Replication stress and cancer

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Abstract | Genome instability is a hallmark of cancer, and DNA replication is the most vulnerable cellular process that can lead to it. Any condition leading to high levels of DNA damage will result in replication stress, which is a source of genome instability and a feature of pre-cancerous and cancerous cells. Therefore, understanding the molecular basis of replication stress is crucial to the understanding of tumorigenesis. Although a negative aspect of replication stress is its prominent role in tumorigenesis, a positive aspect is that it provides a potential target for cancer therapy. In this Review, we discuss the link between persistent replication stress and tumorigenesis, with the goal of shedding light on the mechanisms underlying the initiation of an oncogenic process, which should open up new possibilities for cancer diagnostics and treatment.

Senescence
A sustained growth arrest in which cells are refractory to mitogen stimulation and apoptosis.

Replication fork
DNA region at which unwinding of the double helix and synthesis of the complementary strands occur.

S phase checkpoints
The quality-control mechanisms that guarantee genome integrity during DNA replication.

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Genome instability is the consequence of DNA lesions that can result from errors in DNA replication, from the action of genotoxic compounds including cellular metabolites, or from ultraviolet (UV) and ionizing radiation. Exogenous and endogenous agents can damage DNA, generating approximately 10^5 lesions per day in mammalian genomes[1], with an important source of endogenous DNA damage being reactive oxygen species (ROS). Normal cells respond to DNA lesions by activating the DNA damage checkpoint and by using different repair pathways; the coordinated action of both processes is known as the DNA damage response (DDR). In the case of persistent DNA damage, cells undergo either apoptosis or senescence; otherwise, cancer may develop in conjunction with a long-term cellular state of global genome instability[2].

Every time a mammalian cell divides, billions of nucleotides must be accurately copied in coordination with the cell cycle. Faulty DNA replication can lead to mutations or replication blockage, which can result in breakage, rearrangement and the missegregation of chromosomes. A number of conditions, including those leading to high levels of DNA damage, may interfere with DNA replication and hamper its progression. This phenomenon — termed replication stress — is characterized by DNA synthesis slow down and/or replication fork stalling and is the primary cause of genome instability. Sophisticated mitotic and S phase checkpoint pathways have evolved to respond to potential failures in DNA replication and chromosome transmission. S phase checkpoints are crucial to ensure replication completion, to prevent replication fork breakage and to coordinate the DDR in proliferating cells, thus constituting the surveillance mechanism that prevents genome instability upon replication stress.

Oncogene expression drives cell proliferation by interfering with the regulatory pathways of cell cycle progression control. Several oncogenic features, such as alterations of replication timing and progression, lead to replication stress[3] (FIG. 1). In precancerous lesions from patients, DNA damage signalling is constitutively activated, providing a natural barrier to delay or prevent tumorigenesis through the induction of apoptosis or the establishment of the sustained arrested cell state of senescence[4,5]. Interestingly, analogous constitutive DDR activation is observed upon oncogene-induced replication stress, providing one of the first links between replication stress and tumorigenesis. Furthermore, DDR dysfunction fosters tumour progression upon oncogene-induced replication stress[6,7], which is in agreement with the high occurrence of mutations in DDR factors found in human cancers[8]. The connection between replication stress and tumorigenesis is further strengthened by the findings that aphidicolin-mediated DNA polymerase inhibition leads to micro-deletions that closely resemble those found in human tumours[9], and that the treatment of mice with hydroxyurea, a well-known dNTP-depleting agent, promotes leukaemogenesis by allowing mutated progenitors to outcompete non-mutated cells[10].

Beyond the accumulated evidence for the link between replication stress and tumorigenesis, an understanding of the underlying molecular mechanisms of this link is required to decipher the cause–effect relationship between the two processes. The mechanisms that are responsible for genome instability resulting from replication stress in

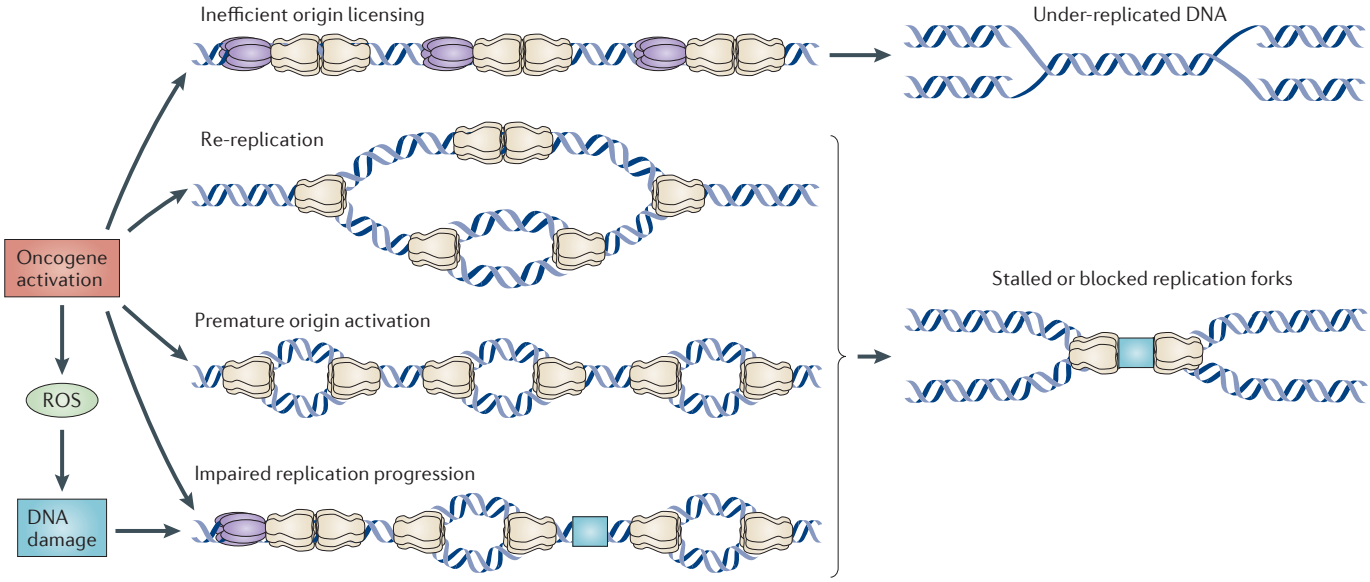


Figure 1 | **Oncogene-induced replication stress.** There are different ways by which oncogene activation can deregulate replication: a decrease in the number of licensed replication origins (as seen upon cyclin E overexpression[35]) reduces the number of active origins, leading to under-replicated DNA; whereas, unscheduled replication initiation causes re-replication and/or premature origin activation (as seen upon expression of cyclin E, cyclin D2 and MYC oncogenes[4,41,45,46]), which could result in replication fork stalling. Alternatively, replication fork stalling induced by oncogenes can be mediated by a direct effect of the oncogenes on replication fork progression (for example, the BCL-2 oncogene[50]) or by an accumulation of reactive oxygen species (ROS) leading to DNA damage with the potential to impair replication (as seen upon MYC overexpression[173]).

eukaryotes, from yeast to mammals, have recently been reviewed[11]. This Review focuses on the factors and processes that are responsible for replication stress in mammalian systems, the effect of replication stress on genome instability and tumorigenesis, and how this knowledge can be used to develop new anticancer therapies.

Replication and S phase checkpoints

Eukaryotic replication is controlled at its initiation stage to ensure that the genome duplicates only once per cell division. For this to take place, cells rely on a series of tightly controlled steps based on the availability of the replicative helicase minichromosome maintenance complex 2–7 (MCM2–7) at replication origins. Binding of MCM2–7 at replication origins is restricted to the G1 phase of the cell cycle and licenses the origin to initiate replication in the following S phase (BOX 1). To initiate replication, cyclin-dependent kinase 2 (CDK2) activates the MCM2–7 helicase to subsequently allow replisome loading on the DNA[12]. Eukaryotic chromosomes have a wide distribution of licensed origins — sites where replication forks can initiate — that can be activated (fired) in S phase (BOX 1). Many of these licensed origins are not used during normal replication but provide backup origins in case of replication slow down or failure[13]. CDKs inhibit re-replication by preventing MCM2–7 from reloading until the next cycle, and the S phase checkpoints act as a surveillance mechanism to preserve the integrity of the replication fork.

