Final report

Our plan was to achieve a better understanding of how repair mechanisms are selected at the stalled replication fork. Normally, our DNA is continuously damaged by endogenous and exogenous agents. The damaged DNA can cause significant problems for the cell at the S phase of the cell cycle because often replicative DNA polymerases cannot handle the damaged template, thus, the replication fork stalls. Prolonged stalling can induce apoptosis and cell death, but the error-prone rescue (damage bypass) of the replication fork may result in point mutations or genome instability, which can lead to cancer formation and progression. Therefore, it is essential to minimalize error-prone damage bypass. Several DNA repair mechanisms have evolved to rescue the stalled replication fork, e.g., the damage tolerance pathway (DDT), the Fanconi anemia (FA) pathway, and homologous recombination (HR), which can bypass the lesion in an error-prone or error-free manner. Currently, there is not enough information about the mechanism of selection between these DNA repair mechanisms. Since the outcome of the bypass affects cancer formation and progression, it is very important to clarify these regulatory mechanisms.

PCNA SUMOylation is an important mechanism inhibiting unexpected HR events. Although we have previously demonstrated its importance in the inhibition of HR in human cells, the biochemical mechanism has not been clarified. As planned, we reconstituted the human D-loop extension system in vitro, modelling replication-associated DNA synthesis, which is the potential site of action of SUMO-PCNA. We tested human orthologues of the yeast Srs2 protein, namely, PARI, FBH1, and RTEL1 in this system because Srs2 is known to contribute to SUMO-PCNA-dependent HR inhibition in yeast. Only one of them, PARI, was able to inhibit HR in the presence of either PCNA or SUMO-PCNA. Both the PCNA-binding motif (PIP) and the SUMO-binding motif (SIM) of PARI were necessary for HR inhibition. RTEL1 and FBH1 did not affect replication-associated DNA synthesis in our in vitro model system. These data are published in the attached article (Burkovics et al. Nucleic Acids Res. 2016 Apr 20;44(7):3176-89).

The other part of the project focused on the function of Ub-PCNA. There are more and more proteins that have Ub-PCNA-binding specificity. We and others have described SPARTAN, the protein in the focus of our research. Another Ub-PCNA-binding protein is Mgs1 in yeast and WRNIP1 in humans. Additionally, in our current research project we identified a new Ub-PCNA-binding protein, FAN1.

FAN1 is an interesting protein because it is a structure-specific endonuclease. Previously, it has been published as a new and important member of the Fanconi anemia (FA) pathway [1-8], which is responsible for interstrand crosslink (ICL) repair and causes a rare genetic disorder called Fanconi anemia, which, for example, displays a cancer-prone phenotype. Based on major facts:

- FAN1 can interact with the ubiquitylated form of FANCD2, which is the major component of the FA pathway in which it recruits downstream effectors to ICLs,
- FAN1-deficient cells are extremely sensitive to crosslinking agents *in vivo,* and
- FAN1 has a structure-specific nuclease activity which is able to process ICL-containing DNA substrates.

Furthermore, later it was published that the absence of FAN1 in humans does not result in a classical FA phenotype, but these patients show Karyomegalic Interstitial Nephritis (KIN) [9]. Moreover, it has been demonstrated in chicken DT40 cells that fan1 does not show any relationship with the Fanconi genes [8]. These facts indicate that FAN1 most probably acts as an FA-independent protein, which could represent a new ICL-specific DNA repair pathway.

We found a PCNA-interacting site in the FAN1 sequence, therefore, we checked its interaction with PCNA. Since they can interact both in vitro and in vivo (Figure 1), we tested the functional relationship between FAN1 and the DNA Damage Tolerance (DDT) pathway. We proved that FAN1 is a member of the DDT pathway and not of the FA pathway. We demonstrated that although FANCD2 ubiquitylation is necessary for the correct targeting of FAN1, Ub-PCNA is also required. We demonstrated that FAN1/Ub-PCNA/Ub-FANCD2 forms a functional protein complex in vivo and in vitro as well (Figure 2).

