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THG-1 suppresses SALL4 degradation to induce stemness genes and tumorsphere formation through antagonizing NRBP1 in squamous cell carcinoma cells

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ABSTRACT

Knockdown of *THG-1* in TE13 esophageal squamous cell carcinoma (ESCC) cells is known to suppress tumorsphere growth. THG-1 was identified as an NRBP1 binding protein, and NRBP1 was reported to downregulate an stemness-related transcriptional factor SALL4, so we decided to examine the possibility that tumorigenic function of THG-1 is achieved by the competition to the tumor-suppressive function of NRBP1. SALL4 was decreased in *THG-1* deficient TE13 cells with reduced tumorsphere formation, while exogenous SALL4 expression in THG-1 deficient TE13 cells recovered expression of stemness genes (*NANOG* and *OCT4*) and partially, but significantly, recovered tumorsphere formation ability. Additionally, we found that NRBP1 induced ubiquitination of SALL4, and THG-1 interrupted the ubiquitination of SALL4 by antagonizing NRBP1 binding to SALL4. These results suggest that THG-1 promotes tumorsphere growth of ESCC cells by the stabilization of SALL4 protein and induction of the target stemness genes through competitive binding to NRBP1.

1. Introduction

Cancer stem cells (CSCs) represent a small subpopulation among cancer cells, that are present within a tumor, and their tumor-initiation properties have been suggested to be the driving force behind metastasis and relapse [1]. Whilst daughter cells from tissue stem cells differentiate into the various types of cells from their tissues or organ of origin, differentiated daughter cancer cells from CSCs form tumors [2]. It has been reported that CSCs are resistant to anticancer drugs. Most of classical anti-cancers drugs target proliferating cells but cancer stem cells can stay in a dormant state are thereby remain and untargeted by conventional anti-cancer drugs. Thus, targeting CSCs has important implications for finding effective therapeutic strategies for cancer patients [3].

Sal-like protein 4 (SALL4) was initially discovered to be an essential stemness regulator in embryonic stem (ES) cells. SALL4 is a transcription factor particularly expressed in embryonic stem cells and is involved in maintaining stem cell pluripotency [4,5]. SALL4 forms a core transcriptional network with OCT4, NANOG, and SOX2 to concurrently activate self-renewal and repress differentiation related genes in murine ES cells [5,6]. SALL4 is suppressed or absent in most adult tissues but is reactivated in many types of cancer cells and is often associated with poor prognosis [7–13]. Therefore, multiple studies have suggested that SALL4 could be a new biomarker for early diagnosis of cancer, and a novel therapeutic target for cancer [11–13]. The molecular mechanism by which SALL4 is regulated in cells remains elusive; however, one molecule called nuclear receptor-binding protein 1 (NRBP1) has

Abbreviations: THG-1, TSC22 Homologous Gene-1; SALL4, Sal-like protein 4; NRBP1, nuclear receptor-binding protein 1.

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been identified to downregulate SALL4 protein [14]. NRBP1 is known to functions as an adaptor for Cullin 5-type E3 ubiquitin ligase. It shuttles molecules between the nucleus and cytoplasm as an adaptor protein [15,16]. Functional domains of NRBP1 such as a protein kinase C domain, two putative nuclear receptor binding motifs, a potential Src homology-2 (SH2) domain, a pseudo-kinase domain, a BC-box, and a myeloid leukemia factor 1 (MLF1)-binding domain have been identified through protein sequence analyses [16–18]. Recent studies reported NRBP1 as a tumor suppressor through downregulation of β-catenin signaling, JNK signaling, and SALL4 [14,19,20].

TSC22 homologous gene-1 (THG-1), also known as TSC-22 domain family member 4 (TSC22D4), was found to interact with NRBP1 [14]. THG-1 has a C-terminal TSC-box adjacent to a leucine zipper motif [21,22]. A murine *Tsc22d4 gene* was first identified as a transcription factor in embryonic pituitary cells and enhanced expression of *Tsc22d4* showed correlation with differentiation of embryonic pituitary cells [23], development of mature Purkinje cells and adult cerebellar granule neurons [24]. Its function in liver metabolism was also reported. THG-1 affected systemic glucose metabolism through regulating transcription of lipocalin 14 (LCN14) and reduced insulin sensitivity [25]. Furthermore, THG-1 overexpression suppressed serum very-low-density lipoprotein and regulated liver metabolism [26].

Esophageal squamous cell carcinoma (ESCC) is the most common type of esophageal cancer and the sixth-leading cause of death by cancer in the world [27,28]. Identification of many diagnostic methods and combinational chemotherapies has brought efficient cure and longer survival for many patients with ESCC. The standard treatment for esophageal cancer patients is surgical resection following radiation therapy or chemoradiotherapy. However, these treatments still show poor prognosis in the advanced stages with low overall survival rate due to metastasis and relapse that is initiated by chemoradioresistant CSCs [29–31].

This study confirmed the interaction between NRBP1 and SALL4. Furthermore, THG-1 was found to antagonize NRBP1 and rescues SALL4 from proteasomal degradation, leading to the induction of stemness gene and promotion of tumorsphere growth in ESCC cells.

