Functions, Regulation, and Therapeutic Implications of the ATR Checkpoint Pathway

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Abstract

The ATR (ATM and rad3-related) pathway is crucial for proliferation, responding to DNA replication stress and DNA damage. This critical signaling pathway is carefully orchestrated through a multistep process requiring initial priming of ATR prior to damage, recruitment of ATR to DNA damage lesions, activation of ATR signaling, and, finally, modulation of ATR activity through a variety of post-translational modifications. Following activation, ATR functions in several vital cellular processes, including suppression of replication origin firing, promotion of deoxynucleotide synthesis and replication fork restart, prevention of double-stranded DNA break formation, and avoidance of replication catastrophe and mitotic catastrophe. In many cancers, tumor cells have increased dependence on ATR signaling for survival, making ATR a promising target for cancer therapy. Tumor cells compromised in DNA repair pathways or DNA damage checkpoints, cells reliant on homologous recombination, and cells with increased replication stress are particularly sensitive to ATR inhibition. Understanding ATR signaling and modulation is essential to unraveling which tumors have increased dependence on ATR signaling as well as how the ATR pathway can best be exploited for targeted cancer therapy.

INTRODUCTION

The genome is constantly bombarded by extrinsic stresses, such as UV light or ionizing radiation, as well as intrinsic stresses, such as reactive oxygen species and replication errors. To cope with these insults, cells require DNA damage-signaling pathways, which are often referred to as checkpoints, to halt the cell cycle and promote the DNA damage response (DDR). Defects in checkpoints allow for an accumulation of genetic alterations that can result in cancer development as well as other diseases. However, checkpoints are necessary for cancer cell proliferation in specific contexts and for this reason have emerged as potential therapeutic targets for cancer therapies. Understanding checkpoint pathways is critical to determining which alterations may lead to tumor development and which checkpoint factors can be targeted for cancer therapy.

In human cells, the ataxia telangiectasia mutated (ATM) kinase and the ATM and rad3-related (ATR) kinase are master regulators of two major checkpoint pathways (16, 70, 95). Whereas ATM primarily responds to double-stranded DNA breaks (DSBs), ATR is activated by a wide spectrum of DNA damage and replication problems. ATM is dispensable for viability but critical for maintaining genomic integrity (95). Mutations in components of the ATM pathway, such as ATM and Chk2, result in a predisposition to cancer development in mouse models and human disease. Furthermore, the ATM pathway is frequently mutated in cancers, suggesting that this pathway acts as an anticancer barrier during tumorigenesis. In contrast to the ATM pathway, the ATR pathway is essential in a number of organisms, and mutations in components of this pathway result in embryonic lethality in mouse models (7, 61, 114). ATR is required to maintain genome integrity during DNA replication and serves a major function to prevent a toxic level of replication stress. Because of these critical functions, cancers rarely harbor loss-of-function mutations in the ATR pathway, and a subset of cancers with specific mutations is more sensitive to inhibition of this pathway than normal cells. This suggests that the ATR checkpoint may be a useful pathway to target for therapeutic purposes (51).

Here, we describe how the ATR pathway is tightly regulated through ATR priming, recruitment, stimulation, and modulation. Furthermore, we describe the important functions of ATR signaling in protecting genomic stability. Finally, we describe particular molecular alterations in cancer cells that result in an increased dependence on ATR signaling and how the ATR pathway can be targeted for therapeutic purposes.

OVERVIEW OF THE ATR PATHWAY

The ATR pathway is activated in response to a broad spectrum of genomic insults, including replication interference, DSBs, and other types of DNA lesions triggering various repair pathways (17, 33, 70). A common effect of these insults is the induction of single-stranded DNA (ssDNA), which acts as a platform to recruit ATR. Following proper priming and recruitment to ssDNA, ATR is stimulated by the concerted action of a group of regulators, including the Rad17 and 9-1-1 (Rad9-Rad1-Hus1) complexes, the MRN (Mre11-Rad50-Nbs1) complex, and TopBP1. With help from mediators, activated ATR then recognizes and phosphorylates a number of downstream effectors, such as Chk1 and RPA, to stall the cell cycle, protect DNA replication forks, signal for DNA repair, and maintain genomic stability.

ACTIVATION OF THE ATR PATHWAY

ATR Priming

ATR functions as a complex with its regulatory partner, ATRIP (20) (**Figure 1**). Even in the absence of genomic insults, the ATR-ATRIP complex is modulated by priming events that prepare

it for efficient DNA damage responses. Priming of the ATR-ATRIP complex requires that ATR and ATRIP be expressed, folded, and stably assembled for activation. The proper translation and folding of ATR-ATRIP requires TEL2 (46, 100). TEL2 interacts with TTI1, TTI2 (TTT complex), and HSP90 to form a chaperone complex, which binds to newly translated ATR to promote proper folding and thus synthesis of mature, functional ATR (**Figure 1**). ATR must then stably associate with its binding partner, ATRIP, which is required for the stability of both ATR and ATRIP. The proper association of ATR and ATRIP requires not only the TEL2 complex but also Nek1 kinase, preparing the ATR pathway for activation (63). Furthermore, post-translational modification, such as SUMOylation, is required to stably glue the ATR-ATRIP complex together (115). ATRIP itself is SUMOylated to stabilize its interaction with ATR, and ATR-ATRIP also requires PIAS3, a SUMO ligase, for sustaining its basal kinase activity (116). Through these modifications and interactions, the ATR-ATRIP complex is primed for full activation.

