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SPARTAN promotes genetic diversification of the immunoglobulin-variable gene locus in avian DT40 cells

Arisa Nakazato[a,1], Kinumi Kajita[a,1], Masato Ooka[a,1], Remi Akagawa[b], Takuya Abe[a,c], Shunichi Takeda[b], Dana Branzei[c,d,*], Kouji Hirota[a,*]

[a] Department of Chemistry, Graduate School of Science, Tokyo Metropolitan University, Minamiosawa 1-1, Hachioji-shi, Tokyo, 192-0397, Japan
[b] Department of Radiation Genetics, Graduate School of Medicine, Kyoto University, Yoshidakonoe, Sakyo-ku, Kyoto, 606-8501, Japan
[c] IFOM, The FIRC Institute of Molecular Oncology, Via Adamello 16, 20139 Milan, Italy
[d] Istituto di Genetica Molecolare, Consiglio Nazionale delle Ricerche (IGM-CNR), Via Abbiategrasso 207, 27100, Pavia, Italy

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ABSTRACT

Prolonged replication arrest on damaged templates is a cause of fork collapse, potentially resulting in genome instability. Arrested replication is rescued by translesion DNA synthesis (TLS) and homologous recombination (HR)-mediated template switching. SPARTAN, a ubiquitin-PCNA-interacting regulator, regulates TLS via mechanisms incompletely understood. Here we show that SPARTAN promotes diversification of the chicken DT40 immunoglobulin-variable λ gene by facilitating TLS-mediated hypermutation and template switch-mediated gene-conversion, both induced by replication blocks at abasic sites. *SPARTAN*$^{-/-}$ and *SPARTAN*$^{-/-}$/*Polη*$^{-/-}$/*Polζ*$^{-/-}$ cells showed defective and similar decrease in hypermutation rates, as well as alterations in the mutation spectra, with decreased dG-to-dC transversions and increased dG-to-dA transitions. Strikingly, *SPARTAN*$^{-/-}$ cells also showed reduced template switch-mediated gene-conversion at the immunoglobulin locus, while being proficient in HR-mediated double strand break repair, and sister chromatid recombination. Notably, SPARTAN's ubiquitin-binding zinc-finger 4 domain, but not the PCNA interacting peptide domain or its DNA-binding domain, was specifically required for the promotion of immunoglobulin gene-conversion, while all these three domains were shown to contribute similarly to TLS. In all, our results suggest that SPARTAN mediates TLS in concert with the Polη-Polζ pathway and that it facilitates HR-mediated template switching at a subset of stalled replication forks, potentially by interacting with unknown ubiquitinated proteins.

1. Introduction

DNA replication is a generally accurate but fragile biochemical reaction that frequently stalls at damage sites and at difficult-to-replicate regions [1–6]. Cells have evolved DNA damage tolerance mechanisms for restarting stalled replication forks and filling in postreplicative gaps that comprise homologous recombination (HR)-mediated mechanisms and translesion DNA synthesis (TLS) [4,7–10]. Replication arrest induces ubiquitination of proliferating cell nuclear antigen (PCNA) [11], which in turn serves as a key regulator for DNA damage tolerance mechanisms [8].

HR promotes switching of the stalled newly synthesized strand from the damaged template to the undamaged sister chromatid, which is often used as template, thereby facilitating error-free bypass replication by template switching [10,12–18]. However, usage of templates other than the sister chromatid in the context of template switching can cause genome rearrangements while also ensuring genetic diversification in specific contexts. By contrast, TLS induces error-prone bypass replication, and is mediated by specialized TLS polymerases, including Polη and Polζ [9,19–23]. The recruitment of TLS polymerases is controlled and favored by PCNA mono-ubiquitination at the conserved K164 residue. The monoubiquitination of PCNA at K164 is mediated by RAD18 in budding and fission yeast, chicken DT40, and mammalian cells [11,24–27]. SPARTAN, which contains a PCNA interacting peptide (PIP) domain, a DNA-binding domain and a ubiquitin-binding zinc-finger 4 (UBZ4) domain in its structure, recognizes ubiquitinated PCNA and it was proposed to promote TLS [28–32]. On the other hand, SPARTAN is also known to recruit the p97 protein segregase to remove Polη from DNA damage sites and thereby to prevent TLS-mediated mutations in response to DNA damage [33,34]. Thus, the roles

* Corresponding authors at: IFOM, The FIRC Institute of Molecular Oncology, Via Adamello 16, 20139 Milan, Italy, and Department of Chemistry, Graduate School of Science, Tokyo Metropolitan University, Minamiosawa 1-1, Hachioji-shi, Tokyo, 192-0397, Japan.
E-mail addresses: dana.branzei@ifom.eu (D. Branzei), khirota@tmu.ac.jp (K. Hirota).
[1] These authors contributed equally to this work.

