Chromosome Research (2007) 15:653–665
DOI: 10.1007/s10577-007-1144-z

Small RNAs and RNAi pathways in meiotic prophase I

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Key words: centromere, chromatoid body, meiosis, piRNAs, prophase I, RNAi

Abstract

RNA interference is involved in many aspects of cell biology, and the recent identification of germ-cell specific small RNAs has led to speculation that RNAi might also be involved in gametogenesis. Work in yeast indicates that RNAi is involved in establishing and maintaining heterochromatin at centromeres, an important component of yeast and mammalian meiosis. Here we review developments in the field of RNAi and relate these to possible roles in mammalian gametogenesis.

Introduction

RNA interference, RNAi, is a homology-dependent gene-silencing mechanism initiated by double-stranded RNA (dsRNA). RNAi involves a number of steps from biogenesis of the trigger RNA, processing of dsRNA to small RNAs and formation of an effector complex containing the small RNA which goes on to guide mRNA cleavage, translational repression and/or chromatin modifications in a sequence-specific manner. In the past few years the discovery of RNAi has led to the identification of several novel classes of small RNAs and protein complexes involved in the pathway. There are many good reviews on the topic of RNAi mechanism (Hannon 2002, Bartel 2004, Tolia & Joshua-Tor 2007). However, in view of the recent emergence of a germ cell-specific small RNA species, and the suggestion that they may play a key role in the regulation of gametogenesis, this review will focus on small RNAs and the role of RNAi pathways in meiosis, with particular reference to meiotic prophase I.

Key players and key concepts

The RNAi phenomenon was first described in *Caenorhabditis elegans* when injected dsRNA resulted in gene silencing (Fire *et al.* 1998). In these initial experiments using dsRNAs of hundreds of nucleotides in length, Fire and colleagues hypothesized that the dsRNAs were guiding a nuclease to target mRNA destruction in a sequence-specific manner. The nuclease responsible for these cleavage events was identified in 2002 as Dicer, a type III RNase, and was shown to cleave dsRNA into small interfering RNAs (siRNAs) (Nicholson & Nicholson 2002, Carmell & Hannon 2004). Since the identification of Dicer, a wealth of information regarding RNAi has been obtained, from proteins involved in the pathway to the small RNAs that trigger the process. The emerging picture is of an evolutionarily conserved system in plants, animals and fungi that regulates a wide variety of cellular processes that include gene silencing (both via transcriptional and translational control), RNA-dependent DNA methylation (RdDM), heterochromatin formation and DNA elimination.

The numerous classes of small RNAs

It has been known for a while that the genome transcribes numerous non-coding RNAs, a large number of which are small RNAs. Several classes of small RNAs have now been described, namely siRNAs, microRNAs, rasiRNAs and, most recently, piRNAs (Table 1).

MicroRNAs

MircoRNAs (miRNAs) are by far the most abundant small RNAs in mammalian systems. They were first identified as repressors of translation in *C. elegans* (Lee *et al.* 1993) and exert their effect by binding to the 3′ UTR of genes. The term miRNA was coined once a large number of these small RNAs were cloned from several model organisms (Lagos-Quintana *et al.* 2001, Lau *et al.* 2001, Lee & Ambros 2001). miRNAs are formed from a non-coding primary transcript (pri-miRNA) that is cleaved by Drosha into a hairpin-shaped pre-miRNA (Lee *et al.* 2003b). Drosha is aided by Pasha/DGCR8 (Denli *et al.* 2004, Gregory *et al.* 2004, Han *et al.* 2004, Landthaler *et al.* 2004). Exportin-5 transports this pre-miRNA out of the nucleus into the cytoplasm (Yi *et al.* 2003) where is it cleaved again, this time by Dicer into 21–22 nt dsRNAs (Grishok *et al.* 2001, Hutvagner *et al.* 2001, Ketting *et al.* 2001, Knight & Bass 2001). miRNAs inhibit translation of target mRNAs through a nuclease complex known as RISC (RNAi-induced silencing complex, discussed later). The diversity of miRNAs, and the fact that they do not have to be 100% complementary to their target mRNA, implicates them in regulating a large number of genes.

siRNAs and rasiRNAs

Another class of small RNAs, known as siRNAs (small interfering RNAs), mediate post transcriptional gene silencing (PTGS) by guiding RISC to degrade perfectly complementary mRNAs. siRNAs arise from a long dsRNA precursor that are cleaved by Dicer into ~21 nt siRNAs (Hammond *et al.* 2000, Zamore *et al.* 2000, Elbashir *et al.* 2001b). Repeat-associated RNAs (rasiRNAs) are a class of siRNAs that, as their name suggests, are derived from regions of repetitive DNA.

piRNAs

PIWI-interacting RNAs or piRNAs are longer than the previously identified siRNAs and miRNAs and they are expressed specifically in the germ lines. piRNAs are discussed in detail later.

