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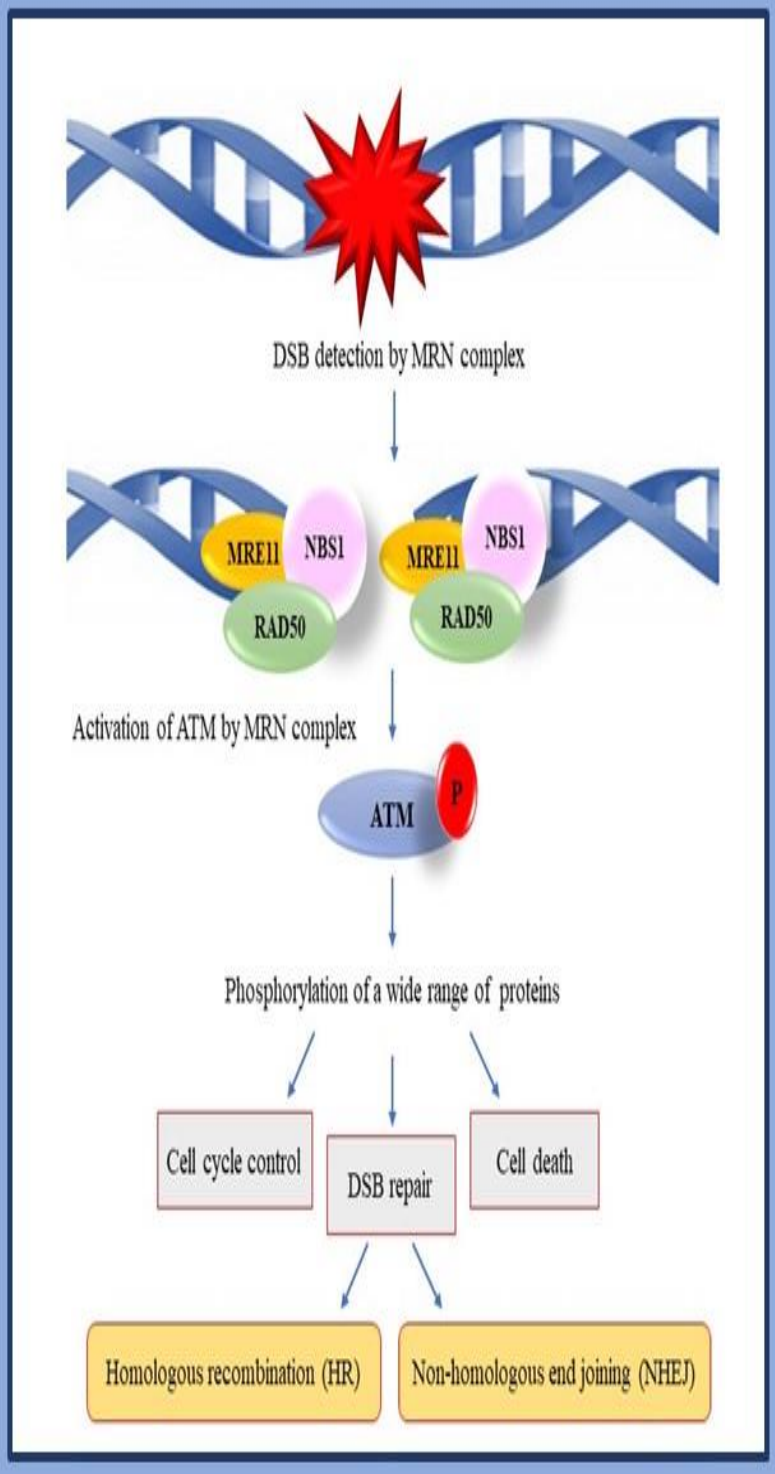
Insights into DNA Damage Response



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Insights into DNA Damage Response

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Genetic paradigms are exposed to various endogenous and exogenous threats. Organisms have evolved powerful DNA repair mechanisms to cope with these genetic lesions. The MRE-11-RAD50-NBS1 (MRN) complex is a key component for sensing and repairing DNA damage, which activates key regulators of these pathways, namely the ataxia-telangiectasia mutated (ATM) protein and ataxia telangiectasia and Rad3-related protein (ATR). ATM, in turn, activates several downstream effectors, such as checkpoint kinase 2 (CHK2) and p53. Furthermore, the chaperone heat shock protein 90 (Hsp90) is involved in regulating signaling pathways in DNA repair in response to double-strand breaks (DSBs). Overexpression of these essential proteins has devastating consequences and is frequently found in tumor cells. To gain new insights into restriction of these overexpression, this study briefly focuses on the signaling pathways, architectures, and conformational aspects of these proteins. The primary goal of this contribution is to share ideas by proposing hypothetical therapeutic approaches to overcome the abnormal phenotypes resulting from aberrations in these proteins. This may lead to minimizing the accumulation of deleterious mutations due to DNA damage and preserving genome integrity.

Keywords: DNA damage response, MRN complex, Protein kinase ATM, Chaperone protein Hsp90, protein phosphatases.

1. Introduction

The human genome encodes the characteristics of life that are proceeded from each organism to its descendants, conserving the genetic information. However, the genome is permanently exposed to endogenous processes induced by cellular metabolism and reactive oxygen species (ROS), as well as exogenous threats arising from chemical reagents, ultraviolet (UV) and ionizing radiations (IR).[1] These genetic lesions can ultimately lead to loss of genome information or wrong transmission to daughter cells. They may cause mutations or chromosomal aberration, cellular senescence, an array of phenotypic consequences, and predisposition to develop malignancies.[2,3] One of these DNA lesions is the double-strand break (DSB), which is the most deleterious lesion. To maintain genome integrity, organisms have evolved multiple mechanisms, such as cell-cycle checkpoints, and potent DNA repair pathways to cope with these lesions by orchestrated cross-talk between these mechanisms. Several DNA damage response (DDR) mechanisms are activated for each lesion. The main

component responsible for DNA repair is the MRE11-RAD50-NBS1 (MRN) complex, which plays a crucial role in sensing and repair DNA damage. In response to DSBs, ataxia-telangiectasia mutated (ATM) protein is considered the key regulator of the DDR pathways, owing to its activation role for a wide range of important downstream substrates, *e.g.*, CHK2 and p53. In addition, the heat shock protein 90 (Hsp90) chaperone regulates various signaling pathways in DNA repair in response to DSBs, due to its role as anti-apoptotic protein. However, its overexpression is commonly found in tumor cells, leading to cancer cell proliferation and inhibition of apoptosis. A better understanding of the signaling pathways, architectures, and conformational aspects of the involved proteins would lay the groundwork for new insights into the treatment of diseases arisen from their overexpression. Therefore, this study briefly casts light on how DNA repair pathways are activated; how the harmonious interaction between the MRN complex, ATM, and p53 works; and how the Hsp90 chaperone participates in DSB repair pathways. This study also offers novel hypothetical therapeutic scenarios for exploring innovative strategies to provide appropriate and effective treatment for cancer and other acute diseases, with the aim of preserving genome integrity by minimizing the accumulation of deleterious mutations.

2. Overview of DNA damage response (DDR) mechanisms

Given the variety of the DNA damages, various DNA damage response (DDR) mechanisms are specifically activated for each lesion (Table 1).[4,5] The main repair mechanisms attributed to the DNA damages include: i) the double-strand break repair pathway (DSBR), with DSB being the most harmful type of DNA lesion, involves homologous recombination (HR) and canonical non-homologous end joining (NHEJ) to restore the sugar backbone of both DNA filaments after their break; ii) the mismatch repair (MMR) occurs when replication and

Table 1. The DNA repair mechanisms.[4,5]

DNA lesions	Common cause	Repair mechanisms
• Double strand breaks	• Ionizing radiation • ROS • Stalled replication forks	DSBR
Replication error: • Base mismatches • Insertion • Deletion	• Inherent in replication	MMR
• 6,4-photoproduct • Intra-strand or DNA-protein crosslinks • Cyclobutane pyrimidine dimer	• UV • Carcinogens	NER
• Basic damage • Oxidised, deaminated, alkylated bases	• UV • ROS	BER
• Single strand breaks	• Ionoizing radiation	SSBR

recombination can cause base-base mismatches and an insertion-deletion loop; iii) the nucleotide excision repair (NER) recognizes helix-distorting lesions to remove bulky adducts and makes a single-stranded excision to remove an oligonucleotide fragment containing the lesion; iv) the base excision repair (BER) restores base lesion arising from oxidation, alkylation, deamination, and depurination/depyrimidination reactions; and, finally, v) single-strand break repair (SSBR) reconstitutes the sugar backbone of the broken single DNA filament.[4,5]

The core component responsible for DNA repair is the MRE11-RAD50-NBS1 (MRN) complex, which has a principal role in sensing damaged proteins and signal other molecules to trigger DDR. This complex is involved in different DNA damage repair pathways *via* interacting with other key players of DDR.[2] The MRN complex is composed of DNA repair protein MRE11, DNA repair protein RAD50 and telomere maintenance protein (NBS1). Mutations in either component of this complex may lead to hypersensitivity to genotoxic agents and predisposition to malignancy, as well as acute diseases such as chromosome instability syndromes – *e.g.* Fanconi anaemia, Ataxia telangiectasia, Nijmegen breakage syndrome, an autosomal recessive disease characterized by immunodeficiency, and Bloom syndrome.[6-8] Mammalian cells have developed a network of proteins to sense and signal DSBs. The members of the phosphatidylinositol 3-kinase (PI3K)-like kinase (PIKK) family: ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3-related protein), and DNA-PK (DNA-dependent protein kinase) are considered the major regulators of the DDR due to their ability to phosphorylate a number of downstream substrates.[9] While ATM and DNA-PK are critical for the signaling of DSBs, being activated in response to ionizing radiation (IR), ATR is mainly involved in response to DNA SSBs, and is activated by UV-induced replication fork stalling. ATM regulates the cell cycle through the phosphorylation of its downstream factors, CHK2 and p53, whereas ATR kinase phosphorylates CHK1 and p53. The MRN complex activates the protein kinase ATM, which in turn phosphorylates the MRN complex, thus producing a positive response. The ATM plays a key role in regulating DNA repair, cell cycle checkpoint activation and DNA damage-induced apoptotic pathways. The DDR is initiated by auto-activation of ATM, which, in turn, phosphorylates many downstream proteins to regulate DDR pathways (Figure 1). The participation of the MRN complex is required to efficiently activate ATM following the induction of DNA breaks.

An important downstream target of ATM is the p53 tumor suppressor that promotes cell cycle arrest and maintains genomic stability and cellular homeostasis. ATM activates p53 regularity by phosphorylating the MDM2 E3 ubiquitin ligase at Ser395 and its homologue, MDMX/4 at Ser403, leading to an increase in p53 protein synthesis, stabilization and activation.[10-12]

On the other hand, Heat shock protein90 (Hsp90) is an ATP-dependent molecular chaperone required for the activation and stabilization of a wide range of client proteins. Hsp90 regulates several signaling pathways in DNA repair in response to DSBs. To fulfill its function, Hsp90 interacts with co-chaperones to form defined binary or ternary complexes that facilitate the maturation of client proteins. As many proteins which control cell survival, proliferation, and apoptosis are client proteins of Hsp90. Hsp90 has cytoprotective functions that are commonly explained by its anti-apoptotic role. Hsp90 is often overexpressed in tumor cells, and plays a role in promoting cancer cell proliferation and apoptosis inhibition.

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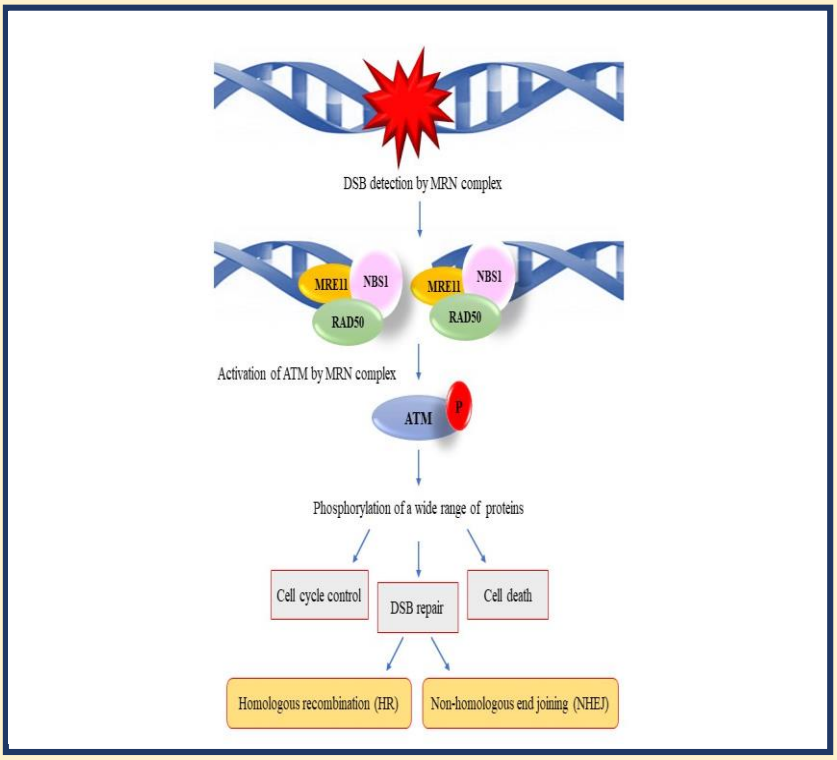

Figure 1. DSB double strand break repair pathway.

3. The DSBs sensing and signaling

The DNA chromosomal DSBs are accidental and potentially dangerous lesions, and defects in their repair pathways results in aneuploidy, genetic aberrations, or cell death, including deletions, translocations and fusions.[4,13] These aberrations can ultimately lead to human disorders including developmental, immunological and neurological diseases, as well as carcinogenesis. These abnormal phenotypes require efficient recognition of DSBs, precise activation of cell cycle checkpoints, and coordinated repair pathways *via* one of several mechanisms that depends on the context in which the break is detected. Among these mechanisms are the NHEJ and HR, which have evolved to repair DSBs. These pathways may either compete or co-operate to repair DSBs. The main difference between these two pathways is that HR can only occur when a homologous template is present and is therefore error-free compared to NHEJ. In fact, HR is most often produce the native DNA, whereas NHEJ is considered error-prone and frequently yields altered DNA sequences, due to the fact that NHEJ is a fast and cell cycle-independent process. The accumulation of genetic lesions arising from error-prone DNA repair leads to cellular apoptosis and senescence.[13] The choice between these two pathways is dependent on the cell cycle stage. In these pathways, the DSBs evoke a rapid change in chromatin structure surrounding the break, as evidenced by a rapid phosphorylation of histone H2AX on serine 139, known as γH2AX (the first observed in cells exposed to γ-rays)[14], allowing both of NHEJ and HR to repair the damage.

