Chapter 25

Noncoding RNAs in Genome Integrity

I. Kovalchuk
University of Lethbridge, Lethbridge, AB, Canada

Chapter Outline

1. INTRODUCTION

Noncoding RNAs (ncRNA) are the RNA molecules that are not encoding for any protein and have diverse functions in the cell. There is a great variety of ncRNAs in terms of size, mechanisms of biogenesis, and functions. Among these ncRNAs, there are well-known ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) that are involved in the process of translation as well as small nuclear RNAs (smRNAs) and small nucleolar RNAs (snoRNAs) playing an essential role in the maturation of mRNA and rRNA [1]. Many other ncRNAs are involved in the regulation of gene expression at all levels, including chromatin, DNA, and RNA. Several ncRNAs are also implicated in the regulation of genome stability or/and DNA repair itself. Such regulation can be indirect (by targeting and changing the expression level of genes involved in DNA repair) or direct (by interfering or aiding the process of DNA repair in a direct manner). A direct role for ncRNAs in DNA repair is supported by the fact that several of them are being able to interact with DNA-repair proteins such as 53BP1 [2], BRCA1 [3], and Ku70 [4]. Moreover, RNA-binding proteins have been shown to be recruited to the site of DNA damage and influence the repair efficiency [5,6]. In this chapter, we describe the involvement of various ncRNAs in the regulation of genome integrity in various organisms, with more emphasis on eukaryotes.

2. TARGETING BACTERIOPHAGE GENOMES BY CRISPR/CAS9

In bacteria, the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated protein 9) system effectively protects bacteria from various bacteriophages by incorporating portions of the bacteriophage genome into the bacterial genome and then produces a single-guide RNA (sgRNA) from the newly incorporated regions. sgRNAs

Genome Stability. http://dx.doi.org/10.1016/B978-0-12-803309-8.00025-2

guide the cleavage of bacteriophage genomes in a sequence-specific manner. Details of the function of the CRISPR/Cas9 system can be found in Chapter 6. The ability of the CRISPR/Cas9 system to recognize a specific sequence and to generate a double-strand break (DSB) is nowadays used for high-precision genome editing (see Chapter 6 for details).

3. DNA ELIMINATION IN CILIATES

In ciliates, various classes of small and long ncRNAs known as scan RNAs (scnRNAs) are involved in the programmed DNA elimination and DNA rearrangement. One of the ncRNA classes, PIWI (P-element–induced wimpy testis)-interacting RNAs (piRNAs), is involved in somatic genome rearrangements in *Tetrahymena* and *Oxytricha*. In *Tetrahymena*, piRNAs arise from the germline and target germline-specific sequences of the developing somatic macronucleus for elimination. In contrast, piRNAs in *Oxytricha* stem from the parental somatic macronucleus; they direct the retention of somatic genes in the mature somatic macronucleus [7]. More detailed information about RNA-directed DNA elimination can be found in Chapter 7.

4. TELOMERASE RNA AND TELOMERE LENGTH

The number of cell divisions for a given cell is limited, and one of the limitation factors is the shortening of chromosome ends—telomeres. With each cell division, the chromosome ends get shorter due to the inability of the polymerase to replicate the leading strand from the first nucleotide (due to the use of RNA primer). The telomerase enzyme also known as telomere terminal transferase adds the missing DNA using telomerase RNA. More details about the role of telomerase RNA in the maintenance of genome stability can be found in Chapter 21.

5. ROLE OF MICRO-RNAS IN THE REGULATION OF DNA REPAIR AND GENOME STABILITY

5.1 A Brief Overview of Micro-RNA Biogenesis

Micro-RNAs or miRNAs are the most abundant and perhaps the most well-described class of ncRNAs generated in plants and animals. Although the mechanism of biogenesis differs in plants and animals, in general, miRNAs are typically transcribed by RNA polymerase II (Pol II) from the loci with a well-defined gene structure [8]. The initial mRNA transcript containing single or multiple miRNAs is 5′-capped and 3′-polyA-tailed; it is typically folded to form the single or multiple hairpin structures with an imperfect pairing. These initial primary transcripts are called pri-miRNA; they are processed to precursor miRNA (pre-miRNA) by RNAse III enzyme DROSHA in the nucleus and once more by DICER in the cytoplasm generating double-stranded RNA with 2-nt overhangs at the 3′-ends [9]. In humans, the DICER complex is associated with two different double-stranded RNA (dsRNA)-binding proteins, a protein activator of PKR (PACT), and a transactivation-response RNA-binding protein (TRBP). Lee et al. demonstrated that PACT together with DICER can inhibit the processing of pre-miRNA substrates [10]. They also showed that PACT and TRBP are nonredundant in the generation of miRNAs with different sizes, the so-called miRNA isoforms (isomiRs), which may have different targets. TRBP protein in humans is phosphorylated by the MAPK/ERK protein kinase and the phosphorylation of TRBP increases the stability of the DICER complex enhancing miRNA production (Fig. 25.1) [11]. In plants, a double cleavage event occurs in the nucleus with the help of a dicer-like 1 (DCL1) protein. In addition, in plants, the generated dsRNAs are methylated (2′-O-methylation) at the 3′-termini by HEN1 methyltransferase [8]. iRNAs regulate gene expression at the posttranscriptional level either by degrading target mRNAs in plants or by interfering with translation (polyA shortening, preventing ribosome loading, and so on, in animals). The degradation in plants requires a perfect homology between the designated miRNA and its target, whereas translation inhibition in animals relies on an imperfect homology, which allows that a single miRNA targets multiple mRNAs or a single mRNA is targeted by multiple miRNAs.

5.2 Indirect Impact of miRNAs on Genome Stability

Being able to regulate multiple independent mRNA targets, miRNAs undoubtedly can have a significant impact on the rate and efficiency of DNA-damage repair. A potential indirect role of miRNAs in DNA-damage response has been demonstrated by the observation that exposure to 2 Gy of radiation in human cells results in an overall decrease in the expression of miRNAs in the first 30 min of exposure, which is paralleled by an increase in the expression of their mRNA targets [12]. Exposure to UV has been shown to change the expression of several miRNAs in human fibroblasts at 4 h time point [13].

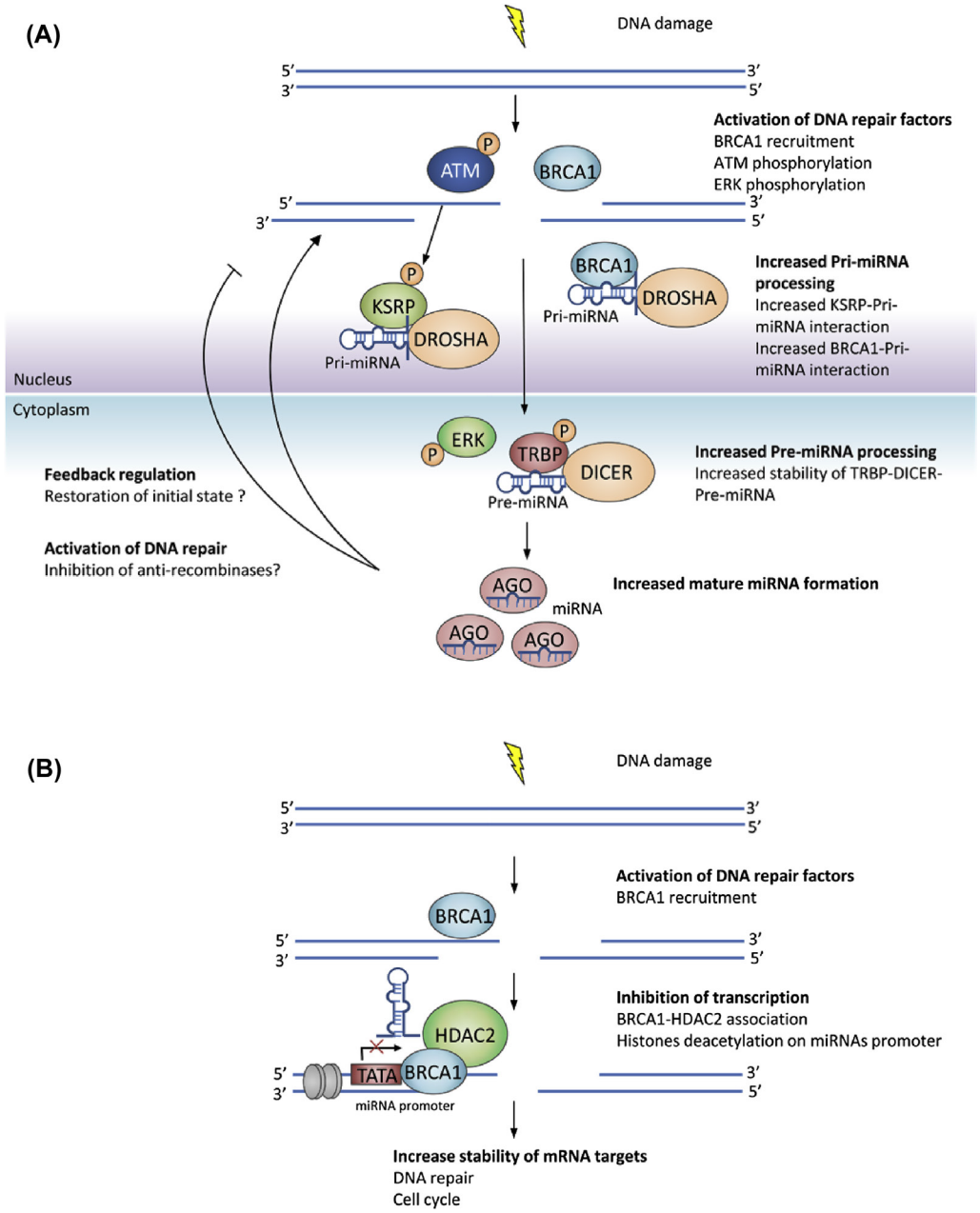

FIGURE 25.1 **Interplay of miRNAs and the DDR.** (A) *DNA damage–induced miRNA biogenesis*. DNA damage activates a signaling cascade which activates the processing of miRNA precursors. DNA damage–induced ATM phosphorylates KSRP and enhances its ability to recruit pri-miRNAs to DROSHA [16]. BRCA1 directly interacts with both pri-miRNAs and the DROSHA complex [18]. Processing by the DROSHA complex allows cytoplasmic export of pre-miRNAs. The MAPK ERK is also phosphorylated after DNA damage [92]. ERK phosphorylates TRBP and phospho-TRBP stabilizes the TRBP-DICER complex to promote pre-miRNAs processing in the cytoplasm [11]. Increased levels of mature miRNAs could play a role in the DNA-damage response by (1) decreasing the levels of anti-repair genes (such as the anti-recombinases, Srs2, PARI, RTEL1 [93]) and (2) down-regulate DDR proteins through a feedback regulation loop to restore pre-DNA-damage levels. (B) *DNA damage–induced repression of miRNA transcription*. BRCA1 associates with HDAC2 which deacetylates histone H2A and H3 on miR-155 promoter, leading to miR-155 transcriptional repression [19]. Transcriptional repression of miRNAs could contribute to the DDR by allowing increased expression of target proteins that are involved in DNA-repair and checkpoint control. (C) *miRNAs impacting DSB repair–pathway choice*. There is interplay of NHEJ and HR pathways during the course of the cell cycle, and this is critical for cell health. NHEJ is known to be active throughout the cell cycle phases and HR activity is maximum in S phase and gradually decreases over G2 phase [93,94]. In G1, NHEJ is promoted by 53BP1 and H2AX which prevent CtIP-mediated resection of the broken end [95]. Resection at a DSB impedes NHEJ and allows HR. In S-phase HR is active and BRCA1 is a key player in the recruitment of HR proteins to DSBs, thereby excluding NHEJ factors like 53BP1 [96]. CtIP promotes ends resection to allow formation of RPA-coated ssDNA at a DSB [97]. BRCA2 is the mediator protein that is essential for replacing RPA with RAD51 and the formation of the RAD51-ssDNA nucleoprotein filament. In normal cells, miRNAs maintain optimal expression of DNA-repair factors allowing efficient repair of DSBs. However, when miRNAs are aberrantly expressed it disrupts the correct choice of DSB-repair pathway. For example, overexpression of miRNAs targeting H2AX (indicated by bold arrow) may allow CtIP-mediated resection in G1 preventing NHEJ. HR-mediated repair in G1 is detrimental to cell health as it would lead to the loss of heterozygosity. Conversely, in S-phase overexpression of miRNAs targeting BRCA1 will impede HR and allow factors such as 53BP1 to direct the DSB to the NHEJ mediated–repair pathway, which in turn leads to higher mutation rates and chromosomal instability. *Reproduced from Chowdhury D, Choi YE, Brault ME. Charity begins at home: non-coding RNA functions in DNA repair. Nat Rev Mol Cell Biol 2013;14(3):181–9, with permission.*

