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

Panagiotis Kotsantis 1, Eva Petermann 2, and Simon J. Boulton 1

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 tumorigenesis. Part of this cellular turmoil involves deregulation of physiologic 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 tumorigenesis 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 tumorigenesis by Hanahan and Weinberg in their celebrated “Hallmarks of Cancer” article (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 tumorigenesis. 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 G 1-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 minichromosome maintenance (MCM2-7) helicases to form the prereplicative 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 G 1 -phase; hence, CDK activity oscillates accordingly, being low during G 1 and high during S/G 2 -phases. Mitogenic signaling by RAS triggers CYCLIN D/CDK4 to phosphorylate retinoblastoma (RB), 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, among other key S-phase genes, which upon binding to CDK2 during G 1/S-phase partake in promoting entry into the S-phase.

Replication initiation occurs as the cell proceeds into the S-phase and requires the concerted action of CDK2 and DBF4/DRF1–dependent CDC7 kinases, which phosphorylate 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 bidirectionally from the origin (Fig. 1A). Under normal conditions, an excess of origins
Figure 1. DNA replication and RS response. A, In late mitosis and throughout G1-phase, the prereplicative complex comprised of 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 the 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 phosphorylates CHK1. CHK1 then organizes 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, repriming, 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.
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is licensed but only a small number of them become activated, with the remaining dormant origins reserved as a backup.

THE DNA RS 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 restoring 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 (9–11). The 9-1-1 complex also interacts with TOPBP1, which triggers ATR–ATRIP kinase activity, leading to the phosphorylation of numerous downstream factors that collectively respond to RS. Recently, ENA1 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 CLASP1 to RPA, which allows ATR to phosphorylate its primary substrate kinase, CHK1, at Ser-317 and Ser-345. In addition, ATR phosphorylates histone H2AX at Ser-319 (H2AX) 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 organizes 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 lesion repair and also prevent premature entry into mitosis with underreplicated DNA. In response to stress, CHK1 phosphorylates the 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 WEEl1, 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). In addition, 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 allowing the firing of dormant origins within active replication factories that experience stress (Fig. 1B; refs. 14, 15).

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 nonhomologous end joining proteins (17).

A common mechanism of fork stabilization 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 Saccharomyces cerevisiae (S. cerevisiae), showed that reversed forks accumulate in response to checkpoint defects (18), which led to the view that fork reversal is a pathologic 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), FANCN (23), FBH1 (24), HHTF (25), and ZRANB3 (26), and are favored 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, repriming, 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 (DSB) 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 laboratories discovered how underreplicated DNA is managed...
if it escapes replication/repair during S-phase. According to their data, regions of underreplicated 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 (CFS) 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 tumor growth, and sensitizes cells to aphidicolin (refs. 41–43; Fig. 2).

**Targeting RS for Cancer Treatment**

Importantly, RS can be exploited for cancer cell killing and thus has been described as an Achilles’ heel of cancer. ATR or CHK1 inhibition induces RS and synthetic lethality in CYCLIN E (45), c-MYC (46), and H/KRAS (47)–overexpressing cells, as well as MYC-induced lymphomas (48), MLL–ENL or NRAS-driven acute myeloid leukemias, and HRAS-expressing fibrosarcomas (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 resulted in fork slowing, replication catastrophe, and synthetic lethality in cells overexpressing HRASV12 or c-MYC and in other cancer cell lines (50).

**Figure 2.** Repair of a stalled fork. Upon encountering an obstacle, the 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, repriming, 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 underreplicated 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.
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Reversing RS as a strategy to alleviate its tumorigenic effect has proved 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 miRNAs that derail their normal physiologic 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 principal mechanisms through which GIN is induced in cancer involve DNA repair defects, heightened RS, 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 unrepaird 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 causes dissociation of BRCA1 from chromatin, which compromises DSBR 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 underreplicated DNA, which leads to accumulation of mutations and GIN. RS manifests in various forms, the most obvious being perturbed fork extension rates and/or heightened fork stalling/collapse, which may prevent complete replication of the genome. Such sites are marked by 53BP1 (57) and often correspond to CSFs, which are more susceptible to breakage by MUS81–EME1 or XPF–ERCC1. Replication through CSFs 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, underreplicated regions often present as ultrafine anaphase DNA bridges (UFB; ref. 59) that are bound by PICH, BLM, and RPA with FANCD2 associated with their extremities. Unresolved UFBs may lead to chromosome breakage and/or missegregation, 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 unsscheduled 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 karyotypic, 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 16 E6/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. In addition, 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 RS, 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 tumor suppressor genes (64–66). Oncogene-induced senescence is ascribed to the actions of the tumor 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. Among 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, the oncogenes RAS, MYC, E2F1, and β-CATENIN and the adenovirus E1A have been shown to upregulate ARF, whereas 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. In addition, 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 from 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 causes fork slowing and replication fork reversal at the same time point, but DSBR formation and DDR signaling 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 RS INDUCTION BY ONCOGENES

In the following section, we will discuss the various mechanisms through which oncogenes induce RS.
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 RS; see text for details. **B**, Activated oncogenes induce a multifaceted set of intertwined activities that deregulate fork progression, leading to underreplicated DNA and genomic instability. In particular, through deregulation of the RB/E2F pathway, licensing factors are increased, which instigates origin refiring 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 underreplicated 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 enhances TRCs.

