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Review

DNA Damage Response: The Players, the Network and the Role in Tumor Suppression

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Abstract. *One of the most common features of cancer is genetic instability. In response to numerous DNA-damaging insults, normal cells have evolved a complex mechanism to monitor and repair DNA damage lesions to maintain genomic integrity. The defects in DNA damage response, indeed, have been shown to associate closely with tumorigenesis. This review provides an overview on the molecular events in DNA damage signaling pathway, including cell cycle checkpoint and DNA repair. The recent research discoveries on how dysfunction in DNA damage response contributes to genomic instability and cancer development are also discussed.*

Cellular responses to DNA damage induced by insults from endogenous and environmental factors are crucial for maintaining homeostasis and preventing cancer development (1). To overcome these attacks and maintain the integrity of the genome, eukaryotic cells have evolved a complex network to detect, signal the presence of and repair DNA damage, which is referred as DNA damage response pathway. DNA damage responses (DDR) are composed of numerous checkpoint and repair proteins that coordinate a complex signaling cascade to assess the damage, then either arrest cell cycle to provide time for DNA repair or trigger apoptosis (2). Other aspects of the DNA damage response include changes in chromatin structure at sites of DNA damage (3) and the transcriptional induction and post-translational modification of DNA repair and checkpoint proteins as well as other proteins that indirectly influence DNA repair (2, 4). In this review the various key features of DNA damage responses and DNA repair are summarized and their impact on cancer development is discussed.

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A Three-tier Signaling Cascade for DNA Damage Response

DNA damage checkpoint events can be linked to a classical intracellular signal transduction pathway. Thus, the stimulus (DNA damage) is detected by a sensor (DNA-damage-binding protein), which triggers the activation of a transduction system composed of upstream (proximal) and downstream (distal) protein kinases, together with a series of adaptor proteins. This kinase cascade amplifies the initial DNA-damage signal and triggers a diverse set of outputs through targeting a wide range of effector proteins (Figure 1). A central issue in the DNA damage response is to understand the early sequence of events that take place between the formation of DNA damage lesions and transducer activation. DNA-damage sensors recruited to the DNA damage sites independently of the phosphatidylinositol 3 kinase-like kinase (PIKK)-containing complexes are known to promote DDR by enhancing the activity of the PIKK proteins and/or by recruiting PIKK substrates to the vicinity of DNA damage, thus facilitating their phosphorylation. Once the DNA damage sensors are activated, the DNA damage signal is mainly activated by two kinases from PIKK family, ATM (Ataxia-Telangiectasia Mutated), and ATR (Ataxia-Telangiectasia RAD3-related). ATM is activated when it is recruited to sites of DNA damage by the MRE11-RAD50-NBS1 (MRN) complex (5-7). Conversely, ATR is proposed to be activated and recruited to the sites of DNA damage by the binding of replication protein A (RPA) that accumulates at sites of single-stranded DNA (ssDNA) (5, 6). A recent study suggests a novel mechanism for ATR activation that is independent of its association with ssDNA-RPA complex. A mediator

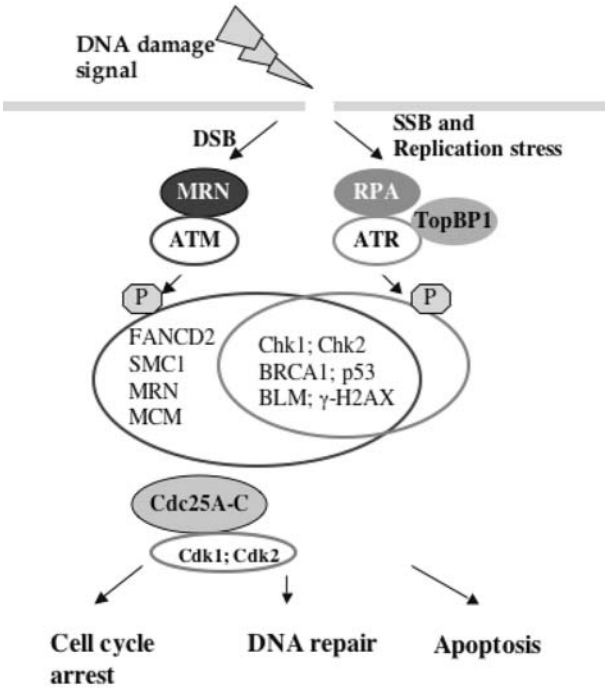

Figure 1. *DNA damage response pathway involves the transduction of DNA damage signal to the complex cellular responses including cell cycle arrest, DNA repair and apoptosis. Two PIKKs, ATM and ATR are on top of this signaling cascade, which phosphorylate a variety of proteins to execute the DNA damage response signaling.*

