Biochemical and Biophysical Research Communications 651 (2023) 30–38

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

SPOP is essential for DNA replication licensing through maintaining translation of CDT1 and CDC6 in HaCaT cells

Sayoko Sanada [a, b, 1], Masashi Maekawa [b, c, 1, *], Sota Tate [b, c], Hiroki Nakaoka [a], Yasuhiro Fujisawa [a], Koji Sayama [a], Shigeki Higashiyama [b, c, d, **]

[a] Department of Dermatology, Ehime University Graduate School of Medicine, Toon, Ehime, 791-0295, Japan
[b] Department of Biochemistry and Molecular Genetics, Ehime University Graduate School of Medicine, Toon, Ehime, 791-0295, Japan
[c] Division of Cell Growth and Tumor Regulation, Proteo-Science Center, Ehime University, Toon, Ehime, 791-0295, Japan
[d] Department of Oncogenesis and Tumor Regulation, Osaka International Cancer Institute, Chuo-ku, Osaka, 541-8567, Japan

ARTICLE INFO

Article history:
Received 20 January 2023
Accepted 3 February 2023
Available online 4 February 2023

Keywords:
SPOP
DNA replication licensing
Translation
CDT1
CDC6

ABSTRACT

Speckle-type pox virus and zinc finger (POZ) protein (SPOP), a substrate recognition receptor for the cullin-3/RING ubiquitin E3 complex, leads to the ubiquitination of >40 of its target substrates. Since a variety of point mutations in the substrate-binding domain of SPOP have been identified in cancers, including prostate and endometrial cancers, the pathological roles of those cancer-associated SPOP mutants have been extensively elucidated. In this study, we evaluated the cellular functions of wild-type SPOP in non-cancerous human keratinocyte-derived HaCaT cells expressing wild-type *SPOP* gene. *SPOP* knockdown using siRNA in HaCaT cells dramatically reduced cell growth and arrested their cell cycles at G1/S phase. The expression of DNA replication licensing factors CDT1 and CDC6 in HaCaT cells drastically decreased on *SPOP* knockdown as their translation was inhibited. CDT1 and CDC6 downregulation induced p21 expression without p53 activation. Our results suggest that SPOP is essential for DNA replication licensing in non-cancerous keratinocyte HaCaT cells.

© 2023 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Speckle-type pox virus and zinc finger (POZ) protein (SPOP) is an adaptor protein for the cullin-3 (CUL3)/RING ubiquitin ligase complex [1]. It acts as a substrate recognition receptor for CUL3 and their substrates, leading to substrate ubiquitination [2]. It has been reported that point mutations in the substrate binding domain (MATH domain) of SPOP occur at a frequency of 6%–15% in cancers such as prostate and endometrial cancers [1,3]. To date, more than 40 proteins have been identified as ubiquitinated substrates of CUL3/SPOP, and cancer-associated SPOP mutations alter their binding affinity to the target substrates [1,4].

SPOP reportedly plays pivotal roles in various cellular processes, such as hormone-induced transcriptional regulation, immune response, X-chromosome inactivation, cell cycle regulation, hedgehog and phosphatidylinositol-3-kinase (PI3K)-AKT signaling pathways, and cellular senescence [4]. Recently, the crucial roles of SPOP in DNA damage response have been discovered [5]. A prostate cancer-associated SPOP mutant impaired homologous recombination (HR) repair in response to DNA double-strand breaks, causing genomic instability [6]. SPOP positively regulates mRNA expression of DNA repair-related genes (e.g., BRCA2, RAD51, CHK1, and ATR), which are essential for efficient DNA repair after exposure to exogenous DNA damages in U2OS sarcoma cells [7]. Phosphorylated SPOP promotes HR repair over nonhomologous end joining in prostate cancer cells by inhibiting 53BP1 activity [8]. SPOP phosphorylation also prevents genomic instability via non-degradative ubiquitination of HIPK2 in prostate cancer cells [9]. SPOP binds to geminin leading to its K27-polyubiquitination followed by the inhibition of minichromosome maintenance (MCM)s/CDT1 interactions, which prevents the origin re-firing in prostate cancer cells [10]. During DNA replication in normally-cultured growing prostate cancer cells, SPOP is required for DNA-protein crosslink repair process through maintaining protein expression of tyrosyl-

* Corresponding author. Division of Physiological Chemistry and Metabolism, Graduate School of Pharmaceutical Sciences, Keio University, Minato-ku, Tokyo, 105-8512, Japan.
** Corresponding author. Department of Biochemistry and Molecular Genetics, Ehime University Graduate School of Medicine, Toon, Ehime, 791-0295, Japan.
E-mail addresses: maekawa-ms@keio.jp (M. Maekawa), shigeki@m.ehime-u.ac.jp (S. Higashiyama).

[1] S.S. and M.M. equally contributed to this work.

https://doi.org/10.1016/j.bbrc.2023.02.012
0006-291X/© 2023 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

DNA phosphodiesterases [11].

DNA replication is an evolutionally-conserved and essential process during cell cycle for the accurate synthesis of genomic DNA in eukaryotic cells [12]. Its dysregulation results in various diseases (e.g., cancers and neurodegenerative diseases), and the irreversible inhibition of DNA replication is a hallmark of cell senescence [13–15]. At the G1-S phase transition, DNA replication initiates with the formation of pre-replicative complexes (pre-RCs) at the replication origins [16]. These pre-RCs comprise specific proteins, origin recognition complex (ORC) proteins (ORC1–6), CDT1, and CDC6 [17]. The ORCs/CDT1/CDC6 protein complex recruits DNA helicases, MCM2–7, that unwind DNA upon activation (DNA replication origin firing) [12]. This process, called DNA replication licensing, ensures that each chromosome is replicated only once per cell cycle to maintain diploidy [16]. The depletion of CDT1 or CDC6 inhibits DNA replication in yeasts and mammalian cells [18–20]. The overexpression of both CDT1 and CDC6 in yeasts and mice generates origin re-firing on the chromosomes, resulting in continuous DNA replication [19,21]. Thus, the regulation of CDT1 and CDC6 expression is critical for proper progress of DNA replication licensing followed by origin firing.

In this study, we demonstrate that SPOP is essential for the proper protein expression of CDT1 and CDC6, critical DNA replication licensing factors, in non-cancerous human keratinocyte-derived HaCaT cells. Knockdown of SPOP in HaCaT cells drastically suppressed DNA synthesis due to the decrease of CDT1 and CDC6, resulting in their cell growth suppression. Mechanistically, translation of CDT1 and CDC6 was inhibited by SPOP knockdown in HaCaT cells. Our results suggest the novel roles of SPOP in DNA replication licensing in non-cancerous keratinocytes.

2. Materials and methods

2.1. Antibodies

The information about the antibodies used in this study is described in supplementary information.

2.2. Cell culture

HaCaT cells were maintained at 37 °C with 5% CO2 in Dulbecco's Modified Eagle Medium (DMEM; Wako) supplemented with 10% fetal bovine serum, 20 U/mL penicillin, and 100 μg/mL streptomycin. Cells were treated with 25 μg/mL CHX (CHX; Sigma) at 37 °C for 4–24 h, 10 μM MG132 (Sigma) at 37 °C for 4 h.

2.3. siRNA transfection

The information about the siRNA duplex oligomers used in this study is described in supplementary information.

