RIBONUCLEOTIDE REDUCTASE
AND
DNA DAMAGE

by

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Ribonucleotide reductase and DNA damage

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Abstract

A prerequisite for a multicellular organism to survive is the ability to correctly replicate and repair DNA while minimizing the number of heritable mutations. To achieve this, cells need a balanced supply of deoxyribonucleoside triphosphates (dNTPs), the precursors for DNA synthesis. The rate-limiting step in de novo biosynthesis of dNTPs is catalyzed by the enzyme ribonucleotide reductase (RNR).

The classic eukaryotic RNR enzyme consists of a large and a small subunit. Together, these subunits form a heterotetrameric RNR complex. The larger subunit harbours active sites whereas the smaller subunit contains a stable tyrosyl free radical. Both subunits are required for RNR activity.

Since failure to correctly regulate de novo dNTP biosynthesis can lead to misincorporation of nucleotides into DNA, genetic abnormalities and cell death, RNR activity is tightly regulated. The regulation of RNR activity involves cell cycle-specific expression and degradation of the RNR proteins, as well as binding of allosteric effectors to the large RNR subunit.

In this thesis, in vitro assays based on purified recombinant RNR proteins, in combination with in vivo assays, have been used successfully to study the regulation of RNR activity in response to DNA damage. I present new findings regarding the function of an alternative mammalian RNR small subunit, and on the role of a small RNR inhibitor protein of fission yeast, during normal growth and after DNA damage. I also show conclusively that there are fundamental differences in the regulation of dNTP biosynthesis between the cells of higher and lower eukaryotes after DNA damage.
Till Hanna
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Introduction

The characteristics of a species as a whole are determined by the genetic code within the cells. Cells store their genetic information in DNA (deoxyribonucleic acid). The DNA molecule forms an unbranched α-helical chain which is synthesized from only four building-blocks: dATP, dCTP, dTTP, and dGTP (Fig. 1).

![Fig. 1. The four building blocks of DNA synthesis: dATP (deoxyadenosine triphosphate), dTTP (deoxythymidine triphosphate), dGTP (deoxyguanosine triphosphate), and dCTP (deoxycytidine triphosphate).](image)

Cells need a balanced supply of all four deoxyribonucleoside triphosphates (dNTPs) during DNA synthesis. Failure to correctly regulate the dNTP pools can lead to misincorporation of nucleotides into DNA, causing genetic abnormalities and cell death [1]. The rate-limiting step in de novo dNTP biosynthesis is catalyzed by the enzyme ribonucleotide reductase (RNR) [1].
In this thesis I present new findings regarding the regulation and enzymatic properties of an alternative mammalian RNR protein. I outline new evidence for how fission yeast cells regulate RNR activity, and how mammalian and fission yeast cells respond to DNA damage in the context of dNTP biosynthesis. At the end, the results are considered in the context of the whole research field.
Background

Before I present the results on which this thesis rests, I will provide the reader with a brief introduction to some of the main concepts.

1.1 The cell cycle

The survival of a multicellular organism depends on its ability to control cell proliferation. To achieve this, cells have developed surveillance mechanisms that constantly monitor the internal and external status of the cell. When a cell receives the right signals, the mother cell becomes irreversibly committed to divide into two daughter cells.

![Fig. 2. The different phases of the cell cycle: the M-phase (mitosis and cytokinesis), the S-phase (synthesis), the G1-phase (gap 1), the G2-phase (gap 2) and the G0-phase (gap 0).](image)

The lifespan of a cell can be described by a sequence of events called “the cell cycle”. Its progression is strictly regulated, where later events of the cycle directly depend on successful completion of earlier events [2]. The cell cycle can be divided roughly into four separate phases: G1, S, G2, and M (Fig. 2). The cell replicates its entire genome during the S-phase. Consequently, the demand for dNTPs is high in S-phase. Segregation of sister chromatids and cytokinesis occur during the M-phase. The S- and M-phase are separated by two intermediate phases. These are the G1- and G2-phases, during which the cell grows in size and prepares itself for DNA replication and mitosis,
respectively. Cells not allowed to proliferate can enter a specialized resting state termed the G₀-phase (Fig. 2).

1.2 The DNA damage response

With time, the mutation of genes controlling cell proliferation can lead to tumour progression. Minimization of the number of dangerous and heritable mutations in cells depends on their ability to recognize unnatural DNA conformations. If a DNA lesion is detected, the appropriate initial response is an attempt to repair the DNA helix. If the damage is too severe, or compromises the integrity of the DNA molecule, signalling cascades activate cellular senescence (permanent withdrawal from the cell cycle) or apoptosis (programmed cell death) [3]. These events are parts of what is known as the “the DNA damage response”. At the molecular level, the DNA damage response is an intricate web of interacting proteins. Based on their individual functions, these proteins have been classified as sensors, transducers, and effectors [3]. Sensors detect the DNA lesion, which activates the transducer proteins. The transducer proteins are central to the DNA damage response, due to their ability to transmit the DNA damage signal from the sensor proteins to the effector proteins. Once the effectors have been activated, they execute the appropriate DNA damage response.

