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Conserved chromosomal functions of RNA interference

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Abstract

RNA interference (RNAi), a cellular process by which small RNAs target and manipulate complementary RNA transcripts, has well-characterized roles in post-transcriptional gene regulation and transposon repression. Recent studies have considered RNAi activity beyond these classical roles and revealed critical roles for RNAi in chromosome function. By guiding chromatin modification, RNAi promotes chromosome segregation during both mitosis and meiosis and regulates chromosomal and genomic dosage response. Small RNAs and the RNAi machinery also participate in the resolution of DNA damage. Interestingly, many of these lesser-studied functions seem to be more strongly conserved across eukaryotes than well-characterized functions such as the processing of microRNAs. These findings have implications for the evolution of RNAi since the last eukaryotic common ancestor and provide a more complete view of the function of RNAi.

Introduction

RNA interference (RNAi) is a post-transcriptional gene silencing (PTGS) mechanism mediated by small RNAs that has been found to play essential roles across eukaryotic organisms in genome defence against viruses and transposable elements[1–4], development[5–7], cellular differentiation[8] and disease progression[9,10]. Effector proteins of the Argonaute family such as AGO and PIWI use the sequence of small RNAs — microRNAs (miRNAs), short interfering RNAs (siRNAs) and PIWI-associated RNAs (piRNAs) — to identify target RNAs with complementary base pairing. The small RNA–effector complex either cleaves the target or recruits other proteins to inhibit the translation or degrade the target RNA.

The strong evolutionary conservation of proteins involved in miRNA biogenesis and RNAi, such as DROSHA–DGCR8, DICER1 and AGO, and the apparent absence of classical miRNAs in the last eukaryotic common ancestor (LECA)[11] suggest that these proteins possess specific qualities that enable non-microRNA precursor RNA interactions to perform a broad range of important, conserved functions. Some of these microRNA-independent, often nuclear, RNAi functions have subsequently been elucidated in great mechanistic detail (as reviewed elsewhere[3,12,13]). Other highly conserved functions of RNAi proteins have consequences specifically for chromosome and genome integrity and emphasize the importance of RNAi outside the production of microRNAs. The molecular mechanisms related to these themes are easier to elucidate in organisms without microRNAs, such as

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the fission yeast *Schizosaccharomyces pombe*, as in other organisms the consequences of losing miRNA-mediated gene repression by mutating or manipulating one of the RNAi components can cloud experimental interpretation. Despite this caveat, we demonstrate that a number of functions and themes of the RNAi machinery are conserved from unicellular eukaryotes, such as *S. pombe*, to higher eukaryotes. Moreover, we explore the possibility that chromatin modification was an early consequence of ancestral function and affected genome integrity at multiple levels.

In this Review, we begin by tracing the evolutionary history of the RNAi components from their origins in prokaryotes to the unification of these proteins into a functional pathway in the LECA. We then provide a brief overview of the known molecular preferences of these proteins to demonstrate the inherent flexibility of RNAi. Next, we detail conserved functions of RNAi in the segregation of chromosomes during cell division. Then we examine the regulation of dosage by RNAi at the RNA, chromatin and DNA levels. We demonstrate how the regulation of dosage at these various stages contributes to chromosome function in general. Finally, we describe the expanding role of RNAi proteins in the detection and resolution of DNA damage. Throughout the review we will draw comparisons to the classic small RNA-driven heterochromatin formation mechanism in *S. pombe* and delineate between mechanisms that seem to be an adaptation of this pathway and those that are independent of small RNAs.

The evolution and molecular targets of RNAi proteins

The production of the various classes of eukaryotic small RNAs — siRNAs, microRNAs and piRNAs — occurs through defined molecular activity on precursor RNA molecules (Fig. 1a–c). The enzymatic activities that produce small RNAs and generate an effect on RNA targets are highly conserved and maintain a general flexibility in substrates. These qualities and their conservation, which will be discussed further in the following sections, are important to recognize to define the broad range of functions of RNAi and of the molecular targets that generate these functions.

The evolutionary history of RNAi

RNAi is an ancient pathway that was present in the LECA. This ancestral RNAi pathway most likely had an Ago-like protein, an Ago-related but distinct Piwi-like protein, a Dicer-like protein and an RNA-dependent RNA polymerase (RdRP)[11,14]. These components and their active domains are highly conserved across eukaryotes (Fig. 1d) and even prokaryotes. For example, the two miRNA-processing enzymes DROSHA and DICER1 (Fig. 1b) possess RNase III domains that are found in bacteria and play important roles in rRNA processing in *Escherichia coli*[15,16]. Eukaryotic RdRP likely derived from a bacteriophage protein and may have been a catalytically inactive cofactor for a ribozyme polymerase[17]. Argonaute proteins are present in all domains of life, and the PIWI domain is always present, indicating the requirement of this domain for Argonaute function[18]. In prokaryotes, it has been proposed that Argonaute is part of a defence system against novel mobile elements[19]. Indeed, given the likely presence of both Ago-like and Piwi-like effector proteins, transcriptional and post-transcriptional repression of transposons and viruses are the proposed functions of the

ancestral RNAi pathway[11,14,18]. Other RNAi pathway components such as the DROSHA–DGCR8 complex, also known as Microprocessor, have been considered to be exclusive to metazoans, although homologues have been identified in unicellular Ichthyosporea (also known as Mesomycetozoea), which is indicative of an earlier than previously predicted evolutionary origin[20].

The lack of conservation of microRNAs, as well as some of the enzymes that are necessary to produce them in metazoans, is also strong evidence that the ancestral function of RNAi may have been genome defence rather than gene regulation. microRNAs have arisen independently in animals[21], plants[22], slime molds[23], multiple lineages of algae[24,25], fungi[26] and Ichthyosporea[20] (Fig. 1d), which suggests that the LECA did not have microRNAs and indicates strong convergent evolution on the microRNA-generating hairpin. The evolutionary origin of microRNA-generating sequences is unknown and is likely distinct in each acquisition, as it is between animals and plants[27]. Many microRNA-like structures evolved from transposons[28] in a number of organisms, including mammals[29,30] and plants[31,32]. In this evolutionary model of gene regulation by RNAi, the ancestral function of genome defence was adapted through molecular rearrangement of the transposon target sequences, resulting in a hairpin-like structure and the production microRNAs. Thus, though RNAi has evolved important gene regulatory functions in higher eukaryotes, the proposed ancestral function of genome defence has been conserved, and we explore other conserved functions that may predate the multiple innovations of microRNAs.

The targeting activity of RNAi proteins

The molecular mechanism of RNAi is dependent on the capacity of its protein components to recognize an RNA target. In animals, the microRNA production pathway begins when the two RNase III domain-containing proteins DROSHA and DICER1 cleave RNA in a structure-dependent manner (Fig. 1b). DROSHA recognizes the junction of the stem loop structure (that is, pri-miRNA) with the single-stranded RNA (ssRNA) base and cleaves approximately 11 base pairs further into the stem to produce the pre-miRNA[33,34]. In coordination with DROSHA, DGCR8 interacts with the double-stranded RNA (dsRNA) stem and apical junction to further stabilize the complex and allow for efficient processing[34]. DICER1 recognizes dsRNA structures in general, including the pre-miRNA hairpin, and preferentially cleaves approximately 21–22 base pairs from the 3’ terminus[35–39].

Despite the clear reliance on secondary structure as the primary means of target recognition, these enzymes can have some primary sequence bias, which varies from one species to another[35,40,41]. The DROSHA–DGCR8 complex shows preferences for a UG motif at the base of the stem loop structure, a UGU motif in the apical loop, and a CNNC motif 16–18 base pairs from the cut site[40]. Although human DICER1 has less preference for sequence motifs than the Microprocessor complex, not all sequences are cleaved equally efficiently and factors such as the presence of a 2 nucleotide (nt) 3’ overhang, which is bound by the PAZ domain, can have a strong influence on human DICER1 processivity[35,36,39]. Additionally, the sequence content of the overhang can influence human DICER1 efficiency[36]. Recent work has identified specific sequence motifs targeted by individual Dicer homologues in the ciliate *Paramecium tetraurelia*[41]. These inherent

preferences of these ciliate Dicers allow the homologues to precisely target sequences for removal in a process called DNA elimination[41], as discussed below.

Many mature microRNA sequences have a terminal 5’ U or A bias. This bias has been attributed to the binding preferences of Argonaute proteins rather than a sequence motif preferentially cleaved by DICER1[42,43]. In general, Argonaute proteins will load dsRNA and then discard one strand (passenger strand), resulting in the single-stranded (guide strand), mature small RNA–AGO protein complex[44]. However, there are indications in mammals and *Drosophila melanogaster* that ssRNA can also be loaded into Argonaute proteins[45,46], and different Ago homologues seem to possess biases for short sequence motifs[45,46]. Additionally, recent evidence points to the wide variety of chemical modifications on RNA molecules (>100)[47]. One modification that has been shown to be functional in small RNAs is 2’O methylation, which stabilizes small RNAs by protecting the molecules from 3’ uridylation[48]. This modification occurs on microRNAs and siRNAs in plants[49], piRNAs in *Tetrahymena thermophila*[50], piRNAs in mammals[51], and on piRNAs[52,53], siRNAs[53–57] and some microRNAs[57,58] in *D. melanogaster*. In fact, in flies, the 2’O methylated siRNAs or microRNAs are associated with Ago2 rather than Ago1[53,57]. Intriguingly, the majority of small RNAs that are 2’O methylated enter the nucleus and participate in small RNA-directed chromatin modification pathways. It will be interesting to determine whether any of the other identified RNA modifications play a role in processing, loading or stability of small RNAs.

In the context of generating microRNAs, DROSHA–DGCR8, DICER1 and AGO proteins principally recognize RNA secondary structure and, to a lesser extent, some primary sequence motifs in selecting their substrates. These qualities of RNAi proteins enable non-microRNA interactions that have a broad range of critical, conserved functions, as we discuss in the following sections.

The role of RNAi in centromere function

Since the discovery of RNAi and of the pathway’s conservation amongst higher eukaryotes, disruption or deletion of RNAi factors has been performed in a wide variety of eukaryotes. Deletion of Drosha–Dgcr8, Dicer or Argonaute homologues is embryonically lethal in many model organisms including mouse[59–61], *D. melanogaster*[62,63], *Caenorhabditis elegans*[64,65], *T. thermophila*[66], *Arabidopsis thaliana*[67], maize[68] and zebrafish[69]. The loss of microRNAs certainly plays a prominent part in this phenotype, as some knockout embryos progress through the early stages of development but fail to properly differentiate cell types, which is a well-defined microRNA function, particularly in mammals[6,8]. However, a closer examination of cellular phenotypes in response to the reduction or deletion of different RNAi proteins demonstrates that these factors have important roles in chromosome function that are unrelated to microRNA-driven gene repression in many eukaryotes.

RNAi and mitotic chromosome segregation in unicellular eukaryotes

S. pombe has single-copy homologues of RdRP, Dicer and Argonaute and their deletions — *rdp1*Δ, *dcr1*Δ and *ago1*Δ, respectively — yield strong chromosomal phenotypes[70–73]. In cycling cells, mutant strains display lagging chromosomes during anaphase and are sensitive

to the microtubule poison thiabendazole[70–73], indicating the presence of compromised mitotic structures. Dicer and Argonaute mutants are particularly poor at chromosome segregation during mitosis[70–73] (Fig. 2a). Additionally, a missegregation phenotype is observed in the cell division that takes place during the transition from cycling cells to quiescent cells in G0 entry caused by nitrogen starvation[74].