ATR Recruitment

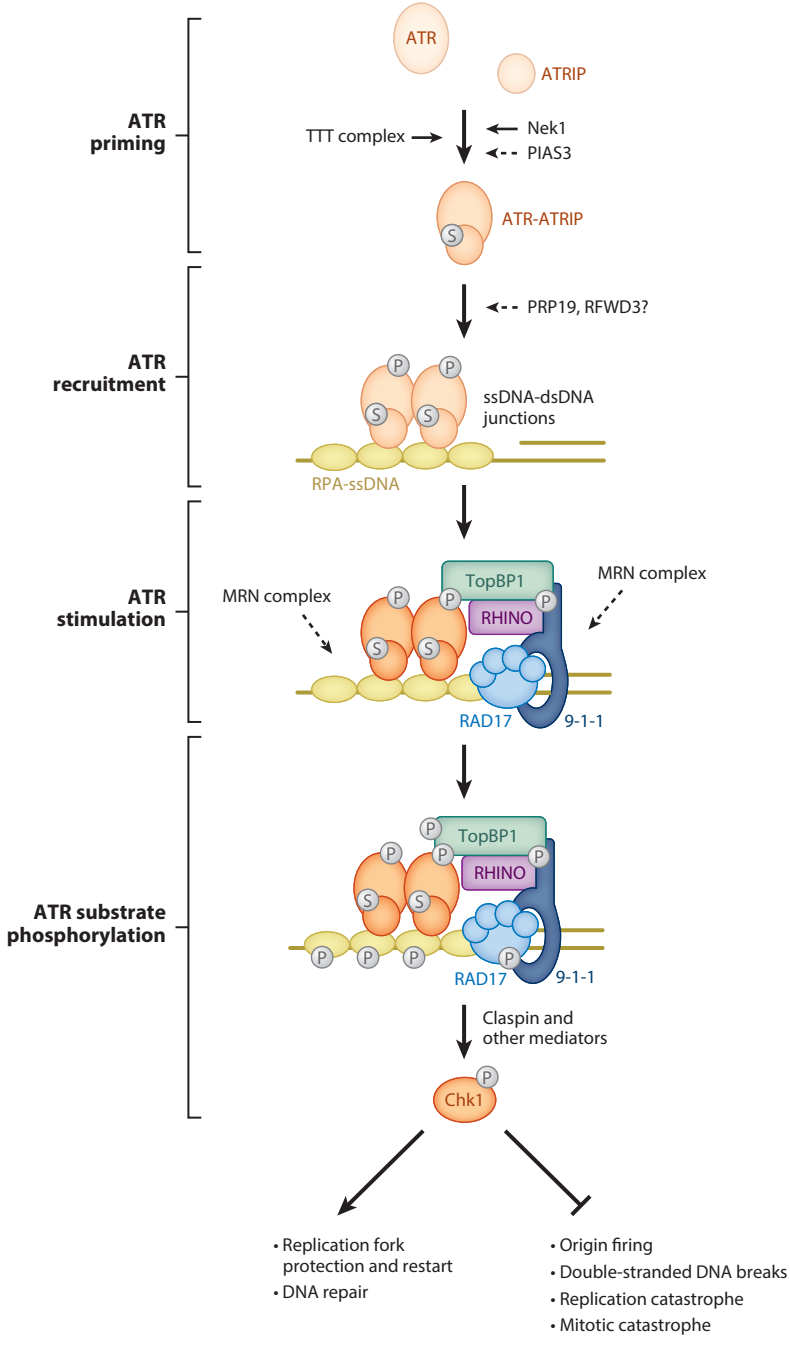
ssDNA exposure following DNA damage or replication inhibition results in recruitment of the RPA complex, consisting of RPA70, RPA32, and RPA14. The formation of RPA-coated ssDNA prevents the generation of DNA secondary structures and protects ssDNA from degradation (14, 26). RPA-coated ssDNA acts as a platform for the DDR and functions to recruit the components necessary for ATR activation. ATR is recruited to RPA through its binding partner, ATRIP (124) (**Figure 1**). A recent study suggested that the MutSβ complex, consisting of Msh2 and Msh3, binds to hairpins in ssDNA and facilitates the recruitment of ATR-ATRIP (9). One of the first markers of ATR pathway activation following ATR recruitment to DNA is phosphorylation of ATR itself on T1989 (64, 80). This critical phosphorylation event requires the basal kinase activity of ATR and the binding of ATR-ATRIP to RPA-ssDNA. The autophosphorylation of ATR presents a docking site for TopBP1, which stimulates the kinase activity of ATR-ATRIP (see below). The RPA-ssDNA-directed ATR autophosphorylation sets up a feed-forward loop wherein ATR molecules can phosphorylate adjacent ATR molecules in *trans* to amplify the DNA damage response. This poises ATR-ATRIP for full activation (64).