The heterodimer Ku70/80 protein, which is a central actor of the NHEJ, rapidly recognizes the DSBs and protects them from nuclease activity. Via its *C*-terminal region, Ku70/80 interacts with the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to form the DNA-PK holoenzyme, which in turn phosphorylates a number of proteins, including the Ku70/80, X-ray-cross-complementation group 4 (XRCC4), XLF, Artemis and DNA polymerase λ (Polλ) for end processing (Figure 2).[4,6,15] Subsequently, XRCC4 and DNA ligase IV, together with other DNA ligase proteins, directly ligate the DSB ends, regardless of DNA loss or mutation. Therefore, the NHEJ is considered as a rapid and error-prone pathway, which is more prominent during the G0, G1 and early S phase. By contrast, HR is a relative slow process occurring only in the late S and G2 phases, as it requires a sister chromatid as a template following DNA replication. HR begins after the generation of a 3' hydroxyl single-stranded DNA (ssDNA) overhang. The 3' DNA tail is coated by replication protein A (RPA), which is in turn replaced by RAD51 (DNA repair protein RAD1 homolog 1) to form a presynaptic filament that is responsible for homology search and subsequent repair.[6,13] The MRN complex has been involved in all

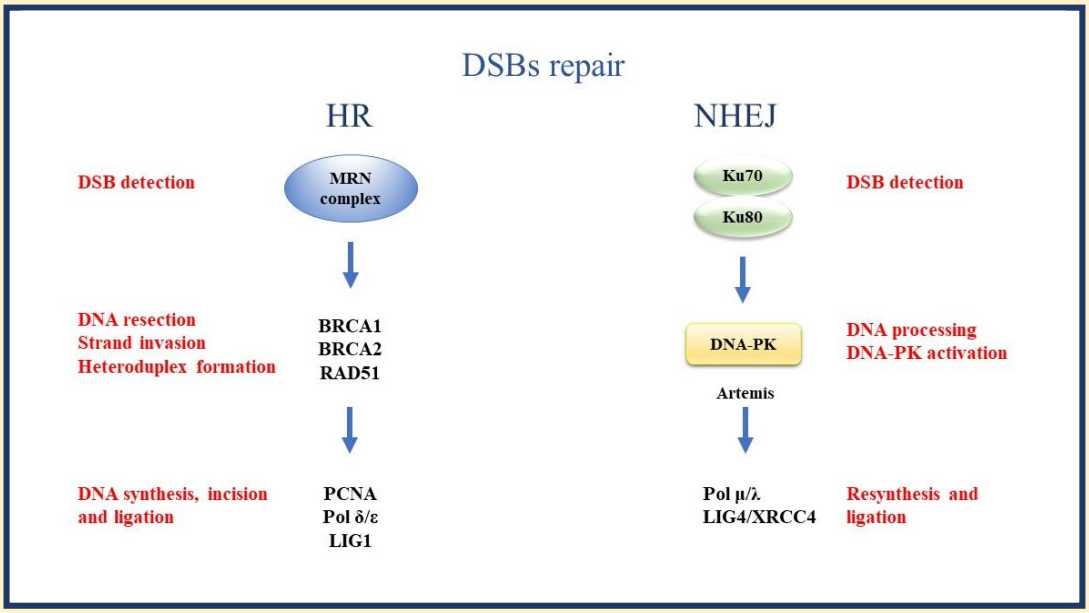

Figure 2. Schematic representation of the DSBs sensing and signaling. Once the cell has sensed the DSB, the DNA repair machinery is recruited to the lesion depending on the cell cycle stage. In G1 phase, cells are primarily repaired *via* the NHEJ repair pathway, while in the G2 phase, the presence of replicated DNA allows repair *via* the HR pathway. The HR pathway recruits the MRN complex to the lesion, which requires the activity of MRE11 or Exo1 exonuclease to resect the ends of the DNA to form a 3' overhang. This structure is stabilized by RPA and then loaded on the homologous DSB region by the strand exchange protein RAD51 and BRCA2, leading to the formation of the Holliday junctions intermediate. The resection of 5′ DNA on either side of the DSB is accomplished by a BRCA1-dependent process, resulting in the exposure of two regions of single-stranded DNA (ssDNA). The tumor suppressor BRCA2 localizes the DNA recombinase RAD51 to the exposed ssDNA regions. RAD51 forms a nucleoprotein filament that can invade the DNA double helix and pair with undamaged homologous sequences. DNA polymerases δ/ε use the homologous DNA sequence as a template and synthesize new DNA. After DNA synthesis occurs, recombination between chromatids can be resolved by endonucleases and the incisions sealed by DNA ligase 1. On the contrary, during the NHEJ pathway, the broken ends are bound by a Ku70/80 heterodimer that recruits DNA-PKcs. After the ends have been processed, the XRCC4/Ligase IV complex completes the final ligation step and the damage is repaired.[15]

aspects of DSB processing, and has emerged as an important regulator of DSB biology, as evidenced by human disorders related to misregulation or inherited mutations in any of its subunits.[16] In the following section, the functions and the structure of this complex would be discussed.

4. The MRE11-RAD50-NBS1 (MRN) complex

As a flexible scaffold, the MRN complex functions as a combined sensor, signaling and effector composite, controlling the biological consequences of DSBs and highly genotoxic lesions through dynamic states. It is regarded as one of the initial sensors and responses to DSB, replication fork (RF) collapse, dysfunction of telomeres and viral invasion. Inability to repair DSBs or protect DNA ends at either replication forks or telomeres can lead to chromosomal rearrangements, loss of chromosome arms or aneuploidy. Mutations in either component of the MRN heterohexamer complex leads to acute diseases, intellectual disability, and cancer predisposition. MRN triggers cell cycle checkpoint response by interacting with ATM and ATR proteins, which are essential components of DDR. MRN complex plays a key role in modulating ATM activity.[2] Moreover, its nuclease activity contributes to DNA end processing for NHEJ and end resection for HR. For example, the suppression of HR-mediated DSB repair may occur in cells through the inhibition of Mre11 complex-mediated DNA resection by B-cell lymphoma 2 (Bcl2) after cellular exposure to high charge and energy particles, leading to tumorigenesis.[6,17] In breast cancer gene 1 (*BRCA1*)-mutant cells, which is a large protein with multiple functional domains and interacts with numerous proteins that are involved in all phases of cell cycle, dynein light chain LC8-type1 (DYNLL1) also inhibits DNA end resection activity by interacting with the MRN complex, although the underlying mechanisms are unclear.[6,18] These evidences reveal the essential role of the MRN complex in DNA repair. In fact, this heterohexamer complex performs three vital roles: i) DNA binding and processing; ii) DNA tethering to bridge DNA over short and long distance; and iii) activation of DDR and checkpoint signaling pathways.[19]

4.1. MRN structural architecture

MRE11 core is crucial for MRN complex formation, DNA binding, and enzymatic processes. The N-terminal of MRE11 contains the phosphodiesterase NBS1-binding sites followed by a cap domain, that can rotate to induce dsDNA unwinding and to orientate the DNA helices for end processing (Figure 3).[2,6] Whereas its C-terminal has a RAD50-binding domain between two DNA-binding domains (DBDs). The MRE11 unit is dimerized through its N-terminal core domains, contributing to the assembly and stabilization of the MRN complex. The N terminal phosphoesterase domain has ssDNA 5'-3' endo nuclease and Mn^{2+}-dependent double-stranded DNA (dsDNA) 3'-5' exonuclease activity, which plays a key role in DNA end-resection to generate 3'-ssDNA overhangs for further repair.[2,6]

The largest component of the MRN complex is the ATP-binding cassette (ABC)-type ATPase RAD50, which belongs to the structural maintenance of chromosomes (SMC) protein family. It contains Walker A and B nucleotide-binding motifs at its N-terminal and C-terminal ends, respectively. The RAD50 ATPase heads are connected by an anti-parallel coiled-coil domain extending toward the Zn^{2+}-chelating CXXC motif (zinc-hook) (Figure 3). The zinc-hook of RAD50 is interchangeable with the SMC hinge, indicating its role as a dimerization

interface. The dimerization of RAD50 *via* the coiled-coil domain assists in the formation of MRN complex and its conformation is controlled by the binding of ATP molecules.[6]

The NBS1 subunit forms the flexible adaptor domain of MRN, and is responsible for protein-protein and protein-DNA interactions. The NBS1 N-terminus has one Forkhead-associated (FHA) domain and two BRCA C-terminal (BRCT) domains,[†] which bind to phosphorylated proteins (Figure 3). For instance, the FHA domain can bind to phosphorylated threonine residues in the Ser-X-Thr motifs of DDR proteins such as Ctp1 (also known as CtIP). It can also recruit DNA damage checkpoint kinases such as Tel1 and ATM, to DNA damage site in order to pause the cell cycle. The NBS1 C-terminus region contains an ATM-binding domain, which helps ATM activation and signaling *via* the adjacent MRE11-interaction domain. The NBS1 protein can be phosphorylated by ATM in turn through Ser-Gln (SQ) motifs in its central part. It also serves as a regulator in the MRN complex, since it can recruit various protein partners to modulate the complex.[6] Moreover, the NBS1 is crucial for localization of MRN to the nucleus, and works in tandem with RAD50 ATP binding and hydrolysis to regulate MRE 11 nuclease activities.[19]

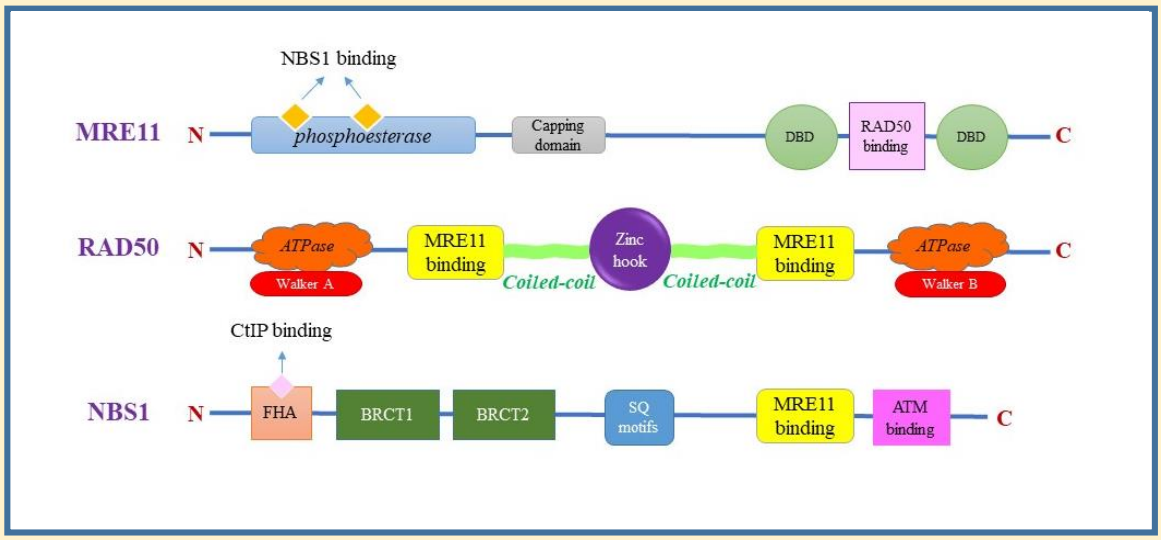

Figure 3. Schematic representation of the basic domain structure of the MRN complex.[2,6]

4.2. The Function of MRN complex

The MRN complex plays a crucial role in the initial detection and responses of DNA damage. The complex

[†] Notably, the BRCA C-terminal (BRCT) domain is the C-terminal of the BRCA1 protein, which cooperates with the BRCA2 protein to maintain genome integrity. BRCA1 is a multifaceted protein and participates in both checkpoint activation and DNA repair, whereas BRCA2 acts as a mediator in homologous recombination.[20] Mutations within the BRCT domain can lead to disruption of the BRCA1 function and ultimately to a predisposition to develop breast and ovarian malignancies.

recognizes and binds to DSBs, which is recruited to DNA damage sites *via* γ-H2AX and RAD17, both of which interact with NBS1. The γ-H2AX interacts with the FHA/BRCT domain of NBS1 following DNA damage *via* the mediator of DNA damage checkpoint 1 (MDC1) to facilitate foci NBS1 formation. In contrast, RAD17 is independent of MDC1 and is phosphorylated by ATM at Thr622 sites, then interacts with NBS1.[6]
Once bound to DSBs, the MRN complex recruits and activates several DDR proteins, mainly ATM through the interaction between ATM and NBS1. ATM is activated by monomerization and autophosphorylation.[2,6]
In HR repair, MRN complex formation and ATM activation need the UFMylation of MRE11 at K282. Such activation of ATM phosphorylates the three subunits of the MRN complex, leading to further activation of downstream signaling pathways.[6]

The MRN complex distinguishes the pathway choice of DSB repair between HR and NHEJ. It is proposed that MRN complex permits HR pathway choice *via* an MRE11 endonuclease cut, which generates 3' ssDNA overhangs that inhibits NHEJ. This is followed by MRE11 exonuclease and EXO1/BLM bidirectional resection toward and away the DNA end, which commits to HR. In HR and NHEJ, DNA tethering and bridging function is predominantly assisted by the Zn-hook in RAD50.[2] In HR, MRN complex is tail-to-tail linked, which such structure facilitates the bridge of sister chromatid and formation of stabilization of displacement loop, a common intermediate in recombination, break-induced replication and telomere maintenance. In NHEJ, the head domains of two MRN complexes bind separate DNA ends, aligning and tethering them after a structure transition.[2]

For proper localization of the MRN complex to the DSBs, the MCM8-9 protein complex is required, which is crucial for DNA resection by MRN complex during HR repair to generate ssDNA. In fact, the minichromosome maintenance (MCM) proteins are a subfamily of hexameric DNA helicases belonging to the AAA+ ATPase family that function in diverse cellular processes, including DNA replication and repair. DNA helicases are motor proteins that move directionally along a nucleic acid phosphodiester backbone, separating two strands of a DNA double-helix. In humans, the MCM helicase family contains eight members (MCM2-9).[21] The homozygous depletion of *MCM9* makes cells hypersensitive to interstrand-crosslinking agents. The association of MRE11 with the MCM8-9 complex at damage sites depends on ATP binding and hydrolysis *via* ATPase motifs, which is facilitated by homologous recombination factor with OB-fold (HROB) protein, possibly by coordinating the conformational changes with DNA translocation and unwinding .[20,21] Genetic or epigenetic inactivation of *MCM8* or *MCM9* are seen in cancers and in a premature ovarian failure (POF) syndrome.[22]
On the other hand, the Bloom syndrome protein (BLM) is also recruited to DSB damage sites, depending on the MRN complex. BLM is a tumor suppressor with multiple functions in DNA damage repair. Its helicase activity contributes to recruiting HR and NHEJ-related proteins in the S and G1 phases, respectively.[6]

5. Ataxia Telangiectasia Mutated protein kinase (ATM)

The ATM is an apical kinase that serves as the mobilizer of the broad signaling network induced by DSBs in the DNA. ATM deficiency has been associated with chromosomal instability, progressive neurodegeneration, endocrine abnormalities, premature ageing, immunodeficiency, cancer predisposition and radiation sensitivity.[23] Moreover, ATM functions as a crucial sensor of oxidative stress in cells and regulates defenses against redox stress by redirecting glycolysis to the pentose phosphate pathway (PPP).[24] ATM also regulates mitochondrial biogenesis, and its deficiency can lead to mitochondrial dysfunction. In addition, it plays a key

role in glucose homeostasis, and is required for the phosphorylation of the insulin-dependent protein kinase, Akt.[24]

ATM resides mainly in the nucleus of dividing cells, where it acts as a transducer in the DDR. ATM is also predominately found in the cytoplasm of non-dividing neuronal cells, where it maintains basal metabolic flux. In other words, it maintains autophagy of these cell types, a catabolic process that transports unnecessary cytoplasmic components to the lysosome for degradation, as well as redox homeostasis. These divergent pathways reveal that ATM can be activated in the cytosol in response to exogenous hydrogen peroxide (H_2O_2) independently of DNA damage response, through the formation of a reversible disulfide bond at the only cysteine site within the protein kinase domain, Cys^{2991}.[24,25] Such intermolecular disulfide bond formation at this residue is crucial for ROS-induced ATM activation.

