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Number of words: 6832 (without references)

Number of figures: 2

Key words: replication stress, cancer, senescence, genome instability, catastrophic mitosis.

Abstract

Alterations of the dynamics of DNA replication cause genome instability. These alterations known as "replication stress" have emerged as a major source of genomic instability in pre-neoplasic lesions, contributing to cancer development. The concept of replication stress covers a wide variety of events that distort the temporal and spatial DNA replication program. These events have endogenous or exogenous origins and impact globally or locally on the dynamics of DNA replication. They may arise within a short window of time (acute stress) or during each S phase (chronic stress). Here, we review the known situations in which the dynamics of DNA replication is distorted. We have united them in four main categories: *i*) inadequate firing of replication origins (deficiency or excess), *ii*) obstacles to fork progression, *iii*) conflicts between replication and transcription and *iv*) DNA replication under inappropriate metabolic conditions (unbalanced DNA replication). Because the DNA replication program is a process tightly regulated by many factors, replication stress often appears as a cascade of events. A local stress may prevent the completion of DNA replication at a single locus and subsequently compromise chromosome segregation in mitosis and therefore have a global effect on genome integrity. Finally, we discuss how replication stress drives genome instability and to what extent it is relevant to cancer biology.

1 Introduction

The maintenance of genome stability is essential to the accurate transmission of genetic information. It relies on the successful duplication of chromosomes, followed by their even segregation during mitosis. Genome instability is not only a hallmark of cancer cells but, maybe more importantly, is also a cause of genomic disorder diseases and cancer predisposition [1]. Errors occurring during DNA replication can impact both on the accurate duplication of chromosomes and on their equal segregation during mitosis (Figure 1) [2]. Thus, replication stress has emerged as a primary source of genome instability [3]. This is clearly the case in cells overexpressing oncogenes such as Cyclin E, Cdc25A, CDC6 or CDT1, which interfere with the DNA replication program and lead to expression of genome instability markers [3-7]. The causes of replication stress are many and it remains challenging to unify them according to their origins or their molecular bases [8]. Pioneering studies on unicellular eukaryotes such as the yeasts *S. cerevisiae* and *S. pombe* have unveiled the molecular events linking alterations of the replication dynamics to the expression of genome instability markers [9]. Replication stress arises from endogenous (*e.g.* inappropriate cellular metabolic conditions) or exogenous (*e.g.* exposure to DNA-damaging or replication-blocking) sources and acts locally (discrete pause sites) and globally on replication dynamics. Here, we review the known sources of replication stress and make an attempt to classify them into four categories: (i) alterations of origins firing, (ii) impediments to forks progression, (iii) conflicts between the transcription and the DNA replication program and (iv) DNA replication in inappropriate metabolic conditions. We also discuss to what extent these causes of replication stress can jeopardize genome integrity and contribute to human diseases.

2 The Eukaryotic DNA replication program

The replication machinery of eukaryotic cells accurately duplicates the genome and is robust enough to carry out this task in face of numerous obstacles. The DNA replication program is temporally and spatially controlled within each individual cell. The initiation of DNA synthesis takes place at thousands of replication origins from which two replication forks progress bi-directionally [10, 11]. The merge of two converging forks occurs randomly within the genome [12]. DNA replication occurs concurrently with many other metabolic processes, such as transcription, mRNA processing and chromatin remodeling. These processes must be tightly coordinated with DNA replication to avoid potential conflicts and ensure the proper transmission of genetic and epigenetic information. The accuracy and robustness of DNA replication relies on key points:

- DNA synthesis is highly accurate owing to the fidelity of replicative polymerases and the association of mismatch repair (MMR) factors to replication factories [13].

- Replication origins fire once and only once per cell cycle [14]. They are activated sequentially throughout the length of the S phase. This sequential activation depends on their accessibility to limiting initiation factors, for which origins compete [15-18]. Moreover, origins are clustered into replication timing domains, in which origins fire stochastically [19].

- The genome contains a large excess of replication origins. All origins are licensed before S phase, but many of them are not activated in unchallenged growth conditions. These origins are referred to as cryptic or dormant origins. They are used to rescue stalled forks under replication stress conditions [19-22].

- The progression of replication forks is escorted by multiples factors that ensure their stability in face to obstacles and promote their restart when needed [8].

Origin-poor regions, such as common fragile sites (CFSs) are particularly at risk and forks travelling through these loci rely on fork-restart mechanism to complete DNA synthesis [23-25]. The same applies to regions that are replicated unidirectionally (such as the ribosomal DNA locus for example), or when two converging forks are simultaneously impeded [26]. Factors promoting the stability and the restart of replication forks are part of the DNA Damage response (DDR) [27]. This cellular response includes the DNA replication checkpoint and the DNA repair and tolerance pathways. Among the DNA repair mechanisms, homologous recombination is particularly important for the robustness of DNA replication [28].

Here, we propose that any event that alters the fulfillment of the DNA replication program can be considered as a source of replication stress. These events include alteration of the initiation and elongation of DNA replication, conflicts between DNA replication and metabolic pathways such as transcription and mRNA processing, as well as inappropriate cellular metabolic conditions, which are not in line with the needs for DNA replication.

