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Author: Agnes Toth Lili Hegedus Szilvia Juhasz Lajos Haracska Peter Burkovics

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The DNA-binding box of human SPARTAN contributes to the targeting of Polη to DNA damage sites

Agnes Toth[1*], Lili Hegedus[1*], Szilvia Juhasz[1], Lajos Haracska[1], Peter Burkovics[1]

[1]Institute of Genetics, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary
*These authors contributed equally to this work

Highlights

- SPARTAN binds ssDNA and fork-like DNA structures
- The DNA binding motif of SPARTAN maps next to the SprT domain
- The DNA binding by SPARTAN is important for Polη targeting to the stalled replication fork

Abstract

Inappropriate repair of UV-induced DNA damage results in human diseases such as Xeroderma pigmentosum (XP), which is associated with an extremely high risk of skin cancer. A variant form of XP is caused by the absence of Polη, which is normally able to bypass UV-induced DNA lesions in an error-free manner. However, Polη is highly error prone when replicating undamaged DNA and, thus, the regulation of the proper targeting of Polη is crucial for the prevention of mutagenesis and UV-induced cancer formation. Spartan is a novel regulator of the damage tolerance pathway, and its association with Ub-PCNA has a role in Polη targeting; however, our knowledge about its function is only rudimentary. Here, we describe a new biochemical property of purified human SPARTAN by showing that it is a DNA-binding protein. Using a DNA binding mutant, we provide *in vivo* evidence that DNA binding by SPARTAN regulates the targeting of Polη to damage sites after UV exposure, and this function contributes highly to its DNA-damage tolerance function.

1. Introduction

UV-induced DNA damage is one of the most common lesions originating from exogenous sources. Bypass of these lesions during the replication process is essential because its failure may lead to either point mutations or replication fork stalling, resulting in genome rearrangements via homologous recombination or cell death [1-3].

The DNA damage tolerance pathway (DDT) is an important pathway in the rescue of the replication fork that stalls when encountering damaged bases such as UV-induced thymine dimers or 6-4 photoproducts [4]. The Rad6/Rad18 ubiquitin ligase complex, whose major function is the monoubiquitylation of PCNA, coordinates these DDT pathways [5-7]. It has been described that in yeast cells the monoubiquitylation of PCNA by RAD18 is essential for translesion synthesis (TLS) [8]. Further processing of Ub-PCNA by RAD5, which results in the K63-linked polyubiquitylation of PCNA, channels the rescue process to the template switching pathway, which can proceed by D-loop intermediate or fork regression [9-17]. Although PCNA ubiquitylation-dependent regulation is tight in yeasts, in human cells, several other components affect pathway selection in DDT besides these posttranslational modifications, resulting in a more flexible regulatory mechanism [18-23].

Spartan is a recently identified member of the damage tolerance pathway whose function is not completely clear yet. The targeting of SPARTAN to the stalled replication fork depends mainly on its PCNA-interacting (PIP) and ubiquitin-binding zinc finger (UBZ) domains, which can mediate its interaction with Ub-PCNA. Additionally, SPARTAN plays an important role in the correct targeting of Polη to the stalled replication fork; in its absence, UV-induced Polη foci formation is highly defective [24-28]. Other publications suggest that SPARTAN is responsible for the targeting of p97 (VCP, vasoline-containing protein), which is an ATP-dependent segregase and facilitates the dissociation of Polη from the site of action [29, 30]. Although the importance of the UBZ and PIP domains in the targeting of Polη is clear, defects in these domains do not completely inactivate SPARTAN function. Additionally, SPARTAN has a putative protease domain (SprT), whose mutation results in serious deficiency in SPARTAN's functions *in vivo* [31, 32]. Recently, the DNA binding and DNA dependent protease activities of WSS1 has been described, and it was suggested to be the yeast functional homologue of human Spartan, based on the domain organization they contain [33]. Given these data, we asked whether human SPARTAN can bind to DNA and whether this activity contributes to the targeting of Polη to the damaged sites after UV irradiation. We demonstrated that SPARTAN preferentially binds to single-stranded DNA, and it is unable to bind to double-stranded DNA. Additionally, we showed that SPARTAN can also bind to replication fork-like structures exhibiting no ssDNA regions. We determined and mutated a DNA-binding box in SPARTAN, which lead to a drastic weakening of its DNA-binding ability. This mutant localized correctly after UV damage, but it showed deficiency in survival after UV exposure and in Polη foci formation. Based on these phenotypes, we conclude that DNA-binding by SPARTAN is an important function in the targeting of Polη to the stalled replication fork.