5.1. The structure and domain mapping of ATM

The ATM protein is a large serine-threonine protein kinase (350 kDa), comprising 3056 amino acids and belongs to the evolutionarily conserved phosphatidylinositol-3-kinase-related protein kinase (PI3K) family. The ATM contains a highly conserved C-terminus FAT (FRAP, ATM and TRRAP proteins),[§] serine/threonine kinase and FATC domain architecture, and an extended N-terminal helical solenoid (multiple α-helical HEAT repeat motifs and a region for ATM interactions with other proteins and DNA) (Figure 4).[26] Under normal conditions, ATM kinase is inactive in cells as a homodimer and quickly undergoes intermolecular autophosphorylation into an active monomer in response to DSB damage to fully activate its kinase activity.[26] Two ATM monomers directly contact head-to-head the FAT and kinase domains. The tandem N-terminal helical solenoid tightly packs against the FAT and kinase domains. From a structural perspective, it was suggested that the dimer interface and the N-terminal helical solenoid could potentially allow tight regulation of the kinase activity by redundantly regulating the bindings of substrates and regulators. The dimer-monomer transition of ATM may therefore expose the substrate and regulator binding domains and allow the formation of active complexes.[26] Indeed, the HEAT motifs of ATM have been found to interact with and recruit a number of proteins to the DNA lesion sites including ATMIN (ATM interacting protein), NBS1 (a component of the MRN complex), and NKX3.1 (a prostate tumor suppressor). In addition to the HEAT motifs, there are other motifs existed on the N-terminal such as a proline-rich area for c-Abl binding, and a leucine zipper.[27] Notably, the catalytic kinase domain of ATM is highly homologous to that of PI3K family. In all PI3K family, the FATC domain always occurs in tandem with the FAT domain. The FATC domain is important for ATM to interact with its partners for activation and control of its kinase activity. The structural motif of FATC domain, in its oxidized form, contains an α-helix and a COOH-terminal disulfide-bonded loop between two completely conserved cysteine residues. Reduction of the disulfide bond resulted in strong conformational changes and increased flexibility of the loop region.[28]

[§] FAT: FRAP (FK506-binding protein 12-rapamycin-associated protein (mTOR), ATM, TRAPP (Transformation/transcription domain-associated protein)) domain.

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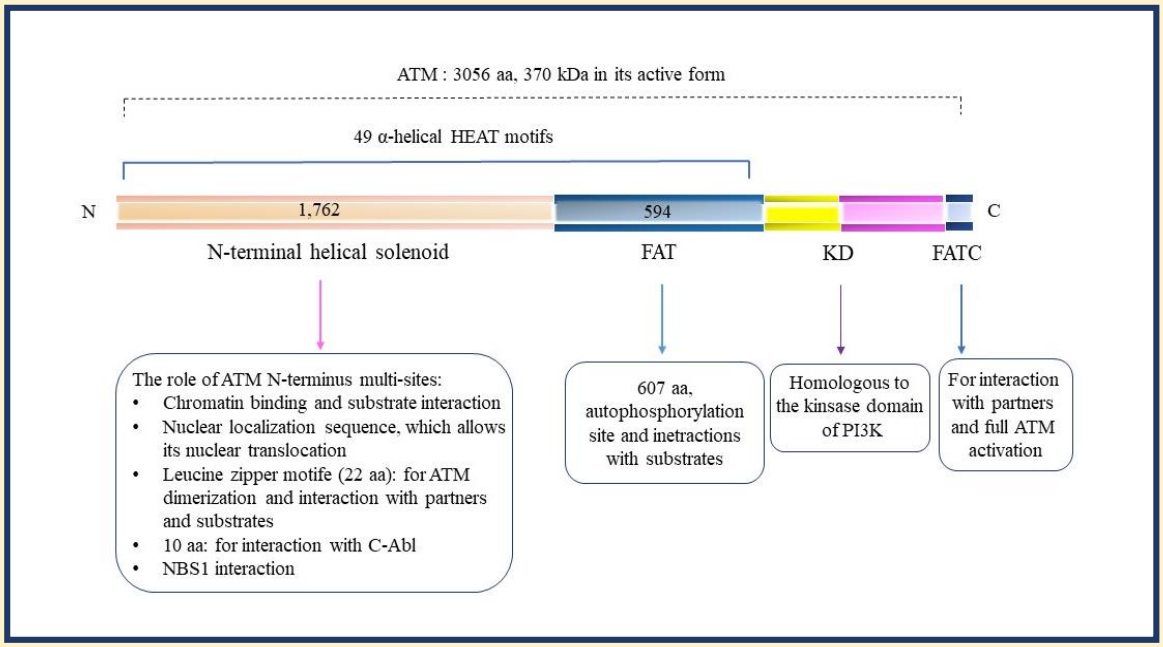

Figure 4. Schematic representation of modular architecture of human ATM's domain map. *ATM* is a large gene with 66 exons and contains multiple domains that enables its protein to interact with numerous regulators, partners, and downstream targets. *ATM* gene encodes a critical serine/threonine kinase essential for genomic integrity. ATM also has 49 α-helical HEAT motifs (huntingtin, elongation factor 3, the A subunit of protein phosphatase 2A, and target of rapamycin 1 (TOR1)). These motifs act as scaffolds, and are important for ATM interactions with substrates and DNA. The number of residues of N-terminal helical solenoid and FAT domains are indicated. [26,27]

5.2. The ATM's function in DNA damage response

ATM activation takes places once it binds to the MRN complex, leading to a conformational change from a relaxing homodimer or polymer state to an active monomer form. Active ATM subsequently employs its kinase activity to phosphorylate a number of downstream targets that are crucial for DNA damage repair, apoptosis, cell cycle arrest, and cell-cycle checkpoints.[27] During this process, ATM undergoes a series of autophosphorylation on Ser367, Ser1893, Ser1981, and Ser2996 as well as acetylation on Lys3016, which are essential for ATM activation. Indeed, activated ATM is recruited to the DNA damage sites, and then activates BRCA1 and ATF2 to promote cascades of DNA damage repair signaling pathways that involve a wide range of sensors, transducers, and effectors. Furthermore, ATM also turns on and stabilizes p53 *via* direct phosphorylation (Figure 5).[27] ATM also phosphorylates MDMX, inducing its degradation, which leads to further p53 stabilization. The p53, in turn, translocates into the nucleus to trans-activate a series of its downstream tumor suppressor target genes. For example, p53 enhances the expression of p21, a potent cyclin-dependent kinase inhibitor. p21 associates with cyclin E, CDK2, CDK4/6 and induces G1/S and G2/M cell cycle arrest, which is critical to prevent unrepaired DNA mutations from passing into daughter cells.[27]

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In addition, p53 directly transactivates Bid, Bax, PUMA to induce apoptosis when DNA damage is too severe for effective repair. This programmed cell death is a major mechanism of tumor suppression.[27]

Furthermore, ATM activates RAD9A to further stimulate the cell cycle checkpoints. CHK1 and CHK2 are induce by ATM through phosphorylation. CHK1 then inhibits TLK1, whereas CHK2 activates the cell cycle inhibitor CDC25A to block CDK2. As a consequence, cell cycle progression is temporarily halted to allow DNA damage repair. ATM is frequently considered a major tumor suppressor whose mutations often lead to significant increase in risk of cancer.[27]

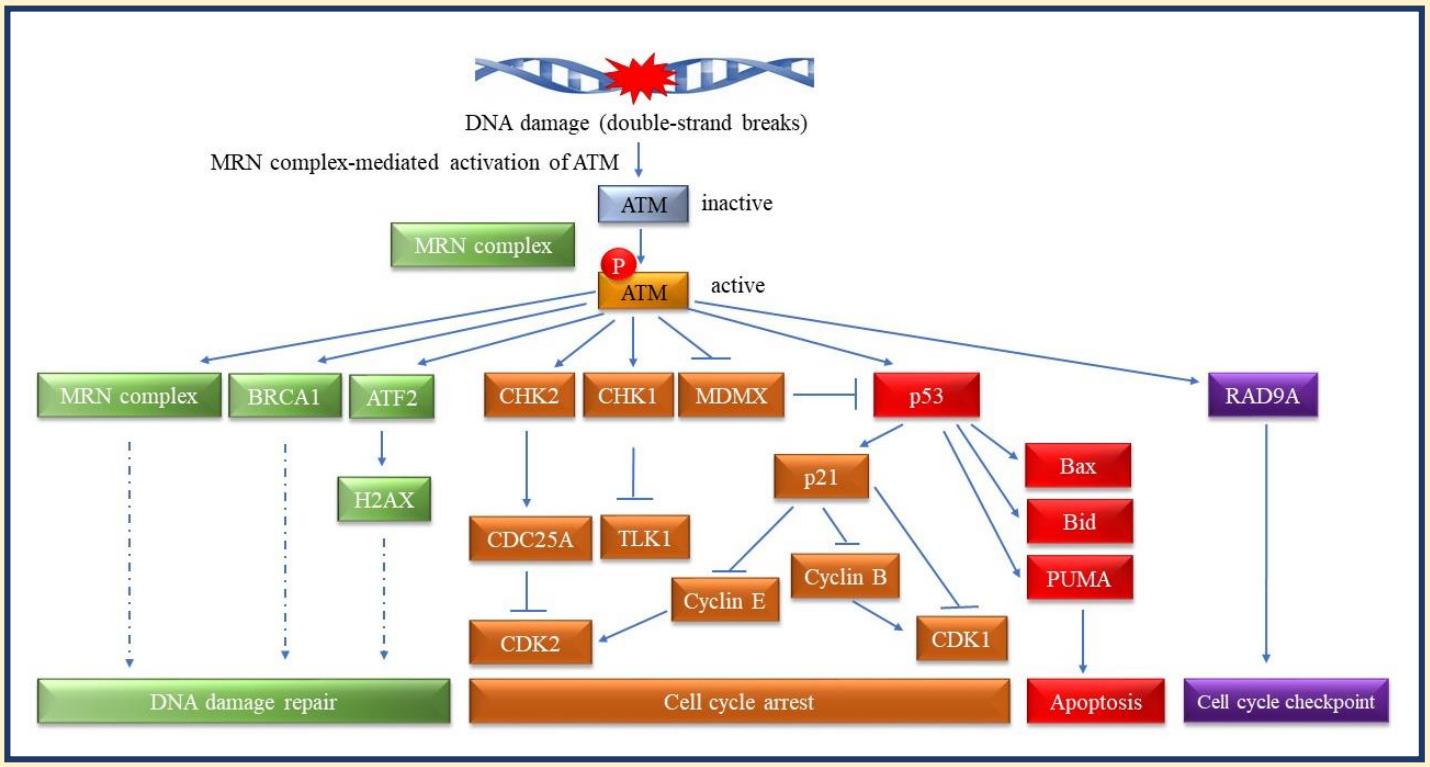

Figure 5. Overview of DNA damage-induced ATM signaling pathway through co-signaling with the MRN (Mre11/RAD50/NBS1) complex at the DNA lesion sites. This ATM canonical signaling pathway phosphorylates several downstream targets, that are vital for DNA damage repair, cell cycle checkpoint, cell cycle arrest, and apoptosis.[27]

5.3. ATM signaling pathways

ATM activation has an essential role in DDR, and is triggered in response to DSB induction and oxidative stress. ATM induces a wide spectrum of signal transduction pathways that connect processes involved in DNA repair, cell metabolism, bioenergetics, and protein translation and transcription.

5.3.1. ATM signaling and DSB induction

ATM signaling could be classified into the canonical pathway and a series array of non-canonical pathways. Several other non-canonical pathways are used by ATM to respond to other types of cellular stresses. In the canonical pathway, ATM shares with the MNR complex for ATM activation and recruitment to DSB lesions. The NBS1 subunit of the MRN complex binds to ATM, and NBS1 ubiquitination promotes ATM recruitment to DSBs. Subsequently, ATM's kinase activity typically targets serine or threonine residues, followed by glutamine (SQ/TQ motif), which phosphorylates several downstream targets to stimulate DNA damage repair machinery.[27]

Strikingly, autophosphorylation at ATM Ser1981, in the presence of MRN complex is not required for ATM function, but ATM is still capable of phosphorylating the downstream histone protein H2AX. This phosphorylation, however, is critical to retain ATM at the DSBs. Subsequently to H2AX phosphorylation, DNA repair complexes containing diverse substrates are recruited to the DSB lesion.[27] For example, phosphorylation of MDC1 (mediator of DNA damage checkpoint protein 1) by ATM recruits RNF8 (an E3 ubiquitin-protein ligase enzyme) and RNF168 (a RING domain E3 ubiquitin ligase), which in turn monoubiquitinate histone H2AX and H2A on Lys13 and Lys15 to stimulate them for additional polyubiquitination reactions. These posttranslational protein modifications are crucial to recruit additional effector repair proteins.[27] Notably, ATM-mediated signaling for DNA damage repair is tightly regulated by a number of feedback mechanisms to ensure proper and effective repair efficiency. For example, as a result of the feedback of p53, ATM activation occurs in a pulse manner.[27]

In non-canonical signaling pathways, chromatin alterations induced by chloroquine, hypotonic cellular stress, among others trigger ATM activation without the need for the MRN complex. The active monomer of ATM binds with ATMIN (ATM interacting protein), that are similar to those of NBS1, to transduce downstream signaling to CHK2, p53, KAP1, and many other proteins, thus promoting genomic integrity and survival.[27] CHK2, the most distinguished effector of ATM signaling, contains seven Ser-Gln/Thr-Gln (SQ/TQ) cluster domains, which are phosphorylated at residue Thr68 by ATM in response to DSB damage.[29] ATMIN is also partially required for ATM-mediated phosphorylation of downstream targets in non-canonical ATM pathways. The DNA damage influences not only expression levels of its target genes, but also exon selection and ultimately their coding potential. The majority of mammalian genes are alternatively spliced to produce multiple mRNA variants from a single gene, expanding thus protein diversity. In non-replicating cells, ATM can be activated by R-loops at transcription-blocking DNA lesions, promoted by chromatin displacement of late-stage spliceosomes. The RNA polymerase pausing at the DNA damaged sites led to spliceosome displacement and R-loop formation, which subsequently activated ATM. R-loop-mediated activation of ATM prevented further spliceosome organization and increased genome-wide ultraviolet-irradiation-induced alternative spilicing.[27,30]

5.3.2. ATM signaling and oxidative stress

ATM also acts as a key redox sensor. After being activated by ROS or oxidation, ATM homodimers form disulfide bonds to maintain their dimer conformations.[27] Similar to active ATM monomers, induced ATM dimers are phosphorylated at Ser1981, but this posttranslational modification is not needed for phosphorylation of downstream targets such as CHK2 at Thr68 or p53 at Ser18.

In response to elevated ROS, ATM dimers activate TSC2 (a negative regulator of mTOR) *via* phosphorylating liver kinase B1 (LKB1) and AMP-dependent protein kinases (AMPKs), blocking mTOR signaling, thereby decreasing ROS levels.[27] ATM, activated by severe hypoxia, also phosphorylates and activates the transcriptional regulator hypoxia-inducible factor 1α (HIF1α), resulting in the upregulation of REDD1, a TSC2 activator. This leads to suppression of mTORC1(mTOR complex 1) signaling and inhibition of ribonucleotide reductase function, resulting in the depletion of deoxynucleoside triphosphates (dNTPs) and replication stress. Consequently, ATM and ATR are activated by severe hypoxia. ATM and ATR then phosphorylate and stabilize HIF1, allowing cell to survive under hypoxic conditions.[31] Bypassing ATM activation plus inactivation in the p53 pathway in early lesions maintains activation of mTORC1 under stress conditions such as hypoxia, which could contribute to tumor progression. In fact, mTORC1 acts as an essential molecular connection between nutrient signals and the metabolic processes, promoting cell growth primarily through the activation of key anabolic processes.[32] There is a correlation between ATM and autophagy in response to nutrient deprivation, ROS, and DNA damage. After being activated by oxidative stress or genotoxic agents, ATM suppresses mTORC1 while inducing autophagy.[27,31]

ATM activation also affects mitochondrial physiology and function by helping to eliminate defective mitochondria through mitophagy, which demonstrates a connection between metabolism and mitochondrial dysfunction. In agreement with this, fibroblasts from individuals with ataxia-telangiectasia have higher ROS levels and are sensitive to oxidative stress, suggesting that ATM not only protects cells from oxidative damage but also maintains low endogenous ROS levels.[33]

Evidences have revealed that ROS-activated ATM support cell migration and invasion *via* interleukin 8 (IL-8), suggesting that ATM might also function to promote tumor progression.[33,34] Although, there is emerging evidence for a role of ATM in promoting tumorigenesis, ATM signaling, early in tumorigenesis, forms a barrier against activated oncogenes and tumor progression, rather than promoting cancer.[33,35]

5.4. ATM signaling dysfunctions and their link with other pathways

ATM is a ploietropic protein kinase reponsible for the phosphorylation of a wide range of proteins in various signaling pathways, thereby fulfilling an important protective role. It participates in protecting the cell against several forms of stress that threaten to perturb cellular homeostasis. Therefore, it is important to provide a brief overview of its dysfunctions and its role in immune regulation, highlighting its role in ataxia-telangiectasia (A-T) and cancer.