**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 RB/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 refiring of the same origin, all of which compromise physiologic fork progression and lead to cells entering mitosis bearing under-replicated or overreplicated DNA that eventually leads to GIN.

Different origins are activated at different time points during the S-phase, and modulation of CDKs affects 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).
Table 1. List of oncogenes and their effect on RS–related response

<table>
<thead>
<tr>
<th>Oncogene</th>
<th>Genetic alteration</th>
<th>Effect on fork progression</th>
<th>DSBs$^a$</th>
<th>ROS$^a$</th>
<th>Transcription/ R-loops$^a$</th>
<th>Affects nucleotide pools</th>
<th>Activates</th>
<th>Induces senescence</th>
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NOTE: All numerals in the table are reference numbers.
Abbreviations: kd, knockdown; oe, overexpression; pm, point mutation; tr, translocation.
$^a$ Positive effect.
$^b$ Indirectly.
$^c$ Negative effect.
$^d$ No effect.
Under physiologic 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 tumor 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 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*Chaos*/Chaos* mouse embryonic fibroblasts (MEF) 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 marks 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 *Xenopus laevis* (*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, which control CDK or CDC7 activity, causes extensive origin firing (77, 78, 90). 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 (86, 90, 91), histones (92), or RPA (93), which hinder fork progression (Fig. 3B).

Many oncogenes disrupt the physiologic origin firing schedule. In particular, Di Micco and colleagues showed RAS to induce a hyperproliferation 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), whereas 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 (87), which again hints at different mechanisms through which oncogene activation leads to RS.

**Refiring**

Disruption of DNA licensing as well as CDK-dependent protective pathways enable re-replication events, which are associated with GIN and tumorigenesis (98). In particular, CDT1 (and to a lesser extent CDC6) overexpression induces refiring that leads to GIN (99), whereas depletion of GEMININ induces refiring and activation of the DDR (100). Moreover, compromising the CUL4-DDB1 ubiquitin ligase, which regulates CDT1 (101) or loss of EMI1 that indirectly regulates GEMININ, also leads to re-replication (102). 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 RB (99, 100, 103). Circumventing the RB/E2F pathway is another way to instigate re-replication, and CYCLIN E and c-MYC facilitate this (104), whereas HPV E7 ubiquitinates RB, marking it for degradation (105).

The main consequences of origin refiring 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 (98). Deregulated origin firing produces ssDNA gaps that promote re-replication at these sites, leading to replication fork breakage (106). 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 upregulates CDC6 (2, 83, 94), 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 relicensing and re-replication (107).

**Transcription**

**Transcription–Replication Conflicts**

DNA transcription is a physiologic process that may turn into an intrinsic source of GIN upon interference with the replication fork machinery. Transcription–replication conflicts (TRC) impede fork progression as a result of head-on or codirectional collisions between the two machines or between replication and R-loops (Fig. 4). TRCs were first visualized by electron microscopy in *Escherichia coli* (*E. coli*), where an inducible origin was inserted upstream or downstream of an rRNA operon (108).
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 favored. A similar orientation bias was also described in the human genome. 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.

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 (ref. 115; Fig. 4C and 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 TFIIIS; (ii) reduction of misincorporated nucleotides by DksA; (iii) RNA removal by the combined actions of ppGpp, DksA, GreA, and Mid, or UvrD and NusA (reviewed in ref. 116); or (iv) transcription regulation by RECQL5 (117).