2.4. Western blotting

Western blotting was performed as described in a previous study [22]. Blot images were analyzed using Fiji software (NIH).

2.5. Reverse transcription-PCR (RT-PCR)

RT-PCR was performed as described in a previous study [23]. The information about the primers used in this study is described in supplementary information.

2.6. 5-Ethynyl-2′-deoxyuridine (EdU) labelling assay

Incorporation of EdU followed by click reactions with fluorophores was performed using Click-IT Plus EdU Cell Proliferation Kit for Imaging Alexa Fluor™ 555 dye (Invitrogen) according to the manufacturer's instructions.

2.7. Cell proliferation assay

Cell proliferation assay was performed as described in a previous study [24].

2.8. ATP assay

Cell survival was determined by ATP assay as described in a previous study [25] using the ATPlite 1-step kit (PerkinElmer) with slight modifications. Briefly, HaCaT cells were seeded into a 96-well plate. Cells were transfected with siRNA on the next day. After a 72-h incubation, 25 μL ATPlite solution was added to each well, and luminescence was measured after incubating for 5 min using Flex Station 3 Multi Plate Reader (Molecular Device).

2.9. Cell cycle assay

The cell cycle of HaCaT cells was analyzed using Cycletest™ Plus DNA Kit (BD) and FACS Aria (BD) according to the manufacturer's instructions.

2.10. Confocal microscopy

Confocal microscopy was performed according to a previous study [26] using the A1R laser confocal microscope (Nikon) with a 60 × 1.27 Plan-Apochromat water immersion lens. Images were analyzed using Fiji software (NIH).

2.11. Statistical analysis

Statistical comparisons were made using one-way ANOVA followed by Tukey's test for post-hoc analysis. Results were considered statistically significant at $p < 0.05$.

3. Results

3.1. SPOP depletion inhibits DNA synthesis in human keratinocyte-derived HaCaT cells

To investigate the roles of SPOP in non-cancerous human keratinocyte-derived HaCaT cells expressing wild-type *SPOP* gene, we silenced *SPOP* by transfecting cells with two kinds of siRNAs targeting *SPOP*. The knockdown efficiencies of both siRNA oligos were confirmed by quantitative PCR (Fig. 1A). Both siRNAs effectively suppressed *SPOP* mRNA levels; however, they did not affect the mRNA expression of SPOPL, an SPOP paralogue [27], excluding the contribution of SPOPL in phenotypic analysis of *SPOP*-knockdown HaCaT cells (Fig. 1B). In normally-growing androgen receptor-positive prostate cancer cells, *SPOP* knockdown caused the accumulation of γH2AX, a marker of DNA double-strand breaks, due to the inhibition of DNA-protein crosslink repair after DNA synthesis during the S phase [11]. γH2AX accumulation, however, was not observed in *SPOP*-knockdown HaCaT cells (Fig. 1C and D). These data suggest that the site of action of SPOP during DNA replication process in non-cancerous keratinocytes is distinct from that in prostate cancer cells.

Although γH2AX was not accumulated by *SPOP* knockdown in HaCaT cells, we noticed that SPOP depletion drastically suppressed cell proliferation (Fig. 1E). This observation was also confirmed by performing ATP assay, in which luminescence signal represents cellular ATP concentrations (Fig. 1F). Next, we examined HaCaT cell