Fission yeast has regional centromeres with an inner centromeric region and outer pericentromeric regions flanking either side. This structure enables accurate specification of the centromeric region used for kinetochore attachment. The pericentromeric region provides a boundary between the inner centromere and the rest of the chromosome. In *S. pombe*, Dicer and Argonaute participate in nuclear co-transcriptional gene silencing at the pericentromeric regions[70,71,75] (Fig. 3a). Bi-directional transcription by RNA polymerase II (Pol II) from the repeated elements of this region forms dsRNA that Dcr1 recognizes and cleaves into double-stranded siRNA precursors[75,76]. To further the reinforcement of this region as a target of siRNAs, Dcr1 and Ago1 interact with the RdRP Rdp1 to generate additional dsRNA and siRNAs from the transcripts of this region[70,77,78] (Fig. 1a). Additionally, another species of siRNAs is produced from hairpin-like structured RNA in pericentromeric transcripts. This secondary structure can be recognized and cleaved by Dcr1 independently of the RdRP[79]. Finally, Dcr1-independent exosome degradation products can be directly loaded into Ago1 and trimmed by the Triman exonuclease to form primal small RNAs (priRNAs)[80]. Thus, there seem to be multiple distinct mechanisms for producing siRNAs from the pericentromere in fission yeast.

The resulting siRNAs are loaded into Ago1 and direct the effector complex, the RNAi-induced transcriptional silencing complex (RITS), to the region. This complex indirectly recruits the histone 3 lysine 9 (H3K9) methyltransferase Clr4 to the region to deposit and spread the methylation mark H3K9me[81]. The methylation can be recognized by several proteins, including Clr4 itself[82], that subsequently induce a state of constitutively repressed heterochromatin. One of these proteins is Swi6, a homologue of the conserved heterochromatin protein 1 (HP1)[83]. This factor recruits the cohesin complex to centromeric heterochromatin for proper cohesion of sister chromatids and faithful segregation of chromosomes during mitosis[84,85]. Furthermore, Clr4, Dcr1 and Swi6 facilitate the deposition of the centromere-specific histone variant Cnp1 (CENP-A in mammals) in the inner core of the centromere that defines the location of kinetochore attachment[86]. Loss of the cohesin subunits Rad21 or Psc3, Swi6, Dcr1, Rdp1 or Ago1 leads to lagging chromosomes and segregation defects during anaphase in fission yeast[70–73,75,84,85]. This cascade of events beginning with Dcr1 recognizing dsRNA of pericentromeric transcripts has an important role in the process of mitosis and chromosome segregation. Interestingly, experimental evolution experiments in other fungi have recently demonstrated a strong correlation between RNAi and the maintenance of a large repetitive regional centromere[87]. This correlation is also seen in the presence of point centromeres and the loss of RNAi in *Saccharomyces cerevisiae* and it may be that the presence of RNAi itself maintains the physical structure of the centromere at the genetic and epigenetic level.

The unicellular eukaryote *Trypanosoma brucei* possesses an RNAi pathway composed of Dicer and Argonaute homologues. The deletion of TbAgo1 in trypanosome cells slows

proliferation and leads to strong mitotic defects that include chromosome missegregation and whole-genome duplication following failed mitosis[88] (Fig. 2b). The trypanosome centromere seems to be regional and of similar size and AT-rich composition as that of higher eukaryotes, but the pattern of loading of a CENP-A homologue has yet to be determined. Furthermore, the centromeric regions are flanked by regions of repetitive DNA derived from transposons[89]. This structure resembles the content of the heterochromatic pericentromeric regions seen in many other eukaryotes. The ciliated protozoa *Tetrahymena* also possesses a fully functional RNAi system and centromeres that have a regional structure[90]. These RNAi homologues have a number of different functions. *Tetrahymena* has a macronucleus that is polyploid (~45n) and transcribed in addition to a micronucleus that is diploid and devoid of transcription in cycling cells[91]. During cell division, the micronucleus, which possesses centromeres, divides mitotically, whereas the macronucleus, which does not have centromeres, divides amitotically, with random distribution of genetic material. Although the deletion of the Dicer homologue Dicer-like protein 1 (Dcl1) is viable, it leads to smaller micronuclei and chromosome lagging and bridging (a continuous piece of DNA between the two segregating chromosome masses in anaphase) during mitosis66 (Fig. 2c). These mutants are also more susceptible to microtubule poisons, as *dcr1*Δ strains are in *S. pombe*, indicating defects in chromosome segregation. Thus, in unicellular eukaryotes, such as fission yeast, trypanosomes and *Tetrahymena*, RNAi plays important roles in chromosome function and segregation.

RNAi and chromosome segregation in higher eukaryotes

Participation of RNAi proteins in chromosome functions such as segregation is conserved in higher eukaryotes, though the evidence for small RNAs or heterochromatin formation in this mechanism remains controversial (Fig. 2). Invertebrate genomes frequently have multiple copies of RNAi genes that have diverged in sequence and function, with one set dedicated to producing microRNAs and the other dedicated to producing endogenous siRNAs (endo-siRNAs) for the purposes of transposon or viral targeting and heterochromatin formation[62,92]. In *D. melanogaster*, which also has regional centromeres, the deletion mutants of the endo-siRNA-producing proteins Dcr-2 and Ago2 have a lagging chromosome phenotype in wing disc cells[93]. S2 cultured cells treated with siRNAs that target Dcr-2 and Ago2 also display the lagging chromosome phenotype[93] (Fig. 2d). Furthermore, deletion of Ago2 in fly embryos results in heterochromatin defects as well as mitotic and chromosomal disruptions[94]. In one accession of the model plant *Arabidopsis thaliana*, mutants in AGO4 have been reported to have lagging chromosomes and bridging in mitosis[95], although this phenotype has not been reported in other studies.

In vertebrate and particularly mammalian cells, the role of RNAi in chromosome segregation is more controversial. The chicken DT40 cell line harbours a human chromosome 21, and conditional deletion of the chicken Dicer homologue results in missegregation of the human chromosome, along with disruption of the centromeric heterochromatin and reduced enrichment of HP1 to heterochromatic loci[96]. These hybrid Dicer knockout cells accumulate long RNAs from the human pericentromeric α-satellite repeats, but they do not produce an abundant small RNA population in the presence of Dicer. This finding may be due to the fact that the small RNA machinery in this cell line is chicken-derived, and there is no RdRP

present. Mouse fibroblasts prepared from *Dicer1* knockout embryos display high incidences of both bridging chromosomes in anaphase and micronuclei, most likely formed from lagging chromosomes failing to enter the new nucleus[97] (Fig. 2e). Mutation of *Dicer1* in mouse embryonic stem cells (mESCs) yields a strong increase in the RNA transcribed from the major and minor satellite sequences, which occupy the pericentromeric and centromeric regions, respectively, accompanied by a reduction in small RNA from these regions[98]. It is important to note that this population was in the 25–30 nucleotide size range and unlikely to be DICER1-dependent. However, these deletion cell lines exhibit either no change[99] or a decrease[98] in H3K9me3 at the pericentromere, without evidence of chromosomal segregation defects. In NIH-3T3 mouse fibroblasts, DICER1 binds directly to major satellite RNA in concert with WDHD1 (WD repeat and HMG-box DNA binding protein 1) and localizes to the pericentromere, as detected by chromatin immunoprecipitation (ChIP)[100]. The association of nuclear DICER1 with satellite loci in human HEK293 cells is also detectable by ChIP[101]. In human RPE-1 cells, the knockdown of *DICER1* or *AGO2* induces lagging chromosomes, an increase in centromeric α-satellite transcripts and mislocalization of a kinetochore-recruiting protein, CENPC1[102] (Fig. 2f). Furthermore, the knockdown of *Dicer1* in HeLa cells also induces lagging chromosomes and a disruption of the localization of a component of the condensin complex[93] (Fig. 2f). These phenotypes are not seen in *Dgcr8* knockout cell lines, which indicates that this phenotype is not due to the loss of canonical microRNAs[102].

The consistent increase in centromeric satellite transcripts upon loss of DICER1 and the localization of DICER1 to centromeric satellite loci in both mouse and human cells suggests that DICER1 binds to either secondary structures in these nascent RNAs or to dsRNA structures arising at the locus from bi-directional transcription. Indeed, both the major and minor satellite transcripts in mouse exist preferentially as dsRNA[103]. In mammals, the long non-coding centromeric and pericentromeric RNAs have a pronounced role in centromere formation, as they are integral parts of the pericentromeric and centromeric heterochromatin[106–109]. The RNAs can interact with a number of heterochromatin-forming and centromere-specific factors such as the H3K9 methyltransferases SUV39H1 and SUV39H2[104–106], HP1[107,108], CENPA[109,110], the CENPA chaperone protein HJURP[109], and AURORA B[111–113], which is necessary for additional epigenetic marks identifying the centromere. Misregulation of satellite RNA levels in mammals can lead to lagging chromosomes and segregation defects[111,113,114]. Similarly, *Dicer1* knockdowns or knockouts can lead to an accumulation of the same satellite RNAs, such as the minor satellite RNA in mouse, but a causal link has not been established[98].

A model built around the regulation of long non-coding satellite RNA transcripts or transcription by DICER1, rather than the loading and targeting by small RNAs, could explain the appearance of lagging chromosomes and segregation defects in mammalian *Dicer1* knockouts (Fig. 3b). Whether the accumulation of long non-coding satellite RNAs in *Dicer1* mutants leads to an alteration of centromeric chromatin by recruiting the previously mentioned factors remains to be seen. Furthermore, in mammals there is conflicting evidence concerning the role of the SUV39-deposited H3K9me3-HP1 complex in recruiting or maintaining centromeric cohesin, which ensures proper chromosome segregation. While some groups report experimental evidence to suggest a role for H3K9me or H4K20me

marks at pericentromeric heterochromatin in centromeric cohesin function in mouse embryonic fibroblast[115], human HeLa cells[116,117], and mESCs[118], others have reported there to be no change in centromeric cohesin following disruption of heterochromatin factors in MEFs[119] or HeLa cells[120]. It may be that these cell types differ in other heterochromatin features such as DNA methylation. There is also conflicting evidence of HP1 proteins interacting with the primary centromeric cohesin maintenance factor shugoshin (SGO1) in mammalian cells with one group reporting a necessary interaction[121] and other groups showing that this interaction is dispensable[117,122].

Small RNAs, whether satellite-derived or not, may participate in heterochromatin formation and/or chromosome segregation in mammals, but evidence is scarce. The fact that a lagging chromosome phenotype is seen in *Ago2* knockdown in one cell line does suggest that small RNAs play some targeting role[102]. However, as mammals lack an RdRP-like enzyme to amplify small RNAs from their precursor transcripts, they do not possess all of the machinery of the co-transcriptional gene silencing mechanism present in *S. pombe* and, therefore, formation, spreading and maintenance of heterochromatin at the pericentromere might occur through different mechanisms. The absence of RdRP might be compensated by an increase in bi-directional transcription and/or by additional hairpin-like structures in the major satellite transcripts. It is important to note that, in humans, a complex involving hTERT (telomerase reverse transcriptase), which is an enzyme that maintains telomere length, exhibits RdRP-like function[123]. Indeed, this complex can produce siRNAs in a Dicer-independent fashion that contribute to heterochromatin formation at centromeres[124]. However, the role of the long non-coding centromeric transcripts in heterochromatin formation appears to be far more pronounced in mammals as detailed above. Additionally, there is evidence in other eukaryotes, such as maize, for long non-coding centromeric transcripts associating with centromeric chromatin[125].

