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Review article

DNA replication: causes of replication stress and DNA damage response pathways

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Abstract: DNA replication is a crucial cellular mechanism that enables proper genetic information to be duplicated and subsequently transferred to the daughter cells. Several perturbations, whether endogenous or external, can impede the accurate advancement and completion of the replication process, endangering genomic integrity, known as replication stress. Secondary DNA structures like alternative non-B form at common fragile sites, oncogenes (RAS, MYC, Cyclin E and CDC6) induction, ribonucleotides (precursors of RNA) incorporation into DNA, RNA:DNA hybrids and other factors have serious implications on cell survival, genome stability and human diseases like carcinogenesis. Cellular DNA damage response as well as DNA repair are crucial for ensuring accurate synthesis of genetic material and preserving genome integrity in case of replication stress. This review aims to highlight the major causes of replication stress as well as the DNA damage response mechanisms that are induced to maintain genomic stability during DNA replication.

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Introduction

During mitosis, a cell must properly duplicate its genome and evenly distribute its newly replicated chromosome in each daughter cell to maintain genome's stability, at the cell division stage. Chromosomal instability (CIN) is referred to as the chromosomal alterations occurring at a greater rate and are the main causes of CIN due to the abnormality in functions related to cell division processes. The IN, as a type of genomic instability can be structural (s-CIN) like loss, gain and/or translocations of parts of chromosomes and numerical (n-CIN) like loss and/or gain of whole chromosome (Geigl et al., 2008). Mitosis is the process by which one cell splits into two genetically identical daughter cells. Mitosis is thought to be the source of n-CIN. Mitotic dysfunctions like defective kinetochore assembly, cohesion defects, spindle monopolarity or multipolarity, altered microtubule stability or dynamics and spindle assembly checkpoint (SAC) function can all cause chromosome segregation errors. The error of merotelic kinetochore attachment arises when a single kinetochore is connected to microtubules originating from both spindle poles, are key causes of whole-chromosome mis-segregation in these settings (Gregan et al., 2011). Despite correcting processes, one of the two kinetochores is attached to both poles; under these conditions, improper attachment may persist, causing mal-oriented chromosomes to lag during anaphase and inhibiting normal segregation (Cimini et al., 2003). The source of s-CIN is characterized as premitotic in the

second case, since structural alterations mostly occur from abnormalities in cellular activities that directly impact integrity of chromosome, like: DNA replication and repair. These two kinds of CIN, however, can be related and frequently coexist in cancer cells (Mitelman et al., 2019). Chromosomal stability and integrity of replicating DNA are continually challenged by various endogenous and external sources that can impede with progression, stability as well as correct restart of replication following fork stoppage. Replication stress is referred as the endogenous DNA damage caused by mistakes during DNA replication, and it mostly influences those genomic loci, at which replication fork (RF) continuation is sluggish or troublesome. Cells have derived a slew of strategies to cope-with various types of DNA damage and safeguard genome's stability throughout replication. Existence of several checkpoint machines and repair mechanisms causes the slow progression of cell cycle or even stops the cell cycle development until damage is corrected. These processes for checkpoint activation, damage repair and DNA replication are highly coordinated and controlled. Errors in any of these activities cause genomic instability, which can result in diseases, linked with genomic integrity loss such as: premature ageing and cancer.

This review addresses the DNA replication process, events that causes replication stress along with the DNA damage response pathways. It focuses on consequences of DNA damage, types of DNA damage response mechanisms and their implications in carcinogenesis.

An introduction to DNA replication

DNA replication begins at specific loci called as origins of replication also called *oriC* for chromosomal genes. Replication begins at numerous origins in the eukaryotic genome like in yeast ranging from a few hundred to thousands in case of humans, which are scattered through each chromosome's length (Sacco et al., 2012). The replication process begins with two-step process that includes the licensing as well as firing of origin. Licensing of origin can begin as preliminary as delayed M or preliminary G1 with the recruitment of a pre-replicative complex (pre-RC) at each preliminary or delayed origin. 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, which includes the mini chromosome maintenance proteins 2–7 (Mcm2–Mcm7), comprise pre-RC (Yamazaki et al., 2013). Mcm2-7 complex activation is involved in second step (origin firing), this complex is confined to cell cycle's (S phase) as well as results in producing a set of oppositely configured RFs containing a single Mcm2-7 helicase (hexamer complex) at vertex of each fork (Boos et al., 2012). Transformation of pre-RC into pre-initiation network proficient of uncoiling as well as synthesizing the DNA is promoted by Dbf dependent kinases (DDKs) and Cyclin dependent kinases (CDKs) (Errico and Costanzo, 2012). Increased activity of CDK causes the conversion of Mcm2-7 double hexamer into two CMG (Cdc45.Mcm2-7. GINS) networks through several additional factors at G1/S transition (Moyer et al., 2006). MCM2–7 is phosphorylated by Cdc7–Dbf4 protein kinase (DDK). Phosphorylation of Sld2 (sharing homology to human RECQ4) and Sld3 (yeast homolog of Treslin in human) occurs by CDK and promotes their interactivity with Dpb11 (yeast homolog of TopBP1 in human). Strong binding of phosphorylated Mcm2-7 to GINS and Cdc45 is permitted by Sld3-Sld2-Dpb11 complex. The CMG unwinds the origin after it has been produced, allowing for the assembly of replisome. RFs then spread bidirectionally from commencement point, until complete genome has been duplicated (Ilves et al., 2010).

Origins of DNA replication stress

Stalling or slowing of RF advancement is referred to as replication stress. It originates from a variety of origins, which are regarded as the replication barriers like: common fragile sites, collision between replication-transcription complexes, misincorporation of ribonucleotides, oncogenic stress, secondary DNA structures, chromatin inaccessibility, hypoacetylation and compaction of chromatin,

telomeres, repetitive sequences, insufficiency of replication factors, DNA-RNA hybrids and dormant replication origins. Following that, we go through the most important origins of replication stress in further depth (Ilves et al., 2010).

Common fragile sites (CFSs)

Areas of genome susceptible to form apparent breaks as well as gaps on the metaphase chromosomes as a result of DNA synthesis disruption, are known as fragile sites. Fragile sites have been classified as rare or common, based on their frequency in the generic population. Rare fragile sites are generated by abnormal expansion of trinucleotide repeat sequences and has been observed in a small fraction of population only. In contrast, all people have common fragile sites (CFSs) (Sinclair et al., 2005). The CFSs are typically linked with copy number variations (CNVs), microdeletions and genomic rearrangement breakpoints in cancer cells (Glover et al., 2017). However, the exact underlying reason of fragility may differ amongst CFSs, but various processes have been suggested to elucidate the susceptibility of CFSs in response to the replication stress. CFSs, on the other hand, are commonly regarded as the final areas of the human genome to be duplicated (Lambert and Carr, 2013). AT-rich sequence compositions of CFSs result in the synthesis of DNA secondary structures which prevent the replisome activity. This, together with their usual absence of dormant/active replication origins, may enhance replication stress during cell cycle's S-phase. CFSs tend to possess actively transcribed genes which are large enough and require one entire cell cycle to transcribe, although, the most noticeable trait of CFSs. As a ramification, a collision occurs between the machineries of replication and transcription on the same template and that is unavoidable. These collisions have the potential to cause DNA damage and/or the creation of pathogenic R-loops (Gao and Smith, 2014). Fragile site 'expression' refers to the formation of gaps and breaks (at CFSs) on metaphase chromosomes (Geigl et al., 2008).