2. Materials and Methods

2.1 Plasmids, cloning, and protein purification

Spartan cDNA was cloned into pENTR2B resulting in pIL 2325. Mutant clones were produced by mutagenic PCR resulting in a partial, N-terminal Spartan protein: Spartan$_{1-310}$ (pIL 2680); a full-length, DNA-binding-site mutant (K220A K221A G222A K223A) named Spartan$_A$ (pIL 2768); and a partial, N-terminal, DNA-binding-site mutant protein: Spartan$_{1-310A}$ (pIL 2704).

For the production and purification of wild-type and mutant Spartan proteins, cDNAs were cloned in N-terminal fusion with glutathione S-transferase (GST) and Flag-tag under the control of a galactose-

inducible phosphoglycerate promoter. The cloning resulted in GST-Flag-Spartan (pIL 2766), GST-Flag-Spartan$_{1-310}$ (pIL2682), GST-Flag-Spartan$_A$ (pIL 2769), and GST-Flag-Spartan$_{1-310A}$ (pIL 2707).

The GST-Flag-Spartan, GST-Flag-Spartan$_{1-310}$, GST-Flag-Spartan$_A$, and GST-Flag-Spartan$_{1-310A}$ proteins were expressed in a protease-deficient yeast strain. Proteins were produced as described previously [26]. Proteins were eluted from the beads with 20 mM reduced glutathione in NT buffer, resulting in GST-Flag-Spartan proteins.

For localization studies, we cloned SPARTAN$_A$ in fusion with an N-terminal Flag-tag in the human expression vector pRK2F, resulting in Flag-SPARTAN$_A$ (pIL 2854). For UV sensitivity and Polη foci formation assays, we used GFP-Polη (pIL 1393) and the previously published mutants Flag-SPARTAN$_{SprT}$ (pIL 2337) and Flag-SPARTAN$_{PIP/UBZ}$ (pIL 2339) and generated double and triple mutants of Flag-SPARTAN in the pRK2F vector by mutating the DNA-binding box in all cases, resulting in Flag-SPARTAN$_{SprT/A}$ (pIL 2825) and Flag-SPARTAN$_{PIP/UBZ/A}$ (pIL 2828). All mutants were sequence verified.

2.2 Gel shift assay

Purified GST-Flag-Spartan, GST-Flag-Spartan$_{1-310}$, GST-Flag-Spartan$_A$, or GST-Flag-Spartan$_{1-310A}$ (50, 150 and 450 nM, respectively) was incubated with 0.7 pmol Fluorescein- or Cy3-labelled DNA substrates (Table 1 and Table 2) in buffer R (25 mM TRIS pH 7.5, 1 mM ATP, 5 mM MgCl$_2$, 1 mM DTT, 10% glycerol, 20 ng/µl BSA) for 60 minutes at 4ºC. Samples were run on a non-denaturing polyacrylamide gel in 0.5% TB buffer and imaged by Typhoon Trio Imager and its software. The binding efficiency was calculated using the ImageJ software. For the supershift assay, purified Spartan was preincubated in buffer R with anti-Flag antibody (Sigma, Cat. No. F7425) without DNA for 30 minutes at 4ºC. After this incubation, DNA was added, and the incubation was continued for another 30 minutes at 4ºC. Samples were run, and the gel was imaged as mentioned before.

Preparation of non-single-stranded substrates was performed according to the following protocol: the selected oligonucleotides were combined in TE buffer and annealed. In order to get rid of the non-annealed oligonucleotides, the samples were run on a non-denaturing polyacrylamide gel and imaged using the Typhoon Trio Imager. The desired substrates were isolated by cutting them out from the gel (Supplementary Figure 1). The isolated gel parts were soaked in water overnight at 4ºC.