The chromosomes of *C. elegans* do not have regional centromeres, but instead are holocentric. Holocentric chromosomes have multiple regions throughout the chromosome that are specified as centromeres and serve as points of kinetochore attachment during cell division. Despite this fundamental difference, much of the machinery in centromere specification and kinetochore attachment seems to be conserved[126], and RNAi also has an essential role in chromosome segregation. *C. elegans* DRH-3, a Dicer associated RNA-helicase, has a chromosome segregation phenotype when knocked down in embryos[127–129]. The worm DCR-1 also participates in proper gamete formation; deletion of DCR-1 results in misshapen oocytes that have high DNA content caused by failure to divide following replication[130]. This phenotype, however, seems to be due to loss of DCR-1 in the soma rather than in the germline[131]. The Argonaute family member CSR-1 has important functions in chromosome segregation and cell division, as both knockdown of CSR-1 and deletion mutants show disrupted chromosome segregation in the early embryo[65,129] (Fig. 2g). In *C. elegans*, CSR-1 and DRH-3 are part of a pathway that includes an RdRP (EGO-1) and a Tudor-domain protein called EKL-1. Knockdown of any of these factors induces lagging chromosomes in the early embryo[129]. Additionally, the CENP-A centromere specific histone homologue HCP-3, components of both Condensin I and Condensin II, as well as two cohesin subunits, SCC-1 and SCC-3, all show a disrupted localization in embryos when these factors are knocked down[129]. The molecular mechanism for ensuring

proper segregation of chromosomes is dependent on the production of 22 nt small RNAs (22G-RNAs) that are ultimately loaded into CSR-1. The 22G-RNAs target a specific subset of genes, but do not lead to the repression of these genes at the RNA or protein level. Instead, these small RNAs guide CSR-1 to the chromatin associated with this subset of protein-coding genes. These genes are widely distributed throughout all chromosomes, except for the X chromosome. Interestingly, the X chromosome is frequently missegregated in meiosis and is the only chromosome whose loss is tolerated by the worm[129].

The CSR-1–22G-RNA pathway appears to act at the chromatin level, though how much it resembles the co-transcriptional gene silencing pathway in *S. pombe* remains to be described. One idea is that the targeting of a subset of protein-coding loci dispersed across chromosomes at the chromatin level may be analogous to targeting the pericentromeric repeats in fission yeast. Furthermore, given the holocentric nature of *C. elegans* chromosomes and the need to define multiple centromeres along the length of the chromosomes and the missegregation phenotypes observed in mutants, CSR-1 targeting has been proposed to play a role in centromere formation. Supporting this notion, it has been shown that the CSR-1 22G-RNA targets are inversely correlated with *C. elegans* HCP-3 occupancy, raising the possibility that this targeting could limit the spread of HCP-3 by establishing chromatin borders[132,133]. The role of CSR-1 targeting seems to be maintenance of Pol II sense transcription of the germline target loci in *cis*. The loss of CSR-1 activity results in a global decrease in sense target loci transcription, an increase in antisense target loci transcription and a concomitant increase in aberrant transcription from normally transcriptionally-silent regions, such as HCP-3-enriched regions[133]. This aberrant transcription may interfere with HCP-3 deposition along the chromosomes, but the general distribution appears to be maintained in *csr-1* mutants[134]. Thus, CSR-1 and 22G-RNAs seem to exert general effects on the transcriptome and the directionality of transcription from the genome of the early worm embryo at the chromatin level. Whether the CSR-1–22G-RNA pathway participates in the chromatin organization of mitotic chromosomes remains to be seen, but the requirement for this pathway for the proper distribution of H3K9me2 on meiotic chromosomes[135] is intriguing. However, the role of the CSR-1/22G-RNA pathway in chromatin modification that promotes chromosome segregation in the early worm embryo is complicated by an indirect genic regulation of KLP-7, which is a microtubule depolymerase that, when misregulated, generates a portion of the chromosome missegregation observed in mutants in this pathway[134].

The role of RNAi in chromosome segregation is widely conserved in unicellular eukaryotes, plants and animals. However, the full small RNA-generating pathway does not always seem to be necessary for this function. The conservation of this role argues that it may have been an important ancestral function of RNAi. The only molecular conservation that is necessary to maintain this function in all of these organisms is Dicer’s recognition of a dsRNA structure that derives from the DNA sequence of the pericentromere or centromere-adjacent regions. This function of Dicer in coordination with small RNAs and/or other proteins can alter chromatin at the region and ultimately assist in defining the true centromeric locus. The establishment of pericentromeric heterochromatin and the proper specification of the centromere then ensures the inheritance of the genetic material on that chromosome.

RNAi and the segregation of meiotic chromosomes

In addition to ensuring the transmission of genetic material during mitosis, this function of RNAi also extends to the transmission of genetic material from the parental generation to progeny. RNAi mutants in *S. pombe*, such as *dcr1*Δ, *rdp1*Δ and *ago1*Δ, show missegregation of chromosomes in the second division of meiosis[73]. This observation is again likely due to malformation of heterochromatin at the centromere and disruption of cohesins. An additional meiotic phenotype of RNAi mutants is increased recombination in the centromere region[136], which is again mirrored in chromatin mutants, but it is not known if this contributes to missegregation. This mirroring of the mitotic chromosome missegregation phenotype in meiosis is a phenomenon seen in many of the organisms already discussed. In *Tetrahymena*, Dicer homologue DCL1 knockouts exhibit disrupted chromosome condensation and missegregation of the micronucleus chromosomes during meiotic divisions[66]. The *C. elegans* CSR-1 small RNA pathway, which is required for mitotic chromosome segregation in the early embryo, is also needed for meiotic chromosome segregation. The germlines of mutants in this pathway show malformed nuclei with abnormal DNA content[127,135,137]. A hermaphrodite mating population displays a sex skewing to XO males as a result of the spontaneous loss of the X chromosome, which strongly implicates chromosome missegregation phenotypes in meiosis of CSR-1 mutants[129]. Furthermore, male DCR-1 mutants have defects in sperm function and X chromosome segregation[138]. The participation of the same pathway in both mitosis and meiosis in *C. elegans* suggests a similar small RNA-directed mechanism may underlie the role of RNAi in ensuring chromosome segregation in cell division.

RNAi components have important roles in meiotic progression in model plants such as *Arabidopsis*, rice and maize. The loss-of-function mutant of the rice Argonaute gene MEIOSIS ARRESTED AT LEPTOTENE 1 (MEL1) disrupts chromosome condensation and loses a class of 21 nucleotide small RNAs called phased siRNAs (phasiRNAs), which are produced from specific loci – PHAS loci – and require a microRNA trigger for biogenesis. The MEL1 mutant is male sterile, but does not show major disruption of the H3K9 methylation of the pericentromeres, as determined by immunocytology[139–141]. In maize, an Argonaute homologue also participates in chromosome segregation during male meiosis. Mutant alleles of AGO104 show disruption of chromosome condensation, chromosome segregation and spindle formation. Early in meiosis I these mutants have abnormal chromosome condensation and spindle defects, whereas later in meiosis multinucleated progeny cells and micronuclei are present[142]. Interestingly, AGO104 seems to be closely related to both *Arabidopsis* AGO9 and AGO4. As previously discussed, ago4 is the Argonaute mutant reported to have both mitotic and meiotic chromosome segregation defects[95], whereas ago9 mutants have diploid female gametes, although most likely due to apospory rather than defects in meiosis[143]. In the maize AGO104 mutant, the centromeric and pericentromeric regions are hypomethylated at the DNA level in a non-CG context, which indicates a disruption of the chromatin in this region[142]. This leads to an increase in transcription of the repetitive elements present at the centromere that AGO104 normally silences. The fact that chromatin disruption at the centromere in these Argonaute mutants leads to chromosomal defects again resembles the consequences of losing co-transcriptional gene silencing in *S. pombe*.

The role of RNAi in regulating dosage

Condensation, alignment and segregation are important chromosome-level functions that RNAi factors clearly play major parts in during cell division. However, chromosomes must also perform the crucial task of supplying the cell with the appropriate dosage of the genic products they encode. Dosage can be determined either at the RNA level by regulating transcripts, the chromatin level by regulating modification, or the DNA level by regulating copy number.

Controlling dosage at the transcript level

RNAi plays a significant role in determining the transcriptional output of loci, including those subject to gene dosage, even at the level of an entire chromosome. Polyploidy is common in plants, and it is important to prevent the formation of deleterious polyploids that arise from the union of a tetraploid-derived diploid gamete with a normal haploid gamete. In *Arabidopsis*, small RNAs are involved in sensing this imbalance in genome dosage. This mechanism begins when a highly conserved microRNA, miR845, targets the $tRNA^{Met}$ primer binding site (PBS) of long-terminal repeat (LTR) retrotransposons in the pollen. This targeting induces 21–22 nt secondary small RNA production by RNA polymerase IV. This class of RNAs is referred to as epigenetically activated small interfering RNA (easiRNA), and their increased abundance in diploid pollen mediates the response to a genomic imbalance[32,144]. In the presence of easiRNAs, triploid seeds abort due to gene misregulation (triploid block). The misregulation seems to take place at the level of genes with a parent-of-origin imprinting expression pattern in the endosperm[145]. It is not currently known exactly how easiRNAs generate this misregulation. Interestingly, miR845 is derived from the truncation and inversion of a transposon-derived 5’ LTR containing a PBS site[32]. Other factors required for the triploid block include the histone H3K9 methyltransferases in plants[146], which suggests that transcriptional regulation is involved in this process. Thus, RNAi is important for sensing the copy number of the genome after meiosis.

A number of sexually reproducing organisms have evolved to use RNAi for a process called meiotic silencing of unpaired DNA (MSUD) or meiotic silencing of unpaired chromatin (MSUC). This phenomenon was first described in the filamentous fungus *Neurospora crassa* in which the unpaired state of a gene causes it to be repressed during meiosis, typically detected via ascospore colour and shape. The RdRP encoded by *SAD-1* is required for MSUD[147], as are the Dicer homologue DCL-1 and an Argonaute homologue SMS-2, all of which generate small RNAs corresponding to unpaired regions[148–150] (Fig. 4a). Homologues of *S. pombe* helicases Hrr1 and Rad54 are also required for MSUD[151,152] and for RNAi-mediated silencing in fission yeast[78,153]. Although little is known about the recognition mechanism that identifies unpaired DNA with remarkable accuracy, the downstream effects clearly employ RNAi to target and silence the detected unpaired regions. Once an unpaired region is specified, it is hypothesized that it is transcribed into an aberrant RNA. This RNA specifying the unpaired region is most likely targeted by SAD-1 for the formation of dsRNA, which is then used as the precursor for the generation of the MSUD-associated small interfering RNAs (masiRNAs) by DCL-1 and other associated factors[154]. SMS-2 will then load the masiRNAs and target any unpaired transcripts. The

mechanism of repression seems to be post-transcriptional, as SMS-2 has been shown to be exclusively perinuclear and cytoplasmic[155]. When an additional copy of a gene is present, but unpaired, all transcripts from all three copies of this gene are silenced, presumably by small RNA silencing. Thus, although MSUD may be a mechanism to control excessive gene dosage resulting from deleterious chromosomal rearrangements, it has also been proposed that this process represses transposons and meiotic drive elements to maintain genomic integrity[147,156,157].