One of the most extensively studied DNA damage response pathways in mammalian cells is the p53 pathway (Fig. 3). As its name implies, the effector protein of this pathway is the p53 protein. Based on its anti-proliferative and genome-guarding properties, the p53 protein has been categorized into a group of proteins called tumour suppressors. The p53 gene is mutated in most human cancers [4], and mice lacking a functional p53 gene develop tumours by the age of 6 months [5]. What is more, mice with unchallenged p53 activity die as embryos [6, 7]. Thus, it is important for a cell/organism to regulate p53 activity. This is mainly achieved by regulated degradation. During normal growth, the p53 protein binds to the Murine-double-minute 2 (Mdm2) protein. Mdm2 is an ubiquitin ligase which targets
p53 for ubiquitin-mediated proteolysis [4]. After DNA damage, the transducer proteins of the p53 pathway—ataxia telangiectasia mutated (ATM), ATM-Rad3-related (ATR), and checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2) (Fig.3)—phosphorylate the p53 protein [8]. Following phosphorylation, p53 loses its affinity for the Mdm2 protein and escapes degradation. Consequently, the intracellular level of p53 increases and p53 can activate transcription of various target genes. Depending on the original magnitude of the DNA damage, the p53 protein executes cell cycle arrest, DNA repair, senescence or apoptosis (Fig. 3) [4].

![p53 DNA damage response pathway](image_url)

**Fig. 3.** The p53 DNA damage response pathway (arrowheads symbolize activating events).

Proto-oncogenes are antagonists of tumour suppressor genes. Proto-oncogenes encode proteins that stimulate proliferation, thereby counteracting the function of tumour suppressor proteins. Gain-of-function mutations of proto-oncogenes are associated with tumour development [9]. Aberrant expression of oncogenes leads to activation of p53.
There are no distinct homologues of the p53 protein in yeast. The Mec1 and Tel1 proteins are budding yeast homologues of the ATR and ATM kinases. In fission yeast, the ATR and ATM homologues are called Rad3 and Tel1. The Chk1 and Chk2 homologues are the Chk1 and Rad53 kinases in budding yeast, and the Chk1 and Cds1 kinases in fission yeast [3].

1.3 DNA repair

Cells encounter a perplexing diversity of DNA adducts. These arise from three main causes. Firstly, environmental agents such as UV radiation and genotoxic chemicals cause alterations in DNA structure. Secondly, endogenous by-products of normal cell metabolism frequently modify DNA molecules. The most physiologically relevant among these by-products are reactive oxygen species. More than 100 different oxidative modifications have been identified in cellular DNA [10]. Thirdly, nucleotides can be spontaneously altered by e.g. hydrolysis of bases [11].

Several cellular DNA repair processes have evolved to deal with the plethora of DNA lesions. The most important DNA repair mechanisms operating in mammalian cells are base excision repair (BER), nucleotide excision repair (NER) and double-strand break repair [12, 13]. In what follows, I will provide you with a brief description of these basic DNA repair mechanisms. For an all-inclusive overview, I refer the reader to Friedberg et al. [11]

Through its removal of damaged or mismatched nucleotides, BER is the main protector against DNA damage of endogenous origin. The BER process is initiated by DNA glycosylases that remove the altered or mismatched base. The resulting abasic (AP) site is recognized by AP endonuclease (APE1) which hydrolyzes the phosphodiester bond at the damaged site. Henceforth, BER has been subdivided into short-patch repair and long-patch repair, the former being the dominant pathway in mammalian cells. In short-patch repair (Fig. 4A), after the incision by the APE1 enzyme,
DNA Polβ catalyzes a one-nucleotide gap-filling reaction and removes the 5’-terminal sugar residue. The remaining nick is sealed by DNA ligase. Long-patch repair requires DNA Polδ/ε and proliferating cell nuclear antigen (PCNA) for repair synthesis (2–10 nucleotides), and also FEN1 endonuclease and DNA ligase for completion of the BER process.

Fig. 4. Four major pathways of DNA repair: A, short-patch base excision repair; B, nucleotide excision repair; C, non-homologous end-joining; D, homologous recombination.
In contrast to BER, NER does not recognize chemically modified bases but detects abnormal backbone conformations (Fig. 4B). NER is divided into global genome NER and transcription-coupled repair. In mammalian cells, at least 25 proteins participate in NER. Briefly, after damage recognition, dual incisions by the XPG (5’-incision) endonuclease and the XPF (3’-incision) endonuclease bracket the lesion to form 24–32 nucleotides long single-stranded gap. Subsequently, the gap is filled by DNA Polδ/ε with the aid of replication accessory proteins PCNA and replication factor C (RFC). The remaining single-stranded nick is sealed by DNA ligase.

