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Replication stress: from chromatin to immunity and beyond

Yea-Lih Lin and Philippe Pasero

Replication stress (RS) is a hallmark of cancer cells that is associated with increased genomic instability. RS occurs when replication forks encounter obstacles along the DNA. Stalled forks are signaled by checkpoint kinases that prevent fork collapse and coordinate fork repair pathways. Fork restart also depends on chromatin remodelers to increase the accessibility of nascent chromatin to recombination and repair factors. In this review, we discuss recent findings on the causes and consequences of RS, with a focus on endogenous replication impediments and their impact on fork velocity. We also discuss recent studies on the interplay between stalled forks and innate immunity, which extends the RS response beyond cell boundaries and opens new avenues for cancer therapy.

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Introduction

DNA replication is a complex process that relies on the sequential activation of thousands of replication origins and on the coordinated action of DNA polymerases, helicases and accessory proteins at replication forks. Replication stress (RS) refers to various events of endogenous or exogenous origin that perturb the execution of this replication program, usually by altering the distribution of origins and/or by blocking fork progression. Fork recovery depends on finely orchestrated repair mechanisms involving homologous recombination (HR) factors [1]. The persistence of stalled or collapsed forks leads to genomic instability and promotes cancer development. Endogenous RS represents also the Achilles' heel of cancer cells by increasing their sensitivity to chemotherapy [2]. In addition, a growing body of evidence indicates that RS triggers an innate immune response through the accumulation of cytosolic DNA and the activation of DNA sensing pathways. In this review, we discuss new findings that expand current views on RS. In particular, these studies show that the responses to RS are not restricted to the cell perimeter but can extend to the microenvironment through activation of the cyclic GMP-AMP synthase/Stimulator of interferon genes (cGAS/STING) pathway, which promotes the induction of type I interferons [3]. This novel link between RS and inflammation could be exploited in cancer therapy to stimulate the rejection of tumor cells by the immune system.

Faster forks as a source of RS

RS is commonly associated with slower or stalled replication forks, but recent evidence indicates that faster forks can also lead to DNA damage. For instance, it has been proposed that the chemical inhibition of the DNA repair factor PARP1 can induce chromosome breaks and lower cell viability by increasing fork speed over a critical threshold [4]. Recent studies indicate that faster fork progression can also cause RS in the absence of treatment. For instance, cells depleted for the Minichromosome maintenance (MCM) complex chaperone MCM-binding protein (MCMBP) activate less origins and compensate by increasing fork velocity, at the expense of genomic instability [5]. Along the same line, the knockdown of AMBRA1 (activating molecule in beclin-1-regulated autophagy) increases cyclin D1 levels and accelerates the G_1/S transition, leading to faster forks and increased DNA damage [6]. Although cells have the ability to compensate for reduced origin activity by increasing fork velocity, this may come at the cost of increased genomic instability, at least when speed reaches a certain threshold, which may vary depending on the physiological state of the cell.

Different mechanisms increasing fork speed in pathological situations have been described. In the chronic inflammatory skin disease *Hidradenitis Suppurativa*, stem cells from hair follicle show faster forks and increase RS in a STING-dependent manner [7]. An unexpected link between inflammation and fork velocity was also established in cells overexpressing the interferon-stimulated gene 15 (ISG15), which is induced by inflammation and increases both fork velocity and DNA damage through the regulation of the RECQ1 helicase [8•]. Interestingly, RECQ1 is a key regulator of fork reversal, a fork protection mechanism that stabilizes stalled forks and generates

a DNA end that can be used as a substrate by the HR machinery to resume DNA replication [9–11].

Fork velocity is typically measured with DNA fiber assays as the average distance traveled by individual forks over the duration of nascent DNA labeling. In the presence of replication impediments, these assays do not measure actual changes in fork velocity, but the time it takes for the fork to overcome the obstacles. For instance, fork reversal involves the extensive remodeling of replication intermediates [12] and depends on several DNA translocases acting in two distinct pathways to stabilize arrested forks [9]. It requires the activity of DNA topoisomerase IIA (TOP2A) for a full extension of the chicken-foot structure [13•]. It also depends on the RAD51 recombinase and on its paralogs RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3 [9,14,15]. Fork reversal is prevented by the dissolution of RAD51 filaments by the ssDNA binding protein RADX [16] and is counteracted by the RECQ1 helicase [1]. The half-life of reversed forks depends therefore on a dynamic equilibrium, affecting the apparent fork velocity measured by DNA fiber analysis [72].

