

## Review

# Mechanisms of Fork Destabilization Under Hydroxyurea: The Interplay of ROS, Checkpoints, and Replisome Integrity

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## Abstract

Faithful DNA replication is essential for genome stability but is constantly challenged by metabolic and oxidative stresses. Hydroxyurea (HU), a widely used antiproliferative drug, is traditionally known to inhibit ribonucleotide reductase and deplete dNTP pools. Recent studies, especially in *Saccharomyces cerevisiae*, reveal that HU-induced replication stress also arises from reactive oxygen species (ROS), which oxidize DNA, impair iron–sulfur-dependent replication enzymes, and disrupt replisome function. These combined effects promote helicase–polymerase uncoupling, accumulation of RPA-coated ssDNA, and activation of the Mec1–Rad53 (ATR–CHK1) checkpoint, leading to strand-specific changes such as PCNA unloading and reduced lagging-strand synthesis. When protective pathways are overwhelmed, HU-treated forks collapse, generating chromosome breaks and genome instability. This review summarizes current understanding of how HU remodels replication forks through both ROS-dependent and ROS-independent pathways and highlights emerging insights into how these mechanisms influence genome stability and may be exploited for therapeutic benefit.

**Keywords:** DNA replication stress; oxidative stress; Hydroxyurea; Mec1/Rad53; DNA polymerases

## 1. Introduction

DNA replication is a foundational process that ensures the precise and timely transmission of genetic information from parent to daughter cells [1]. This process is essential for normal cellular development and maintenance of genome integrity, and its disruption is closely linked to disease susceptibility [2]. However, DNA duplication is continuously challenged by exogenous and endogenous factors, including the dynamic landscape of redox states and cellular metabolites that drive cancer development and aging [3]. Among endogenous stresses, oxidative stress is a major contributor to disruption of multiple cellular processes, including DNA replication. Consequently, cells exposed to chronic endogenous oxidative stress contribute to a significant source of replication stress [4].

Hydroxyurea (HU) is a small molecule recognized in the 1960s as a potent inhibitor of ribonucleotide reductase (RNR), the enzyme that catalyzes de novo dNTP synthesis [5]. Its ability to suppress DNA synthesis led to its early use in cancer chemotherapy, where it demonstrated efficacy against chronic myelogenous leukemia (CML), melanoma, and other malignancies [6]. Today, HU remains widely used in oncology and hematologic disorders and serves as a foundational experimental tool for studying DNA replication stress, checkpoint signaling, and genome instability in both yeast and mammalian systems [7–11].



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Here, we will review the fundamental principles of the DNA replication process and S-phase cell-cycle checkpoint pathways. We will then discuss the current understanding of HU's working role on DNA forks through both reactive oxygen species (ROS)-dependent and ROS-independent pathways and highlight emerging insights into how these mechanisms influence genome stability and may be exploited for therapeutic benefits.

## 2. Molecular Pathways Linking Eukaryotic DNA Replication, Checkpoint Signaling, and ROS-Driven Replication Stress

### 2.1. Components of Core Eukaryotic Replisome

Eukaryotic DNA replication is carried out by a highly conserved molecular machine whose core components are conserved from yeast to humans [12]. The replisome coordinates the leading- and lagging-strand DNA synthesis, and its major structural and enzymatic modules are essential for maintaining replication accuracy and efficiency. At the heart of the replisome is the CMG helicase—an 11-subunit assembly composed of CDC45, the MCM2–7 complex, and the four-subunit GINS complex (Sld5, Psf1, Psf2, and Psf3) [13]. Once activated at replication origins, the CMG helicase translocates along the leading-strand template and unwinds the parental duplex DNA to initiate replication.

