Genetic analysis of PCNA mutants affecting DNA repair and DNA damage tolerance pathways

Main points of the Ph.D. thesis

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Introduction and background:

DNA repair and DNA damage tolerance processes, popular research topics for a while, came even more into the spotlight with the advent of the so called targeted tumor therapies.

The high interest in this processes is not a surprise at all, as some of the DNA repair and DNA damage tolerance genes are tumor supressors, others in turn oncogenes.

A rare, but brilliant example of therapeutical applications of basic DNA repair research results is the use of PARP inhibitors for the treatment of BRCA1 or BRCA2 defective ovarian cancer. Tumors carriing inactivating mutations of the above mentioned genes are inefficient in repairing DNA double strand brakes through homologous recombination. PARP inhibitors in turn contribute to the rise in number of double strand brakes by inhibiting the repair of DNA single strand brakes. This leaves the cancerous cell population affected in DNA damage response capacity exposed to increased number of DNA brakes it can not cope with. Unfortunately success stories like this are still rare to find.

The primary model for DNA repair and DNA damage tolerance studies was the baker's yeast *(Saccharomyces cerevisiae).* As in case of almost every evolutionary conserved processes, most of the yeast DNA repair genes have their human homologues. The haploid genome of the yeast cells can also easily be edited in site specific manner.

Based on the results of yeast genetic studies DNA repair and DNA damage tolerance processes can be divided into several well defined pathways. These pathways differ in their efficiency to recognise and process certain types of DNA damage, as well as in their mechanism of action.

While the classical DNA repair pathways employ mechanisms that physically remove a part of the DNA that contains the damage by excising it, the so called DNA damage tolerance pathways (DDT) do not cut out the damage. They help the cells survive the negative side effects of carriing the damage (examples of DDT pathways are the recombination which copes with the presence of double strand DNA brakes, or the translesion DNA synthesis, which enables the rescue of replication forks stalled at the DNA damage sites). As in case of DNA damage tolerance the goal is to save the cell from immediate danger of apoptosis, and not to preserve the information stored in the DNA, some of this processes (mainly the translesion DNA synthesis) are often mutagenic, causing changes in the information content of the DNA.

It is common in all DNA repair and DNA damage tolerance mechanisms, that at some step they require DNA synthesis. PCNA protein is an indispensable requisite for DNA synthesis, serving as the processivity factor for DNA polymerases, a function which enables it to be present also at

DNA damage sites. Moreover today we can state that it is one of the main regulators of DNA damage response.

Inside the cells PCNA forms a homotrimer encircling the DNA, serving as docking site for various proteins along with DNA polymerases. It was shown that it interacts with several DNA repair proteins. PCNA K164 monoubiquitilation activates a branch of the DNA damage tolerance mechanisms called translesion DNA synthesis (TLS). The protein has three well characterised protein binding surfaces: the N-terminal, the C-terminal and the interdomain connecting loop region, the latter being the most important surfaces which binds most of the known interacting partners.

Besides the above mentioned surfaces there are further large exposed regions on the PCNA protein that haven’t been subjected to detailed genetic and biochemical analysis.

In my thesis I investigated the role of these regions with relatively unknown biological functions in the DNA repair and DNA damage tolerance mechanisms.

Aims:

The PCNA protein is one of the main regulators of DNA repair and DNA damage pathways. Since the regions with well characterised biological functions cover only a fraction of the protein's surface, our goal was to investigate the role of the less characterised regions of PCNA in the DNA repair and DNA damage tolerance processes, hoping to gain deeper insight into DNA damage response regulation.

Implementation of this research process was planned through the following steps:

1. To produce point mutant yeast (*Saccharomyces cerevisiae*) *POL30* (PCNA) genes in which the site directed point mutation causes a desired amino acid replacement.
2. To create yeast strains which express the modified PCNA proteins as a sole PCNA source.
3. To filter out PCNA mutant yeast strains (by exposing the cells to DNA damage and assaying their sensitivity) in which the introduced point mutation interferes with some of the DNA repair or DNA damage tolerance functions of the PCNA protein.
4. To map the effect of the studied point mutation into one of the established DNA damage response pathway using genetic epistasis analysis.
5. To show that the studied amino acid replacements affect a certain biological process (e.g. DNA damage induced mutagenesis, apontaneous mutagenesis, NER) performed by proteins of the pathway it was mapped to.
6. Trying to explain the molecular mechanism by which the PCNA amino acid replacement affects the given DNA repair process, with the use of biochemical assays.

