Review

A direct role for small non-coding RNAs in DNA damage response

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Historically, the role of cellular RNA has been subordinate and ancillary to DNA. Protein-coding mRNA conveys the information content of DNA, and transfer RNAs and ribosomal RNAs allow the polymerization of amino acids into proteins. The discovery of non-protein-coding RNAs (ncRNAs) provided an additional role for RNA in finely tuning DNA expression. However, it has recently become apparent that the safeguard of DNA integrity depends on small ncRNAs acting at the site of DNA lesions to signal the presence of DNA damage in the cell, and on the genes involved in their biogenesis to achieve accurate DNA repair. I review here evidence supporting a role for small ncRNAs, termed DNA damage-response RNAs (DDRNAs) or double-strand break (DSB)-induced RNAs (diRNAs), that are generated at sites of DNA damage and control the DNA damage response (DDR). I also discuss their biogenesis, potential mechanisms of action, and their relevance in cancer.

DNA damage response: a role for RNA

With the exception of RNA-based viruses and their DNA-encoded evolutionary remnants such as the specialized RNA-dependent DNA polymerase telomerase, the information necessary to life is stored in DNA. To avoid risks of information loss or corruption, it is imperative for a cell to preserve the structural integrity of its DNA. To this end, upon DNA damage cells mount a prompt DDR that prevents cell cycle progression of the damaged cell by enforcing cell cycle checkpoints and coordinating DNA damage repair (Box 1 and Figure I).

DDR activation relies on the coordinated recruitment of specialized DDR proteins at sites of DNA damage. DDR factors initially associate directly with DNA lesions. Subsequent protein modifications and additional interactions promote the accumulation of DDR factors, which form nuclear DDR foci large enough to be visualized by standard immunofluorescence techniques. DDR-mediated cellular outcomes may be cell death by apoptosis, a transient cell cycle arrest (checkpoint) followed by repair of DNA damage and resumption of proliferation, or cellular senescence caused by the persistence of unrepaired DNA damage [1,2].

The prevailing view has been that the DDR involves only proteins, and signal generation and amplification is achieved by protein interactions regulated by various protein modifications. RNA becomes involved only in the form of transcriptional outcome of DDR activation at the bottom of the signal cascade. Recently, a paradigmatic shift has changed this view and DDR activation has been shown to depend on small ncRNAs generated at sites of DNA damage. This review will discuss the biogenesis and roles of these RNAs, how they may act in the control of the DDR, and the potential relevance of these findings in cancer.

ncRNAs

It is now realized that the vast majority of the genome is transcribed [3]. Often these transcripts do not code for proteins, but are, nevertheless, biologically functional. Some of these ncRNAs are nuclear and may remain associated with chromatin in a sequence-specific manner to control epigenetic modifications of chromatin [4]. Some ncRNAs may aggregate in membrane-less subcellular structures where they regulate the localization and the activity of proteins or provide structural support, which leads to the regulation of a variety of cellular functions (Box 2). Some long ncRNAs [such as LincRNA-p21, PANDA (promoter of CDKN1A antisense DNA damage activated RNA), and a long ncRNA associated with the cyclin D1 (CCND1) gene], have also been reported to respond to genotoxic stress [5–7]. However, they have not been shown to localize directly and act at sites of DNA damage, suggesting they may act as downstream modulators of gene expression during the DDR.

Various small ncRNAs act in the RNA interference (RNAi) pathway, an evolutionarily conserved machinery whose components are thought to have originally evolved to preserve genomes from attacks by viruses and mobile genetic elements [8]. RNAi precursors are processed by RNases such as DROSHA and DICER (double-stranded RNA-specific endoribonucleases type III) to generate small double-stranded (ds) RNA products [9]. According to their biogenesis and functions, mature RNA products are classified as either small interfering RNAs (siRNAs), repeat-associated small interfering RNAs (rasiRNAs), Piwi-interacting RNAs (piRNAs) [QDE-2 interacting RNAs (qiRNA) in *Neurospora crassa*], and microRNAs (miRNAs)

