Exploiting replicative stress to treat cancer

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Abstract | DNA replication in cancer cells is accompanied by stalling and collapse of the replication fork and signalling in response to DNA damage and/or premature mitosis; these processes are collectively known as 'replicative stress'. Progress is being made to increase our understanding of the mechanisms that govern replicative stress, thus providing ample opportunities to enhance replicative stress for therapeutic purposes. Rather than trying to halt cell cycle progression, cancer therapeutics could aim to increase replicative stress by further loosening the checkpoints that remain available to cancer cells and ultimately inducing the catastrophic failure of proliferative machineries. In this Review, we outline current and future approaches to achieve this, emphasizing the combination of conventional chemotherapy with targeted approaches.

Checkpoints
Signalling events during the cell cycle that prevent further progression.

Cyclin-dependent kinase
(CDK). A class of kinase that associates with partner proteins known as cyclins. Specific CDKs are active at various phases of the cell cycle to promote cell cycle progression.

Replicative stress
The perturbation of DNA replication that interferes with timely and error-free completion of S phase.

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They stumble that run fast. William Shakespeare, *Romeo and Juliet*

How could one halt a running machine with a broken stop button? Pulling the brakes is one option; another is to remove additional control circuits, thereby increasing the running speed to an unsustainable level, overheating the motors and causing breakage. This approach, although destructive, will also stop the machine in a sustainable manner.

Cancer cells are characterized by a loss of control mechanisms for DNA replication (analogous to the 'stop button'), which causes cellular stress. This phenomenon occurs selectively in cancer cells owing to a loss of cell cycle checkpoints. These checkpoints, which are mediated by tumour suppressors and cyclin-dependent kinase (CDK) inhibitors, are lost or overridden during malignant transformation. Conventional chemotherapy frequently enhances replicative stress: for example, by introducing lesions in template DNA or by incorporating nucleoside analogues during replication. Loss of checkpoints can make tumour cells more susceptible to cell death caused by these drugs than most normal cells. It is a common assumption that cancer therapy should aim to attenuate the proliferation of cancer cells. However, in contrast to this view, further increasing replicative stress in a catastrophic manner could become an alternative therapeutic approach. For example, it may be beneficial to push tumour cells through the cell cycle by further lowering their checkpoint barriers, thus promoting cancer cell death.

But how would one enhance replicative stress in a targeted manner? Several key biological transitions and responses are subject to manipulation: the entry into S phase; the stalling of replication forks; the collapse of such replication forks by separation from the replication enzyme complexes; the repair of damaged DNA during S phase; and the premature entry into mitosis. Each of these processes is governed by signalling pathways, and each of these pathways can be manipulated by drug candidates. This raises the possibility of eliminating cancer cells by exaggerating the same checkpoint losses that initially caused their uncontrolled proliferation.

In this Review, we first define what constitutes replicative stress in cancer cells. Second, we describe mechanisms to enhance such conditions using currently available pharmacological approaches, including conventional chemotherapy and targeted inhibitors. Last, we outline strategies to identify and validate additional targets and compounds that enhance replicative stress and that could therefore improve cancer treatments.

What is replicative stress?

Maintaining and copying genetic information are prerequisites for life. Therefore, it is not surprising that sophisticated mechanisms have evolved to preserve the integrity of cellular DNA and to replicate DNA with high accuracy and efficiency. Nonetheless, DNA replication is subject to errors and interruptions, and cells need to control these processes. If the underlying control and

repair mechanisms function suboptimally, damaged DNA will accumulate, thereby preventing cell proliferation and often leading to cell death.

Research into DNA repair mechanisms initially focused on exogenously induced DNA damage, as occurs in response to irradiation or genotoxic compounds. The majority of such DNA lesions also interfere with DNA replication, rendering cells vulnerable to damage when they go through the S phase of the cell cycle. Later, researchers recognized that damaged DNA can also accumulate during regular DNA replication in the absence of external toxic events. These endogenous replicative errors can result from reactive oxygen species (ROS), stochastic misincorporation of nucleotides or topological hindrance of polymerization[1,2].

DNA damage during S phase gives rise to intermediates that are not normally found in other phases of the cell cycle. When trying to replicate damaged DNA, polymerases at replication forks temporarily cease their activity, a phenomenon known as 'fork stalling'. These stalled forks sometimes manage to repair the DNA and continue replication. Alternatively, they may undergo 'fork collapse' and lose the association between the DNA and the replication apparatus. Collapsed forks often undergo endonuclease-mediated DNA cleavage, which leaves behind double-stranded DNA breaks (DSBs)[3,4].

Damaged DNA also triggers specific signalling cascades (FIG. 1). However, the signals induced by replication fork stalling and/or collapse differ somewhat from those that originate from primary DSBs that occur outside S phase. When replication forks are halted, the replicative minichromosome maintenance (MCM) helicase is thought to continue unwinding DNA for a few hundred base pairs directly downstream of the fork, thereby exposing single-stranded DNA (ssDNA). Replication protein A (RPA) coats the ssDNA, which leads to activation of the serine/threonine protein kinase ATR by ATR-interacting protein (ATRIP). The subsequent ATR signalling cascade includes the phosphorylation of checkpoint kinase 1 (CHK1), the cell cycle checkpoint protein RAD17 and histone H2AX. These events are frequently described as 'replicative stress'. Replicative stress can be detected by the accumulation of stalled replication forks, ssDNA and signalling intermediates, such as phosphorylated substrates of ATR (BOX 1). However, of these phosphorylation events, some in fact facilitate the continuation of DNA replication and dampen replicative stress[3–5].

Are tumour cells prone to replicative stress?

In 2005, work from the Gorgoulis and Halazonetis[6] and Bartek[7] laboratories demonstrated that the signalling intermediates of the DNA damage response accumulate in human cancer cells in the absence of genotoxic therapies, even at early stages of tumour development. These findings indicated that such a DNA damage response forms a barrier against carcinogenesis. This barrier is formed, at least in part, by DNA damage-induced senescence[8]. So how does spontaneous DNA damage arise in tumour cells without any detectable exogenous genotoxicity, and how does this contribute to the elimination of pre-cancerous cells or to tumour progression? Indeed, Hanahan and Weinberg[9] added 'genome instability and mutation' to their original list[10] of hallmarks of cancer. This DNA damage could conceivably accumulate as a result of replicative stress, and this hypothesis has been substantiated by studies that have linked oncogene activation with marked replicative stress[11,12].

Several mechanisms might enhance replicative stress specifically in tumour cells (FIG. 2). Tumour cells need to proliferate and hence replicate DNA. This distinguishes them from most cells in differentiated tissue that rarely or never divide. However, this cannot fully explain the DNA damage observed in tumour cells. Some non-malignant cell types — such as haematopoietic progenitor cells, epithelia of the small intestine and hair follicles — proliferate more rapidly than most tumour cells, but no spontaneous, detectable DNA damage has been reported in these cell types.

The uncontrolled activity of oncogenes also seems to contribute to replicative stress. Most tumour cells show enhanced levels or activities of MYC, the transactivating members of the E2F family, and other oncoproteins that stimulate the G1–S transition. This may lead to the premature onset of S phase, and these cells may contain insufficient amounts of key molecules — such as DNA-replicative enzymes and/or nucleotides — that are necessary for accurate and efficient DNA replication[13]. Some oncogenes can promote replicative stress even more directly. Increased levels of cyclin E alter licensing and firing of the replication origin, which gives rise to ssDNA and replication-associated DSBs[8]. Conversely, reduced activity of some tumour suppressors, such as the retinoblastoma-associated protein RB1 (also known as pRb), p53, the CDK inhibitor p16 (encoded by *CDKN2A*) and the p53-activating MDM2 ligand $p14^{ARF}$ (also encoded by *CDKN2A*), can elicit replicative stress by promoting the G1–S transition[13,14].

Tumour cells also seem to have a greater tendency to accumulate ROS, which can be a consequence of increased MYC activity[15]. Paradoxically, hypoxic conditions can also increase the production of ROS by the mitochondria[16], and tumours are often hypoxic owing to insufficient vascularization[17]. Accumulation of ROS can lead to the formation of 8-oxoguanine — the most common DNA base alteration — which causes mismatched base pairing and can be found at high levels in tumour cells. The removal of such oxidized nucleotides from the pool of deoxyribonucleoside triphosphates requires the enzyme mutT homologue 1 (MTH1; also known as NUDT1). Indeed, inhibition of MTH1 function selectively eliminates cancer cells[18–20]. In cancer cells, increased levels of ROS may promote replicative stress by oxidizing nucleotides, which would then cause the replication fork to stall at lesions[21] or cause forks to collide with single-stranded breaks generated by the apurinic/apyrimidinic (AP) endonuclease[22] during the base excision repair process[23].

To further compound this problem, tumour cells often lack efficient DNA repair systems. For instance, an estimated one-quarter of the recurrently mutated genes in cancer have known roles in DNA repair (these are referred to as 'stability genes' or 'caretaker genes')[24]. Of note, some general DNA repair deficiency syndromes

Nucleoside analogues
Compounds with similarity to nucleosides (components of DNA or RNA) that are often used as drugs to interfere with the polymerization of (deoxy) ribonucleotides.

Checkpoint kinase
A protein kinase that is activated by stress signals and halts cell cycle progression.

are associated with increased cancer incidence, and the tumours in these patients also lack the corresponding repair systems. For example, BRCA1 functions in the repair of not only DSBs[25] but also of ultraviolet (UV)-induced lesions that cause replicative stress[26].

