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Jones, Rebecca; Petermann, Eva

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Replication fork dynamics and the DNA damage response

Rebecca M. Jones and Eva Petermann*

School of Cancer Sciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, United Kingdom

*Correspondence to: Eva Petermann; School of Cancer Sciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, United Kingdom; Email: e.petermann@bham.ac.uk

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Synopsis

Prevention and repair of DNA damage is essential for maintenance of genomic stability and cell survival. DNA replication during S phase can be a source of DNA damage if endogenous or exogenous stresses impair the progression of replication forks. It has become increasingly clear that DNA damage response pathways do not only respond to the presence of damaged DNA, but also modulate DNA replication dynamics to prevent DNA damage formation during S phase. Such observations may help explain the developmental defects or cancer predisposition caused by mutations in DNA damage response genes. This review focuses on molecular mechanisms by which DNA damage response pathways control and promote replication dynamics in vertebrate cells. In particular, DNA damage pathways contribute to proper replication by regulating replication initiation, stabilising transiently stalled forks, promoting replication restart and facilitating fork movement on difficult-to-replicate templates. If replication fork progression fails to be rescued, this may lead to DNA damage and genomic instability via nuclease processing of aberrant fork structures or incomplete sister chromatid separation during mitosis.

Introduction

In dividing cells, faithful and complete replication of the genome during S phase is essential for maintenance of genomic stability and cell survival. If the progression of DNA replication forks is impaired, this can lead to formation of aberrant DNA structures and generation of DNA damage including double strand breaks (DSBs). Spontaneous, replication-associated DNA damage may inhibit cell growth during development and promote genomic instability that leads to cancer. Consequently, cellular pathways that prevent and repair damage at replication forks are important during development and can act as tumour suppressors. Thanks to advances in the measurement of replication dynamics using DNA fibre approaches and *Xenopus* egg extracts [1, 2], it has become increasingly clear in recent years that DNA damage response and repair pathways do not only repair damage generated at replication forks, but also modulate the initiation and progression of DNA replication. Pathways that are activated by perturbed or stalled replication forks, such as checkpoint signalling and homologous recombination, modulate replication initiation and fork progression in response to DNA damaging treatments, but they also perform similar functions in the absence of exogenously induced damage. Replication dynamics and the incidence of replication-associated DNA damage are therefore often notably altered in cells with defects in these pathways, which may underlie the developmental and/or cancer-prone genetic disorders that can be associated with such defects. This review will discuss the molecular mechanisms by which the DNA damage response may regulate replication dynamics in vertebrate cells, based on findings from *Xenopus*, chicken DT40 and mammalian systems while also drawing on central findings from yeast and bacteria. We have aimed to concentrate on the most recent findings, as some of these topics have been covered in our earlier reviews [3, 4].

Eukaryotic DNA replication

Replication initiation

DNA replication initiates at genomic sites termed origins of replication. In higher eukaryotes, these are organised into initiation zones or clusters that are activated at different times during S phase [5, 6]. Initially, the DNA double helix is unwound to form a “replication bubble” before two replication complexes are assembled. Two individual replication forks (which form the replicon) then move bi-directionally

away from the origin, unless a replication fork barrier (RFB) is present. Origins are selected through binding of the origin recognition complex (ORC), a six-subunit complex consisting of ORC1-6 [7, 8]. Origin selection seems to be dependent on the ORC1 subunit [9, 10] and, at least in higher eukaryotes, is sequence-independent [11]. Factors influencing the spacing of origin firing include chromatin structure and epigenetics, nuclear organisation or –matrix, developmental stage and gene expression [12]. During G1 and prior to S phase, cell division cycle 6 (CDC6), chromatin licensing and DNA replication factor 1 (CDT1) and the mini-chromosome maintenance 2-7 (MCM) helicase complex are recruited to sites of ORC binding in a coordinated fashion to form the pre-replication complex (pre-RC) [13]. Replication is then initiated in early S phase as the pre-RC becomes specifically phosphorylated by the Cyclin E- cyclin-dependent kinase 2 (CDK2) and Dbf4/Drf1-dependent cell division cycle 7 (CDC7) kinase (DDK) [14, 15]. In budding yeast, the pre-RC components phosphorylated by CDK2 are Sld3 and Sld2 [16, 17], with the possible metazoan homologues of these proteins being (RecQ protein-like 4) RECQL4, geminin coiled-coil domain containing 1 (GEMC1) and Treslin [18-22]. DDK, on the other hand, phosphorylates the MCM2-7 complex, showing preference for the MCM2 subunit [14]. Phosphorylation of these pre-RC components promotes loading of cell division cycle 45 (CDC45) and the go-ichi-ni-san (GINS) complex, which are essential for the actual initiation of replication (origin firing) and fork progression [23-25].

Replication fork progression

The MCM2-7 hexamer and CDC45 form the active replicative helicase required for DNA unwinding during replication. While the MCM complex unwinds the parental DNA in an ATP-dependent manner, the GINS complex maintains protein-protein interactions within the replication complex (replisome) [26-28]. The replisome also contains the clamp loader, replication factor C (RFC), which helps to load the sliding clamp, proliferating cell nuclear antigen (PCNA), onto primed DNA. PCNA tethers the DNA polymerases to the chromosome, enabling processive and high-speed DNA replication [29]. The replisome contains three DNA polymerases, the polymerase α -primase and the replicative polymerases δ and ϵ , which replicate the lagging and leading strands, respectively. RFC and PCNA regulate the switch from the POL α -primase to the replicative polymerases [30], and also act as a loading platform for flap endonuclease-1 (FEN-1) and DNA Ligase I, which process and seal Okazaki fragments.

If replication forks encounter obstacles on the DNA template, they may stall or collapse. Such obstacles can include spontaneous DNA damage or secondary structures that may arise in repeated, A-T-rich or G-C-rich sequences [31, 32]. Replication fork stalling may also occur when the replisome collides with transcription machinery [33, 34] or when it meets an RFB, a DNA-bound protein or protein complex that actively promotes fork pausing or -stalling. Such RFBs promote mating type switching in fission yeast [35] or maintenance of ribosomal DNA repeat copy number from yeast to humans [36, 37]. A stalled replication fork is halted in its movement but able to resume progression when the blockade is removed. A collapsed fork, however, has become inactivated, for example by running into a single-strand break causing generation of a double-strand break or by dissociation of the replication machinery (reviewed in [4]). To stabilise forks, cells must maintain viable fork structures and assembly of the replication machinery until the replication block is removed, as will be discussed below. Replication forks that have been stalled for

many hours become inactivated and are unable to restart. Under these circumstances, global replication restart is achieved by new origin firing [38]. Inactivation of replication forks also coincides with the generation of fork-associated DSBs by the structure-specific endonuclease complex of MUS81 endonuclease homolog (MUS81) and essential meiotic endonuclease 1 homolog 1 (EME1) [38, 39].

Replication termination

Termination of DNA replication in eukaryotes occurs when two opposing replication forks converge. In bacteria, the circular chromosome contains a specific terminator site (TER) opposite to the origin of replication [40]. Efficient termination at these sites requires an RFB posed by the Tus protein, which blocks progression of the replicative helicase [41]. Less is known about replication termination in vertebrates but in *Xenopus* egg extracts at least, it appears that termination occurs at random sites [42]. A large amount of evidence from SV40 and yeast systems suggests that sister chromatids become intertwined (catenated) at replication termination sites and that the resolution of these structures, for successful completion of termination, requires DNA topoisomerase II (TOPII) [43-45]. Similar mechanisms are likely to operate in higher eukaryotes [46].

