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The DNA damage response: the omics era and its impact

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Abstract

The emergence of high density technologies monitoring the genome, transcriptome and proteome in relation to genotoxic stress have tremendously enhanced our knowledge on global responses and dynamics in the DNA damage response, including its relation with cancer and aging. Moreover, '-omics' technologies identified many novel factors, their post-translational modifications, pathways and global responses in the cellular response to DNA damage. Based on omics, it is currently estimated that thousands of gene(product)s participate in the DNA damage response, recognizing complex networks that determine cell fate after damage to the most precious cellular molecule, DNA. The development of next generation sequencing technology and associated specialized protocols can quantitatively monitor RNA and DNA at unprecedented single nucleotide resolution. In this review we will discuss the contribution of omics technologies and in particular next generation sequencing to our understanding of the DNA damage response and the future prospective of next generation sequencing, its single cell application and omics dataset integration in unraveling intricate DNA damage signaling networks.

Keywords

DNA damage response; genomics; transcriptomics; next generation sequencing; proteomics

Introduction to '-omics' technologies

Novel technologies and their applications fuel new insights and discoveries in any field of molecular life sciences, medicine, molecular epidemiology and biotechnology. One of those revolutions represents technologies that monitor a (nearly) complete class of biomolecules in a process of interest. These data-dense technologies have been designated omics technologies, in which the suffix -omics refers to the respective technologies monitoring I) DNA in the context of complete genomes (genomics), II) genome-wide RNA transcript expression levels representing the transcriptome (transcriptomics), III) global protein and/or post-translational modifications (PTMs), designated the proteome (proteomics), or IV) nearly all cellular metabolites, named the metabolome (metabolomics).

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The principle of both proteomics and metabolomics relies on mass differences measured with great accuracy by mass spectrometry due to protein/metabolite levels or the presence of PTMs. Sophisticated and stringent isolation methods of PTMs and stable isotope labeling of amino acids allowing quantitative analysis of protein samples have further propelled proteomics technology. The genome and transcriptome have been extensively investigated by micro-array technology over the past decade. Micro-arrays are based on comparative hybridization of fluorescently labeled DNA or cDNA (in case of RNA expression) under stringent conditions to capture probes (complementary oligonucleotides) printed on a solid surface. This allows the analysis of (tens of) thousands of molecules simultaneously, revolutionizing the scale and depth in which DNA and RNA could be investigated.

The recent emergence of next generation sequencing (NGS) has further changed the landscape of genome and transcriptome analysis. NGS, also named massive parallel sequencing, can sequence hundreds of millions DNA molecules simultaneously. A single NGS run can sequence the human genome ~37 times in 27 hours, thereby tremendously facilitating whole genome (re)sequencing projects and genome analyses such as single nucleotide polymorphisms (SNP), mutation, insertion/deletion and DNA methylation detection. In addition, NGS can map protein–DNA and DNA–DNA interactions at nucleotide resolution. Transcriptomics of large and small RNAs can be performed by simultaneously sequencing millions of cDNA molecules. Since NGS does not rely on capture probe design and their presence on arrays, novel non-coding RNAs, splice variants, post-transcriptional modifications and nascent RNA synthesis can be quantitatively analyzed. In this review, we will discuss the contribution of omics technologies to understanding the DNA damage response (DDR), with the emphasis on genomics and transcriptomics in particular by NGS technologies, and the future prospective of omics research in the DDR research field.

