Original Paper

Sumoylation Negatively Regulates CSR1-Dependent Prostate Cancer Cell Death

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Key Words

Sumoylation • SUMO-specific protease 1 • Cellular Stress Response 1 • Ubiquitination

Abstract

Background/Aims: SUMOylation is a dynamic process and reversed by the activity of SUMO-specific proteases (SENPs) family. SENP1, a member of this family, is highly expressed and plays oncogenic roles in diverse cancers including prostate cancer. However, the SENP1-transgenic mice exhibit aberrant transformation of the mouse prostate gland but do not develop cancer. Cellular Stress Response 1 (CSR1) is a tumor suppressor gene and frequently deleted in prostate cancers. Overexpression of CSR1 in prostate cancer cells inhibits colony formation, anchorage-independent growth and induces cell death. **Methods:** The relationship between CSR1 and SENP1 were determined by immunoprecipitation-based proteomics screen and verified by GST-pull down assay. *In vivo* SUMOylation assay was used to detect the direct effect of SENP1 in the regulation of CSR1. Clustered regularly interspaced short palindromic repeats (CRISPR)-based gene editing was used to generate *Senp1*^{-/-} and *CSR1*^{-/-} PC3 cells. FACS assay was used to determine the apoptosis ratio of cells after transfection. **Results:** CSR1 is SUMOylated at K582 and rapid ubiquitinated and degraded in prostate cancer cells. SENP1 interacts with and deSUMOylates CSR1 to prevent its degradation and enhances CSR1-dependent prostate cancer cell death. **Conclusion:** Thus, our data indicates that CSR1 is a critical SUMOylated substrate of SENP1 that might partially explain the controversial roles of SENP1 in prostate cancer development.

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Introduction

SUMO (small-ubiquitin-like modifier) posttranslational modification (PTM) or SUMOylation is a covalent conjugation occurs on numerous substrates to affect the function, subcellular localization, and/or stability of these proteins [1, 2]. SUMOylation is a dynamic process and has critical roles in both physiological and pathophysiological cellular events

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[3, 4]. It could be reversed by the activity of SUMO-specific proteases (SENPs) family and dysregulation of which often associates with and supports the cancer development [5]. Thus, the SUMO-specific protease family members present attractive subjects for targeted-cancer therapy [6].

SEN1 is one of the six SUMO-specific protease family members and elevation of SEN1 protein is associated with enhanced prostate cancer malignancy [7]. In human prostate cancer cells, SEN1 controls cells proliferation and progression via the regulation of androgen receptor (AR), c-jun, and cyclin D1 [8-11]. However, the SEN1-transgenic mice exhibit aberrant transformation of the mouse prostate gland but do not develop cancer [11], suggesting SEN1 could regulate multiple mechanisms simultaneously, which might also include some tumor-suppressive mechanisms.

Cellular Stress Response 1 (CSR1) is a stress response and tumor suppressor gene located at 8p21, which frequently deleted in prostate cancers [12, 13]. The promoter region of CSR1 is frequently methylated in prostate cancer that associated with prostate cancer relapse [12]. Overexpression of CSR1 in prostate cancer cell lines inhibits colony formation, anchorage-independent growth, and invasion. CSR1 also induces apoptosis and necrotic cell death by inhibiting polyadenylation of RNA through redistribution of CPSF3 [14], a critical polyadenylation enzyme, or preventing the interaction of X-linked Inhibitor of Apoptosis Protein (XIAP) with caspases [13]. However, how to regulate CSR1 at posttranscriptional level is still unknown.

Here, we show that CSR1 is a SUMOylated short-life protein that undergoes rapid ubiquitination and degradation. SEN1 interacts with and deSUMOylates CSR1 to stabilize this protein and enhanced CSR1-dependent prostate cancer cell death.

Materials and Methods

Cell culture

PC3 and LNCap cells were obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CAS, Shanghai, China) and cultured in 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 IU/ml penicillin and 100 mg/ml streptomycin. HEK293T cells were maintained in our lab and cultured in DMEM supplemented with 10% FBS. All cell lines were maintained at 37°C in a humidified atmosphere with 5% CO₂.