Chromatin remodelling during DNA replication

Efficient replication fork progression requires that the replisome gain access to the DNA through remodelling of the chromatin structure. Nucleosomes are disassembled ahead of the replication fork, leading to eviction of parental histones, which are recycled onto the daughter strands behind the replication fork, combined with de novo deposition of newly synthesised histones. Histone eviction likely requires ATP-dependent chromatin-remodelling enzymes, while histone chaperones sequester the released histones to facilitate recycling [47]. Histone chaperones such as anti-silencing function 1 (ASF1) and the FACT complex interact with the replisome and act during replication. FACT may be involved in disrupting nucleosomes ahead of the fork, while ASF1 acts as acceptor of parental and newly synthesised histones and donor for the major histone loader, chromatin assembly factor 1 (CAF-1) [47-51]. CAF-1 deposits histones behind the replication fork, and its interaction with PCNA couples chromatin assembly with DNA replication [47, 52]. ASF1 and CAF-1 are required for S phase progression [53-55], and both the FACT subunit structure specific recognition protein 1 (SSRP1) and ASF1 promote efficient replication fork progression. [49, 50].

The newly replicated sister chromatids need to be tethered together to promote the proper segregation of chromatids during mitosis. This process, termed sister chromatid cohesion, requires Cohesin, a ring-shaped complex consisting of four structural maintenance of chromosome (SMC) subunits [56, 57]. The complex is loaded onto DNA in a CDC7-dependent manner during G1-S phase [58]. Its role in maintaining a close distance between chromosome arms is key in facilitating sister chromatid-dependent modes of DNA damage repair [59]. Recruitment of the complex to sites of DNA damage is checkpoint-dependent [60-62], while mutations in the complex lead to an increase in chromosomal aberrations [59].

DNA damage response pathways at replication forks

Checkpoint signalling

Obstacles on the DNA that specifically affect the progression of the DNA polymerases can lead to uncoupling of the replicative polymerase and helicase

activities, with the helicase continuing unwinding to generate excessively long stretches of single-stranded DNA (ssDNA) [63, 64]. In addition, exonuclease resection of damaged DNA structures such as DSBs can generate ssDNA, which occurs especially during S and G2 phase as resection is controlled by CDK activity [65-67]. This ssDNA is recognised as DNA damage by the cell cycle checkpoint machinery. Specifically, replication protein A (RPA)-coated ssDNA recruits and activates the checkpoint kinase ataxia telangiectasia and Rad3 related (ATR) and its interaction partner ATR interacting protein (ATRIP) [68]. While loss of ATR and other components of the ATR pathway is embryonic lethal, hypomorphic mutations in ATR and other genes of the pathway can cause growth and developmental disorders such as ATR-Seckel syndrome [69, 70]. Activated ATR phosphorylates a large number of downstream targets involved in checkpoint signalling, DNA repair and apoptosis, such as histone H2AX [71] and tumour protein p53 (p53) [72]. One important role of ATR is the phosphorylation and activation of the effector kinase checkpoint kinase 1 (CHK1) [73, 74]. Efficient phosphorylation of CHK1 by ATR is promoted by several other replication and checkpoint proteins, including Rad9 homolog (RAD9), Rad17 homolog (RAD17), topoisomerase (DNA) II binding protein 1 (TOPBP1), Claspin, and the complex of timeless homolog (TIM) and TIMELESS interacting protein (TIPIN) [75-80]. CHK1 in turn phosphorylates the cell cycle phosphatases cell division cycle 25 (CDC25) -A, -B and -C to prevent activation of CyclinE/A-CDK2 and CyclinB-CDK1. This slows progression through S phase and prevents mitotic entry, providing the cell with time to restart or repair the stalled replication forks [81-83]. In budding yeast, the functional homologue of CHK1, Rad53, suppresses late origin firing in response to DNA damage by phosphorylating Sld3 and Dbf4 [84-86]. Other substrates of CHK1 include p53 and DNA repair proteins such as RAD51 homolog (RAD51), breast cancer 2, early onset (BRCA2) and Fanconi Anaemia, complementation group E (FANCE) (see below) [87-90].

The ataxia telangiectasia mutated (ATM) checkpoint kinase, mutated in the neurodegenerative and cancer-prone genetic disorder Ataxia telangiectasia, is activated in response to DSBs. This requires the meiotic recombination 11/ Rad50 homolog/ nibrin (MRE11/RAD50/NBS1) complex in concert with mediator of DNA-damage checkpoint 1 (MDC1), tumour protein p53 binding protein 1 (53BP1) and a host of other signalling and mediator proteins. Its effector kinase is checkpoint kinase 2 (CHK2), which similarly to CHK1 targets CDKs and p53 (reviewed in [91]). ATM is considered to be less important in the response to replication blocks than ATR. However, ATM is upstream of ATR activation by promoting exonuclease processing of DSBs, and can itself be phosphorylated and activated by ATR in response to replication blocks [67, 92]. Indeed, ATM signalling also slows DNA replication in response to DNA damage, likely by inhibiting origin firing, and plays roles in the stabilisation and repair of damaged replication forks [93-96].

Homologous recombination

Homologous recombination (HR) was initially identified as the pathway mediating genetic recombination in meiosis. It has become clear, however, that HR also repairs DNA double-strand breaks, single-strand gaps and damaged replication forks in mitotic cells, as well as having roles during normal DNA replication [97, 98]. HR repairs double-strand breaks by using homologous DNA sequences (the homologous chromosome, sister chromatid or homologous sequences elsewhere in the genome) as a template for DNA polymerase-mediated re-synthesis of the sequence containing the

break (reviewed in [99, 100]). Similar mechanisms are thought to be involved in the functions of HR during replication. In eukaryotes, HR activity is controlled by CDKs, restricting it to S and G2 phases of the cell cycle [65, 66, 101]. HR during S and G2 phases mainly uses the identical sister chromatids, produced by DNA replication, as homologous templates for repair, limiting the potential for large-scale genomic rearrangements.

The central HR factor is RecA in bacteria and RAD51 in eukaryotes. RAD51 is recruited to DNA ends with 3'-single stranded overhangs and possibly single-stranded DNA gaps, where it forms protein-DNA filaments [102]. RAD51 filament formation and -stability is regulated by a large number of RAD51 interacting proteins, including the RAD51 paralogues X-ray repair complementing defective repair in Chinese hamster cells 2 (XRCC2), XRCC3, RAD51 homolog B (RAD51B), RAD51C, RAD51D and BRCA2, the protein product of the breast-cancer susceptibility gene [103-106]. The requirement for single-stranded DNA makes HR repair of double-strand breaks dependent on DNA end resection by nucleases such as MRE11 and exonuclease 1 (EXO1), a process that is controlled by CDK activity and therefore restricted to S and G2 phase of the cell cycle [65, 66, 107]. Once bound to the overhang, RAD51 catalyses homology search and recombines the 3'overhang into the homologous double-stranded DNA to form a D (displacement)-loop, which generates a Holliday Junction (HJ) [108]. Upon completion of DNA repair synthesis, the remaining HJ structures are removed by HJ resolution by HJ resolvases such as Gen homolog 1, endonuclease (GEN1), or HJ dissolution by a complex of Bloom syndrome, RecQ helicase-like (BLM), topoisomerase IIIa (TOPIIIa) and RecQ mediated genome instability 1, homolog (hRMI1) [109, 110].

Efficient HR requires the Fanconi Anaemia (FA) pathway, so called because its components are mutated in patients with the autosomal recessive disorder Fanconi Anaemia. Mutations in the FA pathway cause extreme sensitivity to DNA crosslinking agents, cancer predisposition, developmental defects and anaemia [111]. In the current understanding of the FA pathway, its central factors seem to be FANCD2, which is phosphorylated by ATR in response to replication stress, followed by ubiquitylation by the activated FA core complex [112], and the tumour suppressor BRCA2 (FANCD1). While the FA pathway seems to interact with both translesion synthesis (see below) and HR, its role during HR is characterised better; in particular, BRCA2 directly promotes RAD51 function by facilitating RAD51 filament formation and -stability [113]. BRCA2 also interacts with the FA protein FANCN (PALB2) and together they promote RAD51-mediated D-loop formation [114, 115].

