Mechanisms controlling DNA damage survival and mutation rates in budding yeast

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När folk håller med mig får jag en känsla av att jag har fel.

-Oscar Wilde

The definition of insanity is doing the same thing over and over and expecting a different result.

-Albert Einstein

Till min älskade fru och dotter

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Abstract

All living organisms are made of cells, within which genetic information is stored on long strands of deoxyribonucleic acid (DNA). The DNA encodes thousands of different genes and provides the blueprint for all of the structures and activities occurring within the cell. The building blocks of DNA are the four deoxyribonucleotides, dATP, dGTP, dTTP, and dCTP, which are collectively referred to as dNTPs.

The key enzyme in the production of dNTPs is ribonucleotide reductase (RNR). In the budding yeast *Saccharomyces cerevisiae*, the concentrations of the individual dNTPs are not equal and it is primarily RNR that maintains this balance. Maintenance of the dNTP pool balance is critical for accurate DNA replication and DNA repair since elevated and/or imbalanced dNTP concentrations increase the mutation rate and can ultimately lead to genomic instability and cancer. In response to DNA damage, the overall dNTP concentration in *S. cerevisiae* increases. Cell survival rates increase as a result of the elevated concentration of dNTPs, but the cells also suffer from a concomitant increase in mutation rates. When the replication machinery encounters DNA damage that it cannot bypass, the replication fork stalls and recruits specialized translesion synthesis (TLS) polymerases that bypass the damage so that replication can continue. We hypothesized that elevated dNTP levels in response to DNA damage may allow the TLS polymerases to more efficiently bypass DNA damage. To explore this possibility, we deleted all known TLS polymerases in a yeast strain in which we could artificially increase the dNTP concentrations. Surprisingly, even though all TLS polymerases had been deleted, elevated dNTP concentrations led to increased cell survival after DNA damage. These results suggest that replicative DNA polymerases may be involved in the bypass of certain DNA lesions under conditions of elevated dNTPs. We confirmed this hypothesis *in vitro* by demonstrating that high dNTP concentrations result in an increased efficiency in the bypass of certain DNA lesions by DNA polymerase epsilon, a replicative DNA polymerase not normally associated with TLS activity.

We asked ourselves if it would be possible to create yeast strains with imbalanced dNTP concentrations *in vivo*, and, if so, would these imbalances be recognized by the checkpoint control mechanisms in the cell. To address these questions, we focused on the highly conserved loop2 of the allosteric specificity site of yeast Rnr1p. We introduced several mutations into *RNR1-loop2* that resulted in changes in the amino acid sequence of the protein.

Each of the *rnr1-loop2* mutation strains obtained had different levels of individual dNTPs relative to the others. Interestingly, all of the imbalanced dNTP concentrations led to increased mutation rates, but these mutagenic imbalances did not activate the S-phase checkpoint unless one or several dNTPs were present at concentrations that were too low to sustain DNA replication. We were able to use these mutant yeast strains to successfully correlate amino acid substitutions within loop2 of Rnr1p to specific ratios of dNTP concentrations in the cells. We also demonstrated that specific imbalances between the individual dNTP levels result in unique mutation spectra. These mutation spectra suggest that the mutagenesis that results from imbalanced dNTP pools is due to a decrease in fidelity of the replicative DNA polymerases at specific DNA sequences where they are more likely to make a mistake. The mutant *rnr1-loop2* strains that we have created with defined dNTP pool imbalances will be of great value for *in vivo* studies of polymerase fidelity, translesion synthesis by specialized DNA polymerases, and lesion recognition by the DNA repair machinery.

Keywords: dNTPs, ribonucleotide reductase, translesion synthesis, TLS polymerases

List of papers

This thesis is based on the following papers:

I. Sabouri, N.*, Viberg, J.*, Goyal, D.K., Johansson, E., and Chabes, A. (2008). Evidence for lesion bypass by yeast replicative DNA polymerases during DNA damage. Nucleic Acids Res *36*, 5660-5667.

* Equally contributing authors.

II. Kumar, D., Viberg, J., Nilsson, A.K., and Chabes, A. (2010). Highly mutagenic and severely imbalanced dNTP pools can escape detection by the S-phase checkpoint. Nucleic Acids Res *38*, 3975-3983.

III. Kumar, D., Abdulovic, A.L., Viberg, J., Nilsson, A.K., Kunkel, T.A., and Chabes, A. (2011). Mechanisms of mutagenesis in vivo due to imbalanced dNTP pools. Nucleic Acids Res *39*, 1360-1371.

Abbreviations

4-NQO - 4-nitroquinoline oxide
6-4PP - pyrimidine (6-4) pyrimidone
ADP - adenosine 5'-diphosphate
ATP - adenosine 5'-triphosphate
BER - base excision repair
CDP - cytidine 5'-diphosphate
CPDs - cyclobutane pyrimidine dimers
dATP - deoxyadenosine 5'-triphosphate
dCTP - deoxycytidine 5'-triphosphate
dGTP - deoxyguanosine 5'-triphosphate
DNA - deoxyribonucleic acid
dNTP - deoxyribonucleoside triphosphate
DSB - double stranded DNA break
dTTP - thymidine 5'-triphosphate
GDP - guanosine 5'-diphosphate
HR - homologous recombination
MMR - mismatch repair
MMS - methylmethanesulfonate
MNNG - *N*-methyl-*N´* -nitro-*N*-nitrosoguanidine
NDK - nucleoside diphosphate kinase
NER - nucleotide excision repair
NHEJ - non-homologous end-joining
PRR - post-replication repair
RNR - ribonucleotide reductase
ssDNA - single stranded DNA
TLS - translesion synthesis
TLS polymerases - polymerases involved in TLS
UDP - uridine 5'-diphosphate

Svensk sammanfattning

Alla levande organismer lagrar genetisk information som deoxyribonukleinsyra (DNA). I genomet, som består av DNA, finns tusentals gener som huvudsakligen utgör en organisms egenskaper. Byggstenarna till DNA är deoxyribonukleosidtrifosfaterna: dTTP, dCTP, dATP, dGTP (dNTPs). Regleringen av dNTP koncentrationerna i celler är av stor betydelse vid DNA-replikation, när genomet kopieras, och DNA-reparation. Om den genetiska koden inte blir kopierad på ett exakt sätt, eller om det uppkommit DNA-skador som inte kan repareras, uppstår en mutation. En mutation innebär att den genetiska koden har blivit förändrad från dess ursprungliga komposition.

Den huvudsakliga produktionen av dNTPs hanteras hos alla organismer av enzymet ribonukleotidreduktas (RNR). Vid både DNA-reparation och DNA-replikation är regleringen av dNTP koncentrationerna av stor betydelse då höga och/eller obalanserade koncentrationer ökar frekvensen av mutationer, något som kan kan leda till genetisk instabilitet och cancer. I bakjäst, *Saccharomyces cerevisiae*, är koncentrationerna av dNTPs inte lika höga utan hålls balanserade i ett visst förhållande som framförallt upprätthålls av RNR.

Hos *S. cerevisiae* har man tidigare kunnat visa att den totala dNTP-koncentrationen ökar som svar på DNA-skada. Detta bidrar till en högre tolerans av DNA-skador men leder samtidigt till en högre mutationsfrekvens. När maskineriet som utför replikationen kommer till en DNA-skada som den inte kan syntetisera förbi stannar replikationsgaffeln och speciella polymeraser rekryteras. Dessa speciella polymeraser kallas för *translesion synthesis* (TLS)-polymeraser och kan syntetisera förbi många typer av DNA-skador.

Vi spekulerade att den ökade dNTP-koncentrationen efter DNA-skada kan underlätta för TLS-polymeraser i deras arbete att syntetisera förbi DNA-skador. För att undersöka denna hypotes tog vi bort samtliga TLS-polymeraser i en jäststam samtidigt som vi artificiellt ökade dNTP-koncentrationen. Om det var så att en eller flera av TLS-polymeraserna drog nytta av de ökade dNTP-koncentrationerna för att öka överlevnaden efter DNA-skada skulle en jäststam med avsaknad av dessa ha samma överlevnad oberoende av dNTP-koncentrationen. Vi utförde experiment med denna stam, med och utan förhöjda dNTP-koncentrationer, tillsammans med den DNA-skadande substansen 4-NQO. Resultaten av dessa försök visade att en jäststam med avsaknad av samtliga TLS-polymeraser fortfarande överlever

DNA-skada mycket bättre med ökade dNTP-koncentrationer. Detta antyder att replikationspolymeraser kan vara involverade i att syntetisera förbi vissa typer av DNA-skador vid ökade dNTP-koncentrationer. Vi kunde senare styrka detta genom att i provrör (*in vitro*) visa att DNA polymeras epsilon (Pol ε), normalt associerat med replikation, kan syntetisera förbi vissa typer av DNA-skador med hjälp av höga dNTP-koncentrationer.

