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CACUL1/CAC1 attenuates p53 activity through PML post-translational modification

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ABSTRACT

Promyelocytic leukaemia (PML) is a tumor suppressor protein covalently conjugated with SUMO family proteins, leading to the formation of PML nuclear bodies (NBs). PML-NBs provide a platform for efficient posttranslational modification of targets and protein-protein interaction, contributing to the adjustment of gene expression and chromatin integrity. Although PML SUMOylation is thought to play important roles in diverse cellular functions, the control mechanisms of adequate modification levels have remained unsolved. Here, we report that Cullin-related protein CACUL1/CAC1 (CACUL1) inhibits PML posttranslational modification. CACUL1 interacts with PML and suppresses PML SUMOylation, leading to the regulation of PML-NB size in the nucleus. We also found that Ubc9, a SUMO-conjugating enzyme, binds to CACUL1 and antagonizes the interaction between CACUL1 and PML. Furthermore, CACUL1 attenuates p53 transcriptional activity. These data suggest that CACUL1 is a novel regulator that negatively controls p53 activity through the regulation of PML SUMOylation.

1. Introduction

Promyelocytic leukaemia (PML) is a tumor suppressor protein that localizes to nuclear punctate structures called PML-nuclear bodies (PML-NBs). PML-NBs are proteinaceous structures that are interspersed between chromatins that are involved in the regulation of a variety of cellular functions, including proliferation, survival, senescence, and genomic integrity [1]. PML-NBs are also known to function as repositories for proteins with various activities, which are selectively targeted to or released from PML-NBs depending on their functions. One pathway regulated by either PML or PML-NBs is the p53-signaling cascade. p53 is recruited by PML to the PML-NBs through their binding to promote its acetylation and phosphorylation, enhancing the transcriptional activity of p53 [2]. In addition, PML interacts with p53 ubiquitin ligase, Mdm2, resulting in the regulation of p53 stability [3,4]. Furthermore, other p53 modifiers such as PIASy and HAUSP also accumulate in PML-NBs [5,6], suggesting that a variety of p53 post-translational modifications occur in PML-NBs.

SUMOylation, the process during which the target protein is modified by covalent conjugation of the Ubiquitn-like modifier SUMO, is involved in the regulation of many cellular activities such as cell signaling, stress responses and DNA damage repair [7]. PML is one of the most well-characterized proteins that are covalently conjugated by SUMO family proteins. SUMO modification of PML, as well as noncovalent binding of PML to SUMO, is required for the formation of PML-NBs [1,8]. PML is SUMOylated at three lysine residues (Lys65, Lys160 and Lys490) and in general, SUMO1 attachment onto PML is thought to be important for the PML-NBs formation. However, the loss of SUMO1 in mice does not lead to entire disruption of PML-NBs [9], suggesting that other SUMO family proteins compensate for the absence of SUMO1 in PML-NBs formation. In fact, the alternative SUMO isoform SUMO2/3 also localizes in PML-NBs, and is conjugated onto PML. SUMO1 and

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SUMO2/3 exhibit different organization within the PML-NBs [9]; however the physiological functions of the distinct SUMO organizations within PML-NBs are not completely understood. Besides the formation of PML-NBs, the SUMOylation of PML is also known to facilitate PML ubiquitination by E3 ubiquitin ligase RNF4 [10]. The conjugation of poly-SUMO2/3 chains upon PML acts as a scaffold upon which RNF4 attaches onto, and thereby promotes PML ubiquitination. This ubiquitination is thought to be involved in PML degradation following stress stimulation. Thus, it is likely that SUMOylation of PML is not only important for the formation of PML-NBs, but also for the fates of both PML and PML-NBs and its response to cellular and extracellular stresses.

CACUL1/CAC1 (CACUL1) is a Cullin domain containing protein, which is associated with the regulation of cell cycle and stress response. Recently, we have reported that CACUL1 plays a role in oxidative stress response by regulating Nrf2 ubiquitination states [11]. It has been shown that CACUL1 is upregulated by oxidative stresses, and knock-down in cells led to lower cell viability under stress condition, indicating the importance of this protein in the stress response pathway [12]. Interestingly, CACUL1 is involved in negative regulation of nuclear receptors ERα and RARα, demonstrating that CACUL1-mediated transcriptional activity is another function of CACUL1 [13,14]. Although CACUL1 seems to regulate gene expression associated with several stresses, little is known about the function of CACUL1 in the nucleus.

In this study, we show that CACUL1 is a novel regulator of PML post-translational modification associated with p53 activity. CACUL1 interacted with PML and suppressed PML SUMOylation. CACUL1 expression also lead to the formation of slightly smaller PML-NBs despite no significant changes in the number of PML-NBs in the nucleus. Finally, expression of CACUL1 decreased p53 activity. Taken together, our study suggests that CACUL1 regulates PML posttranslational modification and PML-NBs in the nucleus, involved in the regulation of the p53 pathway.

