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Abbreviations used (footnote):

A-T, ataxia telangiectasia; ATLD, A-T like disorder; Alt-NHEJ, Alternative non-homologous end-joining; ATM, ataxia telangiectasia mutated; BRCA1, breast cancer type 1 susceptibility protein; BRCA2, breast cancer type 2 susceptibility protein; CDK, cyclin-dependent kinase; c-NHEJ, canonical NHEJ; CML, chronic myeloid leukaemia; DDR, DNA damage response; DSB, Double-strand break; EXOI; exonuclease I; HR, homologous recombination; IR, ionising radiation; MMEJ, micro-homology mediated end-joining; MRN, MRE11, RAD50, NBS1 complex; NHEJ, non-homologous end-joining; PALB2, partner and localiser of BRCA2; PLK3, polo-like kinase 3; RPA, replication protein A; WRN, Werner's Syndrome Protein; PolΘ, DNA polymerase theta.

Abstract.

DNA double-strand breaks (DSBs) are a significant threat to the viability of a normal cell, since they can result in loss of genetic material if mitosis or replication is attempted in their presence. Consequently, evolutionary pressure has resulted in multiple pathways and responses to enable DSBs to be repaired efficiently and faithfully. Cancer cells, which are under pressure to gain genomic instability, have a striking ability to avoid the elegant mechanisms by which normal cells maintain genomic stability. Current models suggest that in normal cells DSB repair occurs in a hierarchical manner that promotes rapid and efficient rejoining first, with the utilisation of additional steps or pathways of diminished accuracy if rejoining is unsuccessful or delayed. We evaluate the fidelity of DSB repair pathways and discuss how cancer cells promote the utilisation of less accurate processes. Homologous recombination serves to promote accuracy and stability during replication, providing a battlefield for cancer to gain instability. Non-homologous end-joining, a major DSB repair pathway in mammalian cells, usually operates with high fidelity and only switches to less faithful modes if timely repair fails. The transition step is finely tuned and provides another point of attack during tumour progression. In addition to DSB repair, a DSB signalling response activates processes such as cell cycle checkpoint arrest, which enhance the possibility of accurate DSB repair. We will consider the ways by which cancers modify and accost these processes to gain genomic instability.

Introduction.

Exposure to DNA damaging agents, such as ultraviolet light or ionising radiation (IR), is a well-recognised initiating step for carcinogenesis. Progression from an initiated tumour cell to an aggressive and invasive cancer necessitates the evolution of cells with genomic instability. This is most commonly achieved by enhanced replication and/or oxidative stress coupled with the down-regulation of DNA damage response (DDR) processes, which enhances the potential for endogenous damage to undergo mis-repair and/or enables the survival of damaged cells. Such rapid changes in cancer cells have been proposed to resemble a mutator phenotype capable of adapting to their ever-changing micro-environment [1, 2]. DNA double-strand breaks (DSBs) represent the ultimate lesion arising directly or indirectly from replication and oxidative stress. Although DSBs are a lethal lesion if unrepaired, cells have several pathways that promote their repair or preclude the survival or cell cycle progression of cells harbouring DSBs. Although not always fully appreciated, a normal, untransformed cell has evolved to enable the repair of DSBs with a good degree of accuracy and to minimise inaccurate repair. However, error-prone mechanisms also exist. An emerging notion is that cancer cells can either up-regulate these error-prone DSB repair pathways or modify the normally accurate repair mechanisms to diminish the fidelity of repair. Here we will discuss how cancer cells alter the fidelity of DSB repair. We will first evaluate the fidelity of the DSB repair pathways and factors governing pathway usage. We will also consider other processes which influence the fidelity of DSB repair. Finally, we will overview the mutational changes in cancer cells that impact upon the fidelity of DSB repair.

DSB formation in the context of replication and the role of homologous recombination.

HR exerts its major role in promoting the recovery or repair of lesions that arise during replication and has a less significant role in the repair of non-replication associated DSBs, such as those arising following exposure to IR (Figure 1) [3]. Recovery from replication fork stalling can involve the process of translesion synthesis, which leads to mutation induction, but can also proceed in an error-free manner by the repair of single stranded DNA regions by HR. The process of fork reversal, which helps recovery from stalled forks, also involves some or all steps that function during HR. HR is as well important for repairing one-ended DSBs which arise when replication forks collapse or when the replication machinery encounters single-strand breaks that are formed during base excision repair [4]. Indeed, any attempt by DNA non-homologous end-joining (NHEJ) to repair a one-ended DSB will result in mis-rejoining and a significant function of HR is to prevent the usage of end-joining processes at a one-ended DSB. In contrast, NHEJ has its major role in the repair of two-ended DSBs which arise in a replication-independent manner with HR functioning only to repair a subset of such DSBs in late S/G2 phase [5, 6]. NHEJ-deficient mutants, therefore, display marked sensitivity to agents such as neocarzinostatin and IR, which efficiently induce non-replication associated DSBs. HR, in contrast to NHEJ, is essential for cell viability since replication fork stalling and/or one-ended DSB formation occurs during most cycles of replication, even in the absence of exogenous DNA damage.

