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Replication stress: getting back on track

Matteo Berti and Alessandro Vindigni*

Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University
School of Medicine, St. Louis, MO 63104, USA

Abstract

The replication stress response enables the DNA replication machinery to overcome DNA lesions or intrinsic replication fork obstacles, and is essential to ensure faithful transmission of genetic information to daughter cells. Multiple replication stress response pathways have been identified in recent years, raising questions about their specific and possibly redundant functions. Here, we review the emerging mechanisms of the replication stress response in mammalian cells, and consider how they may influence the dynamics of the core DNA replication complex.

DNA replication forks are frequently challenged and arrested by DNA lesions induced by endogenous or exogenous agents. In addition to DNA lesions, intrinsic replication fork obstacles such as transcribing RNA polymerases, unusual DNA structures, tightly-bound protein-DNA complexes and oncogene activation may also impede DNA replication fork progression¹. Replication stress can be defined as the transient slowing or stalling of replication forks in response to these challenges. In eukaryotes, most replication origins remain “dormant” and are replicated passively. If replication forks stall, dormant origins are activated to complete replication². However, if two converging forks stall in regions lacking dormant origins, cells must restart at least one of these forks to ensure full genome duplication. This is achieved using specific molecular pathways aimed at preserving the stability of perturbed replication forks and promoting their accurate restart.

The choice of the specific fork restart pathway depends on the nature and location of the replication challenge, for example, whether a DNA lesion is located on the leading or lagging template strand. If forks fail to restart, they “collapse.” Fork collapse was initially linked to dissociation of the replisome components³, however, this model has been challenged by recent data⁴. Fork collapse has also been linked to fork breakage—*i.e.*, the formation of a double-strand break (DSB) at the stalled fork. DSBs might result from endonucleolytic cleavage or may arise when the replication fork collides with a lesion in its path, a process commonly known as “replication run-off”. Here, we provide an overview of the emerging mechanisms of the mammalian replication stress response. We also discuss current models and controversies of how replisome dynamics may be altered when replication forks face different types of challenges, and the implications of these changes for replication fork restart.

Correspondence: avindign@slu.edu.

Replication stress response mechanisms

In eukaryotes, DNA replication begins with assembly of a replisome complex at multiple genomic replication origins (Box 1), and DNA synthesis is subsequently initiated in a process called origin firing ⁵. The parental DNA duplex is unwound by a replicative helicase comprised of the CDC45 protein, the hetero-hexameric ring complex MCM2-7 and the tetrameric GINS complex, which together form the so-called CMG complex ⁶. Concomitantly, DNA is replicated by the leading and lagging strands polymerases, Pol ϵ and Pol δ , which are associated with CMG. A common structural determinant linked with replication stalling is the single stranded DNA (ssDNA) formed at replication fork junctions ^{7–10}. This ssDNA may arise from physical uncoupling of the polymerase from the replicative helicase, which continues to unwind the DNA duplex after the polymerase stalls in response to base damage or deoxynucleotide [dNTP] depletion. However, agents that create physical blocks to helicase movement, such as interstrand crosslinks (ICLs), or torsional stress induced by the DNA topoisomerase I cleavage-complex, are not expected to promote uncoupling. The fact that ssDNA is also detected in the presence of these agents suggests that specific nucleases and helicases or translocases actively process stalled forks to create ssDNA at the fork junctions ^{10–12}.

Box 1

Replisome architecture

Key replisome components are displayed schematically. The replisome complex assembles at replication origins during the G1 phase of the cell cycle, and subsequently initiates bidirectional DNA synthesis in S phase in a process called origin firing. Parental DNA is unwound by the CMG helicase complex. CMG is comprised of the CDC45 protein (yellow), the mini-chromosome maintenance 2–7 complex (MCM2-7, green), and the tetrameric GINS complex (green). CMG encircles the ssDNA on the leading strand and unwinds DNA by steric exclusion moving in the 3' to 5' direction. DNA synthesis on the leading and lagging strands is performed by the Pol ϵ (orange) and Pol δ (green) polymerases, respectively, which make contact with CMG. The sliding clamp PCNA (red circle) acts as processivity factor for the polymerases. Additional replisome factors that regulate polymerase functions and coordinate DNA synthesis with unwinding of the template strand CMG are not shown.