Alternative helicases and polymerases

In addition to the MCM2-7 replicative helicase, other DNA helicases play roles at replication forks encountering DNA damage. These helicases can remodel perturbed replication fork structures or difficult-to-replicate secondary structures to aid progression of the replisome. In humans these include two of the known RecQ helicases, Werner syndrome, RecQ helicase-like (WRN) and BLM. Individuals carrying non-functional copies of the BLM or WRN genes suffer from the rare autosomal recessive disorders Bloom and Werner syndrome, respectively, which are characterised by a strong predisposition to all types of cancer [116, 117]. Both BLM and WRN have functions in regulating HR; they can disrupt intermediate structures such as the HJ, thus preventing spontaneous HR and genomic instability at perturbed replication forks [118, 119], and whilst BLM also suppresses genetic rearrangements via its HJ dissolution activity [109]. The SWI/SNF related, matrix associated, actin

dependent regulator of chromatin, subfamily a-like 1 (SMARCA1) helicase, mutated in the genetic disorder Schimke Immunoosseous dysplasia, is an unusual ATP-dependent ssDNA annealing helicase that can potentially reverse previous unwinding by the MCM complex [120]. A further two DNA helicases with roles at perturbed forks are the FA pathway components FANCF/BRIP1 and FANCD1 [121-123]. Both can function similarly to BLM and WRN by remodelling stalled DNA replication forks [121, 123], and FANCF can also by unwind secondary structures [122, 124]. FANCF potentially works alongside specialised polymerases [125], which replace the conventional replicative polymerase in such circumstances; a process known as translesion synthesis (TLS). These polymerases can replicate past a variety of non-coding DNA lesions as their active sites can tolerate a distorted template (reviewed in [126, 127]). So far there are 5 known TLS polymerases: DNA Polymerases η , κ , ι , ζ (the latter comprised of two subunits REV3 and REV7) and REV1 homolog (REV1). REV1 interacts with all other known TLS polymerases via its C-terminus [128] and is believed to be a key regulator of lesion bypass. Because low-fidelity TLS polymerases may also increase the chance of mutations, TLS is only activated at sites of DNA damage through monoubiquitination of PCNA by ubiquitin-conjugating enzyme E2B (RAD6) and the E3 ubiquitin ligase RAD18 homolog (RAD18) [129, 130]. This allows switching from replicative to TLS polymerases as the latter have an increased affinity for monoubiquitinated PCNA through their ubiquitin-binding motifs [131]. Polyubiquitination of PCNA at the same residue by Rad5 in yeast, in contrast, activates not TLS but an alternative “error-free” damage avoidance mechanism thought to involve HR-like activities such as template switching (reviewed in [132]). Mammalian cells contain two Rad5 homologues, helicase-like transcription factor (HLTF) and SNF2 histone linker PHD RING helicase (SHPRH) [133-135].

Regulation of replication by the DNA damage response

Checkpoint signalling controls replication initiation

ATR and ATM signalling pathways down-regulate CDK activity, thereby reducing the overall levels of origin firing during normal S phase, and inhibition of these pathways increases origin firing [136]. This pathway is particularly well studied in the case of ATR-CHK1 signalling. Reduced CHK1 activity leads to accumulation of CDC25A in undamaged cells [82, 83]. This is accompanied by increased Cyclin E-CDK2 activity, increased loading of CDC45 onto chromatin and increased frequencies of origin firing [137-139]. CHK1 also regulates Cyclin A-CDK1 activity, which specifically promotes late origin firing [140]. It has been proposed that the ssDNA that is transiently generated during replication initiation causes low levels of checkpoint activation, thus generating a feedback loop to down-regulate further initiation [136]. In *Xenopus* egg extracts, checkpoint inhibition shortens the overall duration of S phase [136], although this is not necessarily the case in non-embryonic vertebrate cells, possibly due to the simultaneous fork slowing observed [141, 142].

Changes in CDK activity affect the activation of whole replication clusters more strongly than the activation of origins within clusters, and checkpoint inhibition therefore predominantly increases the number of simultaneously active clusters [143]. At the same time, other signalling pathways downstream of the checkpoint counteract the suppression of origin firing, possibly only within clusters. Polo-like kinase 1 (PLK1) is recruited during replication stress in an ATR-dependent manner and can phosphorylate ORC2 to counteract the checkpoint and allow some amount of origin firing even in presence of replication blocks [144, 145]. This is probably necessary

because activation of dormant origins within replication clusters serves as an important mechanism to allow completion of global replication and prevent genomic instability [146-149]. The above observations suggest that the checkpoint selectively allows origin firing within clusters but not activation of new clusters [143]. If, however, replication cluster activation is increased due to checkpoint inhibition, this causes DNA damage [139].

Checkpoint signalling controls replication fork progression

In addition to increased replication initiation, vertebrate cells that are defective in ATR-CHK1 signalling display reduced average speeds of replication fork progression during an unperturbed S phase. This applies to cells with defects in ATR, CHK1, Claspin or TIM [78, 150-153]. These observations suggest that, just as the S phase checkpoint regulates origin firing in unperturbed cells, it also modulates the progression of replication forks during normal replication.

Evidence from our own work suggests that replication initiation control by the checkpoint may underlie its promotion of replication fork progression. Preventing excessive origin firing in CHK1-defective cells by simultaneous inhibition of CDK2 using roscovitine, or siRNA depletion of CDC7, can restore normal speeds of replication fork progression [141]. This suggests that inhibition of CDK (and possibly DDK), to regulate origin firing, may be an important mechanism by which CHK1 promotes normal replication fork progression. In agreement with this, the generation of spontaneous DNA damage in CHK1-depleted cells depends on CDK2 and CDC25A [139, 154]. In another study, overexpression of active Cyclin A2-CDK1 increased origin firing and slowed replication fork progression, whilst loss of CDK1 increased speeds of fork progression [155]. Several mechanisms by which increased origin density might perturb replication fork progression can be envisaged. The increase in active replication forks may deplete replication factors such as nucleotides [156]. Changes in origin density could also interfere with the spatial coordination of replication initiation with transcription, which may increase conflicts between replication and transcription activities [157-160]. In addition to controlling origin firing, ATR-CHK1 signalling may facilitate replication fork progression by promoting fork stability via any of the pathways discussed below; reduced stability of spontaneously stalled forks is likely to result in slower average replication fork speeds.

Intriguingly, evidence suggests that in the presence of DNA damage, the checkpoint can also act to slow down replication fork progression, which would be a useful mechanism to reduce collisions of the replication machinery with DNA lesions. CHK1 and TIPIN have been shown to slow replication fork progression in presence of the DNA damaging agents camptothecin and UV, respectively [78, 161]. The molecular mechanism of checkpoint-dependent replication fork slowing is not yet understood, but it could involve phosphorylation of replication proteins such as the MCM complex [162].

The checkpoint controls replication fork stability

Checkpoint signalling stabilises replication forks i.e. it prevents the accumulation of aberrant fork structures or DNA damage during replication, both in cells treated with replication inhibitors and during unperturbed S phase. Components of both the ATR and the ATM pathways have been shown to stabilise replication forks stalled by replication inhibitors: ATR, ATM, MRE11, CHK1, Claspin, TIM and TIPIN [96, 163-165]. ATR, ATM, MRE11 and CHK1 also protect from DNA breakage during an

unperturbed S phase, possibly by stabilising spontaneously stalled forks in addition to promoting DNA repair [96, 139, 166, 167]. Checkpoint signalling might stabilise replication forks through a variety of downstream mechanisms. Checkpoint kinases phosphorylate the MCM complex [162] and it has been observed that the checkpoint prevents replication proteins from dissociating from stalled or collapsed forks [96]. Other checkpoint targets that promote replication fork stability include the BLM and WRN helicases [168-170]. Further, checkpoint kinases can potentially regulate HR by phosphorylating RAD51, BRCA2, FANCE and especially FANCD2 [87, 89, 90, 112]. In our hands, RAD51 depletion and CHK1 inhibition had an additive effect on inhibiting replication fork restart, suggesting that CHK1 activity stabilises stalled replication forks through mechanisms other than promoting HR [38]. However, the replication fork restart assay cannot distinguish between effects on replication fork progression and –stability [4].