The conserved signal transducers ataxia telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) are the upstream protein kinases of this surveillance mechanism. They regulate the cellular response to replication fork blockage and DNA damage by activating the checkpoint kinases CHK1 and CHK2 and by regulating the timing of replication independently of DNA damage[14]. ATM responds to double-strand breaks (DSBs), and ATR is activated by the presence of single-stranded DNA (ssDNA) generated at stalled replication forks or resected DSBs[15,16]. Upon activation, ATR phosphorylates many downstream targets, including CHK1 and the tumour suppressor p53, to coordinate the DDR. The ATR–CHK1 signalling pathway leads to cell cycle arrest and promotes replication fork stabilization and restart (FIG. 2). Additionally, it activates dormant origins in the vicinity of stalled forks, allowing the complete replication of the affected regions, while late origin firing is inhibited to ensure that limiting replication factors such as replication protein A (RPA) remain available[17]. This pathway is thus crucial to ensure replication completion and to prevent replication fork breakage.

S phase checkpoint activation in cancer

The biological relevance of S phase checkpoints in preventing genome instability is confirmed by several cancer-prone genetic diseases that are caused by mutations in checkpoint genes (BOX 2). ATR and CHK1 kinases are key for the response to replicative stress and are essential for cell viability. However, individuals carrying hypomorphic *ATR* mutations survive but suffer from Seckel syndrome, which is characterized by developmental problems[18]. No cancer predisposition has been reported for patients with Seckel syndrome or linked to CHK1 deficiency. However, the loss of ATR pathway activity is lethal upon oncogene-induced replication stress or a

Replisome
A protein complex involved in DNA replication that moves along the DNA as the nascent complementary strands are synthesized.

Seckel syndrome
A rare autosomal recessive disorder, due to mutations in the *ATR* gene, which is characterized by intrauterine growth retardation, dwarfism, microcephaly with severe mental retardation and a bird-headed facial appearance.

Hypomorphic mice
Transgenic mice carrying a mutation that causes a partial decrease in the activity of the affected gene.

Replication intermediates
DNA structures formed transiently during the process of replication.

DNA primase
Enzyme that catalyses the synthesis of a short RNA segment called a primer that is needed by DNA polymerases to start DNA synthesis.

lack of p53 function[19–21], and upregulation of the ATR pathway is found in several cancers[22]. Therefore, cells undergoing acute replication stress require the ATR pathway to survive[23]. Consequently, the complete loss of ATR or CHK1 compromises the survival of cancer cells. Nevertheless, the role of mutations in the ATR pathway in cancer is not that clear because haploinsufficiency of the ATR pathway by itself leads to carcinogenesis in mouse models. *Atr*- or *Chk1*-heterozygous mice show a mild increase in the incidence of tumours, in particular when other tumour-promoting conditions occur concomitantly, such as oncogene expression, mutations in DNA repair pathways or exposure to chemical carcinogens[24–26] (TABLE 1). In humans, somatic mutations in ATR and CHK1 have been found in tumours with microsatellite instability caused by the loss of mismatch repair activity[27,28]. This can be explained by low activity of the ATR pathway favouring tumorigenesis by enhancing replication defects and mutation selection during constitutive activation of the DNA damage checkpoint[4,5].

Deregulated origin firing and cancer

Licensed origin scarcity. One consequence of deregulated origin activation is licensed origin scarcity during S phase, which generates replication stress. This scarcity can result from the partial depletion of MCM2–7, which is tolerated in normal conditions but which gives rise to DNA breakage in the presence of replication inhibitors such as hydroxyurea or aphidicolin in human cells[29,30]. Similarly, replication stalling and DNA breaks have been observed in MCM2–7 hypomorphic mice, in which dormant origins are not available owing to a lower concentration of MCM2–7 (REF. 31). These DNA damage phenotypes probably arise as a consequence of incomplete DNA replication, as persistent replication intermediates are observed during mitosis (M phase) in cells with reduced chromatin-bound MCM2–7 (REF. 31). Importantly, reduced function of MCM2–7 leads to genomic instability and tumour development in MCM2–7-hypomorphic mice[32] (TABLE 1). Even if a hitherto uncovered function of the MCM2–7 motor helicase in replication fork restart cannot be excluded, failure to activate a sufficient number of origins could increase the distance between forks so that replication cannot be completed before the onset of mitosis. Consequently, incompletely replicated chromosomes could undergo breakage as, indeed, occurs in low-origin-density fragile sites[33,34] (BOX 3). Notably, overexpression of the cyclin E oncogene impairs MCM2–7 binding to chromatin during G1, resulting in cells entering S phase with a reduced number of licensed origins[35] (FIG. 1). Dormant origin paucity thus contributes to oncogene-induced replication stress that may at last impede the completion of replication and promote tumorigenesis.

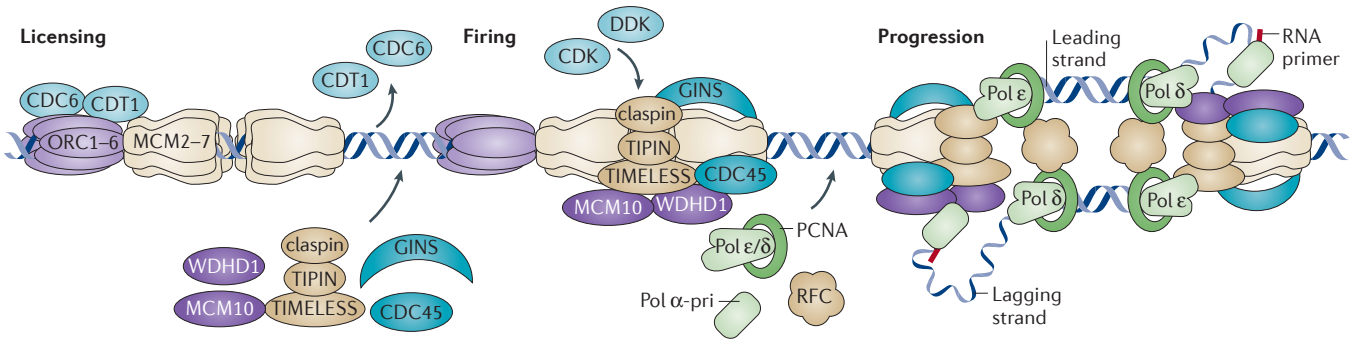
Unscheduled replication. Another source of replication stress is unscheduled replication, which occurs when the timing of origin activation is altered. This can lead to DNA regions replicating more than once in a given

Box 1 | **The basics of DNA replication**

DNA replication occurs exactly once during the cell cycle. This is achieved by controlling replication initiation via the replicative DNA helicase minichromosome maintenance complex 2–7 (MCM2–7), which is loaded at replication origins exclusively during G1 phase, and is activated only during the subsequent S phase. Loading of two MCM2–7 helicases at origins is called licensing and requires the six-subunit origin recognition complex (ORC) and the activities of the cell division cycle 6 (CDC6) ATPase and the chromatin licensing and DNA replication factor 1 (CDT1) protein to constitute the pre-replicative complex (pre-RC) (see the figure). After pre-RC formation, CDC6 and CDT1 are released and ORC and MCM2–7 are retained on the DNA[12].