DNA double-strand breaks are unwanted products caused by reactive oxygen species, ionizing radiation and replication fork collapse. The two major pathways for DNA double-strand break repair are non-homologous end-joining (NHEJ) and homologous recombination (HR). The former is a simple ligation of two chromosome ends (Fig. 4C). The initial step of NHEJ is the binding of the end-binding Ku70/Ku80 complex, leading to the recruitment of DNA-PKcs and XRCC4-ligase4. NHEJ is error-prone, and is mostly used during the G1-phase. HR is a somewhat more complex pathway than NHEJ. It uses the homologous DNA duplex as a source of information, and Holliday junctions are key intermediates during the repair process (Fig. 4D). The two breast cancer-associated proteins, BRCA1 and BRCA2, play an important but still undefined role in HR.

1.4 Ribonucleotide reductase

Cell metabolism constantly generates harmful free radicals as part of the “oxygen paradox” [14]. During evolution, cells have adapted to take advantage of the violent nature of free radicals. By controlled free-radical-chemistry, ribonucleotide reductase (RNR) catalyzes the substitution of the 2´ hydroxyl group of a ribonucleoside diphosphate (NDP) with hydrogen, resulting in a deoxyribonucleoside diphosphate (dNDP) [15]. This is the rate-limiting step in de novo biosynthesis of the four precursors of DNA synthesis (Fig. 1) [1].
Based on the mechanisms by which RNRs employ the free radical, the enzymes have been subdivided into three different classes [15]. This thesis is restricted to the RNR found in almost all eukaryotic cells, the class Ia enzyme.

The classic class Ia enzyme is an α₂β₂ heterotetramer (Fig. 5). In mammalian cells, the α-protein and the β-protein are referred to as the R1 protein and the R2 protein, respectively [15]. The R1 protein (90 kDa) is the “business end” of the enzyme, as it carries the active site and also binding sites for allosteric effectors (Fig. 5) [15]. The smaller R2 protein (45 kDa) contains a Fe-O-Fe center, which generates a tyrosyl free radical (Fig. 5) [16]. During catalysis, the radical is continuously shuttled to a cysteine residue of the active site, where it is obligatory for catalysis [17]. Chemical agents (e.g. hydroxyurea) that destroy the radical are used as antiproliferative drugs [18, 19].

Fission yeast (*Schizosaccharomyces pombe*) has one R1 homologue (Cdc22) and one R2 homologue (Suc22) [20]. Budding yeast (*Saccharomyces cerevisiae*) has two R1 homologues (RNR1 and RNR3) [21] and two R2 homologues (RNR2 and RNR4) [22-25].
1.5 Cell cycle-specific regulation of ribonucleotide reductase

The human genome consists of approximately three billion base pairs of DNA. In order to successfully replicate its DNA, a human cell undergoes a huge demand for dNTPs as it enters S-phase. Consequently, RNR activity is high in S-phase [1]. Both the R1 and R2 genes are transcribed and expressed in an S-phase-specific manner [26-28]. While the R1 protein is constant in proliferating cells (Fig. 6) [29, 30], the R2 protein fluctuates during the cell cycle. The levels of R2 are high in S-phase, low during G1-phase, and considered to be negligible in non-dividing cells (Fig. 6) [15, 31, 32]. The variation in R2 levels is due to cell cycle-specific degradation of the R2 protein in late mitosis. This highly regulated degradation is mediated by the Cdh1-anaphase promoting complex (Cdh1-APC) [32]. If a cell stops proliferating, the level of R1 protein declines, with a half life of approximately 20 hours (Fig. 6) [29, 30].

![Fig. 6. Relative levels of the R1 protein (broken line), R2 protein (dotted line) and RNR activity (solid line) plotted as a function of cell-cycle progression.](image)
Fission yeast also has cell cycle-specific transcription and expression of RNR genes. The Cdc22 transcript reaches maximum levels during the transition from G1- to S-phase whereas the lowest levels are observed in mid-G2-phase [33]. The small RNR protein of fission yeast, Succ22, is encoded by two alternative transcripts [20]. The smaller of the two transcripts is continuously expressed at high levels regardless of the phase of the cell cycle [34]. This expression contrasts with the low S-phase-specific expression of the larger transcript [34]. Both the Cdc22 transcript and the large Suc22 transcript are induced following DNA damage [33, 34].