TopBP1 (Topoisomerase II binding protein 1) has been shown to bind and directly activate ATR kinase activity (8). It is generally perceived that ATR activation is especially critical for the response to lesions generating ssDNA, for instance, stalled replication forks, whereas ATM responds to double-stranded chromosomal breaks (DSBs) (9). However, this distinction is likely to be too rigid as ATR activation has been shown to be regulated by ATM in a cell cycle dependent manner in responses to DSBs. This observation emphasizes the potential crosstalk between the activities of these two kinases (10). Once they are activated, ATM and ATR phosphorylate an array of proteins, which are essential for the signaling cascade in response to DNA damage (11, 12). Substrates of activated ATM include p53, the kinases Chk1(Checkpoint kinase 1) and Chk2 (Checkpoint kinase 2), the Fanconi anemia protein FANCD2, SMC1 (part of the cohesin multiprotein complex involved in sister chromatid cohesion), the DNA helicase BLM1, BRCA1(breast cancer associated gene 1), histone H2AX, the replication fork-associated MCM proteins, and the MRN complex itself. Similarly, ATR phosphorylates Chk1, BRCA1, and BLM. These proteins function as transducers, affecting apoptosis (p53 and Chk2), DNA repair (BLM, BRCA1, H2AX, MRN, and SMC1), cell cycle arrest (p53, FANCD2, SMC1, Chk1, and Chk2).

Spatial Organization of Mammalian Genome Surveillance Machinery in Response to DSBs

A central issue in the DNA damage response is to understand the early sequence of events that take place between the DNA damage formation and transducer activation. In mammalian cells, many proteins that play key roles in the DNA damage response are physically recruited to the chromatin at the sites of DNA damage, resulting in the formation of immunostainable nuclear foci, called IRIF (irradiation-induced foci). IRIF are thought to serve as the platform where repair and checkpoint proteins accumulate to facilitate both DNA repair and propagation of the damage signals (13). A hierarchy of proteins are involved in the assembly of IRIF, which provide a means of ordering the molecular events ensuing from DNA damage detection and signal transduction (14). By utilizing an elegant imaging technology in live mammalian cells to elucidate the temporal order of sensor proteins, MRN complex is found to be recruited first to the sites of DNA damage (15-18). The binding of MDC1 to chromatin requires phosphorylation of histone H2AX (17) and then 53BP1 (19). MDC1 is not first recruited but it is required for sustained binding of MRN and 53BP1 to the damaged chromatin (17, 19).

The next question is how these sensor proteins mediate the next step in the DNA damaging signaling cascade, namely, activation of the transducers. Accumulating evidence indicated that the sensor proteins may mediate the activation of the transducers namely ATM and ATR, in a cyclic process that amplifies the damage signal by repeated interactions among these proteins (18, 20, 21). Thus the signal amplification process depends on the interaction of the sensors and activators with damaged chromatin on the one side and with ATM on the other. An internal spatial organization in the nuclear foci has been established and shown that different classes of proteins occupy specific sub-compartments. ATM is present in DSB-flanking chromatin together with MDC1, MRN, 53BP1 and BRCA1. Single stranded DNA ends demarcate a different subcompartment in which ATR and ATRIP are present together with replication protein A, other portions of MRN and BRCA1, and additional damage-response proteins. This fine spatial organization, which previously seemed to be uniform nuclear foci, reflects the specific function of these foci (22).

