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Mechanisms of Oncogene-Induced Replication Stress: Jigsaw Falling into Place

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ABSTRACT

Oncogene activation disturbs cellular processes and accommodates a complex landscape of changes in the genome that contribute to genomic instability, which accelerates mutation rates and promotes tumourigenesis. Part of this cellular turmoil, involves deregulation of physiological DNA replication, widely described as replication stress. Oncogene-induced replication stress is an early driver of genomic instability and is attributed to a plethora of factors, most notably aberrant origin firing, replication-transcription collisions, reactive oxygen species and defective nucleotide metabolism.

Significance

Replication stress is a fundamental step and an early driver of tumourigenesis and has been associated with many activated oncogenes. Deciphering the mechanisms that contribute to the replication stress response may provide new avenues for targeted cancer treatment. In this review, we discuss the latest findings on the DNA replication stress response and examine the various mechanisms through which activated oncogenes induce replication stress.

INTRODUCTION

Genomic instability (GIN) has been highlighted as a driving force of tumourigenesis by Hanahan and Weinberg in their celebrated ‘Hallmarks of cancer’ paper (1). GIN can result from changes in the number or structure of chromosomes (chromosomal instability), changes in the number of oligonucleotide repeats in microsatellite sequences (microsatellite instability), or base pair mutations, all of which are associated with activated oncogenes. Deregulation of DNA replication, known as replication stress (RS), is linked to GIN and is increased during the early steps of carcinogenesis (2-4). In particular, RS has been associated with chromosomal instability (5) as well as activation of the APOBEC3 family of deaminases (6) which increase the mutagenic load that fuels tumourigeneseis. In this review, we cover the latest findings on the RS response and discuss in detail the various mechanisms through which oncogenes induce RS.

DNA REPLICATION

DNA replication ensures the precise duplication of DNA during each cell cycle. It is a tightly regulated process that consists of two stages: licensing and initiation (reviewed in ref. 7). In eukaryotic cells, the licensing stage is restricted during late mitosis and G1-phase when thousands of replication origins are established along the genome and ensures that DNA replication occurs only once per cell cycle. For an origin to form, the origin recognition complex (ORC) binds at the origin site and recruits CDT1 and CDC6, which in turn facilitate loading of the mini-chromosome maintenance 2-7 (MCM2-7) helicases to form the pre-replicative complex (pre-RC) (Fig. 1A).

Cell cycle progression is controlled by cyclin dependent kinases (CDK) and the retinoblastoma/E2F pathway. CDK activity depends on binding to their regulatory subunits, cyclins, whose levels are regulated throughout the cell cycle by the anaphase promoting complex/cyclosome (APC/C). APC/C activity is high from late M to late G1-phase, hence CDK activity oscillates accordingly, being low during G1 and high during S/G2-phases. Mitogenic signalling by RAS triggers CYCLIN D/CDK4 to phosphorylate retinoblastoma, which renders it inactive, thus alleviating its inhibitory effect on the E2F family of transcription activators. E2F proteins promote expression of *CYCLIN A* and *E*, amongst other key S-phase genes, which upon binding to CDK2 during G1/S-phase partake in promoting entry into S-phase.

Replication initiation occurs as the cell proceeds into S-phase and requires the concerted action of CDK2 and DBF4/DRF1-dependent CDC7 kinases, which phosphorylates the pre-RC allowing recruitment of CDC45 and the GINS complex, and leads to the activation of the replicative helicase (CMG complex). Once this occurs, a replication bubble is formed and replication forks proceed bi-directionally from the origin (Fig. 1A). Under normal conditions, an excess of origins is licensed but only a small number of them becomes activated, with the remaining dormant origins reserved as a backup.

THE DNA REPLICATION STRESS RESPONSE

Replication is susceptible to impediments in DNA caused by both exogenous and endogenous DNA damaging agents and by the intrinsic properties of certain DNA sequences to adopt secondary structures. In particular, fork progression can be hindered due to interference with the transcription machinery, torsional stress or non-

B DNA structures (cruciforms, hairpins, trinucleotide repeats, R-loops, G-quadruplexes).

The ATR/CHK1 pathway

In response to RS, the cell initiates a DNA damage response (DDR) with the aim of resolving the damage or DNA secondary structures and restore fork progression (reviewed in ref. 8). During replication fork stalling, uncoupling between the replicative helicase and polymerase leads to the accumulation of single strand DNA (ssDNA) which is bound by RPA. ssDNA-RPA in turn allows the recruitment of the ATR kinase through ATRIP, as well as RAD17-RFC and RAD9-RAD1-HUS1 (9-1-1). The 9-1-1 complex also interacts with TOPBP1, which triggers ATR-ATRIP kinase activity, leading to the phosphorylation of numerous down-stream factors that collectively respond to RS. Recently, ETAA1 was identified as a novel RPA binding protein that activates ATR in response to DNA damage in parallel to TOPBP1/RAD17/9-1-1, and is involved in fork restart (9, 10). The synergistic action of the TIMELESS/TIPIN complex promotes binding of CLASPIN to RPA, which allows ATR to phosphorylate its primary substrate kinase, CHK1, at Ser-317 and Ser-345. Additionally, ATR phosphorylates histone H2AX at Ser-319 (γH2AX) early in the response. This modification then spreads away from the stalled fork, and is further sustained by two other DDR kinases ATM and DNA-PKcs.

CHK1 organises the cellular DDR by inducing cell cycle arrest, inhibiting late origin firing, activating dormant origin firing, and promoting fork stabilization and fork restart (Fig. 1B). Cell cycle arrest allows sufficient time for the cell to effect lesions repair and also prevent premature entry into mitosis with under-replicated DNA. In response to stress, CHK1 phosphorylates CDK activators CDC25A/C, which leads to

their degradation or nuclear export, thus triggering arrest at S, G2 or G2/M-phases. At the same time, CHK1 phosphorylates and activates the CDK antagonist WEE1 causing G2 delay.

During unperturbed early S-phase, ATR protects the genome from ssDNA formation by inhibiting origin firing and promoting nucleotide synthesis at the same time (11). In general, ATR regulates origin firing by phosphorylating MLL at Ser-516, stabilizing it on chromatin where it methylates histone H3K4, inhibits CDC45 loading and blocks origin activation (12). Additionally, CHK1 regulates replication initiation by binding and phosphorylating TRESLIN, which inhibits CDC45 loading onto origins (13). Recently, a backup pathway of CHK1 activation was identified, when upon ATR inhibition, accumulation of ssDNA produces aberrant DNA structures, that after processing by SLX4-MUS81 induce a DNA-PK-dependent CHK1 activation that inhibits origin firing (11).

In order to complete DNA replication in response to any disturbance, CHK1 inhibits origin firing at new replication factories (late origins), while at the same time allows the firing of dormant origins within active replication factories that experience stress (14, 15) (Fig. 1B).

Replication fork stabilization and reversal

Stabilization of the replication fork has long been considered a CHK1 response to RS, protecting it from deleterious nucleolytic processing. This view has been challenged recently by evidence from yeast, showing that fork stability is retained in the absence of checkpoint kinases (16). In addition, a SILAC-iPOND study in human cells, revealed ATR to be responsible for fork but not replisome stability in response to RS and that ATR protects the fork from various forms of collapse (17). Notably,

RS at an active fork triggers accumulation of homologous recombination proteins, whereas RS arising in the context of an origin that fired due to a checkpoint deficiency triggers accumulation of non-homologous end joining proteins (17).

