DIGITAL ACCESS TO SCHOLARSHIP AT HARVARD
DASH.HARVARD.EDU

HARVARD LIBRARY
Office for Scholarly Communication

The Programming and Assembly of a Transcriptional Silencing Complex

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation	Holoch, Daniel Benjamin. 2015. The Programming and Assembly of a Transcriptional Silencing Complex. Doctoral dissertation, Harvard University, Graduate School of Arts & Sciences.
Citable link	http://nrs.harvard.edu/urn-3:HUL.InstRepos:23845498
Terms of Use	This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA

The Programming and Assembly of a Transcriptional Silencing Complex

A dissertation presented
by

Daniel Benjamin Holoch

to

The Division of Medical Sciences
Program in Biological and Biomedical Sciences

in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
in the subject of
Genetics and Genomics

Harvard University
Cambridge, Massachusetts
August, 2015

© 2015 Daniel Holoch

All rights reserved

Dissertation Advisor: Dr. Danesh Moazed Daniel Benjamin Holoch

The Programming and Assembly of a Transcriptional Silencing Complex

---

Abstract

Argonautes and their small RNA guides form an ancient partnership with diverse roles in controlling gene expression and preserving genome stability. In the fission yeast *Schizosaccharomyces pombe*, the Argonaute Ago1 acts within the RITS complex to target the repetitive DNA elements that flank each centromere for heterochromatic silencing, which is necessary for faithful chromosome transmission. A separate Ago1-containing complex, termed ARC, is also required for pericentromeric silencing but its precise function, and the mechanisms that regulate the movement of Ago1 between ARC and RITS, have remained unclear. This dissertation investigates both of these questions.

By combining distinct approaches, we have defined the role of ARC as that of enabling Ago1 to be programmed with small RNA guides. In an *in vitro* assay using immunopurified proteins, we found that loading of synthetic double-stranded small RNAs into Ago1 requires the ARC subunit Arb1 but not the RITS subunit Tas3. In parallel, we isolated cellular Ago1-associated small RNAs and, by high-throughput sequencing, observed that deletion of ARC components produced read features indicative of nonspecifically-interacting small RNAs. Together, these data indicate that the small-RNA-loading capability of Ago1 is conferred by ARC.

We also discovered using co-immunoprecipitation that the ARC subunits Arb1 and Arb2 are required for the proper association between RITS subunits Ago1 and Tas3, suggesting that small-RNA loading by ARC might license Ago1 for assembly into RITS. Indeed, we went on to

show that Ago1 mutants deficient for small-RNA loading universally fail to interact with Tas3, whereas other non-functional Ago1 variants maintain Tas3 association. We conclude that Tas3 distinguishes between loaded and unloaded Ago1, admitting only the former into RITS.

Our studies have delineated the mechanisms that control the programming and assembly of the RITS complex. The results illuminate the role of ARC in heterochromatic silencing and identify this complex as the machinery required for loading small RNAs into Argonaute in *S. pombe*. Furthermore, we have uncovered small-RNA loading as a checkpoint for the entry of Argonaute into RITS, which may reflect a common discriminatory function of GW-repeat proteins such as Tas3 that precludes the formation of inactive and potentially deleterious complexes.

Table of Contents

Title page ... i
Copyright notice ... ii
Abstract ... iii
Table of Contents ... v
List of Figures and Tables ... vii
Acknowledgments ... viii

Chapter 1 – Introduction ... 1
I. Noncoding RNAs and the regulation of gene expression ... 4
A. Chromosome-associated RNAs that mediate dosage compensation ... 4
i. Xist and X chromosome inactivation ... 4
ii. roX RNAs and hypertranscription of the male X chromosome ... 8
B. Recruitment of chromatin-modifying complexes by noncoding RNAs ... 10
C. Regulation of gene expression by cytoplasmic noncoding RNAs ... 11
D. Antisense noncoding RNAs that repress their sense counterparts ... 12

II. RNA interference ... 15
A. Double-stranded RNA triggers gene silencing ... 15
B. Components of the RNAi machinery ... 17
i. Dicer ... 18
ii. Argonaute ... 18
iii. RNA-directed RNA polymerase ... 19
C. MicroRNAs ... 20
D. Endogenous siRNAs and piRNAs in genome defense ... 24
i. RNA-directed DNA methylation in plants ... 24
ii. piRNAs in *Drosophila* and mammals ... 25
iii. Small RNAs for distinguishing self from nonself nucleic acids in *C. elegans* . 27

III. Argonautes and their GW protein partners in gene silencing ... 29
A. Argonaute slicing ... 29
B. Argonaute loading ... 33
C. GW proteins ... 38
i. GW182 and the mechanism of miRNA-mediated repression in metazoans 38
ii. An evolutionarily-conserved Argonaute-interacting motif ... 40

IV. RNAi-directed assembly of heterochromatin in *Schizosaccharomyces pombe* ... 43
A. *S. pombe* as a model organism for chromatin biology ... 43
B. RNAi protein complexes maintain pericentromeric heterochromatin ... 46
i. RITS ... 46
ii. RDRC ... 49
iii. The RNAi complexes localize to nascent transcripts and directly target H3K9me ... 50
C. Histone methylation and siRNA biogenesis are coupled processes ... 52
i. Elements of the feedback loop ... 52
ii. Initiation of heterochromatin assembly by Dcr1-independent small RNAs ... 55
iii. Restrictions on siRNA-mediated heterochromatin formation ... 56
D. Two Argonaute proteins are required for heterochromatin assembly in *S. pombe* 58

V. References ... 63

Chapter 2 – ARC is Required for Loading Small RNAs into Argonaute in Fission Yeast.. 94
I. Introduction ... 96
II. Results ... 101
A. ARC Associates with Small RNAs Bearing Features of Dicer-Generated Duplex siRNAs ... 101
B. Arb1 Is Required for Argonaute Loading of Small RNAs *In Vitro* and *In Vivo* ... 105
C. Argonaute Overexpression Partially Restores Silencing in Arb1-Deficient Cells Specifically by Overcoming a Small RNA Loading Defect ... 113
III. Discussion ... 116
A. An Argonaute Small RNA Loading Complex in *S. pombe* ... 116
B. On the Role of Arb2 in Small RNA Loading ... 118
IV. Methods ... 121
V. Acknowledgments ... 127
VI. References ... 128

Chapter 3 – Small-RNA Loading Licenses Argonaute for Assembly into a Transcriptional Silencing Complex ... 132
I. Introduction ... 134
II. Results ... 138
A. Arb1 and Arb2 Are Required for Assembly of the RITS Complex ... 138
B. Small RNA Loading onto Argonaute Is Required for Assembly of the RITS Complex but not the ARC Complex ... 139
II. Discussion ... 144
A. Determinants of Argonaute Progression between the ARC and RITS Complexes .. 144
B. GW Proteins as Sensors of Argonaute Loading ... 145
C. Safeguards in the Assembly of GW Protein-Containing Argonaute Silencing Complexes ... 146
IV. Methods ... 150
V. Acknowledgments ... 153
VI. References ... 154

Chapter 4 – Perspectives ... 158
I. The molecular mechanism of small-RNA loading by Arb1 ... 160
II. Models for the contribution of Arb2 to small-RNA loading ... 163
III. An ordered assembly pathway for Argonaute silencing complexes ... 166
IV. References ... 170

Appendix 1 – ARC does not interact with the pericentromeric transcripts whose silencing it controls ... 173
Appendix 2 – Arb1-TAP is a hypomorphic protein but TAP-Arb1 is fully functional ... 176

List of Figures and Tables

Chapter 1:

Figure 1.1. The “nascent transcript” model and a self-reinforcing epigenetic loop in *S. pombe*. 53

Figure 1.2. Schematic illustrating the subunit composition of the RITS and ARC complexes.... 60

Chapter 2:

Figure 2.1. ARC binds small RNAs *in vivo* that bear features of Dcr1-generated duplexes..... 100

Table 2.1. Number of aligned small RNA reads for each sequenced strain 101

Figure 2.2. RITS and ARC bind populations of pericentromeric small RNAs with similar sequences 102

Figure 2.3. Immunopurified Ago1 binds duplex small RNAs *in vitro* in an Arb1-dependent manner 103

Figure 2.4. Arb2 is not required for *in vitro* loading of duplex small RNAs onto immunopurified Ago1 104

Figure 2.5. Arb1 is required for all Ago1 small RNA loading activity *in vivo* 107

Figure 2.6. Arb2 is required for all Ago1 small RNA loading activity *in vivo* 110

Figure 2.7. Overexpressed Ago1 suppresses *arb1*Δ and *arb2*Δ not simply by rescuing protein stability and does not suppress other pericentromeric silencing mutants............. 111

Figure 2.8. *ago1*$^+$ overexpression partially suppresses silencing defect of *arb1Δ* and *arb2Δ* by overcoming the requirement for ARC in small RNA loading 112

Figure 2.9. Ago1 L317A protein does not complement *ago1*Δ but does not show a significant reduction in loading of pericentromeric siRNAs 115

Table 2.2. *S. pombe* strains used in this study 120

Table 2.3. Oligonucleotides used in this study 123

Table 2.4. Plasmids used in this study 126

Chapter 3:

Figure 3.1. ARC subunits Arb1 and Arb2 are required for RITS assembly 137

Table 3.1. Ago1 associates with Arb1 and Arb2 independently of Tas3 139

Figure 3.2. Loading of small RNAs onto Ago1 is required for assembly of RITS but not ARC. 140

Figure 3.3. Ago1 D580A, Ago1 F276A R773E, Ago1 F276A and Ago1 R773E are null-mutant proteins whose small RNA–binding activity correlates with their assembly into RITS 141

Figure 3.4. Regulation of RITS complex assembly by small RNA loading onto Argonaute...... 147

Figure 3.5. *ago1* D580A and *ago1* F276A R773E are dominant-negative alleles when overexpressed 148

Table 3.2. *S. pombe* strains used in this study 149

Table 3.3. Oligonucleotides used in this study 152

Table 3.4. Plasmids used in this study 153

Acknowledgments

In order for this document to become valid in the fulfillment of my degree requirements, it must be read, critiqued and approved by an examination committee consisting of recognized experts in my discipline. I would like to express my sincere and humble gratitude to Drs. David Bartel, Richard Gregory, Scott Kennedy and Mitzi Kuroda for generously agreeing to serve as the members of this committee. The chance to have these scientists evaluate this work and discuss it with me is a great privilege.

Throughout the course of my graduate studies I met regularly with a dissertation advisory committee, which included Drs. Mitzi Kuroda, Stephen Buratowski and Fred Winston. Although I long felt that my progress was too slow, and that the results I discussed with them were not worthy of diverting hours of their time away from their own exciting research programs, Mitzi, Steve and Fred reflected on my reports and presentations with invariably great care and offered the honest feedback and encouragement that I needed. They also suggested some of the experiments that proved critical to the dissertation as a whole. I admire each one of these professors very much, and I am honored to have spent some of the most important moments of my graduate education learning from this eminently gifted and dedicated group.

A portion of my dissertation work was funded by the National Science Foundation Graduate Research Fellowship Program, and I am very thankful for this award and the support it provided.

I carried out all of the experiments reported in this dissertation in the laboratory of Dr. Danesh Moazed, whose students and staff all contributed to my scientific growth and to the advancement of my project. Among them, I reserve the largest share of my thanks for Cecilia Centrella, who prepares virtually all of the media and most of the solutions that are used in the lab. It is difficult to imagine how arduous these studies would have been without her help, and I am also grateful for her indomitable cheerfulness. Tiffany Casey and Cary Hencken oversaw critical aspects of the lab's operation, during distinct periods of my thesis research and in

distinct capacities, but neither spared any effort to advance the lab's mission and both brought tremendous initiative, professionalism and amiability to their work.

The current graduate students and postdoctoral fellows in Danesh's group—Gloria Jih, Ruby Yu, Becky Mathew, Antonis Tatarakis, Marian Kalocsay, Nahid Iglesias, Mark Currie, Keith Connolly, Emily Egan, Kaushik Ragunathan, Reza Behrouzi, Xiaoyi Wang, Vahid Khoddami and Gergana Shipkovenska—have contributed immensely to my training as a researcher and to my wellbeing in the lab. I particularly thank Gloria and Ruby for many years of companionship as fellow students since our earliest days under Danesh's supervision, and for the example they have given me through their creativity and hard work. I am also especially grateful to Nahid for her exceptional instruction in the art of affinity purifications and co-immunoprecipitations, and for keeping me from losing my French. Would that I had the space to acknowledge the others on an individual basis, for I greatly appreciate all that they have done to help me complete this endeavor, mature intellectually, and enjoy the years I have spent as their colleague.

The lab's former students and postdocs—Megumi Onishi, Shane Buker, Karim Mekhail, Geng Li, Mo Motamedi, Mario Halic, Erica Gerace, Aaron Johnson, Corey Morris, Aarti Sevilimedu, Nertila Siuti, Altaf Bhat, Ruchi Jain, Feng Wang, Chenning Lu and Jordi Xiol—have also had an enduring influence on my development as a scientist and a person. I recall as though it were yesterday attending Shane's dissertation defense seminar, not yet suspecting that I would pick up where he had left off, and I am now bound to thank him for initiating the research direction that has provided me with so much fascination these last few years. I learned much of what I know about working with yeast and about the nature of being a graduate student from Erica, whose observations about heterochromatic silencing and about the human condition were ever sharp and insightful. Mario taught me how to prepare next-generation sequencing libraries and analyze their contents bioinformatically, which played a transformative role in my dissertation project. It was thanks to Mo's clear and pedagogical expositions that I began to

master the complexity of chromatin silencing in fission yeast, and his contagious appreciation for the beauty of living systems is a regular reminder of how meaningful this career can be. I am also very thankful to Aaron for catalyzing the intellectual vibrancy of the lab through the combination of his broad interests and his boundless stream of new ideas, and for his constant benevolence as my baymate and senior colleague. Lastly, I thank Jordi for crucial methodological advice on recovering rare small RNAs for sequencing, and for prompting me to consider the big picture with realism and humor.

My first full-time mentor at the bench was Dr. Markus Landthaler in Dr. Thomas Tuschl's group, and the passion and energy he brought to his research further stoked my existing desire to become a scientist and to embark on a PhD. Seven years on, I continue to consider Markus as a role model for his extraordinary creativity and work ethic.

For finishing this work I am also indebted to fellow graduate students from the Biological and Biomedical Sciences and other programs, especially Jeremy Chang, Abby Sarkar, Brian Goodman, Ryan Walsh, Brian Beliveau, Chris Wells, Balthasar Heesters, James Thornton and Marshall Thomas. Their opinions, feedback and camaraderie did much to sustain my motivation and sense of direction, and the excellence of their accomplishments has given me much to look up to. I particularly want to thank Marshall Thomas for the loyalty of his friendship and for embodying the rigorous scientist and thoughtful human being that I aspire to be.

Although this dissertation is the record of a quest to understand a minute part of biology, its completion also depended crucially on support I received from people in my life who are not biologists themselves. I was endlessly encouraged in this pursuit by Florian Hohenberg, David Jousselin, Guillaume Ravilly, Charlie Frogner, Caitlin Zacharias, Socrates Cruz, Nazir Khan, Tabina Bajwa, Sheel Ganatra, Rohit Gupta, Peter Ross, Stephen Alajajian, Kate MacShane, Alice Wen, Kisa Lape, Christina Ng, Rebecca Vernon, Joshua de Bellis, Michael Insler and Rob Tricchinelli, with whom I share eternal bonds of friendship. When I met Caroline Tandetnik on an October evening nearly two years ago, I told her that I was in the process of finishing my PhD.

She has not only forgiven me for grossly transgressing any reasonable interpretation of this timeline, but also cheered me up at every setback and cheered me on at every small success, and I feel unspeakably lucky to be with someone so kind, so patient and so fun.

The help and inspiration given to me by all of the abovementioned people were exceedingly important for carrying out the work of my dissertation, yet they are eclipsed by the role of three individuals who are primarily to thank for what I have been able to do in the last several years. The first is Dr. Danesh Moazed, whom I met for the first time on February 22, 2008, in his newly renovated and utterly vacant office. Although not a single page was present aside from the pad of paper he carried in with him, his ideas, hypotheses and models rapidly filled the room and a universe of molecular mechanisms took shape that I knew intrigued me. I want to thank Danesh not only for allowing me to join his lab, but also for trusting me to explore, for expecting me to work independently, for not depriving me of the joy of working things out on my own. My esteem for his scientific talent is enormous, and my gratitude for everything he has done for me, which I could never repay, will last for the rest of my life.

The second principal acknowledgment I would like to make is to my mother, Bethany Ladimer, who, by enabling me to become bilingual, gave me the single most valuable gift I ever received. She also instilled in me a love of learning from the youngest age, and gave me the lifelong desire to pursue knowledge for its own sake that urged me to undertake these studies.

Finally, I would not have done all of this work, and would not have done any of it, were it not for my father, George Holoch, and the gifts that he left me through his genes and through his nurturing influence. Without these gifts—his affinity for clarity and logic, his curiosity, his patience, his optimism—this dissertation would not exist. I therefore dedicate it to his memory.

Chapter 1

Introduction

To study biology is to gaze into the workings of what is most ordered in the universe. While the inanimate world tends hopelessly toward an ever greater degree of disorder, living things are defined by their singular ability to generate and maintain structured complexity (Boltzmann, 1886; Schrödinger, 1944). This unique capacity for organization and control is what enables life to sustain homeostasis in the face of external changes and to endure and propagate via orchestrated reproduction. Understanding the specific strategies and general principles by which organisms construct and preserve order is the goal of biological science.

And because these aspects of life are a reflection of the environmental pressures that shaped ancestral populations through natural selection, biology is also, through its inferences, a study of history. The features that are most widespread among different living beings are considered to be the most ancient; those that are universal are surmised to have been present in a universal common ancestor whose existence is now widely accepted (Darwin, 1859; Theobald, 2011). Among the characteristics shared between all known organisms are an organization into membrane-bound cells and a system of storage, transmission and expression of hereditary information using DNA, RNA and protein polymers, whose rules are formalized as the Central Dogma of Molecular Biology (Crick, 1958, 1970). The role of RNA in this scheme was long viewed as that of a mere intermediary between the genetic repository (DNA) and its functional products (proteins), with noncoding RNAs (ribosomal and transfer RNAs) simply acting as structural elements in the faithful translation of their coding counterparts (Brenner et al., 1961; Cech and Steitz, 2014). Yet it is difficult to envisage that the DNA-RNA-protein framework that operated in the last universal common ancestor did not evolve from an even earlier system in which RNA could do more than encode proteins; in particular, it is not obvious how the elaborate protein synthesis machinery could have arisen in the first place if RNA could not also have fulfilled other roles in the meantime (Crick, 1968; Orgel, 1968). This suspicion was confirmed perhaps most forcefully by the discovery of catalytic RNA molecules, or ribozymes, in *Tetrahymena* and *Escherichia coli* (Guerrier-Takada, et al., 1983; Kruger et al., 1982), soon

leading to the proposal of a primordial "RNA world" in which both the hereditary material and the enzymes responsible for its duplication consisted exclusively of RNA (Gilbert, 1986). This theory found its most convincing support in the observation that the ribosome itself is a ribozyme, and the notion that such an RNA world once existed is now a matter of consensus (Robertson and Joyce, 2012; Steitz and Moore, 2003). Thus, when the early incarnation of life that gave rise to all the species we now know achieved order in the midst of its disordered surroundings, it did so largely by relying on the informational, structural and catalytic versatility of RNA molecules.

Signs of this legacy pervade modern molecular genetics. RNAs have been studied not only as factories and templates for protein synthesis, but also as enzymes, as templates for the synthesis of telomeric DNA repeats (Greider and Blackburn, 1989), as sensors of metabolite concentrations (Winkler et al., 2002) and, very prominently in recent years, as noncoding *cis* and *trans* regulators of gene expression (reviewed in Wery et al., 2011). It is in this last capacity that RNA will figure in the following pages. This dissertation reports the results of my research on a mechanism of gene silencing mediated by noncoding RNAs (ncRNAs) in the fission yeast *Schizosaccharomyces pombe*. It also reflects on the possible implications of these results for understanding the ordering of biochemical events in a large family of similar phenomena in other organisms.

In this introductory chapter, I will review classical and recent discoveries in the area of genome regulation by noncoding RNAs, with a particular focus on RNA interference (RNAi) and its Argonaute protein protagonists. I will conclude the chapter by discussing the formation of silent chromatin at pericentromeric regions in *S. pombe*, a model which has proved instrumental for elucidating the principles that govern RNAi-directed silencing of transcription, and which served as the context for the studies described in the chapters that follow.

I. Noncoding RNAs and the regulation of gene expression

A. Chromosome-associated RNAs that mediate dosage compensation

The idea that ncRNAs can influence gene expression is epitomized by the chromatin-associated RNAs that control the activity of entire sex chromosomes in the fruit fly *Drosophila melanogaster* and in placental mammals. In both of these well-studied systems, X-linked ncRNAs are transcribed from defined loci in just one of the sexes, and then physically spread to coat the whole X chromosome and mediate wholesale adjustments in gene expression (Kelley and Kuroda, 2000; Stuckenholz et al., 1999). This is critical for dosage compensation, the process that achieves equal levels of X-linked gene products in males and females despite the difference in X chromosome number. Flies and mammals employ opposite strategies for dosage compensation, with the former upregulating transcription from the X chromosome in males (Lucchesi and Kuroda, 2015; Mukherjee and Beerman, 1965) and the latter instead randomly shutting off one of the two X chromosomes in females (Galupa and Heard, 2015; Lyon, 1961). And yet, remarkably, both dosage compensation models depend centrally on ncRNAs. This presents a striking example of convergent evolution and suggests that ncRNAs possess inherent advantages as regulators of gene expression, at least at the level of transcription. These dosage compensation pathways are worthy of a thorough examination, because although not directly related to the studies described in this dissertation, they serve as potent illustrations of several conserved themes in ncRNA-mediated gene regulation.

i. Xist and X chromosome inactivation

The 17-kb ncRNA responsible for X chromosome inactivation in female mammals, called Xist, was first identified through its unusual property of being transcribed exclusively from the inactive X chromosome (Borsani et al., 1991; Brown et al., 1991). The Xist transcript was rapidly shown to lack an open reading frame and to remain localized to the nucleus, coating the length of the chromosome from which it is produced (Brockdorff et al., 1992; Brown et al., 1992; Clemson et al., 1996). This cytological behavior suggested that it played an active role in

mediating X inactivation, which was soon demonstrated genetically (Penny et al., 1996). Importantly, X chromosome inactivation was found to require the presence of the *Xist* gene in *cis* (Penny et al., 1996). This suggested a stepwise mechanism of silencing, involving site-specific initiation and subsequent spreading, that would prove important for framing models of transcriptional repression not only by Xist but also by other ncRNAs.

Interestingly, although Xist is the ncRNA that carries out whole X chromosome inactivation in a direct physical sense, several other ncRNAs are transcribed from the same region, known as the X inactivation center, and also impinge on silencing (reviewed in Lee, 2009). Tsix is a 40-kb ncRNA that spans the *Xist* locus on the opposite strand and whose expression is observed on the opposite, active X chromosome. It acts in *cis* to block the accumulation of Xist RNA, thus antagonizing the initiation of silencing. Consistent with this idea, female cells with a heterozygous *Tsix* disruption show constitutive derepression of *Xist* on the chromosome harboring the mutation and systematic inactivation of that chromosome, in contrast to the ordinarily random choice between the two homologs (Lee and Lu, 1999). However, disruption of *Tsix* is not sufficient to trigger inappropriate X inactivation in males. This intriguing observation betrays the existence of another factor whose role is to promote X inactivation (Lee and Lu, 1999). This molecule also turns out to be a ncRNA, called Jpx, which is encoded by a neighboring locus and is thought to enable *Xist* to overcome Tsix-mediated repression by evicting another *Xist* transcriptional repressor, CTCF (Tian et al., 2010; Sun et al., 2013). Importantly, high levels of Jpx are required to activate *Xist*, which ensures that X inactivation is only triggered when two X chromosomes and thus two copies of Jpx are present (Tian et al., 2010, Sun et al., 2013). Thus, unlike Xist and Tsix, Jpx can act in *trans*. As a clue to how the random choice is made of which X to inactivate, the *Tsix* and *Xist* promoters were recently shown to reside in distinct regions within the three-dimensional space of the nucleus, called topologically associating domains (TADs; Nora et al., 2012; Tsai et al., 2008). This led to a model whereby stochastic fluctuations in the organization of the *Tsix* TAD result in competition

between *Tsix* and another ncRNA locus, *Linx*, for regulatory sequences, which ultimately determines relative Tsix levels on the two homologs and the likelihood of triggering X inactivation (Giorgetti et al., 2014). Finally, three other X chromosome-related ncRNAs are worth mentioning: Xite, which is in reality a set of transcription elements whose products are not so important as the act of their transcription, and which promote Tsix accumulation in *cis* (Ogawa and Lee, 2003); RepA, which represents an independently transcribed 1.6-kb sub-segment of Xist (Zhao et al., 2008) and is discussed further below; and Xact, a recently discovered and very long (251.8-kb) transcript which, by analogy to Xist, coats the entirety of the active X (Vallot et al., 2013).

That such a remarkable array of ncRNAs has evolved to control the inactivation of a whole chromosome underscores the distinctive capacity of these molecules to regulate gene expression. But more concretely, how does Xist spread and how does it bring about silencing? One proposal is that the critical function of Xist is to promote methylation of histone H3 on lysine 27 (H3K27me), which is known to be required for X inactivation (Kohlmaier et al., 2004; Plath et al., 2003), by directly recruiting the Polycomb Repressive Complex 2 (PRC2). The ncRNA RepA has been suggested to target PRC2 during the initiation of silencing, and the corresponding sequence within Xist, called the A repeat, is believed to do the same as Xist spreads across the chromosome (Zhao et al., 2008). Contradicting this view, it has been shown more recently that the A repeat is dispensable for targeting PRC2, albeit necessary for gene silencing (da Rocha et al., 2014). Moreover, the more general notion that ncRNAs physically interact with and recruit PRC2 has been intensely debated (reviewed in Brockdorff, 2013 and discussed later in this chapter), and super-resolution microscopy analyses of cells undergoing X inactivation fail to detect colocalization of PRC2 with Xist (Cerase et al., 2014).

Nevertheless, using PRC2 binding as a surrogate for the spreading of Xist, a genome-wide chromatin immunoprecipitation and next-generation sequencing analysis found that a signature of inactivation appears first at about 150 discrete chromosomal sites, and

subsequently at several thousand additional sites (Pinter et al., 2012). This result illustrates the important role of recent methodological advances in working out dosage compensation mechanisms (reviewed in Ferrari et al., 2014), and suggests that two modes of *cis* spreading exist: long-range targeting to preferred outposts, followed by simple dispersion into adjacent sequences. What is special about the initial localization sites? Mapping Xist binding to chromatin in the course of the inactivation process using capture hybridization analysis of RNA targets and deep sequencing has revealed that Xist first targets gene-rich areas, before spreading into neighboring gene-poor domains (Simon et al., 2013). However, using a similar approach called RNA antisense purification in combination with chromosome conformation capture, another group demonstrated that the selection of initial Xist targeting sites can largely be explained by their proximity in three-dimensional space to the site of Xist transcription (Engreitz et al., 2013). Together these studies indicate that several, non-mutually exclusive influences govern Xist recruitment, and support a hierarchical paradigm of sequential spreading with parallels to the *Drosophila* dosage compensation model (see below).

Lastly, as concerns the mechanism of Xist-dependent gene silencing itself, a major limitation for evaluating alternatives to the PRC2-based model has been the technical challenge of identifying proteins that are specifically associated with Xist. Very recently, however, several groups implemented high-throughput mass spectrometry approaches to systematically discover these interactors and at least one of them, SMRT- and HDAC-associated repressor protein (SHARP, also called SPEN), appears to be especially critical for Xist-mediated silencing (Chu et al., 2015; McHugh et al., 2015; Minajigi et al., 2015). Both SHARP and its interacting histone deacetylase HDAC3 are required for the exclusion of RNA Polymerase II (Pol II) from Xist-coated regions, whereas PRC2 is not required (McHugh et al., 2015). This suggests that the transcriptional silencing event itself occurs upstream of PRC2 activity, but still depends on histone post-translational modifications.

ii. roX RNAs and hypertranscription of the male X chromosome

In contrast to mammals, the critical proteins in fly dosage compensation were identified prior to the discovery of their ncRNA partners. A genetic screen for mutations leading to male-specific lethality (*msl*) found that disruptions of four genes—*mle* (or *maleless*), *msl1*, *msl2* and *msl3*—compromise not only male viability, but also the transcriptional upregulation of the X chromosome (Belote and Lucchesi, 1980). It was later shown that the male X was heavily marked by acetylation of histone H4 on lysine 16 (H4K16ac), a modification known from studies in yeast to promote active transcription, and that this pattern required the MSL proteins (Bone et al., 1994). The enzyme responsible for H4K16ac on the dosage-compensated chromosome was identified as the conserved histone acetyltransferase MOF (Hilfiker et al., 1997), which assembles with the four components discovered in the lethality screen into a complex called MSL (reviewed in Gelbart and Kuroda, 2009).

The binding profile of the MSL complex on the upregulated X chromosome exhibits a preference for the 3' ends of genes (Alekseyenko et al., 2006). Furthermore, the MSL3 subunit recognizes histone H3 trimethylated on lysine 36, a modification that marks the 3' ends of transcribed genes, and this interaction seems to play a critical role in MSL function (Larschan et al., 2007; Sural et al., 2008). Consistent with these observations, the principal mechanism of transcriptional upregulation by the MSL complex appears to involve facilitating the advance of Pol II through gene bodies (Larschan et al., 2011).

The role of RNA in mediating the activity of the MSL complex was portended by a study showing that ribonuclease treatment could cause the MLE subunit to dissociate from the X chromosome (Richter et al., 1996). Soon thereafter two male-specific, MSL-associated ncRNAs were discovered and called roX1 and roX2 (Amrein and Axel, 1997; Meller et al., 1997). The 3.7-kb roX1 and the approximately 1-kb roX2 are largely dissimilar in sequence, yet they act redundantly to mediate MSL complex formation and binding to the X chromosome (Franke and

Baker, 1999; Meller and Rattner, 2002) while, conversely, the MSL subunits stabilize roX1 and roX2 (Amrein and Axel, 1997; Meller et al., 1997, 2000).

The roX RNAs are remarkably reminiscent of Xist: they are noncoding, they are transcribed from the X, they physically spread from their site of synthesis to coat the whole chromosome and they alter levels of gene expression. But important differences are also evident, the most critical being that unlike Xist, the roX RNAs can act in *trans*. For example, when the roX1 locus is moved to an autosome, roX1-containing MSL complexes make their way to the X chromosome, while MSL complexes containing roX2 transcribed from the X are found associated with the autosomal roX1 locus (Meller et al., 1997; Kelley et al., 1999). For Xist to function this way would be self-defeating, since unlike the roX RNAs its mission is to regulate a single X chromosome, not all of the X-linked genetic material in the nucleus (Kelley and Kuroda, 2000). Another possible difference concerns the mechanism of spreading. To be sure, the overall parallels in this regard are striking. The MSL complex targets a few dozen defined “chromatin entry sites” containing a GA-rich sequence motif and then spreads locally (Alekseyenko et al., 2008; Kelley et al., 1999), mirroring the hierarchical logic of Xist spreading. Furthermore, the roX loci themselves act as chromatin entry sites (Kelley et al., 1999; Meller et al., 2000), much as the *Xist* locus serves as a nucleation center for RNA spreading. However, it is not clear whether the roX RNAs themselves play any active role in the spreading mechanism; rather, they seem to act as crucial scaffolds for the assembly and stability of the MSL complex, whose subsequent targeting to non-roX loci may not involve the RNA per se.

Altogether, the *Drosophila* and mammalian dosage compensation systems represent dramatic examples of gene regulation by ncRNAs from which we can draw a number of useful principles. First, ncRNAs can fulfill important structural tasks, such as ensuring the integrity of a protein complex or providing a recognition platform for a histone-modifying enzyme. Second, ncRNAs can act in ways that are largely sequence-independent as suggested by the redundancy between roX1 and roX2, or in ways that involve sequence complementarity as

illustrated by Xist and Tsix. Finally, they can carry out their functions strictly in *cis* (e.g. Xist, Tsix) or on the contrary act in *trans* as diffusible factors (e.g. Jpx, roX RNAs). Insights from the ncRNAs that mediate dosage compensation have informed the study of many other ncRNA-dependent gene regulation phenomena, to which I now turn my attention.

B. Recruitment of chromatin-modifying complexes by noncoding RNAs

Of the mechanisms by which ncRNAs control of gene expression, those that hinge on locus-specific targeting of chromatin-modifying factors are among the best understood. Beyond the examples already discussed, many others involving autosomal loci have been reported over the last decade (reviewed in Mallory and Shkumatava, 2015; Wery et al., 2011; Yang et al., 2014). One interesting class of ncRNAs arises from transcription of mammalian gene enhancer elements (reviewed in Ørom and Shiekhattar, 2013). Termed eRNAs, these transcripts have been found to stimulate the expression of target genes in *cis* by altering chromosome conformation to draw together enhancers and promoters, and by recruiting chromatin-modifying machineries such as Mediator or MLL histone H3 lysine 4 methyltransferases (Lai et al., 2013; Wang et al., 2011). Another fascinating class of ncRNAs that promote gene expression in *cis* consists of transcripts that overlap with protein-coding loci and bind to the DNA methyltransferase DNMT1, somehow preventing it from depositing its silencing mark at local gene promoter sequences (Di Ruscio et al., 2013). A novel ncRNA called Dali performs a similar function but, surprisingly, also acts at many other loci in *trans* (Chalei et al., 2014).

In the majority of the cases reported so far, however, ncRNAs that regulate gene expression through chromatin modifications exert a repressive influence. This includes other DNMT1-interacting RNAs, such as Kcnq1ot1 (Mohammad et al., 2010), ncRNAs that recruit the silencing histone H3 lysine 9 methyltransferase G9a, such as Kcnq1ot1 again as well as Air (Nagano et al., 2008; Pandey et al., 2008) and, perhaps most prominently, ncRNAs that target PRC2. The archetypal molecule in this group is HOTAIR, whose capacity to silence a homeotic gene cluster separate from the one in which it is synthesized represented a foundational

discovery in the field (Rinn et al., 2007). HOTAIR was proposed to bind PRC2 specifically (Rinn et al., 2007), and has since also been reported to act as a scaffold for the concerted recruitment to chromatin, via distinct RNA moieties, of both PRC2 and the repressive histone H3 lysine 4 demethylase LSD1 (Tsai et al., 2010). Several individual ncRNAs have similarly been suggested to silence target genes by specifically recruiting PRC2, albeit in *cis*, including Kcnq1ot1, ANRIL and Xist (Kotake et al., 2011, Pandey et al., 2008; Zhao et al., 2008). Interestingly, PRC2 has been shown in several studies to associate with very large numbers of cellular RNAs (Davidovich et al., 2013; Khalil et al., 2009; Zhao et al., 2010), prompting a vigorous debate as to the genuine specificity of previously reported interactions (Brockdorff, 2013; Cifuentes-Rojas et al., 2014; Davidovich et al., 2013, 2015). The case of HOTAIR is especially perplexing, as the mouse ortholog lacks the proposed PRC2 and LSD1 recruitment motifs found in the human sequence, yet its deletion still results in a loss of H3K27me and a gain of histone H3 lysine 4 methylation, and a consequent homeotic transformation phenotype (reviewed in Diederichs, 2014).

Thus, when evaluating models of ncRNA action it is important to pay close attention to the basis of specificity of RNA-protein interactions. Nevertheless, the work of the past several years clearly indicates that ncRNAs regulate gene expression in a widespread manner by directing chromatin-modifying proteins to (or away from) particular genomic sites. Yet ncRNAs can also control gene expression in its post-transcriptional stages.

C. Regulation of gene expression by cytoplasmic noncoding RNAs

A recent study identified a 3.7-kb ncRNA, called terminal differentiation-induced ncRNA or TINCR, that is strongly upregulated in the course of epidermal differentiation and whose localization is cytoplasmic (Kretz et al., 2013). Disruption of TINCR leads to a differentiation failure through destabilization of many messenger RNAs (mRNAs) critical for epidermal identity, and high-throughput biochemical experiments reveal that these mRNAs contain a TINCR-binding motif that mediates a direct interaction with the ncRNA (Kretz et al. 2013). In turn,

TINCR recruits the STAU1 RNA-binding protein, which would usually promote mRNA decay but in this case mediates a noncanonical stabilization effect (Kretz et al., 2013).

The example of TINCR hints at the possibility that RNA molecules broadly served as *trans*-acting regulators for the activity of other RNA molecules in the former RNA world. For the moment, however, TINCR remains an exception inasmuch as the molecular mechanisms by which most cytoplasmic long ncRNAs regulate gene expression are not understood (Mallory and Shkumatava, 2015), even though they comprise the majority of the long ncRNAs so far identified (Ulitsky and Bartel, 2013).

On the other hand, the cytoplasmic gene-regulatory activities of short ncRNAs have been elucidated in enormous detail, as they are the central players in RNA interference and related pathways, which were first understood as post-transcriptional phenomena. The function of these short ncRNAs depends fundamentally on sense-antisense complementarity with their targets. This places them in a larger context of antisense ncRNA regulators of gene expression, both nuclear and cytoplasmic.

D. Antisense noncoding RNAs that repress their sense counterparts

By analogy to Xist, which can be repressed by the antisense ncRNA Tsix (see above), a number of coding transcripts have also been found to be downregulated by antisense ncRNAs at the transcriptional level. In these situations, a central mechanistic question is whether the antisense RNA molecule has its own role to play in directing changes in sense gene expression, or whether downregulation of the sense RNA is simply the consequence of the passage of antisense RNA polymerases, independently of the transcripts they produce. This latter scheme, called transcriptional interference, is common for example in the budding yeast *Saccharomyces cerevisiae*. In this organism, induction of meiosis requires expression of the *IME4* gene, which is silenced in haploid or vegetatively growing cells by an antisense noncoding transcript (Hongay et al., 2006). Disruption of the antisense promoter leads to constitutive expression of *IME4*, and a transcript identical to the antisense ncRNA but synthesized from an adjacent locus cannot

restore silencing, thus leading to the conclusion that silencing must depend on transcriptional interference (Hongay et al., 2006).

Another example in *S. cerevisiae* seems to offer an alternative mechanistic scenario for ncRNA-mediated repression. The 3’-5’ exoribonuclease complex known as the exosome is responsible for degrading a large class of cryptic ncRNAs that are not detectable in wild-type cells but are revealed by deletion of one of the catalytic subunits, Rrp6 (LaCava et al., 2005; Wyers et al., 2005). Two of these cryptic transcripts are antisense to the *PHO84* gene, and in *rrp6*Δ cells *PHO84* is transcriptionally repressed through the action of these ncRNAs and the histone deacetylase Hda1 (Camblong et al., 2007). The observation that the *PHO84* gene expression outcome depends on the stabilization of the ncRNAs rather than their production per se strongly suggested a function for the RNA molecules themselves in mediating repression (Camblong et al., 2007). But it was shown more recently that, rather than stabilizing the ncRNAs directly, the *rrp6*Δ mutation actually disrupts the termination of PHO84 antisense RNA transcription, and that the elongated ncRNAs thus generated accumulate in the cytoplasm, not at the *PHO84* locus (Castelnuovo et al., 2013). These observations suggest that *PHO84* gene silencing in *rrp6*Δ in fact depends most directly on the transcription across the sense promoter that occurs in the course of synthesizing the elongated antisense transcripts, and not on the RNAs themselves (Castelnuovo et al., 2013).

Nevertheless, in a case where PHO84 antisense RNAs are ectopically introduced and overexpressed, two lines of evidence clearly demonstrate that these molecules can deliver a silencing signal. First, unlike at *IME4*, the antisense RNAs can repress *PHO84* in *trans* and, second, self-cleavage of the RNA abolishes the effect (Camblong et al., 2009). Similarly, a ncRNA that is antisense to the Ty1 retrotransposon is stabilized upon deletion of the 5’-3’ exoribonuclease Xrn1, mediates silencing of Ty1 at the transcriptional level under these conditions, and is also capable of acting in *trans* (Berretta et al., 2008). This is a particularly striking result because Xrn1 is found in the cytoplasm, which suggests that the ncRNA is

trafficked across the nuclear envelope prior to exerting its silencing effect. Interestingly, many other antisense ncRNAs may have similar Xrn1-restricted functions (van Dijk et al., 2011).

Another valuable model for understanding how antisense ncRNAs regulate gene expression is the *FLC* locus in the mustard plant *Arabidopsis thaliana*, which acts as a master regulator in the timing of flowering (reviewed in Ietswaart et al., 2012). *FLC* encodes a repressor whose expression must be silenced before flowering can be initiated. This is accomplished by a set of ncRNAs called COOLAIR, which are induced progressively by prolonged exposure to cold in a process called vernalization that ensures that plants do not flower before the passage of winter (Swiezewski et al., 2009). The antisense promoter sequence is sufficient to confer cold-dependent transcriptional downregulation of a reporter gene, suggesting that antisense transcriptional activity alone could account for silencing, yet the accumulation of native COOLAIR RNAs clearly precedes any changes in FLC mRNA levels (Swiezewski et al., 2009). In addition to vernalization and other external signals, an autonomous pathway also contributes to triggering flowering by *FLC* repression, and this requires the COOLAIR ncRNAs as well (Liu et al., 2010). Interestingly, this autonomous pathway involves a shift in the choice of polyadenylation site in the COOLAIR transcripts that promotes histone H3 lysine 4 demethylation at the *FLC* promoter and consequent *FLC* downregulation (Liu et al., 2010). Thus, although sense mRNA expression is clearly influenced by the transcriptional dynamics of the antisense ncRNAs, the importance of chromatin changes suggests a more subtle mechanism than simple transcriptional interference. Two recent pieces of evidence further argue that the COOLAIR molecules, rather than transcriptional interference, are responsible for *FLC* downregulation. First, in the autonomous pathway, their splicing is critical for their function (Marquardt et al., 2014). Second, in the vernalization pathway, the COOLAIR ncRNAs remain associated with the *FLC* locus (Csorba et al., 2014), in contrast to the PHO84 antisense RNAs in *S. cerevisiae*. Finally, analyses using tiling arrays suggest that upregulation of COOLAIR

does not alter the distribution of nascent FLC mRNAs along the *FLC* gene body, apparently contradicting the transcriptional interference model (Csorba et al., 2014).

The rest of this chapter will concentrate on a category of gene silencing phenomena called RNA interference (RNAi). RNAi is mediated by ncRNAs which, as in the last few examples, are antisense to their targets. But in contrast to the other antisense ncRNA-based mechanisms described in this section, RNAi involves short ncRNAs, and is far more evolutionarily conserved and far better understood. In fact, it represents such a central paradigm that any research concerning antisense ncRNA regulators of gene expression is almost always discussed in relation to RNAi, if not directly within the context of RNAi. More broadly, it is difficult to overstate the significance of RNAi in the history of molecular biology.

II. RNA interference

A. Double-stranded RNA triggers gene silencing

RNAi was first discovered in the nematode worm *Caenorhabditis elegans*, a powerful model organism used to study the genetic basis of development and behavior. In the 1990s, several groups decided to investigate gene function by injecting worms with purified RNA molecules antisense to the genes of interest, reasoning that they would hybridize to the corresponding mRNAs and inhibit protein synthesis (Fire et al., 1991; Guo and Kemphues, 1995; Rocheleau et al., 1997). However, it was noticed fortuitously that sense RNA, used as a control, produced mutant phenotypes at frequencies similar to antisense RNA, indicating that the mechanism of silencing was distinct from that anticipated by the researchers (Guo and Kemphues, 1995; Rocheleau et al., 1997). The phenomenon was dubbed "RNA-mediated interference," abbreviated "RNAi" (Rocheleau et al., 1997). To make sense of the equal efficacy of sense and antisense RNAs, Craig Mello, Andrew Fire and their colleagues hypothesized that each RNA preparation may have contained small amounts of the opposite RNA strand, and that double-stranded RNA (dsRNA) might be responsible for RNAi. Testing this hypothesis directly,

by injecting dsRNA corresponding to specific genes with clear mutant phenotypes, led to a striking discovery: whereas the individual RNA strands produced only mild phenotypes even when provided in very large quantities, comparatively minute concentrations of dsRNA caused complete gene inactivation in the vast majority of animals tested (Fire et al., 1998).

The impact of this finding would prove transformative for many different areas of biological research (Zamore, 2001, 2006). The possibility that the sequence-specific cellular response to dsRNA observed in *C. elegans* might be conserved in other organisms, which was proposed immediately (Fire et al., 1998), opened the door for genetic manipulations in previously intractable organisms. Equally important was the deduction that RNAi reflects an evolutionarily adaptive mechanism that fulfills critical endogenous biological functions (Fire et al., 1998).

The first parallel to be drawn was with plants, in which transgene insertions had been observed to cause post-transcriptional silencing of both the transgene and endogenous gene copies in a phenomenon termed co-suppression (Baulcombe, 1996). A process similar to co-suppression had also been shown to operate in viral resistance, and it had been speculated that the trigger for silencing might involve recognition of dsRNA (Ratcliff et al., 1997). Indeed, the capacity of dsRNA to mediate gene inactivation was directly demonstrated by co-expression of pairs of sense and antisense RNAs, thus mirroring the results in *C. elegans* (Waterhouse et al., 1998).

It is also in plants that the first major mechanistic advance in understanding the workings of RNAi was made, when plants undergoing either transgene- or virus-induced post-transcriptional gene silencing were consistently found to accumulate short RNAs of about 25 nucleotides (nts) that were antisense to the silencing target (Hamilton and Baulcombe, 1999). The generality of this observation was confirmed by a cell-free system recapitulating dsRNA-mediated silencing in *Drosophila* embryo extracts (Tuschl et al., 1999), in which the trigger dsRNA was processed into 21-23-nt RNAs, even in the absence of a target RNA (Zamore et al.,

2000). Together, these data raised the possibility that the short RNAs might represent the direct effectors of RNAi. This idea was further supported by the finding that the target mRNA in the *in vitro* system is endonucleolytically cleaved at 21-23-nt intervals, suggesting that its destruction is guided by the short RNAs (Zamore et al., 2000). Also implicating these short RNAs in the gene silencing event, a dsRNA-induced RNAi activity in another *Drosophila* cell extract system co-purified with 25-nt RNAs, which were proposed to act as sequence specificity determinants (Hammond et al., 2000).

The formal demonstration that RNAi is directed by short RNAs came from experiments in which these RNAs were introduced directly. Crucially, the short RNAs recovered from processing of dsRNA by a *Drosophila* extract were examined and found to be duplexes with 2-nt single-stranded overhangs at the 3' end of each strand (Elbashir et al., 2001b). Providing chemically synthesized versions of these short dsRNAs, termed short interfering RNAs (siRNAs), to a *Drosophila* embryo extract recapitulated the cleavage of sequence-matched target mRNAs previously induced by longer dsRNA (Elbashir et al., 2001b). These experiments also revealed that the endonucleolytic hydrolysis of the target RNA occurs at a site corresponding to the center of the siRNA sequence (Elbashir et al., 2001b). Remarkably, chemically synthesized siRNAs were found to be capable of carrying out RNAi in cultured human cells as well (Elbashir et al., 2001a), speaking to the deep conservation of the RNAi pathway and its mechanisms, and ushering in an era of new genetic tools for studying mammalian gene function, whose power has only recently been surpassed by CRISPR/Cas9 technology (Shalem et al., 2014).

B. Components of the RNAi machinery

The early studies of RNAi delineated an elegant model to explain how cells respond to dsRNA and silence genes. The initiating step involves processing of the dsRNA into small pieces, while the second step consists of a target recognition and cleavage event in which the

small RNAs are directly implicated. The ribonucleases that act at each of these steps were identified even as this model came into being.

i. Dicer

Finding the enzyme that cleaves dsRNA into small RNAs was facilitated by the knowledge that ribonuclease activities directed toward double-stranded species are unusual, with the evolutionarily conserved RNase III family figuring as a major exception. A systematic test of the *Drosophila* RNase III subfamilies for the ability to generate small RNAs from dsRNA *in vitro* led to the discovery of Dicer, an enzyme with two paralogs in *Drosophila* and orthologs in *C. elegans*, *Arabidopsis* and mammals (Bernstein et al., 2001). This initial study demonstrated that both the *Drosophila* and human Dicers are capable of processing of dsRNA into small RNAs, and that the protein is essential for RNAi-mediated silencing (Bernstein et al., 2001). Satisfyingly, the structural features of the small RNAs isolated from dsRNA-treated *Drosophila* extracts that were critical for the functionality of chemically synthesized siRNAs, including the 2-nt single-stranded overhangs, are signatures of RNase III-dependent catalysis (Elbashir et al., 2001a, 2001b). Thus, Dicer provides the conserved molecular basis for the production of the small RNAs that are ultimately responsible for RNAi.

ii. Argonaute

Understanding the details of the silencing step began with the biochemical purification of an RNAi activity from *Drosophila* cells that depends on small RNAs (Hammond et al., 2000). This was named the RNA-induced silencing complex (RISC) (Hammond et al., 2000). The identification of a protein called Argonaute2, both as one of the components of RISC and as a necessary factor for RNAi in *Drosophila* (Hammond et al., 2001), was a milestone for defining the nature of the silencing mechanism. Genes encoding proteins with domain structures identical to Argonaute2 had been reported as essential for RNAi in *C. elegans* (Tabara et al., 1999) and related co-suppression phenomena in the filamentous fungus *Neurospora crassa* and in plants (Catalanotto et al., 2000; Fagard et al., 2000). The convergence of these genetic and

biochemical observations suggested that the key to RNAi might reside in the molecular function of the conserved Argonaute family proteins, and that the role of siRNAs might simply amount to serving as specific guides for target identification by Argonautes.

A unifying feature of the Argonaute family is a characteristic primary structure that includes both PAZ and PIWI domains (discussed in detail later in this chapter). Structural analysis of an archaeal Argonaute protein revealed that the PIWI domain in fact represents a cryptic RNase H-like fold, raising the possibility that Argonautes themselves carry out the destruction of target RNAs in RNAi (Song et al., 2004). In support of this idea, modeling of a small RNA molecule into a positively-charged groove of the Argonaute structure placed the center of the small RNA in close proximity to the proposed catalytic site (Song et al., 2004), which agrees with the early *Drosophila* extract experiments indicating that siRNA-guided hydrolysis of the target mRNA occurs at an equivalent position (Elbashir et al., 2001b). Furthermore, immunoprecipitations of the human ortholog Argonaute2 could mediate siRNA-dependent target cleavage and this was abolished by mutating amino acid residues in the predicted catalytic triad, thus defining Argonaute as "Slicer," or the target-directed endoribonuclease in RNAi (Liu et al., 2004). As a strict demonstration, recombinant Argonaute2 and an siRNA are sufficient for sequence-specific substrate cleavage (Rivas et al., 2005). Certain other human Argonaute paralogs, on the other hand, lack the required catalytic motif and, consistently, do not exhibit slicer activity (Liu et al., 2004; Meister et al., 2004). This observation reflects the existence—in humans and many other species—of a whole host of RNAi-related gene silencing phenomena that do not rely on endonucleolytic cleavage of a target RNA. These will be described later in this section.

iii. RNA-directed RNA polymerase

In addition to Dicer and Argonaute, a third class of proteins is critical for RNAi in certain organisms. When RNAi was first observed in *C. elegans*, it was remarked that a small quantity of dsRNA sufficed to trigger the destruction of a vast excess of target mRNA, "suggesting that

there could be a catalytic or amplification component in the interference process" (Fire et al., 1998). This statement was exceptionally prescient, as it foreshadowed the discovery not only of catalytic Argonaute-siRNA complexes, but also of a role for RNA-directed RNA polymerases (RdRPs) in augmenting the strength of RNAi responses.

Similarly to Argonautes, RdRPs were implicated genetically in RNAi and co-suppression in fungi, plants and animals (Cogoni and Macino, 1999; Dalmay et al., 2000; Mourrain et al., 2000; Smardon et al., 2000) before their contribution was characterized biochemically (Sijen et al., 2001). Although RdRPs with roles in dsRNA-mediated RNAi seem to be absent from flies and mammals, they are otherwise widespread on the evolutionary tree. They have been studied most extensively in *C. elegans*, where, intriguingly, the vast majority of RdRP activity is dedicated to the production of individual small RNAs rather than new long dsRNA substrates for Dicer (Pak and Fire, 2007; Sijen et al., 2007). These small RNAs are termed secondary siRNAs, and are never templated by the exogenous dsRNA but rather exclusively by the target mRNA; accordingly, they are overwhelmingly antisense with respect to the target (Pak and Fire, 2007; Sijen et al., 2001, 2007). A recent study used a clever mismatch-based design to demonstrate that secondary siRNAs cannot themselves direct RdRPs to synthesize tertiary siRNAs in a further round of amplification. Thus, an elegant feedback mechanism allows a robust secondary signal to be generated while simultaneously protecting the organism from a runaway response (Pak et al., 2012).