The checkpoint also down-regulates the activity of nucleases that could otherwise inactivate stalled replication forks by aberrant resection or processing into DSBs: the structure-specific endonuclease Mus81-Eme1 in fission yeast and EXO1 in mammalian cells [171, 172]. Spontaneous DSB formation during replication in mammalian cells deficient in CHK1 or WEE1 (a checkpoint kinase that acts downstream of CHK1) is MUS81-dependent, but a direct regulation of MUS81-EME1 by checkpoint kinases has not yet been found [173, 174].

Another target of the checkpoint is the transcription machinery. In budding yeast, the functional homologue of CHK1, Rad53, prevents stalled forks from reversing into X-shaped structures representing HJ formation [175]. This fork reversal is thought to indicate fork collapse and has been explained by topological strains on stalled replication forks that result from the tethering of transcription to nuclear pores. In response to replication stress, checkpoint signalling targets nuclear pore components to disrupt this tethering and stabilise stalled forks [176].

The checkpoint may also stabilise stalled forks by promoting cohesion [60-62], or regulating histone dynamics at forks. The histone chaperone ASF1 is potentially regulated by ATM and CHK1, which inactivate the tousel-like kinases (TLK1 and TLK2), leading to dephosphorylation of ASF1 [177, 178]. During replication inhibition, ASF1 buffers excess histones so that a pool of histones is readily available for deposition once the block has been removed and replication forks restart [48, 51]. Changes in the regulation of ASF1 could potentially affect its promotion of replication fork progression [50], and evidence from yeast suggests that proper histone supply promotes the stability of replication forks [179].

Claspin, TIM and TIPIN may promote replication independently of ATR signalling

Certain components of the ATR pathway may promote replication fork progression independently of ATR signalling itself. While Claspin promotes replication fork progression in a manner similar to CHK1, it does not appear to control origin firing via CDC25 or CDK and seems to work in a pathway parallel to CHK1 [152, 164]. Accordingly, down-regulating origin firing in Claspin-deficient cells has only a small effect on increasing replication fork speeds [180]. Claspin, as well as TIM and TIPIN, may instead promote replication fork progression through their direct interactions with the replication machinery [181-183]. These proteins are orthologues of budding yeast Mrc1 (Claspin), Tof1 (TIM) and Csm3 (TIPIN), which form a complex that promotes both replication fork progression and –stability [184-186]. One way in which the Mrc1/Tof1/Csm3 complex is thought to achieve this is by helping to couple polymerase and helicase activities to prevent excessive unwinding and ssDNA

formation [186, 187], and a similar function has been proposed for its vertebrate homologues. TIM depletion increases spontaneous formation of ssDNA, supporting the idea that TIM-TIPIN could also counteract polymerase-helicase uncoupling [188]. This function appeared to be independent of ATR signalling, just as some functions of Tof1 and Csm3 are independent of the ATR homologue Mec1 [189]. Current evidence suggests that TIM and TIPIN may promote replication fork progression and -stability by promoting proper sister chromatid cohesion through their interaction with the cohesion factor WD repeat and HMG-box DNA binding protein 1 (AND-1) [165, 190-192]. TIPIN and AND-1 also promote loading of DNA polymerase α , which could facilitate replication fork restart by re-priming downstream of a lesion [190].

Homologous recombination promotes replication fork stability and restart

Our models of HR-dependent pathways of replication fork restart are largely informed by observations from *E. coli* [193, 194]. HR-dependent fork restart involves HJ and D-loop intermediates, which can be formed either directly from a stalled fork structure or after generation of a DSB at the fork. This process allows loading of the replication machinery in *E. coli* and budding yeast [97]. Similarly, HR plays an important role in fork stabilisation and restart in vertebrate cells. Defects in the HR proteins BRCA2, RAD51, MRE11, FANCA, or FANCD2 lead to accumulation of spontaneous and replication inhibitor-induced DSBs, suggesting that these proteins stabilise stalled replication forks [166, 167, 195-197]. The HR proteins MRE11, RAD51 and XRCC3 have been reported to facilitate efficient replication fork restart after treatment with replication inhibitors [38, 96, 198]. MRE11 is recruited to stalled replication forks [199-201] and may perform end processing so as to generate the lagging strand gap, or 3'-overhang, that would be required for RAD51 loading. In addition, MRE11 could perform a number of other functions. It interacts with BLM and WRN and might collaborate with these helicases in functions other than resection (see below) [199, 202]. Budding yeast Mre11 has been suggested to promote fork restart by facilitating sister chromatid cohesion [203].

Once the correct single-stranded overhangs are available, XRCC3 could then promote RAD51 loading for D-loop formation and fork restart. RAD51-mediated fork restart seems to differ from HR-mediated DSB repair in that it does not involve RAD51 foci formation, which would be indicative of very long RAD51 filaments, or detectable long patch recombination in a reporter construct [38]. Recombination-free restart could be supported by the BLM-TOPIIIa- hRMI1 complex, which resolves double HJ in a process that avoids crossing over [109]. If stalled forks collapse and are processed into DSBs after longer replication blocks, RAD51-dependent HR is required for the repair of the breaks [38, 170].

The role of HR at stalled replication forks in higher eukaryotes is complicated by the many additional factors that play roles during HR, such as breast cancer 1, early onset (BRCA1), BRCA2, poly (ADP-ribose) polymerase 1 (PARP-1) and the FA complex. BRCA2 and PARP-1 promote replication fork stabilisation or restart, which is attributed to roles in the recruitment and regulation of MRE11 and/or RAD51. PARP-1, absent in yeast but the major poly(ADP-ribose) polymerase enzyme in vertebrate cells, is activated by DNA damaged structures including ssDNA gaps and has a variety of signalling and recruitment functions in the DNA damage response. PARP-1 interacts with MRE11 [198, 204] and, in response to replication inhibitor treatment, it promotes MRE11 foci formation, ssDNA generation and replication fork restart, suggesting that PARP-1 promotes replication fork restart through MRE11-dependent DNA resection [198].

Supporting the idea that MRE11 may promote resection at stalled replication forks, the tumour suppressor BRCA2 was found to prevent excessive MRE11-dependent resection at stalled forks [205]. RAD51 itself has a similar preventive effect, and BRCA2 is thought to prevent resection by regulating RAD51 loading and the stability of RAD51 filaments [205, 206]. Absence of BRCA2 has not been found to impair replication fork restart, but it leads to fork collapse and increased genomic rearrangements after replication inhibitor treatment [195, 205].

The effects of HR proteins on the speed of replication fork progression are still poorly understood. There is evidence that HR-deficient cells display decreased fork progression during normal S phase [205, 207]. This could result from a requirement for HR to stabilise or restart forks that stall when encountering endogenous obstacles. On the other hand, the presence of RAD51 and its paralogues XRCC3 and XRCC2 actively slow fork progression on templates containing bulky lesions as induced by cisplatin. RAD54, which is involved in later stages of HR, was not required for this fork slowing. It therefore seems that it is RAD51 loading onto the damaged template that slows fork progression directly or indirectly [208]. This suggests that RAD51 activity does not serve to promote fork progression in this situation but rather the opposite, although HR is important for survival of cisplatin damage through its repair function [106].

Fork-remodelling helicases promote replication fork progression and restart

The WRN, BLM, FANCM and SMARCAL1 DNA helicases have all been implicated in replication fork progression and -restart [209-213]. Deficiency in both BLM and WRN causes slow fork progression in untreated cells, as well as defects in the restart of replication forks stalled by replication inhibitors or DNA damage [211-215]. BLM and WRN have the ability to unwind difficult-to-replicate secondary structures in concert with FANCD1, which may aid replisome progression [125, 216, 217]. In addition, BLM has been suggested to promote fork progression by indirectly modulating nucleotide pools. BLM-deficient cells display decreased levels of cytidine deaminase (CDA), which leads to a relative increase in cellular dCTP levels. Re-balancing of pyrimidine pools by expression of CDA or addition of deoxyuridine improved replication fork speeds, but not spontaneous fork stalling in BLM-deficient cells. This suggests that slow fork progression and replication fork restart defect in the absence of BLM may be two separate phenotypes [218]. BLM-dependent replication fork restart is assisted by RAP1 interacting factor homolog (RIF1), a DNA-binding protein of unknown biochemical function [219].