2.3 Cell cultures and cellular protein localization studies

HEK293 cells were grown in Dulbecco's modified Eagle's medium (Sigma, Cat. No. D6429) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, Cat. No. 10270) at 37°C. Transfections were carried out using Lipofectamine 2000 transfection reagent (Invitrogen, Cat. No. 11668) according to the instructions of the manufacturer.

For cellular localization of Flag-SPARTAN and endogenous PCNA, HEK239 or Spartan shRNA-expressing stable cell lines were plated on a glass cover slide, then transfected with Flag-SPARTAN-expressing plasmids. After 48 hours, cells were treated for 8 minutes with a solution containing 10 mM TRIS–HCl pH 7.5, 2.5 mM MgCl$_2$, 0.5% NP-40, 1 mM PMSF and fixed with 3% PFA for 10 minutes, then washed with 0.1% TritonX-100/1x PBS solution for 10 minutes and incubated for 10% FBS containing 0.1% TritonX/1xPBS solution. Immunostainings were carried out using anti-PCNA antibody (Santa Cruz, Cat. No. A1211) diluted 1:200, Cy3-conjugated anti-mouse antibody (Sigma, Cat. No. C2181) diluted 1:500, anti-Flag antibody (Sigma, Cat. No. F7425) diluted 1:200, and FITC-conjugated anti-rabbit antibody (Sigma, Cat. No. F0382) diluted 1:500. Samples were mounted in 25% glycerol in phosphate-buffered saline (PBS) containing 0.5 µg/ml DAPI followed by microscopy using an Olympus FV1000 confocal laser scanning microscope.

For DNA polymerase η localization studies, HEK239 and Spartan shRNA-expressing stable cell line were plated on a glass cover slide, then transfected with GFP polymerase η and control/Flag-Spartan-expressing plasmids. After 48 hours, cells were treated with 0.4% NP40 solution for 1 minute, fixed with

3% PFA for 10 minutes, washed with 0.1% TritonX-100/1x PBS solution for 10 minutes, and mounted in 25% glycerol in phosphate-buffered saline (PBS) containing 0.5 µg/ml DAPI followed by microscopy, using an Olympus FV1000 confocal laser scanning microscope. For quantification, more than 10 polymerase η foci per cell were used as a criteria to classify a cell as positive.

2.4 Cell survival assay

Cell competition-based survival assay was performed as described earlier [26] using GFP-expressing HEK293 cells as control. HEK293 and, in paralell, HEK293 Spartan shRNA-expressing cell lines were transfected with control or Flag-SPARTAN-expressing plasmids and mixed with GFP-expressing HEK293 cells as published previously [34]. After 48 hours, cells were exposed to 20 J/m^2 or 30 J/m^2 UV (as indicated) and cultured for 7 days. The ratio of co-cultivated GFP-positive and GFP-negative cells was measured by Flow Cytometer using FACS Calibur. Quantification was carried out with the WinMDI software. Cell survival rate was calculated from the ratio of GFP-positive and -negative cells.

2.5 Cell cycle analysis

To examine the effect of Spartan expression on the cell cycle, HEK293 and HEK293 Spartan shRNA expressing cell lines were transfected with control or Flag-Spartan-expressing plasmids. After 48 hours, cells were treated with 20 J/m^2 UV as indicated. After 3 hours of incubation, cells were collected and washed with 1xPBS, then resuspended in 0.1% Sodium citrate, 0.1% TritonX-100, 10 µg/ml RNase, 10 ng/ml propidium iodide in 1xPBS containing solution, then incubated for 15 minutes at room temperature. Flow cytometer analysis was carried out with FACS Calibur, followed by quantification measurements with the WinMDI software.

2.6 Statistical analysis

Statistical analysis was carried out using t-student test. Confidence value (p) <0.05 was labelled by a single asterisk. Non-significant differences were labelled by NS on all figures.