Variable density of active replication origins e.g., in case of oncogene activation, chromatin structure like hypoacetylation of histones and differences in intrinsic replication and transcription profiles (in different cell types) contemplates the variability in cell/tissue's specificity of CFSs expression (Miron et al., 2015). Aphidicolin (replicative DNA polymerase inhibitor) is commonly used to promote CFS expression in cultivated cells [30]. Surprisingly, this can cause micro-deletions in CFSs, a condition identical to that observed in actual human carcinomas (Durkin et al., 2008). Oncogene activation occurs during cancer formation, may also drive CFS expression (Miron et al., 2015), and cancer recurrent deletions have been linked to CFSs (Miron et al., 2015). FRA16D and FRA3B are well characterized and most commonly expressed CFSs in human genome which includes WWOX and FHIT tumor suppressor genes respectively (Gao and Smith, 2014). Despite their proclivity to cause chromosomal instability, CFSs have been preserved throughout mammalian evolution (Sinclair et al., 2005). It is unknown why there is such a high level of conservation. Several hypotheses have been suggested. Firstly, miRNA are critical regulatory noncoding sequences, encoded by these loci in addition to specified gene products (Calin et al., 2004). Secondly, CFSs may alert the cell for incomplete genome replication and can function as a sensor. If this is true, it appears likely that this function will be submerged when the cell is subjected to greater levels of DNA replication stress, like in tumorigenesis induced by oncogenes (Sinclair et al., 2005).

Invading organisms like viruses cause replication stress in cells by attempting to destabilize the host's DNA synthesis machinery in order to propagate themselves and CFSs play possible role to alert the cells. ERCC1 (non-catalytic subunit of XPF endonuclease), MUS81 (DNA structure specific endonuclease, BLM helicase, RAD5a recombinase, ATR (main checkpoint kinase during replication stress) and FANCD2 (Fanconi anaemia protein) are DNA damage response and DNA repair proteins implicated in maintenance of CFSs (Naim et al., 2013). We recommend the following reviews for readers interested in learning more about DNA damage response proteins (Hustedt and Durocher,

2017). FANCD2 is a protein that is commonly used as a substitute marker for the location of CFSs in human cell nuclei. It is unclear why this protein binds specifically to CFSs in this manner, but one potential outcome is that it is associated with R-loops formation elsewhere in the genome and at CFSs (Schwab et al., 2015). More information on the proteins needed to improve CFS stability are described elsewhere (Bhowmick and Hickson, 2017).

Replication-transcription complexes

Large cellular resources are utilized by significant nuclear processes like replication and transcription to carry out DNA duplication and gene expression, respectively. In a prevalent manner, RNA polymerase II (RNAP II) is used to transcribe large areas of genome, non-coding genes and protein coding genes in particular (Hangauer et al., 2013). Simultaneously, DNA Replication Forks (RFs) should locate and duplicate every single (nucleotide sequence) base pair of genetic material within the S-limited phase's time window. To avoid an intervention between these two operations like transcription and replication, rigorous monitoring and synchronization are essential at this crucial stage of cell cycle, which would otherwise result in the collisions of replication and transcription machines on same DNA template.

To exclude possible transcription-replication conflicts (TRCs), one credible approach is to limit the transcription process outside of cell cycle's S-phase, hence removing probable intervention with DNA replication. Although, certain gene sets serve critical S-phase-specific tasks like as: ribosomal RNA genes which acts as a constant reservoir of ribosomes (Zaidi et al., 2016), core histone genes and replication factors allowing the newly produced DNA to be assembled into nucleosomes (Kurat et al., 2011) and initiation of other long genes at G1 phase of cell cycle but complete their cycle of transcription cycle in S-phase (Helmrich et al., 2011).

Early microscopic investigations of active replication and transcription sites indicated spatial segregation of the two processes in S-phase nuclei, although at varying extents in cell lines of human cancer. As replication and transcription occur in discrete nuclear foci which contains increased concentrations of replication factors and RNAP complexes, it has been proposed that nucleus is divided by the cells into well-defined entities containing DNA synthesis (replication foci) and increased transcriptional activity (transcription factories). Further confirmation was done by nascent RNA sequencing assay during S-phase, indicated an inverse correlation (globally) between peak transcription of genes and replication timing proposing that cells have evolved strategies to separate the processes spatially and temporally during cell cycle (Meryet-Figuiere et al., 2014).

Transcription/Replication Conflicts (TRCs): An Internal Source of Genomic Instability & DNA Damage

TRCs have been identified as an internal cause of DNA changes, mutagenesis and recombination, consequently posing serious challenges to integrity of genome. However, determining the accurate genomic repercussions of these transitory as well as possibly short-lived processes in vivo, is a difficult endeavor, especially in genomes of higher eukaryotes. Eukaryotic chromosomes have several sources (origin of replication) that fire probabilistically with varying timings and efficiency as compared to bacteria where a single origin copies each gene in predetermined direction (Hyrien, 2015). Furthermore, when replication forks stall, DNA synthesis can be completed by other origins as eukaryotic cells contain an abundance of origins (Ekundayo and Bleichert, 2019). As a result, predicting the site and direction of a collision with perfect precision is challenging in eukaryotic genomes. Moreover, both machines (transcription and replication) can exist in many functional modes. RNAP complexes, for example, can be stopped close to promoters, actively extending through the gene body, or take on alternative forms like backtracking or R-loop creation. Rehybridization of nascent RNA strand with complementary DNA template strand leads to R-loops

formation. These are three stranded (secondary structures of DNA), yielding displaced single stranded DNA as well as RNA/DNA hybrid (Crossley et al., 2019). However, these structures occur spontaneously during transcription and have been assigned a variety of physiological roles in processes of cells, the existence of R-loops in terms of TRCs is assumed to stop transcription upstream of replisome, hence reducing the stability of genome (García-Muse and Aguilera, 2019). In conclusion, replisome collisions with various types of co-transcriptional challenges present concurrently at gene bodies which are highly transcribed and can result in significant changes in the outcome as well as severity of TRC.

During DNA replication, ribonucleotides (RNA precursors), are integrated frequently. However, conventional ribonucleotide excision repair (RER) effectively removes embedded ribonucleotides in genome, RER inactivation generates genomic ribonucleotide buildup, causing numerous aberrations in cells. Mutations in genes producing RER factors are linked to the Aicardi–Goutières syndrome (neuroinflammatory autoimmune illness). Hypomorphic mutations in genes expressing subunits of RNase H2 an enzyme required for the commencement of conventional RER, have been linked to the devastating autoimmune illness Aicardi–Goutières syndrome in humans (AGS) (Crow et al., 2006). Autoimmune phenotype of AGS is thought to be generated by collection of endogenous nucleic acid species that results in stimulation of DNA damage responses and/or intracellular Toll-like receptors, mediated by embedded ribonucleotides, which stimulate production of interferon in compromised RNase H2 cells (Feng and Cao, 2016). RER-deficient mammalian cells cause accumulation of ribonucleotides in genome and exhibit a variety of deformities, including epigenetic dysfunction, continuous stimulation of DDRs, increased DNA damage and DNA replication delay (Reijns et al., 2012). Consequently, accumulation of genomic ribonucleotide has been considered a catastrophic occurrence in organisms, as well as molecular processes enabling genome alteration generated by ribonucleotides, have piqued the interest of researchers during the last decade. Several reviews have properly summarized key findings in this topic (Vaisman and Woodgate, 2018).

