Regulation of Ribonucleotide Reductase and the Role of dNTP pools in Genomic Stability in Yeast *Saccharomyces cerevisiae*

Olga Tsaponina

Department of Medical Biochemistry and Biophysics
Umeå University, Sweden
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Science has made us gods

even before we are worthy of being men

Jean Rostand

To my family with love

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List of Papers.

Papers included in the thesis:

1. **Tsaponina O**., Barsoum E., Astrom S.U., and Chabes A. (2011) Ixr1 is required for the expression of the ribonucleotide reductase Rnr1 and maintenance of dNTP pools. *PLoS Genetics*, 7(5): e1002061

2. Fasullo M., **Tsaponina O**., Sun M., and Chabes A. (2010) Elevated dNTP levels suppress hyper-recombination in Saccharomyces cerevisiae S-phase checkpoint mutants. *Nucleic Acids Research*, 38(4): 1195-203.

Paper not included in the thesis:

1. Rossmann M., Luo W., **Tsaponina O**., Chabes A., and Stillman B. (2011) A common telomeric gene silencing assay is affected by nucleotide metabolism. *Molecular Cell*, 42(1): 127-136

Abbreviations

ADP – adenosine 5'-diphosphate
ATP – adenosine 5'-triphosphate
CDP – cytidine 5'-diphosphate
ChIP – chromatin immunoprecipitation
dATP – deoxyadenosine 5'-triphosphate
dCTP – deoxycytidine 5'-triphosphate
dGTP – deoxyguanosine 5'-triphosphate
DNA – deoxyribonucleic acid
dNTP – deoxyribonucleoside triphosphate
DSB – double-strand break (in a DNA)
dTTP – deoxythymidine 5'-triphosphate
GDP – guanosine 5'-diphosphate
GCR – gross chromosomal rearrangements
HU – hydroxyurea
HMG-box – high-mobility group box
MBF – *MluI*-binding factor
NER – nucleotide excision repair
RF – replication fork
RNR – ribonucleotide reductase
RPA – replication protein A
ssDNA – single-stranded DNA
TLS – translesion synthesis
UDP – uridine 5'-diphosphate

Genetic symbols:

YFG1 (Your Favorite Gene 1) = wild-type allele
yfg1 = mutant allele
yfg1Δ = deletion of a gene
Yfg1 = protein

Abstract

Every living organism is programmed to reproduce and to pass genetic information to descendants. The information has to be carefully copied and accurately transferred to the next generation. Therefore organisms have developed the network of conserved mechanisms to survey the protection and precise transfer of the genetic information. Such mechanisms are called checkpoints and they monitor the correct execution of different cell programs. The DNA damage and the replication blocks are surveyed by the conserved Mec1-Rad53 (human ATM/ATR and Chk2, respectively) protein kinase cascade. Mec1 and Rad53 are essential for survival and when activated orchestrate the multiple cellular responses, including the activation of the ribonucleotide reductase (RNR), to the genotoxic stress. RNR is an enzyme producing all four dNTPs - the building blocks of the DNA - and is instrumental for the maintenance both proper concentration and balance of each of dNTPs. The appropriate concentration of the dNTPs should be strictly regulated since inadequate dNTP production can impede many cellular processes and lead to higher mutation rates and genome instability. Hence RNR activity is regulated at many levels, including allosteric and transcriptional regulation and the inhibition at protein level.

In our research, we addressed the question of the transcriptional regulation of RNR and the consequences of dNTP malproduction in the terms of the genomic stability. In yeast *S. cerevisiae*, four genes encode RNR: 2 genes encode a large subunit (*RNR1* and *RNR3*) and 2 genes encode a small subunit (*RNR2* and *RNR4*). All 4 genes are DNA-damage inducible: transcription of *RNR2*, *RNR3* and *RNR4* is regulated via Mec1-Rad53-Dun1 pathway by targeting the transcriptional repressor Crt1 (Rfx1) for degradation; on the contrary, *RNR1* gene promoter does not contain Crt1-binding sites and is not regulated through the Mec1-Rad53-Dun1 pathway. Instead, we show that intrastrand cross (X)-link recognition protein (Ixr1) is required for the proper transcription of the *RNR1* gene and maintenance of the dNTP pools both during unperturbed cell cycle and after the DNA damage. Thus, we identify the novel regulator of the *RNR1* transcription.