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Box 1. Mechanisms of DDR

DDR is triggered by the recognition of DNA discontinuities in the form of DSBs or exposed regions of single-stranded DNA that are respectively recognized by two specialized DNA damage sensors: the MRN complex [meiotic recombination 11 homolog B (MRE11)–RAD50–Nijmegen breakage syndrome 1 (nibrin/NBS1)] and replication protein A (RPA), together with the 911 complex [RAD9–RAD1–HUS1 (HUS1 checkpoint homolog)] [51]. These DNA damage sensors recruit the apical protein kinases ATM through interaction with the MRN complex, and ATR (ataxia telangiectasia and Rad3-related) through interaction with RPA and the 911 complex, which is associated with the ATR-interacting protein (ATRIP). The activation of these protein kinases in turn modifies a variant of histone H2A known as H2AX on Ser139 (generating the so-called γH2AX) on the chromatin in *cis*, starting from the region most proximal to the DNA lesion and spreading distally for up to hundreds of kb. This amplification mechanism relies on the recruitment of the mediator of DNA damage checkpoint 1 (MDC1) which, together with 53BP1, sustains and amplifies DDR signaling by enforcing further accumulation of the MRN complex and activation of ATM. DDR signal amplification relies on additional mechanisms based on ubiquitylation of DDR factors [52]. Passed its threshold, DDR signaling spreads away from the damaged locus, and this is dependent on engagement of diffusible kinases CHK1 and CHK2 (checkpoint kinases 1/2). Ultimately, signals converge on cell cycle progression regulators such as p21, whose induction is mediated by p53, and on the protein phosphatase CDC25 (cell division cycle 25C). In parallel to checkpoint enforcement, DNA repair mechanisms are activated including homologous recombination (HR) and non-homologous DNA end joining (NHEJ). During HR, a single-stranded 3′ DNA end of the broken DNA invades a dsDNA with a homologous sequence, and restores the damaged DNA by using the intact homologous sequence as a template. Alternatively, during NHEJ, DNA ends are sealed back together by a DNA ligase. The choice between the two repair mechanisms is cell cycle regulated [53] and only upon full DNA repair is cell proliferation allowed to proceed [2] (Figure I).