Besides fork reversal, roadblocks on the leading strand and lagging strand can be bypassed by repriming of DNA synthesis with the PRIMPOL DNA polymerase or by translesion DNA synthesis and by Polα-primase, respectively [17–19]. Since repriming is faster than fork reversal, the choice between these different pathways modulates the resulting fork velocity and the fidelity of DNA synthesis [20•,21]. Nascent DNA at reversed forks and ssDNA gaps is resected by nucleases to generate substrates for HR. This process is tightly controlled by a variety of fork protection factors [22,23] and protein kinases [24–26]. Since the DNA replication checkpoint can actively slowdown forks [27,28], even in the absence of DNA damage [29], the use of fork speed as a direct indicator of RS should be considered with caution.

Endogenous sources of RS

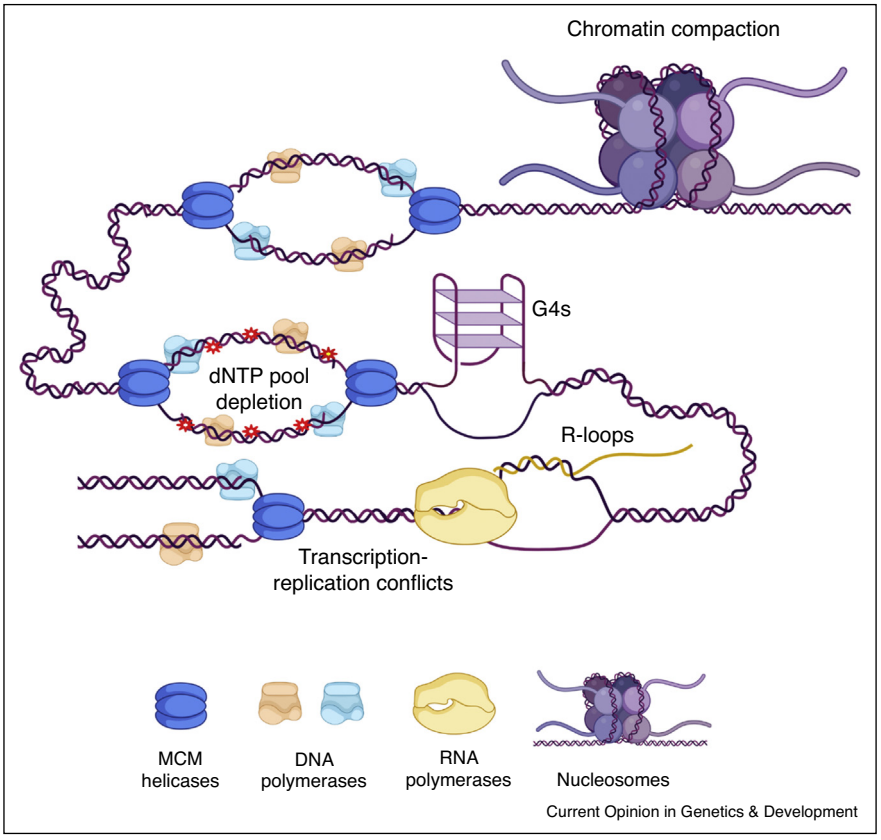
Challenges to replication fork progression can arise from various endogenous sources, including transcription-replication conflicts, structured DNA, condensed chromatin and nucleotide shortage (Figure 1). Transcription-replication conflicts can either be due to frontal collisions between the transcription and replication machineries or to the presence of cotranscriptional R-loops [30]. Recent evidence indicates that complex genomes have evolved strategies to avoid head-on collisions between replication and transcription by activating origins upstream of highly expressed genes to ensure that replication is codirectional with transcription [31•,32].