Vidare frågade vi oss om det skulle vara möjligt att skapa jäststammar med obalanserade dNTP-koncentrationer och i så fall om detta skulle upptäckas av cellens kontrollmekanismer. För att undersöka detta inriktade vi oss på Rnr1-loop2 hos *S. cerevisiae*, som har stora likheter med andra organismers RNR och som är en viktig del i att reglera balansen av dNTPs. Vi introducerade olika mutationer i *RNR1-loop2* som resulterade i förändringar av aminosyrasekvensen hos proteinet. Vi erhöll jäststammar med olika *rnr1-loop2* mutationer som resulterade i olika varianter av obalanserade dNTP-koncentrationer. Alla dessa jäststammar med obalanserade dNTP-koncentrationer medförde en ökad mutationsfrekvens men aktiverade inte cellernas kontrollsystem, den s.k. S-phase checkpoint, såvida inte koncentrationen av en eller flera dNTPer var så låga att DNA-replikation inte kunde fortgå. Med dessa jäststammar kunde vi visa att olika mutationer i Rnr1-loop2 resulterade i specifika dNTP-koncentrationer inne i cellerna. Vi kunde med hjälp av dessa stammar visa att olika typer av obalanser i dNTP-koncentrationen resulterade i unika mutationsspektran. Dessa mutationspektrum kan förklaras av en minskad noggrannhet hos replikationspolymeraserna vid obalanserade dNTP-koncentrationer och en viss typ av DNA-sekvens.

Sammanfattningsvis har vi funnit att DNA polymeras ε vid höga dNTP-koncentrationer har möjlighet att syntetisera förbi vissa typer av DNA-skador *in vitro*. Vi har även visat att obalanserade dNTP-koncentrationer ökar mutationsfrekvensen och att dessa mutagena och obalanserade dNTP-koncentrationer kan undvika att bli upptäckta av cellens kontrollmekanismer så länge det inte är brist på en eller flera dNTPs. Slutligen visade vi att den ökade mutationsfrekvensen kan förklaras av en mindre noggrannhet av replikationspolymeraserna som en följd av de obalanserade dNTP-koncentrationer vi fann i de olika jäststammarna samt den specifika DNA-sekvens som ska syntetiseras.

1. Introduction

All living organisms store genetic information in the form of deoxyribonucleic acid, or DNA. The essential precursors for DNA synthesis are the deoxyribonucleoside triphosphates: dTTP, dCTP, dATP, and dGTP, collectively referred to as dNTPs. The genetic code is written on two strands of DNA that intertwine to form what is known as a double helix. Within these two strands, the nucleotides are always found as pairs, T pairs with A and C pairs with G. The DNA of the budding yeast, *Saccharomyces cerevisiae*, consists of approximately 12×10^6 base pairs, while mammalian cells have approximately 6×10^9 base pairs. The enzyme ribonucleotide reductase (RNR) is the key enzyme in the *de novo* production of dNTPs within the cell. The proteins required for DNA synthesis are called DNA polymerases, and these are needed for both the construction of new DNA helices as well as the repair of existing DNA molecules.

2. Background

In *S. cerevisiae*, it has been demonstrated that DNA damage leads to a six to eight-fold elevation of dNTP concentrations which increase DNA damage tolerance at the cost of higher mutation rates (Chabes et al., 2003). Specialized DNA polymerases involved in translesion synthesis (TLS) increase DNA damage tolerance by bypassing lesions that cause the replicative DNA polymerases to stall when these lesions are encountered. In yeast, TLS polymerases often require higher dNTP concentrations to bind a nucleotide opposite a DNA lesion compared to nucleotide binding opposite an undamaged site. For example, Pol ε, a replicative DNA polymerase, has a K_m of 0.28 μM when binding dCTP opposite dG. Pol η, a TLS polymerase, has a similar binding affinity of 0.21 μM when forming this normal base pair but the K_m of binding dCTP opposite an O^6-methylguanine (m6G) lesion is more than ten-fold higher (5.1μM) (Haracska et al., 2000a; Shimizu et al., 2002). Pol ζ, another TLS polymerase, also has high affinity for forming the dCTP:dG base pair (K_m = 0.36 μM) but the K_m of binding dCTP opposite a m6G or a 7,8-dihydro-8-oxoguanine (8-oxoG) lesion increases to 370 μM and 7.5 μM, respectively (Haracska et al., 2003). Due to these findings, it has previously been suggested that TLS polymerases could benefit from the elevated dNTP concentrations that arise after DNA damage to increase cell survival by more efficiently bypassing DNA lesions (Chabes et al., 2003).

The concentrations of the individual dNTPs in cells are not equimolar and the ratio and overall concentrations differ between species (Buckstein et al., 2008; Traut, 1994). The dNTP concentrations are tightly regulated because imbalances leads to increased rates of mutagenesis (Reichard, 1988). The fact that dNTPs are kept at certain balance suggests that the specific ratio is important. How imbalanced dNTP pools contribute to mutagenesis and possibly interfere with cell cycle progression, however, has not yet been demonstrated.

3. The cell cycle

Living organisms are dependent on the multiplication of cells and the process by which cells reproduce is called the cell cycle. The cell cycle consists of four distinct phases: G_1 (gap 1), S (DNA synthesis), G_2 (gap 2), and M (mitosis) **(Fig.1)**. In G_1 and G_2, the cell grows and prepares for S phase and M phase, respectively. The process of DNA duplication, called replication, takes place in S phase. During M phase, the processes of mitosis (nuclear division) and cytokinesis (cell division) distribute the duplicated cellular components equally between the two new cells.

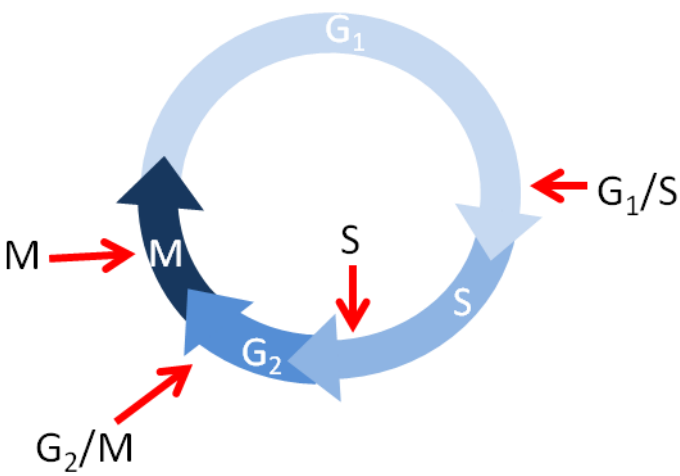

Figure 1. Schematic illustration of the different phases of the cell cycle. The red arrows indicate checkpoints.

The cell has a number of surveillance mechanisms called checkpoints that help to ensure that all of the processes have taken place correctly before the cell moves to the next phase of the cell cycle. The first checkpoint (G_1/S) takes place at the end of G_1 to make sure the cell is ready to start DNA synthesis. The S phase checkpoint ensures that replication is performed accurately and within a certain time limit. The G2/M checkpoint ensures that the cell is ready for mitosis to occur. The checkpoint within M phase (the anaphase checkpoint) ensures that the chromosomes have been properly segregated into each new daughter cell **(Fig. 1)**.

3.1 Activation of the S phase checkpoint

The response to DNA damage is controlled by four groups of proteins: sensors, mediators/adaptors, regulators and effectors. The S phase checkpoint can be activated by double strand DNA breaks (DSBs) or by stalling of replication forks due to either dNTP depletion or the presence of lesions within the DNA (Zegerman and Diffley, 2009).

If the replication fork stalls, single stranded DNA (ssDNA) will be exposed that is then coated by replication protein A (RPA), which is required for the recruitment of the sensor protein complex Mec1p-Ddc2p in *S. cerevisiae* (homologous to human ATR/ATRIP) (Zou and Elledge, 2003). The key step in DNA damage checkpoint activation by DSBs is also the formation of ssDNA. In *S. cerevisiae*, the sensor for DSBs is the Mre11p/Rad50p/Xrs2p (MRX) protein complex, which recruits Tel1p (homologous to human ATM) to the site of the break (Lisby et al., 2004; Nakada et al., 2003; Shroff et al., 2004). The MRX complex initiates degradation of the DNA ends through the 3´–5´exonuclease activity of Mre11p which results in ssDNA that is coated by RPA, which leads to the activation of Mec1p-Ddc2p (Zhu et al., 2008; Zou and Elledge, 2003).

The activation of the Mec1p-Ddc2p complex leads to the activation of a cascade of protein kinases in which Mec1p is the key checkpoint protein. Activated Mec1p activates the regulator protein Rad53p (homologous to human CHK2) resulting in an inhibition of cell cycle progression and late origin firing, stabilization of replication forks, induction of DNA repair genes and activation of Dun1p by phosphorylation (Zegerman and Diffley, 2009).