Continued

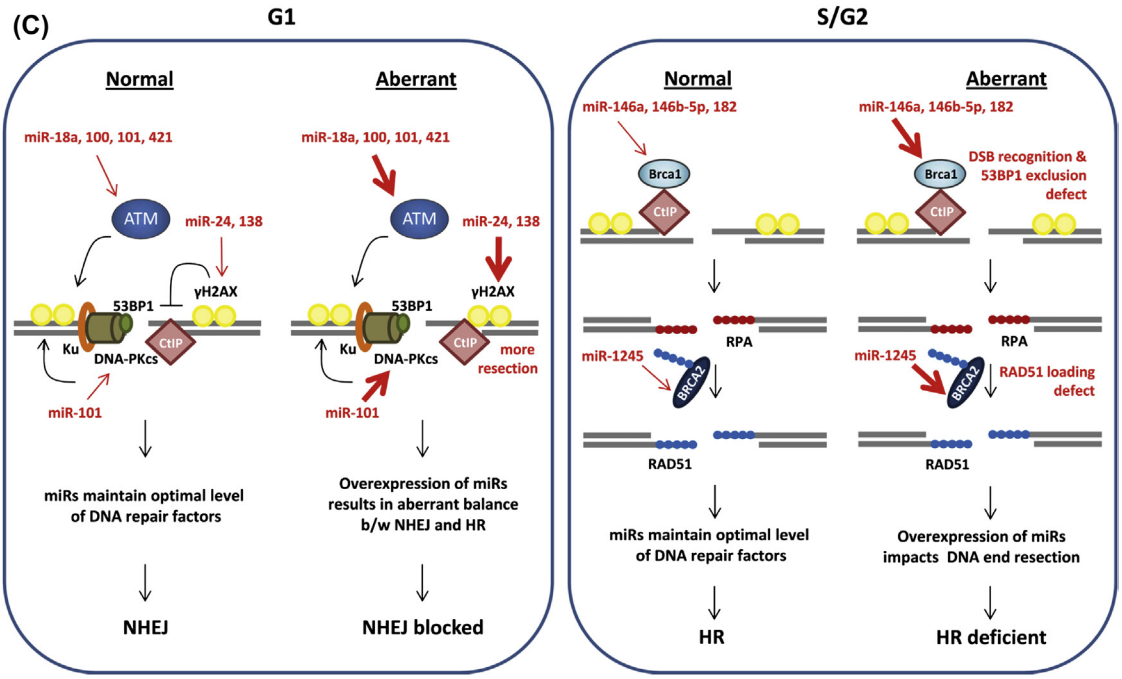

FIGURE 25.1 Cont'd

Similarly, 6 Gy of ionizing radiation (IR) result in an over threefold change in the expression of 22 miRNAs in prostate tumor lines [14]. Among 22 miRNAs, there was miR-521 that targets Cockayne syndrome protein A (CSA) involved in transcription-coupled repair [14].

5.3 DNA-Repair Factors Can Affect miRNA Biogenesis in Response to Stress

DNA-repair proteins can directly regulate biogenesis of miRNAs in response to IR (Fig. 25.1A) [15]. Specifically, in mice, the radiomimetic drug neocarzinostatin induces over 70 miRNAs in an ATM-dependent manner [16]. Biogenesis of miRNAs is dependent on the activity of KH-type splicing-regulatory protein (KSRP), one of the components of DROSHA and DICER complexes, and a direct substrate of ATM phosphorylation (Fig. 25.1A). KSRP phosphorylation significantly increases its activity by allowing a more efficient recruiting of pri-miRNAs to DROSHA for further processing in the nucleus [17]. It remains to be demonstrated whether the same occurs at the level of the pre-miRNA and Dicer complex in the cytoplasm. Therefore, it appears that ATM does not alter the transcription of miRNAs, but rather impacts their biogenesis in the step of conversion of pri-miRNAs.

Another DNA-repair protein that interacts with proteins processing miRNAs is BRCA1. BRCA1 binds DROSHA and several specific pri-miRNAs by regulating their biogenesis in a positive and negative manner (Fig. 25.1A) [18]. In addition, BRCA1 can regulate the expression of specific miRNAs at the level of transcription. For example, BRCA1 represses miR-155 transcription via its association with the histone deacetylase HDAC2 and the deacetylation of promoter region (Fig. 25.1B) [19]. Also, the anti-apoptotic transcription factor NF-Kβ is recruited to miR-21 promoter upon DNA damage, and via its interaction with the signal transducer and activator of transcription 3 (STAT3), it enhances the transcription of miR-21 [20].

5.4 Regulation of the Activity of DNA-Damage Sensors and Effectors by miRNAs

5.4.1 Regulation of Sensors

DNA strand breaks are sensed by several groups of proteins, such as the Mre11–Rad50–Nbs1 (MRN) complex, Ku70/80, and 53BP1. Proteins like ATM and γH2AX (the phosphorylated form of H2AX protein) also play an essential role in the initial damage recognition and signaling because H2AX is one of the first immediate targets of ATM phosphorylation. The repair choice is influenced by this initial binding (see Chapter 14). Therefore, the regulation of the abundance of one or several components of these sensors may significantly influence DNA-repair choice and outcomes.

Two component proteins involved in sensing strand breaks, Nbs1 and Ku80, may likely be regulated by miRNAs as they both contain the long 3′-UTRs with a high number of miRNA binding sites that can serve as a potential target for translation inhibition. Indeed, a 2015 work showed that Ku80 expression could indeed be affected by hsa–miR–526b in nonsmall-cell lung carcinoma (NSCLC) [21]. Hsa–miR–526b was found to be downregulated and Ku80 upregulated in the NSCLC cells compared to healthy tissues. No experimental data exist for Nbs1, but an association study demonstrated that NBS1 as well as Mre11 were likely to be regulated by miRNA; a case–control study revealed the association between the presence of SNPs in binding of several miRNAs at the 3′-UTR of these genes with an increased risk of breast cancer development [22]. Similar data for Nbs1 were observed in case-control studies involving colorectal cancer [23].

The expression of ATM is also regulated by miRNA at the posttranslational level; in neuroblastoma and HeLa cells, miR-421 downregulates ATM activity by modulating cell-cycle checkpoints and changing cell sensitivity to IR [24]. Similarly, miR-100 [25], miR-101 [26], and miR-18a [27] are also likely to regulate ATM because all of them were shown to target the 3′-UTR of ATM and downregulate it. Details of miRNA impact on various steps of DSB repair are shown in Fig. 25.1C [15].

5.4.2 Regulation of Effectors

The expression of effector proteins such as H2AX, BRCA1 and BRCA2, MDC1, RAD51, RAD52, and others, may also be affected by miRNAs. The formation of γH2AX loci, the result of the association of the phosphorylated H2AX histone with DSB lesion, may also be affected by miRNA expression. Several miRNAs that inhibit γH2AX foci formation have been identified [28]. Among them, there is miR-138 that appears to target 3′-UTR of H2AX mRNA by decreasing the number of the formed γH2AX foci and inducing chromosomal instability upon DNA damage [28]. In addition, miR-138 overexpression severely inhibits homologous recombination and increases cell sensitivity to various DNA-damaging agents, including cisplatin and IR.

BRCA1 is one of the essential DSB-repair proteins; its decreased expression is observed in more than 90% of all breast cancers [29]. Several miRNAs, including miR-182, miR-146a, and miR-146b-5p, are known to regulate BRCA1 expression. The overexpression of miR-182 alters homologous recombination and the sensitivity of breast tumors to DNA-damaging agents [30].

Effector proteins Rad51 and Rad52 are also likely to be regulated at the posttranscriptional level; an association study similar to the earlier mentioned one for Nbs1 and Mre11 revealed the correlation between the presence of SNPs in the predicted binding sites for several miRNAs and the chance to get breast cancer [22]. Also, miR-96 expressed from the same polycistronic transcript as miR-182 regulates the expression of RAD51 [31].

The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is recruited to the damaged DNA by the Ku heterodimer. DNA-PKcs and ATM1 are both targeted by the same miRNA—miR-101; the protein amounts were substantially reduced upon miR-101 overexpression sensitizing cancer cells to DNA damage [26].

More details about various miRNAs targeting the essential DNA-repair components can be found elsewhere [15,32].

6. THE ROLE OF PIWI-INTERACTING RNA IN THE MAINTENANCE OF GENOME STABILITY IN THE GERMLINE

The animal genome contains a great number of transposable elements, and many of them are active and expressed in somatic cells. Many such transposons are also expressed in germ cells where their excision or a "copy-and-paste" mechanism can result in genome instability and inheritance of an increased number of such elements. In *Drosophila*, for example, TAHRE, TART, HetA, copia, and the I element are expressed in the germline [33–36], whereas gypsy, ZAM, and idefix are expressed also in somatic cells of the ovary [37–39]. Therefore, controlling the activity of such elements is an important task.

An important discovery was made in 2006 by several independent groups—a specific class of small RNAs was found to be abundant in mouse testes [40–43]. These RNAs were named piRNAs and later on were proposed to control the genome stability in the animal's germline.

piRNAs were first discovered in *Drosophila* as ncRNAs that were involved in silencing transposable elements [44]. Later on, they have been found in multiple metazoan species, including worm, frog, zebrafish, mice, rats, and humans. piRNAs sequences are not conserved among different species and are different even within the same species. Moreover, piRNAs also differ from other small RNAs such as micro-RNAs (miRNAs) and small-interfering RNAs (siRNAs) because they do not have a double-stranded RNA precursor and are not processed by Dicer. In particular, in *Drosophila* and vertebrates, piRNAs are about 26–30-nt long, have a preference for a 5′-uracil, and are 2′-O-methylated at the 3′-(sugar)-end [45]. *Caenorhabditis elegans* piRNAs are rather similar, with the exception of the size—they are 21-nt in length.

6.1 piRNAs in *Drosophila*

PIWI protein was originally identified in *Drosophila* during the analysis of the results of an enhancer trap screen using a P-element. Male *Drosophila* mutants containing the P-element insertion at specific genomic locations in the germline cells had severe defects in spermatogenesis leading to sterility [46]. Further research led to the discovery of three PIWI proteins: PIWI, AUBERGINE (AUB), and ARGONAUTE3 (AGO3). The PIWI protein is localized in the nuclei of somatic follicle and germ cells in the ovary; it functions as a regulator of heterochromatic gene silencing in the transposon regions, and its function requires an interaction with heterochromatin protein 1a (HP1a) and methylation of the DNA at the target locus [47]. AUB protein is expressed in the cytoplasm of germ cells with a partial localization to the nuage, an electron-dense cytoplasmic region located around the nucleus that plays a prominent role in piRNA function. AUB protein is involved in silencing of the repetitive *Stellate* locus via sequence-specific antisense piRNAs [44]. Aub deficiency leads to a loss of anterior–posterior and dorsal–ventral patterning in embryos, which is likely the consequence of double-stranded DNA breaks occurring in the oocyte in the absence of Aub. Finally, AGO3 strictly localizes to the nuage region of the cytoplasm of germ cells [47].

In *Drosophila*, more than 80% of piRNA sequence reads map to the genomic regions harboring transposons [48]. As to the size, two sets of piRNAs are produced in *Drosophila*: 24–28 nt in length associated with AUB and AGO3 proteins and the 29–31 nt–long ones mostly associated with PIWI [49]. piRNAs in *Drosophila* derive from unidirectional or bidirectional clusters of repetitive elements located in the pericentromeric and telomeric regions where piRNAs are encoded by one or two strands, respectively [47]. In the case of bidirectional clusters, convergent transcription may result in stalling of polymerases at the site of conversion, thus contributing to the production of aberrant transcripts [50]. In *Drosophila*, transcripts formed by bidirectional transposon clusters are recognized by the HP1 homolog Rhino (Rhi) [51] and its colocalization partner UAP56 (Fig. 25.2A) [45]. During transcription, Rhi binds the chromatin repressive mark H3K9me3 on bidirectional clusters and recruits the protein deadlock and the transcription termination cofactor cutoff [45]. It is proposed that Cutoff binds the uncapped 5′-end of the piRNA precursor, preventing the degradation or/and splicing of the precursor [52]. In contrast, the transcription of unidirectional clusters occurs through the normal POL II transcription process involving the defined promoters, termination sequences, 5′-capping, and polyadenylation [52].