5.4.1. ATM dysfunctions and their impact on other pathways

As mentioned above, the ATM kinase acts as a DNA damage sensor, which rapidly orchestrates a stress response to DNA repair machinery and activation of cell cycle checkpoints. This allows the damage to be repaired or, in the case of severe damage, induction can occur. ATM can be activated independently of DNA damage signaling pathways through redox dependent mechanisms and participates in a diverse set of signaling pathways involved in metabolic regulation and cancer. ATM-deficient cells exhibit higher accumulation of ROS, aberrant mitochondria, and defective mitophagy, potentially contributing to tumorigenesis.[36]

Although ATM is considered primarily localized in the nucleus, a minority fraction of the protein has been shown to be present in the cytoplasmic of various organelles/vesicles, *e.g.* mitochondria, peroxisomes. In the neurons of the cerebellum, ATM has been reported to be equally distributed between the nucleus and the cytoplasm.[37] Some of the similarities and differences between ATM functions in the cytoplasm and the nucleus are illustrated in (Table 2).[25]

Table 2. Similarities and differences between ATM functions in the cytoplasm and the nucleus.[25]

	Cytoplasmic ATM	Nuclear ATM
Stimulus for activation	Reactive oxygen species	DNA double strand breaks, chromatin structure change, delayed response to replication fork stalls (via ATR)
Autophosphorylation at S1981	Yes	Yes
Activated protein as monomer/dimer	Unknown	Monomer
Signaling pathways	LKB1-AMPK-TSC2-mTORC1	Many pathways, including p53-p21, CHK2 checkpoint responses
p53 dependence	Independent	Primarily p53 dependent
Cell survival/death pathway regulated	Autophagy (may be pro-survival or death mechanism)	DNA repair (cell survival) or apoptosis (cell death mechanism)

Evidence has revealed that ATM deficiency is associated with increased oxidative stress in cell, which may play a crucial role in neurodegeneration, metabolic dysregulation, and oncogenesis. Studies have shown that ATM-deficient cells have a reduced antioxidant response and are sensitive to treatment with oxidizing agents. In fact, accumulated studies have demonstrated that treatment of ATM-deficient cells with antioxidants alleviates proliferation defects and inhibits the activation of stress-associated signaling pathways that initiated by oxidative stress caused by ATM loss.[37]

Cerebellar atrophy is a cardinal feature of both A-T and Ataxia-telangiectasia-like disorder (ATLD), which is caused by mutations in the gene encoding MRE11. This suggests that the defective DNA damage response associated with ATM deficiency might be sufficient to induce the neurological pathology associated with A-T, but the combined oxidative stress and DNA repair defects in A-T patients could potentially increase the rate and severity of neurodegeneration. Purkinje cells in the cerebellum experience high metabolic demand and oxidative stress, making any loss of redox control particularly damaging to this cell type.[37] Interestingly, oxidative stress disrupts DNA binding by MRN and therefore inhibits ATM activation by MRN and DNA, suggesting that oxidation may be the only operational pathway of ATM activation under conditions of high ROS concentrations.[37]

Oxidized ATM is an active dimer in which the two monomers are covalently linked by intermolecular disulfide bonds *via* residue Cys^{2991}, located in the C terminal FATC domain, which is crucial for ATM activation by oxidation. These disulfide bonds are subsequently reduced by thioredoxin 1, returning ATM to its inactive state.[38] Autophosphorylation of ATM activation by oxidation is therefore not required.

Evidence has shown that ATM functions in the regulation of metabolic signaling pathways involved multiple proteins through activation by oxidation.[37,39] In fact, ATM functions as a redox sensor, and has the capacity to phosphorylate several downstream substrates in response to oxidative stress, maintaining cell homeostasis in the face of oxidative and other forms of non-DNA damaging stress. These proteins are involved in insulin signaling, regulation of AMPK, and regulation of glutathione homeostasis, which are activated *via* AMPK, p53 or mTORC. Such events may culminate in either of the cell cycle arrest, autophagy, and senescence or even apoptosis in the case of severe DNA.

ATM functions upstream of the AKT kinase (also known as Protein Kinase B (PKB), in response to insulin stimulation and IR treatment. Following insulin stimulation, AKT is activated *via* the PI3K signaling pathway, promoting cell proliferation and survival. In response to insulin stimulation, ATM is indirectly required for AKT phosphorylation at Ser473 and for translocation of the cell surface glucose transporter 4 (GLUT4). Inhibition of ATM activity has been shown to inhibit cell proliferation and induce apoptosis in cancer cell lines with overactive AKT.[37]

The effects of ATM on insulin function and glucose metabolism may be mediated through p53 phosphorylation. Deletion of the p53 encoding gene, or its mutation to generate p53 variants lacking the primary ATM phosphorylation site, results in elevated ROS levels, glucose intolerance, insulin resistance, reduced AKT phosphorylation and reduced expression of Sestrin proteins, which are involved in the regulation of intracellular antioxidants.[37]

Accumalted studies have revealed that ATM performs cyctoplasmic functions in siganling pathways related to metabolism and mitabolic stress. In response to IR treatment, ATM activates NF-κB signaling, which promotes cell survival and has been linked to metabolic disorders such as type 2 diabetes. Multiple post-translational modifications of the NF-κB essential modulator (NEMO) are induced by ATM activation in the nucleus, followed by translocation of NEMO and ATM to the cytoplasm, activation of IκB kinase (IKK) *via* TGFβ activated kinase (TAK1), and release of I-κBα from NF-κB.[37]

ATM is required for NF-κB activation following exposure to IR and ROS *via* a mechanism that requires the export of ATM from the nucleus to the cytoplasm. In response to IR treatment and elevated ROS levels, ATM phosphorylates p53 at Ser15. ROS-induced p53 phosphorylation either induces apoptosis or reduces ROS levels through upregulation of sestrin proteins, which regenerate peroxiredoxins, glutathione peroxidase 1 (GPX1) and manganese superoxide dismutase (MnSOD). Mutation of Ser15 in p53 to Ala15 results in elevated ROS levels, leading to insulin resistance and impaired glucose metabolism (Figure 6).[37]

ATM regulates mTORC1 activity through AMP-activated protein kinase (AMPK), a key sensor and regulator of cellular energy homeostasis, in response to elevated ROS levels. Elevated ROS levels activate ATM, which in turn phosphorylates and activates LKB1 at Thr366, which then phosphorylates and activates AMPK at Thr172. AMPK phosphorylates the tuberous sclerosis complex 2 (TSC2) tumor suppressor, resulting in TCS2 activation, and then active TCS2 inhibits mTORC1 activity. Furthermore, ATM directly phosphorylates HIF-1α (one of the subunits of HIF-1 that regulates metabolism, mitochondrial function, and angiogenesis under hypoxic conditions), at Ser696 stabilizing the protein, which also promotes TSC2 activity and therefore blocks mTORC1. Under hypoxic conditions, which is common in solid tumors, ATM is activated and found in a diffuse

pattern in the nucleus. Inhibition of mTORC1 activity by TSC2 induces autophagy, a catabolic process that functions as a cellular salvaging pathway during periods of reduced energy supplies within the cell. Autophagy also functions as a tumor suppression pathway by inhibiting cellular growth. Unregulated mTORC1 activity might lead to cancer by promoting excessive cellular growth and cell division. In addition, high levels of mTORC1 activity result in increased ROS production in mitochondria by increasing oxidative metabolism through expression of the mitochondrial transcriptional regulator PGC-1α and other mechanisms (Figure 6).[37] Therefore, ATM activation by the excess ROS generated by high mTORC1 activity may function as a feedback mechanism to regulate mTORC1 activity.

ATM also regulates mitochondrial function in normal cells, as overall mitochondrial respiratory activity is reduced in A-T cell lines and can be rescued by treatment with an antioxidant or by expression of wild-type ATM. The ATM is found endogenously within cardiac myocyte mitochondria, under normoxic conditions, associated with the inner mitochondrial membrane and the electron transport chain.[40] A-T cells have lower activity of cytochrome c oxidase than normal cells, which could explain their reduced respiratory activity; interestingly, treatment of normal cells with an ATM inhibitor also results in reduced cytochrome c oxidase activity.[37]

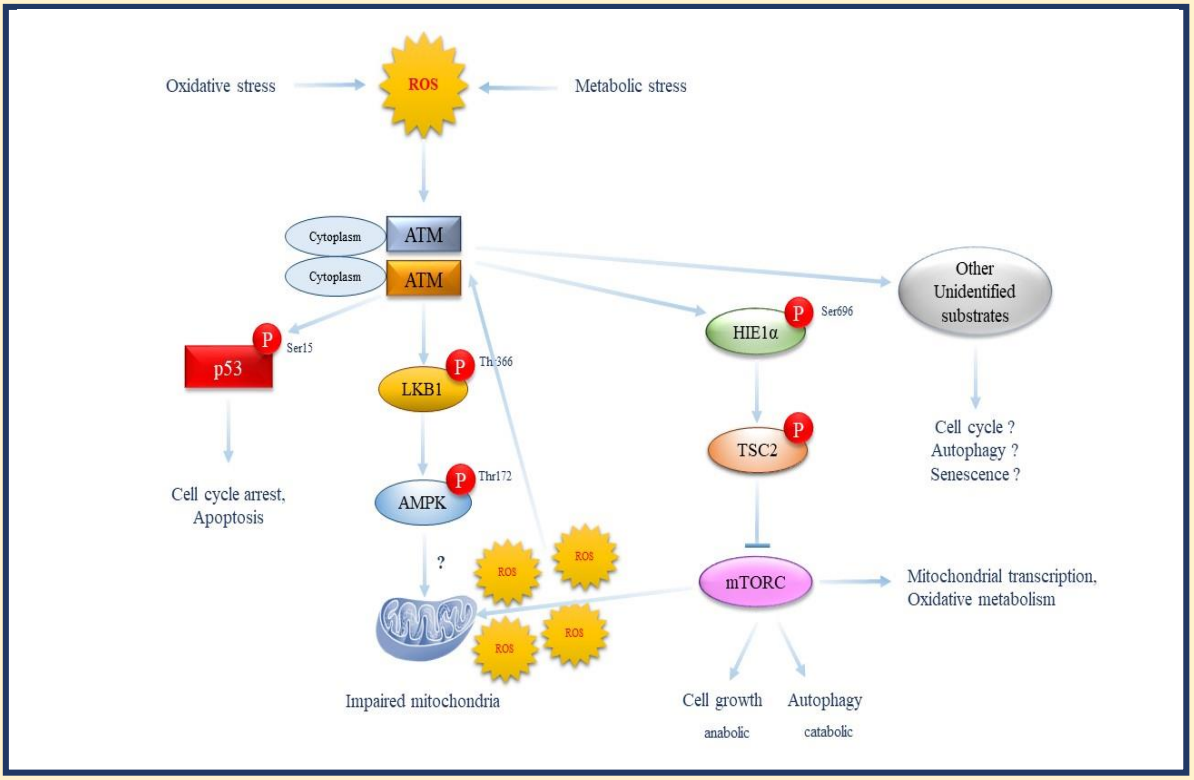

Figure 6. ATM interaction with metabolic signaling pathways.[37,39]

In cancer cells, both ATM and ATR signaling influence tumorigenesis. Indeed, ATM provides a barrier to tumor progression in the early phase of tumorigenesis *via* inducing cell cycle arrest and apoptosis. In precancerous lesions, the ATM pathway is activated, allowing the cell to mount a resistance to tumor development. Furthermore, loss-of-function mutations or deletions of ATM, as well as their reduced kinase activity or expression levels, or deletions of components of their downstream pathways, all promote cell survival and result in a multifold increase in a cell's propensity to become cancerous, and in an acceleration of tumor progression. Strikingly, ATM overexpression, or activation of downstream pathways have been found to cause various human malignancies. Those cancer cells have developed mechanisms to escape ATM-induced cell cycle arrest and apoptosis.[27,33] For example, ATM-mediated activation of NF-κB increases cancer cell survival, blocks apoptosis, and facilitates Epithelial-Mesenchymal Transition, as well as cancer cell migration and metastasis.[27,41]

Decline in ATM functions contributes to the major neurological features of ataxia telangiectasia (A-T). Mutations in the encoding *ATM* gene result in the autosomal recessive disorders A-T. The A-T is a systemic disease characterized by neurodegeneration, hypersensitivity to ionizing radiation, immune-deficiency, metabolic defects and an elevated risk of hematopoietic malignancies. In neural cells, the abundant cytoplasmic ATM in these cells, which is likely to be important for neuroprotection, probably regulates autophagy as a survival measure in response to stress or to maintain redox homeostasis through turnover of damaged ROS-generating organelles, such as mitochondria and peroxisomes. However, such argument may contrast with other proliferative somatic cell types, which must retain the ability to regulate cell cycle progression to maintain genomic stability, and which therefore may favor nuclear ATM signaling and if necessary, induction of apoptosis. These divergent pathways as a result of ATM subcellular localization, different mechanisms of activation and cell survival outcomes may explain some of the pleiotropic phenotypes seen in A-T patients.[25]

5.4.2. The roles of ATM in immune regulation

ATM plays diverse and crucial functions in the immune system. ATM disorder disrupts the cellular immune functions, including thymic hypoplasia, T-cell lymphopenia, abnormal T-cell receptor repertoires, and impaired responses to antigenic stimulation. Patients with ataxia-telangiectasia (A-T) suffer from a deficient ATM function due to nonsense or missense mutations in the *ATM* gene, leading to progressive cerebellar neuronal degeneration, ataxia, immunodeficiency, an increased risk of cancer, and metabolic deficiencies.[36]

ATM plays an important role in lymphocyte biology, particularly in the development and function of T- and B-cells. In the process of V(D)J (Variable, Diversity, Joining) recombination, which is fundamental for generating diverse immunoglobulin (Ig) and T-cell receptor (TCR) repertoires, ATM enhances the stability of coding ends in post-cleavage complexes until joining occurs and maintains coding ends within a stable post-cleavage complex, promoting efficient repair of Recombination-Activating Gene (RAG) complex-induced DSBs and preventing aberrant repair. In addition, ATM plays a key role in Class Switch Recombination (CSR) in developing lymphocytes, which is required for efficient CSR in adaptive immunity, and for introducing mutations in the switch μ region.[36]