Checkpoint activation pathways in humans are clinically relevant since they mediate cellular responses that result in increased survival following DNA damaging events. Functional ATM and ATR pathways increase resistance of cancer cells to chemo- and radiotherapy and have become interesting targets for inhibition to improve the efficiency of anticancer treatments. For example, it has been demonstrated that inhibition of ATM by caffeine results in hypersensitivity of cancer cells to ionizing radiation (Sarkaria et al., 1999). Caffeine has also been demonstrated to inhibit proliferation of untreated breast cancer cells (Alao and Sunnerhagen, 2009).

4. dNTPs – The building blocks of DNA

The precursors of DNA are thymidine 5´-triphosphate (dTTP), deoxycytidine 5´-triphosphate (dCTP), deoxyadenosine 5´-triphosphate (dATP), and deoxyguanosine 5´-triphosphate (dGTP). dTTP and dCTP are known as pyrimidines and dATP and dGTP are known as purines. The dNTPs consist of three parts: a pentose sugar (deoxyribose), three phosphate groups, and one of the nitrogenous bases adenine (A), thymine (T), cytosine (C), or guanine (G) **(Fig. 2)**.

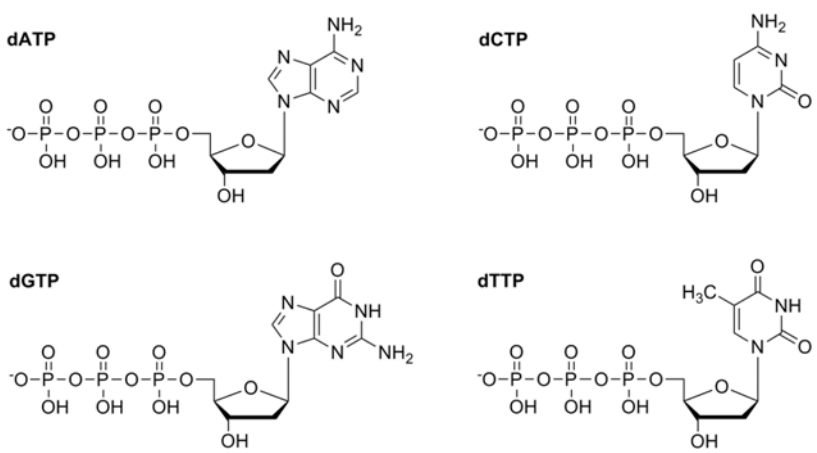

Figure 2. The precursors of DNA, the four deoxyribonucleoside triphosphates (dNTPs).

The building blocks are paired together by hydrogen bonds, A with T, and C with G, to form a helix of two complementary DNA strands **(Fig. 3)**. The concentration of dNTPs at any given moment during DNA replication is only sufficient for synthesis of a small fraction of the genome. For example, the amount of dGTP in a yeast cell at any time point during S phase is only enough for synthesis of approximately 4% of the genome's G bases.

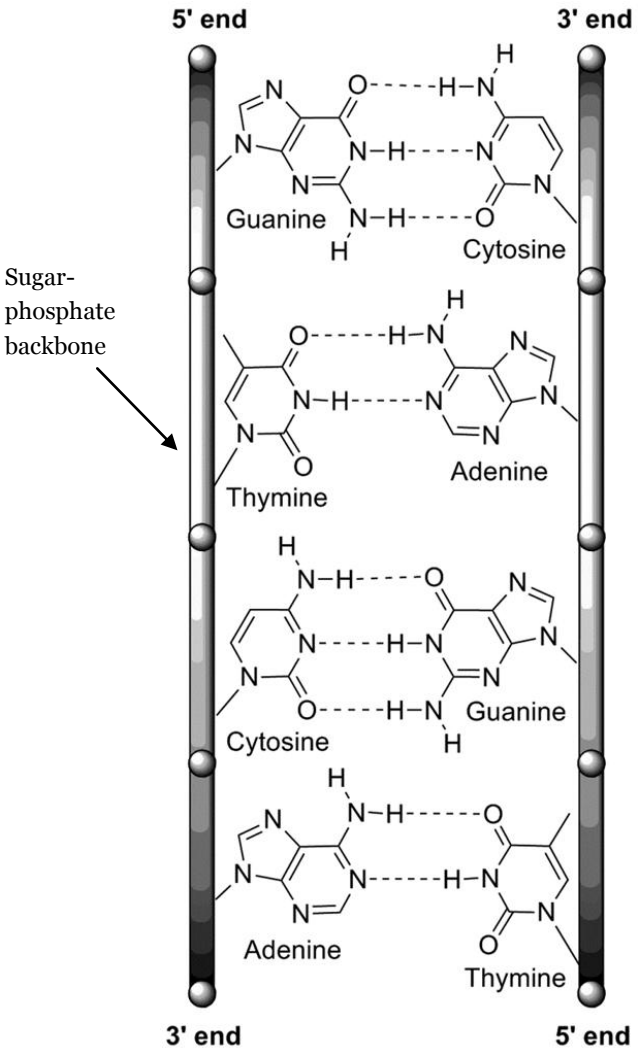

Figure 3. Base pairing between complementary DNA strands. There are two hydrogen bonds (shown as dashed lines) between adenines and thymines and three hydrogen bonds between cytosines and guanines. For simplicity's sake, the sugar-phosphate backbone is not shown.

5. Ribonucleotide reductase

Experiments performed fifty years ago demonstrated that an enzyme from the bacterium *Escherichia coli* could catalyze the formation of a deoxyribonucleotide from a ribonucleotide (Reichard, 1962). This enzyme was named ribonucleotide reductase (RNR). In all organisms, RNR is the key enzyme in the *de novo* production of all four dNTPs.

There are three classes of ribonucleotide reductases based on structural differences as well as on how the free radical that is essential for catalysis is generated. The Class I enzymes are found in all eukaryotes, including *S. cerevisiae*, and form an iron-tyrosyl radical in which the free radical is stabilized on the iron center in the presence of oxygen (Reichard and Ehrenberg, 1983). The Class II enzymes are adenosyl cobalamin-dependent and can function under both anaerobic and aerobic conditions. The Class III enzymes depend on an iron-sulfur cluster to create a free radical and are only active under anaerobic conditions (Reichard, 1993). Additionally, the Class I enzymes are divided into the subgroups Ia and Ib due to differences in the number of allosteric sites, dATP feedback mechanism and electron transport pathway (Jordan et al., 1996).

RNR catalyzes the reduction of ribonucleotides to deoxyribonucleotides using the power of free-radical chemistry and for each round of reduction the free radical is transported from the small subunit to the catalytic site located in the large subunit (Stubbe, 2003). The electron required for reduction is provided by the small proteins glutaredoxin and thioredoxin that have a redox-active thiol and are reduced by glutathione reductase and thioredoxin reductase, respectively, during each round of catalysis. The glutathione reductase and thioredoxin reductase are in turn reduced by NADPH (Jordan and Reichard, 1998). The free radical can be destroyed by the compound hydroxyurea (HU) that directly affects RNR activity (Nyholm et al., 1993).

During S phase in mammalian cells, the active RNR enzyme is composed of the homodimeric R1 (α_2) and R2 (β_2) subunits. The large R1 subunit contains the active site and the small R2 subunit harbors the free radical that is essential for catalysis (Reichard, 1988). However, it has been demonstrated that mammalian R1 also can exist as a hexamer which can interact with R2 to form an enzymatically active protein complex in an $\alpha_6\beta_2$ manner that is either active or inactive depending on whether ATP or dATP is bound (Rofougaran et al., 2006).

When DNA damage occurs, the additional mammalian RNR subunit p53R2 is expressed and has been demonstrated to form an active RNR complex with the R1 protein *in vitro* in a manner that is homologous to the R2 protein (Guittet et al., 2001; Tanaka et al., 2000).

In *S. cerevisiae*, RNR usually exists as a heterotetramer. During normal cell growth, the large subunit exists as a homodimer of Rnr1p (αα) and the small subunit as a heterodimer of Rnr2p and Rnr4p (ββ´) resulting in an $\alpha_2\beta\beta'$ complex. In *S. cerevisiae*, the transcription of the *RNR1* gene is tightly cell cycle regulated and fluctuates 15 to 30-fold reaching maximum expression in late G_1 and early S phase resulting in the highest activity of RNR in S phase (Elledge and Davis, 1990). In response to DNA damage, the non-essential Rnr3p subunit is expressed and has been shown to interact with Rnr1p to form a heterodimer as an alternative large subunit (Domkin et al., 2002; Elledge and Davis, 1990).

The production of dNTPs in *S. cerevisiae* depends entirely on *de novo* synthesis since yeast cells have no ability to salvage nucleosides from their environment. This is because yeast lack both the capacity for exogenous uptake of deoxyribonucleosides as well as nucleoside kinases that can convert nucleosides into 5´-deoxyribonucleoside monophosphates (Vernis et al., 2003). In addition, yeast also lack 5´-nucleotidases thus making it impossible for them to degrade 5´-deoxyribonucleoside monophosphates to nucleosides for subsequent export out of the cell (Reichard, 1988). These attributes make yeast a suitable model organism for studying the effects of dNTP concentrations and imbalances.

