Ubiquitin-Specific-Processing Protease 47, A Novel 53BP1 regulator

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Abstract

DNA double strand breaks (DSBs) are repaired by homology-based repair or non-homologous end joining and multiple sub-pathways exist. 53BP1 is a key DNA double strand break repair protein that regulates repair pathway choice. It is key for joining DSBs during immunoglobulin heavy chain class switch recombination. Here we identify USP47 as a deubiquitylase that associates with and regulates 53BP1 function. USP47 loss results in 53BP1 instability in proteasome dependent manner, and defective 53BP1 ionizing radiation induced foci (IRIF). USP47 catalytic activity is required for maintaining 53BP1 protein level. Similar to 53BP1, USP47 depletion results in sensitivity to DNA DSB inducing agents and defective immunoglobulin CSR. Our findings establish a function for USP47 in DNA DSB repair at least partially through 53BP1.

Introduction

DNA damage occurs due to a number of internal and external insults and sophisticated repair machineries have evolved to repair the damaged DNA and to maintain Genome

instability. DNA damage can manifest in different forms and DNA double strand breaks (DSBs) are some of the most toxic to eukaryotic cells and can lead to cellular death or if non-or mis-repaired to genomic instability and cancer¹. There are a number of DSB repair pathways that are divided in general into two major categories: Homologous recombination (HR) and non-homologous end joining. HR is operational during S and G2 phases of cell cycle when replicated DNA exists for homology search². Multiple proteins are recruited to the damage site to activate the DNA damage response and to regulate the DSB end processing and channel the repair to one of these repair pathways³.

TP53 Binding protein 1 (53BP1) is a key scaffold protein that is recruited early to the repair process and help determine the pathway choice⁴. Thousands of 53BP1 molecules build at the damage site through specific chromatin interaction and oligomerization to form a visible ionization irradiation induced foci (IRIF). 53BP1 recruits a machinery that antagonizes DNA DSB end resection and blocks HR through a pre- and post- resection mechanisms⁵. These 53BP1 foci last for a few hours and ultimately dissolve by an unknown mechanism. A large number of proteins associate with 53BP1 and likely contribute to the repair process through yet to be uncovered mechanisms⁶. Ubiquitin related processes are abundant at the repair sites although the functions of several ubiquitin-associated proteins keep evolving or remain unknown⁷.

USP47 is one of about 100 human deubiquitylases (dubs) that regulate thousands of ubiquitylated events that involve most cellular proteomes in human⁸. It was reported to be involved in Base Excision Repair (BER) through interacting with and deubiquitylating the polymerase Pol β ⁹. USP47 has significant homology to USP7, a deubiquitylase that is involved in a plethora of chromatin and DNA damage processes¹⁰.

We searched for novel 53BP1 Binding proteins that may regulate its function in DSB repair and identified USP47 as a novel 53BP1 regulator.

Results

USP47 associate with 53BP1 after DNA damage

To search for novel 53BP1 binding proteins post DNA damage, we performed immune-precipitation utilizing a high affinity antibody that binds endogenous 53BP1. We subjected the immune-precipitates to mass spectrometry fingerprinting and we identified a number of proteins that existed in 53BP1 Pull down but not in multiple other control pull-downs from our lab and from other non-specific binders reported in literature. We identified known 53BP1 partners such as RIF1, and laminB1. In addition, USP47 was recovered in the 53BP1 IPs and not from control (Figure 1A).

We did 53BP1 IP followed by WB and confirmed the two proteins association utilizing endogenous protein pull-down (Figure 1B) and another IP on Turbo-GFP trap after tGFP-53BP1 expression (Figure 1C). As noted in Figure 1B& C, the two proteins association is enhanced after exposing cells to IR. While USP47 did not form IRIF upon IR exposure, however, we show that the two protein colocalize on a cluster of DNA DSBs triggered by Fok1 nuclease, taking advantage of the more sensitive LacI-Fok1 system (Figure 1D).