Activation of licensed origins, which is termed origin firing, is triggered by the activities of cyclin-dependent kinase (CDK) and CDK-like kinase with its regulatory subunit (CDC7–DBF4). Origin firing depends on the stable association of CDC45 and the DNA replication complex GINS[12], a process that requires additional factors including helicase RECQL4 (mutated in Rothmund–Thomson syndrome), MCM10 and WDHD1 (also known as AND1), which binds to DNA polymerase-α (Pol α)[154]. In mammalian cells, MCM2–7 complexes are loaded in excess on DNA compared with ORC complexes but do not fire during normal S phase, remaining as dormant origins that serve as a backup mechanism in case replication stress arises[29]. Re-replication is averted by high CDK activity and the activity of geminin, which interacts with CDT1 preventing the reloading of MCM2–7 complexes outside the G1 phase.

At each fired origin, two sister replication forks are established that move away from the origin by the activity of the holo-helicase formed by CDC45–MCM2–7–GINS. Synthesis of each new DNA molecule is initiated by the Pol α complex (Pol α-pri), which contains both DNA primase and DNA polymerase subunits. The leading and lagging strands are then extended by Pol ε and Pol δ, respectively, and the activities of PCNA and RFC. Besides the CDC45–MCM2–7–GINS helicase, a key constituent of the replisome is the claspin–TIMELESS (also known as TIM)–TIMELESS-interacting protein (TIPIN) complex, which coordinates DNA unwinding with DNA synthesis, accounting for replication fork progression[154].



Replication fork collisions
Physical encounters between an advancing replication fork and another on-going process, such as transcription, taking place on the same DNA molecule.

Non-B DNA structures
Secondary DNA structures that are different from right-handed double helical B DNA structures.

cell cycle (re-replication) or an increase in origin firing in early S phase (premature origin firing). In *Xenopus laevis*, overexpression of chromatin licensing and DNA replication factor 1 (Cdt1) causes re-replication and checkpoint activation associated with DNA breaks at sites of putative replication fork collisions[36,37]. Unscheduled initiation and re-replication occur in human cells under the upregulation of CDT1 and cell division control protein 6 (CDC6)[38,39]. Abnormal accumulation of these proteins has been observed in early stage epithelial cancer lesions, and injection of premalignant cells expressing human CDC6 and CDT1 led to tumour formation in mice[40]. Other mouse models have confirmed the tumorigenic potential of CDT1 overexpression *in vivo* (TABLE 1). Consistently, some oncogenes induce re-replication, as observed upon overexpression of cyclin E or a mutated form of cyclin D1 (REFS 4,41) (FIG. 1). Notably, re-replication is also observed upon deregulation of the histone H4K20 methyl-transferase SETD8 (also known as PR-Set7), such as that caused by a lack of its degradation in S phase[42] and in tumour cells overexpressing histone lysine-specific demethylase 4A (KDM4A; also known as JMJD2A)[43], suggesting that inappropriate chromatin modifications may participate in tumorigenesis as a consequence of their role in the regulation of origin firing.

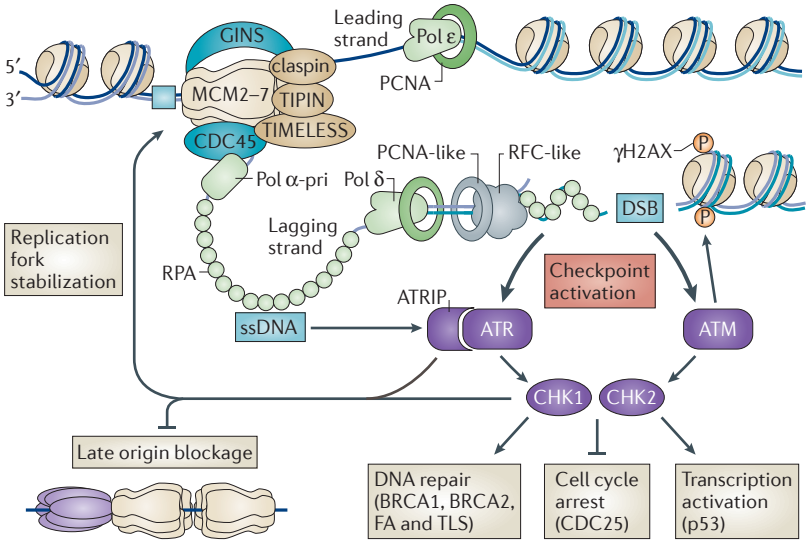


Figure 2 | **Replication impairment activates the checkpoint.** Replication is catalysed by the replisome multi-subunit complex, which is formed by the stable association of the replicative DNA helicase minichromosome maintenance complex 2–7 (MCM2–7) with replication factors CDC45 and the GINS complex. Also part of the replisome is the claspin–TIMELESS–TIPIN complex, which coordinates DNA unwinding with DNA synthesis (carried out by DNA polymerase ε (Pol ε) and Pol δ). DNA polymerases are tethered to DNA by the proliferating cell nuclear antigen (PCNA) replication cofactor. Pol α-pri initiates lagging strand synthesis (BOX 1). Encountering an obstacle can cause replication fork stalling, which results in the uncoupling of leading- and lagging-strand synthesis, generating double-strand breaks (DSBs) and/or single-stranded DNA (ssDNA) gaps. In mammals, DSBs activate the ataxia telangiectasia mutated (ATM) kinase, whereas ssDNA coated with replication protein A (RPA) activates the ataxia telangiectasia and Rad3-related (ATR) kinase via its interacting protein ATR-interacting protein (ATRIP). Further activation requires the PCNA-like complex, which is loaded onto stalled forks by the replication factor C (RFC)-like complex at the boundary between ssDNA and double-stranded DNA. Apical checkpoint kinases ATR and ATM phosphorylate (P) several targets, such as histone H2AX at Ser139 (one of the first signalling steps that helps to recruit DSB repair proteins), and the effector checkpoint kinases CHK1 and CHK2. These kinases coordinate the regulation of a variety of downstream factors to protect the genome by stabilizing the replication fork through the claspin–TIMELESS–TIPIN complex and by blocking late origin activation through the inhibition of origin licensing. The DNA damage response (DDR) is further completed with transcription activation driven by p53, cell cycle arrest targeted by CDK regulators such as phosphatase CDC25, and DNA repair by different pathways (for example, homologous recombination (HR), translesion DNA synthesis (TLS) and Fanconi anaemia (FA)). All of the processes depicted take place in the cell nucleus.

The MYC proto-oncogene is a global regulator of cell growth that promotes proliferation by positively regulating the expression of many genes controlling the cell cycle such as those encoding cyclins, CDKs, dNTP biosynthetic enzymes and replication factors, while repressing anti-proliferative genes, including those encoding CDK inhibitors[44]. Besides transcription regulation, MYC drives cell cycle progression by directly controlling replication initiation. It physically interacts with the pre-replicative complex and co-localizes with replication foci in early S phase regardless of transcription, indicating a non-transcriptional role for MYC in the initiation of DNA replication[45]. In cells overexpressing MYC, the spatiotemporal timing of origin activity is affected, inducing premature origin firing[46]. This origin over-activation in early S phase, which is driven by both transcriptional and non-transcriptional functions, contributes to MYC-induced replication stress. Similarly, the oncogene MDM2 induces untimely origin firing, which elicits an early S phase checkpoint that inhibits further origin firing[47].

Re-replication could lead to DNA breakages as a result of replication fork collisions, possibly as a consequence of multi-fork structures or increased fork stalling. Recent work has shown that ssDNA gaps readily accumulate upon licensing deregulation in human cells[48], indicating that the genomic instability that is associated with re-replication might arise from forks encountering ssDNA regions that remain after the previous replication passage. As unscheduled replication leads to a considerable increase in active replication forks, this may deplete cells of replication factors and nucleotides, impeding replication fork progression. In cells in which replication was aberrantly activated by human papillomavirus E6/E7 proteins and cyclin E, both replication dynamics and oncogene-induced transformation could be rescued by exogenously supplied nucleosides[49]. Similarly, the BCL-2 oncogene induces replication stress by inhibiting the ribonucleotide reductase[50]. The importance of an equilibrated balance of active replication forks, replication factors and available nucleotides is further emphasized by the impact of dNTP biosynthesis deregulation or deficiencies in RPA and Flap endonuclease 1 (FEN1) — which are required for both DNA replication and repair — on tumorigenesis in mice (TABLE 1).

