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Contribution of DNA repair and cell cycle checkpoint arrest to the maintenance of genomic stability

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Abstract.

DNA damage response mechanisms encompass pathways of DNA repair, cell cycle checkpoint arrest and apoptosis. Together, these mechanisms function to maintain genomic stability in the face of exogenous and endogenous DNA damage. ATM is activated in response to double strand breaks and initiates cell cycle checkpoint arrest. Recent studies in human fibroblasts have shown that ATM also regulates a mechanism of end-processing that is required for a component of double strand break repair. Human fibroblasts rarely undergo apoptosis after ionising radiation and, therefore, apoptosis is not considered in our review. The dual function of ATM raises the question as to how the two processes, DNA repair and checkpoint arrest, interplay to maintain genomic stability. In this review, we consider the impact of ATM's repair and checkpoint functions to the maintenance of genomic stability following irradiation in G2. We discuss evidence that ATM's repair function plays little role in the maintenance of genomic stability following exposure to ionising radiation. ATM's checkpoint function has a bigger impact on genomic stability but strikingly the two damage response pathways co-operate in a more than additive manner. In contrast, ATM's repair function is important for survival post irradiation.

Introduction.

Cells have evolved elaborate damage response mechanisms to maintain genome stability. During the progression to carcinogenesis, the damage response pathways frequently become down regulated which enhances the opportunity to generate genomic instability and unrestricted cell growth. The damage response mechanisms include processes of DNA

repair, cell cycle checkpoint arrest and apoptosis. A chromosomal translocation, which is the subject of this special issue, represents a particular form of chromosomal instability that is likely to be heritable since it has the potential to escape recognition by the damage response pathways. Most chromosomal translocations arise through aberrant processing of a DNA double strand break (DSB). In this article, we focus on two damage response processes, namely DSB repair and DSB-induced cell cycle checkpoint arrest, that play critical roles in preventing chromosomal instability caused by the induction of DSBs. We briefly overview the two damage response processes and discuss our recent findings aimed at examining the interplay between the two processes.

Overview of radiation-induced DNA damage response mechanisms .

Homologous recombination (HR) and DNA non homologous end-joining (NHEJ) represent the two major DSB repair mechanisms in mammalian cells [1-3]. HR functions primarily to repair lesions at the replication fork, playing only a minor role in repairing DSBs that arise elsewhere in the genome, as suggested by the minor sensitivity of cell lines defective in HR to ionising radiation (IR). In contrast, NHEJ repairs DSBs at all cell cycle stages and mutants lacking NHEJ components are dramatically radiosensitive [4]. Since here we will consider chromosomal instability arising from DSBs induced by IR, our focus will be on NHEJ. Two DNA damage signal transduction responses have been characterised, which are initiated by two distinct but related phosphatidylinositol 3 (PI 3)-kinase like kinases (PIKKs) [5]. Ataxia telangiectasia mutated (ATM) is activated by the presence of DSBs whilst Ataxia telangiectasia and Rad3 related (ATR) is activated by single stranded regions of DNA. Once activated both kinases phosphorylate multiple overlapping substrates and

initiate overlapping although not identical damage responses [5]. These damage responses include aspects of DNA repair, cell cycle checkpoint arrest and apoptosis. Here, our focus lies on ATM since it is the major kinase activated by DSBs.

NHEJ.

Six core components of NHEJ, which assemble as two distinct complexes, have now been identified [1,2]. The DNA-dependent protein kinase complex (DNA-PK) encompasses the heterodimeric Ku protein with subunits, Ku70 and Ku80, and a large catalytic subunit, DNA-PKcs [6]. The Ku heterodimer has two pillars, a head and a base, with a central core that allows the passage of double stranded DNA [7]. Once Ku encircles double stranded DNA, it recruits DNA-PKcs resulting in activation of DNA-PK kinase activity. Although the role of the kinase activity remains to be fully elucidated, current evidence suggests that it regulates the process and facilitates processing of DNA ends (see below) [8]. The assembled DNA/DNA-PK complex recruits the second complex, which includes DNA ligase IV [9]. Until recently, this complex was thought to encompass two tightly associated proteins, DNA ligase IV and Xrcc4. Recently, however, a third protein, designated XLF or Cernunnos, which has homology to Xrcc4, has been identified and shown to co-associate with the DNA ligase IV/Xrcc4 complex [10,11]. These proteins represent the core NHEJ proteins and loss of any of them confers marked radiosensitivity and defective DSB rejoining. Loss of DNA-PKcs has a less dramatic impact on DSB rejoining compared to loss of the other core components and we, therefore, consider it to be a facilitating but non-essential NHEJ component [12]. In support of such a role, some species including *S. cerevisiae* or *Schizosaccharomyces pombe*, carry out NHEJ efficiently despite lacking a

