible beginning at 6 hours post-induction. The effect of inhibition of Hsp90 activity on the recruitment of nascent GFP-hAgo2 to P bodies was then determined by inducing the expression of GFP-hAgo2 with doxycycline in the presence of either 10 µM geldanamycin or an equivalent volume of DMSO for 6 and 10 hours. As shown in Figure 3.1A, and quantitated using Imaris x64 image quantitation software in Figure 3.1B, inhibition of Hsp90 activity with geldanamycin effectively abrogates the recruitment of nascent GFP-hAgo2 to P bodies. Additionally, the fluorescent intensity of GFP-hAgo2 throughout the cytoplasm was measured and found to be reduced in geldanamycin-treated cells (Figure 3.1B).

**Figure 3.1 Inhibition of Hsp90 activity with geldanamycin impairs recruitment of nascent GFP-hAgo2 to P bodies. A.** Expression of GFP-hAgo2 was induced in stably transfected HeLa cells by addition of 1 μM doxycycline (+DOX) for 6 or 10 hours in the presence or absence of 10 μM geldanamycin (Geld or DMSO, respectively). Images were acquired immediately prior to induction with DOX (-DOX) and also at 6 and 10 hours post-induction. The insets at the bottom left are enlargements of the areas within the black squares. **B.** The average numbers of GFP-hAgo2 positive P bodies in DMSO- (6 hr, n=52; 10 hr, n=63) and geldanamycin-treated cells (6 hr, n=64; 10 hr, n=81) were determined. In addition, the average fluorescent intensities of GFP-hAgo2 in the cytoplasm at 6 and 10 hours post-induction with DOX are shown (arbitrary units). Data were acquired using Imaris software. Size bar represents 10 μm. Error bars indicate standard error of the mean, *p* values are displayed.


A.
-DOX
+DOX (6h)
+DOX (10h)
DMSO
-DOX
+DOX (6h)
+DOX (10h)
Geld


B.
Average GFP-hAgo2 Positive PB/cell
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
.0
DMSO
Geld
p = 0.004
p = 0.018
+DOX (6hr)
+DOX (10hr)
Average Intensity of Cytoplasmic Fluorescence
160
140
120
100
80
60
40
20
DMSO
Geld
+DOX (6hr)
+DOX (10 hr)

### *3.2.2 Hsp90 activity is required for the formation and/or stability of GW182-positive P bodies*

Reduced recruitment of nascent GFP-hAgo2 to P bodies following inhibition of Hsp90 activity with geldanamycin could in fact be due to reduced formation and/or stability of these RNPs. To address the potential scenario that P body integrity is dependent on Hsp90 activity, HeLa cells were treated with 10 µM geldanamycin or DMSO for a total of 6 or 10 hours. Following treatment, the cells were processed for indirect immunofluorescence and the number of P bodies was determined using an antibody to GW182, a core component of P bodies. Results in Figure 3.2 show that inhibition of Hsp90 activity for 6 hours reduces the number and intensity of P bodies, while treatment with geldanamycin for 10 hours results in a near complete loss of microscopically visible P bodies. These data indicate that the biogenesis and/or stability of P bodies is dependent upon Hsp90 activity.

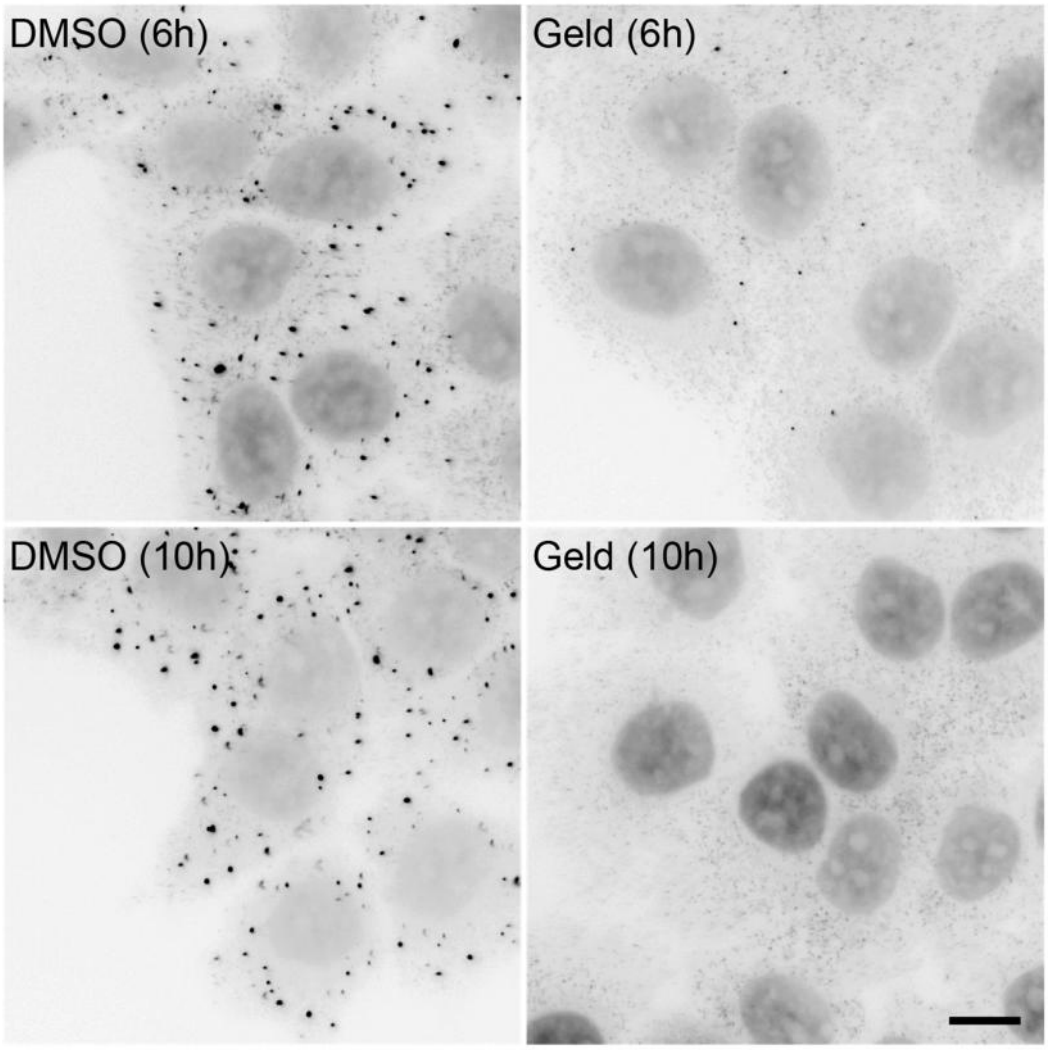



**Figure 3.2 Inhibition of Hsp90 activity with geldanamycin eliminates GW182-positive P bodies.** HeLa cells were treated with DMSO or 10 µM geldanamycin for 6 and 10 hours after which they were processed for indirect immunofluorescence using antibodies specific for the P body resident protein GW182. Size bar represents 10 µm.

### *3.2.3 Hsp90 activity is required for miRNA-mediated post-transcriptional gene silencing*

The results described above, together with recent work suggesting that P bodies are formed as a consequence of RNA-mediated silencing (Eulalio et al., 2007b), support my hypothesis that Hsp90 activity is required for Argonaute function in miRNA- and/or siRNA-mediated gene silencing. The assay used in this study to assess miRNA function is based on the regulation of the level of Ras protein by the let-7 miRNA family (Johnson et al., 2005). Specifically, in a given cell, Ras levels are inversely proportional to the levels of let-7 miRNAs. Accordingly, I predicted that if geldanamycin inhibits the ability of Argonautes to bind let-7 miRNAs, or function after binding, levels of Ras protein would increase in drug-treated cells. HeLa cells were treated with 10 μM geldanamycin or DMSO for a total of 12 hours and protein samples were harvested for immunoblot analysis. Indeed, levels of Ras protein were on average nearly 45% higher in cells treated with geldanamycin (Figure 3.3A and B). These data are comparable to those of Johnson et al who reported that inhibition of let-7c functions increases Ras protein levels by 60-70% (Johnson et al., 2005). The steady state levels of Argonaute proteins were similar in control and geldanamycin treated samples (Figure 3.3A and B), indicating that a drug-induced decrease in Argonaute protein concentration is not the reason for elevated Ras expression. It was also determined whether additional putative miRNA targets were affected when Hsp90 activity was inhibited. Using web-based algorithms (http://microrna.sanger.ac.uk/), we determined that cytochrome *c* is a high confidence target of mi29-b, a miRNA that like let-7c, is highly expressed in HeLa cells (Yeung et al., 2005). It was observed that levels of cytochrome *c* protein were significantly increased in geldanamycin-treated cells (Figure 3.3C). Finally, I monitored Ras protein levels in geldanamycin-treated HepG2 cells. This cell line contains low levels of let-7c miRNA (Johnson et al., 2005) and, as predicted, Ras levels were not significantly affected by this drug (Figure 3.3A and B). These

results suggest that Hsp90 activity is required for efficient miRNA-mediated post transcriptional gene silencing, however, the potential role (or roles) for Hsp90 in the maturation and activity of Argonaute2 is still unclear.

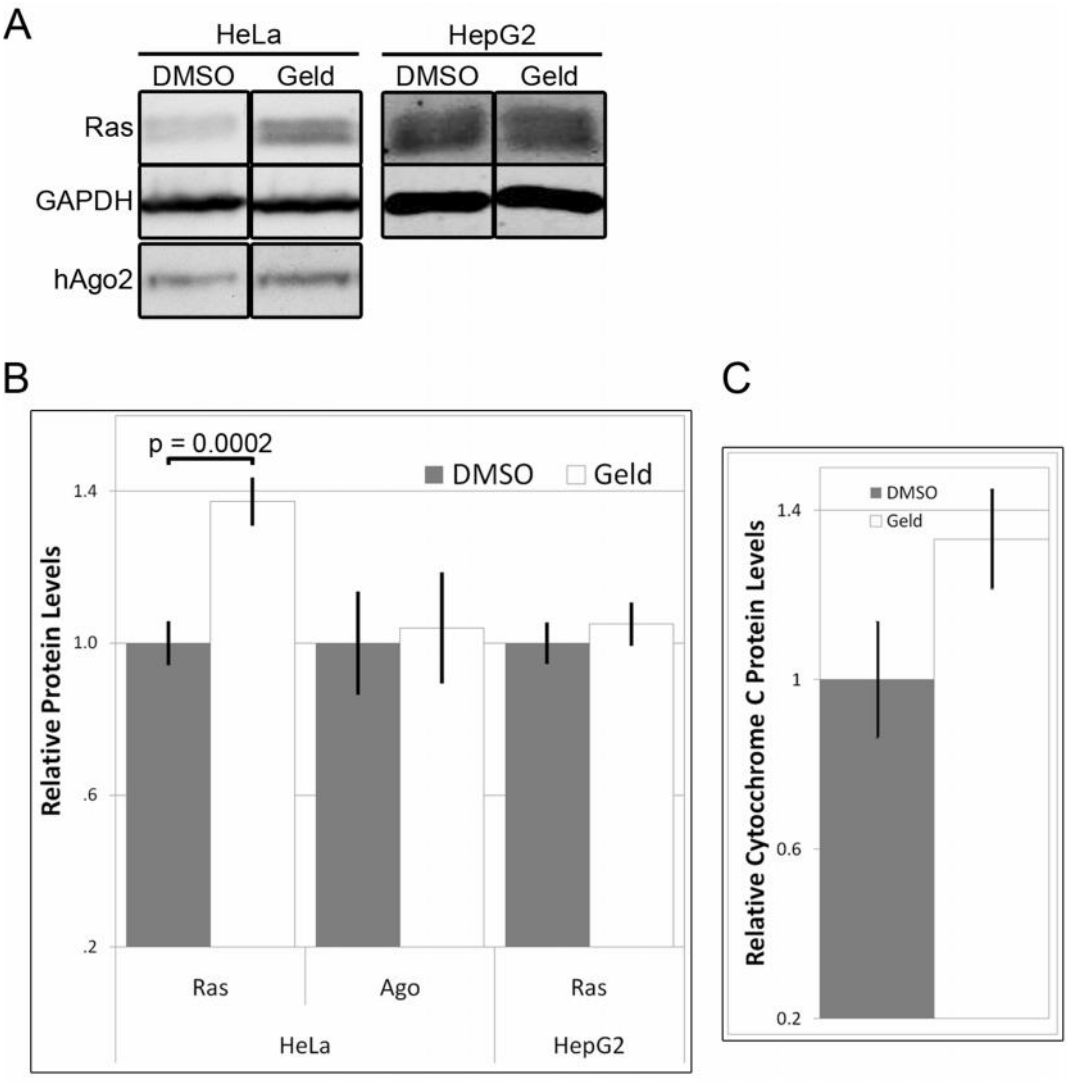


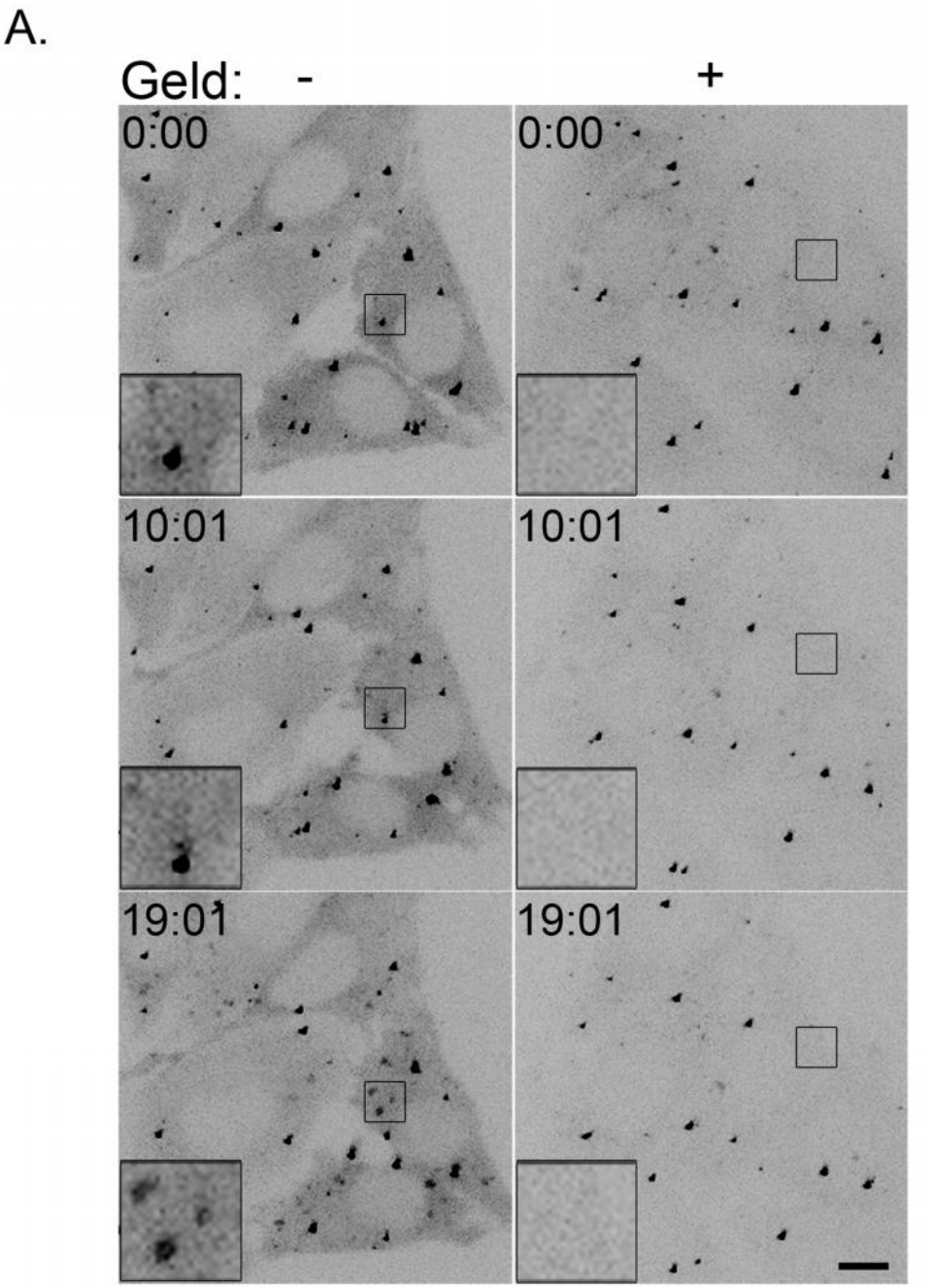

**Figure 3.3 Inhibition of Hsp90 activity with geldanamycin results in increased Ras protein expression. A.** HeLa and HepG2 cells were treated with DMSO or 10 µM geldanamycin (Geld) for 12 hours. The level of endogenous Ras is shown with GAPDH provided as a loading control. Levels of endogenous Argonaute proteins in HeLa cells were determined using a PAZ domain-specific polyclonal antibody. **B.** Quantitation of the level of Ras, normalized to GAPDH and relative to DMSO control is shown for HeLa and HepG2 samples. **C.** HeLa cells were treated with DMSO or 10 µM geldanamycin for 6 hours. Quantitation of the level of cytochrome *c*, normalized to GAPDH and relative to DMSO control is shown. Error bars indicate standard error of the mean, *p* values are displayed..

### *3.2.4 Hsp90 activity is required for the recruitment of GFP-hAgo2 to nascent stress granules*

Stress granules are known to contain stalled translation complexes (Kedersha et al., 2002) and have been implicated in miRNA-mediated post-transcriptional gene silencing due, at least in part, to hAgo2 being a component (Leung et al., 2006). Given that inhibition of Hsp90 blocks the recruitment of nascent GFP-hAgo2 to P bodies and significantly reduces the number of GW182-positive P bodies, the effect of geldanamycin on the recruitment of GFP-hAgo2 to stress granules was investigated.

HeLa cells transiently transfected with pcDNA4/TO/GFP-hAgo2 were pre-treated with 10 µM geldanamycin or DMSO for 2 hours and then stress granule formation was induced during live cell confocal microscopy with 1 µM hippuristanol. Under control conditions, GFP-hAgo2-positive stress granule formation occurred minutes after cells were exposed to hippuristanol (Figure 3.4A). Pre-treatment with geldanamycin appeared to reduce the rate by which GFP-hAgo2 was targeted to stress granules (Figure 3.4A). Quantitation of the data using Imaris x64 image quantitation software confirmed that geldanamycin treatment substantially reduced the total numbers of GFP-hAgo2 positive stress granules as well as the average GFP fluorescence intensity of the individual granules compared to control cells (Figure 3.4B).

**Figure 3.4 Inhibition of Hsp90 activity with geldanamycin inhibits recruitment of GFP-hAgo2 to nascent stress granules in response to hippuristanol.** HeLa cells were transiently transfected with plasmids encoding GFP-hAgo2. At 24 hours post-transfection, cells were treated with DMSO (-) or 10 µM geldanamycin (+) for 6 hours, after which, hippuristanol was added to a final concentration of 1 µM. Cells were then imaged every 30 seconds for a total of 20 minutes. **A.** Still images are presented from beginning, mid and end time points. The insets in the bottom left of the panels are enlargements of the areas within the squares. Size bar represents 10 µm. **B.** The fluorescent intensities of newly forming stress granules in geldanamycin (n=22) or DMSO (control) (n=33) treated samples were measured at regular intervals after addition of hippuristanol.

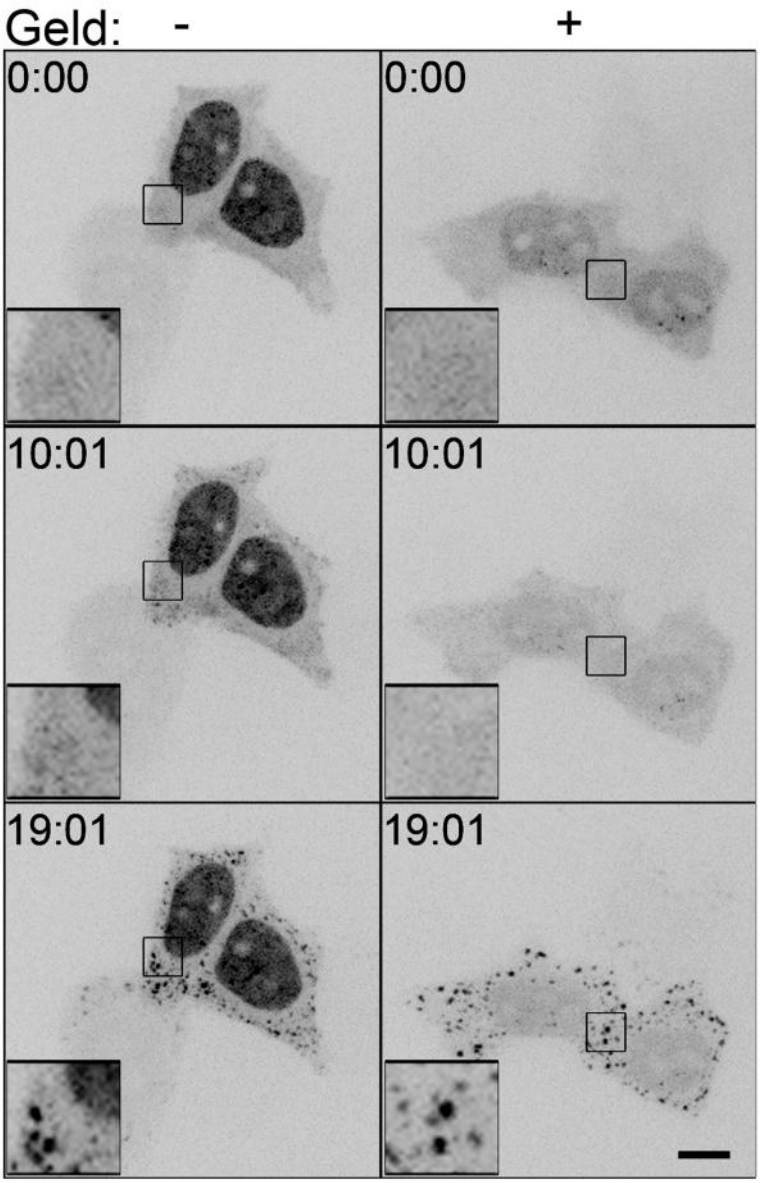

A.
Geld: -
+
0:00
0:00
10:01
10:01
19:01
19:01
B.
Control
Geldanamycin
Number of Fluorescent Stress Granules
+HIPP (5')
+HIPP (10')
+HIPP (15')
+HIPP (19')
Fluorescent Intensity:

### *3.2.5 Inhibition of Hsp90 does not affect the formation of stress granules*

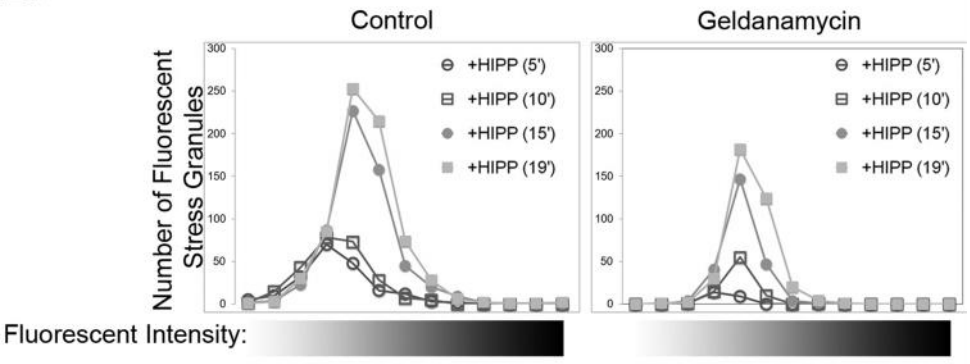
Due to the previously described effects of blocking Hsp90 activity on GW182-positive P bodies, it was necessary to confirm that the inhibition of Hsp90 was not having negative effects on the formation of stress granules. TIA1 cytotoxic granule-associated RNA binding protein (TIA-1) is considered a nucleating factor in stress granule formation (Kedersha et al., 1999) and as such, coalescence of GFP-tagged TIA-1 into nascent stress granules was used to monitor their biogenesis. HeLa cells transiently transfected with pEGFP-C1/TIA1 were pre-treated with 10 µM geldanamycin or DMSO for 2 hours and then stress granule formation was induced during live cell confocal microscopy with 1 µM hippuristanol. In contrast to results with cells expressing GFP-hAgo2, pre-treatment with geldanamycin did not dramatically affect the recruitment of GFP-tagged TIA-1 to stress granules (Figure 3.5A), and this was confirmed by quantitation of the data using Imaris x64 image analysis software (Figure 3.5B).

**Figure 3.5 Inhibition of Hsp90 activity with geldanamycin does not affect formation of GFP-TIA-positive stress granules in response to hippuristanol.** HeLa cells were transiently transfected with plasmids encoding GFP-TIA. At 24 hours post-transfection, cells were treated with DMSO (-) or 10 µM geldanamycin (+) for 6 hours after which hippuristanol was added to a final concentration of 1 µM. Cells were then imaged every 30 seconds for a total of 20 minutes. **A.** Still images are presented from beginning, mid and end time points. The insets in the bottom left of the panels are enlargements of the areas within the squares. Size bar represents 10 µm. **B.** The fluorescent intensities of newly forming stress granules in geldanamycin- (n=20) or DMSO- (control) (n=24) treated samples were measured at regular intervals after addition of hippuristanol.

A.


Geld: -
+
0:00
0:00
10:01
10:01
19:01
19:01

B.


Control
Geldanamycin
Number of Fluorescent Stress Granules
300
250
200
150
100
50
0
+HIPP (5')
+HIPP (10')
+HIPP (15')
+HIPP (19')
Fluorescent Intensity:

### *3.2.6 Hsp90 and a subset of its co-chaperones localize to nascent stress granules*

To determine if Hsp90 complex components are themselves recruited to stress granules and/or P bodies, I examined the relative localizations of Hsp90 and associated co-chaperones under control and stressed conditions. In control cells, Hsp90 was homogenously distributed throughout the cytoplasm and nuclei. However, when translational stress was induced, a pool of Hsp90 was recruited to newly formed stress granules (Figure 3.6A, arrowheads). Similarly, the early and late Hsp90 co-chaperones Hop and Aha1 (Figure 3.6A and B, respectively), as well as the ubiquitous co-chaperone p23 (Figure 3.6C) were recruited to nascent hAgo2-positive stress granules. Hsp90 complexes are defined by the presence of specific co-chaperones and two of the main types are: i) Cdc37-containing, which regulate the maturation of some protein kinases; ii) immunophillin-containing (e.g. FKBP59) that participate in the maturation and transport of steroid hormone receptors (Taipale et al., 2010). In Figures 3.6A and 3.6C it can be seen that Cdc37 but not FKBP59 is recruited to stress granules.