Cell Cycle as the Determinant of the DNA Damage Response

It is vital that cell cycle progression is arrested after DNA damage to provide additional time for DNA repair before the cell enters critical periods of the cell cycle, such as DNA synthesis in S phase or chromosome condensation in G2 phase. The two of the most notable mediators in this process

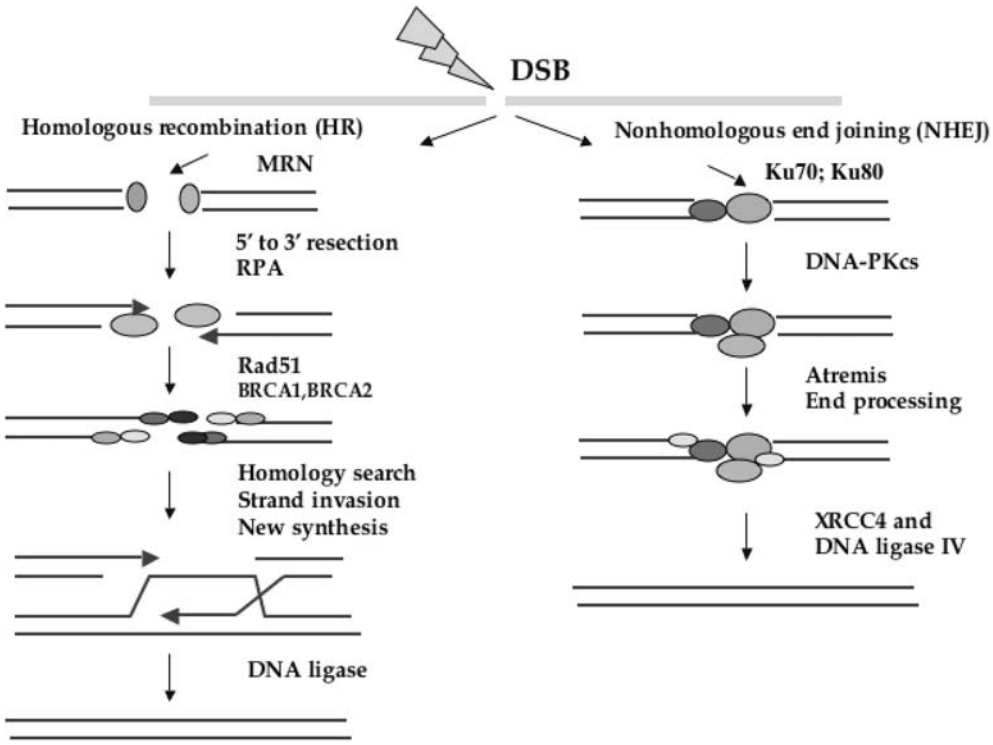

Figure 2. *Two major mechanisms are responsible for the repair of DSB: HR and NHEJ. NHEJ does not require sequence homology and involves a direct ligation of the ends, which usually occurs in G0/G1 phase and has a change in sequence at the break point. HR repairs DSB using the genetic information in the homologous region, which is error free. During HR, upon formation of DSB, the broken ends are recognized and processed by the MRN (Rad50/MRE11/NBS1), which resects 1 strand of broken end from 5' to 3'. The result is 3' ssDNA overhangs. The ssDNA binding protein, RPA, binds to these ssDNA overhangs. Then a large number of proteins such as Rad 54 mediate the replacement of RPA with Rad 51, a DNA recombinase and a key molecule in HR. Recruitment of Rad51 can participate in the search for and invasion of a homologous sequence, usually the sister chromatin. The gapped region in the damage site can be finally repaired by gap repair synthesis and ligation. In contrast to HR, NHEJ pathway involves recognition of DSB by Ku70 and Ku80 and recruitment of DNA-PKcs, which results in the recruitment of Atremis to process the broken end. Then the broken ends can be ligated by XRCC4 and DNA ligase IV complex.*