ATR Stimulation

Following ATR-ATRIP recruitment to RPA-ssDNA and ATR autophosphorylation, ATR is stimulated by TopBP1 through a process mediated by Rad17 and 9-1-1 complexes (**Figure 1**). Rad17 forms an RFC (replication factor C)-like complex with RFC2-5, which recognizes ssDNA/dsDNA junctions and loads the 9-1-1 complex, a heterotrimeric sliding clamp with structural similarity to PCNA (proliferating cell nuclear antigen), onto dsDNA (30, 123, 125). This loading is facilitated by RPA-ssDNA and allows the 9-1-1 complex to interact with TopBP1 (24, 58). TopBP1 contains multiple BRCT (BRCA1 C-terminus) domains and binds to the phosphorylated C-terminal tail of Rad9 and autophosphorylated ATR (58, 64, 101). Additionally, Rad17 also plays a key role in establishing the interaction between 9-1-1 and TopBP1 (56). The interaction between Rad9 and TopBP1 facilitates the accumulation of both proteins at sites of DNA damage in cells (82). In *Xenopus* egg extracts, 9-1-1 is not required for TopBP1 recruitment to ATR-activating DNA structures but is required for TopBP1 function (28). Along with 9-1-1 and TopBP1, efficient activation of ATR requires RHINO, which forms a complex with both 9-1-1 and TopBP1 (21). The internal region of TopBP1 contains the ATR activation domain (AAD), which is both necessary and sufficient for stimulating ATR-ATRIP (53). The AAD of TopBP1 is likely unstructured, and its activity requires two aromatic residues in separate regions. AAD was shown to interact with

both ATRIP and the C terminus of ATR (74), which may induce a conformational change of the ATR-ATRIP complex that increases its kinase activity.

In addition to Rad17 and 9-1-1, MRN is also an important regulator of ATR activation (**Figure 1**). In *Xenopus* egg extracts, MRN is required for recruiting TopBP1 to ATR-activating DNA structures, and it may function in parallel with Rad17 to promote Chk1 phosphorylation



(28, 57). In human cells, MRN plays a minor role in Chk1 phosphorylation but is critical for RPA32 phosphorylation in response to replication-associated DSBs (96). In contrast to MRN, Rad17 is critical for Chk1 phosphorylation but largely dispensable for RPA32 phosphorylation. TopBP1 is required for phosphorylation of Chk1 and RPA32, linking it to both Rad17 and MRN. RPA phosphorylation requires extensive resection of DSBs and binding of Nbs1 to RPA, suggesting that MRN acts on long RPA-ssDNA rather than at ssDNA-dsDNA junctions. These findings suggest that Rad17 and MRN regulate two distinct modes of ATR activation in human cells: Rad17 promotes Chk1 activation at ssDNA-dsDNA junctions, and MRN mediates RPA phosphorylation on long RPA-ssDNA (96). The phosphorylation of Chk1 by ATR is important for DNA damage signaling and the checkpoint response, whereas the phosphorylation of RPA is required for efficient restart of stalled replication forks (78, 96). These distinct modes of ATR activation may allow ATR to phosphorylate different substrates, thereby carrying out diverse functions in the DDR.

ATR Substrates

Once primed, recruited, and stimulated, ATR then phosphorylates a host of downstream substrates (**Figure 1**). One group of ATR substrates is important for DNA damage signaling and the checkpoint response. In particular, ATR phosphorylates Chk1 at S317 and S345, activating this effector kinase (61, 122). The phosphorylation of Chk1 by ATR involves phosphorylation of Rad17 and other mediators, including BRCA1 and Claspin (60, 112). Activated Chk1 then phosphorylates additional effectors, such as Cdc25A and Treslin, contributing to G2 arrest as well as intra-S phase inhibition of origin firing (5, 10, 38, 50, 68). The second group of ATR substrates acts locally at stalled or stressed replication forks to suppress genomic instability. This group of ATR substrates includes RPA, MCMs, And-1, WRN, SMARCAL1, and others (3, 19, 22, 40, 107, 118). The third group of ATR substrates participates in DNA repair in DNA lesion–specific contexts. This group of ATR substrates includes FANCI, Polη, and others (15, 35, 47). The functions of the ATR pathway are further discussed below.

It should be noted that some ATR substrates participate in multiple processes. For example, Chk1 not only controls cell cycle arrest but also regulates origin firing, facilitates replication fork progression, and suppresses replication-associated DNA damage (84, 85). The phosphorylation

Figure 1

Model for ATR (ATM and rad3-related) activation following DNA damage. Newly translated ATR requires the TTT (TEL2-TTI1-TTI2) complex for maturation and optimal interaction with its binding partner, ATRIP. The ATR-ATRIP interaction is also stabilized by Nek1 kinase and SUMOylation of ATRIP. The E3 SUMO ligase PIAS3 is required for sustaining the basal kinase activity of ATR. Following damage, ATR localizes to RPA-coated single-stranded DNA (RPA-ssDNA) through ATRIP. Recruitment of ATR-ATRIP is likely modulated by additional factors, such as ubiquitin E3 ligase PRP19 and possibly RFWD3. Once recruited, ATR undergoes autophosphorylation at T1989. The 9-1-1 (Rad9-Rad1-Hus1) complex is independently recruited to ssDNA-dsDNA (double-stranded DNA) junctions by the Rad17 complex. TopBP1 interacts with the C-term of Rad9 and RHINO. Additionally, the MRN (MRE11-RAD50-NBS1) complex may play a role in recruiting TopBP1 to RPA-ssDNA and ssDNA-dsDNA junctions. The autophosphorylation of ATR helps TopBP1 engage ATR-ATRIP stably, thereby stimulating ATR kinase activity through its ATR activation domain (AAD). ATR kinase activity is represented by increasing red color intensity. This is dependent not only on phosphorylation of ATR itself, but also on stimulation of ATR and amplification of signal by additional factors shown in the figure, as well as additional modifications. Once activated, ATR phosphorylates a number of downstream effectors, such as Chk1 and RPA, allowing the ATR pathway to execute various functions in genome protection.

of FANCI by ATR also affects origin firing and restart of stalled replication forks (15). The ability of ATR to regulate numerous substrates involved in different processes enables it to coordinate a network of events in response to DNA damage, making it one of the master orchestrators of the DDR. Many of the DNA damage–induced phosphorylated proteins identified from proteomics screens may be directly phosphorylated by ATR or Chk1 (71). Understanding the significance of these phosphorylation events will likely extend the functions of the ATR-Chk1 pathway.

