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Charity begins at home: non-coding RNA contributes to DNA repair

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Abstract

During the past decade, evolutionarily conserved non-coding (nc) RNAs, specifically microRNAs (miRNA), have been characterized as regulators of almost every cellular process and signalling pathway. There is now emerging evidence that this new class of regulators also impinges on the DNA damage response (DDR). Both miRNAs and other small ncRNAs are induced at DNA breaks and mediate the repair process. These intriguing observations raise the possibility that crosstalk between ncRNAs and the DDR might provide a means of efficient and accurate DNA repair and facilitate the maintenance of genomic stability.

Introduction

Environmental and endogenous sources constantly trigger DNA damage and, to survive this, cells must rapidly sense and respond to the DNA break. Proliferating cells also have to stop dividing by engaging cell cycle checkpoints. The DDR allows the DNA lesion to be repaired and, if breaks are irreparable, programmed cell death will be induced¹. Efficient repair of double-stranded DNA breaks (DSB) is particularly crucial, as it is believed that a single unrepaired DSB is detrimental for cell health^{2, 3}. Two major mechanistically distinct pathways, homologous recombination (HR) and non-homologous end joining (NHEJ) have evolved to deal with DSBs and are regulated by key components that are conserved from yeast to mammals (Box 1)^{1, 4}. These pathways differ in their DNA template requirements, kinetics and the fidelity of the repair process. HR requires an undamaged homologous DNA template to replace an adjacent damaged DNA strand with high fidelity⁵. In contrast, the untemplated NHEJ pathway is relatively error-prone as it rapidly processes and joins the broken DNA ends⁶. Although DSB repair is largely constitutive, the relative contribution of the two DSB repair pathways differs in the different cell types, and in different phases of the cell cycle⁴. NHEJ is favoured in the pre-replicative (G0/G1) phase whereas HR dominates in the replicative (S) phase. Increasing evidence indicates that the microenvironment of a DSB is critical for the choice of repair pathway (Box 1).

Only ~2% of our genome accounts for protein-coding genes, but only in the past decade there has been significant advancement in understanding the function and relevance of the rest of the genome⁷. Now there is convincing evidence that the ‘junk DNA’ produces non-coding transcripts that are critical for maintenance of cell health, and participate in all major cellular processes⁸. Several classes of ncRNA have been identified, each of which differs in their origin, biogenesis and mode of action (Box 2)^{9–22}. The most widely studied class are microRNAs (miRNAs), are small endogenous ncRNAs that function as post-transcriptional regulators of gene expression^{23–25}. It is predicted that more than 60% of protein-coding

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mRNAs are directly targeted by miRNAs²⁶. The biogenesis of mature miRNAs from longer primary transcripts involve an intricate pathway that includes cleavage by the RNase III enzymes DROSHA in the nucleus and DICER in the cytoplasm to form a dsRNA, 20–25 nts in length^{27, 28}. Ultimately the mature miRNA is incorporated into the RNA-induced silencing complex (RISC) that includes the Argonaute (AGO) proteins and mediates the interaction of the miRNA with its target transcript (for a comprehensive review, see refs^{29–31}).

There is historical evidence that DSB repair proteins including 53BP1³², BRCA1³³ and Ku70³⁴ bind ncRNA and, more recently, RNA-binding proteins have been shown to be recruited to DSB sites^{35, 36} and influence the efficacy of repair. Several studies have also shown that miRNA expression is regulated by DNA lesions and in some instances directly by DDR proteins^{37, 38} (Fig. 1). p53 is important for DNA damage induced expression of miRNAs, and conversely miRNAs regulate cellular levels of p53 (for a comprehensive review, see refs^{39, 40}). From the functional standpoint, it is clear that miRNAs target DNA repair factors and influence DSB repair. There is emerging evidence that other small ncRNAs are induced in response to DSBs in multiple organisms^{19–22}. However there is very limited understanding of how these ncRNAs impacts the DDR. In this Perspective, we highlight the roles of miRNAs and other ncRNAs in the DDR. We speculate on the molecular mechanism of their role in DDR, and propose that ncRNAs directly regulate the process of DSB repair, and broadly influence the choice of DSB repair pathways.

