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# Preserving the Replication Fork in Response to Nucleotide Starvation: Evading the Replication Fork Collapse Point

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/51393>

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## 1. Introduction

Replication fork progression is blocked by a variety of impediments including DNA damage, aberrant DNA structures, or nucleotide depletion [1-3]. The response to replication fork stalling varies according to the type of replication inhibition, the number of stalled forks and the duration of the treatment [3-7]. Stalled replication forks are at increased risk for DNA damage, which can lead to mutation or cell death [7-13]. The cell relies on the Intra-S phase checkpoint and DNA damage response proteins to preserve fork structure to allow recovery and resumption of the cell cycle [5, 10, 14-19]. Thus, the mechanisms that maintain replication fork structure are crucial for genome maintenance, and form a primary barrier to malignant transformation [20, 21].

The drug hydroxyurea (HU) induces a reversible early S-phase arrest by causing deoxynucleotide triphosphate (dNTP) depletion [22-24]. HU is a venerable chemotherapeutic, used for its ability to inhibit cell proliferation, but also because it predisposes proliferating cells to genome instability. The loss of replication fork stability and its associated DNA damage following HU treatment is loosely termed "replication fork collapse". Changes in dNTP pool levels through other mechanisms (*e.g.* exogenous thymidine or 5-bromo-2'-deoxyuridine treatment) are known to cause point mutations [25-27], plasmid instability [28] and polyploidization [29]. Further, dNTP pool changes in human cells may cause hypersensitivity to secondary treatment with alkylating agents [30, 31].

Wild type cells recover from HU arrest and complete S-phase once drug is removed from the culture medium. Alternatively, some cultures may recover from HU arrest prior to its removal.

al by up-regulating nucleotide synthesis and overcoming HU replication inhibition to slowly complete S-phase [17, 32, 33]. The ability to recover stalled replication out of an HU arrest requires restoration of replication forks, restart of DNA synthesis and completion of S-phase.

Whether a replication fork successfully recovers, or collapses with DNA damage, depends in part on the Intra-S phase checkpoint pathway. Cells lacking the checkpoint suffer fork collapse and death. Notably, cells that do not trigger the Intra-S phase checkpoint continue to synthesize DNA despite the presence of HU. Continued synthesis in the presence of low dNTP pools leads to reduced replication rates and increased single stranded DNA (ssDNA) [33-37]. This is a fragile state of "open" DNA that is prone to double strand breaks (DSBs) [38-40]. Further, altered dNTP levels during DNA replication enhance point mutations, in which the base inserted shifts towards that of the dominant pool or away from the lowest pool [25, 41-44]. This explains why the replication checkpoint is a crucial barrier to genome instability.

Thus, replication fork collapse in checkpoint mutants does not occur immediately after HU treatment, detection of decreased dNTP levels, or failure to mount a checkpoint response. Instead, replication fork collapse across a population of forks, within a culture of cells, is a consequence of continued fork activity. The signs and symptoms of replication fork collapse represent a new execution point, the Replication Fork Collapse Point. This metric describes the time at which the majority of replication forks in a cell population become non-functional. In this review, we describe the causes and symptoms of the Replication Fork Collapse Point, with particular regard to the Intra S-phase checkpoint.

## 2. Replication Fork Structure is Maintained During Stalling

The replication fork describes a region of denatured DNA where DNA synthesis is actively occurring, resembling a two-tined fork. The replisome encompasses the forked DNA, and the entire complex is large and dynamic, coupling DNA unwinding and polymerization [45-47]. Unwinding is performed by a conserved hexameric helicase (MCM) and its associated proteins Cdc45 and GINS. The processive helicase produces single strand DNA (ssDNA) which becomes transiently coated with replication protein A (RPA), a ssDNA binding protein homologue. ssDNA is the substrate for leading- (*pol $\epsilon$* ) and lagging-strand (*pol $\delta$*  and *pol $\alpha$* -primase) polymerases.

These functions must be linked to facilitate DNA synthesis. Coupling generation of ssDNA with its use in replication is particularly important, because ssDNA is vulnerable to forming secondary structures, which leads to DNA damage [40, 48, 49], and recombination [50-52]. Thus, fork proteins limit the amount of DNA unwinding and ssDNA [39, 53]. In normal conditions, synthesis may occur rapidly and the goal of minimizing ssDNA production (<200bp) is easily accomplished [54]. However, if either the leading or lagging strand polymerases become stalled or arrested in a slow zone, the helicase must also be slowed down

to prevent it from generating excessive ssDNA and potentially dissociating entirely from the replisome.

Helicase and polymerases are linked by the replication Fork Protection Complex (FPC), which contributes to replication fidelity and later chromosome segregation. Tim1 (*S. pombe* Swi1) and Tipin (*SpSwi3*) are evolutionarily conserved core components of the FPC that are essential for fork stability [11, 55-58](Table 1). This core is joined by AND1 (*SpMcl1*) and CLASPIN (*SpMrc1*), two proteins that bridge the helicase and polymerases. AND1 links the lagging strand primase (pol $\alpha$ ) [59-62], while CLASPIN connects the leading strand polymerase (pol $\epsilon$ ) [58, 63].

Because of its role maintaining replisome structure, the FPC promotes replication fork efficiency and speed, particularly during fork stalling or pausing. While not essential for DNA replication [58, 64-66], the FPC contributes to processivity [67-70], and has additional roles in response to replication stalling [55, 71, 72], and facilitating sister chromatid cohesion, which is essential for faithful chromosome segregation [73-75].

Human FPC component	Homologues	References
TIMELESS (TIM)	Tim1 ( <i>M. musculus</i> , <i>X. laevis</i> ) Tof1 ( <i>S. cerevisiae</i> ) Swi1 ( <i>S. pombe</i> )	[56, 57, 63, 65, 72]
TIPIN (TIP)	Tipin ( <i>M. musculus</i> , <i>X. laevis</i> ) Csm3 ( <i>S. cerevisiae</i> ) Swi3 ( <i>S. pombe</i> )	[11, 56-58, 63, 75, 75-77]
CLASPIN	Claspin ( <i>M. musculus</i> , <i>X. laevis</i> ) Mrc1 ( <i>S. cerevisiae</i> , <i>S. pombe</i> )	[14, 63, 78-82]
AND1	And1 ( <i>M. musculus</i> , <i>X. laevis</i> ) Ctf4 ( <i>S. cerevisiae</i> ) Mcl1 ( <i>S. pombe</i> )	[59, 61, 73, 83]

**Table 1.** Fork Protection Complex Proteins in Various Species.

### 3. Causes of Replication Fork Stalling

DNA replication occurs in a short period during the cell cycle. In yeasts, replication of the ~12 Mb genome occurs within 20 to 30 minutes out of a 2.5 to 3h cell cycle. Human cells require several hours, a fraction of a full cell cycle, to replicate a substantially larger genome. The rate-limiting factor is replication fork velocity at 1–2 kb/min. This is an astonishing rate, considering secondary and tertiary structure of the genome packaged into higher order chromatin domains. The tight links between helicase, polymerase and FPC promote highly processive replication. Importantly, they also contribute to replication fidelity. Disruption of

any one component (if not already lethal) leads to significant disruptions in processivity and/or fidelity. This is particularly true when impediments to replication are encountered.

Replication pausing and stalling is caused by both natural barriers and external factors [3, 84, 85]. Some regions of DNA cause replication fork stalling through sequence elements (*e.g.* DNA secondary structure in repetitive elements), or protein interference (*i.e.* transcription). Replication “slow zones” have been described in many model organisms, and these may contribute to genome instability and chromosome fragility. One characteristic shared by many “difficult templates” is the presence of repetitive sequence elements that cause fork stalling [86-89].

A replication termination sequence (RTS1) at the mating locus of fission yeast also promotes unidirectional fork progression by binding the replication termination factor 1 (Rtf1) [88, 90-95]. Unidirectional DNA replication is required to establish an imprint that directs mating type switching. RTS1 replication fork pausing is polar, meaning that forks approaching the barrier from one direction will be affected; forks from the opposite direction continue replication [93, 96].

Similarly, ribosomal DNA (rDNA) arrays are an example of a natural, repetitive element that is at risk for fork pausing. Each of the rDNA repeats contains a polar replication terminator, which ensures that forks proceed unidirectionally through each element [86, 97-100]. This occurs as a response to the binding of a fork arrest protein. For example, in fission yeast the Reb1 protein binds the replication termination element Ter3, which promotes long-range DNA interactions with other chromosomal Ter sequences [101, 102]. Localized to the nucleolus, this may nucleate a zone for replication termination [103]. Based on similarity to prokaryotic replication terminators, Reb1-Ter binding may stop the MCM helicase from creating more ssDNA leading to fork pausing and stalling. Pausing of the fork at this site also depends on FPC proteins Swi1 and Swi3.

Replication termination at rDNA is also seen in budding yeast and mammals. In *S.cerevisiae*, the FOB1 protein binds to ribosomal fork barrier elements and arrests progression of the replication fork so that replication is in concert with rDNA transcription [104, 105]. In mice, transcription termination factor 1 (TTF1) binds to termination sites in the rDNA and causes fork arrest [106-108]. It is suggested that Reb1/FOB1/TTF1 binding to their specific rDNA elements blocks the replicative MCM helicase and arrests forks.