Next, we show that the depletion of dNTP pools negatively affects genome stability in the hypomorphic *mec1* mutants: the hyper-recombination phenotype in those mutants correlates with low dNTP levels. By introducing even lower dNTP levels the hyper-recombination increased even further and conversely all the hyper-recombination phenotypes were suppressed by artificial elevation of dNTP levels.

In conclusion, we present Ixr1 as a novel regulator of the RNR activity and provide the evidence of role of dNTP concentration in the genome stability.

Introduction

One of the main features of all living organisms is reproduction. The information about how an organism is built is encoded in its deoxyribonucleic acid (DNA) by combinations of just four letters representing the deoxyribonucleoside triphosphates (dNTPs). The dNTPs are organized into sequences of different length, forming genes; genes and intergenic regions are in turn organized into chromosomes. A set of chromosomes in a cell is called a genome and contains the information required for the organism to function. Differences in nucleotide sequences create all the incredible diversity of species inhabiting our world. Information as important as the DNA sequence certainly must be carefully maintained, precisely duplicated, and correctly transferred to the daughter unit(s). Therefore, organisms have developed a number of mechanisms to protect, repair, and preserve genetic information. For example, multiple mechanisms strictly regulate the concentration and relative balance of the DNA building blocks, the dNTPs; furthermore, cells possess mechanisms to prevent and correct any unwanted changes in their DNA. In this thesis, I explore a new pathway of the regulation of dNTP production and investigate how changes in dNTP concentrations can affect genome stability.

Background

1. The cell cycle and the checkpoint

Cells reproduce by dividing into two daughter cells in the process known as the cell cycle. The cell cycle consists of four phases: G_1 (Gap 1), S (DNA replication), G_2 (Gap 2), and M (Mitosis) followed by cytokinesis (Figure 1). In the G_1 phase, cells grow; in the S phase, they duplicate their DNA; in the G_2, they prepare for division; finally, they divide in the M phase (Figure 1).

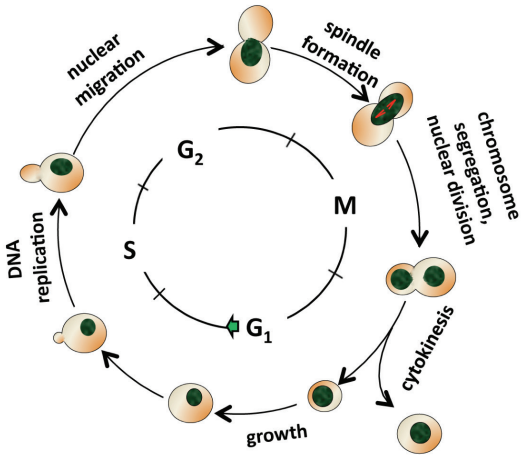

Figure 1. The cell cycle of budding yeast *S. cerevisiae*.

A number of surveillance mechanisms called checkpoints strictly regulate the timing of the cell cycle. The function of checkpoints is to verify that the program of the current phase is executed successfully, thereby controlling entry into the next phase. If a cell does not finish the program for any reason, a checkpoint delays progression of the cell cycle until all requirements of the current stage are fulfilled. If yeast cells experience irreparable damage, they die or eventually override the checkpoint in a process called adaptation [1, 2]. After adaptation, cells can divide for several generations before they finally die.

2. dNTP production

In the S phase, the genome of a cell is duplicated according to the semi-conservative principle of DNA synthesis [3, 4]. Four dNTPs are required to replicate the DNA: deoxyguanosine 5'-triphosphate (dGTP), deoxyadenosine 5'-triphosphate (dATP), deoxycytidine 5'-triphosphate (dCTP), and deoxythymidine 5'-triphosphate (dTTP). An enzyme called ribonucleotide reductase (RNR) produces all four dNTPs *de novo* by catalyzing the reduction of ribonucleotides to deoxyribonucleotides [5]. RNR maintains an adequate concentration of all four dNTPs at each phase of the

cell cycle ([6] and reviewed in [7]). In *S. cerevisiae*, the dNTP concentration is increased by approximately three times upon S phase entry, but the relative concentration of each dNTP remains the same — dTTP is highest, dCTP and dATP are approximately two times lower, and dGTP is the lowest [6, 8, 9]. This relative dNTP concentration may have evolved together with the kinetic properties of eukaryotic polymerases.