3 The cellular response to replication stress

A hallmark of replication stress is the accumulation of single stranded DNA (ssDNA) at replication forks, even though compromised DNA replication without excess of ssDNA has also been reported [29-31]. Stretches of ssDNA are exposed at replication forks as a consequence of replicative helicases continuing to unwind the parental DNA while the replicative DNA polymerases are stalled [32]. This

uncoupling between helicase and polymerase activities is probably not the sole cause of accumulation of ssDNA at stalled forks. It can be also generated by the degradation of newly-synthesized DNA through the combined action of nucleases and DNA helicases, such as MRN (MRE11, RAD50, NBS1), *S. cerevisiae* Sae2 and its human homologue CtIP, EXO1 and the BLM (BLOOM Syndrome Mutated) [33-36]. These enzymes are best known for their role in the resection of DNA ends at DSBs (Double Strand Break), but could also act at stalled forks [37]. Primed ssDNA, together with ssDNA coated by the protein RPA (Replication Protein A), act as a signaling platform to recruit numerous factors involved in the cell response to replication stress and *in fine* activate the central ATR kinase (Ataxia-Telangectasia mutated and Rad3 related) [38]. ATR orchestrates the cellular response to replication stress by phosphorylating several targets that control the stability of replication forks, the firing of replication origins, mRNA metabolism and cell cycle progression. Altogether, the ATR pathway limits genomic instability during S phase and contributes to cell survival in response to replication stress [39]. In neoplasic lesions, this pathway acts as a barrier to both genomic instability and uncontrolled proliferation [3].

Two main functions of ATR can be distinguished: *i*) stabilization of stalled replication forks (local effect) and *ii*) cell-cycle arrest and repression of late origins (global effects) (Figure 2). At the global level, ATR ensures an optimal management of the resources essential to fork progression, such as dNTP pool and histone supply [39, 40]. When dNTP levels run down, the ATR pathway prevents the activation of late-firing origins but allows the activation of dormant origins to complete DNA synthesis at early domains [19, 41-43]. The local activities of ATR at replication forks remain elusive at the molecular level. Studies in yeast have established that ATR maintains the replisome in a replication-competent state by phosphorylating some of its components [44-50]. Whether similar mechanisms operate in mammalian cells is currently unclear [34, 51]. An emerging target of ATR at mammalian replication forks is the SMARCAL1 helicase involved in the remodeling of stalled forks [52-54]. Inhibition of ATR during an acute replication stress results in fork collapse, associated with the accumulation of ssDNA and DSBs at replication forks (Figure 2). ATR phosphorylates SMARCAL1 to prevent fork remodeling and thus fork collapse [34]. Defects in SMARCAL1 cause the human diseases Schimke immunoosseous dysplasia, characterized by growth retardation, immunodeficiency and cancer predisposition [55, 56].

An early function of the DDR at replication stress sites is the phosphorylation of the histone H2A in yeasts and H2AX in mammals (named γ-H2A and γ-H2AX, respectively) [57]. In mammalian cells, γ-H2AX results from the activities of three kinases: ATM (Ataxia-Telangectasia Mutated), ATR and DNA-PK (DNA-dependent Protein Kinase) and thus reflects a local activation of the DDR within the nucleus. γ-H2AX is frequently used as a replication stress marker, together with the formation of

53BP1 foci, another component of the DDR [58-60]. However, both γ-H2AX and 53BP1 foci also occur at DNA damage, in particular DSBs, in a replication-independent manner [57].

Other ATR-dependent phosphorylation events, such as the phosphorylation of RPA on Serine 33 and the phosphorylation of the checkpoint kinase Chk1 on serine 345, are also used as markers of replication stress [31]. However, it should be noted that certain types of replication stress remain undetected by the ATR pathway because they do not generate enough ssDNA at forks, either because they affect a small number of forks or because uncoupling between helicases and polymerases does not occur [18, 38, 61, 62]. Replication stress activates the ATR pathway in a stepwise manner. Sensors and mediators are first recruited to forks to control their stability. Then, this local checkpoint signal is amplified through the activation of the Chk1 kinase to delay mitosis [5, 61]. Thus, localized alterations of fork progression may escape cell cycle surveillance and jeopardize the stability of the genome.

Impediment to fork progression leads to ssDNA gaps and to replication-associated DSBs [63-65]. The origin of these DSBs remains elusive. They might form as a consequence of fork passage through a nick in the parental DNA or as a consequence of enzymatic processing of gapped replication forks [66-69]. In some cases, genome-wide alteration of replication dynamic leads to fork reversion, a 4-branched DNA structure in which parental DNA strands are annealed. Genetic data in bacteria are indicative of the existence of such fork structures that have been visualized in yeast and mammalian cells by electronic microscopy (EM) [50, 65, 70]. Fork reversion has also been identified as a major consequence of the topoisomerase 1 inhibitor campthotecin (CPT) on replication dynamics [71]. Fork reversion reflects the activities of replication-restart pathways rather than the formation of pathological intermediates from which DNA synthesis cannot resume [72]. Overexpression of the Cyclin E oncogene leads to the expression of replication stress markers, including slow-down of fork progression, ATR activation, fork reversion and massive chromosome breakages [73]. Remarkably, fork reversion precedes DSB formation, which results from the Mus81-dependent cleavage of reversed forks. Mus81 activity is normally restricted to late G2 or mitosis and conversion of reversed forks into DSBs is a consequence of premature entry of into mitosis [73, 74]. Thus, the concomitant inactivation of the DDR accelerates chromosomes breakages induced by replication stress. Alternatively, the accumulation of ssDNA at replication forks might sensitize them to endonucleases, thus resulting in the formation of replication-associated DSBs.