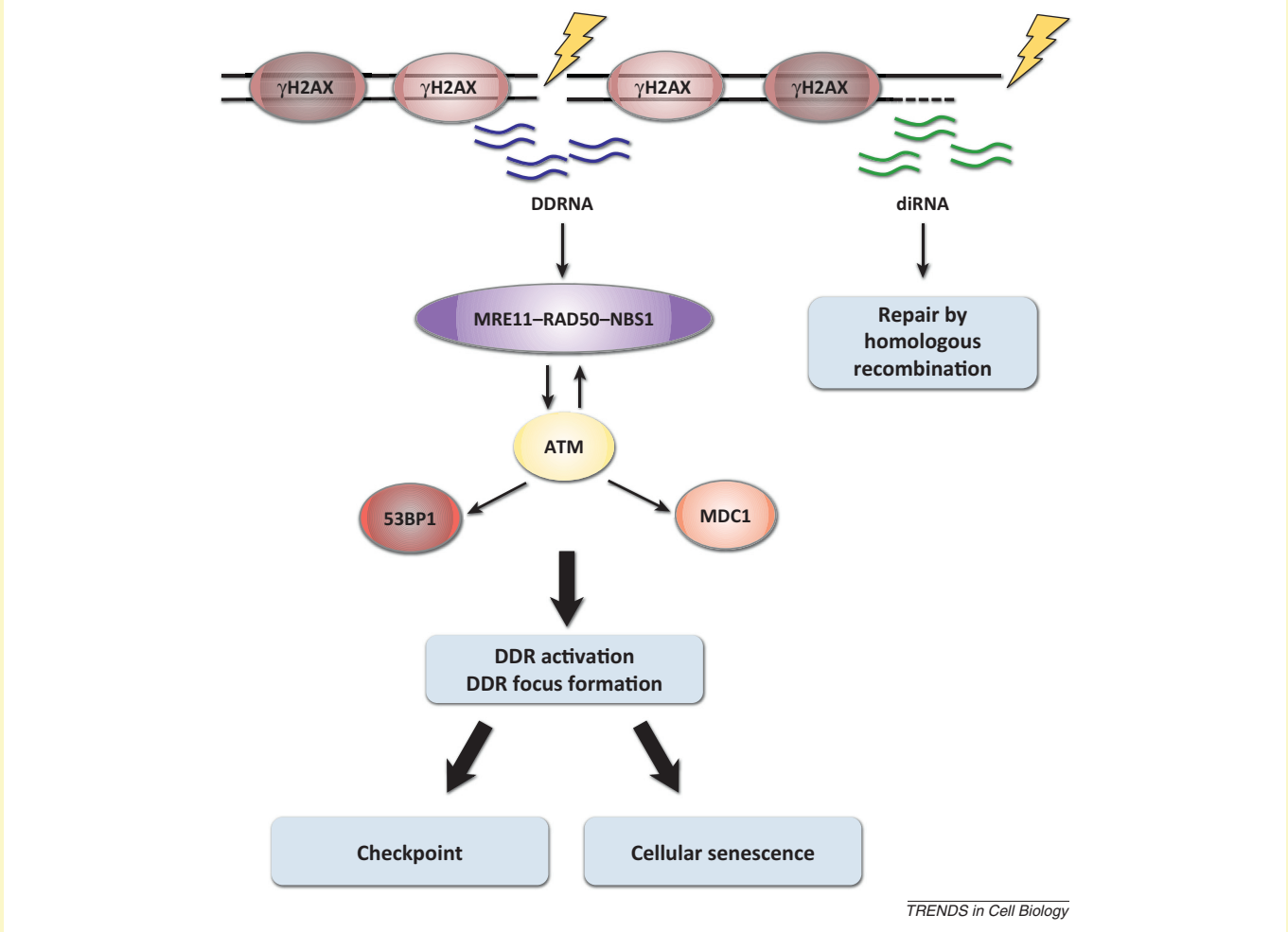

Figure I. Mechanisms of DNA damage response (DDR). Abbreviations: ATM, ataxia telangiectasia mutated; 53BP1, tumor protein p53 binding protein 1; DDRNA, DNA damage-response RNA; diRNA, double-strand break (DSB)-induced RNAs; γH2AX, γ-H2A histone family, member X; MDC1, mediator of DNA-damage checkpoint 1; MRE11, meiotic recombination 11 homolog B; NBS1, Nijmegen breakage syndrome 1 (nibrin); RAD50, DNA repair protein RAD50 homolog.

[9]. Only miRNA maturation is considered dependent on both DROSHA and DICER endoribonucleases. In mammals, miRNAs modulate gene expression by their ability to regulate mRNA translation and stability through their Argonaut (Ago) effector proteins.

There is evidence of interplay between small ncRNA and DDR. miRNAs control, among several targets, the expression of DDR genes including *ATM* (ataxia telangiectasia mutated) [10], *PRKDC* (protein kinase, DNA-activated, catalytic polypeptide, also known as DNA-PKcs) [11], *BRCA1* (breast cancer 1) [12], *H2AX* (H2A histone family, member X) [13] and *RAD51* (RAD51 recombinase) [14]. Furthermore, DDR factors can directly modulate the biogenesis of miRNAs by controlling their maturation: ATM phosphorylates KSRP (KH-type splicing regulatory protein), a DROSHA interactor [15], BRCA1 interacts with DROSHA

Box 2. DDR foci, nuclear bodies, speckles and paraspeckles: role of RNA in the organization of membrane-less subnuclear structures

DDR foci are discrete subnuclear globular structures associated with chromatin at the DNA damage site where DDR signaling originates and is amplified. Constituents of DDR foci are several copies of individual upstream DDR factors. Although DNA damage sensors and their associated kinases directly detect DNA lesions, their accumulation (and the accumulation of additional DDR factors) – to the extent of forming detectable nuclear foci up to a few μm in size – indicates that additional recruitment mechanisms are involved. DDR focus formation depends on γH2AX, which spreads hundreds of kb from the lesion and provides a nucleating platform for focus building by secondary recruitment of multiple copies of DDR factors. Within foci, DDR factor accumulation is dynamic [35]. The high concentration of interacting proteins, enzymes, and substrates within the limited confines of the focus likely facilitates their function. Indeed, tethering some factors to an undamaged locus is sufficient to trigger full DDR focus formation and signaling [54].

DDR foci are not the only known discrete subnuclear domains. Numerous nuclear bodies, distinguishable by the proteins that preferentially inhabit them, have been involved in a variety of nuclear functions [55]. RNA is often a component of nuclear bodies, either acting as the guest and being the substrate of processing reactions within them, or acting as the host and being a crucial architectural and seeding component. Examples of the latter include histone mRNAs that are tethered to ectopic genomic loci and foster histone locus body (HLB) formation through recruitment of specific HLB protein components [56]. Similarly, paraspeckles, subnuclear structures involved in RNA metabolism, can be nucleated by the ncRNA NEAT1 [nuclear paraspeckle assembly transcript 1 (non-protein coding), also known as Men ε/β] which plays an essential structural role by binding specifically to key paraspeckle proteins [55,57].

Although evidence of RNAs seeding such macromolecular structures abounds, self-assembly of RNA binding proteins may rely on proteins alone, with the contribution of protein domains such as the low complexity (LC) domains, unstructured regions with little amino acid heterogeneity. Recently these protein domains have been shown to form hydrogels, semi-solid structures capable of holding their shapes [58], at room temperature. *In vitro* generated hydrogels can bind and retain the same RNAs bound in living cells. The intriguing observation that hydrogel formation is sensitive to the phosphorylation of an amino acid residue targeted, at least *in vitro*, by a DNA damage kinase suggests that post-translational modifications, and more enticingly those occurring upon DNA damage, can control the stability of these aggregates.