RISC

Dicer is a type III RNase containing two catalytic RNase domains, a helicase domain and a PAZ domain. Once generated by Dicer, the double-stranded siRNAs and miRNAs are incorporated into RISC and go to guide sequence-specific mRNA degradation and/or repression of translation. Target sequences show homology to only one strand of the dsRNA incorporated in RISC, thus another function of RISC is to unwind the double-stranded siRNA or miRNA, discard one strand (known as the passenger strand) and retain the other strand, the guide strand, to be used for homology searching. RISC is often referred to as the effecter complex with Dicer and associated proteins as the initiation complex.

The main components of RISC that have been best characterized are members of the Argonaute family. Members of this family contain two signature domains, a PAZ domain (also found in Dicer) and a PIWI domain. The Argonaute family is divided into two classes of proteins–those that are more closely related to *Arabidopsis thaliana* AGO1, Ago1-4 in mouse (also known as EIF2C1-4) and those that are more similar to *Drosophila melanogaster* PIWI, namely MILI, MIWI and MIWI2 in mouse. The Ago class binds siRNA and miRNAs and uses them in the traditional RNAi pathway. The role of PIWI-class proteins in RNAi pathways is still unclear (discussed later).

The PAZ domain of Ago proteins is the domain that binds the 3′ end of guide miRNA/siRNAs whereas the PIWI domain binds the 5′ end (Lingel *et al.* 2003, 2004, Song *et al.* 2003, Yan *et al.* 2003, Ma *et al.* 2004). Interestingly, of the mammalian AGO orthologs, only Ago2 is capable of cleaving target mRNAs despite the presence of PIWI domains in all Ago proteins. Ago1, 3 and 4 are loaded with small RNAs but are not able to catalyze cleavage (Liu *et al.* 2004, Meister *et al.* 2004), the reasons and biological significance of this difference in cleavage activity are unknown.

Other proteins have been identified in RISC, mainly from studies in *D. melanogaster*, but their

Table 1. Key terms and components of the RNAi pathway

Abbreviation	Full name	Function	References
RNAi	RNA interference	Homology-dependent gene silencing mechanism initiated by double-stranded RNA	Fire *et al.* 1998
RISC	RNA-induced silencing complex	Effecter complex that guides sequence-specific mRNA degradation	Hammond *et al.* 2000
RITS	RNA-induced transcriptional silencing complex	Effecter complex in yeast required for heterochromatin assembly	Verdel *et al.* 2004
RdDM	RNA-dependent DNA methylation	Small RNAs are able to direct DNA methylation	Wassenegger *et al.* 1994
RdRP	RNA-dependent RNA polymerase	An enzyme that uses RNA instead of DNA as a template for RNA. Proposed to supply a continuous source of siRNAs from dsRNA templates via RNAi	Volpe *et al.* 2002
Dicer	Dicer	An enzyme with RNaseIII activity responsible for cleaving dsRNAs into siRNAs and pre-miRNAs into miRNAs	Nicholson & Nicholson 2002, Carmell & Hannon 2004
AGO	Argonaute	A family of proteins containing PIWI and PAZ domains. In mammals AGO2 is incorporated into RISC and slices the target mRNA	Liu *et al.* 2004
Drosha	Drosha	Enzyme responsible for cleaving pri-RNAs to pre-miRNAs	Lee *et al.* 2003b
miRNAs	micro RNA	Translation repression Targeting of mRNA degradation	Lee *et al.* 1993 Yekta *et al.* 2004
siRNAs	short interfering RNA	Targeting of mRNA degradation	Hammond *et al.* 2000, Zamore *et al.* 2000, Elbashir *et al.* 2001b
rasiRNAs	Repeat-associated short interfering RNA	Establishment and maintenance of heterochromatin	Mette *et al.* 2000, Aravin *et al.* 2001, Hall *et al.* 2002, Hamilton *et al.* 2002, Llave *et al.* 2002, Pal-Bhadra *et al.* 2002, Reinhart & Bartel 2002, Volpe *et al.* 2002, Vagin *et al.* 2006
piRNAs	PIWI-interacting RNA	Function unknown, germ-line specific expression pattern.	Aravin *et al.* 2006, Girard *et al.* 2006, Grivna *et al.* 2006a, Lau *et al.* 2006, Watanabe *et al.* 2006

functional significance is not full understood (Caudy *et al*. 2002, 2003, Ishizuka *et al*. 2002). These include *Fxr*, the *Drosophila* homolog of fragile-X mental retardation protein FXMR (Caudy *et al*. 2002, Ishizuka *et al*. 2002), *Vig*, vasa intronic gene (Caudy *et al*. 2002, Ishizuka *et al*. 2002) and *Tsn*, tudor-staphylococcal nuclease (Caudy *et al*. 2003). Additional proteins are also expected to be identified to account for all of the functions of RISC, including an 'unwindase' which would unwind the double-stranded siRNA. Armitage is a candidate protein for this activity in *D. melanogaster* (Tomari *et al*. 2004).

PIWI proteins and piRNAs

The most recently identified class of small RNAs are the Piwi-interacting RNAs (piRNAs) (Aravin *et al*. 2006, Girard *et al*. 2006, Grivna *et al*. 2006a, Watanabe *et al*. 2006, Lau *et al*. 2006). These are of particular interest to reproductive biologists due to their specific expression in male germ cells. As their name suggests, they associate with members of the Piwi protein family.

