



Published in final edited form as:

Mol Cell. 2022 June 16; 82(12): 2298–2314. doi:10.1016/j.molcel.2022.05.004.

Hallmarks of DNA Replication Stress

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SUMMARY

Faithful DNA replication is critical for the maintenance of genomic integrity. While the DNA replication machinery is highly accurate, the process of DNA replication is constantly challenged by DNA damage and other intrinsic and extrinsic stresses throughout the genome. A variety of cellular stresses interfering with DNA replication, which are collectively termed replication stress, pose a threat to genomic stability in both normal and cancer cells. To cope with replication stress and maintain genomic stability, cells have evolved a complex network of cellular responses to alleviate and tolerate replication problems. This review will focus on the major sources of replication stress, the impacts of replication stress in cells, and the assays to detect replication stress, offering an overview of the hallmarks of DNA replication stress.

eTOC blurb

This review discusses the major sources of DNA replication stress, the direct and indirect effects of replication stress on the genome, and the assays to detect replication stress in human cells, providing an overview of the hallmarks of DNA replication stress.

In eukaryotic cells, DNA replication is a fundamental cellular process in which the entire genome is duplicated once and only once during S phase of the cell cycle. Accurate DNA replication is essential for the faithful transmission of genetic information through cell divisions. DNA replication is initiated from numerous genomic loci termed replication origins (Bell and Dutta, 2002; Fragkos et al., 2015; Gilbert, 2010). The replisome, a multi-protein machinery containing the replicative DNA helicase and polymerases, unwinds template DNA and synthesizes nascent DNA on both leading and lagging strands, generating

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Author contributions

S.S. and L.Z. wrote the manuscript. Both authors approved the manuscript.

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Declaration of interests

The authors declare no competing interests. L. Z. is a member of the advisory board of Molecular Cell.

DNA replication forks (Burgers and Kunkel, 2017; Waga and Stillman, 1998). The progression of replication forks on DNA duplicates both strands of template DNA in a semi-conservative manner. DNA replication is finished by replication termination, which involves the convergence of replication forks, disassembly of the replisome and resolution of the daughter DNA molecules (Dewar and Walter, 2017).

While the DNA replication machinery is highly accurate, the fidelity of this process is often threatened by stresses of both exogenous and endogenous origins, resulting in altered replication fork progression, reduced replication fidelity, and DNA breaks (Techer et al., 2017; Zeman and Cimprich, 2014). This phenomenon, which is broadly termed replication stress, is an important source of genome instability in pre-neoplastic lesions and a hallmark of cancer cells (Bester et al., 2011; Burrell et al., 2013; Hanahan and Weinberg, 2011; Macheret and Halazonetis, 2015). Hence, elucidating the molecular basis of replication stress is essential to reaching a comprehensive understanding of tumorigenesis. In recent years, tremendous efforts have been made to define the causes of replication stress, ranging from oncogene activation to depletion of nucleotide pools, transcription-replication conflicts, and inherently difficult-to-replicate regions (Magdalou et al., 2014; Zeman and Cimprich, 2014). Depending on the type of stress interfering with replication forks, cells activate multiple response pathways to stabilize, repair and restart the forks, in order to ensure timely genome duplication and maintain genome stability.

In general, DNA replication stress induces three major intertwined responses at replication forks: (1) activation of the replication checkpoint, (2) remodeling of stressed or stalled forks, and (3) engagement of DNA repair or tolerance pathways. For instance, replication stress can perturb the coupling between the replicative helicase and polymerases, increasing the exposure of single-stranded DNA (ssDNA) at forks (Byun et al., 2005). The ssDNA at stressed forks is coated by replication protein A (RPA) and serves as a platform to recruit and activate the ATR checkpoint kinase (Zou and Elledge, 2003). The ATR activated by replication stress stabilizes stressed forks, suppresses origin firing, and promotes cell cycle arrests, thereby alleviating the deleterious effects of replication stress (Saldivar et al., 2017). Replication forks also undergo remodeling in response to stress. For example, through a remodeling process called fork reversal, stressed forks are converted into four-way junctions to regulate fork speed and facilitate DNA damage tolerance (DDT) and repair (Quinet et al., 2017b). Furthermore, several DNA repair or tolerance pathways are activated at stressed or stalled forks to enable them to bypass or recover from the stress. These pathways include translesion synthesis (TLS), PrimPol-mediated repriming, template switching (TS), break-induced replication (BIR), homologous recombination (HR), and others (Berti et al., 2020; Cortez, 2019). Together, these concerted responses at replication forks help ensure the timely completion of DNA replication and keep genomic instability at a tolerable level in the presence of replication stress.

Here, we review the various causes of replication stress in eukaryotic models, with an emphasis on evidence from human cells. In addition to the known barriers to fork progression, we discuss recently emerged sources of replication stress, such as dysregulated origin firing, excessive repair intermediates, and aberrant structures arising from DNA replication. We also discuss the cascading impacts of replication stress, from local effects at

stressed replication forks, to global effects on DNA synthesis and chromosome segregation, and to trans cell-cycle effects caused by persistent DNA lesions. Finally, we provide an overview of the assays to detect and quantify replication stress in human cells.

SOURCES OF REPLICATION STRESS

Replication stress arises from a variety of sources. These include direct barriers to fork progression such as DNA lesions and secondary DNA structures, R-loops, nucleotide imbalance, etc. (reviewed in (Zeman and Cimprich, 2014)). Indeed, the prevailing model has historically considered that replication stress acts *in cis* at replication forks. However, recent studies have identified replication-associated stresses that are spatially and/or temporally separate from active replication forks, raising the possibility that replication stress can also act *in trans*. Another emerging concept is that the intermediates or products of certain DNA repair processes act as roadblocks to DNA replication, suggesting that dysregulated repair can also be a source of replication stress. Here, we discuss these distinct types of replication stress in detail.

Replication fork barriers

Any obstacle that perturbs the progression of replication forks is generally considered to be a form of replication stress. Among the most commonly recognized replication blocks are DNA lesions or adducts (Ashour and Mosammaparast, 2021; Ciccia and Elledge, 2010). These DNA lesions/adducts arise from a variety of endogenous and exogenous sources, including chemical mutagens, UV radiation, reactive oxygen species (ROS), non-canonical nucleotides, or byproducts of cellular metabolism (Tubbs and Nussenzweig, 2017). Many different types of DNA lesions/adducts can interfere with replication forks either directly or indirectly. DNA lesions induced by UV or DNA alkylating agents (e.g. methyl methane sulfonate) present roadblocks to replicative DNA polymerases, forcing forks to bypass these lesions using TLS (Lehmann et al., 2007). DNA adducts and inter-strand crosslinks (ICLs) generated by reactive aldehydes or DNA crosslinkers (e.g. cisplatin, mitomycin C), as well as DNA-protein crosslinks (DPCs) formed by various endogenous, environmental, and chemotherapeutic agents that trap proteins on DNA (e.g. poly [ADP-ribose] polymerase (PARP) and topoisomerase I (TOP1) inhibitors), also act as potent blocks to fork progression (Ide et al., 2011; Vare et al., 2012; Voulgaridou et al., 2011). In addition, encounter of forks with DNA single-stranded breaks (SSBs) generated by ROS and other causes can lead to replication fork run-off and formation of one-ended double-strand breaks (DSBs) (Vrtis et al., 2021) (Figure 1A).

Secondary DNA structures such as hairpins, cruciform structures and G-quadruplexes can form in the genomic DNA during physiological processes that generate ssDNA, such as replication, transcription, and repair. When encountered by replication forks, these structures pose a physical obstacle, causing replication stress (Kumar et al., 2021; Mirkin and Mirkin, 2007; Voineagu et al., 2008) (Figure 1A). Some of the common fragile sites (CFSs), which are regions of the genome that exhibit chromosomal breakage under conditions of mild replication stress, are predicted to form stable DNA secondary structures (Thys et al., 2015). Notably, repetitive DNA sequence elements with high structure-forming potential,

such as microsatellites, inverted retroelement repeats, and quasi-palindromic AT-rich repeats, are more reliant on ATR for stability, possibly owing to their inhibitory effects on DNA replication (Shastri et al., 2018). A number of specialized helicases and structure-specific nucleases are involved in dismantling these structures to allow replication fork progression, and the loss of these enzymes is associated with a wide range of genetic disorders characterized by chromosomal instability (Sharma, 2011; van Wietmarschen et al., 2020).

