Chapter V

Determination of the Cellular role of the Rfc1 BRCT Region
Abstract

Replication Factor C is a five subunit protein that is essential for survival in all eukaryotic cells. The primary function of RFC is to load the PCNA sliding clamp onto DNA using the hydrolysis of ATP. RFC is also involved in other cellular processes such as cell cycle progression, survival after UV exposure and possibly apoptosis [Johnson et al. 2005; Kelman et al. 1998]. Despite containing highly conserved sequences, the N-terminal region (NTR) of the largest subunit, Rfc1, is not essential for complex formation. Furthermore, deletion of the NTR neither affects the in vitro PCNA loading activity of the resulting RFC complex, nor yields a marked DNA metabolic defect in yeast. Here, yeast genetic analysis was used to gain insight into the biological role of rfc1. An S. cerevisiae strain was created by deleting the NTR (residues 3-273) from the chromosomal copy of the yeast rfc1 gene (equivalent to the first 555 amino acids in humans). Using this strain, designated rfc1-ΔNTR, conditions in which the deletion resulted in a detectable phenotype were found. The rfc1-ΔNtr strain proved moderately sensitive to damage caused by the drug cisplatin, but was resistant to UV light, mitomycin C and X-ray radiation. To begin to investigate the molecular mechanism of the cisplatin sensitivity generated more than thirty double mutants were generated using the EUROSCARF haploid deletion strain collection. The mutations allowed the analysis the effect of the NTR deletion in various DNA repair and replication pathways as well as cell-cycle regulation. Taken together, these results point towards a role of the NTR of the large subunit of RFC in translesion synthesis (TLS). This role is likely regulatory in nature.
Introduction.

In eukaryotic cells, two multi-functional protein complexes, Proliferating Cell Nuclear Antigen (PCNA) and Replication Factor C (RFC), are key players in an intricate network of repair systems that are fully integrated with DNA replication and cell cycle progression. PCNA, also called the sliding clamp, is a ring-shaped homotrimeric protein that encircles the DNA and acts as a mobile docking platform for DNA polymerases to ensure highly processive DNA synthesis (reviewed in [Johnson et al. 2005; Moldovan et al. 2007]). PCNA additionally interacts with numerous DNA repair and regulatory proteins. RFC, alternatively known as the clamp loader, binds the primer-template junction and loads PCNA onto DNA in an ATP-dependent reaction. However, RFC itself is also known to be involved in cell cycle checkpoints and, together with PCNA, is thought to form part of a “sensor” that reports the state of DNA [Kelman et al. 1998], thereby providing two-way communication between the enzymatic and regulatory machinery required for genomic maintenance and to safeguard against the constant genotoxic threat created by metabolic by-products and DNA damaging agents.

The clamp loader RFC is a pentameric protein complex. The amino acid sequence and functionality of each of the five subunits are highly conserved throughout the kingdom of eukaryotes. Each subunit is encoded by a unique gene essential for cell survival. The protein products of the four smallest subunits (encoded by S. cerevisiae genes rfc2-5) have a molecular mass between 40 and 36 kDa and are homologous to the central region of the fifth one (generally referred to below as Rfc1) which ranges from 95 kDa in yeast to 140 kDa in mammals (Figure 5.1). This homology region is essential for complex formation and is comprised of a cluster of seven well-defined, conserved boxes, referred to as RFC boxes II-VIII that encompass sequences homologous to nucleotide-binding proteins and Walker type ATPases. In addition, the Rfc1 subunit contains, at both its N- and C-termini, extensions whose sequences
are unique within the complex. To date, no enzymatic activity has been assigned to the portion of Rfc1 N-terminal to the central homology region (aa’s 1-273 in *S. cerevisiae*, aa’s 1-580 in humans, here referred to as the NTR). Furthermore, deletion of the NTR from the human subunit does not negatively affect the PCNA loading reaction *in vitro* [Uhlmann *et al.* 1997]. In fact, the authors reported that the NTR deleted protein was 2-5 times more active than the corresponding full-length equivalent in catalyzing both PCNA loading and the DNA elongation reaction *in vitro*. Similarly, deletion of the NTR in yeast did not lead to any readily detected phenotype [Gomes *et al.* 2000]. Furthermore it also proved necessary to delete the NTR of Rfc1 in order to obtain crystals of the entire RFC complex [Bowman *et al.* 2004], thus knowledge of the structure and function of this portion of RFC remains extremely limited.