Modulation of ATR signaling

Aside from phosphorylation events, ATR signaling can be further modulated by post-translational modifications such as ubiquitylation and SUMOylation of proteins that function in the ATR pathway (**Figure 1**). In response to DNA damage, the E3 ubiquitin ligase RFWD3 is recruited to sites of DNA damage through its interaction with RPA (29, 36, 62). PRP19, another ubiquitin ligase, is also recruited to RPA-ssDNA at sites of DNA damage (69, 109). Both RFWD3 and PRP19 are required for the efficient phosphorylation of Chk1 and RPA, and for restart of stalled replication forks. Both RFWD3 and PRP19 are also involved in the DNA damage–induced ubiquitylation of RPA, but how they function in this process is still poorly understood. In cells depleted of PRP19, the recruitment of ATRIP to sites of DNA damage and ATR autophosphorylation are reduced, suggesting that this RPA-directed ubiquitin ligase promotes the localization of ATR-ATRIP to RPA-ssDNA. The ubiquitylation circuitry mediated by RFWD3 and PRP19 may function as a signal amplifying mechanism to enhance ATR activation on RPA-ssDNA. In addition to ubiquitylation, SUMOylation also plays a role in modulating ATR signaling. SUMOylation of ATRIP, which is increased by UV, facilitates ATRIP interaction with a number of downstream effectors, including RPA70, TopBP1, and MRN (115). This group effect of ATRIP SUMOylation is reminiscent of that observed for DNA repair proteins in yeast (87), suggesting that SUMOylation of ATRIP helps glue together the signaling complex in this pathway. Thus, although ATR signaling is primarily mediated by phosphorylation, ubiquitylation and SUMOylation are important for the robustness of this process. The mechanisms that modulate ATR signaling may be particularly important for the proper function of this pathway in specific contexts.

Context-Specific Regulators of ATR

The activation of ATR is also influenced by many factors in a context-dependent manner. For example, the activation of ATR at DSBs is controlled by factors involved in DNA end resection, a process that gives rise to ssDNA. A number of proteins involved in resection, including ATM, Mre11, CtIP, EXO1, and BLM, have been implicated in ATR activation by DSBs (37, 49, 79, 91, 105). Interestingly, CtIP, which is required for extensive resection, is important for sustaining Chk1 phosphorylation and progressive phosphorylation of RPA, suggesting that resection is directly coupled to the process of ATR activation (52, 96). Finally, certain ssDNA-binding proteins compete with RPA to suppress ATR activation. For example, POT1, a protein that binds to telomeric ssDNA, prevents ATR activation at telomeres (25). ATR activation is also tuned by factors that accumulated on the chromatin-flanking DSBs or stalled forks. For example, MDC1, a BRCT-containing reader of γH2AX, promotes the accumulation of TopBP1 in HU (hydroxyurea)-induced foci, which is important for amplifying ATR signals (111).

The activation of ATR at stalled replication forks involves a number of replication factors. For example, yeast homologs of Claspin, Timeless, and Tipin are canonical components of the replisome (12). Chk1 may also associate with replication forks even in the absence of DNA damage (48, 117). During replication stress, ATR phosphorylates And-1, which then promotes Chk1

phosphorylation by enhancing the interaction between Claspin and Chk1 (40). The accumulation of ssDNA at stalled forks is important for ATR activation. TRAIP, a PCNA-binding ubiquitin ligase, facilitates RPA-ssDNA induction and ATR activation at stalled forks (44). In contrast, SLFN11, an RPA-binding protein that destabilizes RPA-ssDNA, suppresses ATR activation (75). It should be noted that the presence of ssDNA at or behind replication forks does not always correlate with robust ATR activation (120), which is consistent with the idea that additional DNA structures or dynamic changes of DNA are needed to activate ATR efficiently. This is further demonstrated by mechanical stress on the nuclear envelope or osmotic changes, which can cause relocalization of ATR to the nuclear membrane independent of RPA or DNA damage, resulting in Chk1 phosphorylation (54).

The activation of ATR by different types of DNA damage often requires the specific DNA repair proteins that repair the corresponding DNA lesions. For example, inter-strand crosslinks (ICLs) are repaired by the Fanconi pathway, and members of that pathway (FANCM and FAAP24) are implicated in the activation of ATR by ICLs (18, 45). Methylated DNA can be repaired by the mismatch repair pathway, and the mismatch repair protein Msh2 is required for the activation of ATR by DNA-methylating agents (83, 113, 119). Nucleotide excision repair (NER) proteins repair UV-induced damage, and NER proteins are involved in UV-induced ATR activation in noncycling cells (97, 108). This allows for multiple modes of ATR activation following the generation of various DNA lesions, and may result in phosphorylation of different downstream substrates by ATR. Taken together, it is clear that ATR activation is intertwined with DNA replication and repair in different contexts, highlighting the integral roles of the ATR pathway in these processes.

FUNCTIONS OF THE ATR PATHWAY

Activated ATR performs multiple cellular functions to maintain genomic integrity, including regulation of origin firing, protection and restart of stalled forks, and prevention of replication and mitotic catastrophe. These functions are likely critical for the genomic stability of dividing cells and for proliferation of certain cancers.

