DNA damage-independent interaction of CBX4 with SUMO1

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Sulagna Sanyal[1, 2], Chandrima Das[1*] and Sumita Sengupta (Bandyopadhyay)[2*]

[1]Saha Institute of Nuclear Physics, 1/AF, Bidhannagar Kolkata 700 064, India, [2]Department of Biophysics, Molecular Biology and Bioinformatics, University of Calcutta, 92 A.P.C. Road, Kolkata 700009, West Bengal, India

Abstract
The versatile role of CBX4, an important SUMO E3 ligase has been well studied in different biological processes including DNA damage response, transcriptional regulation and cancer.Although very few reports suggest that CBX4 itself gets SUMOylated by SUMO1, still in depth study to explore the biological implication of this association is yet to be divulged. Here we found that CBX4 indeed interacts with SUMO1 in HeLa cells. Subsequently, DNA damage induction was done using UV radiation to examine the effect on CBX4-SUMO1 interaction. Interestingly, no alteration in the SUMO1 mediated SUMOylation status of CBX4 suggested that the interaction between CBX4 and SUMO1 is quite robust and it works in a DNA damage-independent manner.

Key words: DDR, SUMOylation, UV radiation, polycomb, regulation

Introduction

PcG bodies are considered as hubs for gene repression because polycomb groups of proteins are mainly gene silencing factors involved in regulating various gene expressions. In mammals, flies and plants, a number of PcG components are reported to have a role in maintaining higher-order chromosome structure and function as a SUMOylation hub (1). Different PcG body proteins unite to make complexes responsible for various functions that belong to two distinct families: the Polycomb repressive complexes 1 and 2 (PRC1 and PRC2 respectively).The core part of the mammalian PRC1 complex shows E3 ligase activity because of the presence of E3 ubiquitin ligase RING1 (RING1A or RING1B) along with and one of the six members of polycomb group of RING finger proteins (PCGF 1-6) (2). However, PRC1 complex can further be divided into two sub-families namely canonical PRC1 (cPRC1) having homologous function like *Drosophila* PRC1 and non- canonical PRC1 (ncPRC1) that contains different heterogeneous proteins (3). cPRC1 can be identified by the occurrence of five Chromobox (Cbx) containing proteins (CBX2, 4, 6-8) which are the determinant factors for the recruitment of the complex to chromatin (2). Mammalian CBX proteins show their reader protein like function as a result of the presence of a highly conserved chromo domain at the amino-terminal (N-term) site which specifically recognizes methylated lysine residues. Additionally, the members of the family contain a c-box or PcG box at their C-terminal end which is responsible for its interaction to the catalytic core of the complex, Ring1A/B and thus show an enzymatic property or writer protein like function (4). Chromobox 4 (CBX4) (or Pc2) is an important PcG body protein that acts as SUMO E3 ligase by bringing different substrates and E2 inside the PcG body and thereby making it a SUMOylation hub (5). Before the discovery of CBX4, the enzymatic activities of SUMO E3 ligases were not very clearly known. But in 2005, Kagey *et al.* demonstrated the detailed E3

Corresponding author
Email address: chandrima.das@saha.ac.in
ssbmbg@caluniv.ac.in

ligase activity of the protein (CBX4) and it was found to enhance the SUMOylation status of the transcriptional corepressor CtBP by recruiting both the substrate protein and the E2, Ubc9 inside the PcG body (5,6). Later studies have shown that CBX4 can SUMOylate several other proteins like BMI1, hnRNPK, HIPK2 etc. and recruit them at the DNA damage site by initiating p53 mediated DNA damage response (DDR) pathway (7,8,9). SUMOylation of '*de novo*' methyltransferase Dnmt3a and zinc finger protein CTCF by SUMO E3 ligase CBX4 also contributes majorly to their repressive activity (10,11). In hepatocellular carcinoma and osteosarcoma, CBX4 exerts its effect as a tumor-promoting gene by modulating different biological pathways through its SUMO E3 ligase property (12,13). It was previously reported that besides acting as SUMO E3 ligase, CBX4 itself gets SUMOylated by SUMO1 in MEF (Mouse embryonic fibroblast) and COS-1 cells (14). But in spite of acting as an early DNA damage response (DDR) protein, the effect of DNA damage on the SUMO1 association of CBX4 still remains unknown. In this study, we found that CBX4 interacts with SUMO1 in human cervical cancer cells and this association is not dependent on the DNA damage responsiveness of the protein.

Materials and Methods

Cell culture and treatments

HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (Gibco, Invitrogen) and penicillin/streptomycin (10µl/mL of medium, Gibco, Invitrogen) at 37°C in 5% (v/v) CO_2. UV treatment was done in HeLa cells by placing it insideUV stratalinker (Vilber lourmat) under 30 Joules of radiation for 5mins and then allowed to recover for two hours before doing further experiments.

HeLa cells were transiently transfected with FLAG-CBX4 (for coimmunofluorescence experiment) using Lipofectamine2000 (Invitrogen) as per manufacturer's protocol.