RNRs are divided into three classes according to structural differences and the nature of the radical used for catalysis [10] (http://rnrdb.molbio.su.se). Class Ia dominates in eukaryotes and in *S. cerevisiae* is represented as an α2β2 heterotetramer, where α2 corresponds to a large subunit and β2 corresponds to a small subunit (Figure 2).

Each β polypeptide contains a stable tyrosyl radical [10], and during catalysis, the radical function is transferred to the active center of a large subunit via long-range radical transfer [7]. Some chemicals, such as hydroxyurea (HU), can destroy the tyrosyl radical and in this way directly affect RNR activity [11].

3. Regulation of RNR

An adequate concentration of dNTPs is strictly regulated because imbalance or incorrect concentration can result in a perturbed cell cycle, high mutation rates, and genomic instability (reviewed in [12]). Thus, RNR activity is regulated at many levels.

3a. Allosteric regulation

To maintain an adequate concentration of dNTPs, RNRs have evolved a remarkable scheme of allosteric regulation (reviewed in [13]). Each α subunit possesses a catalytic site and two allosteric sites, respectively the specificity site and the activity site (Figure 2). The allosteric activity site controls the overall enzymatic activity: The binding of ATP switches "on" the enzyme, and the binding of dATP switches it "off." The allosteric specificity site dictates the choice of a substrate: The binding of ATP and dATP promotes reduction of UDP and CDP; binding of dTTP promotes reduction

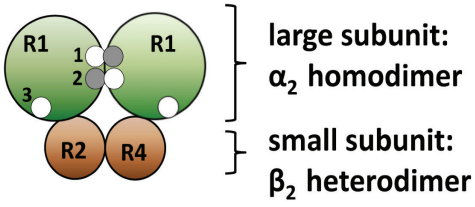

Figure 2. Schematic representation of yeast *Saccharomyces cerevisiae* ribonucleotide reductase. 1 – activity site; 2 – allosteric specificity site; 3 – allosteric activity site. The R2 subunit possesses the tyrosyl radical.

of GDP, and binding of dGTP promotes reduction of ADP. Binding of the effector to the specificity site induces assembly of a large subunit that forms the active complex with a small subunit [14-16] and is another mechanism of regulation of RNR activity.

3b. RNR production is regulated at the transcriptional level

In the yeast *S. cerevisiae*, four genes encode RNR: Two encode a large subunit (*RNR1* and *RNR3* [17]) and two encode a small subunit (*RNR2* [18] and *RNR4* [19]). The large subunit is an Rnr1 homodimer and the small subunit is an Rnr2/Rnr4 heterodimer (Figure 2). Rnr3 is a non-essential Rnr1 paralogue forming Rnr3 homodimers or Rnr1/Rnr3 heterodimers [20]. Transcription of the *RNR2-4* genes is controlled by the DNA damage-dependent Crt1 repressor and the oxygen-dependent Mot1 and Rox1 repressors [21] (Figure 3). Under DNA damage/replication stress or oxygen deprivation, these inhibitors dissociate from the promoters, thereby relieving inhibition of *RNR2-4* transcription [22, 23]. The removal of the Crt1 repressor is controlled by the conserved Mec1-Rad53-Dun1 checkpoint kinase cascade [23], which monitors response to both DNA damage and replication stress (Figure 3) (reviewed in [24]). In contrast, the *RNR1* gene promoter does not contain Crt1, Mot1, or Rox1 binding sites [21]; however, *RNR1* is also DNA-damage inducible [23]. The cell-cycle–dependent expression of *RNR1* is controlled by the dimeric *MluI*-binding factor (MBF) factor consisting of Swi6 and Mbp1 (Figure 3) (reviewed in [25]). Rad53 can

directly phosphorylate Swi6, inhibiting transcription of G1-specific genes in response to DNA damage [26]. However, other reports show that MBF-dependent transcription of G1-specific genes is triggered by the DNA replication checkpoint via removal of the MBF inhibitor Nrm1 [27]. In other words, the exact mechanism of *RNR1* activation in response to DNA damage or replication stress is not known.