miRNA control of the DDR

MiRNAs typically mediate fine regulation of gene expression, ‘tuning’ rather than dramatically altering protein levels. However, any individual miRNA may moderately reduce the amount of multiple proteins and profoundly impact a signaling pathway or cellular process. miRNAs are now being implicated in the DDR, and it is possible that a few miRNAs induced in response to DNA damage regulate the expression of the DDR factors that are necessary for maintaining a specific cell cycle checkpoint, or are involved in a particular DSB repair pathway. Therefore, to gain insight into the cellular response to DNA damage, we need an in-depth understanding of how DNA breaks regulate expression of miRNAs, and how miRNAs influence the DDR.

miRNA induction after DNA damage

The development of miRNA microarrays, RT-PCR platforms and deep sequencing methodologies have resulted in an exponential acquisition of miRNA profiles after the induction of DNA damage from various sources including UV, ionizing radiation (IR), hydrogen peroxide and radiomimetic drugs in an assortment of cell lines^{38, 41}. DNA damage induced change in miRNA expression varies considerably depending on the intensity/type of damaging agents, time after DNA damage, and profiling methods. High doses of damaging agents and longer delays until measurement (6 hours or more) are very likely to have secondary effects on miRNA expression that are not pertinent to the DDR. Keeping these confounding factors in perspective, changes in miRNA expression have been implicated in the initiation of the DDR. When human foreskin fibroblasts are exposed to low doses of radiation (0.1 Gy and 2 Gy of X-rays) and assayed within 30 minutes of exposure⁴², there is an immediate decrease in miRNAs after 2 Gy, and this corresponds with increased expression of predicted target transcripts involved in cell cycle checkpoints and DNA repair. A large subset of miRNAs, including let-7, is transiently altered by 4 hours after UV damage in human primary fibroblasts⁴³. In prostate tumour lines, 22 miRNAs show threefold altered expression within 4 hours after IR (6 Gy)⁴⁴. These include miR-521, which is significantly down-regulated by IR. MiR-521 targets the DNA repair factor, Cockayne syndrome protein A (CSA) and manipulating the cellular level of miR-521 impacts the radiosensitivity of the

tumour⁴⁴. In another study, radiation-induced miRNA alteration was assessed in eight patients in complete remission from AML or CML. A common set of 27 miRNAs were differentially expressed more than twofold in all the patients exposed to 1.25 Gy⁴⁵. Interestingly, there was no obvious overlap between the radiation-induced miRNA expression patterns in the different studies^{42–45}; this may suggest that radiation-induced alterations in miRNA expression are specific to cell lineage. However, in every instance, the miRNAs that respond to radiation are predicted to target factors involved in the DDR.

Direct regulation of miRNA biogenesis by DNA repair factors

The connection between miRNAs and the DDR is supported by the direct role of ATM and BRCA1 in the production of specific miRNAs (Fig. 1A and B). Seventy one miRNAs, representing ~25% of DNA damage induced miRNAs, are upregulated in mouse embryonic fibroblasts (MEFs) after treatment with the radiomimetic drug neocarzinostatin (NCS) in an ATM-dependent manner⁴⁶. Importantly, ATM does not impact the transcription of these miRNAs, but specifically the processing and biogenesis of the mature miRNAs. KH-type splicing regulatory protein (KSRP), a key component of both DROSHA and DICER complexes, is a substrate of ATM. Phosphorylation of KSRP significantly enhances its activity in recruiting target primary miRNAs (pri-miRNAs) to DROSHA for processing⁴⁷. By contrast, BRCA1 regulates miRNA processing directly by binding specific pri-miRNAs⁴⁸. BRCA1 interacts with DROSHA and other proteins in the DROSHA microprocessor complex, facilitating the processing of BRCA1-associated pri-miRNAs. In addition to induction of miRNAs, BRCA1 has also been shown to repress the transcription of particular miRNAs. Specifically, BRCA1 represses miR-155 transcription via its association with HDAC2, which deacetylates histones H2A and H3 at the miR-155 promoter⁴⁹. DNA damage can also enhance transcription of miRNAs, as DNA damage-induced NF- κ B is recruited to the promoter of miR-21 where it cooperates with STAT3 to enhance the transcription of miR-21⁵⁰.