The rDNA elements define one type of genomic sequence that causes replication slowing or pausing sites. Other regions of the genome may also cause fragile sites, which are broadly characterized as replication slow zones that are prone to forming DNA breaks [38, 40, 109, 110]. These may be dependent upon the chromatin context, transcriptional activity, or impairment of the fork by external agents, such as HU [111].

HU inhibits the activity of ribonucleotide reductase, which causes a reduction of dNTP pools [112]. HU is frequently used as a reversible early-S phase block reagent in cultured cells. In this sense, HU response is similar to excess thymidine treatment, which changes dNTP pools and induces an early S-phase arrest in metazoan cells [35]. The size of dNTP pools is intimately linked to cell cycle and checkpoint responses [24, 32, 113-115]. Critically,

checkpoint proficiency allows cells to survive HU arrest, hold forks stable, and efficiently restart during release.

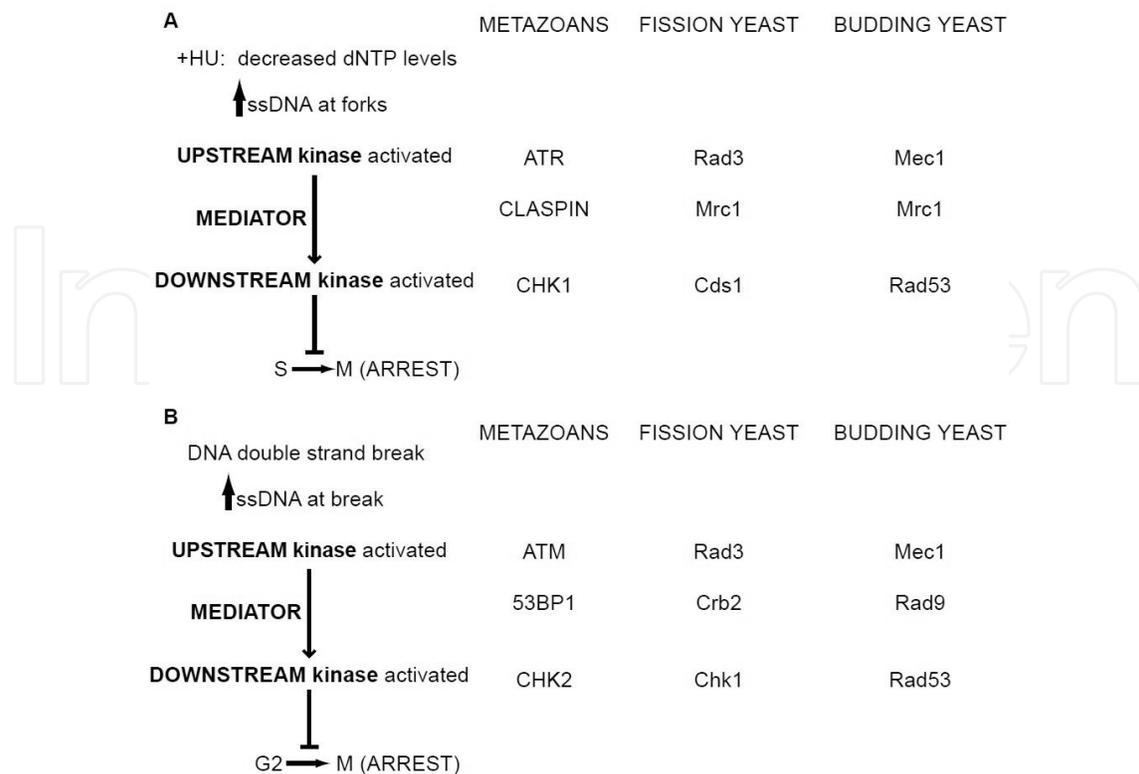
#### 4. Intra-S Checkpoint: keeping things connected

The Intra-S phase checkpoint is a kinase cascade that responds to HU treatment. It serves to stabilize replication forks and arrest replication until dNTP pools recover. The checkpoint also prevents DNA damage from forming, particularly DNA double strand breaks, by restricting endonucleases such as Mus81 that can act on stalled fork structures [9, 10]. In addition, the Intra-S checkpoint regulates recombination enzymes (*e.g.* *SpRqh1/ScSgs1/HsBLM*, Rad60), to preserve stalled forks in a state competent for restart without loss of genetic information [18, 116].

The remainder of this review will focus on the effects of the checkpoint on the replisome itself. During checkpoint activation, the helicase is restrained and stabilized, to prevent excessive unwinding and allow the fork to restart when HU is removed or bypassed. DNA synthesis is also restrained, preventing mutations that may occur during replication in the presence of altered dNTP pools. Late replication origins are prohibited from firing, conserving these “second-chance” origins for later replication restart. These activities help to stabilize established forks after HU treatment, later allowing them to restart. Alternatively, new forks may be established from the late origins in restart to rescue collapsed forks and complete DNA synthesis.

Wild type cells are actively inhibited from DNA synthesis during HU block [10, 17, 36, 58, 65, 117, 118]. That is, the forks do not cease synthesis because they run out of nucleotides. Rather, the checkpoint ensures that the forks are slowed or stopped before such starvation occurs, saving them from the mutagenic effects of dNTP imbalance [34, 42, 119]. These observations are consistent with depletion, rather than exhaustion of specific dNTP pools [22], and extremely slow residual synthesis [33]. This fork arrest is accompanied by inhibition of the helicase [15, 53, 54, 65], which reduces ssDNA accumulation and concomitant RPA binding until HU is removed.

The Intra-S phase checkpoint is a key component of the response to HU and actively restrains forks during replication stress. The initial signal to activate the checkpoint is provided by increased ssDNA created as replication forks stall [39, 53, 54]. ssDNA is bound by RPA and recruits the Rad9-Rad1-Hus1 (9-1-1) complex and ATR family kinases to stalled forks [120, 121]. Thus, the symptom of slow or stalled forks (generation of ssDNA) initiates the checkpoint [4, 120-125]. However, the FPC and checkpoint together ensure that the helicase cannot generate too much ssDNA, which provides one defense against replication fork collapse during HU stalling.



**Figure 1.** The Intra-S phase checkpoint across species. Key components of the checkpoint and their names are described for metazoan (Human, mouse, *Xenopus*), budding yeast (*S. cerevisiae*) and fission yeast (*S. pombe*). A) The replication checkpoint signals the presence of replication fork stalling and ssDNA accumulation through the upstream kinase (ATR/Rad3/Mec1), which activates the downstream effector kinase (CHK1/Cds1/Rad53). CLASPIN/Mrc1 is the mediator of the replication checkpoint and is responsible for efficient dimerization and activation of the downstream kinase. B) Similarities to the DNA damage response checkpoint. When DSBs are generated in G2, ssDNA is created around the break, which recruits the upstream kinase (ATM/Rad3/Mec1). Through the 53BP1/Crb2/Rad9 mediator, the upstream kinase phosphorylates and activates the downstream kinase (CHK2/Chk1/Rad53), which arrests the cell cycle and allows time for repair.

Checkpoint activation is also coupled to the FPC proteins, particularly CLASPIN and its yeast equivalent, Mrc1 [118, 126, 127]. In fission yeast, Mrc1 is phosphorylated by the upstream Rad3/ATR kinase to a checkpoint-active form [128]. This activation recruits the downstream Cds1 kinase to the stalled replication fork and is essential to signal amplification and transmission by activated Cds1. This pathway is conserved: in humans and budding yeast, respectively, Chk1/Rad53 is recruited to stalled forks by CLASPIN/Mrc1 and ATR/Mec1 kinase [6, 14, 16, 124, 129-131].

This S phase checkpoint has a parallel structure to the DNA double strand break (DSB) response: Mrc1 is a replication-specific version of the *Hs*53BP1/*Sc*RAD9/*Sp*Crb2 mediator, which brings together master kinases (ATM/ATR, Mec1 and Rad3) with an effector kinase (CHK2, Rad53 or Chk1) for DSB response [14, 129] (Figure 1).

Fission yeast *cds1* $\Delta$  and *mrc1* $\Delta$  cells rapidly die in HU [117, 118, 127]. These cells lack the Intra-S phase checkpoint and cannot restrain late origin firing or nuclease activity at stalled forks [34, 36, 51, 58, 132]. In contrast to wild type cells, however, these mutants continue

DNA synthesis during HU block [53, 133]. The replication forks develop extensive ssDNA which can be observed by RPA binding. However, the fork proteins do not remain together, suggesting that the link between helicase and polymerase components is lost. Upon release from HU, *cds1Δ* or *mrc1Δ* cells manage a limited amount of further DNA synthesis. Their DNA synthesis proceeds slowly, but cells never achieve a fully replicated amount of DNA. Thus, the forks are failing as they reach the Replication Fork Collapse point, which results in S-phase failure and ultimately, cell death.

## 5. The Rules of Replisome Restraint and Restart, 1: Fork Movement

Considering the phenotype of checkpoint mutants, we infer that an active mechanism restrains the helicase during HU treatment. Genome-wide studies in budding yeast show accumulation of single stranded DNA occurs in checkpoint mutants, adjacent to replication forks upon treatment with HU [39, 58, 133]. Similarly, in fission yeast checkpoint mutants, large masses of RPA can be visualized in whole cells treated with HU, which depend upon the MCM helicase [53].