are the kinases Chk1 and Chk2 (12, 13, 23). As a substrate for both ATM and ATR, Chk1 is a key player in the DNA damage response pathway, the major function of which is to arrest cell cycle at the G2/M and S phase checkpoints. After activation by phosphorylation at Ser345 and Ser317, Chk1 is able to phosphorylate the CDC25A phosphatase. This modification results in CDC25A ubiquitination and subsequent degradation. In its non-ubiquitinated state, CDC25A would otherwise dephosphorylate and activate the cyclin dependent kinases CDK1 and CDK2. This would allow progression through the S phase checkpoint and towards mitosis, which could cause catastrophic consequences in a cell harboring DNA damage. Chk1 also phosphorylates CDC25C at Ser216, leading to its cytoplasmic sequestration by the 14-3-3 proteins and preventing it from acting on CDK1, which results in cell cycle arrest at the G2/M checkpoint. Chk1 may also affect other aspects of DNA damage responses. For example, the function of Chk1 is essential for the maintenance of stalled replication forks, which can often degenerate into DNA damage lesions (12, 13, 23). Like Chk1, Chk2 is a multifunctional serine/threonine kinase, which is activated in response DNA damage. After sensing DSBs in cells, ATM phosphorylates Thr68 on Chk2, which leads to the oligomerization of Chk2 monomers and the trans-phosphorylation at Thr383 and Thr387, resulting in its kinase activity. Chk2 can in turn phosphorylate a number of substrates, for instance, Chk2 can regulate apoptosis in response to DNA damage through phosphorylation of PML, p53, and E2F-1 (12, 24).

Mechanisms of DSB Repair

While cell cycle is arrested in response to DNA damage, the core machinery of the DSB repair pathway is activated. In mammalian cells, two principal pathways, nonhomologous end joining (NHEJ) and homologous recombination (HR) are involved in repairing DSBs (Figure 2) (25). NHEJ is the major route for DSB repair in the G0/G1 phases of the cell

cycle and involves the alignment and ligation of DSB termini. In this process, the KU70 and KU80 proteins form a heterodimer that binds to the ends of a DSB and then recruits DNA-dependent protein kinase (DNA-PKcs), another member of the phosphatidylinositol 3-kinase like kinase family. DNA-PKcs, in turn, recruits and activates another protein (Artemis) that is responsible for processing DNA ends for subsequent ligation. Finally, XRCC4 and DNA ligase IV along with the newly identified component XLF/Cernunnos act to perform the ligation step (26-28). DNA residues lost at the site of the DSB are generally not restored and consequently NHEJ can be mutagenic.

HR, which is favored during S and G2 phases, can be further subdivided into two distinct mechanisms, gene conversion (GC), which is conservative, and single-strand annealing (SSA), which is non conservative. Gene conversion uses an identical sequence, normally the sister chromatid, as a template to copy and replace damaged DNA. In contrast, during SSA, homologous sequences on either side of the DSB are aligned followed by the deletion of the intermediate non-complementary sequence. In complex genomes, these homologous sequences are likely to be repetitive elements. Both GC and SSA are dependent on BRCA1 function (29), and this protein apparently serves multiple functions, including recruitment of additional DNA repair proteins to the DSB, regulation of DNA resection by the MRN complex, and also control of cell cycle checkpoint regulatory proteins (30). In the process of gene conversion, single-stranded 3' overhangs are generated at the DSB by the MRN complex. This ssDNA is then bound by RPA, which is later displaced by RAD51. To enable this displacement, RAD51 is localized to the 3' overhang by the BRCA2 protein, which binds to RAD51 *via* eight evolutionarily conserved binding domains, known as the BRC repeats. The RAD51 nucleoprotein filament then catalyzes the search for homologous target sequences, invades the sister chromatid at the site of homology, and then initiates DNA synthesis using the sister chromatid as template. The final gaps at the end of the newly synthesized sequence are then ligated. The process is completed by resolvases, which remove the links between sister chromatids (31).

It is vital that the core activities of cell cycle arrest and physical DNA repair are integrated when DSBs are processed. Recently, a model proposed by Jazayeri *et al.*, describes the crosstalk between ATM and ATR activation depending on the stage of the cell cycle, which may direct the type of DNA repair used according to the stage at which cell cycle arrest occurs (10). When DSBs are detected in the S and G2 phases of the cell cycle, ATM is activated, which in turn activates the exonuclease activity of the MRN complex. This activity processes DNA at the DSBs such that a stretch of ssDNA is produced. RPA coats this ssDNA, which in turn leads to ATR recruitment and activation. Activated ATR phosphorylates Chk1, which in turn phosphorylates and activates the recombinase protein RAD51 (32). RAD51 is directly involved in the gene conversion pathway that uses RPA-coated ssDNA as a substrate to initiate DSB repair. At the same time, ATM phosphorylates Chk2, which along with activated Chk1 causes cell cycle arrest as described above. Conversely in the G1 phase of the cell cycle, ATM is still activated but has minimal effect on the MRN complex. As minimal exonuclease processing of the DSB occurs, neither ATR nor Chk1 is activated, which leads to the absence of RAD51 phosphorylation and MRN processing. This means that gene conversion is not possible, and NHEJ is then the predominant method for DSB repair. In this phase of the cell cycle, ATM still phosphorylates Chk2, which arrest cell cycle (10). An additional Chk2 substrate is BRCA1 protein. BRCA1 is phosphorylated at Ser988 in response to DNA damage in a Chk2-dependent manner. Mutation of this residue to alanine blocks BRCA1-mediated promotion of HR repair and BRCA1-mediated suppression of NHEJ. This suggests a dual role for Chk2 and BRCA1 in the repair of DSBs (33, 34).