Suppression of Origin Firing and Replication Catastrophe

ATR signaling is important in suppressing cell cycle progression and origin firing following DNA damage. As discussed above, ATR phosphorylates Chk1 following replication stress. One of the critical targets of Chk1 is Cdc25A (10, 50), a phosphatase that activates both Cyclin E-Cdk2 and Cyclin B-Cdk1. Phosphorylation of Cdc25A signals for its degradation and also prevents its association with Cyclin A-Cdk1, Cyclin B-Cdk1, and Cyclin E-Cdk2. This, in turn, results in stalled cell cycle progression, in both G2 and S phase, and suppression of new origin firing (68). Additionally, Chk1 phosphorylates Dbf4, the regulatory partner of Cdc7 kinase, which may contribute to the inhibition of origin firing (43). Chk1 also phosphorylates Treslin, a component essential for Cdc45 loading at replication origins, preventing its association with TopBP1, and decreasing initiation of replication (5, 38). Taken together, it is evident ATR is critical for the suppression of origin firing following DNA damage.

When ATR is inhibited, firing of dormant origins is drastically increased, giving rise to large amounts of ssDNA in the genome. The induction of very high levels of ssDNA could result in the exhaustion of nuclear pools of RPA, leading to massive fork collapse and DNA breakage, which is termed replication catastrophe (103). Cells under high replication stress, such as those treated with HU, are prone to replication catastrophe when ATR is inactive. However, in unperturbed cycling cell populations, only a fraction of S phase cells are prone to replication catastrophe after ATR

inhibition (8). In addition to limiting origin firing, ATR is also important for the accumulation of RRM2, a subunit of the ribonucleotide reductase, in S phase. Consequently, ATR inhibition not only increases origin firing but also reduces deoxynucleotide triphosphate (dNTP) synthesis. This imbalance between dNTP consumption and synthesis is particularly severe in early S phase, rendering early S phase cells prone to replication catastrophe (8).

Replication Fork Protection and Restart

In addition to suppressing replication catastrophe, ATR plays a key role in protecting stalled replication forks. SMARCAL1, a protein recruited to stalled forks following replication stress through association with RPA, is a substrate of ATR (22). The phosphorylation of SMARCAL1 by ATR limits its activity, preventing aberrant fork structures that give rise to DSBs through cleavage by the SLX4-associated nucleases (22). The role of ATR in protecting stalled forks has also been linked to the SUMO-targeted ubiquitin ligase RNF4 and the PLK1 kinase (88). Suppression of RNF4 or PLK1 reduces SLX4-mediated DSB formation following ATR inhibition, suggesting that RNF4 and PLK1 are involved in generation or cleavage of aberrant fork structures. Together, these findings suggest that ATR is important for preventing aberrant remodeling of stalled forks, thereby avoiding excessive nucleolytic processing of the replicating genome.

As discussed above, during S phase ATR promotes RRM2 accumulation and thus dNTP synthesis (8). In fact, increased expression of RRM2 can extend the life span and decrease the chromosomal instability in mouse models of reduced ATR activity (65). Thus, the ability of ATR to coordinate dNTP synthesis and consumption is important for not only preventing replication catastrophe but also suppressing fork failure. ATR plays a role in the restart of stalled forks. RPA mutants lacking ATR-phosphorylation sites compromise fork progression in the presence of HU (78, 107). ATR also phosphorylates WRN, a RecQ helicase, to stabilize WRN's localization to RPA at stalled forks (3). Loss of WRN itself or ATR phosphorylation results in failure to restart forks, fork collapse, and DSBs.

ATR has also been proposed to directly suppress dissociation of the replisome after DNA damage. Total levels of PCNA, POLE, POLD2, and CDC45 associated to chromatin following replication stress are decreased in ATR-deficient cells, suggesting ATR may facilitate replisome stability (88). However, new data resulting from isolation of proteins on nascent DNA (iPOND) have been used to argue that, in fact, inhibition of ATR activity has little effect on replisome stability but rather that ATR acts to maintain the replication competency of stalled forks (27). This may possibly occur through preventing aberrant fork remodeling, as outlined above, as well as through preventing fork collapse associated with increased firing of dormant origins.

Suppression of Mitotic Abnormalities and Catastrophe

In addition to increased genomic instability in S phase, cells compromised for ATR activity also display a number of problems in mitosis (7, 31, 76, 104). These mitotic defects include broken chromosomes, anaphase bridges, and micronuclei. It is possible that these mitotic defects arise from unresolved DNA replication problems in S phase. In the absence of a functional G2/M checkpoint, cells lacking ATR would enter mitosis with increased DNA damage. Alternatively, loss of ATR may compromise the normal transition from S phase to mitosis, leading to premature entry into mitosis before DNA replication is sufficiently complete. ATR loss triggers severe chromosome fragmentation in a fraction of cells with condensed chromosomes (7, 81), showing that ATR suppresses mitotic catastrophe.

Context-Specific ATR Functions

Following specific types of DNA damage that interfere with DNA replication, ATR activation can also play an important role in DNA repair. In response to crosslinking agents, ATR phosphorylation of FANCI is important for the localization of the FANCD2-FANCI complex to sites of DNA damage (47). Furthermore, FANCI phosphorylation is required to suppress dormant origin firing and restart replication forks at ICLs (15). For these reasons, cells expressing FANCI lacking the ATR phosphorylation sites are more sensitive to ICLs generated by MMC (mitomycin C). In a second example, following UV irradiation, Polη is phosphorylated by ATR and is required for postreplicative TLS (translesion synthesis) repair and survival (35). ATR is also known to suppress the genomic instability at fragile sites and telomeres (13, 72). ATR was recently shown to phosphorylate TRF1, a telomere-binding protein, and promote the localization of telomerase to telomeres (106). Through the mechanisms outlined here, ATR protects the genome from replication stress, promotes recovery, coordinates S and G2/M phases, and also plays context-specific roles in genome maintenance.