A simple interpretation is that the helicase becomes uncoupled from the stalling polymerase and unwinds DNA ahead of it. This excessive unwinding generates ssDNA that is prone to breakage, which generates a characteristic DSB marker, phosphorylated histone H2A(X) [15, 39]. In many cases, the RPA signal is associated with markers of DNA synthesis, such as incorporation of the nucleotide analogue BrdU [53], or proximity to replication fork proteins [133]. Importantly, this uncoupling and unwinding occurs at the same time as DNA synthesis during both HU block and release. This suggests a more subtle effect in which leading and lagging strand synthesis is uncoupled, which leads to simultaneous accumulation of ssDNA and markers of synthesis, either because they are in the same region or because the ssDNA is a functional template.

## 6. Rules of Restraint and Restart, 2: Synthesis

The second key to restraint and successful restart is modulating the DNA polymerases. Wild type cells incorporate minimal amounts of nucleoside analogue in the presence of HU. Forks slow but remain stable [7, 17, 34, 54]. The rate of nucleotide analogue incorporation decreases, and DNA content does not increase significantly [36, 117, 134, 135]. In the yeast system, studies suggest that early replication forks extend about 5kb from the origin in the presence of HU before stopping [134, 136]. Decreased dNTP pools slow replication elongation during HU arrest. However, ectopic expansion of dNTPs by expressing ribonucleotide reductase from a plasmid can increase fork velocity even in HU [34]. Upon release from HU, replication rapidly restarts, whether from new origins or reactivation of existing forks, which results in rapid completion of DNA synthesis before cell division.

Budding yeast dNTP metabolism is quite robust and resistant to challenge, sensitive only to high levels of HU or significant NTP imbalance. In contrast, fission yeast [137] and metazo-

an cells are sensitive to low levels of HU, or modest dNTP imbalance, both which are sufficient to provoke replication arrest [7]. In all systems, there is an intimate connection to the Intra-S phase checkpoint.

Surprisingly, checkpoint mutants do not block DNA synthesis in HU, indicating that they are not actually starved for nucleotides, but rather lacking the ability to monitor pool levels [53]. Fission yeast *cds1Δ* mutants continue to synthesize DNA and incorporate analogue. In analysis of chromatin fibers, these can be visualized as extended tracts of newly synthesized DNA despite the presence of HU. Upon release from the drug, *cds1Δ* mutants continue to incorporate some analogue before reaching a plateau, by which time they have accumulated approximately 66% the total amount of DNA incorporated in wild type (continuous labeling, block and release) [53]. These differences can be measured by detection of analogue incorporation, but are obscured by total DNA content analysis, which is prone to artifact [22, 36, 134].

The difference between the two situations is that much of this synthesis occurs *during* HU treatment in the mutant, and only *after release* in wild type [53]. Thus, it is not until the recovery period that the majority of *cds1Δ* forks break down and can no longer synthesize DNA. We define the point at which synthesis ceases as the Replication Fork Collapse Point (see discussion of the Collapse Point in section “The Collapse Point: A Metric for Fork Stability”). Importantly, this is an extended window of time where there is a stochastic probability of forks arresting and suffering collapse. Our data show that regions of DNA synthesis upon HU release have high levels of RPA [53], which indicates that fork collapse is accompanied by accumulation of ssDNA. This may reflect a burden of damage, incurred in HU, which is remembered during release at the Collapse Point.

Polymerase  $\epsilon$  is coupled to the helicase by Mrc1 and the FPC proteins Tof1 (Swi1) and Csm1 (Swi3) [63, 65, 132, 138]. This is thought to stabilize leading strand components at stalled forks in HU. Asynchronously growing *mrc1Δ* cells lack this connection, which leads to intrinsic damage and a higher level of basal Cds1 phosphorylation even without added replication stress [128]. This essentially uncouples leading and lagging strands. *mrc1Δ* cells treated with HU incorporate more nucleotide analogue but in shorter DNA fiber tracts. This is consistent with a role for Mrc1 in modulating origin firing, as well as rate [139-141]. The increase in DNA synthesis is only slightly higher after release, which could be attributed to slower forks or pole uncoupling as in *S. cerevisiae* [81, 141]. These data suggest that in the absence of Mrc1, forks continue to synthesize a low, steady level of DNA and this is independent of Cds1.

Mrc1 brings Cds1 and Rad3 together to phosphorylate Cds1 on threonine 11 [128, 142]. Subsequently, Cds1 activation is amplified by dimerization and autophosphorylation, setting in motion the full Intra-S phase checkpoint [128]. HU treatment induces little Cds1-T11 phosphorylation in *mrc1Δ*; instead, the damage response kinase Chk1 is activated. This suggests that there is conversion of stalled synthesis into DNA damage. Consistent with this, phospho-H2A accumulates in *mrc1Δ* nuclei and replicated fiber tracts after HU release [53]. The damage signal is frequently coincident with areas of synthesis, but often distinct from RPA-

heavy areas. Thus, unwinding and synthesis are likely uncoupled and distinct in *mrc1Δ* with HU treatment.

## 7. Rules of Restraint and Restart, 3: the late origins

An additional function of the Intra-S phase checkpoint is to restrain late origins from firing. Upon release from HU, these origins become competent for replication, and establish “rescue forks” that ensure completion of DNA replication [33, 36, 143-145]. Could these origins explain the post-release DNA synthesis observed in the checkpoint mutants? While late origin firing must contribute to some of the synthesis after release, we suggest that much of the post-release DNA synthesis does not occur from late origin firing, for the following reasons.

First, origin firing is de-regulated in HU blocked checkpoint mutants, which suggests that many late origins have already fired at the time of release, and are not available for this further synthesis. Recent work on dNTP pools in budding yeast suggests that >200 additional origins are fired in a *rad53-11* mutant compared to wild type in HU [34]. Deleting the ribonucleotide reductase inhibitor *Sml1* results in activation of late origins in HU, while a *rad53-11sml1Δ* double mutant shows increased replication tracts in HU [34]. *Sml1* is regulated by the replication checkpoint [114], and *sml1Δ* cells have increased dNTP pools [113, 146]. *sml1Δ* mutant backgrounds are frequently used in replication checkpoint studies because they overcome the lethal effects of *rad53Δ* or *mec1Δ*, but this makes direct comparison of these double mutants with other organisms, which retain controls of NTP pools, difficult. However, HU has also been shown to arrest replication without completely exhausting dNTP pools [22, 34], which suggests that cells sense small dNTP changes. Perhaps the Intra-S phase checkpoint also contributes to fork slowing and stalling, and is not limited to signal transduction at stalled forks.

Second, it is likely that late origins that fire in checkpoint mutants after HU release are incapable of synthesizing more than a short tract length, due to lack of nucleotides [137]. More analogue is incorporated in *cds1Δ* compared to wild type for the first 30 minutes after HU release, suggesting that start-up replication is both different and faster in the mutant cells. Additionally, more origins fire in mutants during HU block [140, 143, 147], suggesting that fewer origins remain to be activated after release. These observations imply that for forks established during HU arrest in *cds1Δ* and *mrc1Δ*, synthesis cannot proceed past a point of increased fork collapse and template damage [9, 15, 116, 148-150]. The role of origin repression, using mutant cells that impede origin firing, is required to confirm the degree to which DNA synthesis occurring in checkpoint mutants after HU release is dependent on late origins.

Together, these observations from multiple systems suggest that wild type cells survive HU block and release through coordination of several mechanisms: control of late origin firing, maintenance of existing replication forks, and later restart of the stabilized forks. Wild type

cells do not encounter the Replication Fork Collapse Point because forks are maintained, replication is successfully restarted, and DNA synthesis completed.

## 8. Converting Stalled Forks to Restart

After HU is removed from culture medium, stabilized replication forks are returned to competence for DNA synthesis. In theory, immediate restart from a stabilized fork may be possible if all components are in place, having been protected from disassembly during HU arrest. In many cases this is likely to involve recombination pathways and the Rad51 recombinase. Rad51 binds to replisome components in HU, and around damaged replication forks [7, 15, 151]. Rad51 binds to ssDNA and promotes homologous recombination by allowing broken DNA to invade a homologous region for repair [52, 152, 153]. Checkpoint mutants have additional ssDNA, and experience “branch migration” of the fork structure [7, 52, 94, 154]. The resulting “chicken foot” structure is at risk for becoming a break or collapsed fork. Alternatively, the cruciform structure can be resolved by exonuclease Exo1, but leads to a partially replicated structure that cannot be replicated without *de novo* polymerase recruitment or break-induced replication [154-158].

