

Chapter 2

Hypoxia and the DNA Damage Response

Monica M. Olcina and Ester M. Hammond

Abstract Gradients of hypoxia occur in most solid tumors. Cells found in these regions are associated with the most aggressive and therapy-resistant fractions of the tumor. Severe levels of hypoxia ($< 0.1\% \text{ O}_2$) have been found to induce a unique DNA damage response (DDR) that includes both ATR- and ATM-mediated signaling. The consequences of the hypoxia-induced DDR include p53-dependent apoptosis and maintenance of replication fork integrity. Interestingly, the hypoxia-induced DDR occurs in the absence of detectable single- or double-strand breaks and in a background of repressed DNA repair. Inhibition of DNA repair in hypoxic cells has been proposed as a mechanism contributing to the increased genomic instability found in hypoxia. Furthermore, an increasing number of novel agents that target the DDR have been described and some are already undergoing clinical testing. Evidence from preclinical studies suggests that the use of some of these agents would be effective at targeting tumor cells in hypoxic regions.

Keywords DNA damage response · Replication stress · Synthetic lethality · PARP · Chk1 · ATM · ATR · p53

2.1 The DNA Damage Response (DDR)

The integrity of the genome is constantly being threatened by a number of endogenous as well as exogenous insults that can lead to the generation of DNA damage (Lindahl and Barnes 2000). This damage will generally be in the form of single- or double-strand breaks (DSBs), which can occur following replication stresses (RSs) such as ultraviolet (UV) radiation or oncogene activation, as well as in response to other genotoxic stresses including ionizing radiation (IR), or chemotherapy (Jackson and Bartek 2009; Ciccia and Elledge 2010). The classical response to DNA damage leads to the activation of a complicated kinase signaling cascade that includes sensing and

E. M. Hammond (✉) · M. M. Olcina

The Department of Oncology, The Cancer Research UK/MRC Gray Institute for Radiation Oncology and Biology, The University of Oxford, Oxford OX3 7DQ, UK
e-mail: ester.hammond@oncology.ox.ac.uk