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SPARTAN plays in TLS are controversial. In addition to its activity as a ubiquitinated PCNA-binding TLS regulator, SPARTAN also mediates the repair of DNA-protein crosslink lesions through its DNA-dependent metalloprotease activity [35–38].

To better define the functionality of SPARTAN in DNA damage tolerance in vertebrates, we generated SPARTAN-deficient cells (*SPARTAN*$^{-/-}$) in avian DT40 cells. DT40 cells provide a unique opportunity for *in vivo* examination of the contributions of TLS-induced mutagenesis and HR template switch-mediated gene-conversion by sequencing the constitutively diversifying immunoglobulin-light-chain-variable (IgVλ) gene. During avian IgVλ diversification, activation-induced deaminase (AID) mediates conversion of dC to dU, which is in turn removed by uracil-DNA glycosylase, leading to a high frequency of abasic sites [39,40], the most common spontaneously arising lesion in mammalian DNA [41]. The resultant abasic sites either induce template switch-mediated gene conversion with one of the 25 copies of upstream IgVλ pseudogenes carrying ~10% mismatch, resulting in HR-mediated diversification/mutagenesis of the immunoglobulin gene, or they induce TLS, resulting in hypermutation at the dC/dG basepairs [5,6,20,24,39,42]. Here we show that SPARTAN works in concert with Polη and Polζ in TLS-dependent hypermutation during immunoglobulin gene diversification. Notably, *SPARTAN*$^{-/-}$ cells exhibited a marked decrease in dG-to-dC transversions and an increase in dG-to-dA transitions, similar to the ones detected in the *POLη*$^{-/-}$/*POLζ*$^{-/-}$ double mutant cells [20]. The results suggest that TLS polymerases other than Polη and Polζ act at abasic sites in the immunoglobuin gene and partly compensate for the absence of SPARTAN. Strikingly, the *SPARTAN*$^{-/-}$ cells exhibited an additional marked defect in gene-conversion. The SPARTAN UBZ4 domain, but not the PIP domain or the DNA-binding domain, was critical for this function. We thus propose that SPARTAN is required for both TLS and template switch-mediated mutagenic gene conversion mechanisms when replication-fork arrest occurs at damaged templates carrying abasic sites in the immunoglobulin-variable gene and possibly at other repeat elements in the genome.

2. Materials and methods

2.1. DT40 cell culture and cell cycle analysis

Culture conditions for DT40 cells, cell counting, and cell-cycle analysis were performed as described previously [43–48]. The DT40 cells were maintained at 39.5 °C under a humidified atmosphere and CO_2 (5%). To analyze cell cycle progression, cells (1×10^6) were exposed for 10 min to 5-bromo-2′-deoxyuridine (BrdU) (20 μM) (Nacalai Tesque, Kyoto, Japan), then harvested and fixed with ethanol (70%). Fixed cells were incubated with HCl (2 M) containing Triton X-100 (0.5%), treated first with mouse anti-BrdUrd monoclonal antibody (BD PharMingen, San Diego, CA), then with FITC-conjugated anti-mouse IgG antibody (Southern Biotechnology Associates, Birmingham, AL). Cells were then resuspended in phosphate-buffered saline (PBS) containing propidium iodide (PI) (5 μg/ml) for subsequent analysis using BD *Accuri*™ C6 flow cytometer.

2.2. Disruption of SPARTAN in DT40 cells

Constructs for targeting and disrupting the chicken *SPARTAN* locus were generated from genomic PCR products combined with *his*^{R} and *neo*^{R} selection-marker cassettes (Fig. S1 A). Genomic DNA sequences were amplified using primers 5′- CAAGGACACGTGACCTCAACTGC TTC-3′ and 5′ CCACCTCAGTTCTATCGCGCATGCGC-3′ for the 5′-arm of the targeting construct and primers 5′- TGGGCTGAGCATCAAGAGAAT TGCGG-3′ and 5′- ATAATGTGGGGCAGTTACTCATGCGC -3′ for the 3′-arm. To generate *SPARTAN*$^{-/-}$ cells, *wild-type* DT40 cells were transfected sequentially with *SPARTAN-His*^{R} and *SPARTAN -Neo*^{R} using Gene Pulser Xcell (Bio-Rad) at 550 V 25 μF. Targeted integration of the constructs was detected by Southern blotting of *Eco*RV- digested genomic DNA and a 0.35 kb probe generated by PCR from the genomic *SPARTAN* locus with primers 5′- CTGGAGTCAGACATCTCCAAGTG AGG-3′ and 5′- GGCCAGCCTGCCTGCAAATCACATTC-3′. The loss of *SPARTAN* transcript was confirmed by RT-PCR using primers 5′- TGG GCTGAGCATCAAGAGAATTGCGG-3′ and 5′- ATAATGTGGGGCAGTTA CTCATGCGC -3′. β-actin transcripts were analyzed as a positive control for the RT-PCR analysis using primers 5′-CGAGAGAGAAATTGTGCGT GAC-3′ and 5′-TGACCTGACCATCAGGGAGTT-3′.

