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Leveraging the replication stress response to optimize cancer therapy

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Abstract

High-fidelity DNA replication is critical for the faithful transmission of genetic information to daughter cells. Following genotoxic stress, specialized DNA damage tolerance pathways are activated to ensure replication fork progression. These pathways include translesion DNA synthesis, template switching and repriming. In this Review, we describe how DNA damage tolerance pathways impact genome stability, their connection with tumorigenesis and their effects on cancer therapy response. We discuss recent findings that single-strand DNA gap accumulation impacts chemoresponse and explore a growing body of evidence that suggests that different DNA damage tolerance factors, including translesion synthesis polymerases, template switching proteins and enzymes affecting single-stranded DNA gaps, represent useful cancer targets. We further outline how the consequences of DNA damage tolerance mechanisms could inform the discovery of new biomarkers to refine cancer therapies.

High-fidelity DNA replication is constantly challenged by endogenous and exogenous sources of genotoxic stress[1] (BOX 1). Endogenous sources of genotoxic stress include abasic sites, improper incorporation of ribonucleotides into replicating DNA, DNA–protein crosslinks, transcription–replication conflicts[2], formation of DNA secondary structures, single-stranded DNA (ssDNA) gaps[3–8], nucleotide imbalances[9,10] and changes in origin firing frequency[11]. Exogenous sources of genotoxic stress include ionizing radiation and DNA-damaging chemotherapy such as alkylating agents, crosslinking drugs, topoisomerase inhibitors and antimetabolites[12] (FIG. 1). The transient slowing or aberrant acceleration of replication forks in response to these challenges is termed ‘replication stress’ and is tightly linked to cancer development[3,13–16].

As part of the replication stress response, cancer cells activate various DNA damage tolerance (DDT) pathways[17]. DDT pathways broadly include translesion DNA synthesis

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Author contributions

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(TLS)[18], template switching (TS)[19] and repriming[20] (FIG. 1). TLS involves specialized polymerases that can replicate through a damaged DNA template[21] and is generally regarded as a lower-fidelity form of DDT because the TLS polymerases recruited to stalled replication forks have a high potential for mutagenesis[18]. TS uses sister chromatid DNA to bypass replication obstacles[22] and, as a result, is less likely than TLS to introduce erroneous nucleotides. One version of TS is fork reversal, which promotes the remodelling of replication forks into four-way junction structures upon encountering DNA lesions[23]. Reversed fork remodelling enables the original lesion to be repositioned ahead of the replication fork junction, facilitating lesion removal before reversed fork restart or lesion bypass through a TS mechanism[24]. Finally, repriming in human cells involves a specialized polymerase-primase enzyme, DNA-directed primase/polymerase protein (PRIMPOL)[25–28], that skips damaged DNA, re-initiating synthesis beyond the lesion and leaving a ssDNA gap between the lesion and the point where synthesis restarts[20]. The ssDNA gap generated by PRIMPOL-mediated repriming can then be filled post-replicatively through TLS or TS[29–32].

The fine-tuning of different DDT mechanisms is an emerging determinant of tumorigenesis and cancer therapy response. Here, we review how the loss of DDT factors can confer an increased cancer risk as DDT proteins are critical for DNA replication in the presence of endogenous and exogenous replication stress. In addition, we describe how the aberrant expression, or indeed the normal function of DDT enzymes upon increased replication stress, can promote the genomic instability that drives cancer development and progression. Many tumours exhibit elevated endogenous replication stress; therefore, we discuss how these pathways can be exploited for cancer cell clearance. Finally, we frame replication stress response mechanisms in the context of current clinical cancer treatments and suggest possible opportunities for biomarker development.

DNA replication stress in tumorigenesis

Translesion DNA synthesis.

Eukaryotic TLS involves polymerases of the Y-family[33] — including REV1, Polη, Polι and Poκ — and the B-family (Polζ)[34]. These Y-family and B-family TLS polymerases lack 3′-to-5′ nucleotide proofreading and exhibit a decreased capacity to distinguish between incoming nucleotides relative to replicative polymerases[21]. As a result, they are more mutagenic than the polymerases that are part of the core replication complex, with error rates of up to 1 in every 10 nucleotides inserted, compared to errors rates as low as 1 in every 10^{10} bases for the replicative polymerases Polε and Polδ[18,35]. Mutagenic events induced by TLS polymerases can contribute to tumorigenesis[36] and impact the response of cancer cells to DNA-damaging chemotherapies[37], highlighting the importance of these mechanisms in the context of tumour treatment.

The recruitment of TLS polymerases to the DNA is mediated by the ubiquitination of proliferating cell nuclear antigen (PCNA), which is an essential processivity factor for replicative DNA polymerases also known as the DNA sliding clamp. PCNA is monoubiquitinated at lysine 164 by the E3 ligase enzyme RAD18 (REF.[38]) (FIG. 1), the activity of which is important for the replication of damaged DNA. RAD18 loss can contribute to genomic instability and increased sensitivity to DNA-damaging agents in non-

malignant mammalian cells[39,40]. However, RAD18-dependent PCNA ubiquitination also drives mutagenic TLS[41], promoting tumorigenesis. Indeed, the expression of RAD18 is high in a variety of cancer types and correlates with worsened survival outcomes[42–44] (TABLE 1). Moreover, RAD18 expression correlates with increased single nucleotide variations in human cancers from The Cancer Genome Atlas, and mutational signatures induced by RAD18 activity in mouse models correlate with mutational landscapes from the COSMIC database[41].

In addition to PCNA monoubiquitination, the Y-family TLS polymerase REV1 functions as a scaffold protein to facilitate the downstream recruitment of other TLS polymerases[45]. In normal cells, REV1 contributes to mitochondrial function[46] and somatic hypermutation[47]. REV1 loss is associated with metabolic dysfunction; a recent study suggested that this dysfunction might be due to the inability of *Rev1*-knockout mice to appropriately respond to endogenous replication stress[46]. Interestingly, REV1 can also function in base excision repair, resulting in a mutational signature enriched for C>G transversions[48], and is upregulated in hepatocarcinomas and select lung cancers[37,49] (TABLE 1).

The B-family polymerase Polζ is involved in the translesion synthesis of DNA adducts that stall replication forks and is composed of the catalytic subunit REV3L and the accessory subunit REV7 (REF.[34]). Loss of REV3L is associated with chromosomal instability[50] and spontaneous tumorigenesis in mouse models[51], suggesting that this TLS polymerase is important in DDT in non-malignant cells. Interestingly, the genomic instability observed in REV3L-deficient cells activates an innate immunity-like response involving upregulation of the cGAS–STING pathway and increased micronuclei formation[52]. When functional, Polζ tends to introduce dinucleotide mutations, with strong preferences for GC>AA or GC>TT mutations[53] (TABLE 1). Several single-nucleotide polymorphisms (SNPs) in *REV3L* have been linked to an increased risk of developing lung cancer in a specific Han Chinese population[54]. Divergent REV3L expression has been reported across different tumour types: REV3L expression is elevated in gliomas[55] and oesophageal squamous cell carcinomas[56,57] but appears downregulated in select colorectal, lung and gastric cancer tissues relative to non-cancer controls[54,58,59] (TABLE 1).

Germline mutations in the Y-family TLS polymerase Polη predispose carriers to skin tumours as this enzyme is critical for efficient and high-fidelity bypass of UV-induced lesions, including cyclobutane thymine dimers[60]. Indeed, patients with xeroderma pigmentosum with non-functional Polη are especially prone to malignancies caused by sun exposure[61]. Polη expression is high in a number of tumour types[37,62,63] (TABLE 1), and increased Polη expression in melanomas, chronic lymphocytic leukaemias and germinal centre B cell lymphomas is associated with a mutational signature enriched at WA/TW motifs (where W is A or T), consistent with Polη mutagenic activity[64,65].

Polι was originally suggested to serve as a back-up of Polη in UV lesion processing, although its unique structural features suggest an independent, albeit still ill-defined, function for this enzyme in DNA damage bypass[66–68]. Although Polι deficiencies in normal cells are not linked with pathologies in humans[68], several SNPs in *POLI* (encoding Polι) have been linked to the development of specific tumour types, including melanoma, prostate

cancer, lung adenocarcinoma and squamous cell carcinoma[69–71]. Cell-based studies have shown that Polι activity increases T>C transitions, T>A transversions or C>A transversions in breast cancer cells exposed to UV damage[72], and Polι expression is elevated in breast cancer cell lines[72], bladder cancer[73] and oesophageal squamous cell carcinoma[56] (TABLE 1). In vivo studies suggest that Polι expression can contribute to oesophageal squamous cell carcinoma cell migration and invasion[74]; however, future research will need to determine the molecular links between Polι expression or activity, migration and invasion, and response to chemotherapy.

Polκ is implicated, along with Polη, in the replication of DNA at common fragile sites[75] and promotes DNA synthesis when replication fork stalling occurs due to nucleotide deprivation[76]. Polκ has a propensity to introduce interrupted mutations and undergo polar pausing[77]; upon hydroxyurea treatment, the mutational signature of Polκ at poly(dA:dT) repeats — sites of fork stalling and collapse in both early and late-replication fragile sites — includes recurrent interruptions of poly(dA:dT) tracts with CC:GG sequences in a mouse cell line model[78]. In general, mutations in common fragile sites have been associated with genomic instability features that drive tumorigenesis[79]. Moreover, *POLK* SNPs are associated with various cancer types, including prostate, breast, lung, melanoma, stomach and large intestine tumours[80], and Polκ expression is elevated in lung cancer[81] (TABLE 1). It is unclear whether there is a direct relationship between Polκ-dependent mutations within particular sequences and tumorigenesis.

Template switching.

TS has been most extensively studied in yeast and bacteria[82,83], and work establishing TS factors and regulators in human cells is limited. In general, TS pathways can lead to genomic instability through genomic rearrangements and sister chromatid exchange (SCE).

TS mechanisms in yeast[84,85] and human cells[86] are associated with K63-linked polyubiquitination of PCNA by the E2-conjugating enzyme UBC13 following PCNA monoubiquitination by RAD18 (FIG. 1). UBC13, expressed at moderate levels in most tumours[87] (TABLE 1), has two E3 ligase partners, helicase-like transcription factor (HLTF) and SNF2 histone linker PHD ring helicase (SHPRH), both of which are implicated in PCNA ubiquitination[88,89]. HLTF expression is generally low across tumour types[90], whereas SHPRH levels appear to be moderate or high in the majority of cancers[91] (TABLE 1). Although there is no clear data indicating the molecular mechanism underlying this difference, we speculate that HLTF might be lowly expressed across tumours because of its reported antiproliferative functions[92–94] in addition to its roles in the DNA replication stress response[95].