The elegance of HR as a two-ended DSB repair process is that it exploits an undamaged homologous template to restore any sequence information lost at the site of the DSB [3, 7, 8]. In normal cells, the homologous chromosome (homologue) is not used as a template for HR, most likely because the lack of homology at intronic sequences poses a barrier to HR, which requires extensive regions of homology. Instead sister chromatids represent the major source of a homologous template driving HR [9]. Following

replication, sister chromatids are in close proximity enhancing the opportunity for the exploitation of recombinational repair. The process of HR has been well described and will not be discussed in detail here [3, 7]. In brief, HR involves 5' to 3' end-resection, the loading of replication protein A (RPA) onto single-stranded DNA tails, the replacement of RPA by RAD51 and invasion of the undamaged strand leading to D-loop and heteroduplex formation [10, 11]. Repair synthesis and branch migration can then take place prior to the resolution of Holliday junction intermediates [8]. DNA end-resection can involve CtIP, the MRE11/RAD50/NBS1 (MRN) complex, exonuclease I (EXOI) and/or BLM/DNA2 as well as the breast cancer type 1 susceptibility protein (BRCA1) [12]. Loading of RAD51 is promoted by breast cancer type 2 susceptibility protein (BRCA2), Partner and localizer of BRCA2 (PALB2), and RAD51 paralogs [13]. Proteins that promote sister chromatid cohesion also facilitate HR [14]. In summary, HR is a complex process, necessitating substantial changes to the DNA duplex and chromatin environment, and can consequently be affected by mutations or copy number alterations in multiple genes. It does, however, play an essential role in maintaining genomic stability during the complex task of replication.

Pathways of end-joining and their fidelity.
The major process that repairs DSBs that are distinct from replication is DNA NHEJ. Canonical NHEJ (c-NHEJ) usually represents an end-joining process that does not involve any resection of the DNA ends but rather promotes their rejoining following end-protection by rapid binding of the Ku heterodimer, the recruitment of the DNA-PK catalytic subunit (DNA-PKcs), and subsequent ligation by a complex involving DNA ligase IV, XRCC4 and XLF [15-17]. NHEJ is routinely described as an error-prone rejoining process. Whilst NHEJ does not encompass the elegant ability of HR to restore sequence information lost on both DNA strands, the complex DSBs that are associated with such sequence information loss may not arise frequently and c-NHEJ, by interfacing with glycosylases and base excision repair, has the capacity to accurately rejoin DSBs with associated base damage [18-21]. Sequence information lost on a single DNA strand can be reconstituted by "fill-in" polymerases. Recent studies have demonstrated that DNA ligase IV is unique amongst ligases in having the capacity to ligate across imperfect ends, such as 1-2 bp gaps or damaged termini [22]. The model proposed is that the NHEJ ligation complex organises the rejoining process to limit end-processing and errors [23]. Any errors introduced can be corrected post rejoining by base excision or mismatch repair. Importantly, such a process will restrict the possibility of translocation formation. The formation of filaments that encompass XRCC4 and XLF may function to promote the efficacy of this process [24]. Chromosome exchanges (as well as chromosome and chromatid breaks) are increased in cells lacking c-NHEJ proteins strongly suggesting that c-NHEJ has a good capacity to rejoin DNA ends correctly [25]. The dramatic radiosensitivity of c-NHEJ-deficient mutants attests also to the significant role played by c-NHEJ in enhancing survival, suggesting that there is good fidelity of rejoining or that the level of mis-rejoining is well tolerated by a mammalian cell [26].

Ku is highly abundant in human cells, has avid end binding capacity and can prevent the activity of exonucleases when bound at the DNA end, which empowers Ku with the capacity to protect DNA ends from resection [27]. Consequently, as will be discussed in further detail below, the available evidence suggests that in G2 cells, where HR can function, NHEJ acts as the pathway of first choice, consistent with the notion that Ku rapidly binds the DSB and resection, the initiating step for HR, only occurs if NHEJ does not ensue [28]. In S/G2 phase, resection is regulated by cyclin-dependent kinase (CDK) phosphorylation [10]. A recent study reported that resection can also occur in G0/G1 phase cells but is of shorter length and differently regulated [29]. One line of evidence suggesting that resection can arise

in G0/G1 is that polo-like kinase 3 (PLK3) can phosphorylate CtIP in a damage inducible manner in G0/G1 rather than CDK1/2, which drives resection in S/G2 phase [30]. Further, depletion or inhibition of PLK3 substantially impedes the repair of DSBs induced by alpha-particle irradiation, which are more complex than X-ray induced DSBs. Interestingly, PLK3 binds to CtIP phosphorylated at S327 via its polo box domain, and this step promotes the subsequent phosphorylation of CtIP at T847. This could reflect a similar situation to G2 phase where ataxia and telangiectasia mutated (ATM) has been reported to initiate CDK-dependent resection [28, 31]. Although this process has to date only been shown to function in DSB repair after alpha particle irradiation, it is required for around half of the X-ray induced translocations, notably those that arise with slow kinetics, demonstrating that the process does function after X-irradiation [29]. Although the precise nature of this process remains to be characterised, these findings provide evidence that end-joining can take place after limited resection. Of relevance here is the finding that the end-joining processes with or without resection causes similar levels of translocations even though the majority of DSBs is repaired by the process without resection. This provides strong evidence that the rejoining process with resection is more error-prone than the process without resection (Figure 2). This observation raises the possibility that tumour cells can gain inaccuracy of rejoining by either (or both) up-regulating the capacity to undergo resection in G1 phase or down-regulating non-resection mediated rejoining.