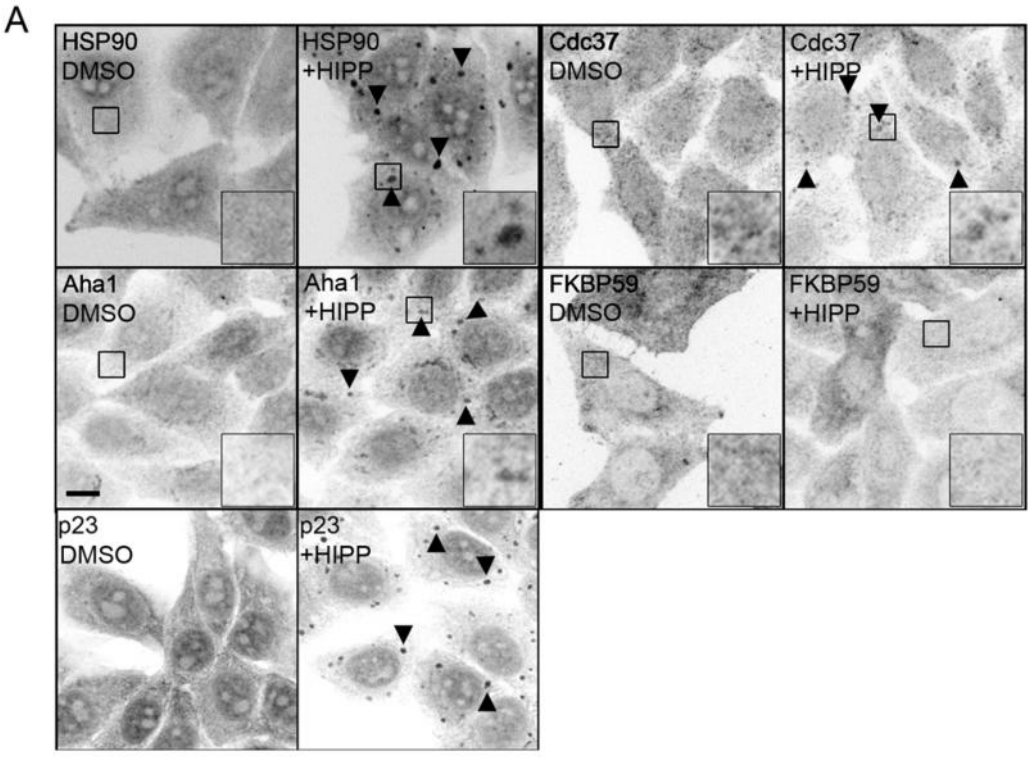
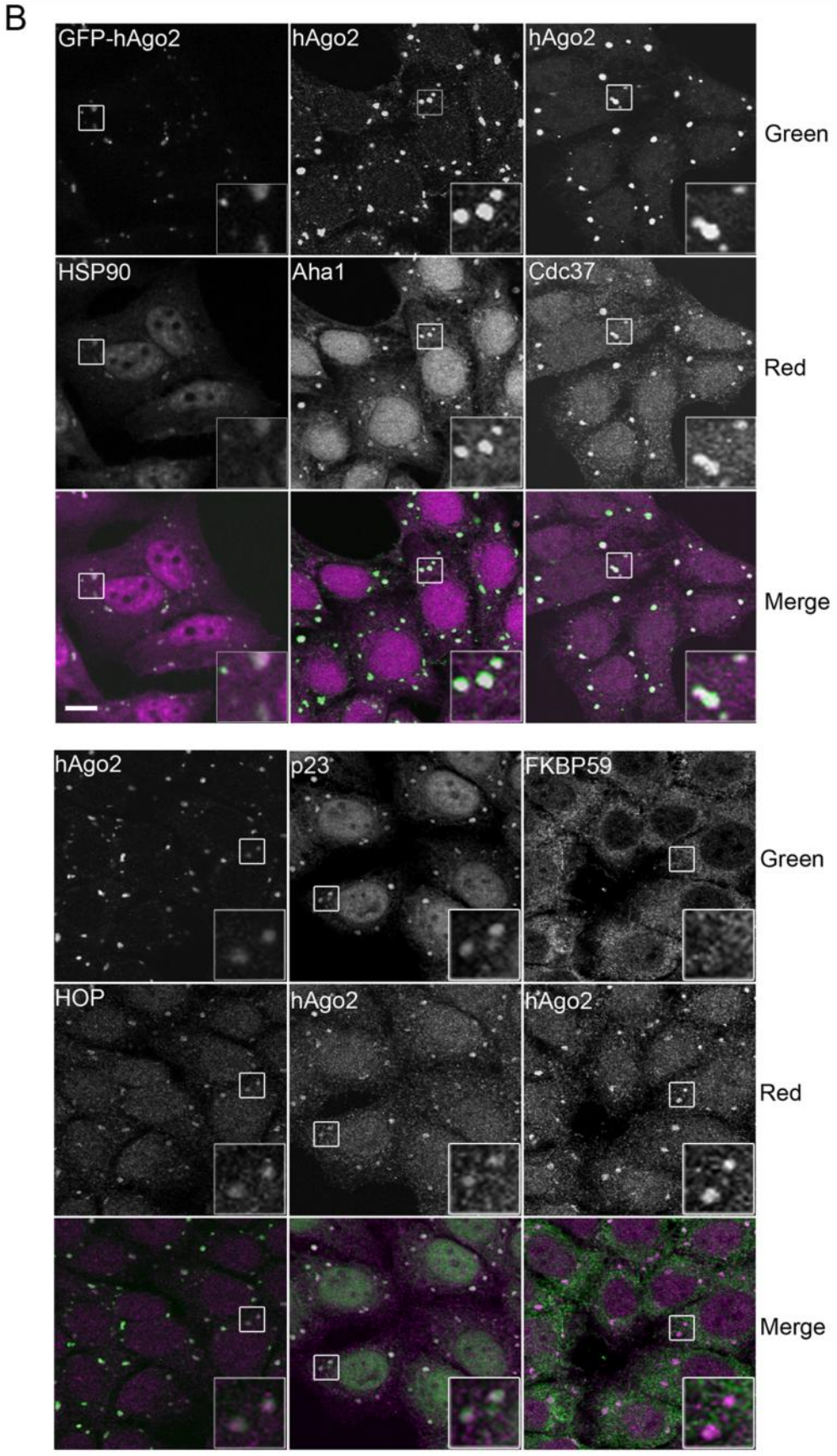


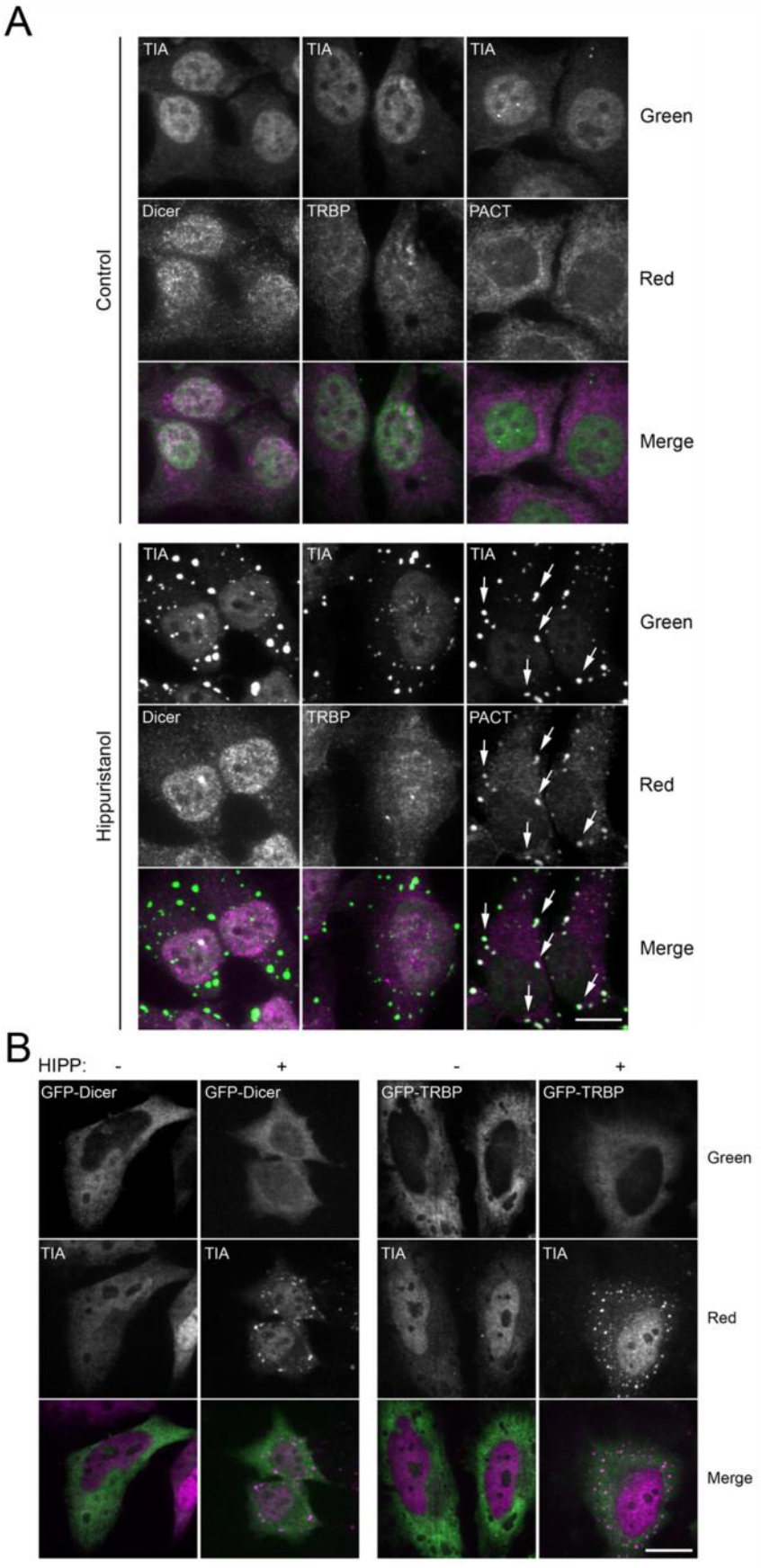

**Figure 3.6 Hsp90 and specific co-chaperones are localized to stress granules formed in response to hippuristanol treatment. A.** HeLa cells were treated with DMSO or 1 µM hippuristanol (+HIPP) for 1 hour before processing for indirect immunofluorescence using antibodies specific for Hsp90, Aha1, Cdc37, p23 and FKBP59. Newly formed stress granules are marked with arrowheads. **B.** Control or stably transfected HeLa cells expressing GFP-hAgo2 were treated with 1 µM hippuristanol for 1 hour. Cells were then processed for indirect immunofluorescence using antibodies specific for hAgo2, Hsp90, Aha1, Cdc37, HOP, p23 or FKBP59. In all cases the inset panels in the bottom right are enlargements of the areas marked by white squares. Size bars represent 10 µm.


B
GFP-hAgo2
hAgo2
hAgo2
Green
HSP90
Aha1
Cdc37
Red
Merge
hAgo2
p23
FKBP59
Green
HOP
hAgo2
hAgo2
Red
Merge

### *3.2.7 PACT, but not Dicer nor TRBP, is recruited to stress granules*

The localization of the hAgo2-binding proteins Dicer, TRBP and PACT in control and stressed cells was also examined as these proteins form a large complex that facilitates maturation of miRNAs and RISC assembly (Lee et al., 2006; Maniataki and Mourelatos, 2005). None of the Argonaute-binding proteins was associated with cytoplasmic foci in control cells, but instead, localized diffusely throughout the cytoplasm and nucleus (Figure 3.7A). The nuclear staining with anti-Dicer and anti-TRBP reagents has been attributed to nonspecific binding because when the experiments were repeated with fluorescently-tagged Dicer and TRBP, it was observed that both proteins were largely confined to the cytoplasm (Figure 3.7B). Similar results were reported by other laboratories (Laraki et al., 2008; Provost et al., 2002). In contrast to Dicer and TRBP, PACT was efficiently recruited to TIA-positive stress granules (Figure 3.7A) indicating that components of the RISC loading complex react differently to translational stress.

**Figure 3.7 PACT, but not Dicer or TRBP, localizes to stress granules formed in response to hippuristanol treatment. A.** HeLa cells were treated with DMSO (Control) or 1 µM hippuristanol for 1 hour before processing for indirect immunofluorescence using antibodies specific for TIA, Dicer, TRBP and PACT. Nascent TIA- and PACT-positive stress granules are indicated with arrows. **B.** HeLa cells were transiently transfected with plasmids encoding GFP-Dicer or GFP-TRBP. Cells were treated with DMSO (-) or 1 µM hippuristanol (+) for 1 hour before processing for indirect immunofluorescence using an antibody specific for TIA. Size bars represent 10 µm.


A
Control
Hippuristanol
TIA
Dicer
TRBP
PACT
Green
Red
Merge
B
HIPP:
-
+
GFP-Dicer
GFP-TRBP

### *3.2.8 PACT and TRBP are not required for the formation of or the recruitment of GFP-hAgo2 to P bodies and stress granules*

The Dicer-binding proteins TRBP and PACT are components of the RISC loading complex, and contribute to efficient miRNA-mediated silencing (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006). Furthermore, it has been suggested that one or both of these co-factors of Dicer may be required for the biogenesis of mature miRNAs (Chendrimada et al., 2005; Lee et al., 2006). To determine whether either of these proteins is required for the formation of P bodies or stress granules, or the recruitment of hAgo2 to stress granules, I used a mouse embryonic fibroblast cells from knockout mice. TRBP- and PACT-deficient cells were treated with either DMSO or 1 µM hippuristanol for 1 hour and then fixed and processed for indirect immunfluorescence using antibodies against GW182, hAgo2 and TIA. Figure 3.8 shows that both GW182-positive P bodies and TIA-positive stress granules are formed in the absence of either TRBP or PACT, and additionally that hAgo2 is efficiently recruited to stress granules in both cases.

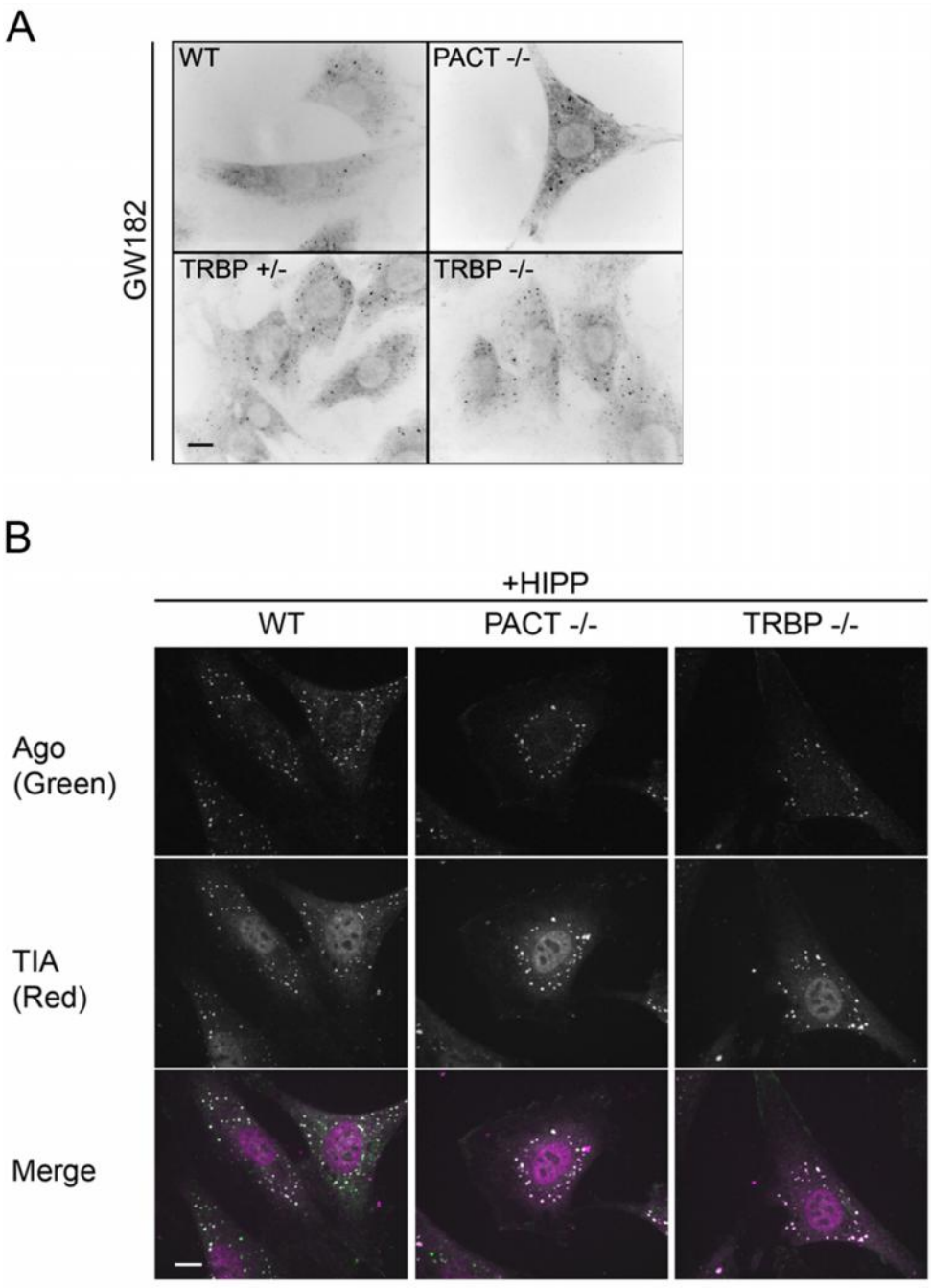



**Figure 3.8 Formation of neither P bodies nor stress granules, and recruitment of endogenous hAgo2 to nascent stress granules, require TRBP or PACT. A.** Mouse embryonic fibroblasts (wildtype (WT), PACT -/-, TRBP +/- and TRBP -/-) were processed for indirect immunofluorescence using an antibody specific for GW182. **B.** Mouse embryonic fibroblasts (wildtype (WT), PACT -/-, and TRBP -/-) were treated with 1 µM hippuristanol for 1 hour and then processed for indirect immunofluorescence using antibodies specific for human Argonaute and TIA. Size bars represent 10 µm.

### *3.2.9 Physical interaction between Hsp90 and human Argonaute 2 is unaffected by inhibition of Hsp90 activity*

To determine whether the co-localization between hAgo2 and Hsp90 complex components at stress granules is due to increased association between hAgo2 and Hsp90, immunoprecipitation of Hsp90 was performed under non-denaturing conditions. Lysates prepared from hippuristanol-treated and control HeLa cells were subjected to immunoprecipitation with mouse anti-Hsp90. Immunoblot analysis was used to determine the relative binding between Hsp90 and Argonaute proteins. Surprisingly, treatment with 1 μM hippuristanol for 90 minutes prior to lysis had no appreciable effect on the amount of Hsp90-associated hAgo2 (Figure 3.9). Similarly, inhibition of Hsp90 activity with 10 μM geldanamycin for 2 hours prior to lysis or treatment with hippuristanol did not significantly affect the binding of Argonautes to Hsp90.

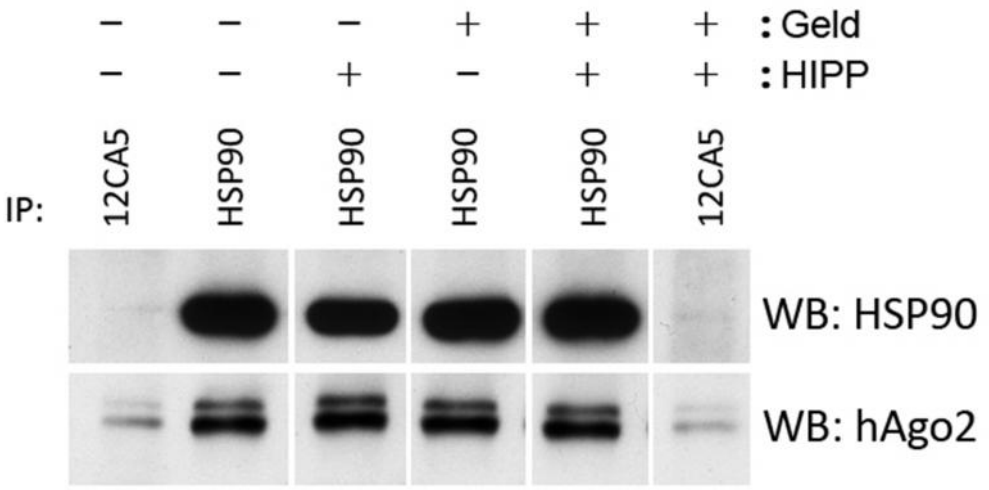
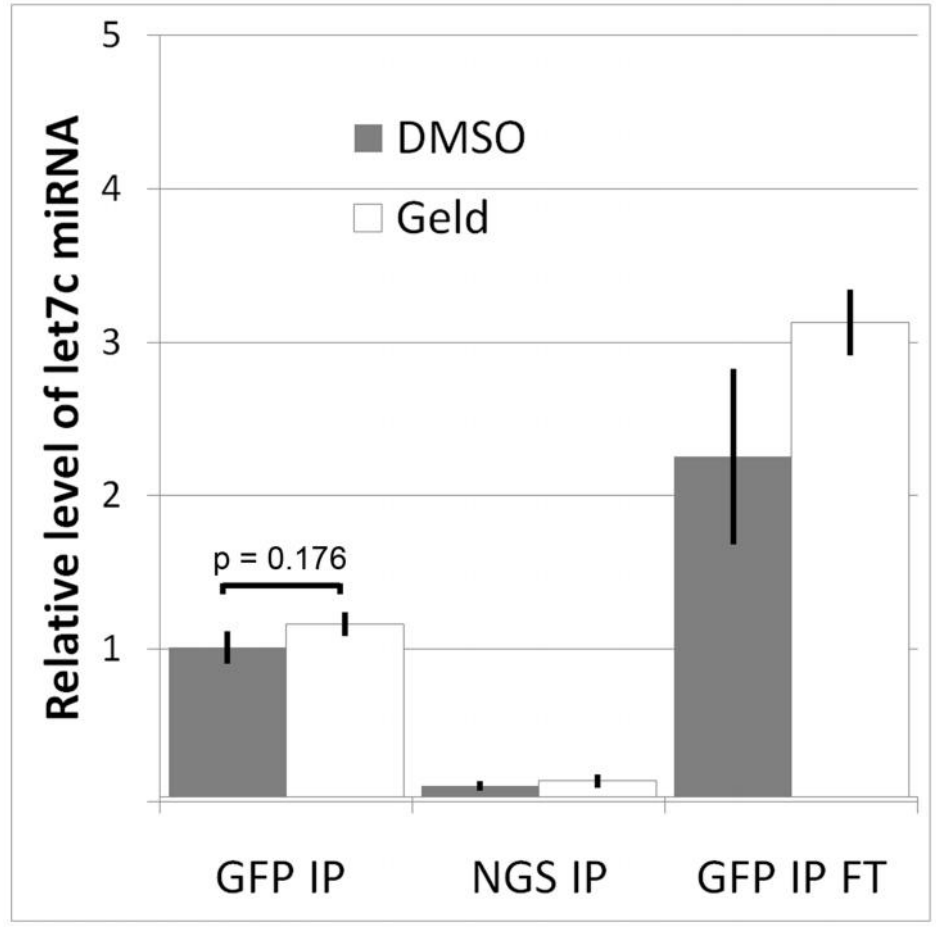



**Figure 3.9 Human hAgo2 interacts with Hsp90 and this interaction is not affected by treatment with geldanamycin or hippuristanol.** HeLa cells treated with DMSO (-), 1 µM hippuristanol for 2 hours (HIPP), 10 µM geldanamycin for 2 hours (Geld) or both drugs were subjected to immunoprecipitation with an antibody specific for Hsp90, followed by immunoblotting with antibodies specific for Hsp90 and human Argonaute. Immunoprecipitation with anti-HA (12CA5) is shown as a control.

### *3.2.10 Association of miRNAs with pre-existing GFP-hAgo2 is unaffected by inhibition of Hsp90*

Although Argonaute complexes can be programmed with small RNAs that do not require Dicer processing (Elbashir et al., 2001a), RISC activation is much more efficient when coupled to Dicer activity (Chendrimada et al., 2005; Gregory et al., 2005; Maniataki and Mourelatos, 2005; Pham et al., 2004). Given that formation of stable Dicer/Argonaute complexes is dependent on Hsp90 activity (Tahbaz et al., 2004), I questioned whether this chaperone is also required for the loading of small RNAs onto Argonaute. To address this question, I compared the amount of let-7 miRNA-associated with GFP-hAgo2 in cells that had been treated with or without 10 μM geldanamycin. Expression of GFP-hAgo2 was induced for 6 hours in stably transfected cells prior to introduction of geldanamycin. Induction of GFP-hAgo2 and inhibition of Hsp90 was continued for an additional 12 hours, after which immunoprecipitations were performed using anti-GFP antibodies. RNA was isolated from load, flow-through and bound fractions and levels of let-7c miRNA were analyzed by quantitative PCR. Results presented in Figure 3.10 show that, while equivalent amounts of GFP-hAgo2 were immunoprecipitated, levels of let-7c miRNA associated with GFP-hAgo2 were unaffected by geldanamycin treatment. Furthermore, the total level of let-7c miRNA remains unchanged after 12 hr of geldanamycin treatment.



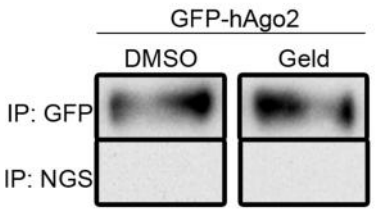




**Figure 3.10 Association of let-7c miRNA with GFP-hAgo2 is unaffected by geldanamycin.** Cells expressing GFP-hAgo2 were treated with DMSO or 10 µM geldanamycin (Geld) for 12 hours after which time GFP-hAgo2 was immunoprecipitated using an antibody specific for GFP or normal goat serum as a control (NGS). Total RNA was isolated from bound (GFP IP), control bound (NGS IP) and unbound (GFP IP FT) and the level of let-7c miRNA present in each fraction was determined by RT-qPCR. Samples were also subjected to western blotting and levels of immunoprecipitated GFP-hAgo2 were determined using an antibody specific to GFP. Error bars indicate standard error of the mean, *p* values are displayed..

### 3.3 Summary

In this section, a putative role for the molecular chaperone Hsp90 during the maturation and/or activation of hAgo2, and potentially other human Argonaute proteins, was described. The effects of geldanamycin on the association of nascent GFP-hAgo2 with existing P bodies, formation and/or stability of existing P bodies and the expression of endogenous miRNA targets (e.g. Ras, cytochrome *c*) strongly suggest a role for Hsp90 in the miRNA pathway. While the mechanism was not fully determined, due to both the reduced association of GFP-hAgo2 with nascent stress granules and that localization of hAgo2 to stress granules is dependent on association with small RNAs (Leung et al., 2006), it was speculated that inhibition of Hsp90 results in a decreased amount of small RNAs associated with RISC. Indeed, a series of papers published following our report show that the loading of RNA duplexes requires both ATP (Yoda et al., 2010) and the activity of the Hsc70/Hsp90 complex (Iki et al., 2010; Iwasaki et al., 2010; Miyoshi et al., 2010). However, our data demonstrating no reduction in the amount of the miRNA let-7c associated with immunoprecipitated GFP-hAgo2 following inhibition of the Hsp90 activity appear to contradict our initial conclusion, as well as the conclusions of the aforementioned reports. It is likely that RISC complexes are very stable once formed; therefore, we may have been unable to detect a difference in loading due to a prevalence of loaded Argonautes formed prior to the addition of geldanamycin. It is also possible that Hsp90 activity is required only to accelerate or enhance the efficiency of loading and, with prolonged treatments with geldanamycin, RISC would still be loaded with miRNAs despite a lack of chaperone activity, albeit with decreased kinetics. Further complicating the conclusion that Hsp90 activity is required for loading miRNAs into hAgo2, and that decreased loading of GFP-hAgo2 is responsible for the impaired recruitment to stress granules, is the observation that stress granules form normally in both PACT-deficient and TRBP-deficient cell lines and that hAgo2 is efficiently

recruited to them. It is clear that a better understanding of the involvement and role of Hsp90 in the maturation and activation of hAgo2 within the context of the miRNA-mediated silencing pathway is required in order to resolve these outstanding issues.

I have also demonstrated that a specific subset of Hsp90 complexes associate with stress granules. As mentioned, two major classes of Hsp90 complexes are classified as containing either Cdc37 or immunophillins. Here I have shown that Cdc37 co-localizes with Hsp90 and hAgo2 at stress granules, while the immunophillin FKBP59 does not. These data led me to speculate that there may be specific sub-groups of Hsp90 co-chaperones that are required for the maturation and/or activation of hAgo2 by Hsp90 and specifically that the Cdc37 co-chaperone may have a role in regulating the function and/or localization of the RNAi machinery.

# CHAPTER 4

Geldanamycin impairs target-binding by hAgo2 *in vitro*

## 4.1 Rationale

After the results detailed in chapter 3 of this thesis were published, a number of groups demonstrated that the loading of RNA duplexes onto Argonaute proteins requires both ATP (Leung et al., 2006) and the activity of the Hsp90 chaperone complex (Iki et al., 2010; Iwasaki et al., 2010; Miyoshi et al., 2010). Iwasaki et al. (2010) reported that, if a siRNA duplex was loaded prior to the inhibition of Hsp90, hAgo2 was able to cleave a target RNA efficiently in the absence of Hsp90 activity. However, the majority of miRNA-mediated silencing in mammalian systems occurs in a cleavage-independent manner (Baek et al., 2008; Guo et al., 2010). Silencing by a cleavage-independent mechanism suggests that the ability of RISC to stably associate with the targeted mRNA is more significant for efficient silencing than its ability to cleave the mRNA.

Endogenous miRNAs can direct the cleavage of an mRNA with a perfectly complementary binding site (Hutvagner and Zamore, 2002; Song et al., 2004; Yekta et al., 2004). As cleavage is a result of complete base-pairing throughout the miRNA sequence, an extensive duplex must form between miRNAs and their target. Moreover, in order for the hAgo2 to cleave the mRNA, the duplex must be incorporated within the structure of hAgo2 such that the substrate is positioned adjacent to the active site within the PIWI domain. However, nascent, unloaded hAgo2 is unable to accommodate a RNA duplex without chaperone activity (Iki et al., 2010; Iwasaki et al., 2010; Miyoshi et al., 2010). In view of this, I hypothesized that the incorporation of a miRNA-target duplex into the structure of hAgo2 would require the activity of Hsp90. Consistent with this prediction, structural studies of *Thermus thermophilus* Argonaute show considerable rearrangement of the amino-terminal and PAZ domains, relative to the MID and PIWI domains, during target-binding (Wang et al., 2008). Therefore, I predicted that inhibition of Hsp90 activity would impair the ability of hAgo2 to stably associate with a targeted mRNA.