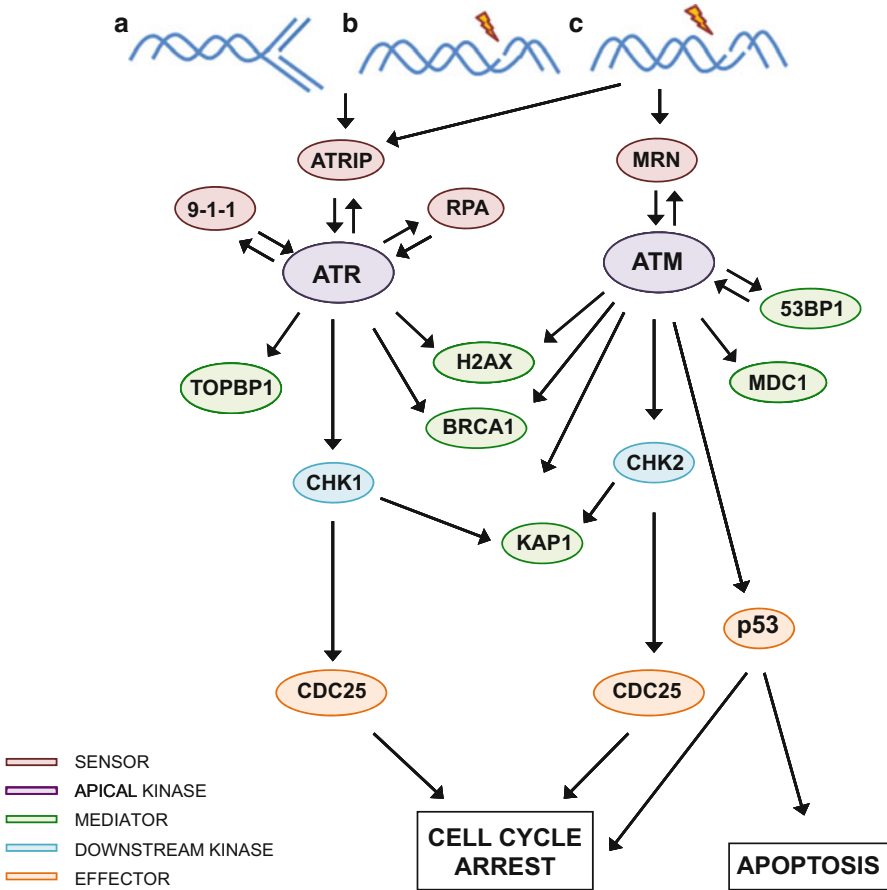


Fig. 2.1 Schematic representation of the DNA damage response. DNA lesions including stalled replication forks (a), single strand breaks (b), and double strand breaks (c) are detected by specific sensors. The apical kinases, *ATR* and *ATM*, are then activated and transduce the signal through mediators to downstream kinases and effectors

responding to the insult, as well as coordinating a number of downstream events generally resulting in cell-cycle arrest, DNA repair, senescence, or apoptosis (shown in Fig. 2.1 and recently reviewed in (Ciccia and Elledge 2010)). The cascade can be envisioned as having three principal kinases, although there is considerable cross-talk between them. If the stress activating the DNA damage response (DDR) leads to the generation of single-stranded DNA (ssDNA), the ATR PI-3K (phosphatidylinositol-3-kinase) like kinase (PIKK) (Ataxia-telangiectasia and Rad3-related) will be activated (Cimprich and Cortez 2008). Alternatively, if the insult leads to the generation of a double-strand break (DSB), two other members of the PIKK family—Ataxia-telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA-PK)—become activated (Jackson and Bartek 2009). ATM is generally regarded as

the main upstream kinase of the signaling pathway activated in response to DSB, while DNA-PK is mainly involved with the repair of DSB in the nonhomologous end-joining (NHEJ) pathway (Collis et al. 2005).

2.2 The DDR and its Relevance to Tumorigenesis

The action of the many players involved in the DDR will result in a coordinated defense mechanism against the original cellular insult. The mounting of an appropriate DDR is essential for the maintenance of genome integrity. Importantly, many studies now point to the importance of the DDR in cancer development, with the DDR acting as a barrier to tumorigenesis (Bartkova et al. 2005, 2006; Halazonetis et al. 2008). Factors involved in the DDR such as γ H2AX are known to be expressed in early-stage tumors (Bartkova et al. 2005). Other studies have demonstrated that loss of DDR activation leads to loss of oncogene-induced senescence and the increase in cellular transformation (Di Micco et al. 2006). Furthermore, ATR haploinsufficiency together with endogenous levels of K-ras in p53 heterozygous mice result in enhanced incidence of lung adenocarcinoma, spindle cell sarcoma, and thymic lymphoma (Gilad et al. 2010). The protective function that might be exerted by the DDR in tumorigenesis may explain some of the common mutations in DDR genes found in cancers (Halazonetis et al. 2008; Gorgoulis et al. 2005; Kastan and Bartek 2004).