USP47 regulates 53BP1 Stability through deubiquitylation

A large-scale screen of ubiquitin pathway proteins' impact on 53BP1 focus generation or persistence identified key proteins that are required for 53BP1 IRIF, such as the ubiquitin E3 ligase RNF168¹¹. The proteasome proteins scored highest positive Z-score in this screen suggesting that the proteasome plays a key role in antagonizing 53BP1 focus formation. We hypothesized that USP47 depletion can lead into 53BP1 protein instability, and indeed, reducing USP47 protein level by siRNA or through CRISPR knock-out is associated with a decrease in 53BP1 protein levels particularly after IR (Figure 2A, B, C). 53BP1 protein level declines faster in USP47 ^{-/-} cells after halting new protein synthesis consistent with reduced half-life after IR

(Figure 2D). This decline in 53BP1 was at least partially reversed after proteasome inhibition consistent with a proteasome mediated degradation (Figure 3A, B). We looked at 53BP1 ubiquitylation status in human cell lines after proteasome inhibition, and with lysate collection under denaturing conditions to preserve ubiquitylated species. We observed enhanced 53BP1 high molecular weight ubiquitylated species in USP47 deficient cells consistent with enhanced ubiquitylation (Figure 3C). WT USP47 but not catalytically dead mutant was able to reverse 53BP1 protein instability (Figure 3D)

USP47 depletion impairs 53BP1 recruitment and function

Having established an impact of USP47 on 53BP1 protein levels, we investigated in this depletion affects its function in DNA DSB repair. First, we observed significant decline in 53BP1 recruitment to damage sites after USP47 depletion (Figure 4). 53BP1 deficient cells are sensitive to DNA damaging agents that generate DSBs⁴. Similarly, we observed radiation and cisplatin sensitivity to cells depleted of USP47 compared to control isogenic cells (Figure 5A, B). A major function of 53BP1 is to facilitate long range DSB repair of cuts generated during immunoglobulin heavy chain class switch recombination (CSR)¹². Some believe 53BP1 evolved to facilitate this process⁴, and 53BP1 deficiency leads to a dramatic CSR defect. We investigated USP47 contribution to CSR through a murine lymphocytic cell line that class switch upon cytokine stimulation. We depleted USP47 by lentivirus shRNA, and we identified significant impairment in CSR although not to the same profound impairment that is seen with 53BP1 total loss by CRISPR (Figure 5C, D).

Discussion

We identify here another protein that contributes to DNA DSB through at least interacting with and stabilizing 53BP1. Consistent with a DNA repair function USP47 is phosphorylated at defined residues post IR and etoposide¹³. USP47 was previously implicated in another DNA

repair pathway (BER) by stabilizing the polymerase Pol β ⁹. Since some DNA repair proteins function in multiple pathways, it is not surprising that USP47 operates in two distinct pathways. As a deubiquitylase, it probably handles dozens of substrates given limited number of deubiquitylases that have to regulate all the ubiquitylated proteome. FEN1 is another protein that function in both BER and DSB through microhomology mediated end-joining (MMEJ)¹⁴. It is interesting that large scale proteomics reveal an association between FEN1 and USP47, and FEN1 and 53BP1^{15,16}. One of these proteomic studies also show an association between USP47 and MDC1^{15,16}, a major partner of 53BP1.

53BP1 regulation by ubiquitin and ubiquitin like proteins is likely extensive given the number of ubiquitin and sumo associated proteins that are discovered in proximity to 53BP1⁶. Future research will likely identify which ubiquitin ligase destine 53BP1 to the proteasome and will further shed light into other hidden substrates/ functions of USP47.

Figures

Figure 1: USP47 is a novel 53BP1 interacting partner.

A. Selected lists of proteins associated with 53BP1 analyzed by mass spectrometry after pull-down using 53BP1 high affinity antibody. **B.** Immunoprecipitation of USP47 using high affinity antibody against endogenous 53BP1. **C.** Pull-down of USP47 after overexpression of tGFP-53BP1 in U2OS cells. **D.** Schematic representation of FokI DSB-reporter cell line. **E.** FokI DSB-reporter cell line transfected with tGFP-53BP1 and treated with 4-OHT and Shield1 for 4hrs to induce DSBs by mCherry-Lac1-FokI. USP47 co-localizes with 53BP1 on DSBs locus. Scale bar is 20µm.

Figure 2: USP47 regulates 53BP1 Stability through deubiquitylation.

A-B. Transient knockdown of USP47 through siRNA in U2OS and A549 cells, respectively. **C.** USP47 CRISPR-generated A549 knockout cell line. **D.** A549 proficient and deficient USP47 cells treated with Cycloheximide and collected after IR after indicated time points.

Figure 3: USP47 antagonizes proteasome degradation of 53BP1.

