RAD18-dependent Activation of FAN1 by Ub-PCNA to Rescue DNA Interstrand crosslinks along with TLS Pol η

Summary of Ph.D. Dissertation

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Introduction

Cytotoxic covalent bonds, DNA interstrand crosslinks (ICLs), can be formed by antitumor agents such as cisplatin and psoralens. DNA ICL lesions can stall the ongoing replication fork and can further cause genomic instability. Fanconi anemia (FA) pathway is one of main DNA repair pathways that bypass the cellular ICLs so that the stalled replication fork can be restored. Interruption of FA repair pathway can lead to Fanconi anemia, which is a rare genetic disorder characterized by abnormalities of the bones and skin, bone marrow failure, endocrine disorders and hypersensitivity to crosslinking agents. Replication-associated ICL repair is tightly controlled by the phosphorylation and ubiquitination signal transduction mechanisms. When the replication fork stalls due to an ICL, ssDNA is coated by the replication protein A (RPA), which is a signal for the autophosphorylation of the ataxia telangiectasia and Rad3-related (ATR) protein at Thr-1989. The activated phosphorylated ATR, as a protein kinase, can further activate the serine 1045 residue of FANCM (FAAP250). Phosphorylated FANCM acts as an anchor protein for the FANC core complex assembly and further induces the monoubiquitination of FANCD2/FANCI (ID2) in the FA pathway. The monoubiquitinated ID2 complex is able to interact with ID2-associated nuclease 1 (FAN1) via its UBZ domain. The activated FAN1 can digest the ICL-neighboring region due to its structure-specific endonuclease activity thus facilitating the bypass of the lesion. Proliferating cell nuclear antigen (PCNA) is a homotrimer DNA clamp protein, which plays an essential role during DNA replication. PCNA is loaded onto the leading strand of the DNA replication machinery with the help of replication factor C (RFC). The Rad18 DNA damage tolerance (DDT) pathway can resolve stalled replication forks during DNA replication. Upon DNA lesion, the lysine 164 residue (K164) of PCNA can be monoubiquitinated by Uba1 (E1), Rad6 (E2), and Rad18 (E3). Monoubiquitinated PCNA acts as central hub to recruit UBZ domain-containing TransLesion synthesis (TLS) polymerases. Previous studies indicate that FAN1 also possesses an uncharacterized PIP-box, and its potential function with Ub-PCNA needs to be demonstrated. Moreover, previous evidences suggest that TLS polymerases play a role in the process of unhooking ICLs. Therefore, we are also going to examine which TLS polymerase can facilitate the bypass of ICL lesions together with FAN1.

Aims

The main goal of this study is to investigate whether FAN1 operates in the RAD18 DDT pathway and contributes to ICL bypass in cooperation with Ub-PCNA and TLS polymerases. The specific research questions are the followings:

-Does FAN1 interact with Ub-PCNA via its PIP box or UBZ domain?

-Can Ub-PCNA interaction affect the nuclease activity of FAN1?

- Are TLS polymerases able to cooperate with FAN1 for coordinated ICL lesion bypass and rescue of the stalled replication fork?

-Finally, to understand detailed molecular role of FAN1. I plan to reconstitute ICL bypass *in vitro* using substrates mimicking ICL and highly purified repair proteins.

In order to achieve the above goals, the following specific experiments are proposed:

- Purification of FAN1, FAN1 mutants' proteins and other DNA repair proteins (PCNA, TLS polymerases (η, κ, ι) by affinity chromatography
- Determining the domain that is responsible for the interaction between FAN1 and PCNA/Ub-PCNA by comparing FAN1 mutant derivatives:
    I. Examining physical protein-protein interactions with pull-down assays
    II. Investigating the effect of FAN1 nuclease activity in the presence of PCNA or Ub-PCNA and various DNA substrates
- Examining which Y-family TLS polymerase can cooperate with FAN1 in the rescue of the stalled replication *in vitro*
- Characterizing the function of TLS polymerase η with FAN1 in bypassing TMP-induced ICL substrates