Both types of transcripts are likely transported through a nuclear pore with the aid of UAP56 [53], and then processed with the mitochondrial surface protein Zucchini (Zuc); Zuc likely trims the 5′- and possibly 3′-ends of the piRNA precursors, although very little detail is known about this process. Among other factors that seem to be involved in the process of biogenesis is CG2183 (Gasz); this protein colocalizes with Zuc and is believed to function as an adapter protein that recruits Piwi proteins to mitochondria for further piRNA maturation [54]. Next step of piRNA maturation requires 2′-O-methylation with the piRNA methyltransferase Pimet, the homolog of *Arabidopsis* methyltransferase HEN1 [55].

Mature piRNAs then bind to PIWI, AUB, and AGO3 proteins that initiate piRNA amplification via a cytoplasmic ping-pong mechanism. The participating proteins have a preference for a different set of small RNAs. While PIWI and AUB have a preference for transposon-derived antisense piRNAs with a 5′-uridine (U), AGO3 preferentially binds sense transposon piRNAs with no enrichment for 5′-U [47]. The binding of antisense piRNAs associated with AUB to a complementary transposon transcript (a length of 10 nt is typically sufficient) results in the endonucleolytic cleavage of the target between the 10th and 11th nt position of the piRNA. The product of this cleavage is a new piRNA with a 10 nt 5′-overlap in the antiparallel orientation (the sense strand) with the initial antisense piRNA; these new piRNAs have an adenosine residue at position 10 complementary to uridine at the initial antisense piRNA [45]. The 3′-end of a newly formed piRNAs is further processed, modified and picked up by AGO3. AGO3-associated piRNAs target primary piRNA cluster transcripts [47]. The cleaved piRNA transcript (antisense) is then picked up by the pair of PIWI/AUB proteins that further trim the transcript. These antisense piRNAs are identical to original antisense piRNAs produced by the initial binding and processing by PIWI/AUB. The complex of antisense piRNA/PIWI/AUB is then ready to enter a new ping-pong cycle or is available for the regulation of translation either in a negative manner by cleavage of target transposon transcripts (which is most common) or in a positive manner by the activation of transcription (which is much less frequent). The earlier mentioned ping-pong mechanism is also found in mice and zebrafish [56,57].

piRNAs in *Drosophila* function both in the cytoplasm and in the nucleus. Studies in *Drosophila* have revealed that the nuclear localization of the Piwi protein is essential for the establishment of chromosomal marks, whereas a slicer activity of the Piwi protein is not [58]. Moreover, loss of Piwi in *Drosophila* results in a substantial loss of the repressive H3K9me3 histone mark and an increased occupancy of POL II at transposable elements [59]. The overexpression of piRNAs against a specific genomic locus leads to the accumulation of the H3K9me3 mark, a decreased POL II occupancy and the recruitment of the heterochromatin protein HP1 [59]. Based on this information, it can be suggested that as a part of the piRNA-induced–silencing complex (pi-RISC), Piwi together with piRNAs translocate to the nucleus where they interact with DNA

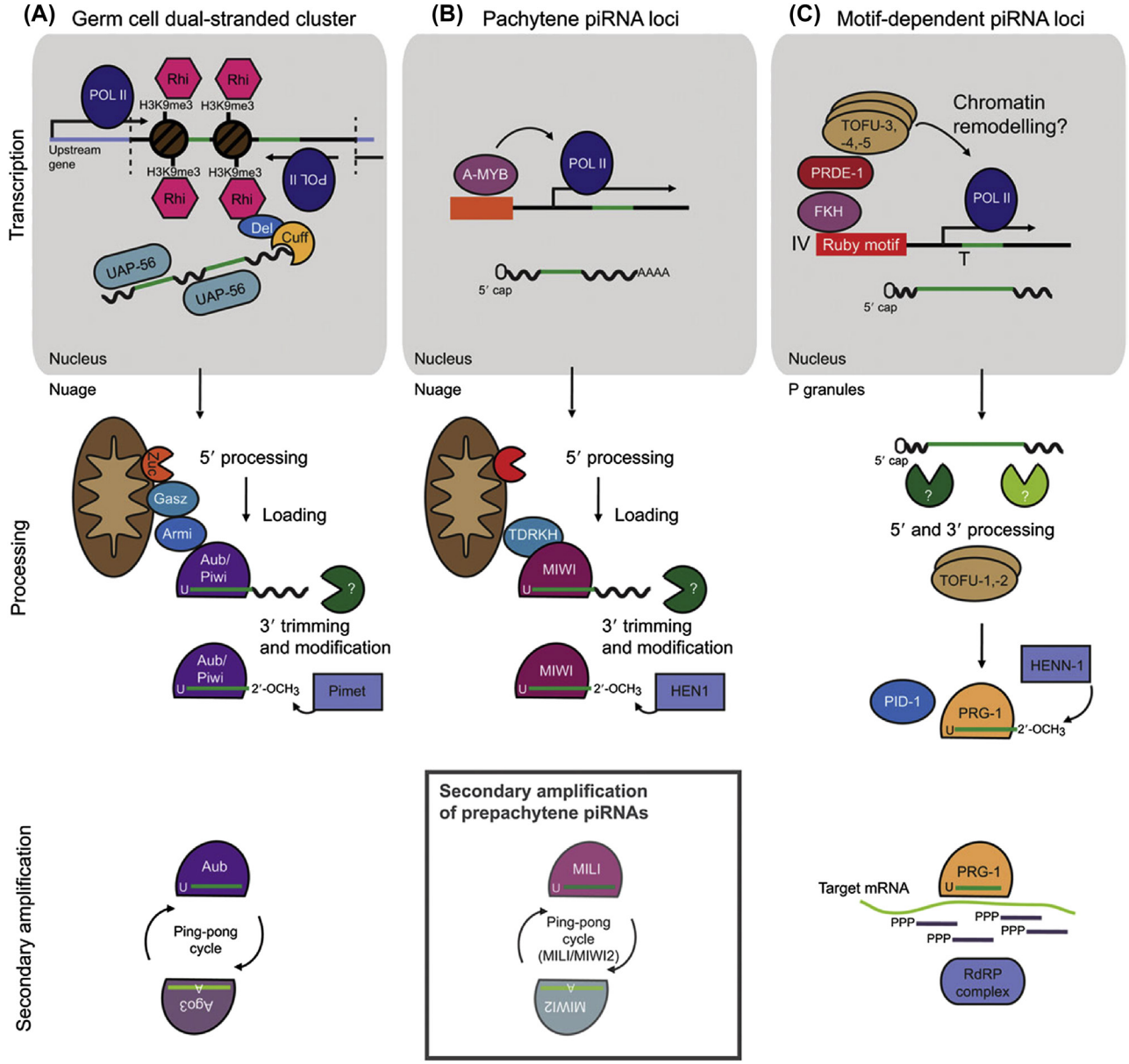

FIGURE 25.2 Mechanisms of piRNA biogenesis in different organisms. (A–C) Models of piRNA generation from dual-stranded clusters in *D. melanogaster* (A), from pachytene piRNA loci in *Mus musculus* (B), and from Ruby motif-containing loci in *C. elegans* (C). (A) Convergent transcription from neighboring genic loci generates piRNA precursors from dual-stranded clusters upon binding of the heterochromatin protein Rhino (Rhi) to H3K9me3 on cluster loci. Rhi in turn associates with Deadlock (Del) and Cutoff (Cuff), the latter of which is thought to protect the 5′-end of the noncanonical precursor transcript from degradation. Nuclear export of the piRNA, mediated by UAP-56, is followed by 5′-end processing, likely mediated by mitochondria-associated nuclease Zucchini (Zuc). Additional factors (eg, CG2183 (Gasz) and Armitage (Armi)) lead to Piwi protein recruitment, piRNA loading, and 3′-end processing, which likely involves an unknown trimmer activity as well as the action of methyltransferase Pimet. Extensive secondary piRNA amplification occurs via the ping-pong cycle, which takes place in *Drosophila* germ cells. (B) The transcription factor A-MYB (MYBL1) binds to a canonical promoter motif and initiates piRNA precursor transcription by POL II while simultaneously inducing expression of piRNA pathway genes (eg, Miwi and Mitopld). The precursor transcripts are 5′-capped and poly-A-tailed and, after export from the nucleus, processed by the murine homolog of Zuc, MITOPLD. Loading onto MIWI is likely followed by 3′-end trimming of the precursor and 2′-O-methylation by murine HEN1. For conceptual comparison of secondary amplification mechanisms in different organisms, the MILI-MIWI2 ping-pong cycle occurring only for prepachytene piRNAs is included here (inset). This process does not take place as part of the biogenesis of pachytene MIWI-bound piRNAs shown here. (C) The conserved Ruby motif is bound by Forkhead proteins (FKH) and possibly additional factors, and transcription of 5′-capped 28 or 29 nt precursors is initiated. Transcription and/or stability of these precursors depend on PRDE-1, TOFU-3, TOFU-4, and TOFU-5. After 5′- and 3′-end processing of the precursor, a process that may be mediated by TOFU-1 and TOFU-2, 2′-O-methylation of the 3′ end of the piRNA by HENN-1 takes place. PID-1 is another novel factor involved in piRNA biogenesis or stabilization, possibly acting at the same level as the *C. elegans* Piwi protein PRG-1. PRG-1:piRNA:target RNA interaction leads to the generation of secondary 22G-RNAs carrying a 5′-triphosphate (indicated as PPP) by a multi-protein machinery containing RNA-dependent RNA polymerases (RdRP). These small RNAs are incorporated into a secondary Argonaute and mediate target silencing. Question marks indicate unknown factors or functions; green lines represent piRNA sequences; and blue lines represent upstream sequences. Although the role of *D. melanogaster* UAP-56 in the targeting of piRNA precursors for nuclear export has been described, analogous mechanisms in mice and *C. elegans* have not yet been discovered; therefore, the sequence of events showing export of a long precursor from the nucleus to the cytoplasm in these organisms is speculative. Biogenesis of uni-stranded clusters in *D. melanogaster*, which occurs concomitantly with dual-strand cluster expression in germ cells and is the only mode of piRNA generation in somatic follicle cells, may be similar to canonical POL II transcription of protein-coding genes. It is currently less well studied and not depicted here. The same is the case for primary biogenesis of prepachytene piRNAs, which are expressed in the fetal germline in *M. musculus*, and for Ruby motif-independent piRNAs, which make up a small proportion of the overall piRNA population in *C. elegans* adults. Ago3, Argonaute3; Aub, Aubergine; RdRP, RNA-dependent RNA polymerase; TDRKH, a tudor domain protein. *Reproduced from Weick EM, Miska EA. piRNAs: from biogenesis to function. Development 2014;141(18):3458–71, with permission.*

or a primary transcript in a sequence-specific manner. This binding recruits other repressive elements, thus leading to the formation of heterochromatin and silencing of the target region (Fig. 25.3A).

piRNAs in *Drosophila* are predominantly derived from transposon regions and target transposon elements transcriptionally and posttranscriptionally. Nevertheless, it is likely that certain piRNAs are also able to target mRNAs transcribed from nonrepetitive genomic regions. For example, the level of the protein-coding transcript Fas3 was found to be regulated by piRNAs generated from the 3′-UTR [60]. This regulatory mechanism likely includes deadenylation. The evidence provides the following facts: NOS mRNA is deadenylated by the CCR4–NOT complex; the CCR4–NOT deadenylation complex interacts with AUB and AGO3 proteins which bind secondary piRNAs; some piRNAs have been identified that contain homology to the 3′-UTR of the NOS transcript.

6.2 piRNAs in Mammals

In contrast to *Drosophila*, in an adult mouse, about 93% of piRNA sequence reads are mapped to a single defined site in the genome. They appear to originate from unidirectional clusters; they are transcribed either from single strands or from two nonoverlapping strands. The chromatin immunoprecipitation analysis showed that piRNAs are derived from RNA Pol II transcripts (Fig. 25.2B) [48].