In addition to cell cycle-specific regulation of the RNR1 gene [21], budding yeast induces expression of all four RNR genes in response to DNA damage [21-25]. Budding yeast also regulates RNR activity using a small (11.8 kDa) RNR inhibitor protein, the Sml1 protein (suppressor of Mec1 lethality) [35, 36]. Outside S-phase—or in the absence of DNA damage—Sml1 binds to the RNR1 protein, thereby inhibiting RNR activity [35, 36]. When the demand for dNTPs increases the Sml1 protein is degraded, which releases RNR from inhibition. The degradation of Sml1 depends on the Mec1/Rad53 pathway [37]. A possible Sml1 homologue has been found in fission yeast [38, 39] whereas there have been no reports of a mammalian homologue of the Sml1 protein.

Recently it has been suggested that budding and fission yeasts partly regulate RNR activity through subcellular localization of RNR subunits [39-42]. Outside S-phase or in absence of DNA damage the Suc22 and RNR2/RNR4 proteins are localized in the nucleus, whereas the large subunits are localized in the cytoplasm. As the need for dNTPs arises the small proteins are relocalized to the cytoplasm, where active RNR complexes presumably are formed. In contrast to yeast and regardless phase of the cell cycle, both the mammalian RNR proteins—the R1 protein and the R2 protein—are localized in the cytoplasm [43, 44].
1.6 Allosteric regulation of ribonucleotide reductase

An appropriate balance of the four DNA building blocks is important for accurate DNA replication and repair [1]. This is achieved by delicate allosteric regulation of RNR activity (Fig. 7). Overall enzymatic activity is controlled by an activity site. Binding of ATP stimulates enzyme activity, whereas binding of dATP inhibits enzyme activity [15, 45]. A specificity site dictates the choice of substrate. Binding of dATP and ATP promotes reduction of pyrimidine ribonucleoside diphosphates (UDP, CDP). Binding of dTTP to the specificity site promotes reduction of GDP, whereas binding of dGTP stimulates reduction of ADP [45].

Fig. 7. How RNR (grey box) is allosterically regulated. Forward reactions are shown with solid arrows, stimulatory effects are shown with dotted arrows, and inhibitory effects are shown with broken lines.
dTTP is not directly synthesized by RNR (Fig. 8). In mammalian cells, the de novo biosynthesis of dTTP largely depends on dCMP deaminase. This allosterically regulated enzyme deaminates dCMP to dUMP. Subsequently, thymidylate synthase converts dUMP to dTMP, which is phosphorylated to dTTP. dCMP deaminase is activated by dCTP and inhibited by dTTP [1].

![Fig. 8](image)

**Fig. 8.** Synthesis of dTTP involves RNR, dCMP deaminase (1), UTPase (2), and thymidylate synthase (3). Allosterically regulated enzymes are represented by grey boxes, forward reactions by solid arrows, stimulatory effects by dotted arrows, and inhibitory effects by broken lines.

### 1.7 Substrate cycles and salvage of deoxyribonucleosides

As a complementary route to de novo dNTP biosynthesis, mammalian cells have substrate cycles for maintenance and fine-tuning of the dNTP pools [1, 46]. If the dNTPs exceed optimal concentrations for DNA synthesis, they are dephosphorylated by 5’-nucleotidases and excreted from the cell as deoxyribonucleosides. If, on the other hand, there is a shortage of dNTPs, deoxyribonucleosides are salvaged by the cell and phosphorylated to dNTPs. In addition to maintaining and fine-tuning the dNTP pools, salvage of deoxyribonucleosides has proved to be vital for mtDNA replication [1, 46], and mutations in the salvage pathways can cause mitochondria-derived diseases [47-49]
The key enzymes of the salvage pathway in mammalian cells are four deoxyribonucleoside kinases: thymidine kinase 1 (TK1), thymidine kinase 2 (TK2), deoxyguanosine kinase (dGK), and deoxycytidine kinase (dCK). These enzymes cover the first phosphorylation step of all four deoxyribonucleosides needed for DNA synthesis [50]. TK2 and dGK are mitochondrial, whereas TK1 and dCK are localized in the cytoplasm. Deoxyribonucleoside kinases are of medical interest, since they activate nucleoside analogues used as anti-viral or anti-proliferative drugs [50]. Mice lacking the TK1 gene die from kidney failure within a year [51].
Results

In this section, the three papers (I–III) that constitute this thesis are summarized.

2.1 Paper I

Mammalian p53R2 protein forms an active ribonucleotide reductase in vitro with the R1 protein, which is expressed both in resting cells in response to DNA damage and in proliferating cells.

Mammalian cells need dNTPs for DNA repair. A prerequisite for an active mammalian RNR complex is that an R1 subunit interacts with a radical supplying the small RNR subunit [15]. Since the R2 protein is degraded in mitosis [31, 32], an intriguing question is how G1-phase and terminal differentiated cells generate dNTPs for DNA repair.