Following PCNA polyubiquitination by UBC13, TS might involve molecular steps resembling those of canonical homologous recombination (reviewed in REF.[96]). The central recombinase factor RAD51 and Nijmegen breakage syndrome 1 protein (NBS1), which is a key component of the MRE11–RAD50–NBS1 (MRN) complex, have been implicated in TS across from abasic sites and benzo[a]pyrene adducts[29,97]; in this context, RAD51 might potentially mediate TS by promoting strand invasion and branch migration between sister chromatids, whereas the MRN complex might be required to process the stalled

replication intermediate. Homologues of the human Bloom syndrome protein (BLM), such as the ATP-dependent helicases SGS1 in *Saccharomyces cerevisiae*[98] and hus2/rqh1 in *Schizosaccharomyces pombe*[99], promote TS by facilitating the dissolution of D-loop structures although there are no mechanistic studies that conclusively demonstrate the function of BLM in TS in human cells. Interestingly, many of these putative TS factors are highly expressed in different cancer types[100,101] (TABLE 1).

TS pathways can lead to gross chromosomal rearrangements and gene amplifications[102,103], which could in turn affect cancer progression, chemoresponse and clinical survival outcomes[102,103]. Copy number variations and gene amplifications are likely to occur when replication-associated TS events bypass genomic regions containing a high number of repetitive sequences such as telomeres, tRNA genes and triplet repeats[102]. Identifying these TS-dependent copy number variations or gene amplifications could uncover targets to improve chemoresponse or reverse resistance resulting from these genomic rearrangements. For example, gene amplification of the HER2 receptor is currently used to inform clinical treatment of specific breast cancers and other *HER2*-amplified tumours with the HER2-specific antibody trastuzumab[104]. It is notable that break-induced replication, which is another fork recovery pathway that rescues collapsed or broken replication forks by promoting a TS-like mechanism[105], is also an important source of gross chromosomal rearrangements in cancer cells; an in-depth review of the molecular consequences of break-induced replication can be found in REF.[105].

Fork reversal.

Replication fork reversal is activated in response to various replication challenges and promotes re-annealing of complementary daughter strands to form a four-way reversed fork structure[24].

Several DNA translocases, including Rad5 in budding yeast[106] and RAD54 (REF.[107]), SMARCAL1 (REFS.[108,109]), FANCM[110], ZRANB3 (REFS.[111,112]) and HLTF[113,114] in mammalian cells, can promote fork reversal, although their exact mechanisms are unclear (FIG. 1). Biallelic *SMARCAL1* mutations cause Schimke immunoosseous dysplasia, and these clinical phenotypes are linked to the defective replication-associated DNA damage response observed in SMARCAL1-deficient cells[115,116]. High SMARCAL1 expression has been observed in pancreatic, testis, breast, prostate and thyroid cancer samples[117] (TABLE 1). Germline mutations in *FANCM*, a member of the Fanconi anaemia (FA) complementation group, lead to increased cancer predisposition, consistent with established roles for FA proteins in genome stability[118,119]. In the context of human malignancy, *ZRANB3* variants have been observed in endometrial cancers[120] and *ZRANB3* RNA expression is highest in testis cancers relative to other tumour types[121] (TABLE 1). *RAD54* mutations have been detected in a single case of primary lymphoma and a single case of colorectal cancer[122], and tumour-associated RAD54 mutations have been linked with genomic instability in cell models[123].

The central recombinase factor RAD51 (REF.[24]), the F-box DNA helicase 1 (FBH1; a helicase and RAD51 ubiquitination regulator)[124] and several RAD51 paralogs[125,126] have been implicated in reversed fork formation. Germline *RAD51* mutations confer an increased

cancer risk, particularly for breast and ovarian cancers[127], and RAD51 foci formation has been used to assess the homologous recombination proficiency of cancers[128]. RAD51 protein levels are increased in pancreatic cancer[129], breast carcinomas[130] and cancer cell lines[131] (TABLE 1). As a result, RAD51 inhibitor development[127] has emerged as a chemotherapeutic strategy, particularly in combination with targeted therapies such as poly(ADP-ribose) polymerase (PARP) inhibitors (PARPi).

Resolution of reversed fork structures is mediated in humans by the RECQ1 helicase[132]. The reversed fork restart activity of RECQ1 is regulated by PARP1, which suppresses RECQ1 activity until the damage is repaired[132]. A second mechanism of reversed-fork processing and restart depends on human DNA replication helicase/nuclease 2 (DNA2) and Werner syndrome ATP-dependent helicase (WRN)[133]. RECQ1 and WRN, in addition to BLM, RECQ5 and RECQ4, all belong to the RecQ helicase family, and Bloom syndrome, Werner syndrome and Rothmund–Thomson syndrome arise from germline mutations in *BLM*, *WRN* and *RECQ4*, respectively. Hallmarks of these syndromes include chromosomal instability, developmental abnormalities and increased risk of cancer[134]. Further, a mutation in the zinc-binding domain of RECQ1 that causes a defective DNA replication and DNA damage response following treatment with topoisomerase poisons is associated with RECON syndrome[135]. Interestingly, a rare *RECQ1* mutation has also been correlated with breast cancer susceptibility[136], further emphasizing a role for the RecQ helicases in maintaining genome integrity. BLM and DNA2 expression levels tend to be high across various tumour types[100,137], whereas WRN seems to be moderately expressed in cancers of the testis, thyroid, head and neck[138] (TABLE 1). RECQ1 expression appears moderate or high in lymphomas, thyroid, head and neck, and carcinoid cancers[139] (TABLE 1). Targeting RecQ family helicases might increase sensitivity to DNA-damaging chemotherapeutics by preventing their reported functions in DNA repair and replication, with the caveat that the functional inactivation of these enzymes might be toxic to non-malignant cells.

Fork reversal represents a high-fidelity form of DDT and reversed replication forks must be protected from extensive nucleolytic degradation to preserve genome stability. In addition to their critical roles in homologous recombination, the breast cancer susceptibility proteins BRCA1 and BRCA2 act to protect reversed replication forks[140–142] and, in their absence, nucleases such as MRE11 and exonuclease 1 (EXO1) target the open DNA end of the reversed fork substrates, leading to extensive fork degradation. Replication fork degradation in cancer is linked to chemosensitivity, whereas restoration of fork protection is associated with drug resistance[140,143,144]. Interestingly, extensive fork degradation is not a terminal event as BRCA-deficient cells employ specialized fork recovery pathways to rescue degraded forks and withstand DNA damage. In BRCA2-deficient cancer cells, MUS81 (a structure-specific endonuclease that is expressed at low levels across cancer types)[145] and DNA polymerase-δ subunit 3 (Polδ3) cooperate to facilitate a break-induced replication-like mechanism of fork restart[146,147]. Of note, this break-induced replication-like pathway is not employed in BRCA1-deficient backgrounds[146], suggesting that BRCA1-deficient cells recover resected forks through a different pathway. Indeed, ectopic expression of the E3 ubiquitin-protein ligase RNF168, together with the DDT enzymes RAD18 and SLF1, contributes to a break-induced replication-like mechanism at stalled replication forks in BRCA1-deficient cells[148]. It is unclear whether this axis is also active under

conditions of endogenous RNF168 expression and whether additional factors are required for fork recovery in BRCA1-deficient cancer cells when RNF168 is not overexpressed. Interestingly, RNF168 loss in *BRCA1*-heterozygous mice predisposes these animals to tumour development[149], suggesting that RNF168 might also mediate a similar replication fork stress response mechanism in non-malignant cells.

Recent work implicates the Cockayne syndrome protein CSB in fork recovery mechanisms in both BRCA1-deficient and BRCA2-deficient cells[150]. CSB functions in a break-induced replication mechanism of fork restart that depends on MRE11, MUS81 and RAD52 (REF.[150]). RNA levels expressed from *ERCC6* (encoding CSB) are highest in thyroid and breast tumour samples relative to other cancers[151] (TABLE 1). DNA repair protein XRCC1, which is involved in ssDNA break repair, is also involved in replication restart in cells lacking *BRCA2* (REF.[152]). XRCC1 is highly expressed in a diverse range of cancers and is synthetically lethal with BRCA2 (REF.[153]) (TABLE 1). BRCA2-deficient cells activate XRCC1-mediated microhomology-mediated end joining (MMEJ) in collaboration with MRE11 to facilitate recovery of extensively degraded replication forks[152]. Collectively, these findings underscore the potential of fork recovery mechanisms as possible therapeutic targets.

Repriming and ssDNA gaps.

Repriming is a highly conserved replication stress response that is present across *Escherichia coli*[154], budding yeast[155] and human cells[25–27]. In human cells, repriming is mediated by PRIMPOL[25–27] (FIG. 1), which operates in both mitochondria and nuclei[156]. Although our understanding of mitochondrial repriming is limited, several studies have documented a key role for PRIMPOL during nuclear DNA replication[3,4,28,32,97,156–159]. PRIMPOL repriming is generally activated in conditions of impaired fork reversal (for example, upon PARPi treatment or loss of SMARCAL1, HLTF or CARM1 expression)[3,32,113], increased PRIMPOL expression[4,32] or BRCA deficiency[160,161]. Interestingly, PRIMPOL expression is elevated in thyroid cancers relative to other tumour types[162] (TABLE 1) and the point mutant PRIMPOL-Y100H, which alters unique preference of PRIMPOL for dNTPs[163], has been identified in lung carcinoma as reported in the COSMIC database[164], suggesting that altering PRIMPOL activity could drive tumour formation.

PRIMPOL-dependent repriming introduces ssDNA gaps downstream of the replication obstacle, leaving these gaps to be repaired post-replicatively[4,32,97,113,157]. Recent studies in cells challenged with cisplatin suggest that there are at least two temporally distinct pathways that repair ssDNA gaps: in G2-phase, a TLS mechanism dependent on RAD18, PCNA monoubiquitination, and REV1 and Polζ promotes gap filling, whereas gap filling is mediated by a TS-like mechanism dependent on UBC13 and RAD51 in S-phase[32]. The choice and timing of a particular gap-filling pathway likely varies with genetic background and the replication roadblock bypassed during the initial repriming event[32,165]. Potential risks of these gap-filling mechanisms include mutagenesis in the case of TLS, and SCEs or chromosomal rearrangements that could contribute to genomic instability in TS. Failure to fill the ssDNA gaps leads to persistent ssDNA stretches, which are susceptible to cleavage and nucleolytic processing, potentially contributing to double-stranded DNA

break accumulation. Recent studies revealed that accumulation of ssDNA gaps or impaired gap filling increase chemosensitivity upon treatment with PARPi, particularly in BRCA-deficient cancer cells[32,160,161,166,167]. Recent evidence also implicates the accumulation of ssDNA gaps in contexts where Okazaki fragment maturation and chromatinization is compromised[167,168]. Thus, factors that promote ssDNA gap generation and the subsequent step of gap filling represent attractive targets to modulate chemotherapy response.

