ABSTRACT OF PhD THESIS

STUDY ON EXPRESSION AND FUNCTION OF THE PROTEINS INVOLVED IN MAINTAINING GENOME INTEGRITY

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Key words: Homologous recombination, Rad51, Rad52, Fbh1, Schizosaccharomyces pombe, open reading frame, checkpoint
MOTIVATION AND OBJECTIVES

DNA repair has two contrasting outcomes: if it fails, it can lead to oncogenesis. On the other hand, its suppression can enhance chemotherapy efficiency. (Wang, 2011) If DNA damage involves one strand, the other one is used as template for repair. If both strands are affected the broken ends are connected to each other with no regard to the sequence of the missing fragment (non-homologous end joining). Another repair mechanism uses the sequence of a homologue DNA duplex as template (homologous recombination). (Christmann et al., 2003)

Fig. 1.1 Homologous recombination mechanism (modified from Aylon, Kupiec, 2004) DNA ends are trimmed, resulting a longer 3' end, which will be coated by replicative protein A. Formation of Rad51-DNA duplex leads to invasion of homologous duplex. 3' end can be ligated to the other end leading to formation of Holliday junctions. Resolution of Holliday junctions can lead to genetic conversion with or without crossover phenomena.
**Choice of model organism:**

Study of DNA metabolism and its implications in oncogenesis is best performed on cancer cells. Due to technical and ethical issues, the process can be very difficult. Taking into consideration the high level of conservation of these mechanisms in simpler organisms, studies may be performed on biological models (for instance yeasts) and the results extrapolated. In our lab the biological model is fission yeast *Schizosaccharomyces pombe*, a simple eukaryote organism, with a genome that was entirely sequenced. Its genome has 4282 genes, 50 of which have high homology with those involved in human pathology. 23 genes are homologous to those involved in DNA repair and checkpoint mechanisms. (Wood et al., 2002, Kim et al., 2010)

Our aim was to study the functions of proteins involved in DNA repair by homologous recombination (Rad51, Rad52, Fbh1), by manipulating their coding genes. A gene’s deletion leads to absence of that protein in the cell and the cell’s phenotype demonstrates the role of that protein. Insertion of an active promoter (like *nmt1* in *S pombe*) leads to modified levels of protein expression; the phenotype can explain the role of protein’s level of expression. A fluorescent peptide, attached to a protein, can facilitate that protein’s localization within the cell.

When our study began, in the Biochemistry lab of UMF Iaşi a few of these experiments had already been done: deletion of open reading frames in order to study the phenotype (Petrescu et al 2005), deletion of genes *ste11* (Voicu et al 2007) and mitochondrial superoxid dismutase (Stoica et al., 2011), tagging of protein Rad13 (Petrescu et al 2006).

Scientific literature reports the overexpression of Rad51 protein in many types of cancer cells, overexpression associated with resistance to genotoxic agents. We aimed to reproduce those conditions in *S pombe*, by inserting an active promoter upstream the coding gene. Details are presented in chapter V of this thesis.

During our experiments, we obtained an unexpected result: protein tagging with GFP peptide led to the insertion of an extra sequence, which contained two open reading frames, in phase with
the main gene’s ORF. That situation did not lead to the lack of gene’s expression. The mechanisms that could explain the protein’s expression under these circumstances are presented in chapter VI.

We also noticed that cells with Rad52 hipofunction did not arrest their cell cycle following hydroxiurea treatment. That observation led to testing Rad52 involvement in checkpoint mechanism. Details are presented in Chapter VIII.

CHAPTER IV. Genomic modification of rad51/rhp51 locus

IV.1 Introduction
Study of Rad51/Rhp51 protein required construction of strains with genomic modification of rad51/rhp51 locus:
1. a strain with rad51/rhp51 overexpression, characterized by a high amount of Rad51/Rhp51 protein in the cell;
2. a strain with rad51/rhp51 deletion, with no Rad51/Rhp51 protein expressed in the cell;
3. a strain with rad51/rhp51 overexpression combined with deletion of fbh1 gene (fbh1 codes a helicase that acts as a down regulator of Rad51/Rhp51)

IV.2 Materials and methods
In order to obtain strains with deletion and overexpression of Rad51/Rhp51 protein, we used the protocols that were described by Werler et al., in 2003 and applied in our lab for genomic modification of other S pombe strains. (Petrescu et al., 2005, Petrescu-Dănilă et al., 2006).

IV. 3 Results
IV.3.1 Deletion of rhp51 gene from S pombe genome
Rhp51 is located in chromosome 1, between nucleotides 2698110 – 2700283 (Pombase). Using rhp sequence, we designed three pairs of primers, which match the upstream and downstream gene sequence:
- one pair of primers was used to amplify a sequence of approximately 500 nucleotides upstream start codon and the first 20 nucleotides of \textit{rhp51} replacement gene;
- another pair of primers was used to amplify the replacement gene for \textit{rhp51} (\textit{ura4}), a gene that codes a protein involved in pirimidines’ synthesis. The primers matched the \textit{ura4} ends;
- the third pair consisted in a primer that matched the first 20 nucleotides of \textit{ura4} and one primer that matched a sequence situated at approximately 500 nucleotides downstream \textit{rhp51} stop codon.

The primers were used to build three DNA sequences, by PCR technique. For fragments I and II we used colony PCR (chromosomal DNA was used as template). For fragment II we used a plasmid that carried \textit{ura4}.