Co-immunoprecipitation (Co-IP)

Cross-linked cells were subjected to Co-IP as described elsewhere(15). In brief, after cross-linking, 20 mM freshly prepared N-ethylmaleimide (NEM) was added to the lysis buffer to block the degradation of SUMOylation and then the cells were lysed with 50mM HEPES (pH7.5), 150mM NaCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 0.5% Sodium deoxycholate, 5% Glycerol, 1mM DTT along with complete protease inhibitor cocktail and incubated on ice for 1hr followed by centrifugation at 13000 rpm for 10mins at 4°C. After pre-clearing the lysates, immunoprecipitation was done with anti-CBX4 antibody (ab4189) followed by washes with the same buffer and immunoblotting with anti-SUMO1 (S8070).

Western blot analysis

Whole cell extracts were prepared using Laemmli Buffer (4% SDS, 20% Glycerol and 120mM Tris-HCL pH 6.8) and sonicated for complete lysis followed by boiling at 100°C. The samples were then electrophoresed on 11% or 15% SDS-PAGE and transferred on the nitrocellulose membrane followed by blocking with non-fat skimmed milk and probed with anti- ϒ-H2AX (ab11174) and anti-H3 (ab1791) antibodies.

Coimmunofluorescence and confocal microscopy

Coimmunofluorescence staining was performed following the standard protocol (16). Briefly, the cells were fixed with 4% Paraformaldehyde, permeabilized with 1% Triton X-100 and blocked with 3% BSA. Cells were then incubated with anti- ϒ-H2AX (ab11174), anti-FLAG (F1804) and anti-SUMO1 (S8070) antibodies for 1 hour. Following washes with PBST the cells were incubated with Alexa fluor 488 and Alexa fluor 594 conjugated secondary antibodies for 1hr at room temperature. The coverslips were again washed with PBST and mounted with DAPI-containing Prolong Gold antifade mounting medium

(Invitrogen). Andor Spinning Disk Ti-E confocal scanning microscope with A1RMP scanner head (Nikon) was used for confocal imaging.

Results and Discussion

CBX4 itself interacts with SUMO1 in a DNA damage independent manner

CBX4-mediated SUMOylation of BMI1, another important member of the PRC1 complex, helps in the recruitment of the protein to the laser micro-irradiated DNA damage site and can initiate the PARP mediated DNA damage response pathway(7). Interestingly, DNA damage also triggers theSUMOylation of heterogeneous nuclear ribonucleopeotein K (hnRNP K) by CBX4 which is required for transcriptional activation of p53 (8). This DDR property of CBX4 as a SUMO E3 ligase led us to check the effect of DNA damage on the SUMOylation status of the protein itself. To induce DNA damage, HeLa cells were subjected to 30J/m^2 of UV radiation for 5 mins and then allowed to recover for two hours before doing the experiment (1A). DNA damage induction was further confirmed by checking the level of ϒ-H2AX through immunofluorescence and western blotting (Figure 1B, panel I and panel IV). Co-immunoprecipitation assay was then performed in untreated and UV treated HeLa cells with anti-CBX4 antibody and the association of the protein was checked with SUMO1 (Figure 1C). Fascinatingly, in both the cases CBX4 showed robust association with SUMO1 and the extent of interaction was also similar for untreated and UV-treated cells. Further through co-immunofluorescence, this interaction was visualized by overexpressing FLAG-CBX4 in HeLa cells and significant co-localization was found in between FLAG-CBX4 and SUMO1 in untreated as well as UV-treated condition (Figure 2A and 2B, panel IV). Additionally, calculated Pearson's coefficient was greater than 0.5 in both the conditions which strengthened our observation.

