Cell-cycle checkpoints and cancer

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All life on earth must cope with constant exposure to DNA-damaging agents such as the Sun's radiation. Highly conserved DNA-repair and cell-cycle checkpoint pathways allow cells to deal with both endogenous and exogenous sources of DNA damage. How much an individual is exposed to these agents and how their cells respond to DNA damage are critical determinants of whether that individual will develop cancer. These cellular responses are also important for determining toxicities and responses to current cancer therapies, most of which target the DNA.

The DNA contained in every mammalian cell is under constant attack by agents that can either directly damage one of its three billion bases or break the phosphodiester backbone on which the bases reside. For example, free oxygen radicals, which can cause both base damage and DNA breakage, arise as a consequence of normal cellular metabolism or can be created when the organism is exposed to external sources of ionizing radiation in the environment. Life on Earth has evolved to deal with both metabolic and external sources of DNA-damaging agents through the development of elegant mechanisms that repair damage to the DNA.

Cellular responses to DNA damage constitute one of the most important fields in cancer biology. First, damage to cellular DNA causes cancer. We know this from epidemiological studies[1], from animal models and from the observation that many human-cancer-susceptibility syndromes arise from mutations in genes involved in DNA-damage responses[2]. Second, DNA damage is used to cure cancer. Most therapeutic modalities that we currently use to treat malignancies target the DNA, including radiation therapy and many chemo-therapeutic agents. Third, DNA damage is responsible for most of the side effects of therapy. Bone marrow suppression, gastrointestinal toxicities, and hair loss are all attributable to DNA-damage-induced cell death of proliferating progenitor cells in these tissues. So, from the perspective of cancer, DNA damage causes the disease, it is used to treat the disease, and it is responsible for the toxicity of therapies for the disease.

Among the mechanisms that cells have developed to cope with this constant attack on their DNA are elegant but not perfect DNA-repair processes. Because there are various types of DNA lesion that can occur, a variety of different repair mechanisms exist. In addition to directly repairing DNA breaks or adducts, cells respond to DNA damage by halting cell-cycle progression or by undergoing programmed cell death. Although we have a limited understanding of how the processes of cell-cycle arrest or apoptosis are coordinated with the process of DNA repair, such coordination must take place to optimize the outcome for the cell or the organism. In addition to damage to the DNA, cells must cope with other stresses, such as intermittent or prolonged exposure to abnormally low levels of oxygen or nutrients. Although cells use different aspects of the signalling pathways to deal with these types of change in their microenvironment, there are commonalities in the steps that cells use to deal with DNA damage.

The term 'cell-cycle checkpoint' refers to mechanisms by which the cell actively halts progression through the cell cycle until it can ensure that an earlier process, such as DNA replication or mitosis, is complete[3]. Here, we focus on some of the mechanisms by which cells modulate progression through the cell cycle in the face of DNA damage and other stresses that affect DNA replication. Although we focus on signalling pathways that have been characterized in mammalian cells, lessons learned from studying lower eukaryotes (in particular, yeast), have been instructive and reflect the considerable evolutionary conservation of these pathways. Finally, current concepts about how sporadic or inherited mutations in genes in these pathways contribute to cancer development will be explored.

The signalling pathways

Signal initiation by different stresses

DNA can be damaged in a variety of ways. First, energy released by free oxygen radicals, generated either by normal metabolic processes or by exposure to an external source of ionizing radiation, can break the phosphodiester bonds in the backbone of the DNA helix. When two of these breaks are close to each other, but on opposite DNA strands, a double-strand break (DSB) is present in the DNA and the cell faces a particularly challenging situation for repair. Second, alkylating chemical moieties can modify purine bases and the size of the chemical adduct determines what repair process is used[2]. Bifunctional alkylating chemicals can cause intra-strand or inter-strand crosslinks that require additional molecular interventions for them to be reversed. Third, inhibitors of DNA topoisomerases can lead to enhanced single or DSBs depending on which topoisomerase is inhibited and on the phase of the cell cycle[4].

Each type of DNA damage requires a specific set of cellular responses to deal with the specific nature of the damage. Different mechanisms are required to repair the damage to the DNA backbone or to the DNA bases and the challenges of repairing the DNA can vary in the different phases of the cell cycle. To optimally repair DNA damage, the cell must also control other cellular processes before or during the repair, such as DNA replication or mitosis. Cells that are damaged when they are already in the middle of the process of DNA replication face particular challenges, but would still probably benefit from halting or slowing DNA replication until the damage has been repaired. So, there should be advantages for a eukaryotic cell to transiently halt progression through the cell cycle after DNA damage, which presumably include limiting heritable mutations in daughter cells and enhancing viability of the damaged cells.

Initiation of the activities of the PI(3)K (phosphatidylinositol-3-OH kinase)-like kinases (PIKKs), ATM (ataxia

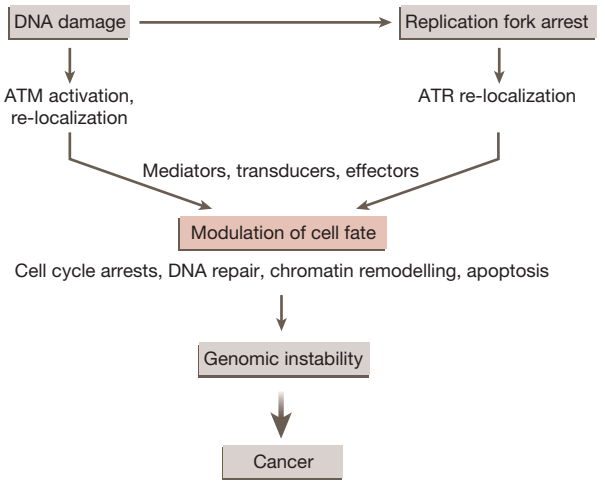

Figure 1 General scheme of responses to DNA damage or replication-fork arrest and the impact on cell fate, genomic instability and cancer development. Replication-fork arrest stimulates the initiation of cellular ATR activity, whereas DNA damage can directly activate ATM and can lead to replication-fork arrest, thereby also activating cellular ATR kinase. Once active, both the ATM and ATR kinases, functioning in combination with other proteins and substrates, help determine the outcome of the cell. If genomic instability ensues, this can contribute to cellular transformation.