The three fragments were used to build a fusion fragment, which transformed the \textit{S pombe} strains for \textit{rhp51} deletion. Ura4 – (negative) strains grow only on uracil enriched medium. We used Bahler’s (Bahler et al., 1998) protocol to prepare the strains for transformation. We transferred the cells to medium lacking uracil and considered the colonies that grew on that medium to contain \textit{ura4} gene.

\textbf{IV.3.2 Construction of Rhp51 overexpression strain}

We used the protocol described by Werler (Werler et al., 2003), which led to gene tagging and insertion of an active promoter. The technique implies the use of PCR and Cre-lox systems.
Fig. 4.1. The fragment used for placing \textit{rhp51} under \textit{nmt1} promoter control. The fragment contains a selection marker \textit{sup3}-5 (confers ability to grow on media with no adenine) and an artificial promoter \textit{nmt1}, placed between two \textit{loxP} sites. In a subsequent step \textit{Cre} recombinase removes the fragment between \textit{loxP} sequences and the gene \textit{rhp51} is placed under the natural promoter’s control. (modified from Werler et al., 2003, Petrescu-Dănilă et al., 2006)

**Excision of DNA sequences between \textit{loxP} sites.**

Excision of these sequences was performed based on the protocol proposed by Iwaki and his collaborators. (Iwaki, Tkegawa, 2004). Strains that contained the insert were transformed with the PW7 plasmid, containing the gene encoding the \textit{Cre} recombinase.
The plasmid was obtained from Prof. AM Carr, from the University of Sussex. The action led to the attachment of EGFP tag in phase with rhp51 gene initiator codon. The plasmid contained a selection marker gene, which conferred the ability to grow on media without uracil to strains that integrated it into the genome.

The transformed strains were selected by growing the cells on a medium lacking uracil and containing a limited amount of adenine (10 mg / l – allows the growth of cells that have lost the marker sup3-5).

IV.4 Discussions
Rad51 plays the main role in the invasion of the homologous DNA duplex. The protein polymerises at the 3' end of the DNA and mediates migration and coupling of the resulting complementary chain. This invasion forms a D loop structure and Holliday juctions, which will be resolved and the duplex structures restored. (Raji, Hartsuiker, 2006)

We intended to study the effect of genotoxic agents on cells expressing different levels of Rad51 protein. Rad51 is involved in repair mechanisms of injuries induced by these agents. For this purpose, in addition to the strain used as a control (expressing unaltered levels of Rad51), we constructed a strain in which the gene rhp51/rad51 is deleted (which has the effect of absence of Rad51 protein from cells) and one in which the gene is placed under the control of an artificial promoter (that results in a overexpression of the protein in the cells).

IV.5 Conclusions
We presented the genomic modifications that we made at the rad51/ rhp51 locus in order to carry out the experiments presented in the following sections, experiments requiring strains with hyper or hypofunction of Rad51/ Rhp51 protein. Hyperfunction was obtained by inserting a very active transcription promoter (nmt1) upstream of the gene coding protein Rad51. Hypofunction has been obtained by deletion of the gene.
CHAPTER V. Resistance to genotoxic agents of strains with modification in Rad51 expression

V.1. Introduction

Rad51 is the protein that catalyzes the strand exchange in the process of double strand break repair by homologous recombination. Recent publications (edited after 2002) report the overexpression of Rad51 in many (over 50%) types of cancer (Klein, 2008). In those types of cancer the expression of protein (measured as the amount of protein produced) exceeds 2-7 times the level of expression of normal cells.

Rad51 overexpression has causes and consequences. It is important to know both the causes and consequences for prevention and therapy of cancer. An opportunity to discover them is to seek for other genomic changes that usually accompany them. Some of these are: loss-of-function mutations in p53, BRCA1, BRCA2, or genomic changes with gain of function like the oncoprotein BCR / ABL (Klein, 2008). Overexpression of Rad51 increases the resistance of cancer cells to chemotherapeutic agents (Yang et al., 2012).

Causes and consequences of Rad51 overexpression could be addressed by using a simple organism like fission yeast, genetically manipulated to induce an overexpression of the Rad51 / Rhp51 together with a mutation of the presumed causes or consequences. Rad52 / Rad22 is the functional equivalent of the S pombe BRCA2 and we have a strain partially defective in of Rad22 function. In S pombe we can also test the effect of Rad51 overexpression on resistance to chemotherapeutic agents.

V.2 Materials and methods

Testing the viability and sensitivity / resistance to genotoxin:

In order to test the viability and sensitivity to genotoxic agents we used a semiquantitative method, the one of serial dilutions (explanation in the legend to Fig. V.1)
Table 5.1. *S. pombe* strains used in this study