2.6 SPARTAN *in vivo* chromatin-binding assay

The SPARTAN *in vivo* chromatin-binding assay was carried out as described earlier [35] Spartan shRNA-expressing cells were transfected with Flag-Spartan-expressing plasmids. After 48 hours, cells were exposed to 20 J/m^2 UV light and incubated for 3h, then collected and washed with 1xPBS. For the isolation of the non-chromatin-bound fraction, cells were resuspended in 100 mM NaCl, 300 mM sucrose, 3 mM MgCl$_2$, 10 mM PIPES (pH 6.8), 1 mM EGTA, 0.2% TritonX, and protease inhibitor (Sigma, Cat. No. S8820) containing solution followed by incubation on ice with gentle shaking for 5 minutes, then centrifuged at 14.000 rpm for 5 minutes at 4°C. The supernatant, which contains the free, non-chromatin-bound fraction, was collected. For the isolation of the chromatin-bound fraction, the pellet was resuspended in 50 mM Tris-HCl (pH7.5), 150 mM NaCl, 5 mM EDTA, 1% TritonX-100, 0.1% SDS, and protease inhibitor (Sigma, Cat. No.: S8820) containing solution. The samples were incubated on ice for 10 minutes, then sonicated, followed by a centrifugation at 14.000 rpm for 5 minutes at 4°C. The supernatant with the chromatin-bound fraction was collected. Both the free and the chromatin-bound fraction was boiled and analysed by Western blot. Quantification was carried out using the ImageJ software.

2.7 Western blot

Flag-SPARTAN expression was verified with Western blot analysis. Cells were collected, lysed, and boiled in 2xSDS-containing buffer for 10 minutes. Electrophoresis was carried out using 10% denaturing polyacrilamide gel. After ON blotting, the expression level was detected using anti-Flag HRP-conjugated antibody (Sigma, Cat. No. A8592) diluted 1:5000. As control, we used anti-PCNA HRP-conjugated antibody (Santa Cruz, Cat. No. D1811) diluted 1:3000, anti-pol δ antibody (Santa Cruz, Cat. No. J2511) diluted 1:2000, and anti-mouse HRP conjugated antibody diluted 1:5000 (Bio Rad, Cat. No. 170-6516). Detection was carried out using Kodak Imager.

3. Results and Discussion

3.1 SPARTAN binds single-stranded DNA and replication fork-like structures

We and others have described previously that Spartan has an important function at the stalled replication fork in the targeting of Polη to the site of DNA damage induced by UV irradiation [24-28]. Since it has also been suggested that Spartan is the human homologue of yeast WSS1, which is a DNA-dependent protease [33], we decided to test whether SPARTAN can bind DNA and whether this biochemical activity affects its function after UV damage.

To test its DNA-binding activity, we assayed purified human SPARTAN (Figure 1A) on ssDNA substrates of different lengths (Figure 1B). The 24-nt- and the 31-nt-long ssDNA were not preferred binding substrates of SPARTAN, while the 42-nt- and the 75-nt-long ssDNA were appropriate for binding. To confirm the specificity of SPARTAN's DNA binding and exclude the possibility of aggregation, we verified the specific binding by supershift assay (Figure 1C).

Spartan was not able to bind the dsDNA form of the 75-nt-long oligonucleotide (Figure 1D, lanes 5-8), indicating its specificity for ssDNA regions. To further test the DNA-binding property of SPARTAN, we used a Y-fork DNA substrate whose binding was as efficient as that of the 75-nt-long ssDNA (Figure 1D, lanes 9-12) even though the single-stranded arms were only 30-nt long. Additionally, we tested SPARTAN's DNA binding on a replication fork-like structure, which had dsDNA arms only. To our surprise, SPARTAN was able to bind to this replication fork substrate as well (Figure 1D, lanes 13-16), indicating that perhaps it can bind to the junction point of forks. In summary, we describe here that Spartan is a DNA-binding protein which has an affinity to bind to long single-stranded DNA regions and to the junction point of the replication fork.

3.2 The N-terminal part of SPARTAN is responsible for the DNA-binding activity

The domain structure of SPARTAN consists of an extremely conserved N-terminal SprT domain and a C-terminal part containing many unstructured regions and SHP, PIP, and UBZ domains. Since this conserved N-terminal sequence encompasses the SprT domain, which shows similarity to the catalytic domain of WSS1 [33, 36, 37], we hypothesized that this region might contain the DNA-binding site. To map the DNA-binding domain, we generated and analysed a C-terminally truncated form of SPARTAN named SPARTAN$_{1-310}$ (Figure 2A). As we hypothesized, the DNA-binding activity of SPARTAN$_{1-310}$ was similar to that of the full-length protein (Figure 2B). Interestingly, in all of the experiments where ssDNA was present, a double band of DNA binding appeared. According to our explanation, the higher mobility band is a single SPARTAN-bound DNA and the slower mobility band is caused by a second SPARTAN protein binding. Since binding of the first SPARTAN decreases the accessible surface of the ssDNA, and SPARTAN has a much lower affinity to bind short ssDNA, a smear also appears between the two bands. Searching for possible DNA-binding motifs in the sequence of SPARTAN$_{1-310}$, we identified a putative