telangiectasia mutated) and ATR (AMT- and Rad3-related) are the first steps characterized to date in the activation of signal transduction pathways that inhibit cell-cycle progression after DNA damage. The ATM kinase seems to primarily be activated following DNA damage whereas the ATR kinase seems to be critical for cellular responses to the arrest of DNA replication forks — the DNA structures formed during replication. This is the case whether the arrest of replication-fork progression is due to DNA damage or to other stresses[5,6]. Because many types of DNA damage result both in the direct damage of the DNA and the arrest of DNA replication forks, ATM and ATR seem to participate together in many cellular-stress responses and complex joint responses must be coordinated (Fig. 1).

Signal initiation by ATM and ATR

To accomplish the physiological goal of minimizing the adverse effects of a stressful physiological situation, an arrest of cell-cycle progression should be engaged very rapidly after exposure to the stress. ATM and ATR are both extremely large (predicted molecular mass of 350 and 301 kilodaltons, respectively) protein kinases that phosphorylate numerous substrates to achieve their physiological goals[7]. It is a mechanistic challenge to tightly control the activities of these large kinases so that they do not stimulate growth-suppressive pathways in the absence of an appropriate stress, but can be activated instantaneously following exposure to the stress.

Patients, mice and cells lacking ATM are viable, suggesting that the ATM kinase is not essential for critical cellular functions such as normal cycle progression or cellular differentiation[5]. ATM kinase activity is minimal or low in unstressed cells and primarily is engaged to help cells deal with cellular stresses that affect DNA or chromatin structure. The identification of a single, major damage-induced phosphorylation site (serine 1981) led to the demonstration of a new mechanism of ATM regulation that permits a rapid and sensitive switch for checkpoint pathways[8]. In unstressed cells, ATM is present as a homodimer in which the kinase domain is physically blocked by its tight binding to an internal domain of the protein surrounding serine 1981. The introduction of a DNA DSB leads to a conformational change in the ATM protein. This stimulates the kinase to phosphorylate serine 1981, causing the dissociation of the homodimer[8]. The activated ATM monomer can now phosphorylate its numerous substrates, whether they are nucleoplasmic, like p53, or at the sites of DNA breaks, like NBS1 (Nijmegen breakage syndrome 1), BRCA1 (breast cancer 1), and SMC1 (structural maintenance of chromosomes 1). The conformational change that induces the extremely rapid and extensive intermolecular autophosphorylation event in ATM does not seem to require the binding of the ATM dimer to sites of DNA damage, but instead results from some change in higher-order chromatin structure that the ATM dimer can sense at some distance away from the site of the DNA break[8]. The nature of this chromatin structure change and how ATM senses this change, including whether it is a direct or an indirect 'sensing' mechanism, remains to be discovered. Recent observations that the multiprotein complex MRE11(meiotic recombination 11)/RAD50/NBS1 (MRN) contributes to the activation of ATM after ionizing radiation — at least at low doses of ionizing radiation — may shed light on the mechanisms by which this activation process occurs[9–13].

The phosphorylation of substrates by the ATM kinase requires more than the dissociation of the ATM homodimer and the release of the blocked ATM kinase domain. The activated ATM monomer must also get to the sites in the cell where the substrates are present, such as at DNA breaks. It was recently demonstrated that MRE11 binds to ATM and enhances its ability to phosphorylate substrates *in vitro* in the presence of an appropriate mimic of DNA breaks[14]. Such a function is consistent with the observation that ATM can be activated by exposure to ionizing radiation in cells lacking NBS1 or BRCA1, but fails to migrate to the sites of DNA strand breaks. Once recruited to the DNA break, the activated ATM can then phosphorylate critical substrates like NBS1, BRCA1 and SMC1, which accumulate at these sites[9]. If ATM is activated but fails to get recruited to DNA breaks, as happens after ionizing radiation in cells lacking NBS1 or BRCA1, or if chromatin structure changes occur in the absence of DNA breaks, then ATM is still able to phosphorylate nucleoplasmic substrates, such as p53. Thus, DNA damage leads to ATM activation and substrate phosphorylation by two distinctive steps: (1) chromatin structure change induces intermolecular ATM autophosphorylation and homodimer dissociation; and (2) activated ATM monomer is recruited to its substrates, some of which localize to sites of DNA damage (Fig. 2). In this model, ATM activation and recruitment of MRN and BRCA1 to sites of DNA breaks are two distinct events.

