Aperito Journal of Cellular and Molecular Biology

Received: Sep 13, 2014
Accepted: Oct 20, 2014
Published: Oct 24, 2014

http://dx.doi.org/10.14437/AJCMB-1-101 Short Communication Eri Yuasa and Hisato Saitoh, Aperito J Cell Mol Biol 2014, 1:1

Sumo-2/3 and Rnf4 Localization to DNA Replication Foci in Cultured Human Cells Over expressing the P150 Subunit of Chromatin Assembly Factor 1

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Abstract

We previously reported that the largest p150 subunit of chromatin assembly factor 1 ($p150^{CAF1}$), which promotes chromatin assembly in the context of DNA replication, interacts directly and preferentially with small ubiquitin-related modifier-2/3 (SUMO-2/3). In this study, using 5-ethynyl-2′-deoxyuridine, we demonstrate accumulation of SUMO-2/3 and the ubiquitin ligase RNF4 at DNA replication sites in a human cell line stably over expressing Flag-tagged $p150^{CAF1}$ (Flag-$p150^{CAF1}$ cells). As the SUMO-RNF4 pathway remains elusive, this cell line may serve as a valuable tool for clarifying the pathway regulation and function in chromatin dynamics.

Abbreviations: CAF1: Chromatin Assembly Factor1; DAPI: 4', 6-DiAmidino-2-Phenyl Indole; EdU: 5-Ethynyl-2′-deoxy Uridine; PBS: Phosphate Buffered Saline; RNF4: RiNg Finger protein 4; SUMO-2/3: Small Ubiquitin-related MOdifier-2/3

Keywords: Histone Chaperone; RNF4; Replication; SUMO-2/3; Ubiquitin

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Introduction

Post-translational modification mediated by Small Ubiquitin-related MOdifiers (SUMOs) is an important mechanism in the control of the structures and functions of nuclear proteins [1-3]. Although the contribution of the SUMO pathway to DNA replication, repair, recombination and maintenance of genome/epigenome is well established, detailed mechanism(s) concerning regulation of the pathway in chromatin dynamics remains largely uncharacterized.

Chromatin Assembly Factor1 (CAF1) is composed of three subunits (p150, p60, and p48) and functions to coordinate histone deposition as well as non-histone protein assembly during DNA synthesis [4, 5]. We previously showed that the p150 subunit of CAF1 ($p150^{CAF1}$) bound preferentially to SUMO-2/3 via its N-terminal SUMO-interacting motif [6]. Moreover, we established a cultured human embryonic kidney HEK293 cell line (Flag-$p150^{CAF1}$ cells) that constitutively over expressed Flag-tagged $p150^{CAF1}$ approximately six times higher than that of

endogenous $p150^{CAF1}$, and identified accumulation of SUMO-2/3 at foci where the homotrimeric sliding clamp and DNA polymerase accessory factor Proliferating Cell Nuclear Antigen (PCNA) was concentrated [6]. Suggesting the role of SUMO-2/3 modification at DNA replication sites in these cells [6]. However, because one could argue that PCNA foci were artificially assembled as a result of Flag-$p150^{CAF1}$ over expression, and thus might not represent activity of DNA synthesis per se, additional experiments, besides PCNA-staining, were required to definitively conclude SUMO-2/3 localization at DNA replication sites in this cell line.

In this study, we applied a thymidine analogue, 5-Ethynyl-2'-deoxyUridine (EdU), and confirmed SUMO-2/3 accumulation at replication sites in the Flag-$p150^{CAF1}$ cell line. Additionally, we found co-localization of Really Interesting New Gene (RING) Finger protein 4 (RNF4), a SUMO-targeted ubiquitin ligase, at EdU-positive replication foci in the cell line. So far no paper describing an association of SUMO and RNF4 at replication sites in higher eukaryotic cellular systems. Thus we believe that our data report the first description of the cell line which is applicable to investigate the SUMO-RNF4 pathway at the sites where DNA synthesis and chromatin assembly take place.

Materials and Methods

Cell culture, drugs and siRNA transfection

Cells were cultured under the standard conditions [6]. The siRNAs against RNF4 (siGENOME SMARTpool siRNA reagent M-006557-03-0005) were obtained from Thermo Fisher Scientific. Transfections were performed in 3.5 x 10-mm tissue culture dishes with 40 nM siRNA duplex and 6 µl of Lipofectamine RNAiMAX (Invitrogen). To confirm depletion of RNF4 expression in the siRNA transfected cells, immunoblot analysis was carried out as described previously [6]. The experiments were repeated at three times and at least 100 cells were counted within individual experiments. The ratio of cells in early and mid-to-late S-phase was presented as the mean ± SD.