The transcription-replication conflict (TRC) is another source of replication stress. R-loops, which are three-stranded polynucleotide structures consisting of DNA-RNA hybrids and displaced ssDNA, also pose a barrier for fork progression (Aguilera and Garcia-Muse, 2012; Helmrich et al., 2013) (Figure 1A). R-loops levels are affected by the orientation of transcription-replication collisions; head-on collisions increase R-loops, whereas co-directional collisions decrease R-loops (Hamperl et al., 2017). R-loops impede replication forks and are cleaved by specific nucleases, which underlies their deleterious effects on the genome (Gan et al., 2011; Hamperl et al., 2017; Matos et al., 2020; Sollier et al., 2014). Hence, cells have evolved multiple pathways to resolve R-loops and avoid TRC. For instance, TOP1 prevents the collision of replication forks with R-loops at transcription termination sites by removing DNA supercoiling (Promonet et al., 2020). R-loops are also suppressed by RNA processing/splicing factors that prevent DNA-RNA hybridization during transcription (Stirling et al., 2012). Once R-loops are formed, they can be removed by the RNase H family of enzymes and DNA-RNA helicases (Skourti-Stathaki et al., 2011; Wahba et al., 2011), or excised by XPF and XPG nucleases (Sollier et al., 2014). Mutations in the genes involved in these pathways result in increased R-loops and genomic instability, and mutations in some of the R-loop removing factors are associated with neurological disorders and cancers (Richard and Manley, 2017). Aberrant R-loop accumulation is also associated with mutations in certain RNA splicing factors and loss of certain DNA repair proteins, which may contribute to the replication stress and genomic instability in cancer cells (Crossley et al., 2019; Nguyen et al., 2019).

In addition to secondary structures and R-loops in the template DNA, compacted chromatin could also pose a barrier for DNA replication and repair. Heterochromatin regions of the genome such as centromeres, peri-centromeres, and telomeres are inherently hard-to-replicate, and require specialized mechanisms for chromatin remodeling to ensure normal replication fork progression (Ivessa et al., 2003; Miller et al., 2006; Sfeir et al., 2009; Zaratiegui et al., 2011). Loss of a chromatin-remodeling activity in cells harboring mutations in SMARCA4/BRG1, a core component of the SWI/SNF chromatin-remodeling complex, increases heterochromatin-associated replication stress and confers susceptibility to ATR inhibition (Gupta et al., 2020; Kurashima et al., 2020). Loss of ARID1A, a core subunit of the BAF chromatin-remodeling complex, increases replication stress through TRC and R-loops (Tsai et al., 2021). ATRX, another chromatin-remodeling protein of the SWI/SNF family, is required for protecting stalled replication forks in heterochromatin (Huh et al., 2016), and loss of ATRX increases telomere replication stress and instability (Flynn et al., 2015; Li et al., 2019). Paradoxically, loss of chromatin compaction can also induce replication stress. For example, reduced chromatin compaction in histone H1-depleted cells elevates TRC, leading to accumulation of stalled forks (Almeida et al., 2018). Thus, while increased chromatin compaction could interfere with replication fork progression,

loss of repressive chromatin could also compromise fork progression, possibly by elevating transcription or origin firing.

Defects in replication forks

Perturbations in cellular nucleotide pools are another source of replication stress (Figure 1A). Intracellular deoxyribonucleoside triphosphate (dNTP) pools must be tightly aligned with replication dynamics to preserve genome integrity. Indeed, alterations in dNTP pools are associated with increased mutagenesis, genomic instability, and tumorigenesis (Bester et al., 2011). High dNTP levels compromise the fidelity of DNA replication by reducing the proofreading efficiency of polymerases (Weinberg et al., 1981). In contrast, low dNTP levels caused by hydroxyurea (HU), an inhibitor of the ribonucleotide reductase, or by excessive origin firing following inhibition of ATR, Chk1, or WEE1, induce replication stress by compromising polymerase activities (Beck et al., 2012; Buisson et al., 2015). Intermediate levels of dNTP starvation, while not posing a global block to DNA replication, can have a more pronounced effect on specific genomic regions such as fragile sites (Pai and Kearsey, 2017). Notably, not only the overall levels of dNTPs, but also the quality of dNTP pools and the balance among individual dNTPs, is critical for genome stability. Incorporation of oxidized dNTPs caused by inefficient sanitation of dNTP pools generates replication-associated DNA damage (Gad et al., 2014), and dNTP imbalance induced by 5-FU or deregulation of enzymes involved in dNTP synthesis induces fork slowing (Saxena et al., 2018). Furthermore, DNA replication under suboptimal dNTP levels may lead to increased misincorporation of ribonucleoside monophosphates (rNMPs). If unrepaired, the rNMPs in DNA can cause replication stress in the subsequent cell cycle as DNA polymerases tend to stall at such sites (Reijns et al., 2012).

Mutations of the replisome components also compromise genome stability by reducing the number, stability, or fidelity of replication forks (Bellelli and Boulton, 2021) (Figure 1A). For instance, in both mouse and human cells, DNA polymerase δ hypomorphic mutants cause inefficient replication initiation, resulting in replication stress, growth retardation, and tumor predisposition (Bellelli et al., 2018). Similarly, hypomorphic alleles of the genes encoding subunits of the MCM helicase are associated with a reduction in active forks, leading to increased replication stress and embryonic lethality (Alvarez et al., 2015; Kunnev et al., 2010; Shima et al., 2007). Haploinsufficiency of RPA, the ssDNA-binding protein at replication forks, also causes defects in DNA replication and high rates of cancer in mice (Hass et al., 2010). Mutations in the FEN1 nuclease, which is critical for Okazaki fragment maturation, generate replication stress and promote tumorigenesis (Zheng et al., 2011). In addition to the core replisome components, loss of accessory replisome factors TIMELESS, TIPIN and CLASPIN (also known as the fork protection complex) results in replication fork slowdown (Petermann et al., 2008; Somyajit et al., 2017). Similarly, loss of AND-1/CTF4, which bridges the MCM helicase and DNA polymerase α , slows down forks and increases ssDNA (Abe et al., 2018). Furthermore, loss of CAF-1, which is essential for replication-coupled chromatin assembly, compromises replication and activates the replication checkpoint (Hoek and Stillman, 2003).

Dysregulated origin firing

It has been long appreciated that origin firing is regulated by the cellular response to DNA damage and replication stress in eukaryotes (Ge and Blow, 2010; Yekezare et al., 2013). However, recent studies suggest that alterations in the origin firing program are not just a consequence, but also a source of replication stress.

To achieve complete and timely duplication of large genomes, metazoan cells license many folds more origins than are used during each round of replication. These licensed but normally dormant origins can be activated if replication forks are stalled in their vicinity, providing a 'backup' mechanism to complete DNA replication (McIntosh and Blow, 2012). Hence, regions in the genome without sufficient licensed origins lack this backup mechanism and are prone to under-replication (Figure 1B). Indeed, in very large and highly transcribed genes poor in replication origins, replication forks cannot cope with TRC adequately, resulting in CFSs (Brison et al., 2019). Moreover, MCM mutants that decrease the number of licensed origins and reduce the rescue of stalled forks (Kawabata et al., 2011) are shown to cause cancer in mouse models (Shima et al., 2007), suggesting that reduced origin licensing and usage could promote tumorigenesis.