2.3 ATR-Mediated DDR

Following RS, areas of ssDNA become coated with replication protein A (RPA) (Cortez et al. 2001; Zou et al. 2003; Fanning et al. 2006). ATR-interacting protein (ATRIP) then associates with the RPA and ultimately recruits ATR to the sites of ssDNA. RAD17 will be directed to these sites where it will load the 9-1-1 complex (composed of RAD9-RAD1-HUS1). The activity of ATR is then enhanced by the phosphorylation of RAD17, 9-1-1, and a specific activator of ATR, topoisomerase II binding protein I (TOPBP1). ATR plays a critical role in the maintenance of replication fork integrity and checkpoint response. Accordingly, many studies have shown that if ATR function is lost, DNA repair and viability are severely compromised (Cliby et al. 1998; Hurley et al. 2007). One of the most characterized ATR substrates is checkpoint kinase 1 (CHK1). Phosphorylated and active CHK1 can direct cell cycle regulation by mediating a checkpoint response (Liu et al. 2000; Dai and Grant 2010; Sanchez et al. 1997). In response to a DNA damage signal (including aberrant replication structures), checkpoints act to halt the cell cycle to allow enough time for any damage incurred by the cell to be repaired. For example, the G₁/S checkpoint is maintained by p53, which is stabilized by the DDR. Phosphorylated p53 (at serine 15) activates cyclin-dependent kinase inhibitor 1A (p21). p21 interacts with CDK2 and CDK4 hindering the interaction between CDK2/cyclin B and CDK4/cyclin E (Sancar et al. 2004). This prevents key cellular events, such as chromosome segregation or DNA replication, being carried out in the presence of DNA damage or during repair (Cortez 2001).

2.4 ATM-Mediated DDR

A single DSB can be lethal to the cell so it is important that these lesions are detected and dealt with appropriately (Jeggo and Lobrich 2007). DSBs are generally sensed by the Mre11-RAD50-NBS1 (MRN) complex (Moreno-Herrero et al. 2005). Following the initial detection of the break, a number of signaling events will be coordinated. For instance, the ATM kinase undergoes autophosphorylation and becomes active (Bakkenist and Kastan 2003). Activation and retention of ATM at the sites of damage then leads to the phosphorylation and recruitment of other factors including mediator of DNA damage checkpoint 1 (MDC1) and 53BP1, which will further enhance ATM retention and signaling (Stewart et al. 2003; Goldberg et al. 2003). Specific chromatin changes also occur during the initiation of the DDR and serve to amplify the signaling and facilitate repair. Phosphorylation of the histone variant H2AX (γ H2AX) occurs in an ATM-dependent manner following DSB generation, and as a result is often used as a marker of DNA damage (Rogakou et al. 1998). This modification is important for the recruitment of MDC1, which further enhances accumulation of γ H2AX (Stucki et al. 2005). MDC1 has been shown to bind to γ H2AX and initiates a feedback loop that encourages further MRN recruitment and sustained ATM activation (Stucki et al. 2005). Once active, ATM initiates the DDR signaling cascade leading to checkpoint activation, repair, or apoptosis (Lavi and Kozlov 2007). One of the key downstream targets of ATM is checkpoint kinase 2 (CHK2), which mediates cell-cycle arrest (Matsuoka et al. 2000). ATM signaling can also facilitate DNA repair by allowing the recruitment of repair factors. ATM-dependent phosphorylation of DNA repair protein BRCA1, for instance, is critical for the response to IR-induced DSB (Cortez et al. 1999). BRCA1-deficient cells have defective homologous recombination (HR)-mediated repair and display hypersensitivity to IR pointing to the importance of BRCA1 in directing repair following DSB formation (Chen et al. 1998).

2.5 Hypoxia-Induced p53-Dependent Apoptosis

One of the key effectors of the DDR is the tumor suppressor protein, p53, which can induce both cell-cycle arrest and apoptosis (Vousden and Lane 2007). p53 is, therefore, regarded as a critical tumor suppressor with significant roles to play in preventing cancer (Lane 1992). Hypoxia can also lead to the induction of the DDR, and p53 activation is indeed a principal consequence of the hypoxia-induced DDR (Riley et al. 2008; Green and Kroemer 2009; Yee and Vousden 2005; Harper and Elledge 2007). In response to hypoxia, p53 is phosphorylated at a number of residues including serine 15, which has been shown to be important in mediating nuclear accumulation of p53 (Hammond et al. 2002). The initial stabilization of p53 in hypoxia is also facilitated by phosphorylation on residues 6, 9, 20, 37, and 46 (Hammond and Giaccia et al. 2005). Interestingly, hypoxic conditions, which lead to the activation of p53, tend to induce apoptosis as opposed to a cell-cycle arrest in G₁. p53 activation is, in fact, critical for hypoxia-induced apoptosis, and loss of