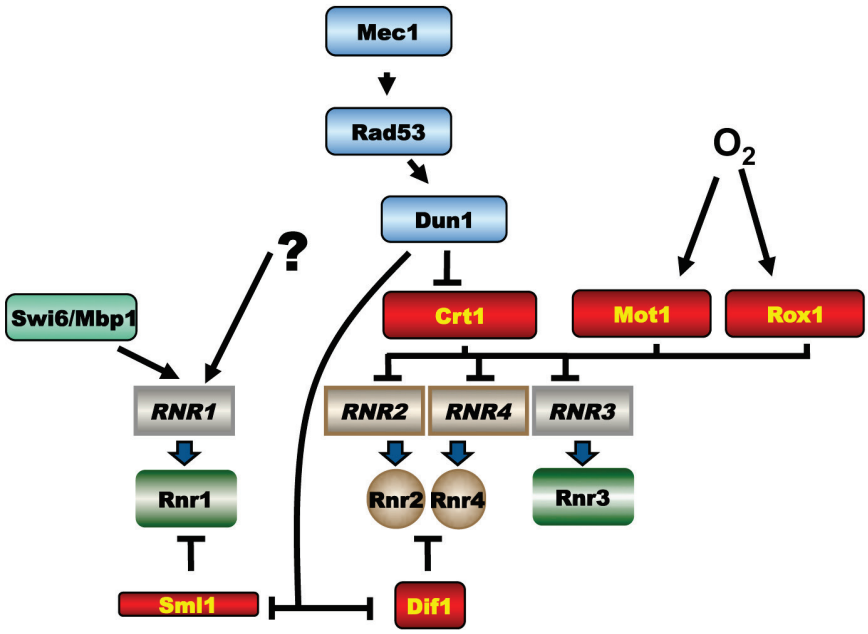

Figure 3. The regulation of *S. cerevisiae* ribonucleotide reductase

3c. RNR activity is regulated at the protein level

Two protein inhibitors of *S. cerevisiae* RNR activity are known: Sml1 and Dif1 (Figure 3). The short peptide Sml1 binds to the Rnr1 subunit, thus inhibiting RNR activity [28, 29], and Dif1 regulates the nuclear compartmentalization of Rnr2 and Rnr4 [30, 31]. Upon DNA damage or replication stress, activated Dun1 phosphorylates Sml1 and Dif1, targeting them for degradation, which allows the formation of the active cytoplasmic RNR complex [30-32].

3d. Relationship between RNR activity and redox status of a cell

RNR depends on the external electron donor systems because a disulfide formed in the active site by two cysteines after each round of catalysis has to be regenerated [33]. A protein called thioredoxin is a possible electron donor, but in turn, its sulfhydryl groups should be regenerated for the next catalytic cycle in an NADPH-dependent manner by thioredoxin reductase [34]. Thus, the efficiency of RNR catalysis depends upon many factors, including the redox status of the cell.

4. Replication fork and genome stability

Eukaryotic cells regulate genomic replication in a highly complex manner. DNA replication is tightly monitored to ensure that the genome is replicated once per cell division and that DNA replication is complete before mitosis starts. Cells also must couple replication with other cellular processes such as chromatin reassembly, the inheritance of epigenetic information, and the establishment of cohesion between sister chromatids.

Replication initiates from multiple regions distributed along chromosomes called origins. At each origin, two sister replication forks (RFs) associated with the replisome are established that move away from each other. The replisome is a multi-component protein complex that includes, among other proteins, a replicative helicase complex (MCM2-7), DNA polymerases (α, ε, and δ), polymerase-associated factor (processivity factor PCNA), and the Mrc1 and Tof1/Csm3 complex required for RF pausing (reviewed in [35]). The progression of an individual RF can be stopped by physical impediments such as DNA–protein complexes, DNA damage, natural elements such as fragile sites and unusual DNA structures, or dNTP depletion. Proteins Mrc1 and Tof1 limit progression of the replisome under such circumstances [36], and the checkpoint kinases Mec1 and Rad53 stabilize stalled RF [37, 38]. When Mec1 or Rad53 are not functional, under the replication stress, the replisome dissociates from the stalled RF, causing its collapse.