Characteristics of piRNAs

piRNAs were identified in mouse and rat testes as a species of small RNAs longer in length than the previously known miRNA and siRNA populations. Two groups visualized these RNAs on polyacrylamide gels (PAGE) stained either with SYBR gold or ethidium bromide (Girard *et al*. 2006, Grivna *et al*. 2006a). Immunoprecipitation studies demonstrated that these RNAs were also found to associate with MILI and MIWI (Aravin *et al*. 2006, Girard *et al*. 2006) and were markedly decreased in *Miwi*$^{-/-}$ mice (Grivna *et al*. 2006a). Cloning revealed two populations of piRNAs differing in size and protein association, the first group being 29–31 nt in length and interacting with MIWI (Grivna *et al*. 2006a,b), and the second being 26–28 nt and associating with MILI (Aravin *et al*. 2006). A class of small RNAs ranging in size from 26 to 30 nt were cloned by another group and named germline small RNAs. The expression pattern of gsRNAs and their size indicates that these could be related to, or the same as, piRNAs (Watanabe *et al*. 2006).

piRNAs appear to be more abundant than microRNAs (as determined visually by PAGE); Aravin *et al*. (2006) have estimated that there are approximately one million piRNAs per mouse spermatocyte. The populations of piRNAs are extremely diverse: 26% of the 52 934 piRNAs cloned by Girard *et al*. are unique and 6% of the 1673 MILI-immunoprecipitated piRNAs cloned by Aravin *et al*. are unique. piRNAs are expressed exclusively in testes but are not detected in mature sperm.

The genomic location of piRNAs appears to be uneven. Some chromosomes are enriched for piRNAs, while others are under-represented. For example the X chromosome has few piRNAs mapped to it but the X chromosome represents 5.5% of the genome (Grivna *et al*. 2006a) compared to some 17.6% of all piRNAs are encoded by chromosome 17 which represents only 3.1% of genomic DNA. piRNAs have been identified in exonic, intronic, intergenic and repeat sequences, although there are a few found within centromeric sequences (Girard *et al*. 2006).

There is a tendency for piRNAs to cluster and orient in the same direction to each other, which might suggest that many are derived from a single primary transcript, as is the case for miRNAs. Whereas miRNAs are derived from characteristic hairpin loop structures, similar hairpin structures were not identified in piRNA transcripts as determined by computational analysis. How piRNAs are generated is not understood at this time, but it has been suggested that they might be formed from a very long primary transcript capable of forming a double-stranded structure (Aravin *et al*. 2006) or that an antisense strand is transcribed but at low, undetectable levels (Watanabe *et al*. 2006).

Like miRNAs and siRNAs formed by Dicer cleavage, piRNAs have a strong preference for a uridine base at their 5′ end, indicating that piRNAs might also be processed by Dicer. piRNAs have a 5′ phosphate group and a 3′ hydroxyl group, reminiscent of miRNAs and siRNAs. The physical distance between the PAZ (RNA-binding) domain, and the RNaseIII domain in Dicer represents 21–24 nt, which coincides with the size of miRNAs and siRNAs. However, since piRNAs are longer than the 21–24 nt required for Dicer activity, it is possible that Dicer is not the piRNA effector molecule, or that the processing of piRNAs by Dicer is different to that of miRNA/siRNAs. A smaller testes-specific isoform of Dicer has been identified (Fortin *et al*. 2002) that

may be involved in piRNAs, although there is no scientific evidence for this. It would be interesting to see if piRNAs are present in a testes-specific knockdown or deletion of Dicer.

piRNAs interact with members of the Argonaute family

Suggestion of piRNA involvement in the RNAi pathway comes from studies of the proteins that they associate with, MIWI and MILI. MIWI and MILI are expressed in germ cells of adult testes, with MILI detected from the primordial germ cell stage up until the pachytene stage of meiosis, and MIWI being abundant from pachytene to early round spermatids (Kuramochi-Miyagawa *et al.* 2001, Deng & Lin 2002). Both MILI and MIWI proteins are essential for spermatogenesis but dispensable for oogenesis; *Miwi*$^{-/-}$ and *Mili*$^{-/-}$ males are sterile whereas the females are fertile (Deng & Lin 2002, Kuramochi-Miyagawa *et al.* 2004). In *Miwi*$^{-/-}$ males spermatogenesis is arrested at the spermatid stage (Deng & Lin 2002) while spermatogenesis in *Mili*$^{-/-}$ males is disrupted earlier at zygotene or early pachytene (Kuramochi-Miyagawa *et al.* 2004). Both proteins are cytosolic and initial analysis indicated that they associate with RNAs (Kuramochi-Miyagawa *et al.* 2001, Deng & Lin 2002), which may include piRNAs.