Although the activity of ATM in *in vitro* kinase assays is increased after immunoprecipitation from irradiated cells, there is no measurable change in the kinase activity of ATR — even in the face of stresses where ATR is required to sustain normal cellular responses[6]. It seems that ATR kinase may be constitutively ready to phosphorylate substrates but have its cellular functions largely controlled by subcellular localization. ATR exists in a complex with the ATR-interacting protein (ATRIP), both before and after exposure to stresses such as ultraviolet irradiation[15–17]. The observation that replication protein A (RPA), a single-stranded DNA (ssDNA)-binding protein involved in DNA replication, stimulates the *in vitro* binding of ATRIP to ssDNA led to a model in which ATR becomes localized to sites of replication-fork arrest by means of binding of ATRIP to RPA (ref. 16). In this model, any stimulus or stress that leads to an abnormal stretch of ssDNA, such as an arrested replication fork, would be decorated with RPA. The accumulation of RPA on the ssDNA would then lead to the recruitment of the ATRIP protein, and its heterodimeric partner, ATR. Once the active ATR kinase is localized to the ssDNA region, it can phosphorylate critical substrates, such as RAD17 and CHK1 (Fig. 2). Although the critical importance of RPA in the recruitment of ATR to ssDNA has been questioned[17], the importance of this change in ATR localization is generally accepted.

As with ATM, the presence of an active ATR kinase in the cells is not sufficient for ATR to carry out its cellular functions. In addition to ATR, several other proteins and protein complexes must be recruited to the ssDNA site as well. These include the clamp-loading, RAD17-containing complex, RSR, which participates in the loading of the RAD9–RAD1–HUS1 (9–1–1) sliding clamp onto chromatin, and the claspin protein, which is independently recruited to chromatin[18–20]. All these events are required for the phosphorylation of CHK1 by ATR and for the activation of the appropriate cell-cycle checkpoints (Fig. 2).

Whereas cells tolerate the absence of ATM, cells and animals lacking ATR seem to be non-viable[21,22]. These observations suggest that ATR is probably required for normal progression through the cell cycle, even in the absence of cellular stress. Consistent with this concept, recent results suggest a critical role for ATR in the normal progression of DNA replication forks[23]. Given the binding of ATR to regions of ssDNA, a role in normal replication-fork progression is perhaps not surprising.

In addition to its apparent roles in normal replication-fork progression, ATR is probably engaged in the cellular responses to many other types of cellular stress because so many of them affect the rate of replication-fork progression. ATR has been implicated in cellular responses to hypoxia[24] and to DNA-replication inhibitors[16,25]. It is also critical for responses to DNA-damaging events that affect the progression of replication forks, particularly agents that introduce bulky DNA adducts, such as ultraviolet irradiation and alkylating agents or crosslinking agents. So, whereas ATM seems to become engaged in signalling pathways primarily following the introduction of DNA breaks, ATR has a critical role in virtually all cellular stress responses that share inhibition of replication-fork progression as a common mechanism. ATR even seems to be engaged in cellular responses to DNA breaks, possibly compensating for ATM: many ATM substrates eventually get phosphorylated after exposure to ionizing radiation in cells lacking ATM protein.

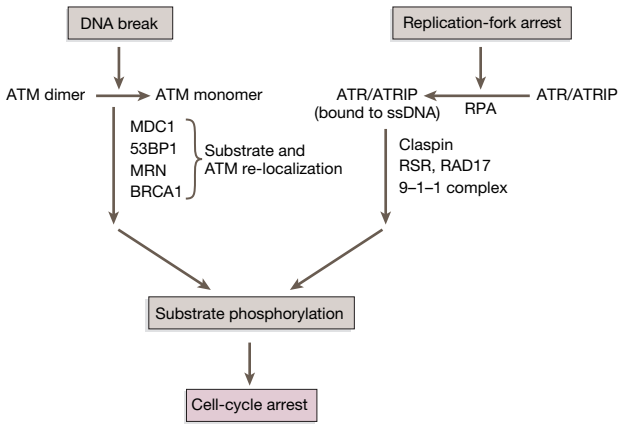

Figure 2 Scheme of mechanisms that lead to the induction of ATM- and ATR-directed cellular activities. DNA strand breaks lead to the dissociation of the inactive ATM dimer. The appropriate localization of both the ATM monomer and the ATM substrates is modulated by several proteins, including the MRN complex, MDC1, 53BP1, and Brca1. The ATR/ATRIP complex is recruited to sites of ssDNA, perhaps by RPA. Optimal substrate phosphorylation and the engagement of cell-cycle arrest depends on other proteins such as claspin, the RSR complex and the 9-1-1 complex. As illustrated in Fig. 1, these pathways may often operate in concert and there may be cross-talk between the pathway components shown here.

Transducing the signal

To efficiently spread the alert signal and orchestrate the global cellular response to DNA damage that is usually inflicted to only a few sites within the vast genome, the proximal checkpoint kinases ATM and ATR (ref. 26) cooperate closely with two other classes of proteins. These are the so-called checkpoint mediators (also known as adaptors)[12,27,28] and the transducer kinases CHK1 and CHK2 (ref. 29; Fig. 3). Regulatory phosphorylations of the downstream checkpoint targets — diverse effector proteins that function at the interface between the cell-cycle, DNA-repair and cell-death machineries — may be carried out by the proximal kinases or transducer kinases alone. Alternatively, distinct residues of these same effectors are targeted by ATM/ATR and CHK1/CHK2, respectively[26,29] (Fig. 3).

It is remarkable how rapidly (within seconds after the focal injury to DNA), the global checkpoint networks become activated and the local events at the damage site are coordinated with more distant cellular processes. For example, in response to only a few potentially harmful lesions, such as DSBs in proliferating cells, not only must the lesions be processed locally, but the whole cell must be alerted to delay the most vulnerable processes, such as DNA replication or initiation of chromosome segregation in a coordinated, 'pan-cellular' manner. Such speed and spatio-temporal coordination reflect the fact that the initial checkpoint responses operate through post-translational modifications, re-localizations, dynamic interactions, and changes of conformation and/or stability of pre-existing proteins, all phenomena that are jointly governed by these three classes of checkpoint regulators.