Functional impact of miRNAs on DSB repair

miRNAs regulate the expression of DNA repair proteins and broadly affect the sensitivity of cells to DNA damage. miRNAs can target genes that are involved in the initial steps of DSB recognition (sensors/mediators), or in other cases work downstream on effector genes. This can make a significant difference to the choice of DSB repair pathway and the process of repair itself (Fig. 1C).

DSB sensors and mediators

DSB sensors detect DSBs and are immediately activated to initiate DNA repair pathways. Examples include the Mre11-Rad50-Nbs1 (MRN) complex and Ku70/80, which are crucial for pathway choice. 53BP1 has also now been added to this category⁴. Transcripts of the DSB sensor genes have long 3'UTRs (for example, the Nbs1 3'UTR is 2246 bases and the Ku80 3'UTR is 1101 bases) with many predicted miRNA binding sites. The high number of miRNA binding sites may suggest that these genes are post-transcriptionally regulated by miRNAs, but it is noteworthy these predictions have not been experimentally validated. Interestingly, a moderate reduction in the 'sensor' proteins can have significant physiological relevance. The loss of a single 53BP1 allele in mice leads to aberrant DNA repair and increased incidence of lymphoid malignancies^{51, 52}. Furthermore a subset of human diffuse large B-cell lymphoma (DLBCL)s have a single copy loss of 53BP1⁵³. This 53BP1 dosage effect may reflect its structural role as a scaffolding protein during DSB repair, but these results strongly suggest that a miRNA-mediated 'moderate' decrease in 53BP1 may significantly impact DSB repair. Indeed, overexpression of miRNAs targeting 53BP1 alters the DNA damage sensitivity of cells, and influences the choice of DSB repair pathways (unpublished data, Y.C. and D.C.).

DSB mediators propagate DNA damage signals primarily via posttranslational modifications, and recruit effector proteins to DNA lesions. Multiple studies demonstrate that miRNAs regulate the mediator proteins ATM and DNA-PK. ATM is a validated target of miRNA-421, miRNA-18a, miRNA-101 and miRNA-100^{54–57}. Overexpression of these miRNAs reduces ATM levels and correlates with aberrant DNA repair, disrupted cell cycle checkpoints, and enhanced radiosensitivity. MiRNA-101 also suppresses the catalytic subunit of DNA-PK(DNA-PKc) in various cancer models *in vitro* and *in vivo*, significantly altering the radiosensitivity of tumours⁵⁶. Another mediator, ATR (a PI3-like kinase) is vital for cell survival and biallelic loss of ATR is not tolerated in any cell type. However, ATR haploinsufficiency results in significant genomic instability, hypersensitivity to genotoxins, and increased cancer predisposition⁵⁸. Considering its importance for cell health, a modest change in ATR levels could have a profound effect on the DDR, and miRNAs are thus ideal candidates for ‘fine-tuning’ ATR expression.