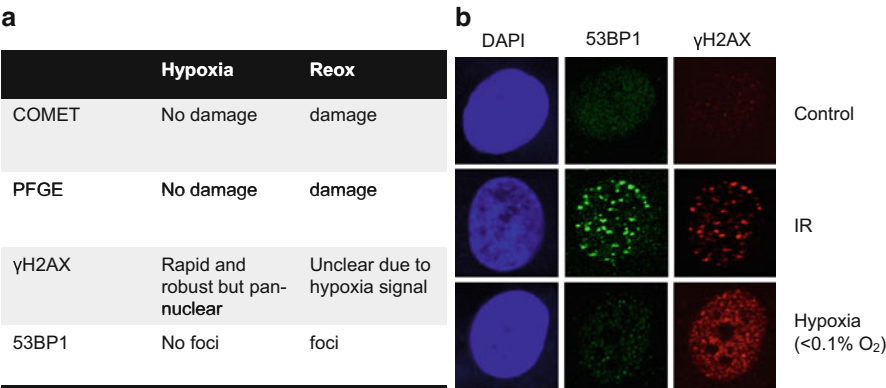


Fig. 2.2 Hypoxia does not induce detectable DNA damage. Standard assays for DNA damage detection demonstrate a lack of damage in hypoxic conditions. In contrast, reoxygenation after hypoxic exposure leads to abundant DNA damage (a). An example of cells exposed to either hypoxia or irradiation (IR) and stained for γ H2AX or 53BP1 is shown (b)

p53 decreases hypoxia-induced apoptosis (Graeber et al. 1996). Regions of hypoxia have been elegantly shown to correlate with high apoptosis in tumors with wild-type p53, whereas low apoptosis levels are observed in hypoxic tumor regions that have lost p53 (Graeber et al. 1996). A strong selection pressure for p53 mutations and subsequent apoptosis has been described in hypoxic cells (1996; Levine 1997). The preferential selection of cells with apoptosis defects in hypoxia may provide an explanation for the increased resistance of many solid tumors to certain forms of cancer therapy, particularly those forms of chemotherapy that rely on p53-dependent apoptosis for their efficacy (Graeber et al. 1994).

2.6 Initiation of DDR Signaling in Hypoxia

As described, hypoxia triggers a DDR, characterized by p53 accumulation and apoptosis (Graeber et al. 1996). Initially, it was unclear exactly what the hypoxia-induced signal was that initiated the DDR. This was primarily because standard assays for DNA damage demonstrated that these hypoxic conditions did not induce DSBs (Hammond et al. 2002). For example, comet assays or staining for the presence of 53BP1 foci demonstrated that hypoxia, in the absence of reoxygenation, does not induce DNA damage or at least damage that is detectable using the assays indicated (Fig. 2.2). One complexity to this conclusion was provided by the observation that H2AX is robustly phosphorylated in response to hypoxia as this histone modification has been correlated with the presence of DSBs (Bencokova et al. 2009). However, as shown in Fig. 2.2, the γ H2AX signal observed in hypoxic conditions is diffuse and pan-nuclear as opposed to forming discreet nuclear foci as seen in response to irradiation. This pattern of γ H2AX staining is reminiscent of the response to RS

