Summary of the Ph.D. Thesis

Def1 promotes polymerase exchange at stalled replication forks upon DNA damage

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INTRODUCTION

The stalling of the replication machinery that occurs as a consequence of encountering unrepaired DNA damages is a challenging problem for cells. Stalled replication forks can undergo DNA breakage and recombination that can lead to chromosomal rearrangements and cell death. To ensure survival, cells have evolved different mechanisms that can sustain DNA replication on damaged templates. These, so called DNA damage tolerance, or DNA damage bypass processes allow replication to continue on damaged DNA without actually removing the damage. DNA damage tolerance is achieved through two main mechanisms: template switching and translesion synthesis (TLS). Template switching is inherently error-free, since replication continues by using the undamaged nascent sister chromatid as template for the bypass of the lesion, while during TLS specialized polymerases take over the nascent primer end from the replicative polymerase and carry out synthesis opposite the DNA lesion in an error-free or error-prone way.

Rad6 and Rad18 are key mediators of DNA damage tolerance in the yeast *Saccharomyces cerevisiae.* They govern at least three different pathways for the replication of UV light-damaged DNA: 1. Rad5-dependent error-free DNA damage bypass; 2. Rad30-dependent error-free translesion synthesis; 3. Rev3-dependent error-prone translesion synthesis. Upon UV-treatment, the Rad6/Rad18 ubiquitin-conjugase-ligase complex mono-ubiquitylates proliferating cell nuclear antigen (PCNA) at lysine-164. Monoubiquitylated PCNA activates the Rev3, and the Rad30-dependent sub-pathways involving TLS polymerases, while further polyubiquitylation of PCNA on the same residue by the Rad5/Mms2/Ubc13 ubiquitin-conjugase-ligase complex activates the Rad5 subpathway.

In this study we identify Def1 as an indispensable regulator of induced mutagenesis. We show that Def1 promotes the proteasomal degradation of the catalytic subunit of the replicative DNA polymerase after DNA damage treatment. We demonstrate that the non-catalytic subunits of the replicative polymerase are not affected by UV-induced degradation and that they can form a complex with the TLS polymerase Rev1. Based on our results we propose a new model for polymerase exchange at stalled replication forks.

AIMS OF THE STUDY

The major aim of this thesis was to look for additional factors that affect DNA lesion bypass. For this purpose we have examined the *DEF1* gene, since deletion of *DEF1* rendered cells sensitive to UV-radiation, and the *def1 rad18* double deletion strain showed the same UV-sensitivity as the *rad18* single mutant suggesting an epistatic relationship, where the function of *DEF1* was dependent on *RAD18*.

To examine the involvement of *DEF1* in the *RAD6* pathway, we planned the following steps:

1. To analyse the genetic relations between *DEF1* and memebers of all three branches of the *RAD6* pathway upon DNA damage.
2. To check the interaction between *DEF1* and members of the *RAD6* pathway.
3. To determine the function of Def1

MATERIALS AND METHODS

1. DNA techniques

- Plasmid contruction for yeast two hybrid assay, for gene blaster generation and for epitope tagging
- Generation of deletion strains in *Saccharomyces cerevisiae* by one step gene replacement method

2. Yeast genetics

- Yeast two hybrid assay
- Quantitative and qualitative UV and MMS sensitivity assay
- UV-induced mutagenesis

3. Biochemistry

- Cell synchronisation
- Whole cell extract and western blotting
- GST fusion protein expression and pulldown assay

RESULTS

To better understand how DNA damage bypass functions our main goal was to search for additional factors that affect this process. We decided to investigate *DEF1* gene, as a possible new player in DNA damage bypass, since deletion of *DEF1* renders cells sensitive to UV-radiation, and the *def1 rad18* double deletion mutant shows the same UV-sensitivity as the *rad18* single mutant suggesting an epistatic relationship, where the function of *DEF1* is dependent on *RAD18*.

To explore the *in vivo* function of *DEF1* in the *RAD6* pathway, we performed genetic analysis between *DEF1* and members of all three branches of the *RAD6* pathway upon DNA damage. The result of these experiments showed that *DEF1* was epistatic to *RAD6* and *RAD18* and showed no epitasis with members of the error-free pathways like *RAD5*, *MMS2*, and *RAD30*, indicating that *DEF1* acted outside of error-free pathways of the PRR. Nevertheless, the *def1, rev3* strain exhibited the same sensitivity to UV and MMS as the *def1* single mutant suggesting that *DEF1* might act in the *REV3* branch of the *RAD6/Rad18*-dependent DNA damage tolerance pathway.

The TLS polymerases of the *REV3* branch are responsible for virtually all damage-induced mutagenesis. To verify that *DEF1* belonged to the *REV3* branch, we measured the rate of UV-induced mutations in different strains. While the wild type strain displayed a slight increase in the level of mutagenesis in accordance with the applied UV doses, the *def1* strain showed a complete defect in induced mutagenesis, even in combination with the *mms2* deletion that by itself caused high mutagenesis. Ectopic expression of *DEF1* from a plasmid in *def1* cells restored the level of mutagenesis close to wild type, confirming that the immutability was due to the absence of *DEF1*.