## 4.2 Results

### *4.2.1 Inhibition of Hsp90 activity with geldanamycin impairs target-binding by hAgo2 in vitro*

Hutvagner et al. (2004) reported that modified antisense oligonucleotides can be used to block small RNA-mediated post-transcriptional gene silencing (Hutvagner et al., 2004). A modification replacing the hydroxyl group at the 2' position of the ribose with an O-methyl group renders an oligonucleotide containing these bases resistant to endonucleolytic cleavage (Inoue et al., 1987). These modified oligonucleotides can also be used in sequence-specific purification of RISC (Aoki et al., 2007; Fabian et al., 2009; Jannot et al., 2011; Mayr et al., 2007; Yigit et al., 2006). Specifically, a biotinylated 2'-O- methylated oligonucleotide antisense to guide miRNA is bound by RISC and pulled down using streptavidin- coupled magnetic beads.

The human hepatoma cell line, Huh7.5, is commonly used as a cell culture model for Hepatitis C virus infection. This cell line was chosen for these experiments as the liver-specific miRNA miR-122 is the predominant miRNA species in human liver and Huh7.5 cells (Randall et al., 2007). In human liver, miR-122 accounts for greater than 70% of all miRNA species cloned, whereas in the Huh7.5 hepatoma-derived cell line it represents 23% of all cloned miRNAs (Randall et al., 2007). We anticipated that this biased expression of a single miRNA species would provide the basis for isolating a significant portion of RISC.

A biotinylated 2'-O-methylated oligonucleotide antisense to miR-122 of *Homo sapiens* was adsorbed to streptavidin-coupled magnetic beads. An oligonucleotide antisense to *Caenorhabditis elegans* miR-35 was used as a control. Huh7.5 cells were lysed by dounce homogenization. Lysates were aliquoted and treated with either DMSO or 100 µM geldanamycin immediately prior to incubation with oligonucleotide-coated beads. The relative level of bead-associated RISC was determined by quantitative western blot analysis. Inhibition

of Hsp90 activity significantly reduced the association of hAgo2 with the 2’-O-methylated oligonucleotide-adsorbed beads (Figure 4.1).

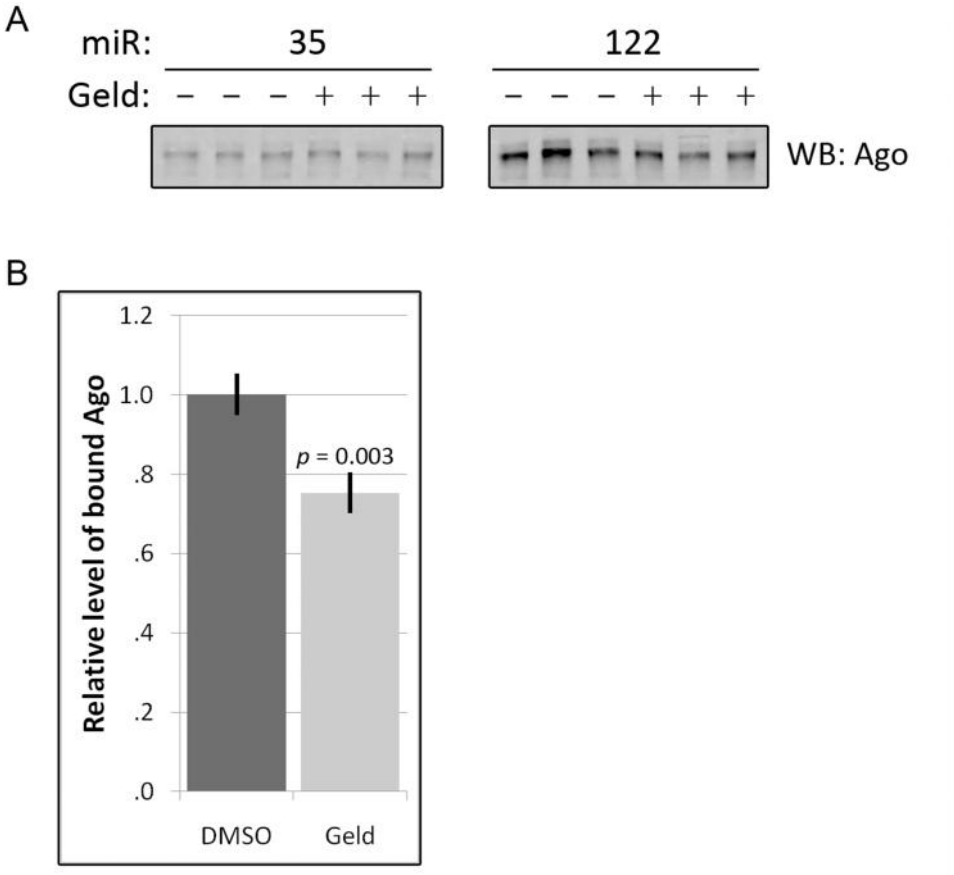



**Figure 4.1 Inhibition of Hsp90 activity with geldanamycin impairs target-binding by hAgo2 *in vitro*. A.** Lysates of Huh7.5 were treated with DMSO (-) or 100 µM geldanamycin (+) prior to pulldown with a 2'-O-methylated oligonucleotide antisense to miR-122. An oligonucleotide antisense to *Caenorhabditis elegans* miR-35 was used as a control. The levels of bound hAgo2 were determined using a PAZ domain-specific polyclonal antibody. **B.** Quantitation of the relative level of hAgo2 is shown for the DMSO- and geldanamycin- treated (Geld) samples. Amount of hAgo2 non-specifically associated with miR-35 beads was subtracted as background. Error bars indicate the standard error of the mean, *p* values are displayed..

## 4.3 Summary

Establishing a stable interaction between miRISC and a targeted mRNA is predicted to be an important factor in maintaining miRNA-mediated post transcriptional gene silencing (Bartel, 2009). This stable association is expected to involve the formation of a partially complementary duplex between the guide strand of the miRNA and the target mRNA. Given the requirement for Hsp90 chaperone activity for efficient loading of a miRNA duplex onto Argonaute proteins, I hypothesized that the stable interaction of RISC with a target mRNA would also require Hsp90 activity. In this chapter, I demonstrate that inhibition of Hsp90 impairs the interaction of hAgo2 with a target RNA in vitro.

As this effect was examined using a single miRNA-target interaction, the universal requirement for Hsp90 activity in target binding remains unclear. It is tempting to speculate that as the complementarity between the guide and the target decreases, resulting in duplexes with increasingly bulged structures, Hsp90 activity may be vital for establishing stable association of hAgo2 with a targeted mRNA. This hypothesis posits that a perfectly complementary duplex would be more readily incorporated into the structure of the Argonaute protein than would a more bulged duplex.

These results suggest that hAgo2 can bind to a target RNA when ATP-binding by Hsp90 is inhibited. However, it is important to note that while chaperones often increase the efficiency of specific processes, they may not be absolutely required for these processes to occur. Moreover, as geldanamycin cannot displace ATP from the binding pocket of Hsp90, these results cannot account for target-binding by hAgo2 in pre-existing complexes with ATP-bound Hsp90. As a result, a more detailed understanding of the requirement for Hsp90 activity in the target-binding by RISC *in vivo* is required.

# CHAPTER 5

Specific Hsp90 co-chaperones are required for efficient small RNA-mediated post-transcriptional gene silencing

## 5.1 Rationale

In chapter 3, I described the identification of a subset of Hsp90 co-chaperones that co-localize with hAgo2 to stress granules. These include the kinase-specific co-chaperone Cdc37 as well as the generic co-chaperone Aha1. Additionally, the immunophillin FKBP52 was shown not to localize to these granules. Generally, the function of co-chaperones is to modulate the biochemical activity of Hsp90 by guiding the initial recognition of specific clients in addition to regulating different aspects of the chaperone cycle. Therefore, I hypothesize that one or more of the co-chaperones that are recruited to stress granules are necessary for hAgo2 function.

A short-hairpin RNA (shRNA)-based approach was used to knock down the expression of Aha1, Cdc37 and FKBP52 in order to study the effects of these co-chaperones on the RNAi pathway. If my hypothesis is correct, knocking down the expression of a co-chaperone required for the maturation and function of hAgo2 would decrease the efficiency of small RNA-mediated post-transcriptional gene silencing. In the present chapter, I identify a role for two of these co-chaperones in the siRNA- and miRNA-mediated post-transcriptional gene silencing pathways.

## 5.2 Results

### *5.2.1 Reporters for assaying the efficiency of small RNA-mediated gene silencing*

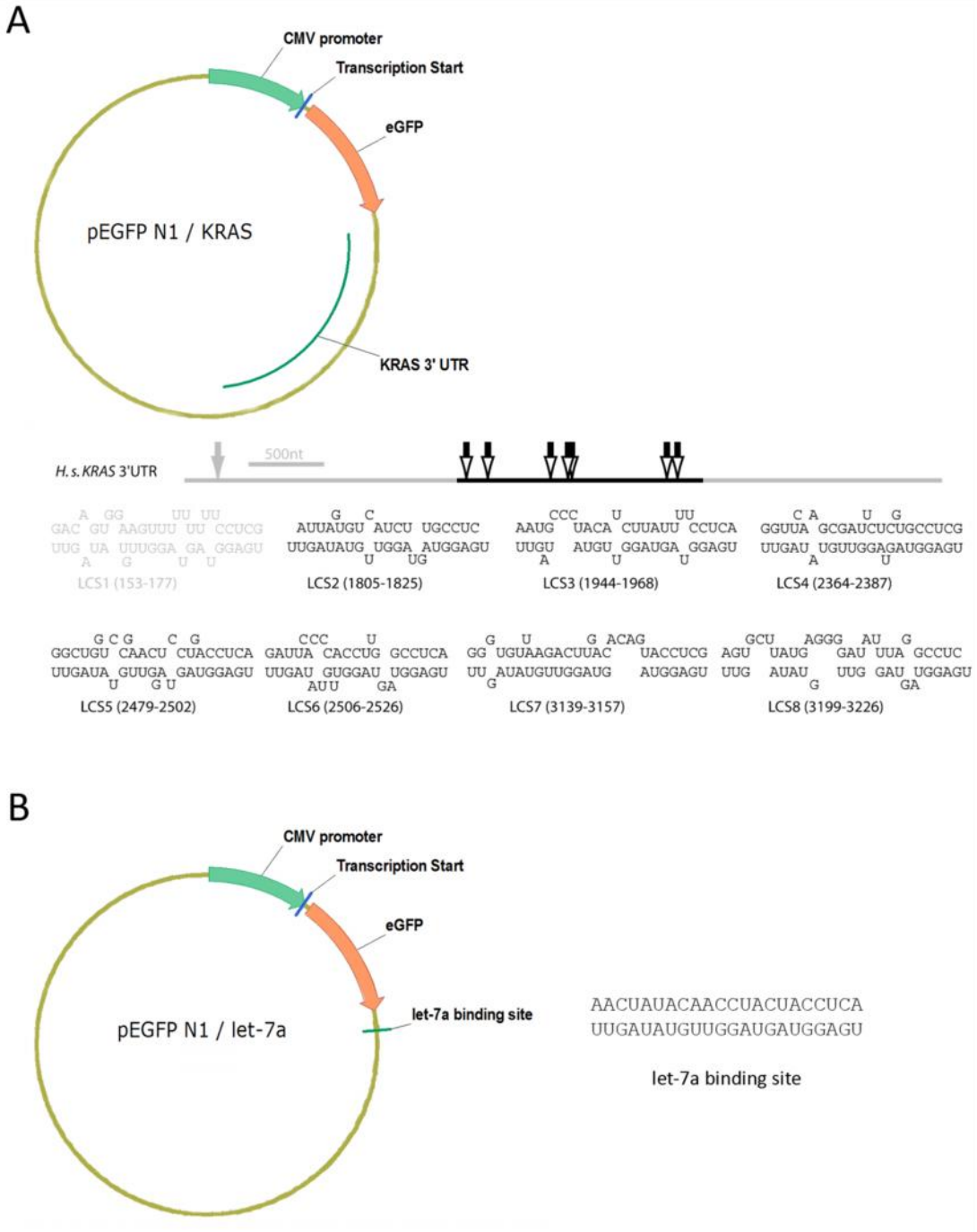
A number of reporter-based systems have been used to assay miRNA-mediated silencing (McClure et al., 2011; Sullivan and Ganem, 2005). Generally, the 3' untranslated region (UTR) of an endogenous miRNA target is subcloned downstream of the open reading frame of a reporter such as luciferase or GFP. As a result of the miRNA-binding sites within the 3' UTR, the level of translation

from this mRNA is significantly reduced as compared to an mRNA transcribed from a control construct that lacks the miRNA target site. This reduced protein expression is attributed to miRNA-mediated, cleavage-independent, post-transcriptional gene silencing. In this study, a reporter containing the 3' UTR of the KRAS mRNA, sub-cloned into the 3' UTR of a GFP reporter was used (GFP-KRAS, Figure 5.1). This 3' UTR contains binding sites that are targeted by members of the let-7 family of miRNAs and is sufficient to reduce the translation of a reporter mRNA (Johnson et al., 2005) (Figure 5.2).

A second type of reporter system relies on a cleavage-dependent mechanism of silencing. Generally, a sequence with perfect complementarity to an endogenous miRNA is sub-cloned into the 3' UTR of the reporter construct. Endogenous miRNAs guide hAgo2-containing RISC to mRNAs produced from this plasmid resulting in cleavage by the endonuclease activity of hAgo2. In this study, a cassette encoding a sequence with perfect complementarity to the let-7a miRNA was cloned into the 3' UTR of a GFP reporter (GFP-let7a, Figure 5.1). This strategy significantly reduces the expression of the reporter protein by destabilizing the mRNA (Figure 5.2).

It should be noted that, in this study, pEGFP-C1 was used as a GFP only control. The GFP protein encoded by pEGFP-C1 is 26 amino acids larger than the GFP encoded by pEGFP-N1, accounting for thedifference in size as detected by immunoblot analysis.

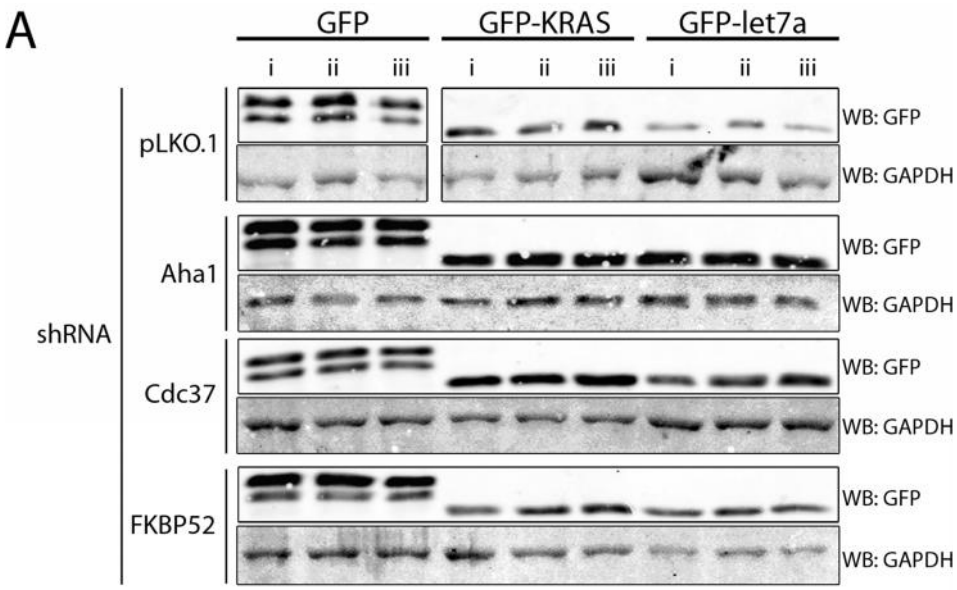
**Figure 5.1 GFP-based reporters for assaying the efficiency of miRNA-mediated gene silencing. A.** Schematic representing the reporter plasmid encoding the 3' UTR of KRAS subcloned downstream of a GFP reporter. The full-length human KRAS 3' UTR has eight potential let-7 complementary sites. Hypothesized duplexes formed by mRNA sites (top) and let-7a miRNA (bottom) are shown. Region of KRAS 3' UTR used in reporter vector and sites within that region are shown in black. **B.** Schematic represents the reporter plasmid encoding the cassette with perfect complementarity to let-7a, subcloned downstream of a GFP reporter. Duplex between the mRNA site (top) and the let-7a miRNA (bottom) is shown. Adapted from (Johnson et al., 2005).

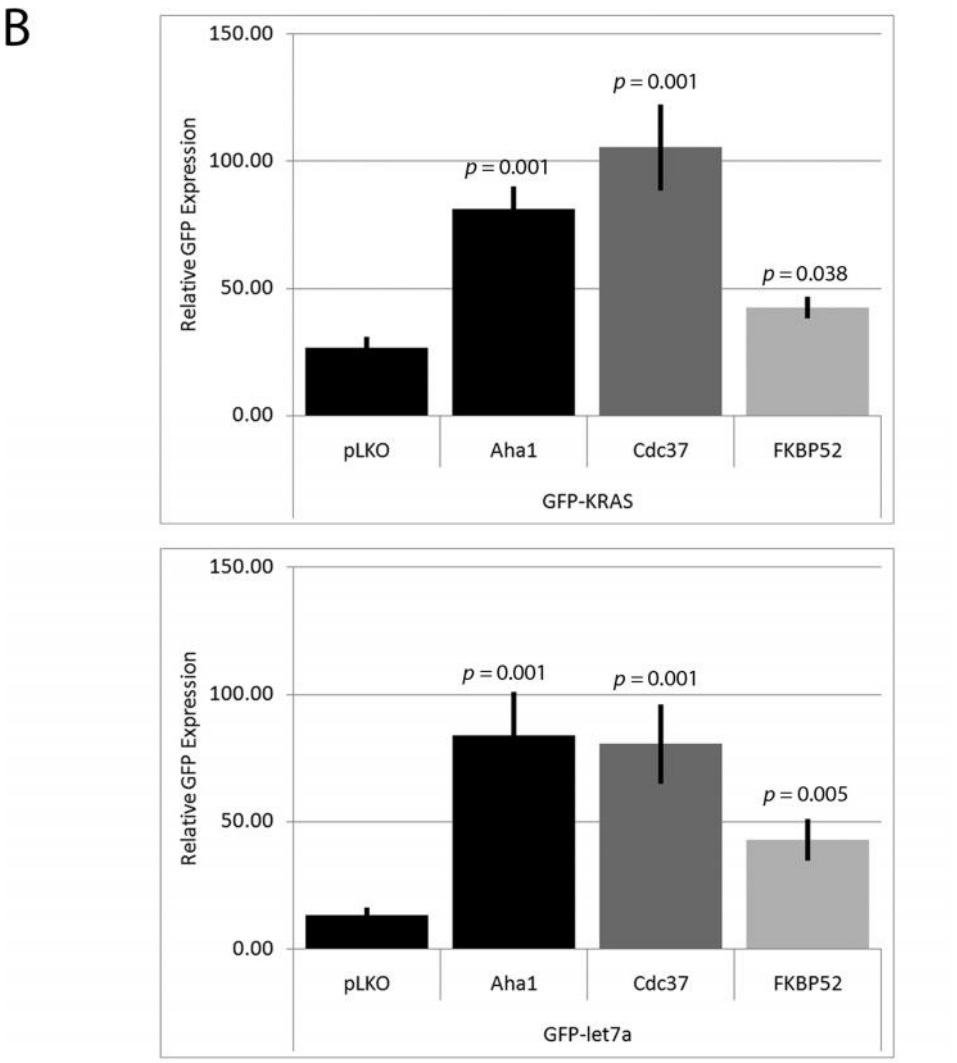

A
CMV promoter
Transcription Start
eGFP
pEGFP N1 / KRAS
KRAS 3' UTR
H. s. KRAS 3'UTR
500nt
LCS1 (153-177)
LCS2 (1805-1825)
LCS3 (1944-1968)
LCS4 (2364-2387)
LCS5 (2479-2502)
LCS6 (2506-2526)
LCS7 (3139-3157)
LCS8 (3199-3226)
B
CMV promoter
Transcription Start
eGFP
pEGFP N1 / let-7a
let-7a binding site
AACUAUACAACCUACUACCUCA
UUGAUAUGUUGGAUGAUGGAGU
let-7a binding site

### *5.2.2 Knockdown of Aha1 and Cdc37 impairs small RNA-mediated gene silencing*

To determine if specific Hsp90 co-chaperones are required for efficient miRNA- or siRNA-mediated post-transcriptional gene silencing, I used the GFP-based RNAi reporters detailed above in combination with shRNA-based knockdown of co-chaperones. It was determined empirically that the most robust knockdown of Aha1 and FKBP52 was seen at 72 hours post-transfection with the shRNA plasmids. The co-chaperone Cdc37 is essential for long-term viability (Tatebe and Shiozaki, 2003; Westwood et al., 2004) and therefore, cells treated with Cdc37 shRNAs were harvested at 48 hours to limit cell death. Control cells transfected with pLKO.1 (the parent vector lacking shRNA cassette) show efficient miRNA-mediated gene silencing of the GFP-KRAS and GFP-let7a reporters (Figure 5.2A and B). Knocking down expression of the Hsp90 co-chaperones Aha1 and Cdc37, but not FKBP52, resulted in a near complete loss of miRNA-mediated, cleavage-independent silencing of the GFP-KRAS reporter (Figure 5.2A and B). Depletion of these co-chaperones had a similar effect on cleavage-dependent silencing of the GFP-let7a reporter (Figure 5.2A and B). Unexpectedly, knocking down FKBP52 expression also reduced the efficiency of miRNA-mediated silencing of the GFP-let7a construct, albeit to a lesser extent than knockdown of Aha1 and Cdc37 (Figure 5.2A and B).

**Figure 5.2 Knockdown of Hsp90 co-chaperones Aha1 and Cdc37 impairs miRNA-mediated silencing of a GFP reporter. A.** HeLa cells were transiently transfected with plasmids encoding GFP, GFP-KRAS or GFP-let7a and shRNAs to silence the expression of Aha1, Cdc37, FKBP52, or vector only control (pLKO.1). 24 hours following transfection, cells containing shRNA plasmids were positively selected by growth in media containing 2.5 μg/mL of puromycin. Cells knocked down for Cdc37 were harvested 48 hours post-transfection while all other samples were harvested 72 hours post-transfection. GFP expression was determined by quantitative western blot analysis. GAPDH levels are shown as loading controls. Lower case Roman numerals indicate triplicate samples. **B.** Quantitation of the level of GFP, normalized to GAPDH and relative to the GFP control, is shown for the GFP-KRAS (-KRAS) and GFP-let7a (-let7a) constructs for each co-chaperone knockdown. Error bars indicate standard error of the mean, *p* values are shown.


A
GFP
GFP-KRAS
GFP-let7a
i
ii
iii
shRNA
pLKO.1
Aha1
Cdc37
FKBP52
WB: GFP
WB: GAPDH


B
Relative GFP Expression
150.00
100.00
50.00
0.00
p = 0.001
p = 0.001
p = 0.038
pLKO
Aha1
Cdc37
FKBP52
GFP-KRAS
Relative GFP Expression
150.00
100.00
50.00
0.00
p = 0.001
p = 0.001
p = 0.005
pLKO
Aha1
Cdc37
FKBP52
GFP-let7a

### *5.2.3 Knockdown of Aha1, Cdc37 or FKBP52 does not affect the ability of tethered hAgo2 to silence translation*

miRNAs function to guide hAgo2-containing protein complexes to the 3' UTR of mRNAs. Following the interaction of hAgo2 with the targeted mRNA, additional protein factors (e.g. GW182) are recruited, leading to a decrease in translation as well as a destabilization of the mRNA. Artificially tethering hAgo2 to the 3' UTR of a reporter mRNA leads to a decrease in the translation of the reporter in a miRNA-independent manner (Eckhardt et al., 2011; Pillai et al., 2004). This can be done using tandem repeats of the BoxB hairpins cloned into the 3' UTR of a reporter (e.g. GFP) mRNA (GFP-BoxB). When co-expressed with HA-tagged hAgo2 fused to the lambdaN protein (NHA-hAgo2), the lambdaN protein will bind to the tandem BoxB hairpin repeats in the 3' UTR of the GFP-BoxB mRNA, thereby tethering hAgo2 to the 3' UTR. HA-tagged fusion of LambdaN and the LacZ protein (NHA-LacZ) was used as a non-silencing control, and HA-tagged hAgo2 (HA-hAgo2) served as a non-tethering control. Using this system, I investigated whether the additional protein factors recruited to the message, following hAgo2 binding, were still able to efficiently silence the translation of an mRNA.

Expression of specific Hsp90 co-chaperones was knocked-down using shRNAs as described above. In all cases, the knockdown of Hsp90 co-chaperones had no effect on the ability of the lambdaN-HA-tagged hAgo2 to silence expression of the GFP reporter (Figure 5.3A and B).