Exciting insights into these highly dynamic events have recently been obtained using new technologies for real-time imaging of fluorescently labelled checkpoint proteins in live cells, and phospho-specific antibodies that recognize proteins modified in response to DNA damage[30–32]. Furthermore, the significance of proper checkpoint signalling for the prevention of cancer is underscored by the fact that most checkpoint kinases and mediators are either established or emerging tumour suppressors — gene products whose decreased expression or loss-of-function mutations contribute to tumorigenesis (Fig. 3). So, what are the roles and the underlying molecular mechanisms of action of the checkpoint mediators and the signal-transducing kinases?

The emerging role of checkpoint mediators

Although the precise mechanisms of action of this important class of checkpoint factors are largely unknown, they seem to modulate the activity of ATM/ATR, facilitate the interactions between ATM/ATR and their substrates, and in a broader sense 'mediate' spatio-temporal assembly of multiprotein complexes in the chromatin regions surrounding the sites of DNA damage. There are currently three known members of this class of checkpoint factors involved in the signalling by ATM; so far only one such protein is known to modulate the response by ATR (Fig. 3). As most of the mediators are initially recruited to sites of DNA damage and/or replication blockade independently of ATM and ATR, they might also be involved in 'sensing' such lesions. Alternatively, the mediator proteins could be recruited through their interaction with the candidate DNA-damage sensors[33,34].

The ATM-related mediators include MDC1 (mediator of DNA damage checkpoint 1; also known as NFBD1), 53BP1 (p53 binding protein 1) and BRCA1 — large multi-domain proteins that contain two tandem BRCT (Brca1 carboxy-terminal) domains at their C terminus[9,27,33,35–41]. Interestingly, the BRCT domains have recently been shown to serve as protein-phosphoprotein-binding modules[42,43], suggesting a possible mechanism for how the mediator proteins could promote the transient multiple interactions of checkpoint and repair proteins near the DNA-damage sites. Indeed, unlike the initial, largely ATM-independent, recruitment of the mediators to sites of DNA damage, their accumulation into the microscopically visible 'foci' depends on ATM-mediated phosphorylation of histone H2AX[12,27,30–32], a modification that marks chromatin regions spanning megadaltons of DNA flanking each DSB (ref. 44). The MDC1 protein

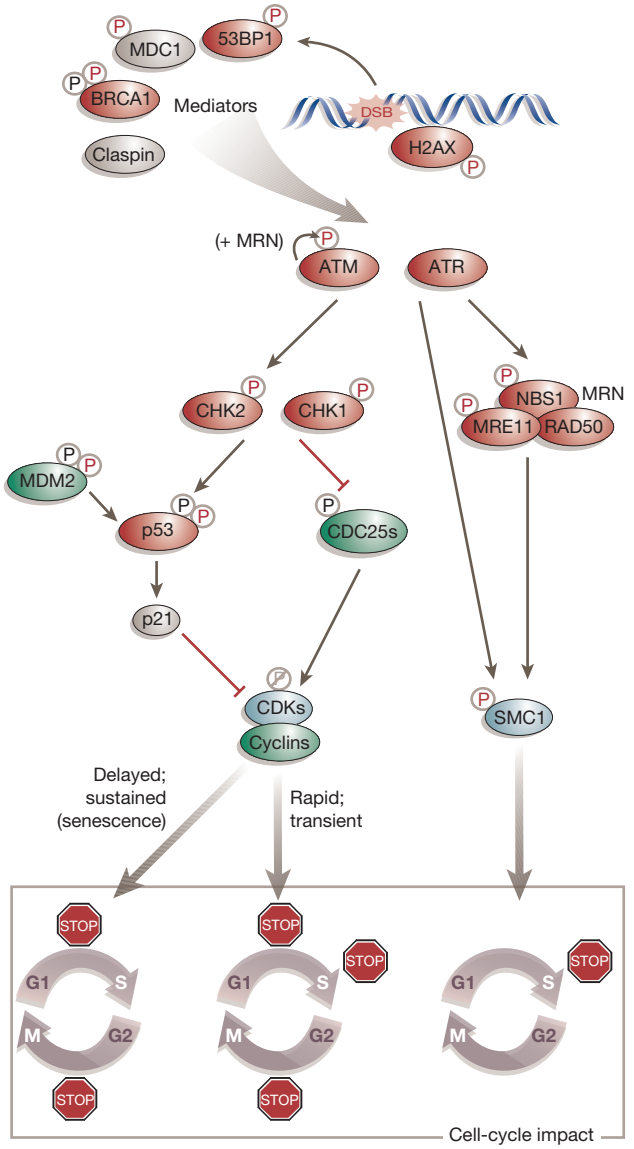

Figure 3 A simplified scheme of cell-cycle checkpoint pathways induced in response to DNA damage (here DSBs), with highlighted tumour suppressors shown in red and proto-oncogenes shown in green. The proximal checkpoint kinases ATM and ATR phosphorylate diverse components of the network, either directly (red 'P') or through the transducing kinases CHK2 and CHK1 (black 'P'). (For simplicity, some candidate damage sensors and several ATM/ATR and CHK1/CHK2 substrates have been omitted.) The BRCA1 protein also contributes to cell-cycle arrest and DNA repair by homologous recombination, whereas p53 controls genes involved in cell death and DNA-repair mechanisms. The cell-cycle phase and the duration of the blockade affected by the effector pathways are indicated, including the potential permanent arrest (senescence), as mediated by p53. The global checkpoint network regulated by ATM/ATR and CHK2/CHK1 also affects cellular responses other than cell cycle progression, including DNA repair, transcription, chromatin assembly and cell death.