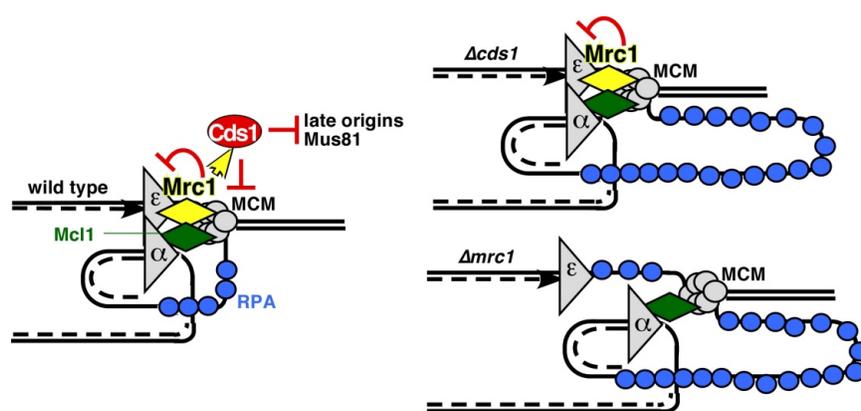
The amount of time in HU until release has different effects in yeast and metazoan cultures. Both budding and fission yeast begin to arrest in HU within the first hour of HU exposure (e.g. [53, 134, 144, 159]). After a few hours at normal growth temperature, adaptation occurs, probably through changes in ribonucleotide reductase activity. In budding yeast, long-term HU exposure causes normal replication profiles to proceed at a glacial pace [33]. Similarly, human cells show increased sensitivity to HU over time, where fewer forks are observed with extended HU dose [7]. Peterman *et.al.* (2010) demonstrated that restarting long-HU treatment forks depends on both Rad51 and the repair protein XRCC3. Thus, protracted HU exposure may change that repair pathway used in fork repair, slowing the entire replication program [7, 153]. Slightly later than ssDNA accumulation, we detect Rad52 foci in live cells, increasing during HU block and release but lagging behind RPA accumulation [53]. Intriguingly, MRN/MRX- components co-localize at the replication fork and are important for fork stability (e.g. [35, 149, 160-162]), pointing at the essential role of recombination repair in restarting stalled replication forks.

## 9. The Collapse Point: A Metric for Fork Stability

The concept of replication fork “collapse” encompasses the observations that DNA damage and broken forks lead to loss of replication. ssDNA accumulates at susceptible forks and is a marker of increased risk of collapse [39, 54, 133, 137]. The DNA damage created at a stalled fork at or before collapse may not simply be DSBs. In fact, single strand breaks may form an

important part in the damage process, converted to DSBs either during fork regression or in a second S-phase [163-165].

We propose the Collapse Point as the time when the balance between replication fork processivity and instability tips toward disaster. The time when the majority of forks in a cell have irreversibly, irrecoverably failed and replication will not be completed. Ongoing synthesis in checkpoint mutants during HU treatment sets the forks on a course to destruction, but actual collapse does not occur until the attempt to recover. We suggest some replication forks retain activity and undergo a shortened replication restart after HU release. This is consistent with data in fission and budding yeasts that fork components are retained and move during HU arrest in checkpoint deficient cells [53, 133].



**Figure 2.** Model of the Replication Fork in HU before the Collapse Point.

Model of how replication fork architecture is affected by HU treatment in wild type, *cds1Δ* or *mrc1Δ* fission yeast cells. Left, wild type forks are stabilized through the Fork Protection Complex of Mrc1 (yellow diamond) and Mcl1 (green diamond), and the Intra-S phase checkpoint. As part of the FPC, Mrc1 forms a link between polymerase epsilon ( $\epsilon$ ), while Mcl1 links the helicase to polymerase alpha ( $\alpha$ ). A small loop of ssDNA forms at the fork in response to HU. This signals through the mediator function of Mrc1, activating Cds1 kinase. Cds1 kinase stabilizes and stalls the helicase activity of the replisome. Cds1 also inhibits Mus81 endonucleolytic activity and late origin firing, maintaining wild type cells in a state where replication can be restarted and/or finished from late origins after HU is removed. Right, top; when Cds1 is absent, a larger ssDNA loop forms from failing to slow the helicase. ssDNA is coated with replication protein A (RPA, blue circles) which form large foci in nuclei during HU block and release. Mrc1 is present, likely stabilizing pole, but the ssDNA may serve as template for lagging strand synthesis by pol  $\alpha$ . Note that a ssDNA loop is presumed on the *cds1Δ* leading strand, but is omitted for clarity. The Intra-S phase checkpoint is not activated, late origins fire, and nucleases are not regulated.

Right, bottom; in the absence of Mrc1 the helicase is deregulated and potentially detached from polymerases. Large amounts of ssDNA form at individual forks and are coated by RPA. Although there is a great deal of ssDNA template, the length of *mrc1Δ* tracts in HU suggests that replication is slow, and that ssDNA areas are used as template during block and release up to a point where damage is encountered and forks collapse. In both *cds1Δ* and *mrc1Δ* cases, loss of replicative activity correlates with increased ssDNA foci, which build during HU block and release, suggesting that both mutants reach the Replication Fork Collapse Point during release.

The Replication Fork Collapse Point has no meaning for an individual fork; instead, it is the emergent property of the sum behavior of forks in a cell. The Collapse Point will generally be *estimated* by ensemble averaging across all cells in a culture.

While forks in *cds1Δ* and *mrc1Δ* retain synthesis activity, they are not necessarily the same as wild type (Figure 2). The amount of ssDNA and DNA damage signal (phospho-H2A(X)) is increased proximal to *cds1Δ* and *mrc1Δ* replication forks [39, 53], which could represent uncoupling of leading and lagging strand synthesis in advance of replication fork collapse. Together, these observations suggest that replication fork activity in checkpoint mutants shapes their stability. The inability of the mutant cells to restrain replication during HU and throughout release contributes to extensive ssDNA, DNA damage, and eventual collapse.

These results point to the *cds1Δ* and *mrc1Δ* Replication Fork Collapse Points occurring later than previously expected, and largely during HU release as cells attempt to resume the cell cycle. Thus, forks do not immediately collapse, but instead retain synthesis activity. The ends of new synthesis in release bear the marks of ssDNA and DNA damage. We conclude that fork collapse for these mutants is delayed, but its seeds are sown during HU block, only coming into full effect during release. The Replication Fork Collapse Point may be used as a descriptor for other genotypes to describe both how and when the majority of replication forks are destroyed in a population of cells. This is an execution point: while the cells are not viable by this time, the Collapse Point signals the time at which decay leading to death is fully established. We anticipate that the Collapse Point will be much later for *rad51Δ* or other repair-deficient mutants, which collapse by failing to properly restart. In contrast, Fork Protection Complex mutants may show an intermediate timing, or an incomplete Fork Collapse Point.

In turn, these studies prompt further questions. Do dNTP pools recover after release in *cds1Δ* and *mrc1Δ* cells? If this is the case, as suggested by the increase in replication after release in *cds1Δ* and *mrc1Δ* cells, what are the additional defining features of replication fork collapse?

## 10. Conclusions

Monitoring replication competency, accumulation of ssDNA and DNA damage signals around replication forks permits modeling to determine how replication forks respond to HU arrest and recovery. This, in turn, indicates what role checkpoint proteins Cds1 and Mrc1 play in fork stability and effective restart. The Replication Fork Collapse Point incorporates the signs and symptoms of fork collapse and attempts to put a time to when the majority of replication forks undergo collapse. This is likely different for different genetic backgrounds missing key components of checkpoint signal, fork stabilization and replication restart. Future work will dissect replication fork proteins in HU and release, and take the genome-wide data from microarray and sequencing, moving into monitoring patterns at individual replication forks. Since replication stability and fidelity is a key barrier to malignancy, defining when and how replication forks collapse in the absence of checkpoint will allow insights into the development and prevention of cancer.

## Acknowledgements

We thank Forsburg and Aparicio lab members (USC) for helpful discussions, and Marc Green and Ruben Petreaca for manuscript comments. This work funded by a National Institutes of Health grant R01 GM059321 to SLF.

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## References

- [1] Torres-Rosell, J., De Piccoli, G., Cordon-Preciado, V., Farmer, S., Jarmuz, A., Machin, F., et al. (2007). Anaphase onset before complete DNA replication with intact checkpoint responses. *Science*, 315(5817), 1411-1415.
- [2] Rothstein, R., Michel, B., & Gangloff, S. (2000). Replication fork pausing and recombination or "gimme a break". *Genes Dev*, 14(1), 1-10.
- [3] Labib, K., & Hodgson, B. (2007). Replication fork barriers: pausing for a break or stalling for time? *EMBO Rep*, 8(4), 346-353.
- [4] Lucca, C., Vanoli, F., Cotta-Ramusino, C., Pellicoli, A., Liberi, G., Haber, J., et al. (2004). Checkpoint-mediated control of replisome-fork association and signalling in response to replication pausing. *Oncogene*, 23(6), 1206-1213.
- [5] Meister, P., Taddei, A., Vernis, L., Poidevin, M., Gasser, S. M., & Baldacci, G. (2005). Temporal separation of replication and recombination requires the intra-S checkpoint. *J Cell Biol*, 168(4), 537-544.
- [6] Tourriere, H., & Pasero, P. (2007). Maintenance of fork integrity at damaged DNA and natural pause sites. *DNA Repair (Amst)*, 6(7), 900-913.
- [7] Petermann, E., Orta, M. L., Issaeva, N., Schultz, N., & Helleday, T. (2010). Hydroxyurea-stalled replication forks become progressively inactivated and require two different RAD51-mediated pathways for restart and repair. *Mol Cell*, 37(4), 492-502.
- [8] Mao, N., Kojic, M., & Holloman, W. K. (2009). Role of Blm and collaborating factors in recombination and survival following replication stress in *Ustilago maydis*. *DNA Repair (Amst)*, 8(6), 752-9.