To avoid increased rates of mutagenesis the specific balance and the overall concentration of dNTPs need to be tightly controlled (Reichard, 1988). Therefore it is understandable why RNR activity is extensively regulated. The regulation of RNR in *S. cerevisiae* will now be discussed in more detail.

5.1 Allosteric regulation

RNR maintains a balanced concentration of dNTPs through a process of allosteric regulation. Each Rnr1p monomer possesses a catalytic site, an allosteric activity site and an allosteric specificity site **(Fig. 4)**. The allosteric activity site controls the overall activity of the enzyme; when ATP is bound, the enzyme is turned "on", and when dATP is bound, the enzyme is turned "off". Binding of dATP or ATP to the allosteric specificity site promotes the reduction of UDP and CDP in the catalytic site while a high concentration of dATP shuts off RNR activity through the activity site. dGTP binding to the allosteric specificity site promotes reduction of ADP, and dTTP binding promotes reduction of GDP (Thelander and Reichard, 1979). The dNDP products are then phosphorylated by the nucleoside diphosphate kinase (NDK) to create the corresponding dNTPs. In *S. cerevisiae*, the concentrations of dNTPs are allowed to increase six to eight-fold after DNA damaging events due to relaxed feedback inhibition (Chabes et al., 2003).

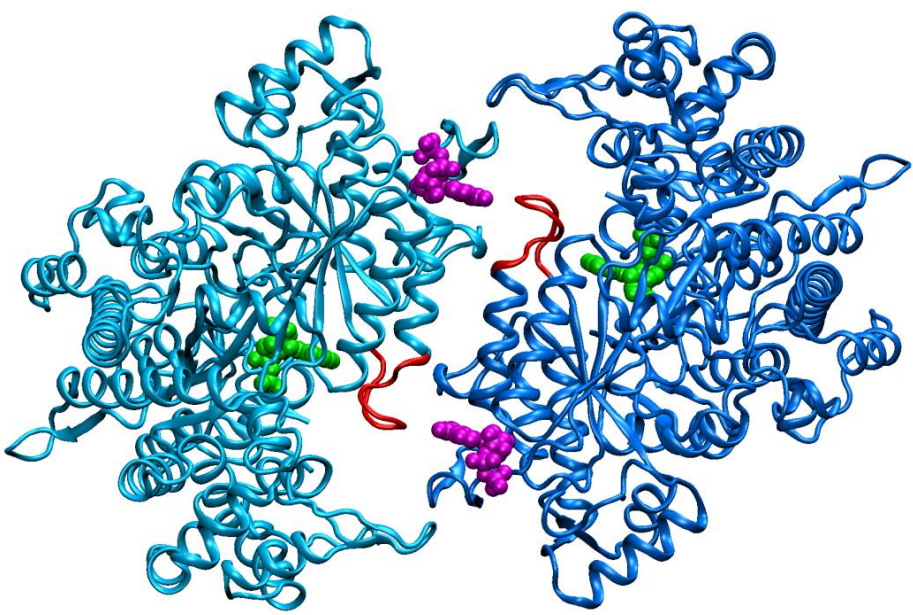

Figure 4. A model structure of the Rnr1p homodimer in yeast (PDB ID: 2CVX, Xu et al., 2006). One monomer is shown as a light blue ribbon and the other in dark blue. dGTP (purple spheres) is bound at the specificity site while ADP (green spheres) is bound at the catalytic site. Loop 2 of the specificity site is shown in red.

5.2 Regulation of RNR at a transcriptional level

The expression of the *RNR2*, *RNR3* and *RNR4* genes is suppressed by the DNA binding protein Crt1p, which inhibits transcription by recruiting the general repressors Ssn6p and Tup1p (Huang et al., 1998). In response to replication stress or DNA damage, the Mec1-Rad53-Dun1 checkpoint mediates a kinase cascade pathway that leads to the inactivation of Crt1p, which relieves the transcriptional repression of the *RNR2-4* genes (Huang et al., 1998; Navadgi-Patil and Burgers, 2009). The *RNR1* gene is also DNA damage inducible, however, *RNR1* transcription is not repressed by Crt1p (Klinkenberg et al., 2006). It has recently been shown that Ixr1p is required for appropriate *RNR1* expression both during normal cell growth and in response to DNA damage. Ixr1p, however, is not controlled by Dun1p or Crt1p but is instead suggested to be regulated indirectly by Rad53p (Tsaponina et al., 2011) (**Fig. 5**).

5.3 Regulation of RNR activity by inhibitor proteins

The cell cycle-regulated small protein Sml1p binds to the large subunit of yeast RNR and thereby inhibits its activity. During replication stress or after DNA damage, the Mec1-Rad53-Dun1 kinase pathway inactivates Sml1p relieving the inhibition of the large subunit (Chabes et al., 1999; Zhao et al., 1998; Zhao and Rothstein, 2002). The protein Dif1p binds to the small subunit of RNR and re-localizes it to the nucleus. The Mec1-Rad53-Dun1 pathway inactivates Dif1p which results in the release of the small subunit into the cytoplasm where it can form an active RNR complex with the large subunit (Lee et al., 2008; Wu and Huang, 2008) (**Fig. 5**).

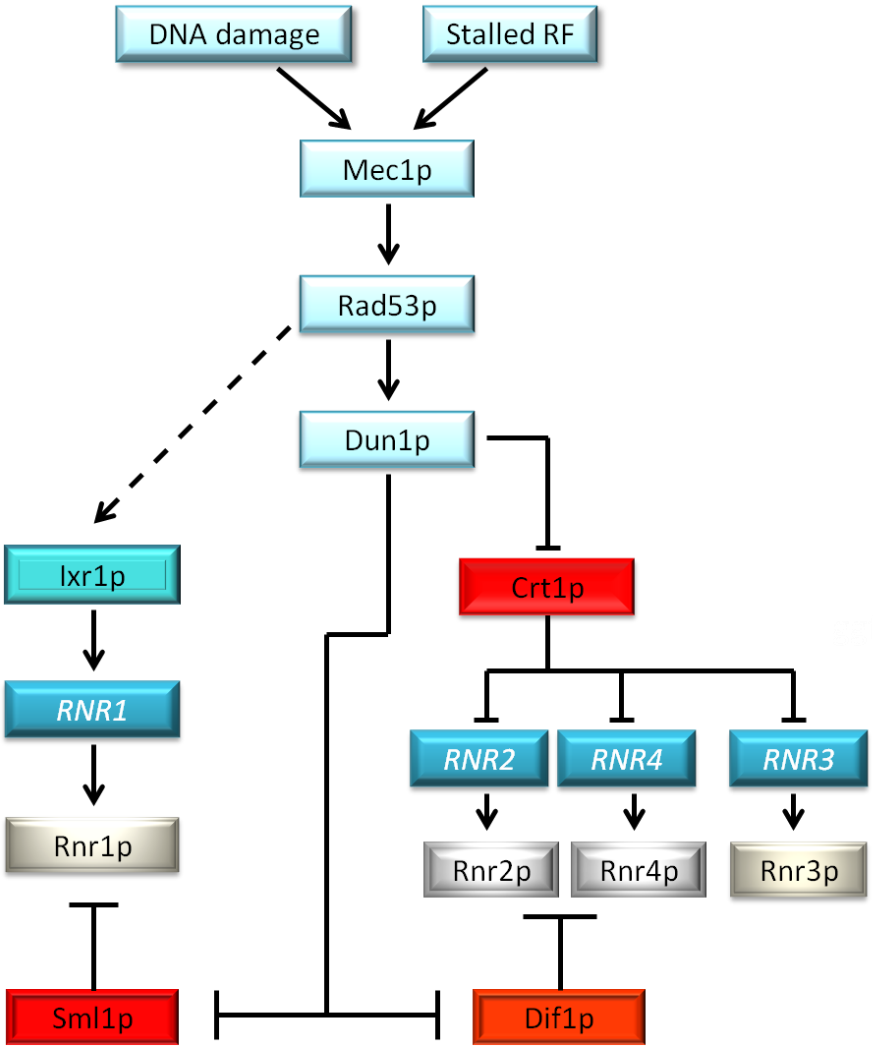

Figure 5. Activation of the S phase checkpoint in response to DNA damage or stalled replication forks. Activated Dun1p relieves the transcriptional inhibition of *RNR2-4* by inactivating Crt1p. Dun1p also inactivates Sml1p and Dif1p and releases the inhibition of the Rnr1p and Rnr2p/Rnr4p complexes, respectively. The expression of *RNR1* is dependent upon Ixr1p, which is suggested to be regulated indirectly by Rad53p.