functions as a molecular bridge between the phosphorylated H2AX (γ-H2AX) and the NBS1 component of the MRN complex[31], and helps provide a platform for a myriad of dynamic interactions for these and additional checkpoint and DNA-repair proteins (including the activated ATM and BRCA1) within the vicinity of the damage sites. Although the mediator proteins are unlikely to initially target the activated ATM to sites of DNA damage (this might be the role of the candidate damage sensors such as the MRN complex)[9–11,13,45,46], the sustained multiprotein interactions mediated by MDC1, 53BP1 and BRCA1 seem to facilitate ATM signalling and the processing/repair of the lesions, thereby contributing to the biological outcome of the checkpoint responses (Fig. 2)[9,12,27,33,35–41]. Consistent with this concept, mammalian cells that lack any of these three mediators show enhanced sensitivity to DNA-damaging agents such as ionizing radiation, and impaired intra-S-phase and G2/M cell-cycle checkpoints.

Reminiscent of the roles of MDC1, 53BP1 and BRCA1 in proper localization, timing and velocity of the ATM-controlled signalling, the ATR-controlled checkpoint signalling, at least towards the Chk1 kinase that is activated by ATR, relies on claspin[28]. Claspin is a mediator/adaptor protein that is structurally unrelated to the mediators involved in response to DSBs. Claspin selectively interacts with chromatin structures created by active replication forks, and is required for ATR-mediated phosphorylation, and so for proper activation, of CHK1 (Fig. 2)[28,47].

Effector kinases CHK1 and CHK2

Prominent among the substrates of the apical checkpoint kinases ATM and ATR are the checkpoint-transducer serine/threonine kinases (also known as effector kinases) CHK2 and CHK1 (ref. 29). Despite some 'cross-talk' between ATM and CHK1, the ATM- and ATR-mediated phosphorylations trigger preferentially the activation of CHK2 and CHK1 (Fig. 3), respectively[29]. Given that the ATM–CHK2 and ATR–CHK1 signalling modules share many substrates among the checkpoint effector proteins[26,29], it is striking that ATM and CHK2 are dispensable for pre-natal development, whereas complete absence of either ATR or CHK1 results in early embryonic lethality[26,29]. As mentioned above for ATR, a plausible explanation for such a fundamental biological difference emerges from recent evidence that supports a role for ATR–CHK1 in the regulation of some essential processes during unperturbed cell cycles, including the control of DNA replication[23,48] or initiation of mitotic events on centrosomes[49]. More mechanistic insights into how the checkpoint kinases ATM and ATR — in concert with the extremely mobile messenger kinases CHK2/CHK1 (refs 29, 30) — trigger cell-cycle delays at various transitions of the cell-division cycle is the subject of the next section.

Affecting the cell cycle

During unperturbed proliferation, mammalian cells can only withdraw from the cell cycle on experiencing growth-factor deprivation or growth inhibitory signals in early-to-mid G1 phase (see review in this issue by Massagué, page 298). This is before the cells pass through the RB (retinoblastoma protein)/E2F (transcription factor)-controlled restriction point, after which they are committed to a round of DNA replication and cell division[50,51]. However, the ATM/ATR–CHK2/CHK1-controlled checkpoint network response to genotoxic stress can transiently delay cell-cycle progression in G1, S or G2 phases, or even impose prolonged, durable cell-cycle arrests in either G1 or G2, before entry into the subsequent S phase or mitosis, respectively. Given the critical significance of error-free DNA replication and chromosome segregation for the maintenance of genomic integrity and the prevention of cancer, it is not surprising that these most vulnerable stages of the cell-division cycle are protected by a wider spectrum of checkpoint effector mechanisms, the identity of which are briefly discussed below.

The G1 and G1/S checkpoint responses

The dominant checkpoint response to DNA damage in mammalian cells traversing through G1 is the ATM(ATR)/CHK2(CHK1)–p53/MDM2-p21 pathway (Fig. 3), which is capable of inducing sustained, and sometimes even permanent G1 arrest[7,29,52]. Although the expression of ATM and CHK2 is relatively constant during the cell cycle, the concentrations of ATR and CHK1 are low in the early-to-mid G1, and their activities become important only closer to the G1/S transition. ATM/ATR directly phosphorylate the p53 transcription factor within its amino-terminal transactivation domain, particularly

on serine 15. Threonin 18 and serine 20 in the same domain, along with probably some additional p53 sequence(s), are also targeted by CHK1/CHK2 (refs 7, 26, 29, 52, 53). In addition, the ubiquitin ligase MDM2 that normally binds p53 and ensures rapid p53 turnover, is targeted after DNA damage by ATM/ATR (ref. 54), as well as by CHK2/CHK1 (N. Motoyama, personal communication). These modifications of p53 and Mdm2 contribute to the stabilization and accumulation of the p53 protein, as well as to its increased activity as a transcription factor. The key transcriptional target of p53 is the p21CIP1/WAF1 inhibitor of cyclin-dependent kinases[7,52], which silences the G1/S-promoting cyclin E/Cdk2 kinase and thereby causes a G1 arrest. This leads not only to the inability to initiate DNA synthesis, but it also preserves the RB/E2F pathway in its active, growth-suppressing mode, thereby causing a sustained G1 blockade (see also review in this issue by Massagué, page 298). Thus, the G1 checkpoint response targets two critical tumour suppressor pathways, governed by p53 and pRB. These are arguably the two mechanisms that are most commonly deregulated in human cancer[7,50–52].