- [9] Froget, B., Blaisonneau, J., Lambert, S., & Baldacci, G. (2008). Cleavage of stalled forks by fission yeast Mus81/Eme1 in absence of DNA replication checkpoint. *Mol Biol Cell*, 19(2), 445-456.
- [10] Kai, M., Boddy, M. N., Russell, P., & Wang, T. S. (2005). Replication checkpoint kinase Cds1 regulates Mus81 to preserve genome integrity during replication stress. *Genes Dev*, 19(8), 919-932.
- [11] Noguchi, E., Noguchi, C., Du, L. L., & Russell, P. (2003). Swi1 prevents replication fork collapse and controls checkpoint kinase Cds1. *Mol Cell Biol*, 23(21), 7861-7874.
- [12] Bryant, H. E., Petermann, E., Schultz, N., Jemth, A. S., Loseva, O., & Issaeva, N. (2009). PARP is activated at stalled forks to mediate Mre11-dependent replication restart and recombination. *Embo J*, 28(17), 2601-2615.
- [13] Bernstein, K. A., Shor, E., Sunjevaric, I., Fumasoni, M., Burgess, R. C., Foiani, M., et al. (2009). Sgs1 function in the repair of DNA replication intermediates is separable from its role in homologous recombinational repair. *Embo J*, 28(7), 915-925.
- [14] Alcasabas, A. A., Osborn, A. J., Bachant, J., Hu, F., Werler, P. J., Bousset, K., et al. (2001). Mrc1 transduces signals of DNA replication stress to activate Rad53. *Nat Cell Biol*, 3(11), 958-965.
- [15] Bailis, J. M., Luche, D. D., Hunter, T., & Forsburg, S. L. (2008). Minichromosome maintenance proteins interact with checkpoint and recombination proteins to promote s-phase genome stability. *Molecular and cellular biology*, 28(5), 1724-1738.
- [16] Branzei, D., & Foiani, M. (2007). Interplay of replication checkpoints and repair proteins at stalled replication forks. *DNA Repair (Amst)*, 6(7), 994-1003.
- [17] Lopes, M., Cotta-Ramusino, C., Pelliccioli, A., Liberi, G., Plevani, P., Muzi-Falconi, M., et al. (2001). The DNA replication checkpoint response stabilizes stalled replication forks. *Nature*, 412(6846), 557-561.
- [18] Marchetti, M. A., Kumar, S., Hartsuiker, E., Maftahi, M., Carr, A. M., & Freyer, G. A. (2002). A single unbranched S-phase DNA damage and replication fork blockage checkpoint pathway. *Proc Natl Acad Sci U S A*, 99(11), 7472-7477.
- [19] Tsang, E., & Carr, A. M. (2008). Replication fork arrest, recombination and the maintenance of ribosomal DNA stability. *DNA Repair (Amst)*, 7(10), 1613-1623.
- [20] Bartkova, J., Horejsi, Z., Koed, K., Kramer, A., Tort, F., & Zieger, K. (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature*, 434(7035), 864-870.
- [21] Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., et al. (2006). Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature*, 444(7119), 633-637.

- [22] Koc, A., Wheeler, L. J., Mathews, C. K., & Merrill, G. F. (2004). Hydroxyurea arrests DNA replication by a mechanism that preserves basal dNTP pools. *J Biol Chem*, 279(1), 223-30.
- [23] Matsumoto, M., Rey, D. A., & Cory, J. G. (1990). Effects of cytosine arabinoside and hydroxyurea on the synthesis of deoxyribonucleotides and DNA replication in L1210 cells. *Adv Enzyme Regul*, 30-47.
- [24] Bianchi, V., Pontis, E., & Reichard, P. (1986). Changes of deoxyribonucleoside triphosphate pools induced by hydroxyurea and their relation to DNA synthesis. *J Biol Chem*, 261(34), 16037-16042.
- [25] Goodman, M. F., Hopkins, R. L., Lasken, R., & Mhaskar, D. N. (1985). The biochemical basis of 5-bromouracil- and 2-aminopurine-induced mutagenesis. *Basic Life Sci*, 31, 409-423.
- [26] Hakansson, P., Dahl, L., Chilkova, O., Domkin, V., & Thelander, L. (2006). Thelander L. The Schizosaccharomyces pombe replication inhibitor Spd1 regulates ribonucleotide reductase activity and dNTPs by binding to the large Cdc22 subunit. *The Journal of biological chemistry*, 281(3), 1778-1783.
- [27] Kunz, BA, Kang, X. L., & Kohalmi, L. (1991). The yeast rad18 mutator specifically increases G.C---T.A transversions without reducing correction of G-A or C-T mismatches to G.C pairs. *Molecular and cellular biology*, 11(1), 218-225.
- [28] Kohalmi, S. E., Haynes, R. H., & Kunz, B. A. (1988). Instability of a yeast centromere plasmid under conditions of thymine nucleotide stress. *Mutat Res*, 207(1), 13-16.
- [29] Potter, C. G. (1971). Induction of polyploidy by concentrated thymidine. *Exp Cell Res*, 68(2), 442-448.
- [30] Meuth, M. (1981). Role of deoxynucleoside triphosphate pools in the cytotoxic and mutagenic effects of DNA alkylating agents. *Somatic Cell Genet*, 7(1), 89-102.
- [31] Meuth, M. (1983). Deoxycytidine kinase-deficient mutants of Chinese hamster ovary cells are hypersensitive to DNA alkylating agents. *Mutat Res*, 110(2), 383-391.
- [32] Mulder, K. W., Winkler, G. S., & Timmers, H. T. (2005). DNA damage and replication stress induced transcription of RNR genes is dependent on the Ccr4-Not complex. *Nucleic Acids Res*, 33(19), 6384-6392.
- [33] Alvino, G. M., Collingwood, D., Murphy, J. M., Delrow, J., Brewer, B. J., & Raghuraman, M. K. (2007). Replication in hydroxyurea: it's a matter of time. *Molecular and cellular biology*, 27(18), 6396-6406.
- [34] Poli, J., Tsaponina, O., Crabbe, L., Keszthelyi, A., Pantesco, V., & Chabes, A. (2012). dNTP pools determine fork progression and origin usage under replication stress. *The EMBO journal*, 31(4), 883-894.

- [35] Bolderson, E., Scolah, J., Helleday, T., Smythe, C., & Meuth, M. (2004). ATM is required for the cellular response to thymidine induced replication fork stress. *Hum Mol Genet*, 13(23), 2937-2945.
- [36] Feng, W., Collingwood, D., Boeck, M. E., Fox, L. A., Alvino, G. M., Fangman, W. L., et al. (2006). Genomic mapping of single-stranded DNA in hydroxyurea-challenged yeasts identifies origins of replication. *Nature cell biology*, 8(2), 148-55.
- [37] Vassin, V. M., Anantha, R. W., Sokolova, E., Kanner, S., & Borowiec, J. A. (2009). Human RPA phosphorylation by ATR stimulates DNA synthesis and prevents ssDNA accumulation during DNA-replication stress. *J Cell Sci*, Pt 22, 4070-4080.
- [38] Letessier, A., Millot, G. A., Koundrioukoff, S., Lachages, A. M., Vogt, N., Hansen, R. S., et al. (2011). Cell-type-specific replication initiation programs set fragility of the FRA3B fragile site. *Nature*, 470(7332), 120-123.
- [39] Feng, W., Di Rienzi, S. C., Raghuraman, M. K., & Brewer, B. J. (2011). Replication stress-induced chromosome breakage is correlated with replication fork progression and is preceded by single-stranded DNA formation. *G3 (Bethesda)*, 1(5), 327-35.
- [40] Durkin, S. G., & Glover, T. W. (2007). Chromosome fragile sites. *Annu Rev Genet*, 41-169.
- [41] Chabes, A., Georgieva, B., Domkin, V., Zhao, X., Rothstein, R., & Thelander, L. (2003). Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase. *Cell*, 112(3), 391-401.
- [42] Davidson, M. B., Katou, Y., Keszthelyi, A., Sing, T. L., Xia, T., Ou, J., et al. (2012). Endogenous DNA replication stress results in expansion of dNTP pools and a mutator phenotype. *The EMBO journal*, 31(4), 895-907.
- [43] Fasullo, M., Tsaponina, O., Sun, M., & Chabes, A. (2010). Elevated dNTP levels suppress hyper-recombination in *Saccharomyces cerevisiae* S-phase checkpoint mutants. *Nucleic acids research*, 38(4), 1195-1203.
- [44] Kumar, D., Abdulovic, A. L., Viberg, J., Nilsson, A. K., Kunkel, T. A., & Chabes, A. (2011). Mechanisms of mutagenesis in vivo due to imbalanced dNTP pools. *Nucleic acids research*, 39(4), 1360-1371.
- [45] Muzi-Falconi, M., Giannattasio, M., Foiani, M., & Plevani, P. (2003). The DNA polymerase alpha-primase complex: multiple functions and interactions. *ScientificWorld-Journal*, 3-21.
- [46] Langston, L. D., Indiani, C., & O'Donnell, M. (2009). Whither the replisome: emerging perspectives on the dynamic nature of the DNA replication machinery. *Cell Cycle*, 8(17), 2686-2691.
- [47] Hubscher, U. (2009). DNA replication fork proteins. *Methods Mol Biol*, 521-19.