Materials and Methods

***In vivo* approaches:**

- Cell culture and cell transfection
- Co-Immunoprecipitation
- Immunostaining and microscopy
- Biomolecular fluorescence complementation assay
- Resazurin-based cell viability assay

***In vitro* approaches:**

- Cloning of plasmids for protein expression
- Mutagenesis PCR
- Protein purification by affinity chromatography
- Pull-down assay
- Generate site-specific interstrand-crosslinked oligonucleotides
- Nuclease assay
- TLS polymerase assay
- Western Blot

Results:

FAN1 is involved in the RAD6/RAD18 DDT pathway:

We have applied various *in vivo* approaches such as co-immunoprecipitation and colocalization study. The results indicated that FLAG-FAN1 immobilized on the anti-FLAG-beads along with RAD18. FAN1 does not form nuclear foci in undamaged cells, while it forms clear nuclear foci and colocalizes with ubiquitinylated PCNA upon cisplatin treatment. However, the various mutants completely lost DNA-damage induced FAN1 foci formation and did not colocalize with ubiquitinylated PCNA.

Pull-down assay and purification of the FAN1 protein and FAN1 mutants by affinity chromatography:

In order to investigate the direct protein-protein interaction, we have carried out the pull-down assay that based on the affinity chromatography. We found that FAN1 bound the ubiquitinated form of PCNA preferentially and that the mutation in the FAN1 UBZ domain completely disrupted this binding. The PIP mutations of FAN1 impaired FAN1 binding to PCNA, its ability to bind to Ub-PCNA remained partially, indicating that FAN1 can bind to PCNA via its two PIP domains and to ubiquitin via its UBZ domain

The nuclease activity of FAN1 can be stimulated by PCNA via PIP-box:

We have applied highly purified proteins for the nuclease assay, and by using RFC protein to load PCNA on the 5' flap DNA substrate. The results indicated that the enhanced nuclease activity of FAN1 WT with increased PCNA concentration, but FAN1 PIP mutant does not exhibit the enhanced nuclease activity.

Both PIP-box and UBZ-domain of FAN1 is regulated by Ub-PCNA:

After loading Ub-PCNA by the help of RFC in the nuclease assay, FAN1 nuclease activity was significantly more than in the case of unmodified PCNA. To confirm my finding, I have applied FAN1 PIP/UBZ in the nuclease assay. Mutations in the UBZ domain of FAN1 impaired the robust Ub-PCNA-dependent stimulation of the FAN1 nuclease.

PCNA can spatially regulate FAN1 function:

During our nuclease assay, when we examined the nuclease activity of FAN1 in the presence of PCNA, we could observe that the PCNA was able to alter the specificity of FAN1 incision site near to the 5' end of the flap to 4 nt 3' to the branch point.

TMP-induced interstrand crosslink substrate:

In order to mimic the cellular process of FAN1 bypassing ICL, we have generated 5'-AT dinucleotides as cross-link site containing 73 nt 5' flap DNA substrate by 4,5′,8-Trimethylpsoralen.

FAN1 and Pol η can bypass ICL in the presence of Ub-PCNA:

We applied highly purified proteins and reconstitute the whole process of ICL lesion bypass *in vitro*. By comparing with different TLS polymerases, we found that bypass can occur by the coordinated action of FAN1, Ub-PCNA and Pol η.

FAN1 processes ICL lesions and recruits TLS Pol η on the lesion site:

Upon cisplatin treatment, FAN1 can functionally interact with Pol η as YFP positive cells in the BiFC assay. And this functional interaction was further demonstrated by sensitivity assay. The results show that in the absence of either Pol η or FAN1 alone the HEK293 cells showed increased sensitivity to cisplatin compared to the shControl cells, however silencing both Pol η and FAN1 did not sensitize the cells further, reflecting epistatic relationship between FAN1 and Pol η.