MSUC occurs in other organisms that need to silence unpaired sex chromosomes in male meiosis. In *C. elegans*, the aforementioned CSR-1–22G-RNA small RNA pathway clearly acts to silence the unpaired male X chromosome. Loss of CSR-1 or other associated factors leads to a global reduction of H3K9me2 on the male X chromosome, concomitant with an increase in H3K9me2 on the autosomes[135]. Interestingly, further analysis of CSR-1–22G-RNA targets indicated that the X chromosome is relatively devoid of small RNA targets[129]. There may be other X chromosome-specific factors that regulate the deposition of H3K9me2 in concert with CSR-1–22G-RNA targeting. The loss of this targeting would then generate additional free H3K9 methylation machinery that acts on the autosomes. In any case, RNAi seems to direct H3K9 methylation in this context as in *S. pombe*. Additionally, in mouse, AGO4 might direct small RNAs for the purpose of MSUC in early prophase of meiosis[158].

In somatic cells of animals with an XY sex chromosome pair, one of the two sexes needs to alter the gene expression from its X chromosome to generate the correct dosage of X-linked genes. The solution is to either increase dosage from the X chromosome in males to match the levels of XX females, or silence one of the X chromosomes in females to reduce dosage to that of a single X. Sex chromosome dosage compensation in Drosophila employs the former strategy by upregulating genes on the X chromosome in males. A protein complex called Male Specific Lethal (MSL) works in concert with two long non-coding RNAs, roX1 and roX2, to achieve this compensation[159]. The long non-coding RNAs are required for proper localization of the MSL complex as deletion of both roX1 and roX2 will lead to mislocalization of the complex to autosomal loci[160]. In addition, RNAi has an integral role in localizing the complex to the X chromosome (Fig. 4b). Mutations in *Dcr-2* and Ag*o2* display synthetic lethality with *roX1– roX2* double mutants, indicating that siRNAs contribute to X chromosome recognition[161]. These siRNAs are derived from the 1.688^{X} satellite repeat, which is highly enriched on the X chromosome[162]. Expression of a hairpin from this satellite repeat is sufficient to generate siRNAs and substantially suppresses the male lethality phenotype in *roX1–roX2* mutants. Ago2 is enriched at the 1.688^{X} satellite repeat loci and recruits Clr4 H3K9 methyltransferase homologue Su(var)3–9 to deposit H3K9me2[163]. This chromatin mark, although generally considered a repressive mark, may direct the MSL complex to the X chromosome to increase expression. The dosage compensation mechanism in Drosophila seems to be an adaptation of the RITS complex for the purpose of transcriptional activation rather than silencing. The reversal of the output occurs downstream of the histone methyltransferase and may be due to a direct or indirect interaction with the MSL complex. The ability of RITS-like complexes to effectively identify specific DNA loci makes it highly useful in processes related to chromosome function.

The proclivity of RNAi to recognize transposon sequences, which is the proposed ancestral function of the pathway, can of course serve to protect the integrity of the genome from these potential invaders, but this function has clearly been adapted for other purposes. The preceding instances exemplify the ways in which eukaryotic genomes, through the capabilities of the RNAi pathway, have co-opted transposon and other repetitive sequences for broad chromosomal functions such as the recognition of interspecies or interploidy hybridization events and regulation of chromosome dosage.

DNA elimination – controlling chromosome content and function through RNAi

Tetrahymena and other ciliates possess both a polyploid macronucleus that is transcriptionally active and a diploid micronucleus that remains transcriptionally silent until meiosis. During conjugation, which is the sexual reproduction phase of *Tetrahymena*, meiosis takes place but only the micronucleus divides to form two haploid pronuclei. After fertilization, the diploid zygote unites the two pronuclei, divides twice to produce a new macronucleus and a new micronucleus, and then eliminates the remaining parental macronucleus. During the formation of the new macronucleus, a process called DNA elimination takes place. This process involves a small RNA-mediated mechanism of the removal of internal DNA segments or internal eliminated sequences (IESs)[164] (Fig. 4c).

DNA elimination begins in the meiotic prophase when the micronucleus is transcribed bi-directionally. DCL-1 recognizes the dsRNA products of this transcription and cleaves them to produce ‘scan RNAs’ (scnRNAs)[66]. The *Tetrahymena* Argonaute homologue TWI1 then loads the scnRNAs for target recognition[165]. The primed TWI1 enters the parental macronucleus searching for complementary target transcripts. This initial scnRNA targeting is a method of negative selection. Any scnRNAs that find target transcripts in the macronucleus are degraded. This degradation prevents subsequent targeting of potentially essential transcripts and facilitates the inclusion of these transcript loci in the new macronucleus. The remaining scnRNAs that have no target transcripts in the parental macronucleus translocate to the new macronucleus[166]. TWI1, loaded with these pre-selected scnRNAs, targets nascent transcripts and recruits the histone methyltransferase EZL1. scnRNAs can also target IESs containing similar sequences in *trans*[167]. This in *trans* targeting confers redundancy and robustness to the scnRNA network. The recruitment of EZL1 nucleates the formation of heterochromatin through the addition of H3K9me and H3K27me[168]. An HP1 homologue, PDD1, recognizes these marks and binds to these regions[169–171]. This step marks the nucleation of heterochromatin in these regions. This state is further reinforced by additional transcription and small RNA production specifically from the regions flanking these newly marked heterochromatic regions. TWI1 loads these new small RNAs, termed late-scnRNAs, and reinitiates the process so that the heterochromatic state spreads over the intended IES[172]. Exactly how the boundaries of this region are maintained is yet to be described. A domesticated piggyBac transposase, TPB2, recognizes the heterochromatic regions in the new macronucleus and removes them from the new genome[173,174]. Similar RNAi and heterochromatin driven mechanisms of DNA elimination take place in other ciliates. In *Paramecium tetraurelia*, the DNA elimination process is entirely dependent on a very similar scnRNA-based mechanism of targeting chromatin modifications to indicate sequences to be removed as in *Tetrahymena*

thermophila[175,176], whereas in *Oxytricha trifallax*[177,178] and *Stylonichia lemnae*[177,178] piRNAs are used to label sequences for retention in the new macronucleus rather than for removal.

These DNA elimination mechanisms closely resemble the small-RNA directed heterochromatin formation strategies that take place at the pericentromeric regions of *S. pombe* and other organisms. Instead of marking the genetic material for proper segregation as in fission yeast, this heterochromatin formation mechanism has been adapted in ciliates to alter the content of their chromosomes via DNA elimination. Interestingly, many of the IESs that are removed correspond to transposon sequences and so it seems this mechanism is also an adaptation of the proposed ancestral function of RNAi and a strategy for maintaining genomic integrity[179]. Despite the obvious difference in outcome, the ultimate function of RNAi in both chromosome segregation and DNA elimination is to maintain chromosome stability and ensure the faithful segregation and propagation of the necessary genetic material.

Maintaining copy number

In *S. pombe*, the Dicer homologue Dcr1 interacts with RNA polymerases and associated factors to facilitate transcription termination and polymerase release[74,180]. These interactions have important roles in maintaining the genetic and epigenetic stability of the genome. Loss of Dcr1 results in the failure of Pol II to release from the 3’ end of transcripts and this leads to DNA damage at the ribosomal DNA (rDNA) locus, pericentromeric regions, tRNA-encoding DNA loci (tDNA) and highly-expressed genes[180,181]. These phenotypes are due to the collisions of the replisome and the transcription machinery during S-phase at these highly-transcribed loci. In the absence of Dcr1 the stalled replication forks are restarted through the activity of homologous recombination (HR), which leads to loss of heterochromatin[180]. Outside of heterochromatic regions, small RNAs are produced in wild-type strains from the regions where Pol II stalls in *dcr1* mutants, but there is little change to the H3K9me2 level at these loci, indicating that the small RNAs are not being loaded into Ago1 and directing heterochromatin formation. In the absence of Dcr1, R-loops accumulate at these sites caused by polymerase collision[181]. R-loops are three-stranded structures formed when a nascent transcript invades the DNA duplex[182]. One consequence of the failure to properly resolve the collisions of the replication and transcription machinery is an accumulation of DNA damage at these sites and the loss of copy number at tandem arrays such as the rDNA[181] (Fig. 4d). The dramatic copy number loss of the rDNA in Dcr1 deletion mutants occurs as a result of the recruitment of HR machinery to the site, demonstrated by ChIP with Rad52. However, the catalytic activity of neither Dcr1 nor Ago1 was required to maintain copy number[181], suggesting interaction with other structures, such as R-loops. Thus, in *S. pombe* Dcr1 is required to maintain the copy number of highly transcribed tandem repeat regions in the genome, such as the rDNA.

S. pombe can be induced to enter a non-proliferative, quiescent G0 state through nitrogen starvation, and RNAi is required to maintain the G0 state over time. Deletion of Dcr1 has the most dramatic effect on G0 maintenance[74], and a number of genetic suppressors of this phenotype have been identified. The suppressors of the G0 maintenance phenotype of

Dicer deletion point to the suppression of toxic H3K9me heterochromatin build-up at the rDNA that leads to cell death. Ultimately, Dcr1 is responsible for the effective release of RNA polymerase I (Pol I) at the rDNA[74]. The failure to release Pol I leads to stalling, the build-up of γH2A.X, the toxic H3K9 methylation, and an eventual loss of viability due to decreased dosage of the transcribed rRNA. Thus, in quiescence, the RNAi and heterochromatin pathways genetically interact, albeit in a different mechanistic manner than in proliferating cells.

Other organisms also rely on RNAi for genome integrity and copy number maintenance at sites such as rDNA loci. For example, the ciliate *Oxytricha trifallax* has a reduction in rDNA copy number in *dcl-1* mutants[183]. This loss of rDNA copies is part of a broader mechanism of small RNA-regulated DNA dosage and genome maintenance that occurs during asexual growth of *O. trifallax*. *Neurospora crassa* utilizes RNAi to respond to DNA damage at the rDNA via siRNAs, sometimes also known as quelling-associated qiRNAs[184]. Mutants in this pathway exhibit a decrease in copy number at the rDNA without altering H3K9me levels[185], closely resembling RNAi mutants in *S. pombe*[181], although levels at embedded reporter genes have been reported to go down[186]. The role of RNAi in the genomic maintenance of rDNA copy number has been proposed to be due to the interaction of siRNAs with the HR repair machinery[187]. In *Drosophila melanogaster*, mutants in RNAi lead to an increase in the numbers of nucleoli (dense nuclear structures composed of rDNA repeats); Dcr-2 mutants have reduced H3K9me2 levels at the rDNA and increased extrachromosomal circular DNA (eccDNA) derived from the rDNA[188]. eccDNA formation is suppressed by mutation of the non-homologous end joining (NHEJ) factor Lig4 and should also result in the loss of copies of rDNA from the array, although this has not been determined experimentally in *Drosophila melanogaster*. The discrepancy between H3K9me changes at the rDNA in *Drosophila melanogaster*, *Neurospora crassa* and *S. pombe* likely indicates that additional mechanisms maintain H3K9me2 at the rDNA in fungi in the absence of RNAi, whereas DNA repair might be more directly impacted in all three organisms. RNAi maintains genomic integrity and thus the dosage of tandemly repeated regions largely by preventing or responding to DNA damage.

RNAi and DNA damage – maintaining genome integrity

In addition to protecting the genome from invaders, RNAi protects the genome from other insults. There is a clear link between RNAi and DNA damage. For example, knockout or knockdown of Dicer promotes the increase in γH2A.X DNA damage signalling in *S. pombe*[74,180,181], *Drosophila melanogaster*[189], zebrafish[190], human HEK293T cells or fibroblasts[190–192], mouse cerebellum, or NIH2/4 cells[190,193] and mESCs[193]. This section will focus on the functions of RNAi in the general DNA damage response.