6. Pathways of DNA repair

Cells are constantly exposed to endogenous and exogenous agents with the capability of damaging the cell's DNA. The cell has many mechanisms to repair DNA before, during, and after DNA synthesis. DNA repair can either reverse the DNA damage or remove the damaged portion of the DNA with subsequent re-synthesis and ligation of the DNA. An example of a DNA damage-reversing enzyme is the methylguaninetransferase encoded by *MGT1* in *S. cerevisiae* (homologous to human *MGMT*) that can irreversibly remove the methyl group of m6G lesions (Brent et al., 1988; Sassanfar and Samson, 1990). The systems that detect and remove altered nucleotide structures are the base excision repair (BER) pathway that detects and repairs damaged DNA bases, and the nucleotide excision repair (NER) pathway that is adapted to repair DNA lesions that distort the double helix (Memisoglu and Samson, 2000; Prakash and Prakash, 2000). The mismatch repair (MMR) pathway is adapted to replace nucleotides that have been incorrectly inserted into DNA by a DNA polymerase and thereby increases the replication fidelity (Friedberg, 2006). Although different repair mechanisms are listed separately here, DNA repair proteins are often involved in more than one pathway suggesting that there is a crosstalk between the different repair pathways.

Defects in repair mechanisms often lead to the development of different cancers. Mutations in the MMR system can result in hereditary non-polyposis colorectal cancer (HNPCC), defects in BER lead to increased risks of colorectal adenomas and carcinomas, and defects in the NER system lead to Xeroderma pigmentosum (XP), a condition of extreme sensitivity to sunlight-induced DNA damage that results in an increased risk of developing malignant melanoma (Cleaver, 1968; Hakem, 2008).

The most severe DNA damaging event is the creation of double stranded DNA breaks (DSBs) that can result in large chromosomal losses and rearrangements if not repaired correctly. DSBs can be repaired either by homologous recombination (HR) in a process that requires a homologous partner, or by non-homologous end-joining (NHEJ) in situations where no sister chromatid is available with a homologous DNA sequence (Friedberg, 2006). However, not all DNA damages are repaired with 100% efficiency and when a replication fork stalls due to the presence of a lesion or obstruction that the fork cannot bypass; specialized TLS polymerases are recruited to bypass the lesion.

7. The replication machinery

DNA replication is a tightly controlled process that has evolved to assure that the genome is only copied once and that it is completed before mitosis begins. Replication starts at multiple regions called origins. To ensure that the genome is only copied once per cell cycle, the assembly of the pre-RC, which prepares the origin for firing, takes place in G_1 phase and cannot be reformed until next cell cycle. Activation of the pre-RC and the start of replication then occurs in S phase (Gilbert, 2001). In the *S. cerevisiae* genome, there are sequences called autonomously replicating sequences (ARS) to which the origin recognition complex (ORC) binds in the G_1 phase. This is the first step in the assembly of the pre-replicative complex (pre-RC) (Bell and Stillman, 1992). The final step in the formation of the pre-RC is the loading of helicase Mcm2-7p complex onto the ORC by Cdt1p/Cdc6p (Araki, 2010). Interestingly, it has been demonstrated that constitutively high dNTP concentrations affect activation of ORCs, delay activation of pre-RCs, and arrest cell cycle progression in the G_1 phase (Chabes and Stillman, 2007).

After the assembly of the pre-RC, the switch from G_1 to S phase results in activation of cyclin-dependent kinases (CDKs) that phosphorylate Sld2p/Sld3p causing them to bind Dpb11p (Tanaka et al., 2007; Zegerman and Diffley, 2007). Also during this switch, Dbf4p activates the protein kinase Cdc7p that then phosphorylates the Mcm2-7p complex as a step in recruiting Cdc45p to the pre-RC (Masai et al., 2006). The association of phosphorylated Sld2p with Dpb11p promotes the formation of the pre-loading complex (pre-LC) containing Pol ε, the GINS complex, Dpb11p and Sld2p (Muramatsu et al., 2010). The delivery of GINS to the pre-RC forms the active CMG helicase, which consists of Cdc45p, Mcm2-7p, and the GINS complex.

After the assembly of the CMG complex, the DNA is unwound and DNA Pol α, which has both a primase and a DNA polymerase activity, is recruited to synthesize the RNA-DNA hybrid primer that is required by Pol δ and Pol ε to start replication (Baker and Bell, 1998). When the RNA-DNA primer is synthesized, the clamp loading complex replication factor c (RFC) loads the sliding clamp proliferating cell nuclear antigen (PCNA), which forms a ring around the DNA. Association between PCNA and Pol δ or Pol ε tethers the polymerases to the DNA and thereby increases their processivity (Garg and Burgers, 2005).

At each origin, two replication forks are assembled that move in opposite directions. Due to the fact that polymerases can only synthesize DNA in the 5´–3´ direction, one strand of DNA is synthesized continuously (the leading strand) while the other is synthesized discontinuously (the lagging strand). The discontinuous synthesis of the lagging strand results in short pieces of DNA called Okazaki fragments that are ligated together after the replication fork has passed. Recently, it has been shown that Pol ε participates in leading strand synthesis while Pol δ synthesizes the lagging strand (McElhinny et al., 2008; Pursell et al., 2007) **(Fig. 6)**.

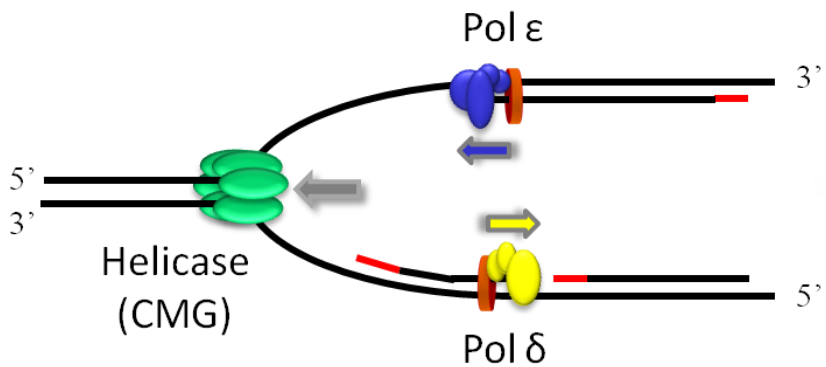

Figure 6. DNA synthesis at the replication fork. The grey arrow shows the direction of the fork and the blue and yellow arrows indicate the direction of Pol ε and δ, respectively. The red lines indicate the primer synthesized by Pol α. The helicase, CMG, consists of Cdc45p, Mcm2-7p, and the GINS complex.

The structure of DNA polymerases resembles a right hand; the fingers function to bind DNA template and dNTPs, the palm possesses the catalytic activity, and the thumb holds the newly synthesized duplex DNA (Steitz, 1999). Pol ε and δ have an intrinsic 3´–5´ exonuclease activity that increases replication fidelity by allowing the enzymes to correct recently made nucleotide misinsertions (Thomas et al., 1991). Experiments with mice have shown that an exonuclease deficient Pol δ or Pol ε leads to an increased mutator phenotype and an increase in spontaneous cancers. In addition, the different types of cancer that arise between the two exonuclease deficient polymerases indicate that Pol δ and Pol ε participate in tissue specific pathways *in vivo* to prevent spontaneous cancers (Albertson et al., 2009). The fidelity, or accuracy, of the replicative polymerases is in decreasing order δ > ε > α (Table 1). *In vitro* experiments have shown that imbalanced dNTP pools reduce the fidelity of replicative polymerases (Kunz et al., 1994).

7.1 Replicative polymerases in DNA repair

The DNA polymerases involved in replication have a high accuracy for inserting the correct nucleotide and they can correct insertion mistakes due to their 3´–5´exonuclease activity. Consequently, it was believed that replicative polymerases would not have any role in the bypass of DNA lesions. However, it has been demonstrated that Pol δ is able to insert dAMP opposite an abasic site, a location in DNA where a base is lost, and that Pol ζ can extend from the inserted nucleotide (Haracska et al., 2001). It has also been shown that Pol δ can replicate through an 8-oxoG lesion, although very inefficiently. However, when Pol δ inserts dAMP opposite an 8-oxoG lesion Pol ζ can efficiently extend the primer and complete the bypass (Haracska et al., 2003).

Alkylating agents are drugs commonly used in the treatment of different cancers. One type of alkylating agent is MNNG, which causes m6G DNA lesions that block synthesis by DNA polymerases. However, *in vitro* experiments with MNNG have shown that Pol δ can bypass m6G lesions in an error-prone manner by inserting a T opposite the lesion instead of a C (Haracska et al., 2000a). After the insertion opposite a m6G lesion by Pol δ, the extension of the primer can be synthesized by either Pol ζ or Pol η (Haracska et al., 2003).

8. The translesion polymerases

Even though a number of repair mechanisms exist in the cell, they are not 100% effective and some DNA lesions will escape repair by these systems. Cells have adapted to this problem with the evolution of specialized TLS polymerases that play an important role in bypassing DNA lesions that stall replication forks. Thus these specialized polymerases increase the ability of the cell to tolerate DNA damaging events.