A common mechanism of fork stabilisation involves the annealing of the parental DNA strands, followed by binding of the newly synthesized strands, thus forming a reversed fork structure, also known as regressed fork or a ‘chicken foot’ (Fig. 1B). Early studies in *S. cerevisiae*, showed that reversed forks accumulate in response to checkpoint defects (18), which led to the view that fork reversal is a pathological response. However, an expanding body of evidence has shown that reversed forks are also important for fork stability and protection from collapse. Reversed forks are formed through the action of many proteins, including RAD51 (19), PARP1 (20), BLM (21), WRN (22), SMARCAL1 (22), FANCM (23), FBH1 (24), HLTF (25) and ZRANB3 (26) and are favoured by positive supercoiling. Reversed forks are protected by BRCA1/2 (27), RAD51 (27), TOP1 (20), FANCA/B (28), FANCD2 (28), REV1 (29), WRN (30), BOD1L (31), RECQL5 (32) and WRNIP1 (33), in whose absence they are susceptible to the activity of nucleases MRE11 (27), DNA2 (34) and EXO1 (35) or resolvase Yen1 (36). Despite its positive role in resolving stalled forks, if unrestrained, fork reversal can cause fork collapse (37), highlighting the need for a regulated balance between reversal and restart.

Replication fork restart

Once a fork is stalled, different pathways, including homologous recombination, re-priming, template switching, translesion synthesis and break-induced replication may occur to allow replication restart (Fig. 2). The details of these mechanisms have been described elsewhere (38) and are not the focus of this review. Alternatively,

stalled forks may be resolved by an incoming fork from an adjacent origin. Evidence in yeast has shown that terminally arrested forks that are unprotected by Rad52/Rad51, cannot merge with a converging fork and appear in the ensuing mitosis as anaphase bridges (39). If fork stalling is sustained the forks often collapse into double strand breaks (DSBs) by the combined nucleolytic activities of SLX4 and nucleases MUS81-EME1, XPF-ERCC1 and EXO1 (reviewed in ref. 40).

Recent insights from the Hickson and Halazonetis labs discovered how under-replicated DNA is managed if it escapes replication/repair during S-phase. According to their data, regions of under-replicated DNA are processed by MUS81-EME1 during G2 and with the help of RAD52, POLD3 polymerase and SLX4-MUS81 perform mitotic DNA synthesis (MiDAS) to repair the collapsed forks (41-43). Furthermore, RECQL5 helicase is recruited to common fragile sites (CFSs) by MUS81 to remove RAD51 filaments from stalled replication forks to allow processing by MUS81-EME1 and enable MiDAS (44). Lack of MiDAS increases 53BP1 bodies, anaphase bridges and chromosomal rearrangements, promotes tumour growth and sensitizes cells to aphidicolin (41-43) (Fig. 2).

Targeting replication stress for cancer treatment

Importantly, RS can be exploited for cancer cell killing and thus has been described as an Achille's heel of cancer. ATR or CHK1 inhibition induces RS and synthetic lethality in CYCLIN E (45), c-MYC (46) and H/K-RAS (47) overexpressing cells, as well as MYC-induced lymphomas (48), MLL-ENL or NRAS-driven acute myeloid leukaemias and HRAS-expressing fibrosarkomas (46). Furthermore, WEE1 inhibition confers synthetic lethality in H3K36me3-deficient cancer cells by instigating deoxyribonucleoside triphosphate (dNTP) starvation and RS (49). In an intriguing

recent report, a combination of ATR and CHK1 inhibitors, result in fork slowing, replication catastrophe and synthetic lethality in cells overexpressing $HRAS^{V12}$ or c-MYC and in other cancer cell lines (50). In this case, CHK1 inhibition causes increased CDK-dependent origin firing that depletes dNTP pools, leading to fork slowing and ssDNA accumulation that is normally protected by an ATR-dependent deposition of RPA; the subsequent ATR inhibition deprotects the forks and kills the cell (50).

Reversing RS as a strategy to alleviate its tumourigenic effect has proven to be more complicated, as an extra CHK1 allele can reduce RS but surprisingly increases transformation (51). Nevertheless, replication fork stability is a significant contributing factor to chemotherapeutic drug resistance. This is exemplified by the fact that alleviation of MRE11-dependent GIN upon treatment with replication poisons in BRCA-deficient cells, by loss of PTIP, CHD4 or PARP1 confers synthetic viability and chemoresistance (52). All of the above, highlight the importance of RS as a hallmark and driver of cancer.

ONCOGENES

Normal cells become cancerous through a complex process known as oncogenic transformation. Transformation is driven by altered expression of oncogenes, tumor suppressors or microRNAs that derail their normal physiological function (reviewed in ref. 53). A proto-oncogene is a gene that under unperturbed conditions generally encodes a protein implicated in cell growth, differentiation or apoptosis. Either through point mutation, chromosomal translocation or copy number amplification, expression of the proto-oncogene is misregulated resulting in an activated oncogene. Oncogenes are translated into oncoproteins, which are classified as

growth factors, growth factor receptors, transcription factors, signal transducers, chromatin remodelers and apoptosis regulators. As such, oncogene activation may cause massive changes in the genome by deregulating cell cycle, metabolism, replication-timing or transcription, which ultimately drive GIN.

The principle mechanisms through which GIN is induced in cancer involve DNA repair defects, heightened replication stress and telomeric dysfunction (Fig. 3A). In hereditary cancers, GIN is commonly attributed to germline mutations in genes involved in DNA damage repair. These mutations give rise to unrepaired or inaccurately repaired DNA that lead to GIN (reviewed in ref. 54). Certain oncogenes can also cause a similar DNA repair defect. In particular, overexpression of oncogenic RAS and BRAF cause dissociation of BRCA1 from chromatin, which compromises DSB repair leading to DNA damage and senescence (55). Furthermore, wild type HRAS and NRAS modulate DDR to support the tumorigenic activity of oncogenic KRAS (56).

Oncogenes can also induce DNA replication defects and under-replicated DNA, which leads to accumulation of mutations and GIN. RS manifests in various forms, the most obvious being perturbed fork extension rates and/or heighted fork stalling/collapse, which may prevent complete replication of the genome. Such sites are marked by 53BP1 (57) and often correspond to CFSs, which are more susceptible to breakage by MUS81-EME1 or XPF-ERCC1. Replication through CFSs is exacerbated by mild RS, partly due to the absence or aberrant activation of dormant origins within these regions (58). During chromosomal segregation in mitosis, under-replicated regions often present as ultra-fine anaphase DNA bridges (UFBs) (59) that are bound by PICH, BLM and RPA with FANCD2 associated with

their extremities. Unresolved UFBs may lead to chromosome breakage and/or mis-segregation, resulting in micronuclei formation in daughter cells (59).

Finally, telomeric dysfunction can also lead to GIN. Telomeres are comprised of repeated sequences that maintain and protect chromosome ends from deleterious processing and/or unscheduled DNA repair. With each cell cycle, telomere repeats progressively shorten due to the 'end-replication problem', oxidative damage and RS associated with oncogenic activation. Telomeric dysfunction is linked to extended chromosomal shattering and rearrangements known as chromothripsis and kataegis, which drive GIN in cancer (60). Telomeric erosion is counteracted by the expression of telomerase that synthesizes *de novo* telomeric repeats at chromosome ends. Reports that HPV 16E6/E7-induced anaphase bridges (61) and oncogene-induced senescence (62) are ameliorated upon activation of telomerase activity, highlight the connection between telomeric integrity and GIN. Additionally, HRAS causes telomeric fork stalling, as well as telomeric fragility and loss of telomere repeats (63). Therefore, telomeric erosion can be linked to oncogene-induced telomeric replication stress, although it can also be attributed to oncogene-induced telomerase deregulation (61).