The DNA damage response

It has been estimated that DNA acquires ten thousands of lesions every day already from endogenous sources alone such as reactive oxygen species and metabolic products. In addition, several exogenous sources also produce DNA lesions, e.g. ultraviolet (UV) light from the sun, ionizing radiation and numerous environmental and man-made chemicals. DNA lesions can interfere with vital the DNA metabolic processes replication and transcription as well as with associated chromatin reorganization. In contrast to RNA, proteins and metabolites, DNA is the only cellular component that cannot be replaced upon damage and therefore solely relies on repair. It is also the largest molecule in the cell, and when paternal and maternal alleles are considered separate, it is unique in most cells. Moreover, since DNA is at the top of the informational hierarchy, unrepaired DNA lesions or incorrectly repaired DNA damage can have lasting consequences (1). Indeed, incorrect DNA repair results in mutations, insertions, deletions or chromosomal aberrations, which eventually lead to cancer development. Many spontaneously tumors as well as hereditary cancer syndromes have defects in DNA repair and response genes, hence illustrating the importance of maintaining genome integrity. On the other hand, studies in human progeroid syndromes and corresponding transgenic mouse models indicate that accumulation of unrepaired DNA damage contributes significantly to aging and numerous age-related

pathologies, again pointing towards the significant role of DNA damage in health and disease.

To deal with the adverse effects of DNA damage, cells have an arsenal of DNA repair mechanisms, each recognizing and repairing its own spectrum of lesions. In addition to DNA repair systems, cell cycle checkpoints are activated that halt cell proliferation to provide a time window to repair. When damage is beyond repair, cell death or cellular senescence, a permanent cell cycle arrest, is induced to remove the damaged cell from the tissue or to prevent it from replicating, with enhanced risk of mutations and cancer. All DNA repair systems, cell cycle checkpoints and additional pathways whose activity changes upon DNA damage are collectively known as the DDR. It is of utmost importance that the DDR is tightly controlled, since there is a delicate balance between incorrect repair driving carcinogenesis and hyperactivation, inducing apoptosis or senescence that leads to loss of tissue homeostasis, a contributing factor to aging and age-related pathologies (1-4). Moreover, the amount and type of DNA lesions, but also context (e.g. cell type, proliferation vs post-mitotic), determine the cellular outcome of DNA damage signaling. It is therefore not surprising that cells have an ingenious DDR that maximizes survival and decides on cell fate. Studies in the last two decades have presented a schematic overview of DDR signaling layers that coordinate the cellular response to DNA damage (Figure 1). The first step involves detecting DNA lesions by a class of sensor proteins. These sensors are required for recruiting various factors to the site of damage such as DNA repair factors, but also transmit a signal to so-called transducer proteins, of which ATM and ATR checkpoint kinases are the most prominent examples. These transducers in turn diversify and amplify the damage signal to the third layer, which are so-called effectors, which control the activity of several cellular processes and pathways, such as cell cycle arrest and apoptosis. Sensor and transducer signaling primarily relies on protein interactions and alterations in protein activity by PTMs such as phosphorylation, ubiquitination, etc. Several effectors however, are transcription factors, e.g. p53, or microRNAs, which demonstrates that the RNA component within the DDR is also essential. While the basic DDR as drawn in Figure 1 already consists of >100 genes, transcriptomics and proteomics have discovered that hundreds of additional proteins are targets of checkpoint kinases and more than a thousand genes are differentially expressed upon DNA damage as a result of transcription factor/microRNA regulation. Thus, transcriptomics and proteomics have tremendously expanded our view of the DDR.

Proteomics

Mass spectrometry after protein complex isolation has been instrumental to identify novel protein-protein interactions and modifications and boosted various branches of the molecular life sciences, including DDR research. In addition, specialized proteomics screens dramatically expanded the components and repertoire of PTM events in the DDR. PTMs are an integral step in signal transduction and within the DDR, including phosphorylation, acetylation, (poly)ADP-ribosylation, ubiquitination, sumoylation and neddylation (5, 6). Since checkpoint kinases ATM and ATR are central nodes in the DDR, one of the first proteomics screening approaches aimed at identifying target proteins. ATM and ATR phosphorylate S and T residues in target proteins at a conserved SQ or TQ motif. Antibodies specifically raised against these phosphorylated motifs were used to isolate ATM/ATR

target proteins phosphorylated after DNA damage, which was followed by mass spectrometry analysis (7). Interestingly, more than 500 ATM/ATR target proteins were identified, which were not only known targets involved in DNA repair and checkpoint function, but also many proteins from processes previously not linked to the DDR such as RNA processing factors. Additional proteomics screens identified numerous proteins phosphorylated after DNA damage independent from ATM/ATR (8-10). These screens together disclose an extensive network of phosphorylation events, crosstalk between ATM/ATR and several other signal transduction pathways (e.g. insulin/IGF1 receptor signaling) and identified additional effectors that control RNA expression programs.