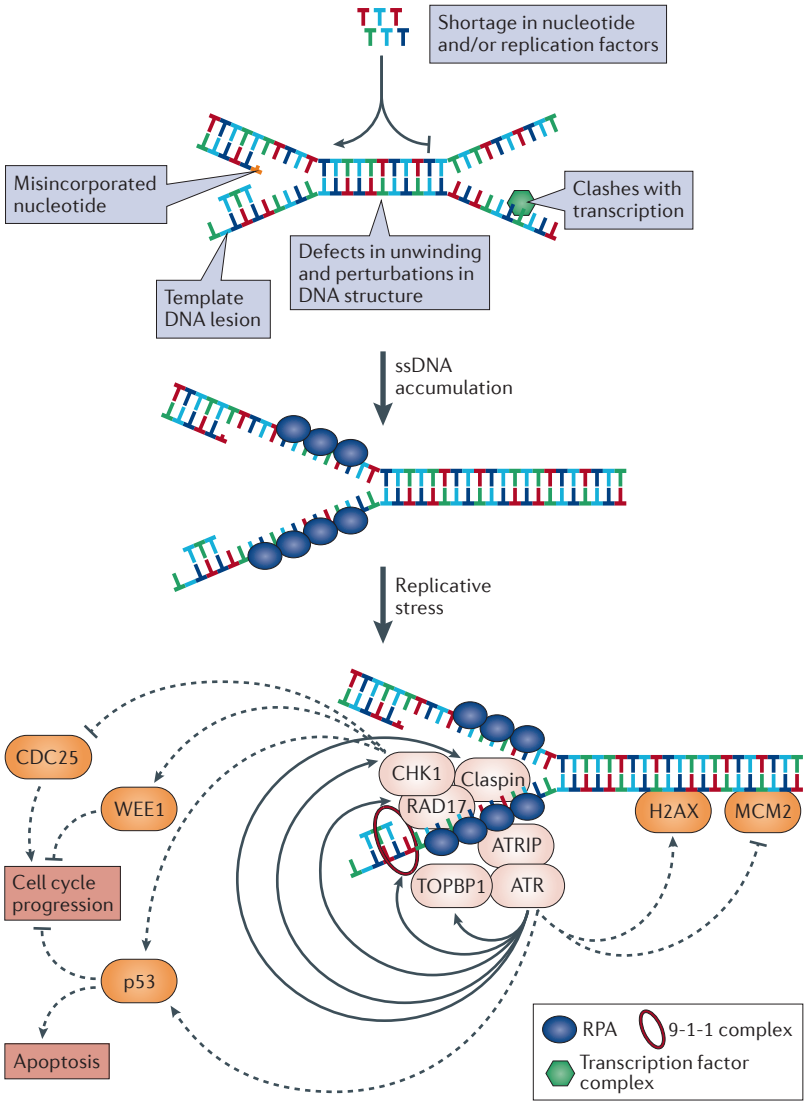

Figure 1 | **Generation of replicative stress and the resulting signalling cascades.** Replicative stress results from endogenous or exogenous obstacles to DNA replication. These include the incorporation of incorrect nucleotides or defects in DNA unwinding, each of which results in a structural hindrance to fork progression; other similar obstacles include lesions in the template DNA or the presence of protein complexes that are involved in transcription. A shortage of nucleotides or replication factors can also impair the progression of ongoing DNA replication. As a result, DNA helicases move ahead of DNA polymerases, and single-stranded DNA (ssDNA) accumulates. This ssDNA recruits replication protein A (RPA), which is the primary trigger for the signalling response to replicative stress. The recruitment of response factors results in activation of the kinase ATR, which in turn phosphorylates not only the regulatory factors in the initial complex but also additional factors that regulate the firing of replication origins, cell cycle progression or apoptosis. The solid arrows indicate phosphorylation events that occur at the stalled fork, and the dashed arrows indicate phosphorylation of proteins that act elsewhere. This response has been reviewed previously[281–283]. 9-1-1, RAD1–HUS1–RAD1; ATRIP, ATR-interacting protein; CDC25, cell division cycle 25; CHK1, checkpoint kinase 1; MCM2, minichromosome maintenance protein 2; TOPBP1, DNA topoisomerase 2-binding protein 1.

Constitutive *BRCA1* deficiency increases the likelihood of breast cancer and other malignancies. Replicative stress can be suppressed by partner and localizer of BRCA2 (PALB2), a key enzyme in the BRCA1–BRCA2–RAD51 homologous recombination repair pathway[27]. Similarly, p53 enhances the expression of the ribonucleotide reductase gene *P53R2* (also known as *RRM2B*), thereby facilitating the repair of damaged DNA[28,29]. Unsurprisingly, this mechanism is attenuated in the large proportion of cancer cells that have malfunctioning p53.

Several replication factors, such as the replication-licensing factors cell division cycle 6 (CDC6)[30] and CDT1 (REF. 31), and alternative DNA polymerases[32] can also be deregulated to promote genome instability and tumorigenesis. Indeed, *CDC6* was listed in the 'census of amplified and overexpressed human cancer genes' (REF. 33).

An insufficient supply of nutrients, such as deoxynucleoside triphosphates (dNTPs) or dNTP precursors, may also decrease the processivity of enzymes at DNA replication forks. Replicative stress has been shown to occur during nutrient depletion in yeast but may also occur in tumours that grow in poorly vascularized areas[34].

Enhancing replicative stress with approved drugs

Most of the currently used anticancer drugs damage DNA and therefore directly or indirectly enhance replicative stress (FIG. 3). The contribution of replicative stress to drug efficacy has recently been elucidated.

Alkylating agents (such as cyclophosphamide, ifosfamide, busulfan, mitomycin C, dacarbazine and temozolomide)[35] and platinum compounds (such as cisplatin, carboplatin and oxaliplatin)[36] function by directly modifying DNA, which typically leads to the formation of intrastrand or interstrand crosslinks between bases. These crosslinks form a barrier against DNA replication and delay the progression of replication forks[37]. Intrastrand crosslinks in the template strand make it difficult to achieve proper base pairing during the incorporation of nucleotides into the newly synthesized strand, a situation that requires translesion synthesis by a specific set of DNA polymerases[38]. By contrast, interstrand crosslinks prevent the first step of replication: that is, the unwinding and separation of strands[39]. Moreover, dacarbazine and temozolomide also cause replicative stress by base methylation and subsequent base excision repair[40], which can result in ssDNA breaks induced by the AP endonuclease[22].

Nucleoside and base analogues induce replicative stress by several mechanisms. Gemcitabine, like hydroxyurea, inhibits ribonucleotide reductase, whereas 5-fluorouracil inhibits thymidylate synthetase. Both drugs reduce the size of the available dNTP pools that are needed for DNA synthesis. In addition to the overall concentrations, the relative amounts of the four dNTPs can be altered by this class of drugs. Both effects enhance replicative stress by reducing the speed of DNA synthesis at individual replication forks[41,42]. Moreover, these analogues can be directly incorporated into nascent DNA strands[43]. This often results in chain termination, although most clinically used nucleoside analogues and their metabolites still contain the 3′ hydroxyl group that is needed to add the next nucleotide. However, by steric distortion of the

Box 1 | **Experimental strategies to detect replicative stress and its mechanisms**

Several technologies have been developed to detect replicative stress and its consequences. These are of crucial importance to the development of new drug candidates to enhance replicative stress.

Detection of replication intermediates

DNA replication can be directly assayed by the incorporation of nucleoside analogues that form epitopes for antibodies. An important method for doing so are the 'DNA fibre assays' (see REF. 257 for a review). Their use in the detection of eukaryotic DNA replication dates back to the 1960s[258], but they have recently been refined by replacing radioactive labelling with fluorescent labelling[259]. Proliferating cells in culture are incubated sequentially (20–120 minutes each) with two different nucleoside analogues, typically chlorodeoxyuridine (CldU) and iododeoxyuridine (IdU). During the second label, a replicative stress-inducing factor (such as a chemotherapeutic) can be added. This is followed by lysing the cells, spreading the DNA on glass slides (either through fluid dynamics or by combing on silanized surfaces[260]), fixing and immunofluorescently staining the DNA with antibodies that bind either to CldU or to IdU. The two colours and the length of their labels on single-stranded DNA (ssDNA) then enable the determination of DNA replication speed and the number of active origins[261]. Slow replication and hyperactive origin firing typically indicate replicative stress.

Another assay that reflects replicative stress is the detection of ssDNA[62], which is located downstream of stalled replication forks as DNA unwinding continues. ssDNA can be detected by antibodies against bromodeoxyuridine (BrdU). Cells are incubated with BrdU and left for a full cell cycle until the cells are in S phase again. At this time, the cells are fixed and stained with antibodies against BrdU. These antibodies detect BrdU only when the DNA is single-stranded. Indeed, when BrdU antibodies are used to quantify cells in S phase, the DNA is denatured under acidic conditions before the antibody is added. Under non-denaturing conditions, BrdU antibodies will only detect DNA that is single-stranded before fixation, as occurs during replicative stress.

Biochemical characterization of replication forks

To identify cellular factors that are not only associated with chromatin but enriched on replication forks (proceeding, stalled or collapsed), a method known as isolation of proteins on nascent DNA (iPOND) has been developed[193,194,262–264]. This technique involves the labelling of newly synthesized DNA with a modified, chemically reactive nucleoside, fixation of chromatin on the DNA (similar to the procedure for chromatin immunoprecipitation) and immobilization of the labelled DNA on beads. Precipitated proteins are then analysed by mass spectrometry and/or immunoblot analysis.

Detection of the cellular response to replicative stress

To analyse the response to replicative stress, conventional analysis of protein modifications (such as detecting phosphoproteins using antibodies) is commonly used. In addition, the relocalization of damage sensor proteins to the sites of stalled replication forks — detected by fluorescence labelling and high-resolution microscopy — is an important hallmark of replicative stress. Examples of this include the relocalization of replication protein A family members and the MRE11–RAD50–NBS1 complex[265].

DNA, they can stall replication forks, particularly when several analogous nucleosides have been incorporated in a row[43]. Furthermore, decitabine is a DNA methyltransferase (DNMT) inhibitor used in the treatment of haematological diseases. Decitabine is incorporated into DNA, where it traps DNMT. This induces the DNA damage response and the formation of chromatid breaks and radial fusion chromosomes during active replication, suggesting that trapped DNMT promotes the collapse of replication forks[44]. Many nucleoside analogues, including 5-fluorouracil[45] and fludarabine[46], can also be incorporated into RNA, and this substantially contributes to their cytotoxicity. Perturbing rRNA synthesis in this manner releases ribosomal proteins from the nucleolus, thus inhibiting the ubiquitin ligase activity of MDM2 and activating p53 (REF. 47). This could arrest cells in the G1 phase or G2 phase, thus decreasing the proportion of cells that replicate their DNA and counteracting the induction of replicative stress. In general, p53 may protect cells from drug-induced replicative stress and enhance drug selectivity for p53-mutant tumour cells.