4 The causes of replication stress

Each time a cell enter S phase, it must replicate a large number of loci that represent a challenge to fork passage and thus a potential source of local replication stress. Some parts of the genome are also more sensitive to global replication stress of endogenous or exogenous origin. These loci are collectively referred to as "intrinsically-difficult to replicate" sites. In mammals, paradigms for difficult to replicate sites are found at CFSs, which are defined as loci that accumulate beaks or gaps, when condensed in response to mild replication stress [75, 76]. CFSs are usually replicated late and thought to remain unreplicated when cells enter mitosis [2]. The molecular bases of CFSs fragility include a defect in replication origins and the presence of non-canonical DNA structures or very large genes [23-25, 77, 78]. The past decade has seen the term "fragile site" used as a generic term to refer to "difficult-to-replicate" loci in many organisms. However, distinct mechanisms are likely underlying the genetic instability of these fragile sites, even within a given organism.

4-1 inappropriate firing of replication origins: excess and deficiency

The DNA replication program ensures that origins fire sequentially throughout the length of the S phase. To maintain genome stability during DNA replication, it is critical that: *i*) a sufficient amount of origins fires to complete DNA replication, *ii*) origins fire in a sequential order and not all at the same time and *iii*) origins fires once and only once per S phase. The sequential firing of replication origins is orchestrated by a limited amount of initiation factors and by the availability of dNTP [8, 16, 79]. Both in mammals and yeast, the replication fork speed in a key determinant of the temporal DNA replication program [79-81]. The rates of initiation and elongation are modulated by the level of dNTPs. When dNTP levels are low, replication forks are slower and more origins fire to ensure the completion of DNA replication. If dNTP pools drop below a critical level, ssDNA accumulates at stressed forks, the DNA replication checkpoint is activated and interrupts the replication program [79]. In all eukaryotes, the genome contains an excess of origins to buffer the consequences of fork stalling [10]. As forks slow down, dormant origins fire to offset the completion of DNA replication. In mammals, this compensatory mechanism has been reported in many situations in which DNA replication is challenged and contributes to cell resistance to replication stress [21, 22, 82, 83]. Alterations of the dNTP pools are associated with increased level of genome instability and cancer development during DNA replication [84, 85].

Replication origins are not equally distributed along the genome and their efficiency varies widely. Some CFSs are origin-poor over megabases of DNA and are thus unable to compensate for slower

forks by activating dormant origins [23-25]. In response to a mild replication stress, duplication of CFSs relies on the ability of slow-progressing forks to synthetize DNA over a long distance. Cells are thus prone to enter mitosis with unreplicated chromosomal segments [2]. As the DNA replication program is cell-type specific, the fragility of CFSs is also cell-type specific [23]. Other CFSs contains elements that can impinge on fork progression [78]. Most CFSs overlap with very long gene (>300 kb) whose transcription might favor collisions with the replication machinery [77]. While the initial cause of replication stress may differ from one CFS to another, the lack of efficient origins could be a common reason of fragility. Importantly, 50 % of recurrent deletions observed in cancer cells originate from CFSs associated with large genes [86].

Importantly, an excessive firing of origins is also deleterious to cell viability. In budding yeast, overexpression of limiting initiation factors allows the simultaneously firing of most origins in early S-phase [16, 18]. As a consequence, the pool of dNTP becomes rapidly limiting, with deleterious consequences for cell viability. An excess of origins firing could also consume other limiting replication factors and ultimately destabilize replication forks (Figure 2). Upon replication stress and in the absence of the main checkpoint kinase ATR, a large fraction of replicons are activated and exhibit an increased level of ssDNA covered by RPA at replication forks [34]. As the number of active origins increases within a cell, the amount of RPA becomes limiting, ssDNA remains unprotected and forks are more susceptible to breakage [87]. The overexpression of RPA appears sufficient to prevent such fork breakage. Similarly, re-replication of chromosomal segments due to deregulated origins firing also results in fork reversal, massive recruitment of RPA at ssDNA gaps, which are then converted into DSBs during the next round of replication [88]. Many factors involved in origin licensing are overexpressed in cancer cells and re-replication could contribute to cancer progression [89].

While the execution of the temporal DNA replication program relies on a limited amount of replication resources, genomes have also evolved an excess of origins and replication factors to buffer replication stress. An excess of origins firing is a factor of acute replication stress by exhausting critical factors that contribute to fork integrity.

4-2 Obstacles to fork progression

A wide variety of obstacles can impede on the progression of replication forks, either by altering the activity of the replicative helicase or the ability of replicative polymerase to incorporate nucleotides. These obstacles include DNA lesions (abasic sites, damaged bases, inter or intra-crosslinks…), DNA-

protein complexes, DNA sequences prone to form secondary DNA structures and highly-transcribed genes [8]. These impediments to fork progression are often referred to as RFBs (Replication Fork Barriers). Some of them result from exposure to exogenous genotoxic agents, including anti-cancer chemotherapy. Other RFBs known as natural pause sites or intrinsic RFBs are caused by normal cellular events and occur during each S phase. For example, discrete fork arrests occur at centromeres, telomeres and tRNA genes in yeast. Natural RFBs can also be programmed to avoid collisions between the replication and transcription machineries or to facilitate sexual differentiation in yeast [8]. The biology of intrinsic RFBs has been mainly explored in yeast models but their frequency and importance in mammals remain poorly evaluated. However, a recent study has revealed the existence of specific loci suffering from recurrent replication stress. These loci are mainly replicated early, are enriched for highly-expressed genes, repetitive elements and CpG dinucleotide. Importantly, these loci are also hot spots for breaks and genome instability in an ATR-dependent manner [90]. The chromatin status has been also proposed to be a source of impediment to fork progression. The histone mark γ-H2AX is enriched at intrinsic RFBs, but also at repressed genes associated with hypo-acetylated histones [58]. Also, the fission yeast heterochromatin at centromeres and telomeres is enriched for γ-H2AX, supporting a link between hypo-acetylated chromatin and replication stress [59].