The size of the NTR varies from species to species, being approximately 30 kDa in yeast and a little more than 50 kDa in humans. This difference is likely to reflect additional functionality, perhaps related to processes such as cell differentiation, that do not occur in yeast. However, all eukaryotic Rfc1 NTRs contain a highly conserved BRCT domain [Bork *et al.* 1997]. Interestingly, although some BRCT domains have been reported to have DNA binding functionality, the BRCT domain from Rfc1 is the only family member with a clearly defined DNA binding activity described so far. Indeed, previous studies [Allen *et al.* 1998; Kolpashchikov *et al.* 2001; Kobayashi *et al.* 2006] have shown that the BRCT region of Rfc1 has an unusual specificity for the 5’ phosphorylated terminus of double-stranded DNA (*K*<sub>D</sub> approximately 100 nM), an intermediate in DNA replication and repair processes. It has also been reported that the BRCT region of Rfc1 can function as a coregulator of transcription in conjunction with some “bZIP” domain transcription factors [Anderson *et al.* 2003; Hong *et al.* 2001]. More recently, [Levin *et al.* 2004] described a physical and functional interaction between DNA ligase I and the non-catalytic
NTR. Taken together, these results suggest that the large subunit of RFC plays a role in regulating DNA replication and/or repair. Also consistent with this regulatory function is the observation that an *S. cerevisiae* yeast strain carrying a chromosomal *rfc1* deletion and expressing only an NTR truncated form of Rfc1 provided in *trans* did not show any marked DNA metabolic defect [Gomes *et al.* 2000]. However, the nature of the role of the Rfc1 NTR is, at this point, uncertain.

Here, yeast genetic analysis was applied in an attempt to better understand the biological role of the NTR. An *S. cerevisiae* haploid yeast strain was constructed in which the entire NTR was deleted from the chromosomal copy of the *rfc1* gene (*rfc1-ΔNTR* strain). This deletion results in the truncation of the Rfc1 region encompassing the residues 3-273 (equivalent to the amino acids 1-555 in humans). Initial phenotypic characterization confirmed the absence of any apparent marked DNA metabolic defect as reported by Gomes and colleagues [Gomes *et al.* 2000]. However, the *rfc1-ΔNTR* strain did prove moderately sensitive to damage caused by the drug cisplatin. To investigate the nature of this sensitivity, a series of double mutants were constructed using the EUROSCARF deletion strain collection. Analysis of cell survival in single and double mutants suggested that the ΔNTR mutation was epistatic with a number of genes involved in trans-lesion synthesis.
Methods

Strains and media

The *S. cerevisiae* precursor and key strains used in this study are listed in Table 5.1. All other single-gene deletion strains used were taken from the EUROSCARF MATα haploid deletion strain collection (BY4741 genetic background) where the open reading frames of non-essential genes have been replaced by the kanamycin resistance cassette *kanMX4*. The one exception, a MATΔrad18 deletion strain originally present in the collection, was switched to a MATα phenotype resulting in the replacement of the *kanMX4* cassette by an *URA3* marker. All double deletion mutants studied here were generated by crossing the relevant single mutants followed either by tetrad dissection and appropriate spore selection (for rad5, rad18, rad30, rad52, rev1, rev3, hpr5 and siz1 mutants) and/or by using a modified version of the described SGA procedure [Tong et al. 2001]. Briefly, diploids were patched on glucose-rich (5% glucose, 3% nutrient broth, 1% yeast extract, 2% agar) presporulation medium and incubated for 1 day at 30°C prior to being patched on