Replicative DNA polymerases, which synthesize DNA with a high degree of accuracy, are blocked by lesions that distort the geometry of DNA. TLS polymerases tend to have a more open and less sterically constrained active site that allows them to synthesize past DNA lesions that are blocks to replicative polymerases. The trade-off for the ability of these specialized polymerases to bypass distorting lesions is that they often do so in an error-prone manner that leads to an increase in mutation rates (Prakash et al., 2005). In addition, all of the known TLS polymerases lack 3´–5´ proofreading activity, which increases the mutation rate of the polymerases

even further. It has, however, been shown that terminal misinsertions performed by Pol η directly after the bypass of thymine dimers can be corrected by the 3´–5´ proofreading function of Pol δ or Pol ε (McCulloch et al., 2004). Cells often prefer error-prone TLS repair to the more serious risk of replication fork collapse that can result in DSBs and subsequent genomic instability (Waters et al., 2009).

The TLS polymerases can be specialized for inserting nucleotides or extending from an inserted nucleotide, and some of the TLS polymerases perform both of these steps. Depending on the particular combination of TLS polymerase and DNA lesion, bypass of the damaged DNA can occur in an error-free or an error-prone manner. The TLS polymerases known in *S. cerevisiae* are Rev1p and Pol η (Y-family polymerases), Pol ζ (a B-family polymerase), and Pol 4 (an X-family polymerase) (Prakash et al., 2005; Prasad et al., 1993).

When the replication machinery stalls due to a DNA lesion that it cannot bypass, the arrest leads to exposure of ssDNA that is rapidly coated with RPA that, in turn, attracts the ubiquitin ligase Rad18p (Davies et al., 2008). Rad18p is a DNA binding protein that, together with the ubiquitin-conjugating enzyme Rad6p, modifies PCNA by monoubiquitination. This modification of PCNA is suggested to result in the switch from replicative polymerases to TLS polymerases at the stalled replication fork (Hoege et al., 2002; Kannouche et al., 2004). The monoubiquitination of PCNA can be removed by Usp1p, a de-ubiquitinating enzyme. UV-irradiation inhibits Usp1p thus enabling the modification of PCNA to promote TLS (Huang et al., 2006).

Y-family TLS polymerases are also substrates for monoubiquitylation. Experiments have shown that, after nucleotide insertion, Rev1p can be monoubiquitylated, which is thought to recruit Pol ζ to the replication fork to perform primer extension of the inserted nucleotide (Bienko et al., 2005; Guo et al., 2006). Due to the low processivity of TLS polymerases, it has been suggested that TLS polymerases fall off the DNA shortly after lesion bypass resulting in a switch back to replicative polymerases to continue replication (Kannouche et al., 2004).

In *S. cerevisiae*, it has recently been demonstrated that TLS takes place not only in S phase but also in G_2 phase (Daigaku et al., 2010; Karras and Jentsch, 2010). Previously, it was shown that replication forks could skip template regions that contained UV-induced DNA lesions and leave behind ssDNA that could persist into G_2 phase where it was repaired by post-replication repair (PRR) pathways (Torres-Ramos et al., 2002).

The activation of PRR is suggested to depend upon polyubiquitination of PCNA, which requires Mms2p-Ubc13p-Rad5p in addition to the previously mentioned Rad6p and Rad18p (Hoege et al., 2002).

PCNA can also be modified by a small ubiquitin-related modifier protein (SUMO) and it has been demonstrated that SUMOylated PCNA cooperates with the helicase Srs2p (Pfander et al., 2005). *In vitro* experiments have demonstrated that Srs2p inhibits the function of Rad51p, a protein that mediates DNA strand exchange during HR (Krejci et al., 2003; Veaute et al., 2003). Based on these findings, it has been suggested that the SUMOylation of PCNA upon DNA damage recruits Srs2p to the replication fork to prevent unwanted HR during replication (Pfander et al., 2005).

8.1 Rev1

Rev1p has a dCMP transferase activity and utilizes an amino acid to direct insertion of dCTP instead of using the DNA strand as a template like other polymerases (Nair et al., 2005). The most commonly formed DNA lesion is the abasic site that can be formed spontaneously through depurination. Due to the dCMP transferase activity of Rev1p, it is able to insert dCMP opposite abasic sites. Pol ζ can then extend the primer resulting in error-free bypass when the abasic site is formed by the loss of dGMP (Nelson et al., 1996a). It has been suggested that Rev1p and Pol ζ together contribute to TLS, resulting in an increased rate of spontaneous mutations (Lawrence, 2002).

The smallest subunit of Pol δ, Pol32p, has been demonstrated to physically interact with Rev1p but not with Pol ζ, and is suggested to be involved in the formation of a Rev1p-Pol32p complex as a step in recruiting Pol ζ to the stalled replication fork (Acharya et al., 2009). The hypothesis is that Rev1p makes the insertion opposite the lesion and Pol ζ extends from the insert to complete the bypass.

8.2 Polymerase ζ

Pol ζ consists of two subunits, the catalytic Rev3p subunit and the accessory subunit Rev7p, and contributes to mutagenesis by creating and extending nucleotide mismatches (Nelson et al., 1996b; Zhong et al., 2006). Pol ζ belongs to the B-family of polymerases but it lacks 3´–5´exonucleus activity and has a high error rate for single base substitutions compared to other B-family polymerases (Table 1) (McCulloch and Kunkel, 2008).

Experimental data indicate that Pol ζ and Pol η work cooperatively to bypass UV-induced DNA damage, primarily cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone (6-4PPs) adducts (Abdulovic and Jinks-Robertson, 2006). However, it was recently demonstrated that Pol ζ has the ability to act alone in the TLS synthesis of abasic sites, CPDs, and 6-4PPs, particularly in the presence of the elevated levels of dNTPs that are generated in response to DNA damage (Stone et al., 2011).

DNA polymerase	Single base substitution ($\times 10^{-5}$)	Deletion ($\times 10^{-5}$)	Reference:
Pol α	9,6	3,1	(Shcherbakova et al., 2003)
Pol δ	<1,3	1,3	(Fortune et al., 2005)
Pol ε	<2.0	<0,05	(Shcherbakova et al., 2003)
Pol ζ	130	4,4	(Zhong et al., 2006)
Pol η	950	93	(McCulloch et al., 2007)

Table 1. A comparison of the rates of single base substitutions and deletion events for yeast DNA polymerases.

In vivo experiments with mice showed that loss of Pol ζ function resulted in early stage embryonic lethality implying that Pol ζ has other functions besides TLS (Bemark et al., 2000; Esposito et al., 2000; Wittschieben et al., 2000). Previously, it has been shown that Pol ζ has a high mutagenic activity and that it is responsible for the increase in base substitution errors near DSBs (Holbeck and Strathern, 1997; Morrison et al., 1989). In addition to its role in TLS, these mutagenic properties of Pol ζ have led to suggestions of a role in somatic hypermutation of immunoglobulin genes (Kim and Storb, 1998).

8.3 Polymerase η

Pol η is encoded by the *RAD30* gene and it has been demonstrated to insert two adenines opposite a thymine dimer as efficiently as when processing undamaged T-T residues (Johnson et al., 1999). Pol η can also bypass 8-oxoG lesions, one of the most commonly occurring DNA lesions, in an error-free manner by inserting dCMP (Haracska et al., 2000b). It has also been shown that Pol η can bypass m6G lesions by insertion of dCMP opposite the lesion resulting in an error-free bypass, however, Pol η occasionally inserts dTMP instead resulting in error-prone TLS (Haracska et al., 2000a).

A group of XP patients have been identified with a fully functional NER system but showing defects in bypass and repair of UV-induced DNA damage (Lehmann et al., 1975). These XP variant (XP-V) patients have mutations in the gene encoding Pol η, which lead to the inability to bypass UV-induced thymine dimers in an error-free manner (Johnson et al., 1999; Masutani et al., 1999).

8.4 Pol 4

The function of Pol 4 in *S. cerevisiae* regarding TLS is unclear. It has, however, been demonstrated to bind and function together with the endonuclease FEN-1 (*RAD27*) in repair of DSBs by NHEJ (Tseng and Tomkinson, 2004).

9. The *CAN1* forward mutation assay

In *S. cerevisiae*, the gene *CAN1* encodes an arginine permease that allows L-Arginine to be imported into the cell. The toxic analogue to L-Arginine, L-Canavanine, is indistinguishable to this enzyme and cells die when grown on media containing this toxin. However, if a mutation has occurred in the *CAN1* gene resulting in a non-functional arginine permease, the cells can survive even in the presence of L-Canavanine since it cannot be imported. In the papers included in this thesis we have used the *CAN1* forward mutation assay to calculate mutation rates. Sequencing of the *can1* gene gave the mutation spectra of the yeast strains with different imbalanced dNTP concentrations.