ATM deficiency severely impairs both B and T-lymphocyte development. In T-cells, ATM plays a role in T-Cell Receptor (TCR) signaling and regulates the responses to ROS generated during TCR stimulation. This

process in which the lack of ATM triggers apoptosis instead of proliferation. In B-cells, ATM is involved in B-cell receptor signaling, and is a key mediator in NF-κB signaling and other transcription factors, inducing a broad genetic program that enhances lymphocyte survival during antigen receptor loci rearrangement.[36] ATM deficiency causes unrepaired RAG-initiated IgH DSBs in developing pro-B-cells to persist in mature B-cells, potentially contributing to chromosomal translocations.[36] Furthermore, ATM plays a vital role in regulating cytokine production and type I interferon responses, which its deficiency leads to spontaneous production of type I interferons, priming the innate immune system for an enhanced anti-viral response. In fact, the deficiency of ATM enhances innate immune responses to various Pattern Recognition Receptor (PRR) ligands, including those for Toll-like receptors (TLRs) and cytoplasmic sensors.[36]

As a redox sensor, ATM is crucial for regulating cellular responses to ROS generated during T-cell activation.[36] The immunodeficiency associated with A-T may result from the inability of ATM-deficient T cells to control responses to ROS following T-cell receptor (TCR) stimulation.[36,42]

Inhibition or depletion of ATM typically increases sensitivity to cGAS-STING-mediated type I interferon production in inflammatory diseases and tumorigenesis.[36]

The regulation of inflammasome activation, a key component of the innate immune response, is contrlled by ATM. Its deficiency negatively impacts inflammasome activation, resulting in reduced IL-1β production.[36] ATM plays a vital role in macrophage proliferation, differentiation, and function. In bone marrow-derived macrophages, ATM mediates DNA damage response triggered by reactive oxygen intermediates. ATM-deficient macrophages show decreased IL-1β responses to bacterial infections and inflammasome agonists, while maintaining normal IL-6 production. The lack of ATM in macrophages impairs ASC oligomerization, a process where the apoptosis-associated speck-like protein (ASC) self-assembles into large, amyloid-like structures called oligomers, and ASC Caspase-1 foci formation, which are crucial for inflammasome activation. ATM also mediates the DNA damage response in bone marrow-derived macrophages triggered by reactive oxygen intermediates, without requiring the formation of DSBs.[36]

In dendritic cells (DCs), ATM protein levels increase dramatically during DC differentiation from bone marrow stimulated with granulocyte macrophage colony-stimulating factor (GM-CSF). ATM deficiency hinders DC development, resulting in reduced cell proliferation and increased constitutive apoptosis.[36]

ATM mutations contribute to the development of lymphoid malignancies, and are frequently observed in various sporadic lymphoid malignancies.[36]

In immune-related disorders, particularly A-T, ATM mutations lead to complex immunological phenotypes, affecting both humoral and cellular immunity.[36]

One of the important protein that coordinates the spatial and temporal order of protein interactions, and regulating several signaling pathways in DNA repair is the heat shock protein 90 (Hsp90). In the following section, I will discuss how the Hsp90 can participate in DNA repair in response to DSB, including its structure, conformational dynamics, and the correlation between ATM and Hsp90.

6. The Heat shock protein 90 (Hsp90)

Heat shock protein 90 (Hsp90) is an evolutionary conserved and an ATP-dependent molecular chaperone for numerous proteins and plays a role in cellular homeostasis, transcriptional regulation, chromatin remodeling, and DNA repair. It has the ability to correlate protein assembly to protein degradation, which is considered as a quality control mechanism, and provides plasticity for dynamic protein complexes.[43] In fact, Hsp90 is involved in DNA repair pathways and interacts with DNA metabolic proteins *via* the p23 co-chaperone or upon phosphorylation. The Hsp90 chaperone machinery plays a pivotal role in orchestrating the spatial and temporal order of protein-protein interactions, which facilitates the final maturation of specific proteins, termed clients, without the need for *de novo* protein folding as with other chaperones. Hsp90 clients include protein kinases, transcription factors such as p53, and steroid hormone receptors (SHRs).[44] There are two major isoforms in the cytoplasm of mammalian cells: Hsp90α and Hsp90β. Hsp90α is inducible under stress conditions, while Hsp90β is constitutively expressed.[44]

To ensure the proper protein assembly, the Hsp90 chaperone machinery performs three main functions: (i) it specifically interacts with a vast array of clients through adapter co-chaperones (*e.g.*, p23 and Cdc37); (ii) it stabilizes specific folding intermediates that allows clients to interact with specific binding partners; and (iii) it regulates the ubiquitin-mediated proteasome degradation.[43] The Hsp90 activity is regulated at several levels, including the ATPase cycle, the association with conformation-specific co-chaperones, and post-translational modifications. Co-chaperones modulate client protein recognition by Hsp90, regulate the ATPase activity of Hsp90, and modulate the client activities. For example, Cdc37 plays a crucial role in cell cycle regulation; p23 is a key player in the DNA repair processes, and Aha1 activates Hsp90 ATPase activity.[43] In fact, Hsp90 is one of the essential eukaryotic phosphoprotein, and any modification in its phosphorylation status has a serious impact on its functions.

Hsp90 and its co-chaperones form a super-chaperone complex, *i.e.*, the “activated state” of the protein, and the client proteins exhibit a stable association with Hsp90. In contrast, under normal conditions, clients interact with Hsp90 with low affinity since the super-chaperone complex is not formed, Hsp90 being in the so-called “latent state”.[43,45] Increased expressions of Hsp90 above the level observed in normal cells is common feature of both solid tumors and hematological malignancies, since Hsp90 is the molecular chaperone of numerous oncoproteins, and is, therefore, considered a key facilitator of oncogene addiction. Therefore, inhibition of the ATPase activity of Hsp90 impairs client protein recognition, leading to client’s degradation.[43] In fact, the impairment of Hsp90 function has been shown to enhance the cytotoxicity of a number of chemical and physical DNA damaging agents (*i.e.*, ionizing radiation), inhibiting, for example, ATM-dependent repair mechanism.[43]

6.1. The Structure and conformational dynamics of Hsp90

Hsp90 is a homodimeric protein of ~90 kDa exhibiting ligand-dependent close and open conformations. Its structure and related chaperons are featured by three domains and two disordered regions: (i) the N-terminal domain contains the ATP binding site and is crucial for dimerization; (ii) the variable, disordered, and charged linker region, which connects the N-terminal to the middle domain, and participates in the recognition of

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nucleotide, co-chaperone, and client protein, and finely modulates the Hsp90 activity acting as a "rheostat"; (iii) the middle domain is involved in the hydrolysis of ATP and participates in co-chaperon recognition;(iv) the C-terminal domain is responsible for Hsp90 dimerization; and (v) the unstructured C-terminus region, ending with the highly conserved MEEVD (Met-Glu-Glu-Val-Asp) motif, serves as the docking site for the interaction with co-chaperones which contain a tetratricopeptide repeat (TPR) clamp. (Figure 7).[43,44]

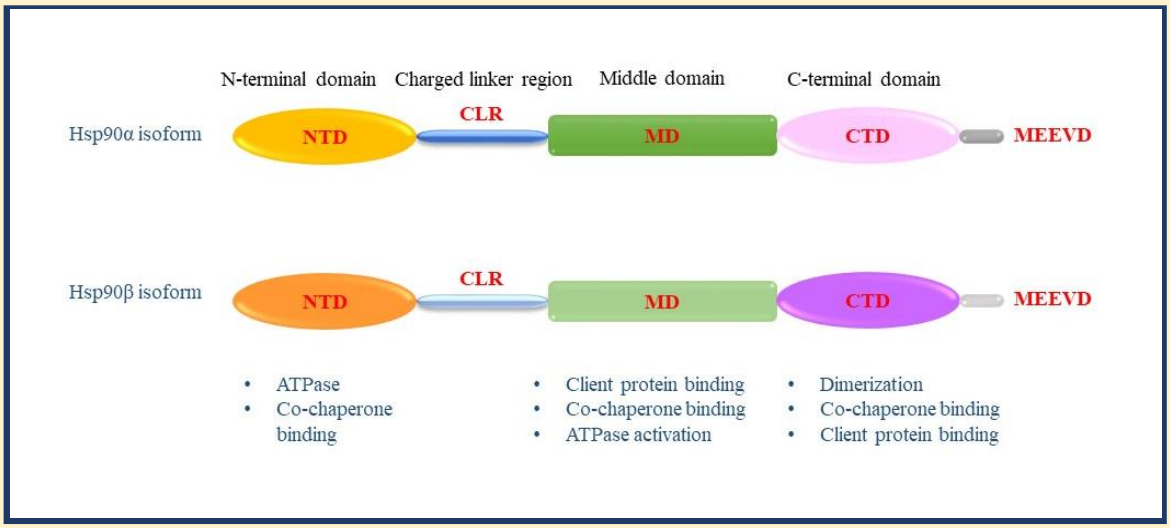

Figure 7. Schematic illustration for the structure of heat shock protein 90 (Hsp90).

Hsp90 adopts structurally distinct conformations, which appear to be in a dynamic equilibrium. Nucleotide binding induces directionality and a conformational cycle. In the apo-state, Hsp90 adopts a "V"-shaped form, termed "open conformation" (Figure 8). ATP binding triggers a series of conformational changes, including repositioning of the N-terminal lid region, that formed by several conserved amino acids residues. This lid region closes over the nucleotide binding pocket in the ATP-bound state, but is open during the ADP-bound state. ATP binding also causes a dramatic change in the N-M domain orientation. Finally, Hsp90 reaches a more compact state, termed "close conformation", in which the N-domains are dimerized.[44]
Notably, Hsp90 also performs ligand-dependent close and open conformations. The interaction with co-chaperones and client protein influences the conformational rearrangement of Hsp90, revealing that there may be a dynamic equilibrium between the different conformations of Hsp90. This conformational plasticity is functionally crucial, since it may allow Hsp90 to adapt to different client proteins.[44]

6.2. Role of Hsp90 in DSB response and its correlation with ATM

The efficacy of the DDR is influenced by the nuclear levels of the DNA repair proteins, which are regulated by balancing between protein synthesis and degradation, and by the control of the nuclear protein import and export. Molecular chaperones play an essential role in protein homeostasis (or proteotasis), regulate protein folding and functions, and mitigate the harmful effects of endogenous and exogennous stressors on the

proteome. Hsp90 represents a proteotasis hub that coordinates both protein assembly and degradation. Hsp90α has a unique feature that is not required for the *de novo* protein folding, but rather facilitates the final maturation of proteins to allow their interaction with binding partners.[46]

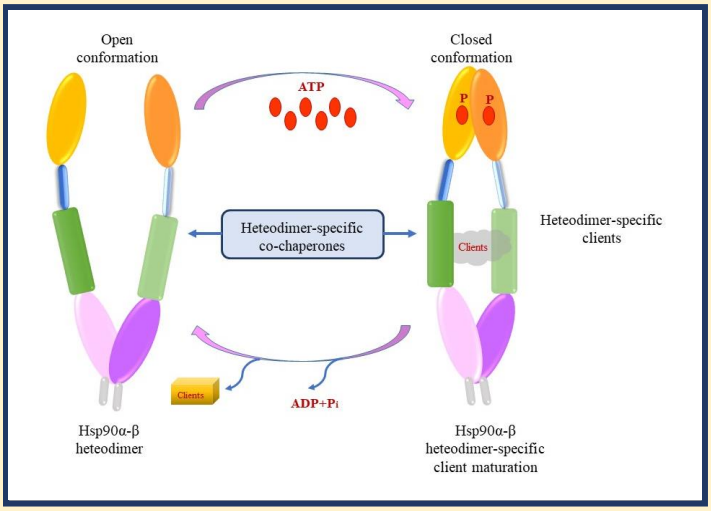

Figure 8. Schematic representation of "open" and "closed" conformation of Hsp90.

Several components of DSBs repair machinery, including MRN complex, RAD51, BRCA1, BRCA2, CHK1, DNA-PKcs, and members of the Fanconi anemia (FA) pathway, have been described as client proteins of Hsp90, which serves as a regulator of various DDR pathways, and is phosphorylated by DNA-PK and ATM at the Thr5 and Thr7 residues, depending on the DNA damage inducer.[43] Hsp90 plays a key regulatory role on the DDR, controlling ATM and NBS1 stability and influencing the DSBs signaling and repair.

Evidence revealed that both of ATM and NBS1 are clients of Hsp90, and form a complexe with the chaperone both in unstressed and irradiated cells. This allows the maintenance of ATM and NBS1 stability that is needed for MRN-dependent ATM activation and ATM-dependent phosphorylation of both NBS1 and Hsp90α in response to IR-induced DSBs.[46] In the presence of DSBs, NBS1 shuttles from a complex with Hsp90α to a complex with ATM, whose kinase activity phosphorylates NBS1 at Ser343, leading to NBS1 dissociation from Hsp90α. This allows NBS1 relocalization at the DSBs together with MRE11 and RAD50, where it promotes the activation of further ATM molecules (Figure 9).[46] However, while phosphorylated NBS1 dissociates from Hsp90α and translocates at the DSBs, Hsp90α phosphorylated at the Thr5 and Thr7 residues partially co-localizes with γ-H2AX at the DSB sites.

In response to IR-induced DNA damage, the cooperation of both ATM and NBS1 is essential for the rapid activation of CHK2 checkpoint kinase. Thus, Hsp90 acts upstream of the ATM-NBS1-CHK2 signaling axis, regulating its function(s). Moreover, the inhibition of Hsp90α ATPase activity influences the nuclear localization of MRE11 and RAD50, impairs the activation of the DDR signaling pathway (*e.g.*, BRCA1 and

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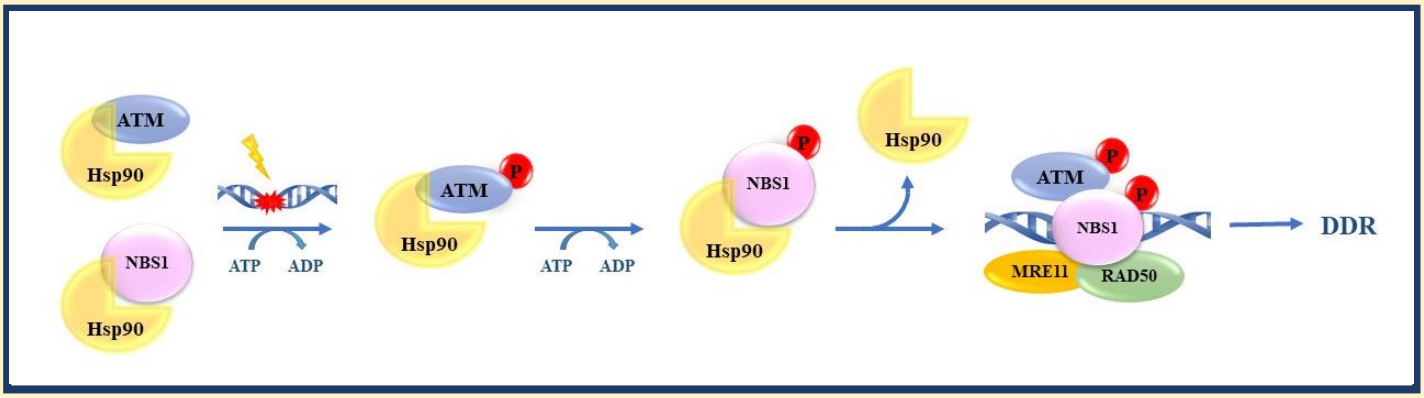

Figure 9. Schematic illustration of the interaction between Hsp90α, ATM and NBS1. In unstressed cells, Hsp90α-ATM and Hsp90α-NBS1 form separate two complexes. Upon IR-induced DSBs, ATM becomes phosphorylated and active, and in turn it rapidly phosphorylates NBS1 and Hsp90α molecules present within the nucleus. The phosphorylated Ser343-NBS1 form dissociates from Hsp90α and relocalizes to the DSBs together with MRE11 and RAD50, where it promotes the DDR.[46]