DNA-PKcs homologue. DNA-PKcs does not simply represent a late evolutionary addition to NHEJ, however, as homologues have been found in arthopods [13].

Most DSBs rarely arise exogenously or endogenously as 5'P and 3'OH ends, the prerequisite for ligation by all known DNA ligases. Thus, the majority of DNA ends must undergo end-processing prior to ligation. Roles for polynucleotide kinase (PNK), DNA polymerase μ and DNA polymerase λ in end-processing have been reported [14,15]. More recently, Artemis has been reported to play a role in modifying a subset of DNA ends prior to ligation by NHEJ [12]. Artemis is a member of the β-lactamase superfamily and has 5' to 3' exonuclease activity. In the presence of DNA-PKcs, Artemis can also function as a 5' and 3' endonuclease and can cleave hairpin junctions [16]. In distinction to cells lacking core NHEJ components, Artemis-defective cells rejoin the majority of IR-induced DSBs normally but fail to rejoin approximately 10 % of X-ray induced DSBs [12] (Fig 1). Interestingly, the Artemis-dependent DSBs are those rejoined with slow kinetics in normal cells**.** Significantly, Artemis is dispensable for rejoining DSBs induced by etoposide, a topoisomerase II inhibiting anti-cancer drug. Since etoposide-induced DSBs are unlikely to have associated base and sugar damage, it has been proposed that Artemis functions to process a subset of DNA ends, although the precise structure of these ends remains to be determined [17]. Intriguingly, Artemis-dependent DSB rejoining also requires ATM, the Mre11/Rad50/Nbs1 (MRN) complex, 53BP1 and H2AX [12]. These findings demonstrate an interplay between NHEJ and ATM-dependent signalling.

Cell cycle checkpoint arrest.

Damage response checkpoints have been identified at the G1/S and G2/M boundaries as well as during S phase and potentially in mitosis [18]. ATM and ATR are upstream activators of damage-inducible checkpoint arrest [19]. The prevailing evidence suggests that in response to a DSB, ATM either directly or via Chk2 phosphorylates p53, which transcriptionally activates the Cdk inhibitor, p21, which serves to prevent entry from G1 into S phase [18]. G2/M arrest functions via the transducer kinases, Chk1 and Chk2. There is strong evidence that ATM and ATR primarily phosphorylate Chk2 and Chk1, respectively. However, the precise overlap between the PIKKs and transducer kinases is still unclear. The Cdc25 phosphatases, which are required to remove inhibitory Cdk phosphorylation and hence promote progression from G2 into mitosis, are phosphorylation targets of Chk1 and Chk2. The precise mechanisms whereby phosphorylation regulates the activity of the phosphatases are currently unclear but include inhibition of activity and ubiquitin mediated degradation [18].

Apoptosis.

Two pathways of apoptosis have been described, mitochondria-mediated apoptosis and a process initiated by receptor signalling. The onset of apoptosis after DNA damage is highly cell type dependent with some cell types having a low threshold for activation and others rarely undergoing apoptosis. In this chapter, we will mainly consider the response of primary human fibroblasts, which rarely undergo apoptosis after exposure to IR, but instead undergo permanent cell cycle arrest. Thus, we will neither consider the process of apoptosis, nor its impact to any extent.

The role of ATM in DNA damage response processes after exposure to IR.