- [48] Lopez-Contreras, A. J., & Fernandez-Capetillo, O. (2010). The ATR barrier to replication-born DNA damage. *DNA repair*, 9(12), 1249-1255.
- [49] Glover, T. W., Arlt, M. F., Casper, A. M., & Durkin, S. G. (2005). Mechanisms of common fragile site instability. *Hum Mol Genet* [2], R197-R205.
- [50] Wang, X., & Haber, J. E. (2004). Role of Saccharomyces single-stranded DNA-binding protein RPA in the strand invasion step of double-strand break repair. *PLoS Biol*, E21.
- [51] Alabert, C., Bianco, J. N., & Pasero, P. (2009). Differential regulation of homologous recombination at DNA breaks and replication forks by the Mrc1 branch of the S-phase checkpoint. *Embo J*, 28(8), 1131-1141.
- [52] Sugiyama, T., & Kantake, N. (2009). Dynamic regulatory interactions of rad51, rad52, and replication protein-a in recombination intermediates. *J Mol Biol*, 390(1), 45-55.
- [53] Sabatinos, S. A., Green, M. D., & Forsburg, S. L. (2012). Continued DNA synthesis in replication checkpoint mutants leads to fork collapse. *submitted*.
- [54] Sogo, J. M., Lopes, M., & Foiani, M. (2002). Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. *Science*, 297(5581), 599-602.
- [55] McFarlane, R. J., Mian, S., & Dalgaard, J. Z. (2010). The many facets of the Tim-Tipin protein families' roles in chromosome biology. *Cell Cycle*, 9(4), 700-705.
- [56] Gotter, A. L., Suppa, C., & Emanuel, B. S. (2007). Mammalian TIMELESS and Tipin are evolutionarily conserved replication fork-associated factors. *J Mol Biol*, 366(1), 36-52.
- [57] Noguchi, E., Noguchi, C., Mc Donald, W. H., Yates, J. R. 3rd, & Russell, P. (2004). Swi1 and Swi3 are components of a replication fork protection complex in fission yeast. *Mol Cell Biol*, 24(19), 8342-8355.
- [58] Katou, Y., Kanoh, Y., Bando, M., Noguchi, H., Tanaka, H., Ashikari, T., et al. (2003). S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. *Nature*, 424(6952), 1078-1083.
- [59] Williams, D. R., & McIntosh, J. R. (2002). mcl1+, the Schizosaccharomyces pombe homologue of CTF4, is important for chromosome replication, cohesion, and segregation. *Eukaryot Cell*, 1(5), 758-773.
- [60] Miles, J., & Formosa, T. (1992). Evidence that POB1, a Saccharomyces cerevisiae protein that binds to DNA polymerase alpha, acts in DNA metabolism in vivo. *Mol Cell Biol*, 12(12), 5724-6735.
- [61] Gambus, A., van Deursen, F., Polychronopoulos, D., Foltman, M., Jones, R. C., & Edmondson, R. D. (2009). A key role for Ctf4 in coupling the MCM2-7 helicase to DNA polymerase alpha within the eukaryotic replisome. *Embo J*, 28(19), 2992-3004.
- [62] Im, J. S., Ki, S. H., Farina, A., Jung, H., Hurwitz, J., & Lee, J. K. (2009). Assembly of the Cdc45-Mcm2-7-GINS complex in human cells requires the Ctf4/And-1, RecQL4, and Mcm10 proteins. *Proc Natl Acad Sci U S A*, 106(37), 15628-15632.

- [63] Bando, M., Katou, Y., Komata, M., Tanaka, H., Itoh, T., Sutani, T., et al. (2009). Csm3, Tof1, and Mrc1 form a heterotrimeric mediator complex that associates with DNA replication forks. *J Biol Chem*, 284(49), 34355-34365.
- [64] Gambus, A., Jones, R. C., Sanchez-Diaz, A., Kanemaki, M., van Deursen, F., & Edmondson, R. D. (2006). GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. *Nat Cell Biol*, 8(4), 358-366.
- [65] Nedelcheva, M. N., Roguev, A., Dolapchiev, L. B., Shevchenko, A., Taskov, H. B., Shevchenko, A., et al. (2005). Uncoupling of unwinding from DNA synthesis implies regulation of MCM helicase by Tof1/Mrc1/Csm3 checkpoint complex. *J Mol Biol*, 347(3), 509-21.
- [66] Calzada, A., Hodgson, B., Kanemaki, M., Bueno, A., & Labib, K. (2005). Molecular anatomy and regulation of a stable replisome at a paused eukaryotic DNA replication fork. *Genes Dev*, 19(16), 1905-1919.
- [67] Hamdan, S. M., Johnson, D. E., Tanner, N. A., Lee, J. B., Qimron, U., Tabor, S., et al. (2007). Dynamic DNA helicase-DNA polymerase interactions assure processive replication fork movement. *Molecular cell*, 27(4), 539-549.
- [68] Kim, S., Dallmann, H. G., Mc Henry, C. S., & Marians, K. J. (1996). Coupling of a replicative polymerase and helicase: a tau-DnaB interaction mediates rapid replication fork movement. *Cell*, 84(4), 643-650.
- [69] Stano, N. M., Jeong, Y. J., Donmez, I., Tummalapalli, P., Levin, M. K., & Patel, S. S. (2005). DNA synthesis provides the driving force to accelerate DNA unwinding by a helicase. *Nature*, 435(7040), 370-373.
- [70] Tougu, K., & Marians, K. J. (1996). The interaction between helicase and primase sets the replication fork clock. *The Journal of biological chemistry*, 271(35), 21398-21405.
- [71] Unsal-Kacmaz, K., Chastain, P. D., Qu, P. P., Minoos, P., Cordeiro-Stone, M., Sancar, A., et al. (2007). The human Tim/Tipin complex coordinates an Intra-S checkpoint response to UV that slows replication fork displacement. *Mol Cell Biol*, 27(8), 3131-3142.
- [72] Yoshizawa-Sugata, N., & Masai, H. (2007). Human Tim/Timeless-interacting protein, Tipin, is required for efficient progression of S phase and DNA replication checkpoint. *J Biol Chem*, 282(4), 2729-2740.
- [73] Errico, A., Cosentino, C., Rivera, T., Losada, A., Schwob, E., Hunt, T., et al. (2009). Tipin/Tim1/And1 protein complex promotes Pol alpha chromatin binding and sister chromatid cohesion. *Embo J*, 28(23), 3681-3692.
- [74] Tanaka, H., Kubota, Y., Tsujimura, T., Kumano, M., Masai, H., & Takisawa, H. (2009). Replisome progression complex links DNA replication to sister chromatid cohesion in *Xenopus* egg extracts. *Genes Cells*, 14(8), 949-963.

- [75] Leman, A. R., Noguchi, C., Lee, C. Y., & Noguchi, E. (2010). Human Timeless and Tipin stabilize replication forks and facilitate sister-chromatid cohesion. *J Cell Sci*, Pt 5, 660-670.
- [76] Errico, A., Costanzo, V., & Hunt, T. (2007). Tipin is required for stalled replication forks to resume DNA replication after removal of aphidicolin in *Xenopus* egg extracts. *Proc Natl Acad Sci U S A*, 104(38), 14929-34.
- [77] Tanaka, T., Yokoyama, M., Matsumoto, S., Fukatsu, R., You, Z., & Masai, H. (2010). Fission yeast Swi1-Swi3 complex facilitates DNA binding of Mrc1. *The Journal of biological chemistry*, 285(51), 39609-22.
- [78] Kumagai, A., & Dunphy, W. G. (2000). Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in *Xenopus* egg extracts. *Mol Cell*, 6(4), 839-849.
- [79] Kumagai, A., Kim, S. M., & Dunphy, W. G. (2004). Claspin and the activated form of ATR-ATRIP collaborate in the activation of Chk1. *J Biol Chem*, 279(48), 49599-45608.
- [80] Lee, J., Gold, D. A., Shevchenko, A., Shevchenko, A., & Dunphy, W. G. (2005). Roles of replication fork-interacting and Chk1-activating domains from Claspin in a DNA replication checkpoint response. *Mol Biol Cell*, 16(11), 5269-5282.
- [81] Lou, H., Komata, M., Katou, Y., Guan, Z., Reis, C. C., Budd, M., et al. (2008). Mrc1 and DNA polymerase epsilon function together in linking DNA replication and the S phase checkpoint. *Molecular cell*, 32(1), 106-117.
- [82] Osborn, A. J., & Elledge, S. J. (2003). Mrc1 is a replication fork component whose phosphorylation in response to DNA replication stress activates Rad53. *Genes & development*, 17(14), 1755-1767.
- [83] Williams, D. R., & McIntosh, J. R. (2005). Mcl1p is a polymerase alpha replication accessory factor important for S-phase DNA damage survival. *Eukaryotic cell*, 4(1), 166-177.
- [84] Arlt, M. F., Mulle, J. G., Schaibley, V. M., Ragland, R. L., Durkin, S. G., & Warren, S. T. (2009). Replication stress induces genome-wide copy number changes in human cells that resemble polymorphic and pathogenic variants. *Am J Hum Genet*, 84(3), 339-350.
- [85] Mirkin, E. V., & Mirkin, S. M. (2007). Replication fork stalling at natural impediments. *Microbiol Mol Biol Rev*, 71(1), 13-35.
- [86] Krings, G., & Bastia, D. (2004). swi1- and swi3-dependent and independent replication fork arrest at the ribosomal DNA of *Schizosaccharomyces pombe*. *Proc Natl Acad Sci U S A*, 101(39), 14085-90.
- [87] Noguchi, C., & Noguchi, E. (2007). Sap1 promotes the association of the replication fork protection complex with chromatin and is involved in the replication checkpoint in *Schizosaccharomyces pombe*. *Genetics*, 175(2), 553-566.