Excessive and dysregulated origin firing also results in catastrophic genomic instability and, potentially, tumorigenesis (Thakur et al., 2021). The level of CDK2 kinase activity is a key determinant of origin firing efficiency (Fagundes and Teixeira, 2021). During S phase, CDK2 activity is restricted by ATR, Chk1 and WEE1 kinases, which prevent excessive origin firing. Compromised activities of these kinases increase origin firing and reduce fork speed (Beck et al., 2012; Buisson et al., 2015; Petermann et al., 2010b), possibly due to limitations in dNTPs or replication factors (Zhong et al., 2013) (Figure 1B). Moreover, in checkpoint-defective cells, increased origin firing in the presence of replication stress generates an excessive amount of ssDNA that exhausts the nuclear pool of RPA. This exhaustion of RPA causes insufficient protection of replication intermediates and induces genome-wide breakage of replication forks, a phenomenon termed replication catastrophe (Toledo et al., 2017; Toledo et al., 2013) (Figure 1B). There is also evidence that the forks initiated from newly fired origins in checkpoint-defective cells are prone to collapse, leading to rapid recruitment of non-homologous end joining (NHEJ) factors such as DNA-PKcs, KU70, and KU80 to these forks (Dungrawala et al., 2015). The importance of regulated origin firing for genome stability is further supported by the findings that activation of oncogenes such as *Cyclin E* alters the spatial and temporal regulation of origin firing (Bester et al., 2011; Dominguez-Sola et al., 2007). This type of dysregulated origin firing elevates TRC, impairing replication fork progression and generating DNA damage (Jones et al., 2013).

The complete and precise genome duplication in proliferating cells requires the entire genome to be replicated once and only once per cell cycle. To avoid re-replication in the same cell cycle, eukaryotic organisms have established several conserved mechanisms to maintain a strict temporal separation of origin licensing and firing (Blow and Dutta, 2005; Hook et al., 2007). Loss of these regulatory mechanisms leads to extensive re-replication of DNA in human cells (Fujita, 2006). Re-replication forks exhibit slow elongation rate, and even re-firing of multiple origins does not result in full replication of the genome (Fu

et al., 2021). Moreover, cells undergoing rereplication exhibit DSBs and activate the DNA damage checkpoint. If the checkpoint is abolished, cells enter mitosis with a partially re-replicated genome, resulting in chromosome breaks and fusions and dying cells with sub-G1 ploidy (Melixetian et al., 2004). The DSB formation and chromosome fragmentation during re-replication are consistent with “head-to-tail” collisions of replication forks (Davidson et al., 2006), supporting the idea that re-replication is a source of replication stress (Figure 1B).

DNA repair intermediates

Mammalian cells have evolved multiple mechanisms to maintain fork stability and promote the repair and restart of stalled replication forks, and loss of these protective pathways is associated with elevated replication stress (Liao et al., 2018). For instance, HR is critical for the restart of stalled replication forks, and HR deficient cells exhibit genome-wide slow replication kinetics and genome instability (Spies et al., 2021; Wilhelm et al., 2014). The ATR kinase and its downstream effectors such as Chk1 help stabilize and restart stalled replication forks (Dungrawala et al., 2015; Saxena et al., 2019). Consequently, loss or inhibition of ATR or Chk1 increases fork collapse and DNA damage (Couch et al., 2013; Petermann et al., 2006; Ragland et al., 2013). Loss of proteins involved in the fork protection pathway, such as BRCA1/2, FANCD2 and RAD51 paralogs, results in increased fork degradation and elevated levels of spontaneous replication stress (Hashimoto et al., 2010; Schlacher et al., 2011; Schlacher et al., 2012; Somyajit et al., 2015). Inhibition of PARP1 impairs lagging strand maturation, causes accelerated fork progression, and prevents the timely repair of ssDNA gaps (Genois et al., 2021; Hanzlikova et al., 2018; Maya-Mendoza et al., 2018; Simoneau et al., 2021).

While repair pathways help to alleviate replication stress, excessive and dysregulated DNA repair can become a source of replication stress. An example of this type of replication stress comes from base excision repair (BER). Base modifications and adducts in the genome are primarily repaired by the BER pathway, which creates several intermediates, such as abasic (AP) sites and SSBs. When the levels of DNA lesions are tolerable and BER occurs efficiently, these intermediates are beneficial to the maintenance of genomic stability. However, when the levels of damage are too high or BER is incomplete, these intermediates become roadblocks to replication forks (Fugger et al., 2021; Thompson and Cortez, 2020) (Figure 1C). Moreover, due to their reactive chemistry, AP sites can give rise to other fork-blocking lesions including DSBs, ICLs and DPCs (Dutta et al., 2007; Prasad et al., 2014). Being ‘faceless’ lesions, which provide no coding information, AP sites are also a potent source of mutation (Simonelli et al., 2005). Similarly, in the absence of efficient ribonucleotide repair (RER), ribonucleotides misincorporated into the genome can be cleaved by BER glycosylases to form mutagenic abasic ribonucleotides (rAP-sites), which are further excised by the BER endonuclease APE1, resulting in SSBs (Malfatti et al., 2017).

DDT pathways employed by replication forks to bypass DNA lesions also generate intermediates conferring replication stress. For instance, when replication forks encounter bulky DNA lesions that block DNA polymerase on the leading strand, they use PrimPol, an RNA/DNA primase-polymerase, to reprime for DNA synthesis ahead of the stalled

polymerase, thereby preventing fork stalling but leaving ssDNA gaps behind (Garcia-Gomez et al., 2013). Alternatively, stalled forks can undergo reversal to generate a stable ‘chicken foot’ structure. The backtracking and re-annealing of nascent DNA strands prevent fork progression across DNA lesions, thus avoiding fork collapse (Quinet et al., 2017b). Reversed forks can also be processed by structure-specific nucleases such as MUS81, providing intermediates for fork restart (Hanada et al., 2007). Although replication fork reversal is beneficial to cells when DNA damage is tolerable, it becomes a source of replication stress when DNA damage is excessive or fork protection is compromised (Fugger et al., 2013). In this situation, reversed forks are degraded by MRE11 and EXO1 nucleases or cleaved by MUS81 (Lemacon et al., 2017), leading to the loss of nascent DNA or toxic levels of DSBs.

Collapsed replication forks with one-ended DSBs can be recovered by BIR (Kramara et al., 2018). During BIR, one nascent DNA strand is synthesized by the extension of DNA ends in D-loops, whereas the second DNA strand is generated using the first nascent strand as a template (Figure 1C). Thus, the DNA replication complex functioning in BIR is significantly different from the canonical replication forks. BIR is known to be error-prone and induce genomic duplications in cancer cells (Costantino et al., 2014; Deem et al., 2011). At telomeres, BIR is associated with increased replication stress and contributes to telomere fragility (Yang et al., 2020; Zhang et al., 2021). Hence, BIR can be viewed as an aberrant replication process that is both a consequence and a cause of replication stress.

Temporal and spatial separation of stress from replication forks

Recent evidence suggests that the replication stress generated at replication forks can be temporally and spatially separated from DNA replication. As discussed above, while PrimPol-mediated repriming allows replication forks to avoid stalling at DNA lesions, it generates post-replicative ssDNA gaps behind the forks. These gaps are repaired by TS and TLS pathways in S and G2 phases, respectively (Taglialatela et al., 2021; Tirman et al., 2021). However, in the absence of efficient gap filling, these gaps can persist into mitosis and even the next cell cycle. Unrepaired ssDNA gaps can interfere with chromosome segregation in mitosis (Ait Saada et al., 2017). Upon encountering active replication forks in the next S phase, the ssDNA gaps cause fork collapse and one-ended DSBs, resulting in trans cell-cycle replication stress (Simoneau et al., 2021).

IMPACTS OF REPLICATION STRESS

Replication stress interferes with the process of DNA replication, thus compromising the fidelity and timely completion of genome duplication. Replication stress can generate DNA breaks in S phase and subsequent cell cycle stages, impair chromosome segregation during mitosis, and even induce genomic instability in the following cell cycle. Here, we discuss the major consequences of replication stress, ranging from local effects on the forks directly affected by replication barriers, to checkpoint activation and its distal effects on origin firing and genome-wide DNA replication, and finally to the global effects on genomic stability and cell survival.