Ataxia telangiectasia (A-T) is a human disorder conferred by mutations in ATM [20]. A-T cell lines have long been known to be defective in IR-induced cell cycle checkpoint arrest, failing to induce G1/S phase arrest, intra-S phase arrest, which confers the well known radioresistant DNA synthesis phenotype, and G2/M phase arrest [19]. More recently, A-T cells have also been shown to display a DSB repair defect identical to that shown by Artemis-defective cells [12,21]. Epistasis-like analysis using an ATM inhibiting drug has demonstrated that ATM and Artemis lie in a common pathway for DSB repair. Additionally, Artemis is an ATM phosphorylation target [12,22-24]. The model proposed is that ATM is required for Artemis-dependent end-processing and hence Artemis-dependent DSB repair [12]. Thus, ATM has dual functions in the response to DSBs, activation of cell cycle checkpoint arrest and activation of an end-processing mechanism required for a component of DSB repair [25]. Intriguingly, as mentioned above, the DSBs that are rejoined in an ATM/Artemis-dependent manner are those rejoined with slow kinetics in control cells. Thus, those DSBs that require ATM for rejoining are those that gain most benefit from ATM-dependent checkpoint arrest. Here, we discuss the contribution of these two distinct ATM regulated DNA damage responses to the maintenance of chromosomal stability.

Checkpoint arrest and DNA repair co-operate in a more than additive manner to maintain chromosome stability.

Checkpoint arrest after DNA damage has two potential impacts. It allows additional time for repair to take place before cell cycle progression and it can permanently prevent

proliferation of severely damaged cells. Thus, loss of checkpoints enhances chromosome aberration formation (breaks per cell) in mitotic cells consistent with the notion that checkpoints serve to prevent cells with an excessive level of damage from entering mitosis[26]**.** Although one study has suggested that Artemis-defective cells fail to maintain arrest at the G2/M checkpoint after IR [23], we have observed Artemis-defective cells to be checkpoint proficient and indeed to maintain a prolonged G2/M arrest after IR, compared to control cells, consistent with their DSB repair defect (Deckbar, manuscript submitted). Thus, we argue that Artemis represents a cell line defective in ATM-dependent DSB repair but proficient for ATM-dependent cell cycle checkpoint arrest. Although Chk2 is phosphorylated by ATM in response to DSBs, we and others have observed that Chk2-deficient cells effect G2/M checkpoint arrest proficiently after IR (although they have been reported in other studies to be defective in the maintenance of this arrest) [27,28](Deckbar, manuscript submitted) . The failure of Chk2-deficient cells to arrest is most likely due to overlapping functions of Chk1 and Chk2 [29]. Consistent with this notion, treatment of cells irradiated in G2 with a Chk1/Chk2 inhibiting drug abrogated ATM's G2/M checkpoint arrest but had no impact on ATM's repair function, thereby providing a repair proficient but checkpoint-defective situation. Using these tools, we recently undertook a study to examine the impact of ATM's repair versus its checkpoint function on the maintenance of chromosome stability following irradiation in G2 (Deckbar, manuscript submitted). We examined chromosome breaks as a monitor of chromosome instability. We found that following irradiation in G2 (and using conditions that prevented S phase cells progressing to G2 or mitosis), the number of chromosome breaks per cell was elevated to similar extents in cells lacking either ATM's repair or checkpoint function. Abrogation of

both, either by examination of A-T cell lines or by treatment of Artemis cells with the Chk1/Chk2 inhibiting drug resulted, as might be anticipated, in a further increase in chromosome aberrations per cell. However, the very impact of checkpoint arrest results in fewer cells reaching mitosis. Thus, we argued that simply estimating the number of chromosome breaks per cell underestimates the impact of checkpoint arrest. We, therefore, attempted to estimate the total number of mitotic chromosome breaks by considering the number of mitotic cells in addition to the number of chromosome breaks per cell. Firstly, we estimated the number of cells entering mitosis (ie the magnitude of checkpoint arrest) by FACs analysis. We then estimated the total number of mitotic breaks based on the estimated number of mitotic cells times the number of breaks per cell. When the ability of checkpoints to prevent mitotic entry was considered, we found that Artemis-defective cells had no elevated chromosome instability relative to control cells whereas A-T cells have a level of instability at least ten fold greater. In other words, although the repair defect has the potential to lead to elevated chromosome breaks, checkpoint arrest efficiently prevents this taking place (Fig 2).When the checkpoint inhibiting drug was added to control or Artemis-defective cells, enhanced total chromosome aberrations were observed, partially for control cells and to a level similar to A-T cells for Artemis-deficient cells. Thus, loss of checkpoint arrest has a greater impact on repair-defective cells relative to repair proficient cells. Thus, the dual impact of ATM's repair and checkpoint functions is greater than the sum of the two individual impacts (Fig 2). We conclude that ATM's repair and checkpoint functions act synergistically to maintain chromosome stability following irradiation in G2. Moreover, our findings show, perhaps surprisingly, that loss of ATM's repair function alone makes little contribution to genomic instability.