CRISPR/Cas9 KO cell lines

The KO plasmids, including SEN1 CRISPR/Cas9 KO Plasmid (h2): sc-417768-KO-2 CSR1, SEN1 HDR Plasmid (h2): sc-417768-HDR-2, CRISPR/Cas9 KO Plasmid (h): sc-409127 and CSR1 HDR Plasmid (h): sc-409127-HDR, were purchased from Santa cruz (Santa cruz, CA, USA). PC3 cells were co-transfected with both plasmids and selected by puromycin. The single clones were chose and western blot was used to verify the knockout efficiency.

Western blot

Cells were harvested and lysed with cold lysis buffer (150 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS and 10% glycerol). Proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes. Western blot assay was then performed by using the following antibodies: anti-CSR1, anti-SEN1, anti-GST, anti-His, anti-GAPDH (Santa Cruz, CA, USA), anti-Cyclin D1 (Cell Signaling, Boston, MA, USA), anti-Flag M2 and anti-HA (Sigma, St Louis, MI, USA).

SUMOylation and Ubiquitination assays

293T cells were co-transfected with indicated plasmids for 48 hours. Cells were sonicated in lysis buffer containing 8M urea and 10mM imidazole. His-Ubiquitin-conjugated or His-SUMO- conjugated proteins were recovered with Ni-NTA resin (Qiagen), washed eight times in urea lysis buffer containing 20mM imidazole, and eluted with buffer containing 5% SDS, 0.72M DTT, and 200mM imidazole. Proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes and visualized with antibodies.

Protein purification and mass spectrum

For purification, eight 10-cm dishes of LNCap cells expressing Flag-SEN1 or control Flag-vector were lysed in cold NETN buffer (100 mM NaCl, 1mM EDTA, 20 mM Tris-HCl, pH 8.0, 0.5% NP-40) for 45 min at 4°C. Cell lysates were centrifuged, and the supernatants were incubated with M2 beads overnight at 4°C. The beads were washed eight times with NETN buffer, and the bound proteins were eluted with Flag peptide. The eluates were digested with trypsin and subjected to mass spectrometric analysis.

FACS assay

Apoptosis assay was carried out by Annexin V-FITC Apoptosis Detection Kit according to manufacturer's instruction (BD, San Diego, CA, USA). Cells were collected, washed twice with cold PBS, resuspended in binding buffer and incubated with Annexin V-FITC at room temperature, followed by addition of propidium iodide for 5 min. Fluorescent intensities were determined on flow cytometry (Beckman Coulter, Miami, FL, USA).

Statistical analysis

The data shown represent the mean $\pm$ standard (SD) of three independent experiments. The GraphPad Prism Version 6.0 was used to evaluate for statistical significance via the Student's t-test. Significance was analyzed (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Results

CSR1 is associated with SEN1 in prostate cancer cells

To search for potential substrates of SEN1 in prostate cancer cells, we performed immunoprecipitation (IP) experiments with Flag-tagged SEN1 in LNCap cells followed by mass spectrometry. Consistent with previously reports, the androgen receptor and histone deacetylase 1 (HDAC1) were identified as SEN1-interacting proteins. Besides these proteins, CSR1 and XIAP were identified in the immunoprecipitates (Fig. 1A). The interactions between SEN1 and CSR1 or XIAP were further confirmed by co-immunoprecipitation (co-IP) assays (Fig. 1B-C). However, GST pull down assays only confirmed the interaction between SEN1 and CSR1, but not XIAP (Fig. 1D and data not shown), suggesting that the direct interaction of SEN1 with CSR1 and the indirect interaction with XIAP. Taken together, these results revealed that CSR1 is a novel SEN1-associating protein.

Fig. 1. CSR1 is associated with SEN1 in prostate cancer cells. (A) A partial list of proteins identified by mass spectrometry analysis. LNCap cells transfected with control or Flag-SEN1 plasmids were subjected to Flag M2 beads affinity purification. Bound proteins were digested by trypsin, followed by mass spectrometry.

(B) 293T cells were transfected with Flag-SEN1 and HA-CSR1, followed by immunoprecipitation with M2 beads and immunoblotting with indicated antibodies. (C) 293T cells were transfected with Flag-SEN1 and HA-XIAP, followed by immunoprecipitation with M2 beads and immunoblotting with indicated antibodies. (D) GST pull-down assays showing that CSR1, but not XIAP, is directly interacts with SEN1. Purified GST-SEN1 immobilized on Glutathione Sepharose beads were incubated with bacterial purified His-CSR1 or His-XIAP. The beads were washed and eluted with 2x SDS loading buffer and subjected to western blot with indicated antibodies.