In mammals, piRNAs are also processed by the PIWI clade proteins: MIWI (PIWIL1), MILI (PWIL2), and MIWI2 (PWIL4). Each of these proteins has a very specific expression pattern during the development of male gametes. The expression of MILI starts at 12.5 days of embryonic development and persists into adulthood, whereas the expression of MIWI2 starts at about 14 days of embryonic development and persists until 3 days postpartum. Finally, the expression of MIWI does not start until 14 days postpartum, which coincides with the beginning of the pachytene stage of meiosis of male gamete development.

In accordance with PIWI protein expression, in germ cells of mice, two different populations of piRNAs have been developed, pre-pachytene and pachytene piRNAs. The expression of pre-pachytene piRNAs in early stages of spermatogenesis originates from transposon and gene-derived sequences; their expression coincides with the expression of two out of three PIWI family proteins—MIWI2 and MILI. MILI-bound piRNAs are 26 or 27 nt, whereas MIWI2-bound piRNAs are 28 nt. In contrast, pachytene piRNAs are 30 nt in length, and their production depends on MAEL and MIWI proteins [61]; they arise from intergenic loci unrelated to repeat sequences [48,62]. Pre-pachytene piRNAs are involved in silencing of transposon regions through de novo DNA methylation, thus contributing toward the stabilization of the genome.

The function of pachytene piRNAs was not clear for a long time. It was demonstrated in 2014 that pachytene piRNAs play a critical role during spermatogenesis by targeting and eliminating large amounts of mRNAs in spermatids. Together with MIWI and the catalytic subunit of the CCR4-CAF1-NOT deadenylation complex CAF1, pachytene piRNAs form pi-RISC. The function of pi-RISC is to deadenylate and degrade specific mRNA targets. A critical importance of MIWI and CAF1 has been demonstrated by knocking down these proteins in the elongating spermatids (ES); about 5000 genes are upregulated in cells with knockdown, with about 90% of them being the genes known to be regulated by MIWI and CAF1 [63]. The immunoprecipitation analysis has shown that over 60% of all mRNAs found in ES cells are physically associated with MIWI, likely as a part of pi-RISC. The fact that the majority of mRNAs physically associated with MIWI have a counterpart match in the form of a specific set of piRNAs is therefore not surprising. Moreover, the levels of these mRNAs inversely correlate with the expression of piRNAs with a sequence-specific match.

Similarly to piRNA-mediated silencing of transposons initially documented in *Drosophila melanogaster*, piRISC is also formed in mice by MILI and MIWI2 [64]. As MIWI2 localizes both in the cytoplasm and the nucleus, it has been proposed to bind secondary piRNAs in the nuage into the cytoplasm and shuttle them into the nucleus in the form of piRISC (Fig. 25.3B).

piRNAs are predominantly mapped to transposon regions, and the depletion of the PIWI protein results in a massive enrichment of transposon mRNAs. piRNAs are most abundant in germline tissues of animals, and they appear to be important for fertility; animals lacking Piwi exhibit various fertility defects [46,57,65]. At the same time, several studies demonstrated an essential role of piRNAs in various somatic tissues, especially during early embryogenesis. In mammals, an evidence for the existence of piRNA in somatic tissues is not yet certain, although the expression of the PIWI protein has been clearly demonstrated, and it has been shown to be even elevated in several human cancers (reviewed in [7]).

6.3 piRNAs in *C. elegans*

Unique features of piRNAs in *C. elegans* include the fact that piRNAs are 21-nt in size and that *C. elegans* has a single PIWI homolog—PRG-1. Because of their unique size and a bias in the location of U at the 5′-end, piRNAs in *C. elegans* are also referred to as 21U-RNAs. Curiously, these RNAs do not match to transposon elements. Most of the primary piRNA

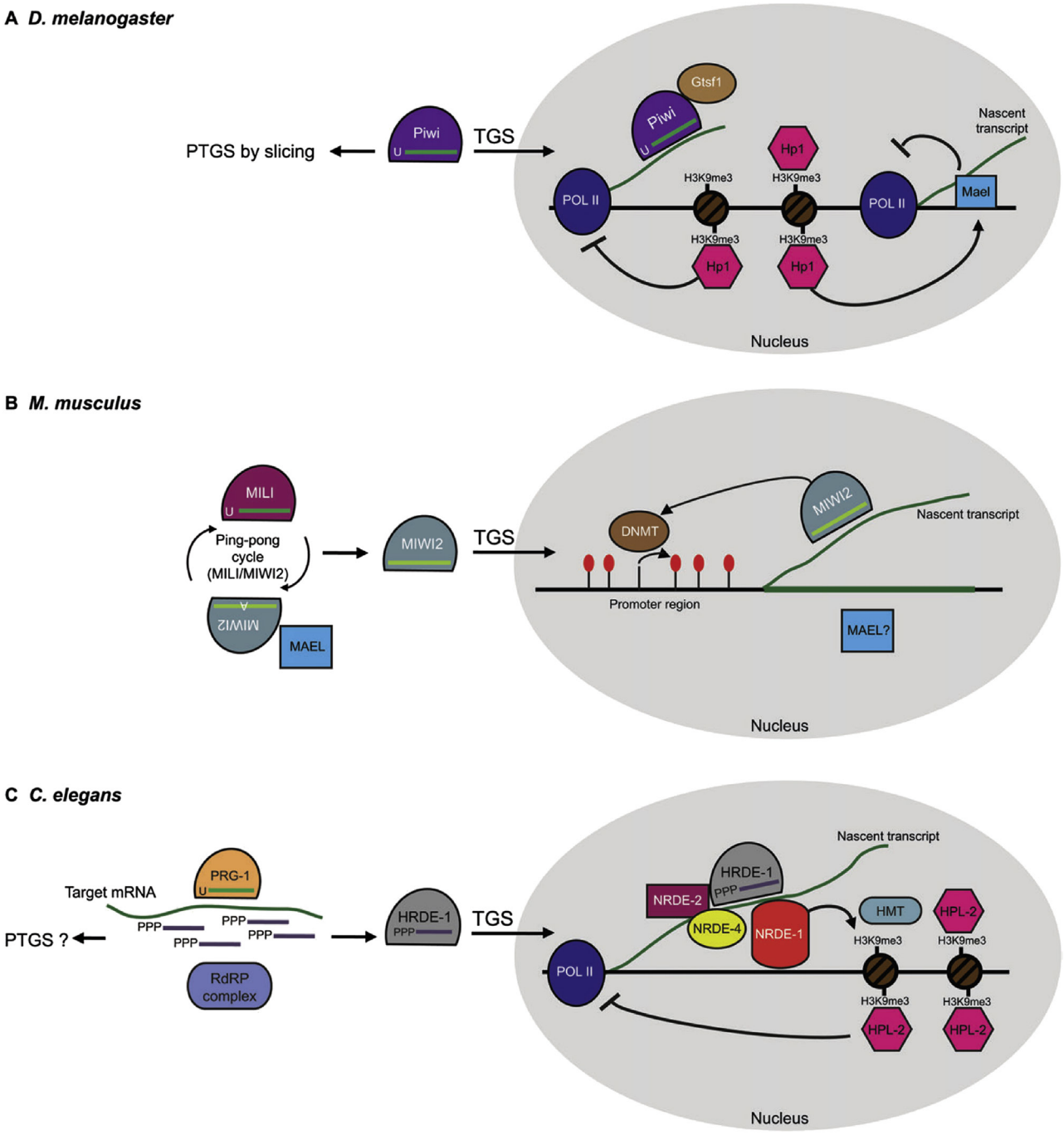

FIGURE 25.3 **Mechanisms of piRNA-mediated transcriptional silencing.** (A) In *D. melanogaster*, Piwi localizes to the nucleus and initiates repressive histone H3K9 trimethylation and RNA polymerase II stalling. Whether Piwi interacts with the nascent transcript or directly with DNA is not understood. The zinc-finger protein Gtsf1 likely directly interacts with Piwi, whereas the heterochromatin protein Hp1 binds to H3K9me3. Mael acts downstream of H3K9me3 methylation and is required for POL II repression; however, its mechanism of action also remains to be determined. In parallel to transcriptional gene silencing (TGS), posttranscriptional gene silencing (PTGS; ie, slicing) plays a well-defined role in *D. melanogaster* piRNA-mediated transposon silencing. (B) In *Mus musculus*, MIWI2 engages in the ping-pong cycle with MILI and translocates to the nucleus where it initiates CpG methylation of promoter elements upstream of transposon loci by DNA methyltransferase (DNMT) action. The murine MAEL homolog is found in the cytoplasm at MIWI2 sites; a role for this protein in the nucleus analogous to that described in *D. melanogaster* remains to be determined. Interaction of MIWI2 with the nascent transcript is speculative. (C) Secondary siRNAs are generated upon PRG-1:piRNA:target RNA interaction by RNA-dependent RNA polymerase (RdRP). These small RNAs are incorporated into the germline secondary Agonaute HRDE-1 which translocates to the nucleus to initiate H3K9me3 methylation and POL II stalling, likely by interacting with pre-mRNA and nuclear RNAi (NRDE) factors. For clarity, this model depicts establishment of repressive histone methylation marks by histone methyltransferases (HMT) as preceding POL II repression; the actual order of events remains to be determined experimentally and the reverse may also be the case. In *C. elegans*, target slicing is not essential for piRNA function; however, as of 2016, other mechanisms of PTGS have not been experimentally investigated. HPL, a H3K9me3-binding protein. *Reproduced from Weick EM, Miska EA. piRNAs: from biogenesis to function. Development 2014;141(18):3458–71, with permission.*

transcripts in *C. elegans* are likely generated by POL II from two major clusters containing over 16,000 genes. A large fraction of piRNAs are produced by transcription from specific promoters (motifs), since 2006 has been referred to as the Ruby motif (by the name of the first author of the publication describing such motifs) (Fig. 25.2C) [45,66].

These piRNA precursors are 28–29 nt in length and contain a 5′-cap. The processing of these precursors occurs through cleavage of the first 2 nt at the 5′-end and about 4–5 nt at the 3′-end [45]. The cleaved precursors are further methylated at the 3′-end by the *C. elegans* HEN1 ortholog HENN-1.

The PRG-1 protein may not be the only protein needed for biogenesis of piRNAs in nematodes since the *prg-1* mutant still contains low levels of piRNAs. Nevertheless, homologs of murine (or *Drosophila*) proteins involved in piRNA biogenesis are not yet found in *C. elegans*. Moreover, a ping-pong mechanism of the generation of secondary piRNAs is also not documented in nematodes. Instead, the secondary piRNAs (siRNAs) of 22 nt in size in worms, known as 22G-RNAs, are generated by RNA-dependent RNA polymerases (RdRPs) (Fig. 25.3C). The secondary siRNAs are picked up by the Argonaute homolog, the nuclear Argonaute NRDE-3 (which is RNAi deficient), and this complex (somewhat similar to pi-RISC in mice) shuttles into the nucleus where it is involved in transcriptional gene silencing. The complex binds pre-mRNAs in a sequence-specific manner and recruits two more proteins, NRDE-2 and NRDE-1. These proteins are involved in the establishment of the repressive H3K9me3 methylation mark that is further reinforced by the *C. elegans* homolog of the H3K9me3-binding protein HP1, HPL-2 [67,68] at the target site (Fig. 25.3C). The additional mechanism includes a direct suppression of transcription by POL II stalling during the elongation phase of transcription.

There are many targets of 21U-RNAs, including transposable elements and many protein-coding genes. Transcriptional gene silencing in nematodes occurs through imperfect binding to pre-mRNAs, allowing targeting of many different transcripts. Distinct classes of piRNAs may target different sets of proteins. piRNAs produced from promoters that lack specific sequence motifs (motif-less piRNAs) target mRNAs encoding immune-response genes. This suggests that there might be a specific mechanism producing a specific class of 21U-RNAs that regulate specific biological mechanisms.