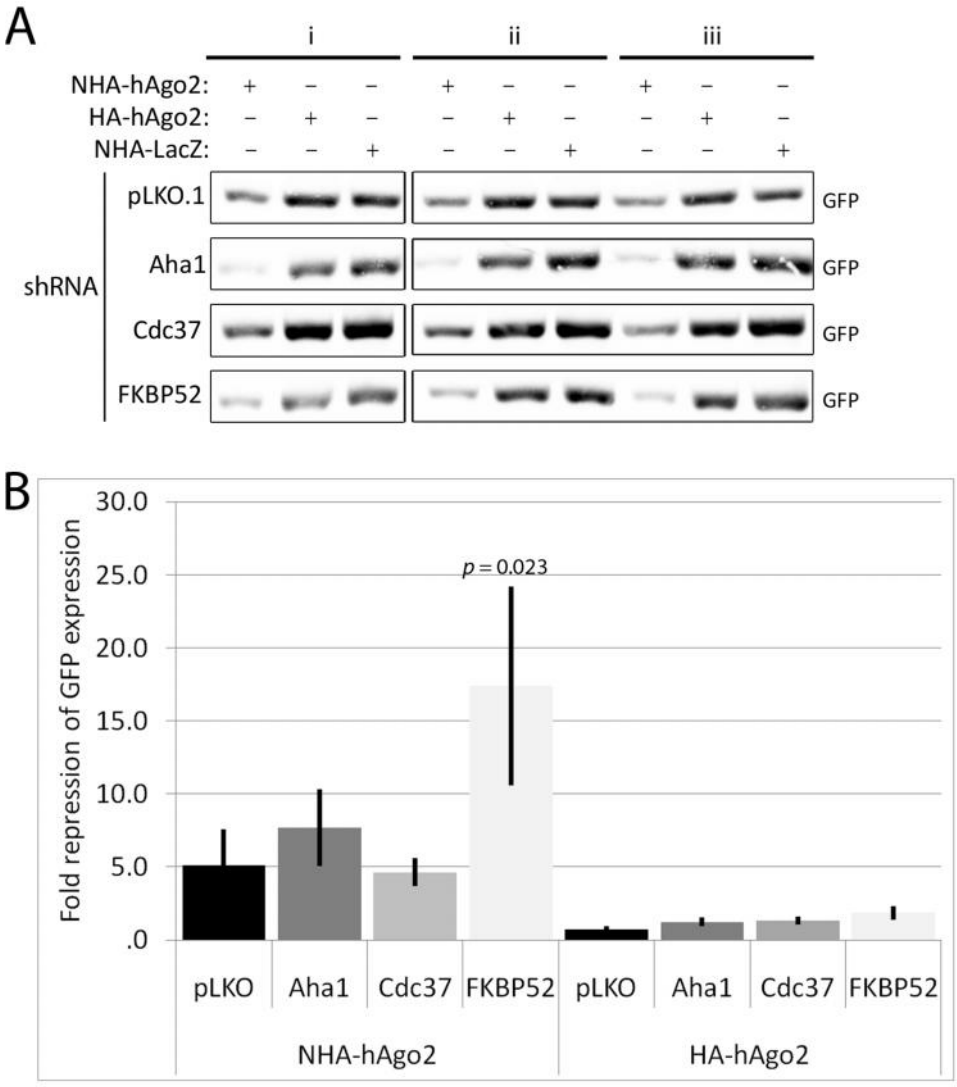


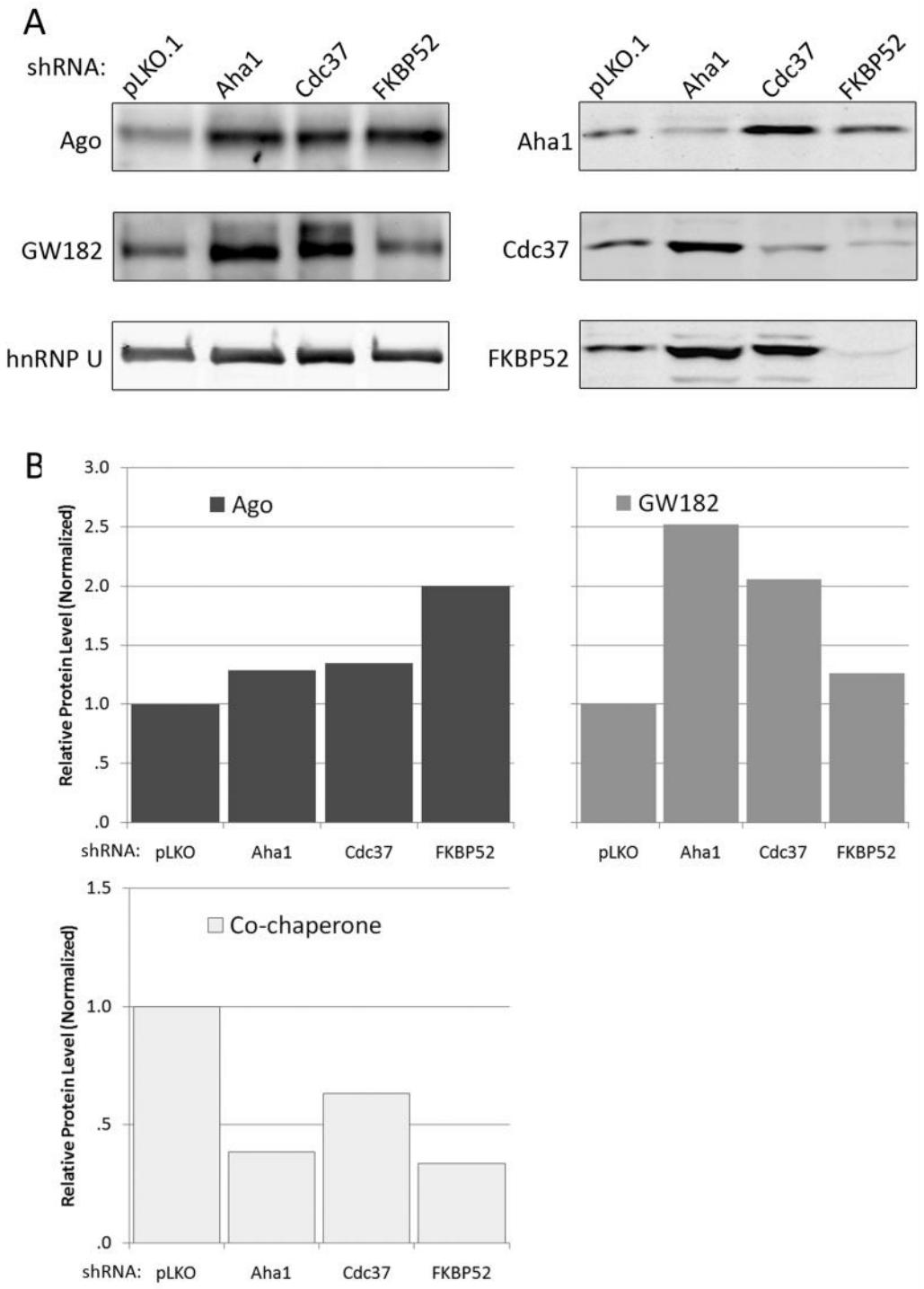

**Figure 5.3 Knockdown of Aha1, Cdc37 or FKBP52 does not impair silencing of a GFP reporter when hAgo2 is artificially tethered to the mRNA. A.** HeLa cells were transiently transfected with plasmids expressing NHA-hAgo2, NHA-LacZ, HA-hAgo2 along with the GFP-BoxB reporter. Also included were shRNAs to silence the expression of Aha1, Cdc37, FKBP52 or vector only control (pLKO.1). 24 hours following transfection, cells containing shRNA plasmids were positively selected by growth in media containing 2.5 µg/mL of puromycin. Cells knocked down for Cdc37 were harvested 48 hours post-transfection while all other samples were harvested 72 hours post-transfection. Level of GFP expression was determined by quantitative western blot analysis using a polyclonal antibody specific for GFP. **B.** The fold-repression of expression observed, relative to the NHA-LacZ non-silencing control, is shown for each co-chaperone knockdown. Error bars indicate standard error of the mean, *p* values are displayed.

### *5.2.4 The levels of hAgo2 and GW182 proteins are unaffected by knockdown of Aha1, Cdc37 or FKBP52*

The GFP-let7a reporter construct is transcribed to generate a mRNA that is targeted for cleavage by hAgo2-containing RISC by virtue of containing a miRNA-binding site with perfect complementarity to the let-7a miRNA. In the case of the GFP-KRAS reporter construct, the expression of GFP is limited by miRNA-mediated repression of translation, via a mechanism that is independent of mRNA cleavage by hAgo2. Decreased hAgo2 protein levels could, in theory, account for the reduced efficiency of both cleavage-dependent and cleavage-independent silencing described in Section 5.2.1. Moreover, GW182 interacts with Argonautes and is necessary for cleavage-independent miRNA-mediated silencing (Jakymiw et al., 2005; Liu et al., 2005a; Meister et al., 2005). Therefore, an alternative explanation for the reduced efficiency of silencing, which is not mutually exclusive with a reduction in hAgo2 levels, is reduced accumulation of GW182.

To determine if the levels of either hAgo2 or GW182 were affected by the knockdown of Aha1, Cdc37 or FKBP52, whole cell lysates were analyzed by quantitative western blot. Immunoblotting for Aha1, Cdc37 and FKBP52 showed efficient knockdown of each co-chaperone (Figure 5.4A and B). Surprisingly, reduction of Aha1 and FKBP52 resulted in increased steady-state levels of hAgo2 and GW182 (Figure 5.4A and B). While unexpected, these data show that the observed impairment of post-transcriptional gene silencing is not the result of decreased steady-state levels of hAgo2 or GW182.

**Figure 5.4 Knockdown of Aha1, Cdc37 or FKBP52 does not reduce the steady-state levels of hAgo2 or GW182. A.** HeLa cells were transiently transfected with plasmids expressing shRNAs to silence the expression of Aha1, Cdc37, FKBP52 or vector only control (pLKO.1). 24 hours following transfection, cells containing shRNA plasmids were positively selected by growth in media containing 2.5 µg/mL of puromycin. Cells knocked down for Cdc37 were harvested 48 hours post-transfection while all other samples were harvested 72 hours post-transfection. The levels of co-chaperones, hAgo2 (Ago) and GW182 expression were determined by quantitative western blot analyses. The level of heterogeneous nuclear ribonucleoprotein U (hnRNP U) is shown as a loading control. **B.** Quantitation of the levels of hAgo2, GW182 and co-chaperones, normalized to hnRNP U and relative to pLKO.1-transfected control, are shown for Aha1, Cdc37 and FKBP52 knockdowns.


A
shRNA:
pLKO.1
Aha1
Cdc37
FKBP52
Ago
GW182
hnRNP U
Aha1
Cdc37
FKBP52
B
Ago
Relative Protein Level (Normalized)
3.0
2.5
2.0
1.5
1.0
.5
.0
shRNA: pLKO Aha1 Cdc37 FKBP52
GW182
pLKO Aha1 Cdc37 FKBP52
Co-chaperone
1.5
1.0
.5
.0
shRNA: pLKO Aha1 Cdc37 FKBP52

### *5.2.5 Knockdown of Aha1, Cdc37 or FKBP52 does not affect steady-state levels of the let-7 miRNA family*

Hsp90 activity mediates the loading of small RNA duplexes onto Argonaute proteins (Iki et al., 2010; Iwasaki et al., 2010; Miyoshi et al., 2010). As critical modulators of Hsp90, I speculated that certain co-chaperones are required for this step in the activation of hAgo2. I also predicted that inhibiting the loading of miRNA duplexes onto Argonaute proteins by knocking down these required co-chaperones would result in decreased miRNA stability and accumulation. As the reporters for RNAi-mediated silencing used in this study were targets of the let-7 family of miRNAs, my analysis of miRNA levels focused on this group of 14 miRNAs.

Following shRNA-mediated knockdown of Hsp90 co-chaperones, the level of miRNAs was monitored by microarray profiling. Pure RNA of high integrity (i.e. no detectable degradation), isolated from cells in which Aha1, Cdc37 or FKBP52 levels were reduced, was submitted to the Alberta Transplant Applied Genomics Centre for analysis. Microarray profiling of microRNA expression showed no changes outside the threshold of greater than two-fold in the relative levels of let-7 family members, as compared to the pLKO.1 control (Figure 5.5).

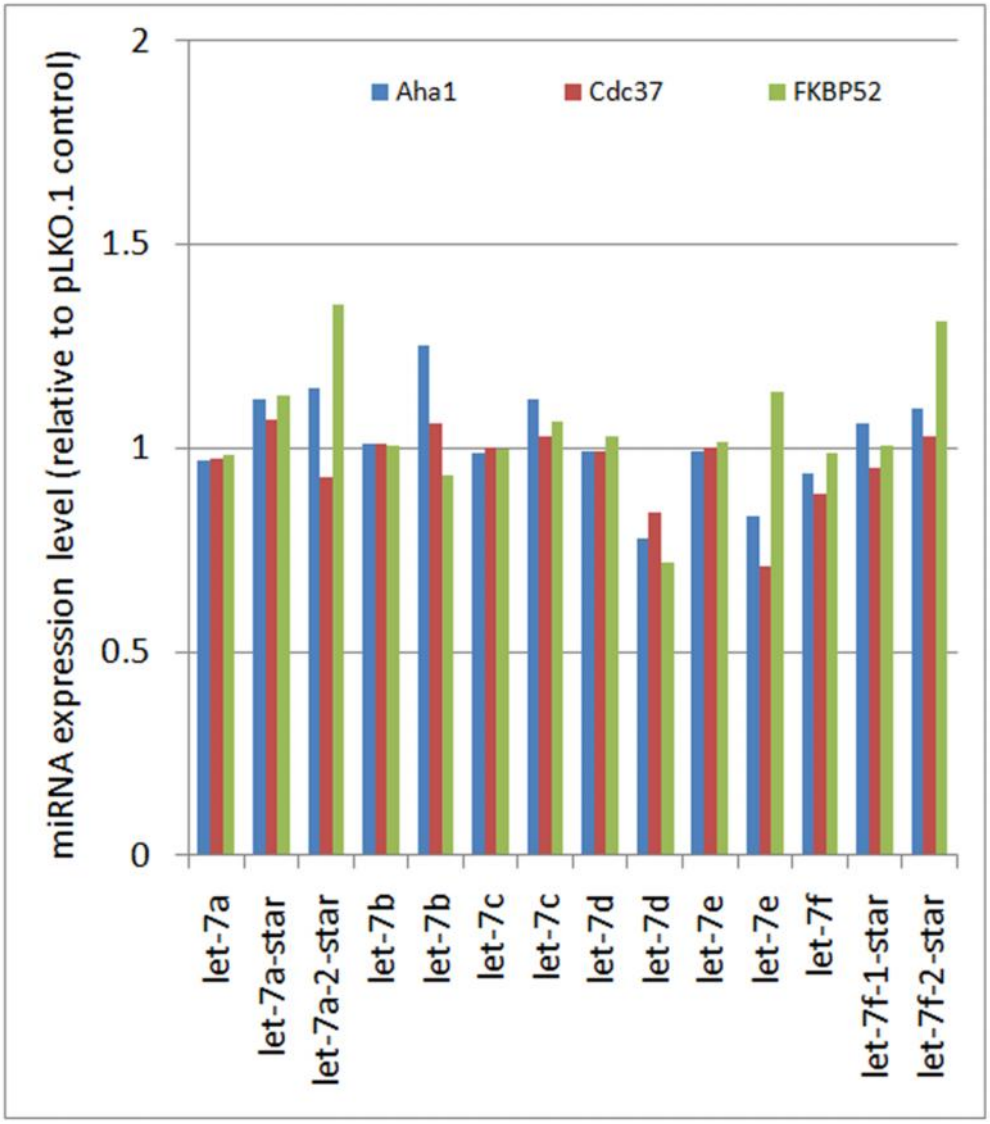



**Figure 5.5 Knockdown of Aha1, Cdc37 or FKBP52 does not reduce the steady-state levels of the let-7 miRNA family.** HeLa cells were transiently transfected with plasmids expressing shRNAs to silence the expression Aha1, Cdc37, FKBP52 or vector only control (pLKO.1). 24 hours following transfection, cells containing shRNA plasmids were positively selected by growth in media containing 2.5 µg/mL of puromycin. Cells knocked down for Cdc37 were harvested 48 hours post-transfection while all other samples were harvested 72 hours post-transfection. miRNA expression profiles were determined by microarray analysis. Levels of let-7 family miRNAs are shown relative to the pLKO.1 control.

### *5.2.6 Knockdown of Aha1, Cdc37 or FKBP52 does not affect pre-miRNA levels of the let-7 family*

In *Drosophila*, miRNA biogenesis requires the Dcr-1 co-factor Loquacious (Loqs) (Forstemann et al., 2005; Jiang et al., 2005; Saito et al., 2005). Northern blot analysis of Loqs knockout *Drosophila* revealed an accumulation of pre-miRNAs; however, mature miRNA levels were differentially affected with some miRNAs decreasing and others showing no change relative to wildtype *Drosophila* (Liu et al., 2007). These findings suggest that perturbations of the RISC loading machinery may not cause a global, predictable decrease in the level of mature miRNA level. As an alternative to my prediction in section 5.2.5, it is possible that knockdown of Hsp90 co-chaperones required for loading miRNA duplexes onto Argonaute proteins would result in pre-miRNA accumulation with no detectable change in mature miRNA level.

To address whether pre-miRNAs were accumulating in the absence of a detectable effect on mature miRNAs, the level of pre-miRNAs following shRNA-mediated knockdown of Hsp90 co-chaperones was determined. Total RNA, isolated from cells in which Aha1, Cdc37 or FKBP52 levels were reduced, was submitted to the Alberta Transplant Applied Genomics Centre for analysis. Microarray profiling of pre-miRNA showed no changes outside the threshold of greater than two-fold in the relative levels of pre-miRNA of the let-7 family, as compared to the pLKO.1 control (Figure 5.6).

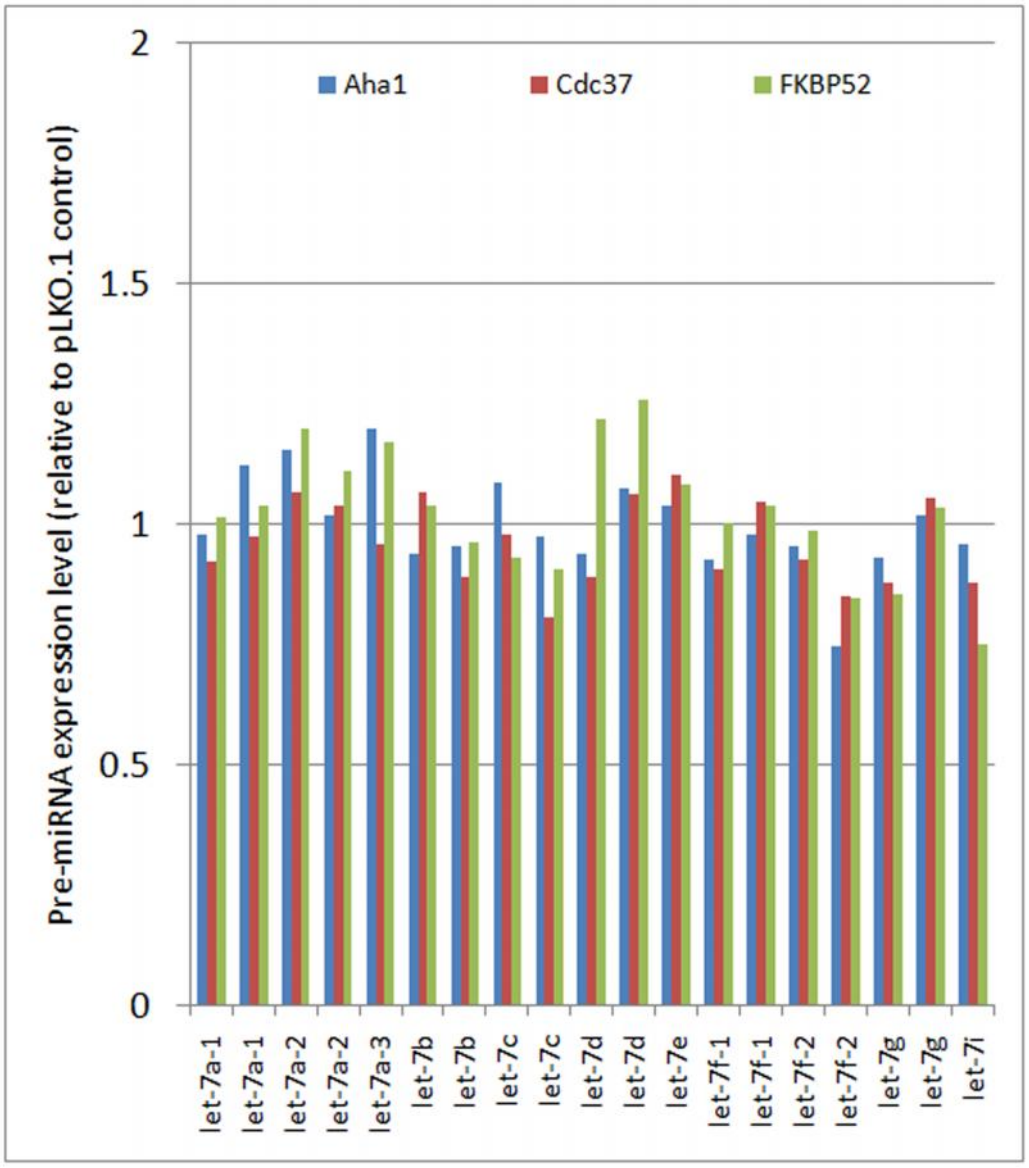



**Figure 5.6 Knockdown of Aha1, Cdc37 or FKBP52 does not affect the pre-miRNA levels of the let-7 family.** HeLa cells were transiently transfected with plasmids expressing shRNAs to silence the expression Aha1, Cdc37, FKBP52 or vector only control (pLKO.1). 24 hours following transfection, cells containing shRNA plasmids were positively selected by growth in media containing 2.5 µg/mL of puromycin. Cells knocked down for Cdc37 were harvested 48 hours post-transfection while all other samples were harvested 72 hours post-transfection. Pre-miRNA expression profiles were determined by microarray analysis. Levels of pre-miRNAs of the let-7 family are shown relative to the pLKO.1 control.

## 5.3 Summary

In this chapter, a role for the Hsp90 co-chaperones Aha1 and Cdc37 in mediating efficient post-transcriptional gene silencing by small RNAs was demonstrated. Knocking down the expression of these Hsp90 co-chaperones caused a significant increase in expression from reporters designed to assay the efficiency of miRNA- and siRNA-mediated gene silencing. This evidence strongly suggests that these co-chaperones participate in the activation of hAgo2 and potentially other Argonautes. Furthermore, the effect on RNAi efficiency was observed without a decrease in the levels of effectors such as hAgo2 or GW182 and miRNAs. Evidence from Loqs knockout *Drosophila* indicates that defects in miRNA biogenesis do not lead to a predictable decrease in mature miRNA levels but do result in a global accumulation of pre-miRNAs (Liu et al., 2007). To address the possibility of a defect in miRNA biogenesis caused by co-chaperones knockdown, I examined the level of pre-miRNAs of the let-7 family and found no increase. These data suggest that miRNAs are efficiently processed into mature miRNAs in the absence of these Hsp90 co-chaperones. Taken together, the results presented in this chapter are consistent with Hsp90 activity being required for loading the miRNA duplex onto Argonaute and/or RISC stably associating with a target mRNA.

# CHAPTER 6

Discussion

## 6.1 Overview

The focus of this thesis was to understand the role of Hsp90 in the activation of hAgo2 within the small RNA-mediated gene silencing pathway. This pathway requires that small RNAs associate with an Argonaute protein and, once loaded and unwound, they guide RISC to mRNAs in a sequence-specific manner. Once bound to a targeted mRNA, translational repression requires stable association of Argonaute with the targeted mRNA. I hypothesized that the above protein-RNA interactions are mediated by Hsp90. Consistent with this prediction, crystallographic studies of *Thermus thermophilus* Argonaute (TtAgo) showed considerable structural rearrangement of the protein is required in order to bind a 21-base guide strand (Wang et al., 2008). Moreover, the interaction of loaded TtAgo with a 20-base target RNA required additional transitioning of its structure (Wang et al., 2008). Consistent with my earlier findings (Pare et al., 2009), recent studies from other laboratories have demonstrated Hsp90 activity is required for miRNA loading (Iki et al., 2010; Iwasaki et al., 2010; Miyoshi et al., 2010); however, the full extent of Hsp90 involvement in hAgo2 maturation remains unclear.

## 6.2 A role for Hsp90 in the RNAi-mediated silencing pathway

### *6.2.1 Localization of silencing machinery*

There are many examples where compartmentalization is used to increase efficiency of cellular processes and, within eukaryotic cells, membrane-bound organelles are a common example. However, there are also structures that are devoid of membranes which function to concentrate macromolecules involved in related pathways. Ribonucleoprotein structures (RNPs) are an example of this style of compartmentalization and the machinery of the miRNA-

mediated post transcriptional gene silencing pathway localizes to at least two of these different RNPs (Leung et al., 2006; Liu et al., 2005b; Sen and Blau, 2005).

#### *6.2.1.1 Processing bodies*

Originally identified as concentrations of the exonuclease Xrn1 (Bashkirov et al., 1997), P bodies contain a number of proteins involved in mRNA decay and degradation (Andrei et al., 2005; Cougot et al., 2004; Sheth and Parker, 2003; Stoecklin et al., 2006; Wilczynska et al., 2005). My data show that inhibition of Hsp90 activity both disrupts P body integrity and prevents the association of nascent GFP-tagged hAgo2 with existing P bodies. Previous work has shown that inhibition of small RNA-mediated silencing prevents P body formation, leading to speculation that the formation of P bodies is a consequence of active RNA-mediated silencing (Eulalio et al., 2007b). Therefore, I propose that the loss of P bodies in geldanamycin-treated cells is a reflection of a decrease in the activity of Argonaute proteins.

#### *6.2.1.2 Stress granules*

Formed in response to various cellular stresses, stress granules are believed to store and repress the translation of mRNAs encoding non-essential proteins as a means of conserving energy (Buchan and Parker, 2009). The mechanism for regulating the association of Argonaute proteins with stress granules remains poorly understood; however, the presence of small RNAs (Leung et al., 2006) and the ability of hAgo2 to bind to miRNAs [our unpublished data, Appendix] are required. Based on these observations, recruitment of hAgo2 to stress granules likely requires that it be actively engaged in RNA-mediated silencing. Therefore, consistent with my investigations of P body stability, I conclude that impaired association of GFP-hAgo2 with stress granules is a consequence of a decrease in small RNA-mediated post-transcriptional gene silencing.

### *6.2.2 RNAi-mediated silencing of endogenous miRNA targets*

Many lines of evidence indicate that the localization of Argonaute proteins to cytoplasmic RNA granules depends on their activity within the RNA-mediated silencing pathway (Chu and Rana, 2006; Eulalio et al., 2007a; Eulalio et al., 2007b; Franks and Lykke-Andersen, 2008). Moreover, I have shown that inhibition of Hsp90 activity by geldanamycin disrupts this localization of hAgo2, and concluded that Hsp90 activity is required for the RNAi function of hAgo2. Consistent with this conclusion, treatment with geldanamycin increases the level of endogenous Ras, a target of the let-7 family (Johnson et al., 2005), and cytochrome *c*, a high confidence target of miR-29b. The observations that geldanamycin leads to an increase in the level of protein expressed from mRNAs targeted by miRNAs; decreased P body biogenesis and/or stability; and inhibited recruitment of hAgo2 to stress granules, together strongly support a model in which Hsp90 activates the RNAi-mediated silencing pathway.

### *6.2.3 Loading Argonaute proteins with small RNAs*

There are a number of steps within the RNAi pathway that could be expected to require chaperone activity. Intriguingly, localization of Argonautes to both stress granules and P bodies is dependent on their ability to bind small RNAs, suggesting that Hsp90 activity may mediate the association of Argonaute with small RNAs. Indeed, the inhibition of Hsp90 impairs the loading of small RNA duplexes onto Argonaute proteins *in vitro* (Iki et al., 2010; Iwasaki et al., 2010; Miyoshi et al., 2010). While I was unable to demonstrate an effect of geldanamycin on the loading of GFP-tagged Argonaute, it is plausible that the Hsp90 chaperone machinery functions to increase the efficiency of small RNA loading *in vivo.*

#### *6.2.3.1 The existing model for Hsp90 involvement in loading small RNAs onto Argonaute proteins*

The current model for Hsp90-mediated loading of an Argonaute protein posits that the structure of nascent Argonaute proteins is unable to accommodate a bulky RNA duplex (Czech et al., 2008; Iki et al., 2010; Miyoshi et al., 2010). In an ATP-dependent manner, the Hsp90 machinery is predicted to alter the tertiary structure of the Argonaute protein, presenting the RISC loading complex with a conformation which is able to accommodate the small RNA duplex (Figure 6.1). Given that addition of geldanamycin after RISC loading does not inhibit the subsequent unwinding of the miRNA duplex, it was proposed that the removal of the passenger strand occurs in an Hsp90-independent manner (Iwasaki et al., 2010). Iwasaki et al. also showed that cleavage of a target RNA was not inhibited by geldanamycin after loading hAgo2 with an siRNA (Iwasaki et al., 2010); therefore, the authors concluded that Hsp90 functions exclusively at the level of loading. However, it is important to note that geldanamycin functions by inhibiting the initial association of ATP with Hsp90. Once bound to ATP, the nucleotide-binding pocket will remain occupied until after ATP hydrolysis and the completion of the chaperone cycle. An alternative hypothesis to the one proposed in Iwasaki et al. is that the Hsp90-dependent steps of RISC maturation (e.g. loading, miRNA unwinding, target binding) occur within a single chaperone cycle. Regardless of which model is ultimately proven correct, further investigation is required in order to determine the extent of the involvement of Hsp90 in the maturation of Argonaute proteins and the activation of RNA-mediated silencing.

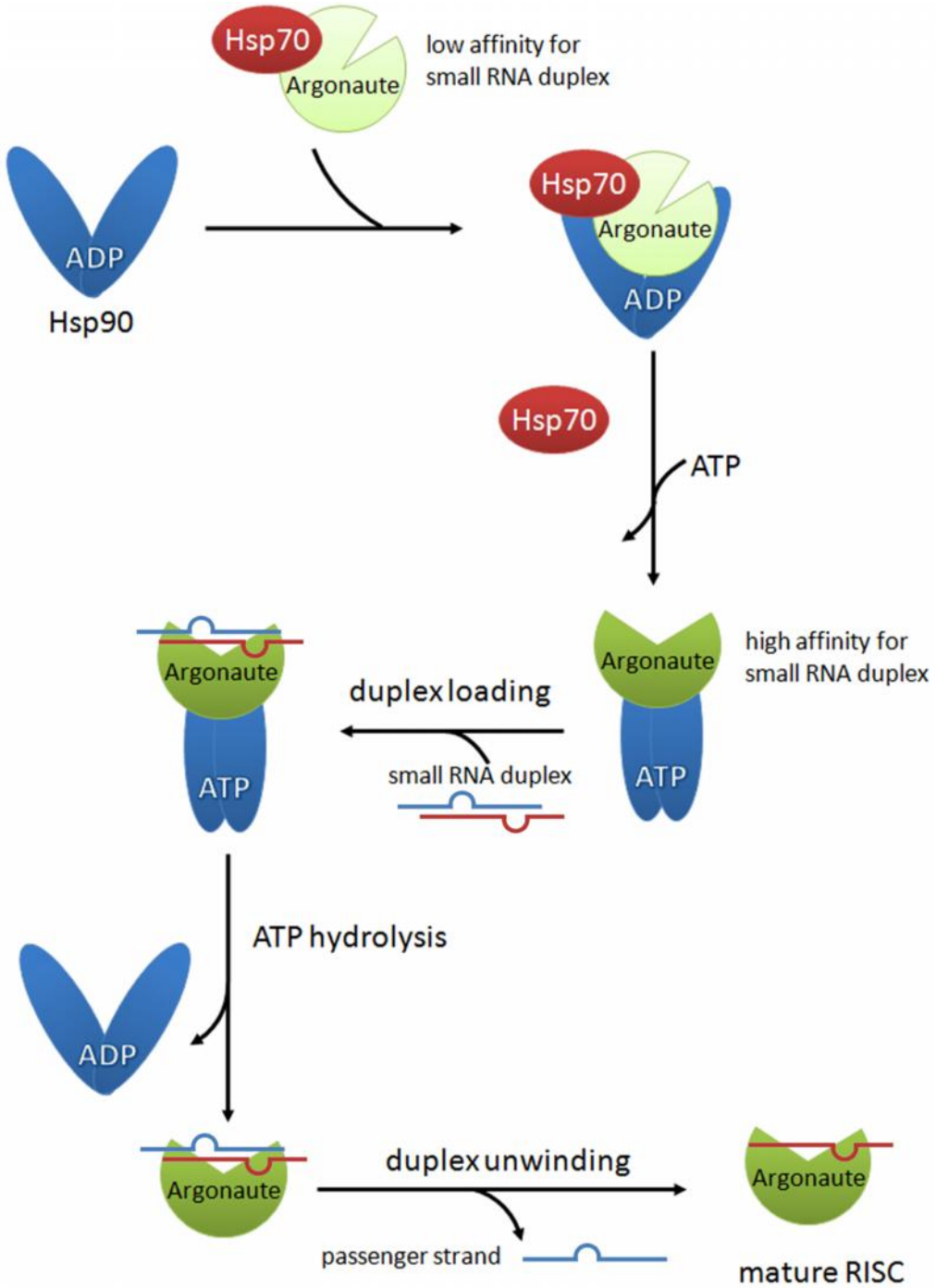



**Figure 6.1 Preliminary model for Hsp90-mediated activation of Argonaute proteins.** Nascent Argonaute with low affinity for a small RNA duplex binds Hsp90. An Hsp90-mediated, ATP-dependent conformational change in Argonaute increases its affinity for small RNA duplexes. ATP hydrolysis releases the Hsp90 machinery from Argonaute loaded with a duplex. Following release from Hsp90, the loaded duplex is unwound and mature Argonaute targets mRNAs for translational silencing.