CHK2 phosphorylation), and delays the DSBs repair.[46] Such a direct link between Hsp90α functions in the nucleus and the DDR demonstrates the radio-sensitizing effects of Hsp90α inhibitors. This is further supported by severely affected cell capability to resist the DNA damage *via* the Hsp90α dependent regulation of ATM-NBS1-CHK2 axes.[46] Strikingly, Hsp90α is spontaneously phosphorylated in several human tumors.[46,47] Thus, Hsp90 has become an attractive therapeutic target in cancer therapy, since its inhibition can induce the degradation of clients and promoting enhanced host natural killer cell-mediated tumor killing.[43] In particular, inhibitors that bind the N-terminal region containing the ATP pocket represent the most effective anti-cancer drugs.[48]

7. The current therapeutic strategies targeting of the ATM pathway

ATM primarily functions as a tumor suppressor, and serves as an essential barrier to tumor initiation by limiting the proliferation of cells with genomic abnormalities, largely through the engagement of the p53 tumor suppressor pathway.[36] However, aberrant upregulation of its signaling can have opposing effects, and can paradoxically promote tumor progression, therapy resistance, epithelial-mesenchymal transition (EMT), and cancer cell metastasis. On the other hand, ATM deficiency might enhance damage tolerance, evading cell death, but promote changes in the microenvironment, and can be associated with immune activation *via* intrinsic/extrinsic mechanisms.[36] Among therapeutic strategies targeting the multifaceted roles of ATM in cancer therapy include: i) inhibition of ATM kinase activity, which is anticipated to delay the repair of DSBs, and potentially impair its activity in regulating cell cycle checkpoints, thereby increasing cancer cell sensitivity to ionizing radiation and DNA-damaging chemotherapeutic agents. Furthermore, ATM inhibition might stimulate interferon signaling and enhance anti-PD1 immunotherapy. Strikingly, ATM inhibition has been shown to enhance immune checkpoint blockade therapy by targeting mitochondrial DNA (mtDNA) leakage

into the cytoplasm and activating the cGAS/STING pathway, ultimately leading to increased responsiveness to type I interferon (IFN-I) production in both inflammatory conditions and cancer development, the detailed molecular mechanisms of which are poorly understood;[36] ii) combination of ATM inhibitors with the enzyme poly ADP ribose polymerase (PARP) inhibitors;[36] and iii) targeting the ATR-checkpoint kinase 1 (CHK1) cascade in ATM-deficient tumors to overcome drug resistance. These ATR/CHK1 inhibitors would enhance killing of tumor cells through cytotoxic drugs or by radiotherapy through blocking cell cycle checkpoints, especially in p53-deficient cells. Such a strategy would sensitize cells to a wide variety of DNA-damaging agents, including IR, antimetabolites, DNA cross-linking agents, topoisomerase I and II poisons, and alkylating agents.[49]

These approaches have shown promising results in sensitizing cancer cells to DNA-damaging therapies, with several inhibitors in clinical development. However, targeting the multifaceted functions of ATM presents challenges, including potential toxicity. The unmet need to overcome therapy resistance, the potential applications in managing inflammation and improving immunotherapy responses calls for further development of potential therapeutic approaches to mitigate normal tissue toxicity and optimize combination therapies. In the next section, I will present some potential approaches, that may inhibit the ATM mutations, and potentially overcome some of the challenges in neutralizing ATM.

8. Hypothetical strategies for neutralizing ATM signaling

Based on the aforementioned study, one could consider the following approaches for neutralizing ATM signaling: i) regulating the protein phosphatase landscape, in particular PPM1A (magnesium-dependent phosphatase 1A); ii) stimulating the ATM kinases to adjust their activation by promoting an environment, that disrupts their overexpression; iii) reducing the oxidative stress caused by elevated reactive oxygen species to initiate a favorable DNA-binding MRN pathway and a disciplined regulation of ATM and ATR proteins; and iv) inhibiting the ATP activity of Hsp90 to impair the recognition of client substrates, thus promoting their degradation.

8.1. Protein phosphatases

Phosphorylation plays an essential role in a wide range of cellular processes, both in physiology and in diseases. Reversible protein phosphorylation is also a vital post-transcription modification that, together with protein kinases, regulates cellular activities. A disruption of the balance between protein kinases and protein phosphatases can have devastating consequences. It is therefore crucial to briefly focus on the regulatory role of protein phosphatase.

8.1.1. The canonical regulatory role of protein phosphatase

I have previously reported that the level of phosphorylation of proteins on their serine (Ser), threonine (Thr), or tyrosine (Tyr) residues depends on the relative activity of protein kinases and protein phosphatases. Protein phosphatases catalyze the removal of phosphate groups from the proteins *via* hydrolysis, which regulates a plethora of physiological processes and signaling pathways (Figure 10).[50] These protein phosphatases belong

to the protein phosphates 2C (PP2C) family of Mg^{2+}/Mn^{2+}-dependent protein phosphatases (PPMs), which comprises 16 distinct enzymes in humans. Protein serine/threonine phosphates (PPM1A) belong to this family.[51]

PPM1A consists of two protein domains, an N-terminal catalytic domain and a 90-residue C-terminal domain. The center of its catalytic domain is a β-sandwich containing two manganese (Mn^{2+}) ions surrounded by α-helical structures. The Mn^{2+} ions bind phosphate groups of the substrate in the binuclear enzymatic center. The activity of PPM1A depends on Mn^{2+} or Mg^{2+}. Various posttranslational modifications, tissue- or cell specific expression, subcellular localization, and degradation also play important roles in the regulation of PPM1A activity. A third metal ion could stabilize the natural structure of the enzyme, where the binding of Mg^{2+} to Asp-146/Asp-239 subunit was found to be essential for catalysis, limiting the conformational mobility of the active site and the specific region of the flip subdomain.[52]

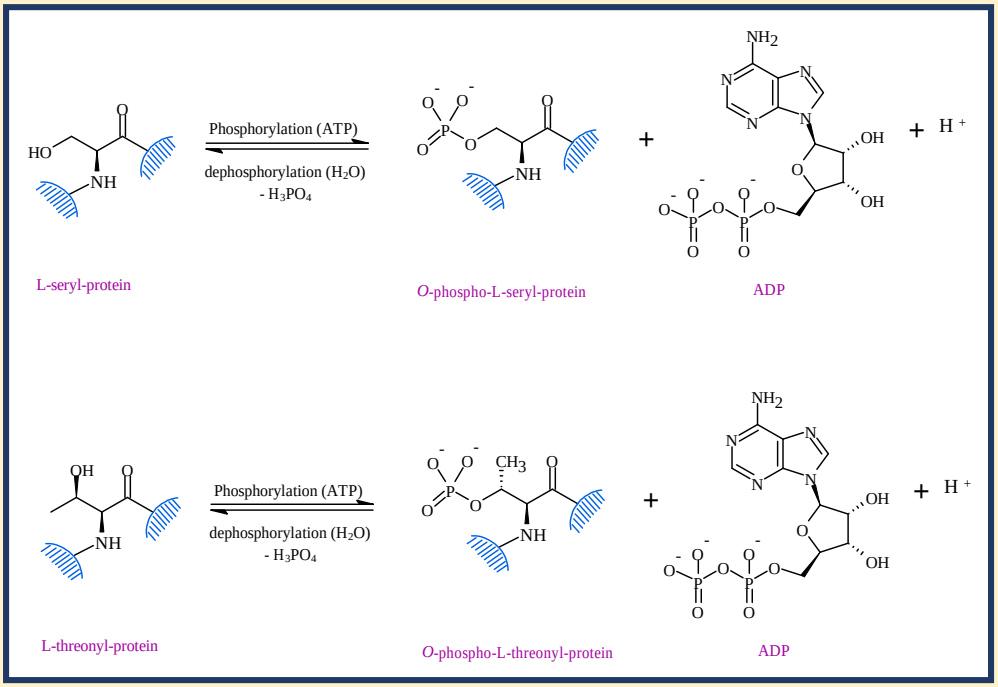

Figure 10. The phosphorylation and dephosphorylation of L-serine and L-threonine residues of the protein.

PPM1A can be modified by myristoylation, a process essential for PPM1A to fulfill its role in the dephosphorylation of its physiological substrates in cells. N-myristoylation is an evolutionarily conserved lipidation that is essential for cell viability in various organisms, including eukaryotic cells and viruses. In fact, some N-myristoylated proteins in small RNA viruses and retroviruses are essential for virus assembly during viral replication or production of infectious viral particles, which may play a role in the survival and propagation

of pathogens. Furthermore, some pathogens need to utilize host cellular machinery to replicate within host cells due to deficiency in viral NMTs.[50]

The hydrophobic myristoyl motif facilitates protein binding in membranes, leading to conformation changes and improved the hydrophobicity of the protein. Moreover, it influences protein localization and the ease with which a protein binds to substrates. The N-myristoylation is catalyzed by N-myristoyltransferase (NMT), and proceeds through two pathways: i) Cotranslational modification, with the most accurate steps occurring after the removal of the methionine initiator by methionine aminopeptidase (MetAP); and ii) Posttranslational modification on an internal glycine exposed by caspase cleavage during apoptosis.[50]

In addition, the cellular senescence-inhibited gene (CSIG), a nucleolar protein, functions as a regulator of PPM1A myristoylation and TGF-β signaling. CSIG is essential for the binding between PPM1A and NMT1 (an isozyme of NMT). The CSIG enhances the dephosphatase activity of PPM1A by promoting its myristoylation, resulting in inhibition of TGF-β signaling, while CSIG knockdown leads to the translocation of PPM1A from its original location in the nucleus to the cytoplasm.[50]

8.1.2. The non-canonical regulatory role of protein phosphatase

In addition to the canonical phosphorylation of the three hydroxyl amino acids in proteins (serine (Ser), threonine (Thr) and tyrosine (Tyr)), the protein phosphorylation landscape includes six of the non-hydroxyl amino acids: histidine (His), arginine (Arg), lysine (Lys), aspartate (Asp), glutamate (Glu) and cysteine (Cys), which can be phosphorylated in proteins *via* non-canonical phosphorylation pathways. The six non-canonical phosphoamino acids function as protein modifications. The phosphohistidine (pHis) is unique in being the only phosphoamino acid that has two isoforms, 1-pHis and 3-pHis, with phosphate linked to either the N-1 or N-3 position on the histidine imidazole ring, respectively (Figure 11).[53]

Because the free energy (ΔG^o) of hydrolysis of the pHis phosphoramidate (P-N) bond is -12 to -14 kcal/mol, considerably higher than that of the phosphomonoester bonds in phosphorylated serine/threonine/tyrosine, the phosphate on pHis serves as an excellent leaving group, and can function as a powerful phosphate donor. The phosphate from a pHis enzyme intermediate can thus be transferred to a various acceptor amino acids, including Asp, Ser or His itself, in a second protein, as well as to small molecules, such as nucleoside diphosphates.[53] Site-specific His phosphorylation can, in principle, serve as a reversible post-translational modification that regulates protein function.[53]

The eukaryotic His kinases belong to the NME family of nucleoside diphosphate (NDP) kinases. These enzymes catalyze phosphate transfer from 1-pHis enzyme intermediate to an NDP molecule to regenerate the corresponding NTP (ATP + NDP ⟷ ADP + NTP), thus maintaining cellular nucleoside triphosphate (NTP) homeostasis, particularly of NTPs other than ATP. The NMEs are highly conserved mammalian proteins, which consist of 10 members in humans, designated NME1-10. They are existed in various cellular locations from cytoplasm to mitochondria, and exhibit diverse enzymatic activities and patterns of subcellular localization. Most of NMEs are known to autophosphorylate on their active site His. They are essential for nucleotide balance, cell signaling, and development, though some members lack the core NDP kinase activity, serving as

scaffolds or having unique roles in processes such as metastasis suppression (NME1) or mitochondrial function (NME6). Their functions also include protein phosphorylation, DNA binding, and regulating G-proteins. The NME1 and NME2 isoforms have been identified as potential canonical transcription factors that regulate gene transcription through their DNA-binding activities. This regulatory function has been proposed to underlie their well-recognized ability to suppress the metastatic phenotype of cancer cells.[54] In addition, NME1 and NME3 have been shown to be involved in repair of both single- and double-stranded breaks in DNA, suggesting that reduced expression of NME proteins could contribute to the genomic instability that drives cancer progression.[54] The chemical phosphorylation of His sites in proteins and peptides can be readily achieved in vitro under physiological conditions with phosphoramidate ($O=P(OH)_2(NH_2)$). Some target His residues in proteins can probably be phosphorylated in cells by small molecule phosphoramidates, such as creatine phosphate.[53]

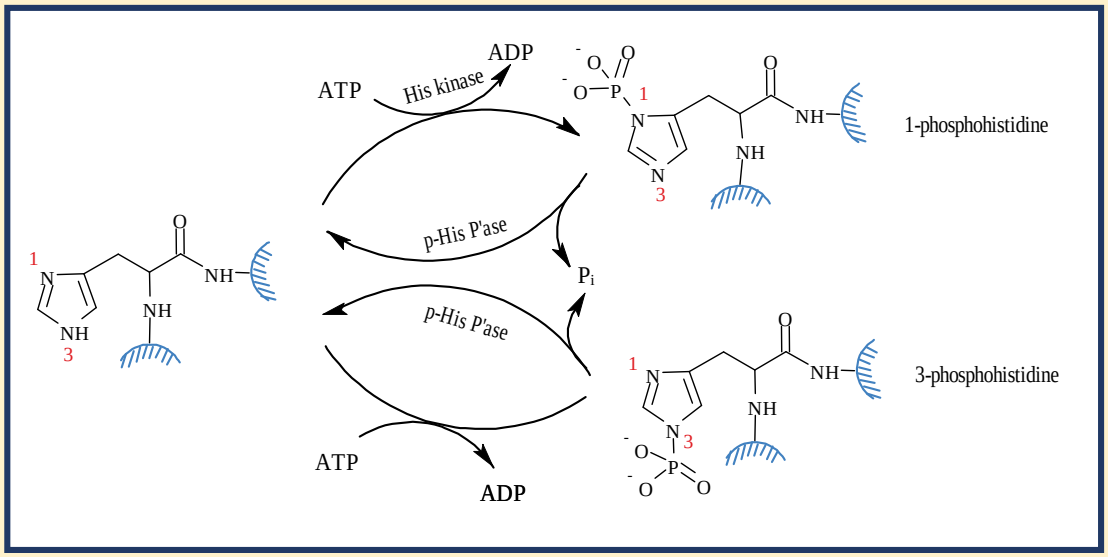

Figure 11. Enzymatic phosphorylation and dephosphorylation of histidine residues in proteins. Histidine protein kinases transfer the γ-phosphate from ATP onto the 1- or 3-nitrogren of the imidazole ring of histidine residues in proteins. The NME1/2 histidine kinases transfer the phosphate in a two-step process *via* a phosphoenzyme intermediate resulting in inversion of phosphate chirality. Phosphohistidine phosphatases (PHPs) hydrolyze the phosphate from the 1- or 3-position of the histidine imidazole ring releasing a free phosphate ion (Pi). In the case of the PGAM5 PHP, the P-N bond in the target pHis is attacked by the active site His of PGAM5, forming a transient pHis intermediate that is then hydrolyzed.[53]

On the other hand, protein-histidine phosphatase activities reverse the action of the His kinases. Three pHis phosphatases (PHPs) can dephosphorylate pHis: PHPT1, PGAM5, and LHPP, a haloacid dehalogenase (HAD) superfamily hydrolase that has been genetically linked to major depression.[53] PHPT1 and PGAM5 dephosphorylate pHis in proteins *in vitro*, although the only pHis protein that PGAM5 is known to dephosphorylate the pHis in NME2 itself. PGAM5 can also act as a pSer/pThr phosphatase, with dephosphorylation proceeding through a pHis intermediate.[53]

His phosphorylation functions as a regulatory mechanism, regulating a wide range of protein functions. Studies have revealed that cell cycle progression, signal transduction, and RNA splicing/processing can be regulated by His phosphorylation. Gene ontology analysis of pHis sites in neuroblastoma cells has linked His phosphorylation to glycolysis, focal adhesion and cell migration, kinase signaling, and protein translation.[53, 55] His phosphorylation plays a role in cancer. The protein kinase activity of NME1 has been shown to act as a potential metastasis suppressor. However, recent studies have also suggested that His phosphorylation promotes carcinogenesis, with increased levels of 1- and 3-pHis in proteins found in both hepatocellular carcinoma and other cancer cells.[53]

8.1.3. The role of histidine phosphorylation - discussion

The role of phosphorylated His in cells raises several questions. Would histidine kinases phosphorylate other proteins involved in DNA damage repair and thus compete with the phosphorylation role of ATM, such as the NBS1 protein *via* the Ser-Gln motif in the central portion? If this were to occur, for example, the regulatory function of NBS1 in the MRN complex would be disrupted, which in turn would affect several of its protein partners and the localization of MRN in the nucleus.