Experiments involving the rejoining of restriction enzyme induced DSBs as well as studies using chicken cells have also pointed to the existence of end-joining mechanisms involving micro-resection [32, 33]. Such micro-homology mediated end-joining (MMEJ) exploits small regions of homology. Deletions must arise during this process, although they could arise by cleavage of single-stranded DNA overhangs rather than end-resection. Although the pathway giving rise to MMEJ remains unclear, the process can occur in cells proficient for c-NHEJ proteins and thus could represent a form of resection-mediated, Ku-dependent NHEJ. Whether MMEJ is related to the PLK3-dependent resection process described above is presently unclear. Alternative NHEJ (Alt-NHEJ) represents another form of MMEJ where resection can be greater in length and larger regions of micro-homology are utilised [34]. Alt-NHEJ has been defined as a process requiring PARP1, XRCC1 and DNA ligase I or III, most likely presenting two single-strand break joining events [27, 35]. Alt-NHEJ is a highly error-prone form of end-joining characterised by high frequencies of large deletions, tracks of micro-homology at the rejoining site and can give rise to translocations in mouse cells, although it does not appear to play a major role in normal human cells [36, 37].

The impact of DNA damage response signalling on DSB repair.
Above we have focused on the inherent fidelity of the available DSB end-joining processes. However, studies over the last decade have revealed that a signalling response involving ATM activates changes to the chromatin around the DSB, and that this also substantially influences aspects of DSB repair, although the DDR signalling proteins are not essential for the process of NHEJ itself [38, 39]. Significantly, the available evidence suggests that the signalling proteins which assemble at the site of a DSB can influence both the level of unrepaired DSBs and the fidelity of DSB repair, although these could, of course, be related.

The DDR signalling response has been reviewed previously and only the points of relevance to the fidelity of repair will be discussed here [17, 38, 39]. Although Ku rapidly binds to double-stranded DNA ends, initiating repair by NHEJ, the signalling response is activated independently but in parallel, most likely by the binding of the MRN complex to the DSB end [40]. The NBS1 component of MRN recruits ATM and, together with MRE11, promotes the activation of its kinase activity [41]. ATM phosphorylates the histone variant, H2AX, resulting in recruitment of MDC1, which in turn interacts with MRN, thereby

promoting the further tethering of activated ATM [42]. In this way, the spreading of H2AX phosphorylation (γ-H2AX) occurs over large regions, extending the initial signal. MDC1 also recruits the ubiquitin ligases, RNF8 and RNF168, which together result in the formation of ubiquitin chains on H2A, as well as the ubiquitylation of other histones such as H4 [43]. Collectively, these phosphorylation and ubiquitylation modifications can impact upon the exposure of methylated histones and/or result in new histone methylation events. Critically, these epigenetic modifications at the DSB site lead to the recruitment of 53BP1, the most down-stream of the mediator proteins [43]. Of significance in the present context, 53BP1 likely via its binding partners, PTIP and RIF1, appears to promote c-NHEJ and inhibit HR by preventing resection [44]. One way this can be achieved is by recruiting Artemis, a PTIP-binding protein, to the break site [45]. For HR to take place in G2 phase cells, the barrier posed by 53BP1 is relieved by BRCA1, with the available evidence suggesting that BRCA1 repositions 53BP1 to the periphery of enlarged foci, allowing RPA and RAD51 foci to form in a core region devoid of 53BP1 [46, 47]. Indeed, loss of 53BP1 relieves the need for BRCA1 for HR [48, 49]. What remains currently unclear is whether 53BP1 and/or BRCA1 also exert functions in inhibiting/relieving the smaller level of resection that has been described to take place in a PLK3-dependent manner in G1 phase [29]. 53BP1 is a tumour suppressor and mediates both productive and mutagenic DSB repair in distinct ways dependent upon the situation [50, 51]. BRCA1 is essential for HR (to reposition 53BP1) but it also influences MMEJ, although its precise role in this process has not yet been defined [52].

The assembly of DDR signalling proteins at DSBs affects the chromatin environment around the DSB and the recruitment of proteins such as 53BP1 as described above. However, the process additionally results in enhancing the tethering of ATM at the DSB site. Whilst this is not essential for ATM activation, the process serves to amplify the ATM signal. Thus, cells lacking H2AX can activate ATM but fail to tether it at the DSB (i.e. foci of phosphorylated ATM are not observed). ATM activation regulates several responses including cell cycle checkpoint arrest and apoptosis. Indeed, consistent with the notion that the ATM signal is inefficiently amplified in the absence of the DDR signalling, cells lacking H2AX show diminished cell cycle checkpoint arrest at low doses, where the amplification of the signal is specifically required [53]. It is possible that the assembly of DDR proteins into foci are either required for ATM to efficiently contact and phosphorylate its substrates or that tethering ATM at the DSB enhances its activation.