ssDNA is rapidly coated by the single-stranded DNA binding protein Replication Protein A (RPA) (Fig. 1a,b). RPA-coated ssDNA stimulates activation of the DNA damage checkpoint kinases ATR and Chk1. Once activated, the ATR/Chk1 checkpoint response recruits accessory proteins to stabilize the halted fork and ensure rapid resumption of DNA synthesis^{13,14}. ATR-mediated signaling orchestrates different pathways at stalled forks: in addition to inhibiting cell-cycle progression and regulating intracellular dNTP levels to ensure proper fork repair and restart, ATR phosphorylates and thereby regulates the activity of several replisome components and fork-remodeling enzymes^{13,15,16}. For example, ATR promotes the association of the *Fanconi Anemia* (FA) protein FANCD2 with the MCM replicative helicase, and this interaction slows DNA synthesis and prevents formation of long ssDNA stretches under conditions of reduced nucleotide pools¹⁷. ATR activation has both a positive and negative effect on replication origin firing in response to replication stress; it prevents new origin firing by inhibiting replication initiation, but it also promotes firing of dormant origins within pre-existing replication factories to complete DNA synthesis in the vicinity of perturbed replication forks^{18,19}. Indeed, unscheduled origin firing in ATR-deficient cells generates a large excess ssDNA that exhausts the cellular pool of RPA, leading to breakage of unprotected ssDNA²⁰. ATR-dependent phosphorylation of FANCD2 also inhibits dormant origin firing while promoting replication restart²¹. Whether ATR is also required when forks are unable to efficiently resume DNA synthesis, for example to facilitate fork termination or fusion with another fork approaching from the opposite direction, remains unclear.

Fork repriming

Base modifications restricted to one strand of the DNA template do not present a physical block for the moving replicative helicase, but can stall polymerases and uncouple helicase unwinding from DNA synthesis. While lagging strand DNA lesions are well tolerated due to the inherently discontinuous nature of Okazaki fragment synthesis and maturation, leading strand lesions represent a major obstacle for processive DNA synthesis²². In these cases, DNA damage tolerance (DDT) mechanisms ensure that replication continues with a minimal effect on fork elongation, either by using specialized DNA polymerases or by postponing their repair. Fork progression may be facilitated by specialized polymerases called Translesion Synthesis (TLS) polymerases—POLH, REV1, POLK, POLI, REV3L/REV7,

POLN and POLQ— that have the ability to replicate through the damaged template, albeit with lower fidelity (reviewed in ²³). Alternatively, the replisome may skip the damaged DNA, leaving an unreplicated ssDNA gap to be repaired after replication. The bacterial replisome is able to reinitiate DNA synthesis downstream from a leading strand lesion by *de novo* priming and stalled replicative polymerase recycling or exchange ^{24,25}. This mechanism appears to efficiently restart replication in eukaryotes also, as proteins capable of “repriming” DNA synthesis beyond a lesion have recently been identified ^{8,26} (Fig. 1e). The human primase PrimPol ensures resumption of DNA synthesis after UV irradiation and under conditions of dNTP shortage ^{27–29}. Interestingly, PrimPol has also TLS activity, though it is currently uncertain whether its fork repriming or its lesion bypass activity is important for fork restart ²⁷. Defining the mechanisms that orchestrate the choice between repriming and TLS thus is an important subject of future investigation.

After repriming, the replisome resumes DNA synthesis, leaving a ssDNA gap behind it (Fig. 1e). This gap is usually filled by an error-free, Homology Directed Repair (HDR)-mediated process or by specialized TLS polymerases ^{30–32}. PCNA monoubiquitination and polyubiquitination may direct the repair of these ssDNA gaps by TLS synthesis or HDR, respectively ³³. Post-replicative gap repair is crucial for genome stability, since unrepaired ssDNA gaps may be converted to DSBs ²⁰. Based on recent studies suggesting that under-replicated regions lead to aberrant mitotic structures, we speculate that an excess of ssDNA gaps might overwhelm the repair and filling mechanisms operating in G2, leading to chromosomal aberrations and breaks during mitosis or during the following replicative round ^{34,35}.