The ability of soluble proteins to assemble into semi-solid structures is reminiscent of other phase transition events including P granules. P granules are RNA and protein-rich discrete subnuclear structures that have been reported to be liquid [59]. P granules display behaviors typical of viscous liquid droplets such as deformability, dripping and fusing. Their ability to self-assemble from a pool of soluble factors into an aggregate, in which subunit components exchange constantly, is akin to a classical phase transition.

Thus, the ability of RNA, based on networks of multivalent weak interactions, to seed the formation of macromolecular complexes, including morphologically distinct membrane-less subcompartments within the cell, appears to be an overarching theme across several cellular functions/events including DDR.

[16], p53 binds the RNA helicase DDX5 (DEAD box helicase 5) [17], and an ATM-dependent phosphoproteome screen identified components of the DROSHA complex [18].

Although the above results highlight how DDR factors may interact with proteins involved in ncRNA maturation, scattered evidence also indicate that some DDR factors, such as 53BP1 (tumor protein p53 binding protein 1), BRCA1, Ku, and ATR (ataxia telangiectasia and Rad3-related), can bind directly to ncRNAs [19–22]. In support of their role in mediating DNA damage, piRNAs and qiRNAs have well-established roles in genome stability maintenance, and these RNAs in ciliated protozoans have been shown to guide the deletion or the retention of homologous genomic sequences during genome rearrangements [23]. Furthermore, studies suggest a potential template role for RNA in DNA repair events [24,25]. Collectively, these studies suggest that RNA molecules directly modulate DDR signaling and DNA repair events. Recently, ncRNAs have been shown to be involved in the DDR of higher eukaryotes, which I discuss below.

DDRNA and DDR in vertebrates

Inactivation of DICER or DROSHA in a variety of animal systems was reported to lead to impaired DDR activation [26]. In this study, oncogene-induced DNA replication stress or ionizing radiation (IR) was induced in cells to promote the formation of DDR foci. Knockdown of DICER or DROSHA, but not the three GW182 (TNRC6A, trinucleotide repeat containing 6A)-like proteins, which are downstream effectors of RNAi-mediated translational repression, reduced the formation and maintenance of DDR foci containing upstream signaling factors such as the activated forms of ATM, 53BP1, and MDC1 (mediator of DNA-damage checkpoint 1). γH2AX focal signals were mildly reduced, likely due to their redundant phosphorylation by DNA-PKcs, its paralog involved in DNA repair, in addition to ATM and ATR. The RNA endonuclease activity of DICER is essential for DDR control, as demonstrated by the evidence that wild type DICER, but not an endonuclease mutant, can rescue DDR activation in DICER knockdown cells [26]. Although untested, DROSHA is likely to act similarly. In addition to reduced accumulation in DDR foci, ATM autophosphorylation and activation are impaired upon DICER or DROSHA inactivation, as determined in cell lysates [26]. Presently it is unknown whether ATR is also affected. Similar observations were made in cell lines carrying stable hypomorphic alleles of DICER [26]. DICER was also shown to control DDR in various tissues of zebrafish larvae, as demonstrated by reduced ATM activation; however, in this animal γH2AX is affected by DICER loss, likely due to the apparent lack, or poor conservation, of DNA-PKcs. Furthermore, impaired DDR activation upon DICER and DROSHA knockdown results in the loss of G1/S and G2/M cell cycle checkpoints and in the escape of oncogene-expressing cells from cellular senescence [26], a tumor-suppressive mechanism.

Given the role of DICER or DROSHA in miRNA maturation and the pleiotropic impact of miRNA misregulation, these observations may be the indirect outcome of altered gene expression – although the lack of a role of GW128-like proteins on DDR suggests a mechanism of action distinct from that of miRNA (Table 1). Experimental evidence suggests a direct role for RNA in DDR foci formation. Irradiated cells depleted of cellular RNA after treatment with RNase A and a transcription inhibitor resulted in reduced maintenance of DDR foci, with the exception of