6.4 piRNAs in Transgenerational Response

Several studies in different animals have demonstrated an interesting feature of piRNAs—the capacity to induce silencing across generations—transgenerational silencing. The first phenomenon of transgenerational transmission of information that later on was proved to have piRNAs as an essential component was described in *Drosophila*. Crosses between wild-caught males and laboratory-strain females resulted in infertile animals, whereas reciprocal crosses gave fertile flies. This phenomenon referred to as hybrid dysgenesis was believed to occur due to the presence of *P*-element or *I*-element transposons in wild animals. Since no sterility was observed in wild females, it was suggested that the suppressing factor was of a cytoplasmic origin and was transmitted by female gametes. It was later on demonstrated that the maternally deposited piRNAs provide the antisense piRNA component of the ping-pong loop, mounting an active defense response against transposons [69].

In 2012, piRNAs were shown to mediate the repression of the LacZ transgene cluster that has lasted for over 50 generations of flies without the presence of the allele that initiates silencing [70]. The trans-silencing effect (TSE) was achieved using repeat clusters of P-element-derived LacZ transgenes by exposing the nonsilencing cluster to the cytoplasm of a *Drosophila* strain carrying a cluster that exhibited strong TSE activities. Such type of silencing based on the transient interaction between the silenced and active alleles is similar to a paramutation phenomenon reported in plants and mice [71,72].

The involvement of piRNAs in transgenerational silencing has also been well documented in *C. elegans*. Feeding transgenic *C. elegans* that express the GFP transgene in the germline with *E. coli* that overexpress double-stranded RNAs (dsRNAs) with the homology to the transgene results in transgene silencing that has been observed in the germline for four consecutive generations [67]. Curious is the fact that once established, the transgenerational memory becomes independent of the piRNA trigger, although it still requires the nuclear RNAi/chromatin modification machinery described earlier. Reestablishing silencing in each generation requires piRNA transmission across generations via the germline followed by the amplification mechanism in somatic tissues.

In 2015, it was demonstrated that double-stranded RNA (dsRNA) generated in somatic tissues (neurons) of *C. elegans* can be transported to germline cells and trigger silencing of genes of matching sequence in germline cells [73]. Silencing within the germline can persist for over 25 generations.

Despite the similarity in the role of piRNAs in triggering transgenerational silencing in *Drosophila* and worms, the maintenance of silencing across generations may be different. Silencing in *Drosophila* is triggered by piRNAs deposited maternally and is maintained by using maternal piRNAs. In contrast, in worms, the maintenance phase can occur in both sexes. However, it should also be noted that all transgenerational effects of silencing were demonstrated by using transgenes and by exposing animals to silencer alleles. It remains to be shown whether the effects are equally strong in the naturally occurring silencing effects.

7. THE ROLE OF SMALL INTERFERING RNAs IN THE MAINTENANCE OF GENOME STABILITY

siRNAs, also referred to as short interfering RNAs, are a group of double-stranded ncRNAs that are usually perfectly complementary, 20–24 nt in size, have a 2-nt overhang, and contain the phosphorylated 5′-ends and the hydroxylated 3′-ends. They are typically processed from either larger endogenous double-stranded RNA molecules, hairpin RNAs, or exogenous (viral) double-stranded RNA molecules. siRNAs play the major role in transcriptional and posttranscriptional gene silencing via the establishment of a repressive chromatin state (via DNA methylation or repressive histone marks, predominantly in plants) or translational inhibition (mostly in animals) or transcript cleavage and degradation (mostly in plants).

The involvement of siRNAs in the RNA interference (RNAi) process has been first reported in plants as a phenomenon of cosuppression of both the integrated transgene and the endogenous homologous sequence [74]. Later on, RNAi has been documented in many different species in response to transgene overexpression, the activation of transposon elements or infection with viruses [75–77]. Therefore, RNAi is believed to be one of the ancient immune mechanisms of protection for the cell and genome, in particular against foreign genetic elements.

7.1 siRNAs in *Neurospora crassa*

In *Neurospora crassa*, siRNAs are involved in quelling, the posttranscriptional gene-silencing mechanism that occurs in response to the insertion of multiple copies of a transgene. Since transgenes often integrate as multiple, frequently truncated, and inverted copies, they are likely treated as repetitive transposable elements. *Neurospora* does not appear to have many copies of repetitive elements, and therefore RNAi against transgenes is considered to be a defense mechanism. Indeed, RNAi has also been shown to suppress the proliferation of multiple copies of LINE-like transposons [78]. *Neurospora* has several other RNAi-like genome defense mechanisms functioning during the sexual cycle. These are repeat-induced point mutation (RIP) and meiotic silencing by unpaired DNA (MSUD, also known as meiotic silencing) [79]. RIP causes C→A mutations in repetitive sequences, whereas MSUD triggers sequence-specific silencing when unpaired DNA is present and two haploid nuclei are fused into a diploid nucleus.

The quelling pathway requires the function of quelling deficient-1 (QDE-1), a protein with the activity of DNA-dependent RNA polymerase (DdRP) and RNA-dependent RNA polymerase (RdRP) [80] (Fig. 25.4). First, RNA Pol II transcribes complex transgenes producing aberrant RNAs. Then, QDE-1 converts aRNAs into dsRNA [80]. These dsRNAs are further processed by Dicer, and the resulting siRNAs of about 25 nt in size are loaded onto the Argonaute homolog QDE-2 to target homologous RNAs and induce posttranscriptional gene silencing in the form of RISC [81]. dsRNAs consist of two strands, the guide strand and passenger strand. While the former one guides the cleavage of mRNA targets, the latter one has to be degraded to activate RISC. The activation of RISC requires two steps: first, the QDE-2 protein nicks the passenger strand, and second, the QIP exonuclease degrades it. An active RISC consists of the guide siRNA strand and the QDE-2 protein.

7.1.1 qiRNA: DNA Damage–Induced siRNAs in Neurospora

DSB repair is a complex process that depends on the concerted action of various DNA-repair and chromatin-modifying proteins. Evidence suggests that the repair process also requires a new subclass of siRNAs. In *Neurospora*, they are produced in response to DNA damage; while studying the regulation of QDE-2, Lee et al. [82] observed that adding histidine rather than any other amino acids to the growth medium increased *qde-2* expression. Since histidine is known to induce DNA damage and mutation rates, it was hypothesized that DNA damage is likely the cause of the induction of *qde-2* expression. Indeed, treatment with other DNA-damaging agents like ethyl methanesulfonate, hydroxyurea, or methyl methanesulfonate also induced *qde-2* expression. The exact mechanism of qiRNA production is not clear, but it is possible that the presence of tandem repetitive DNA sequences within rDNA loci results in the formation of aberrant DNA structures that are recognized by the *recQ* DNA helicase QDE-3. However, a 2015 work demonstrated that the presence of a tandem repeat itself is not sufficient for the generation of qiRNAs and that a strand break in the region of tandem repeats is required for the initiation of aRNA production [83]. QDE-3 unwinds dsDNA to produce ssDNA, and RPA proteins stabilize the structure (Fig. 25.4). QDE-1 is then recruited to this complex to produce aRNA and convert it to dsRNA. RNA polymerase I (Pol I), which is normally required for the transcription from rDNA, is not needed for the generation of DNA damage–induced aRNAs because they are still produced in an RNA Pol I mutant [82]. Similarly, Pol II and III are also not needed for aRNA generation. These qiRNAs are shorter in size than those produced during quelling, they are about 20–21 nt in length and have a strong preference for uridine (93%) at the 5′-end and for A (49%) at the 3′-end. Most of the qiRNAs (86%) were stemming from the nucleolus organizer region (NOR) formed by about 200 copies of ribosomal DNA (rDNA) repeats. In addition, qiRNAs originate from the external and internal transcribed spacer regions of rDNA loci as well as from the intergenic

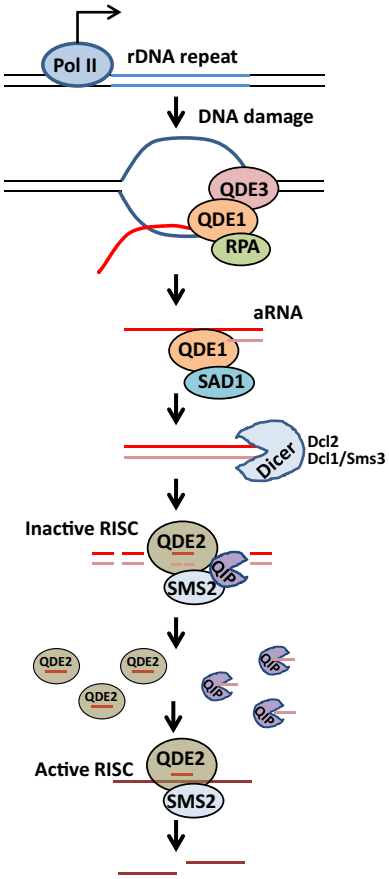

FIGURE 25.4 Mechanisms of quelling and qiRNA biogenesis. Mechanism of biogenesis of siRNAs in quelling and qiRNAs appears to be the same. Locus containing repetitive sequences of transgenes or transposons in quelling and rDNA in qiRNA generation is transcribed by QDE-1 protein, polymerase with high affinity to both DNA and RNA. QDE-3 is a helicase that unwinds dsDNA and stabilizes transcription. DNA damage is an essential component for generation of qiRNAs but not siRNAs in quelling. RPA protein likely aids QDE-1 in transcription by recruiting to the locus and by blocking the formation of DNA/RNA hybrids. QDE-1 then uses its RdRP activity to synthesize the second RNA strand using aRNA as a template. QDE-1 may be assisted by SAD-1 (suppressor of ascus dominance 1), a putative RdRP protein. Dicer protein (DCL2 or DCL1, although known as Sms3) dices dsRNA into small duplexes. RISC complexes consist of Argonaute homolog QDE-2, exonuclease QIP, and possibly Sms-2 (suppressor of meiotic silencing-2) protein, another Argonaute homolog, although it might only function in sexual stage of *Neurospora* development. RISC is activated by removing the passenger strand of the siRNA duplex by QDE-2 cleaving the passenger strand and QIP degrading the cleaved remnants. The guide strand is then loaded onto RISC and executes silencing by binding mRNA and cleaving it in a sequence-specific manner.

regions (6.57%), open reading frames (ORFs, 4.37%), and tRNAs (1.45%). Despite differences in size of siRNAs produced in quelling and upon DNA damage, it is believed that the mechanism of biogenesis is the same (Fig. 25.4).

The role of DNA damage–induced qiRNAs is not very clear, but it is possible that they inhibit rRNA biogenesis and/or the translation process after DNA damage. Indeed, protein synthesis is inhibited upon DNA damage in *Neurospora*, and this inhibition is partially removed in *qde-1* and *qde-3* mutants. The authors who discovered qiRNAs proposed that the production of qiRNAs is one of the mechanisms employed by fungi functioning similarly to DNA-damage checkpoints. Indeed, since the G2 phase requires intensive protein synthesis, its inhibition delays cell division, thus giving more time for DNA repair. Later on, it has been shown that qiRNA production requires homologous recombination (HR) [84]; the introduction of a site-specific DNA break initiates a homologous recombination event. HR at repetitive elements may generate recombination intermediates that are recognized by QDE-3. The QDE-3 helicase may function as a resolvase, by resolving the recombination intermediates into ssDNA. Therefore, it is possible that HR at the site of DNA damage functions to produce qiRNAs only from the repetitive DNA loci, thus distinguishing between genomic regions formed by transposon replication and other nonrepetitive genomic regions.

7.2 DNA Strand Break–Induced Small RNAs or diRNAs Are Involved in DSB Repair

DNA strand breaks—single stranded and double stranded (SSB and DSB, respectively)—occur as a result of oxidative damage due to the normal metabolic activity or as a result of a response to environmental pressures. Regardless of whether

DNA damage is direct or indirect, the outcome is the disruption of the nucleotide chain that has to be fixed. Two major repair pathways are competing for strand break nonhomologous end joining (NHEJ) and homologous recombination (HR). While the former one is a frequent but error-prone repair, the latter one is relatively rare and mostly error free. Cells utilize NHEJ in most of the cases, whereas they use HR mostly in the late S phase and G2 phase largely to avoid the loss of heterozygosity of a given deleterious mutation when sister chromatids are not available in the G1 phase. NHEJ plays a very important role in quiescent cells (G0) by taking care of most of the generated strand breaks.