Finally, topoisomerase inhibitors are also capable of increasing replicative stress. Topoisomerases control DNA supercoiling and entanglement by catalysing nicking and religation of DNA strands[48]. Topoisomerase inhibitors form complexes with their target enzymes as they bind to DNA and thus form a physical obstacle to ongoing replication forks. Subsequent DSBs can arise from replication forks that proceed into ssDNA breaks — which occur owing to inhibition of religation — or through the action of DNA endonucleases as they attempt to resolve the perturbed DNA structure[49]. The cell responds to topoisomerase I inhibitors by reducing DNA replication[50] and by fork reversal (forming a chicken-foot structure)[51]; these structures can be resolved by increasing the activity of ATP-dependent DNA helicase Q1 (REF. 52). Inhibition of CHK1 (see below) can increase the cytotoxic activity of topoisomerase inhibitors, which provides evidence that replicative stress is at least partially responsible for the efficacy of these drugs[53]. Similarly, topoisomerase II inhibitors (such as etoposide) activate CHK1 and interfere with DNA replication[54] by causing transient DSBs. The combination of topoisomerase II inhibitors and CHK1 inhibitors may therefore be worth testing for possible synergies.

Emerging ways to enhance replicative stress

Traditional chemotherapeutics induce replicative stress by directly affecting DNA integrity. The signalling cascades that are induced by DNA damage provide additional targets for intervention (FIGS 2,4). Inactivation of these signalling intermediates with therapeutic agents can increase replicative stress and thereby eliminate cancer cells, and some of these compounds have reached advanced stages of preclinical and clinical development (TABLE 1).

Platinum compounds
A class of chemotherapeutics that include cisplatin, oxaliplatin and carboplatin. Platinum compounds form intrastrand and interstrand crosslinks on DNA.

Translesion synthesis
DNA synthesis by specific enzymes — DNA polymerase-ζ and DNA polymerase-η — to bypass small lesions in the template strand.

Topoisomerase inhibitors
A class of drugs that bind to topoisomerase I (rotating DNA around a nick to relax DNA supercoiling) or topoisomerase II (passing one portion of DNA through another). These inhibitors typically associate with the topoisomerases while bound to DNA, which creates a barrier to DNA replication.

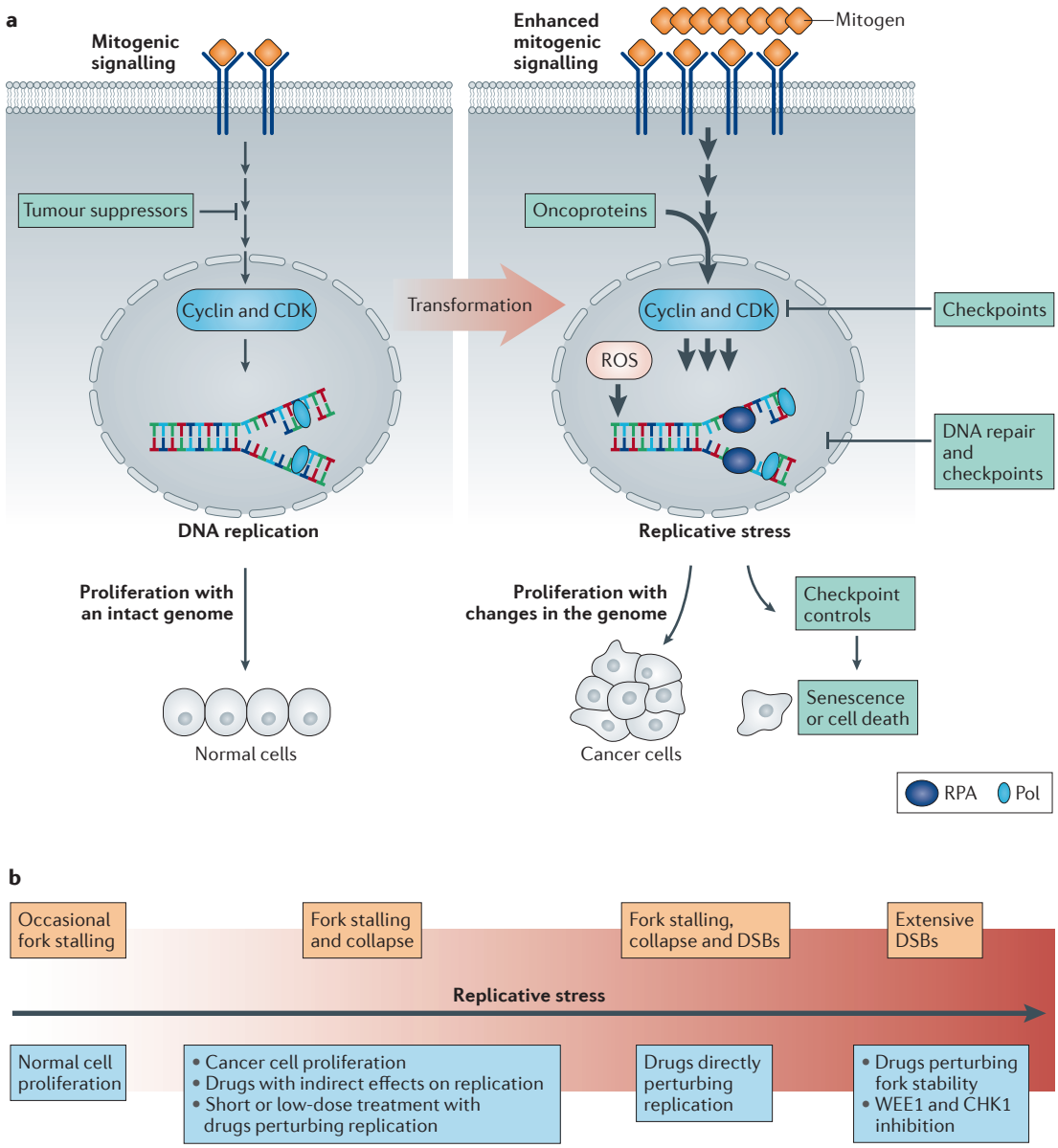

Figure 2 | **Generation and levels of replicative stress in tumour cells.** **a** | The generation of replicative stress through enhanced mitogenic signalling. Proliferative stimuli, especially when permanent, induce replicative stress, unless checkpoint signalling and/or DNA repair are activated. Replicative stress can activate checkpoints, which typically induce senescence or cell death. If such checkpoints fail, transformation and carcinogenesis result. **b** | Levels of replicative stress. Replicative stress is not an on–off phenomenon but can be observed at different levels depending on the extent of mitogenic signalling and the activity of checkpoint and repair mechanisms. CDK, cyclin-dependent kinase; CHK1, checkpoint kinase 1; DSB, double-stranded DNA break; Pol, DNA polymerase; ROS, reactive oxygen species; RPA, replication protein A.

Poly(ADP-ribose) polymerase (PARP) inhibitors were initially found to work particularly well on tumour cells that lack a functional *BRCA1* or *BRCA2* gene product, such as breast carcinomas arising in patients with germline mutations of *BRCA1* (REF. 55). The most clinically advanced PARP inhibitors were recently evaluated in Phase III clinical trials and approved by the US Food and Drug Administration (FDA) for the treatment of advanced ovarian cancer (see the report on the FDA website). In Phase II trials, improved progression-free survival, but not overall survival, was observed in patients with platinum-resistant ovarian cancer[56]. The polymerization of ADP-ribose often occurs in the immediate environment of DNA lesions and facilitates the accumulation of proteins that mediate the DNA damage response at these sites. This includes the association of scaffold attachment factor B1 with chromatin[57], which requires PARP activity and regulates the access of damage response factors that prevent replicative stress. The activation of CHK1 is enhanced by poly(ADP-ribose)[58],

Poly(ADP-ribose) polymerase
(PARP). A class of enzyme that is involved in facilitating DNA repair.

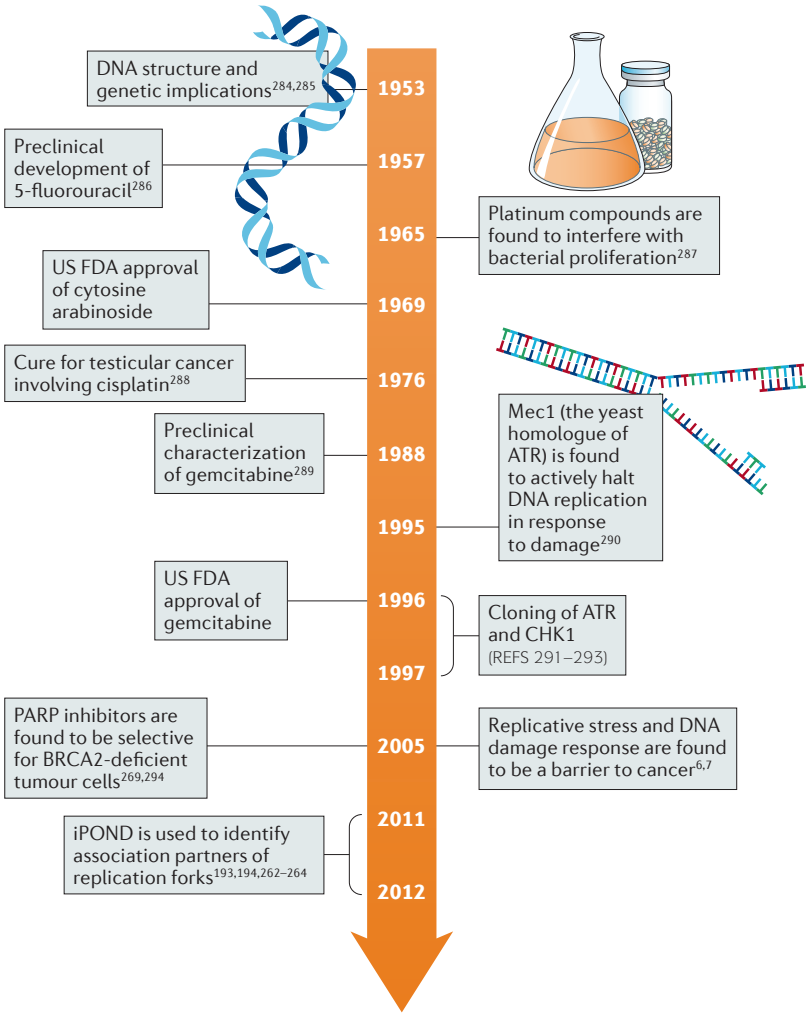

Figure 3 | **Timeline: a history of discoveries leading to drugs and drug candidates that increase replicative stress.** A general overview of the history of chemotherapy is provided in REF. 295. CHK1, checkpoint kinase 1; FDA, Food and Drug Administration; iPOND, isolation of proteins on nascent DNA; PARP, poly(ADP-ribose) polymerase.

which strongly suggests that inhibition of PARP will also increase replicative stress by diminishing the activation of CHK1. Of note, however, not all PARP inhibitors show the same degree of synergism with conventional chemotherapeutics, perhaps because of their differential ability to 'trap' PARP in conjunction with DNA[59].