In late G1, as part of the activated E2F-dependent S-phase-promoting transcriptional programme, the expression of ATR and CHK1 increases. Cyclins E and A, and the activator of the cyclin E(A)/CDK2 kinase — the CDC25A phosphatase — are also induced in late G1. The ATR/CHK1 module (but not ATM/CHK2), through moderate constitutive phosphorylation of CDC25A on its several serine residues, then maintains an appropriate abundance of CDC25A through its ubiquitin-dependent, proteasome-mediated turnover during unperturbed proliferation[29,55]. In response to genotoxic stress, this physiologically operating mechanism becomes enhanced through increased activity of CHK1 and CHK2, leading to downregulation of CDC25A and consequently to the inhibition of cyclin E(A)/CDK2 complexes[29,55,56]. Importantly, despite the simultaneous phosphorylation of CDC25A and p53 by checkpoint kinases (Fig. 3), the impact of these events on cell-cycle machinery is faster in the CDC25A-degradation cascade that unlike the slower-operating p53 pathway, does not require the transcription and accumulation of newly synthesized proteins. Thus, the CHK1/CHK2–CDC25A checkpoint is implemented rapidly, independently of p53, and it delays the G1/S transition only for a few hours, unless the sustained p53-dependent mechanism prolongs the G1 arrest.

The S-phase checkpoint pathways

The intra-S-phase checkpoint network activated by genotoxic insults causes largely transient, reversible inhibition of the firing from those origins of DNA replication that have not yet been initiated. It seems that there are at least two parallel branches of this checkpoint that slow down the ongoing DNA synthesis, both of which are controlled by the ATM/ATR signalling machinery. One of these effector mechanisms operates through the CDC25A-degradation cascade described in the previous section. The inhibition of CDK2 activity downstream of this pathway blocks the loading of CDC45 onto chromatin. CDC45 is a protein required for the recruitment of DNA polymerase α into assembled pre-replication complexes, so the inhibition of CDK2 activity prevents the initiation of new origin firing[29,55].

The other branch of the intra-S-phase checkpoint reflects the impact of ATM-mediated phosphorylations of NBS1 on several sites, in particular serine 343 (refs 7, 26) and serines 957 and 966 of the cohesin protein SMC1 (refs 9, 57, 58). A better mechanistic understanding of this pathway, whose proper function also depends on BRCA1 and FANCD2 (Fanconi anaemia, complementation group D2) proteins[9,59,60], should be particularly rewarding because the observed hypersensitivity to radiation in cells that are defective in NBS1 or SMC1 seems attributable to the inability of ATM to phosphorylate the two critical residues of the SMC1 effector[9].

The concept of the two above-mentioned parallel effector branches of the intra-S-phase checkpoint has been documented for responses to both ionizing radiation (ref. 61) and to ultraviolet light[62]. Whether the recently reported targeting of CDC7 — another kinase involved in regulation of DNA replication through an ATR-dependent DNA-damage response[63] — represents yet another parallel mechanism to delay DNA synthesis remains to be established.

Apart from the inhibition of replication-origin firing, another critical function provided by S-phase checkpoints (particularly the so-called 'replication checkpoint' activated by stalled replication) is to protect the integrity of the stalled replication forks. Such maintenance of fork stability, achieved through yet-to-be discovered effector mechanisms, helps prevent the conversion of primary lesions into DNA breaks and facilitates the subsequent recovery of DNA replication[18,55].

The G2 checkpoint

The G2 checkpoint (also known as the G2/M checkpoint) prevents cells from initiating mitosis when they experience DNA damage during G2, or when they progress into G2 with some unrepaired damage inflicted during previous S or G1 phases[64,65]. The accumulation of cells in G2 may also reflect a contribution of the so-called DNA-replication checkpoint (often referred to as the S/M checkpoint) that may sense some of the persistent DNA lesions from the previous S phase as being inappropriately or not fully replicated DNA.

The critical target of the G2 checkpoint is the mitosis-promoting activity of the cyclin B/CDK1 kinase, whose activation after various stresses is inhibited by ATM/ATR, CHK1/CHK2 and/or p38-kinase-mediated subcellular sequestration, degradation and/or inhibition of the CDC25 family of phosphatases that normally activate CDK1 at the G2/M boundary[56,65–68]. In addition, other upstream regulators of CDC25C and/or cyclin B/CDK1, such as the Polo-like kinases PLK3 and PLK1 seem to be targeted by DNA-damage-induced mechanisms[65]. Analogous to the role of the checkpoint mediators in the S-phase checkpoint, 53BP1 and BRCA1 are also involved in the regulation of the G2-checkpoint responses[39,41,69].

The maintenance phase of the G2 checkpoint probably partly relies on the transcriptional programmes regulated by BRCA1 and p53, leading to the upregulation of cell-cycle inhibitors such as the CDK inhibitor p21, GADD45a (growth arrest and DNA-damage-inducible 45 alpha) and 14–3–3 sigma proteins[65,70]. The fact that even tumours defective in other checkpoints, such as those with mutant p53, tend to selectively accumulate in G2 after DNA damage, indicates that p53-independent mechanisms are sufficient to sustain the G2 arrest. At the same time, this phenomenon has inspired efforts to interfere with the G2 checkpoint as a potential strategy to sensitize cancer cells, which are deficient in their G1/S checkpoint pathways, to radiation- or drug-induced DNA damage[71].