One possible answer to this question was put forward by two independent laboratories in the year 2000. In a search for p53 target genes, Tanaka et al. [52] and Nakano et al. [53] cloned a novel 5.5-kilobase transcript that, following DNA damage, was induced in p53 wild-type cells but not in p53 null mutant cells. This novel p53-inducible gene encoded a protein with 81% amino acid sequence homology to the mouse R2 protein. The protein was given the name p53R2. Both research groups concluded that p53R2 could be a functional homologue to the R2 protein.

Our original intention was to clarify whether this alternative mammalian small RNR protein could substitute for R2 in an active RNR complex. To begin with, we expressed and purified recombinant mouse and human p53R2 proteins. According to the general dogma, a class Ia small
RNR protein requires the ability to generate a stable tyrosyl free radical in order to support RNR activity. In collaboration with the group of Astrid Gräslund, we next used electron paramagnetic resonance (EPR) spectroscopy to show that our recombinant p53R2 proteins contained tyrosyl radicals. As with the R2 proteins, the p53R2 proteins rapidly lost the free radical on incubation with hydroxyurea.

The interaction between the R1 and R2 subunits partly depends on a conserved C-terminal heptapeptide (-FTLDADF) of the R2 protein [54]. From the sequence analysis, it was clear that the C-terminal heptapeptide—and also the amino acids required for efficient radical transfer—are conserved between the R2 and p53R2 proteins. This sequence homology, combined with the fact that p53R2 contained a tyrosyl radical, strongly indicated that the p53R2 protein should form an active RNR complex with the R1 protein. By using a well-established CDP reduction assay [55], we did indeed detect RNR activity in a reaction mixture containing R1 and p53R2 proteins. The specific activity of the recombinant mouse and human p53R2 protein was approximately half of the specific activity of the mouse R2 protein. These results conclusively showed that the p53R2 protein was a functional homologue of the R2 protein in vitro.

Tanaka et al. [52] has shown convincingly that the p53R2 gene is transcriptionally activated in cells that have encountered DNA damage. Since the p53R2 protein needs to be in a complex with a large RNR subunit to support de novo dNTP biosynthesis, we asked whether the R1 gene is also transcriptionally activated following DNA damage. To answer this question, we stably transformed mouse fibroblasts with an R1 promoter-luciferase reporter construct containing 5.7 kilobase pairs of the mouse R1 promoter. We used the activity of the reporter protein as an indirect measure of the transcriptional activity of the R1 promoter. By exposing G0/G1-phase cells transformed with this construct to UV irradiation, we measured a threefold increase in reporter protein activity compared to non-irradiated cells. Control cells stably transformed with an R2 promoter-luciferase reporter construct,
earlier shown to have elevated promoter activity upon entry into the S-phase [31], showed no elevated promoter activity following UV irradiation.

In order to determine whether the R1 protein is expressed after DNA damage, we took advantage of an R1 protein-specific monoclonal antibody. By using this antibody in immunoblot analyses, we could demonstrate that UV-irradiated cells had a twofold increase in their level of R1 protein, as compared to non-irradiated cells. Induced expression of the R1 protein was correlated in time with increased reporter protein activity. Taken together, our results show that mammalian cells have DNA damage specific transcription and expression of the R1 gene.

2.2 Paper II

Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells.

Having shown that the p53R2 protein is enzymatically equivalent to the R2 protein in vitro (I), we set out to investigate the regulation and function of the p53R2 protein in vivo.

The R2 and p53R2 proteins are 81% homologous at the amino acid level, but the p53R2 protein has a 33 amino acid truncation in the N-terminal domain compared with the R2 protein [I]. Within this region, a KEN box targets R2 for degradation in late mitosis [32]. If mutated, the R2 protein escapes degradation and becomes detectable in G1-phase [32]. By analogy with an R2 protein lacking a functional KEN-box, we assumed that the p53R2 protein would be detectable in G1-phase. By running immunodetection analyses using a p53R2-specific antibody, we proved this assumption to be correct. The level of p53R2 protein was constant throughout the cell cycle of undamaged cells. Furthermore, both G1- and S-phase cells induced expression of the p53R2 protein after DNA damage.
To our surprise, induced expression of p53R2 did not significantly elevate the dNTP pools of logarithmically growing fibroblasts. What is more, the dNTP pools of a G₁-phase-enriched cell culture, with only 3% of cells in S-phase, were largely unaffected by DNA damage and p53R2 induction. Even so, the same damaged G₁-phase-enriched cell culture had 20-fold smaller dNTP pools compared to the dNTP pools of an S-phase-enriched cell culture. We were confounded by these results. From earlier studies, we knew that the p53R2 and R1 protein could form an active RNR complex [1]. We and others [56, 57] had thus assumed as a matter of course that induced expression of the p53R2 and R1 protein should give elevated dNTP pools.