Local effects at stressed replication forks

Obstructions to replication fork progression cause fork stalling, which has been recognized as a major contributor to genomic instability (Gaillard et al., 2015) (Figure 2A). Some types of DNA damage such as ICLs simultaneously block DNA synthesis on both leading and lagging strands, whereas others affect one of the strands stochastically. Some leading strand damage uncouple the replicative helicase and polymerase, resulting in long stretches of ssDNA (Byun et al., 2005). In contrast, lagging strand damage prevents Okazaki fragment maturation, generating smaller ssDNA gaps (Marians, 2018; Pasero and Vindigni, 2017). When both leading and lagging strands are blocked, ssDNA can be generated through fork remodeling and nucleolytic processing (Quinet et al., 2017b). Stalling of replication forks triggers activation of the ATR-mediated replication checkpoint, remodeling of stalled forks, and activation of DDT pathways (Berti and Vindigni, 2016; Yeeles et al., 2013). These responses help stabilize stalled forks and promote their recovery. Alternatively, replication forks from origins adjacent to stalled forks can help complete duplication of the replicons, alleviating the deleterious effects of fork stalling (Cortez, 2015).

ssDNA is a physiological intermediate at replication forks and is coated by RPA. While RPA-ssDNA has critical roles in replication, it is a transient structure at progressing forks due to the rapid conversion of ssDNA into dsDNA by DNA polymerases. However, stalling of DNA polymerases increases the exposure of ssDNA, allowing RPA-ssDNA to serve as a platform for recruiting the ATR-ATRIP kinase complex to stalled forks (Cortez et al., 2001; Zou and Elledge, 2003) (Figure 2B). The activation of ATR at stalled forks relies on two parallel pathways involving TOPBP1 and ETAA1, both of which harbor ATR-activating domains (AADs) (Bass et al., 2016; Haahr et al., 2016; Kumagai et al., 2006). The TOPBP1-mediated ATR activation depends on the RFC-like RAD17 complex and the PCNA-like RAD9-RAD1-HUS1 (9-1-1) complex, which recruit TOPBP1 to ssDNA-dsDNA junctions (Delacroix et al., 2007; Lee et al., 2007). In contrast, ETAA1 binds RPA-ssDNA directly (Bass et al., 2016; Haahr et al., 2016). Once activated, ATR phosphorylates numerous substrates, including the effector kinase Chk1 (Liu et al., 2000; Matsuoka et al., 2007). This ATR-Chk1 signaling cascade orchestrates the replication stress response through several distinct mechanisms, such as arresting the cell cycle, suppressing origin firing, stabilizing replication forks, and promoting fork repair and restart (Simoneau and Zou, 2021) (Figure 2B).

Another common effect of replication stress is the formation of DSBs at stalled replication forks. Prolonged stalling of replication forks or inhibition of ATR or Chk1 leads to fork collapse and DSBs (Dungrawala et al., 2015; Petermann et al., 2010a). DNA replication across SSBs and ssDNA gaps generates one-ended DSBs; two-ended DSBs arise when two replication forks converge at SSBs or ssDNA gaps. DSBs are also generated by structure-specific nucleases at stalled or reversed forks (Hanada et al., 2007; Lemacon et al., 2017; Pepe and West, 2014) (Figure 2A). When the levels of DSBs at stalled forks exceed repair activities, they cause genomic instability and even cell death. Consistent with this idea, difficult-to-replicate chromosomal loci such as CFSSs, poly dA/dT tracts, and R-loops cause fork stalling and breakage, representing a common source of genomic instability with significant ramifications for human diseases (Glover et al., 2017; Ozeri-Galai et al., 2011).

Repair-associated effects at forks

Some of the local effects of replication stress at replication forks are generated by replication-coupled remodeling or repair processes. Reversal of replication forks slows fork progression but helps forks bypass barriers (Marians, 2018; Zellweger et al., 2015). DNA translocases including SMARCAL1, ZRANB3, and HLTf are recruited to stalled forks to catalyze fork reversal (Bai et al., 2020; Kolinjivadi et al., 2017; Vujanovic et al., 2017) (Figure 2A). The recombinase RAD51 is also required for fork reversal (Quinet et al., 2017b; Zellweger et al., 2015). The RECQ1 helicase can resolve reversed forks to allow resumption of fork progression (Berti et al., 2013). The activity of RECQ1 at reversed forks is inhibited by PARP1, which stabilizes reversed forks (Berti et al., 2013). Reversed forks can be cleaved by the MUS81-EME2 nuclease, which may promote fork restart through the POLD3-mediated BIR pathway (Lemacon et al., 2017; Pepe and West, 2014). A DNA2 and WRN-mediated mechanism also promotes the processing and restart of reversed forks (Thangavel et al., 2015). Notably, reversed forks contain a 'regressed' arm formed by both nascent DNA strands, which resembles a DSB and must be protected by BRCA1/2, RAD51, RAD51 paralogs and other factors to prevent degradation of nascent DNA (Schlachter et al., 2011; Schlachter et al., 2012; Somyajit et al., 2015; Thakar and Moldovan, 2021). The cleavage of reversed forks by MUS81 and other SLX4-associated nuclease activities is also a source of DSBs when ATR is compromised (Couch et al., 2013; Ragland et al., 2013) (Figure 2A).

Another mechanism to bypass DNA damage is through repriming by PrimPol. As discussed above, PrimPol generates *de novo* DNA primers ahead of stalled polymerases, restarting DNA synthesis beyond the lesion (Garcia-Gomez et al., 2013; Mouron et al., 2013). Repriming at replication forks leaves ssDNA gaps behind, which can be repaired by TS or TLS (Tirman et al., 2021). During TS, the stalled nascent strand dissociates from the damaged template and anneals with the complementary nascent strand, allowing extension of the stalled strand (Ashour and Mosammaparast, 2021). During TLS, specialized DNA polymerases are used to bypass DNA lesions or fill the gaps (Sale, 2013) (Figure 2A). Recent evidence also implicates HR proteins in the repair of replication-born ssDNA gaps (Somyajit et al., 2021; Tirman et al., 2021). Some of the repair or replication proteins, such as BRCA1/2 and PCNA, are involved in multiple stress response pathways. The choice of stress response pathways may be influenced by the causes of fork stalling and the length or position of ssDNA at or behind forks, but the details remain to be elucidated.

ATR also contributes to the effects of replication stress at forks. ATR phosphorylates many replisome components and accessory factors in response to replication stress (Errico and Costanzo, 2012; Lossaint et al., 2013; Matsuoka et al., 2007). The phosphorylation of some of these ATR substrates, such as RPA, BLM, WRN, and FANCI, facilitates fork recovery from stress (Ammazzalorso et al., 2010; Chen et al., 2015; Davies et al., 2004; Murphy et al., 2014). The phosphorylation of SMARCAL1 by ATR restricts its fork remodeling activity (Couch et al., 2013). The function of PrimPol is promoted by Chk1-mediated phosphorylation (Mehta et al., 2022). ATR is also required for suppressing DSB formation at stalled forks (Dungrawala et al., 2015).

Distal effects on origin firing and fork speed

One key outcome of replication stress and subsequent ATR activation is the suppression of origin firing (Zeman and Cimprich, 2014). Under low levels of replication stress, ATR preferentially inhibits the activation of new replication factories while allowing dormant origins to fire within the existing factories experiencing replication stress, thus minimizing fork stalling (Ge and Blow, 2010). Upon high replication stress, ATR suppresses origin firing within the same replication initiation zones, preventing the accumulation of stalled forks in damaged regions (Moiseeva et al., 2019) (Figure 2B). ATR suppresses origin firing through several mechanisms. The activation of Chk1 negatively regulates CDK-dependent phosphorylation events at origins, blocking the loading of CDC45 and other pre-initiation complex (pre-IC) factors (Zhao et al., 2002). The inhibition of CDK by Chk1 also indirectly reduces CDC7 kinase activity through the phosphatase PP1 (Moiseeva et al., 2019). Chk1 also phosphorylates Treslin, which limits CDC45 binding to origins (Guo et al., 2015). Furthermore, ATR phosphorylates and stabilizes the MLL protein, which methylates histone H3 Lys4 (H3K4me) at origins and blocks CDC45 loading (Liu et al., 2010). The suppression of origin firing by ATR limits the number of replication forks, thereby preventing excessive ssDNA accumulation, exhaustion of the nuclear RPA pool, and replication catastrophe (Buisson et al., 2015; Toledo et al., 2013). Whether and how ATR exerts its effects on distal and local origins through distinct mechanisms still needs further investigations.