If the phosphorylation of hydroxylamino acids in proteins were mediated by His kinases, would the specificity of their binding sites change, for example, the threonine residues in the Ser-X-Thr motifs of Ctp1?

Could the activities of protein histidine phosphatase (PHP), *i.e.*, PGAM5, overlap with or negate the activity of the protein phosphate 2C (PP2C) family, specifically PPM1A, by dephosphorylating ATM substrates *via* a pHis intermediate? If so, PP2C proteins would accumulate in cells. Several studies have shown, for example, that overexpression of PPM1F, a member of the PP2C family of Mg^{2+}/Mn^{2+}-dependent protein phosphatases (PPMs), occurs in various types of human cancer. PPM1F plays a prominent role in the regulation of integrin function during mammalian development, and this integrin phosphatase plays a crucial role in tumor cell invasion.[56] A recent study demonstrated that the integrin-directed activity of PPM1F plays a dominant role and ultimately determines the ability of transformed cells to overcome extracellular matrix barriers and invade tissue. Furthermore, they found that genetic ablation of PPM1F completely abolishes the invasive ability of glioblastoma cells, while re-expression of PPM1F completely restores the metastatic phenotype without affecting cell proliferation.[56]

NME1 and NME2 function as histidine kinases, transferring a phosphate group from their catalytic histidine to a histidine on other proteins. These enzymes have been shown to bind coenzyme A (CoA) or acyl-CoA, suggesting that they play a role in the regulation of CoA/acyl-CoA-dependent metabolic pathways, in close correlation with the cellular NTP/NDP ratio. Furthermore, NME1/2 has been shown to regulate the cellular processes that consume acetyl-CoA, such as histone acetylation and fatty acid synthesis. NME1/2 regulates histone acetylation, which in turn mediates an important transcriptional response to metabolic changes, such as those that occur following a high-fat diet (HFD).[57] In other words, NME1/2 can be considered as both carriers of cellular CoA and short-chain acyl-CoAs (SCA-CoAs) and enzymes involved in cellular nucleotide homeostasis, and the local concentrations of NTPs and CoA/SCA-CoAs would determine the function of these enzymes. Moreover, the regulatory mechanisms based on CoA/acetyl-CoA binding by NME1/2 are also susceptible to modulating classical functions of these proteins, based on their NDP kinase activity, such as

endocytosis and vesicle trafficking.[57] In light of these facts, the question arises whether NME1/2, as cellular CoA/SCA-CoA transporters, and the role of CoA in protein acylation and its stabilization of proteins may influence the function of ATM proteins in cellular responses to stress.

8.2. RNA- and phosphorylation-induced protein coacervation dynamics

Transient interactions between proteins with their multiple binding sites can cause them to coalesce, forming a dense, liquid-like phase, or droplet, alongside a dilute phase (known as liquid-liquid phase separation (LLPS)). These LLPS are membrane-less organelles, that have been found to be enriched in repetitive sequences of charged and polar amino acids. LLPS plays an important role in the organization of cellular functions, and exhibits diverse roles ranging from signaling pathways to metabolic processes, such as gene regulation, DNA repair, and RNA metabolism. Dysfunctional of LLPS is linked to neurodegenerative diseases and cancer. LLPS can be divided into self-coacervation (SC) or complex coacervation (CC), which are broadly categorized based on the mode of formation as associative LLPS. SC is caused by electrostatic interactions between differently charged molecules or protein domains, while CC is driven by the absorption or repulsion of similarly or oppositely charged (macro)molecules or polymers (polyanions), such as nucleic acids. Several proteins exhibit an uneven charge distribution, with positively charged residues primarily clustering in the middle of the sequence and negatively charged residues clustering at the two terminal regions, such as tau protein. This biophysical trait, attributed to the intrinsically disordered sequence and uneven charge distribution of proteins, significantly influences their susceptibility to LLPS.

Recently, a study investigated the role of phosphorylation in LLPS of tau. Tau is naturally disordered protein with uneven charge distribution throughout its structure. Its dysfunction is associated with extensive hyperphosphorylation at different residues, leading to the instability and disassembly of microtubules, and ultimately, dystrophic neurites.[58] The study has revealed that phosphorylation is a crucial modulator of LLPS, significantly influencing tau's biophysical properties and its aggregation potential. Phosphorylated tau (p-tau) with 2-9 phosphate groups, particularly in the proline-rich domain and C-terminal domain, showed an increased tendency for LLPS. The stability of these LLPS droplets is influenced by factors, such as pH, temperature, ionic strength, which impact their intermolecular interactions. Indeed, phosphorylation indirectly promotes aggregation by facilitating LLPS, creating a favorable environment for nucleation and protein-protein interactions.[58] Moreover, p-tau has been shown to adopt a more exposed conformation, suggesting that LLPS-mediated conformational changes play a critical role in aggregation processes.

The researcher further demonstrated that the introduction of RNA induces a heterotypic CC, wherein external negatively charged RNA facilitates further phase separation. Regarding the mechanisms and timeframes governing phosphorylation-induced SC and RNA induced CC of tau, they revealed that RNA significantly impacted the stability and morphology of tau condensates, driving their transition toward a gel-like state, which exacerbated aggregation over time. RNA play an essential role in modulating tau's condensation and aggregation tendencies within cellular environments.[58] Furthermore, the sensitivity of p-tau to electrostatic and hydrophobic interactions under varying ionic and pH conditions further emphasizes the complexity of LLPS dynamics.[58] They also found that p-tau SC can induce the aggregation of tau repeat domains at the nuclear envelope, as observed in Alzheimer's disease (AD) brain sections, implying that p-tau might play a vital role in AD pathogenesis.[58]

Given the significant role of RNA- and phosphorylation-mediated protein coacervation dynamic, what possible interpretation can be made to explain such mechanisms with other proteins? As a rational interpretation, two possibilities can be considered: a dynamic state and a quiescent state. A dynamic state defines the formation of an LLPS system, mediated by phosphorylation of proteins, as a transiently regulation of cellular functions, leading to rapid exchange of contents with the surrounding cell fluid and subsequent degradation. A quiescent state, on the other hand, defines LLPS-mediated phosphorylation of proteins as a long half-life during which the formed LLPS droplets persist, and further phosphorylation and/or mutation of other residues takes place, leading to the formation of metastable micro-droplets, that transform into solid nanoassemblies. These nanoassemblies eventually develop into a tumor over time.

8.3. Hypothetical approaches for therapeutic intervention

Canonically, ATM plays an essential role in sensing DSBs and is recruited and activated by MRN through the interaction with NBS1, thereby orchestrating their repair in mammalian genome. Furthermore, ATM plays a diverse and key role in the immune system. Overexpression and mutation of the autophosphorylation sites disrupt ATM signaling in human cells. As such, ATM inhibition could promote microenvironmental changes, that could potentiate immune activation *via* intrinsic/extrinsic pathways.[59,60] Transiently inhibition of ATM would, therefore, reduce its overexpression and potentiate the immune activation mechanisms. This strategy requires synergistic combinations of molecules to mask the N-terminus of NBS1, on the one hand, delaying ATM' recruitment to DNA damage site to pause the cell cycle and transiently deactivate its interaction with ATM. On the other hand, such molecules have the ability to initiate deposphorylation/ hydrolysis of the ATM's active site. In addition, shielding the N-terminus of ATM, which contains proline-rich region, from non-canonical phosphorylation, would likely be helpful.

In response to oxidative stress, ATM is crucial for maintaining cellular redox homeostasis. Therefore, a potent antioxidant molecule is needed to dissociate the intermolecular disulfide bonds of the residue Cys^{2991}, and to reduce ROS concentrations in cellular processes.

Furthermore, inhibiting Hsp90 activation is emerging as another strategy to degrade the clients and enhance immune activation. By shielding its N-terminus region, which contains the ATP binding site, from interaction with clients using metal cations, its conformational dynamics would be constrained.

ATM regulates a wide range of key effectors of downstream substrates. The main goal of these proposals is therefore to stimulate ATM to adjust its functions, and ultimately boost cellular immunity. I have previously described conceptual scenarios for neutralizing TGF-β to diminish the affinity of αv integrin for RGD motif of TGF-β associated peptide (LAP) chain by complexing it with the complex ion catalyst *cis* β-hydroxoaquatriethylenetetramine cobalt(III), $[Co(trienH2O)OH]^{2+}$; dissociating the disulfide-bonded homodimer of the LAP peptides by coordinating selenium-containing nanomaterials with ROS-inducting properties; and inhibiting the phosphorylation process of SMADs and protein kinase residues by the hydrolyzing ATP, which is susceptible to the catalytic action of a variety of divalent metal cations (M^{2+}).[50]

Moreover, I previously reported a hypothetical approach for key kinases controlling centrosome cell-cycle fidelity, based on imposing a dynamic activation environment. This ultimately leads to motivating and adjusting the activation of centrosome cell-cycle proteins using 2-thiohistidine, which may be phosphorylated and

hydrolyzed with a tunable half-life. This molecule possesses unique antioxidant, radical-scavenging, and metal-chelation properties.[61] These conceptual strategies could be used in the design of multifaceted therapeutic agents for neutralizing ATM proteins.

8.3.1. Aquohydroxo-cobinamide

An increased ratio of oxidized to reduced ATM is indicative of oxidative stress. Therefore, there is a need for a potent, effective and versatile antioxidant molecule capable of treating diseases with elevated oxidative stress. The cobalamin (vitamin B_{12}) analog cobinamide is a powerful antioxidant, that can neutralize a wide range of oxidants, such as superoxide, hydrogen peroxide, and peroxynitrile. It functions as both a superoxide dismutase and catalase mimetic. It has a high affinity for ligands, and functions as an efficient cyanide and sulfide scavenger. Moreover, it is water-soluble and stable in aqueous solutions. It has a reduction potential of +270 mV, comparable to that of superoxide dismutase enzymes (~300 mV), and counteract lipid and protein oxidation, and DNA damage.[62] A study revealed that cobinamide was more effective in its +2-oxidation state than in its +3-oxidation state at reducing H_2O_2-induced JNK phosphorylation. *In vivo*, cobinamide may exist primarily in +2-oxidation state, as +3-oxidation state is rapidly reduced to the +2-oxidation state *via* ascorbate, cysteine, and GSH.[62] Due to cobinamide's strong antioxidant properties, I hypothesize it could be potentially useful in regulating proteins affected by increased oxidative stress and could dramatically reduce ATM oxidation.

Cobinamide was synthesized from cobalamin by base hydrolysis using freshly prepared cerium hydroxide (from cerium chloride) and purified over two reversed-phase resin columns. In aqueous solutions at neutral pH, a water molecule and a hydroxyl group are coordinated to the cobalt atom, affording aquohydroxocobinamide (Figure 12).[63]

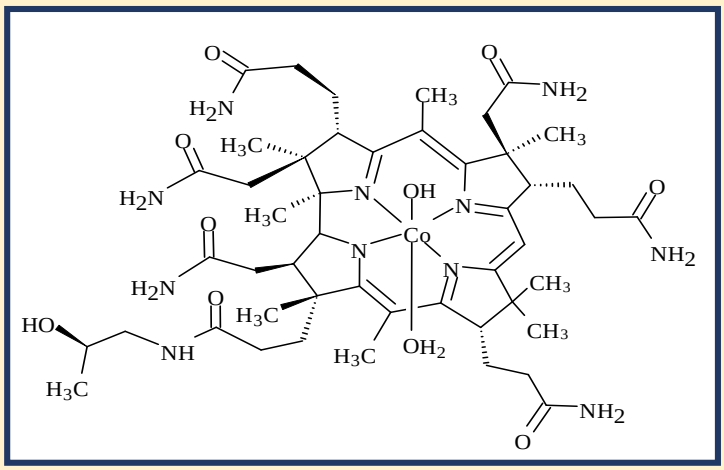

Figure 12. Aquohydroxocobinamide structure.

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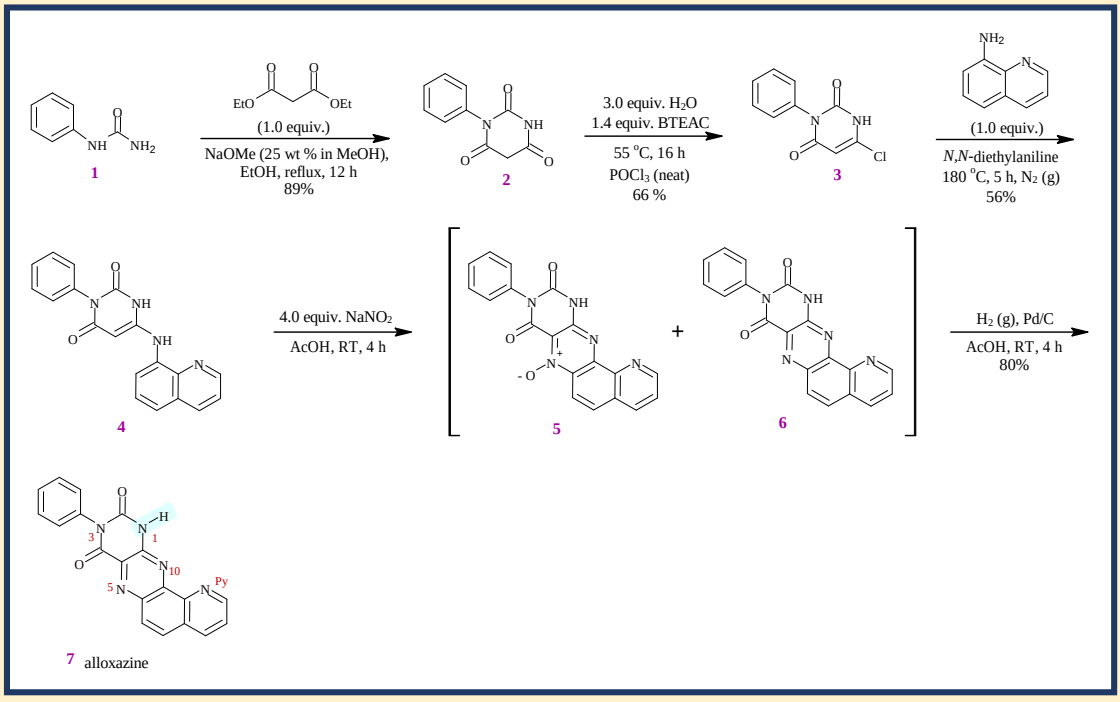
8.3.2. Metalloflavin-like complexes

To diminish the overexpression of ATM kinases, I hypothesize that a metal-organic complex could shield the active site of the kinases by disrupting the local hydrogen bonding; hydrophobic interactions; and the conformational dynamic of the area, imposing a restricted fold conformation on the kinases. Here, I propose metalloflavin-like complexes as multifunctional ligands that enable precise control over coordination environments, leading to advanced catalysis and chemical sensing. Flavins are found in cells, and are almost entirely bound into the structure of proteins. They mediate the transfer of protons and electrons in biological systems, and a diverse range of proteins and enzymes require flavins to perform many essential chemical processes in cells.