Cell Cycle Checkpoint arrest and the fidelity of DSB repair.
Of relevance here, it is well appreciated that cell checkpoint arrest can substantially influence the fidelity of DSB repair. Indeed, loss of checkpoint arrest has a more significant impact on genomic stability than on radiosensitivity [54, 55, 56]. One route by which checkpoint arrest influences the accuracy of DSB repair is by preventing progression into the next cell cycle phase until repair is complete. If replication or mitosis occurs in the presence of DSBs, this will likely result in separation of the spatial proximity of the DSB ends, resulting in a diminished possibility for accurate rejoining. Interestingly, CDKs are active in S phase, potentially allowing resection to arise more avidly. Once replication has taken place, this allows for HR to be utilized but it is currently unclear how avid resection is prevented at DSBs in pre-replicated regions when a sister is not available. The manner by which checkpoints can influence genomic instability has been discussed previously and will not be discussed in depth here [55, 57].

How ATM influences the fidelity of DSB repair.

Ataxia telangiectasia (A-T), the syndrome caused by mutations in ATM, is one of the most radiosensitive human disorders known, yet the underlying basis was enigmatic for many years since the process of DSB repair appeared to take place normally [58]. Eventually, the light dawned that A-T represented a cell cycle checkpoint disorder [59]. With current knowledge, we now appreciate that ATM lies at the centre of the DSB signalling response and activates a plethora of responses, some of which are still poorly understood. Following IR exposure, A-T cells are characterised by increased levels of chromosome or chromatid aberrations for cells irradiated in G1 or G2 phase, respectively [58]. A major contribution to this phenotype is the role of ATM in activating checkpoint arrest (Figure 3). However, there is also evidence that ATM has a role in influencing the fidelity of repair even in non-cycling cells. One line of evidence comes from studies examining the formation of chromosome breaks or rearrangements in G0 cells using fusion with mitotic cells to promote premature chromosome condensation or by analysing chromosome-type aberrations in mitosis that were formed during G0/G1 phase [60-62]. The characteristic phenotype of A-T cells is an increased level of chromosome breaks and rearrangements. Perhaps surprisingly, the mechanism underlying increased chromosome rearrangements arising from loss of ATM remains unexplained but may relate to the failure of A-T cells to effect a proper signalling response. Another line of evidence comes from the study of antigen receptor diversity arising through assembly of TCR and Ig genes from V, D and J segments. ATM is not essential for steps during V(D)J recombination but it cooperates with the recombination activating gene (RAG) proteins, which induce the DSBs, to hold the ends in a post-cleavage complex [63]. Recent studies have shown that at the IgK loci, ATM can inhibit RAG expression to suppress further rearrangements, leading to a feature called allelic exclusion. In the absence of ATM, increased aberrant V-to-DJ rearrangements are observed [64, 65]. ATM deficiency also allows the survival of cells with unrejoined V(D)J junctions due to a failure to activate checkpoint arrest [63]. Interestingly, dicentric translocations can lead to oncogenic translocations via cycles of breakage bridge fusion [66].

Although ATM is dispensable for the repair of most IR-induced DSBs, it is essential for the repair of a subset of DSBs in G1 phase representing the slow component of DSB repair [67, 68]. The current model is that these represent DSBs located within regions of heterochromatin (HC), that compacted HC is a barrier to rapid NHEJ and that ATM phosphorylation promotes localised HC relaxation [17] (Figure 3). A critical observation in this context is that depletion of any one of several components critical for the chromatin superstructure can bypass the need for ATM for DSB repair in G1 phase. The underlying mechanism involves the localised and concentrated phosphorylation of KAP-1 by ATM, and possibly CHK2 as well [69, 70]. Intriguingly, the nuclease Artemis is also required for HC-DSB repair although Artemis does not affect HC superstructure and the need for Artemis is not rescued by loss of HC components [71]. Thus, the slow component of DSB repair has distinct genetic requirements to the fast repair process, although both require the core NHEJ proteins. Given the requirement for Artemis, it is possible that the slow component represents a process involving end-processing or end-resection. Whether it is related to the PLK3-dependent process described above is currently unclear [29].

Finally, recent studies have shown that ATM, together with the PBAF chromatin remodelling complex (also termed SWI/SNF-B), has a role in silencing transcription in the vicinity of a DSB, and that a failure to activate this response delays the repair of a subset of DSBs at early time points [72, 73] (Figure 3). Transcriptional silencing and rapid early repair require an ATM phosphorylation site on BAF180, a PBAF subunit. Given that transcriptionally active regions have an open configuration and that there is strong evidence for transcription factories, it is possible that the silencing of transcription flanking DSBs involves chromatin compaction and that a failure to promote the required chromatin changes

leads to translocations or, at least, impairs the fidelity of repair. Further studies are required to understand whether ATM influences the fidelity of repair of DSBs within transcriptionally active regions.

Role of additional chromatin remodelling factors in DSB repair.
There is mounting evidence that chromatin remodelling complexes are required for aspects of DSB repair. The strongest evidence is the role of chromatin remodelling complexes during HR, which is a process that requires extensive unwinding of chromatin, engagement with the sister chromatid and potentially unwinding on the undamaged chromatid. In most instances, the recruitment of chromatin remodelling factors is ATM dependent and is thus encompassed within the DDR signalling response. This subject has also been reviewed previously and will not be discussed in further detail here [74-77].