CSR1 is SUMOylated at K582

We then determined whether CSR1 is a SUMOylated protein. HEK293T cells were co-transfected with his-SUMO1 and HA-CSR1, CSR1 displayed a higher molecular weight (WM) species in the presence of his-SUMO1 by western blot assay (Fig. 2A), suggesting this high MW band shift might represent a SUMOylated form of CSR1. To further test this hypothesis, CSR1 were co-transfected with his-SUMO1 and either SENP1 wild-type (WT) or SENP1 inactive point mutant (MUT, SENP1-C603S) into LNCap cells. The global SUMO1 proteins were purified by Ni²⁺ beads. The higher molecular weight band was absent in the presence of SENP1 WT but not SENP1 MUT, demonstrating that

this band shift represents the SUMOylated CSR1 protein form (Fig. 2B). We further used clustered regularly interspaced short palindromic repeats (CRISPR)-based gene editing to generate *Senp1*^{-/-} PC3 cells, which were then transfected to produce HA-CSR1. Exogenous CSR1 was heavily SUMOylated in *Senp1*^{-/-} PC3 cells when compared to control KO cells (Fig. 2C). SUMOylation often occurs at lysines in the consensus sequence ΨKX(D/E) [15]. Using a prediction algorithm (GPS-SUMO) [16], we identified a single lysine (K582) as a high-confidence SUMOylation site. To determine whether K582 is the SUMOylation site, we generated CSR1 K582R mutant. When expressed in HEK293T cells, HA-CSR1-WT SUMOylation was clearly observed, whereas SUMOylation of HA-CSR1 K582R could not be detected (Fig. 2D), indicating that K582 is the major SUMOylation site in CSR1.

SUMOylation regulates the stability of CSR1

We then asked whether SUMOylation regulates CSR1 cellular function. In consistent with a previous study, we found that overexpression of HA-CSR1-WT in PC3 cells caused noticeable cell death [14]. However, overexpression of HA-CSR1-K582R mutant resulted in greater cell death, suggesting SUMOylation prevents CSR1-induced PC3 cell death (Fig. 3A). During the transfection process, we observed that HA-CSR1-K582R was easier to be detected than HA-CSR1-WT by western blot in PC3 cells with the same amount of plasmids, suggesting that CSR1-K582R could be more stable than HA-CSR1-WT. To test this possibility, we used Cycloheximide (CHX) chase assay to determine the half-life of proteins. Following treatment with 50 µg/ml CHX, the expression of CSR1-WT was rapidly decreased, however, the half-life of CSR1-K582R was greatly extended (Fig. 3B). In consistent with these data, the ubiquitination of CSR1-K582R was decreased in the presence of the proteasome inhibitor MG132 when compared with CSR1-WT (Fig. 3C). Taken together, these data suggested that the stability of CSR1 was regulated by K582 SUMOylation.

Fig. 2. CSR1 is SUMOylated at K582. (A) HEK293T cells were co-transfected with his-SUMO1 and HA-CSR1, followed by immunoprecipitation with HA antibody and immunoblotting with indicated antibodies. (B) LNCap cells were co-transfected with HA-CSR1, his-SUMO1 and either SENP1 wild-type (WT) or SENP1 inactive point mutant (MUT, SENP1-C603S), followed by Ni²⁺ purification and immunoblotting with indicated antibodies. (C) SENP1 WT and SENP1 KO PC3 cells were transfected with HA-CSR1 and subjected to western blot with indicated antibodies. (D) HEK293T cells were co-transfected with his-SUMO1 and either HA-CSR1 WT or HA-CSR1 K582R mutant, followed by Ni²⁺ purification and immunoblotting with indicated antibodies.

Fig. 3. SUMOylation regulates the stability of CSR1. (A) PC3 cells were transfected with indicated plasmids, cell apoptosis ratio was analyzed by FACS assay. (B) PC3 cells were transfected with either HA-CSR1 WT or HA-CSR1 K582R mutant, and treated with 50 µg/ml CHX for the indicated time. Cells were harvested and subjected to western blot with indicated antibodies. (C) PC3 cells were transfected with indicated plasmids, followed by Ni²⁺ purification and immunoblotting with indicated antibodies.