5-Ethynyl-2′-deoxyUridine (EdU) staining

EdU staining, in which a terminal alkyne group replaces the methyl group in the 5 position, was purchased from Life Technologies (Carlsbad, CA, USA). EdU is readily incorporated intocellular DNA during DNA replication. The terminal alkyne group is then detected through its reaction with fluorescent azides in a Cu (I)-catalyzed [3+2] cyclo addition, providing a highly sensitive, very fast, and damage-free method for detection of DNA replication sites [7]. EdU staining was carried out according to the manufacturer's instructions.

Indirect-immuno fluorescence analysis

Cells grown on glass cover slips were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Then cells were permeabilized with 0.2% TritonX-100 in PBS for 5 min on ice. After the permeabilization, cells were blocked in 0.2% bovine serum albumin, 0.1% TritonX-100 in PBS for 5 min 3 times and incubated with primary antibody for 1 hr, followed by an appropriate secondary antibody. The cover slips were mounted on slide glasses using glycerol-DABCO (Wako Pure Chemical Industries) and samples were analyzed with a DP72 microscope (Olympus). DNA was visualized with 4', 6-Di Amidino-2-Phenyl Indole (DAPI). The antibodies against SUMO-1 and SUMO-2/3 were obtained from Cell Signaling Technology (Danvers, MA, USA). The mouse monoclonal anti-RNF4 antibody (mab 11-3.1) was described previously [8].

Results and Discussion

To confirm SUMO-2/3 localization at replication sites in Flag-$p150^{CAF1}$cells, cells were cultured under the standard conditions, followed by exposure to 5 µM EdU for 15 min. As shown in Figure 1, many, but not all, Flag-$p150^{CAF1}$ cells exhibited EdU-positive fluorescent signals. Based on previous studies [6, 9], we grouped EdU staining patterns into three categories with respect to the status of cell-cycle progression: i) non-S-phase pattern, in which there were no detectable EdU signals; ii) early S-phase pattern, in which highly dense small foci were spread throughout the nuclear volume; and iii) mid-to-late

Figure: 1

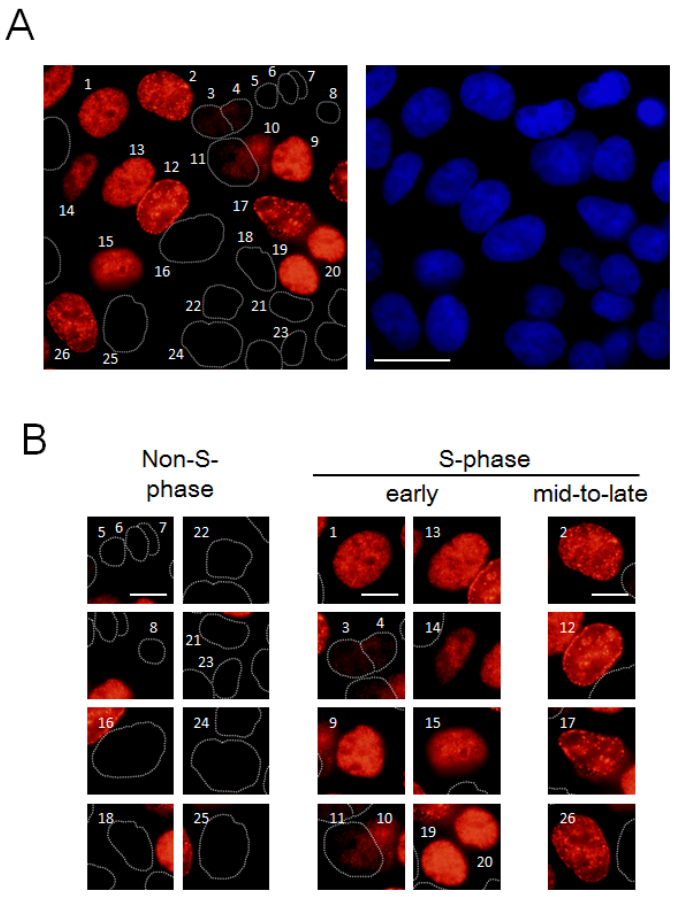

Figure 1: EdU detection of Flag-p150 ^{CAF1}cells undergoing S-phase.