Small RNA and the DNA damage response pathway

Eukaryotic genomes have evolved a number of redundant pathways for repairing DNA after damage. For instance, double strand breaks (DSBs) can be repaired by either NHEJ or HR. NHEJ takes the two free DNA ends and then resects and/or adds bases by polymerase activity before ligating the ends back together. This process does not require a long

homologous template and therefore can be more error-prone than HR. However, it is the primary mechanism of repair in mammalian cells because it can occur at any phase of the cell cycle. The HR process requires the homologous sequence from the sister chromatid to faithfully reproduce the original sequence that was damaged and, thus, can only occur in G2 or S phase[194]. HR can take a number of different paths depending on the type of damage and on the machinery that identifies and repairs the break. Briefly, the molecular events following a DSB begin with the phosphorylation of H2A.X (γH2A.X) by ATM and the recruitment of MDC1[194]. Depending on the method of repair, more proteins will accumulate such as BRCA1, 53BP1 and RIF1 in HR. BRCA1 resects the ssDNA ends, and then RAD51 binds these ends to mediate the exchange of strands[194]. From here there are a number of options for finishing the repair. All pathways involve recognition and copying mechanisms that may or may not result in a crossover of genetic information from one sister chromatid to the other. Importantly, the first step of NHEJ and HR is recognition of the site of damage. It may be at this stage that RNAi assists.

Small RNA and DNA damage in lower eukaryotes—In *S. pombe*, the knockouts *dcr1*Δ and *ago1*Δ are sensitive to the genotoxic agent hydroxyurea (HU)[195] and small RNAs from centromeric repeats are strongly induced on HU treatment, accumulating in the S phase[196]. The *dcr1*Δ mutant accumulates high levels of DNA damage and double mutants of either *dcr1*Δ or *ago1*Δ with *rad51*Δ are synthetic lethal, whereas double mutants with the fork protection complex are lethal under genotoxic stress[180]. Mutants inRad51/54 DNA damage machinery also lose silencing at the centromere[153], whereas rDNA accumulates very high levels of Rad52 in RNAi mutants[181]. In *Neurospora crassa*, exogenous DNA damage induces the expression of the Argonaute homologue QDE-2 and a class of small RNAs termed QDE-2-associated (qiRNAs)[184] (Fig. 5a). The qiRNAs are 21–22 nucleotides long and are distinct in size from other siRNAs in *Neurospora*, which are on average 25 nucleotides and silence multi-copy transgenes by a process called quelling[197]. The production of these small RNAs is dependent on the Dicer homologues DCL-1 and DCL-2, the RdRP homologue QDE-1, and a RecQ DNA helicase homologue QDE-3[184]. These small RNAs are loaded into the Ago QDE-2[185]. The vast majority of these small RNAs are derived from the rDNA locus and are also dependent on the homologues of the HR machinery, such as the classic DNA damage resolution proteins Rad51, Rad52, and Rad54, and other chromatin remodellers that participate in HR[198]. The fact that HR factors are required for the production of qiRNAs at repetitive loci may be because these sequences are the only ones in the genome that can offer many donor templates for the HR machinery. Interestingly, loss of the fork protection complex, which is responsible for the prevention of fork collapse in regions where transcription and replication machinery collide, results in a significant increase in qiRNA production[198], consistent with the genetic interactions of *rad51*Δ and the fork protection complex with RNAi in *S. pombe*[180]. The rDNA is potentially the most important region of the genome for fork protection as it is both highly transcribed and composed of repeated tandem arrays. Although HR seems to be required for qiRNA production, qiRNA production may also be required for efficient HR repair, as mutations in the qiRNA–quelling pathway lead to a decrease in rDNA copy number[185], as they do in *S. pombe*[181].

Small RNA and DNA damage in higher eukaryote models—DNA damage-induced small RNAs have also been found in *Arabidopsis thaliana* and termed DSB-induced small RNA or (diRNAs) (Fig. 5b). These diRNAs are dependent on Dicer homologues, RNA polymerase IV, and the PI3 kinase ATR[199]. In general, small RNA of this size in *Arabidopsis* are generated beginning with Pol IV transcription. The RdRP homologue RDR2 synthesizes dsRNA from the Pol IV transcript and Dicer-like proteins cleave the dsRNA. The diRNAs are then loaded into AGO2. The regulation of diRNAs by ATR indicates they may also be responsive to replication fork failure, as ATR is a primary response mechanism for stalled replication forks[199]. diRNAs play a notable role in DSB repair, as mutants in diRNA-generating proteins such as Dicer perform significantly worse in a DSB reporter assay[199]. However, the diRNAs do not seem to be required for the incorporation of γH2A.X, which is an early signal for DNA damage. These diRNAs probably function downstream or alongside of the original signalling of the occurrence of a DSB. The diRNAs that are produced are from both strands and are complementary to the region immediately surrounding the break. In *Arabidopsis*, these diRNAs do not seem to require HR as in *Neurospora*[200]. They certainly play a role in DNA repair, but this role is not specific to one pathway. It should be noted that a follow up study did not find that diRNAs were required for DNA repair as the original report[200].

In cultured *Drosophila melanogaster* cells, damage-induced small RNAs are produced through the canonical endo-siRNA pathway (Fig. 5c). They require Dcr-2 for generation and Ago2 as the effector protein. These small RNAs derive from the region around the break and act in *trans* through the normal RNAi mechanisms[201]. The production of these small RNAs may rely on a unique property of *Drosophila melanogaster* RNA polymerase. At the site of the DSB, the polymerase initiates and transcribes the regions around the break[201]. Presumably, the double-stranded product of the break-induced RNA and the normal transcript can produce dsRNA for Dcr-2 to act on. However, at this time it is not known if this dsRNA is the relevant substrate of Dcr-2. This mechanism for producing these damage-induced endo-siRNA would require that the break take place in a region that is transcribed to begin with. The generation of these small RNAs does seem to be affected by splicing machinery as well. Substantially more endo-siRNAs were induced in regions in which a break was downstream of an intron compared to non-intronic regions[201]. The stalling of the splicing machinery seems to be able to serve as mechanism for promoting damage-induced endo-siRNA by providing an unspliced template RNA that can lead to dsRNA by convergent transcription[202]. However, these endo-siRNAs do not seem to be required for the repair of DSBs by HR, according to cell culture-based artificial assays[203].

Small RNA and DNA damage in mammals—The role of RNAi in the DNA damage response seems to be conserved in mammals. Multiple exogenous insults, such as oncogenic stress, ionizing radiation and site-specific endonucleases, activate the DNA damage response in human and mouse cells dependent on enzymatic activity from DICER1 and DROSHA[190] (Fig. 5d). DNA damage induces the phosphorylation of DICER1 and subsequent shuttling of the protein to the nucleus in mouse and human cells[192,204]. As in *Arabidopsis thaliana*, the involvement of RNAi factors in the DNA damage response in mouse[205] and human[206,207] cell culture is independent of γH2A.X incorporation at the site of damage. Instead, DICER1

and DROSHA promote the accumulation of other DNA damage response proteins, namely 53BP1[206,208] and MCD1[206,208]. In the NIH2/4 mouse cell line, the DSB response factor 53BP1 is recruited to breaks in a RNA-dependent fashion[205]. When a DSB forms, the MRN complex (MRE11-RAD50-NBS1) recognizes the break and may promote the association of Pol II on the free ends[205]. Additionally, the CTD of RNA Pol II is Tyr1-phosphorylated by ABL1, and strand-specific transcription occurs from the site. The transcripts seem to form RNA:DNA hybrids at the site of the break and lead to the appearance of DROSHA and DICER1 RNA substrates[208]. These precursor transcripts lead to a species of 22–23 nucleotide small RNAs that are dependent on DICER1 and produced from the local region of DSBs[190,199]. The role of DICER1 and DROSHA is not a primary response, as the loss of DICER1 or DROSHA leads to a decline in the specific DNA damage response mediators MDC1 and 53BP1, which are generally considered to be the secondary level of response to a DSB[206,209]. These interactions take place on a timescale of hours after the formation of a DSB and are, therefore, not likely to be related to the production of microRNAs by these enzymes because microRNA repression will in general still be active.

HR-based repair is inhibited by the knockdown of *DICER1* and *AGO2* in human cells as measured by the repair of a GFP marker[199]. The role of AGO2 at the break occurs through interactions with Rad51. Without AGO2, the accumulation of Rad51 at DSBs is greatly reduced[207]. Therefore, the guiding of Ago2 by diRNAs assists in the localization of Rad51 and promotes proper resolution of the DSB. A number of histone modifications also have a role in identifying the site of DSB. In human cells *DICER1* or *DROSHA* knockdown reduces the levels of H4K20me2, H4K20me3 and H4K16ac at the site of the DSB[210]1. The reduction in these modifications is caused by a decrease in the localization of the H4K20 methyltransferase MMSET and the H4K16 acetyltransferase Tip60 to the DSB. The localization of these chromatin modifiers is dependent on their interaction with AGO2[210]. Furthermore, the binding of AGO2 to diRNAs is responsible for this localization. Ultimately, the modification of chromatin assists in directing RAD51 and BRCA1 to the DSB for resolution of the break. In human U2OS cells, the loss of DROSHA also reduces the accumulation RAD51 and BRCA1 and the extent of resection at DSBs[209]. Small RNAs from the site of DSBs were not observed after the induction of breaks in endogenous loci. This may indicate that diRNAs are specific for repetitive or highly transcribed loci such as artificial reporters. In these human cells, DROSHA promotes DNA repair, accompanied by RNA invasion by DSBs, although an indirect role via microRNA has not been ruled out[209]. Interestingly, it has also been demonstrated that antisense transcription generated by the formation of R-loops induces dsRNA formation and the subsequent recruitment of DICER1, AGO1, AGO2, the H3K9 methyltransferase G9a, and HP1γ to form heterochromatin over Pol II pausing and termination sites[211]. Thus, the relationship between RNAi and R-loops in mammals may be more complicated. An R-loop-based HR mechanism is also present in *S. pombe*[212] where Dcr1 has a role in RNA:DNA hybrid formation and/or stability as well as in copy number maintenance[181].

Dicer and nucleotide excision repair: localization of chromatin machinery leads to resolution

The previous sections have described the role of RNAi components and small RNAs in the repair of DSBs. However, Dicer also responds to other forms of DNA damage such as the formation of 6–4 photoproducts and cyclobutene pyrimidine dimers (CPDs) caused by exposure to ultraviolet light as will be discussed below. The primary method for repairing this type of damage is called nucleotide excision repair (NER)[213]. It is a mechanism of scanning the genome for these bulky helix distortions, removing and repairing them. One method of NER that can operate in any region of the genome is termed global genome NER (GG-NER). The other method is called transcription-coupled NER (TC-NER) and takes place specifically when a polymerase runs into one of these adducts.

