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Review Article

DNA replication stress: Causes, resolution and disease

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ABSTRACT

DNA replication is a fundamental process of the cell that ensures accurate duplication of the genetic information and subsequent transfer to daughter cells. Various pertubations, originating from endogenous or exogenous sources, can interfere with proper progression and completion of the replication process, thus threatening genome integrity. Coordinated regulation of replication and the DNA damage response is therefore fundamental to counteract these challenges and ensure accurate synthesis of the genetic material under conditions of replication stress. In this review, we summarize the main sources of replication stress and the DNA damage signaling pathways that are activated in order to preserve genome integrity during DNA replication. We also discuss the association of replication stress and DNA damage in human disease and future perspectives in the field.
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Abbreviations: pre-RC, pre-replicative complex; CFS, common fragile site; DSB, DNA double strand break; CMG complex, Cdc45.Mcm2–7.GINS; ERFS, early-replicating fragile site; DDR, DNA damage response; ssDNA, single-stranded DNA; IR, ionizing radiation; HGPS, Hutchinson–Gilford progeria syndrome; SIOD, Schimke immune-osseous dysplasia; AOA1, Apraxia Oculomotor Ataxia 1; FA, Fanconi anemia; iPOND, Isolation of protein on nascent DNA; BLESS, direct in situ breaks labeling, enrichment on streptavidin and next-generation sequencing; CRISPR, Clustered regulatory interspaced short palindromic repeats

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Introduction

Several exogenous and endogenous sources constantly challenge the integrity of replicating DNA, and can pose a serious threat to chromosomal stability by interfering with progression, stability and proper resumption of replication after fork arrest. DNA damage generated endogenously by errors during DNA replication is often referred to as replication stress and particularly affects genomic loci where progression of replication forks is slow or problematic. Cells have evolved a panoply of mechanisms to deal with different kinds of DNA damage that ensure the integrity of the genome during replication. Various repair mechanisms and different checkpoint machineries exist, which stop or slow down cell cycle progression until the damage is repaired. These DNA replication, repair and checkpoint activation pathways are highly regulated and coordinated. Defects in any of these functions leads to genomic instability and may lead to cancer, premature ageing or disorders associated with loss of genomic integrity.

Overview of DNA replication

DNA replication is initiated at defined loci known as replication origins. In the eukaryotic genome, replication begins at multiple origins, ranging from a few hundred in yeast to thousands in humans. These are distributed along the length of each chromosome [1]. Initiation of replication comprises a two-step process: origin licensing and firing. Origin licensing starts as early as late M or early G1 with the assembly of a pre-replicative complex (pre-RC) at each origin (early or late). The pre-RC consists of the origin recognition complex (ORC1–6 proteins), cell division cycle 6 (Cdc6), cell division cycle 10-dependent transcript 1 (Cdt1) and the core replicative helicase component Mcm2–7, consisting of the minichromosome maintenance proteins 2–7 (Mcm2–Mcm7) [2,3]. The second step, origin firing, involves the activation of the Mcm2–7 complex which is restricted to S phase and culminates in the formation of a pair of oppositely oriented replication forks that contain a single Mcm2–7 helicase hexamer complex at the apex of each fork [4]. Cyclin dependent kinases (CDKs) and Dbf dependent kinases (DDKs) promote the conversion of the pre-RC complex into a pre-initiation complex capable of unwinding DNA and carrying out DNA synthesis [5]. At the G1/S transition, when CDK activity rises, numerous additional factors cooperate to convert the MCM2–7 double hexamer into two CMG (Cdc45. Mcm2–7.GINS) complexes [6]. In particular, Cdc7–Dbf4 protein kinase (DDK) phosphorylates MCM2–7. CDK phosphorylates Sld2 (sharing homology to human RECQ4) and Sld3 (the yeast homolog of Treslin in human), promoting their interaction with Dpb11 (the yeast homolog of TopBP1 in human). The Sld3–Sld2–Dpb11 complex enables the stable binding of Cdc45 and GINS to phosphorylated MCM2–7. Once formed, CMG unwinds the origin, allowing replisome assembly. Replication forks then travel bidirectionally outwards from the origin until the entire genome is replicated [7–10].