- [88] Dalgaard, J. Z., & Klar, A. J. (2000). *swi1* and *swi3* perform imprinting, pausing, and termination of DNA replication in *S. pombe*. *Cell*, 102(6), 745-751.
- [89] Razidlo, D. F., & Lahue, R. S. (2008). *Mrc1*, *Tof1* and *Csm3* inhibit CAG.CTG repeat instability by at least two mechanisms. *DNA Repair (Amst)*, 7(4), 633-640.
- [90] Ahn, J. S., Osman, F., & Whitby, M. C. (2005). Replication fork blockage by RTS1 at an ectopic site promotes recombination in fission yeast. *The EMBO journal*, 24(11), 2011-2023.
- [91] Eydmann, T., Sommariva, E., Inagawa, T., Mian, S., Klar, A. J., & Dalgaard, J. Z. (2008). *Rtf1*-mediated eukaryotic site-specific replication termination. *Genetics*, 180(1), 27-39.
- [92] Codlin, S., & Dalgaard, J. Z. (2003). Complex mechanism of site-specific DNA replication termination in fission yeast. *The EMBO journal*, 22(13), 3431-3440.
- [93] Dalgaard, J. Z., & Klar, A. J. (2001). A DNA replication-arrest site RTS1 regulates imprinting by determining the direction of replication at *mat1* in *S. pombe*. *Genes & development*, 15(16), 2060-2068.
- [94] Lambert, S., Mizuno, K., Blaisonneau, J., Martineau, S., Chanet, R., Freon, K., et al. (2010). Homologous recombination restarts blocked replication forks at the expense of genome rearrangements by template exchange. *Molecular cell*, 39(3), 346-359.
- [95] Vengrova, S., Codlin, S., & Dalgaard, J. Z. (2002). RTS1-an eukaryotic terminator of replication. *Int J Biochem Cell Biol*, 34(9), 1031-1034.
- [96] Lee, B. S., Grewal, S. I., & Klar, A. J. (2004). Biochemical interactions between proteins and *mat1* cis-acting sequences required for imprinting in fission yeast. *Molecular and cellular biology*, 24(22), 9813-9822.
- [97] Coulon, S, Noguchi, E, Noguchi, C, Du, LL, Nakamura, TM, & Russell, P. (2006). Rad22Rad52-dependent repair of ribosomal DNA repeats cleaved by Slx1-Slx4 endonuclease. *Mol Biol Cell*, 17(4), 2081-2090.
- [98] Kaplan, D. L., & Bastia, D. (2009). Mechanisms of polar arrest of a replication fork. *Mol Microbiol*, 72(2), 279-285.
- [99] Krings, G., & Bastia, D. (2005). *Sap1p* binds to *Ter1* at the ribosomal DNA of *Schizosaccharomyces pombe* and causes polar replication fork arrest. *J Biol Chem*, 280(47), 39135-39142.
- [100] Maric, C., Levacher, B., & Hyrien, O. (1999). Developmental regulation of replication fork pausing in *Xenopus laevis* ribosomal RNA genes. *J Mol Biol*, 291(4), 775-788.
- [101] Biswas, S., & Bastia, D. (2008). Mechanistic insights into replication termination as revealed by investigations of the *Reb1-Ter3* complex of *Schizosaccharomyces pombe*. *Mol Cell Biol*, 28(22), 6844-6857.

- [102] Zhao, A., Guo, A., Liu, Z., & Pape, L. (1997). Molecular cloning and analysis of *Schizosaccharomyces pombe* Reb1p: sequence-specific recognition of two sites in the far upstream rDNA intergenic spacer. *Nucleic acids research*, 25(4), 904-910.
- [103] Singh, S. K., Sabatinos, S., Forsburg, S., & Bastia, D. (2010). Regulation of replication termination by Reb1 protein-mediated action at a distance. *Cell*, 142(6), 868-78.
- [104] Bochman, M. L., Sabouri, N., & Zakian, V. A. (2010). Unwinding the functions of the Pif1 family helicases. *DNA Repair (Amst)*, 9(3), 237-49.
- [105] Bairwa, N. K., Zzaman, S., Mohanty, B. K., & Bastia, D. (2010). Replication fork arrest and rDNA silencing are two independent and separable functions of the replication terminator protein Fob1 of *Saccharomyces cerevisiae*. *The Journal of biological chemistry*, 285(17), 12612-9.
- [106] Evers, R., & Grummt, I. (1995). Molecular coevolution of mammalian ribosomal gene terminator sequences and the transcription termination factor TTF-I. *Proceedings of the National Academy of Sciences of the United States of America*, 92(13), 5827-31.
- [107] Gerber, J. K., Gogel, E., Berger, C., Wallisch, M., Muller, F., Grummt, I., et al. (1997). Termination of mammalian rDNA replication: polar arrest of replication fork movement by transcription termination factor TTF-I. *Cell*, 90(3), 559-567.
- [108] Langst, G., Becker, P. B., & Grummt-I, I. (1998). TTF-I determines the chromatin architecture of the active rDNA promoter. *The EMBO journal*, 17(11), 3135-45.
- [109] Arlt, M. F., Durkin, S. G., Ragland, R. L., & Glover, T. W. (2006). Common fragile sites as targets for chromosome rearrangements. *DNA repair*, 5(9-10), 1126-1135.
- [110] Howlett, N. G., Taniguchi, T., Durkin, S. G., D'Andrea, A. D., & Glover, T. W. (2005). The Fanconi anemia pathway is required for the DNA replication stress response and for the regulation of common fragile site stability. *Hum Mol Genet*, 14(5), 693-701.
- [111] Bermejo, R., Capra, T., Gonzalez-Huici, V., Fachinetti, D., Cocito, A., Natoli, G., et al. (2009). Genome-organizing factors Top2 and Hmo1 prevent chromosome fragility at sites of S phase transcription. *Cell*, 138(5), 870-884.
- [112] Yarbrow, J. W. (1992). Mechanism of action of hydroxyurea. *Semin Oncol*, (9), 1-10.
- [113] Tsaponina, O., Barsoum, E., Astrom, S. U., & Chabes, A. (2011). Ixr1 is required for the expression of the ribonucleotide reductase Rnr1 and maintenance of dNTP pools. *PLoS Genet*, 7(5), e1002061.
- [114] Zhao, X., Chabes, A., Domkin, V., Thelander, L., & Rothstein, R. (2001). Thelander L, and Rothstein R. The ribonucleotide reductase inhibitor Sml1 is a new target of the Mec1/Rad53 kinase cascade during growth and in response to DNA damage. *The EMBO journal*, 20(13), 3544-3553.
- [115] Huang, A., Fan, H., Taylor, W. R., & Wright, J. A. (1997). Ribonucleotide reductase R2 gene expression and changes in drug sensitivity and genome stability. *Cancer Res*, 57(21), 4876-4881.

- [116] Miyabe, I., Morishita, T., Shinagawa, H., & Carr, A. M. (2009). Schizosaccharomyces pombe Cds1Chk2 regulates homologous recombination at stalled replication forks through the phosphorylation of recombination protein Rad60. *J Cell Sci*, Pt 20, 3638-3643.
- [117] Lindsay, H. D., Griffiths, D. J., Edwards, R. J., Christensen, P. U., Murray, J. M., Osman, F., et al. (1998). S-phase-specific activation of Cds1 kinase defines a subpathway of the checkpoint response in Schizosaccharomyces pombe. *Genes & development*, 12(3), 382-395.
- [118] Zhao, H., Tanaka, K., Nogochi, E., Nogochi, C., & Russell, P. (2003). Replication checkpoint protein Mrc1 is regulated by Rad3 and Tel1 in fission yeast. *Mol Cell Biol*, 23(22), 8395-8403.
- [119] Kumar, D., Viberg, J., Nilsson, A. K., & Chabes, A. (2010). Highly mutagenic and severely imbalanced dNTP pools can escape detection by the S-phase checkpoint. *Nucleic acids research*, 38(12), 3975-3983.
- [120] Zou, L., Liu, D., & Elledge, S. J. (2003). Replication protein A-mediated recruitment and activation of Rad17 complexes. *Proc Natl Acad Sci U S A*, 100(24), 13827-13832.
- [121] Kanoh, Y., Tamai, K., & Shirahige, K. (2006). Different requirements for the association of ATR-ATRIP and 9-1-1 to the stalled replication forks. *Gene*, 377-388.
- [122] Kemp, M., & Sancar, A. (2009). DNA distress: just ring 9-1-1. *Curr Biol*, 19(17), R733-R734.
- [123] Yan, S., & Michael, W. M. (2009). TopBP1 and DNA polymerase-alpha directly recruit the 9-1-1 complex to stalled DNA replication forks. *J Cell Biol*, 184(6), 793-804.
- [124] Branzei, D., & Foiani, M. (2006). The Rad53 signal transduction pathway: Replication fork stabilization, DNA repair, and adaptation. *Exp Cell Res*, 312(14), 2654-2659.
- [125] Yan, S., & Michael, W. M. (2009). TopBP1 and DNA polymerase alpha-mediated recruitment of the 9-1-1 complex to stalled replication forks: implications for a replication restart-based mechanism for ATR checkpoint activation. *Cell Cycle*, 8(18), 2877-2884.
- [126] Tanaka, K., & Russell, P. (2001). Mrc1 channels the DNA replication arrest signal to checkpoint kinase Cds1. *Nat Cell Biol*, 3(11), 966-972.
- [127] Tanaka, K., & Russell, P. (2004). Cds1 phosphorylation by Rad3-Rad26 kinase is mediated by forkhead-associated domain interaction with Mrc1. *J Biol Chem*, 279(31), 32079-32086.
- [128] Xu, Y. J., Davenport, M., & Kelly, T. J. (2006). Two-stage mechanism for activation of the DNA replication checkpoint kinase Cds1 in fission yeast. *Genes Dev*, 20(8), 990-1003.
- [129] Osborn, A. J., Elledge, S. J., & Zou, L. (2002). Checking on the fork: the DNA-replication stress-response pathway. *Trends Cell Biol*, 12(11), 509-516.