Origins of ribonucleotide incorporation

Depending on comparisons of amino acid sequences, eukaryotic DNA polymerases have been divided into six families like: (AEP, RT, Y, X, B and A) (Shanbhag et al., 2018), AEP family includes PrimPol, RT family includes telomerase, Y family includes (pols η, κ, ι, and Rev1), X family includes (pols β, λ, μ, and TdT), B family includes (pols α, δ, ε, and ζ) and A family includes (pols γ, θ, and ν). The majority of pols have a conserved "steric gate" (amino acid residue), that precludes integration of ribonucleotide into DNA (Brown and Suo, 2011). Both pols such as (β and λ) use a segment of protein backbone to differentiate between sugars, despite the lack of amino acid chain with aromatic steric gate (Bebenek et al., 2014). However, discrimination mechanism has been possessed by pols against rNTPs, pols may integrate rNTPs at non-negligible rate into DNA. During DNA synthesis, rNTPs are incorporated, 500 times less frequently than dNTPs in case of human replicative pol α from B family (Richardson et al., 2000). Other replicative pols like: δ and ε, are more likely to include rNTPs at concentrations of physiological nucleotide, comparable to yeast replicative pols, which integrate thousands of deoxyribonucleotides for every one ribonucleotide (Clausen et al., 2013). As a result, millions of ribonucleotides might be integrated in human genome. Interestingly, pols are possessed by the 3’-exonuclease activity and are incapable of effectively removing the incorporated ribonucleotides (Clausen et al., 2013), implying that proofreading does not safeguard the genome against erroneous incorporation of ribonucleotide during replication.

Depending on the sequence of nucleotides, mitochondrial pol γ, a component of A family, differentiates rNTPs with a fold preference of 1000- to 77,000- for dNTPs. 3’-exonuclease activity is also possessed by pol γ (as seen in B family pols), does not ensure the conservation from inclusion of ribonucleotide (Forslund et al., 2018). Based on prior research, pol γ is estimated to integrate around

10–20 ribonucleotides, for mitochondrial DNA (mtDNA) of 16.5 kb during replication. Furthermore, the quantity of ribonucleotides was found to be substantially greater than predicted in mtDNA. As 1 mtDNA molecule of mouse liver, HeLa cells and human fibroblasts is found to have (65, 36 and 54) ribonucleotides respectively (Forslund et al., 2018). This discrepancy is likely to be caused by the effect of variable concentrations of nucleotide in mitochondria and/or existence of different pols involved in mtDNA replication (Forslund et al., 2018).

Pols of family X, are implicated in DNA repair mechanisms including non-homologous end joining (NHEJ) as well as base excision repair (BER), may also play a role in introducing ribonucleotides into DNA. In contrast to rNTPs, substrate selectivity is possessed by the pols (β and λ) for dNTPs ranging from 3,000 to 50,000-fold (Brown and Suo, 2011). Despite the fact that they significantly differentiate against ribonucleotides, latest research found that pol β instead of λ, has an effect on activity of an oxidatively damaged base in cellular extracts (Berglund et al., 2017). Furthermore, pol β uses (oxidative ribonucleotide 8-oxo-rGTP) as a substrate for synthesis of DNA (Cilli et al., 2015). Particularly, contrary to the other pols, pol TdT and μ preferentially integrate rNTPs into DNA (Brown and Suo, 2011).

Oncogenic stress

To preserve genome integrity, DNA replication needs to be properly controlled during the cell cycle. An enormous collection of research has effectively revealed that oncogene activation causes replication stress at vulnerable genomic regions via several molecular mechanisms. The ramifications of primary human oncogenes are identified to generate DNA replication stress are discussed in depth in the following sections (Cilli et al., 2015).

Rat Sarcoma (RAS)

Oncogenic *RAS* has been linked to DNA replication stress. *N-RAS*, *H-* and *K-* are proto-oncogenes included in *RAS* gene family and act as small GTPase signal transducers. *RAS* proteins are important elements of a system that connects the receptors of cell surface to intracellular proteins in order to control metabolism, survival as well as growth of cells among other processes. Upon GTP binding, G proteins activate as well as switch on the downstream effectors that control many mitogenic pathways such as: PI3K/AKT and RAF/MEK/ERK pathways. Somatic RAS alterations activate it intermittently, triggering the activation of effectors that enhance metabolic reprogramming, cell proliferation and apoptosis suppression. RAS mutations are prevalent in human malignancies, particularly K-RAS mutations has been identified in around 20 percent of lung adenocarcinomas and 40 percent of colorectal tumors (Pylayeva-Gupta et al., 2011). Prolonged mitogenic activation by RAS (H-RASV12) oncogene has a direct impact on DNA replication, causing replication stress via a variety of ways. Di Micco and colleagues demonstrated that increased origin firing and production of asymmetric replication forks are caused by RAS oncogene, which results in replication stress in a leading-edge study forks (Di Micco et al., 2006). It is assumed that enhanced origin firing is caused by licensing factor CDC6 and has an impact on DNA replication. Excessive expression of RAS gene has been demonstrated to promote CDC6 levels. Premature cessation of RFs and dNTP pool depletion are the consequences of RAS oncogene's interference with cellular dNTP levels by decreasing the expression of ribonucleotide reductase subunit M2 (RRM2) (Aird et al., 2013). These findings, along with others, have led to the concept that inevitable cell cycle interruption, a phenomenon called as oncogene-induced senescence (OIS) and DNA damage response (DDR) stimulation are the results of replication stress induced by oncogene (Di Micco et al., 2006).

As a result of oxidative stress, RAS oncogene may also produce replication stress. Initially, the acceleration of RFs and hyperproliferation are caused by expression of RAS oncogene. Excessive expression of RAS for prolonged periods of time, on the other hand, produces cellular metabolic

alterations and slows fork advancement. It has been proven that enhanced generation of reactive oxygen species (ROS) causes RAS-induced senescence (Mitelman et al., 2019), which results in synthesis of H_2O_2 and nucleotide oxidation . Different techniques can be used to mitigate these oxidative shocks and preventing cellular senescence and DNA damage. Consequently, oxidative stress may give rise to replication stress induced by RAS through double strand breaks (DSBs) formation and reservoir oxidized DNA precursors (Leikam et al., 2008; Maya-Mendoza et al., 2015).

Enhanced global transcription is another way of replication stress generated by RAS. RAS proteins enhance proliferation of cells by overexpression of generic transcription factors which promote RNA synthesis (Pylayeva-Gupta et al., 2011). Moreover, RAS oncogene has been linked to increased TBP transcription factor (TATA-box binding protein) expression as well as transcriptional activity. Collisions between transcription and replication machinery, as well as generation of R-loops, result in increased RNA production, which slows replication forks and damages DNA (Kotsantis et al., 2016). TBP overexpression, on the other hand, has the ability to enhance transcription while also causing DNA damage and replication stress, summarizing the consequences of oncogenic RAS (Tu et al., 2011).