DSB effectors

DSB effector proteins actively drive DSB repair and include MDC1, H2AX, BRCA1, BRCA2, RAD51, CHK1 and CHK2. There is convincing evidence that miRNAs target some of these effector proteins and impact the DDR. DNA repair is considered to be a ‘house keeping’ function. However, terminally differentiated cells normally down-regulate overall DNA repair and have decreased levels of repair proteins; miRNAs may contribute to this control. In an *in vitro* model of hematopoietic cell differentiation, miR-24 and miR-182 suppress DSB repair in terminally differentiated blood cells by targeting H2AX and BRCA1, respectively^{59, 60}. Decreased expression of the BRCA1 gene is common in sporadic basal-like breast cancer and sporadic tumours account for more than 90% of the total breast cancer burden⁶¹. Since BRCA1-deficiency correlates with increased response to DNA damaging agents, miRNAs downregulating BRCA1 expression may significantly impact the scope of cancer therapy. MiR-182 regulates BRCA1 expression in triple-negative breast tumour lines, and manipulating miR-182 expression influences homologous recombination, and sensitivity of breast tumours *in vitro* and *in vivo* to DNA damaging agents⁶⁰. This is the first example of a miRNA that specifically impacts a DSB repair pathway. BRCA1 is also a critical factor in the genesis and therapy of ovarian tumours, and miR-182 also targets BRCA1 in high-grade serous ovarian carcinoma patients⁶². Interestingly, miR-96 that is generated from the same polycistronic transcript as miR-182 targets another HR factor, RAD51⁶³. Other miRNAs that target H2AX, BRCA1 and BRCA2 and cause genomic instability and aberrant DNA repair include: miR-138 in osteosarcoma cells; miR-146a, miR-146b-5p and miR-1245 in breast tumour lines; and miR-1 in prostate tumours^{64–67}.

The optimum expression of DNA repair proteins is required for efficient DSB repair and the appropriate choice of repair pathways. Although there is no formal evidence that miRNAs directly regulate the choice of homologous recombination versus non-homologous end joining (NHEJ)-mediated repair of a DSB, it is feasible that miRNAs target ‘recognition factors’ for a pathway (for example, BRCA1 for homologous recombination or 53BP1 for NHEJ) and this may significantly affect pathway choice.

Expanded role of non-coding RNAs in DDR

The link between miRNAs and the DDR is just the tip of the iceberg as the connection of other ncRNAs with this process remains largely unexplored. There is long standing evidence that short ncRNAs regulate chromatin structure and transcription in lower organisms⁶⁸. As the interactions of the DNA repair machinery with chromatin is an intrinsic aspect of the repair process, it is likely that ncRNAs participate in the DDR.

Damage induction of non-coding RNAs

DNA damage induces the production of ncRNAs other than miRNAs. This was first observed in *Neurospora Crassa*, in which DNA lesions induce expression of the AGO protein, QDE-2 and a novel class of associated small single-strand RNA (20–21 nt), named qiRNAs¹⁹ (Fig. 2). QiRNA biogenesis in response to damage requires initial production of a precursor aberrant RNA (aRNA) from the ribosomal DNA (rDNA) locus, a process that depends on the RNA-dependent RNA polymerase QDE-1 and the Werner/Bloom RecQ DNA helicase homologue QDE-3. The aRNAs, which contain both the sense and antisense strands, are processed by DICER-like proteins to produce qiRNAs. The AGO protein, QDE-2 associates with the qiRNAs which have specific nucleotides at both the 5' (uracil) and 3' (adenosine) ends, indicating that they do not arise from non-specific degradation of rRNA. *Neurospora* mutants in any of these RNA processing proteins exhibit increased sensitivity to DNA damage, suggesting a role for qiRNAs in the DDR¹⁹.

The production of non-coding small RNA in response to DSBs is evolutionarily conserved in plants, flies and mammalian cells. Unlike qiRNAs, these small RNAs are produced from sequences near the DSBs^{20–22}. In *Arabidopsis thaliana*, the biogenesis of DSB-induced small RNAs (diRNAs) requires the PI3-like kinase ATR, RNA polymerase IV (Pol IV), Dicer-like proteins and RNA-dependent RNA polymerases (RdRP)²⁰. AGO2, a component of the RISC complex, binds mature diRNAs, and loss of AGO2 significantly reduces diRNA levels and impedes DSB repair²⁰. However, diRNA expression does not affect RNA-directed DNA methylation or histone H2AX phosphorylation in response to damage²⁰. Nor does it alter the levels of key DNA repair proteins, so the mechanism for its effects on DNA repair is still unclear.