BLM and WRN might both promote fork restart after replication blocks through their modulation of HR. Both helicases could promote D-loop-mediated restart by facilitating fork regression to form a HJ [220, 221]. BLM is also involved in one of the two MRE11-dependent pathways of DNA resection, which may promote RAD51 loading during fork restart [107].

While promoting RAD51 function appears important for replication fork restart, BLM and WRN also possess anti-recombinogenic activities that could promote fork restart if HR activities are detrimental for restart under some circumstances (see above). BLM and WRN could prevent HR by reversing fork regression through their HJ migration activity and repressing aberrant formation of RAD51 filaments at stalled or collapsed forks [118, 119, 222]. Interestingly, ATR and ATM differentially phosphorylate WRN in response to replication blocks: ATR phosphorylation of WRN stabilises stalled forks early during replication blocks, while ATM phosphorylation promotes HR repair of collapsed forks after long replication blocks [170]. This could

suggest that WRN has an anti-recombinogenic function at stalled forks, and raises the possibility that preventing HR can be a requirement for fork stability under some circumstances. BLM- and WRN-deficient cells treated with replication inhibitors display increased RAD51 foci formation, which could be interpreted as elevated, unscheduled RAD51 filament formation [223]. Alternatively however, increased RAD51 foci formation could indicate an increased need for the repair of forks collapsed into DSB, as MUS81-dependent DSB formation at forks was observed in WRN-deficient cells [213], or a failure to complete HR. When remodelling replication forks, BLM and WRN may work together with the annealing helicase, SMARCAL1. SMARCAL1 is recruited to ssDNA-containing lesions such as stalled replication forks, and its absence leads to both ssDNA accumulation and defective replication fork restart, supporting the idea that SMARCAL1 may promote fork stability and -restart by re-annealing long stretches of ssDNA generated at stalled forks [210, 224].

The FANCM helicase seems to have particularly intriguing effects on replication. DT40 cells deficient in FANCM (but not other FA proteins) displayed not decreased, but increased replication fork restart after release from camptothecin that was dependent on ATR-, CDK- and PLK-activity [225]. FANCM-deficient cells also displayed increased ATR-dependent phosphorylation of MCM2 and increased origin firing. This suggests that the ATR- and PLK1-dependent pathway of promoting origin firing during replication stress becomes activated in absence of FANCM to allow replication restart in the vicinity of stalled forks [144, 225]. Human cells lacking FANCM, or FANCM ATPase activity, displayed increased replication fork speeds and reduced accumulation of ssDNA during unperturbed S phase and in the presence of replication inhibitors, suggesting that FANCM helicase activity slows replication fork progression [209]. In contrast to the first report, however, this group found that absence of FANCM reduced replication fork restart when cells were treated with camptothecin [209]. One potential reason for this discrepancy could be that new origin firing was able to rescue replication in DT40, but not human (HeLa) cells.

REV1 and HLTF facilitate replication fork progression and restart

The TLS pathway can maintain replication fork progression on templates containing bulky adducts by enabling lesion bypass directly at the fork (early response), but it also performs post-replicative filling of ssDNA gaps left behind the replication fork (late response) [226]. The REV1 polymerase in particular has been implicated in performing TLS directly at the fork, as it promotes replication fork progression on templates containing bulky adducts in chicken DT40 cells and mouse embryonic fibroblasts [226, 227]. It seems that, in DT40 cells at least, this REV1-mediated mode of fork-associated lesion bypass works independently of RAD18 and PCNA ubiquitination, which instead promote post-replicative gap filling by TLS [227]. This process is aided by the WRN helicase [228]. REV1 can also promote fork progression through secondary structures in the DNA, together with FANCI [125, 229]. In REV1-deficient cells, which are unable to bypass such lesions directly and rely on post-replicative gap filling instead, histone deposition at the site of lesions becomes uncoupled from replication fork progression. In this instance, specific silencing histone modifications are lost, which, should the lesion be located around a promoter region, can result in loss of gene silencing [125, 229].

The RAD5 homologue HLTF, which may be involved in the alternative “error-free” mechanism, has also been implicated in replication fork progression. Like RAD5, HLTF is a RING-domain ubiquitin ligase and SWI/SNF helicase. Via their helicase

activity, Rad5 and HLTF can branch-migrate HJ and reverse replication forks in vitro, which has been suggested to enable template switching during the alternative pathway [230, 231]. Indeed, the HLTF helicase activity promotes timely replication fork restart after release from the methylating agent MMS [231]. In apparent contradiction to this, MMS treatment also leads to degradation of HLTF, which in turn promotes the activity of the second Rad5 homologue SHPRH to suppress MMS-induced mutagenesis [133]. Since promotion of fork restart by HLTF was observed at earlier time points than HLTF degradation [133, 231], it seems possible that HLTF and SHPRH may be involved in early and late responses to MMS damage at forks, such as fork progression and post-replication gap filling, respectively.

Resolution of termination intermediates prevents genomic instability

Proper replication termination, and resolution of catenated DNA structures that may arise at termination sites, is ultimately required for the correct separation of sister chromatids during mitosis. Incomplete sister chromatid separation can be observed as DNA bridges that connect the daughter nuclei during anaphase. Especially the late-replicating centromeres frequently form such anaphase bridges even during normal cell growth, though the bridges disappear towards telophase, suggesting that replication intermediates are still resolved during mitosis [232]. In addition to TOPII, the complex of BLM, TOPIII α and hRMI1 is required for the resolution of anaphase bridges [232]. It was suggested that the latter complex helps resolve termination structures by a similar mechanism to its dissolution of double HJ structures during HR [232, 233].

Interestingly, there is increasing evidence that additional anaphase bridges can arise from impaired replication fork progression or a failure to activate dormant origins [147, 233, 234]. This presumably occurs because cells enter mitosis without having completed replication. Anaphase bridges resulting from replication inhibition do not localise to centromeric regions and are marked by foci of FANCD2 and FANCI, suggesting that the FA pathway is activated by the perturbed replication structures that give rise to these bridges [233, 234]. The FA pathway seems to promote BLM recruitment to help resolve non-centromeric bridges [234]. Increased levels of unresolved replication intermediates and anaphase bridges have been implicated in the generation of DNA damage and genomic instability. Inhibition of replication fork progression can cause symmetrical DNA lesions during the following mitosis and G1 phase [235]. Breakage of anaphase bridges can lead to the exclusion of chromosome fragments from the daughter nuclei to form micronuclei, resulting in loss of genetic material [147, 234]. That this could be a common mechanism underlying genomic instability is supported by the observation that anaphase bridges and micronuclei localise to common fragile sites, late-replicating genomic loci that frequently display instability in cells treated with replication inhibitors and are often re-arranged in FA, Bloom's syndrome and cancer cells [233, 234, 236].

Conclusions

Our insights into the effects of DNA damage response factors on the initiation and progression of DNA replication have greatly increased in recent years, mainly due to advances in measuring replication dynamics. We are also starting to gain a better understanding of how changes to replication dynamics cause DNA damage and genomic instability. The emerging picture suggests that DNA damage response pathways are activated during normal S phase by both endogenous DNA lesions and ssDNA intermediates at replication forks. The DNA damage response then promotes

the proper progression of replication forks by i) regulating aspects of replication such as initiation, stability of the replication complex and sister chromatid cohesion, ii) stabilising transiently stalled forks by regulating a variety of cellular pathways such as transcription to preventing aberrant re-arrangements and processing of these forks, iii) promoting replication restart by re-modelling of damaged replication forks and promoting firing of dormant origins and iv) taking over from the standard replication machinery to facilitate fork movement on difficult-to-replicate templates. If fork progression is impaired, this can lead to increased generation of DSBs at forks through the action of fork-processing nucleases. In addition, prevention of normal replication termination due to impaired fork progression and –restart can lead to incomplete sister chromatid separation followed by generation of DNA damage and genomic instability during mitosis. However, while we can observe the effects of the DNA damage response on replication dynamics, the exact nature of the molecular transactions at forks that underlie these observations are still largely subject to speculation, and further investigations and improved methodologies will be needed to test these speculative models.