The ATR–CHK1 signalling cascade has a crucial role in suppressing replicative stress and is targeted by several specific inhibitors. Most notably, targeting CHK1 itself interferes with DNA replication and enhances the DNA damage response during S phase[60,61]. At least one explanation is that CHK1 suppresses CDK activity during S phase, thereby securing the orderly activation of replication origins. In the absence of CHK1, replication is inappropriately initiated from multiple origins, which exhausts replication factors and leads to fork stalling and subsequent collapse[62,63]. Furthermore, CHK1 can phosphorylate and stabilize claspin, which monitors DNA replication[64,65]. Therefore, inhibition of CHK1 would decrease claspin levels and activity, thus preventing smooth fork progression. The promiscuous kinase inhibitor 7-hydroxystaurosporine (UCN-01) — of which CHK1 is a target — was tested in Phase I and Phase II trials and had an acceptable safety profile but uncertain benefit[66,67]. Additional CHK1 inhibitors are currently being evaluated in Phase I trials. Interestingly, the combination of a CHK1 inhibitor and irinotecan was successful in a human-in-mouse model of triple-negative breast cancer, particularly when p53 was mutant[68].

CHK1 activity is strongly enhanced by ATR-mediated phosphorylation; therefore, inhibition of ATR produces similar (but not identical) responses to those observed with inhibition of CHK1 (REF. 69). In addition, ATR phosphorylates SMARCAL1 (a SWI/SNF family member that has annealing helicase activity), thereby limiting its fork regression activity and preventing the collapse of stalled replication forks[70]. Initially, most available small-molecule ATR inhibitors also interfered with the activity of ATM; however, more recently, specific ATR inhibitors — namely, VE-821 — were discovered[71], but clinical trials have not yet been reported. Another ATR inhibitor, AZD6738, is in Phase I trials for lymphatic malignancies (ClinicalTrials.gov identifier: NCT01955668) and advanced solid tumours (NCT02264678 and NCT02223923).

An alternative or additional approach to inhibit CHK1 is to target WEE1. This kinase phosphorylates CDK1 and CDK2, rendering them less active. When WEE1 is inhibited by drugs, CDK activity is enhanced and cells in S phase can be induced to enter mitosis prematurely, even before DNA replication is complete[72]. Moreover, the burst in CDK activity after inhibition of WEE1 rapidly increases the initiation of replication. This leads to a shortage of nucleotides required for DNA replication, which reduces replication fork speed and is followed by DSBs mediated by the endonuclease MUS81 (REF. 73). Hence, inhibition of WEE1 could be a powerful means to force cancer cells with high levels of replicative stress to enter mitosis prematurely and subsequently cause cell death[74]. Numerous clinical trials that involve the WEE1 inhibitor MK-1775 have been registered, but the results have not yet been reported. Inhibitors of heat shock protein 90 (HSP90) and its chaperone function may affect WEE1 activity and could therefore provide an alternative or additional approach to target WEE1 indirectly. WEE1 associates with and is stabilized by HSP90, and this association is enhanced by WEE1-mediated phosphorylation of HSP90. Thus, WEE1 inhibitors interfere with HSP90 function, but direct HSP90 inhibitors also destabilize WEE1 (REFS 75,76).

Replicative stress can also be increased by inhibiting a ubiquitin-like modification known as neddylation. Neddylation can be inhibited by targeting NEDD8-activating enzyme (NAE) — the only known E1 ligase for NEDD8 — using small-compound inhibitors such as MLN4924 (REF. 77). Neddylation is a prerequisite for the activity of cullin-like ubiquitin ligases. As a result, inhibitors of neddylation indirectly inactivate a large class of ubiquitin ligases. Consequently, the substrates of these ubiquitin ligases accumulate. One of these substrates is CDT1, a factor that forms a complex with the replication-licensing factor geminin. When CDT1 is

non-ubiquitylated and therefore accumulates, endoreduplication occurs as DNA is replicated more than once per S phase[78,79]. In preclinical models with various cancer cell lines, MLN4924 induced cell death and delayed xenograft tumour growth[80,81], but escape mutants of NAE were also observed[82,83]. Clinical trials have been registered.

Another interesting emerging target to increase replicative stress is maternal embryonic leucine zipper kinase (MELK), one of the AMP-activated serine/threonine protein kinases. MELK has been found at increased levels in multiple malignancies[84], and its removal reduces clonogenic survival of tumour cells[84,85]. It can contribute to a stem cell-like phenotype, which could promote tumour formation[86]. More recently, however, the inhibition or knockdown of MELK was found to increase the accumulation of stalled replication forks and induce a DNA damage response during S phase in glioma cells[87]. Although the mechanisms remain to be elucidated, recently developed, orally administrable MELK inhibitors[88,89] hold promise as enhancers of replicative stress in tumours in a clinical setting, and a Phase I trial has been registered (ClinicalTrials.gov identifier: NCT01910545).

In addition to targeting regulatory pathways, recently developed small molecules inhibit replication by a more direct mechanism to increase the stress burden. CDC7, along with its activator DBF4, was extensively described as a mediator of replication origin assembly and firing through its ability to phosphorylate MCM proteins[90,91]. Accordingly, inhibition of CDC7 using small compounds, such as PHA-767491 (REF. 92), was proposed as a strategy to halt DNA replication without interfering with ongoing replication forks, thus avoiding DNA damage[93]. Interestingly, however, its inhibition not only prolonged S phase but also eliminated tumour cells, especially those that lacked functional p53 (REFS 94,95). Since then, CDC7 has been found to have a role in the response to replicative stress. CDC7 is required for the recruitment of the E3 ubiquitin ligase RAD18 to sites of stalled replication forks, and RAD18 enables the recruitment of the translesion DNA polymerase-η[96]. Therefore, inhibition of CDC7 could be a useful strategy to increase replicative stress by disabling translesion synthesis[97].

Inhibitors of the bromodomain and extra-terminal (BET) family of proteins have attracted widespread attention as a means to manipulate the transcriptional profile of tumour cells and thereby impair tumour progression[98,99]. JQ1, a BET family inhibitor, was recently found to suppress the progression of replication forks[100]. As this effect could be observed within 1 hour of drug addition, long-term regulation of transcription cannot be held accountable for this effect. It therefore remains to be determined whether JQ1, perhaps by interfering with BET protein–chromatin interactions during S phase, imposes replicative stress on tumour cells.

Similar observations were made with inhibitors of histone deacetylases (HDACs). These inhibitors were primarily developed to manipulate tumour cell transcription, but they also impaired tumour growth[101]. Currently, one HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), has been approved by the FDA for the treatment of cutaneous T cell lymphoma. Surprisingly, a 4-hour treatment of cells with SAHA is sufficient to decrease replication fork speed and trigger the compensatory firing of previously dormant replication origins[102]. Again, it will be of interest to find out whether these effects are triggered indirectly by short-term changes in gene expression or directly by altered chromatin dynamics; moreover, the question of how much this phenomenon contributes to the anticancer efficacy of HDAC inhibitors remains to be answered.

Drug combinations

The above-mentioned drugs can be used in combination with each other or with conventional chemotherapeutics to further boost replicative stress. Synergistic effects have been observed in several cases.

The specific targeting of checkpoint signalling combined with nucleoside analogues has been extensively studied. Gemcitabine efficacy was cooperatively increased when combined with inhibitors of CHK1 (REF. 103), ATR[104], WEE1 (REFS 72,105,106) or NAE[107]. A Phase I clinical trial was completed with the CHK1 inhibitor AZD7762, with or without gemcitabine, but revealed unexpected cardiotoxicity[108,109]; therefore, alternative compounds for this target should be tested.