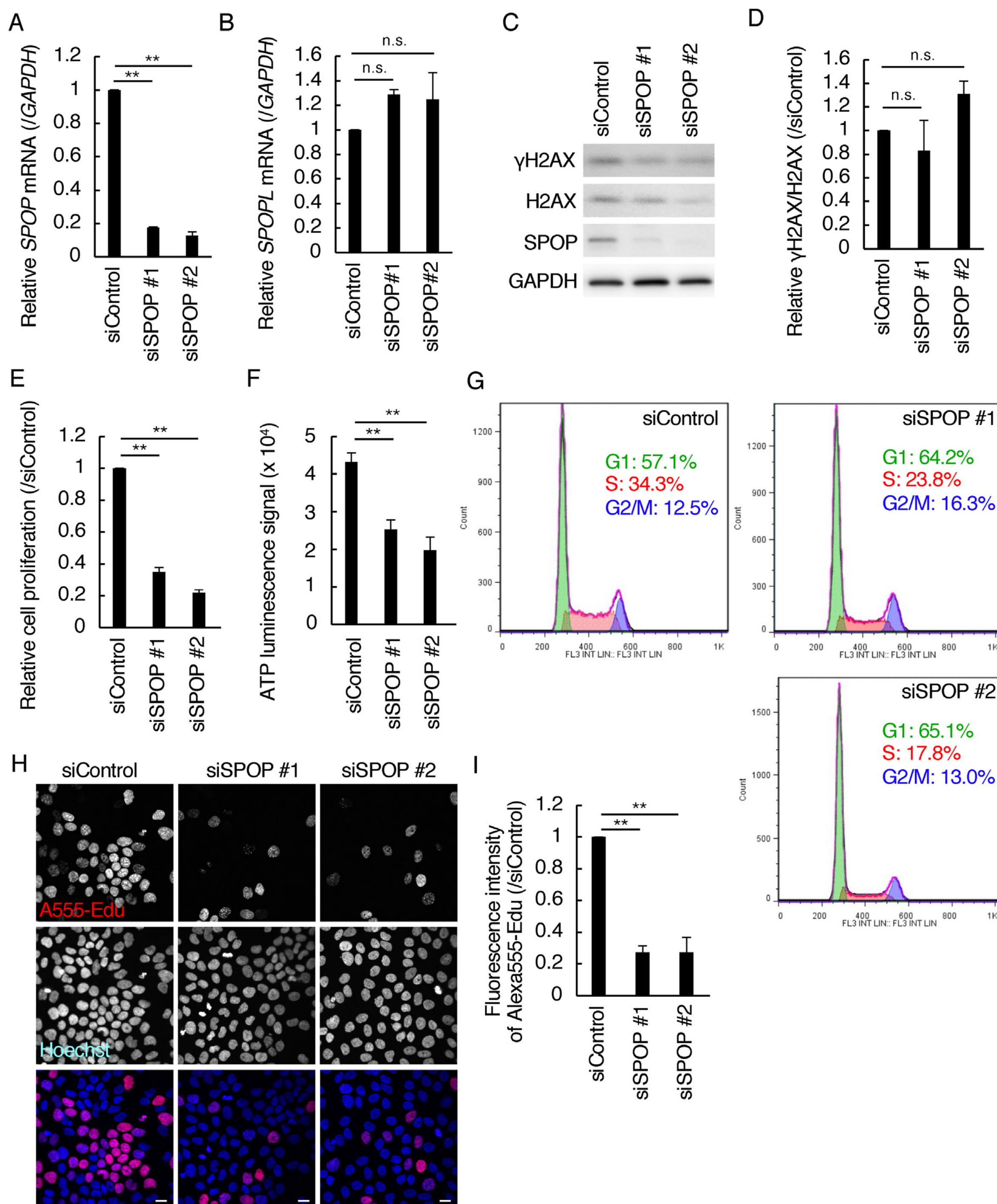

Fig. 1. SPOP knockdown suppresses cell proliferation and DNA synthesis without generation of γH2AX in HaCaT cells. (A and B) The mRNA levels of SPOP (A) and SPOPL (B) in HaCaT cells from three independent experiments were analyzed by RT-PCR at 72 h post-transfection of cells with siRNAs. Data are normalized to siControl. Data represent mean ± SEM (**, $p < 0.01$; n.s., not significant). (C) Western blotting of HaCaT cell lysates 72 h post-transfection of cells with siRNAs. (D) Quantitation of (C). Ratio of γH2AX/H2AX was analyzed based on three independent experiments. Data are normalized to siControl. Data represent mean ± SEM. (n.s., not significant). (E) HaCaT cells were treated with indicated siRNAs for 48 h. Trypsinized cells (total 0.5×10^5 cells) were then replated and treated with the same siRNA the following day. Cell number was counted 72 h after replating. Data are mean ± SEM based on three independent experiments (**; $p < 0.01$). (F) HaCaT cells were treated with indicated siRNAs for 72 h. Cellular ATPase activity was used to measure cell viability. Data are mean ± SEM from six independent experiments. **, $p < 0.01$. (G) HaCaT cells were transfected with indicated siRNA. After 72 h incubation, cells were collected and stained with PI. The cell cycle was then analyzed using flow cytometry. The numbers indicate percentages of cell population in the G1, S and G2/M phases. (H) Confocal images of HaCaT cells incorporated EdU (10 μM) for 4 h before fixation. Cells were fixed at 72 h post-transfection of siRNAs followed by the click reaction to label Alexa555 (A555) to EdU. Bars; 10 μm. (I) Quantitation of (H). Nuclear fluorescence intensity of Alexa555 in more than one hundred cells from three independent experiments was analyzed. Data are normalized to siControl. Data show the mean ± SEM (**, $p < 0.01$).

populations in the G1, S, and G2/M phases under the *SPOP* knockdown condition. As depicted in Fig. 1G, we found that cell populations decreased in the S-phase and increased in the G1-phase upon *SPOP* knockdown in HaCaT cells. We also examined the effects of *SPOP* knockdown on DNA synthesis using EdU, a thymidine analogue that effectively incorporates into newly-synthesized DNA [28]. We observed that EdU incorporation drastically decreased on SPOP depletion in HaCaT cells (Fig. 1H and I). Collectively, these observations indicate that *SPOP* knockdown inhibits HaCaT cell proliferation and DNA synthesis.

3.2. SPOP knockdown reduces CDT1 and CDC6 protein expression in HaCaT cells

During DNA replication licensing, CDT1 and CDC6 form a protein complex with ORCs on chromosomes that is followed by the recruitment of other factors such as MCM helicases [12]. The overexpression of both CDT1 and CDC6 results in DNA rereplication in yeast and mice [19,21], whereas their knockdown in human fetal foreskin fibroblasts reduces cellular DNA synthesis [20]. Since CDT1 and CDC6 are essential for DNA replication licensing, we examined their expression in *SPOP*-knockdown HaCaT cells. CDT1 and CDC6 expression in HaCaT cells significantly decreased (approximately 80%) on *SPOP* knockdown (Fig. 2A–C). In contrast, *SPOP* knockdown in HaCaT cells did not significantly or less affect the expression of MCM helicases, MCM2–7 (Fig. 2A, 2D–I). Given the critical roles of CDT1 and CDC6 in the formation of preRCs, *SPOP* knockdown would suppress their formation. As expected, binding of both MCM2 and MCM5 to chromatin was decreased by the depletion of SPOP in HaCaT cells (Supplementary Fig. 1A). These data suggest that reduced level of CDT1 and CDC6 proteins may cause the inhibition of DNA replication in *SPOP*-knockdown HaCaT cells.

On the contrary, *CDT1* and *CDC6* mRNA expression tended to decrease to some extent, but not significantly, on *SPOP* knockdown (Fig. 2J and K). The depletion of SPOP affected the mRNA expression levels of MCM components to some extent (Fig. 2L–Q). Decreased mRNA level of *CDT1* and *CDC6* including the promotion of their mRNA decay may partially contribute to the decreased amount of those proteins in *SPOP*-knockdown cells. However, the decrease in *CDT1* and *CDC6* mRNA levels was not sufficient to achieve the drastic downregulation of CDT1 and CDC6 observed at the protein level. We thus hypothesized that SPOP regulates CDT1 and CDC6 degradation and/or synthesis at the protein level.

3.3. CDT1 and CDC6 downregulation induces p53-independent p21 expression and cell growth arrest in HaCaT cells

We further examined cell cycle check-point effectors to block cell growth cycle progression, such as cyclin-dependent kinase inhibitors. Compared with control cells, the mRNA expression of p21, a cyclin-dependent kinase inhibitor, significantly increased (approximately four folds) in *SPOP*-knockdown HaCaT cells (Fig. 3A); its protein level also significantly increased (Fig. 3B and C). Considering that p21 inhibits the G1/S transition [29], these data suggest that SPOP acts at the initial steps of the S phase progression in HaCaT cells.

It is well known that p21 mRNA expression is mediated by p53 [30]. Therefore, we analyzed p53 protein level in HaCaT cells under *SPOP*, *CDT1*, and *CDC6* knockdown conditions. The knockdown of these genes had no effect on p53 protein levels (Fig. 3B, D–F), suggesting that the increase in p21 mRNA expression induced by *SPOP* knockdown was p53-independent. However, *CDT1* and *CDC6* knockdown in HaCaT cells elevated p21 protein expression level, but not vice versa, indicating that CDT1 and CDC6 levels regulate p21 expression (Fig. 3E and G). The downregulation of CDT1 and CDC6 protein expression observed upon *SPOP* knockdown was not restored by the depletion of p21 (Fig. 3E, H, 3I). The knockdown of p21, CDT1, or CDC6 did not affect protein expression of SPOP (Fig. 3E and J). As shown in Supplementary Figs. 1B and 1C, p21 knockdown in SPOP-depleted HaCaT cells did not restore the suppression of Edu incorporation. Taken together, these data suggest that SPOP depletion decreases CDT1 and CDC6 protein levels followed by the upregulation of p21 in a p53-independent manner, and at least, the inhibition of DNA replication in SPOP-depleted HaCaT cells is not due to p21 upregulation.

3.4. SPOP knockdown induces CDT1 and CDC6 downregulation in a degradation-independent manner

To investigate the mechanisms of CDT1 and CDC6 downregulation at the protein level in *SPOP*-knockdown HaCaT cells, we firstly treated HaCaT cells with MG132, a proteasome inhibitor. As presented in Fig. 4 (A–C), the treatment of control siRNA-treated cells with MG132 caused a significant accumulation of CDT1 and CDC6 proteins. These results indicate that both CDT1 and CDC6 are degraded via the proteasomal pathway in HaCaT cells. The depletion of SPOP did not result in the accumulation of CDT1 or CDC6 upon MG132 treatment (Fig. 4A–C). We also treated HaCaT cells with CHX, an inhibitory agent that interferes with the translation step in protein synthesis, to monitor the degradation of CDT1 and CDC6. The degradation rate of both CDT1 and CDC6 was not significantly enhanced by *SPOP* knockdown in HaCaT cells (Fig. 4D–F). Collectively, these data suggest that *SPOP* knockdown does not promote CDT1 and CDC6 degradation.