<table>
<thead>
<tr>
<th>Name and genotype</th>
<th>Complete genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>rad51+ rad22+ fbh1+</td>
<td>ade6-704 ura4-d18 leu1-32 h-</td>
</tr>
<tr>
<td>fbh1-d</td>
<td>fbh1::kanr ade6-704 ura4-d18 leu1-32 h+</td>
</tr>
<tr>
<td>rad51-d</td>
<td>rad51::kanr ade6-704 ura4-d18 leu1-32 h-</td>
</tr>
<tr>
<td>nmt1-rad51</td>
<td>nmt1-rad51 ade6-704 ura4-d18 leu1-32 h-</td>
</tr>
<tr>
<td>nmt-rad51 fbh1-d</td>
<td>nmt1-rad51 fbh1::kanr ade6-704 ura4-d18 leu1-32</td>
</tr>
<tr>
<td>E-rad22</td>
<td>E-rad22 ade6-704 ura4-d18 leu1-32 h-</td>
</tr>
<tr>
<td>E-rad22 nmt1-rad51</td>
<td>E-rad22 nmt1-rad51 ade6-704 ura4-d18 leu1-32 h+</td>
</tr>
<tr>
<td>E-rad22 nmt1-rad51</td>
<td>E-rad22 nmt1-rad51 ade6-704 ura4-d18 leu1-32 h-</td>
</tr>
</tbody>
</table>

V.3 Results

V.3.1 Lack of Rad51 function decreases cell viability of *S. pombe*.

V.3.2 Overexpression of *rad51* sensitizes cells to hydroxyurea and camptothecin.

V.3.3 Deletion of *Fbh1* gene alleviates the hypersensitivity to camptothecin of strains overexpressing Rad51.
Fig. V.1, A, B, C. Viability of S. pombe strains with and without mutations at rad51, rad22, or fbh1 loci. Cells were grown to stationary phase and then serially diluted to have $10^5$, $10^4$, $10^3$, $10^2$ or 10 cells/5 µl and then 5 µl of each dilution, in the decreasing order from left to right, was dropped in a row on plates containing the solid medium YEA with or without the genotoxins in concentrations as mentioned. The plates were photographed after 5 days of incubation at 30°C. Panel A: Strains overexpressing Rad51 are hypersensitive to hydroxyurea and camptothecin. Panel B: The Rad22/Rad52 loss-of-function cannot be compensated by overexpression of Rad51. The strains 2 and 4 were identically mutated at rad22 but they were derived independently and had slightly different genetic backgrounds. The same observation is available for strains 3 and 5. Panel C: Deletion of the gene fbh1 alleviates the hypersensitivity to camptothecin of the strain overexpressing Rad51.

V.4 Discussions

The experiments conducted in our laboratory showed a relative decrease in cell viability in Schizosaccharomyces pombe strains overexpressing the recombinase Rad51/ Rhp51, which confirmed previously published results (Kim et al., 2001). We also found an increased sensitivity of the same strain to hydroxyurea and camptothecin (opposed to the results obtained from mammalian cells overexpressing Rad51). Hypersensitivity is maintained in strains in
which Rad52 / Rad22 function is reduced (again, a different situation from cancer cells, where combination of Rad51 overexpression with reduction of BRCA2 function - equivalent functional Rad22 - leads to resistance to chemotherapy). However, if Fbh1 helicase is also missing, the strains appear relatively resistant, which suggests that this helicase has an important function in determining sensitivity / resistance to genotoxins.

**V.5 Conclusions**

Our experiment aimed to obtain a correlation between the level of Rad51 protein expression and resistance to some genotoxic agents used in oncological therapy. We showed that overexpression of Rad51 induced increased sensitivity to two genotoxic substances. This increased sensitivity is maintained even when associated with the lack of function of a protein that mediates recombination, Rad22 /Rad52.

Deletion of *fbh1*, a gene encoding a protein with a destabilizing effect on Rad51 filament, led to increased resistance to camptothecin.

**CHAPTER VI Aspects regarding rad51/rhp51 open reading frame, transcription and translation**

**VI.1 Introduction**

During genomic modifications described in Chapter IV, a construct that we call "long insert " EGFP -loxP - sup3-5 - nmt1-loxP was copied from a plasmid (pPW9-EGFP, Werler et al., 2003) to be inserted at Rad51 locus and then "shortened" by the reaction in vivo of the Cre recombinase. The result was the elimination of loxP - sup3-5 – nmt1, which caused EGFP gene to merge with Rad51. The expected result is shown in the following tabs:
A. *rad51* locus in non-transformed strains

<table>
<thead>
<tr>
<th>Upstream flank</th>
<th>Promotor <em>rad51</em></th>
<th><em>rad51</em></th>
</tr>
</thead>
</table>

B. *rad51* locus in strains containing the long insert

<table>
<thead>
<tr>
<th>Upstream flank</th>
<th>Promoter <em>rad51</em></th>
<th>egfp</th>
<th>loxP</th>
<th>sup3-5</th>
<th>nmt1</th>
<th>loxP</th>
<th><em>rad51</em></th>
</tr>
</thead>
</table>

C. Expected structure for *rad51* locus, after Cre recombinase action

<table>
<thead>
<tr>
<th>Upstream flank</th>
<th>Natural promotor <em>rad51</em></th>
<th>egfp</th>
<th>loxP</th>
<th><em>rad51</em></th>
</tr>
</thead>
</table>

Fig. 6.1 Expected structures for *rad51* locus, before and after Cre recombinase action.

### VI.2. Materials and methods

The protocol of this genomic modification was described by Werler et al (Werler et al., 2003); the plasmids required for this process were provided by the authors. DNA sequencing was done by Sanger method, at lab AM Carr laboratory at Sussex University UK (G. Bordeianu).

### VI.3 Results

Between the expected sequence and the one that we actually obtained there was a difference: the obtained sequence was 303 nucleotides longer than projected.