RNAi is generally understood to denote gene silencing induced by foreign dsRNA, but the cellular machineries that carry out this silencing and that I have just described also play important biological roles in conjunction with endogenously-encoded short RNA molecules.

C. MicroRNAs

The first example of endogenous gene regulation by an antisense ncRNA was discovered in *C. elegans* in the course of analyzing mutants with defects in the timely formation of cell lineages. Researchers had identified the *lin-4* gene as a negative regulator of the *lin-14*

gene based on their opposite phenotypes and epistatic relationship, but, surprisingly, *lin-4* was found not to encode a protein (Lee et al., 1993). Instead, *lin-4* gives rise to very short 22- and 61-nt ncRNAs, and the *lin-14* 3' untranslated region (UTR) contains repeated motifs complementary to the ncRNAs that confer post-transcriptional inhibition of protein synthesis (Lee et al., 1993; Wightman et al., 1993). Thus, the results suggested that *lin-4* ncRNAs acted in *trans* on the *lin-14* mRNA to inhibit its translation, but the mechanism was enigmatic. Interestingly, another developmental regulator, *lin-28*, was also found to be regulated by *lin-4* through a complementary element in its 3' UTR (Moss et al., 1997), and another, much more conserved small ncRNA, *let-7*, was then identified and shown to regulate *lin-14*, *lin-28* and several other genes in a similar manner (Pasquinelli et al., 2000; Reinhart et al., 2000). The *lin-4* and *let-7* gene products were first called small temporal RNAs but are now known as microRNAs (miRNAs).

A key breakthrough in understanding the origin and mechanism of action of miRNAs was the discovery that their biogenesis in worms, flies and human cells requires Dicer (Grishok et al., 2001; Hutvágner et al., 2001; Ketting et al., 2001) and, concurrently, that miRNA function in *C. elegans* requires two genes encoding Argonaute homologs (Grishok et al., 2001). The dependence on Dicer for the generation of miRNAs could be neatly explained by the complementary stem-loop structure of their precursors (Grishok et al., 2001; Hutvágner et al., 2001; Ketting et al., 2001). However, unlike the siRNAs generated during RNAi, miRNAs were not believed to direct the destruction of their targets (Wightman et al., 1993); that Argonaute proteins were necessary for miRNA function would therefore, in retrospect, constitute the first evidence that they possessed slicer-independent activities guided by small RNAs. The failure of miRNAs to trigger Argonaute catalysis was quickly realized to result from their imperfect complementarity to their targets (Hutvágner and Zamore, 2002).

The cooperation between ancient RNAi proteins and a highly conserved and developmentally important short RNA suggested that many other miRNAs, besides *lin-4* and *let-*

7, might exist. Indeed, dozens of additional miRNAs were rapidly identified in *C. elegans*, *Drosophila* and vertebrates (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001), and the ranks of known miRNAs have now swollen into the thousands (Ghildiyal and Zamore, 2009). Much like *lin-4* and *let-7*, their regulatory function depends on complementary sequence elements within the mRNAs that they target. Complementarity in the segment spanning nucleotides 2 through 7 from the 5' end of the miRNA, or the miRNA "seed," acts as a particularly critical parameter for targeting (Lewis et al., 2003). More than half of mammalian protein-coding gene sequences exhibit signs of natural selection to preserve complementarity to miRNAs (Friedman et al., 2009); this observation is a testament to the diversity of biological processes that are influenced by these molecules.

In metazoans, cleavage by Dicer actually represents the second of two processing steps in the biogenesis of miRNAs: first, the stem-loop precursor is released from a primary transcript by another RNase III family enzyme, Drosha (Lee et al., 2003). This reaction also requires a Drosha-associated dsRNA-binding protein, called Pasha in *Drosophila* and DGCR8 in humans; together the two proteins form the Microprocessor complex (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004). A very recent study uncovered an additional, upstream cleavage step for one primary transcript, which is carried out by the endonuclease CPSF73 and is necessary to create an efficient Microprocessor substrate (Du et al., 2015). For a small minority of miRNAs encoded within the introns of genes, however, the stem-loop precursor is generated not by Microprocessor but by the mRNA splicing apparatus, thus bypassing the canonical biogenesis pathway but still relying on Dicer for maturation of the small RNA (Okamura et al., 2007; Ruby et al., 2007). Finally, a Dicer-independent miRNA biogenesis mechanism has been reported for miR-451, which in both zebrafish and mice requires the endonucleolytic activity of Argonaute2 to initiate processing of the mature miRNA from the stem-loop (Cheloufi et al., 2010; Cifuentes et al., 2010). Trimming by the exonuclease PARN concludes this unusual miRNA generation pathway (Yoda et al., 2013). It is worth emphasizing,

however, that the vast majority of animal miRNAs are produced by the sequential actions of Drosha and Dicer (Ameres and Zamore, 2013; Ha and Kim, 2014). In contrast, miRNAs in plants are processed from stem-loop-containing primary transcripts solely by the Dicer homolog DCL1 (reviewed in Rogers and Chen, 2013).

Another difference with animals is that Argonaute-mediated slicing is the primary (albeit not exclusive) mode of gene regulation by miRNAs in plants (Rhoades et al., 2002; Rogers and Chen, 2013). In contrast, miRNA-mediated repression in animals, with very rare exceptions, does not involve target slicing. What, then, is its mechanistic nature? As noted above, the first miRNA, *lin-4*, was found to inhibit the translation of the target *lin-14* mRNA into protein without affecting its abundance (Wightman et al., 1993). Since then, many lines of experimental evidence have indicated that miRNAs can influence not only the translation efficiency of target mRNAs but also their turnover, and the relative importance of each of these two regulatory modes became a subject of intense debate (reviewed in Huntzinger and Izaurralde, 2011). In mammalian cells, the most recent data suggests that target mRNA degradation represents the major mechanism of repression (Huntzinger and Izaurralde, 2011; Jonas and Izaurralde, 2015). A study combining genome-wide analysis of mRNA abundance with ribosome profiling, a technique to map the positions of translating ribosomes on mRNAs, demonstrated that the vast majority of miRNA-dependent gene downregulation occurs through mRNA destabilization, and that changes in translation efficiency are rare (Guo et al., 2010). Thus, inhibition of translation does not seem to be a trick that miRNAs rely upon widely for silencing at steady state; however, there is strong evidence that it may act at the onset of miRNA-mediated silencing in both vertebrates and invertebrates, in a time window that is rapidly followed by mRNA degradation but nevertheless remains distinct (Bazzini et al., 2012; Djuranovic et al., 2012). Both translational repression and mRNA destabilization by miRNAs involve members of the conserved GW protein family (reviewed in Braun et al., 2013), which will be discussed later in

this chapter, although a miRNA-mediated mechanism of translational inhibition that does not require GW proteins has also been reported (Fukaya and Tomari, 2012; Fukaya et al., 2014).

The rise of microRNAs in both the plant and animal lineages illustrates the remarkable potential for components of the RNAi machinery to be co-opted for the regulation of cellular protein-coding genes. Nevertheless, it is widely accepted that the more universal endogenous function of small RNAs is to defend the genome against foreign or unstable genetic elements (reviewed in Malone and Hannon, 2009; Moazed, 2009).

D. Endogenous siRNAs and piRNAs in genome defense

i. RNA-directed DNA methylation in plants

All organisms are confronted with the challenge of parasitic DNA sequences called transposable elements (TEs) or transposons, whose ability to mobilize and re-insert at random positions poses a threat to genome stability. In flowering plants, TEs are kept in check through DNA cytosine methylation, an evolutionarily conserved signal for silencing of transcription (reviewed in Slotkin and Martienssen, 2007). All *de novo* DNA methylation in *Arabidopsis*, as well as maintenance of DNA methylation in asymmetric sequence contexts, requires an RNA-directed DNA methylation (RdDM) pathway (reviewed in Law and Jacobsen, 2010). Interestingly, the RNA in question is a population of endogenous siRNAs whose precursors are synthesized by a plant-specific RNA polymerase, Pol IV (Herr et al., 2005; Onodera et al., 2005), and processed by the RdRP homolog RDR2 and the Dicer enzyme DCL3 (reviewed in Matzke and Mosher, 2014). These siRNAs guide the Argonaute protein AGO4 to target transcripts generated by yet another plant-specific polymerase, Pol V (Wierzbicki et al., 2009). AGO4 is physically associated with the *de novo* DNA methyltransferase DRM2 (Gao et al., 2010; Zhong et al., 2014), an interaction which establishes the direct link between siRNAs and transcriptional silencing. RdDM exhibits a remarkable array of self-reinforcing features which combine to form a robust positive feedback (Johnson et al., 2014; Law et al., 2013; Liu et al., 2014).

ii. piRNAs in *Drosophila* and mammals

Endogenous siRNAs that provide protection against TEs have also been reported in mouse ooctyes (Tam et al., 2008; Watanabe et al., 2008) and in *Drosophila* somatic cells (Chung et al., 2008; Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008). But the most extensively studied line of defense against TEs in metazoans consists of a distinct class of small RNAs, which do not require Dicer for their biogenesis and are termed piRNAs because they interact with members of the gonad-specific Piwi clade of the Argonaute protein family (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Vagin et al., 2006). In both the mouse and *Drosophila* germlines, piRNA biogenesis is coupled with the destruction of TE transcripts in an amplification loop known as the ping-pong cycle (Aravin et al., 2007; Brennecke et al., 2007; De Fazio et al., 2011; Gunawardane et al., 2007). In *Drosophila*, genomic clusters containing inactive transposon sequences give rise to piRNAs that are antisense to TEs and bind to the Piwi paralog Aubergine. The Aubergine-piRNA complex is then able to recognize and cleave sense transcripts, and in so doing it generates the 5’ ends of sense piRNAs that bind to another Piwi paralog, Ago3. In turn, Ago3-bound piRNAs direct the cleavage of antisense transcripts and the 5’-end formation of new Aubergine-bound piRNAs (Brennecke et al., 2007; Gunawardane et al., 2007). In this context, expressed TE transcripts with homology to the piRNA-generating clusters are efficiently eliminated, while also contributing to further piRNA production. Importantly, both the piRNA-encoding repositories and the piRNAs themselves are required to sustain the amplification cycle and mediate TE immunity in the germline (Brennecke et al., 2008). Extending this observation, a recent study demonstrated that exposure to homologous piRNAs is sufficient to permanently transform any chromosomal region into a piRNA-generating locus (de Vanssay et al., 2012). This result is a concrete illustration of how the small RNA repertoire that defends the genome against TEs can be constructed over time.

Details of the *Drosophila* piRNA biogenesis mechanism have continued to emerge in recent reports. In the nucleus, the piRNA clusters that are involved in ping-pong are packaged into repressive chromatin domains containing methylated histone H3 lysine 9 (Sienski et al., 2012), but they are nevertheless transcribed to give rise to piRNA precursors. As it turns out, this noncanonical transcription is mediated by Rhino, a member of the conserved HP1 family of proteins that recognize methylated histone H3 lysine 9 and are more often thought of as transcriptional repressors (Klatenhoff et al., 2009; Le Thomas et al., 2014; Mohn et al., 2014; Zhang et al., 2014). Importantly, Rhino complex activity is itself dependent on piRNAs and the nuclear Piwi protein (Le Thomas et al., 2014; Mohn et al., 2014), suggesting that small RNA generation is reinforced by a positive feedback loop. The Rhino complex also acts to inhibit splicing of the piRNA precursor transcripts, and this is another critical element for proper piRNA accumulation (Zhang et al., 2014). In the cytoplasm, ping-pong amplification by Aubergine and Ago3 occurs in a perinuclear body termed nuage, but its mechanistic organization was only recently uncovered. As demonstrated in an elegant study, amplification is physically coordinated within nuage by the RNA helicase Vasa, which orchestrates the transfer of newly-sliced target RNAs from each Piwi paralog into the reciprocal paralog to serve as piRNA guides for the next round of slicing (Xiol et al., 2014). While the generation of piRNA 5' ends has been known to result from repeated cycles of slicing by Aubergine and Ago3 during ping-pong amplification, or from the very initial cleavage of piRNA precursor transcripts by the Zucchini nuclease (Ipsaro et al., 2012; Nishimasu et al., 2012), piRNA 3'-end formation has remained enigmatic (Kawaoka et al., 2011). Two newly published studies have revealed that, in fact, the 3' ends of piRNAs are also generated by Zucchini as it cleaves the products of Ago3 slicing at successive phased intervals each corresponding to one piRNA length (Han et al., 2015; Mohn et al., 2015).

Fewer of the specifics are known in the case of the mammalian piRNA pathway, but one important difference with flies is that piRNA amplification is initiated by sense TE transcripts from dispersed sites in the genome, rather than by antisense transcripts from piRNA-producing

loci gathered in a few large clusters (Aravin et al., 2008). Another difference is that piRNAs silence TEs by specifying *de novo* DNA methylation (Aravin et al., 2007, 2008) and, interestingly, this does not involve a positive feedback loop, since piRNA generation is unaffected in DNA methylation mutants (Aravin et al., 2008). There is also evidence, for at least certain TEs, that the contribution of piRNAs to DNA methylation is not sufficient to yield full repression, and that piRNAs must additionally provide an essential post-transcriptional layer of silencing (Di Giacomo et al., 2013).

iii. Small RNAs for distinguishing self from nonself nucleic acids in *C. elegans*

C. elegans also possesses piRNAs, called 21U-RNAs (Ruby et al., 2006; Batista et al., 2008; Das et al., 2008), which, unlike their counterparts in insects and mammals, are synthesized as individual transcription units (Cecere et al., 2012; Gu et al., 2012b), and appear to play a comparatively limited role in TE silencing (Batista et al., 2008; Das et al., 2008). Instead, they constitute a more general surveillance system that scans the germline transcriptome for foreign RNA. This was revealed by the observation that transgenes from unrelated organisms inserted into the *C. elegans* genome can undergo heritable silencing in a manner that depends on the Piwi homolog PRG-1 (Ashe et al., 2012; Lee et al., 2012; Shirayama et al., 2012).

C. elegans small RNA pathways employ a hierarchy of Argonautes, with trigger small RNAs binding to primary Argonautes and secondary, RdRP-dependent small RNAs binding to secondary Argonautes (Yigit et al., 2006). For example, siRNAs derived from Dicer-dependent processing of exogenous dsRNA bind to the Argonaute RDE-1, and the secondary siRNAs whose synthesis is instructed by RDE-1-siRNA complexes then bind to different, secondary Argonautes. Similarly, analysis of small RNAs in worms with a transgene subject to PRG-1-dependent silencing shows an accumulation of RdRP-dependent secondary siRNAs (also called 22G-RNAs) that map near sites in the transgene that bear imperfect complementarity to endogenous 21U-RNAs (Lee et al., 2012; Shirayama et al., 2012). This suggests that PRG-1

and its 21U-RNA guides search for nonself transcripts in the germline, and direct the heritable silencing of these transcripts by inducing the synthesis of corresponding 22G-RNAs that interact with a secondary Argonaute. The secondary Argonaute responsible for 21U-RNA-initiated silencing turns out to be the germline-specific HRDE-1 (Ashe et al., 2012; Bagijn et al., 2012; Lee et al., 2012; Shirayama et al., 2012), which acts in the nucleus to promote histone H3 lysine 9 methylation in partnership with the Argonaute-associated silencing factor NRDE-2 (Ashe et al., 2012). Interestingly, HRDE-1 and NRDE-2 are also required for the transgenerational inheritance of dsRNA-mediated silencing (Buckley et al., 2012; Burton et al., 2011; Gu et al., 2012a), and NRDE-2, which halts gene transcription during the elongation phase, is also found in somatic cells where it associates with the Argonaute NRDE-3 (Guang et al., 2010).

The PRG-1-HRDE-1 system for heritable silencing of nonself nucleic acids exhibits two notable features. The first is that it represents a truly epigenetic phenomenon, as removal of PRG-1 after the onset of silencing does not affect the ability of the silencing to be maintained and inherited indefinitely (Ashe et al., 2012; Lee et al., 2012; Shirayama et al., 2012). In other words, HRDE-1-bound 22G-RNAs sustain stable silencing even in the absence of the factors that first trigger their production. The second striking property of the PRG-1-HRDE-1 pathway is that, as indicated by genetic crosses in which transgene expression is restored, the *C. elegans* germline contains *trans*-acting factors that can prevent or reverse the establishment of silencing (Shirayama et al., 2012). Such a countervailing system appears particularly critical given the sequence diversity of the 15,000 21U-RNAs encoded in the genome (Batista et al., 2008; Ruby at al., 2006) and their ability to trigger stable silencing even with imperfect base-pairing to their targets (Lee et al., 2012; Shirayama et al., 2012). To protect the worm's own genes from promiscuous silencing, a parallel small RNA-based strategy has evolved alongside PRG-1-HRDE-1 that identifies "self" sequences and allows them to evade PRG-1 activity. This consists of the Argonaute CSR-1 and its 22G-RNA partners, which map to all germline-expressed mRNAs and, in a manner that is exceptional among Argonaute-siRNA complexes, do not

repress their targets (Claycomb et al., 2009). Instead, recent studies have demonstrated that CSR-1-bound 22G-RNAs can block the targeting of homologous transcripts by PRG-1, and even restore the expression of homologous HRDE-1-repressed genes (Seth et al., 2013; Wedeles et al., 2013). These dual forces set the piRNA model in *C. elegans* apart from that of *Drosophila* and mammals: by safely marking self transcripts for clemency from the silencing machinery, worms can apply a more sweeping surveillance mechanism with a more relaxed specificity, and therefore defend their genomes against foreign nucleic acids innately, without the need for prior exposure (Seth et al., 2013).

As was foreseen when RNAi was first discovered, the experimental observation of "potent and specific genetic interference by double-stranded RNA" implied the existence of endogenous pathways that carry out gene silencing using the same machineries (Fire et al., 1998). The realization that RNAi was mediated by shorter RNAs identified a common mechanistic thread for these pathways. In many of them, the biogenesis of the small RNAs relies vitally on Dicer, RdRP or both, but in other cases these components are dispensable (e.g., piRNAs in *Drosophila* and mammals). Argonautes, however, are the universal effectors in RNAi-related small RNA silencing mechanisms, and the data reported in this dissertation concern the regulation of their function. The next section therefore describes the activities and structural features of these proteins, and introduces a class of Argonaute-associated factors that play important and diverse roles in small RNA-mediated silencing.

III. Argonautes and their GW protein partners in gene silencing

A. Argonaute slicing

As we saw earlier, when Argonaute proteins were definitively linked to RNAi it was because of their role in the endoribonucleolytic cleavage or "slicing" of RNAi targets (Liu et al., 2004). The first crystal structure of a full-length Argonaute protein, from the archaeal species *Pyrococcus furiosus*, revealed the three-dimensional arrangement of the family's four

characteristic domains: the N (amino), PAZ (Piwi-Argonaute-Zwille), MID (middle) and PIWI (P-element-induced wimpy testes) domains (Song et al., 2004). The PAZ domain, which is also present in most Dicer proteins, binds 3'-hydroxyl ends of small RNAs and specifically recognizes the duplex structures with 2-nt single-stranded overhangs that are found in siRNAs and miRNAs (Ma et al., 2004; Song et al., 2003). The MID domain contains a pocket that anchors the 5'-monophosphorylated ends of small RNAs (Ma et al., 2005; Parker et al., 2005). The role of the N domain was elucidated more recently, and involves initiating the unwinding of small RNA duplexes bound to the protein. This participates in the release of the non-guide or "passenger" strand and the formation of a mature complex that is competent for hybridization with a target (Kwak and Tomari, 2012). Finally, the RNase H-like endonuclease or slicer activity resides in the PIWI domain (Liu et al., 2004; Song et al., 2004).

More precise details regarding what controls the slicing reaction were provided by several structures of a small-DNA-guided Argonaute protein from the bacterium *Thermus thermophilus*. These analyses showed that two arginine side-chains interact with the phosphate backbone of the guide, holding it in an inactive non-helical conformation that is released upon pairing with a target RNA (Wang et al., 2008a, 2008b). However, these arginines are not conserved in eukaryotes (Wang et al., 2008b). A more generally applicable conclusion from the *T. thermophilus* structures is that the formation of a slicer-permissive, fully double-helical guide-target duplex within Argonaute requires the release of the 3' end of the guide strand from the PAZ domain (Wang et al., 2009b). This observation is consistent with previous results suggesting that the PAZ domain has a relatively lower affinity for RNA and that, in stark contrast to the high-affinity MID domain, its conserved residues are not required for slicing events (Song et al., 2003; Wang et al., 2008b).

Later, long-awaited successes in crystallizing full-length eukaryotic homologs (Elkayam et al., 2012; Nakanishi et al., 2012; Schirle and MacRae, 2012; Schirle et al., 2014) have offered new insights into the mechanisms of Argonaute function, and particularly into the mechanism of

slicing. RNase H family enzymes have long been known to mediate RNA cleavage using a tetrad of catalytic residues, but, until recently, only an aspartate-aspartate-histidine (DDH) triad had been identified in Argonaute proteins with slicer activity (Liu et al., 2004; Nakanishi et al., 2012; Rivas et al., 2005). A study of the crystal structure of the Argonaute from the budding yeast *Kluyveromyces polysporus* was the first to discern the full catalytic tetrad (Nakanishi et al., 2012). This discovery prompted a re-examination of the four human Ago proteins (Argonautes not belonging to the Piwi clade), among which Argonaute2 is the sole protein capable of slicing. Consistent with its competence as an endonuclease, Argonaute2 possesses the required DEDH tetrad, whereas Argonaute1 does not. Argonaute3, on the other hand, bears all four necessary residues and yet cannot cleave target RNAs (Liu et al., 2004; Hauptmann et al., 2013; Nakanishi et al., 2013). Structural and genetic analyses have shown that, in order to slice, Argonaute1 and Argonaute3 would require not only the intact catalytic motif but also features of the N domain present only in Argonaute2, and, in the case of Argonaute1, the elimination of an inhibitory loop that is specific to this paralog (Faehnle et al., 2013; Hauptmann et al., 2013; Nakanishi et al., 2013). Interestingly, the two Ago proteins of *Drosophila*, Ago1 and Ago2, also differ in their slicing activities. Both are able to catalyze slicing in principle, but only Ago2 fulfills this role in practice, in part because Ago1 has a lower rate of cleavage, but also because it is much slower to dissociate from its products (Förstemann et al., 2007).

The clearest functional implication of slicing is that it destroys the target of the Argonaute-small-RNA complex. This event is at the heart of the mechanism of dsRNA-mediated RNAi, and is also central to TE repression in the piRNA ping-pong amplification cycle. In the mouse male germline, piRNA-directed slicing is additionally required for TE silencing independently of its role in piRNA amplification (Reuter et al., 2011). But, as noted earlier, a variety of small RNA pathways manage to achieve silencing in the absence of Argonaute-mediated slicing. This is the case for the vast majority of miRNAs in metazoans, which repress their targets through other means (Huntzinger and Izaurralde, 2011). It is also true of the 21U-

RNA transcriptome surveillance pathway in *C. elegans*: although PRG-1 is competent for slicing (Yigit et al., 2006), it seems that nearly all target recognition events occur in the absence of full complementarity and therefore without cleavage (Lee et al., 2012; Shirayama et al., 2012). Moreover, the secondary effector Argonaute HRDE-1 lacks the catalytic residues that would be necessary for 22G-RNA-directed slicing (Yigit et al., 2006). Perhaps most surprisingly, the very first RNAi phenomenon to be observed—silencing mediated by exogenous dsRNA in *C. elegans*—also does not require the target mRNA to be sliced by an Argonaute protein (Steiner et al., 2009). The Argonaute involved in this pathway, RDE-1, is a catalytic slicer, but this activity is only required for removal of the passenger strand from the siRNA, not for destruction of the actual target (Steiner et al., 2009). It was recently demonstrated that cleavage of the target transcript in classical RNAi is carried out by the endonuclease RDE-8, after its recruitment downstream of RDE-1 (Tsai et al., 2015).

Thus, slicing by Argonaute is inherent to certain mechanisms of repression, and dispensable or even absent in others. But the endonuclease activity of Argonaute can serve other functions as well. One that has already been discussed is the biogenesis of small RNAs, including piRNAs (Aravin et al., 2007; Brennecke et al., 2007; De Fazio et al., 2011; Gunawardane et al., 2007) and, rarely, miRNAs (Cheloufi et al., 2010; Cifuentes et al., 2010). Another role of Argonaute slicing, as alluded to above in the case of RDE-1 in *C. elegans* (Steiner et al., 2009), is to promote the release of passenger strands from small RNA duplexes, a step that is required to generate a functional Argonaute-guide complex that can identify targets through base pairing. This model was first demonstrated in *Drosophila* and human cells, and it rests on the idea that the passenger strand essentially acts as the first target of the small-RNA-guided slicer enzyme (Leuschner et al., 2006; Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005). The products of passenger-strand cleavage are then cleared by the C3PO endonuclease in order to activate the silencing complex (Liu et al., 2009). Slicing of the passenger strand seems to be critical for its removal when it is perfectly complementary to the

guide, consistent with a scenario in which cleavage facilitates duplex melting by reducing the effective energy of hybridization. Inversely, in cases where the small RNA duplex bears mismatches, such as in *Drosophila* miRNAs, it becomes feasible for the passenger strand to be unwound from the guide and evicted through a slicer-independent mechanism (Kawamata et al., 2009). In *Neurospora*, conversion of a perfect duplex to a single-stranded guide requires the exonuclease QIP which, by analogy to the *Drosophila* C3PO endonuclease, eliminates the nicked passenger strand (Maiti et al., 2007). But, interestingly, QIP also participates in the slicer-independent strand separation of duplex small RNAs (Xue et al., 2012).

In sum, slicing as a gene silencing strategy represents a trademark of the Argonaute family, but slicing has also evolved to fulfill other silencing-related tasks, and at the same time distinct modes of silencing that do not rely on slicing have also arisen. Regardless of whether Argonaute slices an RNA molecule or performs an alternative activity in gene silencing, it must first be programmed with a small RNA guide.

B. Argonaute loading

The loading of Argonaute with a small RNA is far from a trivial event, and the mechanisms governing this process have been explored extensively. A foundational discovery in this field was the identification of R2D2, a *Drosophila* protein that is tightly associated with Dicer-2, but whose contribution to RNAi occurs downstream of Dicer-2 catalysis, specifically in the formation of an siRNA-containing complex competent for target cleavage. It was proposed that R2D2 actively hands siRNAs over from Dicer-2 to the silencing effector complex (Liu et al., 2003). Also supporting the existence of active loading mechanisms, it was noticed that both for siRNA duplexes arising from dsRNA cleavage and for miRNA duplexes processed from stem-loop precursors, the strand chosen as the guide in the active silencing complex was consistently the one whose 5' end was least stably paired (Khvorova et al., 2003; Schwarz et al., 2003). It was then demonstrated that, for *Drosophila* siRNAs, this thermodynamic selection process is directly mediated by R2D2 (Tomari et al., 2004b). Furthermore, R2D2 acts as a gatekeeper by

excluding from being loaded into Argonaute any siRNA whose passenger strand does not carry a 5'-monophosphorylated end, thus ensuring that only *bona fide* products of the RNase III-type Dicer activity are admitted into the silencing complex (Tomari et al., 2004b).

R2D2 was so named because of its two tandem dsRNA-binding domains (R2) and its association with Dicer-2 (D2) (Liu et al., 2003). Although it was discovered through biochemical purification, R2D2 is homologous to a genetically identified *C. elegans* protein called RDE-4, which also contains tandem dsRNA-binding domains and also interacts with Dicer (Grishok et al., 2000; Tabara et al., 2002). This suggested a general role for dsRNA-binding proteins in pairing with Dicer and accompanying Dicer-generated small RNAs along their journey into the silencing complex. Indeed, siRNAs in mammalian cells are loaded into Argonaute2 through the action of the dsRNA-binding protein TRBP (Gregory et al., 2005; Maniataki and Mourelatos, 2005). However, although the second step of miRNA biogenesis in *Drosophila* also involves a partnership between Dicer-1 and a dsRNA-binding protein called Loquacious (Förstemann et al., 2005; Jiang et al., 2005; Saito et al., 2005), Loquacious is dispensable for the assembly of miRNA-containing silencing complexes (Liu et al., 2007). This result suggests that no single type of apparatus is universally responsible for loading Argonautes with small RNAs.

Loading of a particular small RNA into a given Argonaute protein is dictated in part by structural and thermodynamic properties, at least in *Drosophila* (Czech et al., 2009; Tomari et al., 2007). In addition to designating as "guide" the strand with the lowest pairing stability at the 5' end and ensuring the presence of a 5' monophosphate on the passenger strand, R2D2 and Dicer-2 also orchestrate a sorting process that destines perfectly complementary siRNA duplexes for Ago2 and excludes from Ago2 typical miRNA duplexes that bear mismatches. The latter are instead the preferred substrates for loading into Ago1 (Tomari et al., 2007). However, complicating this picture, the miRNA strands complementary to those chosen as guides for Ago1 are often found loaded into Ago2 (Czech et al., 2009).

Another parameter that exerts a determining influence on small-RNA loading is the 5'-nucleotide preference of individual Argonaute homologs. This was first demonstrated in plants, where the Argonautes AGO2 and AGO4 load almost exclusively small RNAs beginning with a 5' adenosine while AGO1 and AGO5 discriminate in favor of 5' uridines and cytidines, respectively (Mi et al., 2008; Montgomery et al., 2008). These results confirmed existing suspicions that other Argonautes enforce a 5'-nucleotide-based selection, notably on piRNAs and 21U-RNAs, which consistently bear a 5' uridine (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Ruby et al., 2006). Parallel findings emerged from a genome-wide study of *Drosophila* miRNA duplexes, which showed a 5'-nucleotide basis for strand partitioning into Ago1 and Ago2, with uridine and cytidine respectively prevailing (Ghildiyal et al., 2010). Importantly, a crystal structure of the human Argonaute2 MID domain revealed specific contacts within the 5'-end-binding pocket that account for the protein's preference for 5' adenosines and uridines. This suggests a simple strategy for discriminating among different 5'-terminal nucleotides that other Argonaute family members might also employ (Frank et al., 2010).

One of the most important aspects of the Argonaute loading process is its requirement for energy. It has been proposed, based on structural considerations, that Argonautes must be actively pried open in order to accommodate siRNA or miRNA duplexes (Kawamata and Tomari, 2010). In agreement with this idea, the loading of a miRNA duplex into Ago1 in a *Drosophila* lysate requires ATP hydrolysis, suggesting that energy must be expended to achieve a permissive Argonaute conformation (Kawamata et al., 2009). Further studies identified the conserved chaperone Hsp90 as the ATP-consuming machinery responsible both for loading miRNAs into Ago1 and for loading siRNAs into Ago2 (Iwasaki et al., 2010; Miyoshi et al., 2010). The precise contribution of Hsp90 was recently investigated by a single-molecule approach, which revealed that, in the absence of the chaperone, Ago2 interacts with Dicer-2-R2D2-bound siRNAs only transiently (Iwasaki et al., 2015). Hsp90 enables loading by prolonging the dwell time of this Ago2-siRNA interaction in a manner that depends on the guide-strand 5'

monophosphate (Iwasaki et al., 2015). Interestingly, analysis of *Arabidopsis* AGO1 suggests a distinct Hsp90-dependent mechanism, wherein the loading event requires Hsp90 in its ATP-bound form, but not ATP hydrolysis (Iki et al., 2010).

Studies of the human Argonaute proteins, on the other hand, have consistently failed to detect a requirement for ATP in small-RNA loading (Gregory et al., 2005; MacRae et al., 2008; Maniataki and Mourelatos, 2005; Wang et al., 2009a). Nevertheless, one group has contended that the apparent dispensability of ATP in loading recombinant or immunopurified human Argonautes is the consequence of a bypass pathway in which the RNAs being loaded are actually single-stranded (Yoda et al., 2010). The authors found that, in a HeLa cell lysate, the loading of duplex small RNAs into Argonaute was in fact largely ATP-dependent (Yoda et al., 2010). Reconciling these results is not an obvious task, but one attractive possibility is that human Argonautes are inherently capable of loading small RNA duplexes, and that the process somehow becomes less efficient and more amenable to enhancement by ATP hydrolysis in the context of cells or cell extracts.

In addition to Hsp90 itself, Hsp90 co-chaperones have also been implicated in the proper assembly of Argonaute-small-RNA complexes. This includes the co-chaperones FKBP4 and FKBP5 for miRNAs and siRNAs in mammalian cells (Martinez et al., 2013; Pare et al., 2013), as well as FKBP6 for piRNAs in both mammalian and insect cells (Xiol et al., 2012). Intriguingly, in the ciliated protozoan *Tetrahymena thermophila*, the co-chaperone Coi12p mediates loading of small RNAs onto the Argonaute Twi1p by distinct mechanisms: one that depends on its interaction with Hsp90 and the hydrolysis of ATP, and another that is independent of both (Woehrer et al., 2015). In both cases, Coi12p acts by overcoming inhibition of loading by the Twi1p-interacting protein Giw1p (Woehrer et al., 2015).

As an alternative to the chaperone machinery, the energy of ATP hydrolysis can also be harnessed for Argonaute loading by RNA helicases. One example is RNA helicase A, which interacts with Argonaute2, TRBP and Dicer, and whose depletion in human cells reduces the

association between Argonaute2 and siRNAs (Robb and Rana, 2007). Another potential example is MOV10, a 5' to 3' helicase (Gregersen et al., 2014) shown to associate with Argonaute proteins (Meister et al., 2005). In the testes of mice carrying disruptions in the *Mov10l1* gene, the Piwi clade Argonautes responsible for ping-pong amplification are devoid of piRNAs, suggesting that Mov10l1 functions in loading (Zheng et al., 2010). An analogous role was uncovered for Armitage, the largely germline-specific *Drosophila* ortholog of MOV10 (Tomari et al., 2004a).

Loading an Argonaute with a small RNA guide programs it to recognize target RNAs for repression. But it has other consequences as well. First, both human Argonaute2 and *Drosophila* Ago1 proteins are considerably stabilized by miRNA binding (Elkayam et al., 2012; Martinez and Gregory, 2013; Smibert et al., 2013), indicating that Argonaute levels are limited by miRNA availability in cells and therefore that Argonautes are unlikely to play small-RNA-independent roles. Interestingly, the turnover of unloaded Argonautes seems to be regulated differently in different organisms, with *Drosophila* and mammals using the proteasomal and lysosomal degradation pathways, respectively (Martinez and Gregory, 2013; Smibert et al., 2013). Another consequence of small-RNA loading, in certain systems, is the transport of Argonaute across the nuclear envelope to mediate chromatin-level silencing. Both the *C. elegans* NRDE-3 and the *Arabidopsis* AGO4 function in the nucleus to repress the transcription of target loci, yet both are loaded with siRNAs in the cytoplasm, and it is the loading event that triggers their nuclear import (Guang et al., 2008; Ye et al., 2012). As a variant on this scheme, the *Tetrahymena* Twi1p is loaded with a small RNA duplex in the cytoplasm, and slicing and removal of the passenger strand are necessary before Twi1p can be imported into the developing somatic macronucleus where it marks chromosomal regions for elimination (Noto et al., 2010). The Twi1p-binding protein Giw1p acts as a sensor of small RNA strandedness to regulate this translocation (Noto et al., 2010).

Slicing and loading are central aspects of Argonaute function. Nevertheless, among nature's numerous small RNA pathways, only rarely does gene silencing entail a simple succession of Argonaute loading and target slicing. Instead, the Argonaute-small-RNA complex often serves as a module to identify a specific RNA or genomic locus and mark it as a target for other repressive machineries. In a diverse subset of pathways that spans the animal, plant and fungal kingdoms and beyond, these downstream components are recruited by GW proteins, so named because they contain a conserved Argonaute-binding motif composed of glycine-tryptophan repeats. In all systems comprising GW proteins, their partnership with Argonaute is of fundamental importance for gene silencing.

C. GW proteins

i. GW182 and the mechanism of miRNA-mediated repression in metazoans

The role of GW proteins in Argonaute-mediated events was first recognized in the context of the animal miRNA pathway (reviewed in Braun et al., 2013; Pfaff and Meister, 2013). This began with the observation that the human Argonaute2 protein localizes to previously characterized cytoplasmic foci (Liu et al., 2005b; Sen and Blau, 2005). These structures are termed processing bodies (or P bodies), because they are rich in mRNA decapping enzymes and the exonuclease Xrn1, and execute the turnover of deadenylated mRNAs (Sheth and Parker, 2003). In metazoans, P bodies additionally contain the GW-repeat protein GW182, and have therefore also been called GW bodies (Eystathioy et al., 2003). The finding that Argonaute resides in P bodies suggested that its interactions with P-body factors help coordinate its regulation of miRNA targets. Indeed, mRNAs engineered to contain miRNA binding sites accumulate in P bodies when expressed ectopically in cultured human cells, whereas similar mRNAs lacking miRNA binding sites do not, indicating that recognition by an Argonaute-miRNA complex marks an mRNA for P-body localization (Liu et al., 2005b). Furthermore, components of the P bodies, including both the mRNA decapping machinery and GW182, associate with

Argonaute and are required for miRNA-mediated repression in human and *Drosophila* cells (Liu et al., 2005a; Meister et al., 2005; Rehwinkel et al., 2005).

These observations would appear to suggest that P-body localization is critical for miRNA-dependent gene silencing, but in fact it is dispensable; instead, what is essential is the interaction between Argonaute and GW182. Thus, in cells expressing a GW182 mutant that cannot localize to P bodies but retains the ability to bind Argonaute, Argonaute localization to P bodies is also lost but silencing by miRNAs is unaffected (Eulalio et al., 2009). The Argonaute-GW182 association is therefore at the heart of the miRNA-mediated silencing mechanism in animal cells. There is even evidence that the function of miRNAs might consist exclusively of recruiting GW182 to their targets, as tethering *Drosophila* GW182 or the human GW182 ortholog TNRC6C directly to an mRNA leads to gene repression independently of Argonaute (Behm-Ansmant et al., 2006; Chen et al., 2009).

The Argonaute-binding region of GW182 maps to the N-terminal GW repeats themselves, which recognize the Argonaute PIWI domain (Behm-Ansmant et al., 2006). Overexpressing the Ago1-binding domain of GW182 in *trans* in *Drosophila* cells suppresses miRNA-dependent silencing, suggesting that it competes with full-length GW182 for Ago1, and that the C-terminal region of GW182 mediates an essential silencing function (Eulalio et al., 2008). This is confirmed by domain deletion experiments in *Drosophila* (Eulalio et al., 2009), and by direct tethering experiments demonstrating that the C-terminal domains of the human GW182 paralogs TNRC6A, TNRC6B and TNRC6C can repress the expression of bound mRNAs independently of Argonaute (Lazzaretti et al., 2009).

How does the C-terminal portion of GW182 bring about gene silencing? As discussed earlier (and reviewed in Huntzinger and Izaurralde, 2011; Jonas and Izaurralde, 2015), mRNA decay serves as the major mechanism of miRNA-mediated repression. One of the principal modes of RNA turnover in the cytoplasm involves successive deadenylation and decapping of mRNAs (Yamashita et al., 2005). It was recognized early that GW182 in fact promotes the

deadenylation of miRNA targets via the CCR4-NOT complex (Behm-Ansmant et al., 2006), but a key contribution to GW182-dependent deadenylation also comes from the PAN2-PAN3 complex (Chen et al., 2009). The contacts between GW182 and each of these deadenylation machineries are direct (Braun et al., 2011; Chekulaeva et al., 2011; Fabian et al., 2011), and recent structural studies have revealed that CCR4-NOT is recruited to the C-terminal domain of GW182 via specific binding pockets in the CNOT9 subunit (Chen et al., 2014; Mathys et al., 2014).

Another critical interaction in miRNA-mediated silencing occurs between GW182 and poly(A)-binding protein (PABP) (Fabian et al., 2009; Huntzinger et al., 2010; Jinek et al., 2010; Zekri et al., 2009). The GW182-PABP association is required for deadenylation of miRNA targets by the CCR4-NOT catalytic subunit CAF1 (Fabian et al., 2009) and, consistent with this, PABP also interacts with several subunits of CCR4-NOT (Zekri et al., 2009). Interestingly, PABP overexpression suppresses miRNA-mediated silencing, suggesting a model in which GW182 must displace PABP from the mRNA poly(A) tail in order to initiate deadenylation (Zekri et al., 2009). In strong support of this idea, tethering GW182 to an artificial transcript that cannot be deadenylated leads nevertheless to PABP dissociation (Zekri et al., 2013). Another consequence of PABP eviction is the loss of mRNA circularization through the interaction of PABP with eIF4G, eIF4E and the 5' cap, thereby illustrating a possible mechanism for GW182-mediated translational repression of miRNA targets (Zekri et al., 2013). Thus, PABP lies at the intersection of distinct GW182-dependent effects on protein synthesis and mRNA stability (Huntzinger et al., 2013); nevertheless, it appears to be dispensable in cell-free reconstitutions of miRNA-dependent silencing (Fukaya and Tomari, 2011).

ii. An evolutionarily conserved Argonaute-interacting motif

Although GW182 proteins and their decisive contribution to miRNA function represent the most thoroughly investigated example, many other Argonaute pathways also rely on GW-repeat-containing factors for proper silencing (Azevedo et al., 2011; El-Shami et al., 2007; Till et

al., 2007). These include fungi, as will be addressed in detail in the next section, as well as *Tetrahymena*, where the Twi1p Argonaute associates with two GW proteins that play essential, albeit redundant, roles in small-RNA-specified DNA elimination (Bednenko et al., 2009). And an especially striking array of GW proteins has been uncovered in plants. As mentioned earlier, RNA-directed DNA methylation in plants depends on siRNA-guided recognition of transcripts synthesized by the plant-specific RNA polymerase Pol V (Wierzbicki et al., 2009). But in addition to mediating the sequence-specific recruitment of AGO4 to chromatin via these nascent transcripts, Pol V also binds to AGO4 directly through a GW domain, and this interaction is essential for RdDM (El-Shami et al., 2007). Remarkably, the GW repeats from human GW182 can be substituted for the native *Arabidopsis* repeats to generate a chimeric Pol V protein that retains partial RdDM function (El-Shami et al., 2007). A second GW protein, KTF1, further reinforces the association of AGO4 with PolV-dependent transcripts by binding not only to AGO4 through its GW repeats but also to the nascent RNAs through an adjacent domain (He et al., 2009). This dense network of interactions underscores the self-reinforcing character of the RdDM process.

Other small RNA pathways in plants also feature GW proteins. An siRNA-dependent DNA methylation phenomenon distinct from canonical RdDM was recently reported; instead of AGO4 loaded with 24-nt siRNAs produced by DCL3, it relies on AGO2 and shorter siRNAs made by other Dicers (Pontier et al., 2012). Although this new pathway is not yet well understood, its target loci appear to be designated by the GW protein NERD, which binds to AGO2 as well as histone H3 tails lacking the transcription-associated methylation of lysine 4 (Pontier et al., 2012). Perhaps the most intriguing examples of GW proteins in plant biology are those encoded by viruses. At least two distinct viral pathogens have been reported to use GW-repeat-containing proteins as virulence factors to bind to and inactivate plant Argonautes (Azevedo et al., 2010; Giner et al., 2010). The emergence of this viral strategy to evade the

small-RNA-based defensive mechanisms of the host species speaks to the universal suitability of GW repeats as specific Argonaute-interacting domains.

The precise manner in which GW proteins bind to Argonautes has been elucidated through biochemical as well as structural approaches. As cited above, early studies of GW182 narrowed the minimal GW-interacting region within Argonaute to the PIWI domain (Behm-Ansmant et al., 2006). A systematic test of single-amino-acid-substitution variants of human Argonaute2 for binding to the GW182 ortholog TNRC6B confirmed the importance of several key positions in the PIWI domain, but also pointed emphatically toward a role for the residues in the MID domain that also engage the 5'-monophosphorylated terminus of small RNAs (Till et al., 2007). This observation suggested that the loading of small RNAs into Argonaute proteins might modulate their binding to GW proteins. In an apparent challenge to this idea, several studies have identified mutants of Argonaute whose small-RNA-loading activity is compromised and which are nevertheless able to associate with GW182 (Eulalio et al., 2009; Liu et al. 2005a; Miyoshi et al., 2009). However, these experiments involved overexpression of the Argonaute variants, which may justify some caution in the interpretation of the results. Other studies of both animal and plant Argonautes have found that GW proteins associate exclusively with the fraction that is loaded with small RNAs (Baillat and Shiekhattar, 2009; Giner et al., 2010). Nevertheless, a clear test of whether small-RNA loading impinges on Argonaute recognition by GW repeats has been lacking.

The crystal structure of full-length human Argonaute2 in the presence of free tryptophan has provided a more detailed view how GW proteins might dock onto the surface of Argonaute proteins. Consistent with earlier findings on GW protein interactions with Argonaute (Behm-Ansmant et al., 2006), both tryptophan molecules present in the structure are bound to the PIWI domain (Schirle and MacRae, 2012). Importantly, the tryptophan-binding pockets are lined with residues whose importance for association with TNRC6B was already known from biochemical assays (Schirle and MacRae, 2012; Till et al., 2007). Synthesizing these different observations,

a simple speculative model for the protein-protein interaction is that GW proteins bind directly to the PIWI domain of Argonaute, but that their recognition of the interaction surface requires an allosteric conformational change that is triggered by small-RNA loading. This proposal can account for the loss of binding upon mutation of the MID domain residues that anchor the 5' end of the small RNA, while not attributing a direct role to these residues in interacting with GW proteins, as this is not currently corroborated by other evidence.

The background I have presented thus far sets the stage for the final part of this chapter, in which I will describe what is known about the RNAi pathway of the fission yeast *Schizosacchramoyces pombe*. Not unlike RdDM in plants and the piRNA system in metazoans, which defend these organisms against the threat of parasitic nucleic acids, RNAi in *S. pombe* protects the genome by controlling its expression and preserving its stability, specifically by silencing repetitive DNA elements. As in other RNAi-related phenomena, Argonaute occupies center stage, and the regulation of its slicer activity, small-RNA loading and association with GW proteins prove to be critical aspects of its function.

IV. RNAi-directed heterochromatin assembly in Schizosaccharomyces pombe

A. *S. pombe* as a model organism for chromatin biology

S. pombe is perhaps most widely known in cell biology for its use in landmark studies of the mechanisms that control cell cycle progression and, in particular, the onset of DNA replication and mitosis (Mitchison, 1957, 1990; Nurse, 1997). The networks of proteins that regulate the timing of these events were first elucidated in *S. pombe*, and the logic of these signaling circuits, including the central role of kinases, is conserved throughout eukaryotic life (Nurse, 1997).

S. pombe has also long served as a key model organism for the study of chromatin structure and its influence on gene expression (Allshire and Ekwall, 2015). In particular, it has provided fundamental insights into the assembly of repressive chromosomal domains known as

heterochromatin. Although the origin of the term “heterochromatin” is the observation of darkly staining material in micrographs of interphase nuclei (Heitz, 1928), it is now commonly defined by its molecular features, including a signature of post-translational modifications to the amino-terminal tails of core histones, and silencing of the underlying DNA (reviewed in Grewal and Jia, 2007). Traditionally, heterochromatic silencing has been considered to occur through the restriction of transcriptional activity, but there is evidence that post-transcriptional mechanisms also make essential contributions to gene repression in many cases (Vasiljeva et al., 2008; reviewed in Grewal and Jia, 2007).

In *S. pombe*, constitutive heterochromatin forms at four genomic regions: the pericentromeric, subtelomeric and rDNA repeats, and the silent mating-type cassette (reviewed in Allshire and Ekwall, 2015). Genes with roles in heterochromatin function were first identified in studies of the mating-type locus. Defects in mating-type heterochromatin lead to inappropriate sporulation of haploid cells, which can be readily detected via increased staining by iodine vapors. A second reliable sign of disrupted heterochromatin is the loss of uracil auxotrophy in strains in which the *ura4*$^+$ gene is inserted within the silent mating-type region. These assays made it possible to assign heterochromatin-related functions to a handful of genes that were unlinked to the mating-type locus itself (Ekwall and Ruusala, 1994; Lorentz et al., 1992; Thon et al., 1994; Thon and Klar, 1992). Their mechanism of action, however, remained enigmatic.

Nevertheless, the discovery that these factors are also required for reporter gene silencing at various sites throughout the pericentromeric DNA repeats (Allshire et al., 1995) suggested a common, genome-wide molecular basis for heterochromatin assembly. It was also realized that pericentromeric heterochromatin was coupled to centromere function, as mutations that derepressed the pericentromeric repeats consistently resulted in chromosome segregation defects (Allshire et al., 1995; Ekwall et al., 1999). This observation ascribed a critical biological significance to heterochromatin in preserving the stability of the genome which, given the structural similarity of *S. pombe* centromeres to those of higher eukaryotes (Takahashi et al.,

1992), likely represents a universal property. This connection was later shown to rest on the heterochromatin-dependent recruitment of cohesin to the centromeres, which ensures centromeric cohesion of sister chromatids and proper chromosome segregation (Bernard et al., 2001; Nonaka et al., 2002).

A fundamental breakthrough in understanding the molecular nature of heterochromatin was the discovery that Clr4, one of the factors required for mating-type and pericentromeric silencing, is a catalytic methyltransferase whose activity is highly specific for the lysine residue at position 9 of the histone H3 tail (Bannister at al., 2001; Nakyama et al., 2001; Rea et al., 2000). At the same time, it was found that Swi6—another factor that is essential for silencing and which, like its orthologs of the HP1 family in metazoans, physically localizes to the heterochromatic portions of chromosomes (Eissenberg and Elgin, 2000)—binds to histone H3 tails *in vitro* with a specificity that depends critically on lysine 9 methylation (Bannister et al., 2001). Extending an earlier observation that Swi6 localization to heterochromatin requires Clr4 (Ekwall et al., 1996), Swi6 localization was further shown to depend specifically on the Clr4 methyltransferase enzymatic activity (Bannister at al., 2001; Nakyama et al., 2001). Together, these results defined an enduring framework for understanding the basic mechanism heterochromatin assembly and function in the majority of eukaryotes: histone H3 lysine 9 (H3K9) methyltransferases modify nucleosomes in the genomic regions to be targeted for heterochromatin, and HP1 proteins such as Swi6 then bind to these regions and mediate silencing of the underlying genetic information.

Swi6 and other HP1 proteins recognize methylated H3K9 (H3K9me) through their chromodomain (Bannister et al., 2001; Jacobs and Khorasanizadeh, 2002; Jacobs et al., 2001; Lachner et al., 2001; Nielsen et al., 2002), a common feature of chromatin-associated proteins in many organisms (Cavalli and Paro, 1998). In *S. pombe*, Swi6 is one of four chromodomain-containing proteins; the others are Clr4 itself, Chp1 and Chp2 (Doe et al., 1998; Thon and Verhein-Hansen, 2000). Analysis of the domain architecture of Chp2 indicates that, like Swi6, it

is an HP1 protein, while genetic experiments show that it is required for efficient silencing of all of the constitutive heterochromatin loci (Thon and Verhein-Hansen, 2000). Interestingly, Chp1 is completely dispensable for repression of the silent mating-type locus, yet *chp1*Δ mutant cells exhibit loss of silencing of pericentromeric reporter genes and, correspondingly, chromosome segregation defects (Doe et al., 1998; Thon and Verhein-Hansen, 2000). A number of other mutants with a similar, region-specific phenotype had also been isolated (Ekwall et al., 1999), suggesting the existence of a distinct heterochromatin assembly pathway defined by its singular importance at the pericentromeric repeats.

B. RNAi protein complexes maintain pericentromeric heterochromatin

In a study whose results would prove transformative, researchers generated strains bearing deletions of the single *S. pombe* orthologs of each of the three key RNAi components that had recently been characterized in other species: Dicer (*dcr1*$^+$), Argonaute (*ago1*$^+$) and RdRP (*rdp1*$^+$). Remarkably, all three deletions led to a pronounced derepression of the pericentromeric repeats, with no effect on the silent mating-type locus (Volpe et al., 2002). Importantly, the authors also discovered that pericentromeric H3K9me was disrupted in each of the three mutants, thereby demonstrating a role for the RNAi machinery in specifying heterochromatin assembly at these genomic regions (Volpe et al., 2002). In the wake of these findings, the precise mechanism by which RNAi influences heterochromatin formation became the subject of intense investigation. What was immediately apparent was that role of RNAi was likely to involve direct targeting, as sequences homologous to the pericentromeric repeats could be found among endogenously expressed siRNAs isolated from *S. pombe* cells (Reinhart and Bartel, 2002).

i. RITS

The emergence of a clear model for RNAi-dependent transcriptional silencing began with the demonstration of a physical link between Ago1 and heterochromatin (Verdel et al., 2004). The critical insight that led to this result was that the pericentromere-specific silencing

defects of the *dcr1*Δ, *ago1*Δ and *rdp1*Δ mutants were identical to those which had been observed in cells lacking the chromodomain protein Chp1 (Thon and Verhein-Hansen, 2000). Importantly, and reminiscent of Swi6, Chp1 had been found to associate with the pericentromeric repeats in a manner that required its chromodomain and the Clr4 H3K9 methyltransferase (Partridge et al., 2000, 2002). It therefore stood to reason that Chp1 might represent a connection between the heterochromatic loci to which it localized and whose silencing it controlled, and the RNAi proteins whose mutant phenotypes it mirrored.