3.5. CDT1 and CDC6 mRNA translation is inhibited in SPOP-knockdown HaCaT cells

Next, we examined whether SPOP positively regulates the translation of CDT1 and CDC6 in HaCaT cells. To this end, HaCaT cells were treated with CHX for 24 h and then washed with PBS and cultured in fresh growth medium to monitor the levels of newly-synthesized CDT1 and CDC6. As shown in Fig. 4 (G–I), the synthesis of CDT1 and CDC6 proteins was significantly suppressed in SPOP depleted HaCaT cells. Of note, the early phase (at 4 h after chase) of CDT1 and CDC6 protein synthesis was remarkably inhibited by *SPOP* knockdown (Fig. 4G–I). We also found that *SPOP* knockdown did not affect the mRNA level of *CDT1* and *CDC6* during the chase after cycloheximide treatment (Supplementary Figs. 2A and 2B). These data suggest that the initial process of translation (e.g., *CDT1* and *CDC6* mRNA recognition by ribosomes) may be impaired in SPOP depleted HaCaT cells. Similar results were obtained using click reactions. HaCaT cells were incubated with HPG, a reactive methionine analogue containing an alkyne moiety, in methionine-free medium. The cell lysates were then subjected to a click reaction with biotin-PEG3-azide and subsequently to pull-down assay using streptavidin beads. As shown in Fig. 4J, newly-synthesized CDT1 and CDC6 were detected in control HaCaT cells incubated with HPG. CDT1 and CDC6 band signals were diminished on treating control cells with CHX in the presence of HPG (Fig. 4J), suggesting the reliability of these experiments. The newly-synthesized CDT1 and CDC6 were significantly reduced on depletion of SPOP in HaCaT cells incubated with HPG (Fig. 4J–L). Collectively, our results suggest that SPOP is essential for the translation of CDT1 and CDC6.

4. Discussion

It has been established that SPOP plays crucial roles in a wide

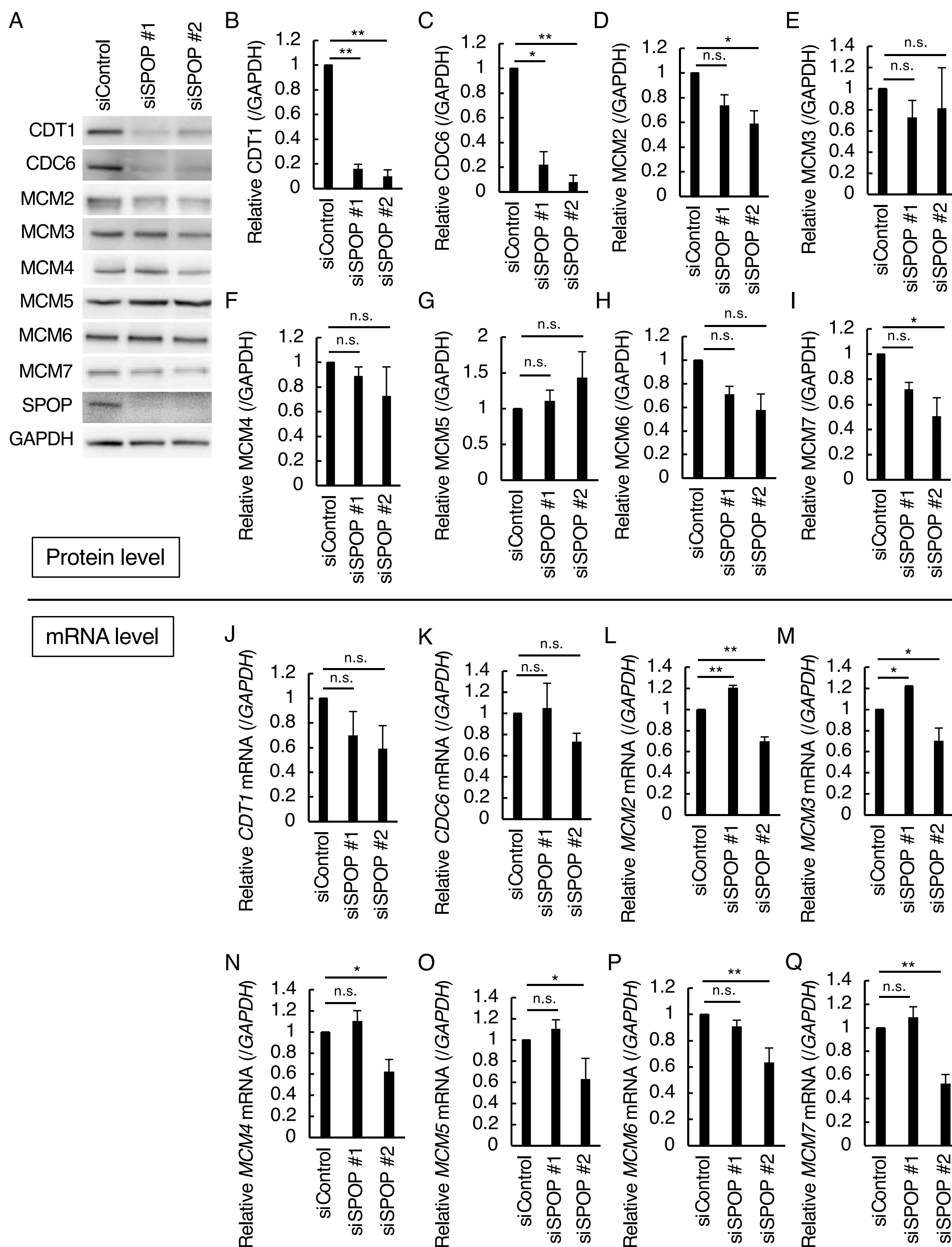

Fig. 2. Depletion of SPOP suppresses the protein expression of CDT1 and CDC6. (A) Western blots of HaCaT cell lysates 72 h post-transfection of siRNAs. (B–I) Quantitation of (A). Ratio of CDT1/GAPDH (B), CDC6/GAPDH (C), MCM2/GAPDH (D), MCM3/GAPDH (E), MCM4/GAPDH (F), MCM5/GAPDH (G), MCM6/GAPDH (H) and MCM7/GAPDH (I) was analyzed from three independent experiments. Data are normalized to siControl. Data represent mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; n.s., not significant. (J–Q) The mRNA level of *CDT1* (J), *CDC6* (K), *MCM2* (L), *MCM3* (M), *MCM4* (N), *MCM5* (O), *MCM6* (P), *MCM7* (Q) in HaCaT cells from three independent experiments was analyzed by RT-PCR at 72 h post-transfection of siRNAs. Data are normalized to siControl. Data represent mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; n.s., not significant).

range of cellular processes, such as steroid hormone receptor signaling, DNA damage response, cell cycle progression, hedgehog/Gli signaling, and PI3K-AKT signaling, by functioning as a substrate recognition receptor of a CUL3-based ubiquitin ligase complex and targets more than 40 substrates [1]. In this study, we demonstrate that SPOP is essential for the initial step of DNA replication, DNA replication licensing, in non-cancerous human keratinocyte-derived HaCaT cells.