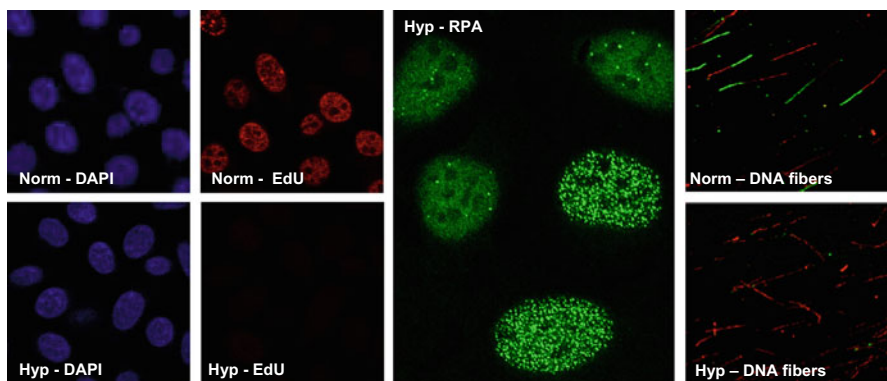


Fig. 2.3 Severe hypoxia induces an S-phase arrest, stalled replication, and a DDR. Representative images are shown for normoxic (*Norm*) and hypoxic (*Hyp*) cells. Hypoxic cells fail to accumulate EdU due to a lack of ongoing S-phase. RPA foci accumulate in the nuclei of hypoxia-treated S-phase cells. Replication stalls and stalled replication forks accumulate in hypoxia as shown by the accumulation of DNA fibers, which have not incorporated the second thymidine analog

for example as initiated by treatment with hydroxyurea or aphidicolin (Toledo et al. 2011). These data suggest that hypoxia induces a DDR, characterized by the robust induction of γ H2AX but that this is more likely to occur as a result of RS rather than DSBs. In support of this a significant hypoxia-induced effect has been described on the S-phase population. In response to levels of hypoxia, which also induce p53 stabilization and γ H2AX, cells rapidly undergo an S-phase arrest, i.e., they fail to incorporate thymidine analogs such as BrdU and EdU (Hammond et al. 2002) (as illustrated in Fig. 2.3).

More recently, hypoxia-induced replication arrest has been shown to correlate with a rapid decrease in the levels of available nucleotides (Pires et al. 2010a). This is predicted to be the result of decreased ribonucleotide reductase activity in these conditions. Ribonucleotide reductase catalyzes the conversion of ribonucleotides to deoxyribonucleotides in an oxygen-dependent manner (since molecular oxygen is required to regenerate a free radical at its active site) (Probst et al. 1989; Reichard and Ehrenberg 1983). Nucleotide levels have been measured *in vitro* and found to fall in response to severe hypoxia in parallel with the S-phase arrest. Importantly, nucleotide levels remain stable in mild hypoxia (for example, 2% O_2) where no replication arrest is observed (Pires et al. 2010a,b). DNA fiber technology has been used to image individual ongoing or stalled forks as well as to measure the rate of ongoing replication fork speeds. This demonstrated a decrease in origin firing and fork speeds as well as stalling of replication forks in response to severe hypoxia. The occurrence of these aberrant replication structures correlates with the formation of RPA foci, which have been shown to form as a result of the coating of regions of single-stranded DNA (Pires et al. 2010a). Together, these data strongly support the hypothesis that, in response to severe hypoxia, nucleotide availability is limited, leading to stalled replication, the accumulation of regions of ssDNA and the DDR (Fig. 2.3).