A’. *rad51* locus in non-transformed strains

<table>
<thead>
<tr>
<th>Upstream flank</th>
<th>Promotor <em>rad51</em></th>
<th><em>rad51</em></th>
</tr>
</thead>
</table>

B’. *rad51* locus in strains containing the long insert

<table>
<thead>
<tr>
<th>Upstream flank</th>
<th>Promoter <em>rad51</em></th>
<th>Supplementary sequence</th>
<th>egfp</th>
<th>loxP</th>
<th>nmt1</th>
<th>Sup3-5</th>
<th>loxP</th>
<th><em>rad51</em></th>
</tr>
</thead>
</table>

C’. Actual structure of *rad51* locus, after Cre recombinase action

<table>
<thead>
<tr>
<th>Upstream flank</th>
<th>Supplementary sequence</th>
<th>Natural promotor <em>rad51</em></th>
<th>egfp</th>
<th>loxP</th>
<th><em>rad51</em></th>
</tr>
</thead>
</table>

Fig 6.2 A, B’,C’ Structure obtained in our lab for *rad51* locus, after Cre recombinase action
The additional part was identified as belonging to the family of plasmids pGEM, which included PW9-EGFP plasmid, the plasmid used in the protocol for modifying rad51 locus.

The new insert was due to mismatch of IIf EGFP primer, used to amplify the sequence-loxP-EGFP-loxP sup3-5-nmt1 from PW9-EGFP plasmid. The ATG initiation codon of the sequence is in phase with the initiating codon of EGFP, but 303 nucleotides downstream (Figure 6.1B). If the translation were to continue, it would provide a hybrid peptide with n extra 101 amino acids at the N end. However, in phase with the initiator codon there is a “stop” codon, TAA, located after only 25 codons. Canonically, translation should stop at this codon, and the strain carrying the insert should synthetise no hybrid protein EGFP-Rad51 and it should lack the Rad51 protein function.

However, there is evidence (direct and indirect) that both the protein and its function are present:

- Insert strain does not exhibit the Rad51 / Rhp51 deletion phenotype.

- Strains containing identical inserts introduced to two other loci do not cause complete lack of function of the affected genes.

- Direct proof: E-Rad51, E-Rad52 and E-Smc6 can be revealed by Western blot at strains bearing insert suppl-EGFP.
Fig. 6.3 E-Rad51 strain with the insert is not sensitive to genotoxin. (A) five-spot rows resulting from the growth of cells on a Petri without or with hydroxyurea and photographed after 4 days of incubation at 30°C. Dead cells do not form colonies, even after ten days. (B) PCR amplicons obtained by amplification of DNA extracted from strain E-Rad51. Track 1, the reaction using primers to obtain an amplicon of 300 pairs of nucleotides (DNA extract positive control). Track 2, amplicon obtained with primers loxP/Rad51-chk. Track 3, lack of amplicon in the reaction using primers nmt1/Rad51-chk. Right: scale of DNA fragments, the shortest one has 500 pairs of nucleotides.

Fig. 6.4 Western blot results. Extracts from strains carrying the E-rad52 (L), E-Rad51 (middle), and E-smc6 (right) were treated with anti-GFP protein. White arrows indicate the position of the protein. Shorter fragments are the result of proteolysis. In the right figure, scale fragments of known sizes (in kDa) subjected to the same separation. (The results were obtained in laboratory AM Carr, Sussex University by G. Bordeianu.)
VI.4 Discussions

The above data provide evidence for the (partial) expression of Rad51, in the presence of genomic changes that have the effect of distancing the transcription promoter codon with 303 nucleotides. Additional sequences between the promoter and the AUG initiator codon have at least one open frame upstream the codon, made up of 26 codons, including the AUG initiator codon and a “stop” codon, TAA. This reading frame is located upstream of the initiator codon of the hybrid gene EGFP-Rad51 (or EGFP-rad52 or EGFP-smc6). According to the rules of protein expression, in these conditions expression would not be possible. We analyzed several hypotheses based on bibliographic data, in order to explain that, however, the expression is however possible. Here is the one considered most likely.

Reinitiation hypothesis

The situation we are analyzing is similar to that described by Hinnebusch group for GCN4 gene expression in Saccharomyces cerevisiae (Hinnebusch et al., 2011) In the present case we assume that the process can be described as follows: Initiation is classic “head-dependent”. Preinitiation complex is formed to the extreme 5' end of the mRNA and it scans the mRNA up to the first initiation codon, which belongs to the supplementary sequence. At this point, the complex associates the large subunit of ribosomes and the synthesis of the peptide starts. This synthesis stops after 25 codons, when it encounters a “stop” codon. The ribosomal subunits dissociate, but part of 40S subunits remain attached to the mRNA and scan forward up to the second initiator codon, located at a distance of 99 nucleotides away from the termination codon of the first cistron. If by the time the complex reaches the second subunit ribosomal initiation codon, there is enough trimeric complex eIF2-GTP-Met-tRNA, translation is restarted from the initiator codon of the second cistron and it is followed by the synthesis of a peptide of 12 amino acids. Translation stops at the second terminator codon TAA, which again leads to dissociation of ribosomal subunits. 40S subunit can still scan, but the probability of reinitiating translation at
the third AUG codon, which belongs to the Rad51-EGFP protein, is very small since the distance between the two cistrons, between termination codon and the beginning of the third cistron, is only 81 nucleotides.