Other pathways might possibly play a role in replication stress induced by RAS. Intervention with DNA repair in one of the possibilities. RAS oncogene has been found to cause BRCA1 protein separation from chromatin, impairing DNA repair as well as result in damage to the DNA (Tu et al., 2011). Deactivation of the BRCA1 protein makes cells prone to subsequent mutation accumulation and eventually cancer formation. Given the many stresses to DNA replication generated by RAS oncogene, it is logical to predict that replication stress induced by RAS may result in instability of genomic. Indeed, activation of RAS has been observed to cause chromosome aberrations like: RF stalling at telomerase which results in aberrant telomeric structures (Suram et al., 2012), telomeric attrition (telomere shortening; progressive erosion of our chromosomes' protective caps), acentric fragments, double minute chromosomes and deletions (Guerra et al., 2003) and genomic mutations at CFS important to human tumorigenesis (Miron et al., 2015).

MYC proto-oncogene (MYC)

MYC is transcription factor's family and consists of three components like: N-MYC, L- and C-. MYC proteins are signaling transduction pathway effectors that regulate an extensive variety of cellular functions such as: call apoptosis, differentiation, proliferation and growth. MYC functions primarily as a transcription factor by dimerizing with DNA binding regulatory proteins and MAX to control a variety of gene transcription processes. Moreover, MYC proteins also have non transcriptional functions in physiology of a cell. MYC oncogene is typically activated through loss of upstream MYC regulators, chromosomal rearrangement and gene amplification, resulting in sustained MYC levels and intervention with important cellular processes. Indeed, c-MYC expression is downregulated in (greater than half of all) human malignancies, and MYC oncogene is linked to invasive colon, prostate and breast carcinoma along with Burkitt lymphoma (Dang, 2012).

MYC-stimulated replication stress is produced by several molecular processes and causes instability of genome and DNA damage during tumorigenesis. Previous research revealed that genomic instability induced by MYC was linked to oxidative stress. Excessive expression of c-MYC alters cellular metabolism, causing enhanced ROS generation, which corresponds with DNA damage (Vafa et al., 2002). Although, MYC oncogene induces replication stress prior to inducing cellular metabolic alterations, in contrast to RAS (Maya-Mendoza et al., 2015). Indeed, multiple studies have now shown that MYC activation causes instability of genome and DNA damage by directly impairing DNA replication dynamics.

MYC-produced replication stress is primarily caused by intervention with origin firing. MYC has been shown to confine to ORIs (origins of replication) as well as interact (physically) with pre-RC

members like: MCMs, CDT1, CDC6 and ORCs during origin licensing. CDC45 is a replication factor required for the start of DNA replication and MYC also contributes to ORI activation by enhancing the binding of CDC45 to genetic material (Srinivasan et al., 2013). Commensurate with, excessive expression of MYC causes enhanced as well as premature origin firing whereas, MYC depletion reduces the quantity of active ORIs. MYC oncogene, once unregulated, causes DNA damage through replication fork stalling, RF asymmetry and ORI hyperactivation. Significantly, it has been demonstrated that MYC's effects on origin firing are no representative of its transcriptional activity. Excessive MYC expression causes alterations in genomic position (from intergenic to intragenic areas representing strong transcriptional activity) of ORI activation, identical to well-known impact of Cyclin E1 oncogene in firing of origin (described below) (Macheret and Halazonetis, 2018). Since excessive expression of MYC enhances origin firing and transcription, being a transcription factor, it is logical to infer that MYC oncogene induces stress by causing complexes between transcription as well as replication machineries. This possible method of replication stress mediated by MYC, although has yet to be established.

MYC proteins have fascinating ability to reverse replication stress by using different mechanisms. MYC transcription factors, as previously stated, promote the expression of multiple genes involved in nucleotide manufacturing pathway, DNA replication and cellular proliferation (Mannava et al., 2008). Surprisingly, c-MYC expression promotes metabolism of purine as well as pyrimidine and supplies enough nucleotide pools to alleviate replication stress caused by significant amount of DNA synthesis when RB-E2F mechanism is disrupted (Bester et al., 2011). In addition, MYC protein elevates expression of particular enzymes implicated in the DNA replication like: MRN nuclease complex (MRE11/RAD50/NBS1), involved in restart of collapsed RF as well as DSB repair and WRN helicase, a protein responsible for resolution of unusual replication intermediates (Petroni et al., 2016). MYC expression results in protecting cells from DNA damage and replication stress through activation of MRN nuclease and WRN helicase.

Cyclin E Oncogene

Replication stress is also caused by Cyclin E which is prototypical oncogene. Cyclin E1 as well as E2 (CCNE1 and CCNE2) are two proteins in Cyclin E family that have identical gene sequences as well as biological activities. Levels of Cyclin E protein typically increase at G1/S conversion and are entirely destroyed by completion of S phase. Cyclin E, in collaboration with CDK2, regulates DNA replication by phosphorylating a number of proteins, including the DNA replication factors like: Treslin, CDC6, and CDT1 as well as RB tumour suppressor. Origin firing and origin licensing require the DNA replication factor's phosphorylation whereas, phosphorylation of retinoblastoma (RB) tumor suppressor causes the release of transcription factor (E2F), increasing the expression of numerous genes necessary for DNA replication. As a result, it is not unexpected that the activation of Cyclin E disrupts DNA replication and progression of cell cycle, resulting in instability of genome and replication stress. Malignancies and premalignant lesions like: leukemias, lung and breast tumours have been found to have CCNE1 excessive expression, amplification, or defective protein degradation.

Many alternative pathways have been implicated in replication stress induced by Cyclin E. During early G1 and late mitosis, levels of Cyclin E1, that are higher than normal impede with pre-RC assembly during delayed mitosis as well as preliminary G1, particularly with attachment of helicase subunits (MCM2, MCM4, and MCM7) to genetic material (Ekholm-Reed et al., 2004). Inadequate pre-RC assembly impedes origin firing, hinders optimal origin licensing and DNA synthesis commencement. In several models, excessive expression of Cyclin E leads to either upregulation (Jones et al., 2013) or downregulation (Liberal et al., 2012) of origin firing. Enhanced Cyclin E expression inhibits replication fork advancement in addition to interfering with origin firing and

licensing. Overexpression of Cyclin E1 results in DSBs, fork collapse and premature termination of RFS. It has been demonstrated that Cyclin E stimulated RF collapse can be restored by homologous recombination pathway which involves, (break-induced replication (BIR), resulting in instability of genome and copy number changes (Costantino et al., 2014). It is critical to identify that Cyclin E produced replication stress is CDK2-dependent, as elevated levels of CDK2 expression are significant to hinder RF advancement and cause DNA damage (Hughes et al., 2013).

The depletion of nucleotide pools is another important factor for replication stress produced by Cyclin E. Enhanced expression of Cyclin E1 promotes cell hyperproliferation due to inadequate nucleotide levels by generating DSBs and disrupting replication fork advancement and via impairment of RB/E2F pathway (Mannava et al., 2008). Surprisingly, activation of nucleotide metabolism or exogenous nucleoside supplementation via c-MYC expression can reduce DNA damage and replication stress caused by excessive expression of Cyclin E1.