- [130] Naylor, M. L., Li, J. M., Osborn, A. J., & Elledge, S. J. (2009). Mrc1 phosphorylation in response to DNA replication stress is required for Mec1 accumulation at the stalled fork. *Proc Natl Acad Sci U S A*, 106(31), 12765-12770.
- [131] Schleker, T., Nagai, S., & Gasser, S. M. (2009). Posttranslational modifications of repair factors and histones in the cellular response to stalled replication forks. *DNA Repair (Amst)*, 8(9), 1089-100.
- [132] Tourriere, H., Versini, G., Cordon-Preciado, V., Alabert, C., & Pasero, P. (2005). Mrc1 and Tof1 promote replication fork progression and recovery independently of Rad53. *Mol Cell*, 19(5), 699-706.
- [133] De Piccoli, G., Katou, Y., Itoh, T., Nakato, R., Shirahige, K., & Labib, K. (2012). Replisome stability at defective DNA replication forks is independent of S phase checkpoint kinases. *Molecular cell*, 45(5), 696-704.
- [134] Kim, S. M., & Huberman, J. A. (2001). Regulation of replication timing in fission yeast. *The EMBO journal*, 20(21), 6115-6126.
- [135] Feng, W., Bachant, J., Collingwood, D., Raghuraman, M. K., & Brewer, B. J. (2009). Centromere replication timing determines different forms of genomic instability in *Saccharomyces cerevisiae* checkpoint mutants during replication stress. *Genetics*, 183(4), 1249-1260.
- [136] Lengronne, A., Pasero, P., Bensimon, A., & Schwob, E. (2001). Monitoring S phase progression globally and locally using BrdU incorporation in TK(+) yeast strains. *Nucleic acids research*, 29(7), 1433-1442.
- [137] Sabatinos, S. A., Mastro, T. L., & Forsburg, S. L. (2012). Nucleoside analogues create DNA damage and sensitivity in fission yeast. *Eukaryotic Cell*, submitted.
- [138] Chin, J. K., Bashkirov, V. I., Heyer, WD, & Romesberg, FE. (2006). Esc4/Rtt107 and the control of recombination during replication. *DNA repair*, 5(5), 618-628.
- [139] Koren, A., Soifer, I., & Barkai, N. (2010). MRC1-dependent scaling of the budding yeast DNA replication timing program. *Genome Res*, 20(6), 781-790.
- [140] Hayano, M, Kanoh, Y, Matsumoto, S, & Masai, H. (2011). Mrc1 marks early-firing origins and coordinates timing and efficiency of initiation in fission yeast. *Molecular and cellular biology*, 31(12), 2380-2391.
- [141] Szyjka, S. J., Viggiani, C. J., & Aparicio, O. M. (2005). Mrc1 is required for normal progression of replication forks throughout chromatin in *S. cerevisiae*. *Molecular cell*, 19(5), 691-697.
- [142] Xu, Y. J., & Kelly, T. J. (2009). Autoinhibition and autoactivation of the DNA replication checkpoint kinase Cds1. *The Journal of biological chemistry*, 284(23), 16016-16027.
- [143] Hayashi, M., Katou, Y., Itoh, T., Tazumi, A., Yamada, Y., Takahashi, T., et al. (2007). Genome-wide localization of pre-RC sites and identification of replication origins in fission yeast. *The EMBO journal*, 26(5), 1327-1339.

- [144] Santocanale, C., & Diffley, J. F. (1998). A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. *Nature*, 395(6702), 615-618.
- [145] Heichinger, C., Penkett, C. J., Bahler, J., & Nurse, P. (2006). Genome-wide characterization of fission yeast DNA replication origins. *The EMBO journal*, 25(21), 5171-5179.
- [146] Chabes, A., & Stillman, B. (2007). Constitutively high dNTP concentration inhibits cell cycle progression and the DNA damage checkpoint in yeast *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America*, 104(4), 1183-8.
- [147] Mickle, K. L., Ramanathan, S., Rosebrock, A., Oliva, A., Chaudari, A., Yompakdee, C., et al. (2007). Checkpoint independence of most DNA replication origins in fission yeast. *BMC Mol Biol*, 8, 112.
- [148] Hanada, K., Budzowska, M., Davies, S. L., van Drunen, E., Onizawa, H., Beverloo, H. B., et al. (2007). The structure-specific endonuclease Mus81 contributes to replication restart by generating double-strand DNA breaks. *Nat Struct Mol Biol*, 14(11), 1096-1104.
- [149] Robison, J. G., Elliott, J., Dixon, K., & Oakley, G. G. (2004). Replication protein A and the Mre11.Rad50.Nbs1 complex co-localize and interact at sites of stalled replication forks. *J Biol Chem*, 279(33), 34802-34810.
- [150] Schlacher, K., Christ, N., Siaud, N., Egashira, A., Wu, H., & Jasin, M. (2011). Double-strand break repair-independent role for BRCA2 in blocking stalled replication fork degradation by MRE11. *Cell*, 145(4), 529-42.
- [151] Ouyang, K. J., Woo, L. L., Zhu, J., Huo, D., Matunis, M. J., & Ellis, N. A. (2009). SUMO modification regulates BLM and RAD51 interaction at damaged replication forks. *PLoS Biol*, 7(12), e1000252.
- [152] Kurokawa, Y., Murayama, Y., Haruta-Takahashi, N., Urabe, I., & Iwasaki, H. (2008). Reconstitution of DNA strand exchange mediated by Rhp51 recombinase and two mediators. *PLoS Biol*, 6(4), e88.
- [153] Wray, J., Liu, J., Nickoloff, J. A., & Shen, Z. (2008). Distinct RAD51 associations with RAD52 and BCCIP in response to DNA damage and replication stress. *Cancer Res*, 68(8), 2699-2707.
- [154] Lambert, S., Froget, B., & Carr, A. M. (2007). Arrested replication fork processing: interplay between checkpoints and recombination. *DNA Repair (Amst)*, 6(7), 1042-1061.
- [155] Aggarwal, M., Sommers, J. A., Morris, C., & Brosh, R. M., Jr. (2010). Delineation of WRN helicase function with EXO1 in the replicational stress response. *DNA Repair (Amst)*, 9(7), 765-776.
- [156] Tinline-Purvis, H., Savory, A. P., Cullen, J. K., Dave, A., Moss, J., Bridge, W. L., et al. (2009). Failed gene conversion leads to extensive end processing and chromosomal rearrangements in fission yeast. *The EMBO journal*, 28(21), 3400-3412.

- [157] Tran, P. T., Fey, J. P., Erdeniz, N., Gellon, L., Boiteux, S., & Liskay, R. M. (2007). A mutation in EXO1 defines separable roles in DNA mismatch repair and post-replication repair. *DNA Repair (Amst)*, 6(11), 1572-1583.
- [158] Lambert, S., Watson, A., Sheedy, D. M., Martin, B., & Carr, A. M. (2005). Gross chromosomal rearrangements and elevated recombination at an inducible site-specific replication fork barrier. *Cell*, 121(5), 689-702.
- [159] Meister, P., Taddei, A., Ponti, A., Baldacci, G., & Gasser, S. M. (2007). Replication foci dynamics: replication patterns are modulated by S-phase checkpoint kinases in fission yeast. *The EMBO journal*, 26(5), 1315-1326.
- [160] Willis, N., & Rhind, N. (2010). The fission yeast Rad32(Mre11)-Rad50-Nbs1 complex acts both upstream and downstream of checkpoint signaling in the S-phase DNA damage checkpoint. *Genetics*, 184(4), 887-897.
- [161] Hashimoto, Y., Puddu, F., & Costanzo, V. (2012). RAD51- and MRE11-dependent reassembly of uncoupled CMG helicase complex at collapsed replication forks. *Nature structural & molecular biology*, 19(1), 17-24.
- [162] Brugmans, L., Verkaik, N. S., Kunen, M., van Drunen, E., Williams, B. R., & Petrini, J. H. (2009). NBS1 cooperates with homologous recombination to counteract chromosome breakage during replication. *DNA repair*, 8(12), 1363-1370.
- [163] Kuzminov, A. (2001). Single-strand interruptions in replicating chromosomes cause double-strand breaks. *Proceedings of the National Academy of Sciences of the United States of America*, 98(15), 8241-8246.
- [164] Caldecott, K. W. (2007). Mammalian single-strand break repair: mechanisms and links with chromatin. *DNA repair*, 6(4), 443-453.
- [165] Hutchinson, F. (1993). Induction of large DNA deletions by persistent nicks: a new hypothesis. *Mutat Res*, 299(3-4), 211-218.