Other PTMs in the context of DNA damage have also been analyzed by proteomics, e.g. ubiquitination (11, 12), sumoylation (13-15), parylation (16) and acetylation (17). These screens identified known DNA repair and checkpoint proteins, but also chromatin remodeling factors and many proteins previously unknown to participate in the DDR, indicating the complexity of signaling networks in the DDR at the PTM level. It is highly conceivable that PTMs in the DDR exhibit crosstalk to fine-tune the cellular response or outcome of DNA damage signaling. The effector protein p53 is among the best-studied examples. p53 is not only phosphorylated at several amino acids, but is also acetylated, ubiquitinated, sumoylated, methylated, neddylated, ADP-ribosylated and glycosylated at several residues (18). Therefore, proteomics screens that quantify multiple PTMs in parallel could unravel such intricate networks. A multilevel proteomics approach was designed to quantify protein phosphorylation, acetylation and abundance in parallel. This study found that the ubiquitination cascade itself is targeted by several phosphorylation events in the DDR (17). In summary, proteomics contributed enormously to our understanding of the complex signaling events in the DDR and the prospect of multi-level PTM proteomics studies will further unravel these elaborate networks (19, 20).

Transcriptomics

The cellular outcome of DNA damage signaling is for a large part determined by transcriptional programs controlled by key effector proteins, including the transcription factor p53 (Figure 1). Transcriptional reprogramming is essential for the execution and outcome of DDR signaling, e.g. transient cell cycle arrest, senescence or apoptosis. Micro-array technology has significantly enhanced our understanding of the transcriptional response associated with DNA damage. Countless micro-array-based transcriptomics studies have been published to date in which cells/organisms were exposed to DNA damage. It is very difficult to compare results between studies and extract common transcriptional changes, because most of these studies were performed under completely different conditions, e.g. cell type/tissue, dose and time after treatment. Moreover, technical variation is induced by choice of micro-array platform, normalization procedure and statistics. Based on all these micro-array studies, we estimate that the expression of up to a few thousand genes is altered after DNA damage, depending on dose, agent, cell type, etc. Overall conclusions could be that besides p53 several additional transcription factors control gene expression after DNA damage and numerous cellular processes and pathways are controlled by the DDR at the transcriptional level (21, 22).

Global gene expression profiling has been very informative to interpret the role of DNA damage in the complex processes of aging (23-25). Human accelerated aging syndromes and corresponding transgenic mouse models with specific DNA repair defects indicated a causal role of DNA damage in aging, which was based on age-related pathology and aging phenotypes at the cellular and tissue level (24, 26). Micro-array analysis revealed that a large part of the transcriptome of naturally aged wild type mice was significantly overlapping with global gene expression profiles from accelerated aging mouse models with defects in transcription-coupled DNA repair. This indicates that transcription-blocking lesions are involved in establishing the aging transcriptional landscape. Moreover, these transcriptomics analyses revealed the presence of a DNA damage-triggered survival response, which includes suppression of the somato- (growth hormone and IGF1), lacto- and thyrotrophic hormonal axes and induction of e.g. the anti- oxidant defense. This response resembles the longevity-promoting response by dietary restriction as seen in transcriptomics, which is constitutive active in long-lived dwarf mutants. Subsequently, micro-arrays generated from cell cultures exposed to UV, which induces transcription-blocking lesions, mimicked these age-related gene expression profiles including the survival response, providing further molecular evidence that DNA damage contributes to aging.