Hierarchy of DSB repair pathway choice.
Above, we have discussed the DSB rejoining pathways as c-NHEJ, HR, Alt-NHEJ (defined as requiring PARP1, XRCC1 and Ligase I/III) and resection-mediated, Ku-dependent end-joining. MMEJ represents rejoining using micro-homology and can arise via Alt-NHEJ or resection-mediated, Ku-dependent NHEJ. It is also possible that end-joining could arise following end-resection without the use of micro-homology, either using fill-in or via blunt end-rejoining of resected ends. Nonetheless, rejoining following resection is likely to have a higher level of inaccuracy compared to c-NHEJ or HR. The question arises as to the mechanism underlying the choice between these distinct repair pathways. Most insight has been gained into the choice between the rejoining of two-ended DSBs by NHEJ versus HR. This has optimally been studied in G2 phase cells, where a sister chromatid is available for HR but where replication is not ongoing, avoiding analysis of the role of HR during replication. Current models are consistent with the notion that c-NHEJ is the pathway of first choice with resection and HR ensuing if c-NHEJ is not rapidly completed [12, 28]. The commitment to HR involves the initiation of resection, most likely upstream of the 5' DSB end, by MRE11 endonuclease activity and CtIP, a step that has the possibility to take place in the presence of Ku although it is unclear whether Ku is transiently released from the end [12, 78]. This is proceeded by the extension of the nick in both the 5' and 3' direction. 5' to 3' resection can be carried out redundantly by EXOI or DNA2/BLM. 3' to 5' resection is proposed to be effected by MRE11 exonuclease activity. Although it is unclear how Ku is removed from the DNA end, the beauty of this proposed mechanism is that it gives rise immediately to an extended 3' overhang which is the structure capable of preventing further binding of Ku. An important question is why c-NHEJ should be the pathway of first choice in G2 cells, when HR is such an elegant and potentially accurate repair pathway. The explanation potentially lies in the degree of chromatin remodelling required to promote HR, a process which involves extensive resection, engagement with the sister chromatid, repair synthesis and possibly branch migration. In contrast, NHEJ is a more compact process, likely requiring little chromatin remodelling. Given the importance of epigenetic modifications to mammalian cells and the likely difficulty of re-establishing the epigenetic code following histone eviction, it becomes unsurprising that c-NHEJ might be the preferred mechanism.

The next question is the mechanism underlying the regulation of pathway choice in G1 phase cells, where HR does not function. Notably, DSBs are repaired with fast and slow kinetics in G1 and G2 phase cells with the fast process repairing approximately 85% of the IR-induced DSBs in both cell cycle phases [5, 68]. In both phases, the fast process appears to represent c-NHEJ without resection. The slow process in G2 phase represents HR. Significantly, the slow process in G1 phase is genetically distinct (ie it does not involve specific HR proteins) but has similar, yet slightly faster, kinetics to the slow process in G2

phase [28]. This argues that the slow process in G1 phase is distinct to HR and also distinct to c-NHEJ, the fast process in G1 and G2. Notably, the slow process in G1 requires the nuclease, Artemis, raising the possibility that it also represents a resection-dependent pathway. This possibility argues that in G1 as in G2, the first choice for pathway usage represents resection-independent c-NHEJ, with the switch to a resection-dependent pathway taking place if repair by resection-independent c-NHEJ stalls. The precise nature of the resection-mediated pathway in G1 remains to be clarified and will not be further discussed.

It is noteworthy that a recent study examining the rejoining steps during c-NHEJ (representing fill-in of 1-2 nucleotide deletions) argued that the precise nature of the rejoining was determined by a hierarchy principle that favours the least number of necessary steps [22]. Similarly, the transition from a resection-independent to a resection-dependent pathway appears to be governed by a hierarchical choice, whereby the simplest and most accurate pathway is utilised first.

The potential benefit of modulating these pathways in cancer cells.
The selective pressure for a cancer cell to rapidly adapt to an ever-changing micro-environment without too dramatically compromising growth potential necessitates the change from a genomically stable normal cell to one with high genomic instability. An indirect consequence of the many changes that can enhance genomic instability, such as enhanced replicative or oxidative stress, is the enhanced formation of DSBs. An added benefit to a cancer cell may be gained if repair of these DSBs proceeds via processes that further promote genomic instability.

Cells that completely lack c-NHEJ are highly radiation sensitive and would be predicted to show compromised proliferation in an environment involving increased DSB formation. Thus, it is unlikely that cancer cells would benefit by fully inactivating c-NHEJ. Since PLK3-dependent resection in G1 phase appears to lead to increased translocations and both MMEJ and Alt-NHEJ lead to deletions and/or translocations, a switch to these pathways may be beneficial for a cancer cell (Figure 1) [29, 37, 79]. Enhancing the switch from c-NHEJ to HR may appear counter-productive for a cancer cell, given the notion that HR is a more accurate DSB repair process than c-NHEJ. However, as discussed above, there may be errors in reconstituting the epigenetic code following HR, a feature which may benefit a cancer cell. Moreover, resected DSBs can be repaired by Alt-NHEJ in G2 phase if HR is compromised or deregulated [80]. Thus, in G2 there may also be a benefit to enhancing the switch from preferential c-NHEJ usage to pathways involving enhanced resection.