Indeed, an affinity purification of Chp1 from *S. pombe* cell extracts was shown by mass spectrometry analysis to contain Ago1 and a newly identified protein, Tas3. Confirming the specificity of these interactions, Chp1 and Ago1 were also recovered in an affinity purification of Tas3. This three-subunit complex was termed RITS, for RNA-induced transcriptional silencing (Verdel et al., 2004). Three additional findings established RITS as the master regulator of RNAi-dependent heterochromatin assembly. First, like Chp1 and Ago1, Tas3 was shown to be required for silencing and H3K9me at pericentromeric reporter genes but not at the mating-type locus (Verdel et al., 2004). Second, Chp1 and Tas3 were found to associate with siRNAs corresponding to the pericentromeric repeats in wild-type but not *dcr1*Δ cells, indicating that, like other Argonaute-containing silencing complexes, RITS is programmed with small RNA guides arising from defined biogenesis pathways (Verdel et al., 2004). Finally, through Chp1 and Tas3 chromatin immunoprecipitation (ChIP) experiments, RITS was shown to localize to pericentromeric regions in a manner requiring the *dcr1*$^{+}$, *ago1*$^{+}$ and *rdp1*$^{+}$ genes (Verdel et al., 2004). Thus, RNAi targets the RITS complex to the pericentromeric repeats to mediate heterochromatin assembly.

As GW repeats began to emerge as a common feature of Argonaute-associated factors, it was recognized that Tas3 also contains a GW-repeat domain in its N terminus that is essential for its interaction with Ago1 and for RNAi-dependent heterochromatin formation (Partridge et al., 2007; Till et al., 2007). Heterochromatic silencing in *S. pombe* therefore joins miRNA-mediated

repression in animals and RdDM in plants in relying on an Argonaute-GW-protein partnership to effectively regulate genome expression. This aspect of the RITS complex is particularly exciting because the genetic tractability of *S. pombe* makes it a powerful model for exploring the mechanisms that control Argonaute-GW-protein interactions. This will be further discussed in Chapter 3.

Interestingly, although it is not required for maintenance of heterochromatin at non-pericentromeric regions, RITS nevertheless also localizes to the other heterochromatic portions of the genome (Cam et al., 2005; Noma et al., 2004). This could be considered a simple consequence of the affinity of the Chp1 chromodomain for H3K9me (Noma et al., 2004), but it also raises the possibility that RNAi could contribute to heterochromatin formation at these loci under certain circumstances. Indeed, a set of proteins including the RNAi factors Dcr1, Rdp1 and Ago1 and the RITS subunits Chp1 and Tas3 acts redundantly with a pair of site-specific transcription factors, Atf1 and Pcr1, to maintain mating-type heterochromatin (Jia et al., 2004; Noma et al., 2004). All of these components are individually required, however, for *de novo* re-establishment of mating-type silencing after drug-induced erasure of heterochromatin (Jia et al., 2004; Noma et al., 2004). Similarly, RNAi and the RITS complex contribute to heterochromatin maintenance in certain subtelomeric regions when the telomere-specific heterochromatin-targeting factor Taz1 is absent (Kanoh et al., 2005), and they mediate *de novo* heterochromatin domain formation at retrotransposons and certain protein-coding loci in cells lacking the exosome RNA degradation system (Yamanaka et al., 2013). However, it should also be noted that Chp1 and Tas3 form a stable sub-complex whose localization to non-pericentromeric heterochromatin does not require Ago1, and it has been determined that this heterodimer carries out RITS-independent functions (Petrie et al., 2005; Schalch et al., 2011).

RITS spreads along the chromosome, and this occurs in at least two ways. Most obviously, the affinity of the Chp1 chromodomain for H3K9me allows it to bind to heterochromatic nucleosomes modified by Clr4 (Noma et al., 2004; Partridge et al., 2000, 2002;

Schalch et al., 2009). A second mechanism of RITS spreading relies on the self-association of Tas3 via its C-terminal alpha-helical motif. Deletion of this motif abolishes Tas3 self-assocation *in vitro* and dramatically reduces silencing at pericentromeric sites distal to those directly targeted by endogenous siRNAs (Li et al., 2009).

ii. RDRC

A second RNAi complex with a role in heterochromatin assembly was identified by mass spectrometry of affinity-purified Rdp1. Called RDRC, it additionally consists of the RNA helicase Hrr1 and the noncanonical poly(A) polymerase Cid12 (Motamedi et al., 2004). Both Hrr1 and Cid12 are required for silencing of the pericentromeric repeats (Motamedi et al., 2004), but the *cid12*Δ silencing phenotype can be suppressed by overexpression of Rdp1 (Halic and Moazed, 2010). It was recently proposed that, in *C. elegans*, 3' uridylation of primary Argonaute cleavage products might act as a signal to stimulate secondary siRNA synthesis by RdRPs using these cleavage products as templates (Tsai et al., 2015). The rescue of *cid12*Δ by Rdp1 overexpression is compatible with an analogous, adenylation-based model also operating in *S. pombe*, but other possibilities are equally conceivable.

The catalytic activity of Rdp1 is required for pericentromeric siRNA accumulation and heterochromatic silencing, and the accepted model for RDRC function is that it initiates siRNA biogenesis by creating dsRNA substrates for the Dcr1 ribonuclease (Motamedi et al., 2004, Sugiyama et al., 2005). In principle, since the pericentromeric repeats are transcribed bi-directionally, base-pairing of complementary transcripts followed by Dcr1 cleavage could give rise to some siRNAs without the need for dsRNA production by RDRC. The idea of a pericentromeric transcript that could self-hybridize to form a long stem-loop substrate for Dcr1 has also been proposed (Djupedal et al., 2009). It turns out, however, that siRNA generation in *S. pombe* is acutely reliant on RDRC, as even siRNAs originating from highly transcribed artificial hairpin constructs require both Dcr1 and RDRC to accumulate efficiently (Iida et al., 2008). Endogenous RDRC-independent siRNAs, or primary siRNAs, are very rare, so much so

that their existence long eluded detection even using high-throughput approaches (Halic and Moazed, 2010), and was confirmed only recently using an instrument with very high sequencing depth (Yu et al., 2014). The abundance of primary siRNAs increases substantially in cells in which Dcr1 is overexpressed, and this partially restores pericentromeric silencing in the *rdp1*Δ background (Kawakami et al., 2012; Yu et al., 2014); however, Dcr1 overexpression also leads to the accumulation of many euchromatic siRNAs (Yu et al., 2014). Thus, *S. pombe* may have evolved to limit the cellular levels of Dcr1 in order to restrict siRNA production to heterochromatic regions (Yu et al., 2014), and RDRC can perhaps be understood as an adaptation that ensures robust generation of pericentromeric siRNAs in spite of low Dcr1 abundance.

The relationship between RDRC and Dcr1 goes beyond the mere complementarity of their enzymatic functions. RDRC and Dcr1 are physically associated in the *S. pombe* cell nucleus, and the association of Dcr1 exerts a stimulatory effect on the catalytic activity of Rdp1 (Colmenares et al., 2007). Physical links between RdRPs and Dicers have also been reported in *Tetrahymena* and *C. elegans*, suggesting that this organizational strategy may be inherently advantageous (Lee and Collins, 2007; Thivierge et al., 2012). The RDRC-Dcr1 association is mediated by a perinuclear protein, Dsh1, and the loss of pericentromeric silencing in *dsh1*Δ cells highlights the importance of this connection (Kawakami et al., 2012).

iii. The RNAi complexes localize to nascent transcripts and directly target H3K9me

How, precisely, do the RNAi complexes promote H3K9me and heterochromatin formation at the pericentromeric repeats? One basic question is whether the small-RNA-guided Argonaute recognizes the DNA repeats themselves, or RNAs that are transcribed from these loci. At least three lines of evidence argue that RNAi targets nascent transcripts in *S. pombe*. First, RITS and RDRC can both be crosslinked to long pericentromeric ncRNAs. Importantly, this requires Dcr1, suggesting that siRNAs guide RITS to the pericentomeric RNAs via base-pairing, and that RDRC associates with RITS to engage the transcripts, perhaps as templates

for dsRNA synthesis (Motamedi et al., 2004). Second, mutations in the Rpb2 and Rpb7 subunits of RNA polymerase II (Pol II) have been shown to disrupt pericentromeric heterochromatin (Djupedal et al., 2005; Kato et al., 2005). Third, artificial tethering of the RITS subunit Tas3 to a euchromatic transcript triggers ectopic formation of heterochromatin over the corresponding locus in a manner that requires Dcr1 and the other RITS and RDRC subunits (Bühler et al., 2006). That the function of RITS in heterochromatin assembly can be recapitulated in this manner strongly supports a nascent transcript model in which siRNAs target RITS to chromatin-associated pericentromeric RNAs, which in turn serve as platforms for siRNA amplification by RDRC. This model finds parallels in other species, such as *C. elegans*, where the nuclear Argonaute NRDE-3 targets nascent RNAs to halt their elongation (Guang et al., 2010), or plants, where AGO4-dependent DNA methylation involves recognition of chromatin-associated Pol V transcripts (Wierzbicki et al., 2009).

The next logical question is how the localization of RITS and RDRC to pericentromeric transcripts results in Clr4-mediated H3K9me in pericentromeric chromatin. Clr4 belongs to a multi-protein cullin E3 ubiquitin ligase complex, called CLRC, all of whose members are also required for H3K9me genome-wide (Buscaino et al., 2012; Hong et al., 2005; Horn et al., 2005; Jia et al., 2005; Kuscu et al., 2014; Li et al., 2005). Very straightforwardly, RNAi targets CLRC to the pericentromeric repeats through direct, Dcr1-dependent interactions between the subunits of RITS and those of CLRC (Bayne et al., 2010; Gerace et al., 2010; Zhang et al., 2008). The CLRC component Rik1 serves as the critical interface with RITS, as artificially tethering it to a euchromatic transcript leads to RNAi-mediated post-transcriptional gene silencing independently of the other CLRC members and H3K9me (Gerace et al., 2010). This demonstrates that Rik1 alone supports a functional interaction with the RNAi machinery. At the native heterochromatic loci, however, RITS must recruit the full CLRC complex and bring about H3K9me in order to achieve silencing.

Conversely, artificially tethering Clr4 to a euchromatic gene locus generates local heterochromatic silencing and functional centromeres in a manner that completely bypasses RNAi (Kagansky et al., 2009). This result, together with the observation that RNAi is dispensable for natural heterochromatin at non-pericentromeric loci, suggests that locus-specific targeting of CLRC activity represents the exclusive role of the RNAi pathway in pericentromeric silencing. Consistent with this idea, RNAi becomes dispensable for maintenance of heterochromatin even at the pericentromeres in the context of specific genetic manipulations (Reddy et al., 2011; Tadeo et al., 2013). Therefore, in a wild-type setting, RNAi serves as no more than a sequence-specific targeting mechanism for CLRC, whose specificity is provided by the small RNA guides loaded into RITS (Moazed, 2011).

In addition to recruiting CLRC, RITS also directly interacts with RDRC on the nascent transcript platform, and the association between the two RNAi complexes requires both Clr4 and Dcr1 (Motamedi et al., 2004). The existence of these multiple physical contacts and their dependence on key constituents of the silencing pathway suggests that RNAi-mediated heterochromatin assembly relies on the cooperation of mutually reinforcing activities. Indeed, there are ample indications of a powerful positive feedback in *S. pombe* linking chromatin modification to the production of small RNAs.

C. Histone methylation and siRNA biogenesis are coupled processes

i. Elements of the positive feedback loop

The direct, siRNA-guided recruitment of the Clr4 H3K9 methyltransferase by the RITS complex has just been described. A critical aspect of this interaction that further reinforces the deposition of H3K9me is the high-affinity binding of Chp1 to H3K9me itself (Noma et al., 2004; Schalch et al., 2009). Indeed, in addition to its targeting via base-pairing between the siRNA and the nascent transcript, RITS localization is also stabilized by its binding to existing H3K9me. And by virtue of its ability to recruit CLRC, RITS can thus prompt the siRNA-dependent spreading of the H3K9me that it encounters. Interestingly, the maintenance of pericentromeric

heterochromatin tolerates mutations that substantially reduce the affinity of Chp1 for H3K9me, but *de novo* establishment of heterochromatin in the case of Clr4 deletion and re-introduction

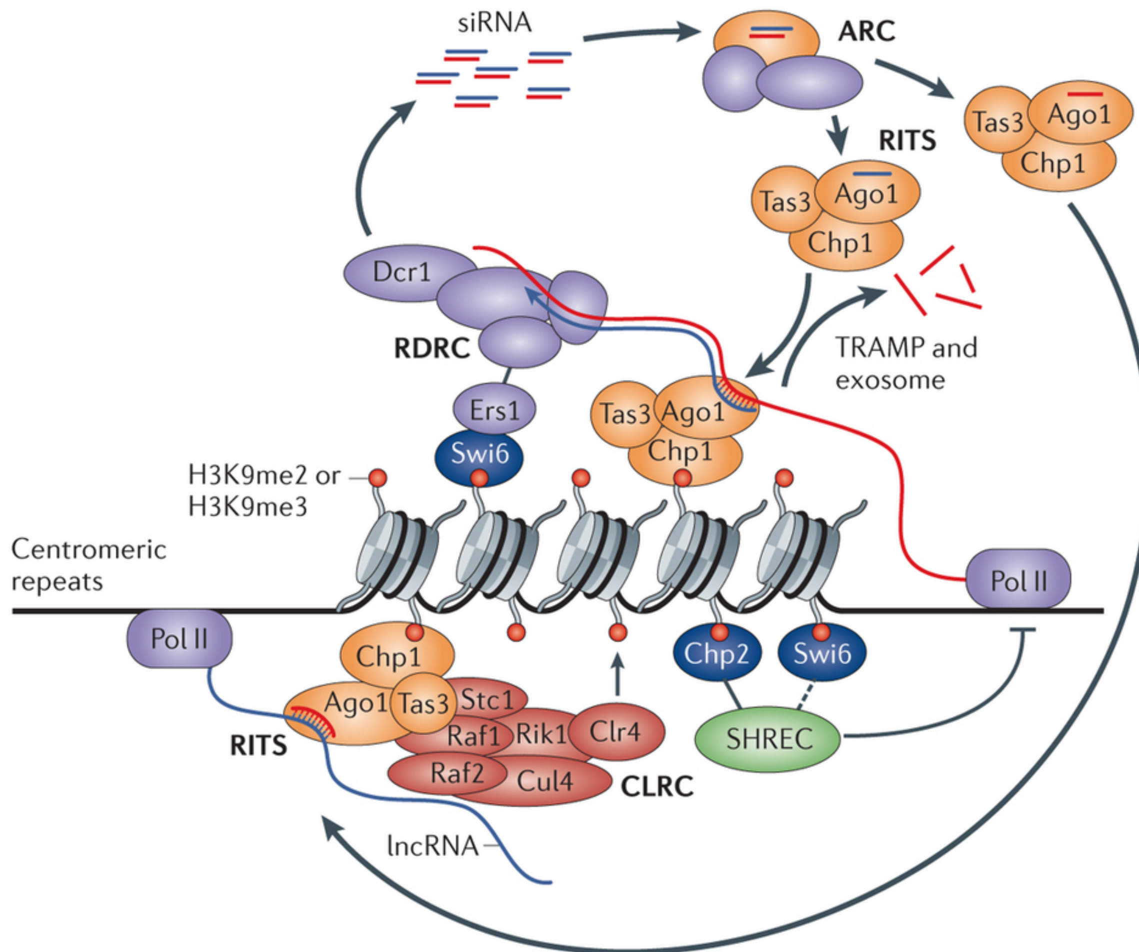

Figure 1.1. The "nascent transcript" model and a self-reinforcing epigenetic loop in *S. pombe*. In *Schizosaccharomyces pombe*, the RNA-induced transcriptional silencing (RITS) complex establishes a physical connection between small interfering RNAs (siRNAs) and heterochromatin by targeting a nascent transcript, and forms the basis of a self-sustaining feedback mechanism that couples siRNA production to chromatin modification. A siRNA-targeted centromeric long non-coding (lncRNA) bound to the RITS complex becomes a template for double-stranded RNA (dsRNA) synthesis by the RNA-directed RNA polymerase complex (RDRC, which is composed of Rdp1, Hrr1 and Cid12) and generation by Dicer 1 (Dcr1) of new siRNAs, leading to further targeting of the RITS complex after passage of Argonaute (Ago1) through the ARC (Argonaute siRNA chaperone) complex. The Chp1 subunit of the RITS complex anchors the complex onto nucleosomes with histone H3 lysine 9 (H3K9) methylation, and the RITS complex recruits the Clr4 methyltransferase complex (CLRC) via Rik1 to promote the further spread of H3K9 methylation. The heterochromatin protein 1 (HP1) homologue Swi6 binds to methylated H3K9 and promotes RDRC recruitment and siRNA biogenesis via the silencing factor Ers1. Together, the RITS complex and the nascent long ncRNA transcript provide a hub for the assembly of machineries that make siRNAs, modify histones and silence gene expression. (Reproduced from Holoch and Moazed, 2015a. Individual CLRC subunits, SHREC, TRAMP, H3K9me2 and H3K9me3 are discussed therein.)

strictly requires the wild-type Chp1 chromodomain, suggesting that this positive feedback element is especially critical in this situation (Schalch et al., 2009).

H3K9me in turn promotes siRNA generation, as pericentromeric siRNA levels are severely reduced in cells lacking Clr4 (Halic and Moazed, 2010; Motamedi et al., 2004). Thus, the key chromatin mark for heterochromatin assembly is not only the consequence of, but also a stimulus for, the production of siRNAs. One simple mechanism that accounts for this observation is the physical interaction between RITS and RDRC. Since RITS binds to H3K9me-containing nucleosomes at heterochromatic loci where it also encounters nascent pericentromeric RNAs, and further recruits RDRC to these RNAs, H3K9me effectively promotes the RDRC-dependent processing of heterochromatic transcripts into siRNAs (Motamedi et al., 2004). H3K9me also performs the very same task through a second mechanism. The silencing protein and HP1 homolog Swi6, which binds to H3K9me at the pericentromeric repeats and other heterochromatic regions, can be crosslinked *in vivo* to nascent pericentromeric transcripts and is required for the efficient association of RDRC with these transcripts, as well as the accumulation of corresponding siRNAs (Motamedi et al., 2008). Thus, H3K9me promotes RDRC-dependent siRNA biogenesis through two chromodomain proteins, Chp1 and Swi6. Interestingly, the interaction between Swi6 and RDRC is directly bridged by Ers1, a factor which, like the RNAi components, is essential for heterochromatic silencing specifically at the pericentromeric repeats (Hayashi et al., 2012; Rougemaille et al., 2008, 2012).

Together, these mechanisms form a remarkably elaborate positive feedback that couples siRNA biogenesis to the spreading and maintenance of H3K9me within the heterochromatic domains of the pericentromeres (Figure 1.1). The importance of this self-reinforcing loop is underscored by the observation that deletion of any one of its members largely compromises siRNA production and H3K9me. This mode of operation also raises two additional questions. First, during the initial establishment of heterochromatin, what is the event that is responsible for priming the positive feedback cycle? And, second, given the existence of

such a robust feed-forward mechanism, what prevents the inappropriate establishment of heterochromatic silencing at genomic loci that need to be expressed?

ii. Initiation of heterochromatin assembly by Dcr1-independent small RNAs

In wild-type cells, deletion and subsequent re-introduction of the Clr4 H3K9 methyltransferase leads to complete loss followed by complete recovery of H3K9me at heterochromatic loci, including the pericentromeric repeats (Sadaie et al., 2004). This demonstrates that the positive feedback loop that maintains RNAi-dependent heterochromatin can be initiated *de novo* as long as all of its components are present. At least two scenarios can be envisioned to explain how this might occur. One possibility is that Clr4 might begin methylating H3K9 in an inefficient, RNAi-independent manner, which could trigger RITS localization, followed by RDRC recruitment, thereby setting in motion the process of siRNA biogenesis and amplification. Alternatively, RITS programmed with small RNAs arising from random degradation of the transcriptome might identify complementary nascent transcripts in the absence of H3K9me, and then nucleate the establishment of heterochromatin by recruiting CLRC to the corresponding loci.

Although it remains a matter of debate (Shanker et al., 2010), the case for small RNAs as the initiators of the heterochromatic silencing loop is by far the more persuasive one (Halic and Moazed, 2010). One key observation is that when Clr4 is re-introduced into *clr4*Δ *ago1*Δ cells, pericentromeric H3K9me levels remain undetectable, whereas, in contrast, an isogenic *ago1*Δ strain constructed without ever removing Clr4 exhibits very low but consistently detectable H3K9me (Ragunathan et al., 2015). This result demonstrates that targeting of the pericentromeric repeats by Ago1 and small RNAs is absolutely necessary to carry out the earliest stages of heterochromatin establishment.

But the most direct evidence indicating that small RNAs prime the positive feedback loop is the discovery of Dcr1-independent primal RNAs (priRNAs) that bind to Ago1 and guide CLRC to the pericentromeric repeats (Halic and Moazed, 2010). Crucially, the level of pericentromeric

H3K9me in *dcr1*Δ cells, while very low, remains reproducibly higher than that observed in *ago1*Δ. This suggests a model in which, in the absence of the RDRC- and Dcr1-dependent siRNA biogenesis pathway, Ago1 can sample and load other cellular RNAs, and then promote *de novo* CLRC activity at sites where it encounters complementary nascent transcripts (Halic and Moazed, 2010). In wild-type cells, these initial events would trigger RDRC recruitment, siRNA amplification and robust H3K9me (Halic and Moazed, 2010).

What is unique about the pericentromeric repeats is that they give rise to overlapping transcripts from both strands; therefore, unlike priRNAs from the rest of the transcriptome, those originating from the repeats would very frequently show antisense complementarity to a nascent transcript and thus be capable of setting off the self-reinforcing heterochromatic silencing loop (Halic and Moazed, 2010). In this way, small RNAs emanating from bi-directionally transcribed loci such as the pericentromeres would be selectively enriched through RDRC-dependent amplification, much as the ping-pong pathway has been suggested to tailor cellular piRNA populations by increasing the representation of sequences complementary to those TEs that are actually expressed (Aravin et al., 2008).

The inherent susceptibility of bi-directionally transcribed regions to targeting by priRNAs may account for the preferential establishment and maintenance of RNAi-dependent heterochromatin at the pericentromeric repeats. But there is evidence that additional features of the pericentromeric transcripts also play a decisive role in directing the RNAi pathway toward these loci and away from ordinary transcription units.

iii. Restrictions on RNAi-directed heterochromatin formation

Generally speaking, Argonaute-small-RNA complexes are considered to function as *trans*-acting factors that can repress complementary RNAs wherever they are found in the cell, and the RITS complex can also work this way in the *S. pombe* nucleus. This is illustrated by experiments in which fragments of the pericentromeric region inserted elsewhere in the genome are automatically targeted for heterochromatin assembly in a Dcr1-dependent manner,

presumably by homologous siRNAs produced from the native heterochromatic loci (Marina et al., 2013). Nevertheless, by contrast, most protein-coding mRNAs are consistently refractory to siRNA-mediated heterochromatin formation. For example, a *ura4*$^{+}$ gene silenced by direct tethering of RITS to the nascent transcript produces abundant *ura4*$^{+}$ siRNAs, but these fail to silence a second *ura4*$^{+}$ locus in *trans*, and they do so only weakly even when their levels are boosted by deleting the siRNA-degrading ribonuclease Eri1 (Bühler et al., 2006; Iida et al., 2006; Kennedy et al., 2004). More perplexing still, hairpin constructs that generate very high levels of Dcr1-dependent siRNAs complementary to the *ura4*$^{+}$ open reading frame are not sufficient to silence the native *ura4*$^{+}$ locus. Instead, in order to become a target for siRNA-directed heterochromatic silencing, *ura4*$^{+}$ must be relocated to a more favorable genomic context (Iida et al., 2008; Simmer et al., 2010). These observations suggest that specific mechanisms have evolved to prevent most loci from undergoing RNAi-mediated heterochromatin formation.

Recent studies have revealed the nature of these mechanisms. In one set of experiments, deletion of the 3' UTR rendered the *ura4*$^{+}$ locus amenable to targeting for H3K9me deposition and silencing, either by hairpin-derived siRNAs or by primary siRNAs resulting from Dcr1 overexpression (Yu et al., 2014). This result suggests that the sequence elements responsible for promoting the transcription termination and 3'-end processing of an mRNA may also allow it to efficiently escape being targeted by the RNAi machinery. Indeed, precise mutations of individual polyadenylation and cleavage signals downstream of the *ura4*$^{+}$ open reading frame were sufficient to confer sensitivity to silencing by hairpin siRNAs (Yu et al., 2014). Importantly, the RNAs transcribed from the pericentromeric repeats that constitute the native targets of RNAi are also terminated and polyadenylated inefficiently. Together, these different observations support a model in which the RITS complex can only target nascent transcripts whose residence time on chromatin is unusually long by virtue of their failure to undergo canonical, efficient termination and release (Yu et al., 2014).

The role of transcription termination and release was confirmed emphatically by a recent genetic screen for mutations that potentiate siRNA-induced heterochromatic silencing in *trans*. This approach demonstrated that the conserved Paf1 complex, which associates with Pol II to promote transcription elongation and 3'-end processing, strongly suppresses siRNA-mediated establishment of heterochromatin (Kowalik et al., 2015). Strains expressing hairpin siRNA constructs homologous to *ade6*$^+$ or *ura4*$^+$ readily exhibit silencing of the corresponding gene when a mutation in any of the Paf1 subunits is also introduced. This effect is clearly attributable to inefficient 3'-end processing and release of the transcript, as inclusion of a self-cleaving ribozyme in the mRNA sequence in order to accelerate its release abolishes siRNA-mediated repression (Kowalik et al., 2015). Remarkably, once initiated in Paf1-mutant cells, gene silencing, H3K9me and siRNAs derived from the target locus all persist indefinitely, even after removal of the original hairpin siRNA trigger. This result potently illustrates the mutually sustaining feedback between siRNAs and H3K9me and shows that it is sufficient for the epigenetic maintenance of a silent state (Kowalik et al., 2015).

Collectively, the studies carried out over the last dozen years and reviewed here have provided a detailed understanding of RNAi-directed heterochromatic silencing in *S. pombe* and the mechanistic principles by which it is governed. Aspects that have emerged as particularly important include the gathering of multiple protein complexes on nascent transcript platforms that exhibit long chromatin residence times, and the self-reinforcing physical and functional cooperation of these different machineries. In the final part of this introductory chapter I concentrate on Ago1, its interacting partners and the regulation of its function, which also constitute the subject of the studies I present in the subsequent chapters of this dissertation.

D. Two Argonaute complexes are required for heterochromatin assembly in *S. pombe*

When the RITS complex was first discovered as the direct physical link between RNAi and heterochromatin, its subunits were identified in affinity purifications from *S. pombe* cell extracts using either Chp1 or Tas3 as the bait (Verdel et al., 2004). However, when subsequent

purifications were conducted using Ago1 as the bait instead, mass spectrometry analysis revealed additional interactors that had not been detected in earlier experiments. Besides Chp1 and Tas3, two novel proteins were also observed to associate with Ago1 and were named Arb1 and Arb2 (Buker et al., 2007). Consistent with the absence of Arb1 and Arb2 from earlier Chp1 and Tas3 purifications, the proteins recovered in Arb1 purifications included both Ago1 and Arb2 but neither Chp1 nor Tas3 (Buker et al., 2007). A co-immunoprecipitation assay also failed to uncover any interaction between Arb1 and Tas3. Together, these results indicated the existence of two biochemically separate Ago1-containing complexes in *S. pombe*: RITS, consisting of Chp1, Tas3 and Ago1, and a second three-subunit complex comprising Ago1, Arb1 and Arb2 (Buker et al., 2007).

Using reporter strains for heterochromatic silencing at the mating-type locus and the pericentromeric repeats, it was shown that, like the RITS subunits and the other RNAi pathway members, Arb1 and Arb2 are required for maintenance of heterochromatin specifically at the pericentromeres. It was therefore concluded that, consistent with their association with Ago1, Arb1 and Arb2 play essential roles in RNAi-mediated heterochromatin assembly (Buker et al., 2007).

A comparison of Tas3- and Arb1-associated pericentromeric small RNAs by non-denaturing Northern blot revealed that, unlike RITS, which contained mainly single-stranded siRNAs, the novel complex bound siRNAs that were exclusively double-stranded (Buker et al., 2007). Consistent with this finding, an *in vitro* assay of siRNA-guided slicer activity by recombinant Ago1 showed that addition of Arb1 inhibits endonucleolytic target cleavage (Buker et al., 2007). A model that assimilates these results is that, as in other systems (Leuschner et al., 2006; Matranga et al., 2005; Rand et al., 2005), perfectly complementary siRNA passenger strands are released from Ago1 only after they have been sliced. Confirming this idea, siRNAs associated with an Ago1 mutant carrying a mutation in the predicted slicer catalytic triad (Ago1-D580A) are also exclusively double-stranded (Buker et al., 2007). Thus, since Arb1 inhibits

slicing, it also inhibits passenger strand release, with the result that the Arb1-containing Ago1 complex contains only double-stranded small RNAs.

Another feature of the second Ago1 complex that distinguishes it from RITS is that although its subunits localize to the nucleus, neither of them exhibits a detectable association with the pericentromeric repeat loci whose silencing they mediate, or with any other chromatin region (Buker et al., 2007; Woolcock et al., 2012). This observation, together with the double-stranded nature of the associated siRNAs, led to the view that Ago1 molecules associate first with Arb1 and Arb2, and are subsequently transferred to the chromatin-localized RITS complex. Once assembled into RITS, conversion of the siRNA duplex to a single-stranded form would activate Ago1 for target recognition (Buker et al., 2007). Because the double-stranded small RNAs bound to the Ago1-Arb1-Arb2 complex represent the native products of the Dcr1 ribonuclease, and because the complex maintains them in this form, it was termed the Argonaute siRNA chaperone complex, or ARC (Figure 1.2) (Buker et al., 2007).

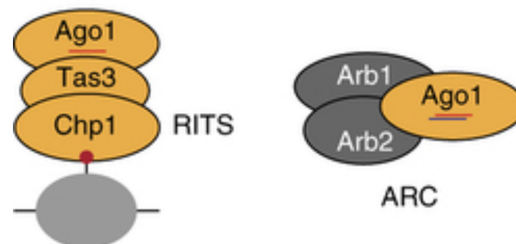

Figure 1.2. Schematic illustrating the subunit composition of the RITS and ARC complexes. RITS is represented carrying a single-stranded small RNA (red line) and is bound via Chp1 to a nucleosome (gray) methylated (red dot) on H3K9, whereas ARC is shown loaded with a duplex small RNA (red and blue lines). (Reproduced from Holoch and Moazed, 2015b.)

At first glance, the ARC complex appears to act as a negative regulator of Ago1 function by inhibiting its slicer activity. It is worth noting that slicing by Ago1 is critical for the spreading and maintenance of H3K9 methylation at the pericentromeric repeats (Buker et al., 2007; Irvine et al., 2006), probably because of its role in generating a mature, single-stranded guide siRNA. Thus, by hindering Ago1 slicing, ARC would presumably delay the formation of a programmed Ago1 complex capable of identifying target transcripts through base-pairing. It is not

immediately clear why proteins performing this type of activity would be required for RNAi-dependent silencing.

The domain structures of Arb1 and Arb2 provide no obvious clues concerning the nature of their contribution to Ago1-mediated heterochromatin assembly. Arb1 contains a C-terminal domain that is homologous to organellar maturases, a class of proteins that facilitates the self-splicing of group II introns in chloroplasts and mitochondria (Buker et al., 2007; Schmitz-Linneweber et al., 2015). Although splicing has been proposed to directly regulate the engagement of nascent pericentromeric transcripts by the RNAi machinery (Bayne et al., 2008; Chinen et al., 2010, Dumesic et al., 2013), recent findings suggest that, in *S. pombe*, the requirement for splicing factors for pericentromeric silencing more simply reflects the necessity of removing introns from mRNAs encoding RNAi factors (Kallgren et al., 2014). The involvement of maturase-domain-containing proteins in other small RNA pathways has not been reported, and the extent to which the maturase domain of Arb1 participates in the protein's overall silencing function is unknown. For its part, Arb2 contains a domain near its N terminus that is very broadly conserved, with homologs in organisms as distant as plants and vertebrates (Buker et al., 2007). This domain is notably found in many class II histone deacetylases, including the *S. cerevisiae* Hda1 and the *S. pombe* Clr3. However, its importance for pericentromeric silencing also remains unknown.

Importantly, neither Arb1 nor Arb2 possesses a clear dsRNA-binding domain. Thus, although it has been hypothesized that ARC may receive siRNAs from Dcr1, its subunits do not share the signature feature of the other proteins known to perform a similar function, such as the dsRNA-binding proteins R2D2 and TRBP (Buker et al., 2007). And furthermore, in contrast to the stable associations that form between R2D2/TRBP and Dicer, mass spectrometry analyses consistently fail to provide evidence that ARC subunits interact with Dcr1 (Buker et al., 2007; Colmenares et al., 2007). In light of these observations, the idea that ARC plays a direct role in siRNA loading has not been considered to represent the most likely possibility. Such a

model was even seemingly ruled out by the detection of considerable levels of Ago1-associated pericentromeric siRNAs in *arb1*Δ and *arb2*Δ cells (Halic and Moazed, 2010). The specific roles of Arb1 and Arb2 have therefore remained enigmatic.

Two major questions motivated the work that is presented in the rest of this dissertation. First, what are the molecular activities of Arb1 and Arb2 that make these proteins essential for RNAi-dependent heterochromatin assembly? Restriction of Ago1 slicer activity alone does not offer a logically satisfying explanation for their requirement in silencing, so it appears probable that a distinct function underlies their critical contribution to heterochromatin. Second, as was posed when ARC was first discovered, what are the mechanisms responsible for controlling the shuttling of Ago1 between the two complexes into which it assembles? These two important unknowns are addressed in part by the experiments described in chapters 2 and 3, respectively.

V. References

Alekseyenko, A.A., Larschan, E., Lai, W.R., Park, P.J., and Kuroda, M.I. (2006). High-resolution ChIP-chip analysis reveals that the Drosophila MSL complex selectively identifies active genes on the male X chromosome. Genes Dev. *20*, 848-857.

Alekseyenko, A.A., Peng, S., Larschan, E., Gorchakov, A.A., Lee, O.K., Kharchenko, P., McGrath, S.D., Wang, C.I., Mardis, E.R., Park, P.J., and Kuroda, M.I. (2008). A sequence motif within chromatin entry sites directs MSL establishment on the Drosophila X chromosome. Cell *134*, 599-609.

Allshire, R.C., and Ekwall, K. (2015). Epigenetic regulation of chromatin states in *Schizosaccharomyces pombe*. Cold Spring Harb. Perspect. Biol. *7*, a018770.

Allshire, R.C., Nimmo, E.R., Ekwall, K., Javerzat, J.P., and Cranston, G. (1995). Mutations derepressing silent centromeric domains in fission yeast disrupt chromosome segregation. Genes Dev. *9*, 218-233.

Ameres, S.L., and Zamore, P.D. (2013). Diversifying microRNA sequence and function. Nat. Rev. Mol. Cell Biol. *14*, 475-488.

Amrein, H., and Axel, R. (1997). Genes expressed in neurons of adult male *Drosophila*. Cell *88*, 459-469.

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., 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.

Ashe, A., Sapetschnig, A., Weick, E.M., Mitchell, J., Bagijn, M.P., Cording, A.C., Doebley, A.L., Goldstein, L.D., Lehrbach, N.J., Le Pen, J., et al. (2012). piRNAs can trigger a multigenerational epigenetic memory in the germline of C. elegans. Cell *150*, 88-99.

Azevedo, J., Cooke, R., and Lagrange, T. (2011). Taking RISCs with Ago hookers. Curr. Opin. Plant Biol. *14*, 594-600.

Azevedo, J., Garcia, D., Pontier, D., Ohnesorge, S., Yu, A., Garcia, S., Braun, L., Bergdoll, M., Hakimi, M.A., Lagrange, T., et al. (2010). Argonaute quenching and global changes in Dicer homeostasis caused by a pathogen-encoded GW repeat protein. Genes Dev. *24*, 904-915.

Bagijn, M.P., Goldstein, L.D., Sapetschnig, A., Weick, E.M., Bouasker, S., Lehrbach, N.J., Simard, M.J., and Miska, E.A. (2012). Function, targets, and evolution of Caenorhabditis elegans piRNAs. Science *337*, 574-578.

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

Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C., and Kouzarides, T. (2001). Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature *410*, 120-124.

Batista, P.J., Ruby, J.G., Claycomb, J.M., Chiang, R., Fahlgren, N., Kasschau, K.D., Chaves, D.A., Gu, W., Vasale, J.J., Duan, S., et al. (2008). PRG-1 and 21U-RNAs interact to form the piRNA complex required for fertility in C. elegans. Mol. Cell *31*, 67-78.

Baulcombe, D.C. (1996). RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants. Plant Mol. Biol. *32*, 79-88.

Bayne, E.H., Portoso, M., Kagansky, A., Kos-Braun, I.C., Urano, T., Ekwall, K., Alves, F., Rappsilber, J., and Allshire, R.C. (2008). Splicing factors facilitate RNAi-directed silencing in fission yeast. Science *322*, 602-606.

Bayne, E.H., White, S.A., Kagansky, A., Bijos, D.A., Sanchez-Pulido, L., Hoe, K.L., Kim, D.U., Park, H.O., Ponting, C.P., Rappsilber, J., and Allshire, R.C. (2010). Stc1: a critical link between RNAi and chromatin modification required for heterochromatin integrity. Cell *140*, 666-677.

Bazzini, A.A., Lee, M.T., and Giraldez, A.J. (2012). Ribosome profiling shows that miR-430 reduces translation before causing mRNA decay in zebrafish. Science *336*, 233-237.

Bednenko, J., Noto, T., DeSouza, L.V., Siu, K.W., Pearlman, R.E., Mochizuki, K., Gorovsky, M.A., and Gorovsky, M.A. (2009). Two GW repeat proteins interact with Tetrahymena thermophila argonaute and promote genome rearrangement. Mol. Cell. Biol. *29*, 5020-5030.

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

Belote, J.M., and Lucchesi, J.C. (1980). Control of X chromosome transcription by the *maleless* gene in *Drosophila*. Nature *285*, 573-575.

Bernard, P., Maure, J.F., Partridge, J.F., Genier, S., Javerzat, J.P., and Allshire, R.C. (2001). Requirement of heterochromatin for cohesion at centromeres. Science *294*, 2539-2542.

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.

Berretta, J., Pinskaya, M., and Morillon, A. (2008). A cryptic unstable transcript mediates transcriptional *trans*-silencing of the Ty1 retrotransposon in *S. cerevisiae*. Genes Dev. *22*, 615-626.

Boltzmann, L. (1886). The Second Law of Thermodynamics. In McGinness, B.F., ed. (1974). Theoretical Physics and Philosophical Problems: Selected Writings. Dordrecht, Netherlands: Springer Netherlands.

Bone, J.R., Lavender, J., Richman, R., Palmer, M.J., Turner, B.M., and Kuroda, M.I. (1994). Acetylated histone H4 on the male X chromosome is associated with dosage compensation in *Drosophila*. Genes Dev. *8*, 96-104.

Borsani, G., Tonlorenzi, R., Simmler, M.C., Dandolo, L., Arnaud, D., Capra, V., Grompe, M., Pizzuti, A., Muzny, D., Lawrence, C., et al. (1991). Characterization of a murine gene expressed from the inactive X chromosome. Nature *351*, 325-329.

Braun, J.E., Huntzinger, E., Fauser, M., and Izaurralde, E. (2011). GW182 proteins directly recruit cytoplasmic deadenylase complexes to miRNA targets. Mol. Cell *44*, 120-133.

Braun, J.E., Huntzinger, E., and Izaurralde, E. (2013). The role of GW182 proteins in miRNA-mediated gene silencing. Adv. Exp. Med. Biol. *768*, 147-163.

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., Malone, C.D., Aravin, A.A., Sachidanandam, R., Stark, A., and Hannon, G.J. (2008). An epigenetic role for maternally inherited piRNAs in transposon silencing. Science *322*, 1387-1392.

Brenner, S., Jacob, F., and Meselson, M. (1961). An unstable intermediate carrying information from genes to ribosomes for protein synthesis. Nature *190*, 576-581.

Brockdorff, N. (2013). Noncoding RNA and Polycomb recruitment. RNA *19*, 429-442.

Brockdorff, N., Ashworth, A., Kay, G.F., McCabe, V.M., Norris, D.P., Cooper, P.J., Swift, S., and Rastan, S. (1992). The product of the mouse Xist gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. Cell *71*, 515-526.

Brown, C.J., Ballabio, A., Rupert, J.L., Lafreniere, R.G., Grompe, M., Tonlorenzi, R., and Willard, H.F. (1991). A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. Nature *349*, 38-44.

Brown, C.J., Hendrich, B.D., Rupert, J.L., Lafreniere, R.G., Xing, Y., Lawrence, J., and Willard, H.F. (1992). The human XIST gene: Analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. Cell *71,* 527-542.

Buckley, B.A., Burkhart, K.B., Gu, S.G., Spracklin, G., Kershner, A., Fritz, H., Kimble, J., Fire, A., and Kennedy, S. (2012). A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality. Nature *489*, 447-451.

Bühler, M., Verdel, A., and Moazed, D. (2006). Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing. Cell *125*, 873-886.

Buker, S.M., Iida, T., Bühler, M., Villén, J., Gygi, S.P., Nakayama, J., and Moazed, D. (2007). Two different Argonaute complexes are required for siRNA generation and heterochromatin assembly in fission yeast. Nat. Struct. Mol. Biol. *14*, 200-207.

Burton, N.O., Burkhart, K.B., and Kennedy, S. (2011). Nuclear RNAi maintains heritable gene silencing in Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA *108*, 19683-19688.

Buscaino, A., White, S.A., Houston, D.R., Lejeune, E., Simmer, F., de Lima Alves, F., Diyora, P.T., Urano, T., Bayne, E.H., Rappsilber, J., et al. (2012). PLoS Genet. *8*, e1002499.

Cam, H.P., Sugiyama, T., Chen, E.S., Chen, X., FitzGerald, P.C., and Grewal, S.I. (2005). Comprehensive analysis of heterochromatin- and RNAi-mediated epigenetic control of the fission yeast genome. Nat. Genet. *37*, 809-819.

Camblong, J., Beyrouthy, N., Guffanti, E., Schlaepfer, G., Steinmetz, L.M., and Stutz, F. (2009). *Trans*-acting antisense RNAs mediate transcriptional gene cosuppression in *S. cerevisiae*. Genes Dev. *23*, 1534-1545.

Camblong, J., Iglesias, N., Fickentscher, C., Dieppois, G., and Stutz, F. (2007). Antisense RNA stabilization induces transcriptional gene silencing via histone deacetylation in S. cerevisiae. Cell *131*, 706-717.

Castelnuovo, M., Rahman, S., Guffanti, E., Infantino, V., Stutz, F., and Zenklusen, D. (2013). Bimodal expression of *PHO84* is modulated by early termination of antisense transcription. Nat. Struct. Mol. Biol. *20*, 851-858.

Catalanotto, C., Azzalin, G., Macino, G., and Cogoni, C. (2000). Transcription: Gene silencing in worms and fungi. Nature *404*, 245.

Cavalli, G., and Paro, R. (1998). Chromo-domain proteins: linking chromatin structure to epigenetic regulation. Curr. Opin. Cell Biol. *10*, 354-360.

Cecere, G., Zhen, G.X., Mansisidor, A.R., Klymko, K.E., and Grishok, A. (2012). Promoters recognized by forkhead proteins exist for individual 21U-RNAs. Mol. Cell *47*, 734-745.

Cech, T.R., and Steitz, J.A. (2014). The noncoding RNA revolution—trashing old rules to forge new ones. Cell *157*, 77-94.

Cerase, A., Smeets, D., Tang, Y.A., Gdula, M., Kraus, F., Spivakov, M., Moindrot, B., Leleu, M., Tattermusch, A., Demmerle, J., et al. (2014). Spatial separation of Xist RNA and polycomb proteins revealed by superresolution microscopy. Proc. Natl. Acad. Sci. USA *111*, 2235-2240.

Chalei, V., Sansom, S.N., Kong, L., Lee, S., Montiel, J.F., Vance, K.W., and Ponting, C.P. (2014). The long non-coding RNA Dali is an epigenetic regulator of neural differentiation. Elife *3*, e04530.

Chekulaeva, M., Mathys, H., Zipprich, J.T., Attig, J., Colic, M., Parker, R., and Filipowicz, W. (2011). miRNA repression involves GW182-mediated recruitment of CCR4-NOT through conserved W-containing motifs. Nat. Struct. Mol. Biol. *18*, 1218-1226.

Cheloufi, S., Dos Santos, C.O., Chong, M.M., and Hannon, G.J. (2010). A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. Nature *465*, 584-589.

Chen, C.Y., Zheng, D., Xia, Z., and Shyu, A.B. (2009). Ago-TNRC6 triggers microRNA-mediated decay by promoting two deadenylation steps. Nat. Struct. Mol. Biol. *16*, 1160-1166.

Chen, Y., Boland, A., Kuzuoğlu-Öztürk, D., Bawankar, P., Loh, B., Chang, C.-T., Weichenrieder, O., and Izaurralde, E. (2014). A DDX6-CNOT1 Complex and W-Binding Pockets in CNOT9 Reveal Direct Links between miRNA Target Recognition and Silencing. Mol. Cell *54*. 737-750.

Chinen, M., Morita, M., Fukumura, K., and Tani, T. (2010). Involvement of the spliceosomal U4 small nuclear RNA in heterochromatic gene silencing at fission yeast centromeres. J. Biol. Chem. *285*, 5630-5638.

Chu, C., Zhang, Q.C., da Rocha, S.T., Flynn, R.A., Bharadwaj, M., Calabrese, J.M., Magnuson, T., Heard, E., and Chang, H.Y. (2015). Systematic discovery of Xist RNA binding proteins. Cell *161*, 404-416.

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.

Cifuentes, D., Xue, H., Taylor, D.W., Patnode, H., Mishima, Y., Cheloufi, S., Ma, E., Mane, S., Hannon, G.J., Lawson, N.D., et al. (2010). A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activ- ity. Science *328*, 1694-1698.

Cifuentes-Rojas, C., Hernandez, A.J., Sarma, K., and Lee, J.T. (2014). Regulatory interactions between RNA and polycomb repressive complex 2. Mol. Cell *55*, 171-185.

Claycomb, J.M., Batista, P.J., Pang, K.M., Gu, W., Vasale, J.J., van Wolfswinkel, J.C., Chaves, D.A., Shirayama, M., Mitani, S., Ketting, R.F., et al. (2009). The Argonaute CSR-1 and its 22G-RNA cofactors are required for holocentric chromosome segregation. Cell *139*, 123-134.

Clemson, C.M., McNeil, J.A., Willard, H.F., and Lawrence, J.B. (1996). XIST RNA paints the inactive X chromosome at interphase: Evidence for a novel RNA involved in nuclear/chromosome structure. J. Cell. Biol. *132*, 259-275.

Cogoni, C., and Macino, G. (1999). Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. Nature *399*, 166-169.

Colmenares, S.U., Buker, S.M., Buhler, M., Dlakić, M., and Moazed, D. (2007). Coupling of double-stranded RNA synthesis and siRNA generation in fission yeast RNAi. Mol. Cell *27*, 449-461.

Crick, F.H. (1970). Central dogma of molecular biology. Nature *227*, 561-563.

Crick, F.H. (1958). On protein synthesis. Symp. Soc. Exp. Biol. *12*, 138-163.

Crick, F.H. (1968). The origin of the genetic code. J. Mol. Biol. *38*, 367-379.

Csorba, T., Questa, J.I., Sun, Q., and Dean, C. (2014). Antisense *COOLAIR* mediates the coordinated switching of chromatin states at *FLC* during vernalization. Proc. Natl. Acad. Sci. USA *111*, 16160-16165.

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

Czech, B., Zhou, R., Erlich, Y., Brennecke, J., Binari, R., Villalta, C., Gordon, A., Perrimon, N., and Hannon, G.J. (2009). Hierarchical rules for Argonaute loading in *Drosophila*. Mol. Cell *36*, 445-456.

da Rocha, S.T., Boeva, V., Escamilla-Del-Arenal, M., Ancelin, K., Granier, C., Matias, N.R., Sanulli, S., Chow, J., Schulz, E., Picard, C., et al. (2014). Jarid2 Is Implicated in the Initial Xist-Induced Targeting of PRC2 to the Inactive X Chromosome. Mol. Cell *53*, 301-316.

Dalmay, T., Hamilton, A., Rudd, S., Angell, S., and Baulcombe, D.C. (2000). An RNA-dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell *101*, 543-553.

Darwin, C. (1859). On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life (ch. 14 “Recapitulation and Conclusion”). London: John Murray.

Das, P.P., Bagijn, M.P., Goldstein, L.D., Woolford, J.R., Lehrbach, N.J., Sapetschnig, A., Buhecha, H.R., Gilchrist, M.J., Howe, K.L., Stark, R., et al. (2008). Piwi and piRNAs act upstream of an endogenous siRNA pathway to suppress Tc3 transposon mobility in the Caenorhabditis elegans germline. Mol. Cell *31*, 79-90.

Davidovich, C., Wang, X., Cifuentes-Rojas, C., Goodrich, K., Gooding, A.R., Lee, J.T., and Cech, T.R. (2015). Toward a consensus on the binding specificity and promiscuity of PRC2 for RNA. Mol. Cell *57,* 552-558.

Davidovich, C., Zheng, L., Goodrich, K.J., and Cech, T.R. (2013). Promiscuous RNA binding by Polycomb repressive complex 2. Nat. Struct. Mol. Biol. *20*, 1250-1257.

De Fazio, S., Bartonicek, N., Di Giacomo, M., Abreu-Goodger, C., Sankar, A., Funaya, C., Antony, C., Moreira, P.N., Enright, A.J., and O’Carroll, D. (2011). The endonuclease activity of Mili fuels piRNA amplification that silences LINE1 elements. Nature *480*, 259-263.

de Vanssay, A., Bouge, A.L., Boivin, A., Hermant, C., Teysset, L., Delmarre, V., Antoniewski, C., and Ronsseray, S. (2012). Paramutation in *Drosophila* linked to emergence of a piRNA-producing locus. Nature *490*, 112-115.

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.

Di Giacomo, M., Comazzetto, S., Saini, H., De Fazio, S., Carrieri, C., Morgan, M., Vasiliauskaite, L., Benes, V., Enright, A.J., and O’Carroll, D. (2013). Multiple epigenetic mechanisms and the piRNA pathway enforce LINE1 silencing during adult spermatogenesis. Mol. Cell *50*, 601-608.

Di Ruscio, A., Ebralidze, A.K., Benoukraf, T., Amabile, G., Goff, L.A., Terragni, J., Figueroa, M.E., De Figueiredo Pontes, L.L., Alberich-Jorda, M., Zhang, P., et al. (2013). DNMT1-interacting RNAs block gene-specific DNA methylation. Nature *503*, 371-376.

Diederichs, S. (2014). The four dimensions of noncoding RNA conservation. Trends Genet. *30*, 121-123.

Djupedal, I., Kos-Braun, I.C., Mosher, R.A., Söderholm, N., Simmer, F., Hardcastle, T.J., Fender, A., Heidrich, N., Kagansky, A., Bayne, E., et al. (2009). Analysis of small RNA in fission yeast; centromeric siRNAs are potentially generated through a structured RNA. EMBO J. *28*, 3832-3844.

Djupedal, I., Portoso, M., Spåhr, H., Bonilla, C., Gustafsson, C.M., Allshire, R.C., and Ekwall, K. (2005). RNA Pol II subunit Rpb7 promotes centromeric transcription and RNAi-directed chromatin silencing. Genes Dev. *19*, 2301-2306.

Djuranovic, S., Nahvi, A., and Green, R. (2012). miRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay. Science *336*, 237-240.