Cotranscriptional R-loops form when the nascent RNA anneals to the template DNA strand, leaving the non-template strand unpaired. It is generally believed that R-loops block fork progression, but direct evidence supporting this view is scarce. As a matter of fact, a recent genome-wide analysis of the distribution of R-loops and RS markers in unchallenged HeLa cells revealed that more than 80% of R-loops are not associated with RS and that the codirectional organization of replication and transcription restricts TRCs to transcription termination sites [32]. Other models to explain the toxicity of R-loops include the formation of RNA:DNA hybrids behind stalled forks [33], which would interfere with fork repair processes. Alternatively, the displaced ssDNA strand in R-loops could facilitates the formation of secondary DNA structures inducing fork arrest. For instance, R-loops at GAA repeats promotes the formation of a replication barrier requiring PRIMPOL-dependent repriming to re-establish processive replication [34].

R-loops could also facilitate the formation of G-quadruplexes (G4) on DNA, which are stacked structures of two or more G-tetrads [35]. G4 structures form spontaneously at newly unwound DNA in between replicative DNA helicases and polymerases [36]. These structures limit the binding of RPA to ssDNA, perturbing the dynamics and organization of replisomes. Their unwinding depends on the FANCJ helicase [36] and on the recruitment of DDX11 by Timeless, a component of the Fork Protection Complex (FPC) [37].

Since chromatin remodeling complexes and histone modifying factors prevent R-loop mediated RS, RNA:DNA hybrids could be particularly toxic to forks when chromatin structure is altered. Thus, depletion of BRG1 (SMARCA4), the main ATPase activity of SWI/SNF complexes, increases fork arrest and R-loop-dependent breaks in human cells [38]. Moreover, deficiency in PBRM1, a subunit of the SWI/SNF complex PBAF, induces elevated levels of R-loops, RS and micronuclei [39]. INO80C, another ATP-dependent chromatin remodeling complex, contributes to processive DNA replication by promoting the resolution of R-loops [40] and by preventing pervasive transcription through replication origins [41]. In *Caenorhabditis elegans*, the histone methyltransferase MET-2 prevents the formation of toxic R-loops by repressing transcription in satellite repeats [42], whereas H3K4 methylation restrains TRCs in budding yeast by decelerating replication forks [43]. The interplay between R-loops, chromatin structure and DNA replication is therefore very complex and deserves further investigation. This is particularly true in the context of cell fate determination since R-loops persist on silent pluripotency genes after differentiation and are associated with repressive chromatin marks [44]. Alterations in these reprogramming mechanisms may also affect DNA replication programs and increase genomic instability [45], stressing the importance of R-loops in the modulation of cell fate memory and cell plasticity.

Figure 1



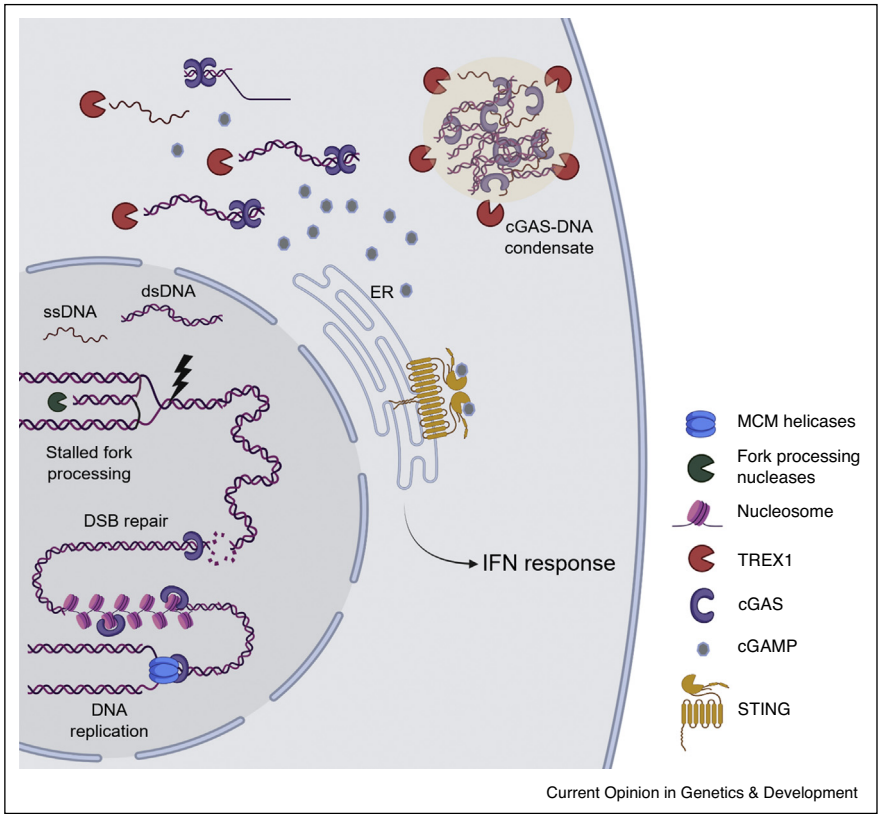
Endogenous sources of replication stress.
The DNA replication machinery frequently encounters obstacles along the DNA. These include RNA polymerases and cotranscriptional R-loops, causing transcription-replication conflicts in an orientation-dependent manner [30]. Secondary DNA structures such as hairpins or G4 structures can also block replication when they form on the displaced ssDNA strand of R-loops or in between the replicative helicase and DNA polymerases [34,36]. Other replication barriers include dense chromatin structures and other tightly bound proteinaceous complexes [38]. Finally, a transient reduction of dNTP pools below optimal levels can also occur during a normal S phase and activate the replication checkpoint by slowing down fork progression [71].