Closely associated with CMG is the primase–polymerase  $\alpha$  complex (Pol  $\alpha$ –primase), which synthesizes short RNA–DNA primers that initiate DNA synthesis on both strands [14,15]. Pol  $\alpha$ –primase maintains stable contacts with multiple replisome components, including Ctf4 (human AND-1) and CMG, ensuring that primer formation is tightly coupled to helicase movement. Although CMG bypasses protein and DNA obstacles predominantly on the leading-strand template, Pol  $\alpha$ –primase functions predominantly on the lagging strand, where repeated primer synthesis is required for Okazaki fragment formation. Following primer synthesis, the two replicative DNA polymerases take over nascent-strand elongation: Pol  $\epsilon$  primarily extends the leading strand, whereas Pol  $\delta$  synthesizes most of the lagging strand [16,17]. Pol  $\epsilon$  maintains a strong and continuous physical association with the CMG helicase [16–18], enabling its rapid and coordinated progression with DNA unwinding. In contrast, the lagging-strand machinery—Pol  $\delta$ , Pol  $\alpha$ –primase, and the clamp loader RFC—interacts more transiently with the replisome as Okazaki fragments are iteratively initiated and extended. DNA synthesis on both strands is further supported by proliferating cell nuclear antigen (PCNA), the sliding clamp that enhances polymerase processivity. Reconstituted yeast replication systems have shown that PCNA strongly stimulates Pol  $\delta$ , increasing its nucleotide incorporation rate by more than tenfold compared with Pol  $\epsilon$  [19]. This differential stimulation contributes to the inherent asymmetry between the leading and lagging strands.

In addition to the replication core components, the evolutionarily conserved fork protection complex (FPC) also plays a critical role in ensuring faithful replication. In budding yeast, the FPC components Mrc1 and Csm3/Tof1 (human CLASPIN and TIMELESS–TIPIN) travel with the CMG helicase throughout the S phase. These proteins stabilize the replisome during both unperturbed and stressed conditions and promote rapid fork progression [20]. Csm3/Tof1 enhances CMG-mediated DNA unwinding, which in turn facilitates stable Mrc1 recruitment. Together, these factors couple helicase activity with DNA synthesis and help preserve replication fork integrity when replication encounters obstacles.

### 2.2. Initiation of the S-Phase Checkpoint: How Replication Stress Generates ssDNA

Cells encounter numerous endogenous and exogenous sources of replication stress, including reactive oxygen species (ROS), dNTP depletion (e.g., HU), DNA lesions, protein–DNA barriers, and defective replication factors [21]. Extensive studies in yeast and metazoans have elucidated how the replisome responds to such stress and how these responses

trigger activation of the S-phase checkpoint. A common consequence of replication stress is uncoupling of the helicase and polymerase. When DNA polymerases stall at lesions or impediments while the CMG helicase continues unwind DNA, extended regions of single-stranded DNA (ssDNA) accumulate [22,23]. Replication protein A (RPA) rapidly coats this ssDNA and serves as the primary signal for checkpoint activation [24]. The impact of DNA lesions or blocks depends strongly on the strand on which they occur. Lagging-strand blocks are often bypassed by CMG, which translocates along the leading-strand template [25,26]. Such lesions typically generate short patches of ssDNA and primarily interfere with Okazaki fragment maturation [27]. In this context, CMG may still advance slowly or transiently, but polymerase stalling results in extensive ssDNA on the leading strand due to helicase–polymerase uncoupling [26]. This configuration is a potent trigger for S-phase checkpoint activation. Together, these scenarios illustrate that RPA-coated ssDNA is the universal initiator of the S-phase checkpoint, regardless of whether stress arises from exogenous damage, intrinsic replisome defects, or metabolic perturbations.

### 2.3. Recognition of ssDNA and Mec1/ATR Recruitment

At stalled forks, RPA-coated ssDNA recruits the yeast Mec1–Ddc2 complex (ATR–ATRIP in humans) [27,28]. Mec1/ATR serves as the primary kinase activated in response to replication stress. Tel1/ATM, which preferentially responds to double-strand breaks, contributes to certain types of damage but plays a minor role at stalled forks [29,30]. Importantly, checkpoint activation in S phase requires more extensive ssDNA than in G1 or G2, ensuring that normal replication intermediates do not inappropriately trigger the checkpoint [31]. Full Mec1/ATR activation also requires loading of the 9-1-1 clamp (Ddc1–Rad17–Mec3; human RAD9–RAD1–HUS1) [32], by the clamp loader Rad24–RFC2-5. The 9-1-1 complex recruits Dpb11/TopBP1, a potent activator of Mec1/ATR kinase activity [33].