In order to understand why induced expression of the p53R2 protein only had marginal (if any) effects on the overall pool size, we set out to investigate the absolute amounts of RNR proteins within the cell. Our prediction was that the actual level of p53R2 protein, both before and after DNA damage, would be lower than the level of R2 protein in S-phase. Such scenario could possibly explain why resting cells, only expressing the p53R2 protein, have 20-fold smaller dNTP pools than S-phase cells. In accordance with our hypothesis, S-phase cells had 30-fold more R2 protein than the p53R2 levels in undamaged cells. Following DNA damage, the absolute amount of p53R2 protein increased approximately threefold. Thus, it is difficult to interpret the effect of p53R2 expression on the average dNTP pool of a cell culture with a few per cent S-phase cells if the average level of R2 protein in the same culture is influenced by the DNA damage.

A breakthrough in our understanding of the p53R2 protein came when we examined how G₁-phase cells exposed to genotoxic agents were affected if the p53R2 protein was inhibited by hydroxyurea. We found that cells exposed to a combination of adriamycin (a chemical drug causing DNA damage [11]) and hydroxyurea have slower S-phase-progression than G₁-phase cells only exposed to adriamycin. Thus, “knocking-down” the p53R2 protein negatively affected the ability of G₁-phase cells to recover from DNA damage. We postulated that this hydroxyurea-dependent phenotype was a direct result of decreased dNTP pools caused by p53R2 inhibition. To
explore this hypothesis, we determined the dNTP pools of damaged G$_1$-phase cells before and after the addition of hydroxyurea. In accordance with our prediction, cells treated with hydroxyurea had a twofold reduction in the purine deoxyribonucleoside triphosphate pools (dATP and dGTP) as compared to untreated cells. The dTTP and dCTP levels were largely unaffected by hydroxyurea, presumably due to more effective salvage of pyrimidine deoxyribonucleosides than of purine deoxyribonucleosides [1].

Encouraged by having tracked down a cellular phenotype resulting from p53R2 inhibition in damaged G$_1$-phase cells, we next asked whether the same phenotype could be seen in undamaged G$_1$-phase cells. That is, do undamaged resting cells exposed to hydroxyurea suffer from dNTP deprivation? This seemed likely, since the p53R2 protein is also expressed in the absence of exogenous DNA stress. By serum starvation of cells followed by cell elutriation, we managed to generate G$_1$-phase cell cultures with less than 0.1% of S-phase cells. These cells had lower dNTP pools than G$_1$-phase cells with induced p53R2 expression, meaning that induction of the p53R2 protein slightly elevates the dNTP pools of resting cells. What is more, hydroxyurea caused reduction of the dATP and dGTP pools in undamaged resting cells. This strongly indicated that there is RNR-dependent ribonucleotide reduction in resting cells under normal conditions.

2.3 Paper III

*The S. pombe replication inhibitor Spd1 regulates ribonucleotide reductase activity and dNTPs by binding to the large Cdc22 subunit*

Our curiosity was drawn to fission yeast for two reasons. Firstly, we wanted to investigate how the dNTP pools of fission yeast are affected by DNA damage. Secondly, we wanted to study the RNR of fission yeast and examine the biological function of a suggested fission yeast RNR inhibitor, the Spd1 (S-phase delayed) protein (14.2 kDa) [38, 39].
To characterize the enzymatic properties of the RNR enzyme in vitro, we reconstituted fully active recombinant fission yeast RNR. The enzyme activity of this complex was abolished by hydroxyurea and the Cdc22 protein was 50% feedback-inhibited by 40 µM dATP. These properties of the fission yeast RNR were in accordance with what could be expected of a class-Ia enzyme. More interestingly, addition of recombinant Spd1 protein to a reaction mixture containing active fission yeast RNR caused inhibition of activity, showing that Spd1 is a potent inhibitor of RNR activity.

Results pointing to the same conclusion, albeit more indirect, have been put forward by the group of Thomas Caspari [39]. They had also proposed a model whereby Spd1-mediated RNR inhibition would act through the Suc22 protein. Having confirmed that the Spd1 protein does indeed inhibit RNR activity, we set about investigating whether Spd1 inhibition acts through the Suc22 or Cdc22 protein. By using a CDP reduction assay, we could demonstrate that Spd1 reduced RNR activity only if the Cdc22 protein was limiting for RNR activity. Furthermore, neither budding yeast RNR nor a heterogeneous RNR complex composed of the Suc22 protein and the budding yeast RNR1 protein was inhibited by the Spd1 protein. These results are contrary to the Caspari model, since our observations indicate that Spd1 inhibits RNR activity by binding to the large—but not the small—RNR subunit.

In accordance with our results from the CDP reduction assays, we did not detect any interaction between the Spd1 protein and the Suc22 protein when using the biosensor technique. Under the same conditions, the Spd1 protein did bind to the Cdc22 protein at a 1:1 molar ratio, with a dissociation constant of 2.4 µM. The interaction between Spd1 and Cdc22 was not significantly affected by the addition of Succ22 or dTTP to the reaction mixture (it is known that dTTP causes dimerization of the large proteins of RNR from mouse and budding yeast [36]).