As discussed above, cell cycle checkpoint arrest both promotes the accuracy of DSB repair and can permanently remove damaged cells from the cycling population. Down-regulation of cell cycle checkpoint arrest is essential for a cancer cell to achieve genomic instability and is an essential step leading from a pre-cancerous state to a proliferating cancer [81]. Although the driving selective pressure is likely to be to promote the proliferation of damaged cells, diminishing the accuracy of DSB repair will be an added benefit.

Finally, replication or oxidative stress is an essential feature by which cancer cells enhance genomic instability and down-regulation of HR is one mechanism by which genomic instability can be gained as a consequence of replication. In other words, down-regulation of HR represents a way to enhance replicative stress. Since enhanced replicative stress is arguably the major process elevating DNA damage in cancer cells, mutations which prevent the usage of HR or hijack the fidelity of the process occur frequently in cancer [82]. However, since HR is an essential process, modifications to HR in cancer are unlikely to be fully inactivating and likely to be accompanied by mutations that down-regulate other DDR surveillance mechanisms (such as apoptosis) or up-regulate alternative mechanisms of continuing replication (such as translesion synthesis or new origin firing).

Modulation of HR in cancer cells.
The first evidence that HR functions as a tumour suppressor process became apparent with the seminal findings that heterozygous mutations in BRCA1 and BRCA2 confer predisposition to breast, ovarian and pancreatic cancer coupled with the evidence that they function during HR [13]. Additionally, mutations in Partner and localiser of BRCA2, PALB2, a BRCA2 interacting protein, also confer breast cancer predisposition [83, 84]. Added to this is the evidence that mutations in genes encoding proteins required for HR are found in hereditary syndromes that display cancer predisposition. Indeed, homozygous mutations in BRCA2 and PALB2 can cause the cancer predisposition syndrome, Fanconi anaemia (FA) [13]. Although the FA pathway confers sensitivity to DNA cross-linking agents, there is a close interface with HR, and proteins such as BRCA2 function in both pathways. Similarly, disorders such as Blooms and Rothman-Thompson Syndrome have mutations in genes that function during HR and display cancer predisposition. Strikingly, mutations in HR genes are commonly found in tumours [85]. Indeed, the integrated genome analysis of ovarian cancer observed that the HR pathway is affected in around half of all such cancers [82].

A striking and significant example of a change in an HR protein observed across many cancers is the overexpression of RAD51 [86]. This particular example is significant because overexpression of RAD51 has been studied in detail and shown to impede normal error-free HR and promote aberrant recombination, including a single-strand annealing mechanism coupled to NHEJ, and the formation of chromosome translocations [86]. In other words, overexpression of RAD51 appears to confer a dominant negative phenotype. Indeed, the level of overexpression of RAD51 correlates in many instances with tumour grading, providing a prognostic marker [87-89]. This useful example demonstrates that it is not simply the loss of HR that occurs but rather hijacking of the process to generate a translocation or error-prone outcome.

Modulation of end-joining in cancer cells.
Several types of tumour cells have now been shown to down-regulate c-NHEJ and exploit the highly error-prone Alt-NHEJ pathway. Rassool and colleagues showed in 2002 that nuclear extracts from both cultured and primary human myeloid leukaemia cells, which are characterised by enhanced translocations and deletions, have an elevated capacity to effect end-ligation compared with peripheral blood lymphocytes from healthy donors [90]. Further, such cells showed enhanced mis-repair involving large deletions, providing strong evidence that end-joining in these cells has gained the capacity for resection and deletion formation. Although this process was initially thought to represent a form of c-NHEJ with diminished fidelity [91], the identification of the Alt-NHEJ pathway prompted an examination of this process. It turned out that key proteins that function in c-NHEJ, such as Artemis and DNA ligase IV, can be down-regulated whilst DNA ligase IIIα, an Alt-NHEJ protein, can be up-regulated in chronic myeloid leukaemia (CML) [92]. Moreover, depletion of DNA ligase IIIα in such cells resulted in the accumulation of unrepaired DSBs. Interestingly, Werner Syndrome Protein, WRN, also appeared to be involved in this process [92]. Significantly, the FMS-like tyrosine kinase 3 (FLT3) receptor, which is frequently expressed in acute myeloid leukaemia cells (AML), appears to regulate the switch from c-NHEJ to Alt-NHEJ and treatment of cells with an FLT3 inhibitor appears able to diminish DNA deletions, demonstrating that AML can also involve such a switch in repair pathway usage and possibly providing a route for treatment [93]. More recently, it has been shown that c-Myc, which is a target of the tyrosine kinases, BCR-ABL and FLT3-internal tandem duplication (FLT3-ITD), plays a significant role in the transcriptional activation and expression of DNA ligase IIIα

and PARP1, in part via the regulation of micro-RNAs [94]. More recent studies have provided evidence that oestrogen and progesterone receptor positive (ER/PR⁺) MCF7 breast cancer cells also display diminished levels of DNA ligase IV, enhanced levels of DNA ligase IIIα, and increased usage of Alt-NHEJ with deletion formation [95].