TARGETING ATR IN CANCER THERAPY

Because of the crucial role of ATR in the cell cycle and the response to replication stress and DNA damage, ATR signaling is likely critical in a number of cancer types. Although complete loss of ATR signaling is not compatible with life, partial inactivation can be achieved through ATR inhibitor treatment. If certain genetic alterations increase a cell's dependancy on ATR signaling, these alterations lead to increased sensitivity to the ATR inhibitor. Many cancers harbor defects in DNA repair or DNA damage signaling pathways, which is advantageous for tumorigenesis, but simultaneously results in an increased dependence on the ATR pathway for viability. In addition, even DNA repair–proficient cancer cells could have elevated levels of genomic instability or use specific DNA repair proteins to cope with particular oncogenic events, rendering cancer cells increasingly reliant on ATR.

Targeting DNA Repair–Defective Tumors

DNA repair defects can result in increased rates of mutations and chromosomal deletions and rearrangements, which are beneficial for cancer cell growth and promote activation of oncogenes or inactivation of tumor suppressor genes. However, if genomic instability is too high, essential genes can be lost, and cells may not be able to survive DNA synthesis through S phase. Because of this, certain cancer cells are dependent on ATR signaling for survival (**Figure 2**). Both ATR and ATM are critical for signaling DNA repair. Perhaps not surprisingly, inactivation of ATM signaling, which results in genomic instability and increased transformation rate, leads to an increased dependence on ATR signaling, and combined inactivation of ATM and ATR is synthetic lethal (55, 89). Interestingly, synthetic lethality also results from inactivation of ATR in combination with additional specific DNA repair components involved in base excision repair (BER), NER, and ICL repair. In ovarian cancer, loss of XRCC1, a factor involved in BER, confers sensitivity to ATR inhibitors (98). XRCC1 loss may increase single-stranded DNA breaks (SSBs) in the genome, which if unrepaired can become DSBs during S phase, presenting a severe problem to replication fork stability especially when origin firing is drastically increased by ATR inhibition. Loss of ERCC1 together with ATR is also synthetic lethal (73). ERCC1, along with XPF, plays an important role in NER and ICL repair and has been proposed as an important biomarker for certain cancers (4, 121). ATR activity is required for cells to progress through S phase after loss of ERCC1, presumably because of the increase in DNA damage resulting from loss of these repair

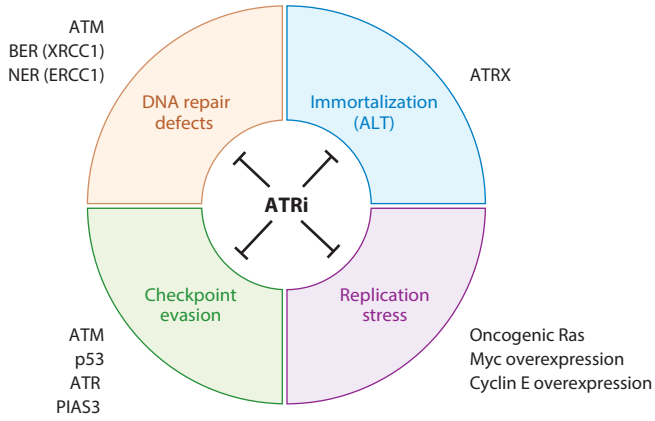


Figure 2

Characteristics of cancers targeted by ATR (ATM and rad3-related) inhibition. Many cancer types harbor mutations that render them particularly dependent on ATR signaling for survival. Cells with DNA repair deficiencies, such as defects in ATM signaling, mutations in base excision repair (BER; e.g., loss of XRCC1), or mutations in nucleotide excision repair (NER; e.g., loss of ERCC1), exhibit an increased dependence on ATR signaling. Cells that have compromised checkpoints, such as those defective for ATM and p53 or those with weakened ATR signaling (low PIAS3 expression), also exhibit an elevated reliance on ATR. Even repair-proficient cancer cells could rely on ATR to achieve immortalization, thereby acquiring an increased dependence on ATR. For example, a subset of cancers uses the ATR-dependent ALT (alternative lengthening of telomeres) pathway to maintain telomeres and bypass crisis, rendering them sensitive to ATR inhibition. Finally, cells with heightened replication stress have increased sensitivity to ATR inhibition. Thus, specific hallmarks of cancer could render tumor cells increasingly sensitive to treatment with ATR inhibitors.

pathways. Taken together, it is clear that inhibition of ATR may prove to be of great therapeutic value to selectively target tumors harboring mutations in specific DNA repair pathways.

Targeting Checkpoint-Defective Tumors

Loss of DNA damage checkpoints, for example through inactivation of tumor suppressors such as ATM and p53, allows cancer cells to proliferate in the presence of damage. However, loss of one checkpoint pathway results in an increased dependence on another checkpoint pathway, namely ATR signaling, to prevent a toxic level of damage from replication stress (**Figure 2**). Both ATM and ATR play a role in activation of the intra-S phase and G2/M checkpoints to delay the cell cycle in response to DNA damage (95). One critical substrate of ATM is p53, which acts to delay the cell cycle following DNA damage in G1. Loss of ATM or p53 results in loss of cell cycle delays following DNA damage, which places additional pressure on the intra-S phase and G2/M checkpoints regulated by ATR. Combined inactivation of ATR and mutation of ATM or p53 results in high levels of DNA damage and cell death (55, 81, 89, 104). Decreased ATR activity itself may also result in higher sensitivity to ATR inhibitors (73). ATR expression is rarely lost in tumors; however, decreased expression of PIAS3, which plays a role in ATR activation, has been found in a number of cancer types (1, 6, 23, 59, 116). These cancers likely have decreased ATR activity and may be more sensitive to ATR inhibition.