Another general manifestation of replication stress is the slowdown of DNA replication throughout the genome (Techer et al., 2017) (Figure 2B). Both the replication stress itself and the ATR checkpoint response contribute to the reduction in DNA synthesis. In S phase cells, inactivation of the ATR pathway elevates overall DNA synthesis, which is primarily a result of increased origin firing (Petermann et al., 2010b). In addition, ATR promotes dNTP synthesis through RRM2 and the deoxycytidine kinase dCK (Buisson et al., 2015; Le et al., 2017). ATR inhibition causes fork slowing, possibly due to the reduced availability of dNTPs and certain replication proteins when more origins are fired (Buisson et al., 2015). However, there is also evidence that ATR promotes slowing of replication forks upon genotoxic stress. For example, ATR is shown to slow forks genome-wide by promoting fork reversal in response to ICLs, and through RAD51 paralogs after dNTP depletion (Mutreja et al., 2018; Saxena et al., 2018). Thus, the effects of ATR on fork speed could be influenced by the level and nature of replication stress.

Global effects during the cell cycle

In the event of replication stress, cell cycle arrest, which is initiated by the checkpoint activation at stalled forks, is a crucial cellular response (Figure 2B). Once activated by ATR, Chk1 phosphorylates the CDC25A phosphatase and promotes its degradation, preventing the removal of inhibitory phosphorylation from CDK2 (Mailand et al., 2000; Sorensen et al., 2003). In S phase cells, activation of ATR and Chk1 reduces CDK2 activity, restricting origin firing and allowing time to resolve the replication stress (Zhang and Hunter, 2014). In addition, the phosphorylation of CDC25C by Chk1 induces its nuclear export and cytoplasmic sequestration, preventing the activation of CDK1 and the G2/M transition (Peng et al., 1997; Sanchez et al., 1997). Thus, Chk1 is a key checkpoint mediator in both S and

G2 phases, which prevents the entry of cells with under-replicated or damaged DNA into mitosis (Xiao et al., 2003).

Despite the functions of cell-cycle checkpoints, under-replicated DNA (UR-DNA) and unresolved replication or repair intermediates can escape checkpoint surveillance and get transmitted to subsequent cell-cycle phases and even to the next cell cycle (Mankouri et al., 2013). Normally, cells deal with such UR-DNA in early prophase through mitotic DNA synthesis (MiDAS), a BIR-like mechanism of conservative DNA synthesis that involves DNA polymerase δ and its accessory subunit POLD3, the nuclease scaffold SLX4, the MUS81-EME1 endonuclease, and the recombinase RAD52 (Bhowmick et al., 2016). Persistent UR-DNA manifests as ultra-fine bridges (UFBs) in late mitosis (Lezaja and Altmeyer, 2021). If segregated into daughter cells, UR-DNA is sequestered into 53BP1 nuclear bodies (53BP1-NBs), which restrain the replication of the embedded genomic loci until they can be repaired by a dedicated RAD52-mediated repair pathway in the next S phase (Harrigan et al., 2011; Lukas et al., 2011; Spies et al., 2019) (Figure 2C).

Genomic alterations resulting from replication stress

The DSBs induced by replication stress can cause mutations and several types of chromosomal alterations, such as deletions, duplications, and translocations. The genomic instability induced by replication stress is at least in part attributed to the error-prone repair pathways activated by stalling or collapse of replication forks, such as TLS, BIR, NHEJ, and microhomology mediated end joining (MMEJ). In addition, replication stress is closely linked to chromosome instability (CIN), another common feature of cancer cells (Burrell et al., 2013). Replication stress leads to UR-DNA persisting into mitosis, resulting in mitotic defects including lagging chromosomes, bulky chromosome bridges, and UFBs (Chan et al., 2009; Wilhelm et al., 2020). These mitotic defects can lead to aneuploidy (numerical CIN) and chromosomal rearrangements (structural CIN) (Burrell et al., 2013). Furthermore, chromosome missegregation gives rise to micronuclei and induces chromothripsis, a process of severe DNA fragmentation and rearrangement in confined genomic regions (Zhang et al., 2015) (Figure 2C).

DETECTION OF REPLICATION STRESS

A number of molecular and cellular assays have been developed to analyze the phenotypes and effects of replication stress. Some of these assays directly analyze the alterations of replication forks, whereas others monitor the indirect effects of replication problems. It is important to note that each of these assays only reflects a specific aspect of replication stress or the stress response. The combination of multiple assays is generally required to obtain a comprehensive understanding of the replication stress and stress responses in specific contexts.

Assays for cellular responses to replication stress

Several assays are used to detect global cellular responses to replication stress, including accumulation of ssDNA, suppression of DNA synthesis, and cell cycle arrests. Several other assays are used to detect replication stress-induced DNA damage and damage responses,

such as phosphorylation and focus formation of DNA repair proteins. These assays provide indirect but quantitative measurements of replication stress in cell populations or individual cells.

The thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU) is a convenient tool to monitor DNA synthesis in cultured cells and *in vivo* (Salic and Mitchison, 2008). Pulse labeling of cells with EdU allows its incorporation into newly synthesized DNA. The EdU intensity in individual cells accurately reflects the levels of DNA synthesis and can be used to identify replicating cells in cell populations. The activation of the ATR-mediated replication checkpoint by replication stress typically results in a reduction of EdU intensity in S phase cells (Fig. 3A). In addition, pulse EdU labeling can be combined with immunofluorescence of specific proteins to study how these proteins behave or function in replicating cells. For example, combined EdU and MCM2 staining was used to reveal how MCM levels affect DNA synthesis (Matson et al., 2019).

As discussed above, replication stress can induce the accumulation of RPA-ssDNA at or behind stalled forks. Hence, the levels of RPA foci and chromatin-bound RPA can be used as markers for elevated replication stress (Buisson et al., 2015; Toledo et al., 2013; Zou and Elledge, 2003). The exposure of ssDNA in the genome can also be detected by native BrdU staining (Buisson et al., 2015; Couch et al., 2013; Toledo et al., 2013) (Fig. 3A). In this assay, cells are cultured in the presence of BrdU for two cell cycles to label the genomic DNA. If BrdU-labeled DNA is exposed as ssDNA under replication stress, it can be visualized by anti-BrdU immunofluorescence under non-denaturing conditions (Fig. 3A).

The activation of ATR pathway also serves as a reliable marker for replication stress. Several ATR-dependent phosphorylation events, such as ATR p-T1989, Chk1 p-S317/S345, and RPA32 p-S33, can be detected by western blot and immunofluorescence using phospho-specific antibodies (Liu et al., 2000; Liu et al., 2011; Shiotani et al., 2013) (Fig. 3A). The phosphorylation of H2AX is also induced by replication stress, which may reflect the activation of ATR at stalled forks or the activation of ATM and DNA-PKcs at replication-associated DSBs (Ward and Chen, 2001). These phosphorylated proteins are commonly used as a proxy for replication stress in cells proficient for replication and DNA damage checkpoints. In addition, ATR-mediated slowdown of the G2/M transition and accumulation of G2/M cells can be detected by propidium iodide staining of DNA and fluorescence-activated cell sorting (FACS) (Fig. 3A). If the S phase cells exposed to replication stress are given enough time to progress to mitosis, a G2/M arrest can be detected by the reduction of mitotic cells marked by histone H3 p-S10 (Liu et al., 2000). If cells with mild replication stress escape the G2/M checkpoint and progress into the next cell cycle, it can trigger a p53-dependent prolongation of G1 (Lezaja and Altmeyer, 2018).

Several DNA replication and repair proteins involved in the replication stress response, such as RPA, RAD51, and FANCD2, form discrete foci in the nucleus (Andreassen et al., 2004; Petermann et al., 2010a). These foci may reflect the remodeling or repair events at stalled or collapsed replication forks (Fig. 3A). The number and intensity of these foci are used as indirect markers of replication stress. Furthermore, nuclear foci or protein levels of ATM p-S1981, Chk2 p-T68, KAP1 p-S824, RPA2 p-S4/8, and RPA2 p-T21 can be used

to indirectly monitor the DSBs formed by prolonged stalling and nucleolytic cleavage of replication forks (Marechal and Zou, 2013; Nakamura et al., 2021; Toledo et al., 2013). The DSBs induced by replication stress can also be directly detected by non-denaturing comet assay or pulsed field gel electrophoresis (PFGE) (Olive and Banath, 2006) (Fig. 3A). It should be noted that the levels of replication-associated DSBs are affected by the levels and duration of replication stress. Prolonged but mild replication stress does not trigger a robust DSB response, but increases DSBs at CFSs (Durkin and Glover, 2007).