Transcription-replication conflicts, can also generate Cyclin E stimulated replication stress, resulting in the generation of permanent R-loops and DNA topological stress. It has been demonstrated that inhibiting transcription elongation reduces DNA damage and alleviates replication stress induced by Cyclin E1 oncogene (Jones et al., 2013). Inhibiting replication initiation frequently recovers normal levels of fork development in presence of elevated amounts of Cyclin E1. These findings suggest that Cyclin E1 oncogene generates replication stress by causing transcription-replication collisions. One possible outcome of these interactions is development of DNA replication intermediates, for example reversed RFs, in relation to topological stress. In fact, excessive amounts of Cyclin E1 cause the formation of abnormal reversed RFs.

In a latest study, the human genome was mapped in terms of replication time and ORI distribution under normal and elevated levels of Cyclin E1 (Macheret and Halazonetis, 2018). ORIs are typically active in intergenic regions, under normal surroundings. However, excessive Cyclin E1 expression results in a shorter G1, a faster S phase entrance, and new origin firing with strong transcriptional activity in intragenic areas. In protein-coding genes, uncontrolled origin firing promotes clashes between replication and transcription machinery, resulting in chromosomal translocations, DSBs and replication fork collapse (Macheret and Halazonetis, 2018).

As previously mentioned, inherent genetic features may predispose cells to replication stress in the face of oncogenic assaults. Dysregulation of Cyclin E1, in fact, permits cells to initiate mitosis with inadequate replication at certain genomic sequences, which leads to mitotic abnormalities like CNAs (copy number variation), anaphase bridges, and chromosomal breaks (Teixeira et al., 2015). Elevated expression of Cyclin E1 causes genomic fragility, which exhibits CFS characteristics like: DNA secondary structures, extremely lengthy genes, late-replicating domains and low origin density. As a result, genomic rearrangements and breakpoints generated by increased expression of Cyclin E1 are observed in vitro, in a high proportion of human malignancies with CCNE1 amplification.

CDC6

CDC6 is required during late mitosis as well as early G1 phase for formation of pre-RCs, as it is a licensing factor of DNA replication. CDC6 particularly enables the binding of MCM helicase to ORIs and can also control gene transcription as well as facilitate the cell cycle checkpoints activation (Borlado and Méndez, 2008). In vitro, abnormal CDC6 expression produces multiple oncogenic features, including tumour development, instability of genome, cellular transformation and DDR activation in vivo. In addition, increased CDC6 levels have been seen in colon carcinoma and advanced stages of non-small cell lung carcinoma (NSCLC).

Deranged CDC6 levels throughout cell cycle development impede with origin activation and/or licensing, as predicted for a protein implicated in pre-RC development. Excessive expression of CDC6

in collaboration with CDT1, stimulates DNA replication as well as origin firing during few hours of S phase, in cells which are deficient of p53, resulting in genomic instability and amplification of large segments of genome, provided the first confirmation for replication stress stimulated by CDC6 (Vaziri et al., 2003). Later, it was discovered that CDC6 oncogene enhances ORI activation at certain locations of genome via genetic material displacement of CTCF. Furthermore, Bartkova et al (2006) demonstrated that increased CDC6 levels stimulate the generation of RPA foci, a representative of ssDNA normally linked to stalled RFs (Bartkova et al., 2006).

Replication stress produced by CDC6 can also arise, as a result of conflicts between transcription and replication machines as well as generation of R-loops (Komseli et al., 2018). Surprisingly, elevated CDC6 expression causes R-loop development in nucleoli, which is justified on the grounds that CDC6 is necessary for transcriptional control of highly repetitive heterochromatic ribosomal DNA (rDNA) (Huang et al., 2016). In diverse scenarios, CDC6 overexpression produces a range of numerical and structural chromosomal abnormalities in response to replication stress, with high proportion of breakpoints situated near CFS. As previously stated, CDC6 overexpression may be caused by Cyclin E1 or RAS oncogene activation, resulting in genomic instability, DDR activation and DNA re-replication instability (Mailand and Diffley, 2005).

Chromatin inaccessibility

Furthermore, natural events like as chromatin compaction, influence DNA accessibility as well as provide challenges to replication machinery. Recent research has found that γH2A is a DSB marker and its replication dependent enrichment reported in heterochromatic regions of yeast (Inagaki et al., 2016). Furthermore, many frequent fragile sites are present in restrictive chromatin settings, and chromatin relaxation minimizes breakage of fragile site (Liberal et al., 2012). These data imply that DSBs are more prevalent in heterochromatic zones. It is unclear whether this is related to the repressive events of chromatin structure on DNA repair pathways or to an increase in replication stress induced by breaks.

Consequences of DNA Damage

DNA damage has a wide range of consequences, most of which are detrimental. Disrupted DNA metabolism leads to the acute effects causing cell death or cell cycle arrest. Irreversible mutations that contribute to inherited genetic diseases as well as tumorigenesis have long-term consequences. Transcription is inhibited by most of the lesions, triggering the formation of a transcription-coupled repair (TCR), specialized repair system, which replaces or eliminates stalled RNA polymerase as well as assures favorable lesions repair inside the transcribed strand expressing genes (Mentegari et al., 2017). DNA lesions-induced transcriptional stress inhibits DNA strand breaks as well as RNA polymerase, driven by stalled RFs or DNA damage, are two important indicators for responses induced by DNA damage, implicating apoptosis (Vaisman and Woodgate, 2018), via both processes such as: p53-dependent and p53-independent (Pryor et al., 2018). Lesions can potentially obstruct replicating DNA. A class of distinctive DNA polymerases has been uncovered, consisting of more than 17 enzymes, dedicated to surviving replication stress caused by damage (Tubbs et al., 2018). These specialized polymerases temporarily replace the halted replicating DNA polymerases. However, translesion polymerases safeguard DNA, this retaliation to stalled RF occurs at the cost of mutations in certain of these polymerases as well as greater replication error rate, promoting cancer susceptibility (Siu et al., 2012). As a result, DNA damages can be detected via specialized sensors, inhibited transcription or replication.

DNA DAMAGE RESPONSE (DDR) PATHWAYS

Initially, DNA damage checkpoints had been identified as monitoring mechanisms that regulate cells' potential to pause cell cycle following DNA damage, possibly enabling time for other cellular activities or repair. Although, these mechanisms implicate proteins that have been demonstrated to regulate the activation of transcriptional responses , DNA repair processes and transport of proteins involved in DNA repair to the locations of damaged DNA (Vaisman and Woodgate, 2018), in addition to influencing cell cycle detention. In case of severe damage, a cell can choose subsequent option of recovery by commencing programmed cell death in order to sustain the organism at risk of eluding a cell (Shanbhag et al., 2018). At molecular level, DDR pathways are well described, it has been viewed as a system of interconnected mechanisms that together implement response (Sacco et al., 2012). A plethora of DNA binding proteins which are damage specific, recognize DNA damage and signal the DDR, either alone or in combination with complexes containing related proteins that are not engaged in DNA repair directly (Reijns et al., 2012). Phosphoinositide-3-kinase-associated proteins implicate the checkpoint kinases (CHEK1 and CHEK2), DNA-PK, ATM-Rad3-related (ATR) proteins, ataxia-telangiectasia mutated (ATM) and others frequently trigger DNA damage signaling pathways (Vannier et al., 2014). ATR reacts to ssDNA sections while ATM originates at DSBs and is important in DNA damage signaling. Most of the protein kinases are candidates for activation and phosphorylation; they subsequently affect downstream gene products important in oncogenesis, like BRCA1 as well as p53. The final targets of this strictly controlled DDR system implicate DNA repair pathways, and while most of DNA repair is intrinsic, a variety of regulative links between DNA repair as well as DDR pathways are developed (Srinivasan et al., 2013). A substantial number of genes are overexpressed (transcriptionally) in mammals and implicated in DNA repair following damaged DNA, proposing that numerous aspects of repair are inferable. Indeed, p53 (TSG) is a key modulator of transcriptional response induced by DNA damage in humans, as well as p53-deficient mammalian cells found to be limited in various facets of DNA repair (Vaziri et al., 2003). As a result, the mammalian DDR system is highly controlled and perfectly alright to decide whether a certain cell type moves to an inspection-point of cell cycle as well as apoptosis or DNA repair, following a severe damage shock. Limitations in any of these processes may disrupt healing and lead to tumorigenesis.