(Table 5.1). Yeast strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>MAT</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y5563</td>
<td>α</td>
<td><em>can1Δ : MFA1pr-His3 lyp1Δ</em></td>
<td>A.Tong</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+</em></td>
<td></td>
</tr>
<tr>
<td>BY4741</td>
<td>α</td>
<td><em>can1Δ : MFA1pr-His3 lyp1Δ</em></td>
<td>EUROSCARF</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+</em></td>
<td></td>
</tr>
<tr>
<td>MGST01</td>
<td>α</td>
<td><em>same as Y5563 plus NATMX4</em></td>
<td>this study</td>
</tr>
<tr>
<td>MGST01</td>
<td>α</td>
<td><em>same as MGST001 but rfc1Δ3-273</em></td>
<td>this study</td>
</tr>
</tbody>
</table>
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agar containing 1% potassium acetate to promote spore formation. After 5 days of incubation at RT, selection for MATa and mutant spores was performed as described [Tong et al. 2001], but in liquid medium.

All strains were kept on selective medium: yeast nitrogen base (0.67% YNB, 2% glucose, 2% bacto agar) supplemented with appropriate markers. For determination of the sensitivity to UV and various chemicals, cisplatin (CisDDP) and X-ray survivals, stationary cultures were obtained by growing cells for at least 3 days in complete medium (YPD, 1% yeast extract, 2% glucose, 2% bacto-peptone) at 30°C under vigorous shaking.

Plasmid and RFC1 gene deletion

Plasmid YIplac211RFC-ΔNTR was created by inserting an SOE-PCR fragment coding for Rfc1 with residues 3-273 deleted, into YIplac211 [Gietz et al. 1988] using the EcoRI and PstI sites. Primers A (5’ CTGTGGTTTTGAAATCTGCCTTT), B (5’ ACTCTAG TGGCTGCAAGCTTGACCATTICCTTCAGCTTAGTTTAT), C (5’ ATAAACTAAGCTGAAGAA ATPGTCAGCTGCAGCTTTGAGTTATG) and D (5’ CATCATCTGCAGCTTTGATATTTTCAT GGTTTATG) were used. In bold and italics are sequences corresponding to the start and Lys274 codons of rfc1 respectively. The EcoRI and PstI sites are underlined. The rfc1 gene was linked to a selectable marker by integrating a natMX4 cassette, amplified by PCR with primers E (5’ TAAAATACTCTTTATTAGTATAGTACACAAAATAATG TACCTGACACATAGGAGGGCCAATACCC) and F (5’ ACAAATGTTAGACCTAAT AAAATGT AATATTTAACGACGGTTATACCAGTACACGACCACCATC) using p4339 as template, into the intergenic region between rfc1 and ste13 in strain Y5563 yielding strain MGST01. Correct integration at the targeted site i.e. the chromosomal DNA regions complementary to the underlined primer sequences, was verified by PCR and Southern blot. The chromosomal N-terminal deletion in rfc1 was made by the popin-popout method using BglII linearized YIplac211RFC-ΔNTR and MGST01.
yielding MGST02. The mutated rfc1 gene was sequenced and correct integration was verified by PCR and Southern blot.

**Sensitivity towards various chemical agents**

Ten-fold serial dilutions of stationary cells were prepared in water and 2 µl drops of each of them were spotted on YPD plates with a concentration ranging from 0 to 0.02% (w/v) for MMS, 0 to 100 mM for HU, 0 to 3% for formamide and 0 to 0.15% for caffeine respectively. Cells were grown for 2-3 days at 30ºC. For mitomycin C and CisDDP, the procedure was similar but instead, 3 to 4-fold dilutions of the stationary cultures and YNB plates supplemented with the appropriate nutrients were used. The range of concentrations tested was 0 to 100 µg/ml for mitomycin C and 0 to 15 µg/ml for CisDDP.