FIGURE 1

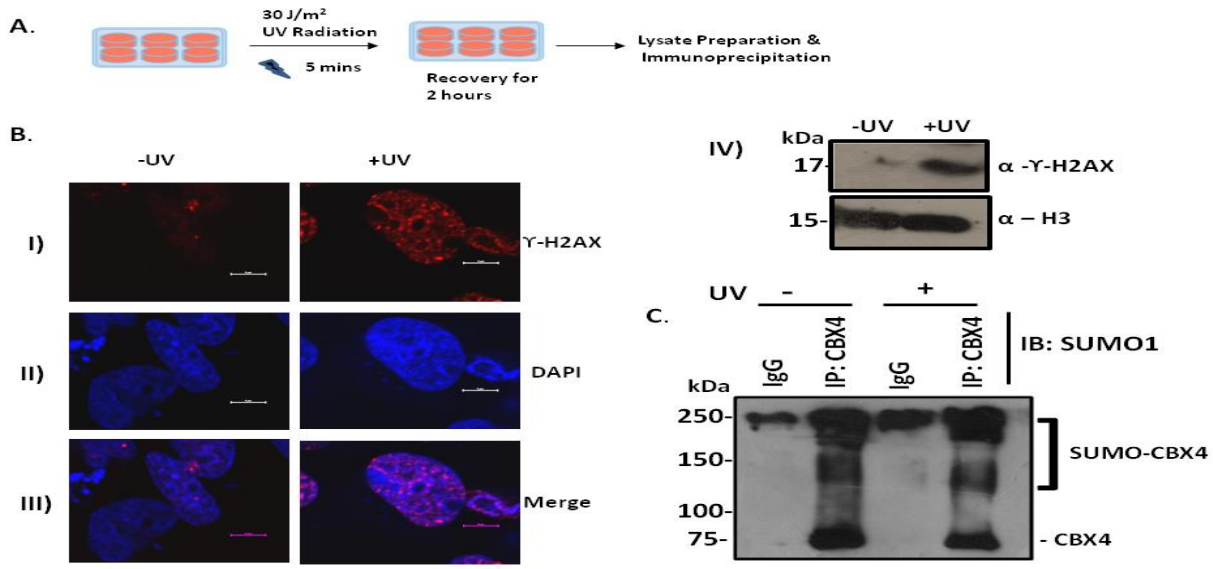

Figure 1: (A) Diagram showing experimental design for UV damage induction. **(B)** Hela cells were immunostained with anti- ϒ-H2AX antibody (**panel I, II, III**) followed by western blotting with anti- ϒ-H2AX and anti-H3 antibodies (**panel IV**) after DNA damage induction. H3 was used as the loading control. **(C)** Co-immunoprecipitaion was done with anti-CBX4 antibody in untreated and UV treated HeLa cells followed by immunoblotting with the anti-SUMO1 antibody

FIGURE 2

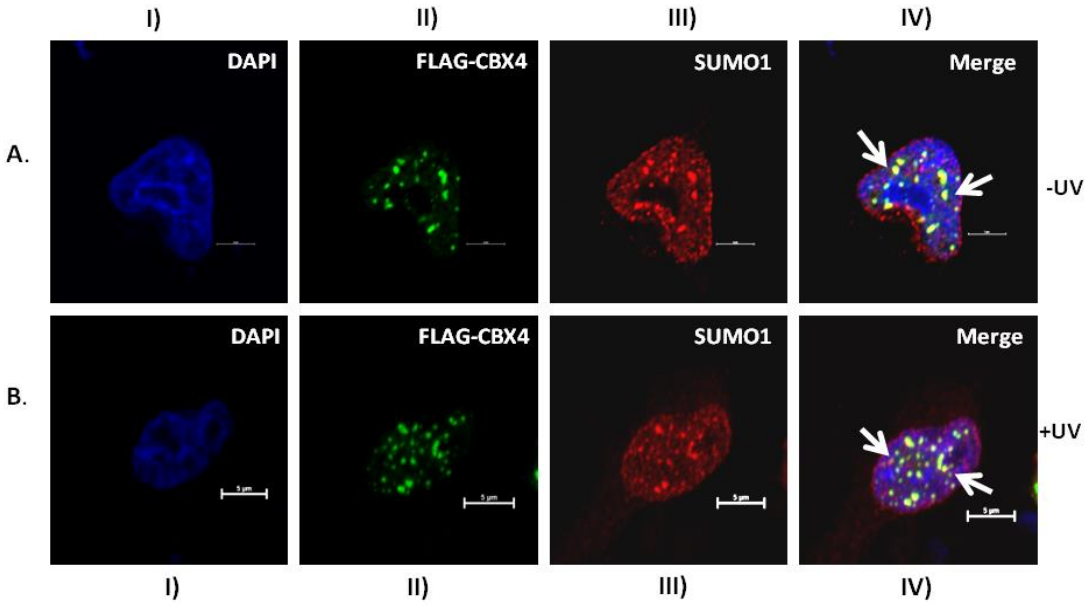

Figure 2: Co- immunofluorescence was done in untreated **(A)** and UV-treated **(B)** HeLa cells after transfecting FLAG-CBX4 with anti-FLAG (**panel II**) and anti-SUMO1 (**panel III**) antibodies. DAPI was used to stain the nucleus (**panel I**). Pearson's coeffiecients was >0.5.

Conclusion

A plethora of proteins, which show cellular response to DNA double-strand breaks (DSBs), are modulated by several highly dynamic and reversible post-translational modifications like methylation, acetylation, ubiquitination and SUMOylation (7). But the role of SUMOylation has always remained elusive in the context of the DDR pathway. CBX4 has been identified as an early DDR protein and DNA damage seems to affect the SUMO E3 ligase property of the protein in several ways (17). However, besides acting as a SUMO E3 ligase, CBX4 itself gets SUMOylated by SUMO1 probably through an autoregulatory mechanism (14)(18). So, we sought to check the effect of DNA damage on this CBX4-SUMO1 interaction. Here we report that, CBX4 indeed gets SUMOylated by SUMO1 in human cervical cancer cells also and this robust CBX4-SUMO1 association is not affected by the DNA damage induction. In the future, detailed investigation based on this observation could be helpful to unravel the underlying mechanism.

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