Although mRNAs are the most studied RNA molecules to date, it is becoming apparent that not-for-protein coding (non-coding) RNAs are abundantly present in cells, even more plentiful than mRNAs (27, 28). One of the best-studied classes of non-coding RNAs are microRNAs, which are small (~22 nucleotides) endogenous non-coding RNAs that repress target gene expression by binding to complementary target sites mainly residing in 3’UTRs, thereby predominantly inducing mRNA degradation (29). MicroRNA micro-array technology identified several differentially regulated microRNAs in response to DNA-damaging agents (30-36). Based on microRNA array time series a hypothesis was postulated that in the DDR microRNAs act in between the fast PTM response and the relative slower gene transcriptional responses via promoter regulation (31, 37). Since a single microRNA can target hundreds of different mRNAs simultaneously, this observation could provide a mechanism to rapidly alter a complete gene expression program followed by more stable changes at the promoter. Subsequent evidence by microRNA arrays demonstrated that a significant part of all microRNA expression after DNA damage was controlled by ATM and its target KHSRP (38). Upon DNA damage, ATM phosphorylates KHSRP, which then binds specific primary microRNAs from the nuclear pool of primary microRNAs and accelerates their biogenesis into mature microRNAs. Thus, microRNAs in the DDR are likely effectors that quickly adapt gene expression programs. The transcription-independent mechanism of microRNA regulation provides a manner to transiently and rapidly alter gene expression upon DNA damage. Importantly, DNA damage responsive microRNAs are frequently misexpressed in human cancer, thereby modulating resistance to genotoxic chemotherapy (35, 36, 39, 40).

Transcriptomics by NGS, also designated RNA sequencing, has identified an enormous amount of non-coding RNAs, both small and long originating from exonic, intronic and intergenic regions (41-47). The overt majority has unknown functions. Standard mRNA sequencing relies on enrichment of poly-adenylated transcripts followed by sequencing. Next to known mature and partially processed RNA species, sequence information also

includes low abundant mRNAs, poly-adenylated long non-coding RNAs and the correct representation of splice variants originating from over 95% of the multi-exonic genes (48). Paired-end sequencing in which sequencing is performed from both ends of the cDNA fragments also detects gene fusion events (49) important for tumorigenesis (50-53). Small RNA sequencing relies on the enrichment of all RNA species smaller than ~30 nucleotides. Sequence information not only detects microRNAs, but also their isoforms (isomiRs), not detectable by array technology. IsomiRs are sequence length modifications of the mature microRNA due to imprecise precursor cropping or dicing (54) or post-transcriptional addition of nucleotides to the 3' end by specialized enzymes (55). Besides microRNAs, small RNA sequencing also detects thousands additional small RNAs of which most have unknown functions. Furthermore, specific protocols have been developed to sequence long non-coding RNAs (28), isolate chromatin-bound non-coding RNAs (47), strand-specific sequencing to identify antisense transcripts (56) or nascent RNA (57, 58) (Figure 2).

Currently, only few mRNA or small RNA transcriptomics studies by NGS in relation to the DDR have been published (59-64) in which the data analysis was mainly focused on mRNAs or mature microRNAs. RNA sequencing identified several long non-coding RNAs that participate in the p53 response by regulating cell cycle arrest and apoptosis (65-68). In another study nascent RNA isolation followed by NGS was performed to monitor the global effect on RNA synthesis by camptothecin treatment, which inhibits topoisomerase I thereby blocking replication and transcription (69). Camptothecin primarily affected transcription elongation and withdrawal led to transcription resumption starting from the 5'-end of genes, while stalled RNA polymerases in gene bodies did not recover. Recovery of RNA synthesis was independent of CSB, an essential component of transcription-coupled repair (TCR), indicating that TCR is not involved in the repair of or RNA synthesis recovery from transcription-blocking Top1 lesions. One of the key advantages of NGS-based transcriptomics is direct sequence information. It was shown that DICER and DROSHA, components of the microRNA biogenesis pathway, are essential for the activation of the DDR at the transducer level. RNA products generated by DICER and DROSHA are required to restore DDR activation. NGS demonstrated that DDR activation requires DICER- and DROSHA-dependent small RNAs originating from the site of the double strand DNA break (70). Taken together, transcriptomics technologies have been extremely powerful in deciphering alterations in the transcriptome after DNA damage and provided several new insights in the DDR.