Fork reversal

Fork reversal is an alternative DDT mechanism in which stalled replication forks reverse their course to aid damage repair, and entails remodeling of replication forks into four-way structures ^{36–39} (Fig. 1f,g). The fork reversal model was proposed almost 40 years ago for replication across UV damaged templates in mammalian cells ⁴⁰, and was later confirmed in prokaryotic systems (reviewed in ³⁶). Fork reversal was subsequently described as a pathological consequence of replication inhibition in checkpoint-deficient yeast cells ⁴¹. However, recent evidence from studies in metazoan cells indicates that fork reversal is a functionally important mechanism that enables DNA replication to pause and then resume without chromosome breakage ^{37,39}. We can thus consider fork reversal as an “emergency brake” that provides time, room and the correct DNA template for the DNA repair machinery to repair damage before replication resumes ³⁸. However, fork reversal can also lead to pathological transactions if the reversed forks fail to restart ³⁸.

Fork reversal occurs in two steps: reversed fork formation by coordinated annealing of the two newly synthesized strands, and restart of the reversed fork structures. Our current understanding of the mechanism of reversed fork formation is very limited. Several DNA translocases, including RAD54 ⁴², SMARCAL1⁴³, FANCM ⁴⁴, ZRANB3 ^{45,46}, Rad5 ⁴⁷ and its mammalian homolog HLTF ^{48,49}, can promote fork reversal *in vitro*. The same *in vitro* reaction is catalyzed by several helicases, including FBH1 ⁵⁰ and the RecQ helicase family members BLM and WRN ⁵¹. However, the *in vivo* function of these helicases has so far only

been confirmed for FBH1⁵⁰. Increasing evidence supports DSB repair-independent roles for several recombination factors in the replication stress response^{52–54}. In particular, the central recombinase RAD51 is required for fork slowing and reversal upon mild genotoxic stress¹⁰. Importantly, there is a strong correlation between the frequency of fork reversal and the amount of ssDNA at fork junctions suggesting that ssDNA formation is required for fork reversal by promoting RAD51 loading¹⁰. By analogy with its well-established role in DSB repair, RAD51 might be recruited to ssDNA at uncoupled forks and promote the initial step of fork reversal by invading the complementary parental strand (Fig. 1f). Future research should determine whether RAD51 also facilitates the recruitment of the motor proteins listed above to drive extensive extrusion of regressed arms. Alternatively, RAD51 may be required to stabilize forks in their reversed state by inhibiting the fork restoration activity of specific branch migration factors⁴².

The subsequent step of reversed fork restart has been elucidated in more detail. In particular, the human RECQ1 helicase drives the restart of reversed replication forks³⁷ (Fig. 2a), and its function is regulated by poly(ADP-ribose) polymerase (PARP1), which suppresses RECQ1 activity until the damage is repaired. Recently, a second human DNA2- and WRN-dependent mechanism of reversed fork processing and restart was identified⁵⁵, supporting earlier findings in *S. pombe*⁵⁶ (Fig. 2b). The DNA2 nuclease and WRN helicase cooperate to resect reversed replication forks with a 5′ to 3′ polarity and mediate fork restart⁵⁵. Interestingly, the role of DNA2 in reversed fork restart is not shared by other human nucleases, including EXO1, MRE11, and CtIP. There are two possible explanations for how DNA2-dependent resection promotes reversed fork restart (Fig. 2c). The 3′-tail generated by partial resection of the reversed arm may be specifically recognized by a motor protein that drives branch-migration-assisted re-establishment of a functional replication fork. For example, the SWI/SNF-related SMARCAL1 DNA translocase efficiently converts four-way junctions into functional replication forks, and displays a preferential polarity for reversed forks with a 3′-ssDNA tail coated by RPA⁵⁷. Alternatively, partially single-stranded DNA structures may activate an HDR-like mechanism of reversed fork restart, as previously suggested⁵². In this scenario, the 3′-overhang on the regressed arm might be coated by RAD51, which would mediate invasion of the duplex ahead of the fork, resulting in a Holliday junction structure that can be resolved by specific resolvases or dissolved by the combined action of the BLM helicase (Sgs1 in yeast) and the type I topoisomerase TOP3⁵⁸.