A study developed a flavin-derived bidentate ligand, that functions as a redox-active, tautomerized platform capable of constructing both primary and secondary coordination spheres. Upon deprotonation, the complex undergoes tautomerization to the isoalloxazine form forming a hydrogen-bonded aqua complex. In this complex, the ligand switches both its ligation type and its role in hydrogen bonding.[64] The synthetic route for flavin-like, redox-active ligand starts from 1-phenylurea **1**. Condensation of **1** with diethyl malonate, followed by regioselective chlorination, afforded 6-chloro-3-phenyluracil **3**. Subsequent nucleophilic aromatic substitution (S_NAr) with 8-aminoquinoline, oxidation with sodium nitrite, and reduction under hydrogen gas furnished the newly synthesized alloxazine ligand **7** as a yellow powder (Scheme 1).[64]



Scheme1. Synthetic route to the Flavin-like ligand.[64]

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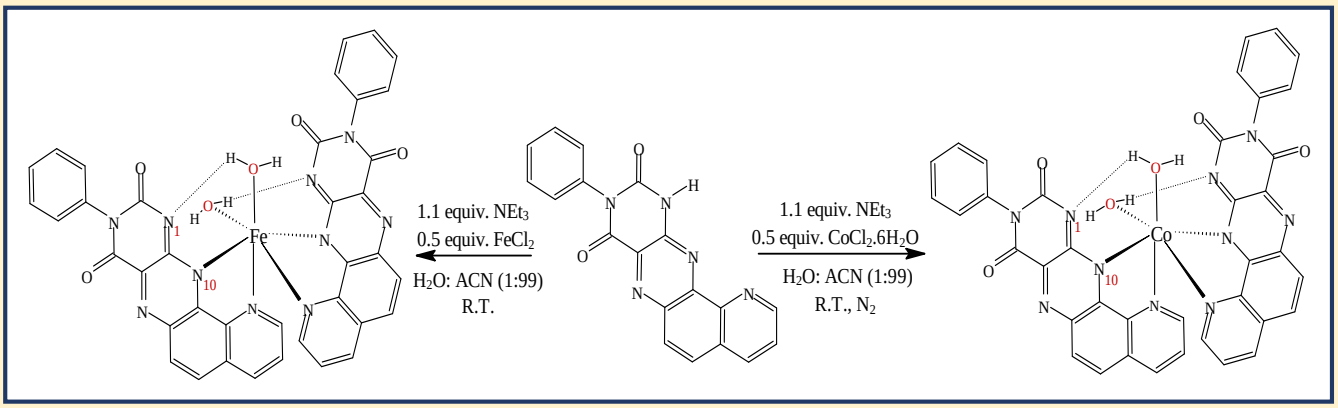

Scheme 2. Base-promoted tautomerization between alloxazine and isoalloxazine in the presence of a transition metal ion. *Note: the synthetic route for metalation with Fe(II) is described in ref. 64, while the metalation with Co(II) is hypothesized by the author*.

Metalation of alloxazine in the presence of trimethylamine base and water as hydrogen-bond donor yielded a brown-colored, paramagnetic species (Sheme 2).[64]

These metal complexes could reduce ATM activation because the iron center under oxidative stress can alternately reduce and oxidize H_2O_2 to H_2O and O_2, while the cobalt ion can play a dual role in the hydrolytic reaction: on the one hand, an orientation or template effect, and on the other, a concentration (of nucleophile) effect at the active site.

8.3.3. Multiple functional nanocomposites

To design therapeutic combinations of multiple functional agents that perform synergistic tasks, structural nanocomposites can enable programmable responses, self-amplifying signals, or feedback controlled delivery. These structural nanocomposites, in which nanotherapeutics are architected with molecular reliability, allow for the fine-tuning of performance metrics, such as circulation time, cellular uptake, and therapeutic potency, while minimizing toxicity. On the other hand, exploring broader sizes increases application potential; smaller nanomaterials (3−10 nm) penetrate cells more efficiently, can more selectively engage key biological targets, penetrate tissues more deeply, and help avoid immune clearance.[65] Larger mimetic structures (300 nm−1 μm), such as red blood cell-mimicking carriers or membrane-coated particles, evade phagocytosis and prolong circulation.[66]

I have previously described novel hypothetical approaches for multifunctional hybrid nanoparticles, including aptamer-functionalized Au@MnO nanoflowers for neutralizing TGF-β;[50] the incorporation of 2-thiohistidine into gold nanoparticles decorated multiwalled carbon nanotubes (MWCNTs); and amphiphilic bacterial cellulose nanocrystals (BCNCs) for transiently blocking the CHK1 kinase and suppressing C-terminus phosphorylation by ATR.[61] These scenarios could also be applied to block the active sites of ATM protein, its downstream substrates, and Hsp90.

8.3.3.1. Bacillus subtilis-templated gold-cobalt nanoparticles

Multifunctional gold-cobalt nanoparticles could enable the application of the same nanoparticle in different domains. The use of bacteria cultures in the preparation of these nanoparticles would allow them to be obtained on a large scale with high colloidal and sedimentation stability. It is therefore hypothesized that gold-cobalt NPs using *Bacillus subtilis* as a biological template could potentially function as biocompatible immobilization materials (Figure 13). Such nanocomposites can be easily functionalized with a number of therapeutic agents by conjugation with nanoparticle morphologies *via* 2-aminoethanethiol ($HSCH_2CH_2NH_2$) grafting.

The chemical composition of the cell wall of *Bacillus subtilis* consists of the cross-linked polymer peptidoglycan (PG), teichoic and teichuronic acids. The long glycan chains of PG are composed of alternating, *β*-1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) subunits. Both anionic polymers, wall teichoic acid (WTA) and lipoteichoic acid (LTA) provide the cell wall an overall negative charge. WTA and LTA have several features: i) they function as a reservoir for mono- and divalent cations; ii) they regulate the activity of autolysins; iii) they act as scaffolds for anchoring cell surface proteins; and iv) WTAs function as receptors for phage binding. Teichuronic acid is free of phosphate, which is available for phosphate binding.[67]

Depending on the *Bacillus* species, a minor form of WTA consists of a polymer chain of *N*-acetylgalactosamine (GalNAc) and glucose phosphate. Teichuronic acid consists of a chain of repeating glucuronic acid-*N*-GalNAc disaccharide residues, coupled to the cell wall *via* a phosphodiester bond similar to teichoic acid. LTA consists of a chain of poly(Gro-P).[67] *Bacillus subtilis* spores are characterized by their stability, safety, and capacity for protein display.

By using a modified method proposed by Kim[68] and Chung,[69] rod-shaped Co@Au nanoparticles could be obtained on the surface of the bacteria. The preparation of rod-shaped Co@Au NPs can be accomplished in four-step procedure: i) synthesis of a microemulsion of hexahydrate nitrate cobalt (II) ($Co(NO_3)_2.6H_2O$) and reducing agent, by mixing equal volumes of cethyltrimethylammonium bromide (CTAB), 1-butanol and isooctane, followed by addition of $Co(NO_3)_2.6H_2O$ and $NaBH_4$ to this mixture; ii) preparation of a gold microemulsion and a reducing agent by mixing equal volumes of CTAB, 1-butanol and isooctane, followed by addition of $HAuCl_4.3H_2O$ and $NaBH_4$; iii) For the preparation of Co@Au NPs, the $Co(NO_3)_2.6H_2O$ microemulsion can be placed in a three-necked flask and purged with argon for 5 min. The miroemulsions are then sequentially injected with a syringe and allowed to stand together in argon atmosphere for 30 min to obtain magnetic Co@Au NPs. The NPs are washed, centrifuged, and dried; and iv) incubation of Co@Au NPs with a suspension of *Bacillus subtilis*, followed by separation and purification of NPs using centrifugation and washing.

The bacteria act as a capping agent for the resulting NPs. Furthermore, they not only function as a carrier of therapeutic agents, but also become fortified bacteria capable of attacking viruses. These *Bacillus subtilis*-templated gold-cobalt nanoparticles may exhibit cytotoxic properties towards human cancer cells, and their magnetic activity could be used for targeted therapies.

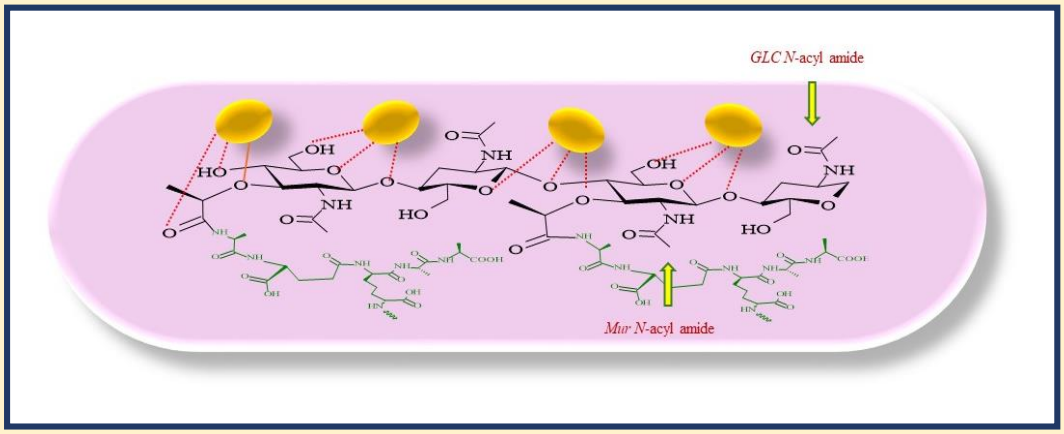

Figure 13. Schematic illustration of *Bacillus subtilis*-templated gold-cobalt nanoparticles.

8.3.3.2. Carbon nanotubes incorporated with cobalt nanoparticles

The incorporation of carbon nanotubes with Co NPs has significant features such as a large surface area and magnetic properties, making them optimal therapeutic agents for treating tumors. One of the methods for preparation such hybrid materials is described by the dissolution of the Co precursor $Co(NO_3)_2 \cdot 6H_2O$) in ethanol together with PVP (polyvinylpyrrolidone) and adding carbon nanotubes to this solution with vigorous stirring. The preparation of carbon nanotube is described in ref. 61. First, the system is sonicated and mixed, then reduced with $NaBH_4$ or ammonia NH_4OH and subjected to ultrasonic treatment at 0 ◦C in an ice bath. The suspension is then stirred for 2 h at 0 ◦C to completely reduce the Co, followed by filtration, washing, and drying at 80 ◦C, to obtain Co_3O_4 NPs. Nanotubes with incorporated cobalt nanoparticles are then calcined at 500 ◦C in an N_2 atmosphere for 1 h to remove PVP.[70]

Studies have shown that Co NPs obtained by the green method exhibit activity against cancer cells.[71] Moreover, it has been proven that for the use of NPs for biomedical purposes, a coating layer is necessary, that ensures the biocompatibility of the particles without changing their magnetic properties. When Co NPs enter the bloodstream, they form a non-magnetic oxide and lose their magnetic properties.[72]

Co NPs are highly cytotoxic towards cancer cells. A study has shown that CoO/Co_3O_4 nanoparticles with a cubic spinal structure, obtained by using fresh leaves of young branches of *Rosmarinus officinalis*, exhibit cytotoxic properties towards human cancer cells. The IC50 value for these NPs is 55 µg/mL to U87 cells, which is the optimal value for cancer therapy. Moreover, NPs exhibit magnetic activity, which can be used for targeted therapies.[73]

8.3.4. Expected consequences of these hypothetical strategies

Based on the strategies proposed above, the following is hypothesized: i) the cobalt center of the corrinoid has a water molecule in the axial site (α), which is readily displaced by nitrogen ligands of proteins. This displacement can lead to significant changes in the protein's activity and selectivity. The hydroxyl group linked to the cobalt can participate in the hydrolysis of amino acids such as serine, threonine, tyrosine, and proline, where it combines with hydrogen atoms to form water, leading to the cleavage of peptide bonds. This dissociation would influence the protein stability and function, and constitute a barrier to enzymatic phosphorylation of these amino acids. Therefore, it is hypothesized that aquahydroxocobinamide may act as a barrier at the N-terminus of NBS1, *i.e.* FHA/BRCT and HEAT motifs and KD of ATM, blocking the formation of NBS1 foci and transiently reducing the activity of ATM proteins; ii) The cobalt of the corrinoid has an affinity to coordinate with sulfide bond, which can lead to the dissociation of the disulfide bond at cysteine Cys^{2991} of the ATM homodimer, which is formed by oxidative stress, and ultimately to the inhibition of ATM kinase; iii) Aquahydroxocobinamide has a powerful antioxidant activity, which has a positive effect on oxidative stress; iv) Metalloflavin-like complexes with their multifunctional ligands can restrict the spatial and conformational environments, thereby blocking the activation of MRN, ATM, and Hsp90; v) Multifunctional nanocomposites imply the availability to design therapeutic combinations of agents with different functionalities to achieve diverse synergistic effects. Incorporating cobalt nanoparticle into these nanocomposites would lead to chelation with several ligands involved in DNA repair proteins and would influence the conformational dynamics; and vi) Involving *Bacillus subtilis* as a capping agent for these nanocomposites architected with therapeutic agents could play an important role in attacking viruses, exerting a cytotoxic effect on tumor cells, enabling their spread to surrounding cells, and its short-chain fatty acids could be beneficial in immunotherapy response, thereby enhancing therapeutic range.

9. Conclusions

Preserving genome integrity requires adjusting and controlling the components involved in DNA damage repair. The key component of DDR is the MRN complex, which triggers cell cycle checkpoint response by interaction with ATM and ART proteins. Controlling ATM kinase activation represents the primary target for repairing deleterious DNA lesions, which influences essential downstream effectors.

This contribution offers a number of new hypothetical approaches for the neutralization of ATM kinases. Aquahydroxocobinamide compounds could shield the active sites of NBS1 and ATM, transiently blocking the activity of ATM kinases *via* forming a barrier, that prevents the interaction between ATM and NBS1 in response to DNA damage. These compounds could dissociate the disulfide bonds of ATM homodimers in response to elevated ROS levels. Moreover, these cobalt compounds possess potent antioxidant activities. Metalloflavin-like complexes could restrict the spatial and conformational dynamics through hydrogen bonding formation and steric hindrance, thereby inhibiting the activation of MRN, ATM, and their downstream effectors. They could also inhibit the phosphorylation processes of these proteins. In addition, they could simulate ATP hydrolysis of Hsp90, leading to reduced its activation. Multifunctional nanocomposites incorporated cobalt nanoparticles could chelate with several ligands participated in DDR, altering the conformational dynamics of these ligands, and also enable the development of combination therapeutic agents, that implement various synergistic tasks.

Capping these nanocomposites with *Bacillus subtilis* could enable them to attack viruses, exert cytotoxic effects on tumor cells, invade surrounding cells, and exhibit a positive immunotherapy response. The bacteria can function as a DNA sensor, enabling multiplexed DNA detection in complex samples, and detecting the DNA released from pre-treated target cells.[74] Furthermore, the magnetic activity of cobalt NPs in these capping nanocomposites could be used for targeted therapies. These ideas present exciting avenues for research in bacterial-assisted molecules as potential drugs for precision lead therapeutic.

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