10. Aims of this thesis

- To investigate to what extent TLS polymerases and replicative polymerases benefit from increased dNTP pools with regards to tolerating damaged DNA.
- To create yeast strains with imbalanced dNTP pools *in vivo* and investigate how imbalances in dNTP concentrations contribute to mutagenesis.

Results & Discussion

Paper I:

Previous studies in *S. cerevisiae* showed that the concentrations of dNTPs increase in response to DNA damage and that this results in higher tolerance of DNA damaging events. This improved DNA damage survival was suggested to depend upon a more efficient bypass of DNA lesions by the specialized TLS polymerases that are involved in DNA repair (Chabes et al., 2003). To investigate this hypothesis, single deletions of *REV1*, *REV3* (the catalytic subunit of Pol ζ), *RAD30* (Pol η), and *POL4* were created. In addition, these single deletions were also constructed together with an extra, inducible *RNR1* gene in the genome. Induction of the additional *RNR*1 gene increased the dNTP concentrations 9- to 13-fold, similar to the dNTP increase seen after DNA damage. If a certain TLS polymerase were to be more efficient in lesion bypass at elevated dNTP concentrations, then a strain with deletion of that polymerase would have equal survival rates after DNA damage regardless of the dNTP concentration.

We tested all four mutant yeast strains in the presence of DNA damage with or without elevated dNTP concentrations. The damaging agent used was 4-NQO, a compound that forms quinoline adducts and 7,8-dihydro-8-oxoguanine (8-oxoG) lesions, one of the most common DNA damages (Kohda et al., 1986). Interestingly, all the strains with deletion of a single TLS polymerase survived DNA damage better when associated with elevated dNTP concentrations. However, the *rev1*Δ and *rev3*Δ (Pol ζ) single deletion strains are more sensitive to DNA damage by 4-NQO compared to a wild type strain, which correlates with more recent findings (Wiltrout and Walker, 2011). Nevertheless, both *rev1*Δ and *rev3*Δ (Pol ζ) strains have increased survival in the presence of elevated dNTP concentrations. The reason for the lower survival of the *rev1*Δ and *rev3*Δ strains compared to wild type is likely due to their involvement in lesion bypass of quinoline adducts produced by 4-NQO, where Rev1p makes the insertion opposite the damage and Pol ζ extends the primer.

We also created yeast strains lacking all known TLS polymerases both with and without an extra inducible *RNR1* gene. Surprisingly, in a strain with all TLS polymerases deleted, elevated dNTP concentrations still increased DNA damage tolerance. These results indicated that in the presence of elevated dNTP concentrations and in the absence of TLS polymerases, replicative polymerases can bypass some of the DNA lesions produced by 4-NQO.

Importantly, it should be noted that the increased survival rates are not due to pre-activation of the DNA damage checkpoint by the elevated dNTP pools (Chabes and Stillman, 2007).

To investigate if replicative polymerases can bypass DNA damage at elevated dNTP concentrations, we performed *in vitro* experiments with Pol ε, the leading strand replicator (Pursell et al., 2007). We performed primer extension assays with Pol ε together with a primer annealed to undamaged, 8-oxoG or m6G templates **(Fig. 7)**.

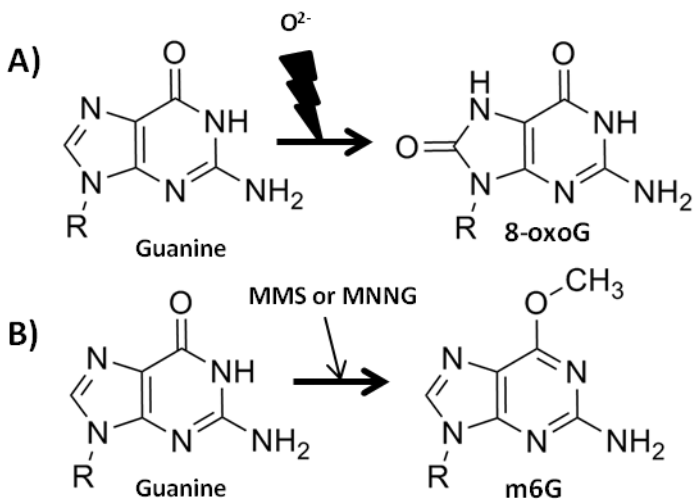

Figure 7. DNA lesions involved in the primer extension assay.
A) A guanine base damaged by oxidative stress leading to a 7,8-dihydro-8-oxoguanine (8-oxoG)
B) A guanine base damaged by an alkylating drug resulting in an O^6-methylguanine (m6G)

The assays were run at three different dNTP concentrations: low (half that of normal S phase concentrations), normal (normal S phase concentrations), and high (dNTP concentrations present after DNA damaging events) with either an excess of Pol ε or under single-hit conditions. Under single-hit conditions, Pol ε could not bypass the m6G or the 8-oxoG lesions at low or normal dNTP concentrations, however at elevated dNTP concentration the lesions were bypassed at 20% and 25%, respectively. In the presence of excess Pol ε such that several rounds of synthesis could be re-initiated, the bypass of m6G and 8-oxoG at normal S phase dNTP concentrations were

22% and 19%, and the bypass with high dNTP concentrations were 61% and 93% respectively. These results showed that at the dNTP concentrations which exist in response to damaged DNA, Pol ε was almost as efficient at bypassing an 8-oxoG lesion as an undamaged G when allowed to reinitiate synthesis from the primer. We identified dAMP as the major nucleotide inserted opposite an 8-oxoG lesion by Pol ε. These *in vitro* results can explain the elevated mutation rates observed (approximately 3-fold) *in vivo* with the yeast strain lacking all TLS polymerases.

In this paper, we showed that a yeast strain lacking all known TLS polymerases still had improved survival after treatment with the DNA damaging drug 4-NQO in the presence of elevated dNTP concentrations. We also demonstrated that Pol ε can bypass 8-oxoG lesions at high dNTP concentrations *in vitro*. Thus, we suggest a new bypass pathway during elevated dNTP levels in *S. cerevisiae* in which the replicative polymerases can bypass less bulky DNA lesions. A similar pathway was recently shown in *E. coli* in which elevated dNTP pools increased the activity of the replicative polymerase, Pol III, while decreasing its proofreading function thus resulting in higher mutation rates (Gon et al., 2011). These data lend support to our findings in *S. cerevisiae* regarding Pol ε.

We demonstrated that Pol ε, in the presence of elevated dNTP concentrations, had a 25% chance of synthesizing past an 8-oxoG lesion without dissociating from the DNA. However, repair of 8-oxoG lesions *in vivo* is often performed in an error-free manner by the BER, which is initiated by removal of the 8-oxoG lesions by the DNA glycosylase Ogg1p (van der Kemp et al., 1996). Thus, it would be reasonable that deletion of *OGG1* would lead to a greater number of 8-oxoG lesions *in vivo* when cells are treated with 4-NQO. If our hypothesis were correct, a strain lacking both *OGG1* in addition to all TLS polymerases would, upon treatment with 4-NQO, have an increased mutation rate due to the misinsertion of dAMP opposite 8-oxoG by Pol ε.

We also showed that m6G lesions can be bypassed by Pol ε *in vitro* but preliminary *in vivo* spot test experiments with MMS (which induce m6G adducts among other damages) did not show any great difference in survival between cells with normal or increased dNTP levels. Notably, it should be pointed out that spot test experiments are an imprecise method for determining DNA damage survival and no further experiments have been done yet. In yeast, the enzyme Mgt1p repairs m6G adducts (Sassanfar and Samson, 1990) and the preliminary results with MMS could be explained if Mgt1p repaired the m6G lesions in the TLS polymerase deletion strain.

Thus, deletion of *MGT1* would increase the number of m6G adducts after MMS treatment and possibly increase DNA damage tolerance in the presence of elevated dNTP pools. Further experiments with MMS and 4-NQO can be done to test our hypothesis of bypass of less bulky DNA lesions *in vivo* by Pol ε.

Furthermore, it has been demonstrated that Pol δ can bypass both 8-oxoG and m6G damages *in vitro* (Haracska et al., 2000a; Haracska et al., 2000b) and that synthesis past 8-oxoG and m6G lesions by Pol δ increased 4.6- and 15-fold, respectively, after the dNTP concentrations were increased from 5 μM to 100 μM (Haracska et al., 2003). Thus, it could be both Pol ε and δ bypassing these lesions *in vivo* in the presence of elevated dNTP concentrations, especially when no TLS polymerases are available.

Our current hypothesis is that replicative DNA polymerases in *S. cerevisiae* can bypass small DNA lesions that can fit in their active sites if given enough time (e.g. to reinitiate) and/or the proper dNTP concentration for a specific DNA lesion.

Paper II:

Previous experiments have demonstrated that imbalanced dNTP concentrations are highly mutagenic and that the ratios of dNTP concentrations are controlled primarily by RNR (Reichard, 1988). This work was the impetus for us to investigate the possibility of creating yeast strains with imbalanced dNTP concentrations *in vivo*, and to test these strains in terms of mutation rates and S phase checkpoint activation.