Platinum compounds frequently function synergistically with checkpoint inhibitors, including inhibitors of CHK1 (REF. 110), ATR[111,112], WEE1 (REF. 72) and NAE[113]. Moreover, chromatin modifiers and drugs that affect them may alter the access of platinum compounds to the DNA[114]. Interestingly, inhibitors of MAP kinase-activated protein kinase 2 (MK2; also known as MAPKAPK2) seem to have different effects depending on whether cisplatin or gemcitabine is used in conjunction with them. MK2 is structurally similar to CHK1 and CHK2 (REF. 115), which suggests that it might function in cell cycle checkpoints. Indeed, removing MK2 sensitizes p53-deficient tumour cells to cisplatin and doxorubicin, perhaps by inducing a premature G2–M transition[116–118]. By contrast, however, inhibition of MK2 rendered cancer cells tolerant to gemcitabine, restoring DNA replication to near-normal levels despite the presence of the drug[41,119]. These contrasting observations may be due to differences in the DNA lesions that were formed with cisplatin versus gemcitabine. Gemcitabine forms lesions only while DNA is being replicated (by inhibition of ribonucleotide reductase as well as incorporation into the DNA), thus only perturbing one of the strands. By contrast, platinum compounds form not only intrastrand but also interstrand crosslinks. It is conceivable that the machineries that cope with each of these lesions are differentially affected by MK2, resulting in opposite effects of MK2 inhibitors. In any case, these observations highlight the need to explore the interactions between signalling inhibitors and conventional chemotherapeutics on an individual basis, taking into account the characteristics of each chemotherapeutic. For example, the efficacy of gemcitabine might be increased by enhancing MK2 activity, thereby increasing replicative stress and causing cell death. One strategy to achieve this would be to inhibit MAP kinase phosphatase 1 (MKP1; also known as DUSP1), thereby activating p38 and its downstream

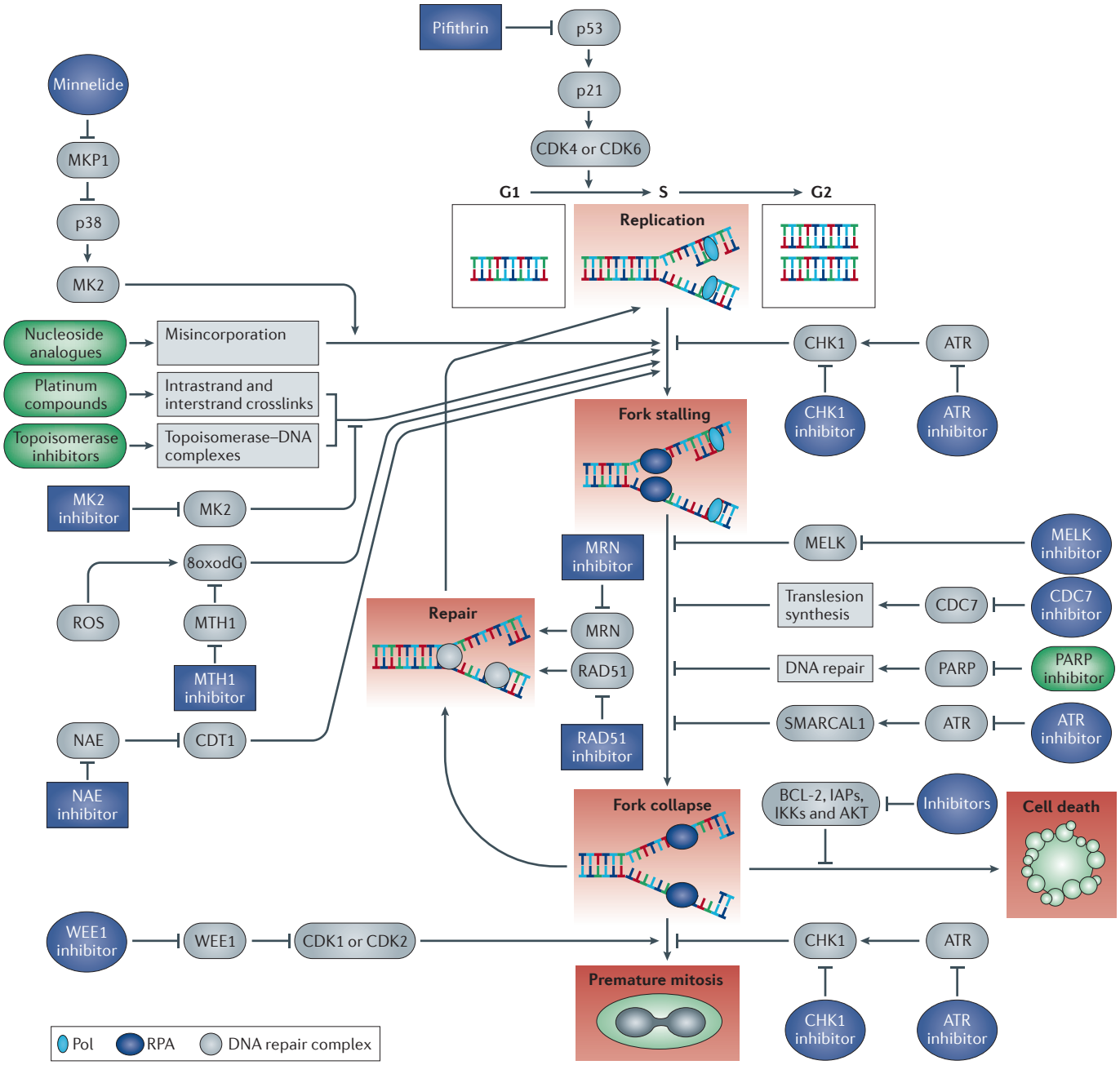

Figure 4 | **Targets to increase replicative stress.** Putative targets (grey) could be used to increase replicative stress using specific inhibitors. Compounds that are approved by the US Food and Drug Administration as drugs are shown in green. Compounds that are in clinical studies but not yet approved are shown as blue ovals, and compounds at preclinical stages of development are shown as blue rectangles. 8oxodG, 8-oxo-deoxyguanosine; CDC7, cell division cycle 7; CDK, cyclin-dependent kinase; CHK1, checkpoint kinase 1; IAP, inhibitor of apoptosis; IKK, inhibitor of nuclear factor-κB kinase; MELK, maternal embryonic leucine zipper kinase; MK2, MAP kinase-activated protein kinase 2; MKP1, MAP kinase phosphatase 1; MRN, MRE11–RAD50–NBS1; MTH1, mutT homologue 1; NAE, NEDD8-activating enzyme; PARP, poly(ADP-ribose) polymerase; Pol, DNA polymerase; ROS, reactive oxygen species; RPA, replication protein A.

effector MK2. Indeed, the MKP1 inhibitor Minnelide (Minneamrita Therapeutics LLC, Minnesota, USA) decreased tumour growth in mouse models of pancreatic carcinoma[120].

Indirect targeting of checkpoint mediators may be useful to overcome their cytoprotective effects. Many of them depend on the activity of HSP90, a druggable chaperone. Indeed, HSP90 inhibitors increase replicative stress by reducing CHK1 and ATR levels and cooperate with gemcitabine to increase DNA damage[121,122]. These phenomena provide an example of how broad-range drugs that each target an essential piece of molecular machinery can be combined to achieve cytotoxic effects in tumour cells by exploiting their vulnerabilities[123].

Targeted inhibitors can also be combined with each other to achieve synergistic effects on tumour cells. The combination of CHK1 inhibitors and WEE1 inhibitors[72,124–128] enhances replicative stress and promotes mitotic catastrophe. Inhibition of CHK1 also suppresses homologous recombination repair, thereby further sensitizing cells to WEE1 inhibitors[129,130]. PARP inhibitors can also be synergistically combined with CHK2 inhibitors[131,132]. This combination supposedly broadens the range of tumours that respond to PARP inhibitors. This presumably also enhances replicative stress, but the exact mechanisms remain unexplored.

Inadvertent inhibition of replicative stress

Drugs that inhibit cell proliferation also reduce the proportion of cells in S phase and would therefore negatively interfere with promoters of replicative stress. Understanding the antinomy between cell cycle arrest and replicative stress might help in choosing the right drug combinations and in determining the correct time to administer these drug combinations to avoid this negative drug interference.

Most chemotherapeutics activate p53, at least when it is still wild type. DNA damage triggers the ATM–CHK2 and/or the ATR–CHK1 signalling pathways, each of which leads to the phosphorylation and subsequent acetylation of p53 (REF. 133). Such modifications interfere with MDM2-mediated degradation of p53 and enable enhanced p53 transcriptional activity. More recently, drugs have been developed that target the ability of MDM2 to inactivate p53, and these are undergoing extensive preclinical and early clinical investigation[134]. Activation of p53 can either induce apoptosis or arrest cells in the G1 phase or G2 phase. Cell cycle arrest occurs through the induction of the CDK inhibitor p21 (also known as CIP1; encoded by *CDKN1A*)[135]. Arrested cells are protected from therapeutic regimens that enhance replicative stress, such as nucleoside analogues. Indeed, activation of p53 by the MDM2 antagonist nutlin 3a[136] potently protects cells from treatment by gemcitabine[137], the lethal effects of UV irradiation[138] and inhibitors of mitosis[139]. Thus, at least in tumours that express wild-type p53, chemotherapy may induce cell cycle arrest and thereby interfere with the activity of nucleoside analogues and other drugs that rely on replicative stress.

Even if p53 is mutant, its transactivating paralogues p73 and p63 might take over its functions to induce cell cycle arrest. For example, cisplatin activates p73 through ABL signalling[140–142], and p63 expression is induced by HDAC inhibitors[143]. In both cases, although apoptosis is induced in a subset of cells, cell cycle arrest compromises replicative stress and limits the efficacy of chemotherapeutics that rely on it.

Cancer drugs can induce cell cycle arrest by means other than p53 activation, such as inhibition of CDKs[144,145]. Again, it should be noted that such compounds may interfere with other drugs by reducing replicative stress. Indeed, although the CDK inhibitor flavopiridol synergized with gemcitabine in breast cancer cells, it only did so when gemcitabine was administered first[146,147]. Thus, CDK inhibitors negatively interfere with the efficacy of nucleoside analogues, unless the analogues are first incorporated into the DNA.

Similar considerations apply to the use of proteasome inhibitors such as bortezomib, which has been approved for the treatment of multiple myeloma. Drugs of this class have many molecular effects that promote cell cycle arrest, including increasing the levels of cellular CDK inhibitors such as p21 (REF. 148) and p27 (also known as KIP1; encoded by *CDKN1B*)[149]. Indeed, a drug screen to find modifiers of p27 activity identified the proteasome inhibitor argyrin[150]. When combinations of bortezomib and gemcitabine were tested on cultured pancreatic cancer cells, the sequential addition of gemcitabine and bortezomib was the most effective at killing cells[151]. Thus, proteasome inhibitors may interfere with the efficacy of nucleoside analogues and other enhancers of replicative stress at least in part by inducing cell cycle arrest.