Sources of DNA replication stress

Replication stress is defined as slowing or stalling in replication fork progression. It arises from many different sources, which are considered as replication barriers such as telomeres, repetitive sequences, DNA lesions and misincorporation of ribonucleotides, secondary DNA structures, DNA–RNA hybrids, dormant replication origins, collisions between replication and transcription complexes, hypo-acetylation and compaction of chromatin, early-replicating fragile sites (ERFSs) and common fragile sites (CFSs). Finally overexpression or constitutive activation of oncogenes such as HRAS, c-Myc and cyclin E is an emerging source of replication stress. Following, we discuss some of the most relevant sources of replication stress in more detail (see Fig. 1). We refer readers to the following review for an overall picture of agents than induce replication stress [11].

Fragile sites

Certain loci in the human genome are particularly difficult to replicate, hence rendering them prone to fragility. Most prominent amongst these are the so-called fragile site loci. As mentioned above, fragile sites can be classed CFSs or ERFSs. The former have a high A/T content, occur at sequences prone to form secondary structures, possess a condensed chromatin structure and replicate late. In contrast ERFSs are G/C rich, have an open chromatin state and replicate early.

Fragile sites are defined as being either common or rare; the former, CFSs, are present in all individuals, whereas rare fragile sites are found in less than 5% of the population [12]. There are over 200 CFSs in the human genome and these regions are quite large, ranging from just under 1 Mb to over 10 Mb in size. CFSs are prone to replication stress-induced DNA double-strand breaks (DSBs) visible in condensed metaphase chromosomes and their occurrence is dependent on the endonuclease activity of MUS81-EME1, in synergy with the resolving action of the BLM helicase to prevent chromosome breakage [13,14]. The most typical inducer of CFSs used experimentally is aphidicolin, an inhibitor of the replicative DNA polymerases α, δ, and ε [15]. The three most frequently expressed CFSs are FRA3B, FRA16D, and FRA6E [16–18]. Several studies in cell culture models have shown that under conditions that induce replication stress, fragile sites are hotspots for sister chromatid exchange, translocations and deletions [19]. The frequent alterations within these regions in multiple cancers have led to the identification of a number of extremely large genes contained within CFSs. Several of these large genes have