One of the known functions of *DEF1* from the literature is to advance the removal of the stalled RNA polymerase by facilitating its proteolytic degradation, when transcription is blocked due to DNA damage. We surmised that it might play a similar role in replication and promote the removal of the replicative polymerase from the stalled replication fork, thereby facilitating the exchange between the TLS and the replicative polymerase at DNA damage sites.

To test this possibility, we followed the fate of the replicative polymerase during DNA damage bypass by monitoring the protein level of Pol3, the catalytic

subunit of the replicative polymerase δ. As opposed to normal growth conditions, we observed an UV-irradiation induced transient decrease in the level of Pol3 in the S phase of the cell cycle in wild type cells and also in *mms2* and *rad30* cells. Importantly, in experiments using a *def1* deletion strain we could not detect any decrease in the level of Pol3. To investigate whether the observed phenomenon was ultimately under the higher control of *RAD6*, we performed the same experiment in a *rad6* strain and found that Pol3 diminution was absent in *rad6* mutants.

The most plausible explanation for the transient decrease of Pol3 would be that Pol3 underwent regulated protein degradation induced by UV. We have supplemented the growth media with the proteasome inhibitor MG132. Indeed, in the presence of MG132, the UV-induced degradation of Pol3 was completely abolished.

Polδ is a heterotrimer and consists of two additional non-catalytic subunits, Pol31 and Pol32, besides Pol3. We aimed to examine whether the whole Polδ enzyme is subject to UV-induced proteolysis, or only the catalytic subunit is affected. We found that contrary to Pol3, Pol31 and Pol32 were not affected by UV-induced degradation and probably remain at the fork. We postulated that a TLS polymerase, like Rev1 could take the place of Pol3 and carry out lesion bypass in complex with Pol31 and Pol32. For this we examined whether Pol31 and Pol32 together could form a complex with Rev1 in *in vitro* assays using purified proteins and we could detect complex formation between GST-Pol32, Pol31, and Rev1, as indicated by the presence of all three proteins in the elution fraction.

DISCUSSION

In this thesis we identify DEF1 as a member of the *REV3* branch of the *RAD6/Rad18*-dependent DNA damage tolerance pathway and a prerequisite for induced mutagenesis in yeast. Def1 is as a key factor for polymerase exchange. We presented evidence that upon UV-irradiation Def1 promoted the proteosomal degradation of Pol3, the catalytic subunit of the replicative DNA polymerase, Polδ, while Pol31 and Pol32, the other two subunits of Polδ were not degraded. We also demonstrated that Pol31 and Pol32 together could form a stable complex with TLS polymerase Rev1.

Our data imply that translesion synthesis polymerases carry out DNA lesion bypass in complex with Pol31 and Pol32, only after the Def1- assisted removal of Pol3 from the stalled replication fork.

Based on these results we propose a new model for polymerase exchange at stalled replication forks

During replication, when Polδ stalls at a DNA lesion, PCNA gets ubiquitylated by Rad6/Rad18. Mono-ubiquitylated PCNA activates the mutagenic pathway where, for translesion synthesis to occur, Pol3 is removed from the stalled Polδ complex by a Def1-dependent manner through proteasome-mediated protein degradation. We assume that a mutagenic TLS polymerase takes over the place of Pol3 and teams up with the remaining Polδ subunits, Pol31 and Pol32, at the stalled fork to form a new complex capable of executing DNA lesion bypass.

We surmise that after lesion bypass and deubiquitylation of PCNA, the TLS polymerase is removed from the primer terminus, Pol3 restores Polδ by regaining its place, and replication continues. Importantly, this finding also gives an explanation for previous genetic results showing that in *pol32* cells induced mutagenesis is severely impaired, and that all subunits of Polδ are necessary for induced mutagenesis.

Our data raises an interesting question: how the *RAD30*-encoded TLS polymerase, Polη can operate independently of Def1? Our results imply that Pol3 does not have to be removed from the stalled fork for Polη-dependent UV-lesion bypass to occur. Polη is mainly specialized for the error-free bypass of cyclobutane pyrimidine dimers, formed upon UV-irradiation. Since UV is one of the most

common DNA damage sources most living things are exposed to, it is reasonable to assume that Polη should have preference over the other error-prone TLS polymerases in the bypass of UV-induced DNA lesions.

Polη, a Y family polymerase, differs from the other TLS polymerases, Rev1 and Polζ, in its way of binding PCNA. While Rev1 and Polζ bind the intermolecular interface at the outer face of the PCNA ring [172, 173], Polη, similarly to Polδ, binds the interdomain connector loop of PCNA through its conserved PCNA-interacting peptide motif. Given, that PCNA is a homotrimer ring, Polδ and Polη could bind the same PCNA ring simultaneously.

We presume that transient conformational changes, probably induced by the stalling of the fork and ubiquitylation of PCNA, could allow Polη to take over synthesis from Polδ, while both remain attached to PCNA. Since Polη synthesizes opposite pyrimidine dimers with the same kinetics as it does opposite undamaged DNA, rapid bypass can occur. Deubiquitylation of PCNA would restore the original conformation and Polδ could continue synthesis. A similar mechanism has already been described in bacteria.