TYPES OF DDR PATHWAYS AND THEIR INVOLVEMENT IN CARCINOGENESIS

DNA repair refers to the cellular reactions linked to the recovery of normal nucleotide sequence following processes damaging or modifying genome (Sfeir et al., 2009). Considering enormous range of damaged DNA that a cell can experience, it is predictable, that there are an equivalent number of repair processes available to deal with these damages. In fact, many of them are extensively interacting and overlapping, with some of them sharing even particular gene products as well as defined methods. Much has been gained about DNA repair in humans from the typically infrequent autosomal recessive hereditary diseases linked to abnormalities in genes for DNA repair (Nambiar et al., 2011).

Nucleotide Excision Repair (NER)

The most diverse as well as widespread methods are available for DNA repair. In these mechanisms, damaged or erroneous section of DNA strand is removed, and subsequent space is filled by repair replication in which complementary strand is used as a template. Excessive genetic information given by duplicate DNA structure is critical to genome preservation via this "cut and patch" method named as excision repair. In case of replication dependent repair, each strand of DNA may function as a template to repair other DNA strand. NER acts by eliminating several forms of lesions, such as: 6-4 photoproducts, cyclobutane pyrimidine dimers (CPDs) induced by UV, intrastrand cross-links (ICLs) and bulky base adducts of chemical carcinogens (Sfeir et al., 2009).

Because of the deformation of helical structure of DNA, these lesions can act as a structural barrier to DNA replication as well as transcription, and they may cause variations if translesions lead to replication process or not restored accurately. Subsequent steps involved in NER are as follows: 1. Identification of damaged section, 2. Cleavage of damaged DNA strand near an area of damage, 3. Excision of a portion of damaged strand incorporating lesion, 4. Repair replication to restore extracted area with a respective stretch of normal nucleotides using complementary strand as a template, 5. Ligation of repaired area with its parental DNA strand at 3' end (Kotsantis et al., 2016). This NER system, known as global genomic repair (GGR), may eliminate damaged DNA from places all around the genome. Most of the human (NER genes) are cloned as well as discovered, and several are reported to be altered in NER-impaired, tumor susceptible genetic disorders (Meryet-Figuiere et al., 2014). A specific difficulty develops when translocating RNA polymerase producing messenger RNA detects a bulky lesion before the enzymes involved in repair have restored undamaged DNA and eliminated the damage. The polymerase can be halted at the location of lesion, preventing repair enzymes to access the damage. Moreover, transcriptional arrest is a powerful indicator for activation of p53 in human cells, which might result in apoptosis (Mailand and Diffley, 2005). In this case, transcription-coupled repair also referred as specific NER process, steps in to displace RNA polymerase as well as effectively fixes the blocking lesion, allowing the cell to survive and transcription to restart (Kurat et al., 2011).

It has been discovered that GGR (NER sub-pathway) is induced by damage as well as strongly regulated by processes like transcription as well as post-translation following DNA damage, in association with apoptosis and cell cycle checkpoints induced by damage . In reality, p53 gene is essential for maintaining genomic integrity in human cells, operates as a transcription factor responsible for (controlling the expression of multiple NER damage detection genes) activated by DNA damage as well as necessary for effective GGR of DNA damage induced by carcinogen and UV light (Kotsantis et al., 2016). Similarly, numerous other critical cancer-related genes, including E2F1 and BRCA1, had been demonstrated to transcriptionally control DNA damage detection NER genes DDB2 as well as XPC (Hangauer et al., 2013; Kato et al., 2012). As a result, the sub-pathway of NER (GGR pathway) appears to be important in inhibiting malignancy induced by DNA damage and is heavily controlled by TSGs. Regardless of the several pathways for DNA repair detailed here, it is probable that replication machinery of cell would experience lesions in template strand of DNA that prevent replication throughout each cell cycle. Translesional synthesis (TLS) is performed by specialized class of error prone DNA polymerases and defined as manufacturing of DNA across the damaged bases and then removed following RF has passed, it comes under DNA damage tolerance (DDT), cell's solution to lesions (Kumari et al., 2015). The halted replicative DNA polymerases are temporarily replaced by DNA polymerases of Y-family, possessing more adaptable base-pairing characteristics allowing for production of translesion DNA, while each polymerase likely tailored for distinct type of damage. However, translesion polymerases safeguard genome, and replication block conclusion comes at risk of greater error rate. For example, genetic abnormalities in polymerase eta (pol η), which is encoded by XPV/POLH/RAD30 gene and plays a central role in relatively perfect circumventing of CPDs induced by UV, create a mutant type of the skin tumor–susceptible condition Xeroderma pigmentosum (XP) (Hustedt and Durocher, 2017).

Base Excision Repair (BER)

Normal cytoplasmic metabolism, including exposure to reactive metabolites and hydrolysis that induce alkylation as well as oxidation of DNA, is a primary cause of DNA damage to the genome of cells. BER pathway is a central repair system implicated in repairing as well as recognizing such lesions and coping with spontaneous purine loss from DNA (Helmrich et al., 2011). The importance of BER for survival is underlined by an experience that, despite the discovery of several BER proteins, only newly has been detected in single human genetic illness that seems to originate from an alteration