**UV dose-response test**

2µl droplets of stationary cultures, diluted 250-fold in water, were spotted on YPD plates and irradiated with UV light at 2 J/m²/s. Different doses ranging from 0 to 140 J/m² were inflicted on individual droplets by masking cells for different periods of time. Cells were grown for 2-3 days at 30ºC in the dark. The same procedure was applied to test the UV sensitivity of exponentially growing cells but, in this case, plates were incubated for 4-5 hours at 30ºC prior to irradiation.

**CisDDP survival rate determination assay**

1 ml of exponentially growing cells (OD₆₀₀≈0.8) was centrifuged, cells were washed twice with 1 ml of water and resuspended in the same volume of water. For each cis-platin dose tested, a 100 µl aliquot of the cell suspension was mixed with 60 µl of water plus the desired volume of a 1 mg/ml CisDDP stock solution in 40 mM NaCl. The final volume of the reaction was 200 µl with a final salt concentration adjusted to 5 mM using a 40 mM NaCl solution. After incubation for 2 hours at 30ºC and 1400
rpm, 800 µl of water was added to the mixture to stop the reaction. Appropriate
dilutions in water were prepared and 50µl of each of each was plated on YPD (in
duplicate or triplicate for dose 0). Plates were incubated for 2-3 days at 30 ºC to
allow formation and counting of colonies.

X-ray survival rate determination assay

15 ml of exponentially growing cells were spun down and resuspended in the
appropriate volume of 0.85% NaCl to prepare a suspension containing 2x10^6 cells/ml
from which a 3 ml sample was submitted to ionizing radiation using a 225 SMART X-
ray source (Andrex SA, Copenhagen, Denmark) at a dose rate of 7.83 Gy/min (200
kV, 4 mA, 1 mm Al1 filter). Aliquots of 100 µl were taken after exposure to 0, 100,
200, 300 and 400 Gy and appropriate dilutions in 0.85% NaCl were prepared.
Duplicates of these cell dilutions (50 µl) were plated on YPD and grown for 2-3 days
at 30 ºC before colonies were counted.

Results.

Chromosomal deletion of the rfc1 NTR in yeast does not induce any detectable
phenotypic defect under normal growth conditions.

An rfc1-ΔNTR S. cerevisiae haploid yeast mutant was constructed in two steps. First,
the rfc1 gene was linked to a selectable marker conferring resistance to the
antibiotic nourseothricin. To accomplish this, a natMX4 cassette was introduced
approximately 165 bp downstream of the rfc1 gene (Figure 5.1). The resulting
intermediate strain MGST01 behaved normally in mating and sporulation tests. This
strain, carrying an intact copy of rfc1, is the parental strain for the deletion construct
and was therefore used as the wild-type control in all experiments. The target
deletion was then introduced by replacing the chromosomal rfc1 copy of MGST01 by
an NTR-deleted version of the gene. The resulting MGST02 strain (referred to as
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(Figure 5.1) Composition of the large subunit of respectively human and yeast RFC.

rfc1-ΔNTR) was designed to chromosomally express the same Rfc1 truncated form (rfc1-Δ3-273) as previously characterized by Burgers and coworkers [Gomes et al. 2000] in a trans-complementation system. The phenotype observed for rfc1-ΔNTR was similar to the earlier report since cells grew normally and exhibited sensitivity to treatment with UV light or hydroxy urea that was indistinguishable from the parental wild type strain. rfc1-ΔNTR cells were also able to mate and sporulate without any detectable defect.

Truncation of the S. cerevisiae Rfc1 subunit residues [3-273] leads to increased cisplatin sensitivity.