Chromatin Modulation and DNA Damage Response

There is a great deal of evidence indicating that DSB formation triggers alterations in chromatin structure, including dynamic and specific post-translational covalent modifications of histone proteins (35). It is hypothesized that the resulting pattern of histone modifications establishes a distinct molecular architecture, which is recognized by and leads to recruitment of different DNA damage effector activities. Thus chromatin modifications that are thought to play critical roles in the surveillance, detection and repair of DSBs include three ways of directly manipulating chromatin structure: covalent histone modifications, ATP-dependent chromatin remodeling and histone variant incorporation (36-38). Histone modifications implicated in the DNA damage response include phosphorylation, methylation, acetylation and ubiquitination.

γ-H2AX in DNA DSB Signaling and Repair

One of the most extensively studied histon modifications is the phosphorylation of serine 139 on the C-terminal tail of histone H2AX. H2AX phosphorylation is induced after DNA DSBs and has become a standard marker for DSBs of DNA. Extensive phosphorylation of H2AX (*i.e.*, γ-H2AX) is an early and ubiquitous event after DNA DSBs, extending over 60 kb in yeast and up to 1 Mb in higher eukaryotes on each side of the break (39). γ-H2AX formation is necessary for the damage-induced focal accumulation of proteins involved in

checkpoint signaling, DNA repair as well as chromatin remodeling. Importantly, this modification is not required for the initial recruitment of key DDR proteins involved in DNA damage sensing, such as NBS1 or 53BP1 to the DNA damage sites (40). Some proteins, for example, MDC1, bind directly to γ-H2AX *via* an interaction between the Ser139 phosphate and the BRCT domains of MDC1 (41). However, direct interaction with γ-H2AX has not been demonstrated for all proteins recruited to the site of DNA damage. The recruitment of these proteins may be facilitated by exposure to other histone modifications or other docking sites by γ-H2AX-dependent chromatin remodeling (42). Proteins recruited in a γ-H2AX-dependent manner are involved in a number of different functions. γ-H2AX is necessary for signal amplification at low doses of damage for tethering DSB ends, DSB-induced cohesion recruitment and chromatin remodeling that are related to checkpoint and/or DNA repair mechanisms (42-45). It is likely that additional roles of γ-H2AX, both related and unrelated to checkpoint and/or DNA repair mechanisms, will be revealed by further study.

The dephosphorylation and removal of γ-H2AX has been recently shown to be a significant step in turning off the DNA damage response. Dephosphorylation of γ-H2AX is catalyzed by PP2A in human cells, which has a high specific activity for γ-H2AX substrates *in vitro* and is localized to DNA damage sites. Suppression of PP2A leads to persistent γ-H2AX foci formation (46, 47). Furthermore, γ-H2AX has been observed to persist on disorganized chromatin in mitosis, which is thought to represent an aberrant chromatin structure caused by illegitimate rejoining (48). This suggests that active dephosphorylation of γ-H2AX near the break is an important step in signaling successful DNA repair. The complex roles played by γ-H2AX in the DNA damage response remain to be fully elucidated.