A. siRNA Transient knockdown of USP47 U2OS cells leads to increase instability of 53BP1. **B.** CRISPR-generated USP47 A549 knockout cell line shows a dramatic phenotype to **A.** **C.** Enhanced ubiquitylation of 53BP1 in USP47 $-/-$ cells compared to USP47 $+/+$ cells. **D.** Overexpression of wild-type USP47 rescues 53BP1 instability, while catalytically dead USP47 does not in CRISPR-generated USP47 deficient cells.

Figure 4: 53BP1 decline recruitment to DNA DSB foci in USP47 deficient cells.

A. A549 CRISPR-generated USP47 proficient and deficient cells show drastic difference between 53BP1 foci formation after IR exposure. **B.** Quantification of 53BP1 foci in USP47 +/+ and USP47 -/- cells after 4hrs of IR exposure. **C.** Measurement of 53BP1 foci intensity in USP47 +/+ and USP47 -/- cells after 4hrs of IR exposure. Scale bar is 10µm.

Figure 5: USP47 deficiency affects 53BP1 function.

A. USP47 proficient and deficient cells exposed to indicated IR dose and left to grow for 14 days. **B.** Similar to **A** except cells treated with Cisplatin at indicated dosages. **C.** Flow cytometry plots measuring CSR to IgG with indicated shRNA targeted gene suppression. **D.** Quantification of flow cytometry plots from **C**.

Materials and Methods

Cell Lines, CRISPR and Gene Silencing

Human HEK293 (ATCC, CRL-1573), A549 (ATCC, CRM-CCL-185) and U2OS (ATCC, HTB-96) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Corning) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Genesee Scientific) in a 5% CO₂ incubator at 37°C. CH12F3-2 and CH12F3-2 53BP1^{-/-} murine B cell lymphoma cells^{17,18} were cultured in RPMI 1640 medium supplemented with 15% FBS, 50nM 2-Mercaptoethanol (BME), 5% NCTC-109 (Life Technologies) and 20 mM HEPES (Corning).

Short-term depletion of USP47 expression was achieved by treating cells with siRNA USP47 (5'-GAA AGC AAA UGA AGG GAA AUU-3' or 5'-AGA UAA AGA UGG UGA ACA AUU-3') from Dharmacon and Lipofectamine RNAiMAX transfecting reagent twice according to the manufacturer's instruction (Invitrogen). All siRNAs were purchased from Dharmacon unless otherwise stated. Other siRNA used in the study are:

53BP1: Sense: 5'-GAA CAG AAG UAG AAA GAA AUU-3'

Scramble from Qiagen Cat No.: 1027310: Sense: 5'-UUC UCC GAA CGU GUC ACG UdT dT-3'

To generate CRISPR deficient USP47 cell line transfection was performed according to IDT's instruction manual with modification. Briefly, using reverse transfection, A549 cells were trypsinized and plated 5x10⁵ cells per well in 6-well plate mixed with 800μL of RNP complex reverse transfection and crRNA sequence are 5'-ACTGTCATCACGGAGTACAA-3' and 5'-GCAATATAGTAGAAACGAGT-3' or proprietary non-targeting sequence for control. After 24hrs, the complex was removed and fresh DMEM supplemented with 10% FBS was added. The cells were trypsinized 48hrs later and sorted through FACS in 96-well plates for colonies. Two week later, the 96-well plates were screened for homozygous knockout of USP47.

Reagents and Plasmids

The proteasome inhibitors MG132 and Carfilzomib were purchased from Sigma-Aldrich and TLC Pharmaceutical Standards Ltd., respectively. Protein synthesis inhibitor cycloheximide was purchased from Sigma-Aldrich. USP47 catalytic domain was mutated by changing cysteine 177 into alanine (C177A) using the sense primer (5'-gtttgcaaaaggctattcaaataaggcagtcattgcttggttactagtcc-3') and Quickchange Lightning Site-directed Mutagenesis Kit from Agilent technologies.