### *6.2.4 Target-binding by hAgo2*

Unlike siRNA-mediated gene silencing, the cleavage-independent miRNA pathway requires stbale association of an Argonaute protein with a targeted mRNA. Initial target recognition relies on sequence complementarity between the mRNA and a stretch of 7-8 bases at the 5' end of the miRNA known as the seed region (Lewis et al., 2003), but changes in the sequence of the 3' end of the miRNA can affect silencing (Brennecke et al., 2005; Doench and Sharp, 2004; Grimson et al., 2007). Moreover, miRNAs can direct cleavage of mRNAs with complete complementarity to the miRNA sequence, and this cleavage takes place outside of the seed region between bases 10 and 11 (Martinez et al., 2002). Taken together, these observations suggest that base-pairing between the guide strand and the target RNA is propagated throughout the length of the miRNA. The proposition that Argonaute proteins cannot accommodate a miRNA duplex without the activity of Hsp90, but can accommodate a guide-target duplex independent of chaperone activity, is paradoxical. I have shown that inhibition of Hsp90 activity causes a modest decrease in the amount of RISC captured by a pulldown using 2'-O-methylated oligonucleotides. These data are consistent with Hsp90 facilitating the incorporation of RNA duplexes within the bilobal structure of hAgo2, and suggest an expanded role for Hsp90 in the RNAi pathway. The nature of this expanded role for Hsp90 activity in mediating target-binding will be discussed further in section 6.4.

## 6.3 Roles for Hsp90 co-chaperones in the RNAi pathway

Hsp90 activity is regulated by no less than 12 co-chaperones (Pearl and Prodromou, 2006), which modulate the chaperone cycle by guiding client protein recognition and either stimulating or inhibiting ATP hydrolysis. Co-localization of a subset of Hsp90 co-chaperones with hAgo2 at stress granules provided preliminary evidence that the RNAi pathway may be regulated by a cohort of

these proteins. In support of this theory, I confirmed that shRNA-mediated knockdown of Cdc37 and Aha1 significantly reduced the efficiency of both cleavage-dependent and cleavage-independent small RNA-mediated silencing.

### *6.3.1 Aha1 and Cdc37 are required prior to the binding of hAgo2 to mRNA*

Following miRNA-mediated binding of Argonaute to the 3' UTR of an mRNA, additional proteins are recruited which function to repress its translation (Fabian et al., 2010). Artificially tethering hAgo2 to the 3' UTR of a reporter mRNA circumvents the requirement for hAgo2 to be loaded with a small RNA (Pillai et al., 2004), the step of the RNAi pathway predicted by the current model to require Hsp90. Following knockdown of Aha1, Cdc37 or FKBP52, efficient repression of a reporter mRNA by tethered hAgo2 confirmed that the effectors of the RNAi pathway are capable of silencing translation in the absence of these co-chaperones. These data suggest that the requirement for Aha1 or Cdc37 in the small RNA-mediated silencing pathway is upstream of hAgo2 interaction with the targeted mRNA, which is consistent with the existing model for Hsp90 involvement.

### *6.3.2 Depleting Aha1 and Cdc37 does not affect steady-state levels of hAgo2 or GW182*

Inhibition of Hsp90 results in the destabilization of many of its clients (Annamalai et al., 2009; Bagatell et al., 2001; Blagosklonny et al., 1995; Jung et al., 2011; Wang et al., 2007), and it has been proposed that Hsp90 serves to stabilize unloaded Argonaute proteins (Johnston et al., 2010). Co-chaperones are required for Hsp90 function and, therefore, lower levels of these proteins may result in decreased stability of client proteins (Smith et al., 2009). Furthermore, a decrease in hAgo2 level is associated with both decreased cleavage-dependent

and cleavage-independent silencing (Mescalchin et al., 2010). However, shRNA-mediated knockdown of Aha1, Cdc37 or FKBP52 did not result in decreased hAgo2 or GW182 levels, strongly suggesting that the defect in RNAi is due to impaired function of hAgo2 rather than loss of protein.

### *6.3.3 Recognition of hAgo2 by the Hsp90 machinery*

A sub-group of Hsp90 co-chaperones are responsible for client recognition and function as molecular bridges between client proteins and the chaperone. For example, Cdc37 participates in the maturation of kinases by facilitating the interaction between Hsp90 and kinase clients. Similar to its role in recruiting kinases, Cdc37 may mediate the interaction between Hsp90 and nascent hAgo2. If so, decreased expression of Cdc37 would lead to a reduced interaction between hAgo2 and Hsp90, resulting in less activated hAgo2. It is also possible that Cdc37 is not specifically required for recruiting hAgo2 to the Hsp90 complex and instead, functions in some other capacity during the chaperone-mediated activation of hAgo2. Regardless of whether it is Cdc37 that is shown to be required, it is likely that the interaction between Hsp90 and hAgo2 is mediated by the action of at least one co-chaperone.

### *6.3.4 ATPase activity and the Hsp90 chaperone cycle*

Knockdown of Hsp90 co-chaperone expression is an alternative experimental approach to pharmacologically inhibiting ATP-binding by Hsp90 that leads to differential defects in the progression of the chaperone cycle. A small number of co-chaperones, including Aha1, activate the ATPase activity of Hsp90 (McLaughlin et al., 2002; Panaretou et al., 2002). The association of these co-chaperones with an Hsp90-client complex likely drives the chaperone cycle forward and promotes the release of the mature, activated client. Meanwhile,

Cdc37 and other co-chaperones can inhibit the ATPase activity of Hsp90 (Hernandez et al., 2002; Panaretou et al., 2002; Prodromou et al., 1999). Specifically, the interaction of Cdc37 with the amino-terminal domain of Hsp90 blocks ATPase activation by inhibiting the interaction of the catalytic arginine residue from the middle domain with the ATP-binding site (Pearl and Prodromou, 2006). By inhibiting the ATPase activity of Hsp90, these co-chaperones stabilize the ATP-bound form and may prolong the step at which the client is held in a conformation with a high affinity for its ligand (i.e. small duplex RNAs). Accordingly, precise regulation of ATP hydrolysis by Hsp90 is a critical determinant of client maturation. shRNA-mediated knockdown of Cdc37, Aha1 and other co-chaperones will arrest the chaperone cycle at discreet stages, leading to an enrichment of Hsp90-client complexes 'locked' at a particular stage of client maturation. Comprehensive biochemical analysis of hAgo2 associated with these complexes will provide valuable information regarding the role for these co-chaperones in the activation of the RNAi machinery.

### *6.4 A revised model for Hsp90 involvement in Argonaute2 activation and function*

Despite widespread efforts, the understanding of Hsp90-mediated maturation of client proteins is limited. Perhaps the best described example of Hsp90 action is the maturation of the glucocorticoid receptor. Hsp90 stabilizes an intermediate conformation of the receptor, which is energetically unfavorable but has greatly increased affinity for the glucocorticoid steroid (Grad and Picard, 2007). ATP is hydrolyzed by Hsp90 following the binding of the hormone to the receptor, leading to the dissociation of the complex. Once released from Hsp90, the mature, active and hormone-bound receptor is able to promote transcription of its target genes. From this relatively well-characterized model of Hsp90 action, parallels can be drawn with the activation of Argonaute proteins. I

propose that nascent Argonaute protein, initially in a complex with Hsp70, is recruited to Hsp90 by the concerted action of one or more Hsp90 co-chaperones (e.g. HOP, Cdc37) (Figure 6.2A). Given our data showing that geldanamycin does not reduce the association between Hsp90 and hAgo2, and consistent with knowledge regarding the initial interaction between Hsp90 and other clients, this step is predicted to occur while Hsp90 is in its ADP-bound state (Figure 6.2A). Subsequent to binding, ADP is exchanged for ATP and additional co-chaperones are recruited. ATP-binding provides the energy required for Hsp90 to stabilize a conformation of the bound Argonaute with an increased affinity for an RNA duplex (Figure 6.2A). The initial steps in this model are similar to the preliminary model (Figure 6.1) and are consistent with geldanamycin inhibiting small RNA loading.

Distinct from the preliminary model, my model proposes that the Argonaute protein remains bound to the chaperone machinery following the loading step. That is to say, the passenger strand is removed while the Argonaute is still in complex with Hsp90. The inability of geldanamycin to inhibit unwinding (Iwasaki et al., 2010) can be explained by Hsp90 maintaining the complex, without ATP hydrolysis, through the unwinding step of RISC maturation. Following the removal of the passenger strand, the guide-target interaction requires the reformation of a duplex. The guide-target duplex is likely incorporated within the structure of the Argonaute; hence, this model predicts that Hsp90 is bound to the mature RISC complex until the Argonaute binds to a target (Figure 6.2B). The ATP-bound conformation of Hsp90 is predicted to be stabilized via the association of one or more co-chaperones which inhibit the ATPase activity of Hsp90 (e.g. Cdc37, p23) (Figure 6.2A, B). Considering that the ability of RISC to bind a target mRNA is modestly impaired by the addition of geldanamycin, a small amount of RISC may be released from the Hsp90 machinery prior to target binding. However, it is predicted that this ‘free’ RISC would require a second round of the chaperone cycle, complete with a second

round of ATP binding, to stably associate with a target mRNA (Figure 6.2C). The association of Argonaute proteins with a targeted mRNA is followed by an exchange of co-chaperones, leading to the activation of the ATPase activity of Hsp90 and hydrolysis of ATP. ATP hydrolysis facilitates the dissociation of the complex and leads to the release of the mature, target-bound RISC (Figure 6.2B).

A number of unanswered questions remain regarding the specifics of Hsp90 involvement in the maturation of Argonaute proteins and RISC. Given the significance of the miRNA pathway in regulating gene expression, a more detailed understanding of the involvement of Hsp90 is warranted. Historically, studying the role of Hsp90 in the maturation of its client proteins has proved difficult, but considerable progress is being made in understanding aspects of the Hsp90 chaperone cycle. The role for co-chaperones in modulating the biochemical activity of Hsp90 puts these accessory proteins at the forefront of investigations into the mechanism and dynamics of Hsp90 activity.

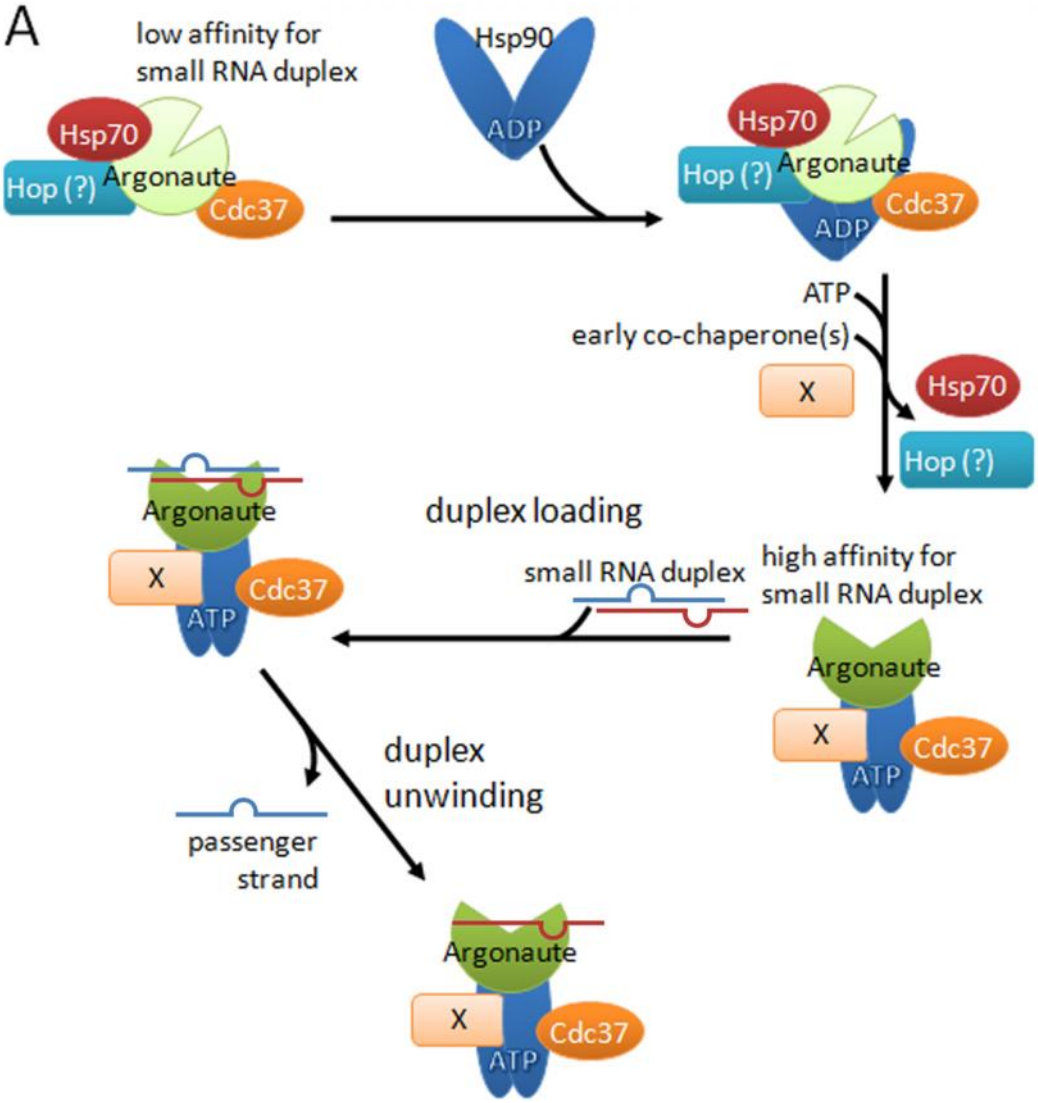
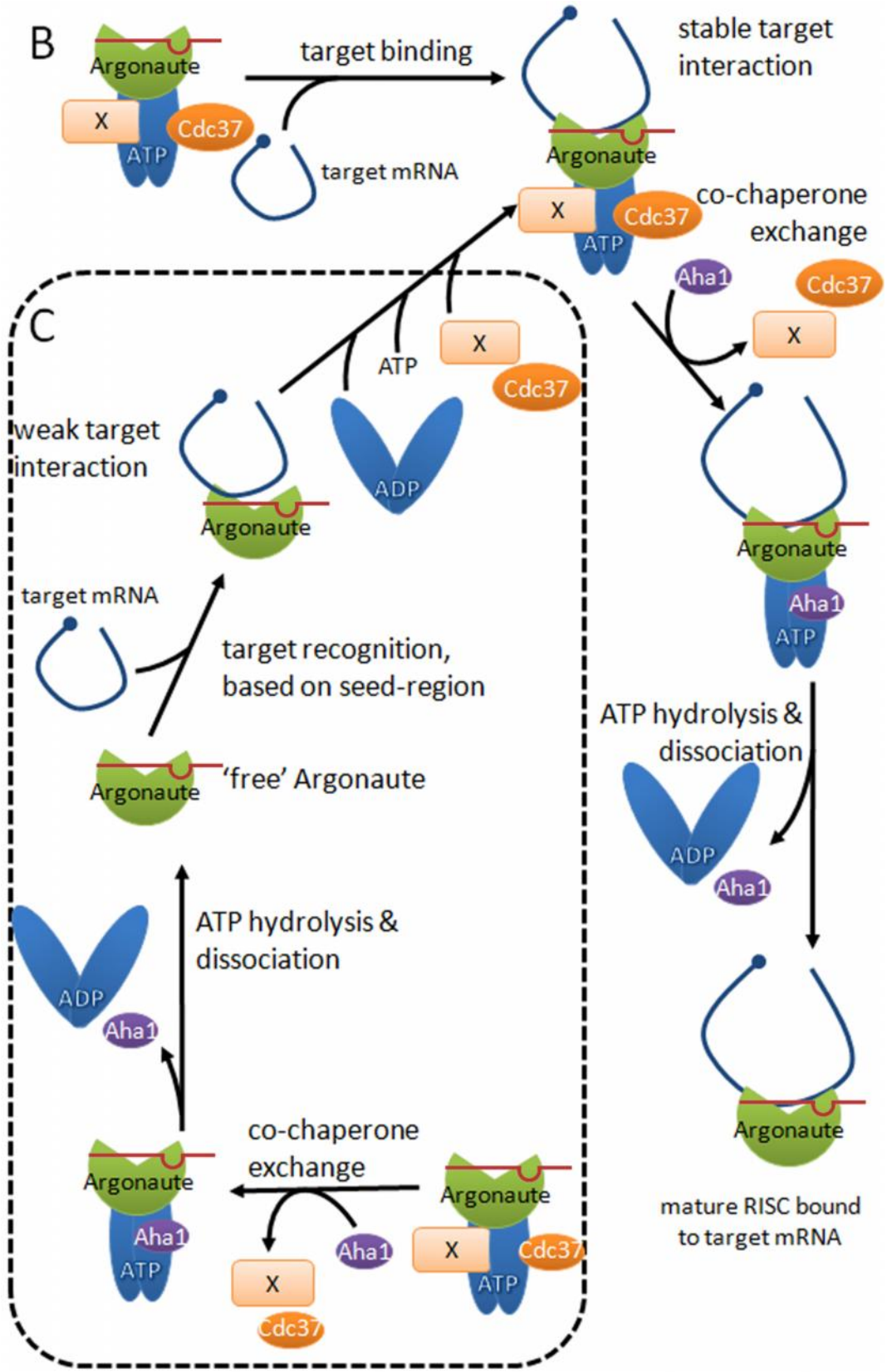



**Figure 6.2 Revised model for Hsp90 involvement in Argonaute activation and function. A.** The complex of Argonaute-Hsp70-Cdc37 binds to Hsp90, potentially via Hop. ATP binding leads to stabilization of the open conformation of the Argonaute which has increased affinity for the small RNA duplex. ATP-bound conformation is stabilized by the association of one or more early co-chaperones (X, Cdc37). Following duplex loading, the passenger strand is unwound and removed. **B.** The primary pathway predicts that an Argonaute binds to a target mRNA subsequent to removal of the passenger strand. Recruitment of Aha1 activates the ATPase activity of Hsp90 leading to ATP hydrolysis and dissociation of the complex. **C.** The secondary pathway predicts that some Argonaute proteins may be released from the Hsp90 complex following passenger strand removal. These Argonaute proteins are competent to target mRNA based on the seed region, but require a second round of the Hsp90 chaperone cycle and ATP-binding in order to stably associate with the mRNA.


B
Argonaute
X
Cdc37
ATP
target binding
target mRNA
stable target interaction
Argonaute
X
Cdc37
ATP
co-chaperone exchange
Aha1
Cdc37
X
C
ATP
X
Cdc37
weak target interaction
ADP
Argonaute
Argonaute
Aha1
ATP
target mRNA
target recognition, based on seed-region
ATP hydrolysis & dissociation
ADP
Aha1
'free' Argonaute
Argonaute
ATP hydrolysis & dissociation
ADP
Aha1
Argonaute
co-chaperone exchange
Argonaute
Aha1
ATP
Aha1
X
Cdc37
Argonaute
X
Cdc37
ATP
mature RISC bound to target mRNA

## 6.5 Future directions & perspective

Various approaches for studying the function of Hsp90 exist and they have different effects on the progression of the chaperone cycle. For example, pharmacological inhibition of ATP-binding leads to the accumulation of Hsp90-client complexes unique from complexes formed in a background of Aha1 depletion. Additionally, Hsp90 mutants that are unable to bind to specific co-chaperones are available. Using complementary approaches, it should be possible to 'trap' Argonaute at discreet stages of maturation, and future investigations will rely on co-immunoprecipitation of Hsp90-associated hAgo2 under these conditions. Identification of co-immunoprecipitated proteins (by mass spectrometry analysis) and characterization of hAgo2-bound small RNAs (by Northern blotting and qPCR) will be required to model hAgo2 maturation. Moreover, these techniques could be used to study the role of Hsp90 in the maturation of other human Argonaute isoforms, as well as Argonaute proteins in other model systems. Understanding the nature of the Argonaute-RNA complexes associated with all stages of the Hsp90 chaperone cycle will unquestionably add to our knowledge surrounding the mechanism of Hsp90-mediated activation of RISC.

From the model of hAgo2 maturation proposed in this thesis, several predictions can be made regarding the nature of hAgo2 that is associated with Hsp90 at the different stages of the chaperone cycle. First, my model predicts that initial recognition of hAgo2 by the Hsp90 machinery is mediated by Cdc37. Demonstrating direct interactions between hAgo2 and Cdc37 will support this hypothesis, and mutations blocking the interaction of Cdc37 with Hsp90 would be predicted to have dominant negative effects on Argonaute maturation and, therefore, impair efficient RNAi. Moreover, my model predicts that inhibition of ATP-binding will not prevent interaction between Hsp90 and hAgo2, and this is supported by observations described in chapter 3 of this thesis. Rather, I suggest

that blocking ATP-binding by the chaperone prevents the conformational change in hAgo2 required for accommodating the small RNA duplex; therefore, Hsp90-associated hAgo2 isolated in the presence of geldanamycin is predicted to be unloaded. Understanding the factors that guide the initial recognition of Argonaute proteins by the chaperone machinery and the consequence of ATP-binding will be critical for establishing any model attempting to detail the Hsp90-mediated maturation of RISC.

Considering that several co-chaperones function to inhibit the ATPase activity of Hsp90 (Hernandez et al., 2002; Panaretou et al., 2002; Prodromou et al., 1999), it may be that one or more of these co-chaperones cause Hsp90 to hold the bound Argonaute in an 'open' conformation. It is tempting to speculate that, by holding the Argonaute protein in this conformation, co-chaperones indirectly promote the subsequent steps of activation. Knockdown of these co-chaperones should result in the premature hydrolysis of ATP, negatively affecting the efficiency of loading, unwinding or target-binding and stimulating the release of an inactive Argonaute. Following target-binding, the successive recruitment of co-chaperones leads to Aha1 binding, which functions to promote the closed conformation of the chaperone (Meyer et al., 2004), stimulate ATPase activity (Panaretou et al., 2002) and ultimately leads to the dissociation of the complex. Accordingly, experiments using either Hsp90 mutants unable to bind to Aha1 or RNAi-mediated knockdown of Aha1 expression should show increased association between Hsp90 and Argonautes loaded with guide miRNAs. Provided that ATP hydrolysis is necessary to promote the dissociation of the Hsp90-client complex, the Argonaute proteins 'locked' in complex with Hsp90 may closely resemble mature RISC in composition even though they are functionally inactive. That is, Argonaute proteins at this late stage of maturation may be able to interact with a target mRNA, but their ability to silence the translation of that mRNA likely requires their release from the Hsp90 chaperone complex.

Hsp90 is required for efficient loading of a small RNA duplex onto Argonaute proteins *in vitro* (Iki et al., 2010; Iwasaki et al., 2010; Miyoshi et al., 2010). In Chapter 4, I presented preliminary evidence suggesting an additional role for Hsp90 in target-binding by hAgo2. It is tempting to speculate that target-binding by RISC occurs as part of a continuous process with miRNA loading and unwinding, and that all facets of this pathway are mediated by a spectrum of Hsp90 chaperone activity. Using fluorescently-labeled guide and target RNAs in conjunction with fluorescent resonant energy transfer (FRET) techniques, it may be possible to further dissect the consequences of treatment with geldanamycin or co-chaperone knockdown on the kinetics of target binding *in vivo*. Future studies of this nature may help reveal an expanded role of Hsp90 mediating the interaction between hAgo2 and the target mRNA.

Many questions remain to be answered regarding the mechanism and extent of Hsp90 involvement in the RNAi pathway. The model proposed in this thesis presents many testable hypotheses regarding the nature of Argonaute proteins associated with various stages of the chaperone cycle. A considerable obstacle in developing this model is the extent to which details of the Hsp90 chaperone cycle are generally unknown. Ultimately, validating or disproving this model will rely on the biochemical characterization of the Hsp90-Argonaute complexes under different conditions, including co-chaperone knockdown, over-expression of mutant Hsp90 and inhibition of ATP binding. My work may serve as a foundation for future experiments designed to elucidate the role of the Hsp90 machinery in not only the RNAi pathway, but also more broadly in the maturation of its other clients.

# REFERENCES

Adams, B.D., Claffey, K.P., and White, B.A. (2009). Argonaute-2 expression is regulated by epidermal growth factor receptor and mitogen-activated protein kinase signaling and correlates with a transformed phenotype in breast cancer cells. Endocrinology *150,* 14-23.

Aizer, A., Brody, Y., Ler, L.W., Sonenberg, N., Singer, R.H., and Shav-Tal, Y. (2008). The dynamics of mammalian P body transport, assembly, and disassembly in vivo. Mol. Biol. Cell *19,* 4154-4166.

Ali, M.M., Roe, S.M., Vaughan, C.K., Meyer, P., Panaretou, B., Piper, P.W., Prodromou, C., and Pearl, L.H. (2006). Crystal structure of an Hsp90-nucleotide-p23/Sba1 closed chaperone complex. Nature *440,* 1013-1017.

Ambros, V., and Horvitz, H.R. (1987). The lin-14 locus of Caenorhabditis elegans controls the time of expression of specific postembryonic developmental events. Genes Dev. *1,* 398-414.

Andrei, M.A., Ingelfinger, D., Heintzmann, R., Achsel, T., Rivera-Pomar, R., and Luhrmann, R. (2005). A role for eIF4E and eIF4E-transporter in targeting mRNPs to mammalian processing bodies. RNA *11,* 717-727.

Annamalai, B., Liu, X., Gopal, U., and Isaacs, J.S. (2009). Hsp90 is an essential regulator of EphA2 receptor stability and signaling: implications for cancer cell migration and metastasis. Mol. Cancer. Res. *7,* 1021-1032.

Aoki, K., Moriguchi, H., Yoshioka, T., Okawa, K., and Tabara, H. (2007). In vitro analyses of the production and activity of secondary small interfering RNAs in C. elegans. EMBO J. *26,* 5007-5019.

Aravin, A., Gaidatzis, D., Pfeffer, S., Lagos-Quintana, M., Landgraf, P., Iovino, N., Morris, P., Brownstein, M.J., Kuramochi-Miyagawa, S., Nakano, T.*, et al.* (2006). A novel class of small RNAs bind to MILI protein in mouse testes. Nature *442,* 203-207.

Aravin, A.A., Hannon, G.J., and Brennecke, J. (2007). The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. Science *318,* 761-764.

Aravin, A.A., Lagos-Quintana, M., Yalcin, A., Zavolan, M., Marks, D., Snyder, B., Gaasterland, T., Meyer, J., and Tuschl, T. (2003). The small RNA profile during Drosophila melanogaster development. Dev. Cell. *5,* 337-350.

Aravin, A.A., Sachidanandam, R., Bourc'his, D., Schaefer, C., Pezic, D., Toth, K.F., Bestor, T., and Hannon, G.J. (2008). A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. Mol. Cell *31,* 785-799.

Aravin, A.A., Sachidanandam, R., Girard, A., Fejes-Toth, K., and Hannon, G.J. (2007). Developmentally regulated piRNA clusters implicate MILI in transposon control. Science *316,* 744-747.