H3K7me in DNA DSB Signaling and Repair

Another covalent histone modification involved in the DNA damage response is the methylation of histone H3 at lysine 79 (H3K79me) (49-51). Unlike γ-H2AX, this modification is not induced by DNA damage and is constitutively present on chromatin. The enzyme responsible for this modification is the evolutionarily conserved histone methyltransferase (HMT), Dot1. In mammals, methylation of lysine 79 on H3 (H3-K79-Me) is important for localization of 53BP1 to the site of DNA DSBs *via* conserved hydrophobic residues in the Tudor domain of 53BP1 (50). Cells deficient in Dot1, the HMT responsible for lysine 79 methylation, are unable to form 53BP1 foci after damage. The requirement for H3- K79 methylation in 53BP1 focus formation is most likely due to a direct interaction between H3 and 53BP1, since 53BP1 can bind H3-K79-Me *in vitro* (50). By recruiting 53BP1, H3K79me is likely to be also involved in both signaling and repair.

Histone acetylation in the manipulation of chromatin structure and recruitment at a DSB residue

A transient increase in the acetylation of several lysine residues in the N-terminal tails of histone H3 and H4 occurs at DSBs introduced by site-specific nucleases. These dynamic changes in histone acetylation are particularly prominent when DSBs are repaired by HR (52, 53). At least part of the H4 acetylation induced at DSBs is mediated by the human Tip60 complex (52-54). Tip60 has recently been shown to acetylate both ATM and DNA-PK, an event that appears to be required early in the activation of both kinases (55, 56). Intriguingly, this PIKK activation function could be dependent on the bromo-domains of Tip60, suggesting that Tip60 would first bind acetylated chromatin before activating these two PIKKs.

DNA Damage Response and Cancer

Significant amount of data have shown that several components of the DNA damage response pathway possess the properties as tumor suppressors (57-61). Recent reports indicate that the ATR/ATM-activated network might serve as an inducible barrier to constrain tumor development in early, premalignant stages, and create environment that selects for mutations in checkpoint genes. Tumor-associated defects in the DNA damage response network, such as those in ATM, Chk2 or p53 may rescue defective cell growth and limit cell death at the expense of genomic instability and tumor progression. These findings suggest an important conceptual ramification in identifying the DNA response machinery as a potent anti-cancer barrier activated early during the multi-step tumorigenesis (62, 63). Recently, our own work demonstrated the role of BRIT1 (BRCT-Repeat Inhibitor of hTERT expression) in DNA damage response and its role in cancer development. BRIT1 was originally identified as an inhibitor of human telomere reverse transcriptase (hTERT) in our laboratory from a genetic screening. Analysis of its protein structure revealed that BRIT1 contains multiple BRCT domains (Breast cancer carboxyl terminus), which are predominantly present in the mediators in DNA response pathway such as BRCA1, 53BP1, MDC1 and TopBP1 (64, 65). BRIT1 gene is located on the human chromosome 8p23.1, a chromosomal region frequently deleted in several malignancies including breast, ovarian and prostate cancer. These evidence strongly supported the hypothesis that BRIT1 might function as a novel tumor suppressor gene through its potential role in surveillance of genomic stability. Our studies reveal that BRIT1 functions as a novel regulator in the ATM/ATR pathway and a proximal factor in DNA damage response controlling the recruitment of multiple sensors and early mediators to the DNA damage sites. In response to the irradiation induced DNA damage, BRIT1 is required for the IRIF formation and chromatin binding of NBS1, 53BP1,