**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 favored by increased transcription activity, polyA tracts, and unmethylated CpG islands (reviewed in ref. 118). 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 (119), transcription regulation (120), IgG class switch recombination (121), telomere maintenance in ALT cells (122), and homologous recombination-mediated DSB repair (123). Nevertheless, accumulation of R-loops can act as an obstacle to replication fork progression either through direct collisions (124) or indirectly through increased chromatin compaction (125), which leads to CFS formation (126) and GIN (ref. 127; 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. 128). 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 (129) 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* shows that it is not length but histone modifications that discriminate between “benign” and “malignant” (130). In particular, Garcia-Pichardo and colleagues 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; ref. 125) that render them culpable for GIN (130). In experiments in *Drosophila* showed that depletion of linker histone H1 facilitates transcription of heterochromatic transcripts, enabling R-loop accumulation (131). Similarly, in *C. elegans*, H3K9 methylation suppresses transcription of repetitive elements that enhance R-loops (132). 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 (127), RNaseH2 (133), SETX (134), DDX19 (135), DDX21 (136), DDX23 (137), DING (138), and PIF1/RRM3 helicases (139) degrade or unwind R-loops. ASF/SF2 (140), TOPO (141), RECQ5 (142), THO/TREX (129), NPL3 (143), AQR (144), and XRN2 (145) prevent R-loop formation. Also, BRCA1/2 (146) and Fanconi anemia proteins remove R-loops (147), whereas BRCA1 forms a complex with SETX at transcription termination pause sites, which collaborate to suppress R-loop accumulation (148). In addition, the chromatin reorganizing complex FACT facilitates resolution of R-loop–dependent GIN, most likely through remodeling chromatin at the sites of conflict (149). 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 (150).

Because oncogenes enhance transcription, it is reasonable that they should also affect R-loop levels. Only recently, HRASV12 overexpression was shown to increase R-loop accumulation that was detrimental to fork progression (97). As part of the same study, HRASV12 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 TRCs**

Orientation of TRCs has a profound effect on DNA integrity. Studies in yeast have shown that head-on collisions are more detrimental than codirectional ones with respect to fork slowing (151). Nevertheless, codirectional collisions also disrupt replication (152). Moreover, in bacteria, codirectional collisions with backtracked transcription complexes cause DSBs, whereas head-on collisions do not (115). Further experiments in bacteria have shown that with both types of collisions, the replisome resumes elongation after displacing RNAP from the DNA, and in the case of codirectional collision it uses the nascent transcript as a primer (reviewed in ref. 153). Using a sophisticated episomal system to induce either head-on or codirectional TRCs, Hamperl and colleagues showed in human cells that replication itself modulates R-loop formation depending on the orientation of the TRC. In particular, codirectional TRCs resolve R-loops and activate the ATM–CHK2 pathway, whereas 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 (124). They also showed that normal replication fork progression suppresses R-loop accumulation, so any type of fork slowing increases R-loop levels (124). According to their proposed model, head-on TRCs cause fork slowing, which leads to increased R-loop levels that in turn activate ATR, whereas codirectional 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 stalls replication, increases mutagenesis, and prevents fork restart (154). 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 (155). Taken together, these data disclose a conserved and complex mechanism that protects the genome from TRC-related GIN.
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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 (ref. 156; 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 (157). Torsional stress is released by the activity of topoisomerases I and II, which relax positive supercoiling by creating nicks in DNA (158). 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 (142). Also, in yeast, Mec1 and Rad53 phosphorylate nucleoprotein Mlp1 and resolve the topologic stress (157). Finally, according to a recent study, pS3 loss causes stabilization of TOP2a on DNA, as well as fork retardation, suggesting a possible role in preventing torsional stress between the two machines (159).

G-quadruplexes

G-quadruplexes are four-stranded structures held by G-G base pairs that are formed cotranscriptionally 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 (160), where they facilitate R-loop stabilization (161). G-quadruplexes are also found within start sites of highly transcribed genes such as MYC (162), KRAS (163), or PTEN and regulate transcription (164). Moreover, the ORC complex has been suggested to bind to G-quadruplexes at putative origin sites of replication initiation (165). 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. 166).

TRC-Associated DDR

Certain areas of the genome are more susceptible to TRCs. Barlow and colleagues identified highly unstable regions of the genome, named early replication fragile sites (ERFs) that are more prone to breakage upon RS than CFSs (167). ERFs 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 (167). In addition, long genes are more prone to TRCs, with frequent R-loop accumulation and induction of CFS instability (126).

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 (112) and, through the synergistic effect of checkpoint sensor Mec1–Ddc2, the chromatin remodeling complex Ino80C, and the transcription complex PaflC, RNApolI is evicted from chromatin and degraded (168). Accordingly, Dcr1 releases RNApolIII from highly transcribed genes that are sites of replication–transcription collisions to prohibit further instability (169). 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 (170). Interestingly, the de Bruin laboratory has shown that the cell responds to RS by upregulating transcription of E2F target genes that maintain checkpoint activation and facilitate DNA damage tolerance (171, 172). 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. Among 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 resolving RAD51-dependent replication intermediates at these sites (173). In S. pombe, Pfh1, a member of the Pif1 family of helicases, facilitates replication across highly transcribed RNApolIII- and RNApolIII-dependent genes (174). Likewise, in S. cerevisiae, another Pif1 family member, Rrm3 allows replication through TRCs (151). Moreover, in E. coli, helicases DinG, Rep, and UvrD collaborate to promote replication through transcription units (138).