Survival represents another important end point following exposure to IR. Previously, we showed that following irradiation in G0 phase, Artemis- and ATM-defective cells show similar marked radiosensitivity [12]. We also examined survival of exponentially growing cells, which we estimate contain approximately 20 % G2 phase cells. Since the survival curves provide no evidence for a resistant component, we conclude that G2 cells display similar radiosensitivity to G1 cells. Importantly, under these conditions, Artemis-defective cells display marked radiosensitivity, demonstrating that ATM's repair function contributes markedly to survival (Deckbar, manuscript submitted).

To summarise, our findings suggest that ATM's repair function makes little contribution to chromosome stability but has a significant impact on survival post IR. In contrast, ATM's checkpoint function provides significant protection against chromosome instability. We have been unable to monitor the impact on survival since abrogation of Chk1 causes cell lethality but the similar survival level of ATM- and Artemis-defective cells suggests that checkpoints do not appreciably contribute to radioresistance at least in a repair-deficient background. Strikingly, however, loss of both damage response processes causes a more than additive impact on chromosome instability. To date, these studies have only examined the impact of repair in G2 and the G2/M checkpoint. Although the relative contribution of ATM's repair versus checkpoint function may differ slightly following irradiation in G1, we anticipate that a similar synergistic effect will be observed.

Other studies examining the interplay between damage response pathways.

Mouse studies have provided an important and complementary approach to examine interactions between damage response pathways. Importantly, mouse studies allow an

analysis of cancer frequency, which is clearly an important endpoint. However, a limitation is that mouse studies do not allow a precise dissection and separation of distinct endpoints such as chromosomal instability and survival. The majority of multiple pathway analysis using mice has focused on genetic crosses involving p53 (see for example [30-34]). Since p53 has critical roles in apoptosis and cell cycle checkpoint arrest, the interplay between defined pathways cannot be established. Nonetheless, these studies have demonstrated that joint defects in DNA repair pathways and p53 confer elevated tumour incidence. Of relevance is the finding of pronounced tumour elevation when defects in p53 are combined with defects in any of the NHEJ proteins, including Artemis. Indeed, Artemis knock out mice display only a minor elevated cancer incidence, which is increased dramatically in a p53 knock out background [34]. Our findings, therefore, are consistent with the mouse studies, namely that DNA repair defects only confer mild genomic instability which is synergistic with checkpoint and/or apoptotic defects. Interestingly, cells from Artemis knock out mice were reported to display genomic instability monitored as chromosome aberrations per mitotic cell but clearly such cells did not progress to enhance tumour frequency dramatically, consistent with the notion that they are frequently removed by cell cycle checkpoint arrest and/or apoptosis [34].

Clinical Significance.

A-T is a disorder characterised by pronounced cancer predisposition [20]. Additionally, ATM mutations have been observed in tumours [35,36]. Additionally, a subset of Li Fraumeni patients, who also display pronounced cancer predisposition, have mutations in Chk2 [37]. Artemis is defective in RS-SCID1, a subset of severe combined

immunodeficiency patients whose cells display radiosensitivity [38]. A considerable number of Artemis-defective patients have now received bone marrow transplants, and subsequently are able to lead healthy lives. There is no reported cancer predisposition in such patients (NB Artemis patients with hypomorphic mutations show elevated frequency of EBV-associated tumours but this may be a consequence of their immune deficiency rather than elevated genomic instability) [39]. Although this analysis of patients has many limitations, the lack of marked cancer predisposition in Artemis patients is consistent with the lack of elevated chromosomal instability suggested from our cellular studies. Further studies, will, however, be required to extend the significance of our cellular studies.