### 2.4. Downstream Effectors: Rad53/CHK2 and Chk1/CHK1

Once activated, Mec1/ATR phosphorylates the effector kinases Rad53 (CHK2) and Chk1 (CHK1). Rad53/CHK2 activation during S phase requires Mrc1/CLASPIN [34]. Mrc1/CLASPIN travels with the replisome, interacting with Pol  $\epsilon$ , MCM, and the Csm3/Tof1 complex [35], positioning it ideally for rapid response to fork stalling. Activated Rad53/CHK2 and Chk1 orchestrate a broad protective program:

- (A) Inhibition of late origin firing (Sld3 and Dbf4 in yeast; CDK-regulated pre-initiation factors in humans) [36].
- (B) Cell-cycle arrest, allowing time for repair; CHK1 additionally blocks G2/M progression by phosphorylating and inhibiting CDC25 phosphatases [37]. In yeast, Rad53 stabilizes the securin Pds1 to prevent premature anaphase onset [38].
- (C) Stabilization and remodeling of stalled forks, including fork reversal.
- (D) Upregulation of dNTP synthesis [36].
- (E) Transcriptional activation of DNA damage response genes.

Through these combined activities, the S-phase checkpoint preserves fork integrity, prevents collapse into double-strand breaks, and ensures that cells do not enter mitosis with under-replicated or damaged DNA.

### 2.5. Reactive Oxygen Species: General Principles and Their Effects on DNA Replication

Reactive oxygen species (ROS) are continuously generated in cells as metabolic by-products and as signaling intermediates. At low or transient levels, ROS function in physiological signal transduction [39]. However, sustained or excessive ROS induce oxidative stress, damaging proteins, lipids, and nucleic acids and promoting replication stress. Elevated ROS levels produce a variety of DNA lesions—including oxidized bases

(e.g., 8-oxoguanine), abasic sites, and single- or double-strand breaks—which can impede replication fork progression and compromise genome stability [4]. Defects in antioxidant pathways or aberrant ROS accumulation are commonly observed in cancers and degenerative diseases, where they contribute to genomic instability and tumor progression [40].

ROS encompass both free-radical and non-radical oxygen species, generated through mitochondrial respiration, inflammatory signaling, oncogene activation, redox reactions, and environmental exposures [41,42]. Examples include superoxide, hydroxyl, peroxy, and alkoxyl radicals, as well as non-radical oxidants such as hydrogen peroxide, ozone, and hypochlorous acid. Interaction with nitrogen metabolism produces reactive nitrogen species (RNS), such as nitric oxide and peroxynitrites [43].

Reactive oxygen species (ROS) damage DNA directly but also affect replisome components, particularly proteins that depend on iron–sulfur clusters (ISCs). Many essential replication enzymes—including DNA polymerases  $\delta$  and  $\epsilon$ , primase, FANCI, and XPD—require ISCs for catalytic activity and structural integrity (Table 1). Because ISCs are highly sensitive to oxidation, ROS can inactivate these proteins, slow fork progression, and increase helicase–polymerase uncoupling. When polymerases stall at oxidative lesions while the CMG helicase continues unwinding, extended regions of single-stranded DNA (ssDNA) accumulate. RPA binding to ssDNA recruits the Mec1–Ddc2 (ATR–ATRIP), activating the S-phase checkpoint to stabilize forks, adjust replication timing, and promote DNA repair. Chronic oxidative stress overwhelms these protective pathways, leading to fork collapse, double-strand break formation, mutagenesis, structural rearrangements, and oncogene activation [44].