The activity of RFBs and their impact on genome stability are strongly dependent on replication fork direction. In mammals, the DNA replication program is cell-type specific [10]. A given locus may thus be replicated in one direction in one cell type and in the opposite direction in another cell type. Therefore, the activity of some RFBs and their impact on genome stability may be cell-type specific. For example, the expansion of CGG repeats in the FRM1 causes the Fragile X syndrome. In embryonic cells, fork stalling occurs at CGG repeats, and cells that are unable to fire origins upstream CGG repeats show repeat instability. It appears that CGG expansion occurs preferentially when the FRM1 gene is replicated such that the CGG strand serves as the lagging strand template [91]. Thus, a developmental switch in the direction of replication fork occurring during cell differentiation may account for the instability of repeated sequences.

The bypass of RFBs requires additional factors than canonical replication components, such as specialized helicases in charge to remove proteins tightly bound to DNA or secondary DNA structures [92]. Also, the bypass of DNA lesions involves additional DNA polymerases activities such as TLS (Trans-Lesions Synthesis) polymerases and PRR (Post-Replication Repair) pathways [93]. An emerging concept in the field of DNA replication is that all genome segments may not be equal in term of factors required for their duplication. The successful duplication of “difficult-to-replicate” loci could

be more dependent on accessory factors than other loci and thus components of the replication machinery might differ according to the replicated locus.

The implication of natural RFBs in diseases is well established for at-risk motifs that can form secondary DNA structures such as tri-nucleotide repeats whose expansion underline many human diseases [94]. While many of the natural RFBs described so far are associated with hot spots of genome rearrangements and recombination, their potential contributions to cancer progression is not yet clearly established [9]. However, cancer cells may be particularly sensitive to natural RFBs because they try to duplicate their genome in inappropriate metabolic conditions (see below).

4-3 Interference between the transcription and the replication programs

Transcription represents a major source of replication stress, both in eukaryotes and prokaryotes [95]. This is due to the fact that DNA replication and transcription machineries share the same DNA template and are therefore prone to collisions. Bacterial genomes are generally replicated from a single origin and genes are organized collinearly with replication to prevent frontal collisions [96]. The situation is more complex in eukaryotes due to the presence of multiple replication origins. To limit interference between these processes, DNA replication and transcription are physically and temporally separated within the nucleus [97]. Yet, a large body of evidence indicates that transcription interferes with DNA replication and induces genomic instability in eukaryotes [31, 95]. The mechanism by which transcription impedes fork progression is currently unclear. One likely possibility is that replication stress is caused by RNA/DNA hybrids that form during transcription when nascent RNA anneals to the template DNA strand, leaving the non-template strand unpaired [98]. Formation of these so-called R-loops is favored by GC-rich DNA sequences and increases when assembly of mRNA-particle complexes (mRNPs) is impaired [99, 100]. R-loops are removed by RNaseH and specialized helicases such as Senataxin [101-103]. Depletion of factors that displace R-loops or prevent their formation induces genomic instability in yeast and in higher eukaryotes [83, 98, 104, 105]. Another source of replication stress is associated to the accumulation of positive supercoiling when transcription and replication converge [83, 106]. In yeast, this is particularly the case of genes physically tethered to the nuclear envelope during mRNA export [107]. In mammalian cells, very large genes are also particularly at risk as they are transcribed throughout the cell cycle [77]. Many of these very large genes are replicated late in S phase and overlap with CFSs. However, their fragility depends on their replication profile and varies widely between cell types [24]. Remarkably, transcription also interferes with DNA replication at early-replicating sequences and a recent study indicates that chromosome breaks occur preferentially at cancer genes under replication stress, for a reason that is still unclear [90, 108]. Altogether, these data indicate that

replication and transcription must be tightly coordinated during S phase to prevent genomic instability. Since both processes are perturbed during oncogene-induced tumorigenesis, it is tempting to speculate that replication/transcription interference represents a major source of genomic instability in precancerous lesions. This view is supported by a recent study indicating that conflicts between replication and transcription increase in cells overexpressing Cyclin E [109].

4-4 Replication in inappropriate metabolic conditions (unbalanced replication)

Accurate chromosome duplication requires adequate resources for DNA replication. As mentioned above, the depletion of dNTP pools results in slow fork progression. While this situation can be artificially induced using replication inhibitors such as hydroxyurea, unbalanced dNTP pools are also associated with genome instability and cancer predisposition in human cells. The expression of HPV-16 E6/E7 (Human Papillomavirus) proteins forces cells to initiate DNA replication by the aberrant activation of the Rb-E2F pathway. This is accompanied with the expression of replication stress markers (slower forks, firing of dormant origins, chromosome instability and breaks) whose expression is largely due to an insufficient dNTP pools [84]. Such type of replication stress refers to unbalanced DNA replication, a situation in which cells initiate replication in inappropriate metabolic conditions. Similarly, overexpression of the Cyclin E oncogene leads to fork slow down due to an inappropriate dNTP levels. To escape unbalanced replication, cells forced to proliferate upregulate genes involved in nucleotide biosynthesis, including c-myc.

The Bloom syndrome, which exhibits a strong association between genome instability and predisposition to all type of cancers, is caused by a mutation in the BLM, a member of the RecQ family of DNA helicases. Patient cells exhibit replication stress markers, including slowed forks and higher number of activated origins [110]. Although these data have been interpreted as a direct involvement of BLM in replication dynamics, patient cells suffer from replication stress due to pyrimidine pool disequilibrium, a consequence of the down-regulation of the CDA gene (Cytidine Deaminase) in BLM-deficient cells [85]. While unbalanced dNTP pools cause replication stress and genome instability, to what extent they are also a general cause of tumor development remains to be determined.