Table 1. miRNA and DDRNA

miRNA	DDRNA
miRNA act by targeting cellular messenger RNAs	DDRNA can act in the absence of cellular mRNA targets: 1. Chemically synthesized DDRNAs allow DDR focus reformation in RNase A-treated cells devoid of any RNA 2. DDRNAs carry the sequences of the lesion sites: they cannot target specific cellular RNAs to modulate DDR 3. DDRNAs with no known homology to mRNA targets were demonstrated to control DDR focus reformation
miRNAs are encoded by cellular genes and act in *trans* by targeting mRNAs	DDRNA are synthesized at sites of DNA damage and act in *cis* at the site of DNA damage
GW182-like proteins family [RNA-induced silencing complex (RISC) components] are necessary for RNAi activity of miRNA	GW182-like proteins family are not necessary for DDR
miRNA are slow: they modulate biological processes indirectly by modulating the expression of mRNAs	DDRNA are fast: they modulate the DDR directly by acting at sites of damage (in RNase A-treated cells, DDRNAs trigger focus reformation within 20 min at room temperature)

γH2AX [26]. Furthermore, upon removal of RNase A and the transcription inhibitor, DDR foci can reform after RNA synthesis resumes, suggesting that both foci dissolution and reformation depend on RNA. Indeed, addition of total cellular RNA to permeabilized RNase A-treated cells allows foci to reform. Fractionation of RNAs according to size indicates that small (20–35 nt) RNAs are sufficient for DDR foci to reform. This result, combined with the observation that RNA extracted from cells lacking functional DICER or DROSHA does not support DDR foci reformation in RNase A-treated cells, indicates that DDR activation at sites of DNA damage is controlled by small RNAs generated in a DICER- and DROSHA-dependent manner.

To determine the genomic locus of origin of such small RNAs, a cell line was used which was generated by the chromosomal integration of a DNA construct carrying bacterial repeats surrounding a unique cut-site for an inducible endonuclease. It was observed that, upon RNase A treatment, the DDR focus does not reform upon addition of RNA from parental cells lacking the integrated locus; instead the focus reforms when RNA from cells carrying the locus is used [26]. Moreover, the generation of random DNA damage in parental cells by IR does not induce the synthesis of RNA active in DDR focus reformation. These results show that the RNAs necessary for DDR focus formation at a given genomic site are not coded by the parental cell, but are instead generated at the damage site. Next-generation sequencing of small RNAs revealed the existence of several small RNAs mapping to this cut locus [26], some with the potential to form double-stranded species. Their number and size distribution change upon induction of the endonuclease and upon Dicer or Drosha knockdown. These features and the observed base-bias at their 5′ and 3′ ends suggest that these RNAs, named DNA damage response RNAs (DDRNAs), are products of a specific enzymatic cleavage and possess biological activity. Indeed, chemically synthesized DDRNAs with the sequence of the cut locus (but not control RNAs of similar size but with a different sequence) allowed dose-dependent and site-specific DDR focus reformation in RNase A-treated cells. Thus, DDR activation at a specific genomic site is associated with the biogenesis of DDRNAs which carry the sequence of the damaged exogenous bacterial DNA. Although mechanisms of DDRNA biogenesis may be shared with those of miRNAs, the mechanisms of action of DDRNAs are distinct (Table 1).

diRNA and DNA repair in plants and mammals

Additional independent evidence has complemented the results described above. Using an *Arabidopsis thaliana* transgenic plant line carrying an inducible DSB repairable by single-strand annealing [SSA – a form of homologous recombination (HR)], it was observed that DSB repair is diminished in plants mutant for ATR or Dicer-like proteins DCL2, DCL3, and DCL4 [27]. Unaltered levels for a number of HR genes were reported, suggesting the effect may not be mediated by canonical miRNA mechanisms suppressing the expression of DNA repair genes tested. Using a probe that spanned a DSB site, Northern blotting detected small (21–24 nt) RNAs termed diRNAs. These diRNAs are specifically induced upon DSB formation and were dependent on ATR and Dicer-like genes. Indeed, deep sequencing confirmed the induction of both sense and antisense transcripts. diRNA abundance and DSB repair was reduced upon impairment of RNA polymerase (Pol) IV, whereas inactivation of RNA Pol V, involved in the synthesis of nascent transcripts targeted by RNA-dependent RNA polymerases in plants, increased diRNA abundance but reduced DSB repair. These observations suggest that at least two distinct RNA polymerases are involved, with distinct roles in diRNA synthesis and DNA repair functions. Furthermore, their RNA products may functionally interact. RNA-dependent RNA polymerases RDR2 and RDR6 also contribute to diRNA biogenesis. Their mutation reduces diRNA levels without affecting DNA repair rates. Searching for effector molecules of diRNAs, the authors observed that Ago2 is the only Argonaute protein induced upon DNA damage in plants. Ago2 is associated with diRNAs and its mutation reduces diRNA abundance and DSB repair. Consistent with some observations in mammals [26], γH2AX foci in irradiated plant cells were unaffected in RNA Pol IV, DCL3, or AGO2 mutants. By the use of a similar cassette carrying an inducible DSB repairable by HR in a human cell line, the authors observed the generation of sense and antisense transcripts in the region surrounding the DSB and decreased DNA repair upon DICER or AGO2 inactivation. Thus, both in plants and humans, DSBs trigger the formation of small ncRNAs at sites of DNA damage, and inactivation of genes involved in