In *C. elegans*, Dcr-1 mutant worms phenocopy strains deficient in NER when exposed to UV treatment[214]. In human HEK293T cells, DICER1 is recruited to chromatin after UV treatment. This interaction is dependent on a known NER-participating protein ZRF1 as well as RNA. Together with PARP1, DICER1 and these associating factors decondense the chromatin in the area surrounding the damage[214] (Fig. 5e). DICER1 brings the methyltransferase MMSET to sites of UV-induced damage, as well as DSBs, and the recruitment of this chromatin modifier is required for efficient NER. MMSET deposits H4K20me2, which in this case provides a signalling mark for the binding of 53BP1 and XPA, which is a factor of the NER repair machinery[215]. This function seems to be independent of DICER1's catalytic activity. The mechanism bears some resemblance to the role of DICER1 in the DSB repair pathway as well with MMSET and 53BP1 as the common recruited factors. It will be interesting to see exactly how DICER1 is recruited to these sites of damage and whether the mechanism is the same in NER as it is in DSB repair. Another RNAi factor, DGCR8, participates in the TC-NER pathway in human cells. This occurs after S153 phosphorylation of DGCR8 and the mechanism is independent of DGCR8 binding to DROSHA or RNA[216]. Although it is clear DGCR8 has an effect on TC-NER, through which factors it operates remains unknown. In *Arabidopsis*, upon UV damage RNAi participates in GG-NER by producing 21 nt siRNAs at intergenic sites of damage. The production of UV-induced siRNAs (uviRNAs) is dependent on RNA POL IV, RDR2 and DCL-4; upon loading into AGO1 in complex with DDB2, they direct repair machinery to DNA lesions like CPDs[217].

DICER1 and DROSHA are clearly involved in the DNA damage response. Some of these roles involve small RNAs bound to an Argonaute effector protein, and are involved in the very initial stages of the DNA damage response, namely the recognition of damage and recruiting other proteins such as the repair proteins themselves or chromatin modifiers, such as MMSET, to further the signal of damage via epigenetic modifications in the region. In the mechanisms that seem to be independent of small RNAs, it is not entirely clear what localizes the RNAi factors to damage. Candidates include RNA or DNA:RNA hybrid structures that form in response to damage, but the catalytic activity or RNA-binding properties of these proteins are not always required for their function in the DNA damage response. The highly conserved nature of these interactions suggests an early role in the DNA damage response independent of the function in silencing.

Conclusions

Although roles for RNAi in chromosomal behaviour and genome integrity were originally described in *S. pombe*, it is clear that these roles are conserved across eukaryotes (Table 1). It may be that the LECA employed RNAi in some capacity for these functions. Or, more likely, RNAi has been independently recruited to perform these functions through convergent evolution. The role RNAi plays in chromosome segregation can most often be attributed to the formation of heterochromatin at the pericentromere. The alteration of chromatin at this locus can disrupt the dynamics of sister chromatid attachment and release in mitosis. This disruption leads to lagging chromosomes and missegregation phenotypes commonly described in RNAi mutants across eukaryotic species. Many of these mechanisms mirror the classic small-RNA directed heterochromatin formation strategy of *S. pombe*. The piRNA-mediated heterochromatin formation in the germlines of *D. melanogaster* and mammals also bears a striking resemblance to *S. pombe* CTGS[218]. While mutants in the piRNA pathway do have dramatic genome instability phenotypes the role of chromosome segregation in these phenotypes is far from clear. However, the evidence for DICER1-generated pericentromere-derived small RNAs directing the formation of heterochromatin in somatic mammalian cells is scarce. Rather, the regulation of long pericentromeric transcripts or the regulation of the act of transcription itself may be sufficient to generate the proper chromatin environment required to accurately segregate chromosomes. Future investigations will be able to more clearly delineate between these mechanisms.

Small RNA-directed heterochromatin formation mechanisms have been adapted to regulate dosage at both the RNA transcript and genome levels in a wide variety of eukaryotes. Many eukaryotes use similar mechanisms for regulating dosage at the transcriptional level by targeting the transcripts themselves. Interestingly, the genomic loci regulated by these dosage response mechanisms often overlap with transposon loci, which suggests that the ancestral function of RNAi is conserved but adapted for specialized chromosomal and genomic functions. In ciliate DNA elimination, the generation of small RNAs from specific genomic loci and subsequent targeting of nascent transcripts can direct the chromatin machinery to epigenetically mark specific loci for removal or retention. Furthermore, the maintenance of genomic dosage of tandem repeat regions, such as rDNA, has revealed important functions of Dicer in preventing DNA damage. In addition to maintaining genome integrity at tandem repeats, RNAi also has important roles in recognition and repair of DNA damage. In both small RNA-targeted and small RNA-independent pathways, RNAi recognizes dsDNA breaks and UV-induced CPDs and recruits chromatin modifiers as well as repair factors to promote the resolution of DNA damage.

The more we examine the conserved functions of RNAi beyond gene regulation by microRNAs, the more we will come to recognize its pivotal roles in chromosome and genome integrity. A better understanding of these roles will undoubtedly further our understanding of the consequences that arise from mutating RNAi components in human diseases such as cancer.

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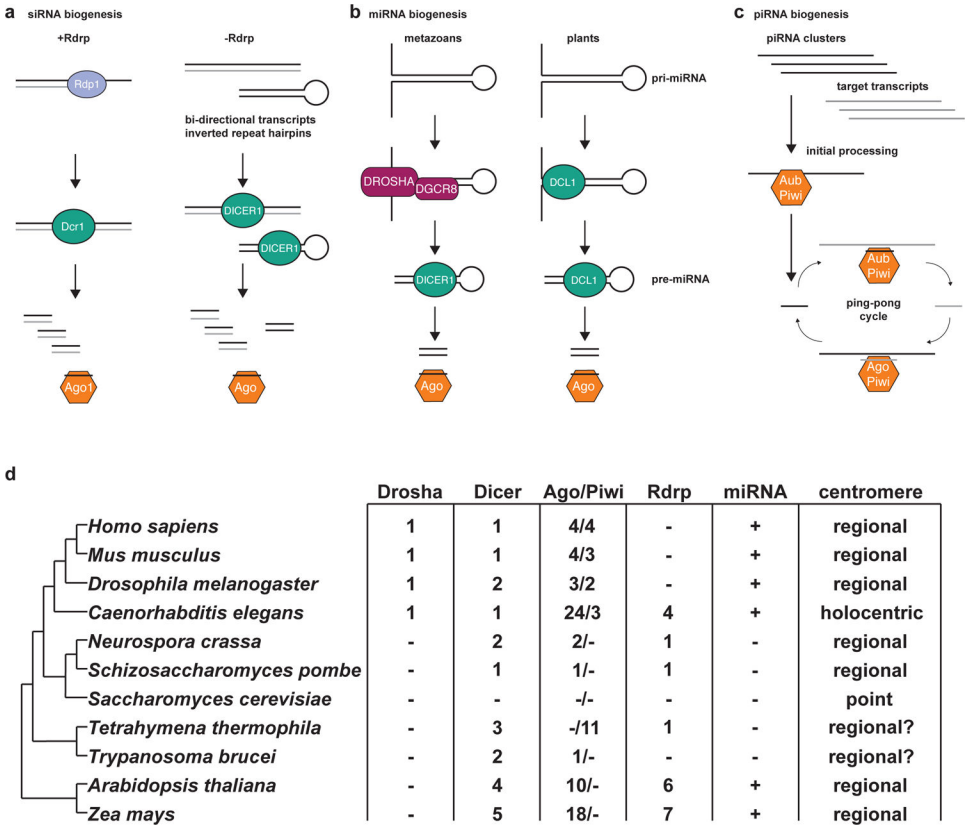

d

	Drosha	Dicer	Ago/Piwi	Rdrp	miRNA	centromere
Homo sapiens	1	1	4/4	-	+	regional
Mus musculus	1	1	4/3	-	+	regional
Drosophila melanogaster	1	2	3/2	-	+	regional
Caenorhabditis elegans	1	1	24/3	4	+	holocentric
Neurospora crassa	-	2	2/-	1	-	regional
Schizosaccharomyces pombe	-	1	1/-	1	-	regional
Saccharomyces cerevisiae	-	-	-/-	-	-	point
Tetrahymena thermophila	-	3	-/11	1	-	regional?
Trypanosoma brucei	-	2	1/-	-	-	regional?
Arabidopsis thaliana	-	4	10/-	6	+	regional
Zea mays	-	5	18/-	7	+	regional

Figure 1 |. Small RNA biogenesis pathways and the evolutionary conservation of their components.

a | Small interfering RNAs (siRNAs) can be produced from either double stranded RNA (dsRNA) generated by RNA-dependent RNA polymerase (Rdrp) or other dsRNA substrates. In *S. pombe* and other eukaryotes possessing a Rdrp (Rdp1), a transcript is targeted for the synthesis of dsRNA, which is subsequently recognized by Dicer (Dcr1) and cleaved into small RNA duplexes. Additionally, dsRNA can occur without the activity of Rdrp in instances such as bi-directional transcription or inverted repeat hairpin formation. The non-Rdrp precursors are also recognized by Dicer and cleaved into small RNA duplexes. In each case the siRNA duplexes are loaded into Argonaute (Ago1) effectors, the passenger strand is discarded, and the guide strand is then used to recognize RNA targets. **b** | microRNAs (miRNAs) are produced from hairpin structures formed in the pri-miRNA transcript. In metazoans, such as humans, the hairpin structure is processed initially by the DROSHA/DGCR8 complex into the pre-miRNA, which is further processed by DICER1 into the miRNA duplex. In plants, the processing of pri-miRNA to pre-miRNA to miRNA duplex occurs through two successive cleavages by Dicer-like 1 (Dcl1). In both metazoans and plants, miRNA duplex is bound by an Argonaute effector protein (AGO), the passenger strand is discarded, and the guide strand can then direct the effector to complementary targets. **c** | PIWI-interacting RNAs (piRNAs) are produced in *D. melanogaster* and *M. musculus* from single-stranded RNA (ssRNA) transcripts from piRNA precursor loci and their complementary target loci. The piRNA precursor transcripts are initially processed by PIWI proteins and then enter the ping-pong amplification cycle in which the recognition of the ssRNA target transcript by the PIWI-piRNA effector complex generates secondary

piRNAs complementary to the piRNA transcripts. These secondary piRNAs are bound by an Argonaute or PIWI effector protein and can again target the piRNA ssRNA precursor transcripts to produce additional piRNAs derived from the piRNA locus. **d** | A phylogenetic diagram of humans and selected model organisms displays the conservation of RNAi proteins, microRNAs, and centromere structure.