Replication fork stalling and cancer

An elongating replication fork may encounter obstacles — such as DNA damage, protein barriers, heterochromatin, non-B DNA structures or transcribed genes — that will hamper its progression (FIG. 3). The resulting replication pause may be transient or may develop into a persistent replication fork stall or collapse, thus triggering the S phase checkpoint[51], particularly in cells treated with genotoxic

Reversed forks
DNA structures that are formed when the nascent leading strand hybridizes to the corresponding lagging strand at a replication fork.

Telangiectasias
Small dilated blood vessels in the outer layer of the skin.

agents that induce replication stress. The replication fork can stall at damaged regions, leading to ssDNA stretches or DSBs that could trigger genome instability, which is exacerbated during dysfunctional DDR[11,52].

Transcription-induced replication stress. The transcription machinery constitutes a natural obstacle to replication fork progression, and collisions between both machineries are an important source of replication stress and genome instability, as shown in bacteria, yeast and mammals[53]. Recombination analyses in Chinese hamster cells provided evidence that transcription-mediated genome instability depends on replication. Transcription-associated recombination only occurs in replicating cells, and transcription inhibitors can partially suppress the high levels of recombination that are observed in cells in which fork progression has slowed[54]. In addition, transcription inhibition suppresses a substantial subset of cyclin E-induced replication stress in human cells[55]. Concomitant transcription and replication, whether resulting in collisions or not, generates a large amount of torsional stress in the DNA that can lead to abundant reversed forks, as supported by structural analyses of replication intermediates in cells overexpressing cyclin E[56]. Reversed forks not only contribute to delaying replication progression but can also be the target of nucleases and other enzymes that might foster genome instability. In addition, during transcription, the nascent RNA may hybridize back to the complementary DNA strand, forming an RNA–DNA hybrid and a displaced ssDNA that is termed R-loop. Notably, conditions leading to R-loop accumulation — such as deficiencies in Topoisomerase I or messenger ribonucleoprotein (mRNP) biogenesis factors — lead to replication fork progression hindrance and DNA breaks in human cells[57–59]. Furthermore, co-transcriptionally formed R-loops have been shown to contribute to the fragility of specific fragile sites[60]. Finally, specific functions are required to replicate through transcribed DNA regions, as supported by the activity of the chromatin-reorganizing complex FACT in promoting replication fork progression during transcription[61]. Although the possible connection between transcription-mediated replication stress and tumorigenesis has not been sufficiently studied, the recently reported link between mutations in the tumour suppressor BRCA2 and R-loop-associated genome instability is consistent with this possibility[62] (see below). Along this line, interference between transcription and replication might be particularly relevant in cells overexpressing MYC, in which global transcriptional activity is markedly increased[63–65], although the possibility that MYC-induced replication stress might depend on transcription has not yet been analysed.

Specific functions with a role at stalled forks. DNA helicases constitute a well-known group of proteins involved in replication fork resumption after stalling, in particular those belonging to the human RecQ-helicase family, three of which — Werner syndrome helicase (WRN), Bloom syndrome protein (BLM) and RECQL4 — are mutated

Box 2 | **Cancer-prone human syndromes related to replication stress**

- Ataxia telangiectasia: a disorder caused by mutations in the *ATM* gene, which encodes a DNA damage checkpoint factor. This syndrome is characterized by cerebellar ataxia, telangiectasias, immune defects and cancer predisposition.
- Li–Fraumeni syndrome: a heterogeneous cancer syndrome caused by mutations in the *TP53* and *CHK2* genes, which encode checkpoint factors. This syndrome is characterized by autosomal dominant inheritance and the early onset of tumours.
- Nijmegen breakage syndrome: a chromosomal instability syndrome caused by mutations in the *NBN* (also known as *NBS1*) gene, which encodes a subunit of the MRN complex. This syndrome is characterized by microencephaly, growth retardation, immunodeficiency and cancer predisposition.
- Xeroderma pigmentosum variant XP-V: a variant form of Xeroderma pigmentosum caused by mutations in the *POLH* gene, which encodes DNA polymerase-η. This syndrome is characterized by increased sunlight sensitivity, DNA repair defects and cancer predisposition.
- Hereditary breast and ovarian cancer syndrome: a familial susceptibility to breast and ovarian cancer owing to mutations in the *BRCA1* and *BRCA2* genes.
- Fanconi anaemia: a heterologous genomic instability disorder caused mutations in the *FANC* genes (*FANCA*, *FANCB*, *FANCC*, *FANCD1*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ*, *FANCL*, *FANCM*, *FANCN*, *FANCO*, *FANCP* and *FANCQ*). This syndrome is characterized by developmental abnormalities, early onset bone marrow failure and a high cancer predisposition.
- Lynch syndrome: hereditary nonpolyposis colorectal cancer caused by heterozygous mutations in mismatch repair genes *MSH2* and *MLH1*.
- Werner syndrome: a premature ageing disorder caused by mutations in the *WRN* gene, which encodes a RecQ DNA helicase. This syndrome is characterized by genomic instability and cancer predisposition.
- Bloom syndrome: a disorder caused by mutations in the *BLM* gene, encoding a RecQ DNA helicase. This syndrome is characterized by proportional dwarfism, sun-sensitive skin, telangiectasia, hyperpigmentation and hypopigmentation, immune deficiency, genomic instability and predisposition to malignancy.
- Rothmund–Thomson syndrome: a premature ageing disorder caused by mutations in the *RECQL4* gene, which encodes a RecQ DNA helicase. This syndrome is characterized by skin atrophy, telangiectasia, hyperpigmentation and hypopigmentation, congenital skeletal abnormalities, short stature and predisposition to malignancy. Some *RECQL4* mutations cause the related RAPADILINO syndrome.

in human syndromes that are characterized by premature ageing and cancer predisposition (BOX 2; TABLE 1). Although RECQL1 and RECQL5 have not yet been associated with genetic diseases, both proteins seemingly have roles in carcinogenesis. RECQL1 is highly expressed in various cancers, and its depletion leads to increased chromosomal instability[66]. This genomic instability is possibly related to replication stress, as RECQL1 associates with replication origins at the onset of S phase and promotes replication fork progression *in vivo* and strand exchange *in vitro*[67,68]. Increased genomic instability is also observed upon RECQL5 depletion — which leads to cancer susceptibility in mice (TABLE 1) — and this is probably due to collisions between replication and transcription machineries[69]. WRN and BLM are required for the efficient rescue of replication fork arrest[70,71], and they convert *in vitro*-engineered reversed fork-like substrates into normal replication fork structures[72].

DSBs can be generated from collapsed replication forks via the action of topoisomerases, structure-specific endonuclease complexes such as MUS81–EME1 and SLX1–SLX4, or the GEN1 Holliday junction 5′ flap endonuclease[73]. Indeed, MUS81–EME1 is involved in DSB formation in response to replication inhibition[74], and the UvrD family helicase FBXO18 (also known as FBH1) participates in the response to replication stress by activating MUS81-dependent cleavage of stalled replication forks and promoting apoptosis[75,76]. In addition, the WRN helicase is necessary for replication fork progression under oncogene-induced replication stress and the resulting DSBs depend on MUS81 (REF. 77). MUS81 and SLX4 deficiencies have been linked to cancer predisposition in mouse models (TABLE 1), but the relevance of replication stress-induced DSBs in tumorigenesis is better supported by homologous recombination (HR) deficiency (see below).