In summary, diverse replication fork stress response mechanisms have different effects on genome stability and tumour development. These findings raise several questions regarding how the differential usage of these pathways affect chemoresponse and clinical outcomes; whether relevant combinatorial treatments could effectively target replication stress response and improve chemosensitivity or combat chemoresistance in a clinical setting; and whether factors involved in replication stress response could serve as useful clinical biomarkers. The next sections outline current research aimed at answering these critical questions.

DNA replication stress in cancer therapy

Targeting TLS and DNA repair polymerases in cancer.

RAD18 has been investigated as a promising target for cancer treatment owing to its elevated expression across many tumour types and role in initiating TLS (FIG. 2). Indeed, targeting RAD18 with a specific microRNA has been shown to sensitize resistant colorectal carcinoma cells to chemotherapy in vitro[169]. Recent data reveal that knockout of *RAD18* in BRCA1-deficient or BRCA2-deficient cancer cells increases DNA damage and formation of unrepaired ssDNA gaps, leading to cell death[161]. Further, RAD18-deficient cancer cells are more sensitive than wild type cancer cells to crosslinking agents, including mitomycin C and cisplatin[170]. The increased sensitivity of RAD18-deficient cells to crosslinking agents could be associated with an additional role of RAD18 in regulating ubiquitination of the FA protein FANCD2 (REFS.[170,171]), which is a central factor involved in inter-strand crosslink repair[172,173]. Following these observations, recent studies have screened for chemical inhibitors of the RAD18 pathway by specifically targeting the interaction between RAD18 and its upstream E2-conjugating enzyme partner RAD6 (REF.[174]), paving the way for future preclinical studies.

PCNA monoubiquitination can be directly targeted using the small molecular inhibitor T2-amino alcohol[175]. Treatment with T2-amino alcohol prevents repair of interstrand DNA crosslinks and increases DNA double-stranded breaks (DSBs) and sensitivity to cisplatin in cell-based assays[176]. Similarly, preventing PCNA monoubiquitination by mutating lysine 164 to arginine decreases cell proliferation[167] and increases sensitivity to UV treatment[167,177]. Loss of PCNA monoubiquitination increases the response of cells lacking BRCA1 or BRCA2 to PARPi and cisplatin[167].

Targeting the deubiquitinase USP1, which removes ubiquitin from monoubiquitinated PCNA, shows promise in exacerbating replication stress and increasing DNA damage in cancer cells[178,179]. As a result, a clinical trial for advanced solid tumours has recently been developed using a first-in-class USP1 inhibitor, both alone and in combination with PARPi (TABLE 1).

TLS polymerases downstream of RAD18-mediated PCNA ubiquitination have been explored as targets to modulate cancer cell survival and improve therapy response. REV1 expression is associated with the development of chemoresistance to platinum-based drugs in ovarian cancer models[180,181] and loss of REV1 in BRCA-deficient cancer cells leads to decreased viability similar to the effects of RAD18 downregulation[161]. However, the degree of epistasis between these two proteins is untested. A newly developed chemical inhibitor of REV1, JH-RE-06, has been shown to bind to the REV1 C-terminal domain and promote REV1 dimerization[182], rendering the enzyme unable to recruit Polζ and initiate TLS. Treatment of mice carrying patient-derived melanoma xenografts with JH-RE-06 in combination with cisplatin substantially reduced tumour burden[182]. Treatment with a different TLS inhibitor, which also inhibits the interaction between REV1 and TLS enzymes, selectively kills cancer cell lines that rely on TLS for replication, including cells expressing FANCJ-S990A (a mutant copy of the helicase FANCJ that is unable to interact with BRCA1) and cells lacking the negative TLS regulator p21 (REF.[166]). In these ‘pro-TLS’ backgrounds, treatment with this TLS inhibitor synergizes with other replication stress-inducing agents, including inhibitors of ATR or WEE1 (REF.[166]), an effect that is attributed to the accumulation of ssDNA gaps. Interestingly, REV1 inhibition preferentially sensitizes BRCA-deficient cancer cells relative to wild type models both in the context of JH-RE-06 monotherapy[161] and in combination with cisplatin and PARPi treatment[32], which might be a result of the formation of ssDNA gaps[161] and decreased gap filling[32] in BRCA-deficient backgrounds.

Notably, REV1 loss or inhibition does not sensitize cells to ionizing radiation, possibly owing to the upregulation of autophagy, which is a hallmark of radioresistance[183]. The complex relationship between autophagy and cancer therapy responses is summarized in REF.[184]. The impact of TLS polymerase inhibition on autophagy should be considered when targeting these proteins for therapeutic benefit.

Promising results have been demonstrated with in vitro optimization of small-molecule inhibitors that target other TLS enzymes such as Polη and Polκ[185–187]. Preliminary studies revealed that targeting of Polκ with a small-molecule inhibitor increases sensitivity to the alkylating agent temozolomide in vitro[185]. Lower Polζ expression is associated with improved response to cisplatin and gemcitabine chemotherapies in head and neck squamous cell carcinomas[63] and increased *POLN* mRNA expression is associated with worsened overall survival in non-small-cell lung cancer (NSCLC)[188]. Studies also indicate that increased Polζ expression is linked to cisplatin resistance in bladder cancers[189] and in ovarian cancer stem cells[62]. Indeed, Polζ and Polκ have both been shown to facilitate replication past platinum adducts[190], suggesting that these TLS polymerases could be targeted to improve platinum-based therapies. A recent study highlighted that Polκ also enables cancer cells to tolerate replication stress resulting from aberrant cyclin-dependent kinase 2 (CDK2) activation[191], which can be induced by cyclin E overexpression or WEE1 inhibition[191]. Consistent with these findings, loss of Polκ or RAD18 sensitizes cancer cells to WEE1 inhibition[191].

Polζ might represent a target for improving chemosensitivity and decreasing drug resistance. Increased expression of REV3L — the catalytic subunit of Polζ — is associated with

cisplatin resistance in gliomas[55] and REV3L loss sensitizes chemoresistant models of NSCLC to platinum-based chemotherapy[192]. Similarly, loss of REV7 — the accessory subunit of Polζ — improved cisplatin response in a mouse model of NSCLC[193], and increased REV7 expression correlates with worsened survival outcomes in patients with diffuse large B cell lymphoma (DLBCL)[194]. Depletion of REV7 also increases the sensitivity of clear cell ovarian carcinoma to cisplatin[195]. However, in BRCA1-deficient backgrounds, REV7 loss contributes to PARPi resistance, an effect attributable to its role in non-homologous end joining (NHEJ)[196,197]. As a result, the impact of REV7 on both TLS and NHEJ must be considered when targeting REV7 across cancers.

Polθ, a member of the A-family of polymerases[198] that functions in MMEJ[199,200], represents a promising therapeutic target, particularly in BRCA-deficient cancers[199–201]. Because of its essential role in MMEJ, Polθ loss is synthetically lethal with deficiencies in other DSB repair pathways, including homologous recombination[199,200] (FIG. 3), and Polθ inhibition in BRCA-deficient cancer cells increases sensitivity to cisplatin and PARPi[199]. In addition to its role in MMEJ, Polθ is implicated in the repair of breaks arising from collapsed replication forks, tolerance of G-quadruplex DNA secondary structures and replication stress response upon fork stalling[202]. Additional work will be critical to define the contributions of each of these mechanisms to chemoresponse as a Polθ inhibitor has recently been combined with PARPi in a clinical trial (TABLE 1). Polθ expression levels and mutational signatures could represent Powerful clinical biomarkers to assess the efficacy of newly developed Polθ inhibitors across a range of tumour types[199,203–205].

Targeting TS and replication fork recovery in cancer.

Linking differential expression levels of UBC13, RAD51, BLM and NBS1 to defects in TS and to cancer chemotherapy response is complicated by the lack of direct methods for investigating homology-mediated TS mechanisms that do not necessarily involve strand transfer. In addition, these proteins have multiple cellular roles: UBC13 promotes DSB signalling and ubiquitinates cytosolic NF-κB pathway targets[206]; RAD51 plays multiple roles in replication fork stability[125]; and RAD51, BLM and NBS1 function in homologous recombination[96]. Consequently, we note that any potential chemotherapeutic benefit associated with targeting these factors cannot be absolutely associated with changes in TS efficiency.

UBC13 upregulation promotes breast and colorectal cancer cell metastasis through JNK and MAP kinase activation[207,208] and melanoma growth through MEK signalling[209] although another study proposes that UBC13 is downregulated in paclitaxel-resistant ovarian cancer cells, with lower expression contributing to worsened outcomes[210]. Interestingly, UBC13 inhibition with a small molecular inhibitor, NSC697923, has been shown to kill neuroblastoma cells[211], DLBCL cells[212] and melanoma cells in vitro[209]. Differences across studies might point to tumour-specific impacts of UBC13 expression and activity and indicate a need to evaluate any off-target effects of UBC13 inhibitors that could contribute to observed cell-killing phenotypes. In addition to UBC13, downstream TS factors might also constitute potential clinical targets and biomarkers.

Loss of the fork recovery factor MUS81 is associated with increased hydroxyurea sensitivity in BRCA2-deficient cancer cells[146] and, similarly, CSB downregulation increases hydroxyurea sensitivity in BRCA1-deficient and BRCA2-deficient backgrounds[150]. Moreover, MUS81 promotes progression of serous ovarian carcinoma[213] and knockdown increases sensitivity of epithelial ovarian cancer to PARPi[214]. Both overexpression and downregulation of RNF168 decrease viability in BRCA1-deficient cancers[215]. These data suggest that the relationship between RNF168 expression and cell survival is distinct from the roles of other recovery factors such as MUS81 and CSB, whose targeting can increase sensitivity to replication stress inducers. Future research should focus on defining whether, in addition to its role in fork recovery[148], the roles of RNF168 in chromatin ubiquitination[216] and DNA damage signalling[149] should be considered when targeting this factor to improve cancer cell chemoresponse.

Leveraging replication stress in PARPi cancer therapy.

The development of PARPi therapies has significantly improved survival outcomes in homologous recombination-deficient cancers. Therapies using PARPi and chemotherapy are proposed to kill homologous recombination-deficient tumours, such as those harbouring *BRCA1* or *BRCA2* mutations, through synthetic lethality[217,218]. PARPi leads to trapping of PARP proteins on DNA and causes an increase in ssDNA breaks, which are converted into irreparable DSBs during replication in BRCA-deficient tumours[218–220] and lead to cell death (FIG. 3). Toxic DSBs might also originate from the degradation and collapse of stalled replication forks upon treatment with DNA-damaging chemotherapy that cannot be adequately protected in the absence of BRCA proteins[140–142] (FIG. 3). Interestingly, PARPi has also shown promise in targeting homologous recombination-proficient cancer cells as loss of RNase H2, which is involved in removal of erroneous ribonucleotides from the DNA, sensitizes BRCA-proficient cells to olaparib[221].