DNA-binding box (KKGK) between amino acids 220 and 223 (Figure 2A).To test the contribution of these amino acids to the DNA-binding activity of SPARTAN, we mutated all of these four amino acids to alanine in SPARTAN$_{1-310,}$ generating the SPARTAN$_{1-310A}$ mutant. When comparing mutants SPARTAN$_{1-310}$ and SPARTAN$_{1-310A,}$ we detected a huge decrease in the affinity of binding all types of DNA substrates, that is, long single-stranded DNA, Y-fork, and double-stranded fork (Figure 2C). We have to note here that this mutation did not completely abolish the DNA binding of SPARTAN. The full-length protein containing the four-amino-acid mutation, SPARTAN$_A$, displayed a similar decrease in the DNA-binding affinity (Figure 2D). In summary, we can conclude that the KKGK box of SPARTAN is one of the main determinant motifs of its DNA-binding activity.

3.3 DNA binding is essential for the DNA repair function of SPARTAN

Since we were able to generate a highly defective DNA-binding mutant, SPARTAN$_A$, we examined how this mutation affects the function of SPARTAN *in vivo*. First, we tested the localization of the SPARTAN$_A$ mutant protein and found that, similarly to wild-type SPARTAN, it was able to form foci that co-localized with PCNA (Figure 3A). Based on this observation, we concluded that the DNA-binding box is not essential for the targeting of SPARTAN. This also indicated that the folding of SPARTAN is not affected by this mutation since both the C-terminal UBZ and the PIP boxes are necessary for its correct targeting to PCNA foci [26]. Moreover, to exclude the possibility that the SPARTAN$_A$ mutant hetero-oligomerizes *in vivo* with its endogenous wild-type form, we repeated the experiment on a SPARTAN-depleted cell line as well (Figure 3B). SPARTAN$_A$ localization was the same as in the wild-type cell line, therefore, we suggest that SPARTAN$_A$ localizes correctly if its DNA binding activity is suppressed. It has been shown previously that the loss of SPARTAN results in a late S or G2/M arrest off the cells [32]. Therefore, we checked the cell cycle profile of the SPARTAN$_A$ mutant to exclude the possibility that the similar localization pattern compared to the wild-type SPARTAN is due to a cell cycle abnormality. Both wild-type and SPARTAN$_A$-containing cells exhibited the same cell cycle progression (Supplementary Figure 2), proving the correct localization of the SPARTAN$_A$ mutant. Additionally, since it has been described that [32] PIP and UBZ mutations do not affect cell cycle progression either, it is possible that SPARTAN's *in vivo* DNA-binding function is cooperative with these domains or the phenotype is not affected because this DNA-binding-site mutation does not cause a complete loss of function.

Since SPARTAN$_A$ localizes normally, we were interested in how it can bind to the chromatin *in vivo*. Therefore, we determined the chromatin-bound fraction of wild-type SPARTAN and the SPARTAN$_A$ mutant in SPARTAN-depleted HEK293 cells as described previously [35]. SPARTAN is partially in the chromatin-bound fraction, and mutation of its DNA-binding domain resulted in a decrease in the amount of SPARTAN in the chromatin-bound fraction (Figure 3C). This result indicates that SPARTAN's DNA-binding site contributes to its anchoring to the DNA. Additionally, since the localization of SPARTAN$_A$ is correct, but the mutation causes an increase in the amount of SPARTAN in the non-chromatin-bound fraction, we suggest that this mutation probably reduces the time SPARTAN can spend at the site of action, at the stalled replication fork. However, the connection between DNA binding and Ub-PCNA binding has to be further analysed.