Apoptosis and senescence act as protective mechanisms that eliminate or halt cells that present with RS and/or GIN. This cancer protection barrier is quite robust as affirmed by the fact that expression of oncogenes alone does not lead to oncogenic transformation, unless combined with other genetic events, most notably, additional expression of other oncogenes or mutation of tumour suppressor genes (64-66). Oncogene-induced senescence is ascribed to the actions of the tumour suppressor p53 and its positive regulator p14/p19 (ARF). ARF inhibits the ubiquitin ligase MDM2 that is normally responsible for p53 degradation, thereby stabilizing

p53 levels. Amongst other oncogenes, p53 is activated in response to RAS, c-MYC, E1A and STAT5A overexpression either directly through ARF or RS-induced ATM activation. In addition, oncogenes *RAS*, *MYC*, *E2F1*, *β-CATENIN* and adenovirus *E1A* have been shown to upregulate *ARF*, while *c-MYC* causes ARF stabilization by inhibiting its ubiquitylation and subsequent degradation by ULF ubiquitin ligase (reviewed in ref. 67).

Of the 803 identified oncogenes in the ONGene database (http://ongene.bioinfo-minzhao.org/) only 27 have been assessed for their impact on RS, the majority of which are most commonly activated in human cancers. Additionally, for those oncogenes that have been studied there is variability in how RS is driven (Table 1). The concept that different oncogenes induce RS through different mechanisms is supported by many reports. In particular, RAS and CYCLIN E create unique landscapes of fragile sites that differ to the sites induced by aphidicolin treatment, which inhibits replication (68). Overexpression of CYCLIN E, but not A or D1, induces chromosomal instability in human cells (69). Overexpression of CYCLIN E and CDC25 also cause fork slowing and replication fork reversal at the same time point, but DSB formation and DDR signalling exhibit vastly different kinetics (70). It is worth noting that not all oncogenes lead to fork stalling, as exemplified by *DEK* that promotes fork progression under RS conditions (71) and *E1A* (72), *SPI1/PU.1* (73) and *LMO2* (74) that increase fork speed.

MECHANISMS OF REPLICATION STRESS INDUCTION BY ONCOGENES

In the following section, we will discuss the various mechanisms through which oncogenes induce RS.

Origin firing dysregulation

As mentioned earlier, replication is a fine-tuned process that ensures faithful DNA duplication once and only once per cell cycle. Dysregulation of CDK activity or mutations in the retinoblastoma/E2F pathway lead to perturbation of licensing or initiation, which in turn cause the unscheduled firing of origins. This may involve increased or decreased firing or re-firing of the same origin, all of which compromise physiological fork progression and lead to cells entering mitosis bearing under- or over-replicated DNA that eventually leads to GIN.

Different origins are activated at different time points during S-phase and modulation of CDKs affect the number of replication clusters rather than the origins within them (75). Interestingly, the level of origin firing inversely correlates with the rate of replication fork progression, which is believed to reflect the availability of essential replication factors (76). Under physiological conditions, origin firing is regulated by ATM and ATR by controlling CDK2 and CDC7 through CDC25A (77). CHK1 is crucial in this regulation, as its inhibition increases origin firing and leads to RS (78).

Decreased firing

Inhibition of DNA licensing reduces origin firing and induces GIN and increased sensitivity to RS-inducing agents (79). Compromised MCM loading due to reduced CDT1, CDC6 and ORC or increased CDT1 inhibitor GEMININ may hinder licensing. In the absence of functional p53, inhibition of DNA licensing in cancer cells allows their entry into S-phase with reduced origin firing (80). Similarly, ORC1 deletion reduces origin firing and increases sensitivity to hydroxyurea in tumour or MYC-expressing cells (81), suggesting a possible therapeutic approach to target certain

cancers. Moreover, CDK deregulation in G1 through inhibition of Cdh1 and Sic1 reduces origin firing and causes GIN (82). In addition, CDKs phosphorylate CDC6 and protect it from APC/C-dependent proteolysis during G1 (83), thus disruption of this mechanism would enable licensing and perhaps origin firing during S-phase.

Oncogenes have also been shown to affect replication origin licensing. In particular, *MYC* deregulates expression of both CDKs and E2Fs and modifies cell cycle progression (reviewed in ref. 84). On the other hand, there seems to be controversial data regarding *CYCLIN E*. In one report, CYCLIN E overexpression impairs MCM2, 4 and 7 loading onto chromatin during telophase and early G1, which reduces the number of active replication origins in early S-phase (85). In contrast to this, other groups have shown that CYCLIN E overexpression increases the number of origins firing in S-phase (86, 87). This discrepancy could be attributed to different properties of the cell lines used in these studies, as CYCLIN E can promote or inhibit pre-RC formation depending on cellular context (88).

Intriguingly, decreased origin firing increases fork speed progression (76), raising the question of how this causes GIN. In mouse, *Chaos3* is a viable mutation that destabilizes MCM4. MCM4$^{Chaos3/Chaos3}$ MEFs exhibit a 60% reduction of all MCM2-7 components, which does not affect origin firing but does reduce the number of dormant origins and increases fork speeds (89). Due to the inability of the cells to repair endogenous RS through firing of dormant origins, fork stalling occurs and RS markers such as γH2AX, pRAD17 and RPA are activated, suggesting that GIN can occur even in the absence of direct fork slowing (89). Following the same pattern, E1A expressing cells exhibit reduced origin firing and increased fork speeds (72).

Increased firing

Untimely origin firing is the result of disruption of initiation control. This has been shown in *X. laevis* where increasing CDK activity accelerates the origin firing pattern (75), as well as in other systems where dysregulation of ATR, CHK1 or WEE1 that control CDK or CDC7 activity, cause extensive origin firing (90, 78, 77). Dysregulation of origin activation increases the possibilities of conflicts with transcription (87) and may lead to enhanced depletion of replication building blocks such as dNTPs (90, 86, 91), histones (92) or RPA (93), which hinder fork progression (Fig. 3B).

Many oncogenes disrupt the physiological origin firing schedule. In particular, Di Micco et al. showed RAS to induce a hyper-proliferation phase accompanied by increased origin firing that contributes to RS (94). HPV E6/7 and CYCLIN E overexpression enhances origin firing leading to GIN (86), while c-MYC overexpression increases origin activity in a transcription-independent (95) but CDC45 and GINS-dependent manner (96). Interestingly, inhibition of origin firing failed to abrogate RAS-induced RS (97) but did rescue CYCLIN E–induced RS (98), which again hints at different mechanisms through which oncogene activation leads to RS.

Re-firing

Disruption of DNA licensing as well as CDK-dependent protective pathways enable re-replication events, which are associated with GIN and tumourigenesis (99). In particular, CDT1 (and to a lesser extent CDC6) overexpression induce re-firing that leads to GIN (100), while depletion of GEMININ induces re-firing and activation of the DDR (101). Moreover, compromising the CUL4-DDB1 ubiquitin ligase, which regulates CDT1 (102) or loss of EMI1 that indirectly regulates

GEMININ, also leads to re-replication (103). The ATR-dependent S-phase checkpoint surveys the genome and prevents re-replication caused by overexpression of licensing factors (but not GEMININ loss), either through p53-dependent activation of p21 or through dephosphorylation of retinoblastoma (104, 101, 100). Circumventing the retinoblastoma/E2F pathway is another way to instigate re-replication and CYCLIN E and c-MYC facilitate this (105), while HPV E7 ubiquitinates retinoblastoma marking it for degradation (106).