MIWI associates with the RNA helicase MVH (mouse VASA homolog) in chromatoid bodies (Toyooka *et al.* 2000, Kotaja *et al.* 2006a) (Figure 1). The chromatoid body is a germ cell-specific cytoplasmic structure that appears at pachynema (Fawcett *et al.* 1970), is capable of moving through intracellular bridges connecting haploid spermatids (Ventela *et al.* 2003) and is thought to be involved with RNA processing and storage (Parvinen 2005). Along with MIWI and MVH, Dicer, Ago2, Ago3 and miRNAs have been detected at the chromatoid body (Kotaja *et al.* 2006a). Whether piRNAs also localize to the chromatoid body is not yet known. MIWI also associates with KIF17b, a testes-specific kinesin that shuttles between the nucleus and cytoplasm and is transiently located at the chromatoid body (Kotaja *et al.* 2006b). The chromatoid bodies of *Miwi*$^{-/-}$ mice are either absent or aberrant (Kotaja *et al.* 2006b), suggesting a role for MIWI in RNA processing through RNAi in the chromatoid body. An excellent model for this has been proposed (Kotaja *et al.* 2006b) in which KIF17b collects mRNAs from the nucleus and transports them to the chromatoid body where MIWI acts as a dock for KIF17b. Other chromatoid body components, including Dicer, Agos and miRNAs, could guide sequence-specific translational repression or degradation of target mRNAs (Figure 1). Dicer and MIWI have been shown to physically interact (Grivna *et al.* 2006b), which makes it the third known protein interaction for MIWI, the other two being KIF17b and MVH (Kotaja *et al.* 2006a,b). Whether these proteins exist as one complex in chromatoid bodies or sequential complexes is not known (Figure 1).

A role for MIWI outside of the chromatoid body has also been proposed. MIWI associates with piRNAs in cytosolic ribonucleoprotein and polysomal fractions where MIWI interacts with the mRNA cap binding complex (Grivna *et al.* 2006a), indicating a role for MIWI in protein translation (Figure 1). piRNAs might act through homology searching as miRNAs do in translational repression of their targets. MIWI is required for expression of a subset of miRNAs since a number of miRNAs that associate with polysomes are absent in *Miwi*$^{-/-}$ mice (Grivna *et al.* 2006a). So in addition to being essential for piRNA biogenesis, MIWI may have a role in miRNA expression or stability, directly or indirectly (Grivna *et al.* 2006a).

In order to understand the biological significance of piRNAs and their associated proteins MIWI and MILI we need to understand the biogenesis and processing of piRNAs and identify the targets of piRNAs. It is easy to speculate that piRNAs play a role during meiosis due to their expression in spermatogenesis. A number of epigenetic changes occur during spermatogenesis. This is a time of genome-wide demethylation and remethylation, and when genomic imprints are erased and re-established in order to depict a paternal epigenotype. Given the expression patterns of piRNAs it is possible that these play a role in some of these global epigenetic changes that occur during gametogenesis. The role they play must be male-specific, because piRNAs have not been identified in oocytes. This may be a reflection on the differing controls of meiotic progression in males and females. It has been suggested that piRNAs might be involved in homology searching and chromosome pairing (Girard *et al.* 2006). Since events such as chromosome synapsis and recombination in female meiosis are not as stringently regulated (reviewed by Morelli & Cohen