Another situation where DSB repair appears to have gained inaccuracy occurs in muscle invasive bladder cancer [96]. Whereas low grade non-invasive papillary bladder tumours are relatively genetically stable, muscle invasive bladder tumours show high frequencies of chromosomal aberrations. Repair assays carried out on extracts from bladder tumours of various grades revealed that whereas non-invasive bladder tumours repair DSBs with accuracy, cell extracts from muscle invasive bladder cancers rejoin DSBs in an error-prone manner, resulting in extensive resection and micro-homology usage. Interestingly, the muscle invasive tumours display reduced Ku DNA binding activity [97]. Provocatively, muscle invasive bladder tumours also display altered regulation of MRE11 expression, although it is unclear whether this correlates with alterations in resection and pathway usage [98].

A similar but somewhat distinct change that has recently been reported in cancer cells is the reciprocal down-regulation of HR with up-regulation of Alt-NHEJ [99, 100]. Central to this work is the finding that DNA polymerase theta (PolΘ; encoded by *Polq* in mice) is an error prone polymerase that functions during Alt-NHEJ [101, 102]. Consequently, depletion of PolΘ suppresses the usage of Alt-NHEJ. As mentioned above, down-regulation of HR is commonly observed in tumours and significantly, it was observed that HR-deficient tumours became up-regulated for PolΘ expression and Alt-NHEJ usage [99, 100]. It was also demonstrated that PolΘ can promote Alt-NHEJ at dysfunctional telomeres [100]. Collectively, these studies provide examples of the interplay between DSB rejoining pathways and how down-regulation of one pathway can lead to up-regulation of a pathway with compromised genomic stability.

Changes in DDR signalling proteins and chromatin modifying factors.
Human syndromes with mutations in the DDR signalling proteins, such as A-T, ATLD, Nijmegen Breakage Syndrome and RIDDLE syndrome, have been described. All these syndromes are characterised by cancer predisposition, attesting to the tumour suppressor function of the genes mutated in these syndromes. Additionally, ATM is down-regulated in a number of tumours, including CML, T-cell pro lymphoblastic leukaemia (T-PLL) and breast cancer [64, 103]. In the case of CML, ATM-deficient tumours have a severe prognosis. Other components required for the DDR are also frequently down-regulated or mutated in cancers (for example [98, 104, 105]). The difficulty in assessing the impact of these changes is that they likely affect multiple endpoints, including the activation of apoptosis and cell cycle checkpoint arrest. Indeed, how ATM maintains genomic stability by its dual function in the slow component of DSB repair coupled with regulating cell cycle checkpoint arrest has been discussed previously [106]. Thus, loss of DDR proteins is likely to have a synergistic impact on genomic instability due to the loss of interfacing DDR processes.

As mentioned above, chromatin remodelling proteins can also affect the outcome of DSB repair, particularly HR, and chromatin remodelling proteins are frequently mutated in cancer [76]. However, as discussed above for the DDR proteins, chromatin remodelling complexes have multiple functions. The mechanism by which the mutations confer enhanced genomic instability will, therefore, be complex to unravel and may arise as a consequence of combined impacts rather than by affecting a single pathway.

The translational potential of understanding these mechanisms.

The modulation of DSB repair pathway usage in tumour versus normal cells has the potential to be a target for therapy. It could also be exploited during radiotherapy, where DSBs are specifically induced. The ability to exploit the down-regulation of HR by the use of PARP inhibitors is arguably one of the most well rationalised anti-cancer treatments [107, 108]. The potential for exploiting the enhanced usage of Alt-NHEJ over NHEJ is already underway [95, 109]. The recent studies of the use of PolΘ in this process provide a further therapeutic avenue for therapy of tumours with down-regulated HR [99, 100]. However, a full understanding of how cancer cells modulate the fidelity of DSB repair requires a deeper understanding of the basis underlying DSB repair pathway usage. Although MMEJ is a well described process, the situations in which it is used and, indeed, the precise mechanism of rejoining that generates such micro-homology usage remains poorly understood. It appears that the initiation of resection is a critical step regulating pathway usage. It is likely that cancer cells enhance the transition to undergo resection but this has not yet been well examined.