Of note, chromatin modifications are also important to facilitate fork restart. In budding yeast, the controlled resection of nascent DNA at stalled forks depends on non-catalytic functions of the Mre11-Rad50-Xrs2 (MRX) complex. MRX acts together with the chromatin modifiers Set1 and Gcn5 to increase the accessibility of nascent chromatin to nucleases and promote the loading of cohesin, ensuring the timely restart of stalled forks [46]. In human cells, fork resection is regulated by the histone acetyl transferase PCAF recruiting the MRE11 and EXO1 nucleases to stalled forks [47]. Finally, SMARCAD1, another SWI/SNF complex component is crucial to stabilize active forks in response to RS [48]. In conclusion, the list of potential replication roadblocks has continued to grow over the past two years, along with the number of chromatin modifiers involved in replication fork arrest and restart.

Signaling RS outside of the cell

The RS response is generally seen as a cell-autonomous pathway coordinating a variety of intracellular processes to ensure the completion of DNA replication. However, recent evidence indicates that cells can signal RS to the microenvironment by activating pro-inflammatory responses (Figure 2). Inactivation of the *SAMHD1* gene, whose mutations are associated with a severe inherited encephalopathy called the Aicardi-Goutières syndrome, results in an aberrant processing of stalled replication forks and in the production of type I interferons [3,49]. This process is initiated with the release of chromosomal DNA fragments into the cytosol, either directly or through the formation of micronuclei [3,49]. These fragments are detected as non-self by the cGAS/STING DNA sensing pathway [50]. When bound to DNA, the cytosolic DNA sensor cGAS undergoes liquid-liquid

Figure 2



Interplay between RS and innate immunity and cGAS DNA sensing.
Aberrant processing of stalled replication forks induces the accumulation of cytosolic DNA [49], triggering the activation of the cGAS/STING pathway and inducing the IFN response [50]. Cytosolic cGAS interacts with dsDNA, forming the cGAS-DNA condensates to prevent the degradation of cytosolic DNA by the 3′-5′ exonuclease TREX1 [51••]. Since cGAS is predominantly a nuclear protein, it is sequestered by nucleosomes to prevent its unscheduled activation in the nucleus [54–58]. Nuclear cGAS also interacts with the MCM helicases to decelerate fork progression [61] and inhibits HR-mediated DSB repair [60]. Depletion of cGAS results in the acceleration of fork speed and increased DNA damage. ER: endoplasmic reticulum; ssDNA: single-stranded DNA; dsDNA: double-stranded DNA; IFN: interferon

phase separation and activates STING to induce a kinase cascade leading to the production of type I interferons (IFNs) [50,51••].