2. Materials and methods

2.1. Plasmids

PML, SUMO2, Ubc9 and RNF4 were amplified from HCT116 cDNA library by PCR and inserted into pDest12-Flag, pcDNA5-FRT-Myc, pcDNA3-HA and pcDNA5-FRT-Flag-RNF4 vectors, respectively. pcDNA-Flag-CACUL1, pcDEF-HA-CACUL1, pSIREN-shCACUL1, pCAGEN-His-Ub and pGL3-p53 reporter plasmid construct has been described previously [11,15]. The primers used were as follows:

PML forward, 5′- AAGGATCCATGGAGCCTGCACCC -3′;
PML reverse, 5′- AAAGGATCCTCAGCTCTGCTGGGA -3′;
SUMO2 forward, 5′- GGCCATGCTAGCATGGCCGACGAAA-3′;
SUMO2 reverse, 5′- CTGAGGTACCTCAGTAGACACCTCC-3′;
RNF4 forward, 5′- ACTCATGCTAGCATGAGTACAAGAAAGCGT-3′;
RNF4 reverse, 5′- ATATGACTCGAGTCATATATAAATGGGGTGGT-3′;
Ubc9 forward, 5′- ACTCATGCTAGCATGAGTACAAGAAAGCGT-3′;
Ubc9 reverse, 5′- ATATGACTCGAGTCATATATAAATGGGGTGGT-3′;

2.2. Antibodies

For immunoblot analysis, anti-Flag (M2, SIGMA), anti-Myc (9E10, Santa Cruz), anti-HA (3F10, Roche), anti-His (Cat.#27-4710-01, GE Helthcare), anti-p53 (DO-1, Santa Cruz), phospho-p53 (Ser15) (Cat.# 9284, CST), anti-Mdm2 (N20, Santa Cruz), anti-Tubulin (DM1A, SIGMA) primary antibodies were used. For secondary antibodies, peroxidase-conjugated anti-mouse and anti-rat antibodies (Jackson Immuno Research LABORATORIES) were used. For immunocytochemical analysis, anti-DYKDDDDK (Cat. #5407. CST), anti-HA (3F10, Roche) and anti-PML (PG-M3, Santa Cruz) antibodies were used with Alexa Fluor 488 or 594 conjugated anti-rabbit, anti-rat and anti-mouse antibodies. Nuclei were stained with Hoechst 33342 (Life Technologies).

2.3. Cell culture and transfection

HEK293T, HEK293 and HeLa cells were cultured in High Glucose Dulbecco's modified Eagle's medium (Wako) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen), and incubated at 37 °C with 5% CO_2. Transfections were carried out by using Polyethylenimine (Polyscience, Inc.).

2.4. Immunoprecipitation and immunoblot analyses

Cells were lysed with ice-cold lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 0.5% NP40, 1 mM EDTA) containing the SUMO protease-inhibitor, 10 mM *N*-Ethylmaleimide (Wako). The lysates were sonicated, incubated on ice for 10 min, and cleared by centrifuge. The cleared supernatant was subjected to immunoprecipitation with anti-Flag M2-agarose (SIGMA), anti-HA agarose beads (SIGMA) or protein G agarose beads (Thermo Scientific) with anti-Myc (9E10 Santa Cruz) antibody and incubated at 4 °C. The beads were then washed with lysis buffer. The protein samples were boiled in SDS sample buffer, separated by SDS-PAGE, and immunoblotted using Western Lightning Plus-ECL reagent (PerkinElmer Life Sciences).

2.5. His-tag pull down assay

Cells were transfected with the indicated constructs and lysed in extraction buffer (6 M guanidinium-HCl, 50 mM sodium phosphate buffer [pH 8.0], 300 mM NaCl and 5 mM imidazole). Cell lysates were sonicated and were then incubated with Talon metal affinity resin (Clontech) at 4 C°. The precipitants were washed with buffer (50 mM sodium phosphate buffer [pH 8.0], 300 mM NaCl and 5 mM imidazole) and then subjected to immunoblot analysis.

2.6. Immunocytochemical analysis

HeLa cells and HEK 293 cells were cultured on cover slips, transfected, and fixed by immersing in ice-cold methanol for 15 min at -80 °C. The coverslips were then blocked in 1% BSA/PBS for 15 min, and incubated with primary antibody for 2 h at room temperature. After washing, cover slips were then incubated with secondary antibodies, and mounted onto slides using the Fluoromout/Plus (Diagnostic BioSystems). Images were obtained using a fluorescence microscope (Keyence model BZ-9000), and then quantified by using the ImageJ software.

2.7. RNA extraction, reverse transcription and qPCR analyses

Total RNA was purified from HEK293 cells using RNeasy Mini Kit (QIAGEN), and reverse transcribed using ReverTraAce (TOYOBO). Quantitative PCR reactions were carried out by THUNDERBIRD SYBR qPCR Mix (TOYOBO) on the Thermal Cycler Dice Real-Time System II (Takara, TP800) to detect PUMA and Actin as control. All procedures were carried out according to the manufacturer's instructions. The primers used were as follows:

PUMA forward, 5′- AGCGGCGGAGACAAGAGGAG -3′;
PUMA reverse, 5′- GGTGCAGAGAAAGTCCCCCG -3′;

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