Doe, C.L., Wang, G., Chow, C., Fricker, M.D., Singh, P.B., and Mellor, E.J. (1998). The fission yeast chromo domain encoding gene chp1(+) is required for chromosome segregation and shows a genetic interaction with alpha-tubulin. Nucleic Acids Res. *26*, 4222-4229.

Du, P., Wang, L., Sliz, P., and Gregory, R.I. (2015). A biogenesis step upstream of Microprocessor controls miR-17~92 expression. Cell, in press. Published online August 6, 2015. 10.1016/j.cell.2015.07.008

Dumesic, P.A., Natarajan, P., Chen, C., Drinnenberg, I.A., Schiller, B.J., Thompson, J., Moresco, J.J., Yates, J.R., 3rd, Bartel, D.P., and Madhani, H.D. (2013). Stalled spliceosomes are a signal for RNAi-mediated genome defense. Cell *152*, 957-968.

Eissenberg, J.C., and Elgin, S.C. (2000). The HP1 protein family: getting a grip on chromatin. Curr. Opin. Genet. Dev. *10*, 204-210.

Ekwall, K., Cranston, G., and Allshire, R.C. (1999). Fission yeast mutants that alleviate transcriptional silencing in centromeric flanking repeats and disrupt chromosome segregation. Genetics *153*, 1153-1169.

Ekwall, K., Nimmo, E.R., Javerzat, J.P., Borgstrøm, B., Egel, R., Cranston, G., and Allshire, R. (1996). Mutations in the fission yeast silencing factors clr4+ and rik1+ disrupt the localisation of the chromo domain protein Swi6p and impair centromere function. J. Cell Sci. *109*, 2637-2648.

Ekwall, K., and Ruusala, T. (1994). Mutations in rik1, clr2, clr3 and clr4 genes asymmetrically derepress the silent mating-type loci in fission yeast. Genetics *136*, 53-64.

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.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001a). Duplexes of 21-nucleotide RNAs mediate RNA interference in mammalian cell culture. Nature *411*, 494-498.

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

Elkayam, E., Kuhn, C.D., Tocilj, A., Haase, A.D., Greene, E.M., Hannon, G.J., and Joshua-Tor, L. (2012). The structure of human argonaute-2 in complex with miR-20a. Cell *150*, 100-110.

Engreitz, J.M., Pandya-Jones, A., McDonel, P., Shishkin, A., Sirokman, K., Surka, C., Kadri, S., Xing, J., Goren, A., Lander, E.S., et al. (2013). The Xist lncRNA exploits three-dimensional genome architecture to spread across the X chromosome. Science *341*, 1237973.

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.

Eystathioy, T., Jakymiw, A., Chan, E.K., Seraphin, B., Cougot, N., and Fritzler, M.J. (2003). The GW182 protein colocalizes with mRNA degradation associated proteins hDcp1 and hLSm4 in cytoplasmic GW bodies. RNA *9*, 1171–1173.

Fabian, M.R., Cieplak, M.K., Frank, F., Morita, M., Green, J., Srikumar, T., Nagar, B., Yamamoto, T., Raught, B., Duchaine, T.F., and Sonenberg, N. (2011). miRNA-mediated deadenylation is orchestrated by GW182 through two conserved motifs that interact with CCR4-NOT. Nat. Struct. Mol. Biol. *18*, 1211-1217.

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.

Faehnle, C.R., Elkayam, E., Haase, A.D., Hannon, G.J., and Joshua-Tor, L. (2013). The Making of a Slicer: Activation of Human Argonaute-1. Cell Rep. *3*, 1901-1909.

Fagard, M., Boutet, S., Morel, J.-B., Bellini, C., and Vaucheret, H. (2000). AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. Proc. Natl. Acad. Sci. USA. *97*, 11650-11654.

Ferrari, F., Alekseyenko, A.A., Park, P.J., and Kuroda, M.I. (2014). Transcriptional control of a whole chromosome: emerging models for dosage compensation. Nat. Struct. Mol. Biol. *21*, 118-125.

Fire, A., Albertson, D., Harrison, S.W., and Moerman, D.G. (1991). Production of antisense RNA leads to effective and specific inhibition of gene expression in *C. elegans* muscle. Development *113*, 503-514.

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.

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

Förstemann, 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 germline stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. PLoS Biol. *3*, e236.

Frank, F., Sonenberg, N., and Nagar, B. (2010). Structural basis for 5'-nucleotide base-specific recognition of guide RNA by human AGO2. Nature *465*, 818-822.

Franke, A., and Baker, B.S. (1999). The *roX1* and *roX2* RNAs are essential components of the compensasome, which mediates dosage compensation in *Drosophila*. Mol. Cell *4*, 117-122.

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.

Fukaya, T., Iwakawa, H.-O., and Tomari, Y. (2014). MicroRNAs block assembly of eIF4F translation initiation complex in *Drosophila*. Mol. Cell *56*, 67-78.

Fukaya, T., and Tomari, Y. (2011). PABP is not essential for microRNA-mediated translational repression and deadenylation *in vitro*. EMBO J. *30*, 4998-5009.

Fukaya, T., and Tomari, Y. (2012). MicroRNAs mediate gene silencing via multiple different pathways in *Drosophila*. Mol. Cell *48*, 825-836.

Galupa, R., and Heard, E. (2015). X-chromosome inactivation: new insights into *cis* and *trans* regulation. Curr. Opin. Genet. Dev. *31*, 57-66.

Gao, Z., Liu, H.L., Daxinger, L., Pontes, O., He, X., Qian, W., Lin, H., Xie, M., Lorkovic, Z.J., Zhang, S., et al. (2010). An RNA polymerase II- and AGO4-associated protein acts in RNA-directed DNA methylation. Nature *465*, 106-109.

Gelbart, M.E., and Kuroda, M.I. (2009). *Drosophila* dosage compensation: a complex voyage to the X chromosome. Development *136*, 1399-1410.

Gerace, E.L., Halic, M., and Moazed, D. (2010). The methyltransferase activity of Clr4Suv39h triggers RNAi independently of histone H3K9 methylation. Mol. Cell *39*, 360-372.

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.

Ghildiyal, M., Xu, J., Seitz, H., Weng, Z., and Zamore, P.D. (2010). Sorting of Drosophila small silencing RNAs partitions microRNA* strands into the RNA interference pathway. RNA *16*, 43-56.

Ghildiyal, M., and Zamore, P.D. (2009). Small silencing RNAs: an expanding universe. Nat. Rev. Genet. *10*, 94-10

Gilbert, W. (1986). The RNA world. Nature *319*, 618.

Giner, A., Lakatos, L., García-Chapa, M., López-Moya, J., and Burgyán, J. (2010). Viral protein inhibits RISC activity by argonaute binding through conserved WG/GW motifs. PLoS Pathog. *6*, e1000996.

Giorgetti, L., Galupa, R., Nora, E.P., Piolot, T., Lam, F., Dekker, J., Tiana, G., and Heard, E. (2014). Predictive polymer modeling reveals coupled fluctuations in chromosome conformation and transcription. Cell *157*, 950-963.

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.

Gregersen, L.H., Schueler, M., Munschauer, M., Mastrobuoni, G., Chen, W., Kempa, S., Dieterich, C., and Landthaler, M. (2014). MOV10 is a 5' to 3' RNA helicase contributing to UPF1 mRNA target degradation by translocation along 3' UTRs. Mol. Cell *54*, 573-585.

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.

Greider, C.W., and Blackburn, E.H. (1989). A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature *337*, 331-337.

Grewal, S.I., and Jia, S. (2007). Heterochromatin revisited. Nat. Rev. Genet. *8*, 35-46.

Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G., and Mello, C.C. (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell *106*, 23-34.

Grishok, A., Tabara, H., and Mello, C.C. (2000). Genetic requirements for inheritance of RNAi in C. elegans. Science *287*, 2494-2497.

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.

Gu, S.G., Pak, J., Guang, S., Maniar, J.M., Kennedy, S., and Fire, A. (2012a). Amplification of siRNA in Caenorhabditis elegans generates a transgenerational sequence-targeted histone H3 lysine 9 methylation footprint. Nat. Genet. *44*, 157-164.

Gu, W., Lee, H.C., Chaves, D., Youngman, E.M., Pazour, G.J., Conte, D., Jr., and Mello, C.C. (2012b). CapSeq and CIP-TAP identify Pol II start sites and reveal capped small RNAs as C. elegans piRNA precursors. Cell *151*, 1488– 1500.

Guang, S., Bochner, A.F., Burkhart, K.B., Burton, N., Pavelec, D.M., and Kennedy, S. (2010). Small regulatory RNAs inhibit RNA polymerase II during the elongation phase of transcription. Nature *465*, 1097-1101.

Guang, S., Bochner, A.F., Pavelec, D.M., Burkhart, K.B., Harding, S., Lachowiec, J., and Kennedy, S. (2008). An Argonaute transports siRNAs from the cytoplasm to the nucleus. Science *321*, 537-541.

Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., and Altman, S. (1983). The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. Cell *35,* 849-857.

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.

Guo, S., and Kemphues, K.J. (1995). par-1, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell *81*, 611-620.

Ha, M., and Kim, V.N. (2014). Regulation of microRNA biogenesis. Nat. Rev. Mol. Cell Biol. *15*, 509-524.

Halic, M., and Moazed, D. (2010). Dicer-independent primal RNAs trigger RNAi and heterochromatin formation. Cell *140*, 504-516.

Hamilton, A.J., and Baulcombe, D.C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. Science *286*, 950-952.

Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature *404*, 293-296.

Hammond, S.M., Boettcher, S., Caudy, A., Kobayashi, R., and Hannon, G.J. (2001). Argonaute2, a link between genetic and biochemical analyses of RNAi. Science *293*, 1146-1150.

Han, B.W., Wang, W., Li, C., Weng, Z., and Zamore, P.D. (2015). piRNA-guided transposon cleavage initiates Zucchini-dependent, phased piRNA production. Science *348*, 817-821.

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.

Hauptmann, J., Dueck, A., Harlander, S., Pfaff, J., Merkl, R., and Meister, G. (2013). Turning catalytically inactive human Argonaute proteins into active slicer enzymes. Nat. Struct. Mol. Biol. *20*, 814-817.

Hayashi, A., Ishida, M., Kawaguchi, R., Urano, T., Murakami, Y., and Nakayama, J.I. (2012). Heterochromatin protein 1 homologue Swi6 acts in concert with Ers1 to regulate RNAi-directed heterochromatin assembly. Proc. Natl. Acad. Sci. USA *109*, 6159-6164.

He, X.-J., Hsu, Y.-F., Zhu, S., Wierzbicki, A.T., Pontes, O., Pikaard, C.S., Liu, H.-L., Wang, C.-S., Jin, H., and Zhu, J.-K. (2009). An effector of RNA-directed DNA methylation in arabidopsis is an ARGONAUTE 4- and RNA-binding protein. Cell *137*, 498-508.

Heitz, E. (1928). Das Heterochromatin der Moose, 1. Jahrb. Wiss. Bot. *69*, 762–818.

Herr, A.J., Jensen, M.B., Dalmay, T., and Baulcombe, D.C. (2005). RNA polymerase IV directs silencing of endogenous DNA. Science *308*, 118-120.

Hilfiker, A., Hilfiker-Kleiner, D., Pannuti, A., and Lucchesi, J.C. (1997). *mof*, a putative acetyl transferase gene related to the Tip60 and MOZ human genes and to the SAS genes of yeast, is required for dosage compensation in *Drosophila*. EMBO J. *16*, 2054-2060.

Holoch, D., and Moazed, D. (2015a). RNA-mediated epigenetic regulation of gene expression. Nat. Rev. Genet. *16*, 71-84.

Holoch, D., and Moazed, D. (2015b). Small-RNA loading licenses Argonaute for assembly into a transcriptional silencing complex. Nat. Struct. Mol. Biol. *22*, 328-335.

Hong, E.J., Villén, J., Gerace, E.L., Gygi, S.P., and Moazed, D. (2005). A cullin E3 ubiquitin ligase complex associates with Rik1 and the Clr4 histone H3-K9 methyltransferase and is required for RNAi-mediated heterochromatin formation. RNA Biol. *2*, 106-111.

Hongay, C.F., Grisafi, P.L., Galitski, T., and Fink, G.R. (2006). Antisense transcription controls cell fate in Saccharomyces cerevisiae. Cell *127*, 735-745.

Horn, P.J., Bastie, J.N., and Peterson, C.L. (2005). A Rik1-associated, cullin-dependent E3 ubiquitin ligase is essential for heterochromatin formation. Genes Dev. *19*, 1705-1714.

Huntzinger, E., Braun, J.E., Heimstädt, S., Zekri, L., and Izaurralde, E. (2010). Two PABPC1-binding sites in GW182 proteins promote miRNA-mediated gene silencing. EMBO J. *29*, 4146-4160.

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

Huntzinger, E., Kuzuoğlu-Öztürk, D., Braun, J.E., Eulalio, A., Wohlbold, L., and Izaurralde, E. (2013). The interactions of GW182 proteins with PABP and dead- enylases are required for both translational repression and degradation of miRNA targets. Nucleic Acids Res. *41*, 978-994.

Hutvágner, G., McLachlan, J., Pasquinelli, A.E., Bálint, E., Tuschl, T., and Zamore, P.D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science *293*, 834-838.

Hutvágner, G., and Zamore, P.D. (2002). A MicroRNA in a Multiple-Turnover RNAi Enzyme Complex. Science *297*, 2056-2060.

Ietswaart, R., Wu, Z., and Dean, C. (2012). Flowering time control: another window to the connection between antisense RNA and chromatin. Trends Genet. *28*, 445-453.

Iida, T., Kawaguchi, R., and Nakayama, J. (2006). Conserved ribonuclease, Eri1, negatively regulates heterochromatin assembly in fission yeast. Curr. Biol. *16*, 1459-1464.

Iida, T., Nakayama, J., and Moazed, D. (2008). siRNA-mediated heterochromatin establishment requires HP1 and is associated with antisense transcription. Mol. Cell *31*, 178-189.

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.

Ipsaro, J.J., Haase, A.D., Knott, S.R., Joshua-Tor, L., and Hannon, G.J. (2012). The structural biochemistry of Zucchini implicates it as a nuclease in piRNA biogenesis. Nature *491*, 279-283.

Irvine, D.V., Zaratiegui, M., Tolia, N.H., Goto, D.B., Chitwood, D.H., Vaughn, M.W., Joshua-Tor, L., and Martienssen, R.A. (2006). Argonaute slicing is required for heterochromatic silencing and spreading. Science *313*, 1134-1137.

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.

Iwasaki, S., Sasaki, H., Sakaguchi, Y., Suzuki, T., Tadakuma, H., and Tomari, Y. (2015). Defining fundamental steps in the assembly of the *Drosophila* RNAi enzyme complex. Nature *521*, 533-536.

Jacobs, S.A., and Khorasanizadeh, S. (2002). Structure of HP1 chromodomain bound to a lysine 9-methylated histone H3 tail. Science *295*, 2080-2083.

Jacobs, S.A., Taverna, S.D., Zhang, Y., Briggs, S.D., Li, J., Eissenberg, J.C., Allis, C.D., and Khorasanizadeh, S. (2001). Specificity of the HP1 chromo domain for the methylated N-terminus of histone H3. EMBO J. *20*, 5232-5241.

Jia, S., Kobayashi, R., and Grewal, S.I. (2005). Ubiquitin ligase component Cul4 associates with Clr4 histone methyltransferase to assemble heterochromatin. Nat. Cell Biol. *7*, 1007-1013.

Jia, S., Noma, K., and Grewal, S.I. (2004). RNAi-independent heterochromatin nucleation by the stress-activated ATF/CREB family proteins. Science *304*, 1971-1976.

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.

Jinek, M., Fabian, M.R., Coyle, S.M., Sonenberg, N., and Doudna, J.A. (2010). Structural insights into the human GW182-PABC interaction in microRNA-mediated deadenylation. Nat. Struct. Mol. Biol. *17*, 238-240.

Johnson, L.M., Du, J., Hale, C.J., Bischof, S., Feng, S., Chodavarapu, R.K., Zhong, X., Marson, G., Pellegrini, M., Segal, D.J., et al. (2014). SRA- and SET-domain-containing proteins link RNA polymerase V occupancy to DNA methylation. Nature *507*, 124-128.

Jonas, S., and Izaurralde, E. (2015). Towards a molecular understanding of microRNA-mediated gene silencing. Nat. Rev. Genet. *16*, 421-433.

Kagansky, A., Folco, H.D., Almeida, R., Pidoux, A.L., Boukaba, A., Simmer, F., Urano, T., Hamilton, G.L., and Allshire, R.C. (2009). Synthetic heterochromatin bypasses RNAi and centromeric repeats to establish functional centromeres. Science *324*, 1716-1719.

Kallgren, S.P., Andrews, S., Tadeo, X., Hou, H., Moresco, J.J., Tu, P.G., Yates, J.R., 3rd, Nagy, P.L., and Jia, S. (2014). The proper splicing of RNAi factors is critical for pericentric heterochromatin assembly in fission yeast. PLoS Genet. *10*, e1004334.

Kanoh, J., Sadaie, M., Urano, T., and Ishikawa, F. (2005). Telomere binding protein Taz1 establishes Swi6 heterochromatin independently of RNAi at telomeres. Curr. Biol. *15*, 1808-1819.

Kato, H., Goto, D.B., Martienssen, R.A., Urano, T., Furukawa, K., and Murakami, Y. (2005). RNA polymerase II is required for RNAi-dependent heterochromatin assembly. Science *309*, 467-469.

Kawakami, K., Hayashi, A., Nakayama, J., and Murakami, Y. (2012). A novel RNAi protein, Dsh1, assembles RNAi machinery on chromatin to amplify heterochromatic siRNA. Genes Dev. *26*, 1811-1824.

Kawamata, T., Seitz, H., and Tomari, Y. (2009). Structural determinants of miRNAs for RISC loading and slicer-independent unwinding. Nat. Struct. Mol. Biol. *16*, 953-960.

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.

Kawaoka, S., Izumi, N., Katsuma, S., and Tomari, Y. (2011). 3’ end formation of PIWI-interacting RNAs in vitro. Mol. Cell *43*, 1015-1022.

Kelley, R.L., and Kuroda, M.I. (2000). The role of chromosomal RNAs in marking the X for dosage compensation. Curr. Opin. Genet. Dev. *10*, 555-561.

Kelley, R.L., Meller, V.H., Gordadze, P.R., Roman, G., Davis, R.L., and Kuroda, M.I. (1999). Epigenetic spreading of the *Drosophila* dosage compensation complex from *roX* RNA genes into flanking chromatin. Cell *98*, 513-522.

Kennedy, S., Wang, D., and Ruvkun, G. (2004). A conserved siRNA-degrading RNase negatively regulates RNA interference in C. elegans. Nature 427, 645-649.

Ketting, R., Fischer, S., Bernstein, E., Sijen, T., Hannon, G., and Plasterk, R. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. Genes Dev. *15*, 2654-2659.

Khalil, A.M., Guttman, M., Huarte, M., Garber, M., Raj, A., Rivea Morales, D., Thomas, K., Presser, A., Bernstein, B.E., van Oudenaarden, A., et al. (2009). Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc. Natl. Acad. Sci. USA *106*, 11667-11672.

Khvorova, A., Reynolds, A., and Jayasena, S.D. (2003). Functional siRNAs and miRNAs exhibit strand bias. Cell *115*, 209-216.

Klattenhoff, C., Xi, H., Li, C., Lee, S., Xu, J., Khurana, J.S., Zhang, F., Schultz, N., Koppetsch, B.S., Nowosielska, A., et al. (2009). The Drosophila HP1 homolog Rhino is required for transposon silencing and piRNA production by dual-strand clusters. Cell *138*, 1137-1149.

Kohlmaier, A., Savarese, F., Lachner, M., Martens, J., Jenuwein, T., and Wutz, A. (2004). A chromosomal memory triggered by Xist regulates histone methylation in X inactivation. PLoS Biol. *2*, e171.

Kotake, Y., Nakagawa, T., Kitagawa, K., Suzuki, S., Liu, N., Kitagawa, M., and Xiong, Y. (2011). Long non-coding RNA ANRIL is required for the PRC2 recruit- ment to and silencing of p15(INK4B) tumor suppressor gene. Oncogene *30*, 1956-1962.

Kowalik, K.M., Shimada, Y., Flury, V., Stadler, M.B., Batki, J., and Bühler, M. (2015). The Paf1 complex represses small-RNA-mediated epigenetic gene silencing. Nature *520*, 248-252.

Kretz, M., Siprashvili, Z., Chu, C., Webster, D.E., Zehnder, A., Qu, K., Lee, C.S., Flockhart, R.J., Groff, A.F., Chow, J., et al. (2013). Control of somatic tissue differentiation by the long non-coding RNA *TINCR*. Nature *493*, 231-235.

Kruger, K., Grabowski, P.J., Zaug, A.J., Sands, J., Gottschling, D.E., and Cech, T.R. (1982). Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of Tetrahymena. Cell *31*, 147-157.

Kuscu, C., Zaratiegui, M., Kim, H.S., Wah, D.A., Martienssen, R.A., Schalch, T., and Joshua-Tor, L. (2014). CRL4-like Clr4 complex in *Schizosaccharomyces pombe* depends on an exposed surface of Dos1 for heterochromatin silencing. Proc. Natl. Acad. Sci. USA *111*, 1795-1800.

Kwak, P.B., and Tomari, Y. (2012). The N domain of Argonaute drives duplex unwinding during RISC assembly. Nat. Struct. Mol. Biol. *19*, 145-151.

LaCava, J., Houseley, J., Saveanu, C., Petfalski, E., Thompson, E., Jacquier, A., and Tollervey, D. (2005). RNA degradation by the exosome is promoted by a nuclear polyadenylation complex. Cell *121*, 713-724.

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature *410*, 116-120.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. Science *294*, 853-858.

Lai, F., Orom, U.A., Cesaroni, M., Beringer, M., Taatjes, D.J., Blobel, G.A., and Shiekhattar, R. (2013). Activating RNAs associate with Mediator to enhance chromatin architecture and transcription. Nature *494*, 497-501.

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.

Larschan, E., Alekseyenko, A.A., Gortchakov, A.A., Peng, S., Li, B., Yang, P., Workman, J.L., Park, P.J., and Kuroda, M.I. (2007). MSL complex is attracted to genes marked by H3K36 trimethylation using a sequence-independent mechanism. Mol. Cell *28*, 121-133.

Larschan, E., Bishop, E.P., Kharchenko, P.V., Core, L.J., Lis, J.T., Park, P.J., and Kuroda, M.I. (2011). X chromosome dosage compensation via enhanced transcriptional elongation in Drosophila. Nature *471*, 115-118.

Lau, N.C., Lim, L.P., Weinstein, E.G., and Bartel, D.P. (2001). An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. Science *294*, 858-862.

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.

Law, J.A., Du, J., Hale, C.J., Feng, S., Krajewski, K., Palanca, A.M., Strahl, B.D., Patel, D.J., and Jacobsen, S.E. (2013). Polymerase IV occupancy at RNA-directed DNA methylation sites requires SHH1. Nature *498*, 385-389.

Law, J.A., and Jacobsen, S.E. (2010). Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nat. Rev. Genet. *11*, 204-220.

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.

Le Thomas, A., Stuwe, E., Li, S., Du, J., Marinov, G., Rozhkov, N., Chen, Y.C., Luo, Y., Sachidanandam, R., Toth, K.F., et al. (2014). Transgenerationally inherited piRNAs trigger piRNA biogenesis by changing the chromatin of piRNA clusters and inducing precursor processing. Genes Dev. *28*, 1667-1680.

Lee, H.C., Gu, W., Shirayama, M., Youngman, E., Conte, D., Jr., and Mello, C.C. (2012). C. elegans piRNAs mediate the genome-wide surveillance of germline transcripts. Cell *150*, 78-87.

Lee, J.T. (2009). Lessons from X-chromosome inactivation: long ncRNA as guides and tethers to the epigenome. Genes Dev. *23*, 1831-1842.

Lee, J.T., and Lu, N. (1999). Targeted mutagenesis of Tsix leads to nonrandom X inactivation. Cell *99*, 47-57.

Lee, R.C., and Ambros, V. (2001). An extensive class of small RNAs in Caenorhabditis elegans. Science *294*, 862-864.

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, S.R., and Collins, K. (2007). Physical and functional coupling of RNA-dependent RNA polymerase and Dicer in the biogenesis of endogenous siRNAs. Nat. Struct. Mol. Biol. *14*, 604-610.

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.

Leuschner, P.J., Ameres, S.L., Kueng, S., and Martinez, J. (2006). Cleavage of the siRNA passenger strand during RISC assembly in human cells. EMBO Rep. *7*, 314-320.

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

Li, F., Goto, D.B., Zaratiegui, M., Tang, X., Martienssen, R., and Cande, W.Z. (2005). Two novel proteins, dos1 and dos2, interact with rik1 to regulate heterochromatic RNA interference and histone modification. Curr. Biol. *15*, 1448-1457.

Li, H., Motamedi, M.R., Yip, C.K., Wang, Z., Walz, T., Patel, D.J., and Moazed, D. (2009). An alpha motif at Tas3 C terminus mediates RITS cis spreading and promotes heterochromatic gene silencing. Mol. Cell *34*, 155-167.

Liu, F., Marquardt, S., Lister, C., Swiezewski, S., and Dean, C. (2010). Targeted 3' processing of antisense transcripts triggers *Arabidopsis FLC* chromatin silencing. Science *327*, 94-97.

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). Micro- RNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat. Cell Biol. *7*, 719-723.

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.

Liu, Y., Ye, X., Jiang, F., Liang, C., Chen, D., Peng, J., Kinch, L.N., Grishin, N.V., and Liu, Q. (2009). C3PO, an endoribonuclease that promotes RNAi by facilitating RISC activation. Science *325*, 750–-753.

Liu, Z.W., Shao, C.R., Zhang, C.J., Zhou, J.X., Zhang, S.W., Li, L., Chen, S., Huang, H.W., Cai, T., and He, X.J. (2014). The SET domain proteins SUVH2 and SUVH9 are required for Pol V occupancy at RNA-directed DNA methylation loci. PLoS Genet. *10*, e1003948.

Lorentz, A., Heim, L., and Schmidt, H. (1992). The switching gene swi6 affects recombination and gene expression in the mating-type region of *Schizosaccharomyces pombe*. Mol. Gen. Genet. *233*, 436-442.

Lucchesi, J.C., and Kuroda, M.I. (2015). Dosage compensation in *Drosophila*. Cold Spring Harb. Perspect. Biol. *7*, a019398.

Lyon, M.F. (1961). Gene action in the X-chromosome of the mouse (*Mus musculus* L.) Nature *190*, 372-373.

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., Ma, E., Zhou, M., Robinson, C.V., and Doudna, J.A. (2008). In vitro reconstitution of the human RISC-loading complex. Proc. Natl. Acad. Sci. USA *105*, 512-517.

Maiti, M., Lee, H.C., and Liu, Y. (2007). QIP, a putative exonuclease, interacts with the Neurospora Argonaute protein and facilitates conversion of duplex siRNA into single strands. Genes Dev. *21*, 590-600.

Mallory, A., and Shkumatava, A. (2015). LncRNAs in vertebrates: advances and challenges. Biochimie, in press. Published online March 24, 2015. 10.1016/j.biochi.2015.03.014

Malone, C.D., and Hannon, G.J. (2009). Small RNAs as guardians of the genome. Cell *136*, 656-668.

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

Marina, D.B., Shankar, S., Natarajan, P., Finn, K.J., and Madhani, H.D. (2013). A conserved ncRNA-binding protein recruits silencing factors to heterochromatin through an RNAi-independent mechanism. Genes Dev. *27*, 1851-1856.

Marquardt, S., Raitskin, O., Wu, Z., Liu, F., Sun, Q., and Dean, C. (2014). Functional consequences of splicing of the antisense transcript *COOLAIR* on *FLC* transcription. Mol. Cell *54*, 156-165.

Martinez, N.J., Chang, H.M., Borrajo, J.D., and Gregory, R.I. (2013). The co-chaperones Fkbp4/5 control Argonaute2 expression and facilitate RISC assembly. RNA *19*, 1583-1593.

Martinez, N.J., and Gregory, R.I. (2013). Argonaute2 expression is post-transcriptionally coupled to microRNA abundance. RNA *19*, 605-612.

Mathys, H., Basquin, J., Ozgur, S., Czarnocki-Cieciura, M., Bonneau, F., Aartse, A., Dziembowski, A., Nowotny, M., Conti, E., and Filipowicz, W. (2014). Structural and Biochemical Insights to the Role of the CCR4-NOT Complex and DDX6 ATPase in MicroRNA Repression. Mol. Cell *54*, 751-765.

Matranga, C., Tomari, Y., Shin, C., Bartel, D., and Zamore, P. (2005). Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell *123*, 607-620.

Matzke, M.A., and Mosher, R.A. (2014). RNA-directed DNA methylation: an epigenetic pathway of increasing complexity. Nat. Rev. Genet. *15*, 394-408.

McHugh, C.A., Chen, C.K., Chow, A., Surka, C.F., Tran, C., McDonel, P., Pandya-Jones, A., Blanco, M., Burghard, C., Moradian, A., et al. (2015). The *Xist* lncRNA interacts directly with SHARP to silence transcription through HDAC3. Nature *521*, 232-236.

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.

Meller, V.H., and Rattner, B.P. (2002). The *roX* RNAs encode redundant *male-specific lethal* transcripts required for targeting of the MSL complex. EMBO J. *21*, 1084-1091.

Meller, V.H., Gordadze, P.R., Park, Y., Chu, X., Stuckenholz, C., Kelley, R.L., and Kuroda, M.I. (2000). Ordered assembly of *roX* RNAs into MSL complexes on the dosage compensated X chromosome in *Drosophila*. Curr. Biol. *10*, 136-143.

Meller, V.H., Wu, K.H., Roman, G., Kuroda, M.I., and Davis, R.L. (1997). roX1 RNA paints the X chromosome of male *Drosophila* and is regulated by the dosage compensation system. Cell *88*, 445-457.

Mi, S., Cai, T., Hu, Y., Chen, Y., Hodges, E., Ni, F., Wu, L., Li, S., Zhou, H., Long, C., et al. (2008). Sorting of small RNAs into Arabidopsis argonaute complexes is directed by the 5' terminal nucleotide. Cell *133*, 116-127.

Minajigi, A., Froberg, J.E., Wei, C., Sunwoo, H., Kesner, B., Colognori, D., Lessing, D., Payer, B., Boukhali, M., Haas, W., et al. (2015). A comprehensive Xist interactome reveals cohesin repulsion and an RNA-directed chromosome conformation. Science, in press. Published online June 18, 2015. 10.1126/science.aab2276

Mitchison, J.M. (1957). The growth of single cells. I. *Schizosaccharomyces pombe*. Exp. Cell Res. *13*, 244-262.

Mitchison, J.M. (1990). The fission yeast, *Schizosaccharomyces pombe*. Bioessays *12*, 189-191.

Miyoshi, K., Okada, T.N., Siomi, H., and Siomi, M.C. (2009). Characterization of the miRNA-RISC loading complex and miRNA-RISC formed in the *Drosophila* miRNA pathway. RNA *15*, 1282-1291.

Miyoshi, K., Tsukumo, H., Nagami, T., Siomi, H., and Siomi, M.C. (2005). Slicer function of Drosophila Argonautes and its involvement in RISC formation. Genes Dev. *19*, 2837-2848.

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.

Moazed, D. (2009). Small RNAs in transcriptional gene silencing and genome defence. Nature *457*, 413-420.

Moazed, D. (2011). Mechanisms for the inheritance of chromatin states. Cell *146*, 510-518.

Mohammad, F., Mondal, T., Guseva, N., Pandey, G.K., and Kanduri, C. (2010). Kcnq1ot1 noncoding RNA mediates transcriptional gene silencing by interacting with Dnmt1. Development *137*, 2493-2499.

Mohn, F., Handler, D., and Brennecke, J. (2015). piRNA-guided slicing specifies transcripts for Zucchini-dependent, phased piRNA biogenesis. Science *348*, 812-817.

Mohn, F., Sienski, G., Handler, D., and Brennecke, J. (2014). The Rhino-Deadlock-Cutoff complex licenses noncanonical transcription of dual-strand piRNA clusters in *Drosophila*. Cell *157*, 1364-1379.

Montgomery, T.A., Howell, M.D., Cuperus, J.T., Li, D., Hansen, J.E., Alexander, A.L., Chapman, E.J., Fahlgren, N., Allen, E., and Carrington, J.C. (2008). Specificity of ARGONAUTE7-miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation. Cell *133*, 128-141.

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.

Motamedi, M.R., Hong, E.J., Li, X., Gerber, S., Denison, C., Gygi, S., and Moazed, D. (2008). HP1 proteins form distinct complexes and mediate heterochromatic gene silencing by nonoverlapping mechanisms. Mol. Cell *32*, 778-790.

Motamedi, M.R., Verdel, A., Colmenares, S.U., Gerber, S.A., Gygi, S.P., and Moazed, D. (2004). Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs. Cell *119*, 789-802.

Mourrain, P., Béclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.-B., Jouette, D., Lacombe, A.-M., Nikic, S., Picault, N., et al. (2000). Arabidopsis SGS2 and SGS3 genes are required for post-transcriptional gene silencing and natural virus resistance. Cell *101*, 533-542.

Mukherjee, A.S., and Beermann., W. (1965). Synthesis of ribonucleic acid by the X-chromosomes of *Drosophila melanogaster* and the problem of dosage compensation. Nature *207*, 785-786.

Nagano, T., Mitchell, J.A., Sanz, L.A., Pauler, F.M., Ferguson-Smith, A.C., Feil, R., and Fraser, P. (2008). The Air noncoding RNA epigenetically silences transcription by targeting G9a to chromatin. Science *322*, 1717-1720.

Nakanishi, K., Ascano, M., Gogakos, T., Ishibe-Murakami, S., Serganov, A.A., Briskin, D., Morozov, P., Tuschl, T., and Patel, D.J. (2013). Eukaryote-specific insertion elements control human ARGONAUTE slicer activity. Cell Rep. *3*, 1893-1900.

Nakanishi, K., Weinberg, D.E., Bartel, D.P., and Patel, D.J. (2012). Structure of yeast Argonaute with guide RNA. Nature *486*, 368-374.

Nakayama, J., Rice, J.C., Strahl, B.D., Allis, C.D., and Grewal, S.I. (2001). Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science *292*, 110-113.

Nielsen, P.R., Nietlispach, D., Mott, H.R., Callaghan, J., Bannister, A., Kouzarides, T., Murzin, A.G., Murzina, N.V., and Laue, E.D. (2002). Structure of the HP1 chromodomain bound to histone H3 methylated at lysine 9. Nature *416*, 103-107.

Nishimasu, H., Ishizu, H., Saito, K., Fukuhara, S., Kamatani, M.K., Bonnefond, L., Matsumoto, N., Nishizawa, T., Nakanaga, K., Aoki, J., et al. (2012). Structure and function of Zucchini endoribonuclease in piRNA biogenesis. Nature *491*, 284-287.

Noma, K., Sugiyama, T., Cam, H., Verdel, A., Zofall, M., Jia, S., Moazed, D., and Grewal, S.I. (2004). RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing. Nat. Genet. *36*, 1174-1180.

Nonaka, N., Kitajima, T., Yokobayashi, S., Xiao, G., Yamamoto, M., Grewal, S.I., and Watanabe, Y. (2002). Recruitment of cohesin to heterochromatic regions by Swi6/HP1 in fission yeast. Nat. Cell Biol. *4*, 89-93.

Nora, E.P., Lajoie, B.R., Schulz, E.G., Giorgetti, L., Okamoto, I., Servant, N., Piolot, T., van Berkum, N.L., Meisig, J., Sedat, J., et al. (2012). Spatial partitioning of the regulatory landscape of the X-inactivation centre. Nature *485*, 381–385.

Noto, T., Kurth, H.M., Kataoka, K., Aronica, L., DeSouza, L.V., Siu, K.W., Pearlman, R.E., Gorovsky, M.A., and Mochizuki, K. (2010). The Tetrahymena argonaute-binding protein Giw1p directs a mature argonaute-siRNA complex to the nucleus. Cell *140*, 692-703.

Ogawa, Y., and Lee, J.T. (2003). Xite, X-inactivation intergenic transcription elements that regulate the probability of choice. Mol. Cell *11*, 731-743.

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.

Onodera, Y., Haag, J.R., Ream, T., Nunes, P.C., Pontes, O., and Pikaard, C.S. (2005). Plant nuclear RNA polymerase IV mediates siRNA and DNA methylation-dependent heterochromatin formation. Cell *120*, 613-622.

Orgel, L.E. (1968). Evolution of the genetic apparatus. J. Mol. Biol. *38*, 381-393.

Ørom, U.A., and Shiekhattar, R. (2013). Long noncoding RNAs usher in a new era in the biology of enhancers. Cell *154*, 1190-1193.

Pak, J., and Fire, A. (2007). Distinct populations of primary and secondary effectors during RNAi in C. elegans. Science *315*, 241-244.

Pak. J., Maniar, J.M., Mello, C.C., and Fire, A. (2012). Protection from feed-forward amplification in an amplified RNAi mechanism. Cell *151*, 885-899.

Pandey, R.R., Mondal, T., Mohammad, F., Enroth, S., Redrup, L., Komorowski, J., Nagano, T., Mancini-Dinardo, D., and Kanduri, C. (2008). Kcnq1ot1 antisense noncoding RNA mediates

lineage-specific transcriptional silencing through chromatin-level regulation. Mol. Cell *32*, 232-246.

Pare, J.M., LaPointe, P., and Hobman, T.C. (2013). Hsp90 cochaperones p23 and FKBP4 physically interact with hAgo2 and activate RNA interference-mediated silencing in mammalian cells. Mol. Biol. Cell *24*, 2303-2310.

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.

Partridge, J.F., Borgstrom, B., and Allshire, R.C. (2000). Distinct protein interaction domains and protein spreading in a complex centromere. Genes Dev. *14*, 783-791.

Partridge, J.F., DeBeauchamp, J.L., Kosinski, A.M., Ulrich, D.L., Hadler, M.J., and Noffsinger, V.J. (2007). Functional separation of the requirements for establishment and maintenance of centromeric heterochromatin. Mol. Cell *26*, 593-602.

Partridge, J.F., Scott, K.S., Bannister, A.J., Kouzarides, T., and Allshire, R.C. (2002). cis-acting DNA from fission yeast centromeres mediates histone H3 methylation and recruitment of silencing factors and cohesin to an ectopic site. Curr. Biol. *12*, 1652-1660.

Pasquinelli, A.E., Reinhart, B.J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Ball, E.E., Degnan, B., Muller, P., et al. (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature *408*, 86–89.

Penny, G.D., Kay, G.F., Sheardown, S.A., Rastan, S., and Brockdorff N. (1996). Requirement for Xist in X chromosome inactivation. Nature *379*, 131-137.

Petrie, V.J., Wuitschick, J.D., Givens, C.D., Kosinski, A.M., and Partridge, J.F. (2005). RNA interference (RNAi)-dependent and RNAi-independent association of the Chp1 chromodomain protein with distinct heterochromatic loci in fission yeast. Mol. Cell. Biol. *25*, 2331-2346.

Pfaff, J., and Meister, G. (2013). Argonaute and GW182 proteins: an effective alliance in gene silencing. Biochem. Soc. Trans. *41*, 855-860.

Pinter, S.F., Sadreyev, R.I., Yildirim, E., Jeon, Y., Ohsumi, T.K., Borowsky, M., and Lee, J.T. (2012). Spreading of X chromosome inactivation via a hierarchy of defined Polycomb stations. Genome Res. *22*, 1864-1876.

Plath, K., Fang, J., Mlynarczyk-Evans, S.K., Cao, R., Worringer, K.A., Wang, H., de la Cruz, C.C., Otte, A.P., Panning, B., and Zhang, Y. (2003). Role of histone H3 lysine 27 methylation in X inactivation. Science *300*, 131-135.

Pontier, D., Picart, C., Roudier, F., Garcia, D., Lahmy, S., Azevedo, J., Alart, E., Laudié, M., Karlowski, W.M., Cooke, R., et al. (2012). NERD, a plant-specific GW protein, defines an additional RNAi-dependent chromatin-based pathway in *Arabidopsis*. Mol. Cell *48*, 121-132.

Ragunathan, K., Jih, G., and Moazed, D. (2015). Epigenetic inheritance uncoupled from sequence-specific recruitment. Science *348*, 1258699.

Rand, T.A., Petersen, S., Du, F., and Wang, X. (2005). Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. Cell *123*, 621-629.

Ratcliff, F., Harrison, B.D., and Baulcombe, D.C. (1997). A similarity between viral defense and gene silencing in plants. Science *276*, 1558-1560.

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C.P., Allis, C.D., and Jenuwein, T. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature *406*, 593-599.

Reddy, B.D., Wang, Y., Niu, L., Higuchi, E.C., Marguerat, S.B., Bähler, J., Smith, G.R., and Jia, S. (2011). Elimination of a specific histone H3K14 acetyltransferase complex bypasses the RNAi pathway to regulate pericentromeric heterochromatin functions. Genes Dev. *25*, 214-219.

Rehwinkel, J., Behm-Ansmant, I., Gatfield, D., and Izaurralde, E. (2005). A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. RNA *11*, 1640-1647.

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.

Reuter, M., Berninger, P., Chuma, S., Shah, H., Hosokawa, M., Funaya, C., Antony, C., Sachidanandam, R., and Pillai, R.S. (2011). Miwi catalysis is required for piRNA amplification-independent LINE1 transposon silencing. Nature *480*, 264-267.

Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B., and Bartel, D.P. (2002). Prediction of plant microRNA targets. Cell *110*, 513-520.

Richter, L., Bone, J.R., and Kuroda, M.I. (1996). RNA-dependent association of the *Drosophila* maleless protein with the male X chromosome. Genes Cells *1*, 325-336.

Rinn, J.L., Kertesz, M., Wang, J.K., Squazzo, S.L., Xu, X., Brugmann, S.A., Goodnough, L.H., Helms, J.A., Farnham, P.J., Segal, E., and Chang, H.Y. (2007). Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell *129*, 1311-1323.

Rivas, F.V., Tolia, N.H., Song, J.J., Aragon, J.P., Liu, J., Hannon, G.J., and Joshua-Tor, L. (2005). Purified Argonaute2 and an siRNA form recombinant human RISC. Nat. Struct. Mol. Biol. *12*, 340-349.

Robb, G.B., and Rana, T.M. (2007). RNA helicase A interacts with RISC in human cells and functions in RISC loading. Mol. Cell *26*, 523-537.

Robertson, M.P., and Joyce, G.F. (2012). The origins of the RNA world. Cold Spring Harb. Perspect. Biol. *4*, a:003608.

Rocheleau, C.E., Downs, W.D., Lin, R., Wittmann, C., Bei, Y., Cha, Y.H., Ali, M., Priess, J.R., and Mello, C.C. (1997). Wnt signaling and an APC-related gene specify endoderm in early *C. elegans* embryos. Cell *90*, 707-716.

Rogers, K., and Chen, X. (2013). Biogenesis, turnover, and mode of action of plant microRNAs. Plant Cell *25*, 2383-2399.

Rougemaille, M., Braun, S., Coyle, S., Dumesic, P.A., Garcia, J.F., Isaac, R.S., Libri, D., Narlikar, G.J., Madhani, H.D. (2012). Ers1 links HP1 to RNAi. Proc. Natl. Acad. Sci. USA *109*, 11258-11263.

Rougemaille, M., Shankar, S., Braun, S., Rowley, M., and Madhani, H.D. (2008). Ers1, a rapidly diverging protein essential for RNA interference-dependent heterochromatic silencing in *Schizosaccharomyces pombe*. J. Biol. Chem. *283*, 25770-25773.

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

Ruby, J.G., Jan, C., Player, C., Axtell, M.J., Lee, W., Nusbaum, C., Ge, H., and Bartel, D.P. (2006). Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in C. elegans. Cell *127*, 1193-1207.

Sadaie, M., Iida, T., Urano, T., and Nakayama, J. (2004). A chromodomain protein, Chp1, is required for the establishment of heterochromatin in fission yeast. EMBO J. *23*, 3825-3835.

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

Schalch, T., Job, G., Noffsinger, V.J., Shanker, S., Kuscu, C., Joshua-Tor, L., and Partridge, J.F. (2009). High-affinity binding of Chp1 chromodomain to K9 methylated histone H3 is required to establish centromeric heterochromatin. Mol. Cell *34*, 36-46.

Schalch, T., Job, G., Shanker, S., Partridge, J.F., and Joshua-Tor, L. (2011). The Chp1-Tas3 core is a multifunctional platform critical for gene silencing by RITS. Nat. Struct. Mol. Biol. *18*, 1351-1357.

Schirle, N.T., and MacRae, I.J. (2012). The crystal structure of human Argonaute2. Science *336*, 1037-1040.

Schirle, N.T., Sheu-Gruttadauria, J., and MacRae, I.J. (2014). Structural basis for microRNA targeting. Science *346*, 608-613.

Schmitz-Linneweber, C., Lampe, M.K., Sultan, L.D., Ostersetzer-Biran, O. (2015). Organellar maturases: A window into the evolution of the spliceosome. Biochim. Biophys. Acta *1847*, 798-808.

Schrödinger, E. (1944). What is Life? (ch. 6 “Order, Disorder, and Entropy”). Cambridge, United Kingdom: Cambridge University Press.

Schwarz, D.S., Hutvágner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P.D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. Cell *115*, 199-208.

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.

Seth, M., Shirayama, M., Gu, W., Ishidate, T., Conte, D., Jr., and Mello, C.C. (2013). The *C. elegans* CSR-1 Argonaute pathway counteracts epigenetic silencing to promote germline gene expression. Dev. Cell. *27*, 656-663.

Shalem, O., Sanjana, N.E., Hartenian, E., Shi, X., Scott, D.A., Mikkelsen, T.S., Heckl, D., Ebert, B.L., Root, D.E., Doench, J.G., et al. (2014). Genome-scale CRISPR-Cas9 knockout screening in human cells. Science *343*, 84-87.

Shanker, S., Job, G., George, O.L., Creamer, K.M., Shaban, A., and Partridge, J.F. (2010). Continuous requirement for the Clr4 complex but not RNAi for centromeric heterochromatin assembly in fission yeast harboring a disrupted RITS complex. PLoS Genet. *6*, e1001174.

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

Shirayama, M., Seth, M., Lee, H.C., Gu, W., Ishidate, T., Conte, D., Jr., and Mello, C.C. (2012). piRNAs initiate an epigenetic memory of nonself RNA in the C. elegans germline. Cell *150*, 65-77.

Sienski, G., Dönertas, D., and Brennecke, J. (2012). Transcriptional silencing of transposons by Piwi and maelstrom and its impact on chromatin state and gene expression. Cell *151*, 964-980.

Sijen, T., Fleenor, J., Simmer, F., Thijssen, K.L., Parrish, S., Timmons, L., Plasterk, R.H., and Fire, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. Cell *107*, 465-476.

Sijen, T., Steiner, F.A., Thijssen, K.L., and Plasterk, R.H. (2007). Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. Science *315*, 244-247.

Simmer, F., Buscaino, A., Kos-Braun, I.C., Kagansky, A., Boukaba, A., Urano, T., Kerr, A.R., and Allshire, R.C. (2010). Hairpin RNA induces secondary small interfering RNA synthesis and silencing in trans in fission yeast. EMBO Rep. *11*, 112-118.

Simon, M.D., Pinter, S.F., Fang, R., Sarma, K., Rutenberg-Schoenberg, M., Bowman, S.K., Kesner, B.A., Maier, V.K., Kingston, R.E., and Lee, J.T. (2013). High-resolution Xist binding maps reveal two-step spreading during X-chromosome inactivation. Nature *504*, 465-469.

Slotkin, R.K., and Martienssen, R. (2007). Transposable elements and the epigenetic regulation of the genome. Nat. Rev. Genet. *8*, 272-285.

Smardon, A., Spoerke, J., Stacey, S., Klein, M., Mackin, N., and Maine, E. (2000). EGO-1 is related to RNA-directed RNA polymerase and functions in germline development and RNA interference in *C. elegans*. Curr. Biol. *10*, 169-178.

Smibert, P., Yang, J., Azzam, G., Liu, J., and Lai, E.C. (2013). Homeostatic control of Argonaute stability by microRNA availability. Nat. Struct. Mol. Biol. *20*, 789-795.

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.

Steiner, F.A., Okihara, K.L., Hoogstrate, S.W., Sijen, T., and Ketting, R.F. (2009). RDE-1 slicer activity is required only for passenger-strand cleavage during RNAi in Caenorhabditis elegans. Nat. Struct. Mol. Biol. *16*, 207-211.

Steitz, T.A., and Moore, P.B. (2003). RNA, the first macromolecular catalyst: the ribosome is a ribozyme. Trends Biochem. Sci. *28*, 411-418.

Stuckenholz, C., Kageyama, Y., and Kuroda, M.I. (1999). Guilt by assocation: non-coding RNAs, chromosmome-specific proteins and dosage compensation in *Drosophila*. Trends Genet. *15*, 454-458.

Sugiyama, T., Cam, H., Verdel, A., Moazed, D., and Grewal, S.I. (2005). RNA-dependent RNA polymerase is an essential component of a self-enforcing loop coupling heterochromatin assembly to siRNA production. Proc. Natl. Acad. Sci. USA *102*, 152-157.

Sun, S., Del Rosario, B.C., Szanto, A., Ogawa, Y., Jeon, Y., and Lee, J.T. (2013). Jpx RNA activates Xist by evicting CTCF. Cell *153*, 1537-1551.

Sural, T.H., Peng, S., Li, B., Workman, J.L., Park, P.J., and Kuroda, M.I. (2008). The MSL3 chromodomain directs a key targeting step for dosage compensation of the *Drosophila melanogaster* X chromosome. Nat. Struct. Mol. Biol. *15*, 1318-1325.

Swiezewski, S., Liu, F., Magusin, A., and Dean, C. (2009). Cold-induced silencing by long antisense transcripts of an *Arabidopsis* Polycomb target. Nature *462*, 799-802.

Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., and Mello, C.C. (1999). The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*. Cell *99*, 123-132.

Tabara, H., Yigit, E., Siomi, H., and Mello, C.C. (2002). The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in C. elegans. Cell *109*, 861-871.

Tadeo, X., Wang, J., Kallgren, S.P., Liu, J., Reddy, B.D., Qiao, F., Jia, S. (2013). Elimination of shelterin components bypasses RNAi for pericentromeric heterochromatin assembly. Genes Dev. *27*, 2489-2499.

Takahashi, K., Murakami, S., Chikashige, Y., Funabiki, H.. Niwa, O., and Yanagida, M. (1992). A low copy number central sequence with strict symmetry and unusual chromatin structure in the fission yeast centromere. Mol. Biol. Cell. *3*, 819-835.

Tam, O.H., Aravin, A.A., Stein, P., Girard, A., Murchison, E.P., Cheloufi, S., Hodges, E., Anger, M., Sachidanandam, R., Schultz, R.M., and Hannon, G.J. (2008). Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes. Nature *453*, 534–538.

Theobald, D.L. (2011). A formal test of the theory of universal common ancestry. Nature *465*, 219-222.

Thivierge, C., Makil, N., Flamand, M., Vasale, J.J., Mello, C.C., Wohlschlegel, J., Conte, D., Jr., and Duchaine, T.F. (2012). Tudor domain ERI-5 tethers an RNA-dependent RNA polymerase to DCR-1 to potentiate endo-RNAi. Nat. Struct. Mol. Biol. *19*, 90-97.

Thon, G., Cohen, A., and Klar, A.J.S. (1994). Three additional linkage groups that repress transcription and meiotic recombination in the mating-type region of *Schizosaccharomyces pombe.* Genetics *138*, 29-38.

Thon, G., and Klar, A. J. S. (1992). The *clr1* locus regulates the expression of the cryptic mating-type loci in fission yeast. Genetics *737*, 287-296.

Thon, G., and Verhein-Hansen, J. (2000). Four chromo-domain proteins of *Schizosaccharomyces pombe* differentially repress transcription at various chromosomal locations. Genetics *155*, 551-568.

Tian, D., Sun, S., and Lee, J.T. (2010). The long noncoding RNA, Jpx, is a molecular switch for X chromosome inactivation. Cell *143*, 390-403.

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.

Tomari, Y., Du, T., Haley, B., Schwarz, D.S., Bennett, R., Cook, H.A., Koppetsch, B.S., Theurkauf, W.E., and Zamore, P.D. (2004a). RISC assembly defects in the Drosophila RNAi mutant armitage. Cell 116, 831-841.

Tomari, Y., Du, T., and Zamore, P.D. (2007). Sorting of Drosophila small silencing RNAs. Cell *130*, 299-308.

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

Tsai, C.L., Rowntree, R.K., Cohen, D.E., and Lee, J.T. (2008). Higher order chromatin structure at the X-inactivation center via looping DNA. Dev. Biol. *319*, 416-425.