We added 4-NQO (a drug that mimics UV-induced DNA damage [11]) to a culture of logarithmically growing fission yeast cells in order to
investigate the effect of DNA damage on intracellular dNTP pools. Within two hours, the drug caused fission yeast cells to elevate their dNTP pools to the same levels as in S-phase cells. This clearly demonstrates that fission yeast cells increase their dNTP levels following DNA stress.
Putting the results into context

The major quest of this thesis has been to explain how mammalian and fission yeast regulate their RNR activity and dNTP levels during normal growth and after DNA damage. To realize our goal, our point of attack was to unravel the biological functions of the p53R2 protein and the Spd1 protein. Frightening though it may be now is the time to ask oneself, what have we learned? In this section, I try to answer this question by putting our results into context. In parts, where our results are ambiguous or less easy to interpret, I will take the opportunity to speculate boldly.

3.1 The p53R2 protein

What is the function of the p53R2 protein? Let us start by pointing out one important observation. Mice lacking a functional p53R2 gene die at a young age from renal failure [58, 59]. Let us further assume that, from an evolutionary point of view, nature would not have kept a radical-containing protein without somehow benefiting from its radical. If this is true and given that the above observation is correct, one possible conclusion might be that the p53R2 protein is important for longevity among mammals—through its ability to support ribonucleotide reduction. The p53R2 protein is not essential for cell proliferation [58]. What then makes p53R2 important for longevity?

Thousands of spontaneous DNA-damaging events occur in each human cell per day. DNA repair is carried out at constant rate regardless of the phase of the cell cycle, and after DNA damage the bulk of repair is accomplished inside a 3-hour time window [60, 61]. During the S- or G2-phase, an R1/R2 RNR complex ensures an adequate supply of dNTPs for DNA repair synthesis [31]. If damage occurs outside these phases, cells must rely on alternative sources for the supply of dNTPs.
If we postulate that an R1/p53R2 RNR complex provides cells outside S- or G2-phase with dNTPs for DNA repair, in this scenario knocking out the p53R2 gene would cause deprivation of the dNTP pools—with impaired DNA repair and shortened lifespan as possible consequences. Our observations strongly support the idea that the p53R2 protein is indeed central for cell cycle-independent DNA repair. Firstly, the p53R2 protein is expressed equally in cells growing under normal conditions irrespective of the phase of the cell cycle [II]. Secondly, the R1 protein forms an active RNR complex with the p53R2 protein \textit{in vitro} (I). Thirdly, knocking-down the p53R2 protein by hydroxyurea negatively affected the ability of G1-phase cells to recover from DNA damage [II]. Finally, exposing G1-phase cells to hydroxyurea causes a reduction in their dATP and dGTP pools, which suggests that G1-phase cells have NDP reduction [II]. Although indirect when taken singly, together these results convincingly demonstrate that one function of the p53R2 protein is to supply cells with dNTPs for “everyday” DNA repair.

Both proliferating and non-dividing cells must replenish their mitochondria, and produce additional mitochondria if the need arises. The mitochondrial DNA (mtDNA) corresponds to only a few per cent of the total cellular genome [62]. Never the less, the demand for dNTPs during replication of mtDNA is not trivial. This demand is (partly) fulfilled by salvage of deoxyribonucleosides through the deoxyribonucleoside kinases. A central role of the R1/p53R2 complex, in addition to supporting nuclear DNA repair with dNTPs, may be to provide cells with building blocks for mtDNA synthesis. This is an important question that will hopefully be addressed experimentally in the near future.

3.2 The Spd1 protein

What is the function of the Spd1 protein? Spd1 was first identified as a cell cycle-regulated protein [38]. In a later publication, it was shown that
over-expression of—or the inability to degrade—Spd1 gave the same phenotypes as treating fission yeast with hydroxyurea [63, 64]. Similar phenotypes, including reduced dNTP pools, defective propagation of mitochondrial DNA, incomplete DNA replication, and cell death had been seen in budding yeast lacking the ability to degrade the RNR inhibitor protein Sml1 [37].

In this thesis, I have shown that the Spd1 protein is a functional homologue of the budding yeast Sml1 protein [III]. Not only do both proteins inhibit RNR activity, both do this by interacting with the large subunit of the RNR enzyme [III, 35, 36]. In part, our results complement the observations of the group of Thomas Caspari [39]. By genetic analysis, these workers could demonstrate indirectly that the Spd1 protein inhibits RNR activity. However, in the same study they put forward a model based on observations from immunofluorescence microscopy experiments. According to this model, Spd1 inhibits RNR activity by anchoring Suc22 inside the nucleus, thereby preventing it from forming an active RNR complex with the cytoplasmic Cdc22 protein. Using the same indirect method, this model was later confirmed by the group of Antony Carr [65].