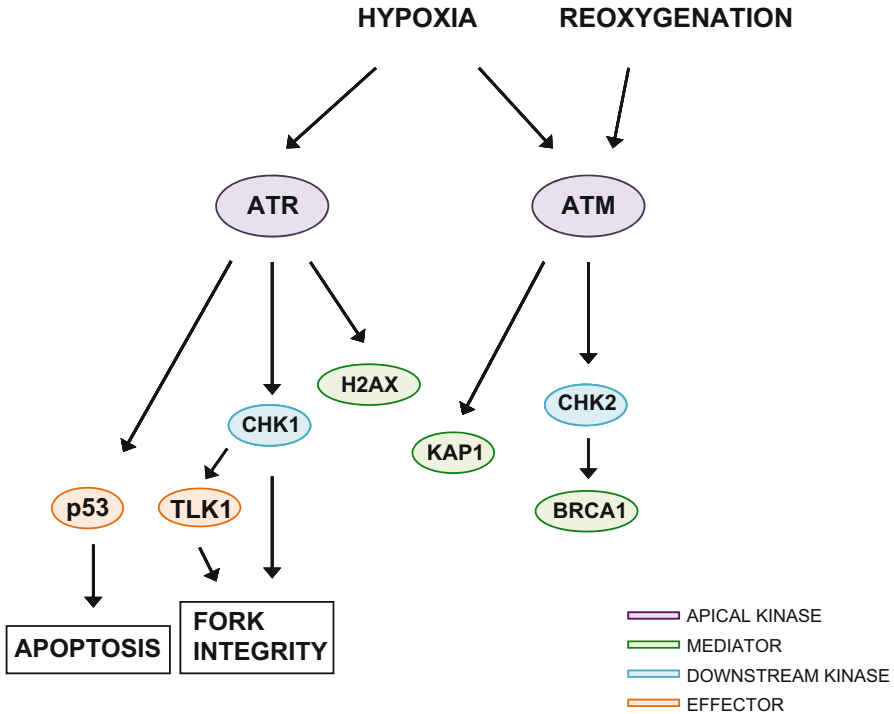


Fig. 2.4 Schematic representation of the hypoxia-induced DDR as described to date. In response to hypoxia, stalled replication initiates a DDR (both ATR- and ATM-dependent), while after reoxygenation DSBs are detected and lead to ATM signaling

2.7 Hypoxia-Induced CHK1 Signaling

ATR has been found to phosphorylate a number of downstream targets in hypoxia, including CHK1 (Hammond et al. 2002, 2003a, 2004) (Fig. 2.4). CHK1 is the key downstream kinase in hypoxia-induced ATR-mediated signaling. ATR and CHK1 have established roles in regulating normal replication and play critical roles in stabilizing stalled replication forks under severe hypoxia. This was demonstrated by showing that while hypoxia did not induce DNA damage, damage did accumulate if ATR or CHK1 were depleted/inhibited (Hammond et al. 2004). In support of the hypothesis that loss of nucleotides leads to replication arrest in hypoxia, loss or inhibition of CHK1 does not play a role in the initial hypoxia-induced replication arrest. If hypoxic cells are allowed to undergo reoxygenation and nucleotide pools are restored following acute hypoxia, replication forks will restart. In this context, an unscheduled increase in the number of new origins will occur if CHK1 is inhibited. CHK1, therefore, maintains genomic integrity following replication restart by delaying origin firing following reoxygenation-induced DNA damage to allow repair before replication can be resumed (Pires et al. 2010a,b). Furthermore, ATR/CHK1 inhibition or

knockdown sensitizes cells to hypoxia/reoxygenation as assayed by colony formation assay (Hammond et al. 2004). The increased sensitivity to hypoxia/reoxygenation observed upon CHK1 loss is thought to be predominantly dependent upon inhibition of CHK1 during reoxygenation since loss of CHK1 activity during both reoxygenation and hypoxia does not lead to additional sensitization. Once active, CHK1 will phosphorylate downstream targets including TLK1 in hypoxia (Pires et al. 2010b). TLK1 is a serine/threonine kinase that is usually inactivated once phosphorylated by CHK1 following DNA damage and is thought to facilitate a number of processes, including DNA replication and chromatin remodeling (Groth 2003). The role of TLK1 in hypoxia has not been fully investigated.