Methods:

Yeast genetics methods and DNA maniplation techniques:

-PCR based site directed mutagenesis for creation of the point mutant *POL30* (PCNA) genes.
-Gene disruption based on homologous recombination in yeast cells.
-Molecular cloning steps: Cloning the point mutant *POL30* genes into yeast centromeric vectors, and creation of other constructs specified in the thsesis.
-Integration of the point mutant POL30 genes into the genome at the original *POL30* locus taking advantage of homologous recombination.
-Qualitative sensitivity assays (spot assays): comparison of the sensitivity of various yeast strains to DNA damaging agents (UV, MMS, HU, X-rays, bleomycin).
-Quantitative sensitivity assays (survival curves): The percentual survival of yeast strains as a function of the DNA damage dose (UV) applied.
-Measurement of spontaneous and UV damage induced mutation rates on L-canavanine contaaining plates.
-Measurement of the effect of UV damage on the gene expression levels using *LacZ* reporter gene.

Biochemical procedures:

-Overexpression and purification of tegged proteins.
-GST-pulldown assays for showing *in vitro* protein interactions.
-Native (SDS free) gradient acrilamide gel electroforesis for multimer stability assesment.

Results and discussion:

1. With PCR based site directed mutagenesis approach we created 10 point mutant yeast *POL30* gene variants. Each carries a different mutation resulting in a single or tandem amino acid replacements to alanines at desired places of the PCNA protein.
2. The created genes were expressed from yeast centromeric constructs as a sole PCNA source in modified yeast strains made defective for the genomic copy of *POL30*.
3. All yeast strains expressing mutant *POL30* variants proved to be viable. Only two of them showed temperature sensitive growth defect which reveals that in this cases the introduced mutations affected the replication promoting function of PCNA. This strains were excluded from further evaluation, as we wanted to examine strains with DNA repair and DNA damage tolerance defects, instead of those with replication defects.
4. Out of the remaining eight strains six showed increased sensitivity to UV and MMS treatment, suggesting that the PCNA amino acid replacements in them inhibited the activity of some DNA repair or DNA damage pathway.
5. In five of these strains with increased sensitivity the amino acid replacement is located near the subunit interface on an exposed beta sheet (*pol30-II99,100AA*; *pol30-D109A*; *pol30-II181,182AA*; *pol30-DI109,167AA* és *pol30-FE103,104AA*), while in the sixth one the modification affects an alpha helix on the inner surface of the PCNA facing the DNA (*pol30-L154A*).
6. Taking advantage of epistasis analysis, we tried to map the effect of the point mutations into one of the existing DNA repair or DNA damage tolerance pathways. Out of the point mutations resulting in amino acid replacement near the subunit interface the effect of *pol30-II99,100AA* could be mapped to the Rad6/Rad18 DNA damage tolerance (DDT, but also known as PRR) pathway, the effect of *pol30-II181,182AA* to the epistasis group of homologous recombination, while the effect of the *pol30-D109A* mutation affected the NER pathway. The *pol30-L154A* allele that causes amino acid replacement on the surface facing the DNA, similarly to the *pol30-II99,100AA* allele showed genetic interaction with the Rad6/Rad18 DNA damage tolerance pathway (PRR).
7. Using functional assays and biochemical experiments we proved that the II99,100AA PCNA amino acid replacement inactivates the mutagenic translesion synthesis (TLS) branch of the Rad6/Rad18 DNA damage tolerance pathway. In the strain producing this mutant form of PCNA, creation of point mutations can not be induced with UV exposure. The observed phenotype is most likely caused by the fact that this PCNA variant is- as opposed to the wild type form- not capable of forming interactions with the Rev1 translesion synthesis DNA polimerase. The D109A PCNA variant interferes with one of the mechanisms of activation of the NER pathway. PCNA which contains the L154A modification inactivates the complete Rad6/Rad18 DNA damage tolerance pathway, the strain producing the mutant form is defective for UV induced mutagenesis. The presence of this mutant allele in the cells supresses

the high UV and MMS sensitivity of the *rad18* gene deletion, a phenotype it shares with only one *POL30* allele known from literature (*pol30-K164R*) which renders the protein defective for ubiquitination. The *pol30-L154A* strain also exhibits a strong mutator phenotype, with increased spontaneous mutation rates comparable to that of DNA polimerase proofreading mutant strains, suggesting that the studied mutant PCNA version could influence the fidelity of the replicative DNA polimerase attached to it.

8. According to results presented in the thesis, the immediate vicinity of the subunit interface is a region of high importance for DNA repair and DNA damage tolerance where certain amino acids despite spatial vicinity modulate different processes.