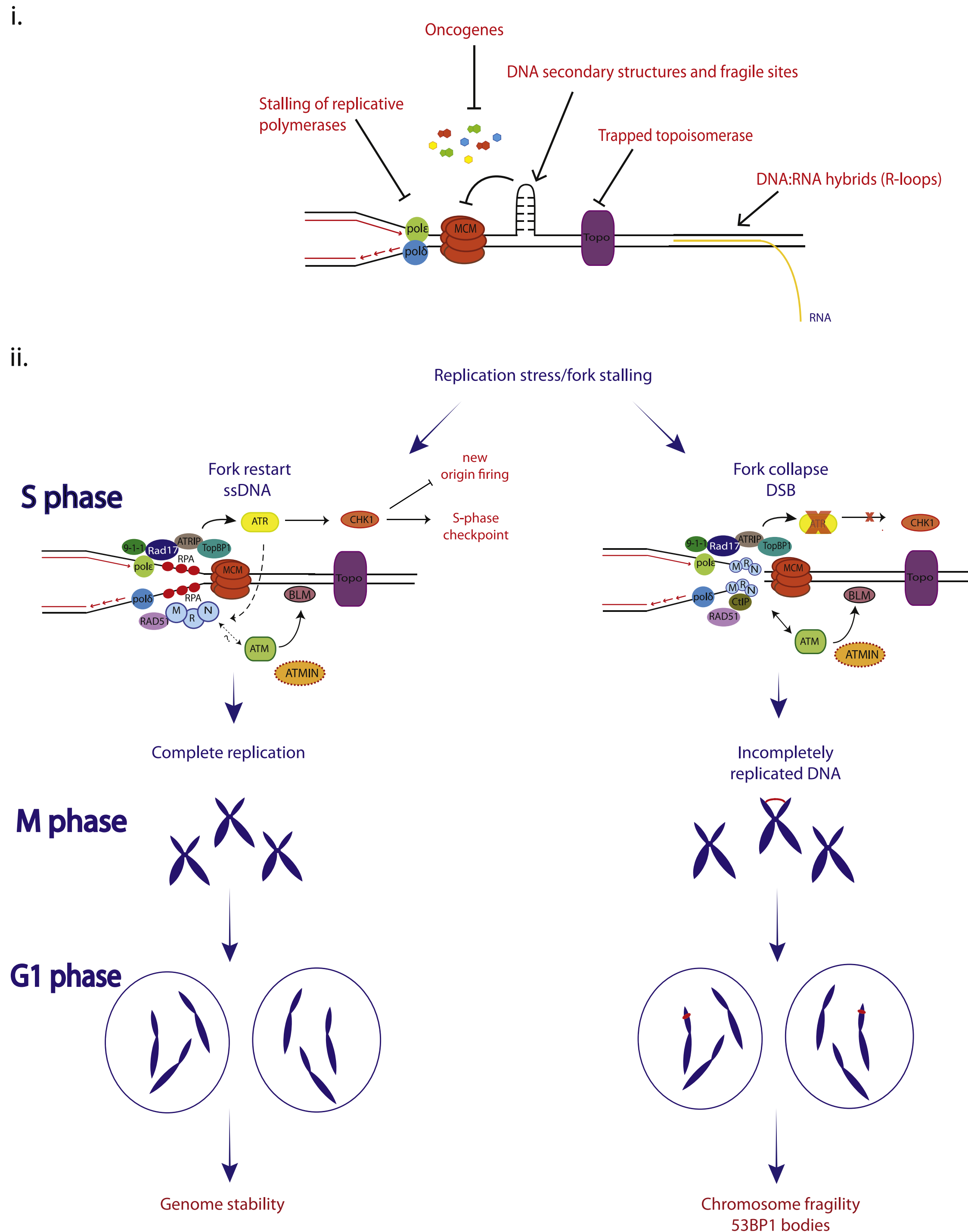

Fig. 1 – Schematic representation of the predominant DNA damage pathways that process replication intermediates. Replication stress induced by various endogenous or exogenous sources results in the generation of single strand DNA bound by RPA protein. RPA recruits ATRIP, Rad17 and 9-1-1 complex which together with TOPBP1 result in the activation of the ATR kinase, which is responsible for phosphorylation of CHK1, suppression of new origin firing and activation of the S phase checkpoint, allowing time for the cell to recover. The MRN complex and RAD51 have also been shown to be recruited at sites of single strand DNA after replication stress and to be required for fork restart. While it is unclear whether ATM has a role in MRN activation under these conditions, ATMIN, which is also an interactor of ATM, may play a role in ATM activation in response to replication stress. ATM can also activate BLM helicase which contributes to the resolution of replication intermediates. When the ATR pathway is compromised, defective checkpoint activation may result to the collapse of the replication fork into double strand breaks which then may be resolved by homologous recombination through recruitment of CtIP and RAD51. However, in the absence of ATR, replication stress can leave regions of the genome incompletely replicated resulting in abnormal DNA structures which if not properly resolved can be transmitted to the next generation in the form of DNA lesions resulting in genome instability.

been demonstrated to function as tumor suppressors involved in the formation of many different cancers including colorectal cancer and oropharyngeal squamous cell carcinomas [20–22]. A recent concept emphasizes the importance of replication origin density in the maintenance of CFS stability [23]. According to this concept, certain common fragile sites are characterized by a reduced number of replication initiation events that limit the number and density of active origins, thus rendering these "initiation-poor" regions susceptible to incomplete replication and fragility.

ERFSs are a new class of fragile sites and have been defined as similar to CFSs. This is because of their susceptibility to chromosome breakage, dependence on ATR signaling and sensitivity to replication stress induced by hydroxyurea, ATR inhibition or deregulated c-Myc expression. Moreover, more than 50% of recurrent amplifications/deletions in human diffuse large B cell lymphoma map to ERFSs [24].