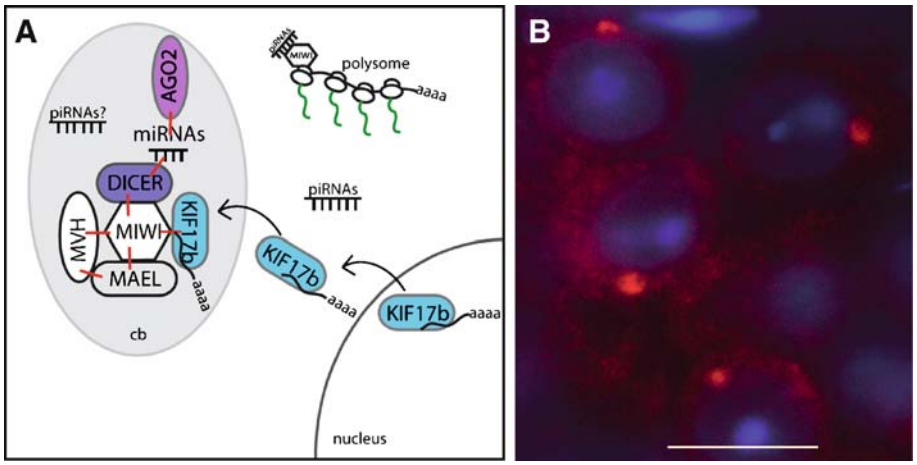

Figure 1. Representation of MIWI interactions in mammalian spermatocytes. (**A**) MIWI is found in the chromatoid body (cb) (Grivna *et al.* 2006b, Kotaja *et al.* 2006a), a mobile structure appearing around late pachynema that moves around the nuclear envelope collecting mRNAs. The chromatoid body is believed to be a site of RNA processing. KIF17b collects mRNAs from the nucleus and transports them to the cb where KIF17b is known to interact with MIWI (Kotaja *et al.* 2006b). The role of MIWI in the cb is not known but MIWI does associate with DICER, MVH and MAEL (although probably not as one large complex as depicted, for simplicity, here.) (Costa *et al.* 2006, Grivna *et al.* 2006b, Kotaja *et al.* 2006a). It is hypothesized that miRNAs present in the cb could guide RNAi with the aid of DICER and AGO2 (also present in the cb). It is not known if piRNAs localize to the cb. MIWI, along with piRNAs, is also associated with the mRNA cap binding complex at polysomes where it may play a role in translation or miRNA expression/stability (Grivna *et al.* 2006b). Red lines indicate known protein-protein or protein-miRNAs interactions. (**B**) MAEL in the chromatoid body. Round spermatids stained with DAPI (blue) and MAEL (red). MAEL localizes to the chromatoid body which lies outside of the nucleus. This illustration was kindly donated by Yael Costa. Scale bar = 10 μm

2005) the absence of piRNAs in oocytes might be one explanation for this.

RNAi, heterochromatin and centromeres

The centromeres of many organisms are structurally complex, heterochromatic, rich in repetitive DNA, and subject to epigenetic modifications. Although centromeres lack sequence conservation between species, a common theme is their repetitive nature, along with the hallmarks of heterochromatin, in particular methylation of histone lysine 9 (H3K9) and recruitment of HP1, heterochromatin protein 1, to methylated H3K9. The importance of repetitive DNA at centromeres is demonstrated from studies deleting *otr*, a heterochromatic repeat region of the centromere in *Schizosaccharomyces pombe*, which resulted in aberrant meiotic sister chromatid attachment (Hahnenberger *et al.* 1991).

Centromeres play a key role in both meiosis and mitosis because they are the site of kinetocore assembly. The kinetocore is a proteinaceous structure that is captured by microtubules and pulls sister chromatids (in the case of mitosis) or homologous chromosomes (meiosis) to opposite poles of the spindle. Coordinated segregation of chromosomes is essential to maintain the correct number of chromosomes during cell division. Given the significant role of centromeres and the unique properties of the DNA with which they are associated, it is important to understand how centromeric DNA and the associated epigenetic modifications are maintained. The question arises as to whether an RNAi-mediated mechanism of heterochromatin formation and maintenance occurs endogenously at centromeres in mammals, as implied by the discovery of a surprising link between RNAi and maintenance of centromeric heterochromatin in *S. pombe*.

The role of RNAi at centromeres in S. pombe

Elegant studies deleting *ago1* and *dcr1* (*S. pombe* Argonaute and Dicer genes, respectively) have shown that the RNAi machinery is essential for epigenetic silencing at centromeres (Volpe *et al.* 2002). Without *ago1* or *dcr1* methylation of H3K9 and therefore Swi6 (the *S. pombe* form of HP1) is

lost at centromeres and transgenes integrated into the centromeres are de-repressed, indicating a switch to a euchromatic state. This was also observed at the silent mating-type region, another heterochromatic domain in *S. pombe* (Hall *et al*. 2002). Links between RNAi and chromatin modifications have since been established in *A. thaliana* (Zilberman *et al*. 2003), *D. melanogaster* (Pal-Bhadra *et al*. 2002) and *Tetrahymena* (Mochizuki *et al*. 2002).

Coinciding with heterochromatin loss in *ago1* and *dcr1* mutants, aberrant accumulation of transcripts derived from both strands of the centromere was observed (Volpe *et al*. 2002). At this time it was proposed that one role of the RNAi machinery is to process dsRNA from centromeric transcripts into siRNAs and guide chromatin modifications and hence heterochromatin formation in a sequence-specific manner (Volpe *et al*. 2002). This idea has been supported by the identification of siRNAs complementary to both strands of centromeric repeats (Reinhart & Bartel 2002) and was further corroborated when a complex known as RITS was identified (Verdel *et al*. 2004). The RITS complex (RNA-induced transcriptional silencing) provides a direct link between siRNAs and heterochromatin. Along with siRNAs, the *S. pombe* RITS complex contains AGO1, CHP1, a chromodomain protein that binds centromeres, and a previously unknown protein TAS3. The RITS complex uses siRNAs to identify target nascent RNA transcripts at specific chromosome regions, leading to epigenetic modifications and heterochromatin formation (Buhler *et al*. 2006, Irvine *et al*. 2006). In order for siRNAs to be produced, a dsRNA precursor must exist. It was initially thought that this precursor would arise through transcription from both strands of the centromere; however, in wild-type cells only the reverse strand of the centromere is actively transcribed, whereas the forward strand is silenced at the transcriptional level (transcription is detectable but at extremely low levels) (Volpe *et al*. 2002). It appears that generation of the dsRNA precursors relies upon RdRP, RNA-dependent RNA polymerase, in a complex which interacts with RITS to produce a continuous supply of siRNAs from an initial dsRNA template (Motamedi *et al*. 2004, Buhler *et al*. 2006, Irvine *et al*. 2006).