Summary

In our current study, we prove that FAN1 is indeed regulated by the Rad 18 DDT pathway *in vivo,* and it colocalizes with Ub-PCNA. We demonstrate *in vitro* that the endonuclease activity of FAN1 can be enhanced by PCNA/Ub-PCNA. Furthermore, we show that both the PIP-box and the UBZ-domain can interact with Ub-PCNA. We could also demonstrate that the digestion specificity of FAN1 can be altered in the presence of PCNA. PCNA is able to position FAN1 to cut at a specific site (after a junction of 4 nt) on the 5' flap substrate, thus it can facilitate ICL bypass. Furthermore, we compare three TLS polymerases (pol η, pol ι, and pol κ) for bypassing the ICL lesion. Pol η is the one TLS polymerase which is able to process the blocking ICL. We also provide evidence for FAN1-pol η epistasis by cell survival assays and FAN1and Pol η have functional interaction upon cisplatin treatment. To sum up, our current results indicate that FAN1 does not only play a role in the FA pathway, but it also participates in the RAD18 DDT pathway, acting in the rescue of stalled replication. Therefore, here we propose a new model for the function of FAN1: upon encountering ICL lesions, the Ub-PCNA-dependent FAN1 nuclease can unhook the covalent bond between the strands of the DNA helix, the unhooked substrate can be further processed by TLS Pol η, and thus stalled replication can be rescued. FAN1 may be a cross-talk protein which regulates two pathways in different cell cycle stages. Our model provides important scientific insight into the repair mechanism of DNA interstrand crosslinks and reveals fine details of the process.

List of Publications

MTMT number: 10061209

1. Mandatory peer-reviewed international publications for the fulfillment of the doctoral process

- Biophysical characterization of histone H3.3 K27M point mutation. Szabolcs Hetey, Beata Boros-Olah, Tímea Kuik-Rozsa, **Qiuzhen Li**, Zsolt Karanyi, Zoltan Szabo, Jason Roszik, Nikoletta Szaloki, Gyorgy Vamosi, Katalin Toth, Lorant Szekvolgyi. **Biochem Biophys Res Commun**. 2017 Aug 26;490(3):868-875. doi: 10.1016/j.bbrc.2017.06.133 (IF: 3.575)
- Coordinated Cut and Bypass: Replication of Interstrand Crosslink-Containing DNA. **Qiuzhen Li**, Kata Dudás, Gabriella Tick and Lajos Haracska.(2021) **Front. Cell Dev. Biol**. 9:699966. doi: 10.3389/fcell.2021.699966 (IF: 6.684)

Summarized IF: 10.259

2. Publications are submitted and in peer-reviewing process

• FAN1 and Pol η constitute a pathway for replicational bypass of DNA interstrand crosslinks under the control of RAD18-dependent PCNA-ubiquitylation. Qiuzhen Li, Mónika Mórocz, Szilvia Juhász, Lili Hegedűs, Alexandra Gráf, Ádám Sánta, Gaurav Sharma, Péter Burkovics, Ernő Kiss and Lajos Haracska

• Ubiquitylate for tolerance: regulatory mechanisms at the stalled replication fork. Lili Hegedűs, Kata Dudás, Qiuzhen Li, Indra Balogh, Gabriella Tick, Lajos Haracska. Peer reviewing in Cancers Journal (IF:6.639).

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Declaration

I declare that the contribution of Qiuzhen Li was significant in the listed publications and the doctoral process is based on the publications listed. The results reported in the Ph.D. dissertation and the publications have not been used to acquire any PhD degree previously and will not be used in the future either.

Nyilatkozat

Kijelentem, hogy Qiuzhen Li hozzájárulása jelentős volt a felsorolt kiadványokban, és a doktori folyamat a felsorolt kiadványokon alapul. A Ph.D. disszertációban közölt eredményeket és a publikációkat korábban nem használták PhD fokozat megszerzésére, és a jövőben sem fogják.

Szeged, 2021.08.24

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