Replication–transcription complex collision

Collisions between transcription machinery and replication forks are an additional source of genome instability. In higher eukaryotes, replication and transcription are coordinated processes, and they occur within spatially and temporally separated domains. Active transcription usually occurs in the G1 phase. When transcription occurs in S phase, it has been suggested to be spatially separated from replication sites [25]. It has been reported that genes of 800 Kb or more in size, often located at CFSs, produce their transcripts over more than one cell cycle, consequently extending transcription into the next S phase, which increases the probability of collisions between replication and transcription complexes and hence formation of DNA–RNA hybrids (R-loops). Multiple strategies are employed to avoid R-loop formation in prokaryotic and eukaryotic cells. Helmrich and colleagues and Wahba and colleagues demonstrate in vivo roles for RNaseH enzymes in maintaining genome integrity, which has potential implications in human disease (see section on diseases associated with defective clearance of replication stress) [26,27]. Interestingly, a recent study showed that BRCA2, a DNA repair protein with tumor suppressive function prevents accumulation of R-loops [28]. Although the exact mechanism is unclear, this important finding further demonstrates the essential role of DNA damage and repair components for efficient dealing with replication stress. Apart from the generation of R-loops transcription can interfere with replication by imposing increased topological stress at sites where newly formed RNA transcripts are tethered to nuclear pore complexes for further processing. Bermejo et al. showed that the ATR-dependent checkpoint counteracts this topological stress by releasing transcribed genes from the nuclear pores, allowing normal progression of the replication fork [29]

Oncogenic stress

Oncogenic stress is a major driving force in the early stages of cancer development [30]. The finding that DNA damage response is activated in hyperplastic tissues and after overexpression of oncogenes such as cyclin E, cdc25A and E2F1 (that deregulate replication) set the ground for linking oncogenes to replication stress-associated DNA damage [31,32]. In studies that followed, further analysis of replication dynamics and the DNA damage response after overexpression of oncogenes confirmed this model, where oncogenes such as cyclin E lead to perturbation of normal replication, activation of the DNA damage response and cell cycle checkpoints that lead to arrest or senescence [33,34]. Cyclin E causes replication stress not only by deregulating cell cycle progression but also by disrupting DNA replication during S phase. Cyclin E overexpression is associated with increased firing of replication origins, impaired replication fork progression and DNA damage. A significant amount of Cyclin E-induced replication slowing is due to decreased nucleotide pools and/or interference between replication and transcription. c-Myc is another oncogene found to directly control DNA synthesis and promotes cell proliferation. Indeed, overexpression studies have indicated that ectopic expression or conditional activation of c-Myc triggers an increase in the percentage of S phase cells in asynchronous populations [35]. The overexpression of c-Myc in a number of in *vitro* cellular systems has been associated with the activation of a DNA damage response (DDR), and increased genomic instability [36,37]. This suggests that elevated c-Myc levels lead to the accumulation of DNA damage, however the molecular mechanism is still not completely defined. One of the suggested mechanisms c-Myc induced genomic instability is its affect on replication fork dynamics [38]. It has been shown that elevated levels of c-Myc increase the number of firing replication origins which are highly asymmetric. This can lead to uneven replication processivity on either side of the replication bubble, which is indicative of replication stress and fork stalling events [35].

DNA structures

It is thought that the accessibility of DNA to replication factors can be influenced by local chromatin structure. Additionally, chromatin structure modulates origin firing time and efficiency [39]. The canonical DNA structure is the right-handed double helix B form of DNA. However, it can adopt several other non-B DNA structures including: cruciforms, hairpins, H DNA, Z DNA and G4. These secondary conformations form in the genome at specific DNA repetitive sequences and present a challenge for progression of DNA replication forks. Impeding normal DNA synthesis, and formation of these alternative forms of DNA structure may threat genome stability and in some instances play a causal role in disease development [40].

The kinases ATR and ATM signal DNA replication stress

In order to cope with the constant challenge of DNA damage encountered by replicating DNA, cells activate a complex network of interacting pathways that lead either to the repair of the damage and resumption of normal cell cycle progression or to programmed cell death. This network coordinates the activation of cell cycle checkpoints, the appropriate DNA repair pathways, and numerous other responses [41]. One of the central components of the DDR is the serine–threonine kinase Ataxia Telangiectasia Mutated protein (ATM), which phosphorylates numerous key players in various branches of the DDR [42,43]. ATM is a member of the phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family, which also includes Ataxia Telangiectasia and Rad3 Related protein (ATR) – refer to Fig. 1. ATM

transduces a response to various stimuli, but most prominently to DNA DSBs. In contrast, ATR is the key kinase in signaling the response to ssDNA, which can occur at persistent DSBs, but more extensively on stalled replication forks [44].