Phenotypic characterization of the rfc1 deletion strain was pursued in order to further investigate whether the NTR is involved in DNA replication or repair processes. First it was determined whether the mild MMS sensitivity observed by the Burgers group [Gomes et al. 2000] could be detected in the chromosomally truncated rfc1-ΔNTR strain used in the present studies. Indeed similar sensitivity was observed (Figure 5.2A) suggesting that the different methods of preparing the truncated rfc1 gene did not affect the function of the protein. In contrast to earlier work [Xie et al. 1999], rfc1-ΔNTR showed no difference with respect to the wild type in either spontaneous or UV-induced mutation rate in a canavanine resistance forward mutagenesis assay, thus ruling out a potential role in mismatch repair.
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(Figure 5.2). Sensitivity of the rfc1-ΔNTR MGST02 strain to cisplatin. Serial three-fold dilutions of a stationary culture of the mutant MSGT02 and control MGST01 strains were spotted on YNB plates supplemented with the appropriate markers and containing 0.02% (w/v) MMS (A) or 15 µg/ml of CisDDP (B). After two days at 30°C, growth of MSGT02 cells which lack the NTR (i.e. Rfc1 residues [3-273]) was clearly more impaired than for MSGT01 cells expressing wild-type, full-length Rfc1.

(MMR, data not shown). Furthermore, exposure of rfc1-ΔNTR cells to double-strand break (DSB)-inducing ionising radiation led to survival rates similar to the control strain, while no effect of the rfc1 deletion was observed when cells were grown in the presence of up to 33 mM mitomycin C (an interstrand crosslinking reagent) or 0.15% caffeine. However, when compared to the parental strain, rfc1-ΔNTR was significantly more sensitive to cisplatin, showing both an impaired growth in the presence of the cross-linking agent as well as a lowered survival rate after drug exposure (Figure 5.2B and below). This phenotype for the Rfc1 NTR deletion allowed the search for interactions with genes coding for other proteins in various DNA repair and replication pathways. In order to do so, epistasis analysis was performed
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**Table 5.2** Cisplatin sensitivity of double deletion mutant strains in comparison to the corresponding single mutant in a drop test assay. Please note: Pol32 also has a role in the processive and error-prone bypass in DNA damage. No sensitivity for CisDDP was observed for Δrad30 cells.

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Pathway</th>
<th>Relative CisDDP sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δrnh1, Δrnh201, Δrnh202, Δrad27</td>
<td>Okazaki Fragment processing</td>
<td>double &gt; single</td>
</tr>
<tr>
<td>Δrad9, Δrad17, Δrad24, Δalg1,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δddc1, Δchk1, Δnft18, Δdcc1,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δpph3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δrad2, Δrad4, Δrad16, Δrad23</td>
<td>NER</td>
<td>double &gt; single</td>
</tr>
<tr>
<td>Δrad10</td>
<td>NER</td>
<td>double ≥ single</td>
</tr>
<tr>
<td>Δpms1, Δmsh2</td>
<td>MMR</td>
<td>double &gt; single</td>
</tr>
<tr>
<td>Δrad52</td>
<td>HR</td>
<td>double &gt; single</td>
</tr>
<tr>
<td>Δhpr5, Δsr22</td>
<td>HR</td>
<td>double &gt; single</td>
</tr>
<tr>
<td>Δpso2, Δsae2</td>
<td>DSB</td>
<td>double &gt; single</td>
</tr>
<tr>
<td>Δpol32</td>
<td>DNA replication</td>
<td>double &gt; single</td>
</tr>
<tr>
<td>Δrev1, Δrev3, Δrad5</td>
<td>PRR, TLS</td>
<td>double = single</td>
</tr>
<tr>
<td>Δrad6, Δrad18, Δsiz1</td>
<td>PRR</td>
<td>double ≥ single</td>
</tr>
</tbody>
</table>