Recent evidence indicates that cGAS is also found in the nucleus [52], so what prevents it from sensing chromosomal DNA? Nuclear cGAS is 200-fold less active than the cytosolic form and is tethered to chromatin to repress its activity [53••]. A series of comprehensive studies revealed the structural basis of how nuclear cGAS is inhibited by nucleosomes. The tight interaction between cGAS and the 'acidic patch' of histone 2A-histone 2B prevent cGAS DNA binding and dimerization, two processes required for cGAS activation [54–58]. Moreover, the N-terminus of cGAS involved in DNA binding, liquid phase separation and chromatin tethering is hyperphosphorylated during mitosis to prevent cytosolic cGAS from sensing mitotic chromosomes [59].

Intriguingly, the nuclear population of cGAS inhibits HR and promotes the formation of micronuclei, independently of its DNA sensing activity [60]. Moreover, it interacts with replication factors such as MCM2, MCM3, MCM7, RFC1 and PCNA, and slows down fork progression [61]. This effect contrasts with the role of ISG15, a downstream effector of cGAS in the RECQ1-dependent acceleration of forks [8•] and stresses the fact that further work is required to fully understand the complex and fascinating interplay between RS and innate immunity.

Exploiting the RS-inflammation axis for cancer therapy

RS is a double-edged sword for cancer cells. While it promotes tumorigenesis by increasing genomic instability, it also hinders their potential to proliferate by destabilizing replication forks [62] and it sensitizes them to

chemotherapy. This vulnerability is classically exploited in cancer treatment to increase RS to unsustainable levels [2,63], but new strategies are emerging, exploiting recently identified specificities of the RS response. These include the inhibition of DNA polymerase α to increase ssDNA gaps, deplete RPA and induce a replication catastrophe [64]. Filamentous actin, which polymerizes in response to RS to promote fork repair, is another promising target for cancer therapy as its protective function is sensitive to ATR and mTOR inhibitors [65]. Moreover, it has been reported that hydroxyurea treatment triggered ATR-CHK1 and the p53-dependent extrusion of monolayer epithelial cells. This process could function as an early checkpoint to eliminate pre-cancerous cells. [66].

The above-mentioned link between RS and immune cell death is also opening new avenues for cancer treatment. Indeed, recent studies indicate that RS can be actioned to trigger the cGAS/STING pathway and attract tumor infiltrating immune cells. These include the use of ATR inhibitors in oxaliplatin-resistant colon cancer [67] and PARP inhibitors in ERCC1-deficient non-small cell lung cancer [68] to increase RS, activate the cGAS/STING pathway and promote antitumor immunity. ATR and PARP inhibitors were also used to induce type I IFNs in cancer cells deficient for the PBRM1 subunit of the SWI/SNF complex PBAF, which display increased R-loops and RS [39]. Moreover, loss of the SWI/SNF complex component ARID1A increases CHK2 degradation, leading to RS and to the activation of a STING-mediated innate immune response, increasing tumor-infiltrating lymphocytes and patient survival [69]. Similarly, inhibition of KDM4A, a histone H3K9me3 demethylase, promotes the formation of liquid-like HP1γ condensates on heterochromatin, stalls replication forks and activates cGAS/STING signaling. Combined with immune checkpoint inhibitors, this strategy prevents the growth and metastasis of squamous cell carcinoma by recruiting CD8+ T cells and efficiently eliminating cancer stem cells [70••].

Conclusion and perspectives

Our view on RS has evolved considerably in recent years. It is no longer limited to fork stalling or collapse, but now includes a wide range of events that alter the distribution of origins and/or the speed of replication forks [5,6,8•]. In addition, RS is no longer always considered as a pathological situation, as recent evidence indicates that it can be used to signal ongoing DNA replication in unchallenged growth conditions [71]. Finally, the sphere of influence of the RS response has considerably grown over the past decade, expanding well beyond the boundaries of the cell. Indeed, it is now well established that cytosolic DNA fragments or micronuclei induced by RS can activate the cGAS/STING pathway and promote the immune rejection of cancer cells through the induction of type I IFN. Furthermore, recent advances in our understanding of the mechanisms of fork protection and restart have revealed that the replisome is incredibly resistant to RS [1,62]. It is therefore likely that chemotherapeutics drugs not only act as cytotoxic agents, but also make tumors more visible to the immune system through RS-mediated inflammation.

Conflict of interest statement

Nothing declared.

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