One major issue that remains to be addressed is the nature of the DNA structures formed at perturbed replication forks *in vivo*, for example whether transactions such as fork regression occur in human cells. Finding ways to answer these questions would constitute a major breakthrough. Further open questions include whether the DNA damage response promotes proper DNA replication by coordinating and integrating it with other cellular mechanisms such as transcription or cell signalling. What are mechanisms and consequences of replication-associated DNA damage in stem cells and during development? How do genetic defects in factors that promote replication fork progression lead to phenotypes from growth failure (e.g. ATR-Seckel syndrome) to cancer predisposition (e.g. Bloom syndrome)? An interesting new light has been shed on the latter question by recent reports that activation of oncogenes can cause perturbed replication fork progression, which may be important for genomic instability during cancer development [237-239]. This suggests that studying replication-associated DNA damage will advance our understanding of cancer development and may open up new possibilities for cancer therapy.

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Conflict of interest

The authors declare no conflict of interest.

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Figure 1. Overview of vertebrate DNA replication

A) Origins of replications are organised into clusters that are activated at different times, and not all origins within a cluster are activated. Origins from during G1 phase, when ORC, CDC6 and CDT1 recruit the MCM2-7 helicase to chromatin (origin licensing). Origins are activated in S phase by phosphorylation of MCM2 by CDC7 and another protein, likely Treslin, by CDK2. This promotes loading of CDC45 and the GINS complex, which are essential for DNA unwinding and replisome loading to initiate replication (origin firing). **B)** The GINS complex maintains interactions within the replisome, which contains the MCM2-7 and CDC45 replicative helicase, the sliding clamp PCNA, and DNA polymerases δ and ϵ , which replicate the lagging and leading strands, respectively. During termination of DNA replication, sister chromatids become intertwined (catenated), and these structures are resolved by the DNA topoisomerase TOP2. **C)** Histones are evicted ahead of the fork and recycled onto the daughter strands, together with newly synthesised histones. The histone chaperones FACT may disrupt nucleosomes ahead of the fork, while ASF1 binds parental and new histones and transfers them to the histone loader CAF-1. The newly replicated sister chromatids are tethered together by the Cohesin complex, which is loaded onto DNA during G1-S phase.

Figure 2. DNA damage response pathways at replication forks

A) Stalled or damaged replication forks recruit and activate ATR, and double-strand breaks (DSBs) activate ATM. ATR and ATM phosphorylate substrates such as histone H2AX and CHK1 or CHK2, respectively. A number of checkpoint factors such as Claspin, TIM, TIPIN or MRE11 promote ATR and ATM activity. Activated CHK1 and CHK2 phosphorylate CDC25A–C, thus preventing activation of CDKs, leading to cell cycle arrest, and also promote other pathways such as DNA repair and apoptosis. **B)** DSBs at collapsed replication forks require homologous recombination for repair. MRE11 and EXO1 resect the DNA end to generate a 5' overhang for RAD51 loading, which is assisted by the RAD51 paralogues such as RAD51C and XRCC3, and the FA pathway including FANCD2 and BRCA2. RAD51 catalyses homology search and D-loop formation, which may allow re-establishing of a replication fork and DSB repair. The resulting Holliday junction (HJ) may be resolved by MUS81-EME1, GEN1 or SLX1-SLX4. **C)** The WRN, BLM and FANCD1 helicases can remodel perturbed replication fork structures. This includes reversal HJ formation by regression of the fork, which may prevent spontaneous HR. SMARCAL1 helicase is an ssDNA annealing helicase that can potentially reverse unwinding by the MCM complex. **D)** Bulky adducts that block the replicative polymerase (Pol ϵ) activate translesion synthesis (TLS) by polymerases such as REV1 and Pol η that can replicate a distorted template. In most cases, TLS is activated through monoubiquitination of PCNA by RAD18, causing polymerase switching. Polyubiquitination of PCNA by Rad5 (yeast) activates an alternative “error-free” damage avoidance mechanism thought to involve template switching.

Figure 3: Control of replication fork progression and –stability by checkpoint factors

ATR signalling down-regulates CDK activity, thereby reducing origin firing which may keep nucleotide (dNTP) levels high for optimal fork progression and prevent conflicts with transcription (RNA Pol). Checkpoint factors such as ATR, CHK1, Claspin, TIM and TIPIN stabilise replication forks through a variety of potential mechanisms such as stabilisation of the replisome, activation of BLM helicase and