Functions of ATR

ATR is activated by its physical recruitment to the ss-DNA binding protein RPA, which independently brings together the two components of the ATR pathway. On one hand, RPA recruits ATRIP, which is in a complex with ATR and thus brings the kinase to the DNA lesion. On the other hand, RPA recruits Rad17, which loads the Rad9–Hus1–Rad1 (9-1-1) complex. This complex is then essential to recruit and position the allosteric activator TopBP1. Within close proximity, TopBP1 activates ATR. In spite of the numerous substrates of ATR, the key event that translates ATR activity into a checkpoint signal may mostly depend on one single target, which is the phosphorylation and activation of CHK1 [44]. ATR and/or CHK1 inhibition results in checkpoint defects and chromosome breakage, further consolidating the notion that cell-cycle checkpoints somehow prevent uncontrolled fork collapse [45]. However, the mechanism governing global regulation of checkpoints via inhibition of new DNA replication origins from firing by the ATR/CHK1 pathway to maintain local fork stability remains unclear. There are two possible mechanisms, firstly checkpoint signaling locally affects replication fork components, which would contribute directly to fork stabilization. Secondly, ATR signaling regulates recombinational repair, which is necessary to restart collapsed forks. Toledo and colleagues demonstrated another mechanism, which explains how ATR protects replication forks locally and suppresses origin firing globally. When ATR is activated by RPA-coated ssDNA generated ahead of the stalled replication fork, CHK1 diffuses globally through the nucleus, where it inhibits new origin firing [46]. This ensures that RPA remains in excess over ssDNA by limiting the number of stalled forks to those that were originally active at the onset of the replication stress. If ATR signaling fails, dormant origins fire, and the newly generated ssDNA progressively depletes nuclear RPA. When all RPA becomes sequestered, every active replicon generates unprotected ssDNA, which is rapidly converted into DSBs [45,46]. Recently, Yamada et al. showed that in human cells activation of the ATR/CHK1 pathway results in stabilization of chromatin-bound Cdc7–ASK kinase complex (human homolog of cdc7–Dbf4) which is necessary for initiation of normal replication and origin firing [47]. However, upon staling of replication, activation of cdc7/ASK complex by the ATR/CHK1 pathway is required for efficient lesion bypass repair, thus preventing fork collapse under conditions of replication stress.

Functions of ATM

Although ATR is considered to be the major kinase mediating the response to replication stress, mainly due to its ability to activate the intra-S phase checkpoint, evidence exists to support a role for ATM activation in response to replication stress. One aspect of ATM function under these conditions could be the activation of the homologous recombination repair pathway, which is important for restart of collapsed replication forks and recovery of replication after induction of replication stress [48]. This function may require the recruitment of the MRN complex at sites of stalled or collapsed replication forks that promote DNA end-resection, the first important step for homologous recombination. Recruitment of the MRN complex to sites of DNA damage is important for ATM activation in response to ionizing radiation (IR)-induced DSBs. The MRN complex members Mre11 and Nbs1 are required for efficient recovery of replication after treatment with replication stalling agents such as hydroxyurea [49,50]. However, the necessity for an interaction between ATM and Mre11 and Nbs1 at sites of stalled forks remains a matter of controversy. Certain studies indicate that recruitment of the MRN complex following replication stress contributes to activation of ATR rather than ATM signaling [51–53]. However, another study suggests that both ATM and ATR are required for efficient Mre11-dependent fork restart and prevention of DSB accumulation during unperturbed replication and after chemically induced replication stress [54]. Apart from homologous recombination, ATM can also influence replication fork restart by directly regulating the DNA helicases WRN and BLM, which are both required for resolution of replication intermediates and are both substrates of ATM [55,56].