2.3. Construction of SPARTAN cDNA-expression vector

Chicken *SPARTAN* cDNA was isolated by PCR amplification using primers 5′- AAAAGCTAGCGCGGCCGCCATGGAGCA -3′ and 5′-AAAAG AGCTCGAATTCGGGCGGCCGCT -3′ and expressed under the RSV promoter [49]. Domain mutations were introduced by PCR as follows. For the PIP mutation, we used primers 5′-AAAGGAAGACGCAACCACCTTT GAAAACGCCGCCATAAAAAAGC-3′ and 5′-GCTTTTTTATGGCGGCGTT TTCAAAGGTGGTTGCGTCTTCCTTT-3′. For the DNA-binding domain mutation, we used primers 5′-CCTGAGAACTTCTCGGCGGCAGCCGCG GAGAAAACCGAGACA-3′ and 5′-TGTCTCGGTTTTCTCCGCGGCTGCCG CCGAGAAGTTCTCAGG-3′. For the UBZ4 mutation, we used primers 5′-AAAACTGTCAGTGCTCCTGTAGCCCAGACTGAGGTT-3′ and 5′-AAC CTCAGTCTGGGCTACAGGAGCACTGACAGTTTT-3′. Correct introduction of the point mutations was confirmed by sequence analysis.

2.4. SCE measurement

Measurement of sister-chromatid exchanges (SCEs) in DT40 cells was carried out as follows. To analyze camptothecin-induced SCEs, cells were incubated with BrdU (10 μM) for 16 h, which corresponds to two cell-cycle periods for DT40 cells. Cells were treated with camptothecin (2 nM) for the last 8 h of the incubation, and with colcemid (0.1 μg/ml), to enrich for mitotic cells, for the last 2 h of the incubation. Cells were then pelleted by centrifugation, resuspended in 75 mM KCl (1 ml) (Nacalai Tesque) for 15 min at room temperature, then fixed in a freshly prepared 3:1 mixture (5 ml) of methanol:acetic acid (Carnoy's solution). The pelleted cells were then resuspended in Carnoy's solution (5 ml), pelleted and resuspended in Carnoy's solution (1 ml), dropped onto clean glass slides, and air dried. Dried slides were incubated with Hoechst 33258 (10 μg/ml) in phosphate buffer (pH 6.8) for 20 min, then rinsed with McIlvaine solution (164 mM Na_2HPO_4, 16 mM citric acid [pH 7.0]). Slides were irradiated with black light (λ = 352 nm) for 60 min and incubated in 2× saline-sodium citrate (SSC) (1 × SSC = 0.15 M NaCl plus 0.015 M sodium citrate) solution at 58 °C for 1 h before staining with HARLECO Giemsa stain solution (5%) (Nacalai Tesque) for 10 min. 50 Giemsa-stained metaphase cells per test were scored at 1000x magnification, with scoring limited to the 11 major macrochromosomes and the Z chromosome.

2.5. Measurement of I-SceI-induced gene-conversion frequency

SPARTAN$^{-/-}$ cells were generated from I-SceI (Tet-ON) cells containing the DR-GFP construct [50]. Cells were incubated with doxycycline (100 ng/ml) for 4 days, after which the mCherry- (I-*Sce*I) and GFP-(targeting) positive fractions were evaluated using the BD *Accuri*™ C6 flow cytometer, as previously described [50].

2.6. AID overexpression by retrovirus infection

Wild-type, *SPARTAN*$^{-/-}$, *POLη*$^{-/-}$/*POLζ*$^{-/-}$ and *SPARTAN*$^{-/-}$/*POLη*$^{-/-}$/*POLζ*$^{-/-}$ cells were inoculated in 96-well plates to obtain single colonies. Single colonies were picked up and genomic DNA extracted. After analyzing the DNA sequences of the V(D)J loci, clones without any mutation or gene conversion in the V(D)J locus were obtained. AID overexpression was carried out by infection of retrovirus containing the *AID* gene, followed by an internal ribosomal entry site and the *GFP*