The identification of the RITS complex helped to explain the observations in *S. pombe ago1* and *dcr1* mutants exhibiting aberrations in mitosis and meiosis (Hall *et al*. 2003). These mutants are hypersensitive to microtubule-destabilizing drugs, show high rates of non-disjunction and lagging chromosomes in mitosis and mis-segregation in meiosis (Hall *et al*. 2003). These phenotypes are thought to be due to a loss of centromeric cohesins, which is a consequence of loss of epigenetic marks. Methylated H3K9 recruits Swi6, which in turn attracts cohesins to the centromere, important for sister chromatid cohesion and chromosome segregation (Bernard *et al*. 2001, Nonaka *et al*. 2002). Without the proper epigenetic marks at centromeres, the *ago1* and *dcr1* mutants are unable to attract cohesins via Swi6 and methylated H3K9 leading to the mitotic and meiotic defects observed.

Does RNAi function at centromeres in mammals?

Two key observations argue against a role for siRNA in maintenance of mammalian centromeres. First an RdRP complex to provide a continuous supply of siRNAs has not (yet) been identified in mammalian genomes. Second, until recently, endogenous siRNAs were not thought to be present in mammalian systems, although this point may now be moot since the identification of rasiRNAs in male and female germ cells (Watanabe *et al*. 2006). However, a number of similarities exist between the mammalian and *S. pombe* systems of heterochromatin formation. Transfection of human cell lines with siRNAs complementary to specific promoters leads to transcriptional gene silencing (TGS) coinciding with H3K9 di- and tri-methylation, both hallmarks of heterochromatin (Morris *et al*. 2004, Ting *et al*. 2005, Weinberg *et al*. 2006). Furthermore, recent studies demonstrate that TGS and H3K9 methylation may be mediated by a member of the Argonaute family, AGO1, and not the expected AGO2 (Kim *et al*. 2006). These experiments suggest that an RNAi-mediated process of heterochromatin formation can occur in mammalian cells when they are given the correct trigger, i.e. siRNAs.

Studies by Wang *et al*., on the other hand, argue against involvement of RNAi in heterochromatin formation using a transgene-induced heterochromatin model. The transgene used in these studies consists of tandem insertion of 1000 copies of inducible transgene which forms a dense chromatin locus with methylation of H3K9, association of HP1 and DNA methylation (Wang *et al*. 2006). Interestingly, small

RNAs with homology to the transgene were not detectable and placing the transgene on a Dicer null background did not affect methylation of H3K9. Moreover, Dicer deletion failed to induce expression of the transgene, suggesting that the transgene remained in a heterochromatic state in the absence of Dicer (Wang *et al*. 2006).

The conflicting results of RNAi involvement in heterochromatin in mammals arose from what might be considered non-physiological models, one inducing an RNAi response by feeding foreign siRNAs into the tissue culture cells (Kim *et al*. 2006) and the other creating a foreign heterochromatin region by the introduction of foreign DNA into mice (Wang *et al*. 2006). As with any tissue culture system predictions of what might happen *in vivo* are established, but these may vary greatly from what actually happens in the animal itself.

Centromere stability in RNAi-deficient mammals

Interesting yet variable data regarding centromeric heterochromatin have come from targeted deletions of *Dicer* in ES cells. Two groups demonstrate increased levels of unprocessed centromeric transcripts (Kanellopoulou *et al*. 2005, Murchison *et al*. 2005), with a decrease in small RNAs homologous to the minor satellite repeats of centromeres (Kanellopoulou *et al*. 2005) and an accumulation of pri-miRNAs (Murchison *et al*. 2005), presumably due to the absence of *Dicer* to cleave longer RNAs into their smaller RNA products. However, decreased epigenetic marks at centromeres were observed in only one of the cell lines (Kanellopoulou *et al*. 2005). A chicken–human hybrid cell line containing a human chromosome 21 and a conditional knock-out of Dicer also shows an accumulation of centromeric transcripts and, interestingly, an absence of the key heterochromatin-binding protein, RAD21, at centromeres (Sonoda *et al*. 2001, Fukagawa *et al*. 2004).

It is intriguing that *Dicer*-deficient ES cells do not exhibit a significant increase in chromosome abnormalities such as aberrations in chromosome numbers and structure, although such analysis was not extensive in these reports (Kanellopoulou *et al*. 2005). Accumulation of centromeric transcripts during meiosis alongside aberrant epigenetic marks at centromeres is likely to impact upon normal meiosis because the centromeres play such a key role in this process. It has not been determined whether HP1 proteins attract cohesins as Swi6 does in *S. pombe*. It seems likely that aberrant methylation of H3K9 or HP1 binding (as seen by Kanellopoulou *et al*.) would affect the heterochromatic structure of the centromere and hence its role in meiosis.

Homozygous deletion of Dicer in mouse results in embryonic lethality at e7.5 (Bernstein *et al*. 2003), making it impossible to analyze the effect of *Dicer* loss on meiosis. However, a number of tissue-specific *Dicer* deletion mice are available (Cobb *et al*. 2005, Harfe *et al*. 2005, Muljo *et al*. 2005, Murchison *et al*. 2005) and a meiosis-specific deletion of *Dicer* would be very interesting to study in terms of centromere function during gametogenesis.