Having found that the targeting of SPARTAN is not affected by the DNA-binding-site mutation, we analysed how this mutation affects the function of SPARTAN. In our experiments, using a stable silenced SPARTAN cell line [26], the sensitivity of the DNA-binding-site mutant SPARTAN$_A$ was comparable to that of the SPARTAN$_{PIP/UBZ}$ double mutant and the SPARTAN$_{SprT}$ single mutant (Figure 4A), indicating that DNA binding has an important function in the repair of UV-induced lesions. To answer the question of how the DNA binding of SPARTAN contributes to its PIP/UBZ and SprT domain-mediated function, we analysed the triple mutant SPARTAN$_{A/PIP/UBZ}$ and the double mutant SPARTAN$_{SprT/A}$. Surprisingly, neither of them were more sensitive than SPARTAN$_A$, SPARTAN$_{PIP/UBZ,}$ or SPARTAN$_{SprT}$ alone (Figure 4A).These results strongly suggest that, most probably, all of these domains

contribute importantly to the repair of UV-induced damage, and the DNA binding of SPARTAN may participate in both the PIP/UBZ and the SprT domain-mediated functions.

UV-induced damage response often acts via TLS, in which Polη is one of the most important players. Its absence is responsible for Xerodema pigmentosum variant form (XPV) in humans, which results in an increased risk of skin cancer development [38, 39]. Previously, we and others have shown that SPARTAN plays a critical role in the regulation of targeting Polη, in which the binding of Ub-PCNA via its PIP and UBZ domains is very important [26-28]. In parallel, it has been proposed that - besides Ub-PCNA binding - another targeting mechanism could be present by which SPARTAN regulates damage bypass [25, 26, 32]. Therefore, we tested how the DNA binding of SPARTAN affects Polη foci formation. As we described previously, the SPARTAN$_{PIP/UBZ}$ double mutant was highly defective in Polη foci formation [26]. Although SPARTAN$_A$ showed only a moderate defect in Polη foci formation, this indicates that the DNA-binding domain contributes to the targeting of Polη (Figure 5A and B). Additionally, the similarity of the effect of SPARTAN$_A$ and SPARTAN$_{PIP/UBZ}$ on UV sensitivity and the difference between their effects on Polη foci formation suggests that the function of SPARTAN's DNA binding is multiform, and it is not limited to the targeting of Polη. The SPARTAN$_{SprT}$ mutant was as defective in Polη foci formation as SPARTAN$_{PIP/UBZ}$, which indicates that this domain is important for the preparation of the stalled replication fork for translesion synthesis. Although at present the real biochemical function of the SprT domain is unknown, it represents the most important question in understanding how SPARTAN contributes to coping with replication stress. In summary, we can conclude that the DNA binding of SPARTAN contributes to the DNA repair function of the protein because its failure results in an increased UV sensitivity of the human cells. Furthermore, our results indicate that the DNA binding of SPARTAN contributes to the targeting of Polη to the damaged site; however, this may not be its only function.

4. Conclusion

Damage bypass during the replication process is one of the major challenges for cells; it is essential for the preservation of genome integrity. SPARTAN was described as an important contributor to damage bypass after replication stress [24-29, 31, 32]. Although its importance is clear, the mechanism by which SPARTAN contributes to the maintenance of genome integrity under strong replication stress has not been clarified yet. The identification of its putative yeast homologue, WSS1, - a DNA-dependent protease - raised the possibility that SPARTAN has a DNA-binding property [33]. In the current study, we characterized for the first time the DNA-binding ability of SPARTAN and its contribution to the bypass of UV-induced DNA lesions. We demonstrate that purified SPARTAN binds mainly single-stranded DNA, but it has binding affinity to replication fork-like structures having no ssDNA regions as well. We mapped the DNA-binding box in the SPARTAN sequence and generated a point mutant SPARTAN that was partially defective in DNA binding. Previously, we and others described that the ubiquitylation of PCNA regulates the subnuclear targeting of SPARTAN [24-26, 28]. We demonstrate that the DNA binding of SPARTAN is not essential for its intranuclear distribution. Although the localization of SPARTAN$_A$ is correct, its function is defective because human cells containing only the DNA-binding-site mutant protein show similar UV sensitivity to those containing the PIP/UBZ or the SprT domain mutant protein. Moreover, the targeting of Polη is also defective in these cells indicating the contribution of SPARTAN's DNA binding to this process. Our results also reveal that DNA binding must have other roles besides contributing to the targeting of Polη; these represent the most intriguing questions to be solved in the near future.