in a gene specific to this pathway (Kotsantis et al., 2016). One of a group of glycosylases which are lesion specific initiates BER by identifying the changed or incorrect base as well as cleaving it (from its sugar moiety) in DNA. Distinct DNA glycosylases repair various types of damage, allowing the process specificity. Phosphodiester backbone is then cleaved 5′ to AP site by AP-endonuclease (APE1), producing a transitional ssDNA break. In certain circumstances, glycosylases which are bifunctional additionally split phosphodiester bond next to injured base averting requirement for APE1 endonuclease activity as well as necessitate a (polynucleotide kinase 3′-phosphatase) alternatively, for end-processing of single-strand break. AP site is replaced by POLB (DNA polymerase) by adding a nucleotide in short-patch process. Finally, ligase (LIG3) produces phosphodiester bond to conclude the repair. DNA polymerase (POLE, POLD or POLB) replaces and multiple nucleotides are added in long-patch process, flap structure-specific endonuclease (FEN1) eliminates replaced nucleotides, completing a repair by ligase (LIG1). PCNA (proliferating cell nuclear antigen) functions as a scaffolding (framework) protein as well as needed for POLD function. Binding and selection of critical mediators is done by poly (ADP-ribose) polymerase (PARP), to the single strand break intermediates. 8-oxo-7,8-dihydroguanine (8-oxoG) is the most frequent oxidized purine lesion, which is both mutagenic as well abundant. Formamidopyrimidines, 5-hydroxycytosine and thymine glycol are examples of oxidized pyrimidines. Oxidized bases, such as thymine glycol and 8-oxoG, have the characteristic of inhibiting DNA replication as well as transcription and it must be repaired effectively to maintain the integrity of genome (Huang et al., 2016). Glycosylases like MYH and APE1 work cooperatively to remove adenines that have been mispaired with an 8-oxoG; hoGG1, removes oxidized purines and hNTH1 targets the oxidized pyrimidines. These glycosylases are implicated in the BER pathway involving strand incision step encoded by certain genes, in mammalian cell (Komseli et al., 2018). DNA damage classes are considered to possess cytotoxic and mutagenic nature and referred as BER substrates, it is plausible that an increased cancer risk would be associated with changes in activity of these pathways (Lambert and Carr, 2013). Tumors from afflicted people indicate that an APC gene exhibits an excess of guanine-cytosine pair transversions to thymine-adenine pair, which is the cause of familial adenomatous polyposis as well as has been linked to colon carcinogenesis. As a result, biallelic inherited MYH mutations cause a polyposis-like disease known as MYH-associated polyposis (MAP). As a result, biallelic inherited MYH mutations cause a polyposis-like disease known as MYH-associated polyposis (MAP). Almost 50 percent of patients with MAP reported to have colon tumor at the time of presentation and typically found to have tens to hundreds of polyps at the age of forty (Kotsantis et al., 2016). Efforts have been made to detect genetic variations which are polymorphic functionally in genes responsible for DNA repair, that influence individual cancer risk as well as regulate activity. Genetic epidemiological researches regarding single-nucleotide polymorphisms (SNPs), notably in BER genes, have proven conflicting thus far. Although, meta-analyses have reported a slight increase in lung cancer risk related with XRCC1 as well as OGG1 SNPs (Lambert and Carr, 2013). Continued developments in high-throughput genotyping and SNP mapping technologies will allow for the investigation of numerous polymorphisms inside many genes using haplotypes; greater sample numbers may allow for clearer risk connections in future research.

Mismatch Repair (MMR)

MMR is an additional excision repair process, employing an identical method for genome preservation. Some forms of base alterations, errors in DNA recombination and replication result in the formation of paired DNA strands which are otherwise complementary, mismatched nucleotides in these strands are repaired by a mechanism known as MMR. This repair method may also handle tiny loops of ssDNA at deletion or insertion sites in double DNA structure. Finding of a lack of this repair system leads to a significant elevation in frequency of spontaneously arising alterations, particularly in highly repetitive DNA's microsatellite sequences, demonstrates the relevance of this

repair process in preserving genetic stability (Kaushal et al., 2019). Most of these spontaneous variations result from errors originated during DNA replication, despite the existence of a "proofreading" machinery that also contributes to replication accuracy. Genetic abnormalities in numerous MMR genes of humans have been associated with spontaneous malignancies characterized by alteration in an area of DNA incorporating short repeating nucleotide sequences, a trait known as microsatellite instability (MSI) as well as Lynch syndrome (hereditary nonpolyposis colorectal tumor).

MMR, like other mechanisms of excision repair, requires four major steps: (1) mismatch identification, (2) recruitment and selection of additional MMR factors, (3) recognition of freshly incorporated DNA strand consisting of mismatched nucleotides, accompanied by their excision, as well as (4) resynthesis of excised region and ligation. This pathway's metabolic workings are best known in bacteria, although, in human cells, a comparable sequence of processes happens. Numerous human genes contributing in MMR have been cloned on the basis of sequence homology to respective yeast genes and functional similarities to their bacterial analogues, including those genes which are homologous to bacterial MutL gene as well as the bacterial MutS mismatch identification protein (hMSH2, hMSH3, and hMSH6) (Costantino et al., 2014). In humans, MSH2/3 dimers identify longer loops, while MSH2/6 heterodimers identify short insertion-deletion loops and single base-pair mismatches. For DNA excision and strand discrimination, MLH1/PMS1 and MLH1/PMS2 heterodimeric complexes interact with replication factors as well as MSH complexes. In repair replication, additional proteins identical to BER and NER, are then selected, on the basis of original DNA template. The MMR mechanism may also interact with damaged DNA as a result of certain intercalating chemicals as well as alkylators, producing comparable structural modifications in DNA such as mismatches, resulting in unsuccessful or erroneous MMR cycles and, eventually cell death. Consequently, functional MMR may offer chemosensitivity to certain chemotherapeutic medicines, but MMR-deficient malignancies may demonstrate drug resistance (Forslund et al., 2018).

Double-Strand Break Repair

Replication, chemicals and ionizing radiations generate DSBs in DNA, over single strand breaks, as well as recombination machinery plays an important role in repairing interstand DNA cross-links. An extremely harmful occurrence is unrepaired DSBs, and even single event in a whole genome is considered to trigger cell cycle checkpoints that halt cell division or attempted DNA synthesis until repair is complete, or programmed cell death if incorrectly restored. DSBs can cause complications during mitosis since undamaged chromosomes are required for correct chromosomal segregation during cell division. As a result, these lesions frequently cause several types of chromosomal abnormalities such as, chromosomal translocations, deletions, and aneuploidy, all are linked to carcinogenesis. The main strategy for coping with DSBs which implicates homologous lengths of nucleotides to be joined at the ends, is genetic recombination. If there is no such homology exists, another method for nonhomologous end joining (NHEJ) exists, which is highly susceptible to inaccuracy. While NHEJ is the most frequent pathway for repair of DSBs in humans. Homologous recombination (HR) is essential for ICL repair in collaboration with NER proteins as well as DSBs repair, generated at collapsed RFs. Recent research has found a protein kinase cascade implicated in signalling biological functions regarding DSBs. Many of them, including as the CHEK2 protein kinase and ATM protein45, have been identified to be impaired in cancer-susceptible diseases characterized by genomic instability (Ekundayo and Bleichert, 2019). The p53 TSG is a primary target for activity of these kinases. When phosphorylated, this protein is activated and implicated in apoptosis as well as G1 arrest following ionizing radiation, and when mutated in germline, it leads to Li-Fraumeni cancer-prone illness. Many additional enzymes required for DSB repair and implicated in original DNA transactions, such as RecQ-like helicases (Rothmund-Thomson, Bloom and Werner syndromes),

BRCA1 and BRCA2 (breast-ovarian cancer syndrome), NBS1 (Nijmegen breakage syndrome) as well as MRE11 (AT-like disease), have been discovered in cancer-prone illnesses (Coté and Lewis, 2008).

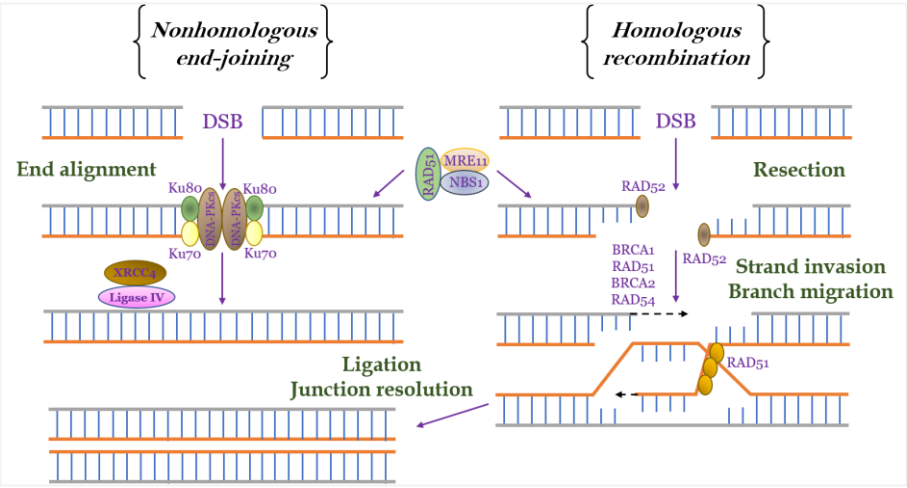

Figure 1. Double strand breaks (DSBs) mechanism.