Targeting ALT Tumors

DNA repair–proficient cancer cells can also develop a dependency on ATR for survival (**Figure 2**). For example, approximately 10–15% of human tumors use the alternative telomere lengthening

(ALT) pathway rather than telomerase to maintain telomeres and bypass crisis (86). Thus, as with activation of telomerase, activation of ALT is an oncogenic event. The activation of ALT is prevalent in specific cancer types, including osteosarcoma, glioblastoma, and neuroendocrine pancreatic tumors (39, 42, 92). Recent cancer genomics studies have suggested that the chromatin modulator ATRX is often lost in cancers with an active ALT pathway (42, 66, 94). ALT is a recombination-mediated process by which telomeres are extended using telomeric DNA repeats in other telomeres or even themselves as templates. Importantly, as with the canonical homologous recombination (HR) pathway (2, 110), ALT is also dependent on ATR (32). Consequently, cancer cells reliant on the ALT pathway are particularly sensitive to ATR inhibitors, providing a new strategy for the treatment of ALT-positive tumors (32).

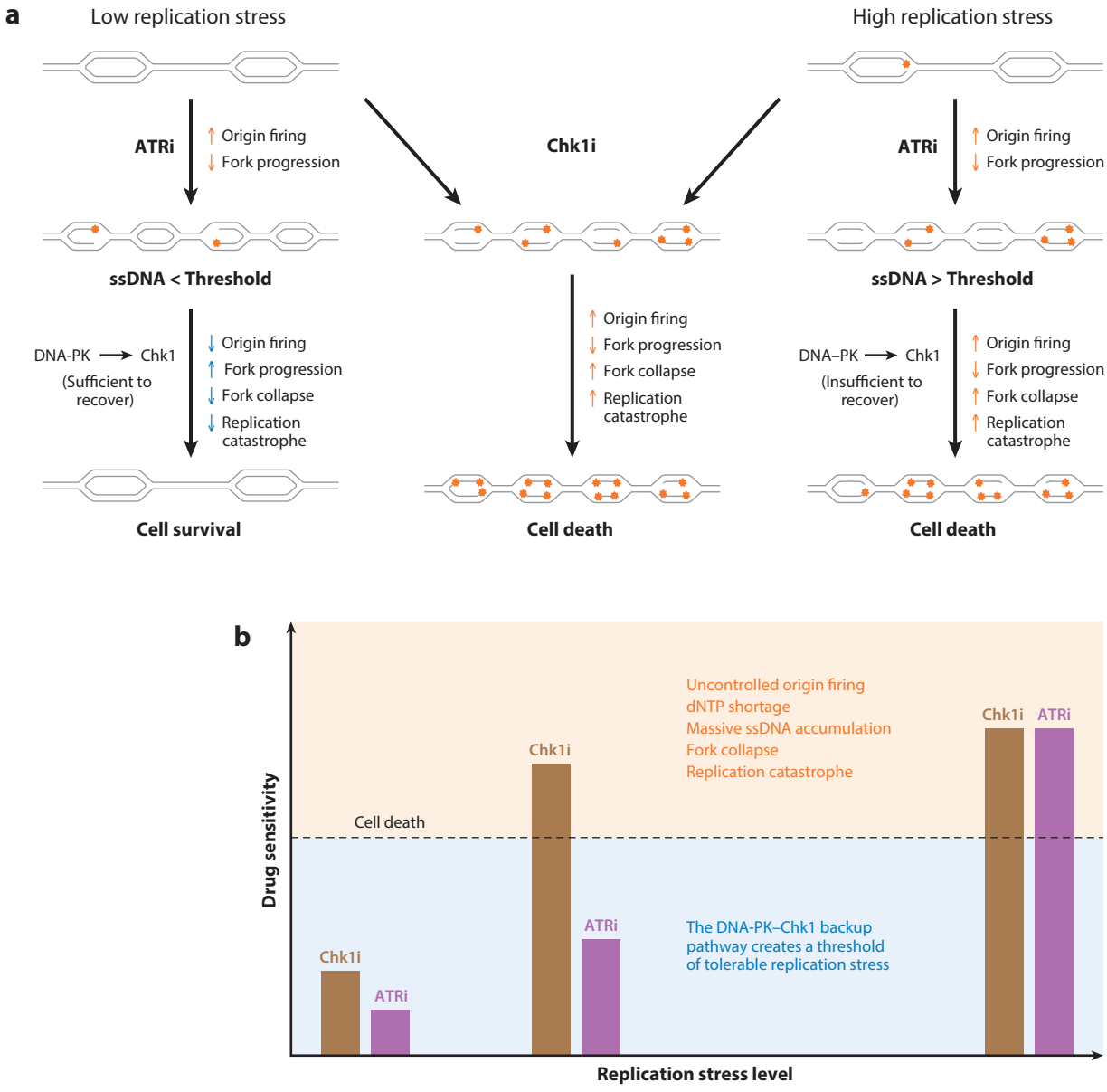
Targeting Cancer Cells with High Replication Stress

The ATR pathway is critical for responding to S phase replication stress in particular. For this reason, cancer cells with high levels of replication stress, such as those harboring mutations in oncogenes such as Ras, Myc, and Cyclin E, rely more heavily on ATR signaling for survival (34, 77, 93, 104) (**Figure 2**). Cells expressing oncogenic Ras experience synergistic increases in genomic instability upon >80% loss of ATR expression, resulting in cell death and decreased tumor burden (34). Amplification of Myc or overexpression of Cyclin E, which drives many cancers, results in high levels of replication stress, rendering cells particularly sensitive to ATR inhibition (77, 104).