How cells choose between fork reversal, TLS, and repriming pathways is still unknown. Interestingly, repriming mechanisms at stalled forks limit extensive fork uncoupling, ssDNA gap formation and fork reversal in *S. cerevisiae* suggesting that these mechanisms are mutually exclusive⁵⁹. Based on emerging evidence, we suggest that PCNA post-translational modification may be a key regulator of pathway choice. For example, the yeast Rad5 homologues HLTF and SHPRH are DNA translocases that also promote PCNA polyubiquitination^{48,49,60,61}. PCNA polyubiquitination might promote fork reversal through the recruitment of translocases with reported fork regression activity, such as ZRANB3^{45,46}. Interestingly, the functions of HLTF and SHPRH appear to be DNA damage-specific, suggesting that cells might differently utilize these factors depending on the type of DNA damage⁶². Alternatively, PCNA monoubiquitination may promote TLS by recruiting specific TLS polymerases to stalled forks^{23,33}. Interestingly, human FANCD2 and RAD51

support PCNA monoubiquitination and TLS⁶³, suggesting that central FA and HDR factors may also act as a switch to balance fork reversal and TLS or repriming events. This notion is supported by the observation that the bacterial RAD51-homolog RecA regulates polymerase occupancy on moving replication forks and promotes TLS polymerase loading on the replisome⁶⁴. A key objective for future research would be to identify specific RAD51-mediators or signaling processes that promote one pathway *versus* the other.

Fork degradation and backtracking

Nucleases play key roles in processing stalled replication intermediates upon genotoxic stress^{53–55,65,66}. Here, it is important to distinguish the limited degradation of nascent DNA strands required for efficient fork restart^{55,66} (Fig. 1a,b) from the extensive degradation of stalled replication intermediates that underlies the pathological effects observed in FA- and BRCA-deficient cancer cells^{53,54,65} (Fig. 1c,d). For example, controlled DNA2-dependent degradation of reversed replication forks is a functionally relevant mechanism to mediate reversed fork restart and provide resistance to prolonged genotoxic treatments⁵⁵. This mechanism is distinct from the pathological MRE11-dependent degradation of stalled replication intermediates detected in the absence of crucial FA/HDR factors, including FANCD2, BRCA2, and BRCA1^{53,54}. The main conclusion of the studies performed in FA/HDR-deficient genetic backgrounds is that these factors stabilize RAD51 filaments at stalled replication forks to protect nascent strands from extensive MRE11-dependent degradation. This conclusion is supported by electron microscopy experiments in RAD51-depleted *Xenopus laevis* extracts showing a high frequency of MRE11-dependent ssDNA gaps at replication forks⁶⁷. Similarly, WRN has a non-enzymatic function in preserving nascent DNA strands from MRE11-dependent degradation at high camptothecin doses⁶⁸. Since MRE11 has limited nucleolytic processing activity, we envision that other nucleases acting downstream of MRE11 promote the extensive degradation observed in these studies. For example, the FA nuclease FAN1 acts downstream of MRE11 to extensively degrade the nascent strands in a FANCD2-deficient background⁶⁶. However, the same study showed that limited FAN1 activity, when properly controlled by FANCD2, is important for fork restart⁶⁶. This suggests that MRE11 can also initiate a limited and controlled nascent strand resection pathway that is beneficial for fork restart, if tightly controlled by FA/HDR factors. Indeed, MRE11 prevents DSB formation upon replication stress⁶⁹. Thus, the limited MRE11-dependent degradation of nascent strands might reflect a role for MRE11 in removing stalled polymerases and promoting repriming past the lesion⁷⁰. Alternatively, limited resection activity at stalled forks might create the proper DNA structure for RAD51 loading when the replication fork stalls. In the absence of key regulatory factors, uncontrolled nuclease activity may lead to extended nascent strand degradation, and the resulting nuclease-dependent ssDNA gaps that form behind the forks could promote reannealing of the parental strands and “fork backtracking” (Fig. 1d). The biological outcome of this effect is still controversial, but surely contributes to the genetic instability observed in FA- and BRCA-mutated cancer cells^{53,54}.