However, if the distance of 99 nucleotides between cistrons 1 and 2 is covered fast, the trimeric complex does not reach the concentration needed in order to reinitiate translation at cistron 2, but re-initiation may occur at third cistron, the protein Rad51-EGFP is expressed. The time required for completing of distance between the cistrons 1 and 2 would be critical amount for the expression of Rad51-EGFP protein. This time depends on the distance scanned (number of nucleotides), as well as on the difficulties caused by secondary structures scan.

We tried to estimate the time required to scan different mRNA segments, taking into account their length and involvement in the chain structure. The chain structure was estimated using CLC Sequence Viewer program. Fragments of 9-15 nucleotides were aligned with the reverse complement of a longer fragment which comprises the additional sup-EGFP and EGFP.

```
  aug gug agc aag ggc gag gaa gcg gaa gag cgc
  cca auu cgc aaa ccg ccu cuc ccc gcg cgu ugg ccg auu
cau uaa UGC AGC UGG CAC GAC AGG UUU CCC GAC UGG AAA
GCG GGC AGU GAG CGC AAC GCA AUU AAU AGU UAG CUC ACU
CAU UAG GCA CCC CAG GCU UUA CAC UUU aug cuu ccc gcu
cgu aug uug ugu gga auu gug agc gga uaa CAA UUU CAC
ACA GGA AAC AGC UAU GAC CAU GAU UAC GCC AAG CUA UUU
AGG UGA CAC UAU AGA AUA CUC AAG CUU GGA UCC –
(supplementary sequence)
aug agu aaa gga gaa gaa cuu uuc acu gga guu guc cca
auu cuu (beginning egfp)
```

Fig. 6.5 Supplementary sequence transcribed into RNA and opportunities of structure formation of nucleotide chain structure of 1, 2, 3 and early cistron intervals between them, the mRNA transcribed construct E-Rad51. Cistrons are lowercase and numbered in order, from right to left. The nucleotides that may be involved in the two-chain structures are highlighted.
Aligns of more than 5 consecutive nucleotides were considered possibly involved in the two-chain structure and are underlined in Fig. 6.5. It should be noted that not all the underlined nucleotides may be involved in such structures at the same time; the neighboring fragments are complementary to portions which are very disparate from other parts of the transcript and we considered them only potentially involved. We used as an arbitrary unit of time the time required to scan a nucleotide which was not involved in the chain structure. The scan of an A or U nucleotide involved in two-chain structures was considered as two units of time, and scanning nucleotid C or G as three units of time. Report 2: 3 complies with the ratio of hydrogen bonds between AU or CG, but the rest of the estimates are approximate. However, they may serve to compare different scan times with each other.

Table 6.I. Number of time units required for scanning of different segments of mRNA E-Rad51

<table>
<thead>
<tr>
<th>Sequence part</th>
<th>Nucleotide s number</th>
<th>Nucleotides potentially included in two-chain structures</th>
<th>Scanning time (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A/U</td>
<td>G/T</td>
</tr>
<tr>
<td>Cistron 1</td>
<td>78</td>
<td>5</td>
<td>21</td>
</tr>
<tr>
<td>Intercistronic 1-2</td>
<td>99</td>
<td>5</td>
<td>19</td>
</tr>
<tr>
<td>Cistron 2</td>
<td>42</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Intercistronic 2-3</td>
<td>81</td>
<td>9</td>
<td>4</td>
</tr>
</tbody>
</table>

The table shows that the scan time for the 2-3 intercistronic fragment is shorter than the one for 1-2 intercistronic fragment which would lead to failure of 3-cistron expression (EGFP-Rad51) if the first two cistrons are translated. The fact that cistron 3 is expressed (see the above results) demonstrates, according to this hypothesis, that the cistron 2 is not translated. Moreover, taking into account the
possibilities of initiating translation outside open reading frame we can say that even at 173 nucleotides away from termination codon, the AUG initiator codon does not restart the translation. If translation could recommence at that level, EGFP-Rad51 expression would not be possible.

VI.5. Conclusions

This chapter discusses some aspects of the way in which the rad51/ rhp51 gene leads to Rad51/Rhp51 protein synthesis. The project originally intended rad51 gene labeling. Because of mismatching of one of the primers used, the final construct contains an additional sequence of 303 nucleotides upstream of the initiator codon of the EGFP tab sequence. The sequence of the plasmid used for transformation belongs to PW9-EGFP and contains two start codon and the two stop codons that are in phase, thus defining three possible reading frames. Although according to the classic theory of translation protein synthesis should stop after the first stop codon, there is direct evidence (evidence of protein by Western Blot) and indirect evidence (no deletion phenotype) which demonstrate that the protein is present in cells.

Given the rules for initiator codon selection found in literature, we proposed several explanations: translation only takes place for the first and third reading frame, or the expression is accomplished only for the last reading frame favored by post-translational modifications. Similar cases have been reported in literature, for preproinsulin gene in mammals and GCN4 gene in yeast.
CHAPTER VII Rad51 involvement in oxidative metabolism

VII.1 Introduction
There is little information in the scientific literature about the involvement of Rad51 protein in oxidative metabolism (Dudásová et al., 2004). We aimed to test the involvement of S. pombe Rad51 protein capacity of neutralizing reactive oxygen species (particularly superoxide radical) using an original in vitro method, based on inhibition of chemiluminescence generated by the xanthine oxidase-allopurinol-lucigenin system. (Stoica et al., 2011).