PARPi efficacy is hampered by the development of resistance[222,223] and several regulators of PARP trapping have recently emerged as key modulators of PARPi[224–226]. Reported mechanisms of PARPi resistance (reviewed in REF.[227]) include *BRCA* reversion mutations that restore homologous recombination in these tumours; upregulation of efflux pumps that clear PARPi from cancer cells; restoration of homologous recombination through the downregulation of NHEJ factors, including tumour suppressor P53-binding protein 1 (53BP1) and the Shieldin complex; diminished PARP trapping via PARP mutations; and rescued PARylation and decreased binding of PARP to DNA through loss of poly-ADP ribose glycohydrolase (PARG)[228], which opposes PARP activity. Another emerging mechanism of chemoresistance is the restoration of replication fork stability in BRCA-deficient cancer cells, independent of the re-establishment of homologous recombination function in these genetic backgrounds[140,229].

ssDNA gaps are frequently formed as a consequence of replication stress and several studies propose that the accumulation of ssDNA gaps in BRCA-deficient cancer cells, exacerbated by treatment with PARPi, modulates cancer cell survival and drug sensitivity[8,32,160,161,166,167,230] (FIG. 3). Therefore, PRIMPOL, which generates ssDNA gaps during repriming and is regulated by the ATR[4] and CHK1 (REF.[231]) kinases (discussed

below), might represent a key regulator of cancer response to PARPi or emerging ATR and CHK1 inhibitors.

While PRIMPOL activity is typically associated with leading-strand ssDNA gaps, recent evidence suggests that aberrant Okazaki fragment processing (OFP) could lead to ssDNA gaps on the lagging DNA strand[5,6,167,168]. Defects in the canonical OFP pathway[232], which involves flap endonuclease 1 (FEN1) and DNA ligase I, or a backup OFP mechanism that relies on PARP, XRCC1 and DNA ligase 3 (REF.[6]) have been implicated in increased sensitivity to cancer therapies, including to PARPi. Restoration of efficient OFP or upregulation of OFP pathways may also contribute to chemoresistance in certain genetic contexts, including BRCA1-deficient cancer cells[8]. Moreover, models of PARPi resistance in BRCA-deficient cancer cells can be re-sensitized to PARPi through depletion of DNA ligase 3 (REF.[233]), which could be explained by an increased reliance of BRCA-deficient cells on DNA ligase 3-mediated OFP or base excision repair. The ssDNA gap accumulation model of chemoresponse[234,235] raises the important possibility that gap-filling mechanisms can be targeted to sensitize BRCA-deficient tumours to PARPi and other DNA-damaging chemotherapy to overcome chemoresistance in these cancers.

There are distinctions to be made between in vitro, in vivo and clinical models of PARPi resistance. Patient data has revealed cases of PARPi resistance caused by reversion mutations in *BRCA1* and *BRCA2* (REF.[227]) as well as by diminished PARP trapping via a *PARP1* mutation in a single instance[227]. Preclinical in vivo model data support PARPi resistance through decreased 53BP1 and Shieldin expression in patient-derived xenografts[236]. However, it remains to be shown whether restoration of replication fork stability or changes in ssDNA gap formation and repair impact clinical PARPi resistance. Translation of findings from in vitro models of chemotherapy resistance to clinical models of disease is complicated by the fact that multiple chemoresistance mechanisms can be activated in the same cell and across cells within the same tumour. In vitro studies must be expanded to other *BRCA*-mutated and wild type tumour types and potentially combine parallel assessments of different mechanisms of chemoresistance found in the clinical setting.

Exacerbating replication stress in cancer with cell cycle-checkpoint inhibitors.

DDT mechanisms are temporally regulated throughout the cell cycle[19] and checkpoint inhibitors are emerging as promising drugs for cancer treatment as they affect the ability of specific DTT pathways to repair or bypass a lesion in S-phase before cells enter G2-phase or reach mitosis.

ATR kinase orchestrates different cellular pathways in response to replication stress, including the enforcement of an S/G2 checkpoint[237], regulation of intracellular dNTP levels and origin firing. ATR might also play a role in replication fork reversal but its contribution is unclear[238–240]. The ATR signalling cascade, involving CHK1 phosphorylation, is activated upon exposure of stretches of ssDNA that form during replication fork stalling and uncoupling[241]. Based on the role of ATR in preserving replication fork stability and enforcing an appropriate cell-checkpoint response, inhibition of ATR kinase and of its downstream CHK1 substrate are relevant strategies to improve cancer chemoresponse

(FIG. 2). Preclinical data show that ATR inhibitors and CHK1 inhibitors can re-sensitize PARPi-resistant, BRCA1-deficient cancer cells to PARPi, making the ATR–CHK1 pathway an attractive therapeutic target in settings of drug resistance[229,242]. CHK1 inhibition also decreases tumour growth in mouse models lacking activating molecule in BECN1-regulated autophagy protein 1 (AMBRA1), which has been uncovered as a key regulator of the cell cycle[243–245]. Multiple clinical trials have used preclinical mechanistic insight to inform combination therapies with ATR inhibitors and either PARPi, platinum-based chemotherapy, antimetabolites or radiotherapy (TABLE 2). Similarly, CHK1/2 inhibitors have been included in clinical trials as a monotherapy or in combination with PARPi, gemcitabine and even PDL1 blockade (TABLE 2). Importantly, the inhibition of ATR appears to have lower toxicity than inhibition of CHK1, which could be related to the non-specificity of some CHK1 inhibitors that target both CHK1 and CHK2 (TABLE 2). Activation of ATR and CHK1 might be a useful biomarker to predict tumour response to emerging targeted and combinatorial therapies that induce replication stress. For a comprehensive review of ATR kinase and its functions at replication forks, we direct readers to REFS.[1,241].

The G2 checkpoint kinase WEE1, which is downstream of ATR and CHK1, shows promise as a therapeutic target (FIG. 2 and TABLE 2). WEE1 inhibitors reduce tumour growth in combination with ATR inhibitors in mouse models of DLBCL[246], are synergistic with CHK inhibitors in acute lymphoblastic leukaemia[247] and improve PARPi response in triple-negative breast cancer[248,249]. WEE1 inhibition contributes to replication fork stress by disrupting nucleotide pools, leading to replication fork collapse and DSBs[250], and by promoting replication fork degradation[251]. In addition, treatment of ex vivo models of ovarian cancer with CHK1 inhibitors or with both CHK1 and WEE1 inhibitors increases sensitivity to PARG inhibition, which induces replication fork catastrophe and increased DNA damage[252]. These data provide mechanistic insight into the potential clinical efficacy of combining CHK1 and WEE1 inhibitors with replication stress-inducing agents such as PARPi and PARG inhibitors.

In the absence of ATR, additional kinases, such as ATM and DNA-dependent protein kinase (DNA-PK), can phosphorylate CHK1 upon replication stress[253]. ATM is also involved in sensing DSBs, including those resulting from collapsed replication forks, and phosphorylates CHK2 as part of the DSB signalling cascade[254]. As a result, ATM and CHK2 inhibitors have entered clinical trials in combination with chemotherapeutic agents that induce replication stress, including PARPi (TABLE 1). Drugs that specifically target CHK2 have been investigated in fewer clinical trials than those that target CHK1, which suggests that they might be less effective as antineoplastics. Indeed, this difference might be connected to *CHK2* being non-essential in cells, whereas *CHK1* is an essential gene[255]. ATM-deficient tumours have been targeted with a wide range of drugs, including platinum-based agents, ATR inhibitors, PARPi and CHK1/2 inhibition strategies[256,257]. In addition to ATR and ATM, DNA-PK plays critical roles both in DSB repair through NHEJ and in the replication stress response. Upon replication stress induction, DNA-PK facilitates ATR–CHK1 checkpoint activation[258], and concurrent ATR and DNA-PK inhibition increases radio-sensitivity in colon and head and neck squamous cell carcinoma cell lines[259]. In vitro data show that DNA-PK inhibition can improve cancer cell response to PARPi, doxorubicin and radiation treatment, and ATM-deficient cancer cells are also highly sensitive to

combined DNA-PK inhibition and PARPi treatment[260]. As a result, DNA-PK inhibitors are in clinical development for the treatment of solid malignancies (TABLE 2).

CDK inhibitors that impair cell cycle progression are increasingly used in the clinic, including as second-line therapy for breast, prostate and ovarian cancer[261]. However, resistance to CDK inhibitors — and particularly to CDK4/6 inhibitors — is seen frequently and the pleotropic effects of many CDK inhibitors make it difficult to pinpoint mechanisms that underlie differential sensitivity[262,263]. CDK inhibitors typically arrest cells in G1/S phase (FIG. 2), which can decrease replication-associated toxicity induced by DNA-damaging chemotherapy or radiotherapy[264,265]. As a result, the blunting of chemotherapy by CDK inhibitors is a relevant concern[266]. Despite these concerns, recent preclinical findings show that CDK4/6 inhibitors have synergistic effects with PARPi[267]. Pre-treatment with the CDK4/6 inhibitor palbociclib sensitizes cancer cells to a wide range of genotoxic agents, such as aphidicolin, camptothecin and doxorubicin, and is associated with prolonged replication stress[268] (FIG. 2). Post-treatment with palbociclib following incubation with DNA-damaging agents, such as gemcitabine, cisplatin and topoisomerase poisons, also enhances cancer cell killing[269]. Although most CDK inhibitors are used as monotherapies, a number of clinical trials have tested combinations of CDK inhibitors, particularly CDK4/6 inhibitors, with platinum-based agents, epirubicin and gemcitabine; however, toxicity with these therapies remains a challenge[270].

Bolstering immunotherapy response in cancer with replication stress induction.

Recent studies have explored a connection between replication fork perturbations, inflammatory signalling and cancer immunotherapy response. The finding that STING inflammatory signalling is upregulated in response to replication stress[271–273] suggests that replication stress might cause the release of DNA fragments from the nucleus into the cytosol, causing cGAS–STING pathway activation. Further studies have implicated the processing of stalled replication forks[271,274,275] and micronuclei[276,277] as sources of these DNA fragments. Upregulation of an interferon-like response by cGAS–STING leads to T cell priming and recruitment and can boost the efficacy of immunotherapies[278], suggesting that combinatorial treatments exploiting STING-inducing replication stress with immune-checkpoint blockade represent a promising therapeutic strategy (FIG. 2).