Depending on the type of aberrant DNA structure that is associated with the stalled replication fork (for example, ssDNA gap, reversed fork, DSB, inter-strand crosslink (ICL), and so on), cells use different pathways to restore replication, including HR, the Fanconi anaemia (FA) pathway or post-replicative repair (PRR). As seen in mouse models, failure in these pathways contributes to and exacerbates persistent replication stress and genome instability[11,52], as well as tumorigenesis (TABLE 1). Interestingly, the FA pathway is activated in MCM2–7-hypomorphic mice, and double deficiency in MCM4 and the Fanconi anaemia complementation group C protein (FANCC) causes perinatal lethality and accelerated tumorigenesis in surviving mice[78].

Cellular responses to stalled forks

Protection of stalled replication forks. One of the first consequences of replication stress is the lower stability of stalled replication forks. Cellular processes that protect stalled replication forks are crucial for responding to replication stress, therefore minimizing its impact and preventing tumorigenesis. Strong evidence for this is provided by tumour-prone FA and hereditary breast and ovarian cancer syndrome that is linked to *BRCA1* and *BRCA2* mutations (BOX 2). Cells deficient in *FA*, *BRCA1* or *BRCA2* genes are extremely sensitive to agents that induce ICLs, such as mitomycin C. This sensitivity is explained by the role of the FA–BRCA pathway in restoring replication fork progression after ICL-induced blockage via the coordination of different steps. Upon ICL-induced replication fork blockage, a key step in FA pathway activation is the monoubiquitylation of the FA group D2 (FANCD2)–FANCI complex. Ubiquitylated FANCD2 allows the recruitment of the FA endonucleases that generate the incisions flanking the ICL lesion[79]. The removal of the ICL lesion would thus generate a DSB and the stalled replication fork would then be repaired through HR. In fact, ubiquitylated FANCD2 is recruited to stalled replication forks via BRCA1 (REF. 80), where it promotes HR by cooperating in DNA resection and by recruiting BRCA2 (also known as FANCD1) and RAD51 (REFS 81,82). Failure to do so causes faulty DNA repair and leads to chromosomal abnormalities. These chromosomal abnormalities can be suppressed in FA-deficient cells by the removal of non-homologous end joining (NHEJ) repair factors[83], which is consistent with the idea that most chromosomal rearrangements occur via NHEJ.

In addition, beyond their role in promoting HR, BRCA proteins protect replication forks. BRCA1 suppresses translesion DNA synthesis (TLS) in favour of S phase checkpoint activation upon UV damage, promoting the re-localization of replication factor C (RFC) at stalled forks[84], and BRCA2 prevents DSBs by stabilizing DNA structures at stalled replication forks[85]. RAD51 also seems to promote replication fork restart in addition to its canonical function in HR repair[86]. Consistently, inactivation of either BRCA protein increases the frequency of aberrant HR resulting from breakage at site-specific replication fork stalls in an engineered replication termination system[87]. Similarly, FANCD2, BRCA1 and BRCA2 proteins protect stalled replication forks from endonuclease-dependent degradation[88,89], and association of FANCD2 with the MCM2–7 helicase enables replication slow down to prevent DNA damage[90].

Therefore, the function of BRCA1, BRCA2 and FA in protecting stalled replication forks probably contributes to their tumour suppressor activity. In support of this idea, p53 is activated in BRCA1- and BRCA2-deficient embryos[91,92], probably in response to DNA damage, and concomitant loss of p53 and BRCA1, BRCA2 or FANCD2 further promotes tumorigenesis in mouse models[93–95] (TABLE 1). In this sense, it is worth noting the recent observations that BRCA1- and BRCA2-deficient cells accumulate R-loops and that BRCA1 functions in preventing or repairing transcription-associated DNA damage[62,96,97]. These findings suggest a novel function for BRCA proteins in preventing R-loop-mediated replication stress that might well be shared by other FA proteins and, more importantly, that R-loops may be a natural source of replication stress in cancerous cells that rely on specific replication fork protection functions to prevent genome instability and tumorigenesis[62]. Notably, overexpression of RAD51 is found in many tumour cells, and seems to correlate with resistance to genotoxic drugs[98]. The recent observation that replication forks are protected in FANCD2-deficient cells by increased RAD51 levels or

Homologous recombination (HR). Error-free double-strand break repair pathway that involves identical or homologous DNA sequences as templates.

Non-homologous end joining (NHEJ). Error-prone double-strand break repair pathway that directly ligates DNA ends.

Table 1 | **A selection of genes involved in replication stress-mediated tumorigenesis in mouse models**

Function	Gene	Mouse model	Relevant phenotypes	Refs
DNA replication				
Replication licensing factors	*CDT1*, *CDC6*	Xenograft of CDT1- and CDC6-expressing papilloma cells in immunocompromised mice	Cell transformation and tumorigenesis	40
	CDT1	Grafts of CDT1-overexpressing cells in immunocompromised mice	Tumorigenesis	175
		T cell-specific expression in p53-deficient mice	Thymic lymphoblastic lymphoma	176
MCM2–7 replicative helicase complex	*MCM2*	Hypomorphic allele	Lymphomas	177
	MCM4	Hypomorphic allele	Genetic instability and mammary adenocarcinoma	32
Replication protein A	*RPA1*	Heterozygosity in one of the three RPA1 DNA binding domains	DSB repair defects, chromosomal instability and lymphoid tumours	119
Structure-specific nuclease	*FEN1*	Heterozygosity in a spontaneous colon cancer mouse model	Increased frequency of adenocarcinomas	178
		Nuclease activity-deficient mice	Strong cancer predisposition	179,180
Nucleotide metabolism				
Ribonucleotide reductase	*RRM2*, *P53R2*	Overexpression of either of the small subunits	Lung carcinogenesis	181
Thymidylate synthase	*TS*	Xenograft of TS-overexpressing cells in immunocompromised mice	Tumour development	182
		Overexpression of the human gene in mouse pancreas	Pancreatic endocrine tumorigenesis	183
Checkpoints				
Apical kinases	*ATR*	Haploinsufficiency	Small increase in tumour incidence	24
		Haploinsufficiency in MMR-defective mice	Early tumour development	184
	ATM	Disruption	Thymic lymphomas	185–187
Effector kinases	*CHK1*	Heterozygosity in p53 haploinsufficient mice	Synergistic enhancement of mammary tumour formation	143
		Hemizygosity in mouse skin	Enhanced carcinogen-induced tumorigenesis in mouse skin	26
	CHK2	Heterozygosity or disruption in *Chk1*$^{+/-}$ mice	Progressive cancer-prone phenotype	188
		Disruption in MRN-deficient mice	Tumour predisposition	189
Translesion and gap-filling synthesis				
DNA polymerase η	*POLH* (*XP-V*)	Disruption	UV irradiation-induced skin cancer	190
DNA polymerase ζ	*REV3L*	Conditional deletion in mice	Spontaneous mammary tumours	191
Replication fork restart and repair				
RecQ helicases	*RECQL5*	Disruption	Strong cancer susceptibility	192
	RECQL4	Helicase domain deletion in a mouse model of intestinal adenomas	Increased cancer susceptibility	193
	BLM	Disruption	Enhanced mitotic recombination and tumour susceptibility	194
Structure-specific endonucleases	*MUS81*	Heterozygosity or disruption in p53-deficient mice	Accelerated tumorigenesis	195
	SLX4	Disruption	Increased incidence of epithelial tumours	196
Single-stranded DNA binding protein	*SSB1*	Conditional deletion in adult mice	Increased cancer susceptibility	118

Table 1 (cont.) | **A selection of genes involved in replication stress-mediated tumorigenesis in mouse models**

Function	Gene	Mouse model	Relevant phenotypes	Refs
Replication fork restart and repair (cont.)				
Breast cancer susceptibility factors	*BRCA1*	Conditional deletion in mammary epithelial cells	Mammary gland tumorigenesis	197
		Homozygous truncation	Lymphomas and late-onset sarcomas and carcinomas	198
		Disruption in *Trp53*$^{+/-}$ mice	Mammary, ovarian and lymphoma tumours	93
	BRCA2	Homozygous truncation	Thymic lymphomas and DNA repair defects	199
		Conditional loss of function in epithelial tissues of p53-deficient mice	Mammary and skin tumours	94
Fanconi anaemia factors	*FANCD2*	Disruption	Increased incidence of late-onset epithelial tumours	200
		Disruption in *Trp53*$^{+/-}$ mice	Early onset mammary and lung adenocarcinomas	95
	FANCC	Disruption in *Mcm4*-hypomorphic mice	Early onset spontaneous tumorigenesis	78

CHO, Chinese hamster ovary; DSB, double-strand break; MMR, mismatch repair; MRN, Mre11-Rad50-Nbs1; UV, ultraviolet. A more detailed version of this table is provided in Supplementary information S1 (table).

stabilized RAD51 filaments may be behind such an overexpression effect[89], as it could provide tumour cells with enhanced proliferative capacity and DNA damage resistance. Thus, although defects in replication fork protection promote tumorigenesis, the compensation of these defects in transformed cells might contribute to their robustness.