VII.2 Materials and methods
We used a kinetic method and the data was recorded on LB 9507 chemiluminometer LUMATIC at 25 °C.

Strains of S. pombe:
1 wild-type strain - a strain with no changes at the rad51 locus used, as control.
2 strain overexpressing Rad51 protein.
3 strain with rad51 gene deletion.

The reaction mixture: 100μL solution containing the cells of interest, glycine buffer 20μL, 100μL solution of allopurinol, lucigenin 100μL, 100μL xanthine oxidase solution.

The results were recorded every 2 seconds over a period of 100 seconds from the moment of lucigenin addition.

VII.3 Results

Following statistical analysis, no significant changes were observed in the values obtained for the three strains if we compared chemiluminescent signals, summed from the beginning and up to 100 seconds, or if we compared areas under the curves. It should be noted that in the case of cell-free control (consisting of the liquid medium YES) the values obtained were 85% from those shown in Figure 7.1. These results suggest a strong interference of the compounds dissolved in the culture medium.
Fig. 7.1 A, B, C: Graphic of inhibition of xanthine oxidase allopurinol—lucigenin chemiluminescence system for the *S. pombe* strains: A - wild type strain B - strain with overexpression of Rad51 protein; C – strains with *rad51*. RLU - relative light units.

Fig. 7.2 Comparison of chemiluminescent signals obtained in the first two seconds of the experiment for the considered strains (* p<0.0005). (RLU-relative light units)
If we compare chemiluminescent emission generated only at the beginning of the experiment (the first two seconds) and substract the value of cell-free medium control, we find statistically significant differences between Rad51 deletion strain and any of the other strains taken into consideration (Figure 7.2, p<0.0005).

VII.4 Discussions

Rad51 is present in the mitochondrial matrix. In the presence of oxidative stress, the activity of Rad51 is required for the regulation of mitochondrial DNA copy number; that action is mediated by Rad51 paralogs, Rad51C and Xrcc3. (Sage et al., 2010) Involvement of Rad51 in oxidative stress was also mentioned in previous studies. Dudásová et al (Dudásová et al., 2004) showed that Rad51 gene deletion in *S. cerevisiae* cells leads to increased sensibility to the action of hydrogen peroxide and defects in double strand breaks repair.

The study presented in this chapter tried to provide answers to two questions:

1. Considering that the peroxide radical derives from superoxide radical, which is the connection between protein Rad51 functions and that radical? To answer this, we used a method addressing mainly the measurement of superoxide radicals.

2. Which would be the advantages of applying a new detection method, known to have a superior sensibility compared to other in vitro methods? To answer this, we used a modified chemiluminescence method, which allows detection of very small amounts of superoxide radicals at the cellular level.

Our results showed no significant differences between the chemiluminescent signals, analyzed in kinetics. However, a statistically significant difference was proven only by comparing the results using an "end point" method. In this case, the strain with Rad51 deletion expressed a significantly lower signal than strains with normal or overexpressed levels of Rad51. This result may be due to a correlation of operation Rad51 with the activity of intracellular and / or mitochondrial superoxide dismutases, if we take into consideration that the method presented predominantly measures
the amount of superoxide radical. Since the interference of culture medium can not be neglected, further studies should eliminate this type of interference. One possibility would be to wash the cells 2 or 3 times before analysis, and to resuspend them (with the control of cell density) in an inert chemiluminescence medium (minimal medium, PBS buffer, etc).

**VII.5 Conclusions**

We tested the possibility of Rad51 protein involvement in the oxidative metabolism, using an original method, able to quantify the levels of superoxide radical formed in the cells. We demonstrated a possible correlation between the levels of Rad51 protein and these radicals.

**CHAPTER VIII Involvement of Rad51 protein in Chk1-dependent checkpoint arrest**

**VIII.1 Introduction**

Taking into consideration that strains of *S pombe* with mutations in *rad22* gene are often elongated, we assumed that this could be due to activation of cell cycle control mechanisms. To test this theory, we constructed double mutant strains, which in addition to a lack of function of Rad22 protein contained deletions of checkpoint genes.

**VIII.2 Materials and methods**

The *S pombe* strains used in this study:

1. Wild type - received from Prof. AM Carr, Genome Damage and Stability in Center, University of Sussex, Falmer, Brighton, UK.
2. E-rad22 - *rad22* gene (orthologous gene of *S cerevisiae* and Homo sapiens rad52) with a specific nucleotide sequence of protein GFP (green fluorescent protein), added at the N-end of the protein, This change genomic change leads to expression of hybrid EGFP-Rad22 protein.
3. rad3-d - rad3 gene deletion strain with rad3 (ATR orthologs in Homo sapiens) deletion, involved in the initial stages of all checkpoint mechanisms of genome integrity or replication.  
4. cds1-d - strain with cds1 gene deletion; the cds1 gene is involved in the replication checkpoint mechanism (prevents cell division when DNA replication is not complete); cds1 has been replaced by ura4, which confers the strain ability to grow on media without uracil.  
5. chk1-d – strain with chk1 deletion; chk1 is involved in the checkpoints mechanism of genome integrity (cell division cycle arrest when DNA damage is present); chk1 was replaced with ura4, which confers the strain ability to grow on media without uracil.  
6. rad3-d E-rad22 – strain mechanisms of control for genome integrity and replication; rad22 is modified by attaching a tab.  
7. Cds1-d E-rad22 - strain lacking genome replication control mechanism, rad22 is modified by attaching a tab.  
8. Chk1-d E-rad22 - strain without genome integrity control mechanism, rad22 is modified by attaching a tab.  