The main consequences of origin re-firing entail the so-called head-to-tail collisions that occur between the leading strand of the secondary fork and unligated Okazaki fragments of an adjacent fork, that cause DSBs and DNA damage checkpoint activation (99). Deregulated origin firing produces ssDNA gaps that promote re-replication at these sites, leading to replication fork breakage (107). Re-replication also causes fork slowing, although it is not clear if this is a consequence of head-to-tail collisions or due to normal forks colliding with the re-replication-induced DSBs. Overexpression of oncogenic RAS, CYCLIN E or MOS upregulate CDC6 (2, 94, 83) and in the case of RAS this causes re-replication (94). Furthermore, overexpression of a constitutively nuclear mutant form of CYCLIN D1 induces CDT1 stabilization that promotes re-licensing and re-replication (108).

Transcription

Transcription-replication conflicts

DNA transcription is a physiological process that may turn into an intrinsic source of GIN upon interference with the replication fork machinery. Transcription-replication conflicts (TRCs) impede replication fork progression as a result of head-on or co-

directional collisions between the two machines or between replication and R-loops (Fig. 4).

TRCs were first visualized by electron microscopy in *E. coli*, where an inducible origin was inserted upstream or downstream of an rRNA operon (109). Organisms have developed various strategies to avoid interactions between the two systems. In prokaryotes, where DNA is circular and there is a single origin of replication, co-orientation of the replisome and the transcription machinery is highly favoured (110). A similar orientation bias was also described in the human genome (111). In order to regulate replication through parts of the genome that are highly transcribed or difficult to replicate, both prokaryotes and eukaryotes have developed replication fork barriers comprising proteins bound tightly to DNA, which serve to limit TRCs. In eukaryotes where replication is mediated through multiple origins, transcription takes place throughout the cell cycle and is regulated spatially and temporally so that it does not clash with replication (112). Furthermore, in budding yeast, tRNA gene transcription is restrained during S-phase by Mec1/Mrc1/Rad53 in order to avoid collision with the replication machinery (113). RNApolII has a unique role in preventing and resolving TRCs, although it is not clear how this is achieved (114). Also, in *E. coli* the transcription factor DksA interacts with RNA polymerase (RNAP) and by altering the transcription elongation complex it prevents TRCs upon nutrition stress (115).

During transcription, RNAP may pause either as part of a regulatory checkpoint (promoter-proximal pausing) or in response to obstacles or misincorporated nucleotides that cause stalling and polymerase backtracking. Backtracking events involve RNAP sliding back and forth across DNA, which dislodges the 3’OH end of the RNA from the RNAP active site allowing it to exit through a secondary channel.

Backtracked RNAPs can hinder replication, as well as transcription, and cause GIN (116) (Fig. 4C, D). To deal with arrested or backtracked RNAPs, cells employ various strategies, such as i) cleavage of the misplaced RNA transcript by GreA/B or TFIIS, ii) reduction of misincoroporated nucleotides by DksA, iii) RNAP removal by the combined actions of ppGpp, DksA, GreA and Mfd or UvrD and NusA (reviewed in ref. 117) or iv) transcription regulation by RECQL5 (118).

R-loops

R-loops are DNA:RNA hybrids that are formed during transcription, when the newly synthesized RNA remains tangled around the template DNA, while the homologous ssDNA is displaced. Mapping of R-loops in mammals revealed that their formation is prevalent, conserved and dynamic across the genome and is favoured by increased transcription activity, polyA tracts and unmethylated CpG islands (reviewed in ref. 119). Information on R-loop forming sequences across the genome of various species can be found online at R-loopDB (http://rloop.bii.a-star.edu.sg). R-loops associate with specific epigenomic signatures at promoters and terminators and are involved in mitochondrial replication (120), transcription regulation (121), IgG class switch recombination (122), telomere maintenance in ALT cells (123) and homologous recombination-mediated DSB repair (124). Nevertheless, accumulation of R-loops can act as an obstacle to replication fork progression either through direct collisions (125) or indirectly through increased chromatin compaction (126), which leads to CFS formation (127) and GIN (128) (Fig. 4G). The ssDNA part of R-loops renders DNA more susceptible to DNA damaging agents, such as activation-induced cytidine deaminase (AID), which regulates class switch recombination and somatic hypermutation in mammalian B cells (reviewed in ref. 129). AID deaminates cytidine

only in ssDNA with base or nucleotide excision repair of these alterations, giving rise to mutations, chromosomal translocations or breaks that in turn may facilitate oncogenic activation.

R-loops may inhibit transcription progression (130) and consequently either on their own or through stalled or backtracked RNAPs act as additional barriers to transcription or replication (Fig. 4D). Their length may vary from a few hundred to over 1 kb and recent evidence in *S. cerevisiae* show that it is not length but histone modifications that discriminate between ‘benign’ and ‘malignant’ (131). In particular, Garcia-Pichardo et al. proposed a two-step model, where at first R-loop formation is facilitated by an altered chromatin configuration, followed by chromatin modifications (including H3S10 phosphorylation (126)) that render them culpable for GIN (131). In addition, experiments in Drosophila showed that depletion of linker histone H1 facilitates transcription of heterochromatic transcripts enabling R-loop accumulation (132). Similarly, in *C. elegans*, H3K9 methylation suppresses transcription of repetitive elements that enhance R-loops (133). Despite the acquired knowledge of R-loop-induced GIN, the exact mechanisms behind it still remain elusive.

R-loops are regulated by a plethora of molecules. In particular, RNaseH1 (128), RNaseH2 (134), SETX (135), DDX19 (136), DDX21 (137), DDX23 (138), DinG (139) and Pif1/Rrm3 helicases (140) degrade or unwind R-loops. ASF/SF2 (141), TOP1 (142), RECQ5 (143), THO/TREX (130), Npl3 (144), AQR (145) and XRN2 (146) prevent R-loop formation. Also, BRCA1/2 (147) and Fanconi anaemia proteins remove R-loops (148) while BRCA1 forms a complex with SETX at transcription termination pause sites, which collaborate to suppress R-loop accumulation (149). In addition, the chromatin reorganizing complex FACT facilitates resolution of R-loop-dependent GIN, most likely through remodelling chromatin at the sites of conflict

(150). Moreover, introns were recently identified as an unexpected source of R-loop regulation, as their presence within highly transcribed regions of yeast or human genomes protects against R-loop accumulation and DNA damage (151).

Since oncogenes enhance transcription, it is reasonable that they should also affect R-loop levels. Only recently, $HRAS^{V12}$ overexpression was shown to increase R-loop accumulation that was detrimental to fork progression (97). As part of the same study, $HRAS^{V12}$ caused stabilization of RNAseH1, which can be interpreted as an intrinsic response to increased R-loop levels. Likewise, CYCLIN E-induced RS was rescued upon RNaseH1 overexpression (87).