Tsai, M.C., Manor, O., Wan, Y., Mosammaparast, N., Wang, J.K., Lan, F., Shi, Y., Segal, E., and Chang, H.Y. (2010). Long noncoding RNA as modular scaffold of histone modification complexes. Science *329*, 689-693.

Tsai, H., Chen, C.C., Conte, D., Moresco, J.J., Chaves, D.A., Mitani, S., Yates, J.R., Tsai, M., and Mello, C.C. (2015). A ribonuclease coordinates siRNA amplification and mRNA cleavage during RNAi. Cell *160*, 407-419.

Tuschl, T., Zamore, P.D., Lehmann, R., Bartel, D.P., and Sharp, P.A. (1999). Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev. *13*, 3191-3197.

Ulitsky, I., and Bartel, D.P. (2013). lincRNAs: genomics, evolution, and mechanisms. Cell *154*, 26-46.

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.

Vallot, C., Huret, C., Lesecque, Y., Resch, A., Oudrhiri, N., Bennaceur-Griscelli, A., Duret, L., and Rougeulle, C. (2013). XACT, a long noncoding transcript coating the active X chromosome in human pluripotent cells. Nat. Genet. *45*, 239-241.

van Dijk, E.L., Chen, C.L., d'Aubenton-Carafa, Y., Gourvennec, S., Kwapisz, M., Roche, V., Bertrand, C., Silvain, M., Legoix-Né, P., Loeillet, S., et al. (2011). XUTs are a class of Xrn1-sensitive antisense regulatory non-coding RNA in yeast. Nature *475*, 114-117.

Vasiljeva, L., Kim, M., Terzi, N., Soares, L.M., and Buratowski, S. (2008). Transcription termination and RNA degradation contribute to silencing of RNA polymerase II transcription within heterochromatin. Mol. Cell *29*, 313-323.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S.I., and Moazed, D. (2004). RNAi-mediated targeting of heterochromatin by the RITS complex. Science *303*, 672-676.

Volpe, T.A., Kidner, C., Hall, I.M., Teng, G., Grewal, S.I., and Martienssen, R.A. (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science *297*, 1833-1837.

Wang, B., Li, S., Qi, H., Chowdhury, D., Shi, Y., and Novina, C. (2009a). Distinct passenger strand and mRNA cleavage activities of human Argonaute proteins. Nat. Struct. Mol. Biol. *16*, 1259-1266.

Wang, K.C., Yang, Y.W., Liu, B., Sanyal, A., Corces-Zimmerman, R., Chen, Y., Lajoie, B.R., Protacio, A., Flynn, R.A., Gupta, R.A., et al. (2011). A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression. Nature *472*, 120-124.

Wang, Y., Juranek, S., Li, H., Sheng, G., Tuschl, T., and Patel, D.J. (2008a). 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. (2009b). 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. (2008b). Structure of the guide-strand-containing argonaute silencing complex. Nature *456*, 209-213.

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.

Waterhouse, P.M., Graham, M.W., and Wang, M.B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc. Natl. Acad. Sci. USA *95*, 13959-13964.

Wedeles, C.J., Wu, M.Z., and Claycomb, J.M. (2013). Protection of germline gene expression by the C. elegans Argonaute CSR-1. Dev. Cell *27*, 664–671.

Wery, M., Kwapisz, M., and Morillon, A. (2011). Noncoding RNAs in gene regulation. Wiley Interdiscip. Rev. Syst. Biol. Med. *3*, 728-738.

Wierzbicki, A.T., Ream, T.S., Haag, J.R., and Pikaard, C.S. (2009). RNA polymerase V transcription guides ARGONAUTE4 to chromatin. Nat. Genet. *41*, 630-634.

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.

Winkler, W., Nahvi, A., and Breaker, R.R. (2002). Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. Nature *419*, 952-956.

Woehrer, S.L., Aronica, L., Suhren, J.H., Busch, C.J., Noto, T., and Mochizuki, K. (2015). A *Tetrahymena* Hsp90 co-chaperone promotes siRNA loading by ATP-dependent and ATP-independent mechanisms. EMBO J. *34*, 559-577.

Woolcock, K.J., Stunnenberg, R., Gaidatzis, D., Hotz, H.R., Emmerth, S., Barraud, P., and Bühler, M. (2012). RNAi keeps Atf1-bound stress response genes in check at nuclear pores. Genes Dev. *26*, 683-692.

Wyers, F., Rougemaille, M., Badis, G., Rousselle, J.C., Dufour, M.E., Boulay, J., Regnault, B., Devaux, F., Namane, A., Seraphin, B., et al. (2005). Cryptic pol II transcripts are degraded by a nuclear quality control pathway involving a new poly(A) polymerase. Cell *121,* 725-737.

Xiol, J., Cora, E., Koglgruber, R., Chuma, S., Subramanian, S., Hosokawa, M., Reuter, M., Yang, Z., Berninger, P., Palencia, A., et al. (2012). A role for Fkbp6 and the chaperone machinery in piRNA amplification and transposon silencing. Mol. Cell *47*, 970-979.

Xiol, J., Spinelli, P., Laussmann, M.A., Homolka, D., Yang, Z., Cora, E., Couté, Y., Conn, S., Kadlec, J., Sachidanandam, R., et al. (2014). RNA clamping by Vasa assembles a piRNA amplifier complex on transposon transcripts. Cell *157*, 1698-1711.

Xue, Z., Yuan, H., Guo, J., and Liu, Y. (2012). Reconstitution of an Argonaute-dependent small RNA biogenesis pathway reveals a handover mechanism involving the RNA exosome and the exonuclease QIP. Mol. Cell *46*, 299-310.

Yamanaka, S., Mehta, S., Reyes-Turcu, F.E., Zhuang, F., Fuchs, R.T., Rong, Y., Robb, G.B., and Grewal, S.I. (2013). RNAi triggered by specialized machinery silences developmental genes and retrotransposons. Nature *493*, 557-560.

Yamashita, A., Chang, T.C., Yamashita, Y., Zhu, W., Zhong, Z., Chen, C.Y., and Shyu, A.B. (2005). Concerted action of poly(A) nucleases and decapping enzyme in mammalian mRNA turnover. Nat. Struct. Mol. Biol. *12*, 1054-1063.

Yang, L., Froberg, J.E., and Lee, J.T. (2014). Long noncoding RNAs: fresh perspectives into the RNA world. Trends Biochem. Sci. *39*, 35-43.

Ye, R., Wang, W., Iki, T., Liu, C., Wu, Y., Ishikawa, M., Zhou, X., and Qi, Y. (2012). Cytoplasmic assembly and selective nuclear import of *Arabidopsis* Argonaute4/siRNA complexes. Mol. Cell *46*, 859-870.

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.

Yoda, M., Cifuentes, D., Izumi, N., Sakaguchi, Y., Suzuki, T., Giraldez, A.J., and Tomari, Y. (2013). Poly(A)-specific ribonuclease mediates 3'-end trimming of Argonaute2-cleaved precursor microRNAs. Cell Rep. *5*, 715-726.

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.

Yu, R., Jih, G., Iglesias, N., and Moazed, D. (2014). Determinants of heterochromatic siRNA biogenesis and function. Mol. Cell *53*, 262-276.

Zamore, P.D. (2001). RNA interference: listening to the sound of silence. Nat. Struct. Biol. *8*, 746-750.

Zamore, P.D. (2006). RNA interference: big applause for silencing in Stockholm. Cell *127*, 1083-1086.

Zamore, P.D., Tuschl, T., Sharp, P.A., and Bartel, D.P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell *101*, 25-33.

Zekri, L., Huntzinger, E., Heimstädt, 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.

Zekri, L., Kuzuoğlu-Öztürk, D., and Izaurralde, E. (2013). GW182 proteins cause PABP dissociation from silenced miRNA targets in the absence of deadenylation. EMBO J. *32*, 1052-1065.

Zhang, K., Mosch, K., Fischle, W., and Grewal, S.I. (2008). Roles of the Clr4 methyltransferase complex in nucleation, spreading and maintenance of heterochromatin. Nat. Struct. Mol. Biol. *15*, 381-388.

Zhang, Z., Wang, J., Schultz, N., Zhang, F., Parhad, S.S., Tu, S., Vreven, T., Zamore, P.D., Weng, Z., and Theurkauf, W.E. (2014). The HP1 homolog rhino anchors a nuclear complex that suppresses piRNA precursor splicing. Cell *157*, 1353-1363.

Zhao, J., Ohsumi, T.K., Kung, J.T., Ogawa, Y., Grau, D.J., Sarma, K., Song, J.J., Kingston, R.E., Borowsky, M., and Lee, J.T. (2010). Genome-wide identification of polycomb-associated RNAs by RIP-seq. Mol. Cell *40*, 939-953.

Zhao, J., Sun, B.K., Erwin, J.A., Song, J.J., and Lee, J.T. (2008). Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. Science *322*, 750-756.

Zheng, K., Xiol, J., Reuter, M., Eckardt, S., Leu, N.A., McLaughlin, K.J., Stark, A., Sachidanandam, R., Pillai, R.S., and Wang, P.J. (2010). Mouse MOV10L1 associates with Piwi proteins and is an essential component of the Piwi-interacting RNA (piRNA) pathway. Proc. Natl. Acad. Sci. USA *107*, 11841-11846.

Zhong, X., Du, J., Hale, C.J., Gallego-Bartolome, J., Feng, S., Vashisht, A.A., Chory, J., Wohlschlegel, J.A., Patel, D.J., and Jacobsen, S.E. (2014). Molecular mechanism of action of plant DRM *de novo* DNA methyltransferases. Cell *157*, 1050-1060.

Chapter 2

ARC is Required for Loading Small RNAs into Argonaute in Fission Yeast

Daniel Holoch and Danesh Moazed

Chapter 2

This chapter consists primarily of a portion of a published report:

Holoch, D., and Moazed, D. (2015). Small-RNA loading licenses Argonaute for assembly into a transcriptional silencing complex. Nat. Struct. Mol. Biol. *22*, 328-335.

All experiments were conducted by Daniel Holoch. The original manuscript was written by Daniel Holoch and Danesh Moazed. The introduction, results and discussion were modified for this dissertation, and some unpublished data have been added.

I. Introduction

Small RNAs embody the common mechanistic elements of a broad category of gene silencing pathways (reviewed in Moazed, 2009). In classical RNA interference, foreign double-stranded RNA (dsRNA) is processed by a Dicer ribonuclease into short RNA segments; these are in turn loaded into Argonaute proteins which repress cellular RNAs containing complementary sequences (reviewed in Zamore, 2006). This response to exogenous dsRNA reflects the existence of related endogenous systems that regulate the genome using the same or similar molecular machineries. For example, taxonomically diverse organisms rely on Argonautes loaded with Dicer-dependent small RNAs known as microRNAs to control the expression of protein-coding genes in ways that are often critical for development and other fundamental processes (Friedman et al., 2009; reviewed in Ghildiyal and Zamore, 2009). The function of small RNAs that is most deeply conserved, however, is to defend the genome against parasitic or repetitive DNA elements that pose threats to its stability (Olovnikov et al., 2013; reviewed in Malone and Hannon, 2009; Moazed, 2009). Small RNAs that fulfill this task include Piwi-interacting RNAs in animal germlines and endogenous small interfering RNAs (siRNAs) in animals, plants, fungi and protozoans.

In the fission yeast *Schizosaccharomyces pombe*, endogenous siRNAs preserve genome stability by repressing the *dg* and *dh* repeat sequences that flank each of the three centromeres (Reinhart and Bartel, 2002; Verdel et al., 2004; Volpe et al., 2002). The siRNA-directed assembly of heterochromatin at these pericentromeric regions is crucial for proper centromere function and protects chromosomes from unequal recombination events (Bernard et al., 2001; Ellermeier et al., 2010; Nonaka et al., 2002). Biogenesis of *dg* and *dh* siRNAs requires dsRNA synthesis by the RNA-directed RNA polymerase complex RDRC and coupled processing by the Dicer enzyme Dcr1 (Colmenares et al., 2007; Kawakami et al., 2012; Motamedi et al., 2004; Yu et al., 2014). The RNA-induced transcriptional silencing (RITS) complex, which contains the Argonaute protein Ago1, uses these *dg* and *dh* siRNAs as guides

for the recognition of complementary nascent transcripts associated with pericentromeric chromatin (Bühler et al., 2006; Motamedi et al., 2004; Verdel et al., 2004). Finally, through direct protein-protein interactions, RITS recruits the Clr4-Rik1-Cul4 (CLRC) complex, which methylates nucleosomes on lysine 9 of histone H3 (H3K9) (Bayne et al., 2010; Gerace et al., 2010; Hong et al., 2005; Zhang et al., 2008). This modification is extensively conserved among eukaryotes as a signal for the assembly of silent chromatin (Bannister et al., 2001; Lachner et al., 2001; Nakayama et al., 2001).

RITS contains not only Ago1, but also the chromodomain protein Chp1, whose binding to methylated H3K9 is important for localization of the complex to heterochromatic regions of the genome, and Tas3, a protein bearing an evolutionarily recurring Argonaute-interacting domain consisting of glycine-tryptophan repeats (Noma et al., 2004; Partridge et al., 2007; Schalch et al., 2009; Till et al., 2007; Verdel et al., 2004). Chapter 3 will report our recent findings on the regulation of RITS complex assembly. Other aspects of RITS function, in particular its coordination of RDRC- and Dcr1-dependent siRNA biogenesis with CLRC-mediated heterochromatin formation, have been studied thoroughly.

Far less understood is the molecular role of the second Ago1-containing complex, which consists of Ago1 and two novel proteins called Arb1 and Arb2 (Buker et al., 2007). Mass spectrometry analyses and co-immunoprecipitation assays consistently suggest that the Ago1-Arb1-Arb2 complex is entirely separate from RITS (Buker et al., 2007; Verdel et al., 2004). Consistent with this notion, immunofluorescence experiments show that Arb1 and Chp1 occupy distinct nuclear foci, while chromatin immunoprecipitation and DamID data indicate that, unlike the subunits of RITS, Arb1 and Arb2 are not associated with the pericentromeric *dg* and *dh* repeats or with any other genomic regions (Buker et al., 2007; Woolcock et al., 2012). Moreover, ARC does not interact with the nascent *dg* and *dh* transcripts targeted by the RITS complex (D.H. and D.M., unpublished data, Appendix 1). Another difference between RITS and the Ago1-Arb1-Arb2 complex is that the latter associates exclusively with double-stranded

siRNAs, which are the immediate products of Dcr1 catalysis, whereas the siRNAs bound to RITS are predominantly single-stranded (Buker et al., 2007). A related observation is that Arb1 inhibits the small-RNA-guided endonuclease or “slicer” activity of Ago1 in an *in vitro* reaction, and that, as in other systems involving perfectly complementary small RNA duplexes, slicing of the non-guide or “passenger” strand is required for its release from Argonaute in *S. pombe* (Buker et al., 2007; Matranga et al., 2005; Rand et al., 2005). Together, these observations suggest that Arb1 maintains the siRNAs within the Ago1-Arb1-Arb2 complex in their original double-stranded form by preventing passenger-strand slicing by Ago1; this complex is therefore termed the Argonaute siRNA chaperone (ARC) complex (Buker et al., 2007).

Arb1 and Arb2 are each required for H3K9 methylation and heterochromatic silencing of the *dg* and *dh* pericentromeric repeats (Buker et al., 2007). At first glance, it is unclear why this should be the case. By delaying the formation of a mature single-stranded siRNA capable of guiding Ago1 to a complementary target, and by limiting the access of Ago1 to chromatin, ARC appears to act as a negative regulator of Ago1 function (Buker et al., 2007). Thus, the requirement for the ARC subunits in pericentromeric heterochromatin assembly is likely a sign that they possess one or more additional molecular activities which have not yet been uncovered.

The primary sequences of Arb1 and Arb2 do not provide clear hints regarding their functions. Arb1 harbors a domain in its C terminus that is homologous to maturases, enzymes that assist in the self-splicing of group II introns in bacteria, mitochondria and chloroplasts, and are considered the distant relatives of eukaryotic nuclear splicing factors (Buker et al., 2007; Schmitz-Linneweber et al., 2015). A more widely conserved domain is found in the N terminus of Arb2, but its functional properties remain unknown (Buker et al., 2007). Proteins bearing these features have not previously been identified as regulators of Argonaute function in other organisms.

The resemblance of ARC-bound siRNAs to the initial products of the Dcr1 enzyme suggests that Ago1 molecules carrying a specific siRNA begin in ARC and progress to RITS. This supposition raises the possibility that Arb1 and Arb2 might act to receive siRNAs from Dcr1 and introduce them into the small-RNA-binding groove of Ago1, in a manner analogous to the *Drosophila* R2D2 and the mammalian TRBP (Gregory et al., 2005; Liu et al., 2003). Four lines of evidence have argued against this type of scenario. First, unlike R2D2 and TRBP, which contain dsRNA-binding regions that are critical for their functions, Arb1 and Arb2 apparently lack such domains (Buker et al., 2007). Second, unlike R2D2 and TRBP, whose interactions with Dicer enzymes are integral to their Argonaute-loading activities, mass spectrometry analyses of proteins natively interacting with Arb1 or Dcr1 have shown no evidence of a stable assocation between either Arb1 or Arb2 and Dcr1 (Buker et al., 2007; Colmenares et al., 2007). Third, a high-throughput sequencing study found substantial levels of pericentromeric siRNAs associated with an overexpressed Ago1 protein in cells lacking either Arb1 or Arb2, suggesting that these factors are dispensable for Argonaute loading (Halic and Moazed, 2010). Fourth, a Northern blot comparing small RNAs from Tas3-TAP and Arb1-TAP immunoprecipitates indicated that ARC associates with vastly lower levels of *dh* siRNAs, which is difficult to reconcile with a possible role of Arb1 and Arb2 in loading siRNAs into Ago1 (Buker et al., 2007). However, a subsequent examination has revealed that *arb1-TAP* is a hypomorphic allele that leads to moderately impaired pericentromeric silencing and a dramatic reduction in the cellular level of *dh* siRNAs. In contrast, a *TAP-arb1* strain exhibits wild-type heterochromatic silencing and a high level of both *dg* and *dh* siRNAs (D.H. and D.M., unpublished data, Appendix 2). Thus, the earlier result suggesting that ARC does not interact with siRNAs was likely the simple consequence of a defect in siRNA biogenesis.

The hypothesis that ARC orchestrates the loading of pericentromeric siRNAs into Ago1, and that its subunits are required for pericentromeric silencing for this specific reason, therefore merits further consideration. Consistent with this idea, we find that cellular ARC associates with

a population of small RNAs bearing several characteristics of Dicer products and that Arb1 is required for loading of small RNA duplexes into immunopurified Ago1 *in vitro*. Furthermore, high-throughput sequencing of Argonaute-associated small RNAs reveals that *bona fide* small RNA loading is abolished in cells lacking Arb1 or Arb2, but not Dicer. Finally, we show that Argonaute overexpression partially restores pericentromeric silencing in ARC-deficient cells, but that this rescuing activity is absent in mutant Argonaute proteins that have a lower binding

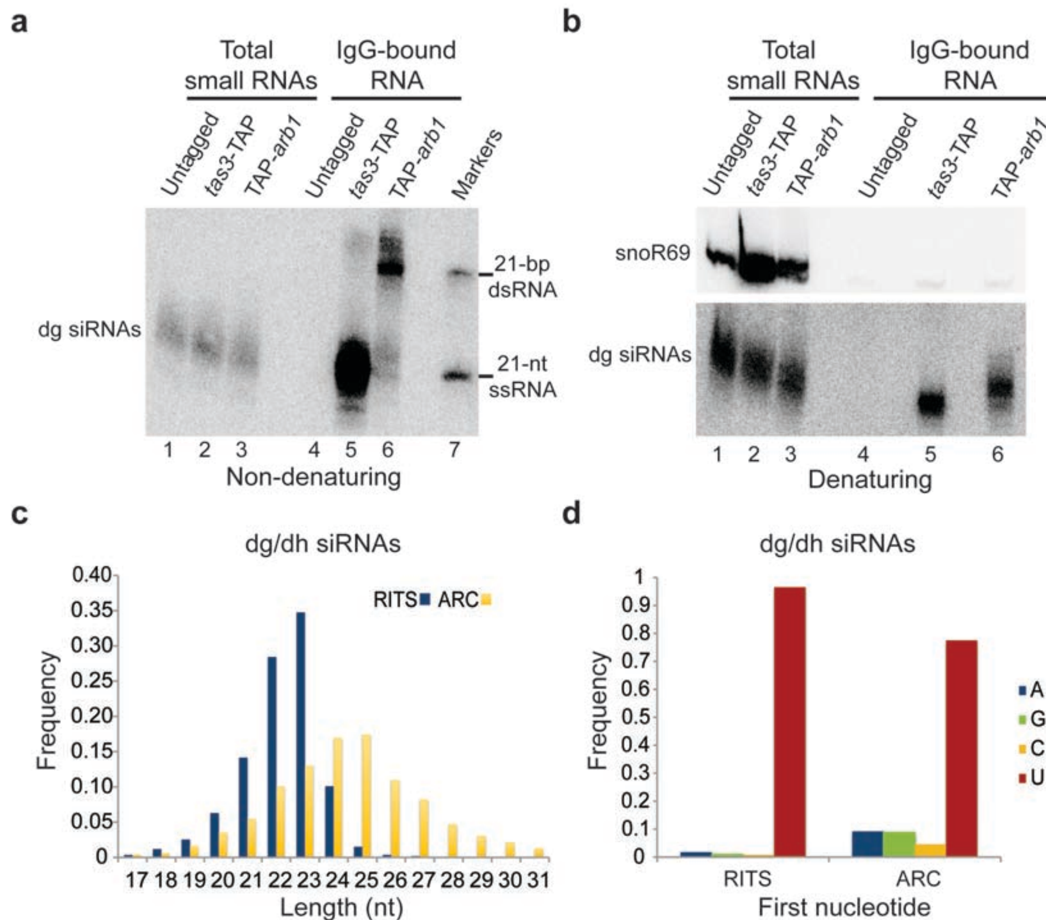

Figure 2.1. ARC binds small RNAs *in vivo* that bear features of Dcr1-generated duplexes.
(**a**) Non-denaturing and (**b**) denaturing Northern blot analyses of *dg* siRNAs contained in RITS and ARC complex purifications. Shown are total small RNA fractions and separately prepared RNA extracted from one-step TAP purifications from cells of the indicated genotypes. Panels (**a**) and (**b**) each represent an independent set of cell cultures. (**c**) Reads from high-throughput sequencing analysis of small RNAs co-purifying with Tas3-TAP and TAP-Arb1 mapping to the *dg* and *dh* repeats are plotted according to their length. (**d**) The percentage of *dg* and *dh* reads beginning with each of the four nucleotides is shown for each complex.

Table 2.1. Number of aligned small RNA reads for each sequenced strain.

Genotype		Aligned reads (millions)
tas3-TAP		19.6
	(without tRNAs, Figure 2.2)	19.1
	(*dg*/*dh* siRNAs, Figure 2.1)	8.6
TAP-*arb1*		19.5
	(without tRNAs, Figure 2.2)	10.2
	(*dg*/*dh* siRNAs, Figure 2.1)	3.9
3xFLAG-*ago1 wild-type* (Figure 2.5)		70.4
ago1$^+$, *trp1*$^+$*::*3xFLAG-*ago1*-F276A Y513A K517A		10.6
3xFLAG-*ago1 dcr1Δ*		27.2
3xFLAG-*ago1 arb1Δ* (Figure 2.5)		10.2
3xFLAG-*ago1 dcr1Δ arb1Δ*		26.8
3xFLAG-*ago1 wild-type* (Figure 2.6)		30.3
ago1Δ, *trp1*$^+$*::*3xFLAG-*ago1*-F276A R773E		33.5
3xFLAG-*ago1 arb1Δ* (Figure 2.6)		34.5
3xFLAG-*ago1 arb2Δ*		35.8

affinity for small RNAs. Collectively, our results identify ARC as the machinery responsible for siRNA loading in *S. pombe*. Thus, Arb1 and Arb2 appear to represent a novel type of loading apparatus whose mechanism of action is likely distinct from that of other known factors with analogous roles.

II. Results

A. ARC Associates with Small RNAs Bearing Features of Dicer-Generated Duplex siRNAs

Experimental results that will be described in Chapter 3 of this dissertation demonstrate that RITS complex assembly is abolished both when the small-RNA loading activity of Ago1 is compromised and upon deletion of either *arb1*$^+$ or *arb2*$^+$. This suggests ARC may regulate RITS assembly by loading small RNAs into Ago1. To test this hypothesis, we first determined the

nature of small RNAs bound respectively to ARC and RITS *in vivo*. We isolated RNA associated with each complex by purifying Tas3-TAP and TAP-Arb1 and performed Northern blot analysis. Confirming our previous data (Buker et al., 2007), a Northern blot carried out under non-denaturing conditions showed that *dg* repeat siRNAs bound to ARC were double-stranded, suggesting that ARC binds duplex siRNAs generated by Dcr1 (Figure 2.1a, lane 6). Intriguingly, a standard denaturing blot showed the ARC-bound *dg* siRNAs to be discernibly longer than those bound to RITS (Figure 2.1b, compare lanes 5 and 6). This is consistent with the idea that they are products of the Dicer ribonuclease, which in *S. pombe* lacks a PAZ domain and consequently produces siRNAs of varying sizes that are subsequently pared by exonucleolytic trimming (Colmenares et al., 2007; Halic and Moazed, 2010, Marasovic et al., 2013). We next examined small RNAs bound to Tas3-TAP and TAP-Arb1 by high-throughput sequencing and found similar populations of pericentromeric reads in the two libraries (Table 2.1 and Figure 2.2). A plot of their length distributions showed that siRNAs mapping throughout the *dg* and *dh* repeats were longer, and displayed a broader size distribution, when bound to ARC than when bound to RITS (Figure 2.1c). Finally, a considerable fraction of *dg* and *dh* small RNA reads from the ARC library did not begin with the 5' uridine typical of Ago1 guide RNAs, unlike their RITS counterparts (Figure 2.1d), which is suggestive of the presence of siRNA passenger strands in ARC.

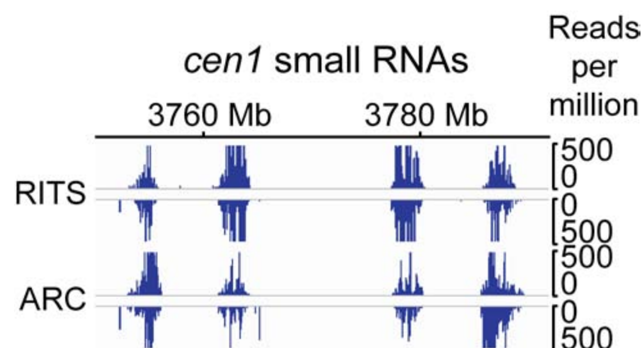

Figure 2.2. RITS and ARC bind populations of pericentromeric small RNAs with similar sequences. Tracks showing the normalized numbers of reads mapping to the *dg* and *dh* repeats flanking the centromere of chromosome 1, for small RNAs co-purifying with Tas3-TAP (RITS) or TAP-Arb1 (ARC), excluding tRNAs.

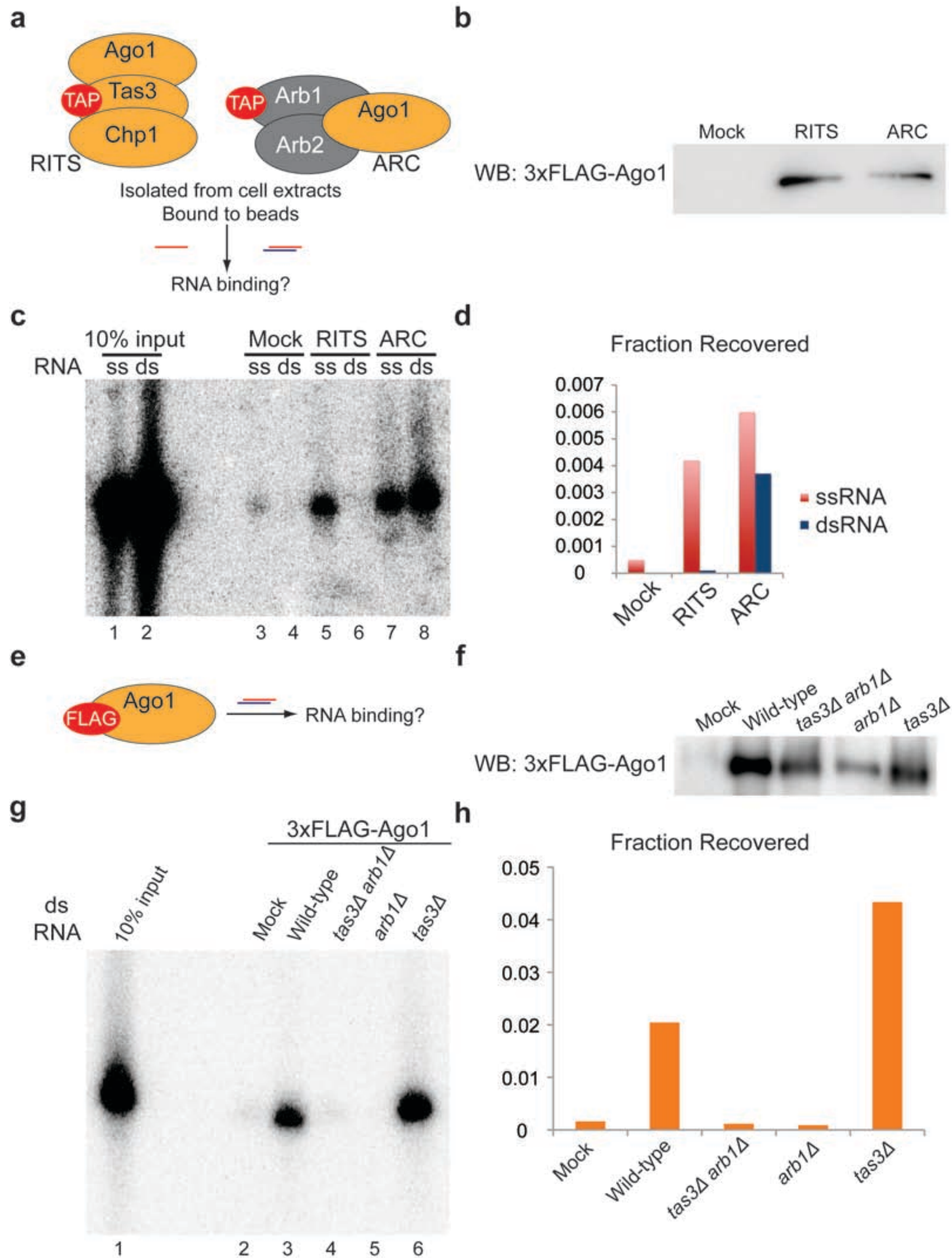

Figure 2.3. Immunopurified Ago1 binds duplex small RNAs *in vitro* in an Arb1-dependent manner. (**a**) Schematic of *in vitro* RNA binding assay. See text for detailed description. (**b**) Western blot showing relative abundance of 3xFLAG-Ago1 in aliquots of beads equal to those used in the binding assay. (**c**) Phosphorimager scan of eluted RNAs after *in vitro* binding to immobilized complexes. Shown is one of two technical replicates. (**d**) Quantification by densitometry of the results shown in (**c**). (**e**) Schematic of *in vitro* assay: Similar to (**a-d**), but using 3xFLAG-Ago1, instead of complexes. See text for detailed description. (**f**) Western blot showing relative abundance of 3xFLAG-Ago1 in aliquots equal to one-eighth of those used in the binding assay. (**g**) Phosphorimager scan of eluted RNAs after *in vitro* binding to immobilized 3xFLAG-Ago1 purified from the indicated wild-type and mutant cells. (**h**) Quantification by densitometry of the results shown in (**g**).

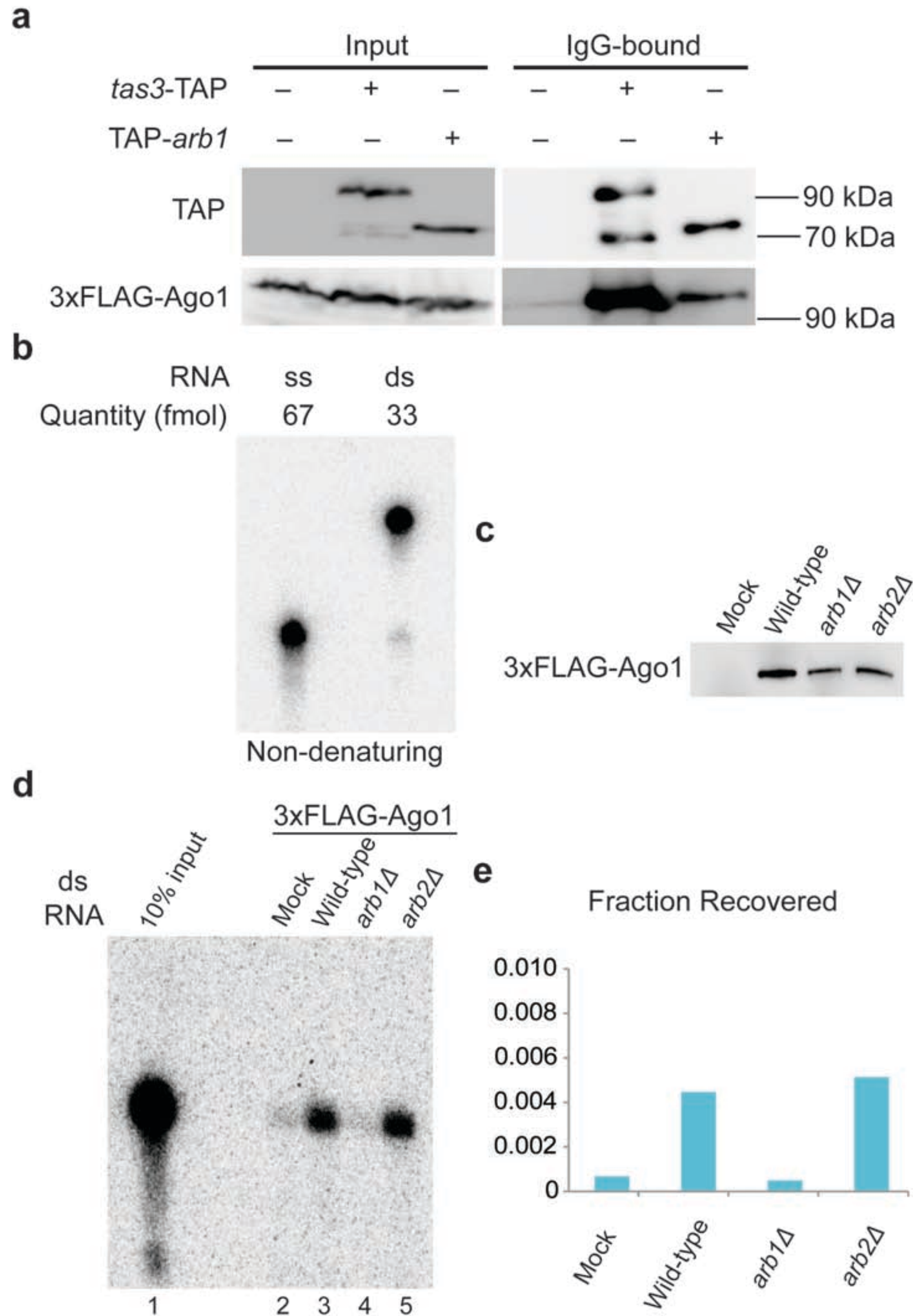

Figure 2.4. Arb2 is not required for *in vitro* loading of duplex small RNAs onto immunopurified Ago1.
(**a**) Western blot analysis of input fractions and IgG magnetic beads after one-step purification of RITS and ARC from *dcr1Δ* cells expressing TAP-tagged subunits Tas3 or Arb1 or no tagged protein. (**b**) Phosphorimager scan of a non-denaturing polyacrylamide gel showing the 5'-end-labeled single-stranded and annealed duplex small RNAs used in *in vitro* binding assays. (**c**) Western blot analysis of the beads from FLAG purifications from the indicated cells, in aliquots equal to one-eighth of those used in the binding assay. (**d**) Phosphorimager scan of eluted RNAs after *in vitro* binding to immobilized 3xFLAG-Ago1 purified from the indicated wild-type and mutant cells. (**e**) Quantification by densitometry of the results shown in (**d**).

B. Arb1 Is Required for Argonaute Loading of Small RNAs *In Vitro* and *In Vivo*

The data above provide new support for an existing presumption—that the products of Dcr1 are loaded onto ARC—but do not clarify whether Arb1 and Arb2 are required for loading. In order to address this question we developed an *in vitro* small RNA binding assay using immunopurified proteins bound to magnetic beads. First we compared binding of immobilized RITS and ARC complexes to single-stranded and duplex RNAs (Figure 2.3a). Although the Ago1 residing in these complexes is expected to be largely occupied with cellular small RNAs, the observation that unloadable Ago1 mutant proteins are somewhat stable (see Chapter 3 and Figures 3.2b,c and 3.3d,e) suggests that some purified Ago1 might be unoccupied and available for loading *in vitro*. In order to increase the chance of recovering such Ago1 molecules we used the *dcr1Δ* background. We confirmed that purifications of Tas3-TAP and TAP-Arb1 co-precipitated 3xFLAG-tagged Ago1 with specificity (Figure 2.4a) and then adjusted each sample with untreated beads to equalize the amounts of Ago1 per bead volume in immunopurified RITS and ARC preparations (Figure 2.3b). Meanwhile, a single-stranded 22-nucleotide RNA and a 22-base-pair duplex RNA with 2-nucleotide 3' overhangs, a signature of Dicer-generated siRNAs, were radiolabeled and separated on a non-denaturing gel to verify their purity (Figure 2.4b). We incubated these RNAs with each complex and with beads from a mock purification, washed away unbound material, and visualized remaining RNA by denaturing gel electrophoresis. Remarkably, while both complexes retained the single-stranded species, only ARC was able to load duplex small RNAs (Figure 2.3c, compare lanes 6 and 8; Figure 2.3d). Consistent with co-immunoprecipitation data shown in Chapter 3 (Figure 3.2b), these results argue that loading of double-stranded Dicer products onto Argonaute must precede RITS formation since this activity was lost once the complex was assembled. Although RITS was able to load new single-stranded small RNAs *in vitro*, this activity is unlikely to contribute significantly to its role in silencing *in vivo*, since most heterochromatic small RNAs are generated as duplexes, which are not converted to single strands until after Argonaute loading.

In principle, when not in RITS, Argonaute could exist within the ARC complex or as a free protein and might load duplex small RNAs in either situation. In order to determine whether the activity is mediated by ARC or is simply inhibited by the other RITS subunits, we performed binding assays using immunopurified Ago1 immobilized using a 3xFLAG tag (Figure 2.3e). We isolated Ago1 from wild-type cells and from *tas3Δ arb1Δ* double-mutant cells, in which Ago1 is associated with neither complex, as well as from the *tas3Δ*, *arb1Δ* and *arb2Δ* single mutants. Western blot analysis of the magnetic beads showed varying stabilities for Ago1 from different backgrounds (Figures 2.3f and 2.4c). However, the binding assay showed that, in contrast to Ago1 isolated from wild-type cells, Ago1 isolated from *tas3Δ arb1Δ* cells was incapable of loading duplex small RNAs (Figure 2.3g, compare lanes 3 and 4; Figure 2.3h). We therefore conclude that Ago1 outside of the RITS and ARC complexes bears no duplex siRNA loading activity, and consequently that duplex siRNA loading strictly requires Arb1. Further substantiating this model, Ago1 isolated from *arb1Δ* single-mutant cells also failed to load the duplex small RNAs, whereas Ago1 isolated from *tas3Δ* cells, though no more abundant than that from *tas3Δ arb1Δ* cells, exhibited excellent loading activity (Figure 2.3f-h). In contrast to Ago1 purified from *arb1Δ* cells, we found that the duplex siRNA loading activity of Ago1 purified from *arb2Δ* cells was similar to that of Ago1 purified from wild-type cells (Figure 2.4d, compare lanes 3 through 5; Figure 2.4e). Thus, an Arb1-Ago1 subcomplex is sufficient for duplex small RNA loading in our assay, and indeed Arb1 and Ago1 remain stably associated in *arb2Δ* cells (see Chapter 3 and Figure 3.1b).

Collectively, the results of these *in vitro* binding experiments support a role for Arb1 in mediating loading of duplex small RNAs onto Ago1. This provides an explanation for the requirement of *arb1* $^{+}$ in the formation of the RITS complex *in vivo* (see Chapter 3 and Figure 3.1a), since we have shown that RITS formation depends on Ago1-small RNA binding (see Chapter 3 and Figure 3.2b). However, if the role of ARC were limited to loading duplex small RNAs, then the *arb1Δ* phenotype for the assembly of RITS should be recapitulated in *dcr1Δ*

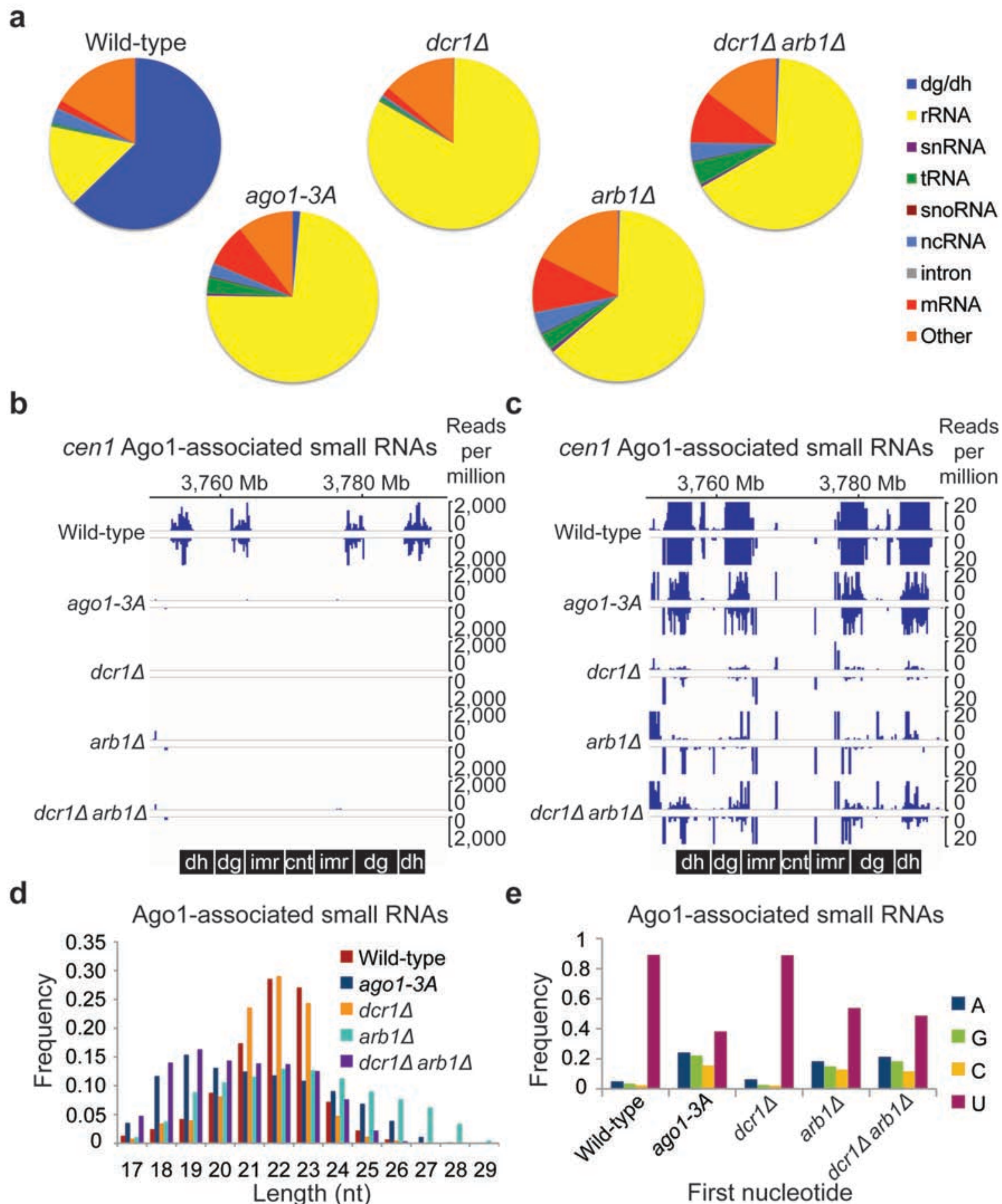

Figure 2.5. Arb1 is required for all Ago1 small RNA loading activity *in vivo*.
(**a-e**) High-throughput sequencing analysis of small RNAs co-purifying with wild-type 3xFLAG-Ago1 in the indicated cells or with the loading mutant 3xFLAG-Ago1-F276A Y513A K517A (Ago1-3A) from cells also bearing wild-type untagged *ago1*$^{+}$. (**a**) Reads are classified as shown in the legend on the right. (**b** and **c**) Tracks showing the normalized numbers of reads mapping to the *dg* and *dh* repeats flanking the centromere of chromosome 1 in each library. The two panels represent the same data plotted on different scales. (**d**) Histogram comparing the genome-wide read length distributions of each library. (**e**) The percentage of reads beginning with each nucleotide genome-wide in each library.

cells. In fact, *dcr1Δ* mutant cells had a much milder RITS assembly defect (Figure 3.1a). This suggests that in *dcr1Δ* cells Dcr1-independent single-stranded primal RNAs (priRNAs) might be loaded onto Ago1 in an Arb1-dependent manner. In order to test this hypothesis, we performed high-throughput sequencing to compare Ago1-bound small RNAs isolated from *dcr1Δ* and *dcr1Δ arb1Δ* cells (Table 2.1). We had previously observed that pericentromeric siRNAs were only modestly reduced in *arb1Δ* cells (Halic and Moazed, 2010), seemingly at odds with the notion that Arb1 is required for Ago1 loading. However, this result was obtained using overexpressed Ago1, and we present evidence below that *ago1*$^{+}$ acts as a high-dosage suppressor of *arb1Δ* and *arb2Δ* with respect to siRNA generation, which in fact is normally lost in these mutants. Therefore, we reasoned that in cells expressing endogenous levels of Ago1, ARC, or at least Arb1, may indeed be required for all small RNA loading. We performed our library preparation accordingly, by isolating RNA co-purifying with 3xFLAG-Ago1 protein expressed at native levels in wild-type, *dcr1Δ*, *arb1Δ* and *dcr1Δ arb1Δ* cells. To assess the background of contaminating cellular small RNAs in the libraries we constructed another library using a 3xFLAG-tagged Ago1 variant with amino acid substitutions that compromise loading of small RNAs into the normal binding groove (F276A/Y513A/K517A, hereafter referred to as Ago1-3A) (Halic and Moazed, 2010) (Figure 3.2a). This allele was inserted under the control of the *ago1*$^{+}$ promoter near *trp1*$^{+}$ (Figure 3.3a) (Iida et al., 2008), leaving an untagged wild-type *ago1*$^{+}$ allele at the native locus in order to maintain abundant levels of pericentromeric siRNAs and to verify that these did not co-purify with Ago1-3A.

We found that pericentromeric siRNAs bound to endogenous Ago1 were severely reduced in *arb1Δ* cells compared to wild-type cells, similar to the levels observed in *dcr1Δ* and *dcr1Δ arb1Δ* cells and even lower than the Ago1-3A-associated background (Figure 2.5a-c). Therefore, in contrast to overexpressed Ago1 (Halic and Moazed, 2010), Ago1 expressed at endogenous levels does not associate with small RNAs mapping to the *dg* and *dh* repeats in cells lacking Arb1. The reads from the mutant libraries consisted primarily of ribosomal RNA

degradation products (Figure 2.5a). In contrast, and as expected, endogenous Ago1 in wild-type cells associated with an abundant population of siRNAs mapping to the *dg* and *dh* repeats (Figure 2.5a-c).

The goal of our sequencing analysis was to determine whether, in addition to mediating loading of Ago1 with small RNA duplexes (Figure 2.3), Arb1 is required for loading Dcr1-independent single-stranded priRNAs *in vivo*. However, this could not be resolved by examining the total numbers of pericentromeric reads, because the levels of priRNAs, defined by their presence in the *dcr1Δ* library, were in the same range as the Ago1-3A-associated background (Figure 2.5a-c). Instead, we used general characteristics of the reads to distinguish between genuine loading events and nonspecific recovery of small RNAs in 3xFLAG-Ago1 immunoprecipitations. The small RNA guides of particular Argonaute proteins exhibit specific lengths as well as a 5' nucleotide bias conferred by the structure of the 5' end binding pocket (Frank et al., 2010; Meister, 2013). *S. pombe* Ago1 binds small RNAs of 22 to 23 nucleotides beginning with a 5' uridine (Bühler et al., 2008) (Figure 2.1c,d). As we have observed previously, the Ago1-bound small RNAs in *dcr1Δ* cells, although depleted in pericentromeric sequences, exhibited the same narrow peak of 22- and 23-nt reads and the same overwhelming 5' uridine preference as the small RNAs bound to Ago1 in wild-type cells (Halic and Moazed, 2010) (Figure 2.5d,e). This indicates that they represent *bona fide* guide molecules loaded into the small RNA binding channel of Argonaute. In stark contrast, the length distributions of the Ago1-associated small RNAs from *arb1Δ* and *dcr1Δ arb1Δ* cells were very broad, strongly resembling the distribution observed for the background small RNAs associating with Ago1-3A (Figure 2.5d). Similarly, the frequency of reads with 5' uridines was dramatically lowered in the *arb1Δ* and *dcr1Δ arb1Δ* libraries, nearly to the extent observed in the Ago1-3A library (Figure 2.5e). These results demonstrate that Arb1 is required for loading both Dcr1-dependent duplex siRNAs and Dcr1-independent single stranded priRNAs into Ago1 *in vivo*.

Intriguingly, we observed apparently higher numbers of Ago1-associated pericentromeric small RNAs in *dcr1Δ arb1Δ* double-mutant cells than in *dcr1Δ* single mutants (Figure 2.5c). One possible explanation is that Arb1-dependent loading of non-pericentromeric small RNAs into Ago1 in *dcr1Δ* cells causes a decline in the normalized share of pericentromeric sequences. An example consistent with this idea is that of the single most abundant sequence in the *dcr1Δ* library, which was non-pericentromeric and made up 15.1 percent of all reads—a ratio that dropped to just 0.36 percent for the *dcr1Δ arb1Δ* library.

Our *in vitro* data suggested that Arb2 does not participate directly in small RNA loading (Figure 2.4d,e). In order to determine whether it might contribute to loading Ago1 in the cellular context, we constructed another set of sequencing libraries from small RNAs present in immunoprecipitations of Ago1. In addition to Ago1 from wild-type cells, and a distinct small RNA loading mutant from cells where it represented the sole source of Ago1 (F276A/R773E) (see Chapter 3 and Figure 3.2a), we compared Ago1 from *arb1Δ* and *arb2Δ* cells (D.H. and D.M., unpublished data, Table 2.1). As we had observed in the case of Ago1-F276A/Y513A/K517A, small RNAs associated with the Ago1-F276A/R773E protein showed a complete collapse of the 22- and 23-nt peak seen in the wild-type read length distribution (Figure 2.6a), as well as a

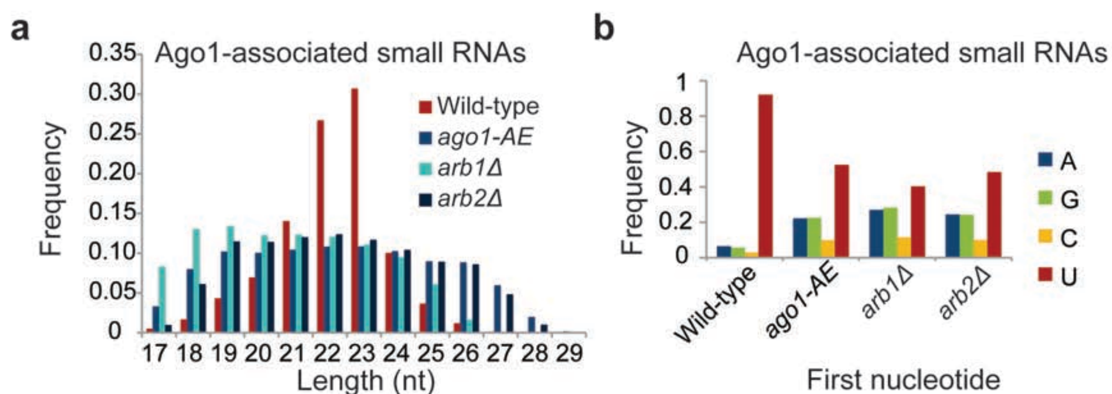

Figure 2.6. Arb2 is required for all Ago1 small RNA loading activity *in vivo*.
High-throughput sequencing analysis of small RNAs co-purifying with wild-type 3xFLAG-Ago1 in the indicated cells or with the loading mutant 3xFLAG-Ago1-F276A R773E (Ago1-AE) from *ago1Δ* cells. (**a**) Histogram comparing the genome-wide read length distributions of each library. (**b**) The percentage of reads beginning with each nucleotide genome-wide in each library.