2.8 Hypoxia-Induced ATM Signaling in the Absence of DSBs

The hypoxia-induced DDR also leads to the activation of ATM (Bencokova et al. 2009). This is somewhat paradoxical since severe hypoxia does not lead to the generation of detectable DNA damage (Fig. 2.2). Following hypoxia-induced RS ATM is phosphorylated at serine 1981 and has been shown to phosphorylate downstream targets including KAP-1 (Krüppel-associated box (KRAB) domain-associated protein 1), CHK2, and DNA-PKcs (Bencokova et al. 2009). The phosphorylation of KAP1 is somewhat surprising as it has previously been described to be entirely dependent on the presence of DSBs (Goodarzi et al. 2009). Furthermore, phosphorylation of p53 at serine 20 and BRCA1 at serine 988 occurs following CHK2 phosphorylation by ATM (Gibson et al. 2006). Hypoxia-induced ATM activity is independent of the MRN complex. As the principal role of the MRN complex is in the initial detection of DSBs, this fact is supportive of DSBs not being the signal which induces the DDR in hypoxia. Similarly to the classical DDR, the mediator protein MDC1 does amplify hypoxia-induced ATM activity and is required for maximal phosphorylation of ATM targets, for example, KAP-1. However, in contrast to the response to *bona fide* DNA damage, BRCA1, 53BP1, and RNF8 are not subsequently recruited to form nuclear foci by MDC1 in hypoxic conditions. ATM is a predominantly nuclear protein, although some reports indicate that it is also active in the cytoplasm (Watters et al. 1997; Ambrose et al. 2000). During hypoxia phosphorylated ATM is found in the nucleus but does not appear to be tightly associated with the chromatin. This is consistent with the fact that it is not activated in response to or recruited to sites of DNA breaks (Toledo et al. 2011). The role of ATM in severe hypoxia is unclear; however, ATM knockdown has been shown to cause sensitization to hypoxia/reoxygenation (Freiberg et al. 2006b).

The mechanism of ATM activation in response to severe hypoxia is still not well understood. It is possible that hypoxia-induced ATR may contribute to ATM phosphorylation and activation. This possibility is supported by the finding that in response to UV, ATR phosphorylates ATM (Stiff et al. 2006). Further investigation of this hypothesis in hypoxia is technically challenging since ATM is activated in response to pharmacological inhibition or genetic knockdown of ATR. As previously mentioned,

this is believed to be due to the collapse of hypoxia-induced stalled replication forks (Toledo et al. 2011). Recently, we demonstrated that a potent ATR inhibitor (VE-821) induced DNA damage, detected by the presence of 53BP1 foci, in hypoxic cells (Pires et al. 2012). Hypoxia is not the only non-DNA damaging stress, which has been shown to induce ATM activity. For example, heat, high salt, and agents which modify chromatin have all been associated with an increase in ATM activity in the absence of DNA damage (Bakkenist and Kastan 2003). The induction of ATM by chromatin-modifying agents suggests the possibility that ATM responds directly to the stalled replication forks in hypoxia, as they would presumably present altered chromatin modifications. In addition, hypoxia is a strong modulator of the chromatin context, with many different chromatin modifications being induced in response to hypoxia (Johnson et al. 2008). Interestingly, the autophosphorylation (on serine 2056) and recruitment of DNA-PK in response to hypoxia (0.1–1 % O₂) have been associated with hypoxia-mediated changes in histone 3 acetylation. In these studies, the authors proposed that DSBs were not the signal for DNA-PK activation since recruitment of the XRCC4-DNA-ligase IV complex did not accompany such activation. DNA-PK was shown to modulate HIF-1-mediated signaling in this study (Bouquet et al. 2011).

2.9 Two Pathways Collide—HIF-1 and the DDR

Recently, it has become clear that ATM is active (phosphorylated at 1981) in milder hypoxic conditions (0.2–1 % O₂) (Cam et al. 2010). In these conditions, ATM has been shown to phosphorylate and stabilize HIF-1 α . ATM-dependent phosphorylation of HIF-1 α leads to the stimulation of a negative mTORC1 regulator, REDD1, and a subsequent reduction in mTORC1 signaling in hypoxia. The activation of ATM in this context, however, was shown to be independent of DDR signaling. This is perhaps not particularly surprising since the milder hypoxic conditions used in this study are not associated with replication arrest, which is thought to be the activating signal for the hypoxia-induced DDR (Hammond, et al. 2002; Cam et al. 2010; Hammond et al. 2003a).