Assays to analyze replication forks and origins

A number of DNA fiber-based assays are developed to analyze the behaviors of replication tracts and alterations in nascent DNA in cells. Several EdU-based assays are used to analyze the proteins associated with replication forks in cell populations or individual cells. These assays provide direct measurements of the effects of replication stress on replication forks.

DNA fiber and combing assays directly measure replication fork dynamics at a single-molecule resolution in cell populations. In these assays, newly synthesized DNA in cells is sequentially labeled with two thymidine analogs—e.g., 5-iodo-2'-deoxyuridine (IdU) and 5-chloro-2'-deoxyuridine (CldU). Cells are lysed on tilted glass slides and genomic DNA is stretched into fibers by gravity. Immunostaining of these fibers enables microscopic visualization of individual replication tracts (Pasero et al., 2002; Techer et al., 2013) (Fig. 3B). These assays can be used under various conditions to monitor fork progression, fork stalling, fork restart, and nucleolytic degradation of nascent DNA (Quinet et al., 2017a). Replication stress is commonly associated with reduced fork speed, which may reflect a reduction in fork function or stability, an increase of barriers in DNA, or defects in fork restart. Acceleration of replication forks can also result in genomic instability, which may be attributed to the aberrant stress response at forks (Genois et al., 2021; Maya-Mendoza et al., 2018; Saxena et al., 2018). DNA fiber assay can also be used to detect newly fired origins and measure the overall levels of origin firing in cell populations. When cells are sequentially labeled with CldU and IdU and subjected to DNA fiber analysis, continuous IdU-CldU-IdU tracts reflect the origins fired during CldU labelling, and IdU-only tracts indicate origins fired during the IdU pulse (Nieminuszczy et al., 2016). In cells with a proficient ATR checkpoint, replication stress typically leads to a reduction in overall origin firing.

While the DNA fiber assay directly analyzes DNA fibers spread by gravity, the DNA combing assay requires a combing apparatus to stretch DNA fibers and hence offers a more accurate measurement of the length of replication tracts. This assay can be used to directly measure origin density by estimating the distance between adjacent initiation events along a DNA fiber, termed inter-origin distance (IOD) (Techer et al., 2013). The activation of ATR by replication stress reduces origin firing, leading to an increase of IOD. However, the IOD determined by DNA fiber/combing assay likely overestimates the actual distance between origins because only the origins fired during the labeling periods can be detected. DNA combing assay can be coupled with site-specific hybridization probes, sequence enrichment strategies, or labeled DNA lesions to analyze the replication tracts in specific genomic

regions, such as telomeres and rDNA repeats, or at sites of ICLs (Huang et al., 2013; Pasero et al., 2002; Sfeir et al., 2009).

The ssDNA gaps induced by replication stress can be detected in DNA fibers by the S1 nuclease, which specifically cleaves ssDNA, leading to shortening of labeled replication tracts (Quinet et al., 2016). The S1 nuclease assay can be used to monitor the formation and repair of ssDNA gaps during and after replication. However, this approach has certain limitations. For instance, S1 nuclease can also cleave ssDNA at secondary DNA structures. Also, ssDNA gaps can only be visualized if they are present on both leading and lagging strands (Mehta et al., 2022). Alternatively, electron microscopy (EM) can be used to directly visualize ssDNA gaps at forks or in the replicated DNA close to forks (Vindigni and Lopes, 2017; Zellweger and Lopes, 2018) (Fig. 3B). This assay has also been successfully used to detect and quantify reversed replication forks (Quinet et al., 2017b; Ray Chaudhuri et al., 2012). However, because EM specifically analyzes the DNA at or close to replication forks, changes in the DNA distal to forks or loss of forks cannot be directly measured by EM.

iPOND (Isolation of proteins on nascent DNA) permits the isolation and analysis of the proteins associated with nascent DNA in cell populations (Fig. 3B). This assay involves pulse labeling of DNA with EdU, followed by the isolation of EdU-labeled DNA and associated proteins (Dungrawala and Cortez, 2015; Sirbu et al., 2011). iPOND can be used to monitor the association of replication proteins with forks during the stress response, as well as the recruitment of repair and signaling proteins to stalled or collapsed forks (Dungrawala et al., 2015). A similar approach, NCC (nascent chromatin capture), relies on biotin-dUTP labelling of nascent DNA (Alabert et al., 2014). In yeast cells, eSPAN (enrichment and sequencing of proteins associated with nascent DNA) offers a strategy to analyze the recruitment of proteins to leading and lagging strands of replication forks (Yu et al., 2014). Another commonly used approach to detect fork-associated proteins is PLA (proximity ligase assay), which combines antibody–oligo conjugates, enzymatic ligation, and rolling-circle amplification to detect the proximity between proteins *in situ* (Soderberg et al., 2008). Recent studies have combined PLA with EdU pulse labeling (Roy and Schlacher, 2019), allowing detection of the proteins in close proximity to nascent DNA in individual cells. Moreover, PLA between RNA polymerase II (RNAPII) and replisome components has been used to analyze TRC (Kim et al., 2020; Matos et al., 2020). It should be noted that iPOND, NCC and eSPAN analyze the association of many proteins with nascent DNA in cell populations, whereas PLA detects the proximity of individual proteins to nascent DNA or replisomes in single cells.

Assays for the effects of replication stress on mitosis and the next cell cycle

As under-replicated or unresolved DNA structures induced by replication stress can persist into mitosis and jeopardize chromosome segregation (Gelot et al., 2015), some of the mitotic assays can serve as a proxy for replication stress in the preceding S phase.

Chromosomal abnormalities resulting from replication stress, such as chromatid breaks, acentric chromosomes, and radial chromosomes, can be measured in metaphase spreads (Wilhelm et al., 2019) (Fig. 3C). DNA breaks at difficult-to-replicate regions such as CFSs manifest as DAPI-negative breaks, gaps, and constrictions on metaphase chromosomes

(Glover et al., 2017). Also, MiDAS-dependent replication of UR-DNA in early mitosis can be visualized as EdU foci on metaphase chromosomes (Bhowmick et al., 2016). Incomplete resolution of replication intermediates can be detected as chromosome bridges. These bridges can be classified into DAPI-positive bulky anaphase bridges and DAPI-negative UFBs, which are characterized by the binding of PICH across the bridge and FANCD2-FANCI at the termini (Chan and Hickson, 2011).

Mitotic catastrophe (MC) is a cell death mechanism in mitosis. MC occurs as a consequence of chromosome nondisjunction or mitotic entry with multiple spindle poles, and is characterized by the formation of giant multinucleated cells (Vitale et al., 2011). Replication stress promotes chromosome nondisjunction and favors mitotic extra centrosomes and multipolar mitosis, thus increasing mitotic catastrophes (Masamsetti et al., 2019). Replication stress also induces micronuclei, which are generated by the entrapment of lagging chromosomes, acentric chromosomes, or chromatid fragments in their own nuclear envelopes upon the completion of mitosis (Xu et al., 2011) (Fig. 3C). Micronuclei serve as a reliable biomarker of replication stress and genomic instability in mammalian cells. Another marker of unresolved replication stress is the presence of large 53BP1 NBs in G1 cells, which contain γ H2AX, MDC1 and OPT (Oct1, PTF, transcription) domains (Harrigan et al., 2011; Lukas et al., 2011; Spies et al., 2019).

Genome-wide assays

In addition to the mechanistic insights from molecular and cell biology approaches, it is critical to obtain a genomic view of replication stress. Genomics approaches are particularly useful to understand the contribution of specific DNA sequence elements to replication stress and map the sites of replication fork perturbation and collapse across the genome.