using the EUROSCARF haploid strain collection as a source of deletion mutants. The NTR deletion was combined with second gene deletions in various DNA repair, cell cycle checkpoint and Okazaki fragment processing pathways. In total, thirty two
double deletion mutants (Table 5.2) were generated from $rfc1\Delta NTR$ using a modified version of the SGA spore selection procedure [Tong et al. 2001] and/or by tetrad dissection and spore analysis. Cisplatin sensitivities were qualitatively assessed and scored compared to the corresponding single mutants by growing all strains in the presence of CisDDP (drop tests). While generating the double mutants a slow growth phenotype was observed for a double mutant of $rfc1-NTR\Delta$ with the deletion of $rad27$ (Figure 5.3A). While cells that contained a deletion of the NTR exhibited a doubling time comparable to wildtype cells, cells containing a deletion of $rad27$ exhibited a significant reduction in doubling time. The doubling time was drastically reduced when both the NTR and $rad27$ were deleted (Figure 5.3B).

Assignment of an epistatic relationship is based on the observation that two single mutants each show a phenotype but that the double mutant is not more sensitive than the most sensitive single mutant. All the double mutants obtained from strains impaired in Okazaki fragment processing or cell cycle progression control were found to be more sensitive to cisplatin than either of the starting single mutants. It seems therefore unlikely that the NTR plays a role in one of these processes. Also no epistasis was observed when homologous recombination (HR), mismatch repair (MMR), double-strand break repair (DB) and nucleotide excision repair (NER) deficient strains were tested with $rfc1\Delta NTR$. Note that, for this last pathway, an epistatic assignment was less clear in the case of $\Delta rad10$ cells. However, the unambiguous results obtained for four additional NER deletion mutants strongly indicate that the NTR is not involved in this mode of repair of cisplatinated DNA.

In contrast, double mutants of $rfc1\Delta NTR$ with either deletion of $rev1$ or $rev3$ did not yield greater cisplatin sensitivity, suggesting that the NTR functions in the same cisplatin survival pathway as the products of these genes. Further, an apparent
Rad27 single and double mutants exhibit a slow growth phenotype. (A) The indicated cell lines were diluted in YPD and growth was observed for 10-12 hours, or when the cells reached a stationary phase. (B) To determine the doubling time of each cell line, samples were taken at the indicated time point, and cells were spotted on YPD plates. After two days of growth at 30 °C, colonies were counted, each colony representing one cell in the original sample. WT and ΔNTR mutants exhibited similar doubling times, while the Δrad27 single and double mutants had significant lower doubling times.
epistatic relationship was observed between ΔNTR and Δrad5, a ssDNA dependent
ATPase that is also involved in ubiquitination and regulation of recombination versus
translesion synthesis [Stelter and Ulrich, 2003]. Double mutants with Δrad6 yielded
irreproducible results because of severely impaired growth, likely due to the
pleiotrophic character of rad6. Finally, no genetic interaction was detected between
the rfc1-ΔNTR and pol32, a subunit of DNA polδ that is known to interact with Polζ
in TLS [Minesinger et al. 2005].

Comparison of the survival rate post cisplatin treatment of specific double deletion
mutants points towards a role of the rfc1 NTR in translesion synthesis.

The drop tests described above are useful to rapidly screen a large number of
mutants for epistasis but can only give a qualitative, indicative result. In order to
further pursue and quantitate these observations, cell survival rates were
determined after treatment with cisplatin for two hours followed by removal of the
drug. A number of additional double deletion mutants were constructed from rfc1-
ΔNTR to get a more complete picture of the role of the NTR in post replication
repair. For each double mutant, a control single mutant was prepared from the
parental MGST01 strain so that any potential effect of the insertion of the natMX4
cassette on the observed phenotype could be ruled out.