The HR-repair pathway consists of several major steps. First, the MRN complex consisting of Mre11, Rad50, and Nbs1 proteins binds the region of strand breaks, and the broken DNA strands are unwound by helicases and resected by nucleases to expose long stretches of 3′-single-stranded DNA (ssDNA). Second, the exposed ssDNAs are covered by RPA. In parallel, the protein kinase ataxia telangiectasia (ATR) that is recruited to the region phosphorylates the proximal histone variant H2AX generating γH2AX; H2AX phosphorylation spreads distally to allow the exposure of a large stretch of DNA sequence [85]. Third, RAD51 displaces RPA from the ssDNA by forming long filaments. The RAD51-ssDNA filament searches for a homologous sequence template located typically on a sister chromatid or (less frequently) a homologous chromosome. The invading ssDNA forms a stretch of dsDNA with a template ssDNA, initiating DNA synthesis to replace the DNA surrounding the former strand break [85]. Fourth, the formed D-loop is resolved via the migration of double Holliday junction intermediate or by simple dissociation of one of the invading strands. Details of the type of HR repair and proteins involved can be found in Chapter 20.

The DNA damage–induced production of ncRNAs was reported in *Neurospora* (see qiRNAs in the preceding section) (Figs. 25.4 and 25.5). The DSB-induced production of ncRNAs has also been demonstrated in other species, including plants, flies, and mammals [86–88].

A class of 21 nt–long small RNAs induced by DSBs that originates in the proximity of the break (named DSB-induced small RNAs or diRNAs) was discovered in 2012 [86]. The authors used a special transgenic *Arabidopsis* recombination reporter line DGU.US consisting of the disrupted version of the GUS gene encoding the beta-glucuronidase enzyme. The transgene contained a recognition site for the rare cutter endonuclease I-SceI. When transgenic plants are crossed with plants carrying this endonuclease, a nick is generated in the transgene, creating a strand break, and the repair of this strand break results in the HR-dependent restoration of the transgene structure, which leads to its expression; its expression can be monitored via a histochemical assay that allows to score the number of recombination events [89]. The authors crossed these lines with various DCL mutants that are known to be involved in siRNA biogenesis (*dcl2*, *dcl3*, and *dcl4*) and demonstrated that the recombination frequency in mutants was decreased by 42%, 90%, and 44%, respectively. The results obtained allowed authors to hypothesize that siRNAs are likely involved in recombination repair. The Northern blot analysis with a probe spanning a region of about 450 nt flanking the I-SceI recognition site showed that indeed there were siRNAs produced from this region; siRNAs were significantly enhanced when the I-SceI endonuclease was introduced by crosses between plants overexpressing the endonuclease and plants containing the recognition site. The deep sequencing analysis confirmed the presence of these siRNAs and demonstrated that they were produced from both strands, thus allowing us to suggest that they were likely produced via dsRNA intermediates. The analysis of mutants showed that siRNAs were depleted in the *atr* mutant and all three DCL mutants [86]. In particular, the production of diRNAs was reduced by 98% in the *dcl3* mutant, which was consistent with the data demonstrating that in these plants, the HR frequency is reduced by about 90%. Furthermore, it was demonstrated that RdRPs (RDR2 and RDR6) and Pol IV (RNA-dependent DNA methylation or RdDM components, see [90]) were found to be involved in diRNA biogenesis; mutations in RDR2 and RDR6 caused 87% and 82% reductions in the levels of diRNAs, respectively. The analysis of other components of RdDM, including AGO4 and DRM2 (the de novo DNA methyltransferase), showed that they were not involved in the diRNA-mediated repair of DSBs. The authors also tested whether other components of RISC are involved in diRNA biogenesis/function. They found that AGO2 expression is induced by radiation; using immunoprecipitation followed by Northern blotting or deep sequencing, they demonstrated that radiation results in an increase in the number of diRNAs associated with AGO2 [86]. To further deduce the function of diRNAs, the authors analyzed whether diRNAs are required for the induction of H2AX phosphorylation and the formation of γH2AX foci; the analysis showed that diRNAs are likely not needed for these steps, and they are functioning downstream of γH2AX focus formation.

This work was extended from *Arabidopsis* to humans using the reporter line similar to the one used in *Arabidopsis*, but with GFP replacing GUS. The analysis of diRNA formation in response to radiation in transgenic human cell lines confirmed that they were induced, and their production was dependent on the presence of Dicer and Ago2 [86].

Finally, it was demonstrated that other components of diRNA biogenesis, such as Ago2, Dicer or Drosha/DGCR8, impaired HR to a similar degree as the knockdown of Rad51 [91]. They also showed that another DSB-repair pathway, NHEJ, did not benefit from diRNAs and was not regulated by them; the NHEJ activity was not altered in Dicer and Ago2 mutants. Their work showed that Ago2 formed a complex with Rad51 and that Rad51 accumulation at DSB loci was

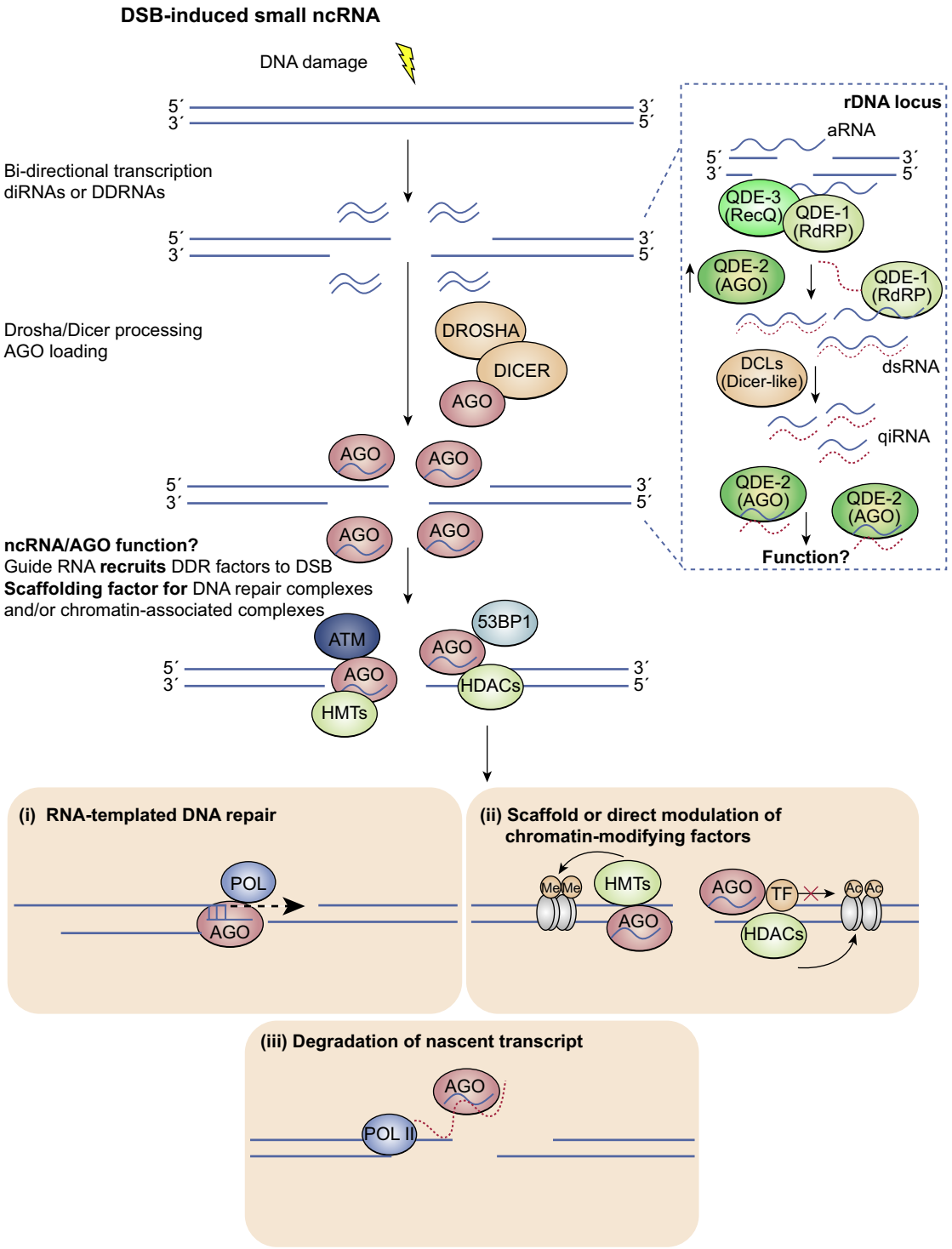

FIGURE 25.5 **Other noncoding RNAs (diRNAs, qiRNAs) in DSB repair.** DSBs can trigger the production of short ncRNA at the site of the DNA lesion. The inset is a schematic of the DNA damage–induced RdRP-dependent (QDE1) production of qiRNAs in *Neurospora crassa* [82]. qiRNAs were identified in complex with QDE2 (AGO homolog), but their role in DDR remains unknown. In higher eukaryotes lacking RdRPs, it is postulated that antisense transcripts lead to the formation of dsRNAs that are processed by the miRNA biogenesis machinery (DROSHA/DICER). The AGO-bound ncRNAs localizes at the DSB, and potentially facilitates the recruitment of DDR factors (53BP1, *p*-ATM) to the DSB site. It is feasible that the homology of the ncRNA to sequences proximal to the DSB allows it to serve as a "guide" for recruiting chromatin-modifying proteins/DDR factors to the DSB [98]. Alternatively, the ncRNA/AGO complex may also serve as a stable scaffold for maintaining the DNA-repair foci and facilitating the process of repair [99]. After the recruitment of the DNA-repair machinery to the DSB sites the precise role of the short ncRNAs is not known. It is speculated that the short ncRNAs could serve as RNA template to fill-in resected DNA during homologous recombination mediated DSB repair. Induced RNAs might play a role in mediating chromatin silenced state at the breaks either by recruiting associated chromatin factors at the breaks [100], or directly by interacting and modulating chromatin-associated factor in cis [101]. The short ncRNA could also act in the conventional siRNA pathway to degrade nascent RNA at the breaks in order to prevent deregulated expression of compromised genes. *Reproduced from Chowdhury D, Choi YE, Brault ME. Charity begins at home: non-coding RNA functions in DNA repair. Nat Rev Mol Cell Biol 2013;14(3):181–9, with permission.*

dependent on the catalytic activity and small RNA-binding capability of Ago2. Other steps of DSB repair, such as DSB resection, RPA and Mre11 loading, were not affected by Ago2 or Dicer depletion. Importantly, the authors demonstrated a direct role of diRNAs in HR repair; HR repair could be restored in Dicer-depleted cells by adding ncRNAs purified from human cells transfected with the I-SceI enzyme [91].

Francia et al. [87] demonstrated a similar diRNA activity in humans, mice, and zebrafish. They induced a strand break at a specific genomic site and by sequencing ncRNAs, they found ncRNA enrichment from the vicinity of the break; these ncRNAs were named DDRNAs. It was further shown that the formation of DDR foci depended on the production of DDRNAs, and DICER and DROSHA were the required components. Similar to the work by Gao et al. [91], a work by Francia et al. [87] showed that DDRNAs, either synthesized chemically or generated in vitro by DICER cleavage, were sufficient to restore the DNA-repair activity in the absence of other cellular RNAs.

Finally, Michalik et al. [88] showed a similar generation of diRNAs in *Drosophila*. They found that the structure of DSB ends (either blunt or with overhangs) did not influence diRNA production. The authors have reported an interesting novel finding. First, they have found that diRNAs are formed more actively when breaks occur in active transcribed genes. Second, they have shown that a DSB within a transcribed gene initiates transcription away from the break site [88]. The formation of such antisense transcripts results in a quick and efficient formation of dsRNA, the activation of RNAs, and a more efficient repair process.

Taking into consideration all the earlier mentioned results, a model for diRNA generation (qiRNAs, DDRNAs) can be proposed. Sequence-specific diRNAs are produced from the regions with DSBs. They are mainly processed by DCL3 (in cooperation with DCL2 and DCL4 in plants and Dicers in animals) from dsRNA intermediates, are picked up by AGO2 (in plants), and are incorporated into diRISC. Being incorporated in diRISC, diRNAs guide Ago2 to promote Rad51 recruitment and/or retention at DSBs to facilitate repair by HR [15].