DNA replication also requires a large supply in histones. Parental nucleosomes are disrupted ahead of replication forks and restored, together with the deposition of newly-synthetized histones, onto daughter strands [111]. Defects in chromatin assembly during DNA replication compromise the transmission of epigenetic marks. More surprisingly, it also affects replication dynamics. In the yeast

S. cerevisiae, DDR is required for degradation of excess histones, which would otherwise compromise genome stability [40]. Defects in replication-coupled chromatin assembly destabilize moving replication forks that ultimately collapse [112, 113]. In mammals, the supply of newly-synthetized histones regulates the rate of fork elongation, presumably by interfering with the recycling of the replication factor PCNA (Proliferating Cell Nuclear Antigen) [30]. Surprisingly, transient shortage in newly-synthesized histones slows down fork progression without activating of the DDR or firing additional origins, probably because the stretches of ssDNA formed at the forks are too short to activate the ATR pathway. However, prolonged defect in histone deposition at the fork leads to DNA damage. Two developmental human diseases (Wolf-Hirschhorn syndrome and congenital dyserythropoietic anemia type I) are associated with a defective histone supply, but the contribution of such type of replication stress to cancer biology remains unexplored [114, 115] .

5 Consequences of replication stress on genome stability and cell fate

Replication stress compromises the completion of chromosomes duplication and impacts on chromosomes segregation at mitosis (figure 1). Incomplete DNA replication, unresolved DNA repair intermediates and intertwined sister chromatid can therefore lead to chromosome breakage due to chromosome non-disjunction. As mentioned above, one of the earliest consequences of replication stress is the formation of RPA-coated ssDNA, which in turn activates the ATR pathway. This pathway ensures that replication forks are able to restart once the source of replication stress is removed. Alternative pathways are required to promote fork recovery at sites of replication stress when the ATR pathway fails to maintain forks in a replication-competent state. The activation of dormant origins is a particularly effective mechanism to complete chromosome duplication both in response to local and global replication stress [19]. However, in regions of the genome that are origin-poor (*i.e* CFSs) or are replicated unidirectionally (*i.e.* telomeres), additional fork restart mechanisms are needed to complete DNA replication. Some of these mechanisms are responsible for inheritable genome modifications, from mutations to chromosomal rearrangements.

5-1 Uneven mitotic chromosome segregation: from a local initiator event to genome-wide effects

Cells reaching mitosis with damaged and/or incompletely-replicated chromosomes

As mentioned above, fork slowdown is generally compensated by the firing of dormant replication origins. However, this mechanism does not operate at origin-poor regions of the genome such as at CFSs, leading to incomplete replication [20, 23, 25]. CFSs exhibit unresolved DNA

repair/recombination intermediates or intertwined sister chromatid at the end of the S phase. This leads to the formation of atypical chromosome segregation events, such as anaphase bridges (UFB) [116, 117]. The fact that CFSs form anaphase bridges in mitosis indicates that partially-replicated chromosomes remain undetected by checkpoint pathways before cells undergo anaphase [60, 118, 119]. This phenomenon could also arise in response to endogenous or low replication stress that escapes cell survey. For example, the phosphorylation of CHK1 is dose dependent and very low doses of HU (such as 10 μM) decelerate replication forks progression without inducing a significant cell cycle arrest or detectable CHK1 phosphorylation [61, 62].

In anaphase, unresolved or unreplicated regions restrain chromosome segregation by creating a physical link between the two sister chromatids, known as anaphase bridges. These structures can be stained with DNA intercalating agents such as DAPI when they result from aberrant chromosomal morphology [2]. In contrast, UFBs do not stain with DAPI but can be detected using immunostaining against UFB-binding proteins such as the PICH or BLM helicases, RPA and some components of the FANC (Fanconi Anemia) complex [116, 117, 120] [121]. A subset of UFBs colocalizes with CFSs and is induced by replication stress [116, 117]. Of note, whereas low levels of UFBs are detected under normal growth conditions, they are very abundant in cells defective for BLM, FANC or homologous recombination [117, 121-124].

Anaphase separation creates an increasing mechanical tension on partially-replicated chromatids, which are then prone to break. Remarkably, CFSs are prone to chromosome breakage under replicative stress [75, 76]. The breakage of anaphase bridges lead to the uneven segregation of broken chromosome arms and thus to potential translocations. While the nature of unresolved replication intermediates or intertwined sister chromatid junctions formed at unreplicated DNA remains unknown, it is generally believed that these structures are cleaved by nucleases such as MUS81-EME1, GEN1 or dissolved by BLM and topoisomerase III alpha [125-128]. It has been proposed that a failure to cleave unresolved sister chromatids leads to chromosome missegregation [2]. Thus, nuclease attacks of unusual replication intermediates persisting after S phase may not be systematically detrimental to genome stability, but may rather protect the genome from the deleterious effects of replication stress.

Genome-wide impact of chromosome segregation defects

Replication stress impedes chromosome segregation locally through the formation of anaphase bridges. Besides this local effect, low levels of replication stress can also lead to the formation of extra centrosomes at mitosis [62]. The mechanisms linking S-phase progression and centrosomes

duplication have only started to emerge and it remains unclear how chronic replication stress promotes centrosomes amplification [129]. Since most of mitotic extra centrosomes are functional, their presence results in multipolar mitosis and unbalanced chromosome segregation [62]. Importantly, extra centrosomes are systematically associated with chromosomes bridges, delayed chromosome condensation and with prolonged metaphase arrest [130].