In response to oncogenic signaling, transcription activity is enhanced (38, 175–179) 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 program that controls cell growth and division. Mitogenic growth signaling may deregulate c-MYC, which can directly enhance RNApolI- (177), RNApolII- (178), and RNApolIII-dependent transcription (176). c-MYC increases transcription indirectly, by upregulating and/or downregulating selective target genes that in turn modify the cellular state and enable global RNA production (180). This transcription amplification has been documented in patient-derived tumors with elevated c-MYC, thus establishing a link to tumorigenesis (178). 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, and senescence and contributes to oncogenesis (97, 181).

A very recent report shed light on the mechanisms underlying oncogene-induced TRCs and GIN. Using sensitive sequencing-based assays, Macheret and Halazonetis mapped replication initiation sites in human cells (182). They showed that under physiologic 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 (182). This highlights the importance of TRCs among 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 synthesized either through the de novo pathway from glutamine, glycine, folic acid, aspartate, 5-phosphoribosyl-1-pyrophosphate, or by nucleotide degradation through the salvage pathway. The reduction of ribonucleosides to dNTPs is catalyzed by the RNR, which is comprised of two catalytic (RRM1) and two regulatory (RRM2, RRM2B) subunits. Oncogenes may target RNR activity or induce hyperproliferation that will both reduce dNTP pool affecting fork progression. Oncogenes also induce production of ROS, including O$_2^-$, H$_2$O$_2$, and OH•. O$_2^-$ is produced either by oxidation of NADPH by NADPH oxidase enzymes (NOX) or through aerobic respiration in mitochondria. H$_2$O$_2$ is generated by O$_2^-$, which is converted by superoxide dismutase (SOD), whereas OH• are produced from H$_2$O$_2$ in the presence of Fe$^{2+}$. It is not clear if oxidized dNTPs affect replication fork speed.

![Nucleotide and ROS metabolism as a cause of RS](image)

Figure 5. Nucleotide and ROS metabolism as a cause of RS. 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 catalyzed by the RNR, which is comprised of two catalytic (RRM1) and two regulatory (RRM2, RRM2B) subunits. Oncogenes may target RNR activity or induce hyperproliferation that will both reduce dNTP pool affecting fork progression. Oncogenes also induce production of ROS, including O$_2^-$, H$_2$O$_2$, and OH•. O$_2^-$ is produced either by oxidation of NADPH by NADPH oxidase enzymes (NOX) or through aerobic respiration in mitochondria. H$_2$O$_2$ is generated by O$_2^-$, which is converted by superoxide dismutase (SOD), whereas OH• are produced from H$_2$O$_2$ in the presence of Fe$^{2+}$. It is not clear if oxidized dNTPs affect replication fork speed.
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**How Do Oncogenes Induce Replication Stress**

or upregulating protein kinase C cause oxidative stress by damaging lipids, proteins, and DNA levels. Basal ROS levels uphold physiologic cellular functions and metabolism (reviewed in ref. 194). ROS include superoxide (O2•−), hydrogen peroxide (H2O2), and hydroxyl radicals (OH•), and their biological effects are proportional to their levels. Basal ROS levels uphold physiologic cellular functions as part of redox biology, whereas increased levels of ROS cause oxidative stress by damaging lipids, proteins, and DNA or upregulating protein kinase C and inducing senescence or apoptosis. O2•− is produced either by oxidation of NADPH oxidase enzymes (NOX) or through aerobic respiration in mitochondria. H2O2 is generated from O2•− by superoxide dismutases and exerts its effect on redox biology or oxidative stress or may be converted to H2O by antioxidant proteins. Finally, OH• are produced from H2O2 in the presence of Fe2+ (ref. 195; 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 (196), which is partially achieved by upregulating NOX4-p22phox (197). c-MYC overexpression also induces ROS and causes DNA damage (198). 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 (199). Moreover, in response to KRASG12D, BRAFV619E and MYCERT2, primary murine cells respond by upregulating the transcription factor NRF2 that regulates an antioxidant program used to detoxify the organism (200). In addition, expression of E6/E7 promotes NOX-dependent generation of ROS and subsequent DNA damage in head and neck cancer cells (201).

It is unclear how ROS affect fork progression. According to the D’adda di Fagagna laboratory, RAS induces ROS and this triggers the hyperproliferation that leads to RS and senescence (94, 196, 202). On the other hand, CYCLIN E–induced hyperproliferation is independent of ROS (2). Another case involves ROS oxidizing dNTPs, as was covered above.

**CONCLUDING REMARKS**

Oncogene-induced RS has long been recognized 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 leads 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 is 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 misincorporation, and oxidized nucleotides in oncogene-induced RS that need to be addressed.

Disclosure of Potential Conflicts of Interest

S.J. Boulton is senior vice president of science strategy at Arturo Pharma Ltd. No potential conflicts of interest were disclosed by the other authors.

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