Our studies may also be important for drug targeting. The development of drugs that target DSB repair pathways, particularly when used in conjunction with radiotherapy, has the potential to inhibit tumour growth with minimal genomic instability. Targets aimed at NHEJ components may, therefore, be more effective than targets aimed at ATM.

Relevance to mechanisms generating chromosomal translocations.

Here, we have discussed the interplay between ATM's DSB repair and cell cycle checkpoint functions in generating chromosomal instability and survival. Chromosomal translocations are a particular form of chromosomal instability that may escape checkpoint arrest particularly if the translocations are balanced. However, most translocations arise concomitantly with acentric chromosome fragments, and thus our studies on chromosome instability will likely provide general rules for the generation of chromosomal translocations. Additionally, translocations involve some form of aberrant DNA repair and to date, our studies have focused on the interplay between ablated repair and checkpoint

functions. Future studies will need to evaluate the impact of hypomorphic mutations that may promote slow but inaccurate repair. Furthermore studies with mutants lacking Ku and other NHEJ components have demonstrated an increase in chromosome rearrangements, suggesting that DSBs that remain unrepaired by NHEJ may undergo aberrant forms of rejoining including telomere-break fusion events and aberrant homologous recombination [40]. Subsequent studies will be needed to address how such events contribute to the generation of translocations.

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Figure Legends.

Figure 1. Damage Responses induced by different classes of DNA DSBs.

DSBs induce cell cycle checkpoint arrest and DSB repair. Checkpoint arrest by DSBs is ATM and Chk1/2 dependent. DSB repair is dependent upon NHEJ proteins and additional factors as indicated in the figure, depending upon the nature of the DSB. The most simple DSBs have 3'OH and 5'P ends, which is the prerequisite for ligation. Slightly more complex DSBs may have 3'P and 5'OH ends that require simple processing (most likely by PNK) prior to ligation. Other ends may be associated with sugar and base damage. Finally, at the furthest extreme, some radiation-induced DSBs are highly complex with multiple associated lesions including DSBs, SSBs and base damage in close proximity. The core NHEJ components alone (Ku, DNA-PKcs, Xrcc4, DNA ligase IV and XLF) are required to repair ~ 90 % of the DSBs. Additional components, as shown in the figure, are required to repair ~ 10 % of radiation-induced DSBs. The precise nature of the DNA end that requires the additional proteins and whether it represents a highly complex DSB or a specific class of DSBs is currently unclear. Thus, ATM regulates two independent functions in the response to DNA damage; Artemis deficiency solely results in a repair defect; abrogation of Chk1/2 causes uniquely a checkpoint defect.

Figure 2. Impact of DNA repair and cell cycle checkpoint arrest on survival and genomic instability after irradiation.

The figure focuses on the impact of irradiation in G2. The frequency of total mitotic chromosome breaks is taken as a monitor of genomic instability. Wild type cells are repair

and checkpoint proficient. Most cells arrest at the G2/M checkpoint until repair is completed. 2-8 h following exposure to 1 Gy, the few metaphases present have low numbers of chromosome break. Abrogation of Chk1/2 does not impact upon DNA repair. However, many cells progress to mitosis before repair has been completed. Thus, there are many metaphases with a slightly elevated number of chromosome breaks per metaphase compared to control cells and the total number of chromosome breaks is moderately increased. Artemis deficiency impairs DSB repair but efficient checkpoint arrest prevents cells entering mitosis. Thus, there are very few metaphases albeit with elevated chromosome breaks but the total number of chromosome breaks remains low. Defects in ATM abrogate both DNA repair and checkpoint arrest. Thus, many cells with unrepaired DSBs enter mitosis causing a big increase in total chromosome breaks. The impact on survival is also shown. It is currently difficult to assess the impact of checkpoint abrogation on survival because Chk1 is required to stabilise replication forks. However, since ATM- and Artemis-deficient cells display similar radiosensitivity, we suggest that checkpoints have only a small impact on survival at least in a repair-defective background. The impact may be more manifest in a repair proficient background, however.