Strains with deletions of genes rad3, cds1 and chk1 (rad3-d, Cds1-d and Chk1-d) were donated to our laboratory by J. Huberman, Roswell Park Cancer Institute, Buffalo New York, USA.  

Double mutant strains rad3-d E-rad22, cds1-d E-rad22 and chk1-d E-rad22 were made in our laboratory. EGFP-Rad22 construct sequencing was done by Sanger method in the AM Carr lab at Sussex University UK by Dr. Gabriela Bordeianu.  

**VIII.3 Results**  
**VIII.3.1 Construction of mutant rad22 strain**  

The results obtained from sequencing the E-rad22 construct demonstrated that one of the primers was misprimed with the plasmid containing the EGFP sequence, which led to insertion of “stop” codons, in phase with the initiating codon.
VIII.3.2 The \textit{rad22} mutant strain has a defect in arresting the cell cycle, following treatment with hydroxyurea

Fig. 8.1 Checkpoint arrest of cell proliferation in hydroxyurea treated asynchronous cultures of \textit{S. pombe} strains carrying or not a mutant allele of \textit{rad22}. Exponential growing cultures of the two strains carrying the \textit{rad22} wild type allele (\textit{rad22+}) and mutant allele (\textit{E-rad22}) were each divided into two samples; to one of the samples hydroxyurea (HU) was added in a concentration of 12.5 mM and all the cultures were incubated at 30°C. At 1 hour intervals the percentage of living cells carrying septa was determined. At least 300 cells were counted for each time point.

Microscopic examination showed that the wild-type cells treated with hydroxyurea grew longer and had less septa than the untreated cells, suggesting the activation of a checkpoint mechanism that arrests the cell cycle. The cells containing the genomic modification E-rad22 (although long, they do not reach the size of the wild-type treated with hydroxyurea) did not show size changes and had a higher percentage of septa (both the living cells and the dead ones).
VIII.3.3 *rad22* belongs to the same epistatic group as *chk1* but different from *cds1*.

VIII.4 Discussions

Strains with mutations in the *rad22* gene are often elongated. One possible explanation could be the activation of cell cycle control mechanisms, explained by the existence of a greater number of lesions in the DNA. Cells with arrested cell cycle continue to grow in length. This happens, for example, when a genotoxic agent like hydroxyurea is added in the environment. After a few hours the cells begin to grow in length and stop mitosis (which is visible by the disappearance of cell septa).
Figure 8.1 shows the septation index (percentage of septate cells reported to all living cells) for wild-type cells and cells with E-rad22 change in the presence and absence of hydroxyurea treatment.

Septation index (equivalent to mitotic index in fission yeast) was small at the beginning (in asynchronous cultures only around 10% of cells were at the septation stage of the cell cycle), but approximately equal in wild types and E-rad22 cells. In hydroxyurea-treated wild-type it dropped to almost zero, was low for about 7 hours of hydroxyurea treatment, then rose again. In E-rad22 cells, treated the same as wild-type and mutant untreated cells during the following 8 h it varied between 8 and 12%, with no tendency to diminish. The temporary dropping of the septation index in wild-type cells under hydroxyurea treatment suggested a checkpoint arrest in wild-type strains and its absence in E-rad22 strains.

This situation may have one alternative explanation (not involving the checkpoint): a cytokinesis defect, since multiseptated cells occurred quite often the E-rad22 strain.

To rule out that explanation we constructed double mutant strains, carrying deletions of checkpoint genes chkl and cds1 then tested single and double-mutants for sensitivity to chronic treatment with hydroxyurea and camptothecin.

We assumed that, if rad22 were involved in a checkpoint mechanism, the combination of deletion of genes involved in checkpoint with rad22 modification (change leading to loss of function of rad22) would lead to increased sensitivity to genotoxic. On the contrary, if rad22 were not involved in that checkpoint mechanism, deletion of a gene belonging to this mechanism would lead to increased sensitivity to genotoxic. In this case the two changes combined would generate more DNA damage. Involvement of rad22 in a different checkpoint mechanism from that involving the deleted gene would lead to similar results.

Figure 8.2 D shows that the strain carrying E-rad22 change has a greater sensitivity to camptothecin compared with wild-type strain. This is explained by deficiencies in the repair of double-strand breaks induced by lack of function of Rad22. High survival of strains carrying deletions of genes involved in checkpoint mechanisms can
be interpreted by taking into account the redundancy of checkpoint mechanisms.

All double mutant strains (with EGFP-rad22 modification combined with deletion of one of the genes involved in checkpoint) have shown similar sensitivity to treatment with genotoxic to that of the strain with only EGFP-rad22 modification. The same observation applies to treatment with hydroxyurea, in concentration of 5 mM. This suggests either that there is an epistasis link (involvement in the same metabolic pathway) between rad22 and all the three checkpoint genes, or the fact that loss of function of rad22 leads to such significant DNA damage that combination with deletion checkpoint genes has little effect. Tests performed using lower concentrations of genotoxic showed that rad3 and Chk1 gene deletion does not change the high sensitivity of EGFP-rad22, while the strain which combines that modification with cds1 deletion has a higher sensitivity than the one conferred by mutation in only rad22 gene (synthetic effect).