Post-replicative DNA repair. Replicative DNA polymerases have evolved to ensure fidelity and do not progress through DNA lesions. However, DNA lesions can be bypassed by alternative low-fidelity DNA polymerases that are responsible for TLS, or by using the newly synthesized strand of the sister DNA duplex as a template in HR-mediated template switching, and both of these constitute the general PRR process (FIG. 4a). TLS is the major pathway by which mammalian cells replicate across DNA lesions[99]. Despite their mutagenicity, TLS polymerases act as suppressors of tumorigenesis, as suggested by the downregulation of TLS polymerases Pol η, Pol ζ, Pol ι and Pol κ observed in several types of cancer[100]. Thus, Pol η co-localizes with tumour suppressors BRCA2 and partner and localizer of BRCA2 (PALB2) at stalled replication forks[101]. Mutations in the gene encoding Pol η (*POLH*) are associated with Xeroderma pigmentosum variant (XP-V), an inherited disorder with a high incidence of skin carcinoma (BOX 2). Individuals harbouring mutations in *POLH* show increased UV-induced mutations owing to the inability of their DNA repair mechanisms to bypass thymine dimers[102], and they have increased chromosome breaks and common fragile site (CFS) expression[103]. Cells lacking Pol ζ accumulate replication-dependent DSBs and chromosomal aberrations[104], and conditional loss of *Rev3l* (encoding Polζ) in adult mice increases tumour incidence that is enhanced in a p53-deficient background (TABLE 1). A key regulator of PRR is the ubiquitylation state of the proliferating cell nuclear antigen (PCNA) clamp. In yeast, PCNA monoubiquitylation, carried out by the Rad6–Rad18 ubiquitin-ligase complex, recruits TLS polymerases to the replisome, whereas polyubiquitylation, mediated by Rad5 and Mms2–Ubc13, promotes template switching[105]. Depletion of the Rad5 human homologues helicase-like transcription factor (HLTF) and E3 ubiquitin protein ligase SHPRH reduces polyubiquitylation of chromatin-bound PCNA upon cell treatment with genotoxic agents that stall replication forks[106], and increases chromosome breaks after treatment with the methylating agent methyl-methane-sulfonate[107]. Interestingly, HLTF is inactivated by hyper-methylation in a considerable number of colon, gastric and uterine tumours, indicating that HLTF silencing may confer a growth advantage and that HLTF could be considered a tumour suppressor[108]. Concordantly, loss of heterozygosity (LOH) at the region containing the *SHPRH* locus is found in melanoma, cervical and ovarian cancers[109]. Therefore, the deregulated activity of TLS polymerases and PRR seems to constitute a tumorigenic threat to replication.

HR repair. Persistent stalled replication forks that are generated under replication stress can collapse, leading to breaks that rely on HR for their error-free repair (FIG. 4a). In the absence of HR, NHEJ and mutagenic repair pathways may take over causing LOH or rearrangements, including translocations that are frequently found in cancer[11] (FIG. 4b). Consequently, HR dysfunction may shift repair towards mechanisms that alter genome integrity and cause tumorigenesis. BRCA proteins are good examples of HR DSB repair proteins that link replication stress and cancer (BOX 2). BRCA1 prevents repair via error-prone NHEJ through the removal of chromatin-associated tumour protein p53 binding protein 1 (53BP1) and by promoting the resection of DNA breaks[110,111].

Loss of heterozygosity (LOH). Loss of the wild-type allele of a diploid cell by deletion, gene conversion or chromosome loss.

Nondisjunction
Lack of chromosome segregation that gives rise to daughter cells with an abnormal number of chromosomes.

Anaphase bridges
String-like DNA fibres connecting two nuclei during chromosome segregation due to unresolved recombination or replication intermediates.

Breakage–fusion–bridge
Cycles of chromosome truncation and rescue by fusion of replicated sister chromatids resulting in chromosome rearrangements.

Cleavage furrow
Infolding of the cell membrane at the equatorial plane of the cell occurring during cytokinesis.

Consistent with the relevance of BRCA1-mediated HR in cancer prevention and cell proliferation, *Trp53bp1* deletion rescues cell growth and HR defects in *Brca1*-null cells and reduces the incidence of mammary carcinoma in BRCA1-deficient mouse models[110,111]. During HR, BRCA2 associates with RAD51 through the BRC-binding motif, several copies of which are found in the central portion of BRCA2, and promotes the replacement of the ssDNA-bound RPA complex by RAD51 (REF. 112). Indeed, bypass of BRCA2 function using a BRCA2–RPA fusion protein reduces spontaneous chromosomal aberrations induced in BRCA2-deficient cells[113]. Thus, both BRCA proteins promote error-free HR repair, a function that presumably contributes to their role as tumour suppressors, provided that NHEJ is involved in carcinogenesis[114]. This is supported by the fact that loss-of-function mutations in PALB2, which mediates BRCA2 recruitment to damaged DNA via BRCA1 binding, cause an increased risk of developing breast cancer similar to the predisposition that is seen with BRCA mutations[115,116]. Another example is provided by the ssDNA-binding protein SSB1, which promotes HR repair at stalled replication forks[117] and functions as a tumour suppressor, as inferred from a conditional mouse model[118] (TABLE 1). It certainly remains to be seen to what degree the roles of these proteins in replication fork protection and DSB repair are responsible for replication stress and tumorigenesis.

The antitumour role of HR as a pathway that attenuates the tumorigenic effect of replication stress is also supported by the elevated cancer incidence in *Rpa1*-mutant mouse models, which are defective in HR-mediated DSB repair[119]. The aberrant chromosome morphology observed in cells from patients with the tumour-prone Bloom syndrome (BOX 2) — who are deficient in the BLM helicase–topoisomerase complex that is required to properly process HR intermediates to complete the repair event[120] — also supports a role for HR in dampening the tumorigenic effect of replication stress.

Incomplete replication at mitosis onset

The presence of incompletely replicated loci or unresolved repair intermediates in mitosis can result in nondisjunction, lagging chromosomes and anaphase bridges. Such missegregating chromatids might break, potentially provoking the breakage–fusion–bridge events that are frequently found in cancer cells[121]. Accordingly, chromosomes that are trapped in the cell cleavage furrow during cytokinesis are frequently damaged, trigger the DDR and cause unbalanced translocations in mammalian cells[122]. Indeed, subtle replication stress or deficient repair can cause cells to enter mitosis with incompletely replicated chromosomes, resulting in prolonged metaphase arrest, anaphase bridges and mitotic extra centrosomes that lead to aberrant mitosis and global unbalanced chromosome segregation[123]. Lagging chromosomes that arise from mitotic errors can generate micronuclei in the daughter cell[124]. Micronuclei DNA replication is defective and leads to extensive DNA damage in G2, including DNA fragmentation upon premature chromosome compaction, which can give rise to chromosome aberrations[125]. Chromosomes within micronuclei, which may carry complex genomic rearrangements, eventually reincorporate

Box 3 | **Breakage-prone sites in cancer cells**

Fragile sites are a paradigm of breakage-prone regions. There are two types: common fragile sites (CFSs) and rare fragile sites. CFSs, late-replicating genome regions where breaks, gaps and constrictions appear repeatedly in metaphase chromosomes from cells undergoing replication stress[155], are the most relevant to cancer. Replication fork progression slow down or impairment at CFSs, as well as a lower density of replication initiation impeding replication completion at mitosis[11,156], have been documented and support the notion that CFSs are the most vulnerable regions to replication stress. Consistently, replication impairment at CFSs is accompanied by the activation of the S phase checkpoint; CFS stability requires ataxia telangiectasia and Rad3-related (ATR), and partial inhibition of ATR causes a fivefold to 20-fold increase in fragility[157,158].