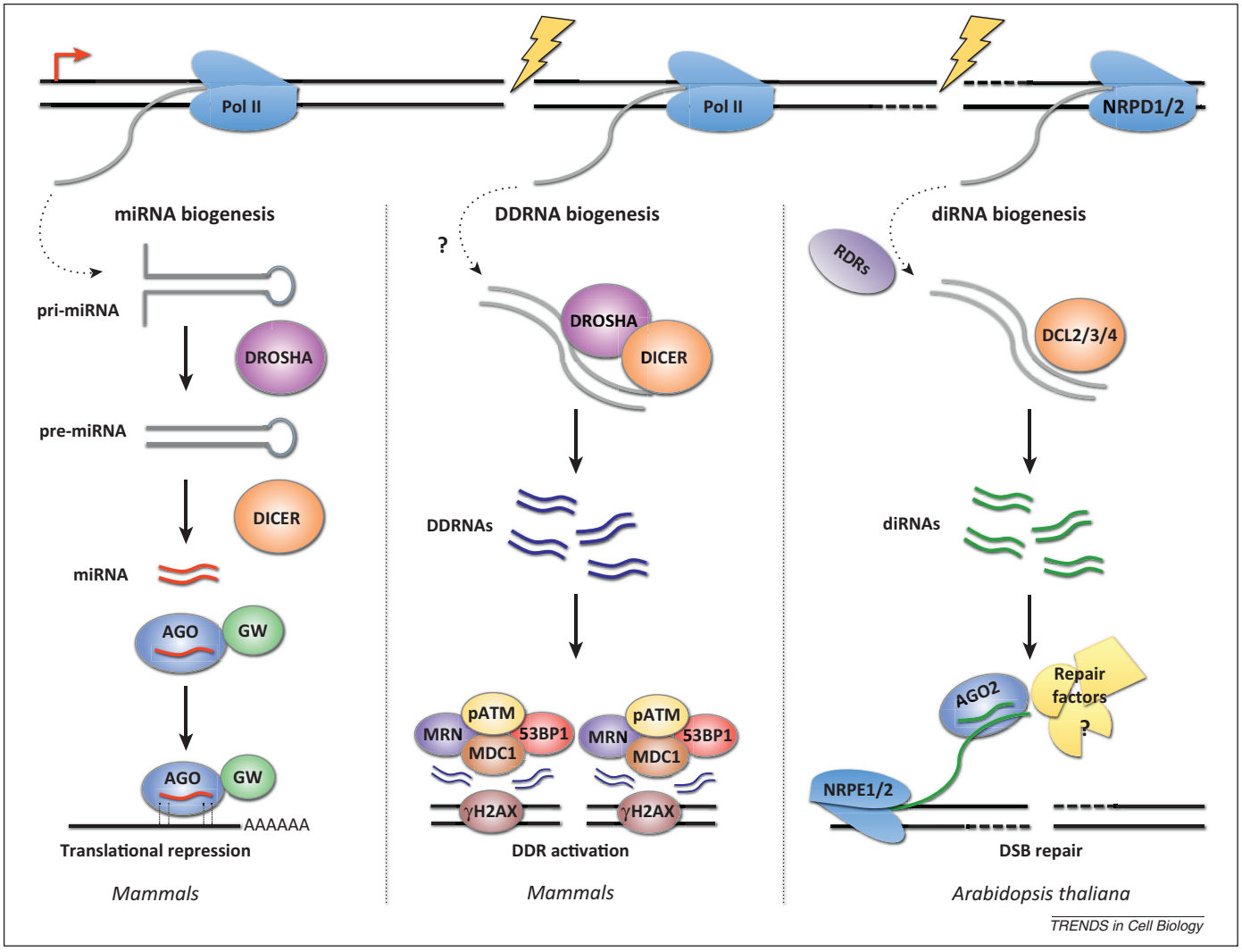

Figure 1. Schematic representation of the biogenesis of microRNAs (miRNAs), DNA damage-response RNAs (DDRNAs), and double-strand break (DSB)-induced RNAs (diRNAs). For further details and abbreviations see text and Figure I legend.