Increased expression of specific TGFβ-responsive genes is associated with immunotherapy resistance in gynaecological cancers[279], and additional work has shown that blocking TGFβ signalling restrains tumour growth in a breast cancer mouse model that is resistant to immune-checkpoint blockade[280]. One study identified that loss of Mediator complex subunit 12 (MED12; a coactivator that functions in transcription) contributes to chemoresistance by upregulating TGFβ signalling and restoring replication fork stability in BRCA-deficient cancer cells[281]. In BRCA-competent cells, BRCA1, which plays roles in both DSB repair and replication fork stability, is downregulated by TGFβ through its interactions with miR-182 (REF.[282]); upon stimulation by TGFβ, this microRNA decreases BRCA1 protein levels in mouse and human cells[282,283]. These data highlight MED12 and the TGFβ signalling axis as promising therapeutic targets for combatting immune therapy resistance and improving drug sensitivity, particularly through promoting replication stress

and decreasing DNA-damage repair capacity. TGFβ preserves genomic stability more broadly by mediating ATM and p53 checkpoint activation and promotion of the DNA damage response and DSB repair through SMAD proteins[284–286]. To refine this preliminary evidence, additional studies must define the contribution of the TGFβ pathway across cancer cell types, especially in cancer stem cell populations in which TGFβ expression has been suggested to both suppress[287] and promote cancer stem cell features in different contexts[288,289].

Several publications broadly suggest that tumour mutational burden (TMB) is linked to immunotherapy response[290,291]. While the impact of TMB on therapy response is not straightforward in every cancer subtype nor with every drug regimen, TMB is generally proposed to correlate with increased neoantigen formation[292]. These neoantigens are recognized by T cells[293] and could boost efficacy of immune-checkpoint blockade and improve tumour killing. To date, and based on the preclinical data described above, numerous clinical trials have combined immune-checkpoint blockade with a range of chemotherapeutics[294,295] (FIG. 2). One study also uncovered a “replication stress response gene expression signature”, which was predictive of immune-checkpoint blockade response in preclinical cancer models[296], suggesting that replication stress-linked TMB or neoantigen formation could be useful as biomarkers. These data emphasize the importance of further mechanistic investigation to exploit the link between replication stress pathways, DNA damage response and immune system activation.

DNA replication stress and clinical biomarkers.

DDT pathways could be useful for the development of new clinical biomarkers (FIG. 4). TLS enzymes are generally elevated across select tumour types[37,56,57,62,63,72,73], possibly because of their contribution to mutagenesis, which drives carcinogenesis and also promotes tumour evolution and resistance to chemotherapy. Increased RAD18, PCNA ubiquitination and TLS polymerase expression relative to normal tissue controls or over time within the same tumour might represent novel biomarkers to predict therapy response and clinical outcomes[43,44,57,69–71,74,297]. Similarly, TS proteins, fork reversal factors and fork recovery enzymes are elevated in a variety of tumours[87,91,100,101,117,121,137–139,145,151,153]. The successful development of these DDT factors as biomarkers will require large-scale studies in a wide range of cancers to assess the tumour specificity of these proteins and establish relevant expression thresholds across cancer types. It should be noted that upregulation of selected DTT factors could be specific to the DNA-damaging agents that tumour cells are exposed to during treatment, which is an important consideration for the clinical applicability of these factors as biomarkers. Further, the replication stress-independent roles of these factors will also need to be explored to determine whether they contribute to cancer cell survival or response to DNA-damaging agents.

Functional assays to assess replication stress activities in tumour samples could be used as biomarkers. For example, single-molecule DNA fibre assays have been used in preclinical studies to monitor replication fork stability in high-grade serous ovarian cancers organoids[298]. TS-dependent SCE or gene amplifications and TLS-mediated mutagenesis could also provide useful readouts to assess possible response to chemotherapy. In addition,

increased accumulation of ssDNA and ATR–CHK1 activation could serve as biomarkers to predict response to drugs that induce replication stress (FIG. 4), including PARPi. Chromatin-bound RPA might be a useful readout of ssDNA gaps in cancer cells[299], although two major challenges are currently associated with this approach: replication-associated ssDNA gaps need to be distinguished from regions of ssDNA generated in other phases of the cell cycle, and background levels of chromatin-bound RPA need to be studied and baseline thresholds established across diverse tumour types.

Recent data have shown that PARP trapping levels could also be used as a biomarker as they are indicative of PARPi sensitivity in cancer cells[300]. Finally, accumulation of cytosolic DNA and activation of the cGAS–STING pathway might also represent novel biomarkers to evaluate the combinatorial benefit of replication stress-inducing chemotherapy with immunotherapy or with other agents that increase TMB and neoantigen formation.

Conclusion

Precision medicine continues to revolutionize cancer care, informing the strengths and limitations of therapeutic strategies and uncovering emerging resistance mechanisms. In parallel, the field continues to identify foundational pathways of replication stress and DNA damage response using genome-wide screens and sequencing techniques[301,302]. These tools and the data they uncover are transforming the way we understand tumours at the molecular level and open new strategies to improve clinical cancer care. Future work needs to identify the tumour or cancer cell-type specificity of these emerging pathways and assess the adequacy of in vitro and in vivo cancer models that are used to elucidate replication stress and DNA repair mechanisms. Further, the feasibility and scalability of possible biomarkers and targets outlined in this Review must be explored given that multiple mechanisms of chemosensitivity and resistance can be activated within a single tumour or within a single cell. These studies are essential to solidify new findings on replication stress that are actively shaping clinical medicine, including the link between replication stress and immunotherapy, which is emerging as a promising direction for cancer treatment[303,304]. We predict that new cross-disciplinary studies will continue to inform the complex interplay of replication stress response mechanisms, DNA damage repair signalling and the tumour microenvironment, better predicting and improving response to therapy.

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Glossary

DNA lesions

Modifications introduced On the DNA helix by different genotoxic agents.

Xeroderma pigmentosum

An autosomal recessive genetic disease caused by biallelic mutations of specific proteins that are involved in molecular mechanisms required to cope with UV-induced DNA lesions, including Polη.

Polar pausing
Transient pausing of the replication fork in response to a unidirectional barrier that only inhibits replication fork progression in one direction.

Schimke immuno-osseous dysplasia
A multi-system autosomal recessive genetic disease caused by inheritance of biallelic *SMARCAL1* mutations, with renal disease being a major cause of mortality in patients with this disease.

RECON syndrome
An autosomal recessive genetic disease caused by biallelic mutations in the *RECQL1* DNA helicase, which functions in the DNA damage response.

Microhomology-mediated end joining
(MMEJ). One of the DNA double-strand break repair pathways, along with homologous recombination and non-homologous end joining, which relies on microhomology sequences (1–16 nucleotides) to anneal and align double-strand break ends for repair.

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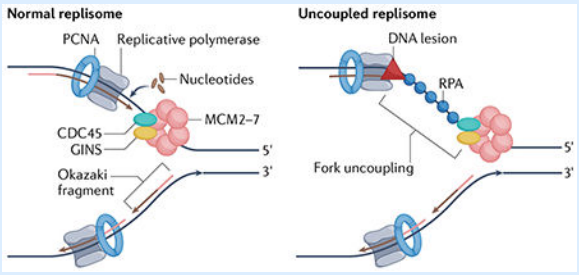
Box 1 |

DNA replication is mediated by a large protein complex known as the replisome that promotes multiple enzymatic activities

In eukaryotic cells, parental DNA is unwound by the CMG complex, which is composed of cell division control protein 45 (CDC45), mini-chromosome maintenance protein homologues 2–7 (MCM2–7) and the go–ichi–ni–san (GINS) complex. It is then replicated by the leading and lagging strand polymerases Polε and Polδ. The DNA sliding clamp proliferating cellular nuclear antigen (PCNA) is a homotrimer that encircles DNA and is essential for processivity of replicative polymerases. DNA lesions or other sources of DNA replication stress (red triangle) can transiently stall the leading strand polymerase, without affecting the movement of the CMG complex. This process is termed replication fork uncoupling and leads to the accumulation of single-stranded DNA stretches that are promptly coated by the single-stranded DNA-binding protein replication protein A (RPA).



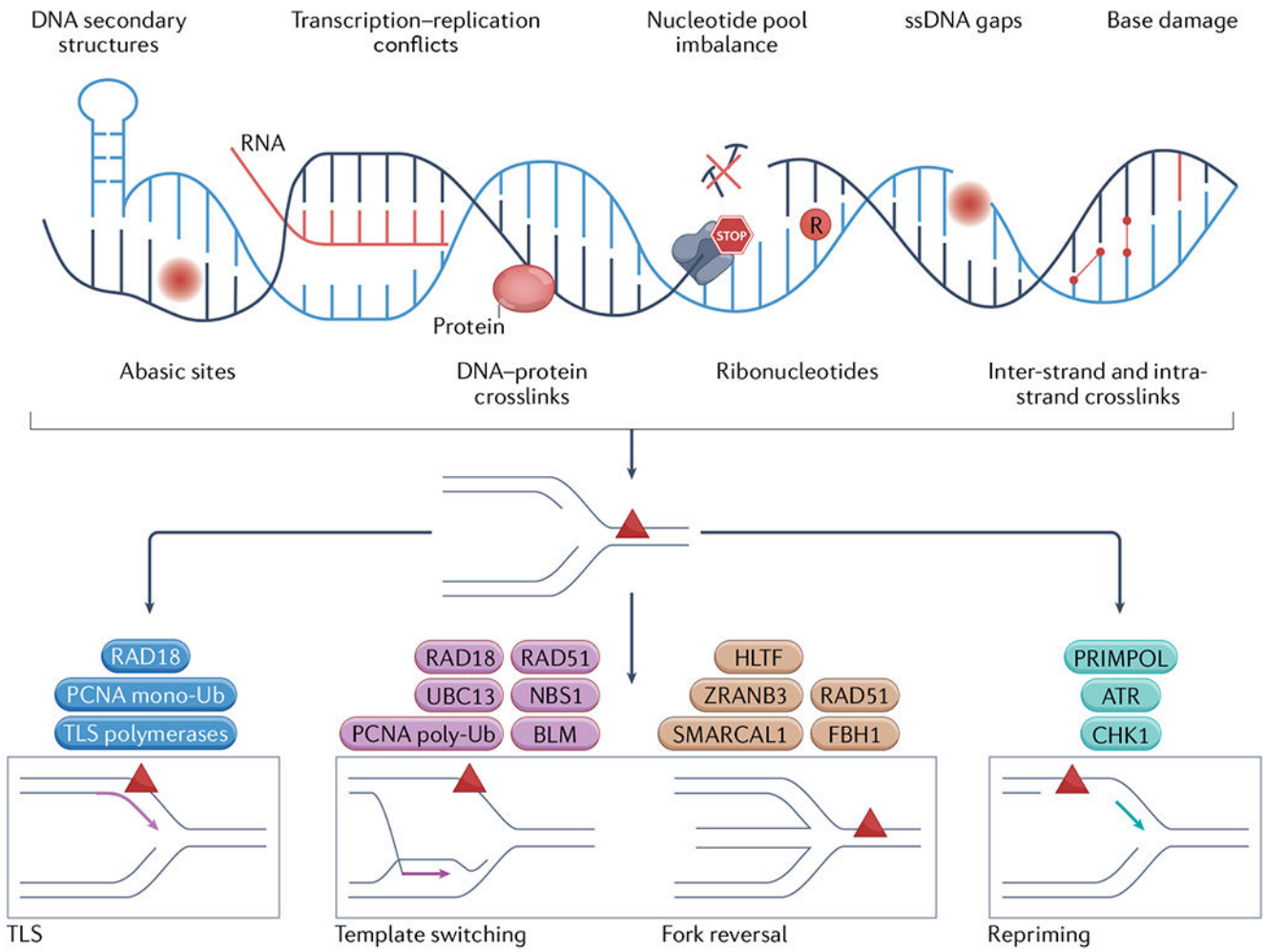


Fig. 1 |. Major mediators of the replication stress response.