Aukerman, M.J., and Sakai, H. (2003). Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. Plant Cell *15,* 2730-2741.

Baek, D., Villen, J., Shin, C., Camargo, F.D., Gygi, S.P., and Bartel, D.P. (2008). The impact of microRNAs on protein output. Nature *455,* 64-71.

Bagatell, R., Khan, O., Paine-Murrieta, G., Taylor, C.W., Akinaga, S., and Whitesell, L. (2001). Destabilization of steroid receptors by heat shock protein 90-binding drugs: a ligand-independent approach to hormonal therapy of breast cancer. Clin. Cancer Res. *7,* 2076-2084.

Baguet, A., Degot, S., Cougot, N., Bertrand, E., Chenard, M.P., Wendling, C., Kessler, P., Le Hir, H., Rio, M.C., and Tomasetto, C. (2007). The exon-junction-complex-component metastatic lymph node 51 functions in stress-granule assembly. J. Cell. Sci. *120,* 2774-2784.

Baillat, D., and Shiekhattar, R. (2009). Functional dissection of the human TNRC6 (GW182-related) family of proteins. Mol. Cell. Biol. *29,* 4144-4155.

Barber, G.N. (2005). The dsRNA-dependent protein kinase, PKR and cell death. Cell Death Differ. *12,* 563-570.

Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. Cell *136,* 215-233.

Bashkirov, V.I., Scherthan, H., Solinger, J.A., Buerstedde, J.M., and Heyer, W.D. (1997). A mouse cytoplasmic exoribonuclease (mXRN1p) with preference for G4 tetraplex substrates. J. Cell Biol. *136,* 761-773.

Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P., and Izaurralde, E. (2006a). mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. Genes Dev. *20,* 1885-1898.

Behm-Ansmant, I., Rehwinkel, J., and Izaurralde, E. (2006b). MicroRNAs silence gene expression by repressing protein expression and/or by promoting mRNA decay. Cold Spring Harb. Symp. Quant. Biol. *71,* 523-530.

Berezikov, E., Chung, W.J., Willis, J., Cuppen, E., and Lai, E.C. (2007). Mammalian mirtron genes. Mol. Cell *28,* 328-336.

Bergerat, A., de Massy, B., Gadelle, D., Varoutas, P.C., Nicolas, A., and Forterre, P. (1997). An atypical topoisomerase II from Archaea with implications for meiotic recombination. Nature *386,* 414-417.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature *409,* 363-366.

Blagosklonny, M.V., Toretsky, J., and Neckers, L. (1995). Geldanamycin selectively destabilizes and conformationally alters mutated p53. Oncogene *11,* 933-939.

Bohmert, K., Camus, I., Bellini, C., Bouchez, D., Caboche, M., and Benning, C. (1998). AGO1 defines a novel locus of Arabidopsis controlling leaf development. EMBO J. *17,* 170-180.

Bordeleau, M.E., Mori, A., Oberer, M., Lindqvist, L., Chard, L.S., Higa, T., Belsham, G.J., Wagner, G., Tanaka, J., and Pelletier, J. (2006). Functional characterization of IRESes by an inhibitor of the RNA helicase eIF4A. Nat. Chem. Biol. *2,* 213-220.

Borkovich, K.A., Farrelly, F.W., Finkelstein, D.B., Taulien, J., and Lindquist, S. (1989). Hsp82 is an Essential Protein that is Required in Higher Concentrations for Growth of Cells at Higher Temperatures. Mol. Cell. Biol. *9,* 3919-3930.

Breitkreutz, B.J., Stark, C., Reguly, T., Boucher, L., Breitkreutz, A., Livstone, M., Oughtred, R., Lackner, D.H., Bahler, J., Wood, V., Dolinski, K., and Tyers, M. (2008). The BioGRID Interaction Database: 2008 update. Nucleic Acids Res. *36,* D637-40.

Brennecke, J., Aravin, A.A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R., and Hannon, G.J. (2007). Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila. Cell *128,* 1089-1103.

Brennecke, J., Hipfner, D.R., Stark, A., Russell, R.B., and Cohen, S.M. (2003). bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. Cell *113,* 25-36.

Brennecke, J., Stark, A., Russell, R.B., and Cohen, S.M. (2005). Principles of microRNA-target recognition. PLoS Biol. *3,* e85.

Brugge, J.S., Erikson, E., and Erikson, R.L. (1981). The specific interaction of the Rous sarcoma virus transforming protein, pp60src, with two cellular proteins. Cell *25,* 363-372.

Buchan, J.R., Muhlrad, D., and Parker, R. (2008). P bodies promote stress granule assembly in Saccharomyces cerevisiae. J. Cell Biol. *183,* 441-455.

Buchan, J.R., and Parker, R. (2009). Eukaryotic stress granules: the ins and outs of translation. Mol. Cell *36,* 932-941.

Carmell, M.A., Girard, A., van de Kant, H.J., Bourc'his, D., Bestor, T.H., de Rooij, D.G., and Hannon, G.J. (2007). MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. Dev. Cell. *12,* 503-514.

Carmichael, J.B., Stoica, C., Parker, H., McCaffery, J.M., Simmonds, A.J., and Hobman, T.C. (2006). RNA interference effector proteins localize to mobile cytoplasmic puncta in Schizosaccharomyces pombe. Traffic *7,* 1032-1044.

Carrello, A., Ingley, E., Minchin, R.F., Tsai, S., and Ratajczak, T. (1999). The common tetratricopeptide repeat acceptor site for steroid receptor-associated immunophilins and hop is located in the dimerization domain of Hsp90. J. Biol. Chem. *274,* 2682-2689.

Caudy, A.A., Myers, M., Hannon, G.J., and Hammond, S.M. (2002). Fragile X-related protein and VIG associate with the RNA interference machinery. Genes Dev. *16,* 2491-2496.

Cerutti, L., Mian, N., and Bateman, A. (2000). Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain. Trends Biochem. Sci. *25,* 481-482.

Chadli, A., Bouhouche, I., Sullivan, W., Stensgard, B., McMahon, N., Catelli, M.G., and Toft, D.O. (2000). Dimerization and N-terminal domain proximity underlie the function of the molecular chaperone heat shock protein 90. Proc. Natl. Acad. Sci. U. S. A. *97,* 12524-12529.

Chapman, E.J., and Carrington, J.C. (2007). Specialization and evolution of endogenous small RNA pathways. Nat. Rev. Genet. *8,* 884-896.

Chavany, C., Mimnaugh, E., Miller, P., Bitton, R., Nguyen, P., Trepel, J., Whitesell, L., Schnur, R., Moyer, J., and Neckers, L. (1996). p185erbB2 binds to GRP94 in vivo. Dissociation of the p185erbB2/GRP94 heterocomplex by benzoquinone ansamycins precedes depletion of p185erbB2. J. Biol. Chem. *271,* 4974-4977.

Chen, C.Z., Li, L., Lodish, H.F., and Bartel, D.P. (2004). MicroRNAs modulate hematopoietic lineage differentiation. Science *303,* 83-86.

Chen, S., and Smith, D.F. (1998). Hop as an adaptor in the heat shock protein 70 (Hsp70) and hsp90 chaperone machinery. J. Biol. Chem. *273,* 35194-35200.

Chen, S., Sullivan, W.P., Toft, D.O., and Smith, D.F. (1998). Differential interactions of p23 and the TPR-containing proteins Hop, Cyp40, FKBP52 and FKBP51 with Hsp90 mutants. Cell Stress Chaperones *3,* 118-129.

Chen, X. (2004). A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. Science *303,* 2022-2025.

Chen, Y., Pane, A., and Schupbach, T. (2007). Cutoff and aubergine mutations result in retrotransposon upregulation and checkpoint activation in Drosophila. Curr. Biol. *17,* 637-642.

Chendrimada, T.P., Finn, K.J., Ji, X., Baillat, D., Gregory, R.I., Liebhaber, S.A., Pasquinelli, A.E., and Shiekhattar, R. (2007). MicroRNA silencing through RISC recruitment of eIF6. Nature *447,* 823-828.

Chendrimada, T.P., Gregory, R.I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K., and Shiekhattar, R. (2005). TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature *436,* 740-744.

Chu, C.Y., and Rana, T.M. (2006). Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54. PLoS Biol. *4,* e210.

Chung, W.J., Okamura, K., Martin, R., and Lai, E.C. (2008). Endogenous RNA interference provides a somatic defense against Drosophila transposons. Curr. Biol. *18,* 795-802.

Cougot, N., Babajko, S., and Seraphin, B. (2004). Cytoplasmic foci are sites of mRNA decay in human cells. J. Cell Biol. *165,* 31-40.

Cox, D.N., Chao, A., Baker, J., Chang, L., Qiao, D., and Lin, H. (1998). A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. Genes Dev. *12,* 3715-3727.

Cox, D.N., Chao, A., and Lin, H. (2000). Piwi Encodes a Nucleoplasmic Factor Whose Activity Modulates the Number and Division Rate of Germline Stem Cells. Development *127,* 503-514.

Csermely, P., Schnaider, T., Cheatham, B., Olson, M.O., and Kahn, C.R. (1993). Insulin induces the phosphorylation of nucleolin. A possible mechanism of insulin-induced RNA efflux from nuclei. J. Biol. Chem. *268,* 9747-9752.

Czech, B., Malone, C.D., Zhou, R., Stark, A., Schlingeheyde, C., Dus, M., Perrimon, N., Kellis, M., Wohlschlegel, J.A., Sachidanandam, R., Hannon, G.J., and Brennecke, J. (2008). An endogenous small interfering RNA pathway in Drosophila. Nature *453,* 798-802.

Deng, W., and Lin, H. (2002). Miwi, a Murine Homolog of Piwi, Encodes a Cytoplasmic Protein Essential for Spermatogenesis. Dev. Cell. *2,* 819-830.

Denli, A.M., Tops, B.B., Plasterk, R.H., Ketting, R.F., and Hannon, G.J. (2004). Processing of primary microRNAs by the Microprocessor complex. Nature *432,* 231-235.

Detzer, A., Engel, C., Wunsche, W., and Sczakiel, G. (2011). Cell stress is related to re-localization of Argonaute 2 and to decreased RNA interference in human cells. Nucleic Acids Res. *39,* 2727-2741.

Dever, T.E. (2002). Gene-specific regulation by general translation factors. Cell *108,* 545-556.

Ding, L., and Han, M. (2007). GW182 family proteins are crucial for microRNA-mediated gene silencing. Trends Cell Biol. *17,* 411-416.

Dittmar, K.D., Demady, D.R., Stancato, L.F., Krishna, P., and Pratt, W.B. (1997). Folding of the glucocorticoid receptor by the heat shock protein (hsp) 90-based chaperone

machinery. The role of p23 is to stabilize receptor.hsp90 heterocomplexes formed by hsp90.p60.hsp70. J. Biol. Chem. *272,* 21213-21220.

Djuranovic, S., Nahvi, A., and Green, R. (2011). A parsimonious model for gene regulation by miRNAs. Science *331,* 550-553.

Doench, J.G., and Sharp, P.A. (2004). Specificity of microRNA target selection in translational repression. Genes Dev. *18,* 504-511.

Dolinski, K., Muir, S., Cardenas, M., and Heitman, J. (1997). All cyclophilins and FK506 binding proteins are, individually and collectively, dispensable for viability in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A. *94,* 13093-13098.

Dunn, J.J. (1976). RNase III cleavage of single-stranded RNA. Effect of ionic strength on the fideltiy of cleavage. J. Biol. Chem. *251,* 3807-3814.

Eckhardt, S., Szostak, E., Yang, Z., and Pillai, R. (2011). Artificial tethering of Argonaute proteins for studying their role in translational repression of target mRNAs. Methods Mol. Biol. *725,* 191-206.

Eisinger-Mathason, T.S., Andrade, J., Groehler, A.L., Clark, D.E., Muratore-Schroeder, T.L., Pasic, L., Smith, J.A., Shabanowitz, J., Hunt, D.F., Macara, I.G., and Lannigan, D.A. (2008). Codependent functions of RSK2 and the apoptosis-promoting factor TIA-1 in stress granule assembly and cell survival. Mol. Cell *31,* 722-736.

Elbashir, S.M., Lendeckel, W., and Tuschl, T. (2001a). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. *15,* 188-200.

Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. (2001b). Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. EMBO J. *20,* 6877-6888.

El-Shami, M., Pontier, D., Lahmy, S., Braun, L., Picart, C., Vega, D., Hakimi, M.A., Jacobsen, S.E., Cooke, R., and Lagrange, T. (2007). Reiterated WG/GW motifs form functionally and evolutionarily conserved ARGONAUTE-binding platforms in RNAi-related components. Genes Dev. *21,* 2539-2544.

Emery, J.F., Floyd, S.K., Alvarez, J., Eshed, Y., Hawker, N.P., Izhaki, A., Baum, S.F., and Bowman, J.L. (2003). Radial patterning of Arabidopsis shoots by class III HD-ZIP and KANADI genes. Curr. Biol. *13,* 1768-1774.

Ender, C., and Meister, G. (2010). Argonaute proteins at a glance. J. Cell. Sci. *123,* 1819-1823.

Eulalio, A., Behm-Ansmant, I., and Izaurralde, E. (2007a). P bodies: at the crossroads of post-transcriptional pathways. Nat. Rev. Mol. Cell Biol. *8,* 9-22.

Eulalio, A., Behm-Ansmant, I., Schweizer, D., and Izaurralde, E. (2007b). P-body formation is a consequence, not the cause, of RNA-mediated gene silencing. Mol. Cell. Biol. *27,* 3970-3981.

Eulalio, A., Helms, S., Fritzsch, C., Fauser, M., and Izaurralde, E. (2009). A C-terminal silencing domain in GW182 is essential for miRNA function. RNA *15,* 1067-1077.

Eulalio, A., Huntzinger, E., and Izaurralde, E. (2008). GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. Nat. Struct. Mol. Biol. *15,* 346-353.

Eulalio, A., Huntzinger, E., Nishihara, T., Rehwinkel, J., Fauser, M., and Izaurralde, E. (2009). Deadenylation is a widespread effect of miRNA regulation. RNA *15,* 21-32.

Eulalio, A., Rehwinkel, J., Stricker, M., Huntzinger, E., Yang, S.F., Doerks, T., Dorner, S., Bork, P., Boutros, M., and Izaurralde, E. (2007). Target-specific requirements for enhancers of decapping in miRNA-mediated gene silencing. Genes Dev. *21,* 2558-2570.

Eulalio, A., Tritschler, F., and Izaurralde, E. (2009). The GW182 protein family in animal cells: new insights into domains required for miRNA-mediated gene silencing. RNA *15,* 1433-1442.

Eystathioy, T., Chan, E.K., Tenenbaum, S.A., Keene, J.D., Griffith, K., and Fritzler, M.J. (2002). A phosphorylated cytoplasmic autoantigen, GW182, associates with a unique population of human mRNAs within novel cytoplasmic speckles. Mol. Biol. Cell *13,* 1338-1351.

Fabian, M.R., Mathonnet, G., Sundermeier, T., Mathys, H., Zipprich, J.T., Svitkin, Y.V., Rivas, F., Jinek, M., Wohlschlegel, J., Doudna, J.A*., et al.* (2009). Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. Mol. Cell *35,* 868-880.

Fabian, M.R., Sonenberg, N., and Filipowicz, W. (2010). Regulation of mRNA translation and stability by microRNAs. Annu. Rev. Biochem. *79,* 351-379.

Filipowicz, W., Bhattacharyya, S.N., and Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat. Rev. Genet. *9,* 102-114.

Filippov, V., Solovyev, V., Filippova, M., and Gill, S.S. (2000). A novel type of RNase III family proteins in eukaryotes. Gene *245,* 213-221.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391,* 806-811.

Forstemann, K., Horwich, M.D., Wee, L., Tomari, Y., and Zamore, P.D. (2007). Drosophila microRNAs are sorted into functionally distinct argonaute complexes after production by dicer-1. Cell *130,* 287-297.

Forstemann, K., Tomari, Y., Du, T., Vagin, V.V., Denli, A.M., Bratu, D.P., Klattenhoff, C., Theurkauf, W.E., and Zamore, P.D. (2005). Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. PLoS Biol. *3,* e236.

Fortin, K.R., Nicholson, R.H., and Nicholson, A.W. (2002). Mouse ribonuclease III. cDNA structure, expression analysis, and chromosomal location. BMC Genomics *3,* 26.

Franks, T.M., and Lykke-Andersen, J. (2008). The control of mRNA decapping and P-body formation. Mol. Cell *32,* 605-615.

Freeman, B.C., Felts, S.J., Toft, D.O., and Yamamoto, K.R. (2000). The p23 molecular chaperones act at a late step in intracellular receptor action to differentially affect ligand efficacies. Genes Dev. *14,* 422-434.

Friedman, R.C., Farh, K.K., Burge, C.B., and Bartel, D.P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. *19,* 92-105.

Fujimura, K., Katahira, J., Kano, F., Yoneda, Y., and Murata, M. (2009). Microscopic dissection of the process of stress granule assembly. Biochim. Biophys. Acta *1793,* 1728-1737.

Gallouzi, I.E., Brennan, C.M., Stenberg, M.G., Swanson, M.S., Eversole, A., Maizels, N., and Steitz, J.A. (2000). HuR binding to cytoplasmic mRNA is perturbed by heat shock. Proc. Natl. Acad. Sci. U. S. A. *97,* 3073-3078.

Garcia-Cardena, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A., and Sessa, W.C. (1998). Dynamic activation of endothelial nitric oxide synthase by Hsp90. Nature *392,* 821-824.

Ghildiyal, M., Seitz, H., Horwich, M.D., Li, C., Du, T., Lee, S., Xu, J., Kittler, E.L., Zapp, M.L., Weng, Z., and Zamore, P.D. (2008). Endogenous siRNAs derived from transposons and mRNAs in Drosophila somatic cells. Science *320,* 1077-1081.

Gilks, N., Kedersha, N., Ayodele, M., Shen, L., Stoecklin, G., Dember, L.M., and Anderson, P. (2004). Stress granule assembly is mediated by prion-like aggregation of TIA-1. Mol. Biol. Cell *15,* 5383-5398.

Girard, A., Sachidanandam, R., Hannon, G.J., and Carmell, M.A. (2006). A germline-specific class of small RNAs binds mammalian Piwi proteins. Nature *442,* 199-202.

Grad, I., and Picard, D. (2007). The glucocorticoid responses are shaped by molecular chaperones. Mol. Cell. Endocrinol. *275,* 2-12.

Gregory, R.I., Chendrimada, T.P., Cooch, N., and Shiekhattar, R. (2005). Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell *123,* 631-640.

Gregory, R.I., Yan, K.P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., and Shiekhattar, R. (2004). The Microprocessor complex mediates the genesis of microRNAs. Nature *432,* 235-240.

Grenert, J.P., Sullivan, W.P., Fadden, P., Haystead, T.A., Clark, J., Mimnaugh, E., Krutzsch, H., Ochel, H.J., Schulte, T.W., Sausville, E., Neckers, L.M., and Toft, D.O. (1997). The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation. J. Biol. Chem. *272,* 23843-23850.

Grimson, A., Farh, K.K., Johnston, W.K., Garrett-Engele, P., Lim, L.P., and Bartel, D.P. (2007). MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol. Cell *27,* 91-105.

Grivna, S.T., Beyret, E., Wang, Z., and Lin, H. (2006). A novel class of small RNAs in mouse spermatogenic cells. Genes Dev. *20,* 1709-1714.

Gunawardane, L.S., Saito, K., Nishida, K.M., Miyoshi, K., Kawamura, Y., Nagami, T., Siomi, H., and Siomi, M.C. (2007). A slicer-mediated mechanism for repeat-associated siRNA 5' end formation in Drosophila. Science *315,* 1587-1590.

Guo, H., Ingolia, N.T., Weissman, J.S., and Bartel, D.P. (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature *466,* 835-840.

Haase, A.D., Jaskiewicz, L., Zhang, H., Laine, S., Sack, R., Gatignol, A., and Filipowicz, W. (2005). TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. EMBO Rep. *6,* 961-967.

Han, J., Lee, Y., Yeom, K.H., Kim, Y.K., Jin, H., and Kim, V.N. (2004). The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev. *18,* 3016-3027.

Han, J., Lee, Y., Yeom, K.H., Nam, J.W., Heo, I., Rhee, J.K., Sohn, S.Y., Cho, Y., Zhang, B.T., and Kim, V.N. (2006). Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell *125,* 887-901.

Hernandez, M.P., Sullivan, W.P., and Toft, D.O. (2002). The assembly and intermolecular properties of the hsp70-Hop-hsp90 molecular chaperone complex. J. Biol. Chem. *277,* 38294-38304.

Hilgers, V., Teixeira, D., and Parker, R. (2006). Translation-independent inhibition of mRNA deadenylation during stress in Saccharomyces cerevisiae. RNA *12,* 1835-1845.

Hock, J., Weinmann, L., Ender, C., Rudel, S., Kremmer, E., Raabe, M., Urlaub, H., and Meister, G. (2007). Proteomic and functional analysis of Argonaute-containing mRNA-protein complexes in human cells. EMBO Rep. *8,* 1052-1060.

Holt, S.E., Aisner, D.L., Baur, J., Tesmer, V.M., Dy, M., Ouellette, M., Trager, J.B., Morin, G.B., Toft, D.O., Shay, J.W., Wright, W.E., and White, M.A. (1999). Functional requirement of p23 and Hsp90 in telomerase complexes. Genes Dev. *13,* 817-826.

Houwing, S., Kamminga, L.M., Berezikov, E., Cronembold, D., Girard, A., van den Elst, H., Filippov, D.V., Blaser, H., Raz, E., Moens, C.B.*, et al.* (2007). A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in Zebrafish. Cell *129,* 69-82.

Humphreys, D.T., Westman, B.J., Martin, D.I., and Preiss, T. (2005). MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. Proc. Natl. Acad. Sci. U. S. A. *102,* 16961-16966.

Huntzinger, E., and Izaurralde, E. (2011). Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat. Rev. Genet. *12,* 99-110.

Hutvagner, G., Simard, M.J., Mello, C.C., and Zamore, P.D. (2004). Sequence-specific inhibition of small RNA function. PLoS Biol. *2,* E98.

Hutvagner, G., and Zamore, P.D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. Science *297,* 2056-2060.

Iki, T., Yoshikawa, M., Nishikiori, M., Jaudal, M.C., Matsumoto-Yokoyama, E., Mitsuhara, I., Meshi, T., and Ishikawa, M. (2010). In vitro assembly of plant RNA-induced silencing complexes facilitated by molecular chaperone HSP90. Mol. Cell *39,* 282-291.

Ingelfinger, D., Arndt-Jovin, D.J., Luhrmann, R., and Achsel, T. (2002). The human LSm1-7 proteins colocalize with the mRNA-degrading enzymes Dcp1/2 and Xrnl in distinct cytoplasmic foci. RNA *8,* 1489-1501.

Inoue, H., Hayase, Y., Imura, A., Iwai, S., Miura, K., and Ohtsuka, E. (1987). Synthesis and hybridization studies on two complementary nona(2'-O-methyl)ribonucleotides. Nucleic Acids Res. *15,* 6131-6148.

Ivanov, P.A., Chudinova, E.M., and Nadezhdina, E.S. (2003). Disruption of microtubules inhibits cytoplasmic ribonucleoprotein stress granule formation. Exp. Cell Res. *290,* 227-233.

Iwasaki, S., Kawamata, T., and Tomari, Y. (2009). Drosophila argonaute1 and argonaute2 employ distinct mechanisms for translational repression. Mol. Cell *34,* 58-67.

Iwasaki, S., Kobayashi, M., Yoda, M., Sakaguchi, Y., Katsuma, S., Suzuki, T., and Tomari, Y. (2010). Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. Mol. Cell *39,* 292-299.

Jakymiw, A., Lian, S., Eystathioy, T., Li, S., Satoh, M., Hamel, J.C., Fritzler, M.J., and Chan, E.K. (2005). Disruption of GW bodies impairs mammalian RNA interference. Nat. Cell Biol. *7,* 1267-1274.

Jannot, G., Vasquez-Rifo, A., and Simard, M.J. (2011). Argonaute Pull-Down and RISC Analysis Using 2'-O-Methylated Oligonucleotides Affinity Matrices. Methods Mol. Biol. *725,* 233-249.

Jiang, F., Ye, X., Liu, X., Fincher, L., McKearin, D., and Liu, Q. (2005). Dicer-1 and R3D1-L catalyze microRNA maturation in Drosophila. Genes Dev. *19,* 1674-1679.

Joab, I., Radanyi, C., Renoir, M., Buchou, T., Catelli, M.G., Binart, N., Mester, J., and Baulieu, E.E. (1984). Common non-hormone binding component in non-transformed chick oviduct receptors of four steroid hormones. Nature *308,* 850-853.

Johnson, S.M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., Labourier, E., Reinert, K.L., Brown, D., and Slack, F.J. (2005). RAS is regulated by the let-7 microRNA family. Cell *120,* 635-647.

Johnston, M., Geoffroy, M.C., Sobala, A., Hay, R., and Hutvagner, G. (2010). HSP90 protein stabilizes unloaded argonaute complexes and microscopic P-bodies in human cells. Mol. Biol. Cell *21,* 1462-1469.

Johnston, R.J., and Hobert, O. (2003). A microRNA controlling left/right neuronal asymmetry in Caenorhabditis elegans. Nature *426,* 845-849.

Jung, G., Barylko, B., Lu, D., Shu, H., Yin, H., and Albanesi, J.P. (2011). Stabilization of phosphatidylinositol 4-kinase type IIbeta by interaction with Hsp90. J. Biol. Chem. *286,* 12775-12784.

Kaufman, R.J. (2004). Regulation of mRNA translation by protein folding in the endoplasmic reticulum. Trends Biochem. Sci. *29,* 152-158.

Kawamata, T., and Tomari, Y. (2010). Making RISC. Trends Biochem. Sci. *35,* 368-376.

Kawamura, Y., Saito, K., Kin, T., Ono, Y., Asai, K., Sunohara, T., Okada, T.N., Siomi, M.C., and Siomi, H. (2008). Drosophila endogenous small RNAs bind to Argonaute 2 in somatic cells. Nature *453,* 793-797.

Kedersha, N., and Anderson, P. (2002). Stress granules: sites of mRNA triage that regulate mRNA stability and translatability. Biochem. Soc. Trans. *30,* 963-969.

Kedersha, N., Chen, S., Gilks, N., Li, W., Miller, I.J., Stahl, J., and Anderson, P. (2002). Evidence that ternary complex (eIF2-GTP-tRNA(i)(Met))-deficient preinitiation complexes are core constituents of mammalian stress granules. Mol. Biol. Cell *13,* 195-210.

Kedersha, N., Cho, M.R., Li, W., Yacono, P.W., Chen, S., Gilks, N., Golan, D.E., and Anderson, P. (2000). Dynamic shuttling of TIA-1 accompanies the recruitment of mRNA to mammalian stress granules. J. Cell Biol. *151,* 1257-1268.

Kedersha, N., Stoecklin, G., Ayodele, M., Yacono, P., Lykke-Andersen, J., Fritzler, M.J., Scheuner, D., Kaufman, R.J., Golan, D.E., and Anderson, P. (2005). Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. J. Cell Biol. *169,* 871-884.

Kedersha, N.L., Gupta, M., Li, W., Miller, I., and Anderson, P. (1999). RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2 alpha to the assembly of mammalian stress granules. J. Cell Biol. *147,* 1431-1442.

Kemp, C., and Imler, J.L. (2009). Antiviral immunity in drosophila. Curr. Opin. Immunol. *21,* 3-9.

Kim, J., Krichevsky, A., Grad, Y., Hayes, G.D., Kosik, K.S., Church, G.M., and Ruvkun, G. (2004). Identification of many microRNAs that copurify with polyribosomes in mammalian neurons. Proc. Natl. Acad. Sci. U. S. A. *101,* 360-365.

Kim, V.N. (2005). MicroRNA biogenesis: coordinated cropping and dicing. Nat. Rev. Mol. Cell Biol. *6,* 376-385.

Kim, V.N., Han, J., and Siomi, M.C. (2009). Biogenesis of small RNAs in animals. Nat. Rev. Mol. Cell Biol. *10,* 126-139.

Kimball, S.R. (2001). Regulation of translation initiation by amino acids in eukaryotic cells. Prog. Mol. Subcell. Biol. *26,* 155-184.

Kirino, Y., Kim, N., de Planell-Saguer, M., Khandros, E., Chiorean, S., Klein, P.S., Rigoutsos, I., Jongens, T.A., and Mourelatos, Z. (2009). Arginine methylation of Piwi proteins catalysed by dPRMT5 is required for Ago3 and Aub stability. Nat. Cell Biol. *11,* 652-658.