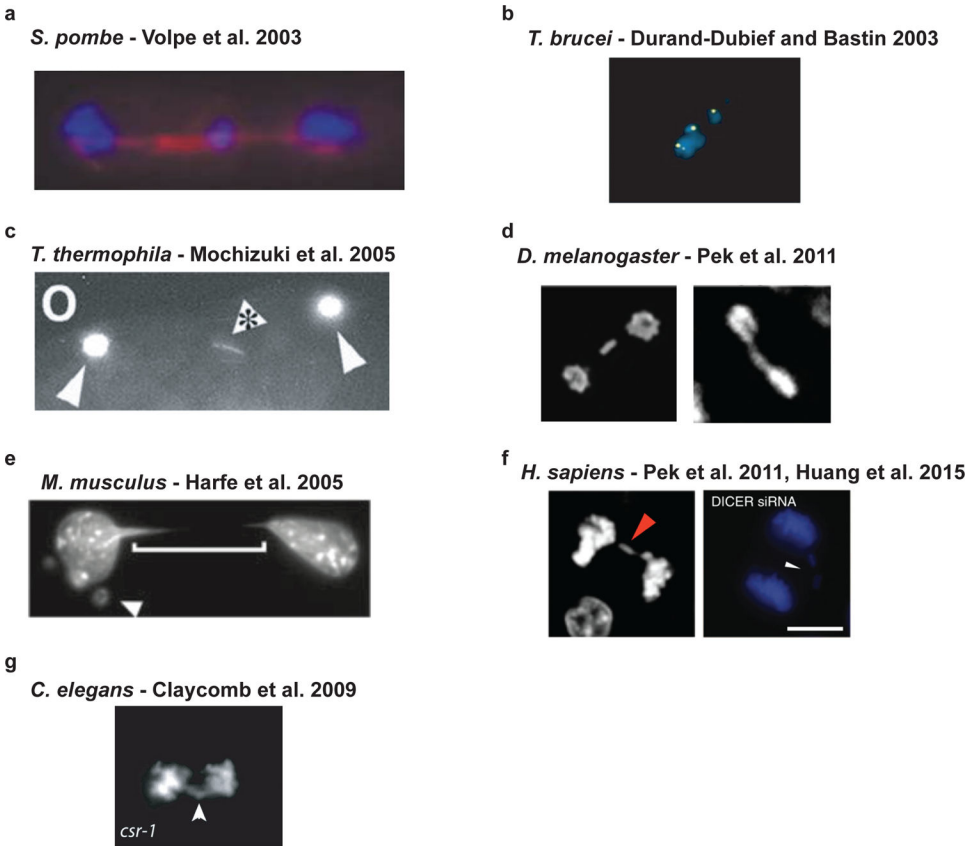

Figure 2 |. Chromosome segregation phenotypes of RNAi are highly conserved.

a| Deletion mutants of either the RdRP, Dicer (shown), or Argonaute homologues in *S. pombe*, result in lagging chromosomes in mitosis and meiosis. **b**| The deletion of an Argonaute homologue in *T. brucei* results in lagging chromosomes and segregation defects in mitosis. **c**| The deletion of a Dicer homologue in *T. thermophila* results in lagging chromosomes and bridging of genetic material in mitosis and meiosis. **d**| While the displayed images are of disruptions of *belle*, the knockout or knockdown of Dicer and Argonaute homologues in *D. melanogaster* leads to lagging chromosomes in the same cell types - wing disc cells and S2 cell culture. **e**| The deletion of *Dicer1* in mouse fibroblasts results in bridging during mitosis and the formation of micronuclei most likely derived from missegregated chromosomes. **f**| In human RPE-1 cells, the knockdown of *Dicer1* or *Ago2* by siRNAs generated lagging chromosomes. In human HeLa cells, the knockdown of *Dicer1* also generated lagging chromosomes. **g**| The knockdown of an Ago homologue (shown) in *C. elegans* or other factors in the CSR-1 22G-RNA pathway results in lagging chromosomes in the early worm embryo.

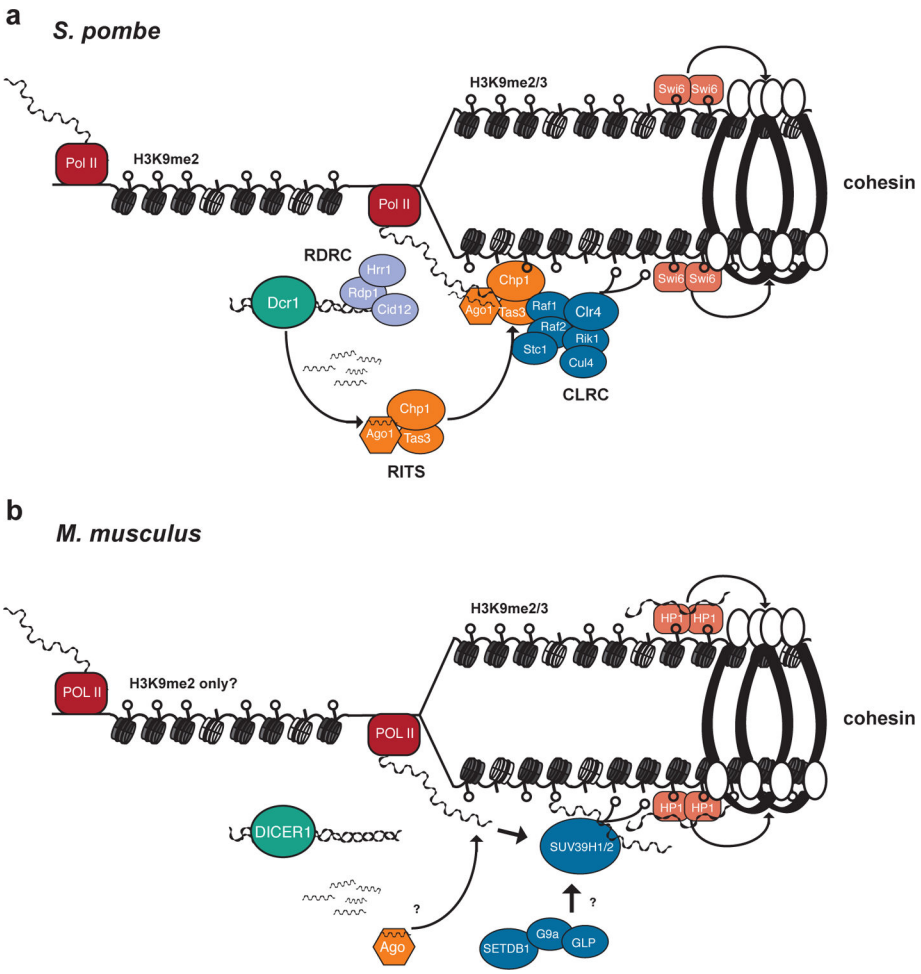

Figure 3 |. Faithful chromosome segregation is ensured by the recruitment and protection of centromeric cohesin.

a| In *S. pombe* the RNAi pathway directs the heterochromatin machinery via small RNAs and the establishment of pericentromeric heterochromatin results in the recruitment and retention of centromeric cohesin during mitosis and meiosis and the proper segregation of chromosomes. The formation of pericentromeric heterochromatin begins with Pol II transcription of long non-coding RNAs (lncRNAs) from the *dg/dh* pericentromeric repeats. The RDRC complex (Rdp1, Hrr1, and Cid12) targets the lncRNAs for the synthesis of dsRNA. These dsRNAs are recognized and cleaved by Dcr1 into siRNAs. These small RNAs are loaded into Ago1 which then directs the RNA-induced transcriptional silencing complex (RITS – Ago1, Chp1, and Tas3) to the pericentromere through the targeting of nascent lncRNAs. Chp1 then recognizes H3K9me and stabilizes the complex at the site. The RITS complex recruits the Clr4-Rik1-Cul4 complex (CLRC – Clr4, Rik1, Cul4, Stc1, Raf2, Raf2), which furthers the spread of H3K9me heterochromatin through the activity of the methyltransferase Clr4. The homologue of heterochromatin protein 1 (HP1) Swi6 binds to H3K9 methylation and reinforces the heterochromatin state. Additionally, it directly recruits the cohesin complex that holds sister chromatids together until anaphase during mitosis. Thus, the combined activity of RNAi and the CLRC complex generate a chromatin state at the pericentromere that results in the proper segregation of chromosomes

during cell division. **b**| In *M. musculus* and other mammals, such as humans, the RNAi pathway ensures the proper segregation of chromosomes presumably through the formation of heterochromatin at the centromeric region. RNA Pol II transcribes the major satellite pericentromeric repeats into lncRNAs in a bi-directional fashion. The resulting dsRNAs are recognized by DICER1 and possibly cleaved into a small RNA population. There is some evidence to suggest that AGO2 may play a role potentially by loading satellite small RNAs, but the effect this has on the chromatin in the region is unknown. Rather the regulation of the major satellite lncRNAs by DICER1 may ensure the appropriate recruitment of recruiting chromatin machinery to the region. The H3K9 methyltransferases SUV39H1 and SUVH39H2 can be recruited directly by major satellite lncRNAs. The H3K9 methylation and major satellite lncRNAs are recognized by HP1, which reinforces the heterochromatic state of the pericentromere. HP1 may play a role in recruiting and/or maintaining cohesin at the centromere to ensure proper separation of sister chromatids, though this function is primarily performed by SGO1 in mammals.

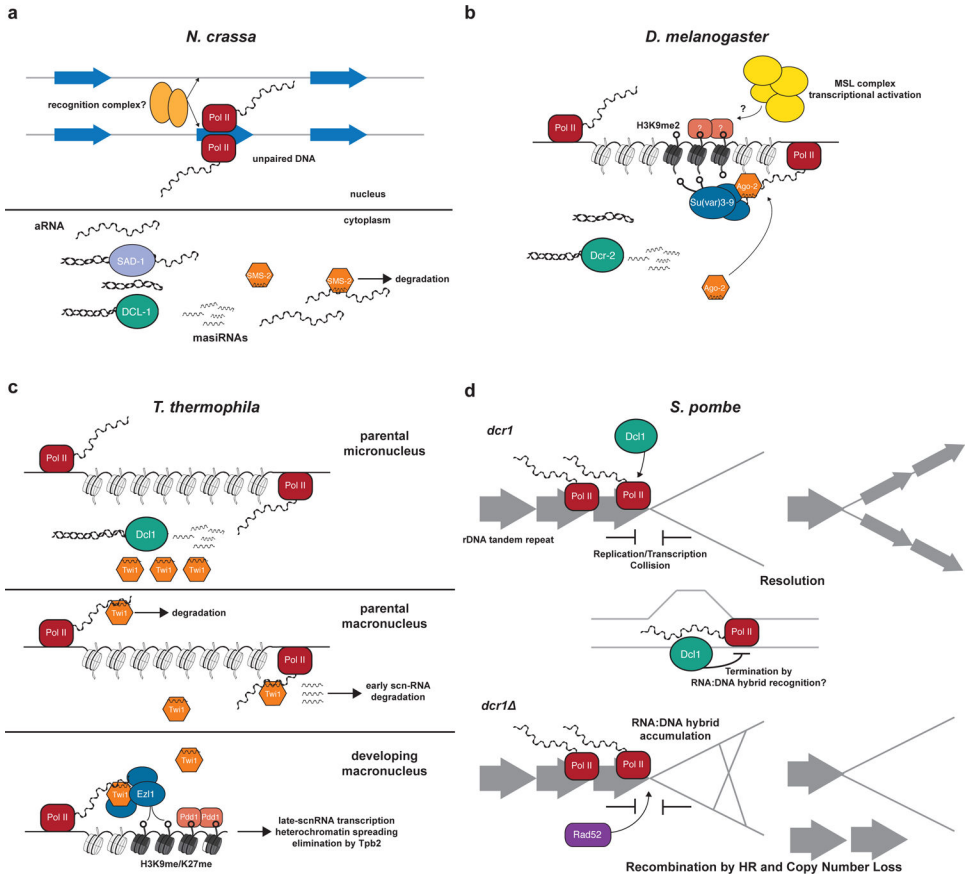

Figure 4 |. RNAi regulates dosage at the transcript, chromatin, and DNA levels.