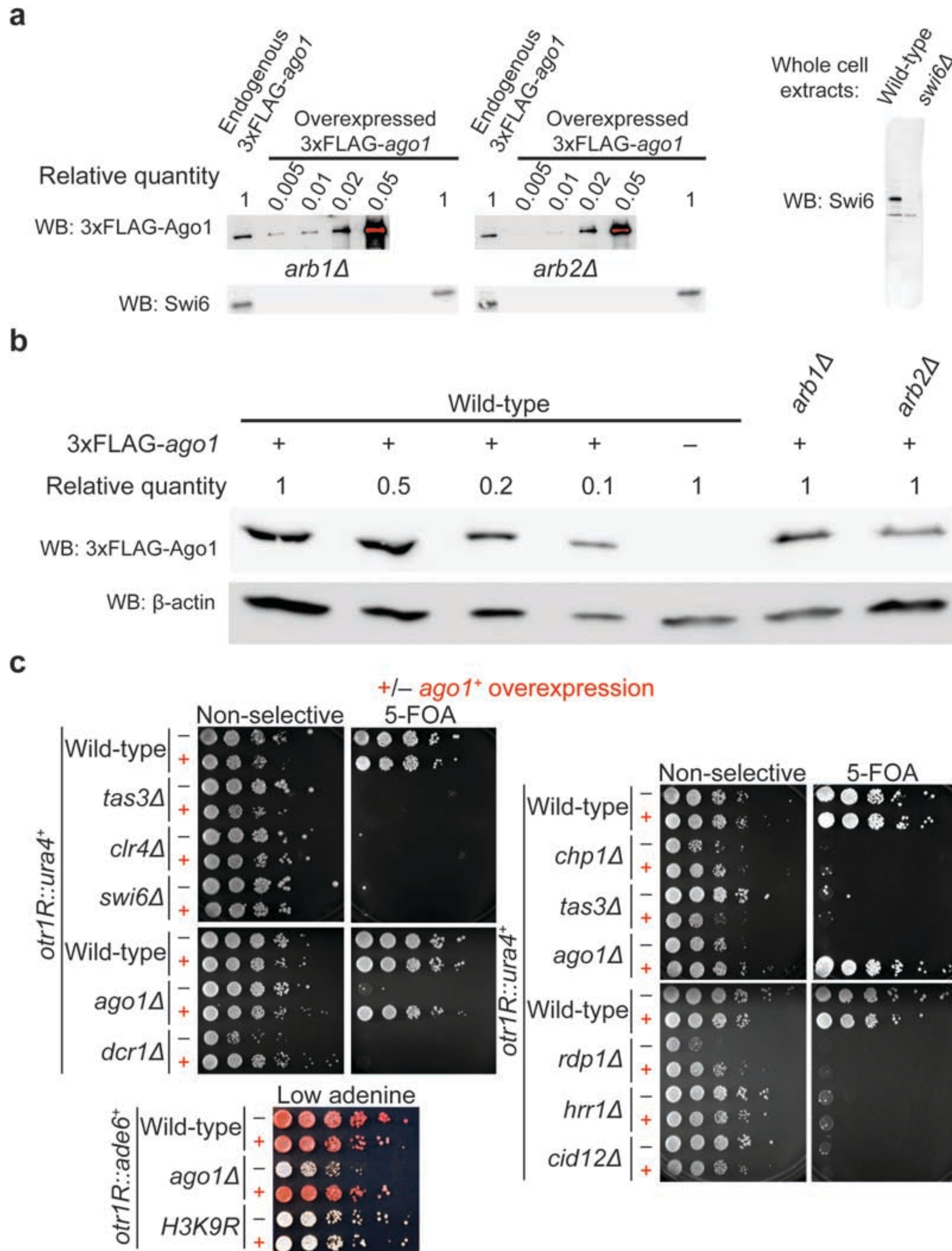

Figure 2.7. Overexpressed Ago1 suppresses *arb1*Δ and *arb2*Δ not simply by rescuing protein stability and does not suppress other pericentromeric silencing mutants.
(**a**) Western blot analysis of total protein prepared from *arb1Δ* and *arb2Δ* cells expressing 3xFLAG-*ago1* either from the endogenous locus or from an overexpression plasmid. Relative quantity of total protein loaded is indicated for each lane. Red pixels indicate saturated signal. (**b**) Western blot analysis of total protein prepared from cells of the indicated genotypes. Relative quantity of total protein loaded is indicated for each lane. (**c**) Tenfold serial dilutions of *otr1R::ura4*$^{+}$ pericentromeric silencing reporter cells of the indicated genotypes, transformed with an empty vector (denoted with a "–") or a 3xFLAG-*ago1* overexpression plasmid (denoted with a red "+"), plated on non-selective medium or medium containing 5-FOA. Similarly, tenfold serial dilutions of *otr1R::ade6*$^{+}$ cells of the indicated genotypes plated on medium containing a limiting concentration of adenine.

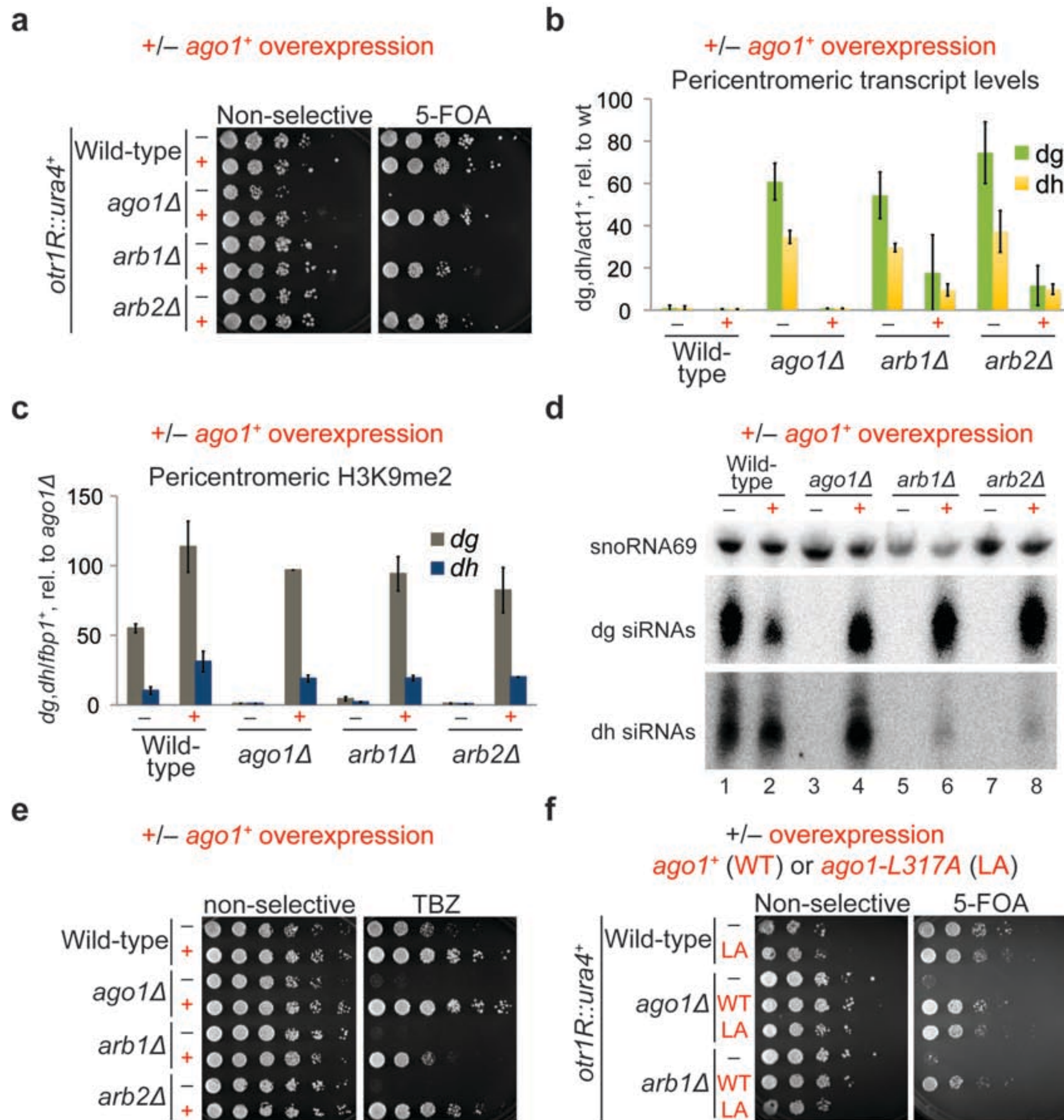

Figure 2.8. *ago1*$^{+}$ overexpression partially suppresses silencing defect of *arb1Δ* and *arb2Δ* by overcoming the requirement for ARC in small RNA loading.
(**a-e**) Analysis of cells transformed with empty vector ("–") or 3xFLAG-*ago1* overexpression plasmid (red "+"). (**a**) Tenfold serial dilutions of cells of the indicated genotypes carrying pericentromeric *ura4*$^{+}$ reporter gene *otr1R::ura4*$^{+}$, plated on the indicated medium. Shown is one of two independent sets of cell cultures. (**b**) Relative levels of *dg* and *dh* transcripts measured by reverse transcription and quantitative PCR, normalized to *act1*$^{+}$ mRNA, with the mean for wild-type cells carrying the empty vector set to 1. Error bars represent s.d. of three independent cell cultures. (**c**) Relative levels of H3K9 dimethylation measured at the *dg* and *dh* pericentromeric repeats by chromatin immunoprecipitation and quantitative PCR, normalized to the euchromatic *fbp1*$^{+}$ locus, with the mean for *ago1Δ* cells carrying the empty vector set to 1. Range bars represent two independent cell cultures. (**d**) Northern blot analysis of small RNAs isolated from total RNA by size fractionation. Shown is one of two replicates performed using RNA isolated from independent sets of cell cultures. (**e**) Fivefold serial dilutions of wild-type and the indicated mutant cells plated on the indicated medium. (**f**) Tenfold serial dilutions of *otr1R::ura4*$^{+}$ pericentromeric silencing reporter cells of the indicated genotypes transformed with empty vector ("–") or plasmid for overexpression of the wild-type 3xFLAG-*ago1* allele (red "WT") or of the small RNA loading mutant allele 3xFLAG-*ago1*-L317A (red "LA") plated on the indicated medium.

striking loss of the 5' uridine preference (Figure 2.6b). Both of these effects were mirrored by the small RNAs associated with wild-type Ago1 protein isolated from cells lacking Arb1 (Figure 2.6), thereby directly replicating the results presented above (Figure 2.5d,e). Surprisingly, Ago1-associated small RNAs from *arb2Δ* cells also failed to exhibit the 22- and 23-nt and 5' uridine enrichment that define genuinely loaded small RNA guides (Figure 2.6). Thus, in sharp contrast to the *in vitro* assay (Figure 2.4d,e), the sequencing experiments indicate that *in vivo*, Arb2 is strictly required for loading Ago1 with small RNAs. We conclude that although Arb2 does not directly mediate the loading event, it is nevertheless required *in vivo* for Arb1-dependent small RNA loading to take place.

C. Argonaute Overexpression Partially Restores Silencing in Arb1-Deficient Cells Specifically by Overcoming a Small RNA Loading Defect

As noted above, our sequencing experiments revealed that pericentromeric siRNA levels in ARC-deficient cells differ considerably depending on whether Ago1 is present at endogenous or overexpressed levels. Without Ago1 overexpression, deletion of *arb1*$^{+}$ leads to a complete loss of siRNAs, consistent with the predicted loss of siRNA amplification in the absence of RITS (Figures 2.5a-c and 3.1a). In contrast, overexpressed Ago1 from *arb1Δ* and *arb2Δ* cells co-purifies with abundant siRNAs mapping to the *dg* repeats and much lower levels mapping to the *dh* repeats (Halic and Moazed, 2010). Thus, ARC becomes partially dispensable for siRNA accumulation when Ago1 is greatly overexpressed. A Western blot analysis comparing serial dilutions of extracts from cells overexpressing 3xFLAG-tagged Ago1 to extracts from cells expressing 3xFLAG-Ago1 from the endogenous locus showed that the level of overexpression from our construct was between 50- and 100-fold (Figure 2.7a).

In addition to rescuing siRNA accumulation, we found that *ago1*$^{+}$ overexpression suppressed the pericentromeric silencing defects of *arb1Δ* and *arb2Δ* cells. The reporter transgene *otr1R::ura4*$^{+}$, whose heterochromatic repression is usually compromised in *arb1Δ* and *arb2Δ* cells, was silenced anew upon *ago1*$^{+}$ overexpression (Figure 2.8a). However, the

noncoding transcripts from the *dg* and *dh* repeats were only partially derepressed under these conditions, suggesting that *ago1*$^{+}$ overexpression does not fully restore silencing in *arb1Δ* and *arb2Δ* cells (Figure 2.8b). Pericentromeric H3K9 dimethylation, normally lost in *arb1Δ* and *arb2Δ* cells, was fully recovered by the action of overexpressed Ago1 (Figure 2.8c). Remarkably, H3K9 dimethylation levels increased upon *ago1*$^{+}$ overexpression even in wild-type cells, suggesting that Ago1 availability was limiting for establishing this modification (Figure 2.8c). A Northern blot analysis of siRNAs mapping to the *dg* and *dh* repeats confirmed our previous sequencing work using overexpressed *ago1*$^{+}$, showing robust recovery of *dg* siRNAs and only partial restoration of *dh* siRNAs in *arb1Δ* and *arb2Δ* cells (Halic and Moazed, 2010) (Figure 2.8d). Although we do not currently understand why *dh* siRNA levels remain lower than their *dg* counterparts, despite similar accumulation of the corresponding transcripts, this pattern appears to be a general feature of many silencing mutants when analyzed by high-throughput sequencing (Halic and Moazed, 2010) and does not represent a specific property of *arb1Δ* and *arb2Δ*. Finally, hypersensitivity to the microtubule poison thiabendazole (TBZ), which occurs in mutants without pericentromeric heterochromatin because of defects in chromosome segregation, was suppressed in *arb1Δ* and *arb2Δ* when *ago1*$^{+}$ was overexpressed, suggesting that the heterochromatin restored in these cells is functional (Figure 2.8e). Ago1 overexpression also reduced the sensitivity of wild-type cells to TBZ (Figure 2.8e), which may be linked to increased levels of H3K9 dimethylation (Figure 2.8c).

Importantly, Ago1 protein accumulation was only modestly affected in *arb1Δ* and *arb2Δ* mutant cells (Figure 2.7b), and inserting an additional copy of *ago1*$^{+}$ under its native promoter to restore wild-type protein levels did not rescue the silencing defect of *arb1Δ* and *arb2Δ* cells (data not shown). Moreover, *ago1*$^{+}$ overexpression failed to suppress a broad array of other mutations in components of the small RNA-mediated heterochromatin pathway (Figure 2.7c). Thus, overexpressed Ago1 acts specifically to circumvent a functional requirement for the ARC complex. In light of our results supporting a role for the ARC subunit Arb1 in mediating small

RNA loading, we hypothesized that small RNA loading constitutes the limiting process in *arb1Δ* cells that is overcome when Ago1 is greatly overexpressed. We reasoned that if this were the case, then the rescue phenomenon might be particularly sensitive to even mild perturbations in the affinity of the overexpressed Ago1 for small RNAs. In order to test this idea, we mutated a conserved leucine residue in the PAZ domain of Ago1, which has been shown in human Argonaute1 to participate in securing the 3' ends of guide small RNAs (Ma et al., 2004). As expected the *S. pombe ago1-L317A* mutation caused a defect in the pericentromeric *otr1R::ura4*$^+$ silencing assay (Figure 2.9a). Nevertheless, a Northern analysis of RNA associated with immunoprecipitated protein showed that Ago1-L317A was still loaded with *dg* repeat siRNAs when overexpressed in wild-type cells (Figure 2.9b) and even when expressed at endogenous levels (Figure 3.3c). Thus, this allele remained partly functional and, consistently, its overexpression complemented the deletion of *ago1*$^+$ in the *otr1R::ura4*$^+$ silencing assay (Figure 2.8f). However, unlike its wild-type counterpart, *ago1-L317A* did not suppress the loss of silencing in *arb1Δ* cells (Figure 2.8f). Thus, although overexpressed Ago1

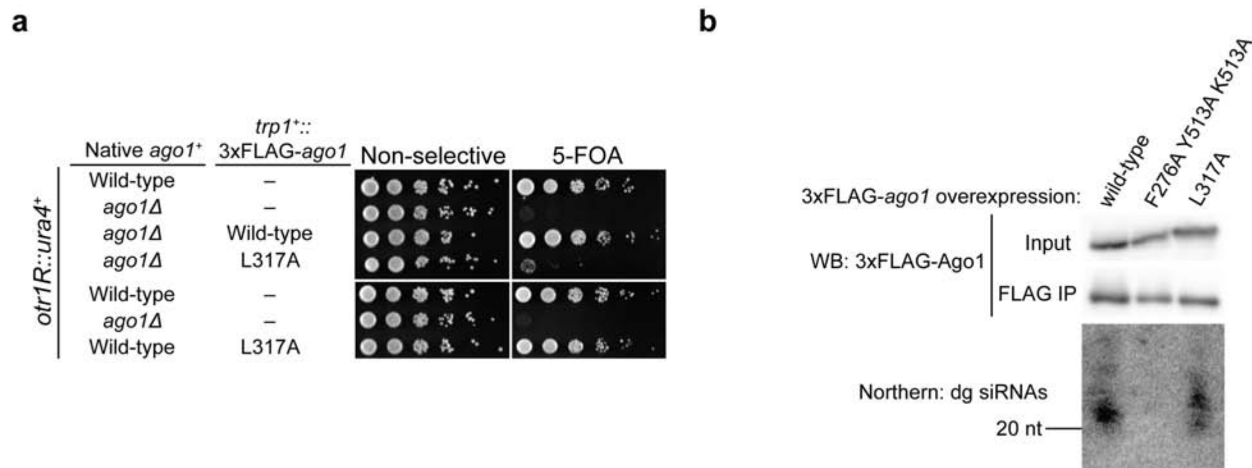

Figure 2.9. Ago1 L317A protein does not complement *ago1Δ* but does not show a significant reduction in loading of pericentromeric siRNAs.
(**a**) Tenfold serial dilutions of *otr1R::ura4*$^+$ or *imr1R::ura4*$^+$ pericentromeric silencing reporter cells of the indicated genotypes plated on non-selective medium or medium containing 5-FOA. (**b**) Western blot analysis of whole cell extracts and FLAG immunoprecipitates prepared from wild-type cells transformed with the indicated 3xFLAG-*ago1* overexpression plasmids, and Northern blot analysis of RNA extracted from each immunoprecipitated sample.

can suppress the silencing defect of *arb1Δ* cells, this suppression requires its intact affinity for small RNAs, which is not necessary for silencing by overexpressed Ago1 when Arb1 is present. This observation lends further support to the idea that silencing is impaired in cells lacking Arb1 specifically because of a disruption in Argonaute loading. Together, our results demonstrate that small RNA loading onto Argonaute, a critical step in siRNA-mediated heterochromatin formation, is directly mediated by Arb1 but also requires the activity of Arb2 *in vivo*.

III. Discussion

In this study we have elucidated the role of the ARC complex in small RNA-dependent heterochromatin assembly. Our data reveal that loading of Argonaute with small RNA molecules in *S. pombe* universally requires the ARC complex, unless Argonaute is vastly overexpressed. But, intriguingly, the ARC subunit Arb2 is dispensable for Argonaute loading *in vitro*.

A. An Argonaute Small RNA Loading Complex in *S. pombe*

Small RNAs must associate with Argonaute proteins in order to accomplish silencing of complementary targets. Studies in many species demonstrate that, rather than automatically binding available small RNAs, Argonautes must actively be loaded with their guides by associated factors. The loading machinery has best been studied in human and *Drosophila*, where the respective double-stranded RNA binding proteins TRBP and R2D2 orchestrate the transfer of duplex small RNAs from the Dicer enzyme that generates them into the Argonaute protein (Gregory et al., 2005; Liu et al., 2003; Maniataki and Mourelatos, 2005). We have previously noted that Arb1 and Arb2 apparently lack double-stranded RNA binding domains and fail to associate detectably with Dcr1 (Buker et al., 2007; Colmenares et al., 2007). Nevertheless, the results in this study clearly identify an Arb1-Ago1 module as the siRNA loading apparatus in *S. pombe*, as only ARC, not RITS, binds duplex small RNAs *in vitro* and all signatures of Argonaute-bound small RNAs vanish in *arb1Δ* cells (Figures 2.3, 2.5 and 2.6).

Argonaute loading in *Drosophila* is ATP-dependent (Kawamata et al., 2009). Early studies using cell-free systems suggested that small RNA loading onto human Argonaute occurs in the absence of ATP (Gregory et al., 2005; MacRae et al., 2008; Maniataki and Mourelatos, 2005), but more recent work has shown that the process is radically enhanced by the addition of ATP, suggesting that ATP is hydrolyzed during physiological loading (Yoda et al., 2010). Further support for a dependence on ATP comes from *in vitro* experiments showing that loading of plant and *Drosophila* Argonautes with duplex small RNAs requires the ATP-dependent Hsc70/Hsp90 chaperone (Iki et al., 2010; Iwasaki et al., 2010; Miyoshi et al., 2010). Nevertheless, recombinant human Argonaute2 was recently shown to be capable of loading duplex siRNAs in the absence of ATP and in a Hsp90-independent manner (Noland and Doudna, 2013), corroborating earlier reports of an ATP-independent loading regime for human Argonaute. Arb1 ostensibly lacks an ATPase domain and we find using our *in vitro* loading assay that ARC mediates small RNA loading onto Ago1 in an apparently ATP-independent manner (data not shown). However, until we reconstitute small RNA loading using recombinant proteins, we cannot decisively rule out a requirement for ATP.

That the RITS complex is able to load single-stranded small RNAs in our *in vitro* system (Figure 2.3c,d) is an observation whose relevance remains unknown. Given that the small RNA binding mutant Ago1-F276A/R773E fails to associate with Tas3 (see Chapter 3 and Figure 3.2b), the simplest explanation for what we observe is that the initial formation of the RITS complex requires Ago1 to be already loaded with a small RNA in an ARC-dependent manner. Then, once within RITS, Ago1 could potentially exchange its RNA cargo for another single-stranded guide as our *in vitro* result suggests. But, as noted above, the siRNAs that guide Ago1 to its pericentromeric targets are generated as duplexes by Dcr1, and are converted into single-stranded form only once they have been loaded onto Ago1 (Buker et al., 2007). Thus, the single-stranded loading ability of RITS may not be important *in vivo*, because the critical small RNA guides are not available to be loaded as single-stranded molecules and must instead be

loaded prior to RITS assembly. Importantly, our sequencing data argue that Ago1 outside of the RITS and ARC complexes fails to load any small RNAs, single-stranded or duplex (Figures 2.5d,e and 2.6). Hence, ARC forms the obligate machinery for *de novo* loading of Argonaute with small RNAs.

B. On the Role of Arb2 in Small RNA Loading

The molecular function of ARC subunit Arb2 remains undefined. Cells lacking Arb2 contain an Ago1-Arb1 subcomplex (see Chapter 3 and Figure 3.1b) which, when isolated, exhibits duplex siRNA loading activity *in vitro* (Figure 2.4d). Therefore, we have excluded a direct biochemical requirement for Arb2 in loading small RNA duplexes onto Ago1. Nevertheless, deletion of Arb2 leads to loss of all loading *in vivo*, as Ago1-associated small RNAs purified from *arb2Δ* cells lack the diagnostic length and first nucleotide profiles of genuinely loaded molecules (Figure 2.6). It can therefore be surmised that while Arb2 does not participate in the loading step itself, it accomplishes some task that is essential for the activity of the Ago1-Arb1 pair in the *S. pombe* cell, and that the nature of this task is most likely related to the physical association between Arb2 and these other ARC subunits.

One possibility is that Arb2 is in fact required for Ago1 and Arb1 to undergo a stable interaction *in vivo*, and that the stable Ago1-Arb1 dimer recovered from *arb2Δ* extracts forms only after cell lysis. In this scenario, loss of Arb2 would effectively abolish any Arb1-mediated loading of Ago1 in living cells, consistent with the identical small RNA loading phenotypes of *arb1Δ* and *arb2Δ* cells. Arb2 might facilitate the Ago1-Arb1 association through direct contacts or, alternatively, might be responsible for mediating the proper subcellular localization of one or both of these ARC components, as a prerequisite for assembly of the full complex. A second model can be envisioned instead, wherein a currently unidentified endogenous *S. pombe* protein systematically inhibits Ago1 loading by Arb1, and Arb2 serves to displace this inhibitor. The inhibitor would also dissociate in an Arb2-independent manner under the purification conditions used to prepare proteins for *in vitro* loading assays, thus allowing Ago1 isolated from

arb2Δ extracts to be loaded with synthetic small RNAs. Although this possibility invokes new and potentially elusive elements and is therefore more difficult to test, it would also be consistent with the observed differences in Ago1 behavior between the *in vitro* and *in vivo arb2Δ* contexts.

Altogether, the data that we have presented in this study demonstrate a direct role for Arb1 in the programming of Ago1 with small RNAs, and suggest that while Arb2 makes a critical contribution to the very same process, its role is subtler and more indirect. Elucidating this role in more detail constitutes an exciting challenge for future investigation.

Table 2.2. ***S. pombe*** **strains used in this study.**

Strain	Genotype
SPY137	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+}
SPY418	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} ago1Δ::kanMX6
SPY552	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} dcr1Δ::natMX6
SPY797	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} natMX6-3xFLAG-ago1
SPY813	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} arb1Δ::kanMX6
SPY815	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} clr4Δ::kanMX6
SPY1098	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} swi6Δ::natMX6
SPY1215	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} tas3Δ::TAP-kanMX6
SPY1319	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} arb2Δ::TAP-kanMX6
SPY1577	leu1-32 ade6-M210 ura4-D18 otr1R::(SphI)::ade6^{+} h3.1/h4.1Δ::his3^{+} h3.3/h4.3Δ::arg3^{+} h3.2-K9R/h4.2
SPY2060	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} natMX6-3xFLAG-ago1 dcr1Δ::kanMX6
SPY2324	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} arb1Δ::kanMX6 hphMX6-3xFLAG-ago1
SPY2327	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} arb2Δ::TAP-kanMX6 hphMX6-3xFLAG-ago1
SPY2421	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+}chp1Δ::TAP-kanMX6
SPY2441	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+}rdp1Δ::TAP-kanMX6
SPY2444	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+}hrr1Δ::kanMX6
SPY2447	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+}cid12Δ::kanMX6
SPY2481	h^{+} leu1-32 ade6-M210 ura4-D18 otr1R(SphI)::ade6^{+} ago1Δ::kanMX6
SPY2556	h^{+} leu1-32 ade6-M216 ura4-D18 imr1R(NcoI)::ura4^{+} tas3-TAP-kanMX6 hphMX6-3xFLAG-ago1
SPY2640	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} ago1Δ::kanMX6 trp1^{+}::natMX6-3xFLAG-ago1
SPY2690	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} natMX6-3xFLAG-ago1 kanMX6-TAP-arb1
SPY3319	h^{-} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} natMX6-3xFLAG-ago1 kanMX6-TAP-arb1 dcr1Δ::hphMX6
SPY3548	h^{+} leu1-32 ade6-M216 ura4-D18 imr1R(NcoI)::ura4^{+} tas3-TAP-kanMX6 hphMX6-3xFLAG-ago1 dcr1Δ::natMX6
SPY4415	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} natMX6-3xFLAG-ago1 tas3Δ::hphMX6
SPY4418	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} arb1Δ::kanMX6 hphMX6-3xFLAG-ago1 tas3Δ::natMX6
SPY4570	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} arb1Δ::kanMX6 hphMX6-3xFLAG-ago1 dcr1Δ::natMX6
SPY4780	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} ago1Δ::kanMX6 trp1^{+}::natMX6-3xFLAG-ago1-F276A R773E
SPY4928	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} trp1^{+}::natMX6-3xFLAG-ago1-L317A
SPY4931	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} ago1Δ::kanMX6 trp1^{+}::natMX6-3xFLAG-ago1-L317A

IV. Methods

A. Accession Codes

The raw and processed small RNA data are publicly available at the NCBI Gene Expression Omnibus under accession number GSE65223. The deposited processed data can be visualized using IGV software.

B. Strain Construction

S. pombe strains used in this study are described in Table 2.2 and were generated using a PCR-based gene targeting strategy (Bähler et al., 1998). All gene deletions were made by replacing the coding region (ATG to stop) with a drug resistance cassette. The N-terminally tagged 3xFLAG-*ago1* allele at the native *ago1*$^{+}$ locus was made by simultaneously inserting a drug resistance cassette 808 bp upstream of the ATG and the 3xFLAG coding sequence immediately upstream of the ATG (Buker et al., 2007). The N-terminally tagged TAP-*arb1* allele at the native *arb1*$^{+}$ locus was made by simultaneously inserting a drug resistance cassette 560 bp upstream of the ATG and the TAP coding sequence immediately upstream of the ATG. The ectopic *trp1*$^{+}$*::*3xFLAG-*ago1* wild-type and mutant alleles were generated as shown in Figure 3.3a in Chapter 3.

C. Western Blotting

Western blots were carried out as described (Yu et al., 2014). Antibodies used were Peroxidase Anti-Peroxidase Soluble Complex (Sigma P-1291), 1:10,000 dilution, FLAG M2-Peroxidase (HRP) mouse monoclonal antibody (Sigma A-8592), 1:5000 dilution, Anti-beta Actin antibody (Abcam 8224), 1:2500 dilution, and custom anti-Swi6 antisera (Covance), 1:5000. Anti-Swi6 is validated in Figure 2.7a and validation information for all other antibodies used is provided on their respective manufacturers' websites.

D. Total RNA and Total Small RNA Isolation

Total RNA was isolated using the hot phenol method (Leeds et al., 1991). Total small RNAs for Northern blots were recovered using the mirVana miRNA Isolation Kit (Ambion)

according to the manufacturer's instructions (Figure 2.1a,b). Alternatively, total small RNAs were recovered by size fractionation of total RNA as described (Figure 2.8d) (Bühler et al, 2006). For RT-PCR assays, total RNA was further purified using the RNeasy Mini kit (Qiagen) following the RNeasy Mini Protocol for RNA Cleanup provided in the manufacturer's handbook, and then treated with RNase-free DNase I (Roche), 25 U for 50 µg RNA in each of two successive reactions performed for 30 min at 37ºC.

E. Isolation of Protein-Associated RNA for Northern Blots and Small RNA Libraries

Tas3-TAP and TAP-Arb1 were purified from cultures of 3 to 6 L grown to a density of ~4 x 10^7 cells/ml. Cells were resuspended in 1 ml extraction buffer (10 mM Tris HCl pH 8, 350 mM NaCl, 10% glycerol, 0.1% NP-40, 1 mM DTT, 1 mM PMSF, Roche Complete EDTA-free Protease Inhibitor Cocktail) per 1 g cells. Extracts were prepared by eleven ten-second cycles of bead-beating in a Biospec bead beater (Model no. 1107900) using a 80-ml-capacity chamber, then cleared by centrifugation at 16,100*g* for 15 min. Supernatants were incubated in two 15-ml tubes with 250 µl prewashed IgG Sepharose 6 Fast Flow (GE Healthcare) for 3 h at 4ºC. Resin was then washed four times with 3 ml extraction buffer and treated with 2% SDS in 300 µl extraction buffer for 10 min at 65ºC. RNA was isolated by phenol:chloroform extraction. Wild-type or mutant 3xFLAG-Ago1 for small RNA sequencing was purified from cultures of 6 L grown to a density of 2 x 10^7 - 3 x 10^7 cells/ml. Cells were resuspended in 1 ml lysis buffer (20 mM HEPES pH 7.6, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 0.25% Triton X-100, 0.5 mM DTT, 1 mM PMSF, Roche Complete EDTA-free Protease Inhibitor Cocktail) per 5 g cells and frozen dropwise in liquid nitrogen. Frozen material was ground into a powder at liquid nitrogen temperatures in a Retsch Cryomill (3 x 3 min at 30 Hz). Anti-FLAG beads were prepared by mixing 300 µl Protein G Dynabeads per sample (Invitrogen), after washing with lysis buffer, with 45 µl anti-FLAG M2 antibody per sample (Sigma) and incubating with rotation. Meanwhile, extracts were prepared by resuspending frozen cell powders in 2 ml lysis buffer per 3 g powder and centrifuging the thawed suspension for 15 min at 16,100 *g*. Extracts were

Table 2.3. Oligonucleotides used in this study.

Oligonucleotide	Sequence	Source
Northern probes		
MB151 (snoRNA69)	CAATGTAAATACTCCGAGTGAGCTGGGTTTAAC	1
censiRNA_a' (dg)	GCGACTAAACCGAAAGCCTC	1
censiRNA_b' (dg)	TACCGTGATTAGCCTTACTCCGCATT	1
IK10 (dg)	GGGAGTACATCATTCCTACTTCGATA	2
DBH45 (dg)	GACTTTCAAAGATGCACA	3
DBH46 (dg)	TTTTCTCTTTCAAAAGTA	3
DBH47 (dg)	CAATTGGAAGTACATCCA	3
DBH48 (dg)	TCAATCCATCATGTACGA	3
DBH49 (dg)	AATTCGATTCCAAGTACA	3
DBH50 (dg)	ATTGTTTCGACAACACGA	3
censiRNA_d' (dh)	TACCGCTTCTCCTTAATCCA	1
censiRNA_e' (dh)	ACACCTACTCTTATCACTTGT	1
censiRNA_f' (dh)	GACGATAAGCAGGAGTTGCGCA	1
censiRNA_g' (dh)	AGTGTGGCGCTATATCTTGTA	1
censiRNA_h' (dh)	TACTGTCATTAGGATATGCTCA	1
censiRNA_i' (dh)	GGGAAATGTATAAATAGGCA	1
censiRNA_j' (dh)	TTTCCCAAGGACTGCTGAGGTAGA	1
censiRNA_l' (dh)	TGGCAGATATTGCAAGTTGTTTA	1
IK9 (dh)	TTTGATGCCCATGTTCATTCCACTTG	2
RNA oligonucleotides		
RNA71	GCGAGCGAGGCAAAGAACAAGA	4
RNA72	UUGUUCUUUGCCUCGCUCGCUG	4
Library barcoding oligonucleotides		
prYU480	CAAGCAGAAGACGGCATACGAGAT**ACATCG**GTGACTG GAGTTCCTTGGCACCCGAGAATTCCA	3
prYU481	CAAGCAGAAGACGGCATACGAGAT**GCCTAA**GTGACTG GAGTTCCTTGGCACCCGAGAATTCCA	3
prYU482	CAAGCAGAAGACGGCATACGAGAT**TGGTCA**GTGACTG GAGTTCCTTGGCACCCGAGAATTCCA	3
prYU483	CAAGCAGAAGACGGCATACGAGAT**CACTGT**GTGACTG GAGTTCCTTGGCACCCGAGAATTCCA	3
prYU484	CAAGCAGAAGACGGCATACGAGAT**ATTGGC**GTGACTG GAGTTCCTTGGCACCCGAGAATTCCA	3
RT-PCR and ChIP		
MB86	AACCCTCAGCTTTGGGTCTT	5
MB87	TTTGCATACGATCGGCAATA	5
AS131 (dg)	AAGGAATGTGCCTCGTCAAATT	6
AS132 (dg)	TGCTTCACGGTATTTTTTGAAATC	6
AS133 (dh)	GTATTTGGATTCCATCGGTACTATGG	6
AS134 (dh)	ACTACATCGACACAGAAAAGAAAACAA	6

1 = Bühler et al., 2006, **2** = Bayne et al., 2010, **3** = this study, **4** = Halic and Moazed, 2010, **5** = Bühler et al., 2007, **6** = Yu et al., 2014

incubated with anti-FLAG beads for 2 h at 4ºC. Beads were washed four times with 1 ml lysis buffer, then RNA was eluted by treatment with Proteinase K (80 U/ml final concentration in 30 mM Tris-HCl pH 8) for 30 min at 37ºC followed by 2% SDS for 10 min at 65ºC and extraction with phenol:chloroform. For Northern blots 3xFLAG-Ago1 was isolated similarly, but from cultures of 3 L grown to a density of ~3 x 10^7 cells/ml (*ago1*$^+$ promoter) or 1 L grown to a density of ~6 x 10^7 cells/ml (overexpressed) and using 70 µl packed EZview Red Anti-FLAG M2 Affinity Gel (Sigma) per sample.

F. Northern Blots

Northern blots were performed as described (Bühler et al., 2006) using total small RNAs or RNA isolated from Tas3-TAP, TAP-Arb1 or 3xFLAG-Ago1 affinity purifications (see above). Oligonucleotide probes are listed in Table 2.3.

G. Small RNA Libraries

RNA was isolated from Tas3-TAP, TAP-Arb1 or 3xFLAG-Ago1 affinity purifications (see above). Small RNAs were size-selected (18-28 nt) by polyacrylamide gel electrophoresis and then were used for library construction as described (Halic and Moazed, 2010). Tas3-TAP- and TAP-Arb1-associated small RNA libraries were each sequenced in individual lanes on an Illumina GAIIx instrument. 3xFLAG-Ago1-associated small RNA libraries carried barcodes added using oligonucleotides shown in Table 2.3 and were sequenced as a mixture in a single lane on an Illumina HiSeq instrument.

H. Analysis of Small RNA Sequences

Small RNA sequencing data were processed and analyzed using custom Perl and Python scripts which are available upon request. The genome sequence and annotation were obtained from PomBase (http://www.pombase.org/). Reads lacking the 3' cloning linker, shorter than 17 nt, or containing ten or more consecutive adenosines were excluded. The remaining reads were aligned to the *S. pombe* genome using NovoAlign (http://www.novocraft.com/). A maximum of either one mismatch or one insertion or deletion of one nucleotide was tolerated.

Reads mapping to multiple locations were randomly assigned. The final numbers of aligned reads for each library are displayed in Table 2.1. Tracks were generated using Integrative Genomics Viewer (IGV) (http://www.broadinstitute.org/igv/).

I. Protein Immobilization for *In Vitro* Binding Assays

IgG-conjugated Dynabeads were prepared from Dynabeads M270 Epoxy (Invitrogen) according to the manufacturer's instructions using rabbit IgG (Sigma). Anti-FLAG beads were prepared by mixing 25 μl Protein G Dynabeads per sample (Invitrogen), after washing with lysis buffer (20 mM HEPES pH 7.6, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 0.25% Triton X-100, 0.5 mM DTT, 1 mM PMSF, Roche Complete EDTA-free Protease Inhibitor Cocktail), with 6.25 μl anti-FLAG M2 antibody per sample (Sigma) and incubating in 120 μl total volume of lysis buffer per sample with rotation. Tas3-TAP and TAP-Arb1 were purified from cultures of 6 L and 3xFLAG-Ago1 was purified from cultures of 3 L, all grown to a density of ~3 x 10^7 cells/ml. Cells were resuspended in 1 ml lysis buffer per 5 g cells and frozen dropwise in liquid nitrogen. Frozen material was ground into a powder at liquid nitrogen temperatures in a Retsch Cryomill (3 x 3 min at 30 Hz). Extracts were prepared by resuspending frozen cell powders in 2 ml lysis buffer per 3 g powder and centrifuging the thawed suspension for 15 min at 16,100 *g*. Extracts containing Tas3-TAP or TAP-Arb1 and control extracts lacking TAP-tagged protein were incubated with 50 μl IgG-conjugated Dynabeads (corresponding to 0.375 mg original dry beads) for 2 h at 4ºC. Extracts containing 3xFLAG-Ago1 and control extracts containing untagged Ago1 were incubated with 25 μl anti-FLAG beads for 2 h at 4ºC. Beads were washed four times with 1 ml lysis buffer and resuspended in lysis buffer containing 0.1 mg/ml BSA: 50 μl (TAP samples) or 25 μl (FLAG samples). For the experiment in Figure 2.6c-e, the lysis buffer was modified to contain 40 mM NaCl instead of 100 mM.

J. *In Vitro* Binding Assays

Tas3-TAP, TAP-Arb1 and 3xFLAG-Ago1 were immobilized on magnetic beads from *S. pombe* extracts and mock beads were prepared in parallel by incubation with control extracts

Table 2.4. Plasmids used in this study.

pDM815	pREP1-*nmt1*$^+$promoter-3xFLAG
pDM817	pREP1-*nmt1*$^+$promoter-3xFLAG-*ago1*
pDM821	pREP1-*nmt1*$^+$promoter-3xFLAG-*ago1*-L317A
pDM1558	pREP1-*nmt1*$^+$promoter-3xFLAG-*ago1*-F276A Y513A K517A

lacking tagged proteins (see above). Aliquots of 4 μl beads for each protein sample were washed with 1 ml wash buffer (30 mM HEPES pH 7.6, 40 mM KOAc, 5 mM $Mg(OAc)_2$, 10% glycerol, 5 mM DTT) and then resuspended in binding buffer (30 mM HEPES pH 7.6, 40 mM KOAc, 5 mM $Mg(OAc)_2$, 0.1 mg/ml BSA, 5 mM DTT, 2 U/μl RNasin (Promega)) containing 0.4 nM single-stranded or duplex small RNA (sequences provided in Table 2.3) 5'-end-labeled with polynucleotide kinase and a fivefold molar excess of γ-^{32}P ATP. After incubating 1 h 30 min at room temperature, beads were washed four times with 1 ml wash buffer. To elute RNAs, beads were resuspended in 8 μl proteinase K reaction buffer (80 U/ml proteinase K (NEB) in 30 mM Tris-HCl pH 8) and incubated 30 min at 37ºC, then 8 μl of formamide was added and samples were incubated 2 min at 95ºC. RNAs were visualized by denaturing polyacrylamide gel electrophoresis and autoradiography, and quantified by densitometry using Quantity One software (Bio-Rad).

K. Growth Assays

Cells were grown to saturation and diluted serially tenfold (except for thiabendazole growth assays which were diluted serially fivefold) so that the highest density spot contained 1.3 x 10^5 cells. Non-selective plates contained Edinburgh Minimal Medium with all standard supplements (Sunrise Science Products) except leucine to ensure maintenance of the transformed plasmids, which are listed in Table 2.4. Selective plates additionally contained 1 g/L 5-fluoroorotic acid or 17 mg/L thiabendazole. Low adenine plates contained 10 mg/L adenine.

L. Reverse Transcription

cDNA was prepared using transcript-specific oligonucleotide primers (Table 2.3) and Superscript III Reverse Transcriptase (Invitrogen). *dg* and *dh* reactions contained primers to reverse transcribe both strands. *act1*$^{+}$ reactions only contained a primer to reverse transcribe forward transcripts. A mock reaction without Superscript III was performed for every sample.

M. Chromatin Immunoprecipitation

ChIP was performed as described (Huang and Moazed, 2003). For each sample 30 µl of Protein A Dynabeads (Invitrogen) and 2 µg Anti-Histone H3-dimethyl K9 (Abcam 1220) antibody were used. Validation information for Anti-Histone-H3-dimethyl K9 is provided on its manufacturer's website.

N. Quantitative PCR

DNA, cDNA or mock cDNA (–reverse transcriptase) was amplified with *Taq* polymerase using oligonucleotide primers listed in Table 2.3 in the presence of SYBR Green in an Applied Biosystems 7900HT Fast Real-Time PCR instrument. A standard serial dilution was included for each primer set on each plate in order to determine amplification efficiency and calculate precise relative quantities among samples. All mock cDNA reactions generated less than one-one thousandth (*act1*$^{+}$) or one-third (*dg*, *dh*) the corresponding +reverse transcriptase signal.

V. Acknowledgments

We thank members of the Moazed laboratory for helpful discussions, E. Gerace, M. Halic, N. Iglesias and J. Xiol for advice on protocols, M. Halic and R. Yu for scripts and advice on bioinformatic analyses, C. Centrella for technical assistance, and E. Egan, N. Iglesias, R. Jain, J. Xiol and R. Yu for critical reading of the manuscript. This work was supported by the U.S. National Science Foundation Graduate Research Fellowship Program (D.H.) and the U.S. National Institutes of Health R01 GM072805 (D.M.). D.M. is supported as a Howard Hughes Medical Institute Investigator.

VI. References

Bähler, J., Wu, J.Q., Longtine, M.S., Shah, N.G., McKenzie, A., III, Steever, A.B., Wach, A., Philippsen, P., and Pringle, J.R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. Yeast *14*, 943-951.

Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C., and Kouzarides, T. (2001). Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature *410*, 120-124.

Bayne, E.H., White, S.A., Kagansky, A., Bijos, D.A., Sanchez-Pulido, L., Hoe, K.L., Kim, D.U., Park, H.O., Ponting, C.P., Rappsilber, J., and Allshire, R.C. (2010). Stc1: a critical link between RNAi and chromatin modification required for heterochromatin integrity. Cell *140*, 666-677.

Bernard, P., Maure, J.F., Partridge, J.F., Genier, S., Javerzat, J.P., and Allshire, R.C. (2001). Requirement of heterochromatin for cohesion at centromeres. Science *294*, 2539-2542.

Bühler, M., Haas, W., Gygi, S.P., and Moazed, D. (2007). RNAi-dependent and -independent RNA turnover mechanisms contribute to heterochromatic gene silencing. Cell *129*, 707-721.

Bühler, M., Spies, N., Bartel, D.P., and Moazed, D. (2008). TRAMP-mediated RNA surveillance prevents spurious entry of RNAs into the Schizosaccharomyces pombe siRNA pathway. Nat. Struct. Mol. Biol. *15*, 1015-1023.

Bühler, M., Verdel, A., and Moazed, D. (2006). Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing. Cell *125*, 873-886.

Buker, S.M., Iida, T., Bühler, M., Villén, J., Gygi, S.P., Nakayama, J., and Moazed, D. (2007). Two different Argonaute complexes are required for siRNA generation and heterochromatin assembly in fission yeast. Nat. Struct. Mol. Biol. *14*, 200-207.

Colmenares, S.U., Buker, S.M., Buhler, M., Dlakic, M., and Moazed, D. (2007). Coupling of double-stranded RNA synthesis and siRNA generation in fission yeast RNAi. Mol. Cell *27*, 449-461.

Ellermeier, C., Higuchi, E.C., Phadnis, N., Holm, L., Geelhood, J.L., Thon, G., and Smith, G.R. (2010). RNAi and heterochromatin repress centromeric meiotic recombination. Proc. Natl. Acad. Sci. USA *107*, 8701-8705.

Frank, F., Sonenberg, N., and Nagar, B. (2010). Structural basis for 5’-nucleotide base-specific recognition of guide RNA by human AGO2. Nature *465*, 818-822.

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.

Gerace, E.L., Halic, M., and Moazed, D. (2010). The methyltransferase activity of $Clr4^{Suv39h}$ triggers RNAi independently of histone H3K9 methylation. Mol. Cell *39*, 360-372.

Ghildiyal, M., and Zamore, P.D. (2009). Small silencing RNAs: an expanding universe. Nat. Rev. Genet. *10*, 94-108.

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.

Halic, M., and Moazed, D. (2010). Dicer-independent primal RNAs trigger RNAi and heterochromatin formation. Cell *140*, 504-516.

Hong, E.J., Villén, J., Gerace, E.L., Gygi, S.P., and Moazed, D. (2005). A cullin E3 ubiquitin ligase complex associates with Rik1 and the Clr4 histone H3-K9 methyltransferase and is required for RNAi-mediated heterochromatin formation. RNA Biol. *2*, 106-111.

Huang, J., and Moazed, D. (2003). Association of the RENT complex with nontranscribed and coding regions of rDNA and a regional requirement for the replication fork block protein Fob1 in rDNA silencing. Genes Dev. *17*, 2162-2176.

Iida, T., Nakayama, J., and Moazed, D. (2008). siRNA-mediated heterochromatin establishment requires HP1 and is associated with antisense transcription. Mol. Cell *31*, 178-189.

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.

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.

Kawakami, K., Hayashi, A., Nakayama, J., and Murakami, Y. (2012). A novel RNAi protein, Dsh1, assembles RNAi machinery on chromatin to amplify heterochromatic siRNA. Genes Dev. *26*, 1811-1824.

Kawamata, T., Seitz, H., and Tomari, Y. (2009). Structural determinants of miRNAs for RISC loading and slicer-independent unwinding. Nat. Struct. Mol. Biol. *16*, 953-960.

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature *410*, 116-120.

Leeds, P., Peltz, S.W., Jacobson, A., and Culbertson, M.R. (1991). The product of the yeast UPF1 gene is required for rapid turnover of mRNAs containing a premature translational termination codon. Genes Dev. *5*, 2303-2314.

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.

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.

MacRae, I.J., Ma, E., Zhou, M., Robinson, C.V., and Doudna, J.A. (2008). In vitro reconstitution of the human RISC-loading complex. Proc. Natl. Acad. Sci. USA *105*, 512-517.

Malone, C.D., and Hannon, G.J. (2009). Small RNAs as guardians of the genome. Cell *136*, 656-668.

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

Marasovic, M., Zocco, M., and Halic, M. (2013). Argonaute and Triman generate dicer-independent priRNAs and mature siRNAs to initiate heterochromatin formation. Mol. Cell *52*, 173-183.

Matranga, C., Tomari, Y., Shin, C., Bartel, D.P., and Zamore, P.D. (2005). Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell *123*, 607-620.

Meister, G. (2013). Argonaute proteins: functional insights and emerging roles. Nat. Rev. Genet. *14*, 447-459.

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.

Moazed, D. (2009). Small RNAs in transcriptional gene silencing and genome defence. Nature *457*, 413-420.

Motamedi, M.R., Verdel, A., Colmenares, S.U., Gerber, S.A., Gygi, S.P., and Moazed, D. (2004). Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs. Cell *119*, 789-802.

Nakayama, J., Rice, J.C., Strahl, B.D., Allis, C.D., and Grewal, S.I. (2001). Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science *292*, 110-113.

Noland, C.L., and Doudna, J.A. (2013). Multiple sensors ensure guide strand selection in human RNAi pathways. RNA *19*, 639-648.

Noma, K., Sugiyama, T., Cam, H., Verdel, A., Zofall, M., Jia, S., Moazed, D., and Grewal, S.I. (2004). RITS acts in *cis* to promote RNA interference-mediated transcriptional and post-transcriptional silencing. Nat. Genet. *36*, 1174-1180.

Nonaka, N., Kitajima, T., Yokobayashi, S., Xiao, G., Yamamoto, M., Grewal, S.I., and Watanabe, Y. (2002). Recruitment of cohesin to heterochromatic regions by Swi6/HP1 in fission yeast. Nat. Cell Biol. *4*, 89-93.

Olovnikov, I., Chan, K., Sachidanandam, R., Newman, D.K., Aravin, A.A. (2013). Bacterial argonaute samples the transcriptome to identify foreign DNA. Mol. Cell *51*, 594-605.

Partridge, J.F., DeBeauchamp, J.L., Kosinski, A.M., Ulrich, D.L., Hadler, M.J., and Noffsinger, V.J.P. (2007). Functional separation of the requirements for establishment and maintenance of centromeric heterochromatin. Mol. Cell *26*, 593-602.

Rand, T.A., Petersen, S., Du, F., and Wang, X. (2005). Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. Cell *123*, 621-629.

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

Schalch, T., Job, G., Noffsinger, V.J., Shanker, S., Kuscu, C., Joshua-Tor, L., and Partridge, J.F. (2009). High-affinity binding of Chp1 chromodomain to K9 methylated histone H3 is required to establish centromeric heterochromatin. Mol. Cell *34*, 36-46.

Schmitz-Linneweber, C., Lampe, M.K., Sultan, L.D., Ostersetzer-Biran, O. (2015). Organellar maturases: A window into the evolution of the spliceosome. Biochim. Biophys. Acta *1847*, 798-808.

Till, S., Lejeune, E., Thermann, R., Bortfeld, M., Hothorn, M., Enderle, D., Heinrich, C., Hentze, M., 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.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S.I., and Moazed, D. (2004). RNAi-mediated targeting of heterochromatin by the RITS complex. Science *303*, 672-676.

Volpe, T.A., Kidner, C., Hall, I.M., Teng, G., Grewal, S.I., and Martienssen, R.A. (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science *297*, 1833-1837.