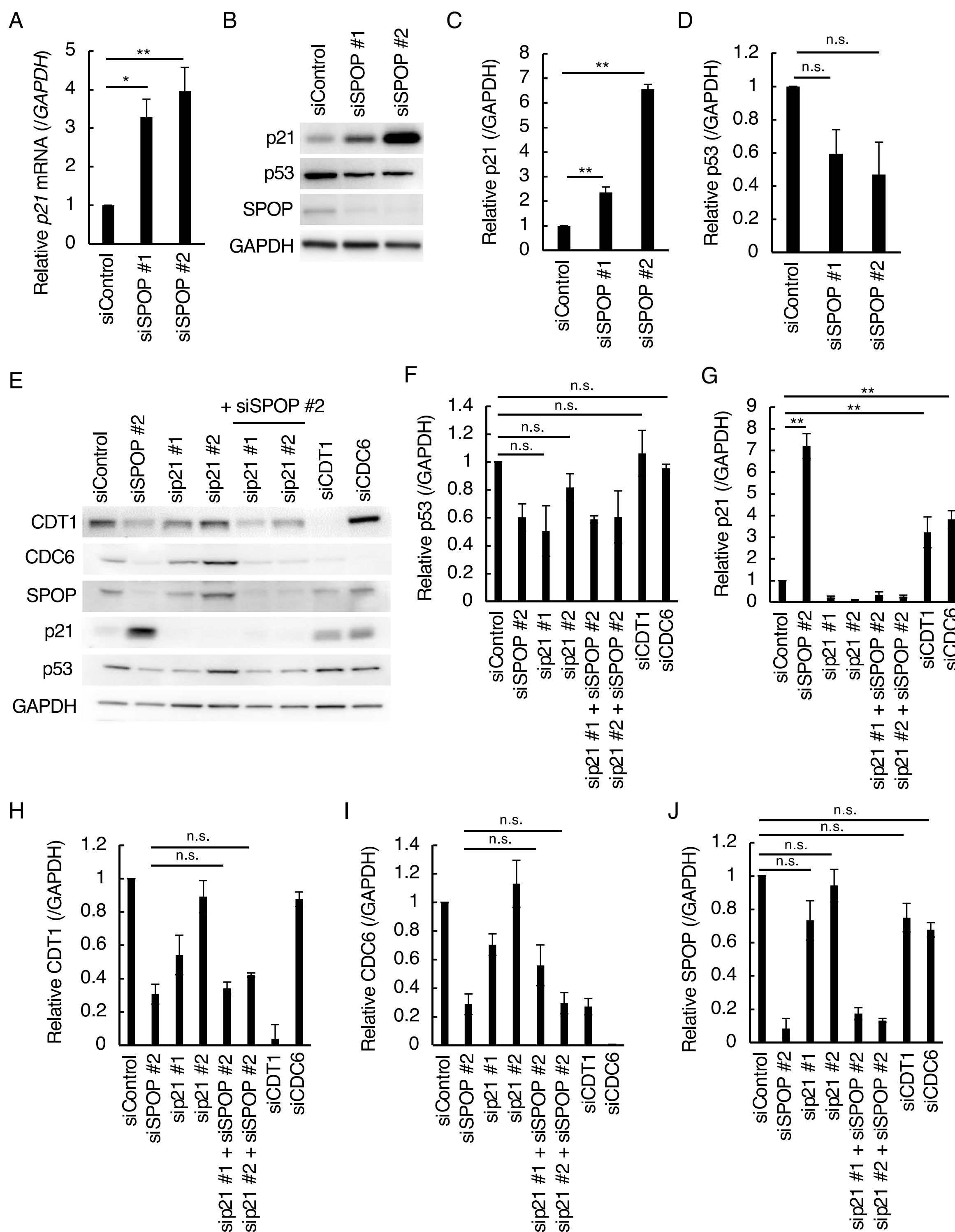

Fig. 3. Depletion of CDT1 and CDC6 resulted in p53-independent upregulation of p21 expression. (A) The mRNA level of p21 in HaCaT cells from three independent experiments was analyzed by RT-PCR at 72 h post-transfection of siRNAs. Data are normalized to siControl. Data represent mean ± SEM. *, $p < 0.05$; **, $p < 0.01$. (B) Western blots of HaCaT cell lysates 72 h post-transfection of siRNAs. (C, D) Quantitation of (B). Ratio of p21/GAPDH (C) and p53/GAPDH (D) was analyzed from three independent experiments. Data are normalized to siControl. Data represent mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; n.s., not significant. (E) Western blots of HaCaT cell lysates 72 h post-transfection of siRNAs. (F–J) Quantitation of (E). Ratio of p53/GAPDH (F), p21/GAPDH (G), CDT1/GAPDH (H), CDC6/GAPDH (I) and SPOP/GAPDH (J) was analyzed from three independent experiments. Data are normalized to siControl. Data represent mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; n.s., not significant).