Homologous recombination (HR) plays a pivotal role in DNA repair and in replication restart, contributing therefore to the accuracy of DNA replication [28]. Defect in HR is also a factor of cancer predisposition. Remarkably, HR-deficient cells display both a genome-wide decrease in replication fork speed, an increased frequency of extra centrosomes and UFBs, suggesting that HR defect result in an endogenous replication stress [62, 82, 122, 123, 131]. In support to this, mitotic defects observed in HR-deficient cells result from slowed fork progression, suggesting that the links between replication stress and mitotic defect potential underline cancer development [62].

Mitotic defects induced by replication stress: an "adaptation-like" process in mammalian cells?

While incomplete DNA replication at specific loci is a checkpoint blind event, mitotic defects due to anaphase bridges activate checkpoints and result in prolonged mitotic arrest [62, 132]. The capacity to bypass the mitotic arrest without resolving its cause has been described in yeast as a process called adaptation [133, 134]. We propose that anaphase bridges are revealed in cells bypassing the mitotic arrest, in a process that is reminiscent to the adaptation process described in yeast. It is tempting to speculate that during the mitotic arrest bypass, opposite forces could be transmitted to the centrosomes in an attempt to force segregation, leading to the splitting of centrioles and multipolar segregations. This model is consistent with the facts that (i) extra centrosomes are detected in mitosis but not in interphase, (ii) extra centrosomes are functional, causing multipolar segregations and (ii) almost all mutipolar segregations are associated with chromatin bridges and with prolonged metaphase arrest [62]. Together, these data indicate that incomplete replication results in local chromosome abnormalities at mitosis such as UFBs fragile sites and that the formation of extra centrosomes amplifies the defects to all the genome by generating multipolar attachments and aberrant segregation of fully-replicated chromosomes.

5-2 Inheritable genome modifications

Replication stress results in inheritable genome modifications, including point mutations and larger chromosomal rearrangements. Some of these rearrangements are particularly complex, including triplication, deletion, inversion and translocation. In cancer cells, the precise mechanisms responsible

for replication stress-induced genome instability remain unclear but they can be determined from the sequence of breakpoint junctions [9].

Both stretches of ssDNA at stalled forks and replication-associated DSBs are likely initiator events of replication-induced genome instability. When forks collapse and eventually break, replication-restart mechanisms occur and faulty replication restart events are causal mechanisms of genome modifications and chromosome rearrangements. Among the pathways ensuring the continuity of replication, HR is an evolutionary conserved mechanisms involved in DSB repair and replication-restart [28]. The purpose of HR is to recombine homologous sequences, usually at sister chromatids, to repair DNA lesions. Occasionally, HR recombines repeated sequences dispersed through the genome and such events refer to NAHR (Non-Allelic Homologous Recombination), a cause of genome rearrangements exhibiting homology at breakpoint junctions [1]. In fission yeast, a single fork arrest is sufficient to induce translocation and genomic deletion in a recombination-dependent manner [135]. Also, slowdown in replication fork progression stimulates fusion between inverted repeats by a recombination pathway in mammals [136]. Replication-borne DSBs can be repaired by BIR (Break-Induced Replication), a type of HR event that can drive translocation in yeast models [137].

Single mutations are by-products of TLS-DNA polymerases that bypass DNA lesions in an error-prone manner. The bypass of DNA lesions can occur either at the fork or behind the fork. Indeed, it has be proposed that DNA synthesis can be re-primed downstream the lesion, leaving a gap in the rear of the moving replisome that is then fill-in by either TLS or HR [138-140]. Clustered mutations surrounding the break-points of somatic rearrangements have been also recently reported in cancer cells; such clustered base substitutions refer to as Kataegis [141]. Long stretches of ssDNA generated during the DSB or fork repair are particularly sensitive to DNA damage, in particular to the activities of cytidine deaminase family [142]. In yeast, chronic replication stress induced by exposure to replication-specific damaging agents leads to damaged ssDNA and clustered mutation over hundreds of kilobases [143].

Error-prone DNA synthesis associated with repair events likely underlies genome modifications. HR requires a DNA synthesis step that is error-prone, mainly due to a lack of processivity: the elongated strand dissociates frequently from the initial template and anneals to another discontinuous template [144-146]. This phenomenon is called template switch and is driven either by significant length of homology or micro-homology (few bases) [147]. The last refers to as MM-BIR (micro-homology mediated break-induced replication) [146, 148]. In fission yeast, replication-restart by HR is associated with an error prone DNA synthesis, liable to replication slippage at microhomologies, thus leading to micro-deletions and insertions [135]. When progressing across repeated sequences,

forks restarted by HR are prone to fold-back inversion, resulting in large chromosome duplication, a possible mechanism to explain fold back inversions observed in pancreatic cancer cells [149, 150]. DNA synthesis associated with BIR events is particularly error prone, resulting in a 2,800 fold increase in frame-shift, base substitution and template switch when progressing over hundreds of kilobases [144, 151]. Remarkably, overexpression of Cyclin E results in genomic duplication with complex breakpoint junctions [152]. Fork progression relies on two key subunits of the DNA polymerase delta that appear dispensable to replication efficiency in cells not overexpressing Cyclin E. The use of reporter assays demonstrated that BIR events are likely to underlie genomic duplication due to serial template switches of the elongated strand [152]. Thus, under replication stress conditions, the continuity of DNA replication relies on accessory DNA polymerase factors that drive genome instability.