Correlation between CFSs and cancer-specific breakpoints was inferred three decades ago[159], and recent data have confirmed that a low but nonetheless considerable number of cancer-associated deletions, and the majority of recurrent cancer-specific translocation breakpoints, map to CFSs[160,161]. CFSs are the preferred targets of oncogene-induced replication stress in pre-neoplastic lesions[162]. Replication stress-inducing chemicals generate DSBs within genes located in the fragile sites FRA10C and FRA10G, and induce rearrangements comparable to those found in papillary thyroid carcinoma[163]. In addition, the boundaries of several oncogene-containing amplified regions, a recurrent feature of tumour cells, coincide with CFSs in several cases, such as the *MET* amplicon, the boundaries of which lie within FRA7G in a human gastric carcinoma cell line[164], or *MYCN* amplicons, the boundaries of which map to FRA2C in neuroblastoma cell lines and primary tumours[165].

Several CFSs overlap with known or putative tumour suppressor genes. The best-known example is FRA3B, the most frequently expressed CFS in lymphocytes, which is located within the fragile histidine triad (*FHIT*) gene[166], a putative tumour suppressor that is frequently rearranged in many cancerous and precancerous lesions[167]. Replication inhibition produces a high frequency of *FHIT* deletions resembling those of tumour cells[9]. Therefore, replication stress can inactivate tumour suppressors located at CFSs. Further examples include the *WWOX* gene located at FRA16D[168], and the *PARK2* gene located at FRA6E[169]. Thus, the contribution of CFSs to tumorigenesis may be essentially driven by their associated gene functions. As CFS expression seems to rely on the actual replication initiation programme, which varies among different cell types[33,170], the contribution of CFSs to tumour-specific rearrangements is likely to be higher than previously suspected. Indeed, reassessment of recurrent cancer deletions revealed that more than 50% of them originate at CFSs[171]. Interestingly, members of a newly discovered class of early-S replicating fragile sites described in B cells frequently coincide with the breakpoints of most B cell lymphoma recurrent rearrangements[172], further strengthening the relevance of replication as a source of genome instability and cancer.

the main nucleus in subsequent cell cycles and may contribute to cancer development. This phenomenon could be the origin of chromothripsis, massive genomic rearrangements that occur in a single catastrophic event[126], although further work will be required to determine the molecular mechanisms involved.

The chromosome missegregation caused by the loss of the RB tumour suppressor reveals a link among replication stress, mitotic defects and tumorigenesis. RB prevents unscheduled replication, controls the expression of many mitotic genes, and mediates chromatin binding of cohesin and condensin complexes[127], which are responsible for sister chromatid cohesion and chromosome condensation, respectively. RB deficiency leads to defects in replication fork progression and mitosis, which can be rescued by either increasing cohesin stability or supplying exogenous nucleosides[128], suggesting that incomplete replication contributes to the mitotic defects associated with RB deficiencies.

During anaphase, some chromosomal regions remain connected by ultra-fine DNA bridges, which are coated by the BLM helicase–topoisomerase complex[129]. These bridges coincide with CFSs and are flanked by foci of the FANCD2 and FANCI members of the FA pathway, which probably represent sister chromatid interaction sites derived from unresolved replication intermediates[130,131]. Thus, BLM and FA pathways might have an additional role beyond replication fork stall and repair. Notably, enzymatic cleavage of entangled CFSs in mitotic cells by ERCC1 and MUS81–EME1 endonucleases contributes to the prevention of chromosome missegregation by promoting sister chromatid disjunction[132,133]. Colorectal cancer cells with chromosomal instability also show impaired replication fork progression and increased replication stress that results in ultra-fine anaphase bridges and in DNA damage in early M phase[134]. Therefore, structural and numerical chromosomal aberrations that occur or that are detected in mitosis during tumorigenesis can also be the result of replication failures and stress, a connection that needs to be investigated further.

Cancer diagnosis and treatment

The fact that replication stress is not observed in normal cells but is a common feature of most precancerous and cancer cells opens up new possibilities for cancer diagnostics by the identification of phosphorylated histone H2AX or 53BP1 foci, or the activation of the S phase checkpoints by phosphorylation of CHK1 or CHK2, among other factors. On the basis of the deficiency of cancer cells to respond to S phase checkpoint activation, new anticancer therapies could be developed that exacerbate this vulnerability of cancer cells without considerably affecting normal cells.

A promising and novel therapeutic approach has been proposed based on promoting the incorporation of damaged dNTPs specifically in cancer cells. This can be

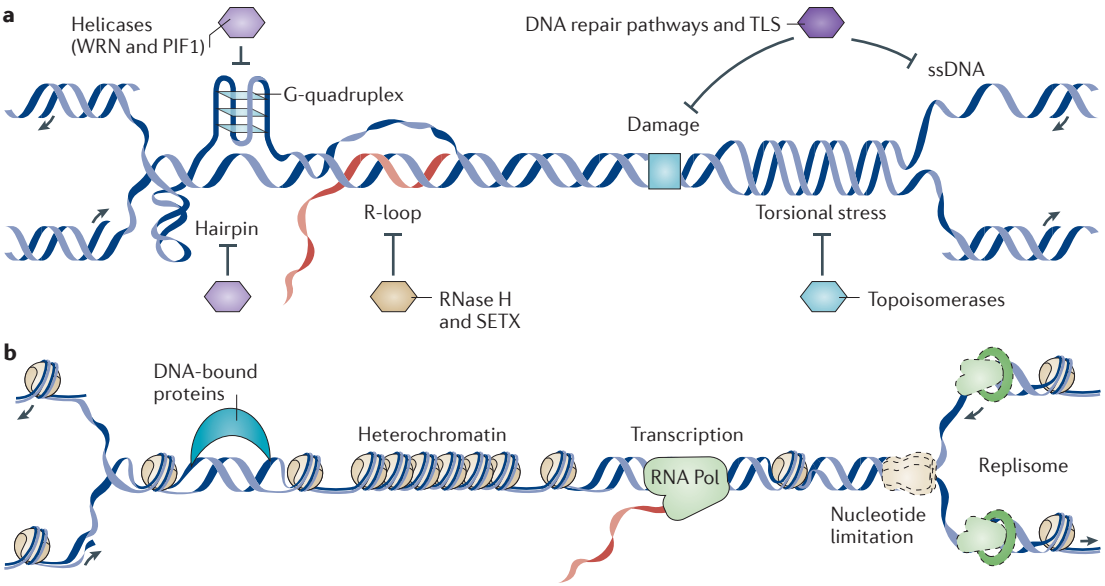


Figure 3 | **Impaired replication fork progression.** **a** | There are several DNA secondary structures able to impair replication fork progression: hairpins (which are favoured by specific DNA sequences such as trinucleotide repeats, palindromic sequences and AT-rich minisatellites), G-quadruplexes, R-loops, altered DNA topology, single-stranded DNA (ssDNA) or double-strand breaks (DSBs). A selection of key factors involved in resolving or removing specific obstacles are shown: DNA helicases such as Werner syndrome helicase (WRN) and PIF1 that remove DNA secondary structures including hairpins and G-quadruplexes; topoisomerases that remove DNA supercoils alleviating torsional stress; RNA-DNA helicase senataxin (SETX) or ribonuclease RNase H, which remove the R-loops by undoing the hybrid or degrading the RNA strand, respectively; DNA repair pathways that mend DNA lesions, gaps or breaks, and translesion DNA synthesis (TLS) that contributes to replication blockage avoidance[11,174]. **b** | In addition to DNA structures, several factors and conditions can also impair replication fork progression, such as tightly DNA-bound proteins (similar to the replication fork barrier protein Fob1 in budding yeast[53]), heterochromatin (that is, condensed chromatin and centromeres), transcribed genes, shortage of replication factors (such as nucleotides and replication protein A (RPA)). RNA pol, RNA polymerase.