Although flies and mammalian cells lack RdRPs, small dsRNAs are generated from regions close to a single DSB^{21, 22}. In the *Drosophila melanogaster* S2 cell line, introduction of linearized plasmid (with a blunt end or 5' or 3' overhang) induced the production of small RNAs (~21 nts), termed endo-siRNAs²²; by contrast, single strand nicks did not elicit endo-siRNAs. The endo-siRNAs are typically encoded from the transcriptionally active region upstream of the cut site and downstream of annotated transcription start sites. In mammalian cells, a chromosomally integrated reporter system has been used to introduce a single site-specific DSB and study the induction of ncRNAs^{20, 21}. These studies show DICER/DROSHA(DD)-dependent production of small RNAs (termed DDRNAs) in response to a single DSB. The precise genomic origin and role of these small ncRNAs remains unclear. One study²¹ suggests that the functional form of the DDRNA is encoded proximal to the DSB site, that is, within a few hundred base pairs. In another study²⁰, RNA sequencing data indicates that the peaks of both sense and antisense transcripts are ~5 kb downstream of the site of the DSB. However, it is not yet clear whether the small RNAs produced distal to the DSB site are involved in the DDR.

Impact of non-coding RNAs on DNA repair

There is considerable evidence that ncRNAs contribute to the maintenance of genomic stability^{69, 70} and there are several intriguing correlations between ncRNAs and DNA repair. For example, several DDR proteins (including 53BP1³², BRCA1³³ and Ku70³⁴) associate with RNA. The activity of DNA-PK can be inhibited by RNA aptamers³⁴ and the activity of ATR is impaired by UV-induced synthesis of human telomerase RNA (hTR)⁷¹. Another ncRNA relevant to telomere biology, telomeric repeat-containing RNA (TERRA) has been involved in the maintenance of genomic integrity and stability^{72, 73}. Several DNA repair proteins including DNA-PK, PARP1 and Bloom's syndrome helicase (BLM) associate with TERRA⁷⁴. Recently it has been suggested that TERRA functions in maintaining proper telomere function by repressing homologous recombination at the telomeres⁷⁵. Further

evidence that ncRNA pathways affect DNA repair has emerged from data showing that mutations or deletions in factors involved in the biogenesis of small ncRNAs, impact the efficacy of DNA repair and sensitize cells to DNA damage in plants, lower eukaryotes and mammals^{19–22}. These studies are suggestive of a multi-faceted role for ncRNA in the DDR.

A direct role for RNA in DSB repair was first shown in *Saccharomyces cerevisiae* in which RNA oligonucleotides serve as templates for homologous recombination-mediated repair of a site-specific DSB⁷⁶. This idea is further supported by RNA-templated DNA rearrangements that occur in another unicellular eukaryote, *Oxytricha Trifalla*⁷⁷. Furthermore, in human and mouse cell lines, the idea of a direct role is supported by the fact that formation of 53BP1 foci in response to IR is impaired by RNase treatment²¹, and that addition of total RNA can rescue this phenotype. Similarly, elegant experiments that demonstrate RNase-mediated abrogation of 53BP1 foci from a site-specific DSB, and foci rescue by synthetic dsRNAs from close proximity (within 500 bps) of the cut site convincingly demonstrate the influence of small RNAs on DSB repair²¹. Interestingly, events preceding the recruitment of 53BP1, such as formation of γ -H2AX, and recognition of the DSB by the MRN complex seem to be independent of RNA²¹. These observations suggest that DSB recognition, and initial DDR signalling occurs prior to generation of small RNAs. It is feasible that, in organisms that lack RdRP and need to synthesize an antisense transcript, processing at the DSB site is necessary to recruit and spatially accommodate a RNA polymerase. Consistent with this, formation of DSB repair foci in mammalian cells is impeded by the addition of the RNA polymerase II inhibitor, α -amanitin²¹. However, transcription is also actively suppressed at DSBs by the DDR machinery^{78, 79}, and it is not yet clear how these observations can be reconciled with this model.