First, it was checked that, as expected from the earlier experiments, the effect of the
NTR deletion on cisplatin survival could be detected. A dose dependent response of
rfc1-ΔNTR was observed that clearly indicated that the truncation strain was more
sensitive to killing by concentrations of cisplatin greater than 100 µg/ml than the
wild type strain (Figure 5.4A). Next the relationship between the rfc1-ΔNTR
truncation and the NER or HR cisplatin repair pathways was investigated. While
rad52 cells are already very sensitive to cisplatin, the double mutant with rfc1-ΔNTR
appears to have an additive effect (Figure 5.4A). A similar effect is observed when
(Figure 5.4) Analysis of cell survival after treatment with CisDDP. Strains deleted for genes in various DNA repair pathways were treated with the indicated amount of CisDDP for 2 hours at 30°C prior to plating for growth. Each point represents an average of at least two independent experiments. (A-B) Analysis of the relationship of NTR to the Homologous Recombination (A) and Nucleotide Excision Repair (B) pathways for CisDDP survival. In each case enhanced sensitivity of the double mutant suggests that NTR operates in a separate repair pathway. (C) Single and double Δrad30 mutants. As expected, the rad30 deletion has no effect on cell survival after cisplatin treatment.

The rfc1-ΔNTR truncation is combined with a rad4 deletion mutant (Figure 5.4B). The enhanced sensitivity of these double mutants suggests that the NTR plays a role in a repair pathway other than NER or HR. Furthermore, the interaction of the rfc1-ΔNTR truncation mutant with a rad30 (Polη) deletion mutant was investigated. The data in Figure 5.4C show that a rad30 strain is no more sensitive than the wildtype parental
strain while a double mutant strain with ΔNTR is no more sensitive to cisplatin than the single mutant \( rfc1-\Delta NTR \). The results of the drop tests suggested a potential epistatic relationship between the \( rfc1-\Delta NTR \) truncation mutant and the PRR pathway which was further investigated using the survival assay. The sensitivity towards cisplatin of \( rev1 \) and \( rev3 \) mutants in conjunction with the \( rfc1-\Delta NTR \) truncation mutant was assayed. Figure 5.5A,B clearly shows that both the \( rev1 \) and \( rev3 \) strains are more sensitive to cisplatin than the parental strain and the \( rfc1-\Delta NTR \) truncation mutant. However, the double mutants are no more sensitive than the single \( rev1 \) or \( rev3 \) mutants. This data confirmed the drop tests that suggested that NTR acts in the same pathway as these translesion polymerases. The switch from replicative to translesion synthesis is regulated by monoubiquitylation of PCNA.

(Figure 5.5) Survival rate for \( \Delta NTR \) and mutants involved in PRR after 2 hours of incubation at 30°C with several doses of CisDDP. Each point represents an average of at least three independent experiments.
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by the Rad6-Rad18 complex [Stelter and Ulrich, 2003; Hoege et al. 2002]. Monoubiquitylation results in the removal of polδ or polε from the daughter strand and its replacement by either polη in the case of bypass of CPD’s or a Rev1 containing complex for more helix distorting lesions. Subsequent polyubiquitylation of PCNA by the Ubc13-Mms2-Rad5 complex leads to a poorly understood response known as DNA damage avoidance [Ulrich and Jentsch, 2000]. In order to confirm a potential role for the NTR in PRR the effect of double mutations of genes coding for proteins involved in the ubiquitylation of PCNA were investigated. As described above, data concerning the rad6 complex was difficult to interpret, therefore a double mutant strain with rad18 was constructed. Cells lacking Rad18p are considerably more sensitive to cisplatin treatment (Figure 5.5C) than the rfc1-ΔNTR truncation mutant. However, the double mutant is no more sensitive to cisplatin than the rad18 single mutant, further supporting a role for NTR in PRR. In order to investigate a potential role for NTR in DNA damage avoidance a possible interaction between Δrad5 mutants and Rfc1-ΔNTR mutants was investigated. Similar results were observed to those obtained with rad18 mutants in terms of sensitivity to cisplatin and apparent epistasis (Figure 5.5D).