The order and timing of drug application may be essential to avoid negative drug interference. Drugs that rely on replicative stress should probably be given first and only then followed by drugs that induce cell cycle arrest outside S phase.

Drugs that induce replicative stress have been in clinical use for many years, and there is considerable knowledge about the mechanisms that enable tumour cells to eventually escape the treatment regimen. These include increased expression or mutation of drug targets as well as reduced drug uptake and increased drug efflux. In addition, however, avoidance or attenuation of replicative stress is an important means of resistance development.

First, drugs that induce replicative stress are not efficient in slowly proliferating cells that can evade the effects of the drug by pausing in the G1 phase of the cell cycle. Cancer stem cells are often characterized by slow proliferation rates, and it has been suggested that this trait underlies part of the difficulty in eliminating these cells[152].

Second, pathways that are modulated in cancers include the WEE1 and ATR–CHK1 pathways, which protect against replicative stress. Accordingly, CHK1 levels are increased in several MYC-expressing tumours[153,154]. These findings have further promoted the interest in targeting the ATR–CHK1 pathway. Mechanisms that increase the tolerance of cells to defective ATR–CHK1 signalling are beginning to emerge: MK2 deficiency restores DNA replication fork rates in cells with impaired CHK1 activity[41], and E3 ubiquitin ligase RNF4 deficiency diminishes replication fork collapse in cells that lack ATR[155]. Furthermore, the inhibition of WEE1 and the resulting replicative stress are better tolerated when nucleotide levels are increased, which normalizes replication speed and reduces the number of DSBs[73].

Last, tolerance of replicative stress can also be expected in situations in which the repair of DNA lesions — in particular, those that cause collapsed replication forks — is activated; this would again render cancer cells more resistant to therapy[156]. These mechanisms of tolerance have been observed in experimental systems but need to be addressed in patients, as they could be used as potential biomarkers.

Table 1 | **Drugs and drug candidates that increase replicative stress**

Target or mechanism of action	Compounds	Clinical stage (ClinicalTrials.gov identifier)	Refs
Incorporation into DNA	• Nucleoside analogues • Cytosine arabinoside • 5-fluorouracil • Gemcitabine • 5-azacytidine • Decitabine	Approved	43
Modification of DNA	• Alkylating agents • Platinum compounds	Approved	35,36
Ribonucleotide reductase	Gemcitabine	Approved	296
Thymidylate synthetase	5-fluorouracil	Approved	45
PARP1	Olaparib and niraparib	Approved by the US FDA in December 2014	297–299
CHK1	7-hydroxystaurosporine (UCN-01)	Phase II completed, but pharmacokinetics results are inconclusive	66
CHK1	AZD7762	Phase I completed and was found to be inacceptable owing to cardiotoxicity	108,109
CHK1	SCH 900776	Phase I completed, with recommendation for Phase II (combination with Ara-C for refractory acute leukaemias)	300
WEE1	MK-1775	Ongoing Phase II for pancreatic cancer (NCT02194829) and ovarian cancer (NCT02151292)	74 (a review of preclinical data)
ATR	VX-970 and others	Ongoing Phase I (NCT02157792) and preclinical	104,301–304
ATR	AZD6738	Ongoing Phase I (NCT01955668, NCT02264678 and NCT02223923)	–
MELK	OTSSP167	Ongoing Phase I (NCT01910545) and preclinical	88
MKP1	Minnelide	Ongoing Phase I (NCT01927965)	120
NAE	MLN4924	Ongoing Phase I (NCT02122770, NCT01814826 and NCT01862328)	77
CDC7	PHA-767491	Preclinical	92
APC/C	TAME	Preclinical	185
p53	Pifithrin	Preclinical	183
MTH1	TH287 and TH588	Preclinical	20

APC/C, anaphase-promoting complex (also known as the cyclosome); Ara-C, cytarabine; CDC7, cell division cycle 7; CHK1, checkpoint kinase 1; FDA, Food and Drug Administration; MELK, maternal embryonic leucine zipper kinase; MKP1, MAP kinase phosphatase 1; MTH1, mutT homologue 1; NAE, NEDD8-activating enzyme; PARP1, poly(ADP-ribose) polymerase 1; TAME, tosyl arginine methyl ester.

Hazards of enhancing replicative stress

Enhancing replicative stress may have toxic effects. A known unwanted effect of nucleoside analogues and other drugs that induce replicative stress is that they destroy rapidly dividing normal cells, which include haematopoietic cells, the epithelia of the gut and hair follicles. Indeed, myelosuppression is the dose-limiting toxicity of gemcitabine (a key nucleoside analogue)[157] as well as of many other DNA-damaging chemotherapeutics[158]. The therapeutic window may be widened by the fact that rapid proliferation does not necessarily mean replicative stress in normal cells. Normal cells, even those that are dividing fast, still contain all of the checkpoint and repair factors; this protective arsenal is incomplete in most tumour cells, conceivably rendering them more susceptible to exogenous enhancement of replicative stress. Nonetheless, the induction of replicative stress in essential and rapidly proliferating normal tissue is likely to be the most substantial barrier against the overall strategy of enhancing replicative stress. This underscores the need to provide specific protection for normal cells (see below).

In situations in which replicative stress is deliberately enhanced, another unwanted effect may be the accumulation of mutations in cells that misincorporate nucleotides but survive therapy. Depending on the genes affected by such mutations, these tumours may become even more aggressive. In some normal cell populations, such as haematopoietic stem cells, mutations that occur in critical growth regulatory genes may also give rise to secondary malignancies or pre-malignancies, such as secondary myelodysplastic syndrome, which often results in acute myeloid leukaemia[159]. Mutations in both primary cancer cells and secondary malignancies have been observed in patients treated with chemotherapy. The contribution of

replicative stress to these outcomes is difficult to assess. However, replicative stress has indeed been shown to result in an increased mutation rate *in vitro*; in particular, the repair of collapsed replication forks can result in gene duplications[160].

Protection of normal cells

To protect normal cells from replicative stress without compromising the cytotoxic effects on tumour cells, characteristic differences between malignant and normal cells can be exploited. One obvious difference is tumour-associated mutations, and the most frequently mutated gene in human malignancies is *TP53*, which encodes p53. Therefore, p53 function could conceivably be exploited in normal cells to confer protection against replicative stress. It is not unreasonable to assume that this is happening with currently used therapies, as their DNA-damaging activities can result in p53 activation and therefore in cell cycle arrest in normal cells. In addition, however, p53 can be pre-activated by non-genotoxic therapeutic approaches: namely, by MDM2 inhibitors such as nutlin 3a[134,136]. At least *in vitro*, using this strategy to activate p53 does not necessarily lead to apoptosis but instead induces cell cycle arrest in the G1 phase. Therefore, pharmacological activators could protect p53-proficient cells from nucleoside analogues[137] and other treatment regimens that affect cells selectively during DNA replication[161–163]. This approach has not yet been evaluated in the clinic, largely owing to delays in the clinical evaluation of several MDM2 antagonists. However, recently developed second-generation MDM2 inhibitors are being investigated in early clinical studies for the treatment of liposarcomas[164] and acute myeloid leukaemia (ClinicalTrials.gov identifier: NCT02098967). Thus, the exploitation of the protective effects of such compounds in normal cells may eventually be feasible.

Integration into treatment strategies

In light of the synergies and antagonisms between current cancer therapies and replicative stress, how could the deliberate induction of replicative stress be part of therapeutic regimens? To be successful, it must be integrated into an overall strategy that includes surgery, adjuvant and neoadjuvant radiotherapy, and immunotherapy.

Irradiation in patients with tumours — often confined to the tumour location — frequently forms part of cancer therapy, raising the question of whether replicative stress contributes to the efficacy of radiotherapy. The effect of ionizing irradiation on DNA replication has long been investigated, and these investigations have often focused on radioresistant DNA synthesis. Normal cells decrease their DNA synthesis (as measured by the incorporation of labelled nucleotides) within 30–60 minutes after ionizing irradiation, whereas cells with defective mediators of the DNA damage response (ATM, CHK2 and CDC25A[165]; NBS1 (REF. 166); CHK1 (REF. 167); and WEE1 (REF. 73)) largely maintain their previous rate of DNA synthesis. The ability of most cells to decrease DNA replication in response to DSB-inducing irradiation implies that this response to DSBs provides an evolutionary advantage. Targeted interference with stress signalling — for example, through inhibition of checkpoint kinases — might therefore augment the detrimental effects of irradiation on tumour cells. Indeed, CHK1 is a key determinant of cell sensitivity to ionizing irradiation[168,169], and inhibition of WEE1 radiosensitizes cancer cells[170,171]. Several deregulated pathways contribute to the effects of checkpoint inhibition, including attempts to replicate DNA with non-repaired DSBs. This can result in collapsed replication forks and/or erroneous chromosomal rearrangements, which can ultimately induce cell death. Moreover, DNA replication after irradiation results in secondary DSBs[172]. Thus, pushing cells into S phase during irradiation may increase tumour cell death.

Historically, most chemotherapeutic regimens were initiated after surgery in an attempt to eliminate those tumour cells that had disseminated before the primary tumour was removed. This is still often the case, and chemotherapy in this 'adjuvant' setting is expected to rely, at least in part, on the induction of replicative stress. However, chemoradiotherapy can also be used to increase the success of subsequent surgery; this is known as neoadjuvant therapy. The mechanistic basis of such regimens is not entirely clear, but clinically neoadjuvant therapy can be more efficacious than therapy in purely adjuvant settings: for example, in patients with rectal cancer[173,174]. It remains to be determined whether replicative stress contributes to such successes and whether deliberately enhancing this stress would further improve the outcome. In principle, it is conceivable that, by targeting the most rapidly proliferating cancer cell populations, replicative stress might decrease the tumour burden that remains to be removed by surgery.

An additional underexplored area is the interplay between therapy-induced replicative stress and the inflammatory and immune response. Some nucleic acids, such as hybrids derived from bacteria or viruses, can induce signalling through several Toll-like receptors[175]. It is tempting to speculate that the stress-associated intermediates of DNA replication or therapy-induced adducts could trigger Toll-like receptor signalling or another inflammatory response, but this has not yet been reported. If so, substantial therapeutic benefit could be gained by enhancing the inflammatory response to tumours at a time when some tumour cells have died and therefore released such DNA fragments into the tumour stroma.