Woolcock, K.J., Stunnenberg, R., Gaidatzis, D., Hotz, H.R., Emmerth, S., Barraud, P., and Bühler, M. (2012). RNAi keeps Atf1-bound stress genes in check at nuclear pores. Genes Dev. *26*, 683-692.

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.

Yu, R., Jih, G., Iglesias, N., and Moazed, D. (2014). Determinants of heterochromatic siRNA biogenesis and function. Mol. Cell *53*, 262-276.

Zamore, P.D. (2006). RNA interference: big applause for silencing in Stockholm. Cell *127*, 1083-1086.

Zhang, K., Mosch, K., Fischle, W., and Grewal, S.I. (2008). Roles of the Clr4 methyltransferase complex in nucleation, spreading and maintenance of heterochromatin. Nat. Struct. Mol. Biol. *15*, 381-388.

Chapter 3

Small-RNA Loading Licenses Argonaute for Assembly into a Transcriptional Silencing Complex

Daniel Holoch and Danesh Moazed

Chapter 3

This chapter is a portion of a published report:

Holoch, D., and Moazed, D. (2015). Small-RNA loading licenses Argonaute for assembly into a transcriptional silencing complex. Nat. Struct. Mol. Biol. *22*, 328-335.

All experiments were conducted by Daniel Holoch. The original manuscript was written by Daniel Holoch and Danesh Moazed. The introduction, results and discussion were modified for this dissertation.

I. Introduction

Small silencing RNA molecules represent an ancient and widespread strategy for regulating gene expression and protecting the genome from foreign and unstable DNA elements (Ghildiyal and Zamore, 2009; Malone and Hannon, 2009; Moazed, 2009; Olovnikov et al., 2013). Small RNAs drive sequence-specific silencing by guiding proteins of the conserved Argonaute family to complementary target RNAs through base-pairing interactions. Argonautes inactivate their targets by RNase H-like endonucleolytic cleavage or by recruiting additional factors to mediate translational repression, RNA turnover or transcriptional silencing through chromatin modification of the corresponding loci (Hutvagner and Simard, 2008; Jinek and Doudna, 2009; Meister, 2013). In most cases Argonautes function within multi-subunit assemblies called the RNA-induced silencing complex (RISC) or, in the nucleus, the RNA-induced transcriptional silencing (RITS) complex (Hammond et al., 2000; Verdel et al., 2004). The mechanisms that control the formation of these protein complexes are not well understood.

In the fission yeast *Schizosaccharomyces pombe*, small RNAs mediate silencing at the transcriptional level. The Argonaute-containing RITS complex is recruited to nascent transcripts at the *dg* and *dh* noncoding repeats that flank the centromeres of each chromosome by endogenous small interfering RNAs (siRNAs) whose biogenesis requires the RNA-dependent RNA polymerase complex RDRC and the Dicer-family ribonuclease Dcr1 (Colmenares et al., 2007; Motamedi et al., 2004; Reinhart and Bartel, 2002; Verdel et al., 2004; Yu et al., 2014). Since siRNAs are initially generated as duplexes, endonucleolytic cleavage of the “passenger” strand by the “slicer” activity of Argonaute, and its release, must occur before the remaining “guide” strand can mediate recognition of pericentromeric nascent transcripts (Buker et al., 2007; Matranga et al., 2005; Rand et al., 2005). Once recruited, siRNA-programmed RITS directs heterochromatic silencing of the *dg* and *dh* repeats via methylation of nucleosomes on histone H3 lysine 9 (H3K9) by the Clr4-Rik1-Cul4 (CLRC) complex (Bayne et al., 2010; Gerace et al., 2010; Hong et al., 2005; Verdel et al., 2004; Volpe et al., 2002; Zhang et al., 2008).

Pericentromeric heterochromatin is important for accurate chromosome segregation and maintenance of genomic stability in *S. pombe* (Bernard et al., 2001; Ellermeier et al., 2010). This requirement is widely shared among multicellular eukaryotes (Probst et al., 2009).

In addition to the single *S. pombe* Argonaute protein Ago1, the RITS complex contains Chp1, a chromodomain protein whose high affinity for methylated H3K9 contributes to targeting RITS to heterochromatic loci, and Tas3, a member of the conserved glycine-tryptophan (GW) motif-containing family (Noma et al., 2004; Schalch et al., 2009; Verdel et al., 2004) (Figure 1.2). Tas3 orthologs bind to Argonautes via their GW or "Ago-hook" domains and participate in different modes of small RNA-mediated silencing in a broad range of organisms (Azevedo et al., 2010; Braun et al., 2013; El-Shami et al., 2007; Till et al., 2007). Whereas in animals GW182 and its homologs serve as a link between microRNA-programmed Argonaute and the deadenylation machinery to trigger turnover or translational repression of target mRNAs in the cytoplasm (Braun et al., 2011; Chekulaeva et al., 2011; Fabian et al., 2011), GW proteins in plants and *S. pombe* connect Argonautes to chromatin to promote transcriptional silencing (El-Shami et al., 2007; Partridge et al., 2007; Pontier et al., 2012; Verdel et al., 2004). Through extensive mutagenesis studies, the GW domain recognition site has been mapped to a region spanning the MID and PIWI domains of Argonaute, and in particular to amino acid residues crucial for anchoring the 5'-monophosphate end of the guide small RNA (Behm-Ansmant et al., 2006; Till et al., 2007). Accordingly, some groups have suggested that a relationship exists between Argonaute's loading of a guide RNA and its association with GW domains, while other studies have contended that the activities are separable (Baillat and Shiekhattar, 2009; Giner et al., 2010; Liu et al., 2005; Eulalio et al., 2009). Association of unloaded Argonautes with GW proteins and downstream factors might be expected to generate inactive complexes that poison the activity of mature small-RNA-programmed complexes and their own ability to interact with downstream factors. Although studies have been carried out using overexpressed proteins, it is

currently unknown whether the interaction of endogenous Argonaute with GW proteins depends on small RNA loading or is regulated by other mechanisms.

Previously, we reported the discovery of a distinct Ago1-containing complex in fission yeast, called the Argonaute siRNA Chaperone (ARC) complex, which includes Arb1, a protein present throughout the fungal lineage that bears homology to organellar maturases, and Arb2, which is broadly conserved in fungi, plants and metazoans but whose function is unknown (Buker et al., 2007). Immunofluorescence assays show ARC to be localized to the nucleus, but mass spectrometry analyses indicate that RITS and ARC are distinct complexes, with only the Ago1 subunit in common (Buker et al., 2007; Verdel et al., 2004). ARC carries duplex siRNAs and despite its nuclear localization appears not to associate with chromatin, suggesting that, in contrast to RITS, it is not directly involved in target transcript engagement (Buker et al., 2007; Woolcock et al., 2012) (Figure 1.2, see also Appendix 1). An explanation for the observation that ARC-associated siRNAs are double-stranded comes from the finding that Arb1 inhibits Ago1 slicer activity, and thus passenger-strand release (Buker et al., 2007). Alone, this result might imply that ARC acts as a negative modulator of the siRNA pathway but, on the contrary, ARC plays a critical positive role in siRNA-directed heterochromatin assembly, as deletions of *arb1*$^{+}$ and *arb2*$^{+}$ cause a similar loss of pericentromeric H3K9 methylation and gene silencing as deletion of *ago1*$^{+}$ (Buker et al., 2007). Moreover, Arb1 and Arb2 are each required for the induction of silencing in *trans* by ectopic hairpin siRNA constructs (Iida et al., 2008; Simmer et al., 2010). However, the specific nature of their contribution to siRNA-dependent heterochromatin formation has remained undefined.

Chapter 2 of this dissertation presents several lines of evidence indicating that Arb1 is strictly required for loading small RNAs into Ago1, whereas Arb2 is necessary *in vivo* but not *in vitro*. In this study we demonstrate that Arb1 and Arb2 are also both required for assembly of the RITS complex. In contrast, RITS persists in cells lacking Dicer, indicating that complex formation requires Arb1 and Arb2 in particular, rather than an intact silencing pathway *per se*.

We further show that loading of Argonaute with a small RNA is required for its association with the GW protein Tas3 but not with Arb1. This suggests that small-RNA loading is a prerequisite for entry of Argonaute into RITS and, consistent with the conclusions of Chapter 2, that small RNA loading occurs within the ARC complex. Premature assembly of Argonautes lacking small RNA guides into effector complexes, such as RITS, is expected to result in undesired competition for limiting downstream silencing factors. The ordered assembly pathway described

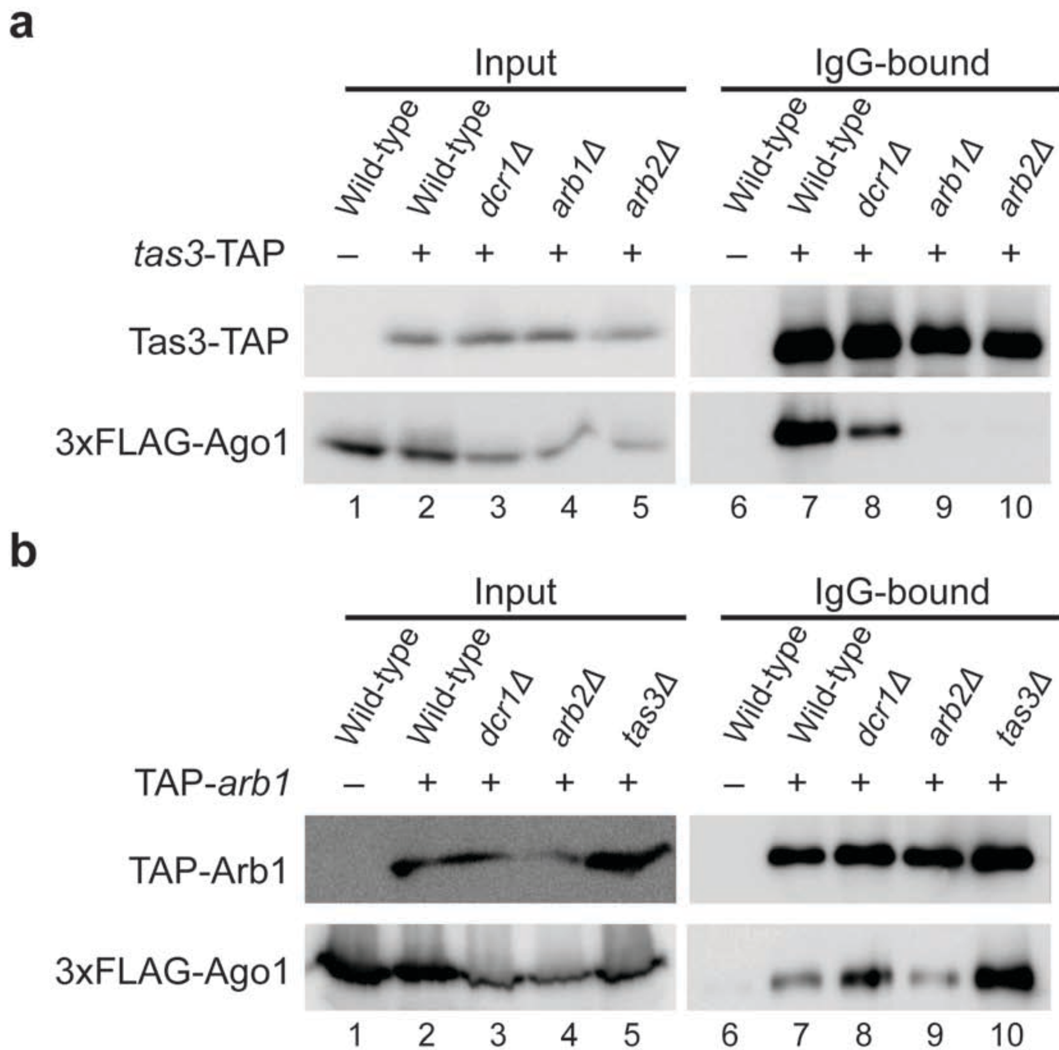

Figure 3.1. ARC subunits Arb1 and Arb2 are required for RITS assembly.
(**a**) Western blot analysis of co-immunoprecipitation experiment to assay Tas3-Ago1 association in the indicated wild-type and mutant cells. Shown is one of two independent experiments. Supplementary Data Set 1 shows uncropped blot images. (**b**) Western blot analysis of co-immunoprecipitation experiment to assay Arb1-Ago1 association in the indicated wild-type and mutant cells.

here is therefore likely to be a central and conserved feature of the mechanisms that regulate the association of Argonautes with their GW protein partners.

II. Results

A. Arb1 and Arb2 Are Required for Assembly of the RITS Complex

Before the studies presented in Chapter 2 were carried out, the requirement for the ARC-specific subunits Arb1 and Arb2 in small RNA-mediated heterochromatin assembly had remained perplexing, because ARC contains duplex siRNAs that cannot base-pair with target pericentromeric transcripts and because no association is detectable between ARC and chromatin (Buker et al., 2007; Woolcock et al., 2012). However, since duplexes represent the initial output of the Dicer enzyme, it has been presumed that individual Ago1 molecules begin their life cycle in ARC before undergoing a transition to the RITS complex. We therefore asked whether Ago1 retains the ability to be assembled into RITS when its passage through ARC is prevented by genomic deletion of *arb1*$^+$ or *arb2*$^+$. Surprisingly, we found that the interaction between Ago1 and the GW protein Tas3 was abolished in *arb1Δ* and *arb2Δ* cells (Figure 3.1a). Importantly, a considerable degree of interaction persisted in *dcr1Δ* cells (Verdel et al., 2004) (Figure 3.1a). This indicates that the common phenotypes of *dcr1Δ*, *arb1Δ* and *arb2Δ* cells—loss of pericentromeric silencing, H3K9 methylation and siRNAs—are not sufficient to explain the loss of RITS integrity. Instead, the data suggest that Arb1 and Arb2 license Ago1 for entry into RITS by an unknown mechanism. In this regard, mass spectrometry analyses of purifications of Chp1, Tas3 and Arb1 consistently fail to detect overlap of complex components beyond Ago1, arguing against a role for Arb1 or Arb2 in directly presenting Ago1 to Tas3 and Chp1 (Buker et al., 2007; Verdel et al., 2004). We also used co-immunoprecipitation to monitor the integrity of ARC in cells lacking Tas3. In contrast to the effect of ARC on RITS assembly, we found that the association between ARC subunits Ago1 and Arb1 was greatly enhanced in *tas3Δ* as well as in *dcr1Δ* cells, and was not dramatically diminished in *arb2Δ* cells (Figure 3.1b). Mixture mass spectrometry analysis of 3xFLAG-tagged Ago1 purifications from wild-type

Table 3.1. Ago1 associates with Arb1 and Arb2 independently of Tas3.

Unique peptides (percent coverage)	Mock (wild-type)	3xFLAG-Ago1 (wild-type)	3xFLAG-Ago1 (tas3Δ)
Ago1	0 (0%)	41 (43%)	33 (38%)
Tas3	0 (0%)	25 (45%)	0 (0%)
Chp1	0 (0%)	8 (14%)	0 (0%)
Arb1	0 (0%)	8 (27%)	10 (35%)
Arb2	0 (0%)	4 (17%)	8 (38%)

and *tas3Δ* cells confirmed that Ago1-Arb1 and Ago1-Arb2 interactions remained intact in the absence of the RITS complex (Table 3.1). Together these results show that Ago1 can assemble with Arb1 and Arb2 into the ARC complex independently of RITS, but that Ago1 can only enter the RITS complex after passage through ARC.

B. Small RNA Loading onto Argonaute Is Required for Assembly of the RITS Complex but not the ARC Complex

The altered efficiency of the protein-protein interactions we observed within each of the two Argonaute complexes in *dcr1Δ* cells was striking (Figure 3.1), and suggestive of a relationship between small RNA levels and complex assembly. We therefore hypothesized that loading of a small RNA onto Ago1 is a prerequisite for RITS assembly, and that Arb1 and Arb2 are required for entry of Ago1 into RITS because they mediate this activity. Indeed, the experimental results described in Chapter 2 demonstrate that all loading of Ago1 with small RNAs depends on Arb1 and Arb2 *in vivo*. Importantly, *S. pombe* cells produce a class of Dicer-independent small RNAs called primal RNAs (priRNAs), whose biogenesis requires the exonuclease Triman and which can direct H3K9 methylation in an Argonaute-dependent

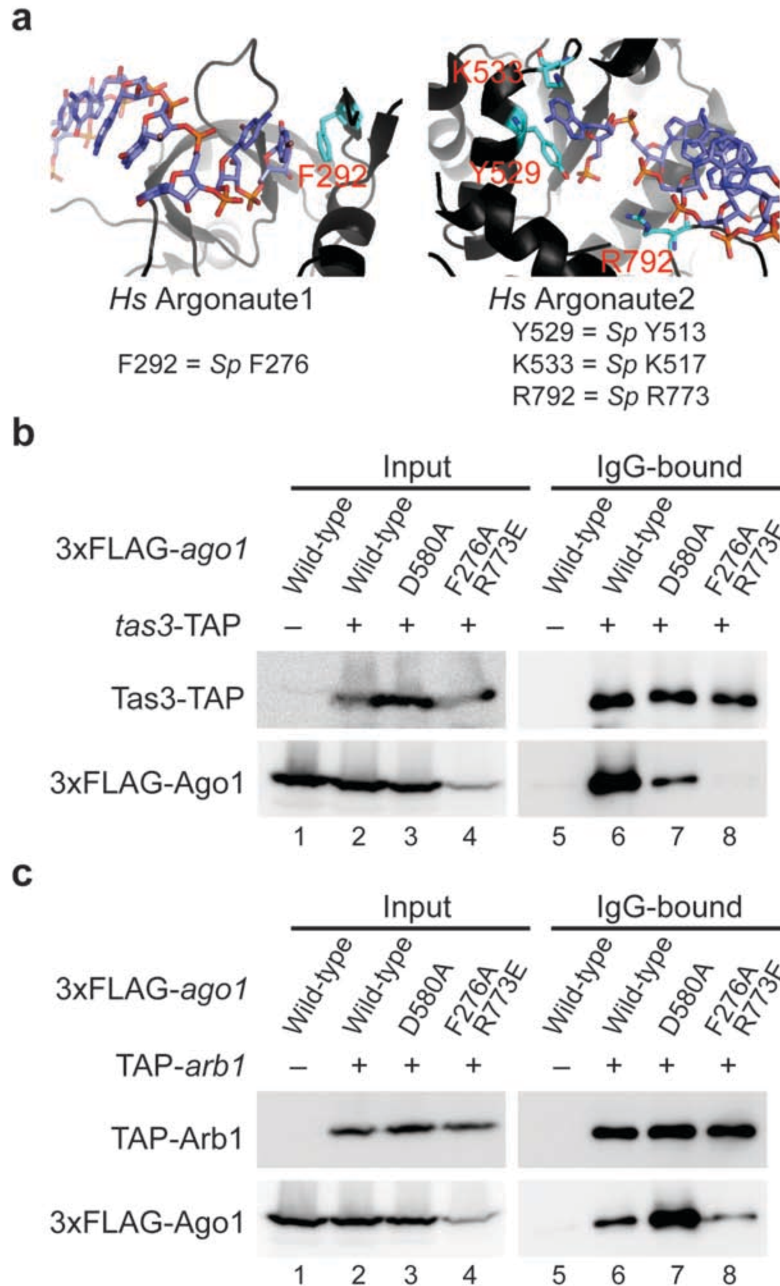

Figure 3.2. Loading of small RNAs onto Ago1 is required for assembly of RITS but not ARC.
(**a**) Views of the human Argonaute1 PAZ domain (Ma et al., 2004) (left) and full-length human Argonaute2 (Schirle and MacRae, 2012) (right) each in complex with a small RNA, illustrating the residues chosen for mutagenesis in the corresponding *S. pombe* Ago1 protein to abrogate small RNA binding. Human Argonaute1 F292 = *S. pombe* Ago1 F276; human Argonaute2 Y529, K533 and R792 = *S. pombe* Ago1 Y513, K517 and R773, respectively. (**b**) Western blot analysis of co-immunoprecipitation experiment to assay association of Tas3 with the indicated Ago1 proteins. Result was identical in a separate experiment substituting Ago1-F276A Y513A K517A for Ago1-F276A R773E (data not shown). (**c**) Western blot analysis of co-immunoprecipitation experiment to assay association of Arb1 with the indicated Ago1 proteins. Result was identical in a separate experiment substituting Ago1-F276A Y513A K517A for Ago1-F276A R773E (data not shown).

manner (Halic and Moazed, 2010; Marasovic et al., 2013). Therefore, the maintenance of RITS in *dcr1Δ* cells (Figure 3.1a) might reflect loading of Argonaute with these remaining small RNAs,

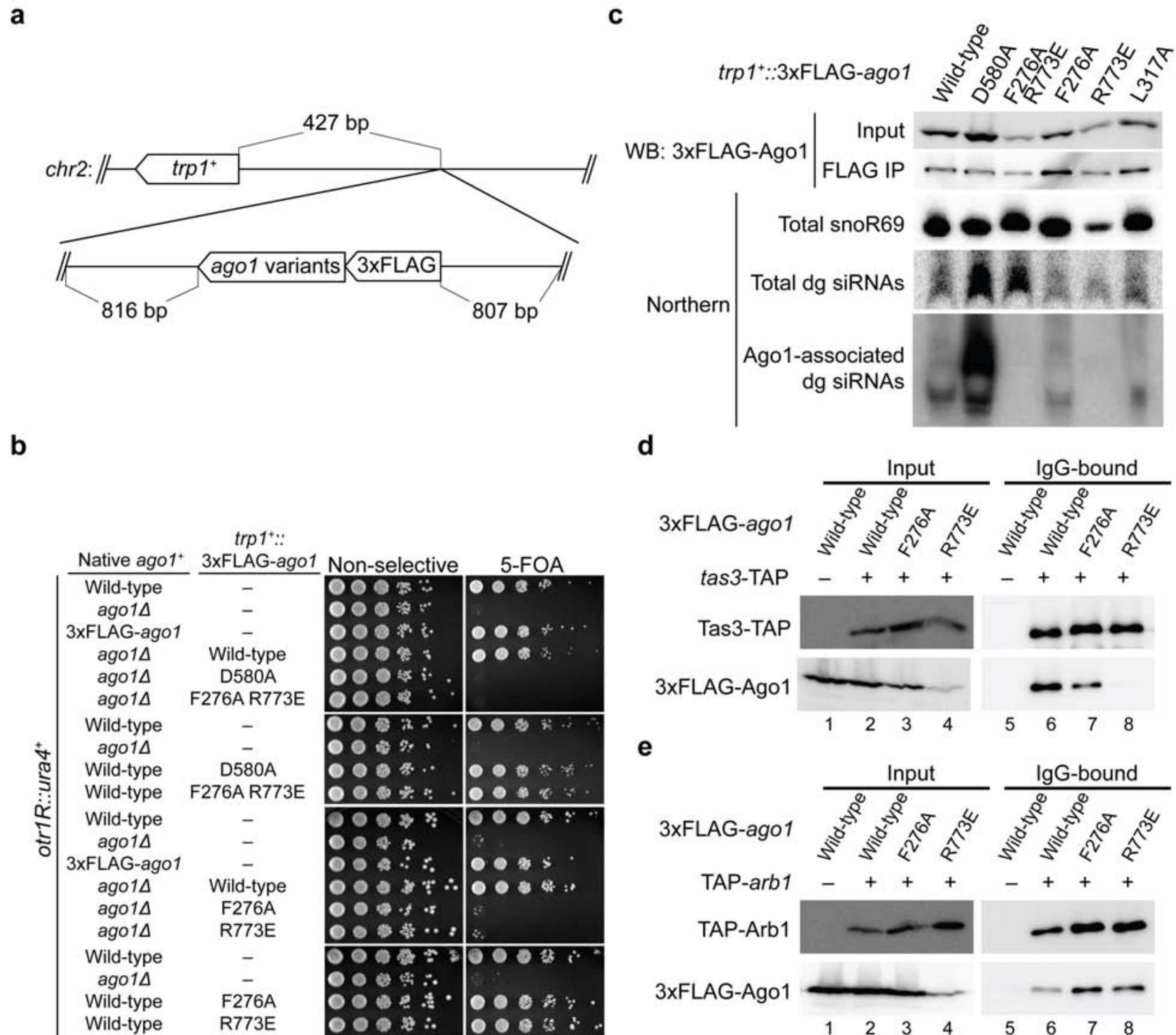

Figure 3.3. Ago1 D580A, Ago1 F276A R773E, Ago1 F276A and Ago1 R773E are null-mutant proteins whose small RNA–binding activity correlates with their assembly into RITS.
(**a**) Schematic illustrating the ectopic insertion of 3xFLAG-tagged alleles of *ago1* with endogenous promoter and terminator sequences near the *trp1*$^+$ locus on chromosome 2. The native *ago1*$^+$ locus harbors either a wild-type untagged allele or a deletion. (**b**) Tenfold serial dilutions of *otr1R::ura4*$^+$ pericentromeric silencing reporter cells of the indicated genotypes, plated on non-selective medium or medium containing 5-FOA. (**c**) Northern blot analysis of *dg* siRNAs in total small RNA fractions and RNA immunoprecipitated with wild-type and mutant 3xFLAG-Ago1 proteins, in cells also expressing untagged wild-type Ago1, and Western blot analysis of input extracts and FLAG-immunoprecipitated material. (**d**) Western blot analysis of co-immunoprecipitation experiment to assay association of Tas3 with the indicated Ago1 proteins. (**e**) Western blot analysis of co-immunoprecipitation experiment to assay association of Arb1 with the indicated Ago1 proteins.

and is compatible with a model in which RITS assembly is strictly contingent on a small RNA binding event.

In order to test this model directly, we generated a mutant of Ago1 that cannot load small RNAs and asked whether it is able to associate with the GW protein Tas3 *in vivo*. A thorough mutagenesis study by Till *et al.* (2007) showed that the region of human Argonaute2 required for recognition by the GW protein TNRC6B corresponds precisely to the MID and PIWI domain residues identified in structural studies as involved in anchoring the monophosphorylated 5' end of the guide RNA (Ma et al., 2005). Although we have previously mutated equivalent residues in *S. pombe* Ago1 (Y513 and K517) to abolish small RNA binding (Halic and Moazed, 2010) (Figure 3.2a, right), for the current experiment we needed to identify mutations that would prevent small RNA binding without altering the conserved Argonaute surface recognized by GW proteins. To this end, we combined a previously used PAZ domain mutation (F276A) which impairs binding of the guide RNA 3' end but only partially reduces loading *in vivo* (Buker et al., 2007; Ma et al., 2004; Partridge et al., 2007) (Figure 3.2a, left) with a mutation in a conserved arginine (R773E) shown in a recent crystal structure of full-length human Argonaute2 to make electrostatic contacts with two phosphates in the small RNA backbone (Schirle and MacRae, 2012) (Figure 3.2a, right). It is critical to note that mutation of the corresponding arginine by itself does not disrupt *in vitro* binding of Argonaute2 to TNRC6B, suggesting that the residue does not directly contact GW domain proteins (Till et al., 2007).

In order to investigate the relationship between Ago1 small RNA loading and Tas3 binding under normal silencing conditions, we left the native *ago1*$^{+}$ locus intact and integrated 3xFLAG-tagged wild-type and mutant alleles at an ectopic site near the *trp1*$^{+}$ gene (Iida et al., 2008) (Figure 3.3a). A wild-type *3xFLAG-ago1* insertion at this locus rescued *ago1Δ* in silencing of a pericentromeric *ura4*$^{+}$ reporter gene (Figure 3.3b). Similarly to the previously studied slicer-dead mutant *ago1-D580A* (Buker et al., 2007; Irvine et al., 2006), *ago1-F276A/R773E* failed to complement *ago1Δ*, but neither allele was dominant negative at single copy number (Figure

3.3b). Moreover, total pericentromeric *dg* siRNA levels were unperturbed by these alleles when *ago1*$^{+}$ was also present (Figure 3.3c). Thus, differences we observe in the association of the Ago1 variants with Tas3 are likely to reflect their intrinsic properties rather than general changes in heterochromatic silencing. Importantly, no *dg* siRNAs co-purified with Ago1-F276A/R773E despite their normal abundance in total cellular RNA, demonstrating that the mutant protein is unable to load small RNAs (Figure 3.3c). We also generated strains expressing the single-mutant proteins Ago1-F276A or Ago1-R773E. Interestingly, while neither restored pericentromeric reporter silencing in *ago1Δ* (Figure 3.3b), only Ago1-R773E was fully deficient for *dg* siRNA loading, whereas Ago1-F276A retained some loading activity, as had been observed previously when overexpressing this allele (Buker et al., 2007). In agreement with earlier results, Ago1-D580A associated with slightly longer siRNAs, suggesting that passenger strand release coincides with an exonucleolytic trimming event (Halic and Moazed, 2010) (Figure 3.3c).

Strikingly, we detected no association between Tas3 and either Ago1-F276A/R773E or Ago1-R773E, suggesting that loading of a small RNA onto Ago1 is a prerequisite for its assembly into RITS (Figures 3.2b and 3.3d). In contrast, the null mutant protein Ago1-D580A, which loads duplex siRNAs but cannot release the siRNA passenger strand (Figure 3.3c) (Buker et al., 2007), was still incorporated into RITS (Figure 3.2b), as was another null protein that nevertheless can load siRNAs, Ago1-F276A (Figure 3.3d). Recent studies have shown that Argonaute protein stability is affected by small RNA availability in different organisms (Martinez and Gregory, 2013; Smibert et al., 2013). In agreement with these reports, Ago1-F276A/R773E and Ago1-R773E were also present at reduced levels in whole cell extracts (Figures 3.2b and 3.3d). However, this does not account for their failure to associate with Tas3, because they associated with Arb1 at levels comparable to the wild-type variant (Figures 3.2c and 3.3e). Unexpectedly, the slicer mutation D580A seemed to shift Ago1 from RITS to ARC (Figure 3.2b,c), suggesting that passenger strand release inhibits the association of Ago1 with Arb1 and

Arb2. What we conclude most clearly from our observations, however, is that small RNA loading into Ago1 is a precondition for its assembly into RITS, but not ARC.

III. Discussion

The results presented in this chapter identify small RNA loading as a critical event that enables Argonaute to assemble into RITS, a GW protein-containing effector complex. We propose that this ordered assembly mechanism has evolved to prevent the premature assembly of Argonautes lacking small RNA guides into non-functional GW protein complexes, which could engage in unproductive interactions with downstream silencing factors. These findings carry broad implications for the diversity of organisms in which small RNA-dependent silencing mechanisms rely on the physical association of Argonaute with a conserved GW motif protein.

A. Determinants of Argonaute Progression between the ARC and RITS Complexes

Our data indicate that loading of Argonaute with a small RNA promotes, and indeed is required for, its progression from ARC to RITS. First, deletion of *dcr1*$^{+}$, which eliminates siRNAs and leaves only a residual population of priRNAs (Halic and Moazed, 2010; Marasovic et al., 2013), leads to a strengthening of the Arb1-Ago1 interaction (Figure 3.1b) and a weakening of the Tas3-Ago1 interaction (Figure 3.1a). Second, abolishing the small RNA binding activity of Ago1 prevents it from associating with Tas3 (Figures 3.2b and 3.3d) while leaving its interaction with Arb1 largely or entirely unaffected (Figures 3.2c and 3.3e).

Another determinant of Argonaute complex progression appears to involve the slicer-dependent conversion of siRNA duplexes to single strands. We found that the Ago1-D580A catalytic slicer mutant exhibits a reduced association with Tas3 and a concomitantly increased association with Arb1 (Figure 3.2b,c), implying that passenger strand release by Ago1 may inhibit its maintenance within ARC. Two observations suggest that transit from ARC to RITS is still possible without slicing: first, some Tas3-Ago1 association persists in the slicer mutant (Figure 3.2b), and, second, we have detected duplex siRNAs bound to RITS in a previous study

(Buker et al., 2007) and to some extent in this study (Figure 2.1a). Nevertheless, our data support a role for the single-stranded state of Argonaute-bound small RNAs in promoting entry into the GW protein-containing silencing complex RITS.

B. GW Proteins as Sensors of Argonaute Loading

More generally, our results underscore the importance of small RNA loading itself in licensing Argonautes for association with GW protein effectors. The most thorough investigation to date of the recognition of an Argonaute by a GW protein was carried out by Till *et al.* (2007). These authors observed a striking concordance between the residues in human Argonaute2 required for TNRC6B binding *in vitro* and those predicted from earlier structural work to secure the 5'-monophosphorylated 5'-terminal nucleotide of a guide RNA (Ma et al., 2005). Although RNA may not be necessary to observe the interaction at the high protein concentrations used *in vitro*, the results suggest that GW proteins may recognize a region of Argonaute whose conformation is modulated by RNA binding *in vivo*. Corroborating the conclusions of early experiments with truncated *Drosophila* proteins (Behm-Ansmant et al., 2006), a recent crystal structure of human Argonaute2 with free tryptophan suggests that GW motifs may in fact bind to a region in the PIWI domain located at a site proximal to, but distinct from, the RNA 5' end binding site (Schirle and MacRae, 2012). Conspicuously, residues of Argonaute2 belonging to the tryptophan-binding pockets resolved by Schirle and Macrae (2012), such as R583, I592, R647, F653 and K660, are also critical for the *in vitro* interaction between Argonaute2 and TNRC6B (Till et al., 2007).

We propose that GW proteins act as sensors of small RNA loading onto Argonaute proteins. Engagement of Argonaute with a small RNA guide, particularly at its 5' end, may propagate a critical conformational change to the GW binding site in the PIWI domain and trigger a permissive state for GW protein association. Consistent with this model, we have shown here that the *S. pombe* GW protein Tas3 does not associate with the unloaded Argonautes Ago1-F276A/R773E and Ago1-R773E *in vivo* (Figures 3.2b and 3.3d). Importantly,

Ago1-F276A/R773E and Ago1-R773E still associate with Arb1 at near wild-type levels (Figures 3.2c and 3.3e), indicating that the mutants remain competent to undergo protein-protein interactions and supporting the notion that their binding to Tas3 is governed by small RNAs. Finally, given the role of Arb1 and Arb2 in small RNA loading (see Chapter 2), our observation that wild-type Ago1 fails to assemble into RITS in the absence of these proteins (Figure 3.1a) lends further support to the idea that GW proteins detect the loading state of Argonautes.

In apparent contradiction with the model of GW proteins as small RNA sensors, two previous studies have identified Argonaute mutants with defects in small RNA binding that still associate with GW proteins in human and *Drosophila* cells (Eulalio et al., 2009; Liu et al., 2005). However, these experiments were performed using overexpressed proteins and it is unclear whether the observed interactions persist at physiological protein concentrations. And indeed, more exhaustive analyses with *Drosophila* AGO1 have revealed that mutants deficient in small RNA loading consistently fail to associate with GW182 in cells, but that certain mutants that do not associate with GW182 still load small RNAs (Boland et al., 2011; Eulalio et al., 2008). Together with our data, these findings suggest that GW protein docking occurs downstream of the programming of Argonaute with a small RNA.

C. Safeguards in the Assembly of GW Protein-Containing Argonaute Silencing Complexes

This mechanism of sensing small RNA loading may have evolved in order to preserve GW proteins from wasteful interactions with unguided Argonautes. Since GW proteins form a critical link between sequence-specific small RNA signals and the machineries that carry out gene silencing, such as the CCR4/NOT deadenylase complex in metazoans and the CLRC H3K9 methyltransferase complex in *S. pombe* (Braun et al., 2011; Chekulaeva et al., 2011; Fabian et al., 2011; Gerace et al., 2010), their availability is expected to be important for ensuring the efficiency of silencing. In this model, association of Tas3 and other GW proteins with unloaded Argonautes would be deleterious because these non-functional complexes could

compete with small RNA-programmed Argonautes for interactions with downstream factors. Thus, by rejecting unloaded Argonaute molecules, GW proteins would prevent them from acting as poisons that would make silencing systems less responsive to small RNA specificity signals (Figure 3.4). In *S. pombe*, the slicer mutant Ago1-D580A illustrates this type of danger: although it is loaded with small RNAs, these remain double-stranded and the mutant Argonaute therefore

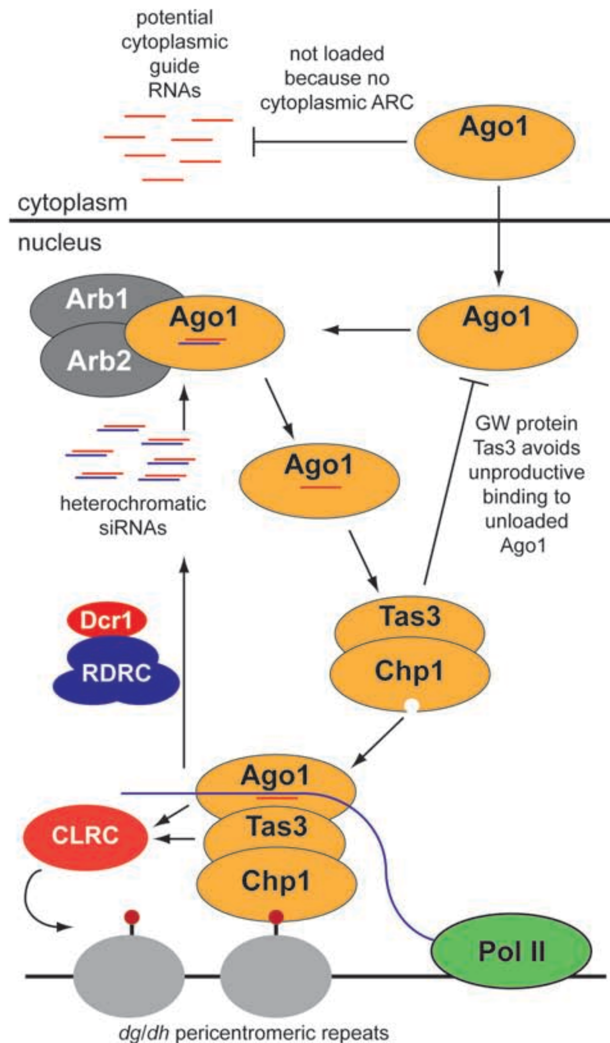

Figure 3.4. Regulation of RITS complex assembly by small RNA loading onto Argonaute.
The requirement for the nuclear complex ARC in loading Ago1 with small RNAs prevents Ago1 from binding guide RNAs in the cytoplasm and mediating post-transcriptional gene silencing. In the nucleus, Ago1 associates with Arb1 and Arb2 which enable its loading with heterochromatic duplex siRNAs generated by RDRC (RNA-dependent RNA polymerase Complex) and Dcr1. Small RNA loading renders Ago1 competent for assembly into the RITS effector complex, whose sequence-specific recruitment to nascent transcripts leads to CLRC-dependent methylation of H3K9 (red circles). The GW motif-containing RITS subunit Tas3 rejects unloaded Ago1, preventing unproductive interactions that would compromise silencing.

renders the RITS complex inactive. This produces a dominant negative phenotype upon overexpression (Figure 3.5) (Buker et al., 2007), suggesting that the exclusion of immature Argonautes from silencing complexes is imperative for proper functioning of the pathway. Finally, fission yeast Ago1 appears to localize both to the nucleus and to the cytoplasm, while Arb1 is predominantly nuclear. This is likely to restrict Ago1 small RNA loading to the nucleus and may provide an explanation for weak post-transcriptional gene silencing in *S. pombe*, even when siRNAs are produced to high levels from long hairpin RNAs (Iida et al., 2008; Simmer et al., 2010) (Figure 3.4). In support of this idea, overexpressed Ago1, which our data imply is loaded in an ARC-independent manner (see Chapter 2 and Figure 2.8), frequently associates with small RNAs derived from the 3' untranslated regions of protein-coding genes (Yu et al., 2014).

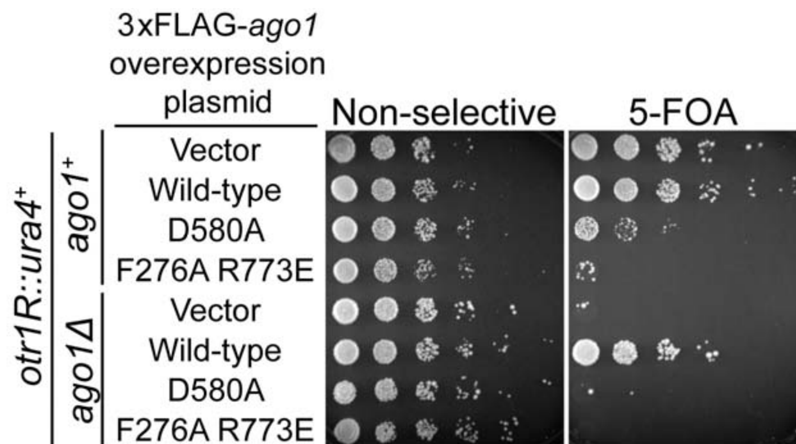

Figure 3.5. *ago1* D580A and *ago1* F276A R773E are dominant-negative alleles when overexpressed.
Tenfold serial dilutions of *otr1R::ura4*$^{+}$ pericentromeric silencing reporter cells of the indicated genotypes, transformed with an empty vector or a 3xFLAG-*ago1* overexpression plasmid (wild-type or mutant as noted), plated on non-selective medium or medium containing 5-FOA.

Table 3.2. ***S. pombe*** **strains used in this study.**

Strain	Genotype
SPY137	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+}
SPY418	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} ago1Δ::kanMX6
SPY797	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} natMX6-3xFLAG-ago1
SPY2556	h^{+} leu1-32 ade6-M216 ura4-D18 imr1R(NcoI)::ura4^{+} tas3-TAP-kanMX6 hphMX6-3xFLAG-ago1
SPY2640	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} ago1Δ::kanMX6 trp1^{+}::natMX6-3xFLAG-ago1
SPY2690	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} natMX6-3xFLAG-ago1 kanMX6-TAP-arb1
SPY3206	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} natMX6-3xFLAG-ago1 kanMX6-TAP-arb1 arb2Δ::hphMX6
SPY3319	h^{-} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} natMX6-3xFLAG-ago1 kanMX6-TAP-arb1 dcr1Δ::hphMX6
SPY3548	h^{+} leu1-32 ade6-M216 ura4-D18 imr1R(NcoI)::ura4^{+} tas3-TAP-kanMX6 hphMX6-3xFLAG-ago1 dcr1Δ::natMX6
SPY3928	h^{+} leu1-32 ade6-M216 ura4-D18 imr1R(NcoI)::ura4^{+} tas3-TAP-kanMX6 hphMX6-3xFLAG-ago1 arb1Δ::natMX6
SPY3931	h^{+} leu1-32 ade6-M216 ura4-D18 imr1R(NcoI)::ura4^{+} tas3-TAP-kanMX6 hphMX6-3xFLAG-ago1 arb2Δ::TAP-natMX6
SPY4314	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} trp1^{+}::natMX6-3xFLAG-ago1
SPY4317	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} trp1^{+}::natMX6-3xFLAG-ago1-D580A
SPY4320	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} trp1^{+}::natMX6-3xFLAG-ago1-F276A Y513A K517A
SPY4321	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} ago1Δ::kanMX6 trp1^{+}::natMX6-3xFLAG-ago1-D580A
SPY4327	h^{+} leu1-32 ade6-M216 ura4-D18 imr1R(NcoI)::ura4^{+} tas3-TAP-kanMX6 trp1^{+}::natMX6-3xFLAG-ago1
SPY4329	h^{+} leu1-32 ade6-M216 ura4-D18 imr1R(NcoI)::ura4^{+} tas3-TAP-kanMX6 trp1^{+}::natMX6-3xFLAG-ago1-D580A
SPY4331	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} kanMX6-TAP-arb1 trp1^{+}::natMX6-3xFLAG-ago1
SPY4334	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} kanMX6-TAP-arb1 trp1^{+}::natMX6-3xFLAG-ago1-D580A
SPY4415	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} natMX6-3xFLAG-ago1 tas3Δ::hphMX6
SPY4421	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} natMX6-3xFLAG-ago1 kanMX6-TAP-arb1 tas3Δ::hphMX6
SPY4778	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} trp1^{+}::natMX6-3xFLAG-ago1-F276A R773E
SPY4780	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} ago1Δ::kanMX6 trp1^{+}::natMX6-3xFLAG-ago1-F276A R773E
SPY4784	h^{+} leu1-32 ade6-M216 ura4-D18 imr1R(NcoI)::ura4^{+} tas3-TAP-kanMX6 trp1^{+}::natMX6-3xFLAG-ago1-F276A R773E
SPY4785	h^{+} leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4^{+} kanMX6-TAP-arb1 trp1^{+}::natMX6-3xFLAG-ago1-F276A R773E

Table 3.2 (Continued).

SPY5186	*h^+ leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4$^+$ trp1$^+$::natMX6-3xFLAG-ago1-F276A*
SPY5189	*h^+ leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4$^+$ trp1$^+$::natMX6-3xFLAG-ago1-R773E*
SPY5192	*h^+ leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4$^+$ ago1Δ::kanMX6 trp1$^+$::natMX6-3xFLAG-ago1-F276A*
SPY5195	*h^+ leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4$^+$ ago1Δ::kanMX6 trp1$^+$::natMX6-3xFLAG-ago1-R773E*
SPY5196	*h^+ leu1-32 ade6-M216 ura4-D18 imr1R(NcoI)::ura4$^+$ tas3-TAP-kanMX6 trp1$^+$::natMX6-3xFLAG-ago1-F276A*
SPY5199	*h^+ leu1-32 ade6-M216 ura4-D18 imr1R(NcoI)::ura4$^+$ tas3-TAP-kanMX6 trp1$^+$::natMX6-3xFLAG-ago1-R773E*
SPY5200	*h^+ leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4$^+$ kanMX6-TAP-arb1 trp1$^+$::natMX6-3xFLAG-ago1-F276A*
SPY5203	*h^+ leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4$^+$ kanMX6-TAP-arb1 trp1$^+$::natMX6-3xFLAG-ago1- R773E*

IV. Methods

A. Strain Construction

S. pombe strains used in this study are described in Table 3.2 and were generated using a PCR-based gene targeting strategy (Bähler et al., 1998). All gene deletions were made by replacing the coding region (ATG to stop) with a drug resistance cassette. The N-terminally tagged 3xFLAG-*ago1* allele at the native *ago1*$^+$ locus was made by simultaneously inserting a drug resistance cassette 808 bp upstream of the ATG and the 3xFLAG coding sequence immediately upstream of the ATG (Buker et al., 2007). The N-terminally tagged TAP-*arb1* allele at the native *arb1*$^+$ locus was made by simultaneously inserting a drug resistance cassette 560 bp upstream of the ATG and the TAP coding sequence immediately upstream of the ATG. The ectopic *trp1*$^+$*::*3xFLAG-*ago1* wild-type and mutant alleles were generated as shown in Figure 3.3a.

B. Co-immunoprecipitation and Western Blotting

Co-immunoprecipitations and Western blots were carried out as described (Yu et al., 2014). Rabbit IgG (Sigma) was used to prepare IgG-conjugated Dynabeads from Invitrogen

Dynabeads M270 Epoxy according to the manufacturer's instructions. Antibodies used were Peroxidase Anti-Peroxidase Soluble Complex (Sigma P-1291), 1:10,000 dilution, and FLAG M2-Peroxidase (HRP) mouse monoclonal antibody (Sigma A-8592), 1:5000 dilutionValidation information for both antibodies is provided on their respective manufacturers' websites.

C. Protein affinity purification and mass spectrometry

3xFLAG-Ago1 was purified from 1 L cultures grown to a density of ~2.5 x 10^7 cells/ml. Anti-FLAG beads were prepared by mixing 37 µl Protein G Dynabeads per sample (Invitrogen), after washing twice with 1 ml Tris-buffered saline (50 mM Tris-HCl pH 7.5, 150 mM NaCl) followed by twice with 1ml lysis buffer (20 mM HEPES pH 7.6, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 0.25% Triton X-100, 0.5 mM DTT, 1 mM PMSF, Roche Complete EDTA-free Protease Inhibitor Cocktail), with 9.25 µl anti-FLAG M2 antibody per sample (Sigma) and incubating in 200 µl total volume of lysis buffer per sample with rotation overnight at 4ºC. Anti-FLAG beads were crosslinked by washing twice with 1 ml 0.2 M sodium borate pH 9, incubating in 300 µl 0.2 M sodium borate pH 9 containing 4 mg/ml dimethyl pimelimidate per sample for 30 min at room temperature, washing twice with 1 ml 0.2 M ethanolamine-HCl pH 8, incubating in 300 µl per sample 0.2 M ethanolamine-HCl pH 8 for 1 h 30 min and finally washing three times with 1 ml lysis buffer. Aliquots of 0.4 g of cells were resuspended in 0.4 ml lysis buffer in 2-ml tubes and lysed with 1 ml acid-washed 0.5-mm glass beads per tube using 4 cycles of 45 s at 5000 rpm on the MagNA Lyser Instrument (Roche). Extracts were cleared by centrifugation for 15 min at 16,100 *g* and incubated with anti-FLAG beads for 3 h at 4ºC. Beads were washed four times with 1 ml lysis buffer, and protein was eluted from beads by incubation in 0.5 ml 0.5 M NH_4OH for 20 min at 37º with shaking. Sixteen percent of each eluate was analyzed by silver stain (Pierce) to confirm specific enrichment of 3xFLAG-Ago1 and 80 percent was analyzed by MS carried out in the Taplin Biological Mass Spectrometry Facility (Harvard Medical School, Boston, MA, USA).

Table 3.3. Oligonucleotides used in this study.

Oligonucleotide	Sequence	Source
Northern probes		
MB151 (snoRNA69)	CAATGTAAATACTCCGAGTGAGCTGGGTTTAAC	1
censiRNA_a' (dg)	GCGACTAAACCGAAAGCCTC	1
censiRNA_b' (dg)	TACCGTGATTAGCCTTACTCCGCATT	1
IK10 (dg)	GGGAGTACATCATTCCTACTTCGATA	2
DBH45 (dg)	GACTTTCAAAGATGCACA	3
DBH46 (dg)	TTTTCTCTTTCAAAAGTA	3
DBH47 (dg)	CAATTGGAAGTACATCCA	3
DBH48 (dg)	TCAATCCATCATGTACGA	3
DBH49 (dg)	AATTCGATTCCAAGTACA	3
DBH50 (dg)	ATTGTTTCGACAACACGA	3

1 = Bühler et al., 2006, **2** = Bayne et al., 2010, **3** = Halic and Moazed, 2010

D. Total Small RNA Isolation

Total small RNAs for Northern blots were recovered using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's instructions.

E. Isolation of Protein-Associated RNA for Northern Blots

Wild-type or mutant 3xFLAG-Ago1 was purified from cultures of 3 L grown to a density of ~ 3 x 10^7 cells/ml (*ago1*$^+$ promoter) or 1 L grown to ~6 x 10^7 cells/ml (overexpressed). Cells were resuspended in 1 ml lysis buffer (20 mM HEPES pH 7.6, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 0.25% Triton X-100, 0.5 mM DTT, 1 mM PMSF, Roche Complete EDTA-free Protease Inhibitor Cocktail) per 5 g cells and frozen dropwise in liquid nitrogen. Frozen material was ground into a powder at liquid nitrogen temperatures in a Retsch Cryomill (3 x 3 min at 30 Hz). Meanwhile, extracts were prepared by resuspending frozen cell powders in 2 ml lysis buffer per 3 g powder and centrifuging the thawed suspension for 15 min at 16,100 *g*. Extracts were incubated with 70 µl packed EZview Red Anti-FLAG M2 Affinity Gel (Sigma) per sample for 2 h at 4ºC. Beads were washed four times with 1 ml lysis buffer, then RNA was eluted by treatment with Proteinase K (80 U/ml final concentration in 30 mM Tris-HCl pH 8) for 30 min at 37ºC followed by 2% SDS for 10 min at 65ºC and extraction with phenol:chloroform.

Table 3.4. Plasmids used in this study.

pDM815	pREP1-*nmt1*$^+$promoter-3xFLAG
pDM817	pREP1-*nmt1*$^+$promoter-3xFLAG-*ago1*
pDM831	pREP1-*nmt1*$^+$promoter-3xFLAG-*ago1*-D580A
pDM1590	pREP1-*nmt1*$^+$promoter-3xFLAG-*ago1*-F276A R773E

F. Northern Blots

Northern blots were performed as described (Bühler et al., 2006) using total small RNAs or RNA isolated from 3xFLAG-Ago1 affinity purifications (see above). Oligonucleotide probes are listed in Table 3.3.

G. Growth Assays

Cells were grown to saturation and diluted serially tenfold so that the highest density spot contained 1.3 x 10^5 cells. Non-selective plates contained Edinburgh Minimal Medium with all standard supplements (Sunrise Science Products) except leucine to ensure maintenance of the transformed plasmids, which are listed in Table 3.4. Selective plates additionally contained 1 g/L 5-fluoroorotic acid.