In human cells DNA replication initiates within broad zones of ~150 kb, termed initiation zones (IZs), which contain clusters of origins used at varying efficiencies (Petryk et al., 2016). Induction of replication stress leads to characteristic changes in replication profiles, including the loss of specific IZs within early replication domains, and global disappearance of the replication timing domain structures (Hayakawa et al., 2021). These genome-wide changes in the replication timing domains can be detected by Repli-seq (Hansen et al., 2010) (Fig. 3D). Another widely used approach to study replication initiation, elongation, and termination in yeast and mammalian cells is OK-seq (Kit Leng Lui et al., 2021). OK-seq detects transitions in fork polarity, and it has produced comprehensive maps of fork polarities in several mammalian cell lines (Petryk et al., 2018; Petryk et al., 2016). In addition, a method called ORM (optimal replication mapping) was developed recently to reconstruct genome-wide replication profiles from extra-long DNA fibers (Wang et al., 2021). This approach will also be useful for analyzing the changes in replication under stress.

As an indirect approach, chromatin immunoprecipitation sequencing (ChIP-seq) of replication and repair proteins can be used to identify difficult-to-replicate regions in the genome. For example, ChIP-seq analysis of RPA, BRCA1, and SMC5 revealed that these proteins colocalize at early replicating fragile sites (ERFS) in the genome (Barlow et al., 2013) (Fig. 3D). Alternatively, END-Seq is a highly sensitive technique which allows

quantitative mapping of DSBs at nucleotide resolution across the genome (Canela et al., 2016). This assay has been successfully used to map the sites of replication fork collapse throughout the genome (Tubbs et al., 2018).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

While replication stress is detrimental to the genome, it is an inseparable part of DNA replication. Even in proliferating normal cells, DNA replication forks inevitably encounter transcription machineries, DNA secondary structures, and intrinsic DNA damage. The cellular responses to replication stress, which involve the checkpoint and DNA repair pathways, are critical for maintaining genomic integrity. In cancer cells, replication stress is increased by a wide variety of cellular alterations, ranging from elevated ROS to mutations in DNA repair proteins. While our general understanding of replication stress has improved dramatically during the past decade, how replication stress arises in different types of cancers remains poorly understood. Several concepts emerging from recent studies will help us better understand the replication stress in normal and cancer cells. First, we now appreciate that replication stress comes in multiple flavors. Several sources of replication stress, such as TRC, R-loops, AP sites, ssDNA gaps, and DPCs, may be upregulated in specific cell types and tissues, during specific developmental stages, or in subsets of cancers. Second, cells use multiple mechanisms to deal with replication stress, and the choice among these mechanisms can vary in different contexts. For example, the choice among fork reversal, repriming, TS, and TLS can be influenced by several factors including PARP, DNA translocases and PCNA ubiquitination. Third, replication stress is a quantitative trait with multiple possible outcomes. For example, the levels of ssDNA at replication forks may dictate which repair or cell death pathway to activate. Fourth, it is important to investigate when and where replication stress occurs in the genome. Different types of replication stress may manifest in distinct regions of the genome, and genome-wide assays such as END-seq will be useful for pinpointing these effects. Finally, replication stress can be spatially and temporarily uncoupled from replication forks in some contexts. For example, the ssDNA gaps generated during replication can persist into later phases of the cell cycle and even the next S phase, allowing them to affect genomic stability in different ways. It is conceivable that future studies will benefit from methods that quantitatively analyze different types of replication stress in the genomic space, in cell populations, and across multiple cell cycles. A better understanding of replication stress will provide a basis to improve the treatment of cancer and other diseases associated with replication-born genomic instability.

Acknowledgements

We apologize to those authors whose work could not be cited due to space constraints. S.S. is supported by the James A. Harting Scientific Scholar Award from the Rivkin Center. L.Z. is the James & Patricia Poitras Endow Chair for Cancer Research and support by the NIH (CA263934).

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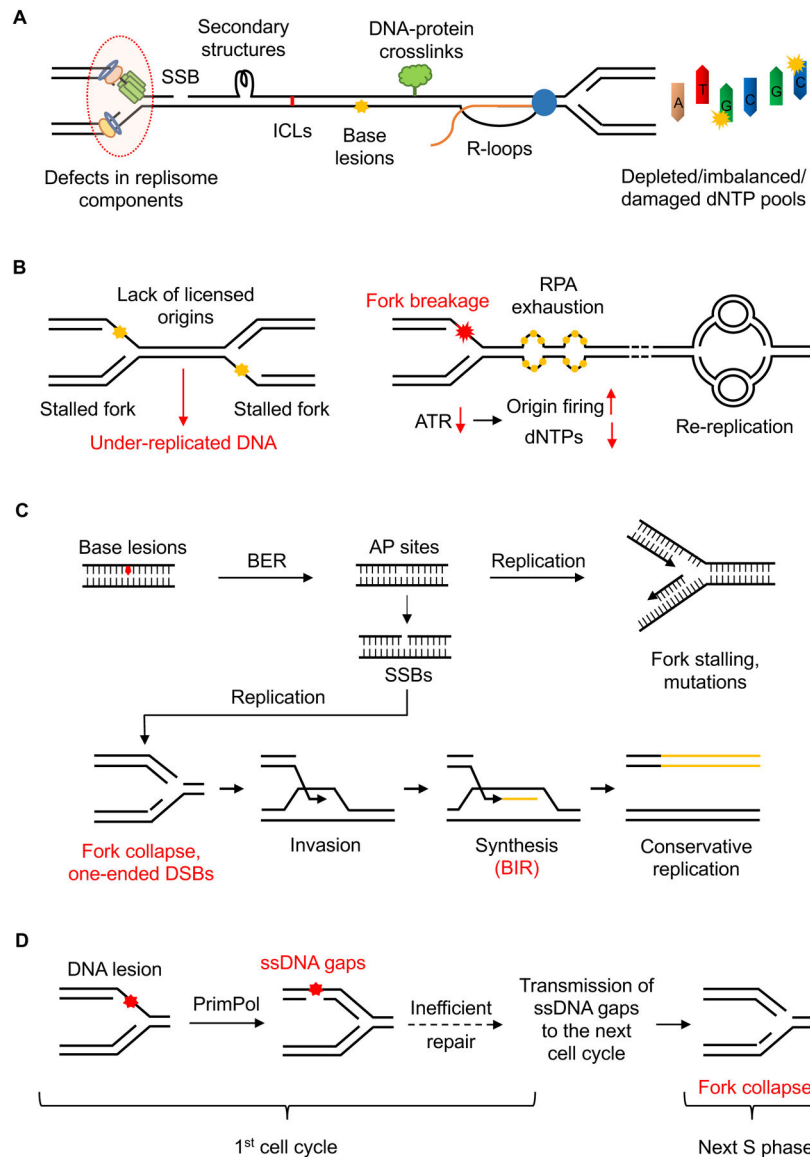


Figure 1. Sources of DNA replication stress.

(A) The progression of replication forks can be impeded by several obstacles including SSBs, DNA secondary structures, ICLs, etc. Defects in replisome components or alterations in dNTP pools can also reduce fork speed, compromise fork stability, and promote mutagenesis. (B) Lack of licensed origins leads to reduced rescue of stalled forks, resulting in under-replicated regions. Increased origin firing reduces fork speed by depleting dNTPs and other replisome factors. It also generates excessive amount of ssDNA, which exhausts the nuclear pool of RPA and induces genome-wide breakage of replication forks. Re-replication forks progress slowly and undergo “head-to-tail” collisions with previously initiated replication forks, resulting in DSBs. (C) Excessive repair intermediates like AP sites and SSBs act as roadblocks to replication forks. Collisions of active forks with SSBs cause fork collapse and one-ended DSBs, which can be recovered by BIR, an aberrant replication process that is error-prone and generates replication stress. (D) Replication forks

can bypass DNA lesions by PrimPol-mediated repriming, which generates ssDNA gaps behind the forks. If unrepaired, these ssDNA gaps can persist into the next cell cycle, where they encounter active forks and cause fork collapse, generating trans-cell cycle replication stress.