Western Blotting, Immunoprecipitation and Antibodies

Cell were washed with PBS (137mM NaCl, 2.7 mM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄) then collected 4hrs after irradiation at a single dose of 6Gy using a Cobalt-60 Source Gamma Irradiator 72hrs after siRNA exposure in lysis buffer (150mM NaCl, 50mM Tris-HCl pH 8, 1mM Dithiothreitol (DTT), 0.5% NP-40, with protease and phosphatase inhibitors (Sigma)), sonicated for 10 seconds twice, and centrifuged at 4°C at 14Kx g for 1h. Protein quantification performed using Bradford Protein Assay (Bio-Rad), and 20μg samples were resolved on a 7% SDS-PAGE gels, transferred to nitrocellulose or PVDF membranes, blocked with 5% non-fat milk or 2% BSA then incubated with primary antibodies overnight. Horseradish peroxidase

secondary antibodies and enhanced chemiluminescence (ECL) substrate from Santa Cruz Biotechnology were used to detect antigen-antigen interaction.

Cells were collected with IP lysis buffer (150mM NaCl, 50mM Tris-HCl pH 8, 1mM EDTA, 1mM DTT, 0.5% NP-40, with protease and phosphatase inhibitors (Sigma)), sonicated for 10 seconds twice at 60Hz, and centrifuged at 4°C 14Kx g for 1hr. Samples were pre-cleared using protein G magnetic beads (Dynabeads, Invitrogen). The lysate then incubated overnight with beads conjugated with corresponding antibodies. The beads were washed four times with IP lysis buffer and four times with PBS then boiled in Laemmli sample buffer.

Antibodies include rabbit anti-53BP1 (ab36823, Abcam and NB100-304, Novus Biologicals), rabbit anti-USP47 (A301-048A, Bethyl Laboratories), mouse anti- β -actin (A2228, Sigma), mouse anti-FK2 (04-263, Sigma), mouse anti-TurboGFP (TA150041, Origene), mouse anti-FLAG M2 (F1804, Sigma) and mouse anti-GFP (sc-9996, Santa Cruz).

Immunofluorescence

Cells grown on coverslips were exposed to 1Gy IR. After 4hrs, the cells were fixed for 10 minutes in 4% formaldehyde and sucrose at room temperature. Then permeabilized for 10 minutes in 0.5% Triton-X in PBS and blocked for 1hr in 4% BSA in PBS at room temperature. The cells were washed three times with PBS before every step. The indicated primaries were diluted in 1% BSA in PBS, while secondary antibodies were diluted in PBS. Both incubated at room temperature for 1hr.

Clonogenic Assay

CRISPR-generated USP47 cells were plated at approximately 500 cells per well in 6-well plate or 60mm dishes. The following day, cells were incubated with cisplatin for 2hrs at indicated concentrations then washed three times with PBS and fresh medium was add to the wells or exposed to IR at indicated dosages and left to grow. After 14 days, colonies (defined as >50 cells) were fixed and stained with 1% Crystal violet solution (Sigma).

In Vivo Deubiquitylation

For *in vivo* deubiquitylation of 53BP1 by USP47, A549 CRISPR-generated USP47 proficient and deficient cells were used to overexpress tGFP-53BP1 using Lipofectamine 2000 according to manufacturer's instructions. 48hrs after transfection, cells were exposed to 6Gy IR dose and treated with 500uM Carfilzomib for 4hrs. Cells were collected in lysis buffer (150mM NaCl, 50mM Tris-HCl pH 8, 1mM DTT, 0.5% NP-40, with protease and phosphatase inhibitors (Sigma)). After 1 h, the reaction was stopped by boiling in SDS-based loading buffer, and the ubiquitylated species was detected by anti-ubiquitin chain FK2 antibody.

Class switch recombination assay

CH12F3-2 and CH12F3-2 53BP1^{-/-} murine B cell lymphoma cells were treated with either shScramble or shUSP47. For induction of CSR, CH12F3-2 cells were incubated in culture medium supplemented with 10ng/mL IL-4 (R&D Systems #404-ML-050), 1ng/mL TGF β (R&D Systems #7666-MB-005) and 1 μ g/mL Anti-CD40 antibody (Thermo Fisher#16-0401-86) for 48hrs. Anti-IgA-PE was used to stain the cells and subsequently PE signal analyzed on BD FACSCanto II machine.

Statistical analysis

All results are from, at least, three independent experiments. Student's t-test was used to compare two groups, while one-way analysis of variance (ANOVA) for comparing more than two groups. Graphs were generated using GraphPad Prism 8.0.2 on Microsoft Windows 10 (La Jolla, CA, USA). P-value less than 0.05 will be considered statistically significant.

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