Discussion

Despite the known involvement of nucleotide excision repair (NER), homologous recombination (HR) and post-replication repair (PRR) pathways, the molecular mechanism of repair of cisplatin lesions in yeast is still poorly understood. About thirty double mutants were therefore generated using the EUROSCARF MATa haploid deletion strain collection to analyze the effect of the NTR deletion in various backgrounds including an extensive set of DNA repair deficient strains. No epistasis was observed with respect to cisplatin sensitivity in the 5 different NER and 2 mismatch repair (MMR) deficient strains tested. Similar results were observed for
double mutants impaired in HR and double-stranded break repair (DSB) pathways, as well as for mutants involved in cell cycle checkpoint regulation. However, one particular mutation is worthy of note. It had been reported that cells lacking the gene \textit{pph3} exhibited a mild sensitivity to cisplatin [Hastie \textit{et al.} 2006]. Recently this protein phosphatase was shown to be involved in DNA damage checkpoint recovery. This study demonstrates that \textit{pph3} mutants have nearly identical sensitivity to cisplatin as do \textit{rfc1-ΔNTR} mutants. However, the increased sensitivity of double mutants of the two genes suggests that they are not involved in the same pathway and it is possible that the sensitivity of the individual mutants is merely coincidental. In contrast, when tested in the same way, mutants with deletion of either \textit{rad5}, \textit{rev1} or \textit{rev3} showed a potential epistatic relationship with the NTR deletion. Rev1 is member of the \textit{Y} family of low fidelity, translesion DNA polymerases that acts in concert with pol\textit{η} and pol\textit{ζ} to bypass certain types of lesions [Waters \textit{et al.} 2009]. Pol\textit{ζ} is a heterodimer of Rev3 and Rev7 subunits. Epistasis with both the \textit{REV1} and \textit{REV3} genes strongly suggests a role in so-called post-replication repair and TLS. This was not observed for polymerase \textit{eta}, which is consistent with previous observations that Pol\textit{η} is primarily required for bypass of cis-syn pyrimidine dimers [Johnson \textit{et al.} 1999; Masutani \textit{et al.} 1999] and is not required for survival after cisplatin treatment [Wu \textit{et al.} 2004]. However, the exact nature of the relationship could not be determined using this approach since the apparent sensitivity of the rad30 mutant to cisplatin was mild. This suggests that the BRCT region of the large subunit of RFC plays a role in certain specific modes of TLS. This role is likely regulatory in nature and may be involved in determining which enzymes have access to the 3’ terminus of DNA. If the NTR of Rfc1 is important for TLS in cisplatin survival, then deletion of NTR should result in a decreased involvement of TLS in cisplatin survival. One would predict a reduction in mutagenesis rates that would result from the \textit{Δntr} but unfortunately, despite several attempts, no conclusive result could be obtained so
far due to the unfavorable balance between the sensitivity of the strain and the relatively low mutagenic character of the drug.

The observed slow growth of Δrad27 in combination with deletion of the NTR suggests that the NTR might be involved in a second pathway of Okazaki fragment processing that acts as a backup when the primary pathway fails. RFC has been shown to stimulate Fen1 in human cells, and is required for Rad27 activity in yeast cells [Cho et al. 2008; Sommers et al. 2008]. However, since ΔNTR cells grow normally, it is unlikely that the NTR is required for Rad27 function. A possible explanation for the slow growth observed for the double rad27Δntr mutant is that when rad27 is absent, another protein might substitute for its role in Okazaki Fragment processing. The data suggests that this protein may require the NTR dependent activity of RFC. For instance, the RNA primer could still be processed by RNaseH, and could require RFC activity at the site of a nick, generated by RNaseHI. Genetic studies have indicated that multiple alternative pathways, including HR and meiotic recombination, can substitute for rad27 in Okazaki fragment processing [Farah et al. 2005; Debrauwere et al. 2001]. The data suggests that NTR is involved in one or more of these alternative pathways for Okazaki fragment processing. The presence of nicks and gapped intermediates in these pathways could require RFC binding [Farah et al. 2005]. Since these gaps or nicks include a 5’ primer terminus, this could be a potential docking site for the NTR [Kobayashi et al. 2006].
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