In *Arabidopsis thaliana* and in a human tumour cell line, reporter-based assays suggest that the small RNA biogenesis pathway affects homologous recombination-mediated repair of DSBs²⁰. This is consistent with the notion from fly studies that anti-sense transcripts at DSB sites would require resection, and would temporally fit the profile of homologous recombination²². The idea of RNA-templated DNA repair in *Saccharomyces cerevisiae* also supports a direct role for small RNAs in this pathway⁷⁶. However, in mammalian cells, the only direct assay using synthetic small RNAs spanning the cut site was conducted using 53BP1 foci as a read-out for DSB repair²¹. Formation of 53BP1 foci is not a surrogate marker for a specific DSB repair pathway, and if at all 53BP1 promotes NHEJ, possibly impeding homologous recombination. Therefore it remains to be seen whether which specific repair pathway is promoted by small ncRNAs.

Concluding Remarks

The avalanche of data on ncRNAs and their putative roles in different cellular processes and signalling pathways has finally reached the field of DDR. Although our current understanding of how crosstalk occurs between the DDR and ncRNAs (including miRNAs) barely scratches the surface of a tremendously complex issue, they do provide important glimpses of its relevance. There is clear evidence that miRNAs control the DDR, although most of these studies were conducted in cancer lines with artificial manipulation of miRNA levels. A key question is whether miRNAs mediate DNA repair in 'normal' cells. One hypothesis is that having too much of DNA repair proteins is detrimental to the process as the stoichiometry of factors in a DSB repair pathway maybe disrupted. MiRNAs would therefore facilitate DNA repair by maintaining optimum levels of repair proteins. Consistent with this idea, miRNAs may also affect the choice of DSB repair pathways; future studies using conditional ablation of specific miRNAs in animal models should allow this to be tested.

Even less is known about the interplay between other ncRNAs and the DDR. A fundamental question is the source of these small ncRNAs, and the mechanisms that lead to their presence at DSBs in mammalian cells. The limited data suggests that anti-sense transcription at DSBs allows the formation of dsRNA precursors that are then processed by the miRNA biogenesis machinery. So are DSBs in transcriptionally dormant intergenic regions repaired without the induction of ncRNAs? If so, are ncRNAs really required for the repair of breaks in euchromatin, or are they simply by-products of the DDR? A careful kinetic assessment of ncRNA biogenesis at site-specific DSBs in intergenic loci will be very informative. The most important question that needs to be addressed is how ncRNAs function at the site of the DNA lesion. The scarce data available suggests that ncRNAs recruit DNA repair proteins to the DSB site or maintain DNA repair foci. On the basis of observations in yeast and other lower organisms, **we speculate** on the role of ncRNAs in DSB repair (Fig. 2). ncRNAs may serve as a template for DNA polymerase that ‘fills in’ the resected DNA and/or be involved in regulating chromatin structure at a DSB site. Another possibility is that ncRNAs in complex with AGO proteins rapidly degrade the nascent RNA synthesized from broken DNA templates and prevent aberrant expression of truncated gene products.

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BOX1 – The cellular response to DNA double-strand break (DSB)

DSBs are produced by various types of genotoxins including ionizing radiation, UV light, reactive oxygen species (ROS), and chemicals and replication fork collapse. Mammalian cells repair DSBs mainly by two DNA repair mechanisms, which are homologous recombination (HR) and non-homologous end joining (NHEJ) [reviewed in Chapman et al, 2012 and Ciccia and Elledge 2010]. Depending on the need for DNA end resection at the damage site, either HR or NHEJ is activated to repair the damage. DSBs are detected by sensor complexes, Mre11-Rad50-Nbs1 (MRN), Ku70/80, and Poly (ADP-ribose) polymerase (PARP). When DNA broken ends can be directly rejoined by NHEJ, Ku70/80 heterodimer is loaded on DSB ends and recruits DNA-PKcs. DNA-PKcs regulates DSB ends stabilization through phosphorylation of ARTEMIS and other substrates. ARTEMIS facilitates end processing and, subsequently, LIG4/XRCC4/XLF