Orientation of transcription-replication conflicts

Orientation of TRCs has a profound effect on DNA integrity. Studies in yeast have shown that head-on collisions are more detrimental than co-directional ones with respect to fork slowing (152). Nevertheless, co-directional collisions also disrupt replication (153). Moreover, in bacteria co-directional collisions with backtracked transcription complexes cause DSBs, while head-on collisions do not (116). Further experiments in bacteria have shown that with both types of collision, the replisome resumes elongation after displacing RNAP from the DNA and in the case of co-directional collision it uses the nascent transcript as a primer (reviewed in ref. 154). Using a sophisticated episomal system to induce either head-on or co-directional TRCs, Hamperl et al. showed in human cells that replication itself modulates R-loop formation depending on the orientation of the TRC. In particular, co-directional TRCs resolve R-loops and activate the ATM-CHK2 pathway, while head-on conflicts between replication and an R-loop (but not an R-loop-less transcription machinery) promote R-loop accumulation and activation of the ATR-CHK1 pathway (125). They

also showed that normal replication fork progression suppresses R-loop accumulation, so any type of fork slowing increases R-loop levels (125). According to their proposed model, head-on TRCs cause fork slowing, which leads to increased R-loops levels that in turn activate ATR, while co-directional TRCs resolve R-loops, but the replisome may collide with backtracked RNAPs left by the R-loops that causes DSBs and ATM activation. Similar results were obtained in *B. subtilis*, where head-on TRCs induce R-loop formation, which stall replication, increase mutagenesis and prevent fork restart (155). These findings were further corroborated in *S. cerevisiae*, where in unchallenged conditions, a mutation in the MCM2-7 complex modulates replication and causes accumulation of R-loops in S-phase (156). Taken together, these data disclose a conserved and complex mechanism that protects the genome from TRC-related GIN.

Topological stress

Topological constraints may also develop between the replication and transcription machines as they move along DNA, causing positive or negative supercoiling, that may act as an obstacle to fork progression (157) (Fig. 4E). Furthermore, it has been suggested that nascent RNA can become tangled around template DNA and form a loop during nuclear export (Fig. 4F). These loops can obstruct the release of torsional stress from incoming replication forks and thus impede fork progression (158). Torsional stress is released by the activity of topoisomerases I and II, which relax positive supercoiling by creating nicks in DNA (159). RECQ5 facilitates SUMOylation of TOP1 which enables TOP1 binding to RNAPolII and at the same time compromises its activity, indicative of a regulatory mechanism of TOP1-induced genotoxicity (143). Also, in yeast Mec1 and Rad53

phosphorylate nucleoporin Mlp1 and resolve the topological stress (158). Finally, according to a recent study, p53 loss causes stabilization of TOP2A on DNA, as well as fork retardation, suggesting a possible role in preventing torsional stress between the two machines (160).

G-quadruplexes

G-quadruplexes are four-stranded structures held by G-G base pairs that are formed co-transcriptionally and impede replication fork progression leading to breakage (Fig. 4H). G-quadruplexes can form within the displaced ssDNA part of an R-loop of an active transcription bubble (161), where they facilitate R-loop stabilization (162). G-quadruplexes are also found within start sites of highly transcribed genes such as *MYC* (163), *KRAS* (164) or *PTEN* and regulate transcription (165). Moreover, the ORC complex has been suggested to bind to G-quadruplexes at putative origin sites of replication initiation (166). In order to avoid potential collisions with the replication machinery, helicases such as Pif1, RTEL1 or DHX9 bind G-quadruplexes and unwind them to maintain genomic stability (reviewed in ref. 167).

TRCs-associated DNA damage response

Certain areas of the genome are more susceptible to TRCs. Barlow et al. identified highly unstable regions of the genome, named early replication fragile sites (ERFSs) that are more prone to breakage upon RS than CFSs (168). ERFSs are usually found within highly transcribed gene clusters and although they may break during normal replication, their fragility is increased in response to hydroxyurea-induced RS, ATR inhibition, *c-MYC* expression or increased transcription (168).

Additionally, long genes are more prone to TRCs, with frequent R-loop accumulation and induction of CFSs instability (127).

In response to DNA damage or TRCs, the cell tends to shut off transcription to reduce further collisions and facilitate replication restart. This is evident in yeast, where genotoxic-induced RS causes tRNA gene transcription repression by Maf1 (113) and through the synergistic effect of checkpoint sensor Mec1-Ddc2, the chromatin remodelling complex INO80C and transcription complex PAF1C, RNAPolII is evicted from chromatin and degraded (169). Accordingly, Dcr1 releases RNApolII from highly transcribed genes that are sites of replication-transcription collisions to prohibit further instability (170). Moreover, in response to transcription-blocking DNA damage, R-loop-dependent ATM activation triggers spliceosome displacement from chromatin, causing widespread splicing changes that presumably facilitate DNA repair (171). Interestingly, the de Bruin lab has shown that the cell responds to RS by upregulating transcription of E2F target genes that maintain checkpoint activation and facilitate DNA damage tolerance (172, 173). These data suggest that there needs to be a fine tuning between decreasing deleterious transcription and retaining and/or upregulating levels of checkpoint proteins.

Various proteins, especially helicases, preserve genomic stability by preventing or resolving TRCs. Amongst them, RECQL5 has a dual role in resolving conflicts between replication and RNApolI/RNApolII-dependent transcription by either promoting RAD18-dependent PCNA ubiquitination or through its helicase activity resolves RAD51-dependent replication intermediates at these sites (174). In *S. pombe*, Pfh1, a member of the Pif1 family of helicases, facilitates replication across highly transcribed RNApolII- and RNApolIII-dependent genes (175). Likewise, in *S. cerevisiae*, another Pif1 family member, Rrm3 allows replication through TRCs (152).

Moreover, in *E. coli*, helicases DinG, Rep and UvrD collaborate to promote replication through transcription units (139).

In response to oncogenic signalling, transcription activity is enhanced (176-178, 38, 179, 180) and collisions between replication and transcription are more likely to occur. This is usually attributed to point mutations in the promoter region of proto-oncogenes that increase transcription activity. c-MYC is itself a transcription factor that under normal conditions drives a transcriptional programme that controls cell growth and division. Mitogenic growth signalling may deregulate c-MYC which can directly enhance RNApolI- (178), RNApolII- (179) and RNApolIII-dependent transcription (177). c-MYC increases transcription indirectly, by up- and/or downregulating selective target genes that in turn modify the cellular state and enable global RNA production (181). This transcription amplification has been documented in patient-derived tumours with elevated c-MYC thus establishing a link to tumorigenesis (179). CYCLIN E overexpression enhances both transcription activity and origin firing, causing increased TRCs and subsequent RS that can be rescued by either transcription or CDK inhibition (87). This is not the case with RAS overexpression, which causes RS, which can be rescued by transcription but not origin firing inhibition (97). RAS-induced enhanced transcription is driven through upregulation of transcription factors such as TBP, which is required for transcription of all promoters (97). Interestingly, overexpression of TBP alone causes RS, DNA damage, senescence and contributes to oncogenesis (182, 97).

A very recent report shed light on the mechanisms underlying oncogene-induced TRCs and GIN. Using sensitive seq-based assays Macheret and Halazonetis mapped replication initiation sites in human cells (183). They showed that under physiological conditions, transcription suppresses intragenic origin firing, but upon

overexpression of CYCLIN E or MYC, G1-phase shortening drives earlier S-phase entry before completion of transcription. This allows origin firing within transcribing genes which leads to TRCs, fork collapse and DSB formation specifically at these sites. Moreover, this study revealed that fork collapse at oncogene-induced intragenic fired origins causes chromosomal translocations in a transcription-dependent manner both in their experimental setup as well as in a large cohort of human cancers (183). This highlights the importance of TRCs amongst the other mechanisms in inducing GIN.