The crystal structure of *S. cerevisiae* Rnr1p was recently solved showing a crosstalk between the allosteric specificity site and the catalytic site (Xu et al., 2006). They demonstrated that binding of a specificity effector rearranges Rnr1-loop2, a polypeptide chain that is a part of the specificity site, resulting in a conformation change that promotes binding of a certain substrate in the catalytic site. They also showed that effector binding leads to a substrate preference primarily through interactions with residues R293 and Q288 of Rnr1-loop2.

We hypothesized that mutations in the amino acid sequence of Rnr1-loop2 (**Fig. 4**) would affect the crosstalk between the specificity site and catalytic site. We chose to make substitutions in Y285, D287, Q288, and R293 of Rnr1-loop2 either because they are conserved or because the structural study showed that they are involved in important interactions with effectors or substrates (Xu et al., 2006). We overexpressed the *rnr1-loop2* mutants using an inducible *GAL1* promoter and these changes resulted in unique dNTP imbalances within the yeast cells that were specific for each mutation. Previously published results demonstrate that continuous expression of *RNR1* from the GAL1 promoter leads to increased dNTP concentrations outside of S-phase that could interfere with DNA damage checkpoint activation and cell cycle progression (Chabes and Stillman, 2007). We therefore replaced the genomic, wild type *RNR1* with the different *rnr1-loop2* mutants. The viable yeast strains we obtained were *rnr1-Y285A*, *rnr1-Y285F*, *rnr1-Q288A*, and *rnr1-R293A*. The observed dNTP pool imbalances in these strains led to an increased mutation rate, however, the mutation rates were not proportional to the degree of dNTP imbalance. The *rnr1-Y285A* and *rnr1-Y285F* had normal growth rates while the *rnr1-Q288A* and *rnr1-R293A* strains had proliferation defects.

To investigate checkpoint activation, we analyzed the levels of Rnr2-4p, proteins that are known to be highly induced after checkpoint activation (Elledge and Davis, 1990; Huang and Elledge, 1997). The *rnr1-Q288A* and *rnr1-R293A* strains had high levels of Rnr2-4p and thus had activated S phase checkpoint; however, the *rnr1-Y285A* and *rnr1-Y285F* strains did not

activate the checkpoint. In the *rnr1-Q288A* and *rnr1-R293A* strains, it is possible that depletion of one or several dNTPs resulted in stalling of replication forks and that this event led to activation of the S phase checkpoint. In the *rnr1-Y285A* and *rnr1-Y285F* strains there were no dNTPs below wild type levels nor were any checkpoints activated. It has been proposed that misinsertion due to imbalanced dNTP pools in mammalian cells results in checkpoint activation through the MMR system (Hastak et al., 2008). However, the imbalanced dNTP pools and increased mutation rates in *rnr1-Y285A* and *rnr1-Y285F* suggest that MMR does not activate checkpoints in *S. cerevisiae*.

In this paper we demonstrated that different Rnr1-loop2 mutations lead to defined dNTP pool imbalances *in vivo*. From the structural studies (Xu et al., 2006), the dNTP imbalances in the *rnr1-Q288A* strain were likely due to decreased binding of all substrates to the catalytic site except GDP, which leads to increased levels of dGTP and dATP. The Y285 residue is involved in binding the guanine base in the dGTP-ADP complex and it is likely that the Y285A and Y285F mutant strains have a weaker affinity for dGTP. dGTP is a negative effector for CDP and UDP reduction and a positive effector for ADP, thus reduced binding of dGTP would result in higher concentrations of pyrimidines in the cell. However, the interaction of the R293 residue with the base of the substrates ADP and GDP cannot explain the dNTP imbalances in this strain. Perhaps a larger conformational change takes place that leads to the defined dNTP imbalances in the *rnr1-R293A* strain. Structural studies of RNR from the *rnr1* mutant strains with different effectors/substrate bound could give more insights into how dNTP imbalances arise in these strains.

Finally, it would be interesting to introduce other mutations in *RNR1-loop2* to look for other types of defined dNTP imbalances. It would also be interesting to use the *rnr1-loop2* mutant strains to study how various mismatches are recognized by the mismatch repair system.

Paper III:

In this paper, we investigated how dNTP imbalances contribute to mutagenesis using the previously obtained yeast strains *rnr1-Y285A*, *rnr1-Y285F*, and *rnr1-Q288A*. Using the *CAN1* forward mutation assay, we collected *CAN1*-positive clones that had mutation(s) disrupting arginine permease in both the wild type and the *rnr1* mutant yeast strains. After PCR amplification, all of the clones were sent for sequencing of the *CAN1* locus. The *can1* mutation spectrum from the wild type strain showed mutations spread throughout the gene, which correlated with earlier findings (Kokoska et al., 2000; Tishkoff et al., 1997). Interestingly, mutation spectra for the *rnr1* mutant strains had mutation hotspots in certain areas of the *CAN1* gene. In this study, we have defined a mutational hotspot as a nucleotide that has more than a 10-fold increase in mutation rate compared to the wild type strain.

For the wild type and the *rnr1-Y285F* mutant strain, the sequence changes were mainly single base substitutions. However, the substitution rate in *rnr1-Y285F* was 3-fold higher compared to wild type but only 17% of the mutations were at a common location between the strains. At 18 locations in the *CAN1*, mutation rates for the *rnr1-Y285A* mutant strain was up to 300-fold higher than wild type and was dominated by insertion-deletions (indels) that occurred at a rate 66-fold higher than wild type. The *rnr1-Y285A* had an 8-fold increase in substitution rate relative to wild type and the majority occurred at G-C base pairs. Surprisingly, the *rnr1-Q288A* strain had a totally different mutation spectrum that was dominated by single base substitutions, of which 65% were transitions.

Previously, it has been shown that replication of the *CAN1* gene originates from the origin ARS507 and moves towards the telomere (Raghuraman et al., 2001; Yabuki et al., 2002). This predicts that the coding strand will be a template for lagging strand synthesis while the non-coding strand acts as a template for leading strand synthesis. Thus, the replication of the non-coding strand would then be performed by Pol ε and coding strand replication by Pol δ (McElhinny et al., 2008; Pursell et al., 2007). The mutation spectrum for the *rnr1-Y285A* mutant strain can be inferred as being the result of both leading and lagging strand replication due to mutations in both the coding and non-coding strands. The mutation spectrum of the *rnr1-Q288A* mutant, on the other hand, can be inferred to result exclusively from lagging strand replication due to mutations only occurring in the coding strand. From the previous paper, we know that *rnr1-Q288A* activates the S phase checkpoint, which provides more time for DNA repair and may provide time for more extensive proofreading by Pol ε thus

selectively preventing replication errors on the leading strand. It would be interesting to further investigate this strand specificity and to examine if mutations appear mostly during the bypass of damaged bases. All of the *rnr1* mutant strains have different, defined dNTP imbalances that result in mutation hotspots which can be explained by dNTP-induced misinsertion, DNA strand misalignment, and mismatch extension at the expense of proofreading.

The dNTP ratio that would explain the specific mutation rates in the different *rnr1* strains correlates with the measured dNTP concentrations in the cell and these results support further use of HPLC analysis to measure biologically occurring dNTP concentrations. Our results suggest that dNTP imbalances increase mutagenesis in a manner that is dependent upon the sequence being synthesized and the particular type of dNTP imbalance.

In *E. coli*, it has recently been shown that dNTP concentrations increase in response to UV-induced DNA damage but the increase is not the same for all four individual dNTPs. After the elevation of dNTP concentrations, the activity of the replicative polymerase, Pol III, increases while there is a concomitant decrease in proofreading function. This results in higher mutation rates (Gon et al., 2011), which is similar to what we observed in yeast.

Our studies in papers II and III have demonstrated that the maintenance of dNTP ratios *in vivo* is crucial for genome integrity. Future experiments would be to measure dNTP pools in malignant cells to look for any disturbances in dNTP pool ratios in these cells. It would also be very interesting to begin looking for genetic disorders in humans that may result in imbalanced dNTP pools. The drug Methotrexate is commonly used to treat autoimmune diseases, such as rheumatoid arthritis and psoriasis, and cancers, especially leukemia and lymphomas. Methotrexate interacts with and inhibits dihydrofolate reductase (Rajagopalan et al., 2002), which is a crucial enzyme in the dTMP synthesis pathway as well as in the salvage pathway of purines. Treatment of cells with a low dose of Methotrexate would likely lead to dNTP imbalances and it would be interesting to measure dNTP pools and calculate mutation rates in cells treated with this drug. Perhaps patients treated with a low dosage of Methotrexate over a long time span, in the treatment of rheumatoid arthritis, for example, have an increased risk of cancer development due to imbalanced dNTP pools. However, from our published data, one could argue that the decrease in concentration of one or more dNTPs would activate the S phase checkpoint resulting in a moderate increase in mutation rate compared to the high mutation rates seen with elevated imbalanced dNTP pools.

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