ligate the broken ends. Typically replication stress induced DNA lesions are recognized by the MRN complex and the signals are transmitted to the mediators, such as ATM and ATR. The mediators rapidly phosphorylate multiple DNA repair factors including H2AX, CtIP, BRCA1, EXO1 etc. Endonucleolytic cleavage by Mre11 at DSBs allows resection mediated by CtIP and EXO1 in the presence of BRCA1 and BLM. H2AX phosphorylation (γ H2AX) spreads around the damage site stabilizing the DNA repair complex. The ssDNA generated by resection is rapidly coated by RPA, and subsequently replaced by RAD51 in the presence of BRCA2. RAD51 nucleofilaments invade the sister chromatid to look for homology and the fidelity of this search is maintained by anti-recombinases (PARI, Srs2 etc). The invading strand is extended by DNA polymerase and ligates to form D loop structures. The final product of the HR-mediated repair is determined by the resolution of the D-loops by anti-recombinases (RTEL1) or resolvases (Mus81/Eme1, Yen1 etc).

DSB Repair Pathway Choice

Cell cycle dependent expression of the key repair proteins could be a mode of regulation, as cellular levels of several HR specific factors like BRCA1, RAD51 and RAD52 increase as cells progress from G1 to S-phase⁹⁴. Conversely, the absence of certain factors also affects the choice; for example, Ku70 and DNA-PKc deficient ES cells show a sharp increase in HR-mediated repair⁹⁵. In chicken cells RAD18 and PARP-1 suppress the access of NHEJ to DSBs and facilitates HR⁹⁶. Resection at the DSB initiates the process of HR, and is also critical for impeding NHEJ. H2AX inhibits CtIP-mediated resection in G1-cells to facilitate NHEJ⁸⁴. In the same vein, BRCA1 promotes resection and excludes 53BP1 from the DSB site to allow HR^{85, 86}. These results highlight the complexity of the DNA damage response (DDR) and suggest that the microenvironment around a DSB is important for pathway choice.

BOX 2 Non-coding RNA

Several classes of non-coding RNAs (ncRNAs), arbitrarily grouped into short (<200nt) and long (>200nt) ncRNAs, have been identified so far and each class differs in their origin, biogenesis and the mode of action. We have highlighted ncRNAs that are relatively well studied and/or have been implicated in maintaining genomic stability.

miRNAs

miRNAs are short ncRNAs that are encoded in intronic regions of protein-coding genes or the intergenic regions of the genome. The primary transcripts (pri-miRNAs) are processed in the nucleus by the DROSHA microprocessor complex to generate ~70bp stem-loop precursor forms (pre-miRNA), exported to the cytoplasm by exportin-5 and further processed by DICER into mature ~22nt products. Mature miRNAs are incorporated in the RNA-induced silencing complex (RISC) which includes Argonaute (AGO) and GW182 families of protein and loaded onto target transcripts. Typically the nucleotides 2–8 from the 5' end of the miRNA (seed region) pairs perfectly with sequences in the 3'UTR of the target mRNA, and the rest of the miRNA/mRNA interaction is not continuous. miRNAs mediate post-transcriptional gene silencing by inhibiting translation or by inducing degradation of target transcripts.

piRNAs

PIWI-interacting RNAs (piRNAs) are the largest class of small ncRNAs (26–31nt) that act as cofactors for the AGO-family protein PIWI. PIWI-piRNA complexes specifically target and silence transposable element in germline cells to maintain genomic stability. PiRNAs possess a predominant uridine at their 5' end and are generated in a DICER- and DROSHA-independent manner. They are transcribed from piRNA clusters, intergenic repetitive elements or transposons and processed by still unknown mechanisms. Sense piRNA/AGO3 complex can cleave antisense piRNA transcripts to generate another functional piRNA. Like miRNA, piRNA base-pairs with target mRNAs and induces their degradation, but can also mediate heterochromatin silencing and DNA methylation.