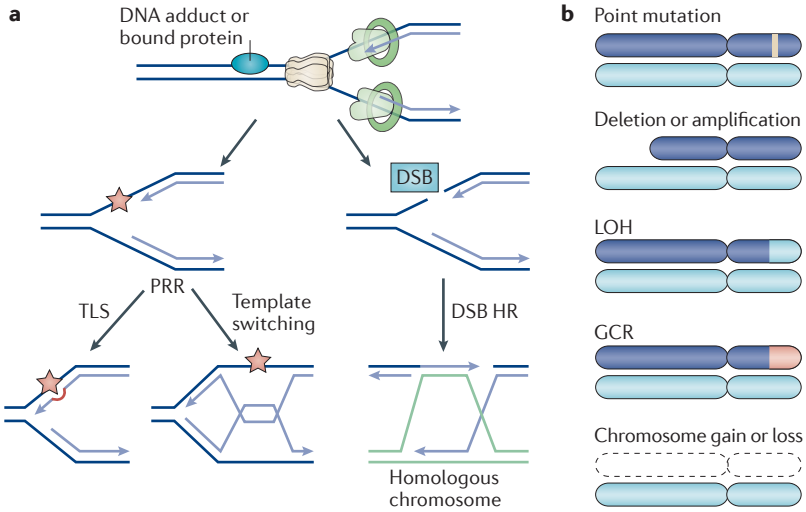


Figure 4 | **Genomic instability resulting from replication stress.** **a** | DNA adducts — DNA sequences covalently bound to a mutagenic chemical residue (blue oval) — or tightly DNA-bound proteins similar to yeast replication fork blocking protein Fob1 can block replication fork progression leading to single-stranded DNA (ssDNA) or double-strand breaks (DSBs) that activate the checkpoint. During replication, base lesions (orange star) can be bypassed via post-replicative repair (PRR) by translesion DNA synthesis (TLS) or by template switching with the sister chromatid using a homologous recombination (HR)-dependent process. Instead, DSBs are repaired by HR primarily using the sister chromatid, although this can also occur with the homologous chromosome (as shown). **b** | A defective response to replication stress by failure in PRR or HR can lead to genome instability, which can be observed as high levels of point mutations, deletions and amplifications, loss of heterozygosity (LOH), gross chromosomal rearrangements (GCRs) and chromosome gain or loss that presumably involve pathways or events such as error-prone DNA synthesis, non-homologous end joining (NHEJ), breakage–fusion–bridge, anaphase bridges, and so on.

achieved by targeting NUDT1 (also known as MTH1), a protein that prevents the misincorporation of oxidized dNTPs during replication and that is dispensable in normal cells but not in cancer cells[135,136]. This approach would thus specifically kill cancer cells. Alternatively, replication stress may be increased by the depletion of licensing factors. Thus, KRAS-positive cancer cells are sensitive to CDC6 depletion, and different tumour-derived cell lines have been hyper-sensitized to hydroxyurea and H_2O_2 upon origin recognition complex subunit 1 (ORC1) depletion[137,138]. An alternative strategy consists of increasing origin firing to induce re-replication, as achieved by depleting the CDT1 inhibitor geminin in cancer cells in which DNA damage has originated from re-replication, and this can trigger apoptosis[139]. As normal cells proliferate without re-replication, drugs that induce re-replication are candidates for anticancer therapy. One such drug is MLN4924, which causes CDT1 stabilization, re-replication, apoptosis and senescence in checkpoint-defective cells[140].

Although deficiencies in S phase checkpoints promote tumorigenesis in normal cells, mutations in the DDR that are acquired in many advanced stage cancers paradoxically make cancer cells dependent on the ATR–CHK1 pathway to proliferate. In other words, escaping the checkpoint system enables cell proliferation despite the accumulation of DNA damage early in tumorigenesis, but at the same time checkpoint-dependent cell cycle arrest is required to prevent transformed cells from entering mitotic catastrophe[141]. This can explain the frequent overexpression of CHK1 found in various tumours and provides a target for cancer therapies. Indeed, the well-known CHK1 inhibitor UCN-01 efficiently enhanced the sensitivity to DNA-damaging agents in p53-deficient cancer cells[142]. In the absence of exogenous DNA damage, CHK1 dysfunction generated either by depletion or by CHK1 inhibitors reduces mammary tumour formation in p53-deficient mice and kills mammary tumour cells[143], as well as MYC-driven lymphomas[19]. Similarly, ATR depletion protects against oncogene- or UV-induced carcinogenesis in mouse models[144], whereas ATR inhibitors sensitize ATM- or p53-deficient cancer cells and xenografts to conventional chemotherapeutic and radio-therapeutic agents[145–147]. Therefore, it is plausible that CHK1 and/or ATR inhibitors may improve the treatment of some cancers.

Finally, targeted inhibition of specific repair pathways can aggravate the toxicity of replicative lesions and jeopardize cell survival, as shown for BRCA-deficient cancers[148,149]. This is the case for poly(ADP-ribose) polymerase (PARP) inhibitors, which, when used as single therapies or in combination with DNA-damaging agents, are particularly efficient against tumours with defects in HR, such as BRCA-deficient tumours, by blocking the HR repair of replication stress-induced DSBs[150]. Along this line, specific inhibitors or the downregulation of error-prone gap filling and TLS Polβ have been shown to sensitize cancer cells[151,152]. The POLD3 subunit of DNA Polδ may also be a suitable target, as it was recently shown to enable the repair of broken replication forks using a type of break-induced recombination, and is thus required for S phase progression in cells undergoing replication stress[153]. Therefore, taking advantage of the persistent replication stress of cancer cells to target replication and DDR functions with specific drugs can open up new and powerful ways to fight cancer.

Conclusions and perspectives

In the past decade, different studies showing that replication stress is not only a major cause of genome instability but also a condition linked to pre-tumour and tumour cells have changed our perspective of cancer. As replication and the DDR are well-conserved processes, our understanding of how replication stress is generated and causes genome instability derives from studies on mammalian systems as well as model organisms such as *Saccharomyces cerevisiae*, *Xenopus laevis* and others. Our knowledge of the mechanisms of replication and the DDR, even though far from complete, has accelerated our comprehension of the early molecular steps in tumorigenesis. Oncogenic cells probably result in their early stages from a multifactorial process fostered by the altered function of several gene products — rather than a single gene product — that control replication stress and genome integrity. This complicates the establishment of a mechanistic relationship between particular gene mutations and cancer predisposition. Nevertheless, even though replication stress can be a prominent source of tumorigenesis, it also has the

potential of being a target for cancer therapy. This finding offers two possible applications: the use of replication stress markers as early molecular diagnostic tools; and the use of factors and cellular processes that are involved in replication stress as targets of antitumour drugs. Some of the important questions to answer in the future include why different tissues and cancer cells respond so differently to each replication stress-inducing condition, and the molecular basis of different types of cancer, as well as careful analyses of the prevalence of replication stress in different cancer types. This should allow us to understand the mechanisms and factors that may help to improve diagnostic and therapeutic approaches.

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Competing interests statement
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