8. CONCLUSION

Here, we provided a relatively short summary of ncRNAs that are directly or indirectly involved in the regulation of genome stability and in DNA repair itself. The role of ncRNAs is essential in prokaryotes where they are involved in the regulation of transcription, DNA repair, and chromatin accessibility to potential DNA lesions as well as in ncRNA-mediated degradation of foreign genomes (phages or other bacteria). It is even more critical in eukaryotes where they are involved in the targeted DNA elimination, the regulation of transposon activity, the regulation of transcription and chromatin structure, the regulation of the activity of DNA-repair components, and their recruitment to DNA lesions, and likely in many other processes. Many questions remain unanswered. It will have to be demonstrated that diRNAs, qiRNAs, and other damage-induced ncRNAs are actually actively involved in DNA repair, rather than being the byproduct of DNA damage. It will be important to establish whether there are ncRNAs that are produced specifically in response to a certain type of DNA damage, or whether they influence (aid) a certain type of DNA repair (although it was shown that diRNAs do not influence NHEJ but rather HR repair). It will also be important to develop an in vitro system that allows to study the kinetics of biogenesis of these ncRNAs and their role in the repair of certain type of lesions. Finally, it is important to demonstrate similarities and differences in the generation of various types of ncRNAs in different organisms.

GLOSSARY

Bidirectional clusters Genetic clusters representing genes (transgenes, transposons, and so on) located on both strands of DNA.

Convergent transcription The simultaneous transcription of bidirectional clusters containing overlapping gene sequences.

diRNAs DSB-induced siRNAs produced in the vicinity of DNA damage; observed in plants and animals.

Hybrid dysgenesis High rates of mutations in germline cells of *Drosophila* obtained upon crossing males carrying autonomous P elements with females without these elements.

MSUD Meiotic silencing by unpaired DNA, also known as meiotic silencing.

Nuage A perinuclear organelle, the site of piRNA processing in *Drosophila*.

Ping-pong mechanism The mechanism of generation of piRNAs using transposon-derived RNAs as a template.

qiRNAs DSB-induced siRNAs typically derived from rDNA loci in the *Neurospora* genome.

Quelling The mechanism of posttranscriptional gene silencing observed in *Neurospora* in response to transgene integration in a clustered repetitive manner.

Transgenerational memory A memory of exposure to certain environmental stimuli, manifesting itself in the form of changes in DNA methylation, histone modifications, DNA-repair efficiency, tolerance to stress, changes in transcriptomes, and so on.

Transgenerational silencing Silencing of transgenes or endogenes observed in the progeny in response to stimuli that occurred in parents.

LIST OF ABBREVIATIONS

CRISPR/Cas9 Clustered regularly interspaced short palindromic repeats/CRISPER-associated protein 9
CSA Cockayne syndrome protein A
DdRP DNA-dependent RNA polymerase
diRNA DSB-induced small RNAs
DNA-PKcs DNA-dependent protein kinase catalytic subunit
DSB double-strand break
isomiRs miRNA isoforms
miRNA Micro-RNA
MRN Mre11–Rad50–Nbs1
ncRNA noncoding RNA
PACT protein activator of PKR
piRNAs (P-element-induced wimpy testis)-interacting RNAs
pre-miRNA Precursor miRNA
PTGS Posttranscriptional gene silencing
qiRNA QDE-2-interacting small RNAs
RdDM RNA-dependent DNA methylation
RdRP RNA-dependent RNA polymerase
RNAi RNA interference
RPA Replication protein A
rRNA Ribosomal RNA
scnRNAs Scan RNAs
sgRNA Single-guide RNA
siRNA Small interfering RNA
smRNA Small nuclear RNA
snoRNA Small nucleolar RNAs (snoRNAs)
SSB Single-strand break
STAT3 Signal transducer and activator of transcription 3
TGS Transcriptional gene silencing
TRBP Trans-activation response RNA-binding protein
tRNA Transfer RNA
TSE Transsilencing effect
Zuc Zucchini

REFERENCES

[1] Kowalczyk MS, Higgs DR, Gingeras TR. Molecular biology: RNA discrimination. Nature 2012;482(7385):310–1.
[2] Pryde F, Khalili S, Robertson K, Selfridge J, Ritchie AM, Melton DW, et al. 53BP1 exchanges slowly at the sites of DNA damage and appears to require RNA for its association with chromatin. J Cell Sci 2005;118(Pt. 9):2043–55.
[3] Ganesan S, Silver DP, Greenberg RA, Avni D, Drapkin R, Miron A, et al. BRCA1 supports XIST RNA concentration on the inactive X chromosome. Cell 2002;111(3):393–405.
[4] Yoo S, Dynan WS. Characterization of the RNA binding properties of Ku protein. Biochemistry 1998;37(5):1336–43.
[5] Adamson B, Smogorzewska A, Sigoillot FD, King RW, Elledge SJ. A genome-wide homologous recombination screen identifies the RNA-binding protein RBMX as a component of the DNA-damage response. Nat Cell Biol 2012;14(3):318–28.
[6] Polo SE, Blackford AN, Chapman JR, Baskcomb L, Gravel S, Rusch A, et al. Regulation of DNA-end resection by hnRNPU-like proteins promotes DNA double-strand break signaling and repair. Mol Cell 2012;45(4):505–16.
[7] Ross RJ, Weiner MM, Lin H. PIWI proteins and PIWI-interacting RNAs in the soma. Nature 2014;505(7483):353–9.
[8] Millar AA, Waterhouse PM. Plant and animal microRNAs: similarities and differences. Funct Integr Genomics 2005;5(3):129–35.
[9] Ha M, Kim VN. Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol 2014;15(8):509–24.
[10] Lee HY, Zhou K, Smith AM, Noland CL, Doudna JA. Differential roles of human dicer-binding proteins TRBP and PACT in small RNA processing. Nucleic Acids Res 2013;41(13):6568–76.
[11] Paroo Z, Ye X, Chen S, Liu Q. Phosphorylation of the human microRNA-generating complex mediates MAPK/Erk signaling. Cell 2009;139(1):112–22.
[12] Maes OC, An J, Sarojini H, Wu H, Wang E. Changes in MicroRNA expression patterns in human fibroblasts after low-LET radiation. J Cell Biochem 2008;105(3):824–34.
[13] Pothof J, Verkaik NS, van IW, Wiemer EA, Ta VT, van der Horst GT, et al. MicroRNA-mediated gene silencing modulates the UV-induced DNA-damage response. EMBO J 2009;28(14):2090–9.
[14] Josson S, Sung SY, Lao K, Chung LW, Johnstone PA. Radiation modulation of microRNA in prostate cancer cell lines. Prostate 2008;68(15):1599–606.

[15] Chowdhury D, Choi YE, Brault ME. Charity begins at home: non-coding RNA functions in DNA repair. Nat Rev Mol Cell Biol 2013;14(3):181–9.
[16] Zhang X, Wan G, Berger FG, He X, Lu X. The ATM kinase induces microRNA biogenesis in the DNA damage response. Mol Cell 2011;41(4):371–83.
[17] Trabucchi M, Briata P, Garcia-Mayoral M, Haase AD, Filipowicz W, Ramos A, et al. The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs. Nature 2009;459(7249):1010–4.
[18] Kawai S, Amano A. BRCA1 regulates microRNA biogenesis via the DROSHA microprocessor complex. J Cell Biol 2012;197(2):201–8.
[19] Chang S, Wang RH, Akagi K, Kim KA, Martin BK, Cavallone L, et al. Tumor suppressor BRCA1 epigenetically controls oncogenic microRNA-155. Nat Med 2011;17(10):1275–82.
[20] Niu J, Shi Y, Tan G, Yang CH, Fan M, Pfeffer LM, et al. DNA damage induces NF-kappaB-dependent microRNA-21 up-regulation and promotes breast cancer cell invasion. J Biol Chem 2012;287(26):21783–95.
[21] Zhang ZY, Fu SL, Xu SQ, Zhou X, Liu XS, Xu YJ, et al. By downregulating Ku80, hsa-miR-526b suppresses non-small cell lung cancer. Oncotarget 2015;6(3):1462–77.
[22] Wu Z, Wang P, Song C, Wang K, Yan R, Li J, et al. Evaluation of miRNA-binding-site SNPs of MRE11A, NBS1, RAD51 and RAD52 involved in HRR pathway genes and risk of breast cancer in China. Mol Genet Genomics 2015;290(3):1141–53.
[23] Li JT, Zhong BY, Xu HH, Qiao SY, Wang G, Huang J, et al. Associations between NBS1 Polymorphisms and Colorectal Cancer in Chinese Population. PLoS One 2015;10(7):e0132332.
[24] Hu H, Du L, Nagabayashi G, Seeger RC, Gatti RA. ATM is down-regulated by N-Myc-regulated microRNA-421. Proc Natl Acad Sci USA 2010;107(4):1506–11.
[25] Ng WL, Yan D, Zhang X, Mo YY, Wang Y. Over-expression of miR-100 is responsible for the low-expression of ATM in the human glioma cell line: M059J. DNA Repair (Amst) 2010;9(11):1170–5.
[26] Yan D, Ng WL, Zhang X, Wang P, Zhang Z, Mo YY, et al. Targeting DNA-PKcs and ATM with miR-101 sensitizes tumors to radiation. PLoS One 2010;5(7):e11397.
[27] Song L, Lin C, Wu Z, Gong H, Zeng Y, Wu J, et al. miR-18a impairs DNA damage response through downregulation of ataxia telangiectasia mutated (ATM) kinase. PLoS One 2011;6(9):e25454.
[28] Wang Y, Huang JW, Li M, Cavenee WK, Mitchell PS, Zhou X, et al. MicroRNA-138 modulates DNA damage response by repressing histone H2AX expression. Mol Cancer Res 2011;9(8):1100–11.
[29] Mueller CR, Roskelley CD. Regulation of BRCA1 expression and its relationship to sporadic breast cancer. Breast Cancer Res 2003;5(1):45–52.
[30] Moskwa P, Buffa FM, Pan Y, Panchakshari R, Gottipati P, Muschel RJ, et al. MiR-182-mediated downregulation of BRCA1 impacts DNA repair and sensitivity to PARP inhibitors. Mol Cell 2011;41(2):210–20.
[31] Wang Y, Huang JW, Calses P, Kemp CJ, Taniguchi T. MiR-96 downregulates REV1 and RAD51 to promote cellular sensitivity to cisplatin and PARP inhibition. Cancer Res 2012;72(16):4037–46.
[32] Tessitore A, Cicciarelli G, Del Vecchio F, Gaggiano A, Verzella D, Fischietti M, et al. MicroRNAs in the DNA damage/repair network and cancer. Int J Genomics 2014;2014:820248.
[33] Brennecke J, Aravin AA, Stark A, Dus M, Kellis M, Sachidanandam R, et al. Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. Cell 2007;128(6):1089–103.
[34] Chambeyron S, Popkova A, Payen-Groschene G, Brun C, Laouini D, Pelisson A, et al. piRNA-mediated nuclear accumulation of retrotransposon transcripts in the *Drosophila* female germline. Proc Natl Acad Sci USA 2008;105(39):14964–9.
[35] Shpiz S, Kwon D, Uneva A, Kim M, Klenov M, Rozovsky Y, et al. Characterization of *Drosophila* telomeric retroelement TAHRE: transcription, transpositions, and RNAi-based regulation of expression. Mol Biol Evol 2007;24(11):2535–45.
[36] Malone CD, Brennecke J, Dus M, Stark A, McCombie WR, Sachidanandam R, et al. Specialized piRNA pathways act in germline and somatic tissues of the *Drosophila* ovary. Cell 2009;137(3):522–35.
[37] Desset S, Buchon N, Meignin C, Coiffet M, Vaury C. In *Drosophila melanogaster* the COM locus directs the somatic silencing of two retrotransposons through both Piwi-dependent and -independent pathways. PLoS One 2008;3(2):e1526.
[38] Mevel-Ninio M, Pelisson A, Kinder J, Campos AR, Bucheton A. The flamenco locus controls the gypsy and ZAM retroviruses and is required for *Drosophila* oogenesis. Genetics 2007;175(4):1615–24.
[39] Pelisson A, Payen-Groschene G, Terzian C, Bucheton A. Restrictive flamenco alleles are maintained in *Drosophila melanogaster* population cages, despite the absence of their endogenous gypsy retroviral targets. Mol Biol Evol 2007;24(2):498–504.
[40] Aravin A, Gaidatzis D, Pfeffer S, Lagos-Quintana M, Landgraf P, Iovino N, et al. A novel class of small RNAs bind to MILI protein in mouse testes. Nature 2006;442(7099):203–7.
[41] Girard A, Sachidanandam R, Hannon GJ, Carmell MA. A germline-specific class of small RNAs binds mammalian Piwi proteins. Nature 2006;442(7099):199–202.
[42] Grivna ST, Beyret E, Wang Z, Lin H. A novel class of small RNAs in mouse spermatogenic cells. Genes Dev 2006;20(13):1709–14.
[43] Watanabe T, Takeda A, Tsukiyama T, Mise K, Okuno T, Sasaki H, et al. Identification and characterization of two novel classes of small RNAs in the mouse germline: retrotransposon-derived siRNAs in oocytes and germline small RNAs in testes. Genes Dev 2006;20(13):1732–43.
[44] Aravin AA, Naumova NM, Tulin AV, Vagin VV, Rozovsky YM, Gvozdev VA. Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the *D. melanogaster* germline. Curr Biol 2001;11(13):1017–27.
[45] Weick EM, Miska EA. piRNAs: from biogenesis to function. Development 2014;141(18):3458–71.
[46] Lin H, Spradling AC. A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the *Drosophila* ovary. Development 1997;124(12):2463–76.