**Table 1.** List of iron–sulfur clusters containing DNA-metabolic enzymes involved in replication and repair.

| Yeast  | Human | Function                                                                         | References |
|--------|-------|----------------------------------------------------------------------------------|------------|
| Chl1   | CHLR1 | Helicase, sister chromatid cohesion, heterochromatin organization                | [45,46]    |
| Dna2   | DNA2  | Helicase/nuclease, DNA repair, Okazaki fragment maturation, telomere maintenance | [47,48]    |
| Absent | FANCI | Helicase, repair of DNA interstrand crosslinks                                   | [49]       |
| Absent | MUTYH | DNA glycosylase, base excision repair                                            | [50]       |
| Ntg2   | NTHL1 | DNA glycosylase, base excision repair                                            | [51,52]    |
| Pol1   | POLA  | Catalytic subunit of polymerase $\alpha$ , DNA replication                       | [53]       |
| Pol3   | POLD1 | Catalytic subunit of polymerase $\delta$ , DNA replication                       | [53]       |
| Pol2   | POLE1 | Catalytic subunit of polymerase $\epsilon$ , DNA replication                     | [53]       |
| Pri2   | PRIM2 | Subunit of DNA primase, DNA synthesis and double-strand break repair             | [54]       |
| Absent | RTEL1 | Helicase, regulation of telomere length, anti-recombinase                        | [46]       |
| Rad3   | XPD   | Helicase, nucleotide excision repair                                             | [46]       |

### 3. ROS-Dependent and -Independent Mechanisms of Hydroxyurea-Induced Replication Stress

#### 3.1. ROS-Independent Pathways

##### 3.1.1. RNR Inhibition

HU primarily acts by inhibiting ribonucleotide reductase (RNR), thereby lowering intracellular dNTP pools and slowing DNA synthesis. In budding yeast, depletion of Mec1—an activator of RNR transcription—results in reduced RNR activity [55], severely diminished dNTP levels, and pronounced replication slowing, ultimately leading to replication fork collapse. Increasing RNR levels suppresses DNA damage in both Mec1- and ATR-depleted cells, highlighting the central role of dNTP availability in fork stability [56].

##### 3.1.2. Checkpoint-Mediated Regulation of Origin Firing

In budding yeast, a high concentration of HU suppresses late origin firing via the checkpoint pathway. In the timely origin system, activation of Mec1 at RPA-coated ssDNA leads to phosphorylation and activation of the effector kinase Rad53, which in turn inhibits key replication initiation factors. Rad53 phosphorylates Sld3 and Dbf4, the regulatory subunit of the Dbf4-dependent kinase Cdc7 (DDK), reducing its ability to activate MCM helicases at unfired origins. Through coordinated inhibition of both Sld3 and Dbf4, the Mec1–Rad53 checkpoint pathway prevents excessive origin firing, conserves limiting replication resources, and ensures that cells focus on stabilizing existing forks rather than initiating new ones [57,58]. This checkpoint-mediated origin suppression is essential for maintaining genome stability during HU-induced replication stress and other conditions that challenge replisome progression. Interestingly, HU's action on late origin fire is RNR-independent.