V. Acknowledgments

We thank members of the Moazed laboratory for helpful discussions, E. Gerace, M. Halic, N. Iglesias and J. Xiol for advice on protocols, M. Halic and R. Yu for scripts and advice on bioinformatic analyses, C. Centrella for technical assistance, and E. Egan, N. Iglesias, R. Jain, J. Xiol and R. Yu for critical reading of the manuscript. This work was supported by the U.S. National Science Foundation Graduate Research Fellowship Program (D.H.) and the U.S. National Institutes of Health R01 GM072805 (D.M.). D.M. is supported as a Howard Hughes Medical Institute Investigator.

VI. References

Azevedo, J., Cooke, R., and Lagrange, T. (2011). Taking RISCs with Ago hookers. Curr. Opin. Plant Biol. *14*, 594-600.

Bähler, J., Wu, J.Q., Longtine, M.S., Shah, N.G., McKenzie, A., III, Steever, A.B., Wach, A., Philippsen, P., and Pringle, J.R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. Yeast *14*, 943-951.

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

Bayne, E.H., White, S.A., Kagansky, A., Bijos, D.A., Sanchez-Pulido, L., Hoe, K.L., Kim, D.U., Park, H.O., Ponting, C.P., Rappsilber, J., and Allshire, R.C. (2010). Stc1: a critical link between RNAi and chromatin modification required for heterochromatin integrity. Cell *140*, 666-677.

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

Bernard, P., Maure, J.F., Partridge, J.F., Genier, S., Javerzat, J.P., and Allshire, R.C. (2001). Requirement of heterochromatin for cohesion at centromeres. Science *294*, 2539-2542.

Boland, A., Huntzinger, E., Schmidt, S., Izaurralde, E., and Weichenrieder, O. (2011). Crystal structure of the MID-PIWI lobe of a eukaryotic Argonaute protein. Proc. Natl. Acad. Sci. USA *108*, 10466-10471.

Braun, J.E., Huntzinger, E., Fauser, M., and Izaurralde, E. (2011). GW182 proteins directly recruit cytoplasmic deadenylase complexes to miRNA targets. Mol. Cell *44*, 120-133.

Braun, J.E., Huntzinger, E., and Izaurralde, E. (2013). The role of GW182 proteins in miRNA-mediated silencing. Ad. Exp. Med. Biol. *768*, 147-163.

Bühler, M., Verdel, A., and Moazed, D. (2006). Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing. Cell *125*, 873-886.

Buker, S.M., Iida, T., Bühler, M., Villén, J., Gygi, S.P., Nakayama, J., and Moazed, D. (2007). Two different Argonaute complexes are required for siRNA generation and heterochromatin assembly in fission yeast. Nat. Struct. Mol. Biol. *14*, 200-207.

Chekulaeva, M., Mathys, H., Zipprich, J.T., Attig, J., Colic, M., Parker, R., and Filipowicz, W. (2011). miRNA repression involves GW182-mediated recruitment of CCR4-NOT through conserved W-containing motifs. Nat. Struct. Mol. Biol. *18*, 1218-1226.

Colmenares, S.U., Buker, S.M., Buhler, M., Dlakic, M., and Moazed, D. (2007). Coupling of double-stranded RNA synthesis and siRNA generation in fission yeast RNAi. Mol. Cell *27*, 449-461.

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.

Ellermeier, C., Higuchi, E.C., Phadnis, N., Holm, L., Geelhood, J.L., Thon, G., and Smith, G.R. (2010). RNAi and heterochromatin repress centromeric meiotic recombination. Proc. Natl. Acad. Sci. USA *107*, 8701-8705.

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.

Fabian, M.R., Cieplak, M.K., Frank, F., Morita, M., Green, J., Srikumar, T., Nagar, B., Yamamoto, T., Raught, B., Duchaine, T.F., and Sonenberg, N. (2011). miRNA-mediated deadenylation is orchestrated by GW182 through two conserved motifs that interact with CCR4-NOT. Nat. Struct. Mol. Biol. *18*, 1211-1217.

Gerace, E.L., Halic, M., and Moazed, D. (2010). The methyltransferase activity of $Clr4^{Suv39h}$ triggers RNAi independently of histone H3K9 methylation. Mol. Cell *39*, 360-372.

Ghildiyal, M., and Zamore, P.D. (2009). Small silencing RNAs: an expanding universe. Nat. Rev. Genet. *10*, 94-108.

Giner, A., Lakatos, L., García-Chapa, M., López-Moya, J., and Burgyán, J. (2010). Viral protein inhibits RISC activity by argonaute binding through conserved WG/GW motifs. PLoS Pathog. *6*, e1000996.

Halic, M., and Moazed, D. (2010). Dicer-independent primal RNAs trigger RNAi and heterochromatin formation. Cell *140*, 504-516.

Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature *404*, 293-296.

Hong, E.J., Villén, J., Gerace, E.L., Gygi, S.P., and Moazed, D. (2005). A cullin E3 ubiquitin ligase complex associates with Rik1 and the Clr4 histone H3-K9 methyltransferase and is required for RNAi-mediated heterochromatin formation. RNA Biol. *2*, 106-111.

Hutvagner, G., and Simard, M.J. (2008). Argonaute proteins: key players in RNA silencing. Nat. Rev. Mol. Cell Biol. *9*, 22-32.

Iida, T., Nakayama, J., and Moazed, D. (2008). siRNA-mediated heterochromatin establishment requires HP1 and is associated with antisense transcription. Mol. Cell *31*, 178-189.

Irvine, D.V., Zaratiegui, M., Tolia, N.H., Goto, D.B., Chitwood, D.H., Vaughn, M.W., Joshua-Tor, L., and Martienssen, R.A. (2006). Argonaute slicing is required for heterochromatic silencing and spreading. Science *313*, 1134-1137.

Jinek, M., and Doudna, J.A. (2009). A three-dimensional view of the molecular machinery of RNA interference. Nature *457*, 405-412.

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

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.

Malone, C.D., and Hannon, G.J. (2009). Small RNAs as guardians of the genome. Cell *136*, 656-668.

Marasovic, M., Zocco, M., and Halic, M. (2013). Argonaute and Triman generate dicer-independent priRNAs and mature siRNAs to initiate heterochromatin formation. Mol. Cell *52*, 173-183.

Martinez, N.J., and Gregory, R. (2013). Argonaute2 expression is post-transcriptionally coupled to microRNA abundance. RNA *19*, 605-612.

Matranga, C., Tomari, Y., Shin, C., Bartel, D.P., and Zamore, P.D. (2005). Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell *123*, 607-620.

Meister, G. (2013). Argonaute proteins: functional insights and emerging roles. Nat. Rev. Genet. *14*, 447-459.

Moazed, D. (2009). Small RNAs in transcriptional gene silencing and genome defence. Nature *457*, 413-420.

Motamedi, M.R., Verdel, A., Colmenares, S.U., Gerber, S.A., Gygi, S.P., and Moazed, D. (2004). Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs. Cell *119*, 789-802.

Noma, K., Sugiyama, T., Cam, H., Verdel, A., Zofall, M., Jia, S., Moazed, D., and Grewal, S.I. (2004). RITS acts in *cis* to promote RNA interference-mediated transcriptional and post-transcriptional silencing. Nat. Genet. *36*, 1174-1180.

Olovnikov, I., Chan, K., Sachidanandam, R., Newman, D.K., Aravin, A.A. (2013). Bacterial argonaute samples the transcriptome to identify foreign DNA. Mol. Cell *51*, 594-605.

Partridge, J.F., DeBeauchamp, J.L., Kosinski, A.M., Ulrich, D.L., Hadler, M.J., and Noffsinger, V.J.P. (2007). Functional separation of the requirements for establishment and maintenance of centromeric heterochromatin. Mol. Cell *26*, 593-602.

Pontier, D., Picart, C., Roudier, F., Garcia, D., Lahmy, S., Azevedo, J., Alart, E., Laudié, M., Karlowski, W.M., Cooke, R., Colot, V., Voinnet, O., and Lagrange, T. (2012). NERD, a plant-specific GW protein, defines an additional RNAi-dependent chromatin-based pathway in *Arabidopsis*. Mol. Cell *48*, 121-132.

Probst, A.V., Dunleavy, E., and Almouzni, G. (2009). Epigenetic inheritance during the cell cycle. Nat. Rev. Mol. Cell. Biol. *10*, 192-206.

Rand, T.A., Petersen, S., Du, F., and Wang, X. (2005). Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. Cell *123*, 621-629.

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

Schalch, T., Job, G., Noffsinger, V.J., Shanker, S., Kuscu, C., Joshua-Tor, L., and Partridge, J.F. (2009). High-affinity binding of Chp1 chromodomain to K9 methylated histone H3 is required to establish centromeric heterochromatin. Mol. Cell *34*, 36-46.

Schirle, N.T., and MacRae, I.J. (2012). The Crystal Structure of Human Argonaute2. Science *336*, 1037-1040.

Simmer, F., Buscaino, A., Kos-Braun, I.C., Kagansky, A., Boukaba, A., Urano, T., Kerr, A.R., and Allshire, R.C. (2010). Hairpin RNA induces secondary small interfering RNA synthesis and silencing in *trans* in fission yeast. EMBO Rep. *11*, 112-118.

Smibert, P., Yang, J., Azzam, G., Liu, J., and Lai, E.C. (2013). Homeostatic control of Argonaute stability by microRNA availability. Nat. Struct. Mol. Biol. *20*, 789-795.

Till, S., Lejeune, E., Thermann, R., Bortfeld, M., Hothorn, M., Enderle, D., Heinrich, C., Hentze, M., 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.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S.I., and Moazed, D. (2004). RNAi-mediated targeting of heterochromatin by the RITS complex. Science *303*, 672-676.

Volpe, T.A., Kidner, C., Hall, I.M., Teng, G., Grewal, S.I., and Martienssen, R.A. (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science *297*, 1833-1837.

Woolcock, K.J., Stunnenberg, R., Gaidatzis, D., Hotz, H.R., Emmerth, S., Barraud, P., and Bühler, M. (2012). RNAi keeps Atf1-bound stress genes in check at nuclear pores. Genes Dev. *26*, 683-692.

Yu, R., Jih, G., Iglesias, N., and Moazed, D. (2014). Determinants of heterochromatic siRNA biogenesis and function. Mol. Cell *53*, 262-276.

Zhang, K., Mosch, K., Fischle, W., and Grewal, S.I. (2008). Roles of the Clr4 methyltransferase complex in nucleation, spreading and maintenance of heterochromatin. Nat. Struct. Mol. Biol. *15*, 381-388.

Chapter 4

Perspectives

Living systems have evolved sophisticated mechanisms of organization to optimize the efficiency of their growth and reproduction, and to contend with the challenges presented to them by their living and nonliving surroundings. Among the most fundamentally important of these strategies are those that faithfully preserve the integrity of the genetic material, which represents a vital necessity for the future of each species. Early in the history of life, Argonaute proteins emerged as participants in this critical aspect of survival (Swarts et al., 2014). In eukaryotes, Argonautes use short ncRNAs to repress repetitive, foreign and mobile genetic elements that pose threats to the stability of the genome (Malone and Hannon, 2009; Moazed, 2009). In addition to this ancestral role, Argonautes in many organisms have also evolved to regulate the expression of endogenous genes. For either of these functions, two major phases of Argonaute protein activity can be distinguished: a programming step that involves the loading of small RNAs into Argonaute, and a silencing step in which Argonaute recognizes an RNA with complementarity to the small-RNA guide and mediates its repression, typically by assembling into a complex with other silencing factors.

The experimental results reported in this dissertation offer novel insights into both the programming and the assembly of Argonaute complexes. Chapter 2 defines two previously identified interacting partners of the *Schizosaccharomyces pombe* Argonaute protein Ago1, the ARC complex subunits Arb1 and Arb2, as the members of an Argonaute loading apparatus whose mode of operation appears to be mechanistically distinct from that of known loading machineries from other species. Chapter 3 presents evidence that the formation of the RITS effector silencing complex in *S. pombe* is governed by the small-RNA loading status of Ago1 and, on the basis of this finding and previous literature, proposes that GW-repeat proteins such as the RITS subunit Tas3 act as sensors of Argonaute loading that ensure the ordered assembly of functional silencing complexes. These conclusions also raise new questions and open fresh avenues for future investigation. In this final chapter I explore some of the unknowns that have arisen from our work and suggest possible strategies to address them.

I. The molecular mechanism of small-RNA loading by Arb1

Arb1 and Arb2 were discovered in the very first mass spectrometry analyses of proteins present in purifications of Ago1 from *S. pombe* cell extracts (Buker et al., 2007). But, as discussed in Chapter 2, their role in small-RNA loading was not immediately obvious. The classic examples of loading factors, R2D2 and TRBP, both bind to Dicer (Liu et al., 2003; Gregory et al., 2005), suggesting that they physically capture small RNAs at the instant of their biogenesis. In contrast, Arb1 and Arb2 apparently do not interact with the *S. pombe* Dicer enzyme Dcr1 (Buker et al., 2007; Colmenares et al., 2007). Thus, Arb1 and Arb2 do not seem to pass siRNAs directly from Dcr1 into Ago1.

Nevertheless, interaction with Dicer is not formally necessary for a loading factor to function by first binding siRNAs and then introducing them into Argonaute. It was recently shown, for example, that the human AUF1 RNA-binding protein binds the microRNA let-7b *in vitro* in the absence of Dicer or other proteins, and promotes its loading into Argonaute2 *in vivo* (Yoon et al., 2015). Although this case involves sequence-specific small-RNA recognition, which is unlikely to apply to *S. pombe*, it illustrates a model wherein a loading factor binds a small RNA separately from Dicer and then transfers it to Argonaute. Our own *in vitro* assays indicate that only Arb1, not Arb2, is directly involved in Ago1 loading (Figure 2.4d,e). Thus, it is conceivable that Arb1 could bind to small RNAs after their release from Dcr1, and then load them into Ago1, mirroring the action of AUF1. However, while the ARC complex immunopurified from *S. pombe* cells via TAP-Arb1 binds double-stranded small RNAs *in vitro* (Figure 2.3c,d), and this activity is also observed for TAP-Arb1 purified from *arb2*Δ cells, which contains associated Ago1 (Figure 3.1b and data not shown), it is absent in TAP-Arb1 recovered from *ago1*Δ cells (data not shown). Thus, under the conditions of our *in vitro* experiments, Arb1 does not exhibit any small-RNA-binding ability of its own. Although it remains possible that Arb1 binds small RNAs directly and does so exclusively when it is in complex with Ago1, the simplest

interpretation of our data rules out the idea that Arb1 mediates the loading of small RNA molecules by first capturing them and then presenting them to Ago1.

Instead, a model that is more consistent with our observations is that Arb1 exerts an allosteric effect on Ago1 that enables it to accept small RNAs. It has been remarked that the conformation ordinarily adopted by Argonaute proteins, as revealed by structural analyses, does not appear capable of accommodating a rigid, perfectly paired small-RNA double helix (Kawamata and Tomari, 2010). This might explain the prominent role of ATP hydrolysis and Hsp90 chaperones in certain well-studied cases of small-RNA loading (Iki et al., 2010; Iwasaki et al., 2010; Kawamata et al., 2009; Miyoshi et al., 2010; Yoda et al., 2010), as significant energy could be required to alter the conformation of Argonaute into a state permissive for small-RNA duplex insertion (Kawamata and Tomari, 2010). In our *in vitro* assay, the small-RNA loading activity of immunopurified ARC appears to be unaffected by the addition of either ATP or an ATP-depleting enzyme, or an inhibitor of Hsp90 chaperones (data not shown). However, these observations may not be meaningful, because we were not able to confirm the efficacy of the added reagents. Further experiments are therefore necessary to draw any conclusions about the necessity or dispensability of ATP for Arb1-mediated loading of Ago1. Future studies should endeavor to recapitulate small-RNA loading using purified, recombinant Ago1 and Arb1, which would allow a test of whether loading can occur in the absence any additional *S. pombe* proteins and a more rigorous assessment of the potential role of ATP hydrolysis.

But facilitating a conformational change that would permit the insertion of small RNA duplexes into Ago1, as is proposed to occur during ATP-dependent loading of fly and human Argonautes (Kawamata and Tomari, 2010), cannot, alone, represent the function of Arb1. Our sequencing of small RNAs associated with Ago1 in *dcr1*Δ and *arb1*Δ *dcr1*Δ cells demonstrates that single-stranded, Dcr1-independent RNAs also depend strictly on Arb1 for loading into Ago1 (Figure 2.5d,e). Therefore, it is reasonable to suggest that Arb1 does not simply enable Ago1 to overcome an energetic barrier toward duplex loading, but plays a more fundamental role in

allowing Ago1 to bind to small RNAs of any kind. This can be considered surprising, given that other full-length eukaryotic Argonaute proteins purified from heterologous expression systems are consistently associated with small RNAs (Elkayam et al., 2012; Nakanishi et al., 2012; Schirle and MacRae, 2012), suggesting that Argonautes sample single-stranded small RNAs promiscuously. Wild-type Ago1 purified from *arb1*Δ (and *arb2*Δ) *S. pombe* cells is markedly different in this respect when not overexpressed (Figures 2.5d,e and 2.6), and the mechanism of Ago1 loading by Arb1 is thus likely to be distinct from those described for other Argonautes.

A critical element in the elucidation of this mechanism will be an understanding of the consequences of Arb1 binding on the structural conformation of Ago1. This could be obtained most precisely and ambitiously by X-ray crystallography analysis of Ago1 compared to Ago1 in complex with Arb1. Such a study would have the potential to reveal Arb1-induced changes in Ago1 that might underlie the Arb1-conferred small-RNA-loading activity. The relevance of specific Arb1 amino acid residues in permitting loading by influencing Ago1 structure could be tested using the *in vitro* loading assay we have described in Chapter 2 and confirmed by *in vivo* measures of pericentromeric silencing. A structural view might also provide insights into the inhibition of Ago1 slicing by Arb1. Another point of interest is whether the Arb1-interacting surface of Ago1 might overlap with the putative GW-repeat-binding pockets uncovered in a structure of human Argonaute2 (Schirle and MacRae, 2012) and corroborated by earlier *in vitro* affinity data (Till et al., 2007). The GW protein Tas3 likely binds to these sites, and whether it competes with Arb1 for the same region of Ago1 carries implications for the mechanism by which the protein is transferred from ARC to RITS.

I have argued that Arb1, the ARC subunit directly responsible for programming Ago1, is unlikely to act by establishing the initial contact with the small RNA guide and then delivering it to Ago1, and rather is more likely to modulate the conformation of Ago1 in a manner that is essential for achieving a loading-permissive state. Although this model remains quite tentative, the role of Arb2 in the Ago1 loading process is even more enigmatic.

II. Models for the contribution of Arb2 to small-RNA loading

Our *in vitro* results demonstrate that the extent of small-RNA binding by immunopurified Ago1 is comparable in the presence and absence of Arb2 (Figure 2.4e,d). We therefore infer that Arb2 does not mediate the loading event itself. Yet no small-RNA loading occurs *in vivo* in *arb2*Δ cells (Figure 2.6). Chapter 2 concluded with speculative explanations for this *in vivo* requirement for Arb2. Here, I propose potential experimental approaches for testing those hypotheses.

Since small-RNA loading exhibits a clear dependence on Arb1 both *in vitro* and *in vivo*, an Ago1-Arb1 pair can be considered to serve as the minimal loading machinery. Perhaps Arb2 is essential for small-RNA loading *in vivo* because it is necessary for the assembly or stability of this Ago1-Arb1 heterodimer. We systematically observe an interaction between Ago1 and Arb1 in *arb2*Δ cell extracts (Figure 3.1b). While such interactions are generally presumed to reflect complexes that exist in living cells, it is formally possible that they form only after cell lysis. In order to test this idea, one could mix cell extracts prepared from independent cultures of *arb2*Δ cells, one containing epitope-tagged Arb1 and the other containing epitope-tagged Ago1, before undertaking a co-immunoprecipitation experiment similar to that shown in Figure 3.1b. Failure to detect an interaction would suggest that Ago1-Arb1 subcomplexes must in fact form inside *arb2*Δ cells, whereas a positive co-immunoprecipitation result would demonstrate that they can in principle assemble *de novo* after cell lysis. While inconclusive, the latter outcome would at least raise the possibility that Ago1 and Arb1 might require Arb2 in order to associate *in vivo*. Although this scenario may appear improbable at first glance, one can also argue that it is even more difficult to explain how hypothetical Ago1-Arb1 subcomplexes in *arb2*Δ cells would avoid loading any small RNAs (Figure 2.6) when the *dcr1*Δ case suggests that essentially any cellular small RNA can undergo Arb1-dependent loading into Ago1 (Figure 2.5c-e).

The most obvious reason why Arb2 might be required for bringing Ago1 and Arb1 together in living cells, but not in cell extracts, is that it may be responsible for transporting one

or both of these other ARC subunits into the nucleus, where they both normally reside (Noma et al., 2004; Buker et al., 2007). Accordingly, we have begun to conduct immunofluorescence experiments to assess the influence of Arb2 on Arb1 localization. Our preliminary results suggest that, in fact, deletion of Arb2 leads to a loss of Arb1 nuclear foci and the appearance of a diffuse cytoplasmic Arb1 signal (data not shown). If these results are further substantiated, we will be able to conclude that Arb2 mediates the nuclear translocation of Arb1. Importantly, however, it does not necessarily follow that this is the means by which Arb2 promotes Ago1-Arb1 association. Indeed, Arb2 could act directly or indirectly to produce the pattern we have observed. In a direct model, Arb2 might bind to Arb1 in the cytoplasm and transport it into the nucleus, where Ago1, imported independently of the other ARC subunits, would await the arrival of Arb1 to form a loading-competent complex. But another, indirect model can be envisioned in which Arb2 is responsible for assembling Ago1 with Arb1 at a step upstream of, and essential for, their nuclear import. Although the requirement for Arb2 in bringing Ago1 and Arb1 together would be more difficult to rationalize in this second case, another aspect of the model is appealing because of its several precedents in the literature. Indeed, if the Arb2-mediated binding of Ago1 to Arb1 in the cytoplasm were to prompt siRNA loading, and siRNA loading in turn were to trigger nuclear import of the ARC complex, this would be highly reminiscent of the coupling between small-RNA loading and nuclear import that is observed for Argonautes from such diverse organisms as *C. elegans*, ciliated protozoans and plants (Guang et al., 2008; Noto et al., 2010; Ye et al., 2012). Cytoplasmic loading of small-RNA guides has even been proposed to constitute a universal property of Argonautes that function in the nucleus (Castel and Martienssen, 2013). Nevertheless, there is strong case to be made that *S. pombe* represents an exception to this rule, as Dcr1 contains a motif that enforces its retention in the nucleus and that this localization is critical for siRNA-dependent heterochromatin assembly (Emmerth et al., 2010). It stands to reason that siRNAs are produced in the nucleus and, absent any evidence to the contrary, that they are not transported to the cytoplasm prior to Ago1 loading. A simple

experiment to test the link between small-RNA loading and nuclear import of the ARC complex would be to use immunofluorescence to compare Arb1 localization in *ago1*∆ cells complemented with either wild-type Ago1 or the Ago1-F276A/R773E mutant which cannot be loaded. If the formation of nuclear Arb1 foci is unaffected by this *ago1* mutation, we can deduce that Arb2 is not required for Arb1 nuclear localization because of an upstream function in Ago1 loading, but rather more likely because it directly carries Arb1 into the nucleus prior to small-RNA loading. On the other hand, if the Arb1 foci are sensitive to the disruption of Ago1 loading, we can conclude that small-RNA loading is in fact a prerequisite for nuclear translocation of Arb1 (and most likely Ago1 as well), and that Arb2 probably controls Arb1 localization through its role in Ago1 loading.

If the contribution of Arb2 to Ago1 loading does not simply amount to coordinating the assembly of an Ago1-Arb1 heterodimer, then some justification must be provided for the failure of this heterodimer to load small RNAs in the absence of Arb2 (Figure 2.6) despite its clear biochemical ability to do so (Figure 2.4d,e). In other words, what is it that might prevent Arb1-mediated loading of Ago1 in *arb2*∆ cells? An Argonaute-associated protein that obstructs small-RNA loading, called Giw1p, has been described in *Tetrahymena*, whose inhibitiory activity must be overcome by the Coi12p co-chaperone in order to successfully program the Twi1p Argonaute (Woehrer et al., 2015). In *S. pombe*, the interaction of a potential endogenous inhibitory protein with the Ago1-Arb1 subcomplex would presumably be quite labile in standard cell lysis conditions, as it would have been washed away from the Ago1 preparations used in our *in vitro* loading reactions, including from *arb2*∆ extracts. This could account for its escape from detection in earlier studies. Nevertheless, for a model wherein the role of Arb2 is to displace this hypothetical inhibitor, which I will call Arb3, a key prediction is that it should be strongly enriched in immunopurifications of Ago1 and Arb1 from *arb2*∆ cells relative to wild-type. This comparative purification strategy, coupled with sensitive mass spectrometry analysis, might identify candidates for Arb3, assuming that it exists. Alternatively, one could consider using a

genetic approach to discover Arb3. Since the loss of Arb2 would compromise pericentromeric silencing because of an inability to overcome the Arb3 inhibitory activity, one could perform a screen for suppressors of the *arb2*Δ pericentromeric reporter gene derepression phenotype. Mutations in *arb3*$^{+}$ would be expected to restore silencing and generate hits. But undertaking such an effort would probably not be warranted without much stronger biochemical evidence of the existence of this type of factor. And, as with all genetic screens, the risk cannot be ruled out of failing to recover the desired mutants because of an inseparable function that is essential for cell viability.

Arb2 plays an essential role in small-RNA loading, and determining the nature of that role presents a significant challenge for future studies. Fortunately, the dispensability of Arb2 for the actual loading process, as revealed by our *in vitro* data, provides a defined framework for further elucidating its function. In addition to examining the programming of an Argonaute protein with small-RNA guides, this dissertation has also investigated the decisive influence of that programming event on the formation of a silencing complex. I therefore spend its final pages reflecting on our proposed model of ordered complex assembly and possible ways to further assess its validity.

III. An ordered assembly pathway for Argonaute silencing complexes

The identification of the RITS complex in *S. pombe* marked the first demonstration of a physical link between the small-RNA-dependent silencing machinery and nucleosomes carrying repressive modifications (Verdel et al., 2004). This discovery set the stage for an extensive series of studies on the mechanisms of small-RNA-directed heterochromatin assembly (reviewed in Moazed, 2009). The original report on RITS also contains a remark that bears special significance in light of the findings presented here in Chapter 3: “Our purification of the RITS complex from *dcr1*Δ cells showed that the protein subunits of the complex remained associated together in the absence of siRNAs. The purification results, together with the ChIP

analysis, indicate that the ‘empty’ RITS complex is inactive and can only associate with its chromosomal target after it is programmed by siRNAs” (Verdel et al., 2004). This conclusion can now be amended. In fact, our new results show that “empty” RITS does not exist, because RITS assembly is strictly controlled by the prior loading of small RNAs into Ago1 (Figures 3.1a, 3.2b and 3.3d). Thus, while it is true that the RITS subunits remain associated in the absence of Dcr1-dependent siRNAs (Figure 3.1a) (Verdel et al., 2004), the complex in this case is still programmed with Dcr1-independent primal RNAs (Figure 2.5d,e) (Halic and Moazed, 2010). The programming of the RITS complex paradoxically occurs before its assembly, and these are carefully ordered events in *S. pombe*.

RITS therefore universally contains small RNAs, and we have proposed that the GW-repeat protein Tas3 is responsible for enforcing the exclusive assembly of programmed complexes by distinguishing between loaded and unloaded Ago1 molecules (Figure 3.4). We have also conjectured that an analogous regulatory principle applies in many other organisms, given the conservation of the Argonaute-GW-protein partnership and previous suggestions of a relationship between GW proteins and Argonaute-small-RNA binding. As examples that ostensibly contradict our model, Argonaute mutants with impaired small-RNA-loading activity have been reported to co-immunoprecipitate with GW182 homologs in human and *Drosophila* cell extracts (Eulalio et al., 2009; Liu et al., 2005). However, the reliance of these experiments on overexpressed proteins significantly undermines their conclusions, and no evidence yet suggests that GW proteins bind to unloaded Argonautes in a physiological setting. In contrast, there are several reports of GW proteins associating preferentially with loaded Argonautes. For instance, a size-exclusion chromatography analysis revealed that GW182 proteins co-fractionate with the subset of Argonaute-containing fractions that also contains miRNAs (Baillat and Shiekhattar, 2009), and a viral suppressor of a plant Argonaute was found to bind specifically to programmed complexes (Giner et al., 2010). In this study, we have presented what to our knowledge constitutes the first direct test, using natively expressed components, of

a causal link between the loading of Argonaute with a small RNA and its recognition by a GW protein.

However, without more detailed structural information on this recognition event, we do not have a precise sense of how the discrimination between loaded and unloaded Argonautes might occur. If Argonaute binding and the sensing of loading status are separable activities, it may be possible to generate a mutant GW protein that lacks selectivity for loaded Argonautes and would permit the assembly of unprogrammed complexes. A prediction of our model is that these complexes would prove deleterious to the silencing pathway as a whole because they would divert downstream components away from the repression of small-RNA targets. The hypothetical GW protein mutant would therefore be predicted to act in a dominant-negative manner. A genetic screen for dominant-negative *tas3* alleles is not a promising method for identifying this type of mutant, however, because other mutations in the GW repeats that abolish Argonaute binding altogether are likely to be much more numerous, and these might also be expected to produce a dominant-negative phenotype. In *S. pombe*, for example, Tas3 interacts with Chp1 through a distinct domain (Debeauchamp et al., 2008; Petrie et al., 2005). Thus, Tas3 mutants that gain the ability to bind to unloaded Ago1, and Tas3 mutants that do not bind to Ago1 at all, would both complex with Chp1 and reduce the available supply of this subunit, which would impair proper RITS formation even if wild-type Tas3 were also present.

The isolation of a Tas3 variant that still interacts with Ago1 but no longer discerns its loading state might be possible using an alternative genetic approach. In a two-hybrid assay adapted to *S. pombe*, one could introduce an Ago1 bait that is either wild-type or defective for loading, and first verify that a plasmid-derived Tas3 interacts with the former but not the latter. One could then mutagenize the Tas3-encoding plasmid and screen the resulting library for rare mutations that confer an interaction with the loading-defective Ago1. A co-immunoprecipitation experiment could then be carried out under conditions of endogenous protein expression to confirm the ability of the Tas3 mutant to associate with un-loadable Ago1, as well as with wild-

type Ago1 in *arb1*Δ and *arb2*Δ cells. A series of tests could then be conducted to evaluate the consequence of such a Tas3 mutation on small-RNA-directed pericentromeric silencing. If the "promiscuous" Tas3 variant exerts a dominant-negative effect, or even if it simply fails to complement *tas3*Δ despite forming complexes with Ago1, one could conclude that the ability of Tas3 to determine the loading state of Ago1 is critical for the efficiency of small-RNA-mediated silencing in *S. pombe*, as we have postulated. Such a finding would also strengthen the case for the widespread evolutionary importance of this discriminatory capacity among GW-repeat proteins, and suggest that the ordered assembly pathway we have uncovered here provides an adaptive benefit.

In undertaking the work I have presented, we sought to understand the roles of two Argonaute-associated factors, Arb1 and Arb2, in a small-RNA-guided silencing mechanism whose ultimate function is to protect the stability of the genome. We were able to ascribe the activity of loading Argonaute with small RNAs to these two proteins collectively *in vivo*, but only to Arb1 *in vitro*. We concluded that Arb1 directly mediates small-RNA loading, and that Arb2 makes an indirect but essential contribution to this Arb1 activity inside the cell. Unexpectedly, we also discovered that disrupting small-RNA loading by various means, either through mutation of Argonaute or deletion of Arb1 or Arb2, completely obstructed the incorporation of Argonaute into its effector silencing complex. Hence, we have found that the programming and the assembly of this silencing complex are strictly ordered events, and we propose that this represents a general organizational principle of small-RNA-mediated silencing. Beyond these findings, the mechanism of Argonaute loading by Arb1, the precise molecular task carried out by Arb2 and the true functional significance of our ordered assembly model all remain largely mysterious. Applying the strategies suggested in this chapter to the investigation of these new questions will be exciting.

IV. References

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

Buker, S.M., Iida, T., Bühler, M., Villén, J., Gygi, S.P., Nakayama, J., and Moazed, D. (2007). Two different Argonaute complexes are required for siRNA generation and heterochromatin assembly in fission yeast. Nat. Struct. Mol. Biol. *14*, 200-207.

Castel, S.E., and Martienssen, R.A. (2013). RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond. Nat. Rev. Genet. *14*, 100-112.

Colmenares, S.U., Buker, S.M., Buhler, M., Dlakić, M., and Moazed, D. (2007). Coupling of double-stranded RNA synthesis and siRNA generation in fission yeast RNAi. Mol. Cell *27*, 449-461.

Debeauchamp, J.L., Moses, A., Noffsinger, V.J., Ulrich, D.L., Job, G., Kosinski, A.M., and Partridge, J.F. (2008). Chp1-Tas3 interaction is required to recruit RITS to fission yeast centromeres and for maintenance of centromeric heterochromatin. Mol. Cell. Biol. *28*, 2154-2166.

Elkayam, E., Kuhn, C.D., Tocilj, A., Haase, A.D., Greene, E.M., Hannon, G.J., and Joshua-Tor, L. (2012). The structure of human argonaute-2 in complex with miR-20a. Cell *150*, 100-110.

Emmerth, S., Schober, H., Gaidatzis, D., Roloff, T., Jacobeit, K., and Bühler, M. (2010). Nuclear retention of fission yeast dicer is a prerequisite for RNAi-mediated heterochromatin assembly. Dev. Cell *18*, 102-113.

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.

Giner, A., Lakatos, L., García-Chapa, M., López-Moya, J., and Burgyán, J. (2010). Viral protein inhibits RISC activity by argonaute binding through conserved WG/GW motifs. PLoS Pathog. *6*, e1000996.

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.

Guang, S., Bochner, A.F., Pavelec, D.M., Burkhart, K.B., Harding, S., Lachowiec, J., and Kennedy, S. (2008). An Argonaute transports siRNAs from the cytoplasm to the nucleus. Science *321*, 537-541.

Halic, M., and Moazed, D. (2010). Dicer-independent primal RNAs trigger RNAi and heterochromatin formation. Cell *140*, 504-516.

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.

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.

Kawamata, T., Seitz, H., and Tomari, Y. (2009). Structural determinants of miRNAs for RISC loading and slicer-independent unwinding. Nat. Struct. Mol. Biol. *16*, 953-960.

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

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

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.

Malone, C.D., and Hannon, G.J. (2009). Small RNAs as guardians of the genome. Cell *136*, 656-668.

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.

Moazed, D. (2009). Small RNAs in transcriptional gene silencing and genome defence. Nature *457*, 413-420.

Nakanishi, K., Weinberg, D.E., Bartel, D.P., and Patel, D.J. (2012). Structure of yeast Argonaute with guide RNA. Nature *486*, 368-374.

Noma, K., Sugiyama, T., Cam, H., Verdel, A., Zofall, M., Jia, S., Moazed, D., and Grewal, S.I. (2004). RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing. Nat. Genet. *36*, 1174-1180.

Noto, T., Kurth, H.M., Kataoka, K., Aronica, L., DeSouza, L.V., Siu, K.W., Pearlman, R.E., Gorovsky, M.A., and Mochizuki, K. (2010). The Tetrahymena argonaute-binding protein Giw1p directs a mature argonaute-siRNA complex to the nucleus. Cell *140*, 692-703.

Petrie, V.J., Wuitschick, J.D., Givens, C.D., Kosinski, A.M., and Partridge, J.F. (2005). RNA interference (RNAi)-dependent and RNAi-independent association of the Chp1 chromodomain protein with distinct heterochromatic loci in fission yeast. Mol. Cell. Biol. *25*, 2331-2346.

Schirle, N.T., and MacRae, I.J. (2012). The crystal structure of human Argonaute2. Science *336*, 1037-1040.

Swarts, D.C., Makarova, K., Wang, Y., Nakanishi, K., Ketting, R.F., Koonin, E.V., Patel, D.J., and van der Oost, J. (2014). The evolutionary journey of Argonaute proteins. Nat. Struct. Mol. Biol. *21*, 743-753.

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.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S.I., and Moazed, D. (2004). RNAi-mediated targeting of heterochromatin by the RITS complex. Science *303*, 672-676.

Woehrer, S.L., Aronica, L., Suhren, J.H., Busch, C.J., Noto, T., and Mochizuki, K. (2015). A *Tetrahymena* Hsp90 co-chaperone promotes siRNA loading by ATP-dependent and ATP-independent mechanisms. EMBO J. *34*, 559-577.

Ye, R., Wang, W., Iki, T., Liu, C., Wu, Y., Ishikawa, M., Zhou, X., and Qi, Y. (2012). Cytoplasmic assembly and selective nuclear import of *Arabidopsis* Argonaute4/siRNA complexes. Mol. Cell *46*, 859-870.

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.

Yoon, J., Jo, M., White, E.J., De, S., Hafner, M., Zucconi, B., Abdelmohsen, K., Martindale, J., Yang, X., Wood, W., et al. (2015). AUF1 promotes let-7b loading on Argonaute 2. Genes Dev. *29*, 1599-1604.

Appendix 1: ARC does not interact with the pericentromeric transcripts whose silencing it controls

Several lines of evidence suggest indirectly that the ARC complex silences the pericentromeric *dg* and *dh* repeats without physically interacting with the long ncRNAs that are transcribed from these loci. First, ARC is loaded with double-stranded small RNAs (Figure 2.1a) (Buker et al., 2007), which in principle should be unable to mediate target RNA identification and

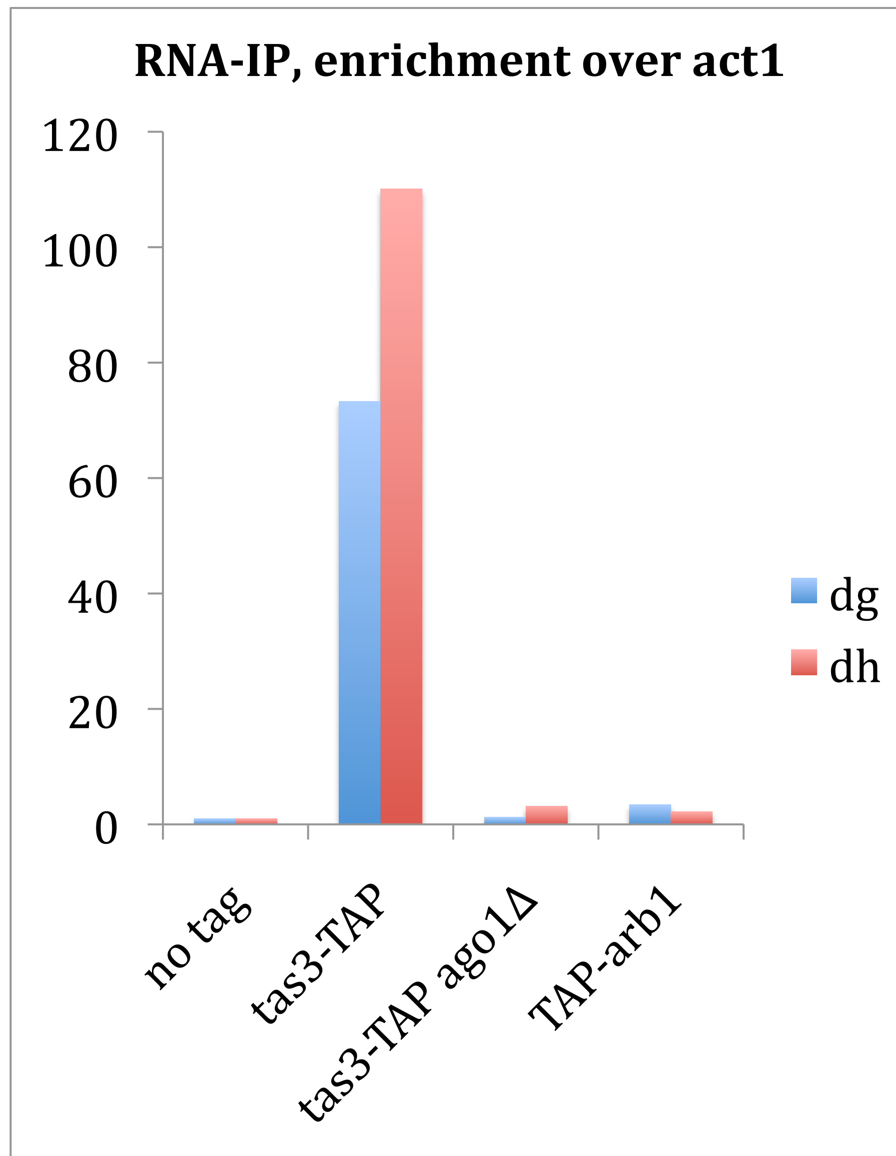

Figure A1. Arb1 does not associate with dg and dh pericentromeric transcripts.
Relative levels of TAP-tagged protein association with the dg and dh pericentromeric transcripts, measured by formaldehyde crosslinking, immunoprecipitation, reverse transcription and PCR, normalized to *act1*$^{+}$ mRNA, with the mean for the untagged sample set to 1.

interaction via base-pairing. Second, while dg and dh transcripts could theoretically be encountered anywhere in the cell, they are widely believed to be retained in proximity to heterochromatin, particularly given the possibility of simultaneous engagement of nascent transcripts and nucleosomes by different subunits of RITS (Motamedi et al., 2004). The apparent lack of assocation between the ARC subunits and chromatin (Buker et al., 2007; Woolcock et al., 2012) therefore does not support a model in which ARC interacts with the dg and dh long ncRNAs, nor does the observation that Arb1 and Chp1 localize to distinct subnuclear foci (Buker et al., 2007).

Nevertheless, we tested this idea directly by RNA immunoprecipitation after formaldehyde crosslinking, a technique that has been employed previously to uncover interactions between several RNAi and heterochromatin components and the pericentromeric transcripts (Bayne et al., 2010; Motamedi et al., 2004, 2008). The results of our assay indicate that, in contrast to RITS subunit Tas3, which interacts very robustly with dg and dh transcripts in an Ago1-dependent manner, ARC subunit Arb1 does not exhibit such an interaction (Figure A1). We therefore conclude that ARC is necessary for silencing the pericentromeric repeats because of an activity that does not directly involve physical association with nascent pericentromeric transcripts. This is consistent with the findings presented in Chapter 2 demonstrating a role for ARC in loading Ago1 with siRNAs.

Methods

RNA immunoprecipitations were performed as described (Motamedi et al., 2004). Reverse transcription and quantitative PCR were carried out as described in Chapter 2.

Strains

Strain	**Genotype**
SPY106	*h^+ leu1-32 ade6-M216 ura4-D18 imr1R(NcoI)::$ura4^+$ tas3::TAP-kanMX6*
SPY137	*h^+ leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::$ura4^+$*
SPY240	*h^+ leu1-32 ade6-M216 ura4-D18 imr1R(NcoI)::$ura4^+$ tas3::TAP-kanMX6 ago1Δ::hphMX6*
SPY2689	*h^+ leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::$ura4^+$ kanMX6-TAP-arb1*

Oligonucleotides

Oligonucleotide	Sequence	Source
MB86	AACCCTCAGCTTTGGGTCTT	1
MB87	TTTGCATACGATCGGCAATA	1
AS131 (dg)	AAGGAATGTGCCTCGTCAAATT	2
AS132 (dg)	TGCTTCACGGTATTTTTTGAAATC	2
AS133 (dh)	GTATTTGGATTCCATCGGTACTATGG	2
AS134 (dh)	ACTACATCGACACAGAAAAGAAAACAA	2

1 = Bühler et al., 2007, **2** = Yu et al., 2014

Acknowledgments

We thank M. Motamedi and R. Yu for technical advice on RNA immunoprecipitation.

References

Bayne, E.H., White, S.A., Kagansky, A., Bijos, D.A., Sanchez-Pulido, L., Hoe, K.L., Kim, D.U., Park, H.O., Ponting, C.P., Rappsilber, J., and Allshire, R.C. (2010). Stc1: a critical link between RNAi and chromatin modification required for heterochromatin integrity. Cell *140*, 666-677.

Bühler, M., Haas, W., Gygi, S.P., and Moazed, D. (2007). RNAi-dependent and -independent RNA turnover mechanisms contribute to heterochromatic gene silencing. Cell *129*, 707-721.

Buker, S.M., Iida, T., Bühler, M., Villén, J., Gygi, S.P., Nakayama, J., and Moazed, D. (2007). Two different Argonaute complexes are required for siRNA generation and heterochromatin assembly in fission yeast. Nat. Struct. Mol. Biol. *14*, 200-207.

Motamedi, M.R., Hong, E.J., Li, X., Gerber, S., Denison, C., Gygi, S., and Moazed, D. (2008). HP1 proteins form distinct complexes and mediate heterochromatic gene silencing by nonoverlapping mechanisms. Mol. Cell *32*, 778-790.

Motamedi, M.R., Verdel, A., Colmenares, S.U., Gerber, S.A., Gygi, S.P., and Moazed, D. (2004). Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs. Cell *119*, 789-802.

Woolcock, K.J., Stunnenberg, R., Gaidatzis, D., Hotz, H.R., Emmerth, S., Barraud, P., and Bühler, M. (2012). RNAi keeps Atf1-bound stress genes in check at nuclear pores. Genes Dev. *26*, 683-692.

Yu, R., Jih, G., Iglesias, N., and Moazed, D. (2014). Determinants of heterochromatic siRNA biogenesis and function. Mol. Cell *53*, 262-276.

Appendix 2: Arb1-TAP is a hypomorphic protein but TAP-Arb1 is fully functional

Initial analyses of ARC-associated small RNAs were carried out using the epitope-tagged protein Arb1-TAP, which appeared to complement the *arb1*$^{+}$ deletion by the measures that were tested (Buker et al., 2007). These experiments used a set of Northern blot oligonucleotide probes corresponding primarily to *dh* siRNAs (Bühler et al., 2006; Buker et al., 2007; Reinhart and Bartel, 2002), and revealed that Arb1-TAP associates with vastly lower numbers of these small RNAs *in vivo* than does Tas3-TAP (Buker et al., 2007). In light of this result, it was far from obvious that Arb1 and Arb2 played a role in loading Ago1 with small RNAs, and we even entertained the possibility that these proteins might act to restrict small-RNA loading to protect Ago1 from being programmed irreversibly with miscellaneous RNA degradation products.

But in fact, the above interpretation of the Arb1-TAP-associated small RNA analysis is undermined by our more recent observation that Arb1-TAP is not fully functional in pericentromeric silencing. A *ura4*$^{+}$ reporter gene inserted in the *dg* repeat on the right arm of chromosome 1 is not silenced in *arb1-TAP* cells to the same degree as in wild-type cells (Figure

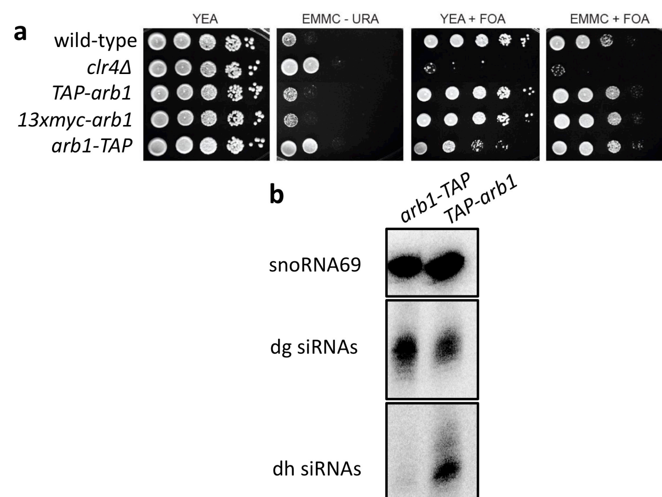

Figure A2. *TAP-arb1*, but not *arb1-TAP*, supports wild-type pericentromeric silencing and siRNA accumulation.
(**a**) Tenfold serial dilutions of cells of the indicated genotypes carrying pericentromeric *ura4*$^{+}$ reporter gene *otr1R::ura4*$^{+}$, plated on the indicated medium. (**b**) Northern blot analysis of small RNAs isolated from total RNA by size fractionation.

A2a). In contrast, an N-terminal TAP-Arb1 fusion fully recapitulates the function of the wild-type Arb1 in this assay (Figure A2a). Strikingly, a Northern analysis of total small RNAs isolated from *arb1-TAP* and *TAP-arb1* cells reveals a strong defect in the accumulation of siRNAs from the *dh*, but not the *dg* repeats, in *arb1-TAP* cells (Figure A2b). Therefore, that the levels of *dh* siRNAs co-purifying with Arb1-TAP are much lower than those associated with Tas3-TAP (Buker et al., 2007) is not a reflection of the siRNA-loading activity of ARC, but rather simply the consequence of a hypomorphic *arb1-TAP* allele. *TAP-arb1* cells, in contrast, accumulate siRNAs from both types of pericentromeric repeats (Figure A2b) and TAP-Arb1-associated siRNAs, accordingly, are as abundant as those co-purifying with Tas-TAP (Figure 2.1a,b). The TAP-Arb1 allele has therefore proved instrumental in defining the role of the ARC complex as the Ago1 loading machinery.

Methods

For growth assays, cells were grown to saturation and diluted serially tenfold so that the highest density spot contained 1.3 x 10^5 cells. Non-selective plates contained Edinburgh Minimal Medium with all standard supplements (Sunrise Science Products) (EMMC) or yeast extract (5 g/L) supplemented with dextrose (30 g/L) and adenine (225 mg/L) (YEA). Selective plates additionally contained 1 g/L 5-fluoroorotic acid. Total small RNAs for Northern blots were recovered using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's instructions. Northern blots were performed as described (Bühler et al., 2006) using the oligonucleotide probes listed below.

Strains

Strain	**Genotype**
SPY137	*h^+ leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::$ura4^+$*
SPY815	*h^+ leu1-32 ade6-M216 ura4-D18 otr1R(SphI)::$ura4^+$ clr4Δ::kanMX6*
SPY855	*h^+ leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::$ura4^+$ arb1::TAP-natMX6*
SPY2689	*h^+ leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::$ura4^+$ kanMX6-TAP-arb1*
SPY2691	*h^+ leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::$ura4^+$ natMX6-3xFLAG-ago1 hphMX6-13xmyc-arb1*

Oligonucleotides

Oligonucleotide	Sequence	Source
Northern probes		
MB151 (snoRNA69)	CAATGTAAATACTCCGAGTGAGCTGGGTTTAAC	1
censiRNA_a' (dg)	GCGACTAAACCGAAAGCCTC	1
censiRNA_b' (dg)	TACCGTGATTAGCCTTACTCCGCATT	1
IK10 (dg)	GGGAGTACATCATTCCTACTTCGATA	2
DBH45 (dg)	GACTTTCAAAGATGCACA	3
DBH46 (dg)	TTTTCTCTTTCAAAAGTA	3
DBH47 (dg)	CAATTGGAAGTACATCCA	3
DBH48 (dg)	TCAATCCATCATGTACGA	3
DBH49 (dg)	AATTCGATTCCAAGTACA	3
DBH50 (dg)	ATTGTTTCGACAACACGA	3
censiRNA_d' (dh)	TACCGCTTCTCCTTAATCCA	1
censiRNA_e' (dh)	ACACCTACTCTTATCACTTGT	1
censiRNA_f' (dh)	GACGATAAGCAGGAGTTGCGCA	1
censiRNA_g' (dh)	AGTGTGGCGCTATATCTTGTA	1
censiRNA_h' (dh)	TACTGTCATTAGGATATGCTCA	1
censiRNA_i' (dh)	GGGAAATGTATAAATAGGCA	1
censiRNA_j' (dh)	TTTCCCAAGGACTGCTGAGGTAGA	1
censiRNA_l' (dh)	TGGCAGATATTGCAAGTTGTTTA	1
IK9 (dh)	TTTGATGCCCATGTTCATTCCACTTG	2

1 = Bühler et al., 2006, **2** = Bayne et al., 2010, **3** = this study

Acknowledgments

We thank Nahid Iglesias for performing the reporter gene silencing assay shown in Figure A2a.

References

Bayne, E.H., White, S.A., Kagansky, A., Bijos, D.A., Sanchez-Pulido, L., Hoe, K.L., Kim, D.U., Park, H.O., Ponting, C.P., Rappsilber, J., and Allshire, R.C. (2010). Stc1: a critical link between RNAi and chromatin modification required for heterochromatin integrity. Cell *140*, 666-677.

Bühler, M., Verdel, A., and Moazed, D. (2006). Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing. Cell *125*, 873-886.

Buker, S.M., Iida, T., Bühler, M., Villén, J., Gygi, S.P., Nakayama, J., and Moazed, D. (2007). Two different Argonaute complexes are required for siRNA generation and heterochromatin assembly in fission yeast. Nat. Struct. Mol. Biol. *14*, 200-207.

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