Genomics

NGS especially impacted DNA research in relation to the DDR. Although DNA micro-arrays have provided valuable information, NGS with the capacity to sequence the genome ~37 times in 27 hours data at nucleotide resolution (compared to hybridization-based micro-array results) dramatically accelerated and quantitatively improved genome research associated with DNA damage (Figure 2, overview NGS technologies). One of the most frequently used applications of whole genome sequencing or exome sequencing, which only sequences known coding areas (71), is the identification of SNP/mutations associated with specific genetic traits or genetic diseases, which have been performed for numerous human diseases. Importantly, SNPs or defects in human DDR genes have been linked by these

studies to e.g. accelerated ovarian aging (72), karyomegalic interstitial nephritis (73) and UV sensitivity syndrome, the last unresolved genetic disorder due to deficiency in nucleotide excision repair (74), linking defects in DDR factors to human age-related pathology.

Evidently, somatic genomic aberrations due to DNA damage, e.g. mutations and chromosomal rearrangements, can be resolved by NGS at nucleotide resolution. Although this appears logical, this approach is met with technical limitations due to the random and infrequent nature of somatic mutations that cannot be separated from sequencing errors. These complications were overcome by performing a sophisticated single cell sequencing approach that rules out these errors and correctly calls somatic mutations by ENU in the Drosophila genome (75). One potential complication of single cell and single DNA sequencing may be the fact that damages may be present in the original DNA molecule, which cause de novo mutations in the sequencing protocol. In addition to mutations, DNA rearrangements are also often masked. This was improved by Strand-seq (76), a single-cell sequencing technique that sequences the original parental DNA template strands in daughter cells following cell division. Both single cell-sequencing techniques will be very useful in determining mutation frequencies of genotoxic compounds, in cancer samples and during aging.

Next to monitoring genetic aberrations, genomics protocols are valuable tools to study basic DDR biology. Specialized NGS methods, chromosome conformation capture sequencing (or its derivatives), analyze nuclear architecture, nucleosome positioning or the 3D chromosomal interaction landscape (77). This sequencing technique has been used to examine whether chromosomal translocations in human cancer originate from selection of random translocations, targeted DNA damage or frequent interactions between translocation partners (78). While location and frequency of recurrent translocations, including those driving B-cell malignancies, is due to targeted DNA break formation, nuclear organization was identified as the main driver in non-targeted rearrangements (78). Another application of chromosome conformation capture sequencing examined distant enhancer elements of the central DDR transcription factor p53, which drives transcriptional programs triggering cell cycle arrest and in a later stage apoptosis or cellular senescence. Genome-wide p53-binding sites were found located far from any known p53 target gene. Chromosome conformation capture sequencing discovered that these p53-bound enhancer regions interact intra-chromosomally with multiple neighboring genes to convey long-distance p53-dependent transcription regulation. Moreover, these regions produced p53-dependent enhancer RNAs that are short RNAs (200 – 1000 nucleotide long) required for efficient transcription of target genes (79). These results illustrate the complexity of the DDR in the context of genomic DNA.

Chromatin immunoprecipitation coupled to NGS, ChIPSeq in short, maps DNA–protein interactions at nucleotide resolution. Using an inducible double strand DNA break (DSB) system, the chromatin landscape of γH2AX around the DSB was mapped and its spreading properties along the damaged chromosome (80, 81). Since chromatin remodeling is essential for a proper DDR, this technology could provide complete chromatin maps from the sites of DNA damage. ChIPSeq is often used to map transcription factor binding sites. ChIPSeq provided a genome-wide profile of p53-binding sites, which revealed stimulus-specific

functions of p53 during differentiation and DNA damage (82). ChIPSeq was also used to map single strand DNA by targeting Rad52 in fission yeast, which binds to single strand DNA formed at DNA lesions (83). This method was applied to identify DNA damage sites in the genome.