Association of Telomer and DNA replication

It is known that chromosomal ends consist of telomeres, which are critical for stability of genome. Composition of telomeres includes paired head-to-tail repetitions of a short G-rich sequence (Zaidi et al., 2016). For instance, human telomeres consist of 2–20 kb of (TTAGGG) n repeats (Vaisman and Woodgate, 2018). T-loop structure is generated, when internal G-rich sequence is displaced by the overhangs of 3'end of chromosome containing G-rich strand in a single strand, invading the inside of telomere, as chromosomal ends are not blunt therefore, chromosomal ends are protected from being identified as DSBs by the cell, in combination to safeguard offered by proteins binding the telomere (Shastri et al., 2018). Eukaryotic chromosomes undergo semiconservative replication including leading strand (constant formation at nascent leading strand's 3' end for net growth) as well as lagging strand (production of Okazaki fragments at nascent lagging strand's 5' end for net growth). Removal of short 5' RNA primer occurs from nascent strand during semiconservative replication of chromosome and the resulting gap is filled with DNA ligating to the surrounding nascent DNA. End-replication problem is defined as the gap left after deletion of 5' terminal RNA primer, towards the end of chromosome, on lagging strand. This gap cannot be filled, and chromosomes may get shorter with each subsequent round of replication, therefore telomerase come to address this issue problem (Gerbi, 2015).

Before telomerase takes effect, there is a phase of semi-conservative replication. RFs in chromosomal DNA were previously assumed to move out from subtelomeric origin in a bidirectional fashion, duplicating the telomere, beginning from an origin next to the telomere repeats (Usdin et al., 2015). In any case, whether DNA replication could begin on a regular basis inside the telomere itself was still an open topic. Drosopoulos et al. has employed single molecule analysis of replicated DNA (SMARD) to provide a solution to this topic (Drosopoulos et al., 2015). Immunofluorescence may be used to identify cells that have replicated using two distinct nucleotide analogues. Initial studies based on SMARD data showed that most replication in human and mouse genomes begins in subtelomeric regions rather than the telomeres. Since the confirmation for bidirectional replication in telomere was limited, it is obvious that replication origin as well as real RF may exist inside the telomere, elongating into the subtelomere over time. It's not yet clear whether chromosomal telomeres other than 14q have a high rate of replication initiation (Zaidi et al., 2016).

Telomeric DNA contains high GC content which is highly repetitive as well as thermally stable. RFs proceed through it due to its tendency to generate secondary structures, causing complications for

DNA replication. It has been shown that Werner syndrome helicase (WRN) as well as Bloom syndrome helicase (BLM) are both involved in supporting telomere replication: WRN reduces abnormalities in production of telomere lagging strand and BLM reduces fragile telomeres which are replication dependent (Teixeira and Reed, 2017). A new study shows that production of leading strand initiating inside of telomere, in BLM-deficient cells is slower to move towards the subtelomere as shown by SMARD imaging. Furthermore, BML deficient cells showed higher replication commencement in 14q subtelomere of BLM-deficient cells and occurred closer to the telomere as compared to the cells that had sufficient amounts of BLM. These findings show that latent origins of replication in 14q subtelomere may become active in BLM-deficient cells when fork advancement is inhibited. As an alternative method to awaken latent origins due to replication stress, an elevation in commencement of subtelomeric replication was observed when RF advancement from telomere was inhibited by aphidicolin. When BLM-deficient cells treated with (PhenDC3) G4 stabilizer, further increase in 14q subtelomeric origin firing occurred (Drosopoulos et al., 2015). As further evidence, BLM helicase plays a role in removing G4 structures, BG4 antibody staining against G4 in telomeres as well as in whole genome was enhanced in BLM-deficient cells (Schwab et al., 2015).

In vitro, WRN helicase is able to unwind G4. Cells which were double deficient of WRN as well as BLM, investigated for replication by SMARD. Sharp decline of red replication signal was observed in 14q telomeres, indicating several functional crossovers between WRN and BLM in case of leading strand formation outside (G-rich) telomere strand (Shanbhag et al., 2018). To support this finding, double deficient (WRN and BLM) cells showed increased BG4 antibody staining against G4 as compared to cells deficient of just WRN or just BLM. Direct evidence of WRN and BLM helicases involvement in G4 structure resolution in vivo has never been seen before. G4 structures are critical for advancement of leading strand formation, which is copied from G-rich strand and commences at telomeric regions (Drosopoulos et al., 2015).

Synthesis of cell free Serum DNA with DNA replication

Cell-free systems have been employed extensively for in vitro protein production in recent decades. In cell-free technology, a wide variety of methods and cellular sources, ranging from prokaryotes to eukaryotes, are currently accessible (Drosopoulos et al., 2015). In contrast, cell-free systems cannot be fully exploited using established procedures. The methodologies and options for optimizing cell-free protein synthesis have been examined. These methods increase transcription, translation, and protein folding in a way that is both stable and instructive, and they may be placed to use in the future to develop the optimum cell-free processes for each lysate batch. Many key molecular processes may be carried out in vitro using cell-free protein synthesis systems (CFPSS), which are produced from lysed cells and allow for growth-independent and reasonably quick in vitro protein synthesis (Clausen et al., 2013). They save a lot of time compared to typical in vivo approaches for prototyping recombinant protein production. Many applications have been developed during the last 60 years for cell-free systems, including, protein activities, decoding genetic, biological computing and biomanufacturing, biosensors. It is possible to buy or make a variety of cell-free systems in lab. In addition to their reduced cost and simplicity of preparation, lab-made cell-free reactions are becoming more popular, opening the door to their optimization versus commercially available ones. A wide range of model organisms have been used for cell-free extracts, including insects (Spodoptera frugiperda), *Bacillus subtilis*, *Escherichia coli*, extracts obtained from mammals, including and sHeLa cell lines, human CHO, and rabbit reticulocytes as well as wheat germ (Batista et al., 2021).

Conclusion

Elaborating on the mechanisms as well as linkages that drive the reaction in case of replication stress offers more information on molecular processes, maintaining genomic stability during replication facing internal replication stress origins. In addition, we will learn more about how external origins or abnormalities in crucial pathway elements may contribute to an enhanced genomic variation. Identifying the exact areas of genome that are specifically damaged by different forms of replication stress will be an important challenge in the coming years. To that aim, current technological breakthroughs can be used to implement an innovative method such as next generation sequencing to sequence particular DNA regions impacted by replication stress as well as the precise protein interactions governing their fragility. Recent advancements in current knowledge about replication stress response will also be a significant step toward more specific therapies as well as generation of novel treatments for illnesses such as cancer.

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