CNVs (Copy Number Variation) are the most frequent genome modifications observed from an individual to another. CNVs are also common to cancer cells and underlie genomic disorders [1, 153]. CNVs are induced by replication stress and independently of the DSB repair pathway NHEJ (Non Homologous End Joing) [153, 154]. The analysis of breakpoint junctions of CNVs has revealed an unexpected complexity, including the presence of microhomologies, small insertions/deletions, frame-shifts and base substitutions [155]. Such a complexity has been interpreted as a consequence of multiple attempts to restart stalled replication forks.

5-3 Cell death, senescence and cancer.

One century ago, Theodor Boveri hypothesized that tumors originate from improper chromosome segregation, thus creating aneuploid cells that undergo clonal expansion. He proposed that in some cases, abnormal chromosome segregation and aneuploidy are caused by extra-centrosome generation, leading to multipolar cells [156]. More recently, the formation of active mitotic extra centrosomes has been correlated to replication stress [62]. Consistently, both replication stress and centrosome abnormalities have also been reported at early stages of malignancy [4, 157-159] . Replication stress provokes an arrest at mitosis through the activation of the mitotic spindle checkpoint. Strikingly, even low or unprocessed endogenous replication stress results in prolonged metaphase arrest. Prolonged mitotic arrest should then lead to mitotic catastrophe and cell death. These processes could therefore select cells able to escape mitotic arrest and cell death, thus with increased genetic instability (see above) and possibly with de-repressed proliferation program.

Endogenous replication stress has been proposed to be involved at the very early steps of senescence and/or malignancy initiation [3, 4, 7, 159, 160]. Beside the induction of tumors, oncogenes can induce senescence in a process called oncogene-induced senescence (OIS). It is proposed that the oncogene activation leads to an hyper-replication state through the stimulation of the cell proliferation program. The hyper-replication increases the endogenous replication stress, which activates the DDR. The persistence of the DDR arrests cells in cell cycle, causing senescence [3, 161]. Note that senescence is generally considered as a tumor suppressor mechanism through its anti-proliferation consequences. However, senescence is a double-edge sword since it can also induce tumorigenesis through the production of inflammatory cytokines [162, 163]. In addition, the induction of senescence allows to select cells able to bypass the proliferation restriction, thus with stimulated proliferation programs, thus constituting a first step toward neoplasia.

The link between replication stress and senescence is supported by several studies. Patients suffering from the Werner syndrome (in which a RecQ-helicase is mutated) or from Fanconi anemia, both syndrome that exhibit markers of endogenous replication stress, also suffer from premature aging and patient cells are prone to senescence [164, 165]. Caloric restriction, which prolongs life span in all eukaryotes, also inhibits DNA replication [166, 167]. Exposure of cells to low doses of HU for two weeks, induce senescence monitored by the lysosomial β-galactosidase activity and the poor ability to incorporate nucleotide analogues [160]. However, the senescence-associated heterochromatin foci (SAHF) were not efficiently induced compared with OIS. Note that OIS-induced SAHFs require DNA replication and ATR [160].

Noteworthy, HR protects against spontaneous endogenous replication stress and is affected in many familial breast cancers. More specifically, most of genes identified to be inactivated in familial breast cancer control the replication/recombination interface [168, 169]. In addition, the oncogene AKT1 is activated in 40 to 60% of sporadic breast and ovary cancer [170, 171]. Interestingly, AKT1 stimulates proliferation, represses cell death and HR and thus should generate high endogenous replication stress [170]. Consistently AKT1 over-activation leads to the formation of mitotic extra centrosomes [172]. Such genetic instability should then increase genetic instability favoring thus the tumor progression, in addition to tumor initiation. This shed light on the importance to secure replication completion and to manage replication stress (even endogenous stresses) prior to mitosis.

6- Concluding remarks

The sources of replication stress are many and varied, but different causes results in similar consequences. It is likely that novel mechanisms of genome instability caused by replication stress will emerge, concerning for instance oncogene-induced alterations of the DNA replication program. Our knowledge of the bases of eukaryotic DNA replication program is a pillar to understand the contribution of replication stress to cancer development. The cellular responses to replication stress have been mainly characterized in response to acute and exogenous stress. When the DDR is defective, acute replication stress leads to a massive deregulation of the DNA replication program, catastrophic genome instability and senescence or cell death. Cells escaping apoptosis are prone to accumulate additional mutations and to initiate clonal proliferation. In another scenario, neoplasic lesions suffer from low levels of chronic and endogenous replication stress. Such type of alteration might be in part checkpoint-blind and thus facilitates the transmission of damaged chromosomal segments to the next cell cycle [60]. Also, adaptation processes might take place to face endogenous and chronic replication stress. Tumor cells could “learn” how to cope with for arrest and duplicate their genome in stress conditions. Indeed, replication stress arises in neoplasic lesions but lingers as well in solid tumors in which it contributes to chromosomal instability such as aneuploidy [5]. The cascade of DDR activation and its components have been largely characterized in response to acute replication stress, but the cell response to chronic and endogenous replication stress remains poorly described, despite the fact that it likely represents the main source of replication-induced genome instability during cancer development. Understanding to what extent and how cells adapt to subtle replication alterations should help identify new targets and open new avenues for anticancer therapeutic strategies.

Acknowledgements

We apologize to our colleagues whose contributions are not cited due to space constraints. Work in the SAEL laboratory is supported by Agence Nationale Recherche grants ANRJCJC10-1203 01 and la Ligue contre le cancer (comité Essonne). IM and BSL were supported by INCa (Institut national du cancer) and by “la Ligue Nationale contre le cancer”. PP thanks ANR, INCa and the Ligue contre le Cancer for support.