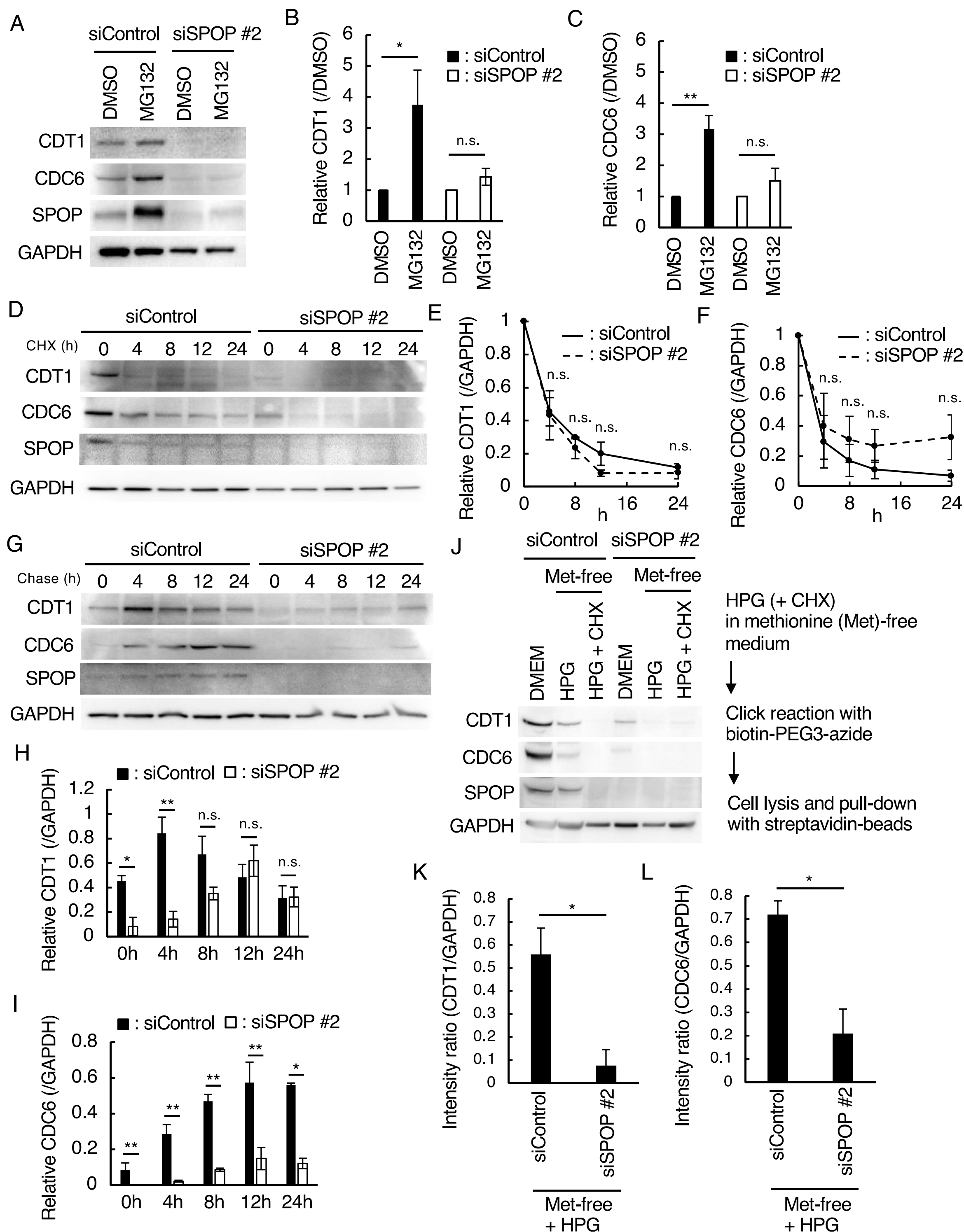

Fig. 4. SPOP is essential for proper translation, but not for their degradation, of CDT1 and CDC6 in HaCaT cells. (A) Western blots of HaCaT cell lysates 72 h post-transfection of siRNAs. Cells were treated with MG132 (10 μM) for 4 h before cell lysis. (B, C) Quantitation of (A). Ratio of CDT1/GAPDH (B) and CDC6/GAPDH (C) was analyzed from three independent experiments. Data are normalized to DMSO. Data represent mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; n.s., not significant. (D) Western blots of control or SPOP-knockdown HaCaT cell lysates in CHX pulse experiments. Seventy-two h post-transfection of siRNAs, cells were incubated with CHX (25 μg/mL) for the indicated time followed by cell lysis. (E, F) Quantitation of (D). Ratio of CDT1/GAPDH (E) and CDC6/GAPDH (F) was analyzed from three independent experiments. Data are normalized to time point 0 h. Data represent mean ± SEM (n.s., not significant). (G) Western blots of control or SPOP-knockdown HaCaT cell lysates in CHX pulse-chase experiments. Forty-eight h post-transfection of siRNAs, cells were incubated with CHX (25 μg/mL) for 24 h. Cells were then washed with PBS, cultured with fresh growth medium for the indicated time followed by cell lysis. (H, I) Quantitation of (G). Ratio of CDT1/GAPDH (H) and CDC6/GAPDH (I) was analyzed from three independent experiments. Data represent mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; n.s., not significant. (J) HaCaT cells were transfected with the indicates siRNAs. At 72 h post-transfection, cells were incubated for 3 h in methionine-free medium containing L-cystine. Cells were then incubated with fresh media containing HPG for 12 h followed by cell lysis. Cell lysates were subjected to click reactions with biotin-PEG3-azide and pull-down with Dynabeads. The newly-synthesized proteins are detected in the lane HPG. The pull-downed samples were analyzed by Western blot. CHX was used as a positive control. See also supplementary information. (K, L) Quantitation of (J). Ratio of CDT1/GAPDH (K) and CDC6/GAPDH (L) of the HPG-treated cell lysates was analyzed from three independent experiments. Data represent mean ± SEM (*, $p < 0.05$).

SPOP knockdown arrested HaCaT cells at the G1/S phase transition and suppressed DNA synthesis, indicating the perturbation of S-phase entry. DNA replication licensing involves the assembly of pre-RCs comprising the ORC component (ORC1–6), CDC6, CDT1, and MCM helicase complex component (MCM2–7). CDC6 and CDT1 are essential for the recruitment of MCMs to ORC on chromosome [12]. In the present study, we showed that SPOP is indirectly involved in DNA replication as it regulates the translation of CDT1 and CDC6 proteins in HaCaT cells (Fig. 4G–L). Given the functions of SPOP as a substrate recognition receptor for the CUL3/RING E3 complex, the CUL3/SPOP E3 complex may ubiquitinate unidentified substrates, which positively regulates the translation of CDT1 and CDC6.

Our results suggest that SPOP controls the specific recognition of *CDT1* and *CDC6* mRNA by ribosomes, leading to their efficient translations. Ribosomal protein L38, a part of the large 60S subunit of the eukaryotic ribosomes, is essential for the specific translation of Homeobox mRNAs during mice development [31]. The ribosome-associated non-coding RNA, rancRNA_s194, inhibits the translation of specific mRNAs in archaea [32]. In yeast, Rps26-depleted ribosomes, generated under the high-salt and high-pH conditions, interact with a distinct set of mRNAs [33]. The profiles of ribosome subtypes during cell cycle, including DNA replication licensing, would shed new light on understanding the specific translation of *CDT1* and *CDC6* mRNAs. We noticed that both 5′-untranslated regions of *CDT1* and *CDC6* mRNAs contains highly-GC enriched sequences (Supplementary Fig. 3. *CDT1*; CGCCGCGCACUCCGCCGCC, *CDC6*; GGCUGCGGGUUCCGGCGAGGCC). The GC rich sequences may form specific three-dimensional structures, which enables to interact with specific RNA binding proteins leading to the efficient and specific translation of *CDT1* and *CDC6* mRNAs.

In case of C4-2 prostate cancer cells that express wild-type *SPOP* gene, the protein expression of tyrosyl-DNA phosphodiesterases (TDP1 and TDP2) was reduced on *SPOP* knockdown without affecting their mRNA levels, leading to the inhibition of DNA protein crosslink repair [11]. The SPOP-dependent regulation of protein translation may also contribute to DNA repair process in prostate cancer cells. Recent studies have shown that a variety of ribosomal proteins are ubiquitinated and deubiquitinated to control ribosomal functions during the translation process [34–37]. For example, the ribosome associated protein quality control requires uS10 ubiquitination followed by the dissociation of 80S ribosomes into 40S and 60S ribosomes [38,39]. The uS3 ubiquitination is essential for the degradation of non-functional 18S ribosomal RNAs [34]. The recognition of mRNA by heterogenous nuclear ribonucleoproteins controls translation efficacy of target mRNAs [40]. SPOP possibly ubiquitinates some RNA-binding proteins that directly interact with the untranslated regions of *CDT1* and *CDC6* mRNAs. The responsible RNA-binding proteins for the CUL3/SPOP E3 complex should be investigated in the future.

p21, a cell cycle checkpoint factor and a marker of cellular senescence [41], was upregulated by *SPOP* knockdown in HaCaT cells in CDC6 and CDT1-dependent and p53-independent manners (Fig. 3), suggesting that SPOP-knockdown HaCaT cells mimic premature senescent cells. It has been also reported that p53-independent p21 pathway in melanocytes induces premature senescence [42]. The dysregulation of DNA replication licensing indicates replicative senescence of keratinocytes [43]. The protein expression of all the components of MCM complex, MCM2–7, which is essential for DNA replication licensing, is reduced as human primary keratinocytes progress into senescence [43]. The enforced expression of a catalytic subunit of human telomerase restored their protein expression in keratinocytes [43]. HaCaT cells could mimic the differentiation of human primary keratinocytes [44]. Given the inactivation of DNA replication licensing due to the down-regulation of CDT1 and CDC6 in *SPOP*-knockdown HaCaT cells, SPOP expression may be reduced during the process of keratinocyte senescence. Keratinocyte senescence is related to various diseases, such as squamous cell carcinoma, melanoma, and solar keratosis [41]. The protein expression of p21 is high in human actinic keratosis tissues, precancerous lesions of the skin [45]. A high expression of CDC6 has been correlated with the development of human oral squamous cell carcinomas (OSCC) [46]. A microRNA, miR-373, negatively regulating SPOP protein expression has been found to be upregulated in human OSCC patients [47]. These previous studies suggest that SPOP may also positively regulate protein expression of CDC6 in OSCC cells. The immunohistochemistry of SPOP in healthy (young *versus* old), cancerous, and precancerous human skin tissues would be necessary to validate the relationship between SPOP expression and keratinocyte senescence.