Sources of endogenous replication stress include abasic sites, transcription–replication conflicts, the incorporation of ribonucleotides into DNA and protein–DNA crosslinks. Sources of exogenous stress include DNA inter-strand and intra-strand crosslinks induced by DNA crosslinking agents and base damage induced by alkylating drugs. Single-stranded DNA gaps or breaks, secondary DNA structures, including hairpins, and nucleotide imbalances that stall replicative polymerases are also considered sources of DNA replication stress. DNA damage tolerance mechanisms are activated when forks encounter these endogenous or exogenous DNA lesions or roadblocks, which are represented as red triangles. Translesion synthesis (TLS) is a DNA damage tolerance mechanism that involves RAD18-dependent monoubiquitination (mono-Ub) of proliferating cellular nuclear antigen (PCNA), which promotes downstream recruitment of TLS polymerases and DNA synthesis across the DNA lesion. Template switching involves RAD18 and UBC13-mediated polyubiquitination (poly-Ub) of PCNA and the downstream factors RAD51, Bloom syndrome protein (BLM) and Nijmegen breakage syndrome 1 protein (NBS1), allowing use of the complementary base pairs to replicate DNA past the DNA obstacle. Fork reversal, another form of template switching, employs the RAD51, SMARCAL1,ZRANB3, helicase-

like transcription factor (HLTF) and F-box DNA helicase 1 (FBH1) enzymes to facilitate replication fork remodelling into a four-way junction structure. Repriming relies on the action of DNA-directed primase/polymerase protein (PRIMPOL), leaving single-stranded DNA (ssDNA) gaps behind the replication forks to be filled post-replicatively. PRIMPOL activity is regulated by ATR and CHK1.

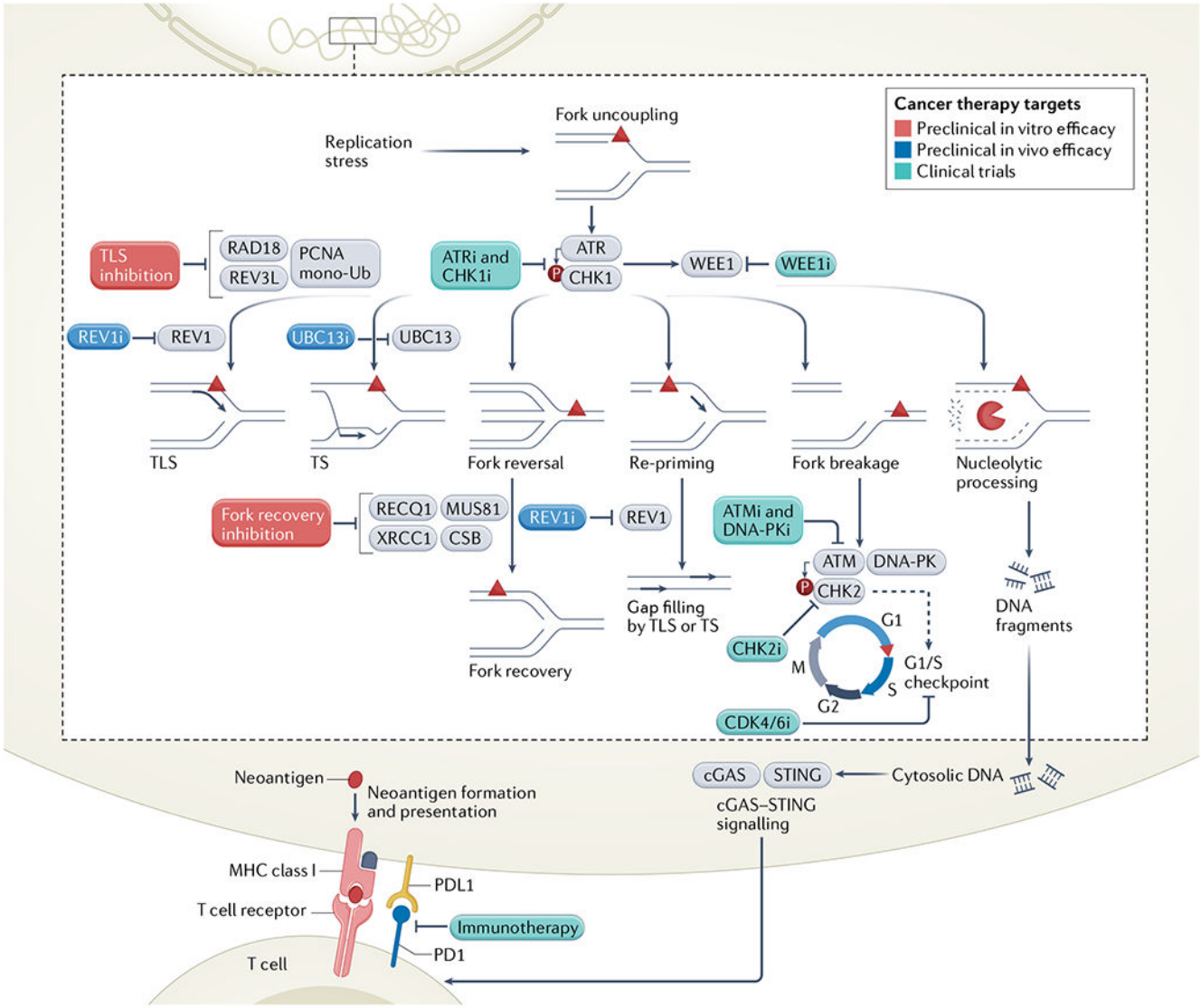


Fig. 2 |. The replication stress response in cancer and emerging therapeutic targets.

Upon replication fork stalling, single-stranded DNA exposure leads to the activation of the ATR checkpoint and the downstream CHK1 and WEE1 proteins. These signalling pathways can be targeted by ATR, CHK and WEE1 inhibitors, which are being tested in clinical trials. Following ATR induction, cells activate diverse replication stress response pathways, including translesion synthesis (TLS), template switching (TS), fork reversal and repriming. Inhibitors of REV1 and UBC13 have been tested in vivo in several cancer types, and REV1 inhibitors could also be used to target single-stranded DNA gap filling following repriming. Additional TLS enzymes and factors involved in replication fork reversal have also shown promise as therapeutic targets in vitro. Replication-associated breaks activate the ATM, DNA-dependent protein kinase (DNA-PK) and CHK2 kinases. Inhibitors of ATM, DNA-PK and CHK2 are being evaluated for anticancer potential in clinical trials, in addition to CDK4/6 inhibitors, which are associated with cell cycle arrest and antiproliferative effects. Stalled replication forks can also be processed by nucleases, and extensive resection can

promote release of DNA fragments into the cytosol, stimulating the cGAS–STINC pathway. cGAS–STINC activation can augment the cancer cell response to immunotherapy, a strategy that is used widely in clinical trials and in the clinic. Inhibitors of DNA replication stress response factors that are already in clinical trial are shown in green, those that have shown preclinical in vivo efficacy are in blue, and those with in vitro efficacy are in red; they are all indicated by the letter i. mono-Ub, monoubiquitination; PCNA, proliferating cellular nuclear antigen.

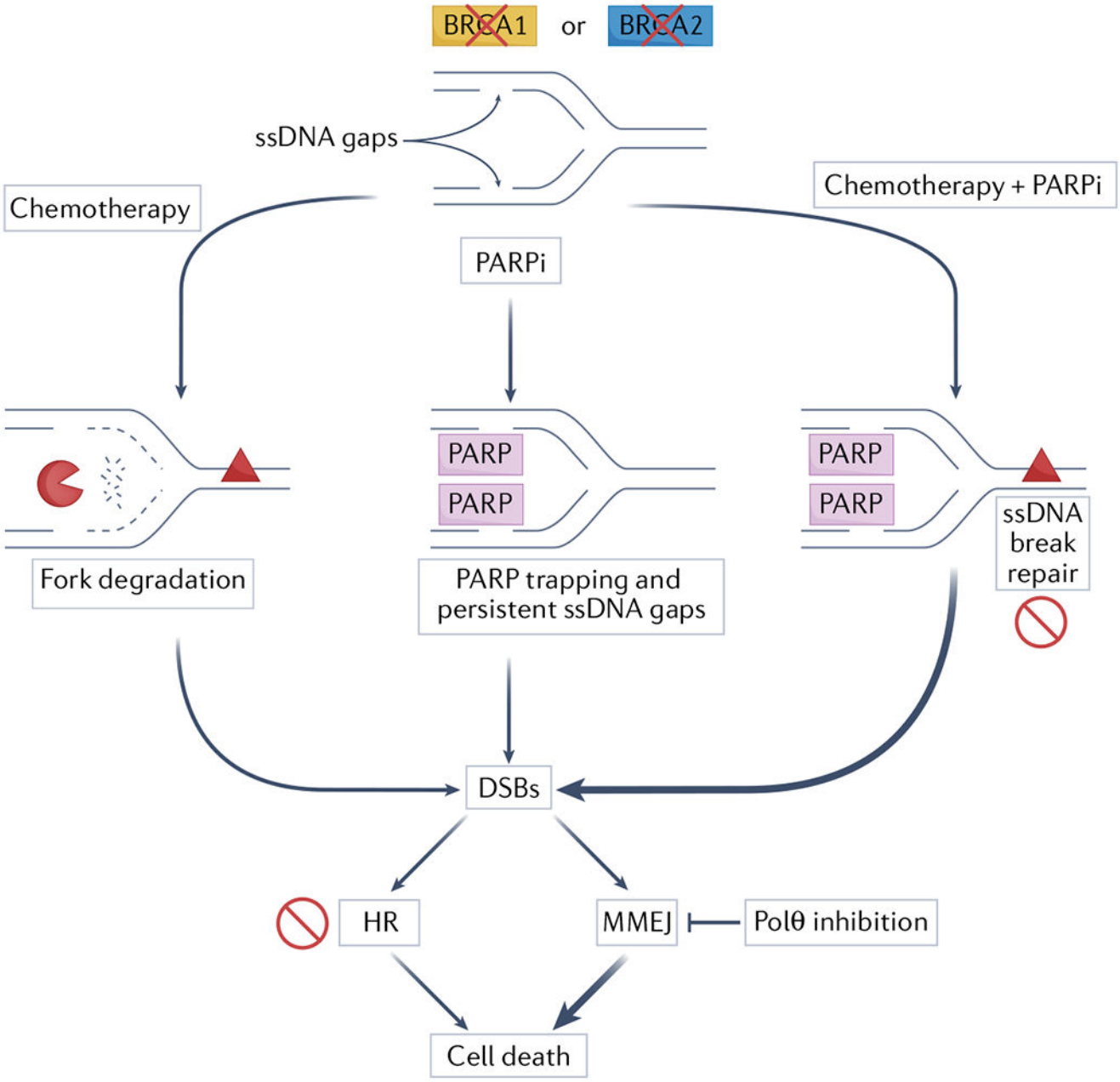


Fig. 3 |. Chemotherapy and PARPi in BRCA-deficient cancers.