lncRNAs

The class of long (<200 nt) non-coding RNAs (lncRNAs) represents the largest group of mammalian non-coding RNA transcripts with tens of thousands different species. They are involved in a broad range of processes including epigenetic regulation, transcriptional and post-transcriptional regulation, RNA splicing and editing, telomere function, development, cancer and other diseases. lncRNAs are encoded in large intergenic loci or regions overlapping protein-coding genes and they resemble mRNAs as most are capped, spliced, polyadenylated, and transcribed by RNA polymerase II. lncRNAs that are transcribed from intergenic regions constitute the specific class of long intergenic non-coding RNA (lincRNAs). Some lncRNAs are transcribed from ultraconserved regions (T-UCR); genomic elements that are completely conserved in mouse, rat and human genomes. T-UCRs are derived from intragenic or intergenic regions with a strong strand preference. Variations in T-UCR expression have been observed in several malignancies and, like miRNAs, T-UCRs are frequently associated with fragile sites and various types of cancer-associated genomic regions.

Name	Machinery	Roles	References
miRNAs	DROSHA DICER RISC	mRNA degradation Translational repression	23–31
piRNAs	PIWI proteins	Transposon repression	9–12
DDRNAs	DROSHA DICER	Accumulation of 53BP1 at DSB DNA repair?	21
diRNAs	RDRs RNA pol IV DCL	DNA repair?	20
qiRNAs	QDE-1 QDE-3 DCL1 DCL2	DNA repair?	19
endo-siRNAs	DCR-1 DCR-2 Loqs-D Ago1 Ago2	DNA repair?	22
lncRNAs	Pol II	X-chromosome inactivation Splicing	8, 13,14
lincRNAs	Pol II	Transcriptional repression Reprogramming of iPS	15, 16
T-UCRs	Pol II?	Tumorigenesis?	17, 18

a. DNA damage-induced miRNA biogenesis

b. DNA damage-induced miRNA transcription repression

c. miRNAs impacting DSB repair pathway choice

Figure 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.⁴⁶

BRCA1 directly interacts with both pri-miRNAs and the DROSHA complex⁴⁸. Processing by the DROSHA complex allows cytoplasmic export of pre-miRNAs. The MAPK ERK is also phosphorylated after DNA damage⁸⁰. ERK phosphorylates TRBP and phospho-TRBP stabilizes the TRBP-DICER complex to promote pre-miRNAs processing in the cytoplasm⁸¹. Increased levels of mature miRNAs could play a role in the DNA damage response by (i) decreasing the levels of anti-repair genes (such as the anti-recombinases, Srs2, PARI, RTEL1⁴) (ii) 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 repression⁴⁹. 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 inter-play 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^{4, 82, 83}. In G1, NHEJ is promoted by 53BP1 and γH2AX which prevent CtIP-mediated resection of the broken end^{84, 85}. Resection at a DSB impedes NHEJ and allows HR. In S-phase HR is active and BRCA1 is a key player in recruitment of HR proteins to DSBs, thereby excluding NHEJ factors like 53BP1^{85, 86}. CtIP

promotes ends resection to allow formation of RPA-coated ssDNA at a DSB^{87, 88}. 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, over expression 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 over-expression 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.

Figure 2. Other noncoding RNAs 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*¹⁹. 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 DSB⁸⁹. Alternatively, the ncRNA/AGO complex may also serve as a stable scaffold for maintaining the DNA repair foci and facilitating the process of repair⁹⁰. After the recruitment of the DNA repair machinery to the DSB sites the precise role (if any?) of the short ncRNAs is not known. We speculate that the short ncRNAs could serve as RNA template to fill-in resected DNA during homologous recombination mediated DSB repair⁷⁶. Induced RNAs might play a role in mediating chromatin silenced state at the breaks either by recruiting associated chromatin factors at the breaks^{91, 92}, or directly by interacting and modulating chromatin associated factor in cis⁹³. 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.

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