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Figure legend

Figure 1: Replication stress jeopardizes the completion of chromosome duplication and successful mitotic commitment. Left panel: the DNA replication program ensures that origins fire in a sequential order, and not all at the same time, thus allowing an appropriate management of the essential resources for accurate and complete DNA synthesis. Right panel: replication stress from either endogenous or exogenous causes (see text for details) compromises the achievement of chromosome duplication, leaving chromosomal segment unreplicated when cells enter mitosis. Unreplicated loci form anaphase bridges and challenge the even chromosome segregation. Replication stress also favors mitotic extra centrosomes and multipolar mitosis, thus amplifying mitotic catastrophes and genome instability to the whole genome.

Figure 2: Consequences of replication stress. Top panel: Alteration of the replication fork speed (whatever the cause, see text for detail) generates stretches of ssDNA either because of uncoupling between the replicative helicase and the DNA polymerase or nuclease attacks. Stretches of ssDNA contribute to the activation of the ATR pathway which in turn acts locally to maintain the replisome in a replication-competent state, and acts globally to delay cell progression, inhibits late-replicated domains and probably slows down the speed of activate forks. Of note, ATR, and more generally the DDR, also impact on many other cellular metabolism processes. Bottom panel: acute replication in ATR deficient cells results in an excess of origin firing, a massive amount of chromatin-bound RPA and an excessive consumption of dNTP. Stretches of ssDNA are thus unprotected and sensitive to breakages. Inefficient replication complexes correspond to replisomes that cannot be maintained in a replication-competent state due to ATR deficiency.

Figure 1

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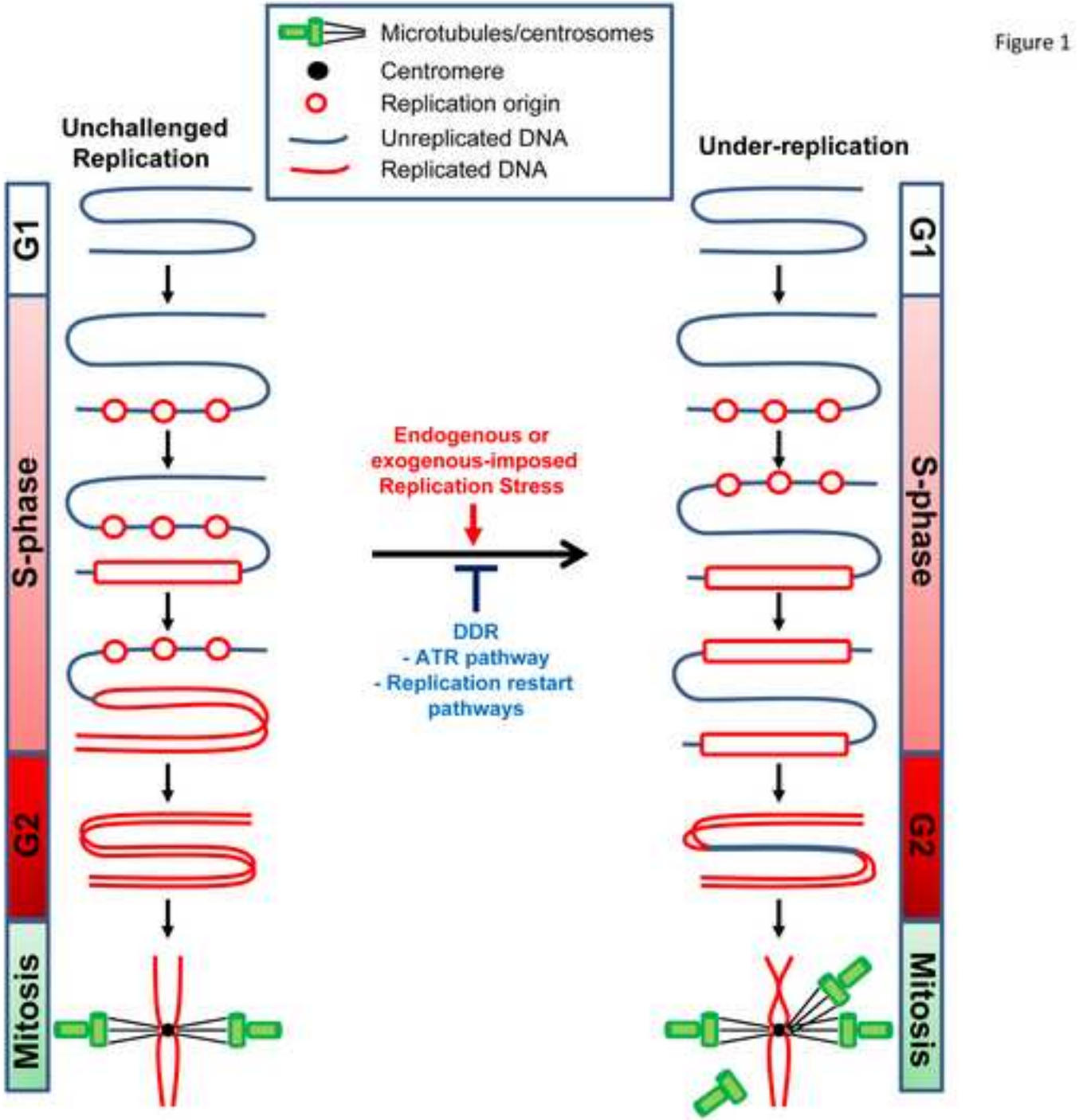

Figure 1

Figure 2

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