Direct detection of DNA damage and mapping its genomic location could be applied to identify hotspots for DNA damage and analyze at which locations DNA repair is most (in)effective. These approaches in ChipSeq are often hampered or limited by the choice of protein and quality of the antibody. Recently, a method has been developed that directly labels DSBs *in situ* with a linker followed by isolation and NGS (84). This approach named BLESS (direct in situ Breaks Labeling, Enrichment on Streptavidin and next-generation Sequencing) maps DSBs at nucleotide resolution. Replication stress-induced DSBs by aphidicolin in human cells identified more than 2000 fragile regions that were overrepresented with genes, satellite repeats and frequently rearranged regions found in human cancer. *In toto*, genomics approaches by NGS constitute important tools to monitor DDR processes at unprecedented nucleotide resolution.

Conclusion & Future Prospective

The emergence of NGS technology has dramatically enhanced genomics and transcriptomics studies. Current throughput that can handle dozens to hundreds of samples in a reasonable amount of time, quantitative results (absiolute number of sequences per genomic location/RNA species) instead of relative hybridization signals and single nucleotide resolution will undoubtedly address many long-standing questions in the DDR and many other research areas. Several dedicated experimental approaches have been developed that isolate specific DNA, RNA or chromatin species followed by sequencing. Examples at the DNA level are not only the relatively general ChIPSeq (85) and chromosome conformation capture sequencing methods (77), but also more specific protocols to monitor DSB sites (84) and single cell analysis of mutations or chromosomal rearrangements (76) (Figure 2). At the RNA level, it is possible to generate quantitative transcriptomes that include non-coding RNAs, splice variants, nascent RNA production and RNA-chromatin interactions (28, 47, 49, 56-58, 71) (Figure 2). We have developed an RNA sequencing method that does not rely on class selection, but monitors all RNA species, large and small, in a single sequence run thereby quantitatively preserving all RNA classes, allowing cross-class comparisons. Upon cisplatin treatment we identified numerous differentially expressed RNAs undetectable by other methods, but also a specific global repression of the class of microRNA and microRNA isoforms (*Derks et al., submitted*). At the level of chromatin, specific properties and changes in chromatin can be monitored using antibodies to specific histone modifications in combination with NGS providing a genome-wide view of epigenetic alterations after e.g. genotoxic treatment. We expect that several additional genomic and transcriptomic methods based on NGS will be developed, which open new perspectives in relation to the DDR.

Although a wealth of omics data has been generated, NGS methods have not been exhaustively used in relation to DDR research. Since DNA and RNA are integral parts of the DDR, we expect several significant discoveries based on NGS data. For example, NGS

genomics studies will provide answers on the 3-dimensional chromosome conformation during DNA repair. Since many DNA-binding proteins and chromatin remodelers are required for sensing, transducing and repairing damage, detailed DNA-protein interaction maps around the site of damage could be generated by ChIPSeq. With current NGS-based transcriptomic methodologies differential RNA expression including non-coding RNAs upon genotoxic stress can be mapped. Systematic quantitative screening in time, including non-coding RNAs as well, comparing the transcriptional response between different DNA lesions in similar experimental conditions (dose, cell type, etc) will produce detailed RNA expression landscapes of the DDR. Because both coding and non-coding RNAs are required for cell fate determination after DNA damage, these maps will assist in unraveling the complex DDR networks and their involvement in cancer and aging. Specialized transcriptomics methods can be used to monitor global nascent RNA production and chromatin-RNA interactions in relation to the DDR.

Cellular heterogeneity in organs, cancer, aging, but also in cell cultures (86), such as different cell types and cell states (e.g. proliferating versus post-mitotic), can result in “noise” in datasets due to various responses to genotoxic stress. This could be addressed by single cell analysis. Several advances in single cell genomics and transcriptomics have been made including mutation and chromosomal rearrangement frequency determination (76, 87). These technologies will be useful to understand the evolution of cancer and metastasis. Furthermore, the relation of stochastic DNA damage in the aging process can be addressed.