Nucleotide metabolism

Nucleotides are the building blocks of the replication machine, therefore any dysregulation of their structure or levels has an immediate effect on fork progression. In mammals, dNTPs are either synthesized through the *de novo* pathway in the cytoplasm or by recycling nucleosides and nucleobases from nucleotide degradation through the salvage pathway, which acts both in the cytoplasm and in mitochondria (Fig. 5). The crucial step in dNTP synthesis is the reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, which is catalysed by the ribonucleotide reductase (RNR). RNR is a tetrameric protein composed of two catalytic (RRM1) and two regulatory (RRM2, RRM2B) subunits. RRM2 levels are rate-limiting for RNR activity and consequently dNTP levels, so in order to retain balanced dNTP pools, RRM2 expression increases in S-phase and is low during G1, G2 and M-phases (184). There is increasing evidence that part of RNR associates with the replication fork and that in response to DNA damage RNR components re-localize towards repair foci to increase dNTP availability (reviewed in ref. 185). In addition, ATR regulates dNTP pools by increasing RRM2 levels in response to DNA

damage (11, 49). Similarly, MEFs from mice carrying an extra allele of RRM2 display increased RNR activity, which reduces chromosomal fragility following ATR inhibition (186).

dNTP levels are inversely related to origin firing and are key determinants of replication fork speed (90, 86, 91). Increased RNR activity accelerates fork progression, while reduced nucleotide pools instigate a transition to a slow replication mode with reduced origin usage, minutes after entry into S-phase (86, 91). RRM2/RRM2B-induced increase in replication fork speed causes misincorporated uracil into DNA and breaks at fragile sites and it has been suggested that dUTPase can sanitize this to avoid GIN (187). Nevertheless, dNTPs need to be regulated within certain levels, as increased dNTP pools will cause aberrant replication and lead to mutations (188).

With regards to the effect of oncogenes on nucleotide metabolism, BCL2 binds to RRM2 and disrupts the RRM1/RRM2 complex inhibiting RNR activity, which reduces nucleotide pool levels and leads to fork slowing (189). Moreover, overexpression of RAS downregulates RRM2 through binding of transcription repressor E2F7 to the RRM2 promoter, causing dNTP pool depletion, RS and senescence (190). In addition, CYCLIN E or HPV-16 E6/E7 overexpression activate cell proliferation but do not affect nucleotide biosynthesis, resulting in dNTP pool depletion and impaired fork progression (86). In contrast, c-MYC upregulates genes involved in nucleotide biosynthesis, increasing dNTP pools and enabling cell proliferation (191).

Upon reduced dNTP levels, there is increased incorporation of ribonucleotides (rNTPs) into DNA during replication. Since rNTPs are more prone to hydrolysis than dNTPs, their integration into DNA affects fork progression and may lead to GIN. Misincorporated rNTPs can be detrimental to genomic stability, as depletion of

RNaseH2 that removes rNTPs causes RS (192). Despite the fact that there is no record of oncogenes promoting misincorporation of rNTPs on DNA, it seems quite likely that this may occur during the hyper-proliferation stage following oncogenic activation.

The integrity of dNTPs is another critical factor that may cause GIN. Reactive oxygen species (ROS) can oxidize nucleotides that when incorporated into DNA trigger breaks, mutations and senescence (193). Oxidized dNTPs trigger fork slowing in *X. laevis*, but this is attributed to a protein kinase C-mediated reduction in DNA replication and not to an actual fork retardation (194). So, although it seems reasonable for oxidized dNTPs to cause fork slowing it still has not been shown definitively (Fig. 5). A hint towards this view lies with MTH1, which is a non-essential enzyme that sanitizes oxidized dNTPs, hindering them from being incorporated into DNA, thus preserving genomic integrity.

Reactive oxygen species metabolism

ROS are an assorted class of free radical species produced under normal conditions as byproducts of aerobic metabolism (reviewed in ref. 195). ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH•) and their biological effects are proportional to their levels. Basal ROS levels uphold physiological cellular functions as part of redox biology, while increased levels of ROS cause oxidative stress by damaging lipids, proteins and DNA or upregulating protein kinase Cδ and inducing senescence or apoptosis. O_2^- is produced either by oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) by NADPH oxidase enzymes (NOXs) or through aerobic respiration in mitochondria. H_2O_2 is generated from O_2^- by superoxide dismutases and exerts its effect on redox biology

or oxidative stress or may be converted to H_2O by antioxidant proteins. Finally, OH• are produced from H_2O_2 in the presence of Fe^{+2} (196) (Fig. 5).

Cancer cells exhibit abrupt alterations in their metabolism to support their rapid growth. These include ATP generation to maintain energy levels, increased synthesis of macromolecules and maintenance of cellular redox status. Oncogenes are known to induce ROS and cause DNA damage. Expression of activated RAS increases intracellular and mitochondrial ROS and leads to senescence (197), which is partially achieved by upregulating NOX4-$p22^{phox}$ (198). c-MYC overexpression also induces ROS and causes DNA damage (199). A comparative study between *RAS* and *MYC* showed that both oncogenes evoke changes in the cellular metabolism patterns and induce different degrees of oxidative stress and RS, highlighting again that different oncogenes follow different mechanisms to cause RS (200). Moreover, in response to $KRAS^{G12D}$, $B\text{-}RAF^{V619E}$ and MYC^{ERT2} primary murine cells respond by upregulating transcription factor Nrf2 that regulates an antioxidant program used to detoxificate the organism (201). In addition, expression of E6/E7 promotes NOX-dependent generation of ROS and subsequent DNA damage in head and neck cancer cells (202).

It is unclear how ROS affect fork progression. According to the D'adda di Fagagna lab, RAS induces ROS and this triggers the hyper-proliferation that leads to RS and senescence (94, 197, 203). On the other hand, CYCLIN E-induced hyper-proliferation is independent of ROS (2). Another case involves ROS oxidizing dNTPs, as was covered in the previous chapter.

CONCLUDING REMARKS

Oncogene-induced RS has long been recognised as an early driver of cancer and investigating the mechanics of this process has been established as a field in its own right. Understanding RS has accelerated cancer diagnosis and assisted the development of more sophisticated anticancer treatments. Nevertheless, the mechanics of how oncogene activation lead to RS present a complicated kaleidoscope of intertwined pathways and to make matters worse, various oncogenes may differentially affect these pathways based on the cell type or the time point their effect is assessed. So, the consensus that different oncogenes exert a different effect at different time points needs to be more widely adopted.

Despite the importance of each of the different mechanisms of oncogene-induced RS presented here, oncogene-induced deregulation of transcription appears to play the most significant role in cancer development, in part, due to its multifaceted nature in inducing GIN. The role of TRCs is especially intriguing, as currently little is known regarding the proteins and pathways involved in their detection and repair, as well as the implication of oncogenes in them. Although there is growing interest in R-loops and their involvement in GIN, there are a lot to be explored on their regulation and role in genome wide RS and telomere erosion and potential connections between them. Finally, there are still unanswered questions on the role of G-quadruplexes, rNTP missincorporation and oxidized nucleotides in oncogene-induced RS that need to be addressed.