[47] Brower-Toland B, Findley SD, Jiang L, Liu L, Yin H, Dus M, et al. *Drosophila* PIWI associates with chromatin and interacts directly with HP1a. Genes Dev 2007;21(18):2300–11.
[48] Li XZ, Roy CK, Moore MJ, Zamore PD. Defining piRNA primary transcripts. Cell Cycle 2013;12(11):1657–8.
[49] Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 2009;10(2):126–39.
[50] Hobson DJ, Wei W, Steinmetz LM, Svejstrup JQ. RNA polymerase II collision interrupts convergent transcription. Mol Cell 2012;48(3):365–74.
[51] Klattenhoff C, Xi H, Li C, Lee S, Xu J, Khurana JS, et al. The *Drosophila* HP1 homolog Rhino is required for transposon silencing and piRNA production by dual-strand clusters. Cell 2009;138(6):1137–49.
[52] Mohn F, Sienski G, Handler D, Brennecke J. The rhino-deadlock-cutoff complex licenses noncanonical transcription of dual-strand piRNA clusters in *Drosophila*. Cell 2014;157(6):1364–79.
[53] Zhang F, Wang J, Xu J, Zhang Z, Koppetsch BS, Schultz N, et al. UAP56 couples piRNA clusters to the perinuclear transposon silencing machinery. Cell 2012;151(4):871–84.
[54] Czech B, Preall JB, McGinn J, Hannon GJ. A transcriptome-wide RNAi screen in the *Drosophila* ovary reveals factors of the germline piRNA pathway. Mol Cell 2013;50(5):749–61.
[55] Saito K, Sakaguchi Y, Suzuki T, Suzuki T, Siomi H, Siomi MC. Pimet, the *Drosophila* homolog of HEN1, mediates 2′-O-methylation of Piwi-interacting RNAs at their 3′ ends. Genes Dev 2007;21(13):1603–8.
[56] Aravin AA, Hannon GJ, Brennecke J. The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. Science 2007;318(5851):761–4.
[57] Houwing S, Kamminga LM, Berezikov E, Cronembold D, Girard A, van den Elst H, et al. A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in Zebrafish. Cell 2007;129(1):69–82.
[58] Klenov MS, Sokolova OA, Yakushev EY, Stolyarenko AD, Mikhaleva EA, Lavrov SA, et al. Separation of stem cell maintenance and transposon silencing functions of Piwi protein. Proc Natl Acad Sci USA 2011;108(46):18760–5.
[59] Le Thomas A, Rogers AK, Webster A, Marinov GK, Liao SE, Perkins EM, et al. Piwi induces piRNA-guided transcriptional silencing and establishment of a repressive chromatin state. Genes Dev 2013;27(4):390–9.
[60] Saito K, Inagaki S, Mituyama T, Kawamura Y, Ono Y, Sakota E, et al. A regulatory circuit for piwi by the large Maf gene traffic jam in *Drosophila*. Nature 2009;461(7268):1296–9.
[61] Castaneda J, Genzor P, van der Heijden GW, Sarkeshik A, Yates 3rd JR, Ingolia NT, et al. Reduced pachytene piRNAs and translation underlie spermiogenic arrest in maelstrom mutant mice. EMBO J 2014;33(18):1999–2019.
[62] Aravin AA, Sachidanandam R, Bourc'his D, Schaefer C, Pezic D, Toth KF, et al. A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. Mol Cell 2008;31(6):785–99.
[63] Gou LT, Dai P, Yang JH, Xue Y, Hu YP, Zhou Y, et al. Pachytene piRNAs instruct massive mRNA elimination during late spermiogenesis. Cell Res 2014;24(6):680–700.
[64] Kuramochi-Miyagawa S, Watanabe T, Gotoh K, Totoki Y, Toyoda A, Ikawa M, et al. DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. Genes Dev 2008;22(7):908–17.
[65] Carmell MA, Girard A, van de Kant HJ, Bourc'his D, Bestor TH, de Rooij DG, et al. MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. Dev Cell 2007;12(4):503–14.
[66] Ruby JG, Jan C, Player C, Axtell MJ, Lee W, Nusbaum C, et al. Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in *C. elegans*. Cell 2006;127(6):1193–207.
[67] Ashe A, Sapetschnig A, Weick EM, Mitchell J, Bagijn MP, Cording AC, et al. piRNAs can trigger a multigenerational epigenetic memory in the germline of *C. elegans*. Cell 2012;150(1):88–99.
[68] Shirayama M, Seth M, Lee HC, Gu W, Ishidate T, Conte Jr D, et al. piRNAs initiate an epigenetic memory of nonself RNA in the *C. elegans* germline. Cell 2012;150(1):65–77.
[69] Brennecke J, Malone CD, Aravin AA, Sachidanandam R, Stark A, Hannon GJ. An epigenetic role for maternally inherited piRNAs in transposon silencing. Science 2008;322(5906):1387–92.
[70] de Vanssay A, Bouge AL, Boivin A, Hermant C, Teysset L, Delmarre V, et al. Paramutation in *Drosophila* linked to emergence of a piRNA-producing locus. Nature 2012;490(7418):112–5.
[71] Chandler V, Alleman M. Paramutation: epigenetic instructions passed across generations. Genetics 2008;178(4):1839–44.
[72] Rassoulzadegan M, Grandjean V, Gounon P, Vincent S, Gillot I, Cuzin F. RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse. Nature 2006;441(7092):469–74.
[73] Devanapally S, Ravikumar S, Jose AM. Double-stranded RNA made in *C. elegans* neurons can enter the germline and cause transgenerational gene silencing. Proc Natl Acad Sci USA 2015;112(7):2133–8.
[74] Napoli C, Lemieux C, Jorgensen R. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell 1990;2(4):279–89.
[75] Sijen T, Plasterk RH. Transposon silencing in the *Caenorhabditis elegans* germ line by natural RNAi. Nature 2003;426(6964):310–4.
[76] Siomi MC, Saito K, Siomi H. How selfish retrotransposons are silenced in *Drosophila* germline and somatic cells. FEBS Lett 2008;582(17):2473–8.
[77] Wang X, Hsueh YP, Li W, Floyd A, Skalsky R, Heitman J. Sex-induced silencing defends the genome of *Cryptococcus neoformans* via RNAi. Genes Dev 2010;24(22):2566–82.
[78] Nolan T, Braccini L, Azzalin G, De Toni A, Macino G, Cogoni C. The post-transcriptional gene silencing machinery functions independently of DNA methylation to repress a LINE1-like retrotransposon in *Neurospora crassa*. Nucleic Acids Res 2005;33(5):1564–73.

[79] Dang Y, Yang Q, Xue Z, Liu Y. RNA interference in fungi: pathways, functions, and applications. Eukaryot Cell 2011;10(9):1148–55.
[80] Lee HC, Aalto AP, Yang Q, Chang SS, Huang G, Fisher D, et al. The DNA/RNA-dependent RNA polymerase QDE-1 generates aberrant RNA and dsRNA for RNAi in a process requiring replication protein A and a DNA. Helicase PLoS Biol 2010;8(10).
[81] Catalanotto C, Pallotta M, ReFalo P, Sachs MS, Vayssie L, Macino G, et al. Redundancy of the two dicer genes in transgene-induced posttranscriptional gene silencing in *Neurospora crassa*. Mol Cell Biol 2004;24(6):2536–45.
[82] Lee HC, Chang SS, Choudhary S, Aalto AP, Maiti M, Bamford DH, et al. qiRNA is a new type of small interfering RNA induced by DNA damage. Nature 2009;459(7244):274–7.
[83] Yang Q, Ye QA, Liu Y. Mechanism of siRNA production from repetitive DNA. Genes Dev 2015;29(5):526–37.
[84] Zhang Z, Chang SS, Zhang Z, Xue Z, Zhang H, Li S, et al. Homologous recombination as a mechanism to recognize repetitive DNA sequences in an RNAi pathway. Genes Dev 2013;27(2):145–50.
[85] Yamanaka S, Siomi H. diRNA-Ago2-RAD51 complexes at double-strand break sites. Cell Res 2014;24(5):511–2.
[86] Wei W, Ba Z, Gao M, Wu Y, Ma Y, Amiard S, et al. A role for small RNAs in DNA double-strand break repair. Cell 2012;149(1):101–12.
[87] Francia S, Michelini F, Saxena A, Tang D, de Hoon M, Anelli V, et al. Site-specific DICER and DROSHA RNA products control the DNA-damage response. Nature 2012;488(7410):231–5.
[88] Michalik KM, Bottcher R, Forstemann K. A small RNA response at DNA ends in *Drosophila*. Nucleic Acids Res 2012;40(19):9596–603.
[89] Orel N, Kyryk A, Puchta H. Different pathways of homologous recombination are used for the repair of double-strand breaks within tandemly arranged sequences in the plant genome. Plant J 2003;35(5):604–12.
[90] Herr AJ, Jensen MB, Dalmay T, Baulcombe DC. RNA polymerase IV directs silencing of endogenous DNA. Science 2005;308(5718):118–20.
[91] Gao M, Wei W, Li MM, Wu YS, Ba Z, Jin KX, et al. Ago2 facilitates Rad51 recruitment and DNA double-strand break repair by homologous recombination. Cell Res 2014;24(5):532–41.
[92] Tang D, Wu D, Hirao A, Lahti JM, Liu L, Mazza B, et al. ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. J Biol Chem 2002;277(15):12710–7.
[93] Chapman JR, Taylor MR, Boulton SJ. Playing the end game: DNA double-strand break repair pathway choice. Mol Cell 2012;47(4):497–510.
[94] Karanam K, Kafri R, Loewer A, Lahav G. Quantitative live cell imaging reveals a gradual shift between DNA repair mechanisms and a maximal use of HR in mid S phase. Mol Cell 2012;47(2):320–9.
[95] Helmink BA, Tubbs AT, Dorsett Y, Bednarski JJ, Walker LM, Feng Z, et al. H2AX prevents CtIP-mediated DNA end resection and aberrant repair in G1-phase lymphocytes. Nature 2011;469(7329):245–9.
[96] Bunting SF, Callen E, Wong N, Chen HT, Polato F, Gunn A, et al. 53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks. Cell 2010;141(2):243–54.
[97] Yun MH, Hiom K. CtIP-BRCA1 modulates the choice of DNA double-strand-break repair pathway throughout the cell cycle. Nature 2009;459(7245):460–3.
[98] Verdel A, Jia S, Gerber S, Sugiyama T, Gygi S, Grewal SI, et al. RNAi-mediated targeting of heterochromatin by the RITS complex. Science 2004;303(5658):672–6.
[99] Tattermusch A, Brockdorff N. A scaffold for X chromosome inactivation. Hum Genet 2011;130(2):247–53.
[100] Grosshans H, Johnson T, Reinert KL, Gerstein M, Slack FJ. The temporal patterning microRNA let-7 regulates several transcription factors at the larval to adult transition in *C. elegans*. Dev Cell 2005;8(3):321–30.
[101] Wang X, Arai S, Song X, Reichart D, Du K, Pascual G, et al. Induced ncRNAs allosterically modify RNA-binding proteins in cis to inhibit transcription. Nature 2008;454(7200):126–30.