Impacting cancer

As the checkpoint and repair pathways facilitate cellular responses to DNA damage, and because there is significant data suggesting that DNA damage from both endogenous and exogenous sources is a major contributor to the development of human cancers, it is reasonable to speculate that alterations in these pathways increase the risk of cancer developing. Data from both animal models and humans strongly support this concept (Table 1). Cells with an intact DNA-damage response frequently arrest or die in response to DNA damage, thus reducing the likelihood of progression to malignancy. Mutations in apoptosis, DNA-damage responses or in mitotic-checkpoint pathways, however, can permit the survival or the continued growth of cells with genomic abnormalities, thereby enhancing the chance of malignant transformation. Although many of the DNA-damage response factors described above have been classified as tumour suppressor genes and oncogenes (see also Fig. 3), the dysfunction of these pathways has not been linked to cancer development in all cases. Sorting out which pathway steps are important in affecting the predisposition to malignancies versus those that are not may provide invaluable insights into the mechanisms responsible for human tumorigenesis. Although germline mutations in mice and humans are used to identify the genes and pathway steps that predispose

Table 1 **Human cancer susceptibility linked to DNA-damage response**

Disease	Gene	Number of mutant alleles inherited	Cancer predisposition	Comments
Ataxia-telangiectasia (A-T)	*ATM*	2	Leukaemia, lymphoma	Most mutations result in null protein phenotype
Nijmegen breakage syndrome (NBS)	*NBS1*	2	Leukaemia, lymphoma	Fragment of NBS1 protein still expressed in some cell types
A-T-like disorder (ATLD)	*Mre11*	2	Leukaemia, lymphoma	Hypomorphic mutations in *Mre11*
Fanconi's anaemia (FA)	*FancD2*, *Brca2* (also known as *FancD1*)	2	Acute myelogenous leukaemias	Other FA genes not directly implicated in checkpoints; *Brca2* — hypomorphic
Familial breast, ovarian carcinoma syndrome	*Brca1*, *Brca2*	1	Breast, ovarian, scattered others	
Li-Fraumeni syndrome	*p53*, *CHEK2*	1	Sarcomas, leukaemias, brain tumours, adrenal tumours, others	

This list does not include syndromes resulting from DNA-repair defects, which includes xeroderma pigmentosum, hereditary non-polyposis colon cancers, Bloom's syndrome and other Fanconi's anaemia complementation groups.

animals to acquiring tumours, it is likely that dysfunction of these steps is also critically important in the development of sporadic tumours, which constitute most human cancers.

DNA-damage signal transducers and cancer

Loss of ATM strongly predisposes both humans and mice to lymphoma development[5], and to a lesser degree to other malignancies[26]. Because the deletion of the Rad52 protein, which is required for homologous recombination, significantly reduces lymphoma development in ATM-deficient mice, it has been suggested that excessive recombination is an important contributor to tumorigenesis in ataxia telangiectasia[72]. Similarly, patients with mutations in NBS1 or MRE11 are predisposed to develop cancer[73–75].

In contrast to the disruption of both alleles of ATR causing embryonic lethality in mice[22], a human disease, Seckel syndrome, was recently associated with hypomorphic mutations in *ATR* that lead to low levels of ATR expression[76]. Interestingly, although these patients show growth retardation, dwarfism, microcephaly and mental retardation, and their cells show chromosome instability after mitomycin C exposure[77], a high incidence of malignancies is not thought to be a prominent part of this inherited syndrome. However, *ATR* haploinsufficiency enhances tumorigenesis in mice that are defective for DNA-mismatch repair[78].

Certain mutations in additional components of these signalling pathways also lead to cancer predisposition. Mice lacking either *H2AX* (refs 79, 80) or *53BP1* (ref. 40) show cell-cycle checkpoint defects and cancer predisposition. Even haploinsufficiency for *H2AX* results in detectable genomic instability and enhanced tumour susceptibility in the absence of p53 (refs 79, 80). Although *H2AX* maps to a cytogenetic region commonly altered in human cancers, 11q23, it is not clear whether *H2AX* abnormalities contribute to human cancer. Although Mdc1 seems to be required for cell-cycle checkpoint function[27,81], mutations in the gene have not yet been linked to enhanced tumour development in mice or humans.

The homozygous-deficient state cannot be tested, but *Chk1* heterozygosity modestly enhances the tumorigenic phenotype of *Wnt1* transgenic mice[82]. As the tumours in these mice did not lose the other allele of *Chk1*, a haploinsufficient tumour suppressor mechanism was suggested. Potential mechanisms underlying the haploinsufficient phenotype were studied using generations of mice in which *Chk1* was conditionally disrupted in mammary epithelial cells. These cells showed inappropriate S-phase entry, accumulation of DNA damage during replication and inappropriate mitotic entry[83]. These observations suggest that checkpoint defects associated with *Chk1* haploinsufficiency can contribute to tumorigenesis. *Chk2*$^{-/-}$ mice do not spontaneously develop tumours[84], but a lack of Chk2 enhances skin tumorigenesis induced by carcinogen exposure. As inherited mutations in one allele of *CHEK2* can be found in some families with the extremely cancer-prone Li-Fraumeni syndrome[85], and *CHEK2* variants predispose individuals to breast and prostate cancer[29], *CHEK2* seems to be a complex tumour suppressor gene.