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oncogene	genetic alteration	effect on fork progression	↑DSBs	↑ROS	↑transcription / R-loops	affects nucleotide pools	activates			induces senescence
							γH2AX	ATR	ATM	
AKT-2	pm, oe			§204						§204
AML1/ETO	oe		205				205		205	205
AURORA A	oe	↓206								207
β-CATENIN	oe						208			208
BCL-2	oe	↓189				189				209
BCR/ABL	oe, tr		210, 205	210, 211			X205		X205	205
BRAF	oe, pm			212		190	190			190, 213, 212, 214
B-MYB	kd	↓215	215				215			216
CDC6	oe		217				3		3	3
CDC5A	oe	↓70	70				2, 218, 70	2, 70	2, 70	
CYCLIN D	oe, tr, kd	↓108					108, 219	X219	108	220
CYCLIN E	oe	↓3, 86, 87, 70	3, 70		§87	86	2, 3, 86, 87, 70, 219	2, 3, 70, 219	2, 70	3
DEK	kd	↑71	221				221, X71	X71	X71	216, 221
E1A	oe	↑72					72	X72	72	
E2F1	oe						2, 218, 222		218, 222	223, 222, 224
EGFR	oe			225			225			225
HPV E6/E7	oe	↓86	226, 202	202		86	86, 226, 224, 219			
LMO1-4	oe, kd	↑74			176, 180					
MDM2	oe	↓227						227		
MOS	oe		3				3		3	3
MYC	pm, oe	↓200, 96	228, 199	200, 228, 199	177-179	191	229, 200, 96	229	228, 96	199
MYH11/CBFB	oe		X205				X205		X205	205
NPM-ALK	oe			230	231		232, 233	232	232	233
RAS	pm, oe	↓234, 97, 200	97, 205, 198	197, 225, 200, 203, 198	97	190	190, 94, 97, 225, 224, 200, 203, 205, 198	234, 94, 97, 224	224,94, 205	190, 94, 225, 224, 203, 66, 205
SPI-1/PU.1	oe	↑73	X73				X73			
STAT5A	oe						224	224	224	224
TAX	oe	↓235	235, 236	236, 237			235, 236			236

TABLE LEGENDS

Table 1. List of oncogenes and their effect on replication stress-related response. §: Indirectly, ↑: Positive effect, ↓: Negative effect, X: No effect, tr: translocation, pm: point mutation, oe: overexpression, kd: knockdown.

FIGURE LEGENDS

Figure 1. DNA replication and replication stress response. **A,** in late mitosis and throughout G1-phase, the pre-replicative complex comprised by the ORC complex, CDC6 and CDT1 is recruited to replication origins to facilitate loading of the MCM2-7 complex. During G1-phase, retinoblastoma (Rb) is bound to E2F rendering it inactive. Phosphorylation of Rb by the Cyclin D-CDK4 complex alleviates its inhibitory effect on E2F. APC/C activity is high from late M- to late G1-phase regulating CDK activity. Upon entry in S-phase, APC/C is inhibited and CDKs are activated throughout S, G2 and early M-phase. CDKs form complexes with E2F-regulated cyclins that collaborate with CDC7 to phosphorylate TRESLIN and MCM2-7 complex, activating the CMG (CDC45-MCM2-7-GINS) helicase complex. Simultaneously, clamp loader RFC and sliding clamp PCNA are recruited and enable polymerases δ and ε to initiate replication in the lagging and leading strands respectively. **B,** when a replication fork is stalled, ssDNA is generated as the CMG complex unwinds DNA. ssDNA binds RPA that recruits ATR (through ATRIP), RAD17-RFC and 9-1-1 complexes at the stalled fork. ATR is then activated by TOPBP1/RAD17/9-1-1 and ETAA1 and phosphorylates H2AX, while through TIMELESS/TIPIN/CLASPIN phoshoprylates CHK1. CHK1 then organises the RS response by arresting the cell cycle, inhibiting new origin firing, enabling dormant origin firing and stabilizing the fork, which can then be reversed by various proteins

in a chicken foot structure. An unprotected reversed fork is susceptible to nucleolytic degradation by MRE11, EXO1 and DNA2. A stalled fork can restart through homologous recombination, re-priming, template switching, translesion synthesis or break-induced replication. Alternatively, it will collapse into DSBs by the combined activity of MUS81-EME1, XPF-ERCC1, EXO1 and SLX4 that will drive the cell to senescence.

Figure 2. Repair of a stalled fork. Upon encountering an obstacle, replication machine arrests and the CMG complex unwinds DNA ahead of the stalled fork, leaving ssDNA behind. Replication can resume through various mechanisms, such as translesion synthesis, template switching, re-priming, break-induced replication or homologous recombination. If this fails, the stalled fork is cleaved by MUS81-EME1 during G2-phase and upon entrance in metaphase RAD52, POLD3 and MUS81-SLX4 collaborate to facilitate replication of the under-replicated DNA through MiDAS. In the absence of MiDAS and during chromosomal segregation in anaphase, UFBs are formed at the CFSs, which will appear as micronuclei or 53BP1 bodies in the daughter cells.

Figure 3. Oncogene-induced genomic instability. **A,** germline mutations in DNA repair genes lead to GIN. Activated oncogenes elicit genomic instability by causing defects in DNA repair, telomere erosion or replication stress; see text for details. **B,** activated oncogenes induce a multifaceted set of intertwined activities that deregulate fork progression leading to under-replicated DNA and genomic instability. In particular, through deregulation of the Rb/E2F pathway licensing factors are increased which instigates origin re-firing that decreases fork speed in response to head-to-tail fork collisions. Deregulation of CDK activity can decrease or increase

origin firing. In the first case, fork speed is initially increased, but the cell ends up with under-replicated DNA due to its inability to rescue endogenous RS by firing dormant origins. Increased origin firing raises the possibility of TRCs and simultaneously may cause depletion of dNTPs, histones or RPA. Oncogene-induced ROS either increase origin firing or oxidize nucleotides that potentially may affect fork progression. Oncogenes also increase transcription activity that either directly or through R-loops enhance TRCs.

Figure 4. Transcription-associated replication stress. **A,** upon head-on conflicts between replication and transcription, R-loop formation is increased and ATR/CHK1 are activated. **B,** upon co-directional conflicts between replication and a stalled or backtracked RNAP, DSBs are formed that activate ATM/CHK2 and R-loops are resolved. **C,** in response to any obstacle an RNAP may pause and backtrack and act as an impediment to fork progression; **D,** this can also occur as a result of an encounter with an R-loop. In both cases, this will cause accumulation of arrested or backtracked RNAPs that hinder fork progression. **E,** while the replication and transcription machineries move, positive supercoiling develops in front of them, that can impede progression of both. **F,** transcribed RNA may get trapped in the nuclear pore complex generating obstacles to fork progression. **G,** R-loops can cause chromatin condensation, marked by H3 phosphorylation at Ser-10, that impedes fork progression. **H,** G-quadruplexes can be formed co-transcriptionally or within the ssDNA part of an R-loop and act as obstacles to fork progression.