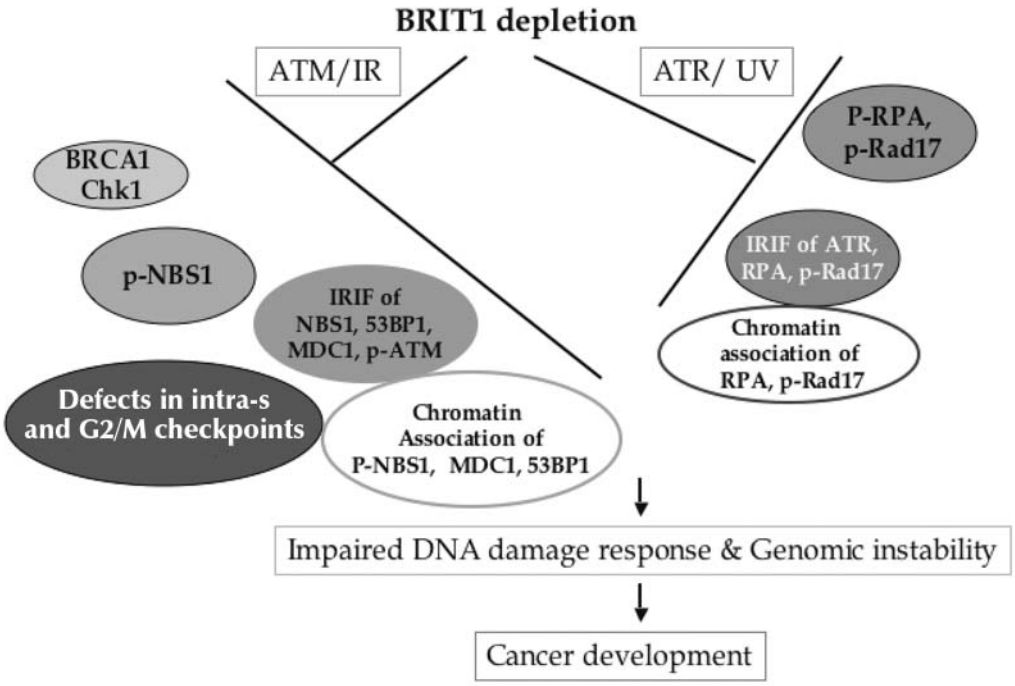

Figure 3. *BRIT1 functions as a key regulator in the proximal DNA damage response pathway. Its dysfunction contributes to the genomic instability and cancer development. In response to IR-induced DNA damage, depletion of BRIT1 results in the defect in intra-S and G2/M checkpoints, which is associated with the decrease of phosphorylation of NBS1, and expression of BRCA1 and Chk1 at transcriptional level. Depletion of BRIT1 also blocks the IRIF formation and chromatin association of early response proteins NBS1, 53BP1, MDC1 and p-ATM. In parallel, upon UV exposure, BRIT1 depletion inhibits the phosphorylation, IRIF formation and chromatin association of RPA and RAD17. Impaired BRIT1 function in DNA damage response leads to genomic instability and the development of multiple cancers.*

MDC1 and p-ATM. BRIT1 is also implicated in the regulation of ATR pathways. It is required for the UV-induced phosphorylation, IRIF formation and chromatin binding of RPA, p-Rad17 and ATR. As a consequence of impaired DNA damage response, depletion of BRIT1 results in chromosomal abnormalities and genomic instability in multiple cell lines. In order to understand whether dysfunction of BRIT1 contributes to the cancer development, by using high-density array comparative genomic hybridization (CGH), we found substantial decreases in BRIT1 DNA copy numbers in ovarian cancer specimens. Expression of BRIT1 are also reduced in breast cancer cell lines and prostate cancer specimens compared to their normal counterparts. It is likely that BRIT1 status correlates with the metastatic potential in breast cancer (66). Collectively, our study suggests that BRIT1, as a potential tumor suppressor gene functions as a key regulator in the DNA damage response pathway (Figure 3). Fully elucidating the function of this intriguing protein using a BRIT knockout mouse model will provide insights into the functions of BRIT1 in DNA damage response and tumorigenesis *in vivo*. In addition, this mouse model will be of great value to identify potential targets for the treatment of BRIT1 deficiency-associated cancer.

Conclusion

Further investigations into the cellular response to DSBs will focus on a comprehensive view of how cells respond to DNA damage, in addition to detailed mechanistic insights into this elaborate response. Each of the three tiers of this signaling system provides many research directions. In particular, several important questions remain unanswered. First of all, what is the nature of the initial signal sent from DNA damage sites to the MRN complex and how does MRN recognize the damage and initiate the binding? Secondly, what is the molecular basis for the activation of ATM? It has been known that activated ATM exists as a form of monomer. Then, what is the nature of the signal that activates ATM and dissociates the inert ATM dimers? Also, where inside the nucleus does this process take place and how localization and phosphorylation of ATM regulates its activity and its downstream signaling. Elucidating the many targets of ATM and its downstream pathways is a continuous endeavor. Understanding the DNA-damage response mediated by this critical kinase and by its interacting pathways through ATR and DNA-PKcs will certainly enhance our knowledge in the key biological processes such as coping with environmental hazards, aging, cancer formation and tumor responses to therapy.

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