Other nucleases are implicated in the recovery from replication fork blockage, but the exact structure of the stalled replication intermediate(s) targeted by these nucleases is unknown^{53,71,72}. The fact that DNA2 nucleolytic processing of nascent DNA strands is not

detected in a RAD51 knockdown background that prevents reversed fork formation strongly suggests that DNA2 specifically targets reversed fork structures. Interestingly, RAD51 depletion seems to prevent both DNA2 and MRE11 nascent strand degradation, while perturbation of RAD51 function by BRCA2 depletion promotes extensive MRE11-dependent degradation⁵³. Our interpretation for this apparent discrepancy is that perturbation of RAD51 function might suffice to prevent fork reversal, hence DNA2-dependent degradation, but still allow residual RAD51 loading to promote MRE11-dependent degradation. Together, these observations suggest that the MRE11-dependent pathway likely attacks unprotected and non-reversed forks that are unable to reverse upon prolonged stalling^{53,54}.

Replication fork breakage

DNA replication is the major source of spontaneous DSBs in dividing cells. Prolonged fork stalling or failure to resume DNA synthesis by the mechanisms described above leads to fork collapse and one-ended DSB formation (Fig. 1h). However, DSBs are not necessarily terminal events for DNA replication, as cells can fix these breaks through an HDR pathway known as Break-Induced Replication (BIR)⁷³. BIR starts with a strand invasion event that can copy hundreds of kilobases of DNA from a donor molecule. This process is particularly important to complete DNA replication close to telomeric ends, which lack replication origins. Surprisingly, studies in yeast suggest that during BIR, the D-loop, formed by the invasion of the broken DNA into its homologous region within the genome, is not immediately resolved to re-establish a functional replication fork. DNA synthesis proceeds instead via a migrating D-loop, where lagging strand synthesis occurs in an unusual conservative-manner by using nascent ssDNA extruded from the migrating bubble as template⁷⁴⁻⁷⁶. The relevance of BIR to repair and restart of broken forks was recently confirmed in human cells by studying replication stress induced by cyclin E overexpression⁷⁷. However, a major problem with BIR is that it is highly error-prone, leading to frequent microhomology-based template switching and chromosomal rearrangements, particularly at repetitive sequences^{78,79}. Moreover, prolonged exposure of the extruded ssDNA during D-loop formation and dependence on the non-canonical polymerase activity of Pol32 in yeast (POLD3 in humans) makes BIR highly mutagenic⁸⁰.

Reversed replication forks that are unable to resume DNA synthesis might also represent a preferred substrate for structure-specific nucleases. Indeed, the MUS81 nuclease is associated with DSB formation upon prolonged fork stalling⁸¹ and reversed fork cleavage upon oncogene-induced replication stress⁸². SLX4-dependent endonucleases can also cleave similar structures under deregulated checkpoint conditions¹⁶. Under normal conditions, the activity of these structure-specific nucleases is restricted to late S- or G2-phases of the cell cycle by several checkpoint regulatory modifications⁸³. Thus, nuclease-mediated replication fork cleavage might represent the last attempt by cells to complete replication and/or to untangle the sister chromosomes before cell division. This view is challenged by studies showing that the endonuclease activity of MUS81 is important to restart stalled forks upon mild replicative stress⁸⁴ and even in the absence of exogenous DNA damage⁸⁵. The mechanism and structure of the targeted replication intermediate(s) is

currently unknown, but it seems that MUS81 associates with EME2 for its replicative function during S phase, while it forms a complex with EME1 when it acts in G2 ⁸⁶.