Replication-associated single-stranded DNA (ssDNA) gaps accumulate in BRCA-deficient cancer cells. Upon treatment with DNA-damaging chemotherapy, replication forks are subject to extensive nucleolytic degradation, leading to fork breakage and formation of one-ended double-stranded DNA breaks (DSBs). Degradation mainly originates from reversed forks (not shown). Poly(ADP-ribose) polymerase (PARP) inhibition impairs reversal (not shown), and causes trapping of PARP proteins and persistent ssDNA gaps, which lead to DSBs. Combination of chemotherapy and PARP inhibitor (PARPi) therapy exacerbates DSB formation by blocking ssDNA break repair of chemotherapy-induced lesions in addition to causing ssDNA gap accumulation and PARP trapping. In BRCA-deficient backgrounds,

these DSBs cannot be processed by homologous recombination, leading to cell death. These cells also become increasingly reliant on microhomoLogy-mediated end joining (MMEJ) to repair breaks, which provides a rationale for targeting Polθ in BRCA-mutant tumours.

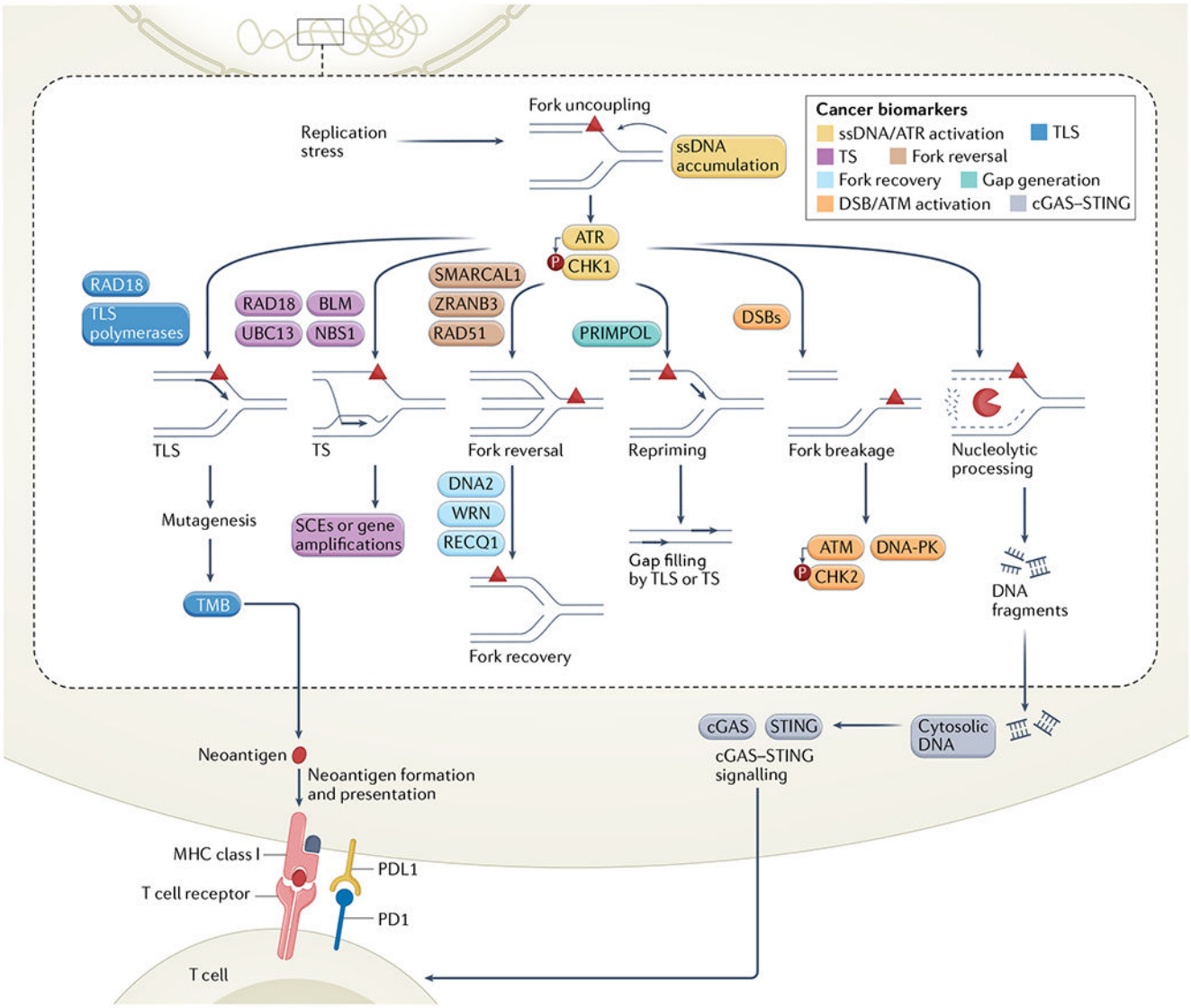


Fig. 4 |. The replication stress response in cancer and implications for cancer biomarkers.
Potential biomarkers are highlighted within the context of different replication stress response pathways. Biomarkers associated with accumulation of single-stranded DNA (ssDNA) and ATR–CHK1 activation in yellow; translesion synthesis (TLS) enzyme expression or activity and TLS-mediated mutagenesis in blue; template switching (TS) enzyme expression or activity and TS-dependent sister chromatid exchange (SCE) or gene amplifications in purple; fork recovery protein expression in light blue; fork reversal protein expression in brown; DNA-directed primase/polymerase protein (PRIMPOL) expression in green and downstream gap filling by TS or TLS-based mechanisms; DNA double strand break (DSB) and ATM activation in orange; and cGAS–STING activation in grey. BLM, Bloom syndrome protein; NBS1, Nijmegen breakage syndrome 1 protein; TMB, tumour mutational burden; WRN, Werner syndrome ATP-dependent helicase.

Table 1 |

Replication stress response in cancer and consequences on genome stability

Replication stress response mechanism	Enzyme	Expression	Activity	Consequences
TLS	RAD18	Moderate or high in most cancers[a,42]	PCNA monoubiquitination[38]	Mutagenesis leading to elevated TMB and increased neoantigen formation, mutagenesis leading to acquired chemoresistance
	REV1	Moderate or high in select liver[a] and lung cancers[a,b,37,49]	TLS scaffold[45], G>C transversions[48]	
	Poζ	Elevated in oesophageal cancers[a,57] and gliomas[b,55], downregulated in CRCs[b], lung[b] and gastric cancers[b,54,58,59]	GC>AA/TT mutations[53]	
	Polη	High in select basal cell carcinomas[a,37] and elevated in head and neck cancers[a,63]	Mutations at WA/TW motifs (where W is A or T)[64,65]	
	Polι	Elevated in breast cancer cells[b,c,72], bladder cancers[a,73] and ESCCs[a,b,56,74]	T>C, T>A or C>A mutations[72]	
	Polκ	Elevated in lung cancers[b,c,81]	CC:GG interruptions on poly(dA:dT) tracts[78]	
TS	UBC13	Moderate in most cancers[a,87]	PCNA polyubiquitination[84,85]	SCEs contributing to gross chromosomal aberrations and increased genomic instability, gene amplification leading to acquired chemoresistance
	HLTF	Low in most cancers[a,90]	PCNA polyubiquitination[88,89]	
	SHPRH	Moderate or high in most cancers[a,91]	PCNA polyubiquitination[88,89]	
	RAD51	Elevated in pancreatic[a,129] and breast cancers[a,130]	Strand invasion, D-loop formation[29,96,97]	
	BLM	Moderate or high in most cancers[a,100]	D-loop dissolution[98,99]	
	NBS1	Moderate or high in most cancers[a,101]	Protein recruitment and regulation[29]	
Fork reversal/ recovery	SMARCAL1	Moderate in select pancreatic, testis, breast, prostate and thyroid cancers[d,117]	Fork reversal[108,109]	Fork reversal consequences include replication fork degradation in specific genetic backgrounds, release of degraded or cleaved DNA into the cytosol, and decreased fork repriming

Replication stress response mechanism	Enzyme	Expression	Activity	Consequences
	ZRANB3	Elevated in testis cancers[d,121]	Fork reversal, interaction with polyubiquitinated PCNA[111,112]	Fork recovery consequences include resumption of DNA synthesis and cancer cell survival
	HLTF	Low in most cancers[a,90]	Fork reversal, PCNA polyubiquitination[113,114]	
	RAD51	Elevated in pancreatic[129] and breast cancers[130]	Fork reversal, fork protection[24]	
	RECQ1	Moderate or high in lymphomas, thyroid, head and neck, and carcinoid cancers[a,139]	Restart of reversed forks[132]	
	WRN	Moderate in select testis, thyroid, and head and neck cancers[a,138]	Stalled fork processing[133]	
	DNA2	Moderate or high in most cancers[a,137]	Stalled fork processing[133]	
	MUS81	Low in most cancers[a,145]	Reversed fork cleavage[146,147]	
	CSB	Elevated in thyroid and breast cancers[d,151]	Stalled fork processing[150]	
	XRCC1	High in most cancers[a,153]	MMEJ at stalled forks[152]	
	RNF168	Downregulated in *BRCA1*-mutated cancers[a,215]	Potential role in break-induced replication-like fork recovery[148]	
Fork repriming	PRIMPOL	Moderate in thyroid cancers[a,162]	Primase, polymerase[25–28]	Increased ssDNA gaps and activation of ssDNA gap filling by TLS or TS, gap processing, and release of DNA into the cytosol

Replication stress response mechanisms with corresponding enzymes, expression levels in cancer and enzyme activities shown. Consequences of each replication stress response pathway are shown on the right. The Human Protein Atlas uses the terms weak, moderate or high to describe the intensity of immunohistochemistry staining on tumour sections. For our purposes, factors expressed at low levels correspond to weak staining, factors expressed at moderate levels correspond to moderate staining and factors expressed at high levels correspond to strong staining. ‘Elevated’ or ‘downregulated’ refers to increased or decreased protein expression relative to either non-malignant controls or other cancer types. BLM, Bloom syndrome protein; CRC, colorectal cancer; ESCC, oesophageal squamous cell carcinoma; HLTF, helicase-like transcription factor; MMEJ, microhomology-mediated end joining; NBS1, Nijmegen breakage syndrome 1 protein; PCNA, proliferating cellular nuclear antigen; PRIMPOL, DNA-directed primase/polymerase protein; SCE, sister chromatid exchange; SHPRH, SNF2 histone linker PHD ring helicase; ssDNA, single-stranded DNA; TMB, tumour mutational burden; TLS, translesion synthesis; TS, template switching; WRN, Werner syndrome ATP-dependent helicase.