Recent advances in omics technologies offered much more complete datasets that monitor the behavior of cellular macromolecules. Intelligent experimental design will allow integration of genomics, transcriptomics and proteomics datasets obtained under identical conditions and provide a holistic view of the complex DDR networks and final cellular outcome of these signaling events. Shifting the focus from single omics datasets to integration of multiple types of omics datasets requires sophisticated systems biology approaches and mathematical modeling. Development of dataset integration and visualization methods is needed to deal with these large and complex datasets. In conclusion, omics technologies, their integration and comparison to aging and cancer will tremendously enhance our knowledge of the DDR and its relation to health and disease.

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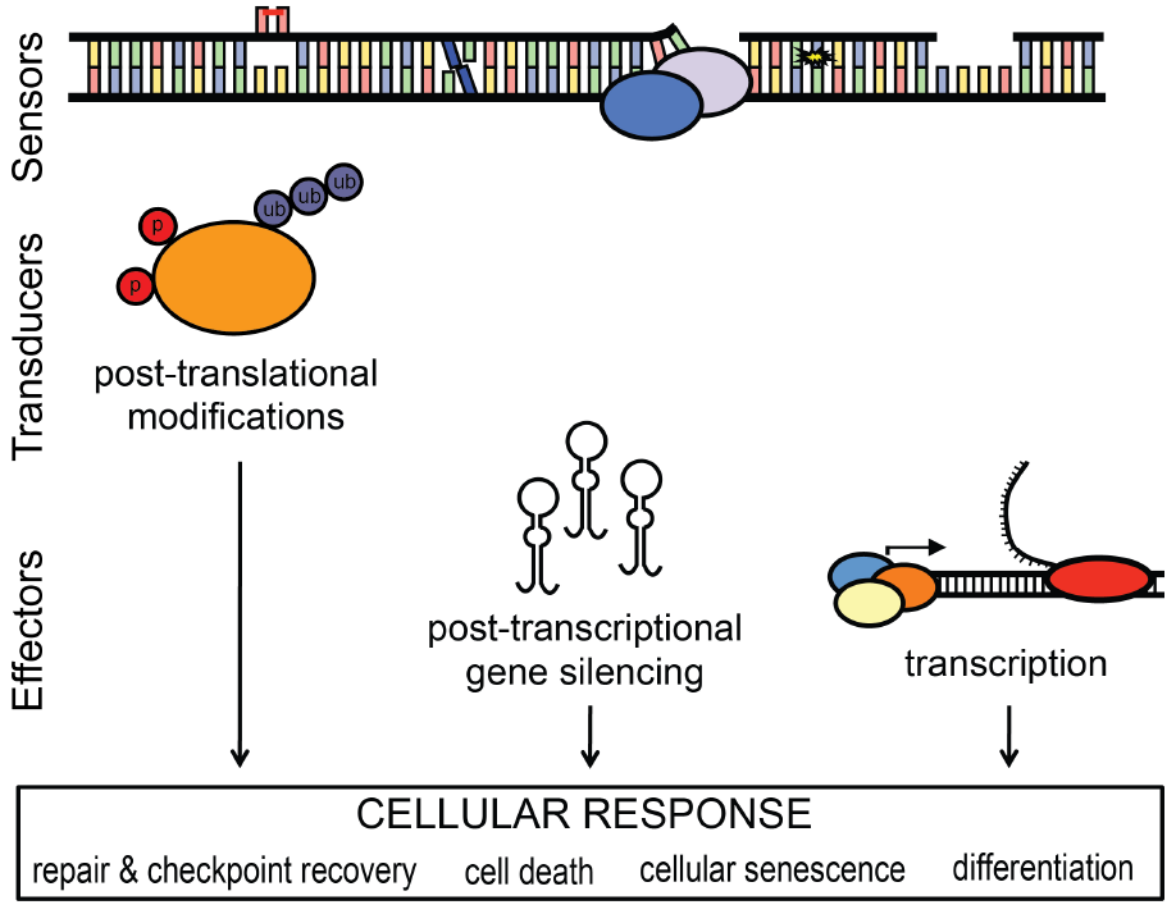

Figure 1. Schematic overview of DNA damage response (DDR)

Components of the DDR have been classified into three steps: sensors, transducers and effectors. Sensors and transducers consist of proteins and their post-translational modifications. Effectors also include microRNAs and gene expression changes by transcription factors. Both protein and RNA responses are required for cell fate determination after DNA damage, i.e. repair & checkpoint recovery, cell death, cellular senescence or differentiation.