Replisome dynamics during replication fork restart

The fate of replication machinery during replication stress is a subject of ongoing and often controversial investigation. In particular, the notion that the core replicative helicase complex (CMG) is a static entity has been recently challenged by the discovery that this complex alters its modular and conformational features to deal with replication stress. The mechanism of CMG loading at replication origins requires extensive conformational changes of each of the protein constituents to assemble a functional complex ⁸⁷. The current model predicts that two MCM ring complexes initially load on replication origins in an “open” conformation to form an inactive helicase complex with the dsDNA duplex threaded through its central channel ^{88,89}. Next, binding of CDC45 and GINS closes the gap between the MCM2 and MCM5 subunits, leading to a “closed” conformation of the active helicase ⁹⁰. At this point, the CMG helicase can melt the DNA duplex by steric exclusion of the lagging strand and translocate along the leading strand with a 3′ to 5′ polarity ^{91,92}. An important implication of this translocation mechanism is that it enables CMG to bypass protein roadblocks on the lagging strand, but not on the leading strand ⁹³. DNA replication stalling induced by roadblocks on the leading strand might therefore cause the reversed transition from the “closed” to the “open” conformation of MCM2-7, releasing the leading strand. However, CMG dissociation from DNA would be prevented by CDC45, which traps the leading strand through its RecJ dead-exonuclease domain ⁹². The functional relevance of this mechanism during replication stress response is debated, but is consistent with several reports pointing to a role for CDC45 in controlling replisome dynamics during replication stress. For example, mutations in the DNA-binding domain of yeast Cdc45 cause hydroxyurea sensitivity, enhanced ssDNA formation and progressive decay of helicase occupancy at early replication origins. On the other hand, early origin binding by the replicative polymerase seems unaffected by the same mutations, suggesting that CDC45 regulates the helicase-polymerase functional coupling during replication stress ⁹⁴. An independent study using *Xenopus laevis* showed that CMG loses the GINS subunit, but not CDC45 and MCM2-7, upon replication fork collapse ⁹⁵. Interestingly, the same authors showed that replisome reloading to restarting replication forks requires RAD51 and MRE11 suggesting that the replisome can be re-established by a recombination-mediated process in an origin independent fashion. As discussed above, PCNA is also emerging as a key regulator between different replication stress response mechanisms and we refer the reader to other reviews for more insights on this topic ³³.

The notion that roadblocks on the leading strand represent an absolute block to replication fork progression was recently challenged by an elegant study which showed that the replicative machinery can traverse ICLs in a process that requires the FANCM translocase in complex with the MHF protein ⁹⁶. Considering that crosslinks represent absolute blocks for the replicative helicase, we posit that the replicative helicases might switch from the ssDNA bound/closed to the dsDNA bound/open conformation to traverse ICLs (Fig. 3). This transition would permit sliding over the block with the help of the dsDNA translocase activity of FANCM and reassembly on ssDNA in a replication-competent state.

Alternatively, the ability of *in vitro* reconstituted MCM2-7 complexes to passively slide on dsDNA suggests that latent/inactive MCM2-7 complexes, loaded in excess on chromatin, might represent a “helicase reservoir” for rescuing stalled replisomes⁸⁹. Despite their differences, both models imply a conformational transition of the MCM2-7 complex from an open to a ssDNA-bound closed conformation in order to resume fork unwinding past the lesion.

Replisome disassembly at perturbed forks is associated with dysfunctions in the ATR or Chk2 checkpoint kinase yeast homologues (Mec1 and Rad53, respectively)^{97,98}. However, this notion was challenged by the finding that the replisome is stably associated with DNA in the absence of the same kinases upon replication stress and that checkpoint-mediated phosphorylation of the replisome might influence its function more than its stability⁴. This model is supported by recent observations that the *Drosophila* CMG complex is negatively regulated by Chk2-mediated phosphorylation *in vitro*⁹⁹. However, the mechanism and outcome of checkpoint-mediated replisome modifications remains poorly understood and we consider this a central question to be addressed in the near future.

The main conclusion of the above studies is that the modular composition and structural plasticity of the CMG complex dynamically changes following genotoxic stress induction, probably in a DNA damage-specific fashion. An important challenge will be to define these conformational changes and uncover the specific pathways and factors that control them. For example, the relationship between replisome dynamics and the fork reversal process remains unclear. Intuitively, the remodeling of a three-way into a four-way junction should require replisome dissociation to allow annealing of nascent strands. However, different replisome conformations might still allow fork reversal without dissociation, as suggested by single-molecule experiments with T4 bacteriophage UvrW helicase¹⁰⁰. Moreover, reversed forks might provide a structural platform to prime the restart of stalled replisomes through the RAD51-mediated formation of a D-loop structure ahead of the fork (Fig. 2c). Interestingly, a similar structure primes origin-independent reloading of the bacterial replisome upon replicative stress²⁴. Further studies are however necessary to define the structural and molecular links between replisome dynamics and different mechanisms of stalled fork processing and restart.