a| Meiotic silencing of unpaired DNA (MSUD) is an RNAi-based mechanism that identifies and silences transcription products of unpaired loci at the transcript level. During the pairing of homologous chromosomes in meiosis an unidentified recognition complex identifies regions that are unpaired. This stimulates bi-directional transcription by RNA Pol II of aberrant RNAs. The aberrant RNAs are exported to the out of the nucleus and then used as a template by RdRP homologue SAD-1 to generate dsRNA. A Dicer homolog, DCL-1, then targets the dsRNA and generates siRNAs called MSUD-associated small interfering RNAs or (masiRNAs). The Argonaute homologue SMS-2 loads the masiRNAs and targets any transcript from the unpaired locus for degradation. **b**| The X chromosome dosage compensation mechanism in *Drosophila* uses endo-siRNAs targeting the 1.688^{X} satellite repeat to direct chromatin modifications that contribute to increasing transcription from the male X chromosome to match the dosage of the two X chromosomes in female flies. The mechanism most likely begins with RNA Pol II bi-directionally transcribing the 1.688^{X} loci. The resulting dsRNA is recognized by Dcr-2, which generates 1.688^{X} endo-siRNAs. Ago2 loads the endo-siRNAs and directs the H3K9 methyltransferase Su(var)3–9 to the 1.688^{X} loci on the X chromosome. The presence of this mark may recruit the Male Specific Lethal (MSL) complex to the surrounding region, which in turn increases transcription from the X chromosome. **c**| The meiotic process of DNA elimination relies on the direction of heterochromatin formation by RNAi to identify sequences to be removed from the new macronucleus. The bi-directional transcription of the parental micronuclear genome, most

likely by RNA Pol II, generates dsRNA that are processed by the Dicer homologue Dcl1 into early scan-RNAs (scn-RNAs). The PIWI-like effector Twi1 binds the early scnRNAs and translocates to the parental macronucleus where the effector protein and small RNA search for complementary sequences on nascent transcripts. If a Twi1/scn-RNA complex finds a complementary transcript the scn-RNA is degraded. If a target is not found the Twi1/scn-RNA complex translocates to the developing macronucleus and again searches for complementary nascent transcripts. When a target sequence is identified, Twi1 recruits the H3K9 and H3K27 methyltransferase Ezl1 to the region. The recognition of H3K9me/H3K27me by the HP1 homologue Pdd1 strengthens the chromatin state of the region by inducing the transcription of late-scnRNAs which then feedback on the region to spread heterochromatin. This leads to the excision of this DNA sequence by Tpb2. **d**| The copy number of the ribosomal DNA tandem array loci is maintained by Dcr1 in *S. pombe* through the resolution of replication/transcription collisions. In the presence of Dcr1 (top panel) the collision of a replication fork with RNA Pol II can be resolved through the action of Dcr1 in Pol II transcription termination and importantly neither the catalytic activity Dcr1 nor small RNAs are required for this function. Rather it may be an RNA:DNA hybrid that stimulates this function of Dcr1. In the absence of Dcr1 (bottom panel), Rad52 and RNA:DNA hybrids accumulate at sites of unresolved replication stress. This leads to repair by homologous recombination and as a consequence a loss of rDNA copy number.

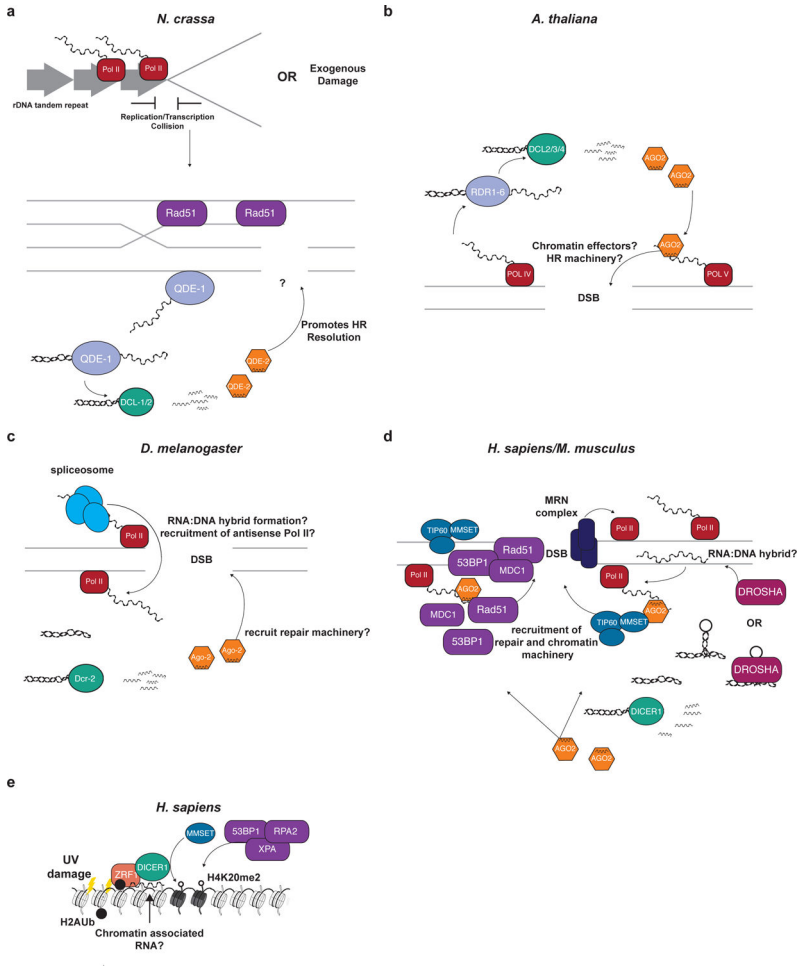

Figure 5 |. The roles of RNAi in the recognition and resolution of DNA damage.

a| In *Neurospora* QDE-2-associated siRNAs (qiRNAs) participate in the DNA damage response generally through the HR pathway. The RdRP homologue QDE-1, which has DNA-dependent RNA polymerase activity as well, transcribes aberrant RNA from sites of damage and then converts it to dsRNA. The dsRNA is recognized by DCL-1/2 and qiRNAs are produced. These small RNAs are loaded into Argonaute homologue QDE-2 and then promote the resolution of damage through HR. **b**| The production of DSB-induced siRNAs (diRNAs) in *Arabidopsis* is dependent on transcription by RNA Pol IV from the site of the damage. These transcripts serve as the template for RdRP homologues RDR2/6 to generate dsRNA. Dicer-like homologues generate the diRNAs, which are then loaded into Ago2. The Ago2/diRNA complex can then recognize nascent transcripts from RNA Pol V and may direct chromatin effectors or repair machinery to the site of damage. **c**| The endo-siRNA pathway of *Drosophila* can generate damage-induced small RNAs (and the production of these small RNAs may be enhanced by the splicing machinery. The production of dsRNA recognized by Dcr-2 is enhanced at sites of active transcription, especially sites where the spliceosome is present indicating that the spliceosome may promote antisense transcription. The diRNAs are loaded into Ago2 and it is proposed that the Ago2/diRNA complex may direct downstream repair machinery to the site of the break. **d**| In mammals, the RNAi

pathway can direct both chromatin modifiers and repair machinery to the site of a DNA double-strand break. Pol II transcribes the regions around the break and it may be recruited there by the early-responding MRN complex. The production of anti-sense transcripts may be promoted by the formation of RNA:DNA hybrids in the region and DROSHA may recognize these hybrids. The production of DNA damage-response RNAS (DDRNAs) is dependent on DROSHA and DICER processing dsRNA from the site of the break. These small RNAs can be loaded into AGO2, which can then target the site of DSB and recruit the chromatin effectors MMSET and TIP60 as well as the DNA damage response factors 53BP1 and RAD51 among others to resolve the damage. **e|** In human cell lines, DICER1 plays a role in the recognition and resolution of DNA damage caused by UV irradiation. In a complex with ZRF1, which can recognize ubiquitinated H2A, DICER1 localizes to the site of damage in an RNA-dependent manner. DICER1 then recruits chromatin effectors, such as MMSET, and repair factors, such as XPA, 53BP1, and RPA2 to the site to promote the resolution of damage.

Table 1 |

The functions and phenotypes of RNAi in eukaryotes

Organism	Chromosomal functions and phenotypes	Genome integrity functions and phenotypes
S. pombe	***dcr1*Δ, *ago1*Δ, and *rdp1*Δ** - Lagging chromosomes and missegregation in dividing cells, quiescence entry, and meiosis **(small RNA)**[71–75] ***dcr1*Δ** - reduction in rDNA copy number[181]	***dcr1*Δ and *ago1*Δ** - sensitive to HU (indicative of replication stress-induced damage), accumulate DNA damage (Rad22 foci), and are synthetic lethal with *rad51A*[180,195,196] ***dcr1*Δ** - damage at repetitive loci and highly expressed genes, loses rDNA copy number[180,181]
N. crassa	**SAD-1 (Rdrp), DCL-1, SMS-2 (Ago)** – required for MSUD post-transcriptional dosage control **(small RNA)**[147–150] **DCL-1/2, QDE-2 (Ago), and QDE-1 (Rdrp)** – loss of this pathway reduces copy number at repetitive loci such as the rDNA **(small RNA)**[185]	**DCL-1/2, QDE-2 (Ago), and QDE-1 (Rdrp)** – generate damage induced siRNAs (qiRNAs) that interact with repair machinery, **(small RNA)**[184]
T. thermophila	**Dcl1** knockout - lagging/bridging during mitosis and meiosis[66] **Dcl1, Twi1P (Ago/Piwi)** drive the DNA elimination pathway **(small rna)**[66,165,172]	
T. brucei	**TbAgo1** knockout – chromosome missegregation, whole genome aneuploidy **(small RNA)**[88]	
H. sapiens	***DICER1* or *AGO2*** knockdown – lagging chromosomes in RPE-1, HeLa cells[93,102]	**DICER1/DROSHA** – knockdown increases DNA damage in fibroblasts (γH2A.X)[190] **DICER1/DROSHA/AGO2** – production of DNA damage-induced small RNAs and direction of chromatin/repair machinery to DSB **(small RNA)**[199,206,207,209,210] **DICER1/DGCR8** – assist in nucleotide excision repair possibly at the chromatin level[214–216]
M. musculus	***Dicer1*** knockout – lagging chromosomes in fibroblasts[97]	**DICER1/DROSHA** – knockdown increases DNA damage (γH2A.X) in NIH2/4 cells, cerebellum, and mESCs[193,190] **DICER1/DROSHA/AGO2** – production of DNA damage-induced small RNAs and direction of repair machinery to DSB in NIH2/4 cells **(small RNA)**[205]
D. melanogaster	**Dcr-2 and Ago2** deletion/knockdown – lagging chromosomes in wing disc, S2 cells, and embryos **(small RNA)**[93,94] **Dcr-2** – mutant has reduced H3K9me2 at rDNA and an increase in rDNA circles[188] **Dcr-2 and Ago2** loss of function mutations – synthetic lethal with roX1/roX2 doubles, indicating role in X chromosome recognition for dosage compensation through 1.688^{X} satellite repeat siRNAs **(small RNA)**[161,162]	**Dcr2** knockout – increase in DNA damage (γH2A.X)[189] **Dcr-2 and Ago2** – generate damage-induced small RNAs **(small RNA)**[201]
C. elegans	**CSR-1 (Ago)** knockdown – chromosome segregation defects in embryo and meiosis **(small RNA)**[65,129] **DCR-1** knockout – misshapen oocytes with high DNA content, X chromosome missegregation in male meiosis[130,131,138] **CSR-1** mutants have reduced H3K9me2 on unpaired X chromosome and increased H3K9me2 on autosomes in early meiosis[135]	**DCR-1** – loss of function mutant shows disruption of nucleotide excision repair upon UV treatment[214]
A. thaliana	**AGO4** knockout – lagging/bridging chromosomes reported during mitosis and male meiosis **(small RNA)**[95] **easiRNAs** (dependent on microRNA to trigger formation) sense whole genome dosage in interploidy hybrids **(small RNA)**[32,144,145]	**DCLs and AGO2** - generation of damage induced siRNAs (diRNAs) **(small RNA)**[199] **RDR2, DCL-4, and AGO1** – production of uviRNAs in GG-NER UV damage response **(small aRNA)**[217]
Z. mays	**AGO104** loss of function mutation – disruption of chromosome condensation and segregation during male meiosis, also shows the formation of micronuclei and multinucleated cells **(small RNA)**[142]	