Figure 5. Nucleotide and ROS metabolism as a cause of replication stress. dNTPs are formed either through the *de novo* pathway from glutamine, glycine, folic acid,

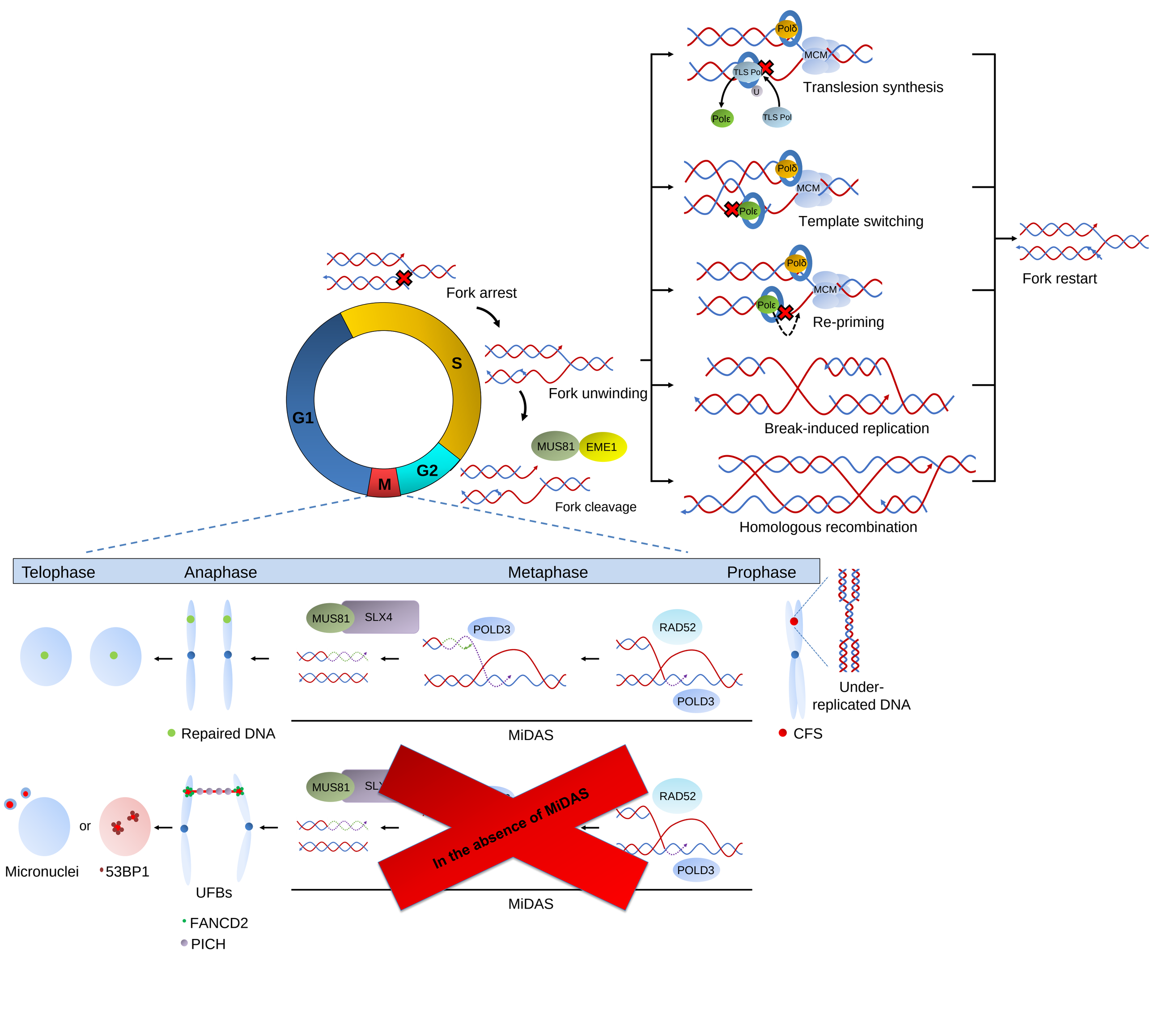
aspartate, 5-phosphoribosyl-1-pyrophosphate or by nucleoside degradation through the salvage pathway. The reduction of ribonucleosides to dNTPs is catalysed by the ribonucleotide reductase (RNR), which is compromised by two catalytic (RRM1) and two regulatory (RRM2, RRM2B) subunits. Oncogenes may target RNR activity or induce hyper-proliferation that will both reduce dNTP pool affecting fork progression. Oncogenes also induce production of ROS, including O_2^-, H_2O_2 and OH•. O_2^- is produced either by oxidation of NADPH by NOX enzymes or through aerobic respiration in mitochondria. H_2O_2 is generated by O_2^- which is converted by superoxide dismutase (SOD), while OH• are produced from H_2O_2 in the presence of Fe^{+2}. It is not clear if oxidized dNTPs affect replication fork speed.

A

GINS
MCM
CDC45
P
TRESLIN
CDC7
E2F active
Cyclin A
CDK2
Rb
E2F
Cyclin E
CDK2
PCNA
RFC
Polε
Polδ
MCM
Cyclin D
CDK4
↑ APC/C
↓ CDK
↓ APC/C
↑ CDK
S
G1
G2
M
Rb
E2F
E2F inactive
CDT1
ORC
CDC6

B

PCNA
Polε
MCM
Polδ
RPA
9-1-1
γH2AX
RAD17
TOPBP1
ETAA1
ATR
ATRIP
TIMELESS
TIPIN
CLASPIN
S
G1
M
G2
Cell cycle arrest
CHK1
Dormant origin firing
Inhibited late origin firing
SMARCAL1, BLM,
WRN, PARP1,
BRCA2, RAD51,
FANCM, FBH1
MRE11,
EXO1,
DNA2
Fork stabilization
Fork regression
Homologous Recombination
Re-priming
Template switching
Translesion synthesis
Break-induced replication
Fork restart
senescence
MUS81-EME1,
XPF-ERCC1,
EXO1, SLX4
Fork collapse-DSBs


Polδ
MCM
TLS Pol
U
Polε
TLS Pol
Translesion synthesis
Polδ
MCM
Polε
Template switching
Polδ
MCM
Polε
Re-priming
Break-induced replication
Homologous recombination
Fork restart
Fork arrest
S
G1
G2
M
Fork unwinding
MUS81
EME1
Fork cleavage
Telophase
Anaphase
Metaphase
Prophase
MUS81
SLX4
POLD3
RAD52
POLD3
MiDAS
Under-
replicated DNA
CFS
Repaired DNA
or
Micronuclei
53BP1
UFBs
FANCD2
PICH
MUS81
RAD52
POLD3
MiDAS
In the absence of MiDAS

A

Germline mutations

Oncogenes

Defective DNA repair

Telomere erosion

Replication stress

BRCA1, *BRCA2*,
PALB2, *RAD50*,
NBS1, *WRN*, *BLM*,
RECQL4, Fanconi
Anaemia genes

Micronuclei

53BP1 bodies

Fork slowing

Telomere fragility

Telomere loss

CFSs
ERFs

UFBs

Genomic instability

B

Oncogenes

↓Firing

Re-Firing

↑Firing

ROS

Transcription

Nucleotide metabolism

R-loops

dNTP oxidation

Transcription conflicts

RPA, histone, dNTP depletion

↑Fork speed

Head-to-tail
fork collisions

Under-replicated DNA

↓Fork speed

Genomic instability

↑R-loops

A

Head-on TRC

R-loop

Polε

MCM

RNAP

Polδ

R-loops increase

ATR

CHK1

ATR-CHK1 activation

B

Co-directional TRC

ATM-CHK2 activation

ATM

CHK2

Backtracked or
arrested RNAP?

R-loops decrease

C

Backtracked RNAP

3’

D

Queue of arrested or backtracked RNAPs

E

Torsional stress

F

Nuclear pore complex-
induced loop

G

H3S10

Increased chromatin
compaction

H

G-quadruplex

Glutamine, glycine, folic acid, aspartate, 5-phosphoribosyl-1-pyrophosphate

Oncogenes

de novo pathway

nucleosides

Salvage pathway

RRM1 RRM2

RRM1 RRM2B

dNTPs

hyper-proliferation

Deregulated dNTP metabolism

NADPH O_2

NOX mitochondria

$NADP^+$ O_2^-

SOD

Fe^{+2}

$OH\bullet$ H_2O_2

normal dNTP pool

reduced dNTP pool

oxidized dNTPs

?