Table 2. Genes tested for small ncRNA biogenesis and functions in DDR

DDRNA in mammals	DDRNAs in Zebrafish (*Danio rerio*)	diRNAs in *Arabidopsis thaliana*	diRNAs in human cells	Small RNAs in *Drosophila*
DICER and DROSHA: necessary for DDR foci formation and maintenance, DDR checkpoints enforcement, and DDRNAs biogenesis	DICER: necessary for DDR activation	Dicer-like proteins DCL2, DCL3, and DCL4: necessary for diRNA accumulation and DSB repair	DICER and AGO2: necessary for diRNA accumulation and DSB repair	Dcr-2, Loqs-D (Loquacious), and Ago2: necessary to decrease GFP signal from linearized plasmid
RNA Pol II: necessary for DDR focus reformation (as determined by α-amanitin sensitivity)		RNA Pol IV (DNA-dependent RNA polymerase): necessary for diRNA accumulation and DSB repair		Ago1 and Dcr-1: partially necessary to decrease GFP signal from linearized plasmid
TNRC6A, B, and C (GW182-like proteins): not necessary in DDR foci formation and maintenance		RNA Pol V (DNA-dependent RNA polymerase): not necessary for diRNA accumulation but necessary for DSB repair		
		RDR2 and RDR6 (RNA-dependent RNA polymerases): necessary for diRNA accumulation but not necessary for DSB repair		
		ATR: necessary for diRNA abundance and DSB repair		
		AGO2: necessary for diRNA accumulation and DSB repair		
		AGO4: not necessary for diRNA accumulation and DSB repair		

their biogenesis or stability reduces DNA repair by HR. In both reports [26,27], small ncRNA sequences could not be accurately and quantitatively mapped due to repeat sequences flanking the DSB. It is therefore presently unclear how far from the break small ncRNAs can be generated.

The higher abundance of diRNAs observed in plants compared to humans is consistent with the strong role of RNAi in plants as a mechanism of innate immunity and the engagement of RNA-dependent RNA polymerases able to amplify their synthesis.

Small RNAs generated at DNA ends in *Drosophila*

In cultured *Drosophila* cells, transfection of a linearized plasmid leads to the generation of small (21 nt) RNAs containing the sequence of the plasmid DNA ends [28]. Concomitant with the generation of these small RNAs is the reduced expression of the marker encoded by the plasmid. Repression of this marker may be the result of the RNA interference activity of these small RNAs acting as endo-siRNAs. Indeed, inactivation of some of the factors involved in this pathway relieves the observed repression. Interestingly, these small RNAs are reported to be able to act in *trans* and repress the expression of genes bearing homologous sequences. Although generated from DNA ends, and as such intrinsically similar to the events taking place at a DSB, the role of these small RNAs in *Drosophila* remains to be clarified.

Although several important features make these short ncRNAs different from canonical miRNAs (Table 1) [26–28], these small ncRNAs may share features among themselves. Indeed, small dsRNAs at DNA ends are generated through partially evolutionarily shared pathways (Figure 1, Table 2). However, it remains to be demonstrated formally that the different functions individually attributed to them (DDR activation, DNA repair, and transcriptional repression) are shared among them, making the differences between them purely semantic.

Concluding remarks

RNA biogenesis near DSB sites

The issue of transcription at DSBs is a very relevant matter because the detection of DDRNAs containing sequences of the DNA next to the DSB, and the involvement of DICER and DROSHA in DDRNA biogenesis, indicates that transcription near the DSB and synthesis of a long RNA precursor must occur.

Transcription at DSBs has been intensely studied by several groups. Transcription by RNA Pol II of a chromosome-integrated exogenous reporter gene is repressed in *cis* in an ATM-dependent manner upon induction of a cluster of DSBs upstream of the reporter [29]. This is consistent with the reported role of MDC1 in silencing sex chromosomes in meiosis and in the exclusion of nascent transcription at DNA lesion sites [30–32]. By contrast, a recent report using a different cell system, in which individual DSB are induced at discrete endogenous sequences, observed that a DSB alone does not affect the transcription of adjacent genes, but does inhibit their transcription, in a DNA-PK-dependent manner, if the DSB occurs within a transcription unit [33]. The observation that DSBs next to, but not within, transcriptional units do not reduce transcription is further supported by the seemingly unaltered transcription within DNA damage-induced γH2AX-containing chromatin regions [34]. Thus, the results obtained may be different because of the different experimental setups exploited. The use of single-cell versus pooled-cell analyses and persistent versus transient DNA lesions may affect the outcome. Importantly, the above analyses focused on 'canonical transcripts', whereas the events associated with short ncRNA biogenesis have not been studied and may be different. Regardless of differences, a precursor transcript must be generated at DNA lesions. It remains to be elucidated whether its generation precedes the occurrence of a break, and DDRNAs arise from precursor processing following DNA damage, or whether the precursor itself is induced by DNA damage. The latter option is supported by the observation that DDR foci are dynamic structures [35] that can persist for long periods of time [2], therefore making their persistence dependent on a single initial RNA precursor seem unlikely. Furthermore, when RNase A treatment removes RNA, DDR foci promptly reform if RNA neosynthesis is allowed, indicating that, at DNA damage sites, RNA is constantly being synthesized, processed, and probably degraded. Of note, because DDR activation and focus formation is expected to occur when DSBs are generated outside well-recognized transcriptional units, the transcriptional events (if any) in these regions remains to be determined, including the potential impact on (or involvement of) 'pervasive transcription' [3].