##### 3.1.3. Replisome Remodeling

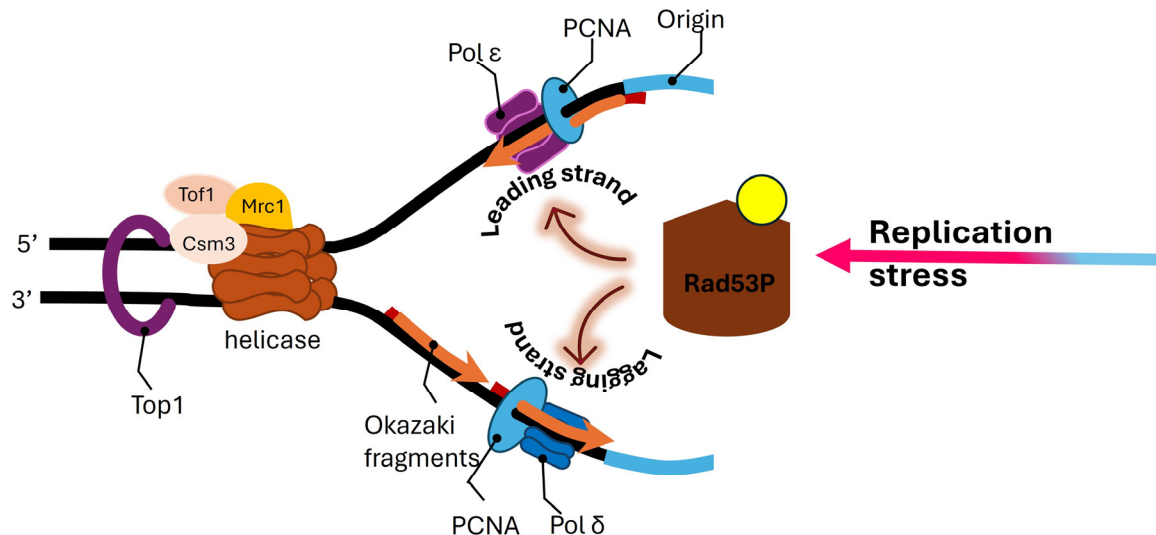
Recent strand-specific nascent DNA profiling approaches, such as eSPAN (enrichment and sequencing of protein-associated nascent DNA), have enabled high-resolution analysis of how leading- and lagging-strand polymerases respond to replication stress [59,60]. Under HU-induced replication stalling, one prominent structural alteration is the selective unloading of PCNA from the lagging strand, which reduces Pol  $\delta$  processivity and slows Okazaki fragment synthesis [61,62]. On the leading strand, Rad53 phosphorylates Mrc1, which weakens the ability of the Mrc1–Tof1–Csm3 fork protection complex to stimulate CMG helicase activity and may dampen Pol  $\epsilon$  progression [36] (Figure 1). As a result, HU-treated forks frequently exhibit asymmetric synthesis, reduced lagging-strand extension, and partial uncoupling between helicase unwinding and polymerase activity. These coordinated structural changes represent protective adaptations that slow fork progression, prevent excessive ssDNA accumulation, and preserve fork integrity until replication can resume following HU removal.

##### 3.1.4. Additional Impact on Replication Stability and Genome Integrity

Under HU, forks typically initiate at early origins and progress only ~8–9 kb before stalling [63]. This stall appears largely stochastic and does not require Mec1 or Rad53 activity [64]. Checkpoint mutants, however, exhibit distinct sensitivities. *mrc1* $\Delta$  cells, which lack both the replication-coupling and checkpoint functions of Mrc1, show slow fork progression and hypersensitivity to HU [65]. By contrast, *mrc1AQ* mutants, which retain Mrc1's replisome-coupling activity but lack its checkpoint function, complete the S phase with near-normal kinetics and display much greater resistance to HU than *mrc1* $\Delta$  cells [66,67]. This distinction emphasizes that fork progression defects in *mrc1* $\Delta$  cells arise primarily from loss of replisome-coupling activity rather than checkpoint deficiency. Similarly, Rad53

mutants display asymmetric DNA synthesis and hypersensitivity to HU, indicating that HU-induced fork slowing occurs independently of Mec1/Rad53-mediated checkpoint activation [23,68,69].

## Checkpoint-mediated replication coupling



**Figure 1.** A model illustrating Rad53-mediated fork stalling during HU-induced replication stress. Under replication stress, Mec1-dependent phosphorylation of Mrc1 activates Rad53, which in turn restrains MCM helicase to limit DNA unwinding and slow replication fork progression. Disruption of helicase–polymerase coupling at stalled fork promotes PCNA unloading, particularly from lagging-strand templates, thereby terminating Pol  $\delta$ -mediated Okazaki fragment synthesis. Together, Mrc1-mediated checkpoint signaling coordinates replisome remodeling to stabilize stalled forks and prevent fork collapse.

### 3.2. HU Contributes to Replication Stress and Genome Instability Through ROS-Dependent Pathways

Hydroxyurea (HU) is widely used in research and cancer therapy and traditionally viewed as an inhibitor of ribonucleotide reductase (RNR). However, growing evidence demonstrates that HU also induces oxidative stress, and that ROS contribute significantly to HU-mediated replication defects [8,70,71].