Knowles, R.B., Sabry, J.H., Martone, M.E., Deerinck, T.J., Ellisman, M.H., Bassell, G.J., and Kosik, K.S. (1996). Translocation of RNA granules in living neurons. J. Neurosci. *16,* 7812-7820.

Kojima, K., Kuramochi-Miyagawa, S., Chuma, S., Tanaka, T., Nakatsuji, N., Kimura, T., and Nakano, T. (2009). Associations between PIWI proteins and TDRD1/MTR-1 are critical for integrated subcellular localization in murine male germ cells. Genes Cells *14,* 1155-1165.

Kolobova, E., Efimov, A., Kaverina, I., Rishi, A.K., Schrader, J.W., Ham, A.J., Larocca, M.C., and Goldenring, J.R. (2009). Microtubule-dependent association of AKAP350A and CCAR1 with RNA stress granules. Exp. Cell Res. *315,* 542-555.

Krishnamoorthy, T., Pavitt, G.D., Zhang, F., Dever, T.E., and Hinnebusch, A.G. (2001). Tight binding of the phosphorylated alpha subunit of initiation factor 2 (eIF2alpha) to the regulatory subunits of guanine nucleotide exchange factor eIF2B is required for inhibition of translation initiation. Mol. Cell. Biol. *21,* 5018-5030.

Kuramochi-Miyagawa, S., Kimura, T., Ijiri, T.W., Isobe, T., Asada, N., Fujita, Y., Ikawa, M., Iwai, N., Okabe, M., Deng, W.*, et al.* (2004). Mili, a mammalian member of piwi family gene, is essential for spermatogenesis. Development *131,* 839-849.

Kuramochi-Miyagawa, S., Watanabe, T., Gotoh, K., Totoki, Y., Toyoda, A., Ikawa, M., Asada, N., Kojima, K., Yamaguchi, Y., Ijiri, T.W.*, et al.* (2008). DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. Genes Dev. *22,* 908-917.

Kwon, S., Zhang, Y., and Matthias, P. (2007). The deacetylase HDAC6 is a novel critical component of stress granules involved in the stress response. Genes Dev. *21,* 3381-3394.

Landthaler, M., Yalcin, A., and Tuschl, T. (2004). The human DiGeorge syndrome critical region gene 8 and Its D. melanogaster homolog are required for miRNA biogenesis. Curr. Biol. *14,* 2162-2167.

Laraki, G., Clerzius, G., Daher, A., Melendez-Pena, C., Daniels, S., and Gatignol, A. (2008). Interactions between the double-stranded RNA-binding proteins TRBP and PACT define the Medipal domain that mediates protein-protein interactions. RNA Biol. *5,* 92-103.

Lau, N.C., Seto, A.G., Kim, J., Kuramochi-Miyagawa, S., Nakano, T., Bartel, D.P., and Kingston, R.E. (2006). Characterization of the piRNA complex from rat testes. Science *313,* 363-367.

Lazzaretti, D., Tournier, I., and Izaurralde, E. (2009). The C-terminal domains of human TNRC6A, TNRC6B, and TNRC6C silence bound transcripts independently of Argonaute proteins. RNA *15,* 1059-1066.

Lee, J.H., Schutte, D., Wulf, G., Fuzesi, L., Radzun, H.J., Schweyer, S., Engel, W., and Nayernia, K. (2006). Stem-cell protein Piwil2 is widely expressed in tumors and inhibits apoptosis through activation of Stat3/Bcl-XL pathway. Hum. Mol. Genet. *15,* 201-211.

Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell *75,* 843-854.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S., and Kim, V.N. (2003). The nuclear RNase III Drosha initiates microRNA processing. Nature *425,* 415-419.

Lee, Y., Hur, I., Park, S.Y., Kim, Y.K., Suh, M.R., and Kim, V.N. (2006). The role of PACT in the RNA silencing pathway. EMBO J. *25,* 522-532.

Lee, Y.S., Nakahara, K., Pham, J.W., Kim, K., He, Z., Sontheimer, E.J., and Carthew, R.W. (2004). Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell *117,* 69-81.

Leung, A.K., Calabrese, J.M., and Sharp, P.A. (2006). Quantitative analysis of Argonaute protein reveals microRNA-dependent localization to stress granules. Proc. Natl. Acad. Sci. U. S. A. *103,* 18125-18130.

Lewis, B.P., Shih, I.H., Jones-Rhoades, M.W., Bartel, D.P., and Burge, C.B. (2003). Prediction of mammalian microRNA targets. Cell *115,* 787-798.

Li, C., Vagin, V.V., Lee, S., Xu, J., Ma, S., Xi, H., Seitz, H., Horwich, M.D., Syrzycka, M., Honda, B.M.*, et al.* (2009). Collapse of germline piRNAs in the absence of Argonaute3 reveals somatic piRNAs in flies. Cell *137,* 509-521.

Li, L., Yu, C., Gao, H., and Li, Y. (2010). Argonaute proteins: potential biomarkers for human colon cancer. BMC Cancer *10,* 38.

Li, S., Lian, S.L., Moser, J.J., Fritzler, M.L., Fritzler, M.J., Satoh, M., and Chan, E.K. (2008). Identification of GW182 and its novel isoform TNGW1 as translational repressors in Ago2-mediated silencing. J. Cell. Sci. *121,* 4134-4144.

Lian, S.L., Li, S., Abadal, G.X., Pauley, B.A., Fritzler, M.J., and Chan, E.K. (2009). The C-terminal half of human Ago2 binds to multiple GW-rich regions of GW182 and requires GW182 to mediate silencing. RNA *15,* 804-813.

Lingel, A., Simon, B., Izaurralde, E., and Sattler, M. (2004). Nucleic acid 3'-end recognition by the Argonaute2 PAZ domain. Nat. Struct. Mol. Biol. *11,* 576-577.

Lingel, A., Simon, B., Izaurralde, E., and Sattler, M. (2003). Structure and nucleic-acid binding of the Drosophila Argonaute 2 PAZ domain. Nature *426,* 465-469.

Lipsich, L.A., Cutt, J.R., and Brugge, J.S. (1982). Association of the transforming proteins of Rous, Fujinami, and Y73 avian sarcoma viruses with the same two cellular proteins. Mol. Cell. Biol. *2,* 875-880.

Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L., and Hannon, G.J. (2004). Argonaute2 is the catalytic engine of mammalian RNAi. Science *305,* 1437-1441.

Liu, J., Rivas, F.V., Wohlschlegel, J., Yates, J.R.,3rd, Parker, R., and Hannon, G.J. (2005a). A role for the P-body component GW182 in microRNA function. Nat. Cell Biol. *7,* 1261-1266.

Liu, J., Valencia-Sanchez, M.A., Hannon, G.J., and Parker, R. (2005b). MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat. Cell Biol. *7,* 719-723.

Liu, Q., and Paroo, Z. (2010). Biochemical principles of small RNA pathways. Annu. Rev. Biochem. *79,* 295-319.

Liu, Q., Rand, T.A., Kalidas, S., Du, F., Kim, H.E., Smith, D.P., and Wang, X. (2003). R2D2, a bridge between the initiation and effector steps of the Drosophila RNAi pathway. Science *301,* 1921-1925.

Liu, X., Park, J.K., Jiang, F., Liu, Y., McKearin, D., and Liu, Q. (2007). Dicer-1, but not Loquacious, is critical for assembly of miRNA-induced silencing complexes. RNA *13,* 2324-2329.

Loschi, M., Leishman, C.C., Berardone, N., and Boccaccio, G.L. (2009). Dynein and kinesin regulate stress-granule and P-body dynamics. J. Cell. Sci. *122,* 3973-3982.

Lu, L., Han, A.P., and Chen, J.J. (2001). Translation initiation control by heme-regulated eukaryotic initiation factor 2alpha kinase in erythroid cells under cytoplasmic stresses. Mol. Cell. Biol. *21,* 7971-7980.

Lu, R., Maduro, M., Li, F., Li, H.W., Broitman-Maduro, G., Li, W.X., and Ding, S.W. (2005). Animal virus replication and RNAi-mediated antiviral silencing in Caenorhabditis elegans. Nature *436,* 1040-1043.

Lund, E., Guttinger, S., Calado, A., Dahlberg, J.E., and Kutay, U. (2004). Nuclear export of microRNA precursors. Science *303,* 95-98.

Lykke-Andersen, J. (2002). Identification of a human decapping complex associated with hUpf proteins in nonsense-mediated decay. Mol. Cell. Biol. *22,* 8114-8121.

Ma, J.B., Ye, K., and Patel, D.J. (2004). Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. Nature *429,* 318-322.

Ma, J.B., Yuan, Y.R., Meister, G., Pei, Y., Tuschl, T., and Patel, D.J. (2005). Structural basis for 5'-end-specific recognition of guide RNA by the A. fulgidus Piwi protein. Nature *434,* 666-670.

Macrae, I.J., Zhou, K., Li, F., Repic, A., Brooks, A.N., Cande, W.Z., Adams, P.D., and Doudna, J.A. (2006). Structural basis for double-stranded RNA processing by Dicer. Science *311,* 195-198.

Mallory, A., and Vaucheret, H. (2010). Form, function, and regulation of ARGONAUTE proteins. Plant Cell *22,* 3879-3889.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R.M. (1995). The nuclear receptor superfamily: the second decade. Cell *83,* 835-839.

Maniataki, E., and Mourelatos, Z. (2005). A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. Genes Dev. *19,* 2979-2990.

Maroney, P.A., Yu, Y., Fisher, J., and Nilsen, T.W. (2006). Evidence that microRNAs are associated with translating messenger RNAs in human cells. Nat. Struct. Mol. Biol. *13,* 1102-1107.

Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R., and Tuschl, T. (2002). Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell *110,* 563-574.

Maruya, M., Sameshima, M., Nemoto, T., and Yahara, I. (1999). Monomer arrangement in HSP90 dimer as determined by decoration with N and C-terminal region specific antibodies. J. Mol. Biol. *285,* 903-907.

Mathonnet, G., Fabian, M.R., Svitkin, Y.V., Parsyan, A., Huck, L., Murata, T., Biffo, S., Merrick, W.C., Darzynkiewicz, E., Pillai, R.S.*, et al.* (2007). MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. Science *317,* 1764-1767.

Mayr, C., Hemann, M.T., and Bartel, D.P. (2007). Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. Science *315,* 1576-1579.

Mazroui, R., Di Marco, S., Kaufman, R.J., and Gallouzi, I.E. (2007). Inhibition of the ubiquitin-proteasome system induces stress granule formation. Mol. Biol. Cell *18,* 2603-2618.

McClure, L.V., Seo, G.J., and Sullivan, C.S. (2011). Reporter-based assays for analyzing RNA interference in mammalian cells. Methods Mol. Biol. *725,* 173-189.

McLaughlin, S.H., Smith, H.W., and Jackson, S.E. (2002). Stimulation of the weak ATPase activity of human hsp90 by a client protein. J. Mol. Biol. *315,* 787-798.

McLaughlin, S.H., Sobott, F., Yao, Z.P., Zhang, W., Nielsen, P.R., Grossmann, J.G., Laue, E.D., Robinson, C.V., and Jackson, S.E. (2006). The co-chaperone p23 arrests the Hsp90 ATPase cycle to trap client proteins. J. Mol. Biol. *356,* 746-758.

Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., and Tuschl, T. (2004). Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol. Cell *15,* 185-197.

Meister, G., Landthaler, M., Peters, L., Chen, P.Y., Urlaub, H., Luhrmann, R., and Tuschl, T. (2005). Identification of novel argonaute-associated proteins. Curr. Biol. *15,* 2149-2155.

Mescalchin, A., Detzer, A., Weirauch, U., Hahnel, M.J., Engel, C., and Sczakiel, G. (2010). Antisense tools for functional studies of human Argonaute proteins. RNA *16,* 2529-2536.

Meyer, P., Prodromou, C., Liao, C., Hu, B., Mark Roe, S., Vaughan, C.K., Vlasic, I., Panaretou, B., Piper, P.W., and Pearl, L.H. (2004). Structural basis for recruitment of the ATPase activator Aha1 to the Hsp90 chaperone machinery. EMBO J. *23,* 511-519.

Minet, E., Mottet, D., Michel, G., Roland, I., Raes, M., Remacle, J., and Michiels, C. (1999). Hypoxia-induced activation of HIF-1: role of HIF-1alpha-Hsp90 interaction. FEBS Lett. *460,* 251-256.

Miyoshi, T., Takeuchi, A., Siomi, H., and Siomi, M.C. (2010). A direct role for Hsp90 in pre-RISC formation in Drosophila. Nat. Struct. Mol. Biol. *17,* 1024-1026.

Mokas, S., Mills, J.R., Garreau, C., Fournier, M.J., Robert, F., Arya, P., Kaufman, R.J., Pelletier, J., and Mazroui, R. (2009). Uncoupling stress granule assembly and translation initiation inhibition. Mol. Biol. Cell *20,* 2673-2683.

Morel, J.B., Godon, C., Mourrain, P., Beclin, C., Boutet, S., Feuerbach, F., Proux, F., and Vaucheret, H. (2002). Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. Plant Cell *14,* 629-639.

Morishima, Y., Kanelakis, K.C., Murphy, P.J., Lowe, E.R., Jenkins, G.J., Osawa, Y., Sunahara, R.K., and Pratt, W.B. (2003). The hsp90 cochaperone p23 is the limiting component of the multiprotein hsp90/hsp70-based chaperone system in vivo where it acts to stabilize the client protein: hsp90 complex. J. Biol. Chem. *278,* 48754-48763.

Morita, S., Horii, T., Kimura, M., Goto, Y., Ochiya, T., and Hatada, I. (2007). One Argonaute family member, Eif2c2 (Ago2), is essential for development and appears not to be involved in DNA methylation. Genomics *89,* 687-696.

Moser, J.J., Eystathioy, T., Chan, E.K., and Fritzler, M.J. (2007). Markers of mRNA stabilization and degradation, and RNAi within astrocytoma GW bodies. J. Neurosci. Res. *85,* 3619-3631.

Moss, E.G., Lee, R.C., and Ambros, V. (1997). The cold shock domain protein LIN-28 controls developmental timing in C. elegans and is regulated by the lin-4 RNA. Cell *88,* 637-646.

Nelson, P.T., Hatzigeorgiou, A.G., and Mourelatos, Z. (2004). miRNP:mRNA association in polyribosomes in a human neuronal cell line. RNA *10,* 387-394.

Nemoto, T., Ohara-Nemoto, Y., Denis, M., and Gustafsson, J.A. (1990). The transformed glucocorticoid receptor has a lower steroid-binding affinity than the nontransformed receptor. Biochemistry *29,* 1880-1886.

Nottrott, S., Simard, M.J., and Richter, J.D. (2006). Human let-7a miRNA blocks protein production on actively translating polyribosomes. Nat. Struct. Mol. Biol. *13,* 1108-1114.

Nover, L., Scharf, K.D., and Neumann, D. (1989). Cytoplasmic heat shock granules are formed from precursor particles and are associated with a specific set of mRNAs. Mol. Cell. Biol. *9,* 1298-1308.

Ohn, T., Kedersha, N., Hickman, T., Tisdale, S., and Anderson, P. (2008). A functional RNAi screen links O-GlcNAc modification of ribosomal proteins to stress granule and processing body assembly. Nat. Cell Biol. *10,* 1224-1231.

Okamura, K., Balla, S., Martin, R., Liu, N., and Lai, E.C. (2008a). Two distinct mechanisms generate endogenous siRNAs from bidirectional transcription in Drosophila melanogaster. Nat. Struct. Mol. Biol. *15,* 581-590.

Okamura, K., Chung, W.J., Ruby, J.G., Guo, H., Bartel, D.P., and Lai, E.C. (2008b). The Drosophila hairpin RNA pathway generates endogenous short interfering RNAs. Nature *453,* 803-806.

Okamura, K., Hagen, J.W., Duan, H., Tyler, D.M., and Lai, E.C. (2007). The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila. Cell *130,* 89-100.

Olsen, P.H., and Ambros, V. (1999). The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev. Biol. *216,* 671-680.

Owens-Grillo, J.K., Czar, M.J., Hutchison, K.A., Hoffmann, K., Perdew, G.H., and Pratt, W.B. (1996a). A model of protein targeting mediated by immunophilins and other proteins that bind to hsp90 via tetratricopeptide repeat domains. J. Biol. Chem. *271,* 13468-13475.

Owens-Grillo, J.K., Stancato, L.F., Hoffmann, K., Pratt, W.B., and Krishna, P. (1996b). Binding of immunophilins to the 90 kDa heat shock protein (hsp90) via a tetratricopeptide repeat domain is a conserved protein interaction in plants. Biochemistry *35,* 15249-15255.

Palatnik, J.F., Allen, E., Wu, X., Schommer, C., Schwab, R., Carrington, J.C., and Weigel, D. (2003). Control of leaf morphogenesis by microRNAs. Nature *425,* 257-263.

Panaretou, B., Siligardi, G., Meyer, P., Maloney, A., Sullivan, J.K., Singh, S., Millson, S.H., Clarke, P.A., Naaby-Hansen, S., Stein, R.*, et al.* (2002). Activation of the ATPase activity of hsp90 by the stress-regulated cochaperone aha1. Mol. Cell *10,* 1307-1318.

Pare, J.M., Tahbaz, N., Lopez-Orozco, J., LaPointe, P., Lasko, P., and Hobman, T.C. (2009). Hsp90 regulates the function of argonaute 2 and its recruitment to stress granules and P-bodies. Mol. Biol. Cell *20,* 3273-3284.

Parker, J.S., Roe, S.M., and Barford, D. (2005). Structural insights into mRNA recognition from a PIWI domain-siRNA guide complex. Nature *434,* 663-666.

Parker, J.S., Roe, S.M., and Barford, D. (2004). Crystal structure of a PIWI protein suggests mechanisms for siRNA recognition and slicer activity. EMBO J. *23,* 4727-4737.

Pearl, L.H., and Prodromou, C. (2006). Structure and mechanism of the Hsp90 molecular chaperone machinery. Annu. Rev. Biochem. *75,* 271-294.

Pelisson, A., Sarot, E., Payen-Groschene, G., and Bucheton, A. (2007). A novel repeat-associated small interfering RNA-mediated silencing pathway downregulates complementary sense gypsy transcripts in somatic cells of the Drosophila ovary. J. Virol. *81,* 1951-1960.

Petersen, C.P., Bordeleau, M.E., Pelletier, J., and Sharp, P.A. (2006). Short RNAs repress translation after initiation in mammalian cells. Mol. Cell *21,* 533-542.

Pham, J.W., Pellino, J.L., Lee, Y.S., Carthew, R.W., and Sontheimer, E.J. (2004). A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in Drosophila. Cell *117,* 83-94.

Pillai, R.S., Artus, C.G., and Filipowicz, W. (2004). Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. RNA *10,* 1518-1525.

Pillai, R.S., Bhattacharyya, S.N., Artus, C.G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E., and Filipowicz, W. (2005). Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science *309,* 1573-1576.

Prodromou, C., Panaretou, B., Chohan, S., Siligardi, G., O'Brien, R., Ladbury, J.E., Roe, S.M., Piper, P.W., and Pearl, L.H. (2000). The ATPase cycle of Hsp90 drives a molecular 'clamp' via transient dimerization of the N-terminal domains. EMBO J. *19,* 4383-4392.

Prodromou, C., Roe, S.M., Piper, P.W., and Pearl, L.H. (1997). A molecular clamp in the crystal structure of the N-terminal domain of the yeast Hsp90 chaperone. Nat. Struct. Biol. *4,* 477-482.

Prodromou, C., Siligardi, G., O'Brien, R., Woolfson, D.N., Regan, L., Panaretou, B., Ladbury, J.E., Piper, P.W., and Pearl, L.H. (1999). Regulation of Hsp90 ATPase activity by tetratricopeptide repeat (TPR)-domain co-chaperones. EMBO J. *18,* 754-762.

Provost, P., Dishart, D., Doucet, J., Frendewey, D., Samuelsson, B., and Radmark, O. (2002). Ribonuclease activity and RNA binding of recombinant human Dicer. EMBO J. *21,* 5864-5874.

Qi, H.H., Ongusaha, P.P., Myllyharju, J., Cheng, D., Pakkanen, O., Shi, Y., Lee, S.W., Peng, J., and Shi, Y. (2008). Prolyl 4-hydroxylation regulates Argonaute 2 stability. Nature *455,* 421-424.

Qin, H., Chen, F., Huan, X., Machida, S., Song, J., and Yuan, Y.A. (2010). Structure of the Arabidopsis thaliana DCL4 DUF283 domain reveals a noncanonical double-stranded RNA-binding fold for protein-protein interaction. RNA *16,* 474-481.

Radanyi, C., Chambraud, B., and Baulieu, E.E. (1994). The ability of the immunophilin FKBP59-HBI to interact with the 90-kDa heat shock protein is encoded by its tetratricopeptide repeat domain. Proc. Natl. Acad. Sci. U. S. A. *91,* 11197-11201.

Randall, G., Panis, M., Cooper, J.D., Tellinghuisen, T.L., Sukhodolets, K.E., Pfeffer, S., Landthaler, M., Landgraf, P., Kan, S., Lindenbach, B.D*., et al.* (2007). Cellular cofactors affecting hepatitis C virus infection and replication. Proc. Natl. Acad. Sci. U. S. A. *104,* 12884-12889.

Ratajczak, T., and Carrello, A. (1996). Cyclophilin 40 (CyP-40), mapping of its hsp90 binding domain and evidence that FKBP52 competes with CyP-40 for hsp90 binding. J. Biol. Chem. *271,* 2961-2965.

Reinhart, B.J., and Bartel, D.P. (2002). Small RNAs correspond to centromere heterochromatic repeats. Science *297,* 1831.

Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R., and Ruvkun, G. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature *403,* 901-906.

Retzlaff, M., Hagn, F., Mitschke, L., Hessling, M., Gugel, F., Kessler, H., Richter, K., and Buchner, J. (2010). Asymmetric activation of the hsp90 dimer by its cochaperone aha1. Mol. Cell *37,* 344-354.

Reuter, M., Chuma, S., Tanaka, T., Franz, T., Stark, A., and Pillai, R.S. (2009). Loss of the Mili-interacting Tudor domain-containing protein-1 activates transposons and alters the Mili-associated small RNA profile. Nat. Struct. Mol. Biol. *16,* 639-646.

Rikhvanov, E.G., Romanova, N.V., and Chernoff, Y.O. (2007). Chaperone effects on prion and nonprion aggregates. Prion *1,* 217-222.

Robertson, H.D., Webster, R.E., and Zinder, N.D. (1968). Purification and properties of ribonuclease III from Escherichia coli. J. Biol. Chem. *243,* 82-91.

Roiniotis, J., Masendycz, P., Ho, S., and Scholz, G.M. (2005). Domain-mediated dimerization of the Hsp90 cochaperones Harc and Cdc37. Biochemistry *44,* 6662-6669.

Ruby, J.G., Jan, C.H., and Bartel, D.P. (2007). Intronic microRNA precursors that bypass Drosha processing. Nature *448,* 83-86.

Rudel, S., Wang, Y., Lenobel, R., Korner, R., Hsiao, H.H., Urlaub, H., Patel, D., and Meister, G. (2011). Phosphorylation of human Argonaute proteins affects small RNA binding. Nucleic Acids Res. *39,* 2330-2343.

Saito, K., Ishizuka, A., Siomi, H., and Siomi, M.C. (2005). Processing of pre-microRNAs by the Dicer-1-Loquacious complex in Drosophila cells. PLoS Biol. *3,* e235.

Saito, K., Nishida, K.M., Mori, T., Kawamura, Y., Miyoshi, K., Nagami, T., Siomi, H., and Siomi, M.C. (2006). Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the Drosophila genome. Genes Dev. *20,* 2214-2222.

Sanchez, E.R., Toft, D.O., Schlesinger, M.J., and Pratt, W.B. (1985). Evidence that the 90-kDa phosphoprotein associated with the untransformed L-cell glucocorticoid receptor is a murine heat shock protein. J. Biol. Chem. *260,* 12398-12401.

Sato, N., Yamamoto, T., Sekine, Y., Yumioka, T., Junicho, A., Fuse, H., and Matsuda, T. (2003). Involvement of heat-shock protein 90 in the interleukin-6-mediated signaling pathway through STAT3. Biochem. Biophys. Res. Commun. *300,* 847-852.

Sato, S., Fujita, N., and Tsuruo, T. (2000). Modulation of Akt kinase activity by binding to Hsp90. Proc. Natl. Acad. Sci. U. S. A. *97,* 10832-10837.

Savitsky, M., Kwon, D., Georgiev, P., Kalmykova, A., and Gvozdev, V. (2006). Telomere elongation is under the control of the RNAi-based mechanism in the Drosophila germline. Genes Dev. *20,* 345-354.

Schisa, J.A., Pitt, J.N., and Priess, J.R. (2001). Analysis of RNA associated with P granules in germ cells of C. elegans adults. Development *128,* 1287-1298.

Schuh, S., Yonemoto, W., Brugge, J., Bauer, V.J., Riehl, R.M., Sullivan, W.P., and Toft, D.O. (1985). A 90,000-dalton binding protein common to both steroid receptors and the Rous sarcoma virus transforming protein, pp60v-src. J. Biol. Chem. *260,* 14292-14296.

Schulte, T.W., Akinaga, S., Soga, S., Sullivan, W., Stensgard, B., Toft, D., and Neckers, L.M. (1998). Antibiotic radicicol binds to the N-terminal domain of Hsp90 and shares important biologic activities with geldanamycin. Cell Stress Chaperones *3,* 100-108.

Schulte, T.W., Blagosklonny, M.V., Ingui, C., and Neckers, L. (1995). Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association. J. Biol. Chem. *270,* 24585-24588.

Schwarz, D.S., Hutvagner, G., Haley, B., and Zamore, P.D. (2002). Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. Mol. Cell *10,* 537-548.

Sen, G.L., and Blau, H.M. (2005). Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. Nat. Cell Biol. *7,* 633-636.

Sepehrnia, B., Paz, I.B., Dasgupta, G., and Momand, J. (1996). Heat shock protein 84 forms a complex with mutant p53 protein predominantly within a cytoplasmic compartment of the cell. J. Biol. Chem. *271,* 15084-15090.

Sheth, U., and Parker, R. (2003). Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. Science *300,* 805-808.

Shpiz, S., Kwon, D., Uneva, A., Kim, M., Klenov, M., Rozovsky, Y., Georgiev, P., Savitsky, M., and Kalmykova, A. (2007). Characterization of Drosophila telomeric retroelement TAHRE: transcription, transpositions, and RNAi-based regulation of expression. Mol. Biol. Evol. *24,* 2535-2545.

Siligardi, G., Panaretou, B., Meyer, P., Singh, S., Woolfson, D.N., Piper, P.W., Pearl, L.H., and Prodromou, C. (2002). Regulation of Hsp90 ATPase activity by the co-chaperone Cdc37p/p50cdc37. J. Biol. Chem. *277,* 20151-20159.

Smith, D.F., Sullivan, W.P., Marion, T.N., Zaitsu, K., Madden, B., McCormick, D.J., and Toft, D.O. (1993). Identification of a 60-kilodalton stress-related protein, p60, which interacts with hsp90 and hsp70. Mol. Cell. Biol. *13,* 869-876.

Smith, D.F., Whitesell, L., Nair, S.C., Chen, S., Prapapanich, V., and Rimerman, R.A. (1995). Progesterone receptor structure and function altered by geldanamycin, an hsp90-binding agent. Mol. Cell. Biol. *15,* 6804-6812.

Smith, J.R., Clarke, P.A., de Billy, E., and Workman, P. (2009). Silencing the cochaperone CDC37 destabilizes kinase clients and sensitizes cancer cells to HSP90 inhibitors. Oncogene *28,* 157-169.

Song, J.J., Liu, J., Tolia, N.H., Schneiderman, J., Smith, S.K., Martienssen, R.A., Hannon, G.J., and Joshua-Tor, L. (2003). The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. Nat. Struct. Biol. *10,* 1026-1032.

Song, J.J., Smith, S.K., Hannon, G.J., and Joshua-Tor, L. (2004). Crystal structure of Argonaute and its implications for RISC slicer activity. Science *305,* 1434-1437.

Squirrell, J.M., Eggers, Z.T., Luedke, N., Saari, B., Grimson, A., Lyons, G.E., Anderson, P., and White, J.G. (2006). CAR-1, a protein that localizes with the mRNA decapping component DCAP-1, is required for cytokinesis and ER organization in Caenorhabditis elegans embryos. Mol. Biol. Cell *17,* 336-344.

Stebbins, C.E., Russo, A.A., Schneider, C., Rosen, N., Hartl, F.U., and Pavletich, N.P. (1997). Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. Cell *89,* 239-250.

Stoecklin, G., Mayo, T., and Anderson, P. (2006). ARE-mRNA degradation requires the 5'-3' decay pathway. EMBO Rep. *7,* 72-77.