Concluding Remarks

Defining these replication stress response pathways is integral to understanding the molecular mechanisms of genome stability and how these mechanisms impact cancer and aging¹. Most notably, cancer cells display elevated DNA damage and depend on replication stress response mechanisms to proliferate and overcome treatment by DNA-damaging chemotherapy agents. The number of newly discovered causes of replication stress and the network of mechanisms that the replication machinery uses to respond to genotoxic insults is constantly growing. The recent discovery of the mechanisms highlighted in this review, and of the many players involved, sheds new light on this process, but also raises several questions: How do cells choose between these apparently redundant mechanisms? Is the choice DNA damage- or DNA locus-specific? What is the fate of the replisome during the replication stress response? How do post-translational modifications regulate these

processes? The recent development of new single-molecule DNA fiber, electron microscopy, iPOND, and whole-genome sequencing technologies to study DNA replication provides new tools to address these pressing questions and we predict that they will lead to major breakthrough discoveries in the future by defining the precise structures and changes in the protein composition of the replication intermediates involved in the different replication stress response pathways.

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Figure 1. Mechanisms of replication fork processing and restart

Different mechanisms may resume DNA synthesis when replication forks are stalled by a leading strand lesion. **(a,b) Fork uncoupling/resection:** Replication fork uncoupling leads to ssDNA accumulation at the fork junction through functional dissociation of the MCM helicase and the stalled polymerase. Alternatively, fork uncoupling may result from nuclease-mediated resection of stalled forks. ssDNA is rapidly coated by the single-stranded DNA binding protein RPA (yellow spheres). **(c,d)** Several factors, including the HDR/FA proteins BRCA1, BRCA2 and FANCD2, regulate the stability of stalled replication forks, and prevent helicase-polymerase uncoupling or nucleolytic degradation of nascent strands^{53,54}. **(e) Fork repriming:** DNA synthesis can be reprimed (green arrow) and reinitiated ahead of a lesion or block. The resulting gaps are repaired post-replicatively by a recombination-based mechanism or by specific Translesion Synthesis (TLS) polymerases. TLS polymerases may also function at stalled replication forks to ensure continued DNA synthesis through damaged templates (not shown). **(f,g) Fork reversal:** A controlled resection/uncoupling event at stalled forks promotes RAD51 loading (orange spheres) and primes fork reversal. The exact location of RAD51 binding within forks is not known. Fork reversal prevents collisions between the moving fork and a block or lesion, allowing the lesion to be repaired by the DNA repair machinery. Alternatively, it may promote lesion bypass via template switching. **(h) Fork breakage:** Prolonged fork stalling promotes fork cleavage by structure-specific endonucleases. Broken forks are able to resume DNA synthesis by the error-prone Break-Induced Replication (BIR) mechanism.

Figure 2. Mechanisms of reversed replication fork processing/restart

Two mechanisms of reversed replication fork resolution have been identified to date, one dependent on RECQ1 helicase³⁷ and the other on DNA2 nuclease and WRN ATPase activity⁵⁵. **(a)** RECQ1 (yellow oval) restarts reversed forks via its ATPase and branch migration activity. PARP activity (green oval) is not required to form reversed forks, but it promotes the accumulation of regressed forks by inhibiting RECQ1 fork-restoration activity, thus preventing premature fork restart. **(b)** DNA2 and WRN (orange and green ovals, respectively) functionally interact to process reversed forks. DNA2 degrades reversed forks with a 5' to 3' polarity. WRN ATPase activity assists DNA2 degradation, possibly by promoting the opening of the reversed arm of the fork. RECQ1 limits DNA2 activity by an ATPase-independent mechanism. **(c)** Following DNA2-dependent processing, branch migration factors (grey oval) specifically recognize the partially resected reversed forks to promote fork restart. Alternatively, the newly formed 3' overhang of the reversed fork invades the duplex ahead of the fork, resulting in a pseudo-Holliday junction structure that can be resolved by specific resolvases or dissolvases to promote fork restart.

Figure 3. Replisome dynamics and ICL bypass

MCM2-7 adopts an open conformation upon replication fork blockage and it is able to slide on dsDNA and bypass the ICL (indicated by a yellow star). The FANCM/MHF complex is required for this process, however the exact molecular mechanism is unclear (not shown)⁹⁶. The question mark indicates that it is also unclear whether CDC45 and GINS are able to bypass the roadblock in complex with MCM2-7. It is thought that the GINS proteins may be released from the complex to allow this transition⁹⁵. The active replisome is reestablished ahead of the block in an origin-independent fashion and the ICL is repaired post-replicatively. MCM2-7 is shown in blue, CDC45 in yellow, and GINS in red.