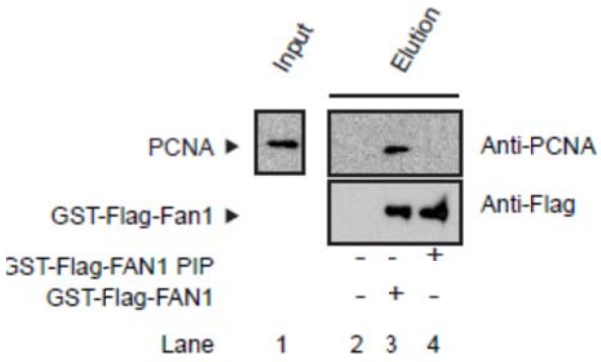

Figure 1: FAN1 binds to PCNA. FAN1 and PIP mutant FAN1 were bound to glutathione beads. PCNA was added. The beads were washed, and the bound fraction was analysed by Western blotting.

A B

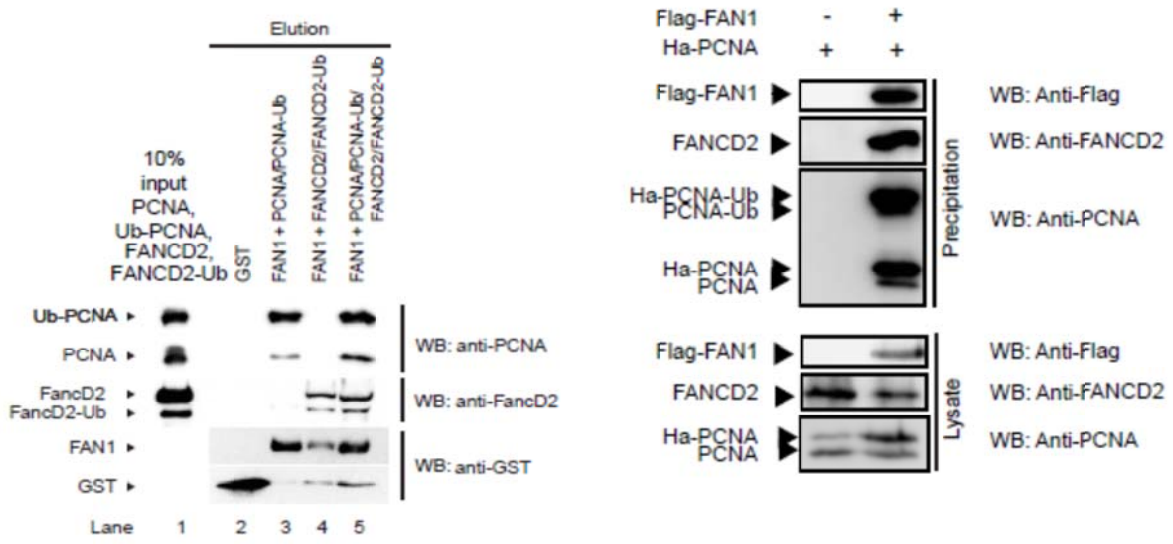

Figure 2: FAN1/FANCD2/Ub-PCNA forms a complex *in vivo* and *in vitro*. A) FAN1 can interact with FANCD2 and UB-PCNA at the same time. FAN1 was bound to glutathione beads. Ub-PCNA and FANCD2 were added. The beads were washed, and the bound fraction was analysed by Western blotting. B) FAN1/FANCD2/Ub-PCNA forms a complex *in vivo*. Human HeLa cells were transfected with Flag-FAN1 and Ha-PCNA. Cell extract was bound to anti-Flag beads. After washing, the bound fraction was analysed by Western blotting.