Stohr, N., Lederer, M., Reinke, C., Meyer, S., Hatzfeld, M., Singer, R.H., and Huttelmaier, S. (2006). ZBP1 regulates mRNA stability during cellular stress. J. Cell Biol. *175,* 527-534.

Sullivan, C.S., and Ganem, D. (2005). A virus-encoded inhibitor that blocks RNA interference in mammalian cells. J. Virol. *79,* 7371-7379.

Sullivan, W., Stensgard, B., Caucutt, G., Bartha, B., McMahon, N., Alnemri, E.S., Litwack, G., and Toft, D. (1997). Nucleotides and two functional states of hsp90. J. Biol. Chem. *272,* 8007-8012.

Sun, G., Wang, Y., Sun, L., Luo, H., Liu, N., Fu, Z., and You, Y. (2011). Clinical significance of Hiwi gene expression in gliomas. Brain Res. *1373,* 183-188.

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.

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.

Taipale, M., Jarosz, D.F., and Lindquist, S. (2010). HSP90 at the hub of protein homeostasis: emerging mechanistic insights. Nat. Rev. Mol. Cell Biol. *11,* 515-528.

Takimoto, K., Wakiyama, M., and Yokoyama, S. (2009). Mammalian GW182 contains multiple Argonaute-binding sites and functions in microRNA-mediated translational repression. RNA *15,* 1078-1089.

Tariq, M., Nussbaumer, U., Chen, Y., Beisel, C., and Paro, R. (2009). Trithorax requires Hsp90 for maintenance of active chromatin at sites of gene expression. Proc. Natl. Acad. Sci. U. S. A. *106,* 1157-1162.

Tatebe, H., and Shiozaki, K. (2003). Identification of Cdc37 as a novel regulator of the stress-responsive mitogen-activated protein kinase. Mol. Cell. Biol. *23,* 5132-5142.

Thermann, R., and Hentze, M.W. (2007). Drosophila miR2 induces pseudo-polysomes and inhibits translation initiation. Nature *447,* 875-878.

Thomas, M.G., Martinez Tosar, L.J., Loschi, M., Pasquini, J.M., Correale, J., Kindler, S., and Boccaccio, G.L. (2005). Staufen recruitment into stress granules does not affect early mRNA transport in oligodendrocytes. Mol. Biol. Cell *16,* 405-420.

Thomson, T., and Lin, H. (2009). The biogenesis and function of PIWI proteins and piRNAs: progress and prospect. Annu. Rev. Cell Dev. Biol. *25,* 355-376.

Till, S., Lejeune, E., Thermann, R., Bortfeld, M., Hothorn, M., Enderle, D., Heinrich, C., Hentze, M.W., and Ladurner, A.G. (2007). A conserved motif in Argonaute-interacting

proteins mediates functional interactions through the Argonaute PIWI domain. Nat. Struct. Mol. Biol. *14,* 897-903.

Tolia, N.H., and Joshua-Tor, L. (2007). Slicer and the argonautes. Nat. Chem. Biol. *3,* 36-43.

Tomari, Y., Matranga, C., Haley, B., Martinez, N., and Zamore, P.D. (2004). A protein sensor for siRNA asymmetry. Science *306,* 1377-1380.

Tourriere, H., Chebli, K., Zekri, L., Courselaud, B., Blanchard, J.M., Bertrand, E., and Tazi, J. (2003). The RasGAP-associated endoribonuclease G3BP assembles stress granules. J. Cell Biol. *160,* 823-831.

Vagin, V.V., Klenov, M.S., Kalmykova, A.I., Stolyarenko, A.D., Kotelnikov, R.N., and Gvozdev, V.A. (2004). The RNA interference proteins and vasa locus are involved in the silencing of retrotransposons in the female germline of Drosophila melanogaster. RNA Biol. *1,* 54-58.

Vagin, V.V., Sigova, A., Li, C., Seitz, H., Gvozdev, V., and Zamore, P.D. (2006). A distinct small RNA pathway silences selfish genetic elements in the germline. Science *313,* 320-324.

Vagin, V.V., Wohlschlegel, J., Qu, J., Jonsson, Z., Huang, X., Chuma, S., Girard, A., Sachidanandam, R., Hannon, G.J., and Aravin, A.A. (2009). Proteomic analysis of murine Piwi proteins reveals a role for arginine methylation in specifying interaction with Tudor family members. Genes Dev. *23,* 1749-1762.

van Dijk, E., Cougot, N., Meyer, S., Babajko, S., Wahle, E., and Seraphin, B. (2002). Human Dcp2: a catalytically active mRNA decapping enzyme located in specific cytoplasmic structures. EMBO J. *21,* 6915-6924.

Vasudevan, S., Tong, Y., and Steitz, J.A. (2007). Switching from repression to activation: microRNAs can up-regulate translation. Science *318,* 1931-1934.

Wakiyama, M., Takimoto, K., Ohara, O., and Yokoyama, S. (2007). Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system. Genes Dev. *21,* 1857-1862.

Wang, B., Love, T.M., Call, M.E., Doench, J.G., and Novina, C.D. (2006). Recapitulation of short RNA-directed translational gene silencing in vitro. Mol. Cell *22,* 553-560.

Wang, B., Yanez, A., and Novina, C.D. (2008). MicroRNA-repressed mRNAs contain 40S but not 60S components. Proc. Natl. Acad. Sci. U. S. A. *105,* 5343-5348.

Wang, K., Ma, Q., Ren, Y., He, J., Zhang, Y., Zhang, Y., and Chen, W. (2007). Geldanamycin destabilizes HER2 tyrosine kinase and suppresses Wnt/beta-catenin signaling in HER2 overexpressing human breast cancer cells. Oncol. Rep. *17,* 89-96.

Wang, Q.E., Han, C., Milum, K., and Wani, A.A. (2011). Stem cell protein Piwil2 modulates chromatin modifications upon cisplatin treatment. Mutat. Res. *708,* 59-68.

Wang, X.H., Aliyari, R., Li, W.X., Li, H.W., Kim, K., Carthew, R., Atkinson, P., and Ding, S.W. (2006). RNA interference directs innate immunity against viruses in adult Drosophila. Science *312,* 452-454.

Wang, Y., Juranek, S., Li, H., Sheng, G., Tuschl, T., and Patel, D.J. (2008). Structure of an argonaute silencing complex with a seed-containing guide DNA and target RNA duplex. Nature *456,* 921-926.

Wang, Y., Juranek, S., Li, H., Sheng, G., Wardle, G.S., Tuschl, T., and Patel, D.J. (2009). Nucleation, propagation and cleavage of target RNAs in Ago silencing complexes. Nature *461,* 754-761.

Wang, Y., Sheng, G., Juranek, S., Tuschl, T., and Patel, D.J. (2008). Structure of the guide-strand-containing argonaute silencing complex. Nature *456,* 209-213.

Watanabe, T., Takeda, A., Tsukiyama, T., Mise, K., Okuno, T., Sasaki, H., Minami, N., and Imai, H. (2006). Identification and characterization of two novel classes of small RNAs in the mouse germline: retrotransposon-derived siRNAs in oocytes and germline small RNAs in testes. Genes Dev. *20,* 1732-1743.

Watanabe, T., Totoki, Y., Toyoda, A., Kaneda, M., Kuramochi-Miyagawa, S., Obata, Y., Chiba, H., Kohara, Y., Kono, T., Nakano, T.*, et al.* (2008). Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes. Nature *453,* 539-543.

Wek, R.C., Jiang, H.Y., and Anthony, T.G. (2006). Coping with stress: eIF2 kinases and translational control. Biochem. Soc. Trans. *34,* 7-11.

Westwood, P.K., Martin, I.V., and Fantes, P.A. (2004). Fission yeast Cdc37 is required for multiple cell cycle functions. Mol. Genet. Genomics *271,* 82-90.

Whitesell, L., Mimnaugh, E.G., De Costa, B., Myers, C.E., and Neckers, L.M. (1994). Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc. Natl. Acad. Sci. U. S. A. *91,* 8324-8328.

Wightman, B., Ha, I., and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell *75,* 855-862.

Wilczynska, A., Aigueperse, C., Kress, M., Dautry, F., and Weil, D. (2005). The translational regulator CPEB1 provides a link between dcp1 bodies and stress granules. J. Cell. Sci. *118,* 981-992.

Wu, H., Xu, H., Miraglia, L.J., and Crooke, S.T. (2000). Human RNase III is a 160-kDa protein involved in preribosomal RNA processing. J. Biol. Chem. *275,* 36957-36965.

Xu, P., Vernooy, S.Y., Guo, M., and Hay, B.A. (2003). The Drosophila microRNA Mir-14 suppresses cell death and is required for normal fat metabolism. Curr. Biol. *13,* 790-795.

Yan, K.S., Yan, S., Farooq, A., Han, A., Zeng, L., and Zhou, M.M. (2003). Structure and conserved RNA binding of the PAZ domain. Nature *426,* 468-474.

Yang, Z., Jakymiw, A., Wood, M.R., Eystathioy, T., Rubin, R.L., Fritzler, M.J., and Chan, E.K. (2004). GW182 is critical for the stability of GW bodies expressed during the cell cycle and cell proliferation. J. Cell. Sci. *117,* 5567-5578.

Yekta, S., Shih, I.H., and Bartel, D.P. (2004). MicroRNA-directed cleavage of HOXB8 mRNA. Science *304,* 594-596.

Yeung, M.L., Bennasser, Y., Myers, T.G., Jiang, G., Benkirane, M., and Jeang, K.T. (2005). Changes in microRNA expression profiles in HIV-1-transfected human cells. Retrovirology *2,* 81.

Yigit, E., Batista, P.J., Bei, Y., Pang, K.M., Chen, C.C., Tolia, N.H., Joshua-Tor, L., Mitani, S., Simard, M.J., and Mello, C.C. (2006). Analysis of the C. elegans Argonaute family reveals that distinct Argonautes act sequentially during RNAi. Cell *127,* 747-757.

Yin, D.T., Li, H.Q., Wang, Y.F., Cao, S.L., Zhou, Y.B., Zheng, L.Y., Jiang, J.H., and Wang, Q.D. (2011). Expression of Piwil2 and its relationship with tumor invasion and metastasis in papillary thyroid carcinoma. Zhonghua Er Bi Yan Hou Tou Jing Wai Ke Za Zhi *46,* 237-239.

Yoda, M., Kawamata, T., Paroo, Z., Ye, X., Iwasaki, S., Liu, Q., and Tomari, Y. (2010). ATP-dependent human RISC assembly pathways. Nat. Struct. Mol. Biol. *17,* 17-23.

Young, J.C., and Hartl, F.U. (2000). Polypeptide release by Hsp90 involves ATP hydrolysis and is enhanced by the co-chaperone p23. EMBO J. *19,* 5930-5940.

Young, J.C., Obermann, W.M., and Hartl, F.U. (1998). Specific binding of tetratricopeptide repeat proteins to the C-terminal 12-kDa domain of hsp90. J. Biol. Chem. *273,* 18007-18010.

Yuan, Y.R., Pei, Y., Ma, J.B., Kuryavyi, V., Zhadina, M., Meister, G., Chen, H.Y., Dauter, Z., Tuschl, T., and Patel, D.J. (2005). Crystal structure of A. aeolicus argonaute, a site-specific DNA-guided endoribonuclease, provides insights into RISC-mediated mRNA cleavage. Mol. Cell *19,* 405-419.

Zekri, L., Huntzinger, E., Heimstadt, S., and Izaurralde, E. (2009). The silencing domain of GW182 interacts with PABPC1 to promote translational repression and degradation of microRNA targets and is required for target release. Mol. Cell. Biol. *29,* 6220-6231.

Zeng, Y., Sankala, H., Zhang, X., and Graves, P.R. (2008). Phosphorylation of Argonaute 2 at serine-387 facilitates its localization to processing bodies. Biochem. J. *413,* 429-436.

Zhang, H., Kolb, F.A., Brondani, V., Billy, E., and Filipowicz, W. (2002). Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. EMBO J. *21,* 5875-5885.

Zipprich, J.T., Bhattacharyya, S., Mathys, H., and Filipowicz, W. (2009). Importance of the C-terminal domain of the human GW182 protein TNRC6C for translational repression. RNA *15,* 781-793.

# APPENDIX

**Synopsis**

Proteins of the Argonaute family are the core components of the RNA-induced silencing complex, the central effector of mammalian RNA interference. Argonaute proteins are known to associate with at least two types of cytoplasmic RNA granules known as processing bodies and stress granules. In addition to Argonautes, these granules contain proteins that function in mRNA degradataion and translational repression. The role of Argonaute proteins in siRNA-directed mRNA cleavage and miRNA-mediated translational repression has prompted speculation that the association of Argonautes with P bodies and stress granules may be a result of their activity within the RNAi pathway. Here we report that targeting of human Argonaute2 to stress granules is dependent upon its ability to bind to small RNAs. These results suggest that hAgo2 is recruited to stress granules as a consequence of its interaction with mRNAs that are targeted by the RNAi pathway.

**Figures and Legends**

Figure 1. Wildtype myc-hAgo2 localizes to P bodies and stress granules. HeLa cells transiently expressing myc-hAgo2 were treated with DMSO or 50 µM arsenite (+ARS) for 60 minutes prior to fixation and processing for indirect immunofluorescence. GW182-positive P bodies (arrowheads) and TIA-1-positive stress granules (arrows) are indicated. Bar, 15 µm.

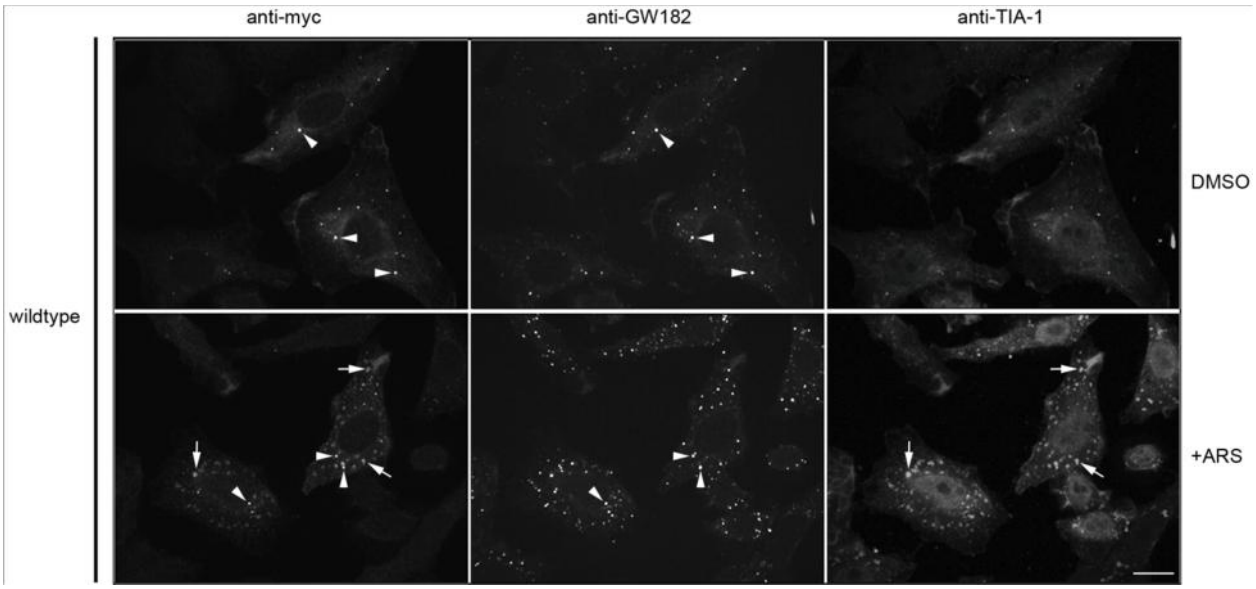


Figure 2. Mutations of the PAZ domain of hAgo2 disrupting small RNA binding impair Ago2 localization to P bodies and stress granules. HeLa cells transiently expressing myc-hAgo2-PAZ-9 (PAZ9) or myc-hAgo2-PAZ-10 (PAZ10) were treated with DMSO or 50 µM arsenite (+ARS) for 60 minutes prior to fixation and processing for indirect immunofluorescence. GW182-positive P bodies (arrowheads) and TIA-1-positive stress granules (arrows) are indicated. Bar, 15 µm.

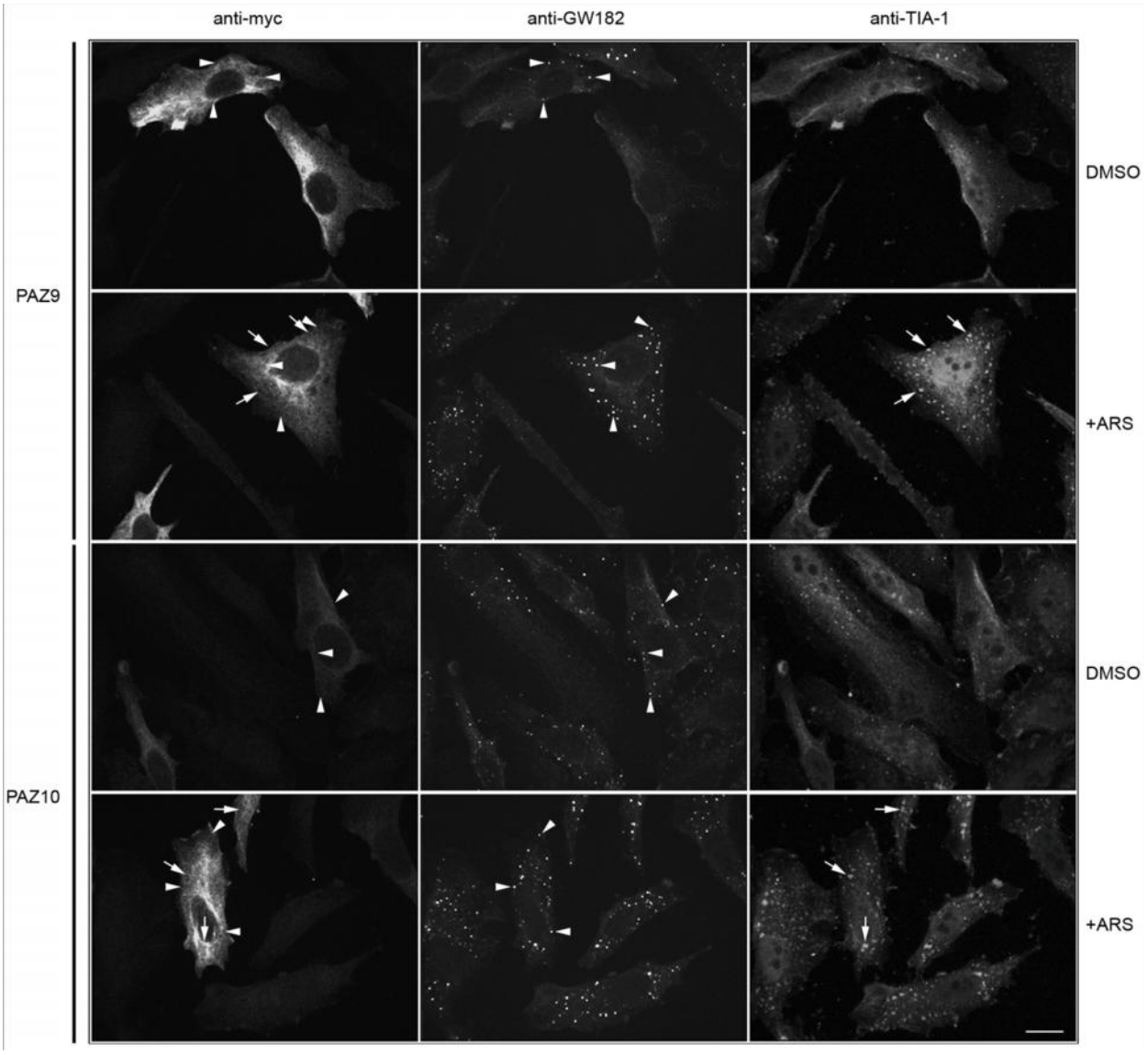


Figure 3. Mutations of the PIWI domain of hAgo2 that disrupt its endonuclease activity do not affect its localization to P bodies or stress granules. HeLa cells transiently expressing catalytically dead myc-hAgo2-Q633R (Q633R), myc-hAgo2-H634A (H634A) or myc-hAgo2-H634P (H634P) were treated with DMSO or 50 µM arsenite (+ARS) for 60 minutes prior to fixation and processing for indirect immunofluorescence. GW182-positive P bodies (arrowheads) and TIA-1-positive stress granules (arrows) are indicated. Bar, 15 µm.

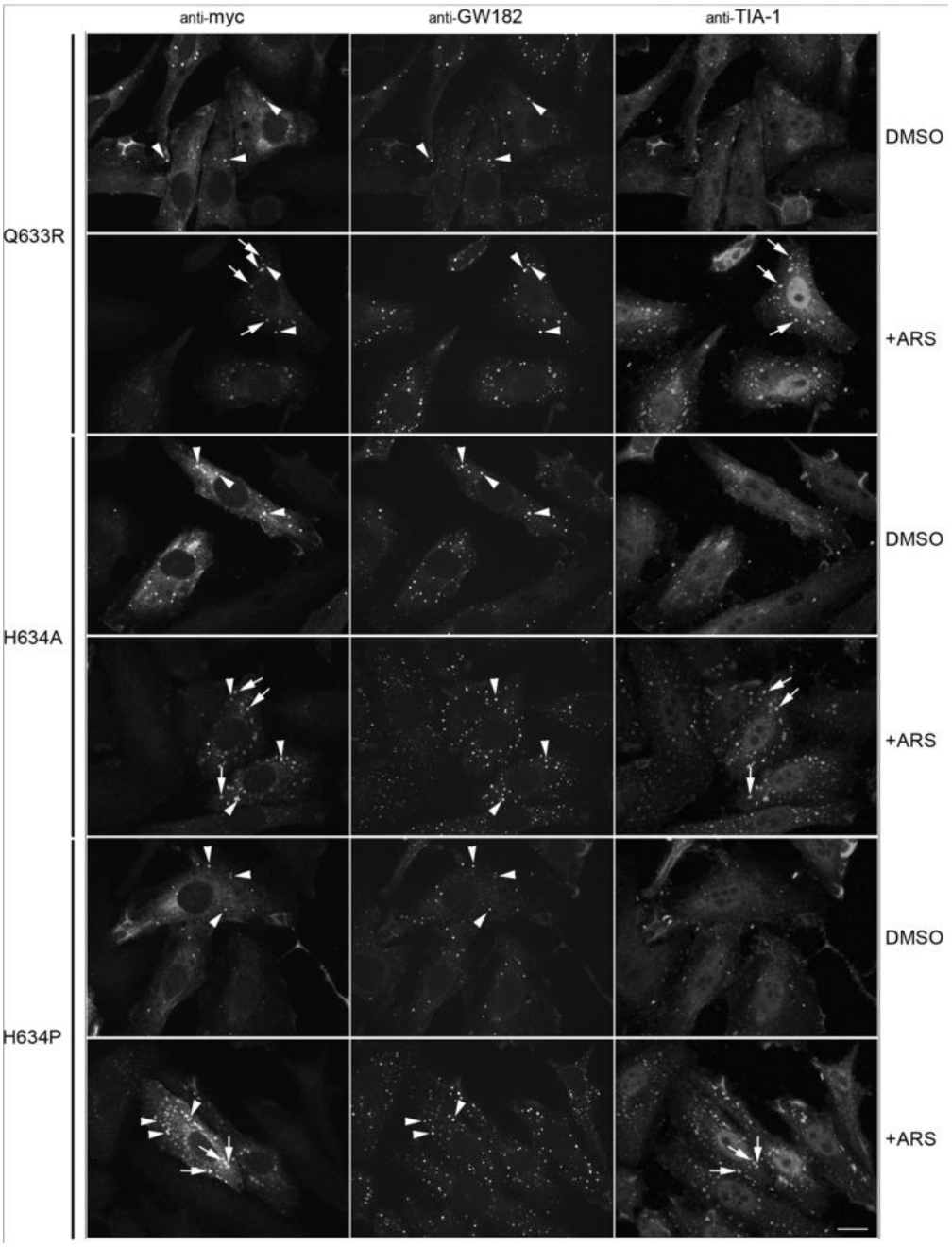


Figure 4. Mutations of the MID domain of hAgo2 disrupting small RNA binding impair Ago2 localization to P bodies and stress granules. HeLa cells transiently expressing myc-hAgo2-Y529E (Y529E) or myc-hAgo2-Y529F (Y529F) were treated with DMSO or 50 µM arsenite (+ARS) for 60 minutes prior to fixation and processing for indirect immunofluorescence. GW182-positive P bodies (arrowheads) and TIA-1-positive stress granules (arrows) are indicated. Bar, 15 µm.

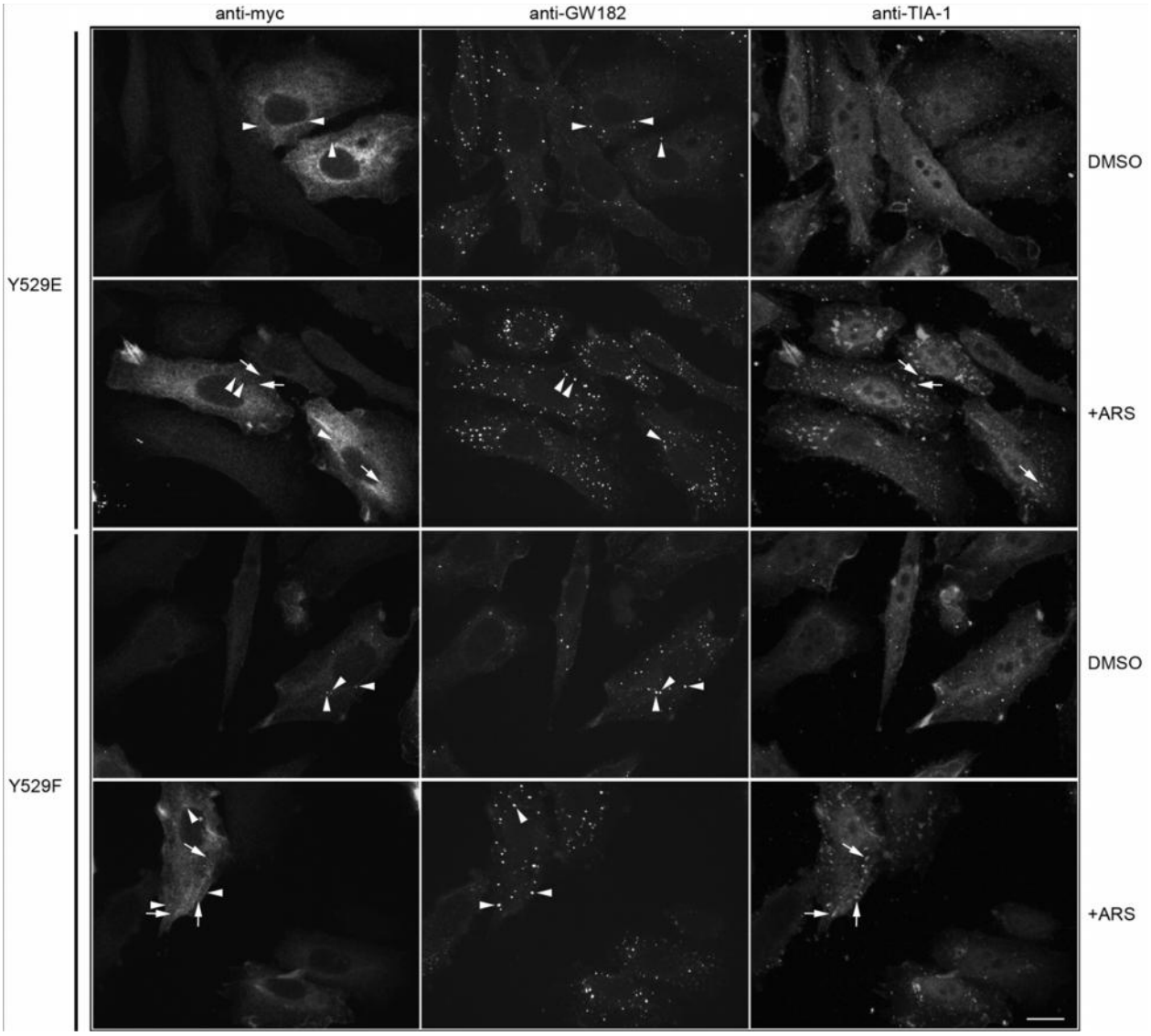


Figure 5. Mutants of Ago2 are expressed to equivalent levels and size, relative to wildtype myc-hAgo2. Total cell lysates were generated from HeLa cells transiently transfected with myc-tagged hAgo2 constructs. Membrane stained with Ponceau S and immunoblotted with an antibody against hnRNP U are shown as loading controls. Level of myc-tagged Argonaute proteins was determined using an anti-myc antibody.