5. Acknowledgements

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Figure legends

Figure 1: **SPARTAN binds longer ssDNA and fork-like DNA substrates.** A) Representation of all purified SPARTAN proteins that we used in our biochemical experiments. B) SPARTAN binds longer ssDNAs. C) Supershift assay. The addition of anti-Flag antibody to the reaction shifted the mobility of the band corresponding to the Flag-SPARTAN DNA complex. D) SPARTAN does not bind to the 75-nt-long dsDNA substrate. It binds to Y-fork DNA with similar affinity as the 75-nt-long ssDNA and also has affinity to double-stranded fork. Increasing amounts of SPARTAN were incubated with different lengths of fluorescently labelled single-stranded oligonucleotides, dsDNA, Y fork, and double-stranded fork, as indicated. Reactions were resolved on native polyacrylamide gels and fluorescently labelled DNA was detected.

Figure 2: **The mapping and function of the DNA-binding box of SPARTAN.** A) Schematic representation of the domain structure of human SPARTAN. The conserved DNA-binding box with the mutated amino acids is indicated. B) The N-terminal part of SPARTAN is responsible for DNA binding. Fluorescently labelled ssDNA, Y-fork, and double-stranded fork substrates were incubated with SPARTAN or SPARTAN$_{1-310}$. C) The DNA-binding-box mutant SPARTAN$_{1-310A}$ is defective in DNA binding. Fluorescently labelled 75-nt-long ssDNA, Y-fork DNA, and double-stranded fork-DNA substrates were incubated with increasing amounts of SPARTAN$_{1-310}$ or SPARTAN$_{1-310A,}$ as indicated. D) The full-length DNA-binding-site mutant SPARTAN$_A$ is highly defective in DNA binding. Fluorescently labelled ssDNA, Y-fork, and double-stranded fork substrates were incubated with SPARTAN or SPARTAN$_A$, as indicated. Reactions were resolved on native polyacrylamide gels and fluorescently labelled DNA was detected.

Figure 3: **The DNA binding site of SPARTAN contributes to the targeting of SPARTAN into the chromatin fraction.** A) Localization of the SPARTAN$_A$ mutant protein in wild-type HEK293 cell line. B) Localization of the SPARTAN$_A$ mutant protein in SPARTAN-depleted HEK293 cell line. C) Determining the amount of SPARTAN$_A$ in the chromatin-associated fraction.

Figure 4: **Spartan$_A$-containing cells are sensitive to UV irradiation.** A) UV sensitivity of Spartan mutants. Different silencing-resistant Spartan mutants were expressed in Spartan-depleted HEK293 cell lines then treated with 20 and 30 J/m^2 UV irradiation when indicated. The survival rate was analysed using a Flow Cytometer. Mean values of triplicates are shown with SD (error bars). Significant difference is indicated by asterisk. B) Western blot analysis of the expression level of the different SPARTAN mutants used in Figure 4A.

Figure 5: **DNA binding by SPARTAN is important for Polη foci formation.** A) UV-induced Polη foci formation in different SPARTAN mutant-expressing HEK293 cell lines. GFP-Polη was expressed in Spartan-depleted stable cell lines that expressed different SPARTAN mutants, as indicated. Cells were treated with 20 J/m^2 UV irradiation. In all cases more than 10 polymerase eta foci per cell were used as a criteria to classify a cell as positive. B) Quantitative analysis of the Polη foci-forming ability of different SPARTAN mutant-expressing cells. Mean values of triplicates are shown with SD (error bars). Significant difference is indicated by asterisk. C) Western blot analysis of the expression level of the different SPARTAN mutants used in Figure 5B.
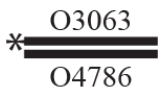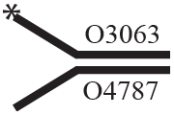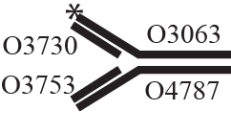
24 nt ssDNA	O4866 ✱━
31 nt ssDNA	O1676 ✱━━
42 nt ssDNA	O4865 ✱━━━
75 nt ssDNA	O3063 ✱━━━━
75 nt dsDNA	O3063 ✱━━━━ O4786
Y fork	✱ O3063 O4787
Double-stranded fork	O3730 ✱ O3063 O3753 O4787