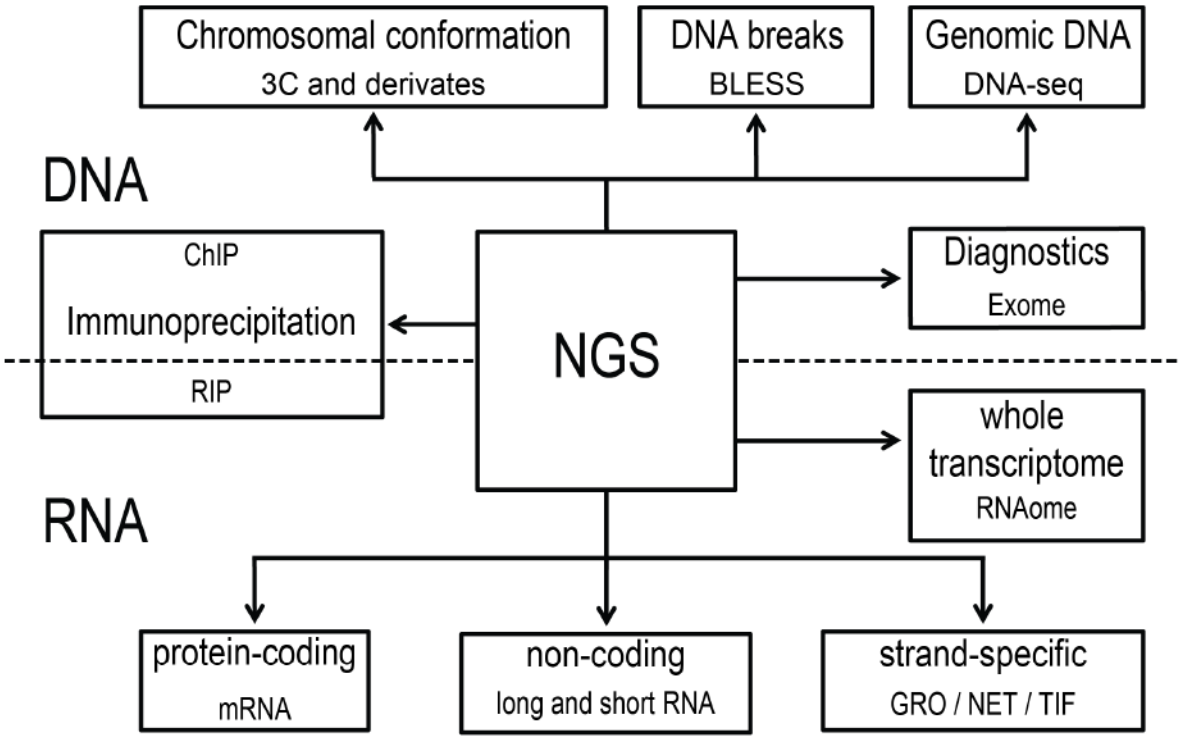

Figure 2. Overview of next generation sequencing (NGS) methods

NGS protocols depicted above the dashed line have been developed to investigate DNA. Detection of DNA-protein (ChIP), DNA-DNA interactions or chromatin conformational changes (3C-sequencing or its derivatives). Nucleotide resolution-mapping of double strand breaks (BLESS). Whole genome sequencing (DNA-seq) or only protein-coding regions of the genome (exome sequencing). NGS protocols below the dashed line have been developed to investigate RNA. RNA-protein interactions by immunoprecipitation of proteins followed by RNA-sequencing (RIP). Protocols that sequence RNA enriched for poly-adenylated transcripts or small RNAs. Protocols for nascent RNA sequencing (GRO/NET/TIF). Ribosomal RNA-depleted total RNA sequencing (RNAome).