Author contributions

S.S. and M.M. designed and performed the experiments, analyzed the data, interpreted the results, and wrote the paper. S.T. performed the experiments. H.N., Y.F. and K.S. analyzed the data. S.H. designed and performed the experiments, interpreted the results, and wrote the paper.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgements

We thank Ms. Mami Chosei, Ms. Teruko Tsuda, Mr. Kenji Kameda, Dr. Tomohisa Sakaue, Dr. Masamoto Murakami, Dr. Hideki Mori, and Dr. Hiroyuki Takeda from Ehime University and Dr. Jun Nakayama from National Cancer Center Research Institute for providing their technical assistance. This study was supported by the Division of Analytical Bio-Medicine, Advanced Research Support Center (ADRES), Ehime University. This work was supported by JSPS KAKENHI Grant Number20K18413 to S.S.; JSPS KAKENHI Grant Number 21K07126, 21H00286, and Nakatomi Foundation, KOSE Cosmetology Research Foundation and Suzuken Memorial Foundation to M.M.; JSPS KAKENHI Grant Number 19K10030 to H.N.; Takeda Science Foundation to Proteo-Science Center; and JSPS KAKENHI Grant Number 21K18262 and The Naito Foundation to S.H.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2023.02.012.

References

[1] M.J. Cuneo, T. Mittag, The ubiquitin ligase adaptor SPOP in cancer, FEBS J. 286 (2019) 3946–3958.
[2] P. Genschik, I. Sumara, E. Lechner, The emerging family of CULLIN3-RING ubiquitin ligases (CRL3s): cellular functions and disease implications, EMBO J. 32 (2013) 2307–2320.
[3] T. Bernasocchi, J.P. Theurillat, SPOP-mutant prostate cancer: translating fundamental biology into patient care, Cancer Lett. 529 (2022) 11–18.
[4] X.M. Li, H.L. Wu, Q.D. Xia, P. Zhou, S.G. Wang, X. Yu, J. Hu, Novel insights into the SPOP E3 ubiquitin ligase: from the regulation of molecular mechanisms to tumorigenesis, Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 149 (2022), 112882.
[5] M. Maekawa, S. Higashiyama, The roles of SPOP in DNA damage response and DNA replication, Int. J. Mol. Sci. 21 (2020).
[6] G. Boysen, C.E. Barbieri, D. Prandi, M. Blattner, S.S. Chae, A. Dahija, S. Nataraj, D. Huang, C. Marotz, L. Xu, J. Huang, P. Lecca, S. Chhangawala, D. Liu, P. Zhou, A. Sboner, J.S. de Bono, F. Demichelis, Y. Houvras, M.A. Rubin, SPOP mutation leads to genomic instability in prostate cancer, Elife (2015) 4.