#### 3.2.1. ROS-Mediated Effects on Ribonucleotide Reductase

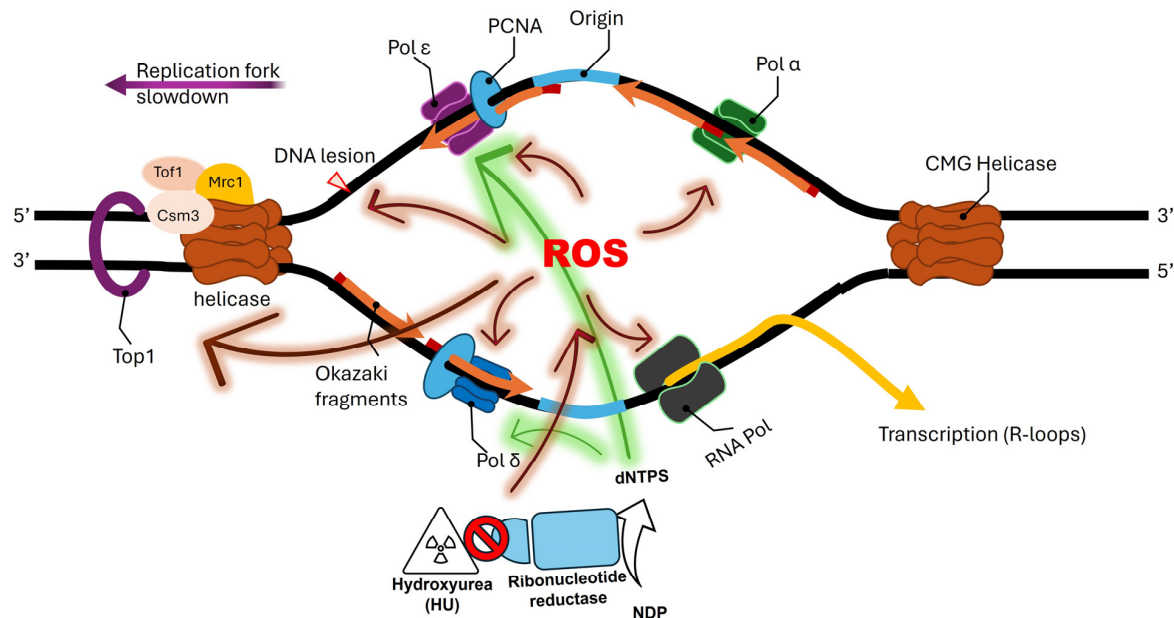
RNR function depends on both the ferric–tyrosyl radical and multiple ISC-containing proteins such as Dre2–Tah18 [72]. Because these cofactors are sensitive to oxidation, HU-induced ROS can further impair RNR assembly and function, amplifying replication stress.

#### 3.2.2. ROS Effects on the Replisome

ROS produced during HU exposure affect replication in several ways. First, they oxidize ISCs within replicative polymerases and other DNA-metabolic enzymes, destabilizing replisome components and slowing fork progression [7] (Figure 2). In budding yeast, HU disrupts cytosolic ISC biogenesis via ROS generation, establishing a direct link between HU treatment and ISC pathway dysfunction [9]. Second, HU-induced ROS modulates replisome regulators. Low oxidative conditions promote oligomerization of peroxiredoxin 2, stabilizing the human TIMELESS–TIPIN complex and supporting fork progression. Higher ROS levels oxidize peroxiredoxin subunits, causing TIMELESS dissociation from chromatin and slowing fork movement. Consistent with redox involvement, HU-induced fork inhibi-

tion can be partially rescued by antioxidants such as N-acetylcysteine or ascorbic acid, but not by exogenous dNTPs [7].

## Replisome–ROS interactions



**Figure 2. Possible roles of ROS (reactive oxygen species) in yeast replication stress response.** Replication stress causes replication fork stalling, and recovery requires preservation of replisome architecture, DNA lesion repair, and coordinated fork remodeling to complete genome duplication. Many key replication and repair enzymes—including Pol $\epsilon$ , Pol $\delta$  and helicases—contain essential iron–sulfur clusters that are crucial for their structural stability, catalytic activity, and redox sensing. Reactive oxygen species (ROS) can oxidize these Fe–S clusters, directly impairing the activity of polymerases, helicases and nucleotide bases, both free and inserted into DNA. This oxidative damage may lead to polymerase dissociation, compromised helicase unwinding, and defective nuclease processing required for fork restart, ultimately destabilizing the replisome. Additionally, Fe–S cluster dysfunction can exacerbate replication stress by promoting transcription–replication conflicts. Hydroxyurea-induced replication stress is strongly linked to ROS accumulation, suggesting that vulnerability of Fe–S-containing proteins represents a central molecular node through which oxidative and metabolic stress impairs fork stability, though the precise mechanisms remain to be fully elucidated.