From the data presented in this thesis, combined with data from the group of Thomas Caspari, we can safely conclude that the biological function of Spd1 is to inhibit RNR activity. Furthermore, as our data convincingly show, Spd1 inhibition acts through the large subunit and not through the small RNR subunit, as earlier proposed [39].

3.3 The dNTP pools

How do mammalian and fission yeast cells regulate RNR activity and the size of dNTP pools during normal growth and after DNA damage? To answer this complicated question, let us start by reviewing how budding yeast cells regulate RNR activity.
The Sml1 protein inhibits RNR activity when the demand for DNA precursors is low. As budding yeast cells progress into S-phase, the Sml1 protein is degraded to a defined extent. The partial degradation of the Sml1 protein releases RNR from inhibition, which results in a three- to fivefold elevation of dNTP pools as compared to the pool size of G1-phase cells [66]. This increase in dNTP pools is limited by a co-operation mechanism between the remaining Sml1 protein and the dATP feedback of the RNR1 protein. If a budding yeast cell undergoes massive DNA damage, the DNA damage response pathway (the Mec1/Rad53 pathway) induces expression of RNR genes, and targets the Sml1 protein for total degradation. Consequently, the dNTP levels become limited only by allosteric feedback inhibition of the RNR1 protein. Due to its relaxed feedback inhibition, the size of the dNTP pools is higher after DNA damage than the dNTP pools of S-phase cells [66].

Mammalian cells, as with budding yeast, induce expression of RNR proteins following extensive DNA damage [II, 52, 53]. Induced expression of the p53R2 protein is mediated by the p53-dependent DNA damage response pathway [52, 53], which is homologous to the Mec1/Rad53 pathway of budding yeast [3]. However, and in striking contrast to budding yeast, induced expression of the p53R2 and R1 proteins after DNA damage does not cause a general increase in the dNTP pools of mammalian cells [II].

Mechanisms ensuring appropriate dNTP concentrations for optimal DNA replication during the S-phase in mammalian cells include: (i) tight dATP feedback inhibition of the R1 protein [67], and (ii) 5´-nucleotidase activity [1]. Thus, cells with higher RNR activity than normal S-phase cells do not have higher dNTP pools than S-phase cells [68]. In contrast, cells carrying a mutated R1 protein defective in dATP inhibition have elevated dNTP pools compared with wild-type cells [69]. Tight dATP feedback inhibition of the R1 protein and 5´-nucleotidase activity directly explain why the dNTP pools after DNA damage not is higher than the dNTP pools of S-phase cells. However, these features cannot explain why damaged resting cells with induced expression of the p53R2 and R1 protein have approximately 20 fold lower dNTP levels than S-phase cells.
Putting the results into context

Two possible strategies for restricting RNR activity, and thereby dNTP pools, after DNA damage could be: (1) much lower specific activity of the p53R2 protein compared to the R2 protein, or (2) limited expression of RNR proteins after DNA damage. Our results strongly favour the latter hypothesis. Firstly, recombinant p53R2 and R2 have similar enzymatic properties in vitro [I]. Secondly, the absolute amounts of p53R2 and R1 proteins in damaged resting cells is only one-tenth of the amounts of R2 and R1 proteins in S-phase cells [II]. Hence, mammalian cells regulate dNTP synthesis by strictly regulating expression of RNR proteins after DNA damage.

Let us turn to fission yeast cells, and how they regulate the size of their dNTP pools. As fission yeast cells enter S-phase or encounter severe DNA damage, the Spd1 protein is degraded [38, 39]. By analogy with the Sml1 protein, if we assume that severe DNA damage leads to a total degradation of the Spd1 protein, fission yeast (as with budding yeast) would have maximal dNTP pools after damage. Our results show that fission yeast has as high dNTP pools in S-phase as after DNA damage [III]. This strongly suggests that both entry into S-phase and DNA damage lead to a total (or to the same extent) degradation of the Spd1 protein. In line with this hypothesis, fission yeast cells have tighter dATP feedback inhibition of RNR activity than budding yeast [III]. Consequently, the amplitude of dNTP fluctuations is more restricted in fission yeast, which presumably explains why the dNTP pools are of the same level in the S-phase as after DNA damage.

Based on our results, I propose a model for how the Spd1 protein regulates de novo dNTP biosynthesis in fission yeast cells. In contrast to budding yeast cells—which partly use the Sml1 protein for tuning dNTP pools—the Spd1 mechanism is like an on/off system. Once the system is on (i.e. Spd1 is not bound to Cdc22), the actual size of the dNTP pool is entirely controlled by the allosteric feedback inhibition of RNR in both the S-phase and after DNA damage.
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References