From BRCA to p53 to cancer

The inheritance of a single mutated allele of either *BRCA1* or *BRCA2* markedly increases the incidence of breast and ovarian cancers in women[86]. As the tumours from these individuals virtually always lose the second allele, both *BRCA* genes conform to the classic pattern of tumour suppressor genes[87]. It is now clear that both *BRCA* gene products participate in cellular responses to DNA damage, but they seem to have distinct roles. As described above, BRCA1 is a target of the ATM, ATR and CHK2 kinases and is required for cell-cycle checkpoint responses in S phase and G2/M (ref. 69). BRCA1 also localizes to sites of DNA breakage, interacts with chromatin remodelling proteins and has been implicated in transcriptional control[87]. Which of these, or other suggested functions of BRCA1, are critical for tumour suppression and which explain the relative specificity for breast and ovarian cancers associated with its mutation remain to be clarified. Mouse models have suggested complex answers to this question. Because bi-allelic disruption of *Brca1* in the mouse results in embryonic lethality, tissue targeting and conditional disruptions have been used to assess the function of Brca1 (ref. 88). Increased

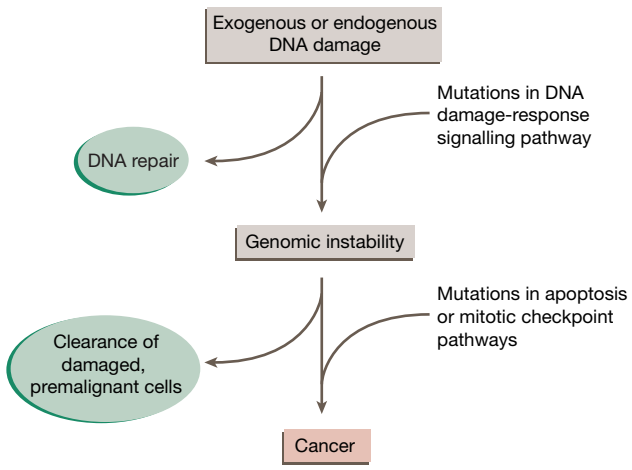

Figure 4 Schematic representation of two main steps that contribute to a spectrum of mutations leading to cancer development. If DNA damage is repaired efficiently, the likelihood of tumour development is low. If cells have mutations in DNA-damage-response signalling pathways — either sporadic or inherited — this will lead to enhanced genomic abnormalities. Cells with damaged DNA frequently arrest or do not survive, thus reducing the probability that they will progress to malignancy. Mutations in apoptosis pathways, DNA-damage, DNA-repair or mitotic-checkpoint pathways can permit the survival or continued growth of cells with genomic abnormalities, thus enhancing the likelihood of malignant transformation.

mammary and lymphoma carcinogenesis is seen in combination with p53 disruption. This suggests that p53-mediated apoptosis normally eliminates cells with enhanced DNA damage associated with *Brca1* disruption[89]. Disruption of *Chk2* is less potent at enhancing the Brca1 effects than disruption of p53, suggesting that some of the p53 tumour suppressor functions are retained in the absence of Chk2 (ref. 90).

BRCA2 binds directly to the RAD51 recombinase and has been linked to the S-phase checkpoint and to homologous recombination functions[91]. A direct link between BRCA2 and the cancer-prone Fanconi's anaemia syndrome arose when patients with the Fanconi's D1 complementation group turned out to harbour biallelic hypomorphic mutations in the *BRCA2* gene[92]. In addition, BRCA1 and the Fanconi's D2 protein interact in DNA-damage signalling pathways (see section 'The S-phase checkpoint pathways' above). Although mice bearing mutations in the Fanconi's A or C genes show chromosome instability and defective germ-cell development, they do not spontaneously develop cancer[93]. In contrast, mice lacking the Fanconi's D2 gene and *Brca2* hypomorphic mice develop epithelial cancers, such as breast, ovarian and liver cancer. Although mice with heterozygous mutations in *Brca2* do not develop tumours at an increased frequency, mice with homozygous truncations of *Brca2* develop thymic lymphomas. Growth arrest and unstable chromosome structure induced by *Brca2* truncation are relieved when cell-cycle checkpoints that are responsive to mitotic spindle disruption are inactivated[94]. This suggests that inactivating mutations in mitotic checkpoint genes might cooperate with Brca2 deficiency in the pathogenesis of inherited breast cancer and potentially other diseases of chromosomal instability, such as Blooms syndrome or Fanconi anaemia. This concept of mutations that cooperate with checkpoint or repair defects to enhance tumour development is likely to be a recurring theme in future studies (Fig. 4).

The BRCA stories suggest that genetic instability caused by altered DNA-damage response pathways may not be sufficient to lead to cancer development, and that cooperating mutations must be present to facilitate continued growth or viability of pre-malignant cells. Similarly, mice bearing hypomorphic mutations in the *Mre11* genes show pronounced chromosomal instability but are not prone to malignancy[95]. However, tumour formation in these mice on a p53-heterozygote background is significantly enhanced, suggesting that the combination of genomic instability and cell-cycle checkpoint defects is a significant risk factor for tumour development. One recent report demonstrated that mice bearing a mutation in p53 that was defective in apoptosis, but retained some cell-cycle checkpoint function, was markedly less prone to tumour development than p53-null mice. This surprising result suggests that the growth arrest and chromosome stability functions of p53 provide tumour suppressor function even in the absence of its role in apoptosis[96]. Finally, the recent observation that ATR haploinsufficiency increases tumorigenesis on a background of mismatch repair deficiency[78] may presage a flurry of new insights into how heterozygous mutations, although seemingly innocuous on their own, can enhance tumour formation when present in certain combinations, such as those controlling checkpoint responses and repair abilities.

Future directions

Animal models and human-cancer-susceptibility syndromes will continue to teach us about the physiological roles of the genes and pathways involved in DNA-damage responses. Many questions remain, such as how the cross-talk between the signalling pathways discussed here, and the processes of DNA repair and apoptosis operate. As these pathways seem to be major determinants of cellular responses to the types of cytotoxic agent that we use to treat tumours, these insights may teach us new ways to more effectively treat tumours. Similarly, because these response pathways seem to be major protectors from cancer development, the study of these pathways could lead to effective and new approaches to the reduction of cancer development. In addition to the prevention of cancer and more effective treatment of malignancies, insights into the mechanisms involved in these response pathways may even shed light on the processes of aging and senescence. □

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