It also remains to be seen whether replicative stress confers greater susceptibility to the adaptive immune response. This would be of particular interest in the context of recently developed enhancers of cytotoxic T cell activity, including antibodies against cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed cell death protein 1 (PD1)[176]. Both the innate and the adaptive immune responses to tumour cells can be enhanced by conventional chemotherapy[177] through the release of damage-associated molecular patterns[178], but the contributions of replicative stress to this phenomenon is currently unknown.

Taken together, the integration of replicative stress into a broader range of anticancer strategies remains an underexplored but promising area for future research.

Future targets to enhance replicative stress

In addition to the currently available compounds and molecules that enhance replicative stress, there are several targets and approaches that could be exploited in the future to achieve the same goal.

Promoting the G1–S transition. Replicative stress requires the onset of DNA replication. Thus, at least under some circumstances, it may be beneficial to push cancer cells into S phase. This view seems to be contradictory to the overall goal of halting cancer progression, as many current strategies aim to arrest the cell cycle with agents — such as CDK inhibitors[144,179] or MDM2 antagonists — that induce p53 activity[134,136]. However, such strategies will only be effective when cell cycle arrest becomes permanent: that is, by inducing senescence. The S phase-promoting approach, although promising, should be limited in time — for example, by metabolization of the drug — to avoid unwanted proliferation and tumour progression.

Cancer relapse and metastasis may occur years and even decades after successful removal of the primary tumour. This can be attributed to residual tumour cells, a phenomenon known as dormancy. Dormant tumour cells are characterized by cell cycle arrest and may be cancer stem-like cells[180,181]. We propose that dormant cancer cells may be made amenable to therapy if they are forced to undergo cell cycle progression, a strategy that is comparable to the attempts to eliminate dormant lentiviruses by forcing virus replication[182]. Currently, no clinically established pharmacological approach exists to push cells into S phase, but a few related concepts are emerging.

Inhibitors of p53 have been developed preclinically[183,184] and are capable of lowering p53 activity, which reduces the levels of the cellular CDK inhibitor p21. Additional ways to push cells to enter S phase are conceivable. The APC/C (anaphase-promoting complex; also known as the cyclosome) has ubiquitin ligase activity that limits the accumulation of cell cycle-promoting factors. A pharmacological inhibitor of APC/C, tosyl arginine methyl ester (TAME), has been described[185]. APC/C is activated by two substrate adaptors, CDH1 (also known as FZR1) and CDC20; CDH1 is mainly active in the G1 phase, and CDC20 is active in mitosis. TAME interferes with APC/C binding to CDC20. The development of CDH1-specific compounds would be useful to probe the potential use of APC/C inactivation to transiently promote entry into S phase. A more direct way to enhance the G1–S transition would be to inhibit the retinoblastoma family of proteins, RB1 and its paralogues RBL1 (also known as p107) and RBL2 (also known as p130). Small DNA tumour viruses have evolved mechanisms that enable them to induce the G1–S transition in previously quiescent cells by inactivating RB1 and related proteins[186]. However, to our knowledge, there is currently no small compound that can function similarly. PARK2 is a ubiquitin ligase that destabilizes cyclin D1 and cyclin E1; therapeutic inactivation of PARK2 may increase the proliferation rate of tumour cells[187].

By enhancing proliferative intracellular signalling, several growth factors and hormones may ultimately increase the readiness of tumour cells to proceed through the cell cycle. For example, the receptors for oestrogen and epidermal growth factor are typically subject to antagonizing therapies to prevent cancer cell growth but could also be targeted with agonists. However, the pleiotropic effects of such agonists, and their possible anti-apoptotic effects, will make their use in cancer therapy difficult.

Taken together, the targeted transient induction of the G1–S transition is mostly theoretical at present but deserves further exploration as a means to sensitize tumour cells to inducers of replicative stress. Enhancing cell proliferation to boost replicative stress also risks promoting tumour growth and increasing the malignancy of quiescent tumour cells, unless proliferating cells can be killed in a comprehensive and reliable manner. Therefore, it is essential to ensure that the induced cell cycle acceleration is transient.

Increasing ROS and their effects on replicative stress. Replicative stress can be caused by the incorporation of oxidized nucleotides (such as 8-oxo-deoxyguanosine) that arise as a consequence of ROS accumulation. Oxidized nucleotide triphosphates are hydrolysed to monophosphates by MTH1, which prevents their incorporation into DNA. Prototype inhibitors of MTH1 have recently been developed and inhibit cancer cell proliferation through DNA damage[19,20]. Oxidative stress is enhanced in tumours[188]; therefore, further increasing oxidative stress is expected to boost replicative stress specifically in tumour cells and less so in normal cells. Conversely, subsets of cancer stem cells were found to have reduced levels of ROS, making them radioresistant[189]; this implies that pharmacological accumulation of ROS could be useful. Various targets and corresponding small molecules exist that can further increase levels of ROS in tumour cells, both by enhancing ROS production and by interfering with ROS removal[188,190]. However, increasing ROS levels as a strategy to prevent tumour progression is not without risk. Under specific circumstances, ROS can function as tumour promoters in mice that carry a targeted disruption of p53 (REF. 191). Thus, the development of ROS enhancers for therapeutic purposes will require a precise understanding of the conditions in which the accumulation of ROS would not only induce replicative stress but also refrain from supporting tumour progression.

Replication fork stalling and collapse. Once the cell enters S phase, the enzymes and regulators of DNA replication are obvious targets for enhancing replicative stress. Most conventional therapeutics alter the processivity of DNA polymerases (see above), but this might be made more efficient with direct enzyme inhibitors. The drug aphidicolin targets DNA polymerase-α; for use in clinical trials, it was replaced with aphidicolin glycinate to enhance its solubility[192], but no study beyond Phase I was subsequently reported. Recent reports have described the isolation of numerous proteins that are bound to DNA replication forks using a combination of DNA labelling and protein crosslinking (known as isolation of proteins on nascent

Isolation of proteins on nascent DNA (iPOND). A technology that involves the labelling of newly synthesized DNA and subsequent purification of proteins that associate with this DNA.

DNA (iPOND))[193–195], which has identified a plethora of potential targets. Moreover, RNase H2 (REF. 196) may be a future target to enhance replicative stress by increasing the incorporation of ribonucleotides in DNA.

Additional cellular machineries that contribute to DNA replication, or their regulators, may also be attractive drug targets to enhance replicative stress. For example, the synthesis of DNA despite lesions in the template strand (translesion synthesis)[197,198] is governed by CDC7 activity[96,97]. The licensing complex[199] that ensures DNA replication occurs only once may also be a suitable target to enhance replicative stress — to some extent, an inhibitor of neddylation, MLN4924, targets one of these complex members, CDT1 (REF. 79) (as explained above). In addition, some specialized components of the DNA replication machinery may be useful to target pharmacologically. These include specific complexes that mediate the replication of strongly transcribed regions[200,201], fragile elements[202] and telomeres[203].

Chromatin modifications in replicative stress. Modifications on chromatin are determinants of replicative stress, and such modifications are already amenable to manipulation by small compounds. Chromatin modifiers can affect replicative stress through the expression of regulatory genes but also by directly affecting the template strand and its function. The histone H4 lysine 20 methyltransferase SETD8 (also known as PR-SET7) has a key role in suppressing replicative stress through interactions with the replication machinery[204–208]. HDAC inhibitors can also increase replicative stress[102].

Damage signalling. Once DNA replication is hindered, replicative stress triggers a signalling cascade (FIG. 1). Manipulation of this cascade provides ample opportunities to further enhance replicative stress. The main kinases involved in this cascade (ATR and CHK1) have already been targeted with small molecules, as described above. A potential target for future evaluation is pre-mRNA-processing factor 19 (PRP19), which is an E3 ubiquitin ligase that assists in the recruitment and activation of ATR by the RPA sensors[209]. ATM and CHK2, as well as DNA-dependent protein kinase (DNA-PK) are activated later during replicative stress, perhaps as a result of secondary DSBs. Small-molecule inhibitors of signalling downstream of DSBs are also available; for example, the MRE11–RAD50–NBS1 complex[210] and RAD51 (REFS 210,211), which is a key factor in homologous recombination repair, are amenable to inhibition[211,212].

Repair of collapsed replication forks and other DNA lesions. Even when replication forks stall or collapse, this does not necessarily confer a death sentence on the cell. Rather, repair mechanisms are available during these situations (BOX 2). The general role of DNA repair in cancer therapy has been extensively reviewed[156,213–215].

Death response to replicative stress. Extensive replicative stress has the potential to trigger cell death. Three major death pathways can be distinguished, and each of them might be further enhanced by drug candidates.

First, premature mitosis and catastrophe can be triggered when the cell tries to separate its chromosomes without having fully replicated its genome. Most notably, interfering with the kinase WEE1 triggers such premature mitosis[72]; inhibition of CHK1 can also do so but usually to a lesser extent. Inhibition of WEE1 induces CDK1 and CDK2 activity, and this is necessary for premature mitosis[216]. Even if completed, premature mitosis will give rise to daughter cells with severe genomic losses.

Second, apoptotic cell death can result from replicative stress. Although p53 is a key mediator of cell death, it also attenuates replicative stress by upregulating p21 (REFS 217–219), which may somewhat reduce drug efficacy. Importantly, p53 also induces the phosphatase WIP1 (also known as PPM1D), and this enzyme is capable of removing phosphate groups from ATM and ATR target sites[220]. Recently, small-molecule inhibitors of WIP1 have been developed[221]; such inhibitors may enhance the death response to DNA damage by increasing the pro-apoptotic activities of p53. The p53 paralogue p73 is also activated through phosphorylation by several kinases that respond to replicative stress and/or are amenable to inhibition by small molecules, such as CHK1, CDK1, p38 and ABL[222]. The kinases p38 (REF. 223) and JNK[224] are activated by replicative stress and often transmit pro-apoptotic signals. However, inhibitor of nuclear factor-κB kinases (IKKs)[225] and AKT[226], as well as inhibitors of apoptosis[227] often provide anti-apoptotic signalling, and they are targeted by available inhibitors. Finally, the intrinsic apoptotic pathway triggered by mitochondria can be induced by small compounds such as BH3-domain mimetic drugs[228] and/or by remodelling the cristae within the inner mitochondrial membrane[229–231] — each of these mechanisms remains to be tested for possible synergisms with inducers of replicative stress.