Similar studies of Argonaute protein deletions in mouse would also be informative. Like $Dicer^{-/-}$ mice, $Ago2^{-/-}$ mice are not viable (Liu *et al*. 2004). While only AGO2 in mouse shows slicing activity, AGO1 and AGO3 are also loaded with siRNAs and incorporated into RISC but are not able to target cleavage of mRNAs (Liu *et al*. 2004). Why only AGO2 has a role in mRNA degradation is unknown, but other roles might exist for AGOs 1, 3 and 4, possibly in TGS as indicated by Kim *et al*. (2006) or heterochromatin formation, as Ago1 is in *S. pombe*.

RNAi and centromeres in other species

RNAi is associated with centromere function in the ciliated protozoan *Tetrahymena* (Mochizuki & Gorovsky 2004a,b, 2005). *Tetrahymena* undergoes genome rearrangement and eliminates large segments of DNA during conjugation. siRNA-like small RNAs known as scnRNAs (scan RNAs) are expressed during conjugation. These are derived from micronucleus transcripts, processed by a Dicer-like protein, Dcl1p, to scnRNAs (Mochizuki & Gorovsky 2005), and associate with Twi1p, a member of the Argonaute family, to eliminate DNA sequences (Mochizuki & Gorovsky 2004a). Dcl1p is also required for proper segregation of micronucleus chromosomes during mitosis and meiosis, probably through an RNAi process at centromeres (Mochizuki & Gorovsky 2005).

Whilst the role of RNAi in centromeres and heterochromatin in mammals is still controversial it remains an interesting field that requires further systematic studies to elucidate its true role. Meiosis is a highly regulated process that relies heavily on centromeres and their associated proteins. Aberrant

centromeres can lead to a number of meiotic defects and understanding the role of RNAi in centromeres will be a major contribution to the field. Furthermore, given the putative role of chromatinization in homologous pairing and synapsis, it seems plausible that RNAi processes may play other roles in the context of prophase I events.

Meiotic silencing of unsynapsed chromatin (MSUC) and unpaired DNA (MSUD)

A link between RNAi and meiotic silencing of unpaired DNA (MSUD) has been identified in *Neurospora crassa*. In the vegetative state, *N. crassa* is haploid but a transient diploid state occurs in the zygote which undergoes meiosis. During chromosome pairing, regions of DNA that are unable to pair are silenced, as well as regions of the genome that show homology to the unpaired region independent of whether the other copies are paired. A mutation in the gene *Sad1* reverses this meiotic silencing (Shiu *et al*. 2001). Cloning and sequencing of *Sad1* revealed it to be an RNA-dependent RNA polymerase (RdRP) (Shiu & Metzenberg 2002). In *N. crassa*, *Sms2*, an Argonaute-like protein, and Sms3, a Dicer-like protein, are also required for meiotic silencing (Galagan *et al*. 2003, Lee *et al*. 2003a). Thus two proteins that are involved in RNAi in other species are involved in meiotic silencing in *N. crassa*. The model proposed by Lee *et al*. (2003a) involves members of the RNAi pathway. Unpaired DNA forms a loop which is transcribed to a ssRNA. This ssRNA is a template for *Sad1* forming a dsRNA. This dsRNA is a target for Dicer to form small RNAs that could be incorporated into a RISC-like complex targeting all homologous transcripts.

Regions of unpaired DNA also occur in mammalian meiosis and form a distinct territory known as the sex body comprising the two sex chromosomes. The X and Y chromosomes pair only at a small region known as PAR (pseudo-autosomal region). During pachytene, unpaired regions of the X and Y are transcriptionally inactive and have hallmarks of heterochromatin (hypoacetylation and methylation of H3K9 (Khalil *et al*. 2004)). If RNAi is involved in centromeric heterochromatin formation in mammals, it is tempting to speculate that heterochromatinization of the X and Y may also be due to an RNAi-based method.

The region around the X and Y (sex body) is rich in phosphorylated H2AX, BRCA1, ATR and XMR (Calenda *et al*. 1994, Baarends *et al*. 1999, Mahadevaiah *et al*. 2001, Turner *et al*. 2004). A novel protein, MAELSTROM, identified in a screen for meiotic genes (Maratou *et al*. 2004) has recently been shown to localize to the sex body (Costa *et al*. 2006). Furthermore MAELSTROM interacts with chromatin-remodeling factors (Costa *et al*. 2006), suggesting a role in heterochromatin formation. MAELSTROM, like BRAC1, ATR and H2AX, also localizes to regions of unpaired chromosomes that are transcriptionally inactive, a process known as MSUC (Turner *et al*. 2005, Costa *et al*. 2006). Costa *et al*. hypothesize a link between MSUC and RNAi by a mechanism of RNAi-dependent heterochromatinization seen in *S. pombe*. In addition to its localization to regions of unpaired chromosomes, MAELSTROM was observed in the chromatoid body where it interacts with MVH, MILI and MIWI (Costa *et al*. 2006) Fig. 1b. A role for MAELSTROM in RNA processing along with RNAi components is speculated and, but remains unconfirmed.