Mechanisms that sense and deal with DNA lesions at hindered RFs have been studied in many systems and excellently reviewed [35, 39-41], so they are not addressed in depth here. Briefly, when a RF encounters a DNA lesion or stalls because of dNTP deprivation, the helicase and polymerase can uncouple, creating long stretches of single-stranded DNA (ssDNA) covered by replication protein A (RPA) (Figure 4). RPA–ssDNA stretches are also generated during nucleotide excision repair (NER) and the processing of double-stranded breaks (DSBs). Extensive RPA–ssDNA complexes trigger the Ddc2-mediated recruitment of Mec1 to the site of damage, leading to extensive Mec1 phosphorylation. Activated Mec1 in turn phosphorylates downstream targets and components of the RF, activating the replication stress/DNA damage response and stabilizing the RF. Next, DNA lesions are bypassed or removed via error-prone or error-free mechanisms based on the type of lesion: bulky DNA lesions are bypassed by translesion synthesis (TLS) or a template switch–mediated mechanism; interstrand cross links are removed by the serial action of incision, TLS, NER, and homologous recombination; and finally, DSBs can be repaired by non-homologous end joining, single strand re-annealing, or homologous recombination.

In conclusion, spontaneous single- and particularly double-stranded DNA breaks generated by replication in the absence of functional checkpoint proteins can result in a hyper-recombination phenotype [42], leading to genomic instability that results in cell death or malignant transformation.

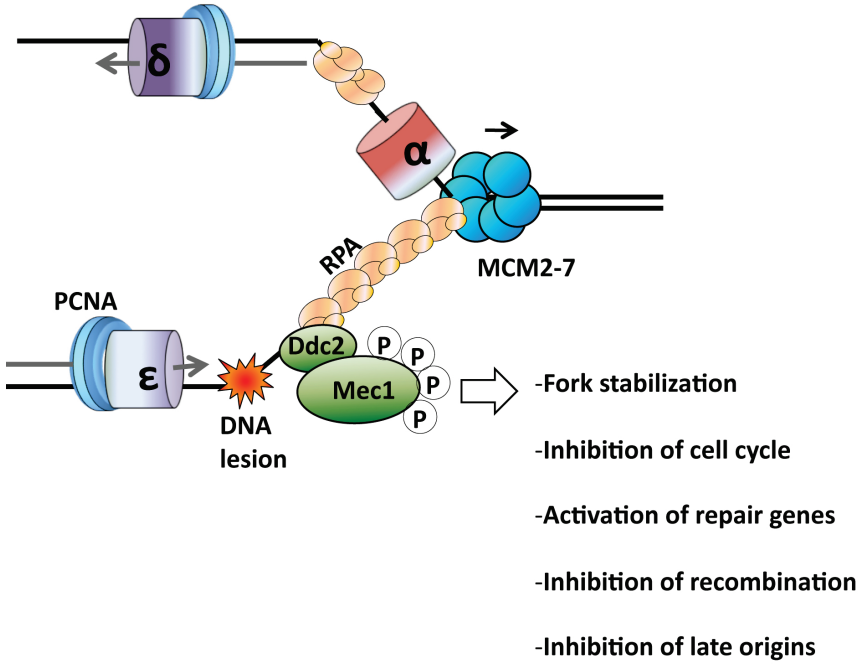

Figure 4. A schematic illustration of a stalled replication fork. Recruitment of Mec1-Ddc2 to the ssDNA-RPA stretches leads to extensive Mec1 phosphorylation and checkpoint activation.