SEN1-mediated CSR1 deSUMOylation regulated prostate cancer cells proliferation

As SENP1 interacts with and deSUMOylates CSR1, we then asked whether SENP1 regulated the stability as well as biology function of CSR1. We found that SENP1-WT, but not SENP1-C603S MUT, increased the expression of endogenous CSR1 (Fig. 4A). Indeed, in the present of exogenous SENP1-WT, the half-life of CSR1 was extended (Fig. 4B). Overexpression of SENP1-WT significantly enhanced CSR1-dependent cell death towards PC3 cells (Fig. 4C). However, overexpression of SENP1-C603S MUT had only minor effect on CSR1-dependent cell death (Fig. 4B). SENP1

Fig. 4. SENP1-mediated CSR1 deSUMOylation regulated prostate cancer cells proliferation. (A) PC3 cells were transfected with either SENP1-WT or SENP1-C603S MUT, followed by immunoblotting with indicated antibodies. (B) PC3 cells were transfected with HA-CSR1 with or without Flag-SENP1-WT, and treated with 50 µg/ml CHX for the indicated time. Cells were harvested and subjected to western blot with indicated antibodies. (C) PC3 cells were transfected with indicated plasmids, cell apoptosis ratio was analyzed by FACS assay. (D) CSR1 WT and KO PC3 cells were transfected with or without Flag-SENP1 and subjected to western blot with indicated antibodies. (E) The cell growth curve of CSR1 WT and KO PC3 cells transfected with or without Flag-SENP1.

functions as an oncogene in prostate cancer cells by modulating the transcriptional activity of ARs and c-Jun through deSUMOylation [17]. SENP1 also promoted prostate cancer cells proliferation by inducing Cyclin D1 expression [5]. However, it's still unclear whether CSR1 plays a role in SENP1-induced prostate cancer cells proliferation. To this end, we generated a CSR1^{-/-} PC3 by CRISPR and we found that knockout of CSR1 significantly decreased SENP1-induced CyclinD1 expression (Fig. 4D) and proliferation in PC3 cells (Fig. 4E).

Discussion

As a powerful posttranslational modification, SUMOylation regulates the functions and fates of hundreds of proteins in nearly all aspect of cell pathways. SUMOylation could be reversed by means of SENP family members that have distinct substrates and cellular functions. In the present study, we found that CSR1 was a SUMOylated protein and a substrate of SENP1. Although we have utilized *Senp1*^{-/-} cells, the endogenous CSR1 SUMOylation is still hard to detect due to low CSR1 expression and the quality of CSR1 antibody. However, by using mutagenesis assay, we demonstrate that CSR1 is SUMOylated at K582 in prostate cancer cells and SUMOylation inhibits its tumor suppressor function, most likely by promoting ubiquitin- and proteasome-dependent degradation of CSR1. Several works have shown that SUMOylation could alter the intracellular localization of its target proteins [18, 19], thus, we could not exclude the possibility that CSR1 SUMOylation might change its intracellular localization to affect its stability.

Accumulated studies suggest that SENP1 expression directly correlates with prostate cancer development. SENP1 expression is regulated both androgen dependent and independent, and increased SENP1 expression could also potentiate both androgen dependent and independent prostate cancer cells growth [20]. A recent study suggested that SENP1 could modulate the stability of the tumor suppressor gene PTEN to regulate prostate epithelial cell apoptosis [21]. SENP1 inhibits ubiquitylation and degradation of PTEN via deSUMOylation, which is similar to the case of CSR1.

In this work, we have identified SENP1 as a novel regulator of CSR1 stability, providing a new perspective of the functional relationship between SENP1 and prostate cancer cells. Despite significant progress in establishing normal and pathologic functions of SENP1, the underlying molecular mechanism of SENP1 in prostate cancers remains enigmatic. Our data suggested that SENP1 associated with and stabilized CSR1 by modulating its SUMOylation status, representing the tumor suppressor function of SENP1 in prostate cancers. SUMO-targeted ubiquitin ligases (STUbLs) mediate the ubiquitylation of SUMOylated proteins to modulate their stability and functions [22]. It will be interested to identify the STUbL which is responsible for the degradation of CSR1 in the prostate cancer cell in the future.

Disclosure Statement

No conflict of interests exists.

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