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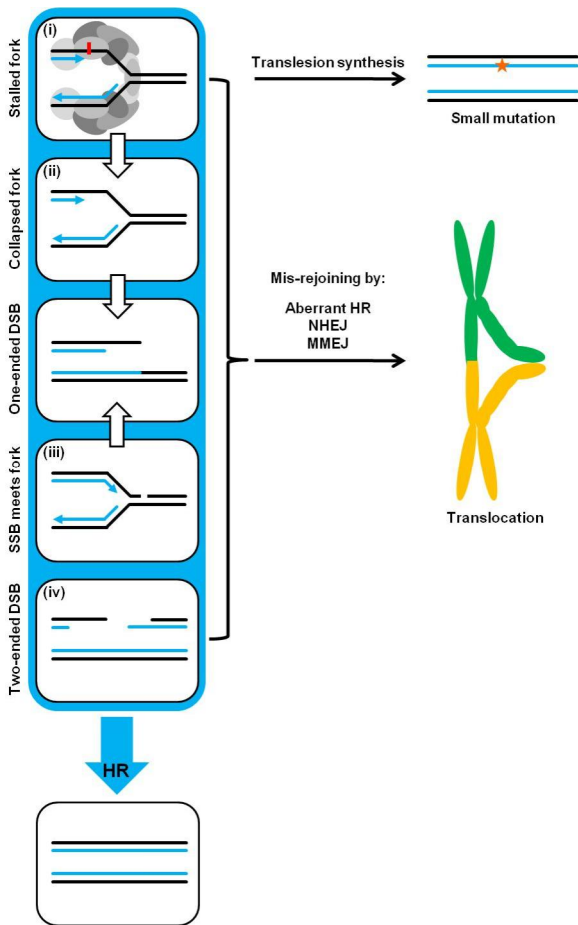
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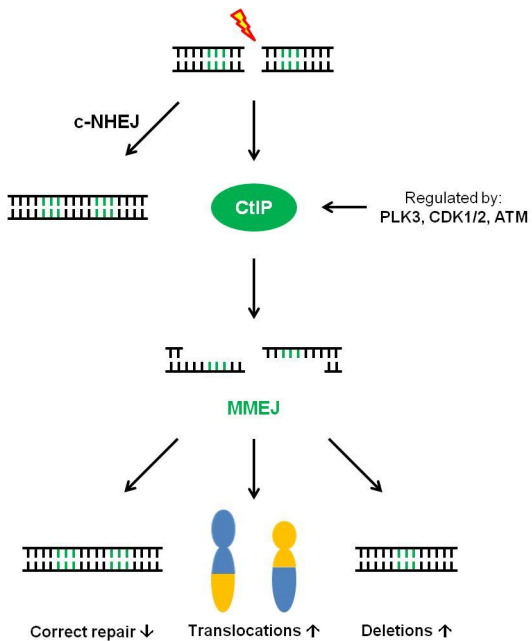
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Figure 1: HR represents a high-fidelity repair process. (i) Replication forks are transiently stalled at DNA lesions. The error-free recovery of such stalled forks by HR involves processes of fork regression/reversal or the post-replicative repair of single-stranded gaps arising at the DNA lesion. Alternatively, translesion synthesis (TLS) by error-prone polymerases can cause mutation induction. (ii) Prolonged replication fork stalling can lead to the disassembly of replication proteins, a process called replication fork collapse. The repair of collapsed replication forks typically involves the generation of a one-ended DSB. (iii) One-ended DSBs can also arise when replication forks encounter unrepaired SSBs either formed as repair intermediates during base excision repair or as a result of topoisomerase cleavage. (iv) Finally, a subset of two-ended DSBs induced by exogenous agents such as IR is also repaired by HR. If any of these events fail, translocations can arise from mis-rejoining by aberrant HR, NHEJ or MMEJ of the resected break ends.
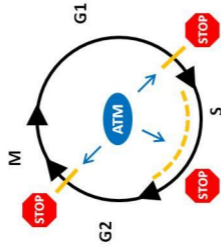
Figure 2: Resection prior to end-joining enhances levels of translocations and deletions. C-NHEJ is the major DSB repair pathway and usually rejoins ends with no resection. More complex DSBs which harbor associated base or sugar damage or DSBs in less accessible genomic regions can undergo some level of resection prior to repair by an end-joining process. The key factor initiating resection is CtIP, whose regulation involves cell cycle-specific and damage-inducible kinases. Since the resection process often exposes regions of micro-homology which facilitate the rejoining step, such processes have been termed MMEJ. In addition to enhanced deletion formation due to loss of the nucleotides between the regions of (micro-) homology, MMEJ is associated with increased levels of translocations. We define Alt-NHEJ as a specific class of MMEJ, which uses larger regions of micro-homology and has a considerable potential to cause translocations.

Figure 3: ATM regulates the fidelity of DSB repair via distinct functions. ATM has a major role in activating cell cycle checkpoints at the border between G1 and S phase, throughout S phase and at the transition between G2 phase and mitosis. A failure to efficiently activate the checkpoints will result in the progression of unrepaired DSBs in subsequent cell cycle phases which diminishes the fidelity of the repair process. ATM also regulates the repair of DSBs which localize to heterochromatic DNA regions. During this process, ATM phosphorylates the heterochromatin building factor Kap-1 which results in disassembly of the heterochromatic superstructure and allows DSB repair to ensue. Finally, ATM promotes the silencing of transcription in the vicinity of a DSB. This role also involves regulation of chromatin remodeling complexes and is required for the efficient repair DSBs in actively transcribed genomic regions.
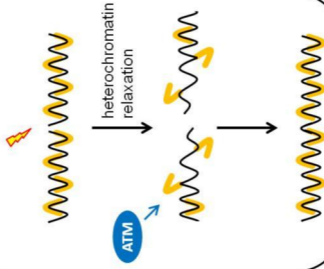
Stalled fork

(i)

Translesion synthesis

Small mutation

Collapsed fork

(ii)

One-ended DSB

Mis-rejoining by:

Aberrant HR
NHEJ
MMEJ

SSB meets fork

(iii)

Translocation

Two-ended DSB

(iv)

HR

c-NHEJ

CtIP

Regulated by:
PLK3, CDK1/2, ATM

MMEJ

Correct repair ↓   Translocations ↑   Deletions ↑

Cell cycle control

G1

S

G2

M

ATM

STOP

STOP

STOP

Heterochromatic DSB repair

heterochromatin relaxation

ATM

Transcription inhibition

active transcription

silenced transcription

ATM

STOP