rasiRNAs

Repeat-associated small interfering RNAs (rasiRNAs) are a type of siRNA that arise from repetitive DNA, particularly transposable elements. Like siRNAs, rasiRNAs are processed from longer dsRNAs and exhibit a preference for a uridine base at their 5′ end. Although less abundant than miRNAs, they have been identified in a number of organisms and are believed in be involved in establishment and maintenance of heterochromatin and silencing of transposable elements in *A. thaliana*, *D. melanogaster* and *S. pombe* (Mette *et al*. 2000, Hall *et al*. 2002, Hamilton *et al*. 2002, Llave *et al*. 2002, Pal-Bhadra *et al*. 2002, Reinhart & Bartel 2002, Volpe *et al*. 2002). In *D. melanogaster*, rasiRNAs are abundant in the germ line and early embryo when silencing of transposable elements is critical.

Until recently endogenous siRNAs had not been detected in mammals. However, artificial siRNAs introduced to mammalian cells can exert sequence-specific mRNA degradation, in the same way as naturally occurring siRNAs do in plants and animals, as evidenced by the recent wealth of studies utilizing siRNA as an experimental tool. Plant and animal

endogenous siRNAs arise from a longer dsRNA precursor that is cleaved into siRNAs by Dicer proteins. Introduction of long dsRNA into mammalian cells leads to an interferon response (Stark *et al*. 1998) but shorter dsRNA duplexes of less than 30 nt in length do not trigger the interferon pathway (Elbashir *et al*. 2001a) which is why siRNAs are such a powerful tool for gene-specific knockdown in mammalian and plant systems. The fact that mammalian cells cannot tolerate long dsRNA might explain why naturally occurring siRNAs had not been detected. However, in mammalian oocytes, which do not exhibit an interferon response, dsRNAs function efficiently through the RNAi pathway (Svoboda *et al*. 2000), presumably because oocytes can tolerate the long dsRNA trigger. Thus, oocytes were the most logical place to search for endogenous siRNAs. Small RNA cloning experiments from oocytes were successful in detecting siRNAs, the majority of which were rasiRNAs (Watanabe *et al*. 2006). It is possible that mammalian rasiRNAs, like lower organism rasiRNAs, function through the RNAi pathway in order to regulate transposable elements and possibly heterochromatin. Supporting evidence for this comes from *Dicer*-deficient ES cells which accumulate retrotransposon-derived transcripts, presumably because they cannot process long dsRNA into smaller rasiRNAs (Kanellopoulou *et al*. 2005).

Discussion

There have been a significant number of small RNA classes identified in the past two decades. It is tempting to speculate that more will be identified in the immediate future. How these RNAs relate specifically to meiosis, particularly in light of the meiosis-specific class of piRNAs, but also to other chromatin-related processes, is the subject of much interest at the current time. In the context of meiosis and germ cell development, the role of the PIWI family of proteins, along with the piRNA species, remains unclear, although the different timing of spermatogenic arrest in *Mili*$^{-/-}$ and *Miwi*$^{-/-}$ mice would suggest roles for PIWI-related events throughout meiosis and spermiogenesis. Interestingly, the male-specific germ cell phenotype of these animals, coupled with the restriction of piRNAs to male germ cells, indicates an important difference between regulatory events in male and female gametogenesis. This male germ cell-specific RNAi mechanism may confer greater stringency during spermatocyte prophase I events, or may reflect a role in sex body formation and/or meiotic sex chromosome inactivation, the latter being suggested by the presence of the chromatoid body-associated, MAEL, within the sex body during meiotic prophase I. Experimental evidence for this needs to be obtained.

Post-meiotic roles for non-coding RNAs are indicated by the later spermatogenic arrest of *Miwi*$^{-/-}$ mice, suggesting roles in spermiogenic events that may include facilitating the necessary changes in chromatinization of the sperm head. Indeed, the histone replacement transition protein 2 (TNP2) mRNA is a target for destruction by the miR-122a (Yu *et al*. 2005), suggesting a role for RNAi in mediating the histone to TNP to protamine transition. The sequential replacement of histones during spermiogenesis has long been considered to be a post-translationally regulated process in view of the earlier termination of transcription in haploid germ cells. In line with this suggestion, the mRNAs for transition proteins and protamines are extremely long-lived, remaining in the cytoplasm for up to 7 days prior to their translation (reviewed by Hecht 1998).

Many of the putative functions of RNAi in gametogenesis require further investigation. In particular, understanding the exact timing and regulation of piRNA synthesis in relation to meiotic and spermiogenic processes will provide further insight into their role in these events. Furthermore, the implications of the sexually dimorphic restriction of RNAi mechanisms to the male germline will be of importance in understanding how gametogenesis is regulated in males and females.

Acknowledgements

Rebecca Holmes is supported by a postdoctoral scholarship from the Center for Vertebrate Genomics, Cornell University. Work in the Cohen laboratory is supported by funding from the National Institutes of Health (HD41012) and by start-up funding from the College of Veterinary Medicine, Cornell University. We thank Yael Costa and James

Turner for contributing part B of Figure 1. We also thank John Dominy for reading this manuscript and for his helpful suggestions.

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