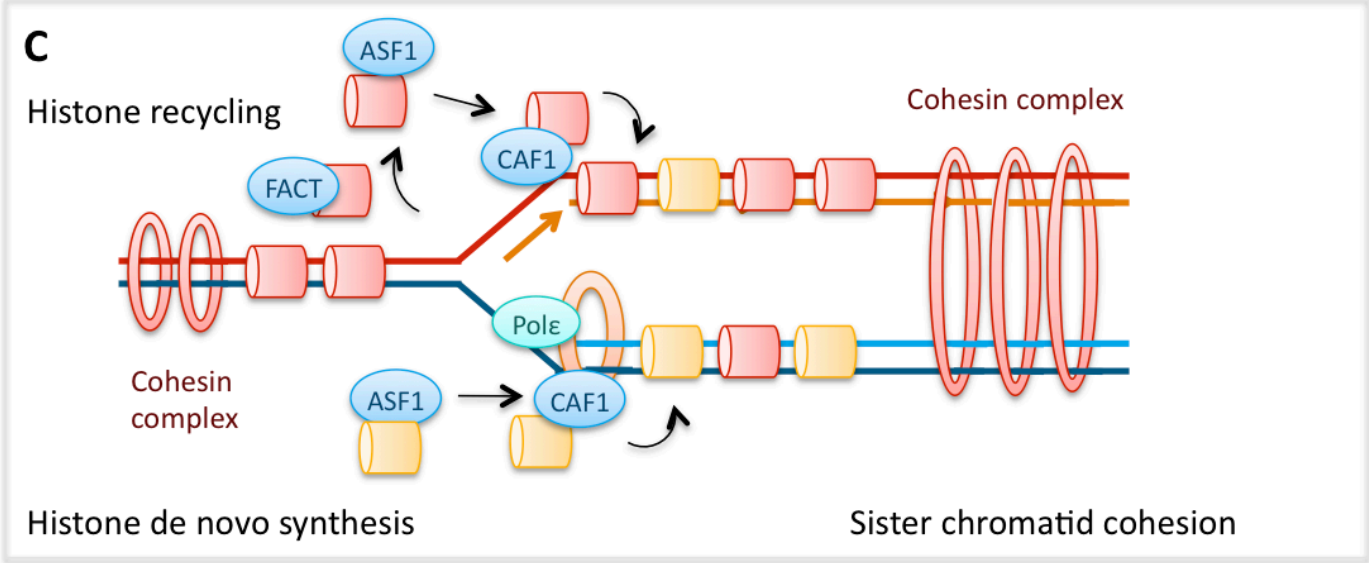
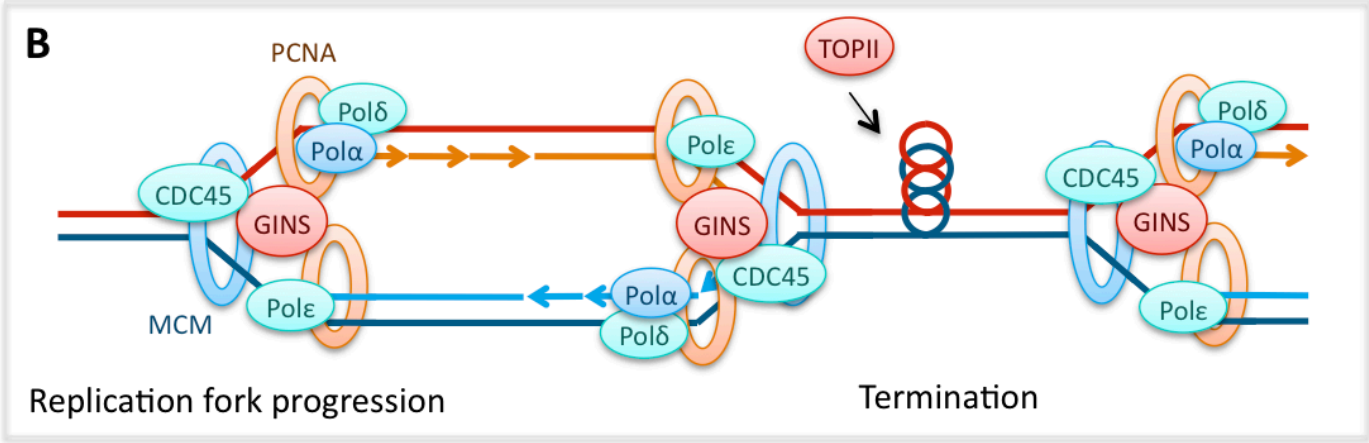
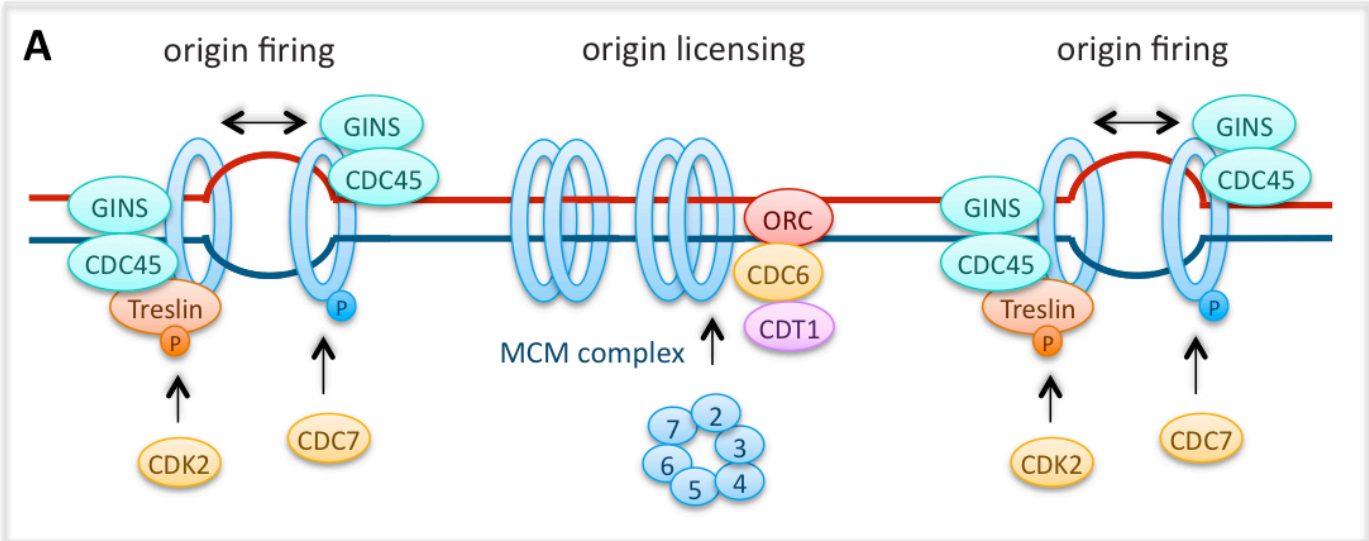
RAD51 to promote homologous recombination and inhibition of nucleases such as EXO1. The checkpoint may also stabilise stalled forks by phosphorylation of nuclear pore components (NUP1) to disrupt this tethering of the transcription machinery and by promoting sister chromatid cohesion (AND-1). **B)** In absence of checkpoint activity, origin firing is increased and replication fork progression is decreased, possibly because high origin firing causes nucleotide (dNTP) depletion and conflicts with transcription complexes. These factors, as well as low stability of the replisome or insufficient cohesion, may lead to replication fork stalling. Topological strains from transcription complexes may promote reversal of stalled forks, which then become prone to processing by nucleases such as MUS81-EME1 and EXO1, resulting in DNA breakage.