Third, replicative stress may also promote cell death by non-apoptotic mechanisms, such as necrosis or autophagy. For instance, alkylating agents promote caspase-independent cell death by stimulating PARP, which then depletes the cell of NAD^+, thus hampering glycolysis. Tumour cells, which strongly rely on glycolysis for energy production, then undergo necrosis in response to decreased glycolysis[232].

Finally, a senescence response may be triggered by replicative stress. This could be acute senescence, a phenomenon triggered by conventional chemotherapeutics[233] that is characterized by markers that are also used to detect replicative senescence. Moreover, the replication of telomeres seems to be particularly sensitive to replicative stress[234], which provides evidence that replicative stress may be especially harmful to telomeres. Along this line, ATR is activated by telomere shortening and prevents further damage at chromosomal ends[235,236].

Areas for future research

Basic methods to study replicative stress are detailed in BOX 1. However, broader approaches are needed to fully exploit replicative stress and counteract malignancies, such as the following.

Box 2 | **Repair of replication-associated DNA damage and opportunities for interfering with it**

If the progress of a replication fork is hindered for a prolonged period of time, the cell has several ways to cope. In the context of enhancing replicative stress for therapeutic purposes, these mechanisms are potential targets for inhibitors.

Restarting stalled replication forks

Stalled replication forks can be restarted and DNA replication can be continued at this site[266]. RAD51, which is mostly known for the role it has in homologous recombination repair, is essential for restarting stalled forks[267]. To prevent the collapse of stalled replication forks, BRCA2 and poly(ADP-ribose) polymerase 1 are also required[268,269]. A recent discovery points to a role for PrimPol, a novel polymerase that mediates translesion synthesis, in circumventing small template lesions and the restart of stalled replication forks[270,271].

Removal and replacement of permanently stalled replication forks

Some DNA lesions do not permit the progression of replication forks, even by translesion synthesis. This is particularly true when interstrand crosslinks have formed, as these create a physical barrier to strand separation and hence fork progression. Under such circumstances, the cell can still use a more complex repair system: in particular, the Fanconi anaemia repair pathway[39,272]. The exact mechanisms of this pathway are still being explored; recently, it emerged that the large complex of Fanconi anaemia proteins involves histone chaperone activity[273] as well as the interaction with choline transporter-like protein[274] to resolve interstrand crosslinks that stall DNA replication.

Enhanced firing of adjacent origins of replication

Before entering S phase, cells provide a firing 'licence' to many origins of replication by enabling them to associate with minichromosome maintenance proteins[275,276]. Certainly, not all of these origins are used during the subsequent S phase. However, when replication forks are stalled, this can lead to DNA replication from licensed but still dormant origins next to the stalled replication fork, thus compensating for the loss[277,278]. Interestingly, depleting the licensing factor origin recognition complex subunit 1 from tumour cells renders them sensitive to hydroxyurea, a compound that blocks ribonucleotide reductase and causes replicative stress[279]. Hence, the capacity of origin licensing may become limiting for tumour cells and is therefore a potential drug target to enhance replicative stress. Conversely, unlimited origin firing may not always reduce replicative stress. Instead, when ATR-mediated suppression of origin firing is absent, cells can exhaust their reservoir of replication protein A family members by excessive origin firing. This then results in the accumulation of single-stranded DNA and eventually in DNA breakage (or replicative catastrophe). This phenomenon provides an explanation for the sensitivity of cancer cells to ATR inhibitors[280].

Target identification to enhance replicative stress. Cell-based assays using small interfering RNAs (siRNAs) are commonly used for target identification[237]. To tailor such screens for the identification of replicative stress modulators, most approaches apply a treatment that is known to induce replicative stress (such as UV irradiation or incubation with nucleoside analogues) and then use a general readout for a DNA damage response (for example, immunostaining of H2AX phosphorylated on serine 139)[41,216,238–240] or cell viability (for example, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT))[241–243]. Such screens do not distinguish between general DNA damage, cell death and specific replicative stress. Hence, the hits need to be re-screened for their effects on DNA replication and its processivity. Future screens may use readouts that are more specific for replicative stress but are nonetheless simple and robust enough for high-throughput strategies. Examples include the accumulation of ssDNA, as determined by staining for (pre-incorporated) bromodeoxyuridine[62] (BOX 1), the detection of ATR–CHK1 signalling or relocalized RPA, and simply the quantification of cells that remain with a DNA content between 2n and 4n (relative to their previous DNA content, which is not necessarily the same as in a euploid primary cell). Other interesting approaches that have been used include the UV-induced destabilization of the replication-licensing factor CDT1 as a screening readout[244] and the identification of the MMS22L–NFKBIL2 (also known as MMS22L–TONSL) complex by screening for siRNAs that alter the transition time through cell cycle phases[245].

Developing small-molecule enhancers of replicative stress. Drug candidates that enhance replicative stress can, in principle, be identified in cell-based screens such as the siRNA screens outlined above. However, it is often desirable to develop such small molecules in a more targeted manner. Many of the components outlined as future targets in the above section represent such opportunities. We expect the combination of targeted and broadly active (such as DNA-damaging) drugs to be most efficient in the elimination of cancer cells through replicative stress, and these combinations deserve to be tested even at early stages of screening.

Improved model systems to validate the anticancer activity of replicative stress-inducing compounds. Preclinical testing often requires the use of animals, preferably those bearing realistic cancer models. In the context of enhancing replicative stress, this raises the question of whether impaired DNA replication and/or stress signalling can be modelled in such animals, thereby enabling the specific interrogation of drug candidates that increase replicative stress. Transgenic mice with deliberately modified replicative stress signalling have been engineered. A recent example includes mice overexpressing CHK1, which resulted in decreased replicative stress and increased tumour formation[246]. Such tumours can now be challenged with drug candidates, with the aim of restoring replicative stress despite the CHK1 barrier. Another example of a useful mouse model is mice that lack full ATR activity, as they show exacerbated replicative stress, at least in part through hampered CHK1 activity[247,248],

which might enhance the sensitivity of assays to stress-inducing drugs. If combined with additional transgenes to induce cancer, such as the overexpression of MYC or mutant RAS[249], such models may give rise to cancers that are particularly susceptible to replicative stress-inducing drugs and therefore provide a proof-of-principle system for preclinical drug testing.

Monitoring replicative stress in patients with cancer. When assessing the efficacy of current or future drugs in clinical studies, it is most useful to measure parameters that faithfully reflect the degree of replicative stress in the tumour cells of patients. Some biomarkers are reasonably established but reflect only the downstream consequences of replicative stress, such as markers of apoptosis (cleaved caspase and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling)) and proliferation (Ki67). Even such indirect readouts usually require tumour biopsies and cannot be taken from easy-to-reach material, such as body fluids. Direct biomarkers of replicative stress, including ssDNA or phosphorylated signalling intermediates, are even less established, as their occurrence is quite transient and difficult to preserve in biopsies. This difficulty is further aggravated when monitoring the levels of unstable metabolites, such as ROS or intermediates of nucleotide metabolism. However, the use of tumour cell DNA in the peripheral blood, known as 'liquid biopsies', has undergone remarkable progress during the past few years[250,251], and it remains to be determined whether replication intermediates (replication forks, ssDNA and incorporated nucleoside analogues) are detectable in the periphery, giving rise to a convenient method to monitor replicative stress during cancer therapy.

Patient selection and accurate prediction of the tumour cell response to replicative stress. In addition to the direct assessment of immediate drug efficacy, it will be important to identify parameters that can predict the therapeutic response to agents that increase replicative stress. Recently, predictive markers have become important for stratifying patients, in particular when using targeted drugs that interfere with specific signalling pathways. For example, PARP inhibitors are currently used only in a highly selected group of patients[55,213], and similar patient selection may be required for at least some of the drugs that exploit replicative stress. There are purely empirical ways to identify such markers, including expression profiling of tumour samples to find genes whose expression correlates with drug response. Such approaches have been used in patients with cancer and also in cancer-derived cell lines[252,253]. However, strongly correlating genes cannot always be found in this way, and even the genes that do correlate with drug sensitivities can function through very indirect mechanisms that have limited predictive potential. Better predictors may be found based on known mechanisms. For example, if the strategy is to further increase replicative stress in a catastrophic manner, p53 status and the proliferative index are among the key markers. Additional obvious candidates include regulators or effectors of DNA replication, translesion synthesis and damage signalling. Whenever targeted drugs are used — for example, to block kinases that are involved in the response to replicative stress — the levels, modifications and activities of such targets should be evaluated, as has been done for growth factor receptors and their pharmacological antagonists[254]. Moreover, chromosomal instability (which is detectable by karyotyping) is often a result of replicative stress[255] and may therefore function as an indirect indicator of the initial stress level. Assuming that this initial level determines the efficacy of pharmacological stress enhancers, the degree of chromosomal aberrations may predict the response of cancer cells to these drugs. Finally, perhaps the most promising way to predict the therapeutic response is by obtaining patient-derived tumour xenografts[256], which can allow direct testing of drug sensitivities and even the assessment of replicative stress parameters.

Conclusion

Exploiting replicative stress for cancer treatment seems to be a promising strategy. Doing the opposite — that is, reducing tumour cell proliferation — does not always lead to sustainable therapeutic success. Conventional chemotherapy often increases the presence of stumbling replication forks. Our rapidly expanding knowledge of DNA replication and its cellular management opens up the potential for targeted strategies to cause cell death through replicative stress. This would turn a major selective advantage of malignant cells — that is, their loss of proliferation control — into a deadly disadvantage.

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Competing interests statement
The authors declare no competing interests.

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