Therefore, we analysed the endonuclease activity of FAN1. FAN1 has been published to be a structure-specific endonuclease that shows preference to 5' flap DNA structures. Our major goal was to analyse how PCNA and FANCD2 affects the enzymatic activity of FAN1. We were able to demonstrate that PCNA stimulates the enzymatic activity of FAN1 and determine its cleavage site (Figure 3). Moreover, we analysed the effect of PCNA ubiquitylation on the endonuclease activity of FAN1. Unfortunately, we were not able to demonstrate any difference in the nuclease activity of FAN1 in the presence of PCNA or Ub-PCNA. We were also not able to observe any difference in the endonuclease activity of Fan1 in the reaction supplemented with FANCD2. Currently, we are optimizing the reaction conditions to obtain positive results using PCNA and FANCD2 together, and we are preparing the manuscript for submission.

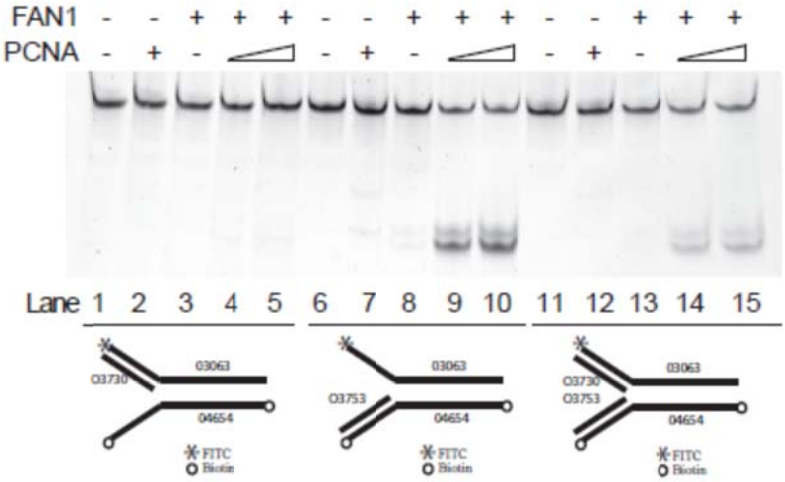

Figure 3: PCNA stimulates the endonuclease activity of FAN1. Fluorescently labelled replication fork-like substrates were incubated with Fan1 and PCNA as indicated. Reactions were resolved on denaturing polyacrylamide gel. Cleaved DNA appeared in lanes 9, 10 and 14, 15.

Our work plan contained the detailed analysis of the function of SPARTAN. SPARTAN is known to be an important protein because homozygous Spartan knock-out mice are lethal [10], and two human patients have been found who display progeria syndrome and are prone to cancer formation [11]. Previously, we and others have published that SPARTAN is a Ub-PCNA-binding protein that is responsible for the correct targeting of Polη to the stalled replication forks [12-16]. Moreover, it is also known that SPARTAN can recruit the p97 protein, which is responsible for the removal of ubiquitylated proteins [17, 18]. Most recently, a yeast protein called WSS1 has been described. The major function of WSS1 is the removal of DNA- protein crosslinks, which is an important and poorly characterized mechanism of the DNA repair pathways. Although there is only very little homology between SPARTAN and WSS1, they were suggested to be homologues [19-21].

During the analysis of the function of SPARTAN, we were the first to demonstrate that SPARTAN has a DNA-binding motif. SPARTAN binds to single-stranded DNA, Y-fork and double-stranded fork as well. A point mutation of the DNA-binding motif weakened the SPARTAN-DNA interaction, but the mutant did not lose its DNA-binding property completely. We demonstrated that cells containing DNA-binding site-mutant SPARTAN are more sensitive to UV damage then wild-type. Additionally, although the intranuclear localization of DNA-binding site-mutant SPARTAN is similar to the wild-type, its affinity to chromatin is decreased, indicating the in vivo functionality of this motif. Moreover, SPARTAN DNA-binding domain-mutant cells show reduced Polη foci formation ability, also indicating the importance of SPARTAN's DNA binding. Comparing several double and triple mutant forms of SPARTAN, we concluded that the DNA-binding site cooperates both with the PIP UBZ domain (with the Ub-PCNA) and the putative protease function (SprT domain). We published our results in the following article: The DNA-binding box

of human SPARTAN contributes to the targeting of Polη to DNA damage sites (Toth et al. DNA repair in Press)