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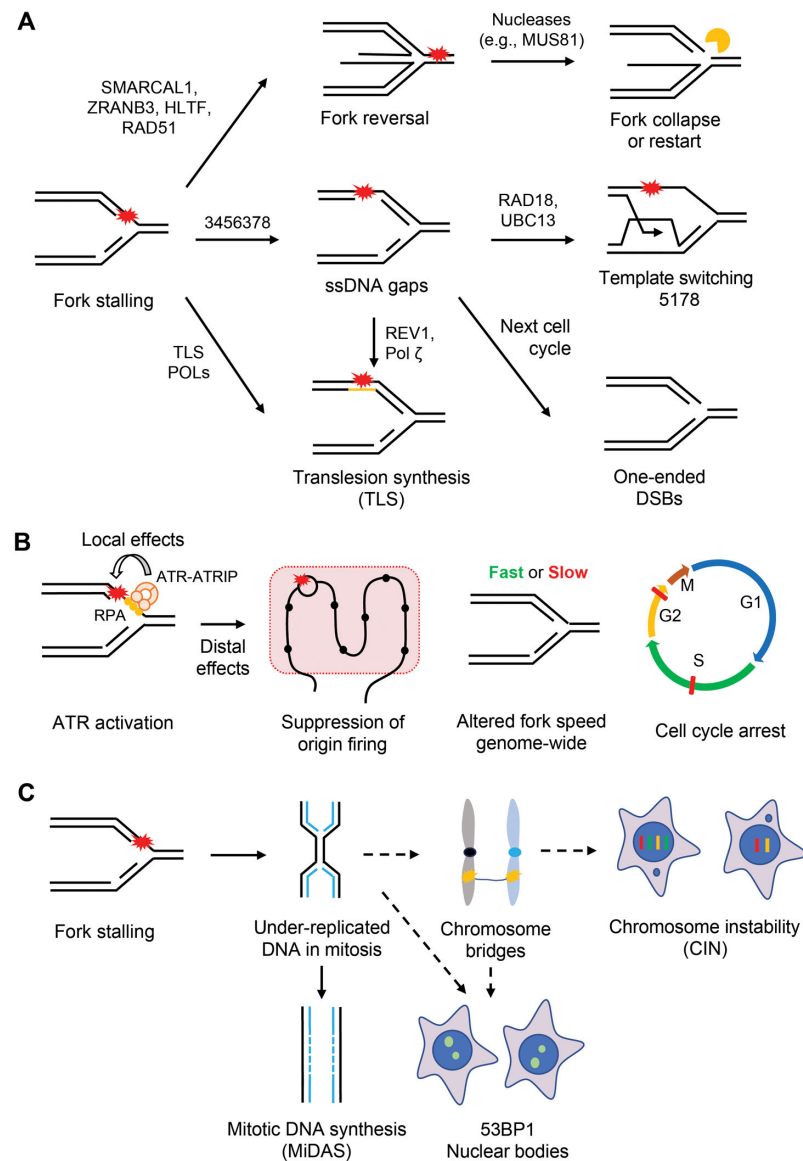
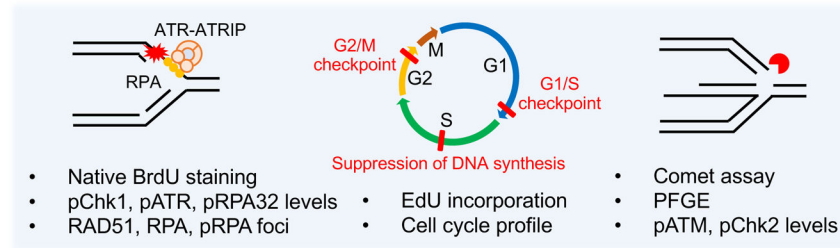


Figure 2. Consequences of DNA replication stress.

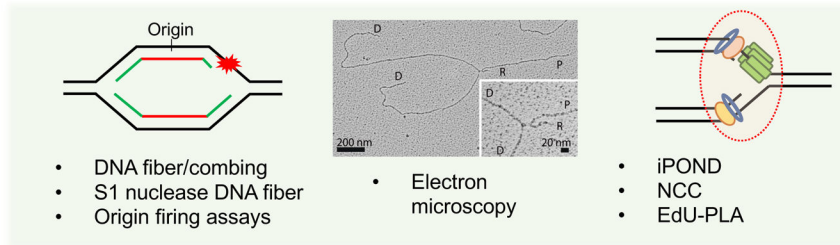
(A) Obstructions to replication progression cause fork stalling. Several replication-coupled repair mechanisms are used to promote the bypass and/or repair of DNA damage. Stalled forks can be reversed by the action of DNA translocases such as ZRANB3, HLTF, and SMARCAL1 and recombinase RAD51. The resulting four-way structure can be cleaved by nucleases like MUS81 to form DSBs. The DSBs at reversed forks can promote fork restart, but can also be toxic when they accumulate at high levels. Alternatively, stalled forks can bypass DNA lesions by PrimPol-mediated repriming. Repriming generates ssDNA gaps behind the fork, which can be repaired by TS or TLS in S and G2 phases. In the event of inefficient repair, these gaps persist into the next S phase and induce one-ended DSBs. Stalled forks can also directly bypass DNA lesions through TLS. (B) Fork stalling generates stretches of ssDNA at stressed forks, which is coated by the RPA complex. ssDNA-RPA in turn recruits the ATR-ATRIP kinase complex to stalled forks. Once activated, ATR

phosphorylates substrates at stalled forks to promote fork stabilization and restart (local effects). Furthermore, ATR suppresses origin firing, alters fork speed genome-wide, and induces cell cycle arrests in S and G2/M phases (distal effects). (C) Under-replicated DNA (UR-DNA) can persist into mitosis and undergo MiDAS. If not replicated by MiDAS, UR-DNA forms chromosome or DNA bridges in anaphase. If these bridges are not resolved by repair proteins, they give rise to chromosome instability (CIN). If UR-DNA is segregated into daughter cells, it is sequestered into 53BP1 nuclear bodies (53BP1 NBs).

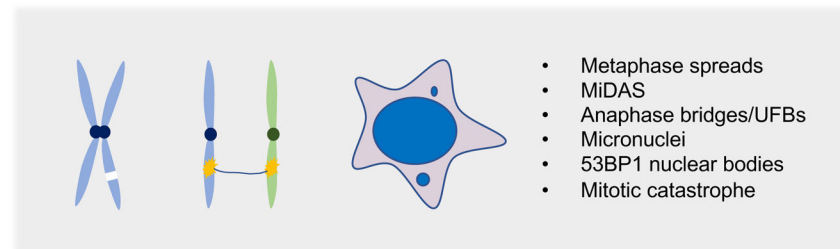
A. Assays for cellular responses to replication stress



B. Assays to analyze replication forks and origins



C. Assays for the effects of replication stress on mitosis and the next cell cycle



D. Genome-wide assays

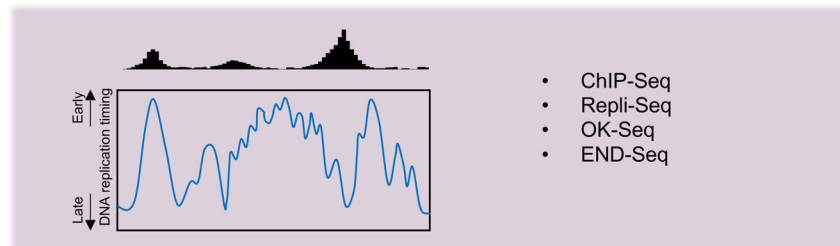


Figure 3. Assays for the detection of replication stress.

(A) Cellular assays to study the global effects of replication stress by measuring the accumulation of ssDNA, phosphorylation of ATR substrates, suppression of DNA synthesis, cell cycle arrests, and generation of DSBs. (B) DNA fiber-based assays directly study the impact of replication stress on progressing forks and origin firing. Replication forks can be directly visualized by EM to study fork reversal and ssDNA gap generation. The EM image is reproduced from (Genois et al. 2021). Other assays including iPOND and PLA are used to analyze factors associated with nascent DNA. (C) Cellular assays that use mitotic defects as a proxy to measure the replication stress in the preceding S phase. These assays study the effects of replication stress on mitosis and the next cell cycle in the form of anaphase bridges, micronucleation, chromosomal aberrations, and 53BP1 NBs. (D) Genomic assays

that use next-generation sequencing (NGS) to obtain a whole-genome view of the effects of replication stress. These assays are used to map the regions of replication perturbation across the genome and to understand the contributions of specific chromatin environments and DNA sequence elements to replication stress.

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