Many elements of DNA lesion bypass proved to be highly conserved from yeasts to humans. The role of TLS polymerase in mutagenesis and in cancer makes it highly important to identify the human homologue of Def1.

PUBLICATIONS

Rentsendorj O, Nagy A, Sinkó I, **Daraba A**, Barta E, and Kiss I- Highly conserved proximal promoter element harboring paired Sox9-binding sites contributes to the tissue- and developmental stage-specific activity of the matrilin-1 gene. **Biochem J.** 2005 Aug 1; 389:705-716.(IF$_{2005}$: 4,224)

Ianovici N, **Daraba A**, Postelnicu M and Kiss I- Studiul Bioparticulelor aeropurtate in Timisoara -România (Study of airborne bio-particles in Timisoara-România). **Revista Lucrări Ştiinţifice. Seria Agronomie (Scientific works Journal. Agronomy series)** 2007, vol. 50, 2: 501-506; ISSN (electronic): 2069-7627; CNCSIS code: 477

Daraba Andreea. Regulation of translesion synthesis. (2007). Szeged University Biology Ph.D. School Seminars, Szeged, Hungary published in *Acta Biologica Szegediensis* 51 (2) p.137-160 Dissertation summary

Daraba A, Gali VK, Halmai M, Haracska L, Unk I (2014) Def1 Promotes the Degradation of Pol3 for Polymerase Exchange to Occur During DNA Damage-Induced Mutagenesis in *Saccharomyces cerevisiae*. **PLoS Biol** 12 (1): e1001771. doi:10.1371/journal.pbio.1001771; (IF$_{2013}$: 12,690).

Conference presentations:

1. **Daraba Andreea** and Kiss Ibolya- Fuctional analysis of the regulatory regions of the matrilin-1gene, International Training Course: Proceedings of the Closing Seminar, Biological Research Center Hungarian Academy of Science, Szeged, Hungary, 2004 August 4-6
2. Blastyák András, Hajdu Ildikó, Szukacsov Valeria, **Daraba Andreea**, Unk Ildikó and Haracska Lajos- Role of def1 gene in the post-replication repair machinery; The 6th Hungarian Genetic Congress, 13th Cell and Development Biology Days - Eger, 2005 April 10-12
3. Szukacsov Valéria, Blastyák András, **Daraba Andreea**, Unk Ildikó and Haracska Lajos- A new member of DNA repair machinery; Straub Days Biological Research Center of the Hungarian Academy of Sciences -2005 november 16-18
4. Ianovici Nicoleta, **Daraba Andreea**, Postelnicu Mihaela, Kiss Izabela and Matis Adriana (2007)– *Study of airborne bio-particles in Timisoara***,** *România,* Scientific Conference "ROMANIAN AGRICULTURE IN THE EU - OPPORTUNITIES AND PERSPECTIVES", University of Agricultural Sciences and Veterinary Medicine, Faculty of Agriculture, Iasi, 18-19 October 2007
5. Halmai Miklós, Gali Vamsi Krishna, **Daraba Andreea** and Unk Ildikó- Rescue of the stalled replication fork; Straub Days Biological Research Center of the Hungarian Academy of Sciences-2008 december 3-5
6. **Daraba Andreea,** Gali Vamsi Krishna, Halmai Miklós and Unk Ildikó- Polymerase exchange at stalled replication forks; Straub Days Biological Research Center of the Hungarian Academy of Sciences-2012 May 23-24
7. **Daraba Andreea,** Gali Vamsi Krishna, Halmai Miklós, Haracska Lajos and Unk Ildikó- DNA damage induced polymerase exchange at stalled replication forks; Hungarian Molecular Life Sciences 2013,5-7 April, Siófok, Hungary
8. **Daraba Andreea,** Gali Vamsi Krishna, Halmai Miklós, Haracska Lajos and Unk Ildikó - Polymerase exchange at replication forks stalled at DNA damage sites; Central European DNA Repair Meeting, 2013, November 8th, Vienna, Austria

Posters:

1. Sinkó Ildikó, Nagy Andrea, Rentsendorj Otgonchimeg, **Daraba Andreea**, Barta Endre and Kiss Ibolya- *Cartilage-specific Sox transcription factors bind to the conserved proximal promoter element of the matrilin-1 gene*; 30th FEBS Congress-9th IUBMB Conference, Budapest, Hungary (2-7 July,2005)
2. **Daraba Andreea** -*New player in the post replication repair*; 1st ITC Alumni Meeting 2006-Modern trends in biological sciences: Seeking an integrative approach, , Szeged, Hungary (19-21 October,2006)
3. **Daraba Andreea,** Gali Vamsi K., Halmai Miklós, Unk Ildikó – Polymerase exchange at replication forks stalled at sites of DNA damage in Saccharomyces cerevisiae; 38th FEBS Congress, „Mechanisms in Biology”; July 6th, 2013; Saint Petersburg, Russia