Because tumors with increased replication stress would be ideal targets for ATR inhibitor treatment, it is important to understand how replication stress can be measured and which proteins can function as biomarkers for ATR inhibitor treatment. Following replication fork stalling, the discoordination of proteins in the replication machinery gives rise to an increase in ssDNA (11). The accumulation of ssDNA is drastically enhanced when the ATR checkpoint is removed, presumably due to increased origin firing and compromised fork protection (8, 103). The levels of ssDNA induced by ATR inhibition may reflect the replication stress in different cell populations, predicting the sensitivity of cancer cells to ATR inhibitors (8). The levels of ssDNA can be measured by staining tumor cells for chromatin-bound RPA. Additionally, tumor cells can be cultured with BrdU, a nucleoside analog, to label genomic DNA. Immunostaining of BrdU-labeled DNA under nondenaturing conditions specifically detects exposed ssDNA in the genome. A second marker of replication stress is provided by 53BP1 foci, which mark sequestered replication stress-induced lesions in G1 (41, 67). 53BP1 nuclear bodies can be used as biomarkers for cells experiencing heightened stress during replication. Finally, γH2AX, typically a marker of DSBs, can also serve as a general marker of genomic instability associated with replication stress (76, 99).

Differences in ATR and Chk1 Inhibition

Both ATR inhibitors and Chk1 inhibitors are currently under investigation in clinical trials for their efficacy in cancer therapy (51). Although Chk1 is a significant downstream effector of ATR, there are several differences in outcomes of treatment with ATR or Chk1 inhibitors. ATR can directly phosphorylate a number of substrates independently of Chk1, potentially making ATR inhibitors more useful across a wide-range of oncogenic contexts. Additionally, a backup pathway mediated by DNA-PK (DNA-dependent protein kinase) and Chk1 comes into action upon ATR inhibition in S phase cells, creating a threshold of tolerable replication stress (8) (**Figure 3*a***). Because of this threshold, ATR inhibition selectively kills cells under high replication stress. In contrast, Chk1 inhibition, which blocks both ATR-Chk1 and DNA-PK-Chk1 pathways, kills cells



at a much lower threshold of replication stress (**Figure 3*b***). Consistent with the idea that ATR and Chk1 do not always function in a linear pathway, a recent study reported that Chk1 inhibition leads to hyperactivation of ATR and that simultaneous inhibition of both ATR and Chk1 results in synthetic lethality (90). In addition, another study showed that Chk1 loss, but not ATR loss, triggers fork slowing through MRE11 and MUS81-mediated DSB formation and ATM activation (102). Although both ATR and Chk1 inhibitors may be useful tools for cancer treatment, a better understanding of the differences between the effects of these compounds will allow more precise use clinically.

Figure 3

Differences between ATR (ATM and rad3-related) inhibition (ATRi) and Chk1 inhibition (Chk1i). (*a*) ATRi results in loss of activated Chk1, leading to stalled replication forks and increased origin firing. In the presence of low levels of replication stress, cells are able to recover from ATRi through a backup pathway in which DNA-PK phosphorylates Chk1. This backup pathway suppresses origin firing, increases fork progression, and limits ssDNA accumulation before it exceeds a tolerable threshold. However, in cells under high levels of replication stress, ATRi quickly induces accumulation of ssDNA (single-stranded DNA) that exceeds the tolerable threshold, and the DNA-PK–Chk1 backup pathway is insufficient to promote recovery. These cells experience uncontrolled origin firing and increased fork stalling, resulting in massive ssDNA accumulation, fork collapse, replication catastrophe, and, ultimately, cell death. In contrast, treatment with Chk1i bypasses the DNA-PK backup pathway, resulting in fork collapse, replication catastrophe, and cell death regardless of the level of initial replication stress. ATRi can selectively target cells with high replication stress, whereas Chk1i has decreased ability to discriminate between cells with high and low replication stress. Orange stars represent fork stalling and DNA damage. (*b*) Owing to the distinct oncogenic events, different strains of cancer cells exhibit various types and levels of replication stress. Oncogenic events such as activating Ras mutations, overexpression of Myc, and overexpression of Cyclin E are known to increase replication stress. Replication stress in cancer cells can be exploited by both ATRi and Chk1i. Unlike ATRi, Chk1i blocks both ATR-Chk1 and DNA-PK–Chk1 pathways, resulting in replication catastrophe and cell death even in cells under low replication stress. For this reason, ATRi may be a more selective targeted therapy than Chk1i for cancers with high levels of replication stress.

PERSPECTIVES

ATR is a central regulator of DNA damage signaling and repair that has been a focal point of cell biology research for many years. The ATR pathway has recently come to light as an important target for cancer therapy. ATR signaling is particularly important following replication stress, which is associated with oncogenic events in many cancers. A better understanding of ATR activation and its diverse functions will allow better insight into the types of cancers that may have an increased reliance on ATR signaling, and thus may be targets for ATR inhibitor treatment. Furthermore, knowledge of the ATR pathway will provide additional targets for cancer therapy as well as biomarkers for assessment of ATR inhibitor sensitivity. It is conceivable that the convergence of basic and translational research on the ATR pathway will bring about an even more exciting time in this critical research area.

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