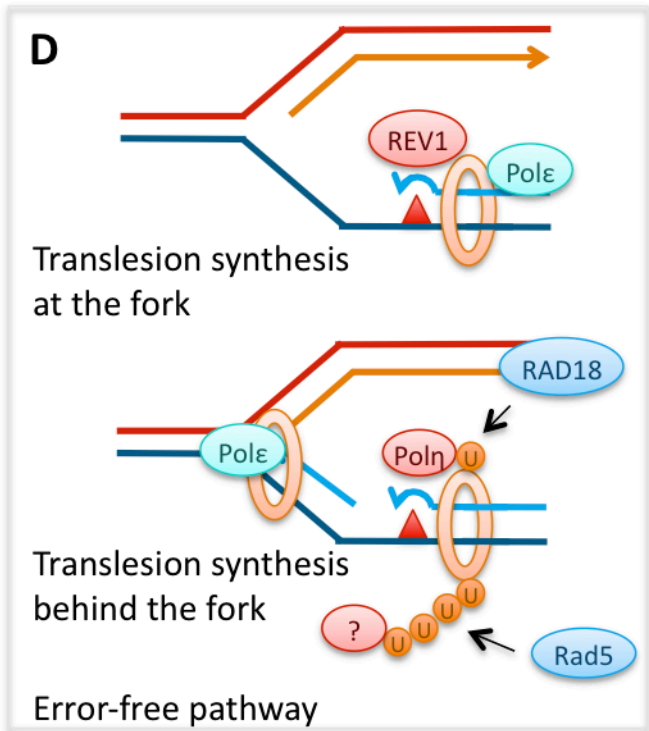
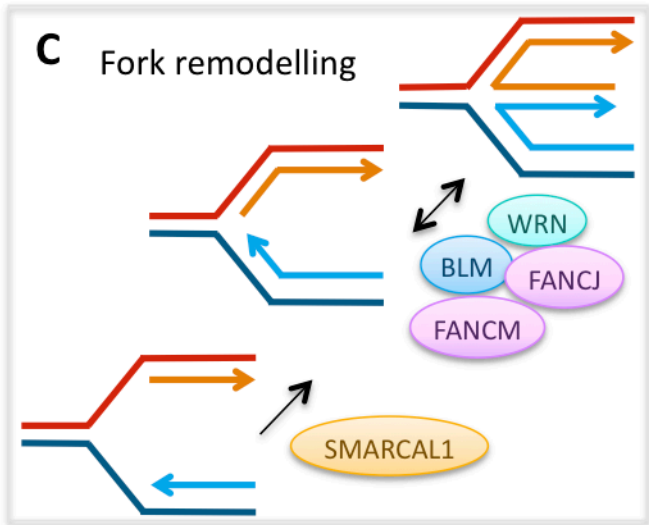
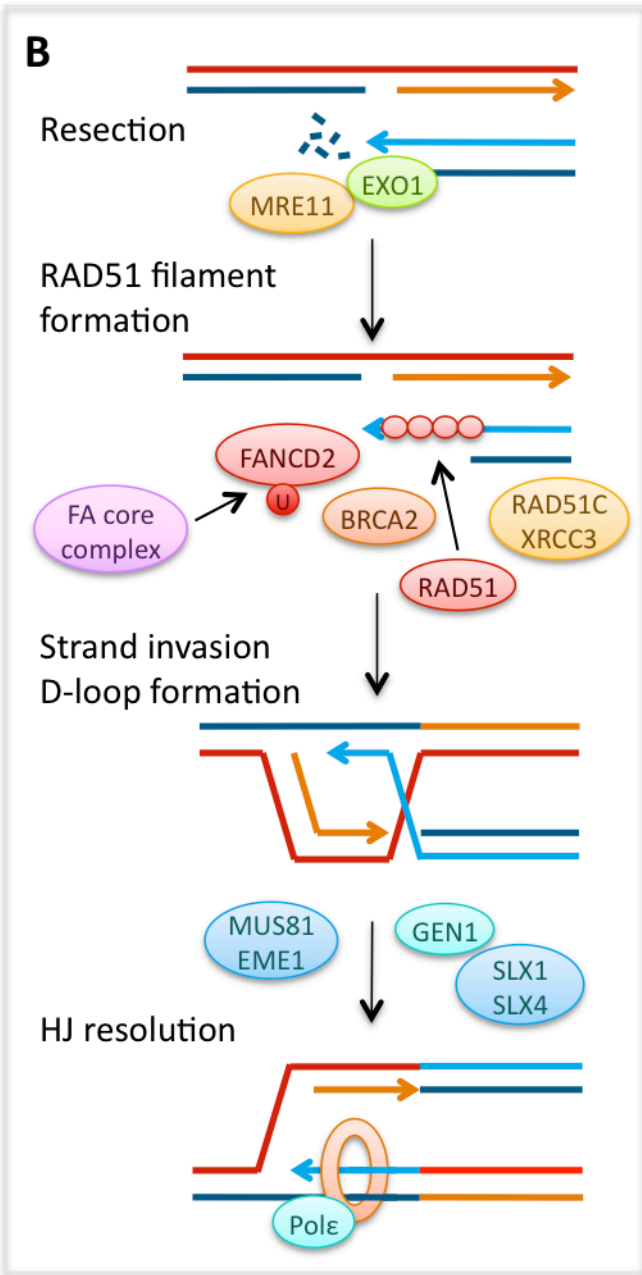
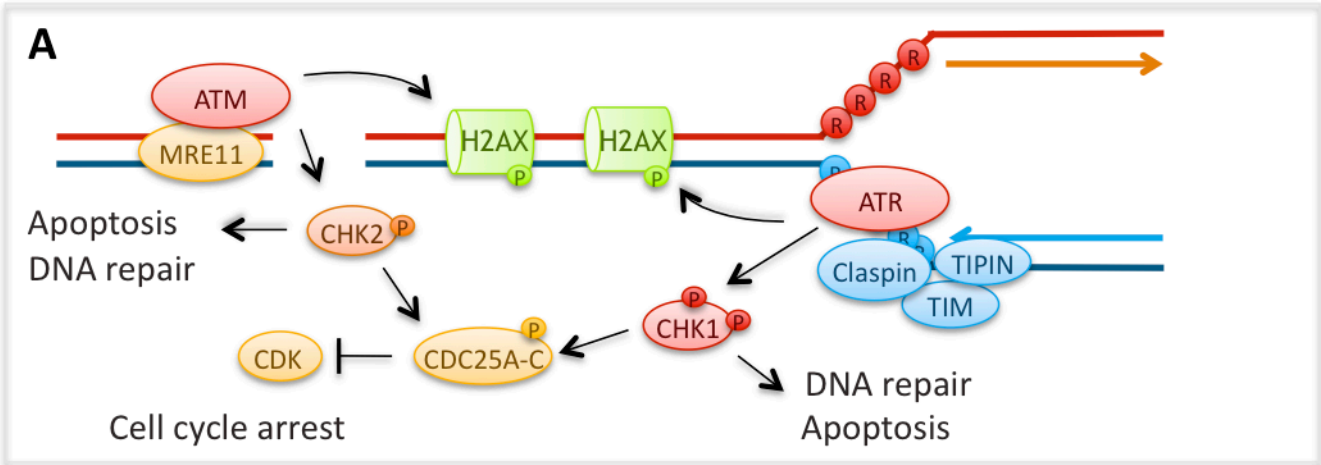
Figure 4: Replication fork restart by homologous recombination

In this model, a stalled and excessively unwound replication fork is re-annealed by SMARCAL1 and reversed into a HJ by BLM and WRN. MRE11 is recruited by PARP-1 to generate 3'-overhang for RAD51 loading. BRCA2, which together with XRCC3 loads RAD51 and stabilises the RAD51 filament, prevents further resection by MRE11. RAD51 promotes D-loop formation and fork restart. Recombination-free restart could be supported by the BLM, which resolves double HJ in a process that avoids crossing over.

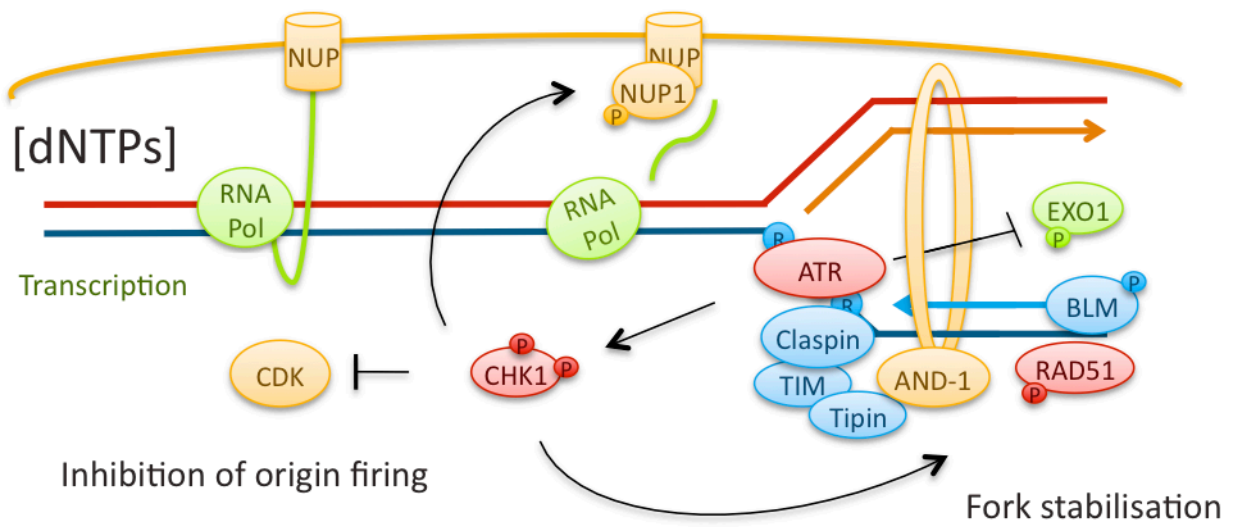
Figure 5: DNA helicases and REV1 promote replication fork progression and restart

A) BLM, WRN and FANCM could promote restart by facilitating fork regression to form a HJ or prevent HR by reversing fork regression and repressing aberrant formation of RAD51 filaments. SMARCAL1 may promote fork stability and -restart by re-annealing long stretches of ssDNA generated at stalled forks. In chicken DT40 cells lacking FANCM, replication restarts by an alternative pathway mediated by PLK1, which phosphorylates ORC2 to promote new origin firing. **B)** BLM and WRN promote fork progression, possibly by unwinding difficult-to-replicate secondary structures together with FANCI. BLM may also promote fork progression by modulating nucleotide pools via controlling cytidine deaminase (CDA). In contrast, FANCM was found to slow fork progression, possibly by remodelling fork structures. **C)** REV1 maintains replication fork progression on templates containing i) bulky adducts or ii) secondary structures, which is aided by WRN or FANCI, respectively. **D)** The HLTF ubiquitin ligase and DNA helicase is implicated in PCNA polyubiquitination and in the "error-free" DNA damage tolerance mechanism. HLTF might help reverse replication forks stalled by a methylating agent to promote fork restart, possibly after removal of the damage by a DNA repair mechanism.





A Active checkpoint



B Inactive checkpoint