These results demonstrate that chk1 and rad22 are in an epistasis relation and suggest that rad22 is involved in the checkpoint mechanisms dependent of chk1 (G2 / M or spindle division checkpoint). Rad22 appears to belong to the same group of epistasis with Rad3, which is not surprising given that Rad3 is known as activation of Chk1. (Furnari et al, 1999; Lin et al., 2012)

VIII.5 Conclusions

We have demonstrated that rad22 is in the same epistasys group with chk1 and cds1 and rad22 belong to different epistasys groups. Rad22 protein may thus be involved in the checkpoint mechanism chk1 dependent.

CHAPTER IX Conclusions and research perspective

A. Contributions to field knowledge

Proof of protein Fbh1 role in the resistance to some genotoxic agents.

Rad51 protein is one of the proteins involved in the repair of DNA damage. Its hyperfunction has been reported in many types of
cancer cells, in correlation with the cancer cells’ sensitivity to genotoxins used in treatment. We tried to reproduce both hyperfunction and hypofunction of Rad51 protein in fission yeast *Schizosaccharomyces pombe*, by using genomic modifications to clarify the causes conditioning resistance / sensitivity to genotoxic agents by this protein. Rad51 hyperfunction was obtained by inserting a very active transcriptional promoter (nmt1) upstream of the coding sequence of Rad51 gene. Hypofunction was obtained by deletion of the gene.

We proved that both deletion and overexpression of *rad51* gene lead to increased cell sensitivity to camptothecin and hydroxyurea, agents frequently used in tumor therapy. This increased sensitivity is maintained when overexpression of Rad51 is combined with loss of function of protein Rad52/Rad22 (functional equivalent of human BRCA2). Deletion of *fbh1* gene, which encodes a helicase that destabilises Rad51 nucleofilament, leads to increased cellular resistance to genotoxic agents, by a more efficient repair of DNA damage by homologous recombination mechanism. The correlation between sensitivity to genotoxic agents and Fbh1 helicase has not been reported so far. Fbh1 relationship with oncogenesis and resistance to therapeutic agents is not yet well known.

**Involvement of Rad51 protein in antioxidant mechanism demonstrated by an original method.**

We have shown that Rad51 protein contributes to an antioxidant mechanism, using an original chemiluminescent method, able to quantify the levels of the superoxide radicals in the cells.

**Hypothesis (supported by experimental evidence) that Rad52 protein may play a role in checkpoint mechanism of genome integrity.**

Starting from an experimental observation (strains with mutations in the gene *rad52* / *rad22* have a phenotype that might be caused by activation of checkpoint mechanisms), we demonstrated that protein Rad52 / Rad22 hypofunction leads to a yet unreported effect: cancellation of cell cycle arrest following DNA damage (genome integrity checkpoint). This suggests the participation of
Rad52 / Rad22 protein in the checkpoint mechanism. The checkpoint pathway involved is this case is the one dependent of Chk1 protein.

B Methodological contributions
Building genomically modified strains that will be used as instruments for future studies.
In order to study resistance to genotoxic agents conferred by Rad51 protein, we used the following strains:
- strains with modified protein expression of Rad51- characterized by deletion or overexpression of the protein,
- strains with an association of Rad51 over expression with partial loss of function of the protein Rad22/Rad52,
- strains with deletion of fblh helicase;
- strains that associate Rad51 protein overexpression with Fbh1 helicase deletion.

Strains with overexpression and deletion of $rad51$ gene were used to test the involvement of Rad51 protein in oxidative metabolism.
The hypothesis of involment of Rad52 protein in cell cycle control mechanisms was tested using strains that associated deletions of genes known to be involved in the checkpoint mechanisms and $rad22 / rad52$ modified with EGFP tap.

Building a strain that could be used as a model for the study of the translation of polycistrionic messages.
The genomic modification we obtained genomic changes led to the situation that Rad51 cistron is expressed even when its natural promoter is distanced from the initiator codon by 303 nucleotides. An additional reading frame between the promoter and initiator codon is also present. Canonically, that situation should have stopped the recombinant protein from translation. But we have presented evidence which proves that the protein is expressed. The mechanism which allows this expression is related to the choice of the reading frame during translation and the translation of polycistronic mRNAs. This construction can be used to study genomic translation (elucidating the mechanism for choosing a reading frame).
Participation in developing an original chemiluminescence method used for free radical detection

To investigate the participation of Rad51 protein in oxidative metabolism we used an original chemiluminescence method. The method was developed for testing the activity of superoxide dismutase and it is based on the mechanism of superoxide radical generation in the allopurinol-xanthine oxidase reaction.

BIBLIOGRAPHY


Stoica BA, Bordaianu G, Stănescu R et al. A new method for the quantification of superoxide dismutase mimics with an allopurinol-


Voicu PM, Petrescu-Danila E, Poitelea M et al. In *Schizosaccharomyces pombe* the 14-3-3 protein Rad24 is involved in negative control of pho1 gene expression. *Yeast* 2007; 24:121-127.


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