Two independent studies show that activated ATM is recruited to chromatin foci at sites of common fragile sites following mild replication stress induced by aphidicolin [57,58]. In line with this, ATM depletion in addition to ATR loss results in increased fragility at CFS compared to depletion of ATR alone, supporting a role for ATM in the maintenance of chromosome stability after replication stress [59]. According to the proposed model, ATM is activated after formation of DNA DSBs that arise at a later stage as a result of replication fork collapse or chromatin breakage at sites of unreplicated DNA during mitosis. If the role of ATM is restricted solely to the activation of checkpoint and DNA repair in response to DSBs that arise as a result of further processing of replication intermediates remains unclear. Evidence challenging this view is limited, nevertheless intriguing, especially considering the lack of information on the exact nature of the specific DNA lesions that arise at sites of stalled forks and incompletely replicated DNA. For example, induction of replication stress by low doses of the topoisomerase I inhibitor camptothecin results in ATM activation in the absence of detectable DSBs [60]. Furthermore recent data from a large-scale analysis of proteins specifically localized at stalled forks after replication stress showed ATM recruitment at nascent chromatin at an early stage of DNA replication [61]. However, a role for ATM in the early response to replication stress has not been confirmed and more studies will be needed to clarify this. Moreover, the type, intensity and duration of the stimulus might be critical factors determining the relative contribution of each pathway to the final response.

In addition to NBS1, ATM possesses a second cofactor; ATMIN (also known as ASCIZ) that has been described [62,63]. ATMIN interacts with ATM using a motif homologous to that of NBS1 [62]. It has previously been shown using siRNA approaches in human cell lines and using ATMIN-deficient mouse embryonic fibroblasts (MEFs) that ATMIN has a complementary function to NBS1 with respect to ATM activation: ATMIN is dispensable for IR-induced ATM signaling, but ATM activation following replication and hypotonic stress is mediated by ATMIN [62]. Hence, NBS1 and ATMIN are required for ATM activation in a signal dependent manner [64].

Unresolved replication intermediates can occur during S/G2 phases of the cell cycle and can be converted into DNA lesions in

M phase in particular into DSBs. It has been shown that a protein that binds to p53, known as 53BP1 [65], is involved in shielding genomic regions exposed to replication stress and is recruited to such sites in an ATM-dependent manner. 53BP1 forms nuclear bodies at such sites of unrepaired DNA lesions in the subsequent G1 phase to shield these regions against erosion [58].

Defects in resolving DNA replication stress: implications in human disease

Identification of mutations responsible for various genetic syndromes has revealed the direct implication of proteins mediating the response to replication stress in the pathology of human disease [11]. Phenotypic characteristics shared among these syndromes include developmental defects, growth retardation and neurological disorders, suggestive for the importance of efficient regulation of replication during processes that require increased cell proliferation. For example, mutations in the pre-replication factors ORC1, ORC2–6 and cdt1, cdc6 that affect licensing of DNA replication are related to the Meier–Gorlin Syndrome, a disease characterized by severe growth retardation and developmental malformations [66].

Many other mutations involved in genetic syndromes affect proteins that also play an important role in the DDR, which is essential for accurate replication of the genetic material. The most prominent example is the Seckel syndrome caused predominantly by mutations in the ATR gene, which is essential for the activation of the intra-S phase checkpoint during replication stress [67]. Interestingly, a different type of Seckel syndrome is associated with mutations found in the RBBP8 gene encoding CtIP, a protein required for DNA-end resection during S phase [68]. More importantly, CtIP is an ATM substrate and interacts with the MRN complex, which also mediates DNA damage signaling and repair during replication. Mutations affecting members of the MRN complex cause syndromes characterized by growth and developmental defects [69]. Apart from ATR and RBBP8, other types of Seckel syndrome are caused by mutations in genes affecting centrosome structure and function including pericentrin (PCNT) [70].

Another type of heterogeneous human diseases, collectively called laminopathies, are the result of mutations in nuclear lamin genes such as LMNA which lead to abnormal nuclear morphology and alterations in chromatin structure. Interestingly, cells expressing a defective form of Lamin A called progerin are more sensitive to replication stress and recently it has been demonstrated that reorganization of the microtubule network inside the nucleus can rescue the nuclear morphology and fitness of laminopathic cells derived from Hutchinson–Gilford progeria syndrome (HGPS) patients [71,72]. The above examples provide a link between microtubule network organization and replication stress and point out the importance of intact centrosome function and nuclear structure for normal replication.