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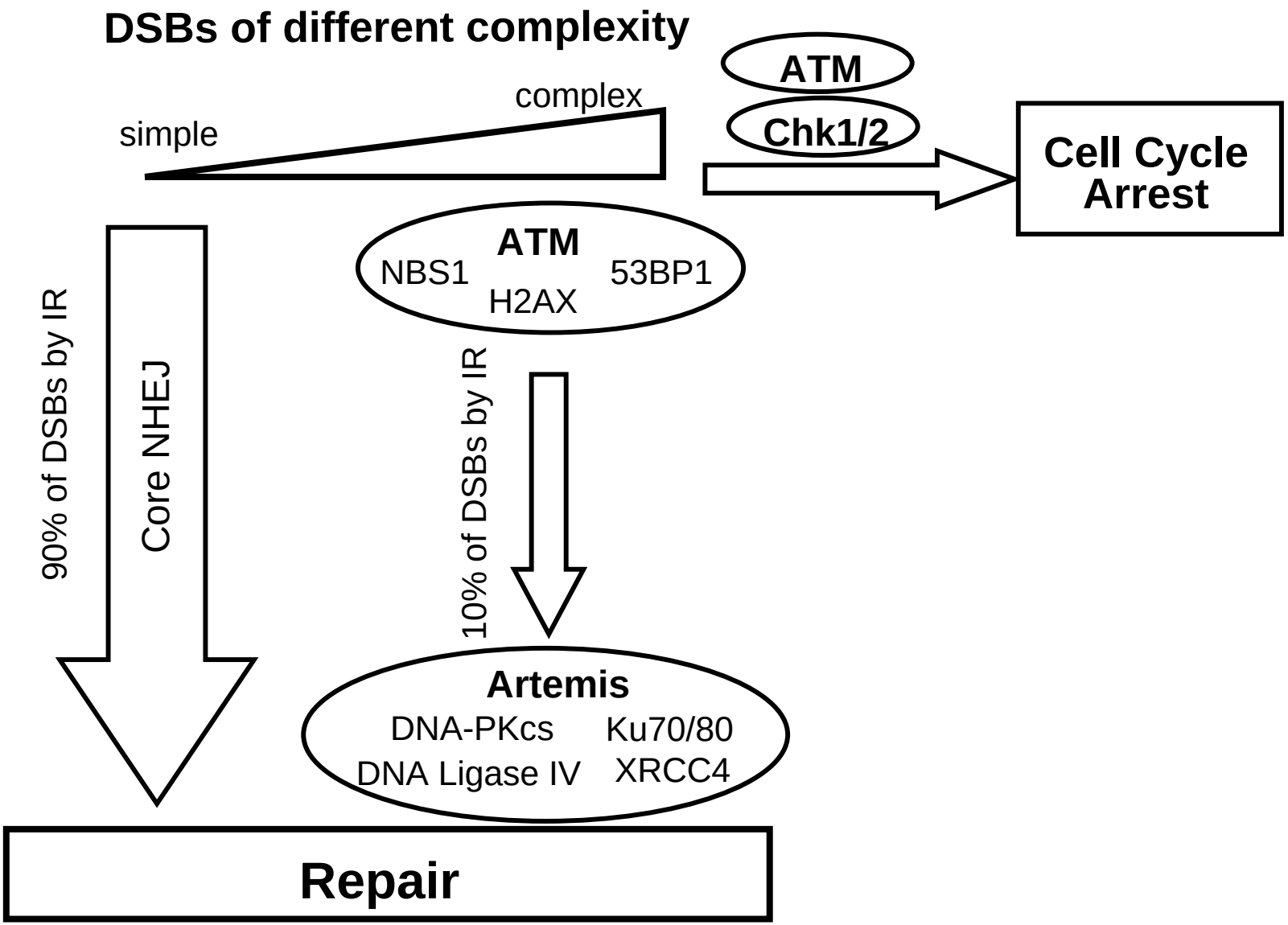
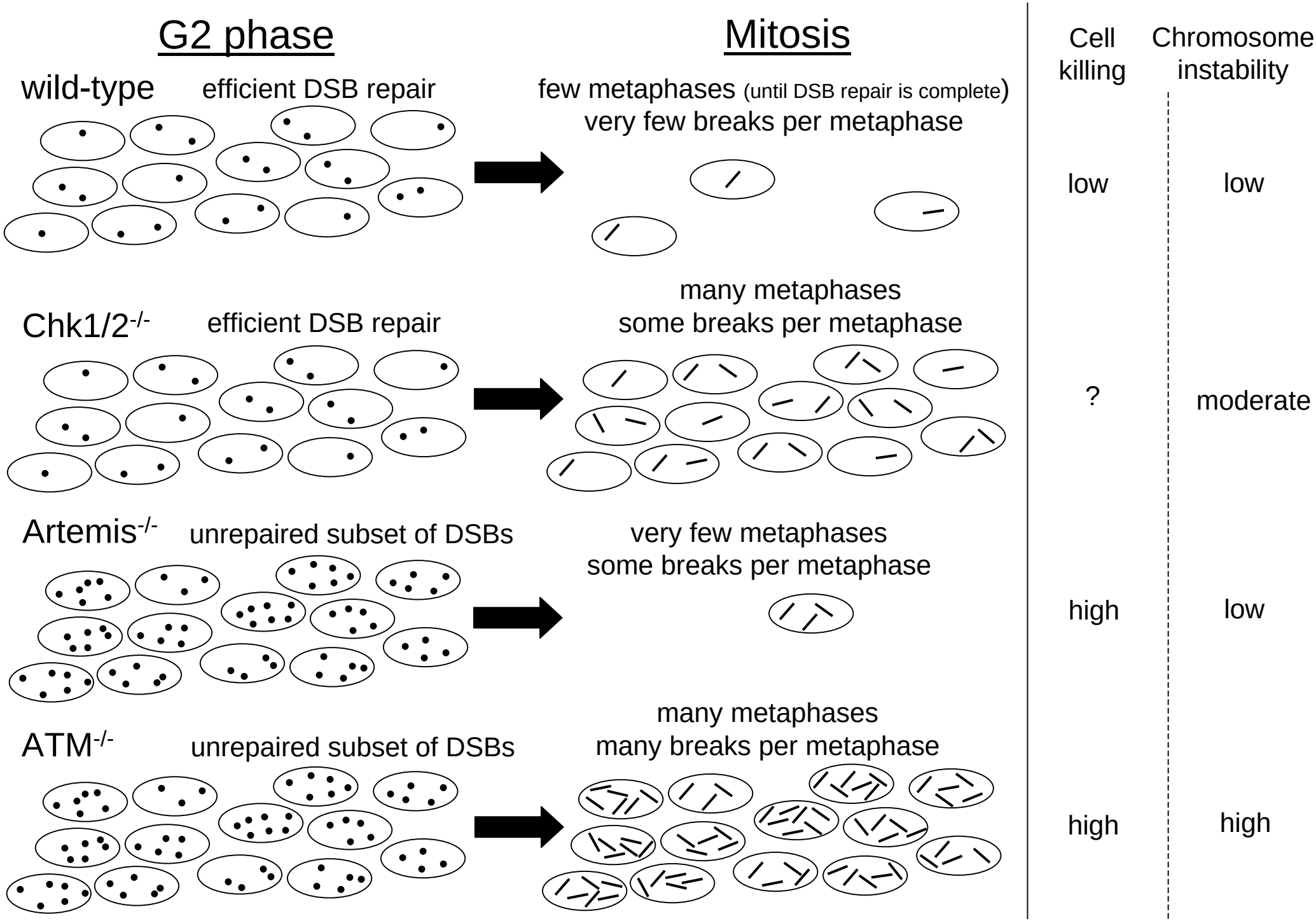

DSBs of different complexity
simple
complex
ATM
Chk1/2
Cell Cycle Arrest
ATM
NBS1
53BP1
H2AX
90% of DSBs by IR
Core NHEJ
10% of DSBs by IR
Artemis
DNA-PKcs
Ku70/80
DNA Ligase IV
XRCC4
Repair


G2 phase
Mitosis
Cell killing
Chromosome instability
wild-type
efficient DSB repair
few metaphases (until DSB repair is complete)
very few breaks per metaphase
low
low
Chk1/2-/-
efficient DSB repair
many metaphases
some breaks per metaphase
?
moderate
Artemis-/-
unrepaired subset of DSBs
very few metaphases
some breaks per metaphase
high
low
ATM-/-
unrepaired subset of DSBs
many metaphases
many breaks per metaphase
high
high