A. Exponentially growing Flag-p150 ^{CAF1}cells were incubated in the presence of 5 µM EdU for 15 min. EdU staining (left) and DAPI staining (right) are shown. Cells were numbered as indicated (1-26). Bar = 20 µm.

B. Based on EdU-staining patterns, the 26 cells numbered in A were grouped into three categories; non-S-phase, early S-phase or mid-to-late S-phase. Bar = 10 µm.

S-phase pattern, in which clear foci and/or large dots were located at the nuclear rim and nucleolar periphery. We then performed indirect immunofluorescence analysis using anti-SUMO-2/3 or -SUMO-1 antibody and examined whether immuno stained signals were merged with EdU-positive replication sites. As shown in Figure 2A, anti-SUMO-2/3 antibody signals, but not anti-SUMO-1 antibody signals, were merged with EdU staining throughout the nucleus in many EdU-positive cells. In particular, cells undergoing mid-to-late S-phase contained many EdU-foci co-stained with anti-SUMO antibody; more than 90% of EdU foci, which might represent mid-to-late replicating chromatin regions, were stained with an anti-SUMO-2/3 antibody (Figure 2B). These results confirmed the previous conclusion that SUMO-2/3 accumulated at replication sites in Flag-p150^{CAF1} cells. Thus, we re-evaluated the value of the human Flag-p150^{CAF1} cell line for elucidating the role of SUMO-2/3 in DNA synthesis and chromatin assembly.

Figure: 2

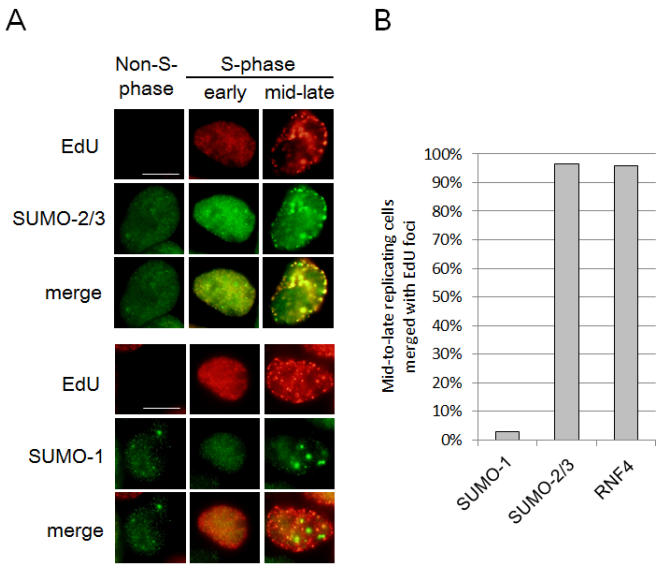

Figure 2: SUMO-2/3 foci merged with EdU-positive foci in Flag-p150 ^{CAF1} cells.

A. Exponentially growing Flag-p150 ^{CAF1}cells were incubated in the presence of 5 µM EdU for 15 min followed by indirect-immunofluorescence analysis using anti-SUMO-2/3 (upper panel) or- SUMO-1 (lower panel) antibody. Based on the EdU-staining patterns, cells were grouped into three categories; non-S (left), early S (middle) or mid-to-late S-phase (right). EdU staining (top), antibody staining (middle) and merged images (bottom) are shown. Bar = 10 µm.

B. EdU foci merged with anti-SUMO-1, -SUMO-2/3 or RNF4 antibody in mid-to-late S-phase cells were counted. The values shown represent the ratio (%) of the EdU foci merged with indicated antibodies. In each experiment, at least 200 EdU-positive foci were observed.