### 3.2.3. ROS-Induced Transcription–Replication Conflicts and Cell-Cycle Perturbations

HU-induced oxidative stress increases transcription and promotes R-loop formation, leading to collisions between transcription and replication machinery, a major source of fork stalling in human cells [8]. Yeast cells respond to oxidative stress by modulating replication timing through ROS-sensitive transcription factors such as Swi6 [73]. Additionally, HU-induced ROS influence cell-cycle progression: oxidative stress can induce G2/M arrest through CHK1 activation or direct oxidation of CDC25 phosphatases [74], whereas specific mitochondrial ROS signals can oxidize CDK2 to promote S-phase entry [75].

Collectively, HU acts through multiple mechanisms to disrupt replication fork integrity and promote genome instability.

## 4. Conclusions, Remarks and Future Perspectives

Hydroxyurea (HU) has long been used as a tool compound and as an anticancer drug, classically defined by its ability to inhibit ribonucleotide reductase, deplete dNTP

pools, and stall replication forks. Studies in budding yeast and mammalian cells now reveal a far more complex picture in which HU-induced replication stress and genome instability arise from an interplay between nucleotide limitation, oxidative stress, and replisome remodeling. HU not only slows forks by reducing dNTP availability but also perturbs redox homeostasis, leading to ROS accumulation, oxidation of DNA and proteins, and disruption of iron–sulfur cluster (ISC)-dependent replication factors. These insults converge on the replication fork, promoting helicase–polymerase uncoupling, excessive ssDNA formation, checkpoint activation, and, when protective responses fail, fork collapse and chromosome breakage. Together, these findings support a model in which HU acts not as a single-target inhibitor, but as a multifaceted stressor that probes the robustness of replication and DNA damage response networks.

Despite significant progress, many important questions remain. At the mechanistic level, we still lack detailed structural and kinetic insights into how individual replisome components—particularly ISC-containing polymerases and helicases—respond to HU-induced ROS *in vivo*, and whether these responses differ between leading- and lagging-strand machinery. Although strand-specific nascent DNA profiling methods, such as eSPAN and related technologies, have revealed highly asymmetric replication patterns under HU, these approaches are largely limited to population-based analysis. As a result, cellular heterogeneity in replication dynamics and fork asymmetry cannot be directly assessed. Further refinement of these tools, particularly toward single-cell or time-resolved resolutions, will be required to elucidate how replication fork architecture and enzymes evolve throughout HU exposure. The concentration- and time-dependent effects of HU on fork reversal, resection, and restart also require deeper exploration, ideally with single-molecule, time-resolved, and *in vitro* reconstitution approaches. Additionally, the interplay among HU, ROS, and transcription–replication conflicts—including R-loop formation and resolution—represents an emerging area where yeast genetics and mammalian models will continue to provide complementary insights.

Clinically, HU remains a frontline therapy for several hematologic malignancies and sickle cell disease, yet patient responses and long-term genomic consequences are variable and incompletely understood. Integrating HU's redox effects with its canonical role in dNTP depletion may help explain differential sensitivity among tumors with defects in checkpoint signaling, antioxidant pathways, or ISC biogenesis. Future work should investigate whether modulation of ROS (for example, via antioxidants or pro-oxidants), targeting ISC assembly factors, or manipulating fork protection pathways can be leveraged to enhance HU efficacy or limit HU-induced genome instability in normal tissues. Ultimately, a deeper mechanistic understanding of HU-induced replication stress in yeast and mammals promises not only to refine therapeutic strategies for HU, but also to illuminate general principles of how cells maintain genome stability under conditions of metabolic and oxidative stress.

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