In addition, ATR has recently been shown to affect HIF-1 α , pointing to a further link between these two signaling pathways (Pires et al. 2012). Cells grown as spheroids treated with the ATR inhibitor VE-821 were found to have lower levels of the HIF-1 α target GLUT1 compared to untreated spheroids. This observation was expanded to show that in the presence of VE-821 HIF-1 α stabilization was delayed in hypoxic conditions and that this manifested in delayed induction of HIF-1 target genes. This effect of ATR inhibition on HIF-1 stability and activity appeared transient and it is likely that this is due to the concomitant induction of ATM, which can also stabilize HIF-1 α as described previously. Despite this, the role of ATR in stabilizing HIF-1 α is of significant interest. An increasing number of human tumors have been shown to harbor ATM mutations, suggesting that ATR inhibition may have prolonged effects on HIF-1 activity in certain tumors. Interestingly, ATR inhibition was shown

to slow down hypoxia-induced cell motility in a HIF-1 α -dependent manner, suggesting that agents of this type might have unexpected impact on tumor spread. A similar role for ATR in regulating HIF-1 signaling has been demonstrated in a recent study where HIF-1 α translation was shown to be regulated by ATR kinase activity at 0.1 % O₂ (Fallone 2012). In addition, Economopoulou et al. have shown that in response to mild hypoxia (1 % O₂), where no replication arrest is observed, H2AX is phosphorylated in an ATR-dependent manner in proliferating endothelial cells. The low levels of DNA damage arising during normal replication were thought to serve as the activating signal for DDR activation and H2AX phosphorylation in this case. These studies showed that γ H2AX was required for pathological neovascularization in hypoxia. Notably, loss of γ H2AX had no effect on developmental angiogenesis (Rankin et al. 2009; Economopoulou et al. 2009).

2.10 Reoxygenation and the DDR

As previously mentioned, reoxygenation following periods of hypoxia leads to the induction of DNA damage. This reoxygenation-induced damage signaling can be inhibited with the use of reactive oxygen species (ROS) scavengers, suggesting that ROS maintains the DDR after reoxygenation. In response to reoxygenation, ATM has been shown to phosphorylate downstream targets, including p53 and CHK2. ATM-dependent CHK2 phosphorylation leads to G₂ arrest (Gibson et al. 2005). In the absence of CHK2, cells undergo elevated levels of apoptosis and the G₂ arrest is abrogated (Freiberg et al. 2006a, 2006b; Hammond et al. 2004). If cells are exposed to acute periods of severe hypoxia (under 12 h) and then are allowed to undergo reoxygenation, they will undergo replication restart (Pires et al. 2010a,b). It is important to note that these cells will restart following the induction of ROS-induced DNA damage and in a context of reduced DNA repair (described below), potentially leading to increased genomic instability. Human tumor cells, with intact p53 function, will undergo p53-dependent apoptosis if exposed to periods of acute hypoxia followed by reoxygenation (Rzymiski et al. 2010). When cells are exposed to longer periods of hypoxia (for example, over 12 h), replication does not restart in response to reoxygenation. Expression analysis determined that numerous key replication factors are repressed in hypoxic conditions. These include members of the MCM family, which act as a complex and are loaded onto chromatin for DNA replication. In addition, several members of this complex such as MCM3, MCM4, MCM5, and MCM6 have been shown to become unbound from chromatin in cells exposed to over 12 h of severe hypoxia. Replication and DNA synthesis restart cannot occur in these cases, even if reoxygenation occurs and nucleotide levels are restored (Pires et al. 2010a).

In addition after reoxygenation, ATM has been shown to be important in regulating pathological angiogenesis by limiting excessive ROS. Loss of ATM results

Hypoxia and Cancer

Biological Implications and Therapeutic Opportunities

Melillo, G. (Ed.)

2014, XIV, 363 p. 53 illus., 36 illus. in color., Hardcover

ISBN: 978-1-4614-9166-8

A product of Humana Press