Additionally, we tried to reveal the interaction network of SPARTAN. Unfortunately, interaction studies between SPARTAN and HLTF/WRNIP1/RAD18 were not successful because we were not able to detect any interaction. Additionally, we determined a residue of SPARTAN - K452 - which is ubiquitylated. Unfortunately, this mutant was only partially defective in the ubiquitylation and the further ubiquitylation sites, and the ubiquitin ligase of SPARTAN not found either.

In 2014, a new UBZ domain-contacting protein, ZBTB1, was characterized as an upstream regulator of the RAD18 pathway and PCNA ubiquitilation [22]. Since it has been suggested previously that BRCA1 and BRCA2 can also affect it [23, 24], we analysed how these proteins contribute to the regulation of the RAD18 function and PCNA ubiquitylation. We found that overexpression of ZBTB1 can partially complement the phenotype in damage tolerance caused by the loss of BRCA1 (Figure 4). In order to understand how the mechanism really works, we have to carry out more detailed experiments.

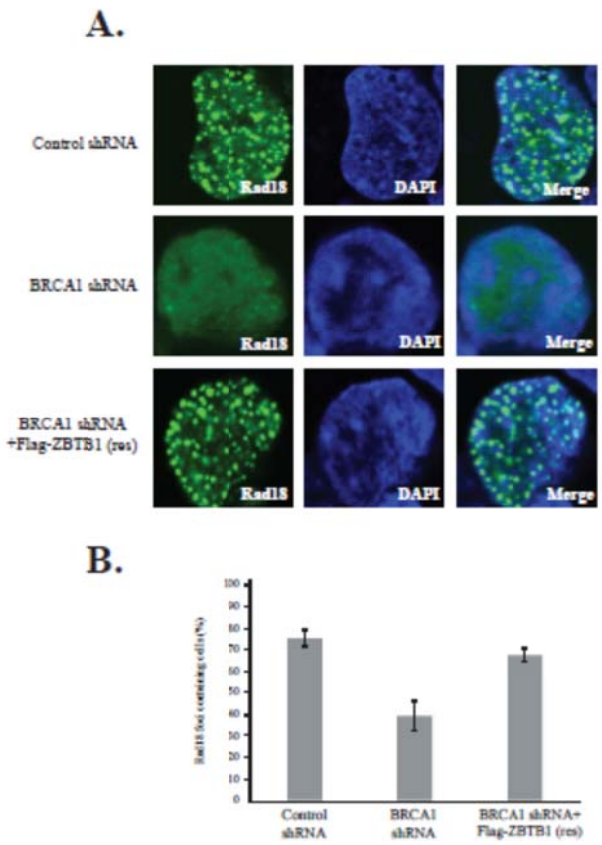

Figure 4: Overexpression of ZBTB1 complements the absence of ZBTB1 in the foci formation ability of RAD18. A) BRCA1-depleted cells were transfected with GFP-RAD18 and Flag-ZBTB1-expressing plasmids as indicated. The foci forming ability of RAD18 was analysed and quantified (B).

Summary

- **We clarified the biochemical basis of how homologous recombination is inhibited at the stalled replication fork.**
- **We explored a new FAN1-dependent ICL repair pathway and characterized its biochemical mechanism**
- **We determined a DNA-binding motif of SPARTAN and analysed its *in vivo* function.**
- **We analysed the ZBTB1 BRCA1 interplay in the DNA damage tolerance pathway *in vivo*.**

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