Whichever mechanism leads to the synthesis of precursor RNA, it also remains to be established how it is processed. The observation that transcripts from both strands are detectable [26–28] suggests that both strands are transcribed to generate a long dsRNA, which is dependent on DICER. The involvement of DROSHA in this process remains to be clarified. In addition, RNA-dependent RNA polymerases, as observed in plants, may also generate a suitable substrate for DICER.

Furthermore, it remains to be determined whether processing of precursor RNA occurs at the DNA lesion sites, seemingly the most parsimonious model, or in the cytoplasm, where most, but not all, DICER molecules can be found [36].

Mechanisms of action

The most daunting question remains the clarification of the mechanisms of action of small RNAs at sites of DNA damage. Unfortunately, their abundance in a cell is presently unclear and thus their stoichiometry with the DNA of the lesion site is not known. Furthermore, it remains to be determined whether they interact with the factors that accumulate at the damaged locus and if or how they modulate their functions – whether they do so directly or through the potential induction of chromatin modification events.

In addition, it will also be crucial to understand their sequence specificity of action. DDRNAs rescue DDR focus formation only when carrying the sequence of the damaged locus. However, each DSB location in the genome is expected to generate a set of small RNAs with a different

sequence. Thus, any potentially specific RNA–protein interaction cannot depend on the RNA sequence. Similarly, the structure of linear small dsRNAs does not seem to bear enough structural information to confer specificity to protein interactions. Although it is conceivable that synthesis at the site of damage may be sufficient to explain their local activity, the observation that exogenous DDRNAs can also fuel DDR activation in a sequence-specific manner [26] suggests that the RNA sequence is sufficient to provide a homing mechanism and mediate their locus-specific activity. However, it remains unclear if sequence complementarity between nucleic acids mediates their specific localization, and which partners may be involved.

Impact on cancer studies

With some exceptions, mature miRNA are generally downregulated in tumors compared to normal counterparts [37]. Consistent with this, germline *DICER* mutations are associated with tumor predisposition [38] and acquired mutations of DICER, DROSHA and interacting partners have been described in human carcinomas [39–43]. Inactivation of various components of DICER and DROSHA complexes stimulate cell transformation, tumorigenesis [44,45], and metastasis [46,47]. Furthermore, loss of one *DICER* allele enhances tumor development in a K-Ras-induced mouse model of lung cancer [48]. The common interpretation of these observations is that DICER, DROSHA, and associated factors suppress tumors by processing functional miRNA. However, individual miRNAs have been reported to regulate the expression of mRNAs encoding proteins with different, even opposite, roles in cell proliferation [49]; therefore, it is unclear how the impairment of miRNA biogenesis favors tumorigenesis. The biogenesis of DDRNAs depends on a set of factors similar to that of miRNAs. DDR is an established tumor-suppressive mechanism that prevents the proliferation of oncogene-expressing cells by imposing cellular senescence [1,50]. The experimental evidence that DDRNAs are essential positive regulators of DDR, and that inactivation of DICER or DROSHA prevents DNA damage-induced checkpoint enforcement and allows oncogene-induced senescent cells to proliferate, suggests a novel interpretation: DICER and DROSHA inactivation may favor tumorigenesis by impeding DDR checkpoint enforcement through DDRNA biogenesis, rather than affecting miRNA maturation. However, this hypothesis at present remains unproven; additional work will be necessary to disentangle the distinct contributions of miRNAs and DDRNAs, and the genes involved in their biogenesis and activities, in cancer suppression.

In summary, although the emerging evidence is very recent, more is likely to follow. DNA can rest assured: RNA is looking after it.

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