Mutations in proteins involved in chromatin remodeling during DNA replication have also been associated with human disease. Mutations in the SMARCAL1 and ATRX genes, which are both related to the SWI–SNF chromatin remodeling complex, are mutated in the Schimke immune-osseous dysplasia (SIOD) and the α-thalassemia/mental retardation syndrome, X-linked (ATR-X) respectively [73,74].

Hypomorphic mutations in the RNase H2 gene cause the Aicardi-Goutieres syndrome that is characterized by severe neurological dysfunction and a congenital infection-like phenotype [75]. As RNase H2 cleaves misincorporated ribonucleotides and DNA:RNA hybrids that arise during replication, it is possible that a defective response to increased replication stress may be the cause of the developmental retardation phenotype. Another protein acting in the same pathway, aprataxin (APTX) is mutated in the neurological disorder Apraxia Oculomotor Ataxia 1 (AOA1), characterized by cerebellar degeneration. Aprataxin deadenylates adenylated RNA:DNA hybrids that arise after cleavage by RNase H2, thus preventing S phase checkpoint activation [76].

Mutations in the FA complementation group are responsible for the heterogeneous genetic disorder Fanconi Anemia (FA) which is characterized by skeletal abnormalities, developmental delay, growth retardation and increased incidence of cancer development, especially in tissues with a high proliferation index [77]. FA proteins are components of the interstrand crosslink DNA repair pathway, while FANCD2 is also essential for maintenance of genome stability during replication [78]. Mutations affecting the RECQ family DNA helicases WRN, BLM and RECQL4, which play an important role in the efficient resolution of replication intermediates and arrested forks, are responsible for the genetic syndromes Werner, Bloom and Rothmund–Thomson respectively [79]. In addition to growth retardation, these syndromes are also characterized by premature aging and predisposition to cancer, phenotypes indicative of increased genomic instability. Cancer susceptibility is also a characteristic of FA patients suggesting a causative link between replication stress and cancer. Confirming this link, replication stress has been shown to be a major source of chromosomal instability (CIN) observed in CIN^+ colorectal cancers [80].

Whether replication stress is a driving force of tumourigenesis or a result of oncogenic mutations that allows further genome instability during cancer development remains to be elucidated. Interestingly, extensive crosstalk between different DNA repair pathways is necessary for the coordination of an efficient response to replication stress. For example, FANCD2 has been shown to mediate part of the ATR response to replication stress, while it also interacts with CtIP and BLM to promote restart of stalled replication forks [81–83]. Although the exact mechanism of the pathological phenotype is not entirely understood, common clinical manifestations of mutations in different genes of these pathways clearly indicates the existing crosstalk and the need for efficient coordination of the replication process with the DNA repair machinery through the DNA damage signaling pathway in order to allow normal progression of DNA replication while preserving genome integrity.

Conclusions and perspectives

Elucidating the pathways and interactions governing the response to replication stress will shed light on the molecular mechanisms that ensure genome integrity during replication under endogenous sources of replication stress. Furthermore, we will increase our understanding of how exogenous sources or defects in critical pathway components can lead to increased genome instability. An important challenge for the years to come will be to identify the specific regions of the genome that are particularly affected

during different types of replication stress. To this end, recent advances in technology can be exploited to apply novel methodology such as next generation sequencing, iPOND or BLESS to map specific DNA regions affected by replication stress and the specific protein interactions that mediate their fragility, respectively [84,85]. Moreover, CRISPR-mediated imaging of specific chromatin loci in living cells offers a powerful opportunity to reveal the dynamic interactions at sites of increased fragility following replication stress [86]. Thus, despite the progress that has been achieved during the last years, extensive studies and novel technology will be needed to boost our current understanding of the mechanisms mediating the response to replication stress and the associated genome instability. Advance in current knowledge regarding the response to replication stress will also be an important step towards more specialized therapies and development of new treatments for diseases including cancer.

Conflict of interest

The authors declare that they have no conflict of interest.

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