Results

Paper I

Ixr1 is a novel regulator of *RNR1* expression

In *S. cerevisiae*, Mec1 and Rad53 are essential and control phosphorylation and activation of non-essential checkpoint kinase Dun1 (Figure 3). The Mec1-Rad53-Dun1 pathway maintains an adequate supply of dNTPs by regulating the activity of RNR both during an unperturbed cell cycle and after DNA damage. Cells lacking Dun1 kinase exhibit a prolonged S phase, decreased dNTP levels, and DNA damage sensitivity but are viable [8, 32, 43]. We performed a large-scale synthetic lethality screen to identify mutants sensitive to decreased dNTP levels in a *dun1* mutant and isolated three mutants synthetic lethal with *dun1*, one identified as *ixr1-S366F*. Ixr1 is an intra-strand cross (X)-link recognition protein implicated previously in aerobic transcriptional repression of *COX5b* [44]; S366 is a conserved high-mobility group-box residue directly interacting with DNA [45]. The *dun1 ixr1* synthetic interaction had been reported before we published our data, but the mechanism of the interaction remained unknown [46, 47].

We demonstrated that *dun1 ixr1* inviability was suppressed by artificial elevation of dNTP levels. The requirement for functional Dun1 in *ixr1* cells implied that the Mec1-Rad53-Dun1 checkpoint was activated. The activation of this checkpoint leads to the induction of expression of *RNR2*, *RNR3*, and *RNR4* genes and Sml1 degradation, which can be used as a sensitive read-out of the checkpoint activation. Indeed, the levels of Rnr3 and Rnr4 were increased and Sml1 levels were decreased in *ixr1* cells, but Rad53 phosphorylation was not observed. We concluded that the Mec-Rad53-Dun1 checkpoint was more active in *ixr1* cells during the unperturbed cell cycle than in wild-type cells but less active than after DNA damage. In contrast to Rnr3 and Rnr4, Rnr1 levels and dNTP concentration were decreased in *ixr1*, which was partially compensated by the downregulation of Sml1 levels and Rnr3 and Rnr4 elevated production. Of note, after DNA damage, levels of Rnr1 in *ixr1* were decreased even further, and this

reduction was continuous. Thus, we concluded that Dun1 was indispensable in *ixr1* cells because these cells cannot execute Dun1-dependent relief of RNR inhibition.

Next, we investigated Rnr1 production in different checkpoint mutants. To our surprise, we found significant downregulation of Rnr1 levels in *rad53* mutant cells and to a lesser extent in *mec1* cells, but not in *dun1* cells. We explained this reduction by a decrease of Ixr1 levels in *rad53* cells, so these cells cannot properly induce expression of *RNR1* when Ixr1 is depleted. By treatment with λ phosphatase, we showed that Ixr1 is a highly phosphorylated protein, implying possible ways of Ixr1 regulation by kinase or phosphatase activities.

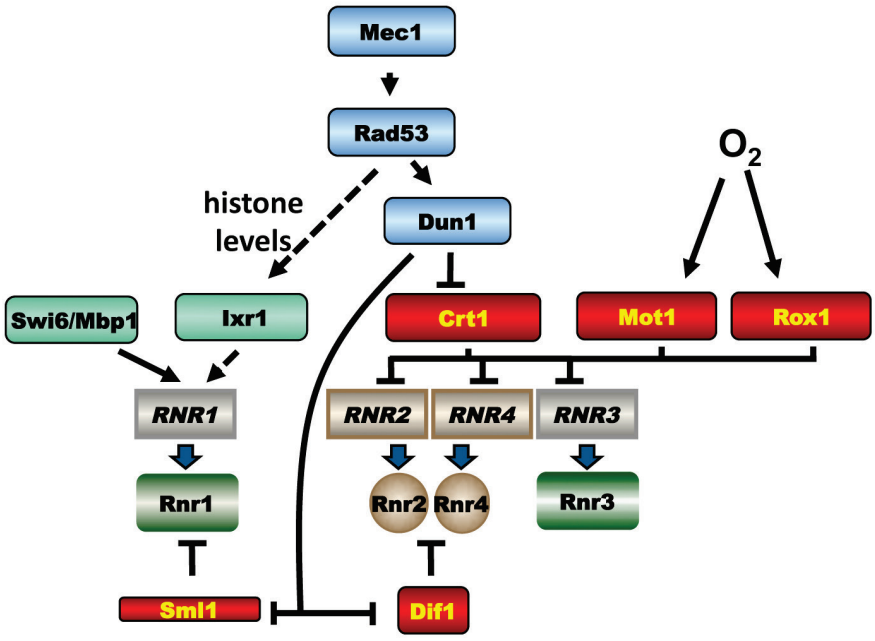

Figure 5. Ixr1 is a novel regulator of *S. cerevisiae* ribonucleotide reductase.