Next, to further identify other proteins, in addition to SUMO-2/3, at replication sites in Flag-p150^{CAF1} cells, we selected several antibodies that recognize components in the SUMO pathway and performed indirect immunofluorescence analysis. Among them, we found that a newly developed mouse monoclonal antibody that recognizes Really Interesting Gene (RING) Finger protein 4 (RNF4 or SNURF) [8] was merged with EdU-positive replication sites (Figure 3A and B); more than 90% of EdU-foci were merged with RNF4 foci in mid-to-late S-phase cells (Figure 2B). Given that RNF4is a member of the SUMO-Targeted Ubiquitin Ligase family (STUbL), which catalyzes the ubiquitylation of proteins that have been modified by SUMO [10, 11], and is involved in multiple aspects of chromatin dynamics, including base-excision repair-mediated, active DNA demethylation and the double-strand DNA damage response [8, 12-19], we considered RNF4 as a good candidate for the regulator at the SUMO-containing replication sites in Flag-p150^{CAF1} cells. Indeed, when RNF4 expression was reduced by RNA interference (Figure 4A and B), numbers of mid-to-late replicating cells were increased, indicating an impact of RNF4-depletion on regulation of S-phase progression in Flag-p150^{CAF1} cells (Figure 4C). Although these results argue for a role of RNF4 in controlling DNA synthesis and/or chromatin assembly in Flag-p150^{CAF1} cells, it should be noted that SUMO-2/3 signals were detected at EdU-positive replication foci in the RNF4 siRNA-treated cells (Figure 4D), implying that SUMO-2/3 might assemble on replication foci in an RNF4-independent manner. Investigations on the effect of RNF-depletion with respect to regulation of DNA replication forks and/or S-phase progression would be one of the important research avenues for future studies.

In summary, here we used our previously established Flag-p150^{CAF1} HEK293 cell line to confirm SUMO-2/3 localization at replication sites. EdU technology enabled us to co-localize proteins of interest to replication sites in a sensitive and simple manner, leading to the identification of RNF4 as a component of SUMO-containing replication foci. We propose that the Flag-p150^{CAF1} cell line will provide a unique opportunity to elucidate the SUMO-2/3-RNF4 pathway in chromatin dynamics, in particular, to study the role of the SUMO-RNF4 pathway at the sites where DNA synthesis and chromatin assembly take place.

Figure: 3

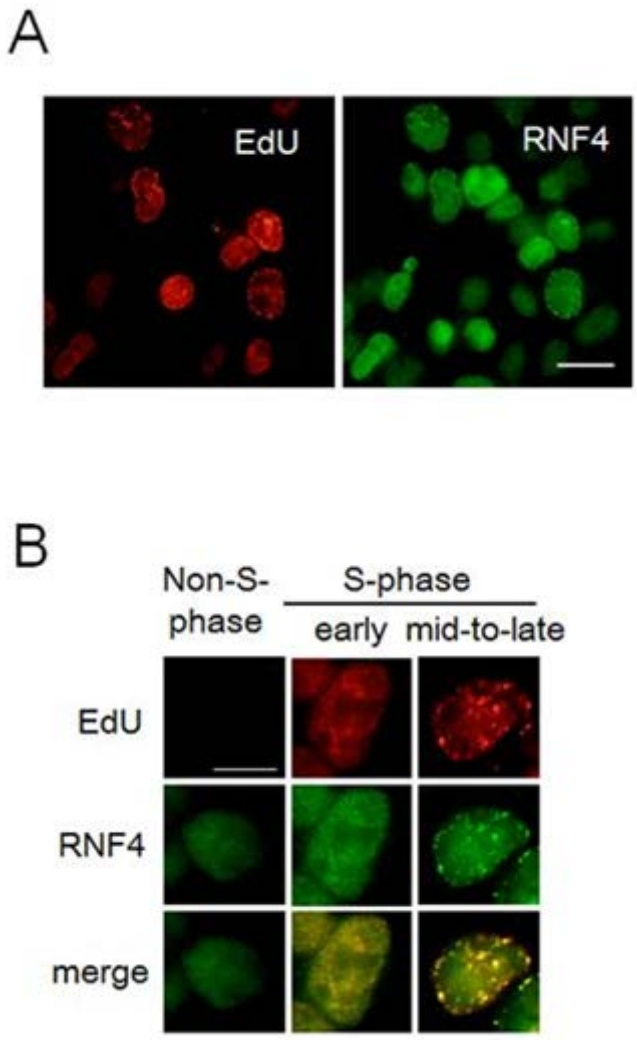

Figure 3: RNF4 was accumulated at EdU-positive DNA replication sites in Flag-p150^{CAF1}cells.

A. Exponentially growing Flag-p150 ^{CAF1}cells were incubated in the presence of 5 μM EdU for 15 min (left) followed by indirect-

immunofluorescence analysis using anti-RNF4 antibody (right). Bar = 10 µm.