Summary:

In our work we created point mutations in the yeast (*Saccharomyces cerevisiae*) *POL30* gene encoding for PCNA, one of the main regulators of DNA repair and DNA damage tolerance pathways. The mutations were introduced with site directed mutagenesis, and cause chosen amino acid replacements at protein level. The locations of the amino acids that have been replaced are in less characterized regions of the PCNA. The replacements always manifest as switches to alanine.
Studies of the yeast strains containing the mutant genes and expressing the mutant proteins revealed that the immediate vicinity of the subunit interface forms a regulatory surface for DNA damage tolerance and DNA repair. Parts of this surface are implicated in the function of various DNA damage response pathways (NER, HR, translesion DNA synthesis).
We also characterized a PCNA amino acid replacement that inactivates the entire Rad6/Rad18 DNS damage tolerance (DDT) pathway. In this case the amino acid replacement happened farther from the subunit interface on the inner surface facing the DNA.

List of publications:

1. The two publications that form the basis of the doctoral procedure:

Halmai M, Frittmann O, Szabó Z, Daraba A, Gali VK, Bálint E, Unk I. Mutations at the Subunit Interface of Yeast Proliferating Cell Nuclear Antigen Reveal a Versatile Regulatory Domain. PLoS One. 2016. doi: 101371/journal.pone.0161307. IF (2015/2016) 3,057

Daraba A, Gali VK, **Halmai M**, Haracska L, Unk I. Def 1 promotes the degradation of Pol3 for polymerase exchange to occur during DNA-damage-induced mutagenesis in Saccharomyces cerevisiae. PLos Biol. 2014. doi: 10.1371/journal.pbio.1001771. IF (2014) 9,343

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Total IF: 12,40

2. Publications in peer reviewed journals:

2.1. Publications supporting the dissertation:

Halmai M, Frittmann O, Szabó Z, Daraba A, Gali VK, Bálint E, Unk I. Mutations at the Subunit Interface of Yeast Proliferating Cell Nuclear Antigen Reveal a Versatile Regulatory Domain. PLoS One. 2016. doi: 101371/journal.pone.0161307. IF (2015/2016) 3,057

2.2. Additional publications:

Daraba A, Gali VK, **Halmai M**, Haracska L, Unk I. Def 1 promotes the degradation of Pol3 for polymerase exchange to occur during DNA-damage-induced mutagenesis in Saccharomyces cerevisiae. PLos Biol. 2014. doi: 10.1371/journal.pbio.1001771. IF (2013) 11,771

3. Other scientific achievements:

Conference lectures:

Miklós Halmai, Zoltán Szabó and Ildikó Unk. PCNA at the crossroad of DNA repair pathways. Straub-Napok, Szeged, 2016.

Miklós Halmai, Orsolya Frittmann, Zoltán Szabó, Vamsi K. Gali, Éva Bálint and Ildikó Unk. Genetic analysis of PCNA mutations in *Saccharomyces cerevisiae.* 6th Central European Genome Stability and Dynamics Meeting, Szeged, 2015.

Andreea Daraba**,** Vamsi K. Gali, **Miklós Halmai**, Lajos Haracska, Ildikó Unk. Polymerase exchange at replication forks stalled at DNA damage sites. 4th Central European Genome Stability and Dynamics Meeting, Vienna, 2013.

Andreea Daraba, Vamsi K. Gali, **Miklós Halmai**, Lajos Haracska, Ildikó Unk. DNA damage induced polymerase exchange at stalled replication forks. Hungarian Molecular Life Sciences, Siófok, 2013.

Andreea Daraba, Vamsi Krishna Gali, **Miklós Halmai**,
Lajos Haracska and Ildikó Unk. Polymerase exchange at stalled replication forks in *Saccharomyces cerevisiae*. Straub-Napok, Szeged, 2012.

Halmai Miklós. A PCNA fehérje szerepe a mutagenezisben. Szegedi Biológus Doktorandusz Konferencia. Szekció előadói díj. 2011

Miklós Halmai, Vamsi Krishna Gali, Andreea Daraba, Ildikó Unk. Rescue of the stalled replication fork. Straub-Napok, Szeged, 2008.

Halmai Miklós, Daraba Andreea, Gali Vamsi Krishna, Unk Ildikó. DNS-hiba tolerancia utak szabályozása. ”Genetikai Műhelyek Magyarországon” VII. Genetikai Minikonferencia, Szeged, 2008.

Posters:

Daraba Andreea, Gali Vamsi Krishna, **Halmai Miklós**, Unk Ildikó. Polymerase exchange at replication forks stalled at sites of DNA damage in *Saccharomyces Cerevisiae*. 38th FEBS Congress *„Mechanisms in Biology”*. Saint Petersburg, Russia, 2013