Then, by a promoter activity assay and chromatin immunoprecipitation experiments (ChIP), we demonstrated that Ixr1 directly regulates *RNR1* expression. Finally, we showed an earlier unknown competition between histones and non-histone Ixr1 protein: In the presence of excessive histone levels, Ixr1 most likely is displaced from DNA and degraded in the cytoplasm.

In conclusion, we identified a new protein controlling the *RNR1* gene: In the absence of Ixr1, *RNR1* becomes uninducible in the same way as *RNR2-4* becomes DNA-damage uninducible in the absence of Dun1 (Figure 5).

Paper II

dNTP levels affect genome stability in checkpoint mutants

As discussed above, Mec1 is an essential protein controlling the S phase checkpoint and preventing RF collapse when RF progression is hindered. As was shown previously [42], the viability of the *mec1* mutant depends on the homologous recombination pathway, suggesting high levels of recombinogenic lesions generated by replication and leading to gross chromosomal re-arrangements and a hyper-recombination phenotype. On the other hand, lethality of *mec1Δ* can be rescued by the artificial elevation of dNTP levels [23, 28, 30, 48, 49], such as the deletion of the *SML1* gene. A number of studies have indicated that changes in dNTP concentration can affect RF speed [50, 51]. Presumably, higher dNTP levels can promote progression of the RF and lead to fewer stalled/collapsed forks in *mec1* mutants. Indeed, we showed that the hyper-recombination phenotype presumably caused by RF impediments correlates with low dNTP levels. Moreover, by deleting *dun1* and thus further lowering dNTP concentration, we demonstrated even higher hyper-recombination rates in corresponding mutants. Conversely, artificial elevation of dNTP levels suppressed the hyper-recombination.

The stabilization of forks in *mec1* cells by higher dNTP levels comes at a cost, however: We showed increased spontaneous mutation rates in the *mec1 dun1 sml1* cells compared to *mec1 dun1* cells. Previous reports have shown that increased dNTP concentration can lead to a mutator phenotype [52-54]. The increased mutation rates in the presence of high dNTP levels could be explained by the ability of replicative polymerases to bypass lesions more efficiently when dNTPs are in excess [55]. We speculate that higher dNTP levels can promote lesion bypass in *mec1* cells where RFs frequently stall, thus promoting RF progression and reducing recombinogenic events (Figure 6).

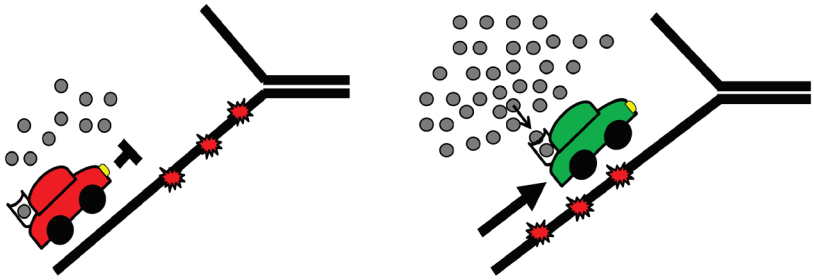

Figure 6. The car represents a replisome. The replisome can progress through obstacles at replication forks more efficiently when fuel (dNTPs) is in excess.

Of note, the correlation among high dNTP levels, greater survival, and higher mutation rates has been reported previously [6]. In summary, these data can be interpreted to mean that in general, elevated dNTP levels can promote RF progression, thus reducing fork stalling and consequent recombinogenic lesions but being associated with higher mutation rates.

Conclusion.

In conclusion, we provide the evidence that Ixr1 regulates RNR activity through controlling the expression of *RNR1*, the major isoform of the large subunit and we confirm the important role of adequate dNTP maintenance in sustaining genome stability.

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