B. Based on the EdU-staining patterns, cells were grouped into three categories; non-S (left), early S (middle) or mid-to-late S-phase (right). EdU staining (top), antibody staining (middle) and merged images (bottom) are shown. Bar = 10 µm.

Figure: 4

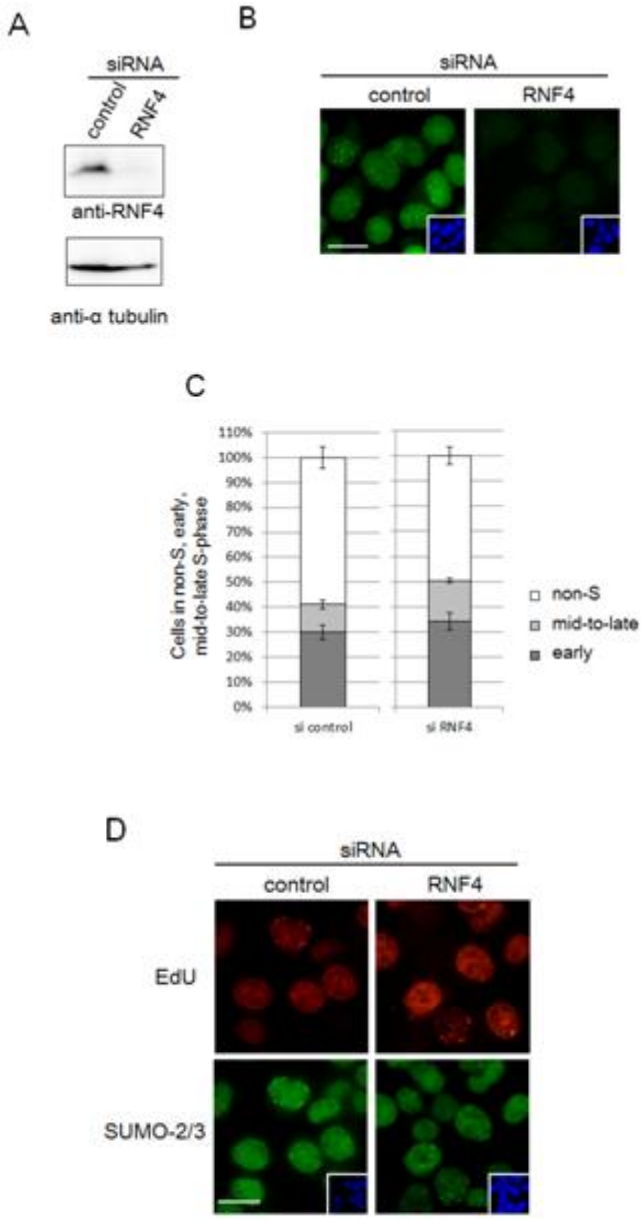

Figure 4: RNF4-depletion using siRNA duplex against RNF4 and its effect on S-phase progression.

A. Using siRNA duplex against RNF4, Flag-p150^{CAF1}cells were transfected and maintained for 48 h. Cells were harvested and subjected to immunoblot analysis using anti-RNF4 (upper) or anti-α tubulin antibody (lower).

B. Flag-p150^{CAF1}cells were transfected with control siRNA or siRNA against RNF4. At 48 h post-transfection, cells were subjected to immuno staining using anti-RNF4 antibody. Bar = 10 µm.

C. Flag-p150^{CAF1}cells were transfected with control siRNA or siRNA against RNF4. At 48 h post-transfection, cells were subjected to EdU staining followed by quantification of EdU-positive cells (100 cells). The cell numbers of the early (dark gray) versus mid-to-late S-phase cells (light gray) were counted, respectively. Bar charts depict the ratio of cells undergoing early or mid-to-late S phase.

D. Flag-p150^{CAF1}cells were transfected with control siRNA (left) or siRNA against RNF4 (right). At 48 h post-transfection, cells were incubated in the presence of 5 µM EdU for 15 min (upper) followed by indirect-immunofluorescence analysis using anti-SUMO-2/3 antibody (lower). Bar = 10 µm.

Acknowledgment

We thank all the members of the Saitoh Laboratory for their helpful discussions. This work was supported by KAKENHI grants (Nos. 23616004 and 26116517) from the Japan Society for the Promotion of Science.

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