Table 1. Suubstrates use ed in the prootein-DNA bbinding assaay

ame	Sequence	ength	Labeling
4866	TTTGCCTCGAGCCAGCCGCAGACG	4	Cy3
1676	CGACGATGCTCCGGTACTCCAGTGTAGGCAT	1	Cy3
4865	GTGAGATGTTGACCATGGGTGCGTCTGCGGCTGGCTCGAGGC	2	FITC
3063	GTTTTCCCAGTCACGACGATGCTCCGGTACTCCAGTGTAGGCATATTACGAATTCTTGAGGCAGGCATGGTAGCT	5	FITC
4786	AGCTACCATGCCTGCCTCAAGAATTCGTAATATGCCTACACTGGAGTACCGGAGCATCGTCGTGACTGGGAAAAC	5	-
4787	AGCTACCATGCCTGCCTCAAGAATTCGTAATATGCCTACACTGGACCGTACTTCGCCTAGTAGACTGCCTTCCCG	5	-
3730	GTACCGGAGCATCGTCGTGACTGGGAAAAC	0	-
3753	CGGGAAGGCAGTCTACTAGGCGAAGTACGG	0	-

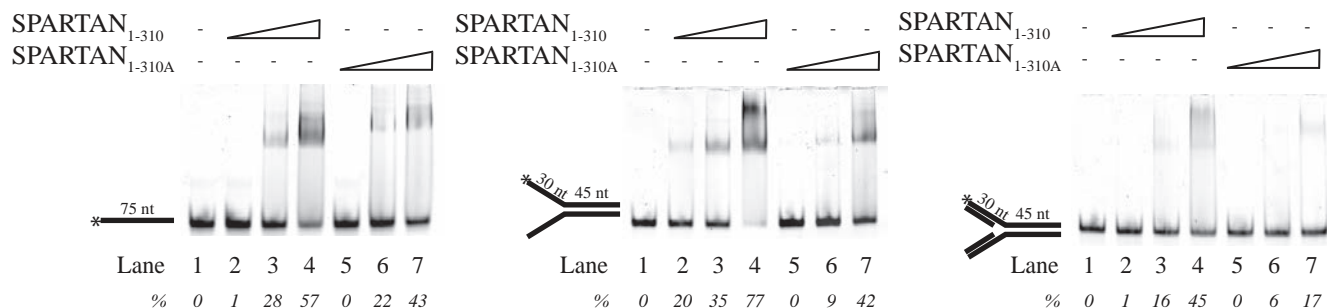Table 2. Sequence of the oligonucleotides used as DNA substrates

A

SPARTAN
SPARTAN$_A$
SPARTAN$_{1-310}$
SPARTAN$_{1-310A}$

100 —
75 —
50 —

C

SPARTAN   - - + +
anti-Flag - + - +

Lane   1   2   3   4

B

SPARTAN

24 nt	31 nt	42 nt	75 nt

Lane   1   2   3   4   5   6   7   8   9 10 11 12   13 14 15 16
%   0   2   4 12   0   4   5 23   0   4 18 44   0 25 52 80

D

75 nt   75 nt   30 nt 45 nt   30 nt 45 nt

SPARTAN   -   -   -   -

Lane   1   2   3   4   5   6   7   8   9 10 11 12   13 14 15 16
%   0 14 48 72   0   6 10 24   0 33 68 95   0 11 47 74

Figure 1

A

SPARTAN

SPARTAN$_{1-310}$

Mutated amino acids

	A A A A	
Homo sapiens	P E N Y S K K G K G K	a K l g K
Mus musculus	P E N Y S K K G r G K T	K a d K
Rattus norvegicus	P E N Y a K K G r G K T	K a g K
Gallus gallus	P E N f S K K p K e K T	e t e K
Xenopus tropicalis	P E N Y S q K r K r n n	d p t K
Danio rerio	P E N Y g K t G K s d k	q r d K

B

C

D

Figure 2

A

B

C

Figure 3

A

B

Figure 4

A

B

C

Figure 5