[7] K. Hjorth-Jensen, A. Maya-Mendoza, N. Dalgaard, J.O. Sigurethsson, J. Bartek, D. Iglesias-Gato, J.V. Olsen, A. Flores-Morales, SPOP promotes transcriptional expression of DNA repair and replication factors to prevent replication stress and genomic instability, Nucleic Acids Res. 46 (2018) 9484–9495.
[8] D. Wang, J. Ma, M.V. Botuyan, G. Cui, Y. Yan, D. Ding, Y. Zhou, E.W. Krueger, J. Pei, X. Wu, L. Wang, H. Pei, M.A. McNiven, D. Ye, G. Mer, H. Huang, ATM-phosphorylated SPOP contributes to 53BP1 exclusion from chromatin during DNA replication, Sci. Adv. 7 (2021).
[9] X. Jin, S. Qing, Q. Li, H. Zhuang, L. Shen, J. Li, H. Qi, T. Lin, Z. Lin, J. Wang, X. Cao, J. Yang, Q. Ma, L. Cong, Y. Xi, S. Fang, X. Meng, Z. Gong, M. Ye, S. Wang, C. Wang, K. Gao, Prostate cancer-associated SPOP mutations lead to genomic instability through disruption of the SPOP-HIPK2 axis, Nucleic Acids Res. 49 (2021) 6788–6803.
[10] J. Ma, Q. Shi, G. Cui, H. Sheng, M.V. Botuyan, Y. Zhou, Y. Yan, Y. He, L. Wang, Y. Wang, G. Mer, D. Ye, C. Wang, H. Huang, SPOP mutation induces replication over-firing by impairing Geminin ubiquitination and triggers replication catastrophe upon ATR inhibition, Nat. Commun. 12 (2021) 5779.
[11] R. Watanabe, M. Maekawa, M. Hieda, T. Taguchi, N. Miura, T. Kikugawa, T. Saika, S. Higashiyama, SPOP is essential for DNA-protein cross-link repair in prostate cancer cells: SPOP-dependent removal of topoisomerase 2A from the topoisomerase 2A-DNA cleavage complex, Mol. Biol. Cell 31 (2020) 478–490.
[12] M. Fragkos, O. Ganier, P. Coulombe, M. Méchali, DNA replication origin activation in space and time, Nat. Rev. Mol. Cell Biol. 16 (2015) 360–374.
[13] J.M. van Deursen, The role of senescent cells in ageing, Nature 509 (2014) 439–446.
[14] M. Macheret, T.D. Halazonetis, DNA replication stress as a hallmark of cancer, Ann. rev. pathol. 10 (2015) 425–448.
[15] A. Berson, R. Nativio, S.L. Berger, N.M. Bonini, Epigenetic regulation in neurodegenerative diseases, Trends Neurosci. 41 (2018) 587–598.
[16] H. Nishitani, Z. Lygerou, Control of DNA replication licensing in a cell cycle, Gene Cell. : devoted to molecular & cellular mechanisms 7 (2002) 523–534.
[17] S.P. Bell, Rethinking origin licensing, Elife 6 (2017).
[18] T.J. Kelly, G.S. Martin, S.L. Forsburg, R.J. Stephen, A. Russo, P. Nurse, The fission yeast cdc18+ gene product couples S phase to START and mitosis, Cell 74 (1993) 371–382.
[19] H. Nishitani, Z. Lygerou, T. Nishimoto, P. Nurse, The Cdt1 protein is required to license DNA for replication in fission yeast, Nature 404 (2000) 625–628.
[20] T.E. Braun, E. Poole, J. Sinclair, Depletion of cellular pre-replication complex factors results in increased human cytomegalovirus DNA replication, PLoS One 7 (2012), e36057.
[21] S. Muñoz, S. Búa, S. Rodríguez-Acebes, D. Megías, S. Ortega, A. de Martino, J. Méndez, In vivo DNA Re-replication elicits lethal tissue dysplasias, Cell Rep. 19 (2017) 928–938.
[22] M. Maekawa, K. Tanigawa, T. Sakaue, H. Hiyoshi, E. Kubota, T. Joh, Y. Watanabe, T. Taguchi, S. Higashiyama, Cullin-3 and its adaptor protein ANKFY1 determine the surface level of integrin beta1 in endothelial cells, Biology open 6 (2017) 1707–1719.
[23] M. Tezuka-Kagajo, M. Maekawa, A. Ogawa, Y. Hatta, E. Ishii, M. Eguchi, S. Higashiyama, Development of human CBF1-targeting single-stranded DNA aptamers with antiangiogenic activity in vitro, Nucleic Acid Therapeut. 30 (2020) 365–378.
[24] K. Nishiyama, M. Maekawa, T. Nakagita, J. Nakayama, T. Kiyoi, M. Chosei, A. Murakami, Y. Kamei, H. Takeda, Y. Takada, S. Higashiyama, CNKSR1 serves as a scaffold to activate an EGFR phosphatase via exclusive interaction with RhoB-GTP, Life sci. alliance 4 (2021).
[25] J. Murai, S.Y. Huang, B.B. Das, A. Renaud, Y. Zhang, J.H. Doroshow, J. Ji, S. Takeda, Y. Pommier, Trapping of PARP1 and PARP2 by clinical PARP inhibitors, Cancer Res. 72 (2012) 5588–5599.
[26] A. Murakami, M. Maekawa, K. Kawai, J. Nakayama, N. Araki, K. Semba, T. Taguchi, Y. Kamei, Y. Takada, S. Higashiyama, Cullin-3/KCTD10 E3 complex is essential for Rac1 activation through RhoB degradation in human epidermal growth factor receptor 2-positive breast cancer cells, Cancer Sci. 110 (2019) 650–661.
[27] M. Gschweitl, A. Ulbricht, C.A. Barnes, R.I. Enchev, I. Stoffel-Studer, N. Meyer-Schaller, J. Huotari, Y. Yamauchi, U.F. Greber, A. Helenius, M. Peter, A SPOPL/Cullin-3 ubiquitin ligase complex regulates endocytic trafficking by targeting EPS15 at endosomes, Elife 5 (2016), e13841.
[28] A. Salic, T.J. Mitchison, A chemical method for fast and sensitive detection of DNA synthesis in vivo, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 2415–2420.
[29] J. Bartek, J. Lukas, Pathways governing G1/S transition and their response to DNA damage, FEBS Lett. 490 (2001) 117–122.
[30] C.L. Brooks, W. Gu, New insights into p53 activation, Cell Res. 20 (2010) 614–621.
[31] N. Kondrashov, A. Pusic, C.R. Stumpf, K. Shimizu, A.C. Hsieh, J. Ishijima, T. Shiroishi, M. Barna, Ribosome-mediated specificity in Hox mRNA translation and vertebrate tissue patterning, Cell 145 (2011) 383–397.
[32] L. Wyss, M. Waser, J. Gebetsberger, M. Zywicki, N. Polacek, mRNA-specific translation regulation by a ribosome-associated ncRNA in Haloferax volcanii, Sci. Rep. 8 (2018), 12502.
[33] M.B. Ferretti, H. Ghalei, E.A. Ward, E.L. Potts, K. Karbstein, Rps26 directs mRNA-specific translation by recognition of Kozak sequence elements, Nat. Struct. Mol. Biol. 24 (2017) 700–707.
[34] T. Sugiyama, S. Li, M. Kato, K. Ikeuchi, A. Ichimura, Y. Matsuo, T. Inada, Sequential ubiquitination of ribosomal protein uS3 triggers the degradation of non-functional 18S rRNA, Cell Rep. 26 (2019) 3400–3415, e3407.
[35] C. Meyer, A. Garzia, P. Morozov, H. Molina, T. Tuschl, The G3BP1-family-USP10 deubiquitinase complex rescues ubiquitinated 40S subunits of ribosomes stalled in translation from lysosomal degradation, Mol. Cell 77 (2020) 1193–1205, e1195.
[36] B. Ossareh-Nazari, C.A. Niño, M.H. Bengtson, J.W. Lee, C.A. Joazeiro, C. Dargemont, Ubiquitylation by the Ltn1 E3 ligase protects 60S ribosomes from starvation-induced selective autophagy, J. Cell Biol. 204 (2014) 909–917.
[37] Y. Takehara, H. Yashiroda, Y. Matsuo, X. Zhao, A. Kamigaki, T. Matsuzaki, H. Kosako, T. Inada, S. Murata, The ubiquitination-deubiquitination cycle on the ribosomal protein eS7A is crucial for efficient translation, iScience 24 (2021), 102145.
[38] K. Ikeuchi, P. Tesina, Y. Matsuo, T. Sugiyama, J. Cheng, Y. Saeki, K. Tanaka, T. Becker, R. Beckmann, T. Inada, Collided ribosomes form a unique structural interface to induce Hel2-driven quality control pathways, EMBO J. 38 (2019).
[39] Y. Matsuo, K. Ikeuchi, Y. Saeki, S. Iwasaki, C. Schmidt, T. Udagawa, F. Sato, H. Tsuchiya, T. Becker, K. Tanaka, N.T. Ingolia, R. Beckmann, T. Inada, Ubiquitination of stalled ribosome triggers ribosome-associated quality control, Nat. Commun. 8 (2017) 159.
[40] E. Matoulkova, E. Michalova, B. Vojtesek, R. Hrstka, The role of the 3' untranslated region in post-transcriptional regulation of protein expression in mammalian cells, RNA Biol. 9 (2012) 563–576.
[41] A.S. Wang, O. Dreesen, Biomarkers of cellular senescence and skin aging, Front. Genet. 9 (2018) 247.
[42] X. Hou, J. Shi, L. Sun, L. Song, W. Zhao, X. Xiong, Y. Lu, The involvement of ERK1/2 and p38 MAPK in the premature senescence of melanocytes induced by H(2)O(2) through a p53-independent p21 pathway, J. Dermatol. Sci. 105 (2022) 88–97.
[43] H. Harada, H. Nakagawa, M. Takaoka, J. Lee, M. Herlyn, J.A. Diehl, A.K. Rustgi, Cleavage of MCM2 licensing protein fosters senescence in human keratinocytes, Cell Cycle 7 (2008) 3534–3538.
[44] I. Colombo, E. Sangiovanni, R. Maggio, C. Mattozzi, S. Zava, Y. Corbett, M. Fumagalli, C. Carlino, P.A. Corsetto, D. Scaccabarozzi, S. Calvieri, A. Gismondi, D. Taramelli, M. Dell'Agli, HaCaT cells as a reliable in vitro differentiation model to dissect the inflammatory/repair response of human keratinocytes, Mediat. Inflamm. 2017 (2017), 7435621.
[45] A. Marinescu, C. Mărgăritescu, A.E. Stepan, A.M. Marinescu, C.E. Simionescu, F.M. Nitu, P21 immunoexpression in actinic keratosis and cutaneous carcinomas, Curr. health sci. j. 43 (2017) 226–230.
[46] C.J. Feng, H.J. Li, J.N. Li, Y.J. Lu, G.Q. Liao, Expression of Mcm7 and Cdc6 in oral squamous cell carcinoma and precancerous lesions, Anticancer Res. 28 (2008) 3763–3769.
[47] X.J. Zhang, Y. Jin, J.L. Song, F. Deng, MiR-373 promotes proliferation and metastasis of oral squamous cell carcinoma by targeting SPOP, Eur. Rev. Med. Pharmacol. Sci. 23 (2019) 5270–5276.