[a]Protein level measured by immunohistochemistry;

[b]RNA measured by quantitative PCR;

[c]Protein level measured by western blot;

$d_{\text{RNA measured by RNA sequencing.}}$

Table 2 |

Clinical trials with emerging targeted therapies

Inhibitor	Cancers	Combination treatments	Clinical trial number[a]
ATR inhibitors			
ART0380	Advanced cancer or metastatic cancer, ovarian cancer, primary peritoneal cancer, fallopian tube cancer	Gemcitabine (antimetabolite) and irinotecan (topoisomerase inhibitor)	NCT04657068
AZD4547	NSCLC, squamous cell carcinoma, adenocarcinoma	Durvalumab (PDL1 blockade)	NCT02664935
AZD6738	High-grade serous carcinoma	Olaparib (PARPi)	NCT03462342
	Advanced solid tumours, advanced pancreatic adenocarcinoma	Gemcitabine (antimetabolite)	NCT03669601
	CCRCC, locally advanced pancreatic cancer, metastatic renal cell carcinoma, metastatic urothelial carcinoma, metastatic pancreatic cancer	Olaparib (PARPi)	NCT03682289
	Breast cancers	Olaparib (PARPi) and durvalumab (PDL1 blockade)	NCT03740893
	Prostate cancer	Olaparib (PARPi)	NCT03787680
	Gynaecological cancers	Olaparib (PARPi)	NCT04065269
BAY1895344	Advanced solid tumours	Pembrolizumab (PDL1 blockade)	NCT04095273
	Advanced solid tumours (excluding prostate cancer) and ovarian cancer	Niraparib (PARPi)	NCT04267939
Berzosertib (also known as M6620 and VX-970)	Ovarian serous tumour, recurrent fallopian tube carcinoma, recurrent ovarian carcinoma, recurrent primary peritoneal carcinoma	Gemcitabine (antimetabolite)	NCT02595892
	Metastatic colorectal, lung, small-cell lung and pancreatic carcinomas; refractory colorectal, small-cell lung and pancreatic carcinomas; unresectable colorectal, small-cell lung and pancreatic carcinomas	Irinotecan (topoisomerase inhibitor)	NCT02595931
	SCLC, high-grade neuroendocrine cancers	Lurbinectedin (transcription inhibitor)	NCT04802174
	Adult leiomyosarcoma	Gemcitabine (antimetabolite)	NCT04807816
	Homologous recombination-deficient cancer, SCLC and advanced solid tumours	Sacituzumab govitecan (topoisomerase inhibitor antibody conjugate)	NCT04826341
	NSCLC, SCLC, ovarian cancers, uterine cervical cancers, neuroendocrine carcinoma, extrapulmonary small cell cancer	Topotecan (topoisomerase inhibitor)	NCT02487095
	Advanced stage solid tumours	Monotherapy or in combination with carboplatin and paclitaxel	NCT03309150
	Oesophageal adenocarcinoma, squamous cell carcinoma, solid tumours	Cisplatin, capecitabine (antimetabolite) and radiation	NCT03641547

Inhibitor	Cancers	Combination treatments	Clinical trial number[a]
	Advanced solid tumours	Topotecan (topoisomerase inhibitor)	NCT05246111
	Refractory solid tumours	Veliparib (PARPi) and cisplatin (crosslinking agent)	NCT02723864
Ceralasertib	Advanced solid tumours, head and neck squamous cell carcinoma, NSCLC, gastric cancer, breast cancer and ovarian cancer	Carboplatin (crosslinking agent)	NCT02264678
	Head and neck squamous cell carcinoma	Olaparib (PARPi)	NCT03022409
	Metastatic triple-negative breast cancer	Olaparib (PARPi)	NCT03330847
Elimusertib	Advanced cancers of the gastrointestinal system	Fluorouracil, irinotecan (topoisomerase inhibitor) and leucovorin (enhances fluorouracil)	NCT04535401
	Various metastatic and unresectable cancers	Irinotecan and topotecan (topoisomerase inhibitors)	NCT04514497
	HPV-mediated oropharyngeal carcinoma	Pembrolizumab (PDL1 blockade) and radiation	NCT04576091
IMP9064	Advanced adult solid tumours	Senaparib (PARPi)	NCT05269316
M1774	Metastatic or locally advanced unresectable solid tumours	Niraparib (PARPi)	NCT04170153
M4344	PARPi-resistant recurrent ovarian cancer	Niraparib (PARPi)	NCT04149145
RP-3500	Advanced solid tumours	Talazoparib (PARPi) and gemcitabine (antimetabolite)	NCT04497116
	Advanced solid tumours	Niraparib or olaparib (PARPi)	NCT04972110
VX-970	Refractory solid tumours	Veliparib (PARPi) and cisplatin (crosslinking agent)	NCT02723864
WEE1 inhibitors			
Adavosertib (also known as AZD1775 and MK-1775)	Glioblastoma and recurrent glioblastoma	Temozolomide (alkylating agent) and radiation	NCT01849146
	Cancers of the nervous system	Irinotecan (topoisomerase inhibitor)	NCT02095132
	Ovarian tumours, recurrent fallopian tube carcinoma, recurrent ovarian carcinoma and recurrent primary peritoneal carcinoma	Gemcitabine (antimetabolite)	NCT02101775
	Metastatic or unresectable pancreatic adenocarcinoma	Gemcitabine (antimetabolite) and paclitaxel (antimicrotubule)	NCT02194829
	Metastatic triple-negative breast cancer	Ceralasertib (ATR inhibitor) and olaparib (PARPi)	NCT03330847
	Cancers of the female reproductive system	Cisplatin (crosslinking agent) and radiation	NCT03345784
	Prostate cancer	Monotherapy	NCT03385655
	Advanced solid tumours	Carboplatin and paclitaxel	NCT02341456
	Head and neck squamous cell carcinoma	Cisplatin and radiation	NCT02585973
	Leukaemias and myelodysplastic syndromes/cancers	Cytarabine (antimetabolite)	NCT02666950

Inhibitor	Cancers	Combination treatments	Clinical trial number[a]
	Metastatic colorectal cancer	Irinotecan (topoisomerase inhibitor)	NCT02906059
	Triple-negative metastatic breast cancer	Cisplatin (crosslinking agent)	NCT03012477
	Hypopharynx squamous cell carcinoma, oral cavity squamous cell carcinoma and larynx cancer	Cisplatin and radiation	NCT03028766
	Epithelial ovarian cancer	Carboplatin (crosslinking agent)	NCT01164995
	Pancreatic adenocarcinoma	Gemcitabine (antimetabolite) and radiation	NCT02037230
	Head and neck squamous cell carcinoma	Cisplatin and docetaxel (antimicrotubule)	NCT02508246
CHK1 inhibitors			
LY2603618	NSCLC	Pemetrexed (antimetabolite) and cisplatin	NCT01139775
LY2880070	Ewing sarcoma and Ewing-like sarcoma	Gemcitabine (antimetabolite)	NCT05275426
Prexasertib[b]	Ovarian cancer, breast cancer, prostate cancer	Monotherapy	NCT02203513
	Advanced cancers	Monotherapy	NCT02873975
	Advanced solid tumours	Olaparib (PARPi)	NCT03057145
	Advanced solid tumours	LY3300054 (PDL1 blockade)	NCT03495323
	Recurrent, refractory, or paediatric brain tumours, medulloblastoma and CNS tumours	Cyclophosphamide (alkylating agent) and filgrastim (biologic agent)	NCT04023669
SCH 900776	Adult leukaemias	Cytarabine (antimetabolite)	NCT01870596
SRA737	Advanced solid tumours	Gemcitabine (antimetabolite) and cisplatin	NCT02797977
ATM inhibitors			
AZD0156	Advanced solid tumours	Olaparib (PARPi), irinotecan (topoisomerase inhibitor) and fluorouracil (antimetabolite)	NCT02588105
AZD1390	Glioblastoma, brain cancers and leptomeningeal disease	Radiation	NCT03423628
CHK2 inhibitors			
PHI-101	Platinum-refractory or resistant ovarian carcinoma, platinum-resistant fallopian tube carcinoma and platinum-resistant primary peritoneal carcinoma	Monotherapy	NCT04678102
DNA-PK inhibitors			
AZD7648	Advanced cancers	Pegylated liposomal doxorubicin (topoisomerase inhibitor)	NCT03907969
Peposertib (also known as nedisertib and M3814)	Advanced solid tumours	Cisplatin and radiation	NCT02516813
	Solid tumours	Avelumab (immune-checkpoint blockade) and palliative radiation	NCT03724890

Inhibitor	Cancers	Combination treatments	Clinical trial number[a]
	Glioblastoma and gliosarcoma	Temozolomide (alkylating agent) and radiation	NCT05002140
	Locally advanced rectal cancer	Capecitabine (antimetabolite) and radiation	NCT03770689
Inhibitors of both ATM and DNA-PK			
XRD-0394	Metastasis, locally advanced solid tumours and recurrent cancer	Palliative radiation	NCT05002140
Polθ inhibitors			
ART4215	Advanced or metastatic cancer and breast cancer	Talazoparib (PARPi)	NCT04991480
USP1 inhibitors			
KSQ-4279	Advanced solid tumours	PARPi	NCT05240898

CCRCC, clear cell renal cell carcinoma; CNS, central nervous system; DNA-PK, DNA-dependent protein kinase; NSCLC, non-small-cell lung carcinoma; PARPi, poly(ADP-ribose) polymerase inhibitor; SCLC, small cell lung carcinoma.

[a] NCT numbers, along with cancer types and drugs, were compiled from completed, recruiting and active trials from ClinicalTrials.gov.

[b] Prexasertib has also shown inhibitory activity against CHK2; however, the biological antitumour activity of prexasertib has been linked to CHK1 inhibition and not CHK2 inhibition.