2. Materials and methods

2.1. Cell lines and cell culture

HEK293T cells were purchased from the American Type Culture Collection. ESCC cells (TE13) were donated by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. Human cell line authenticity of these cells was confirmed in October 2019 at the Department of Human Genetics Forensic Laboratory for DNA-research, Leiden University Medical Center, Leiden, the Netherlands. These cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Life Technologies, Paisley, UK), 50 U/ml penicillin (Wako) and 50 μg/ml streptomycin (Wako) at 37 °C in a 5% CO2 atmosphere. For the indicated experiments, the culture medium was supplemented with a proteasome inhibitor MG132 (Peptide Institute Inc).

2.2. Generation of THG-1 knockdown TE13 cells

RNAi-mediated *THG-1* knockdown vectors were constructed by cloning the targeting sequences into shRNA expression vector using the pSUPER RNAi system (OligoEngine). The following sequences were used for targeting: 5′-GGACGTGTGTGGATGTTTA-3′ for shTHG#2, 5′-GAAGCCTGGTTGGCATTGA-3′ for shTHG#3 and 5′-GCGCGCTTTGTAGGATTCG-3′ for non-targeting control (shControl). TE13 cells were transfected with pSUPER.puro-shTHG-1#2, pSUPER.puro-shTHG-1#3, or pSUPER.puro-control, and selected in the presence of 1 μg/ml of puromycin.

2.3. Plasmid DNA transfection

The cells were transfected with the plasmids indicated in Supplementary Table S1 using transfection reagents, polyethylenimine (PEI) or Lipofectamine 3000 (Invitrogen), according to the manufacturers' recommendations.

2.4. Western blot analysis

Cells were lysed either in HEPES lysis buffer with 50 mM HEPES, 250 mM NaCl, 0.1% SDS, 0.5% Nonidet P-40, and Complete Protease Inhibitor Cocktail (Roche) or in Nonidet P-40 lysis buffer with 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, aprotinin 2000 kallikrein inactivating units (KIU)/ml, and leupeptin (1 μg/ml). After centrifugation at 13200 rpm for 10 min at 4 °C, the protein concentrations of the supernatants were quantitated. Approximately 20–40 μg of the protein extracts were run on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), followed by electrophoretic transfer to a polyvinylidene difluoride membrane (Merck Millipore). After 30 min blocking at room temperature with 5% skim milk, the membranes were incubated with the indicated primary antibodies at 4 °C overnight. The information pertaining to the primary antibodies is shown in Supplementary Table S2. Thereafter, the membranes were incubated for 4 h or overnight at 4 °C with horseradish peroxidase (HRP)-conjugated secondary antibodies specific for mouse IgG, rabbit IgG, or rat IgG. After incubation with the secondary antibodies, the membranes were washed 3 times in Tris-buffered saline, pH 7.6, with 0.1 w/v % Tween 20 (TBS-T). The proteins were detected using an LAS-3000 Image Analyzer (Fuji Photo Film) with a chemiluminescent HRP substrate.

2.5. Coimmunoprecipitation (Co-IP)

The cell lysates were incubated with the indicated antibodies for 2 h at 4 °C following which protein G beads (GE Healthcare Bio-Science) were added to each sample. After further incubation with the beads for 50 min at 4 °C with end-over-end rotation, the beads were washed 3 times. Then, 2 × SDS sample buffer was added to the beads and the samples were boiled at 98 °C for 5 min.

2.6. Tumor sphere-formation assay

Primary tumor spheres were grown from 1500 resuspended single cells per well in 12-well ultra-low attachment plates coated with poly-2-hydroxyethyl methacrylate (Sigma-Aldrich) and cultured for 15 days in serum-free DMEM/F12 (Sigma-Aldrich) supplemented with B27 (20 ng/ml; Life Technologies), epidermal growth factor (EGF) (20 ng/ml; Sigma-Aldrich), and basic fibroblast growth factor (FGF) (20 ng/ml; Wako). The number of tumorspheres was counted and the diameter of each tumorsphere was measured from each replicate well.

2.7. Immunofluorescence (IF) staining

The indicated cells were plated on glass coverslips and cultured for 2 days. Then, the coverslips were washed with phosphate buffered saline (PBS) and fixed with 4% neutral buffered paraformaldehyde for 15 min. After fixation, the cells were incubated with anti-SALL4 antibodies (EE-30, Santacruz) overnight at 4 °C. After washing with PBS, cells were incubated with anti-mouse IgG

conjugated with Alexa Fluor 488 (#1704586; Life Technologies) for 4 h at 4 °C. The coverslips were incubated with Hoechst33342 (#1704586; Life Technologies) for nuclear staining and then mounted onto glass slides with mounting medium (Dako). The cells were observed and photographed using a confocal laser scanning microscope (TCS SP 8; Leica).

2.8. Quantitative real-time PCR

Total RNA was isolated from the indicated cells by use of ISOGEN II (Nippon Gene). First-strand cDNA was synthesized by reverse transcription using High-capacity RNA-to-cDNA Master Mix (Applied Biosystems) in accordance with the manufacturer's protocol. Real-time qPCR was performed using a GeneAce SYBR qPCR Mix α Low ROX (Nippon Gene) and an ABI 7500 Fast Sequence Detection system according to the manufacturer's protocol. Each result of the final quantitation was determined via a standard curve protocol relative to the *β-actin*. For each experiment, all the samples were analyzed in triplicate. The primer sequences are listed in Supplementary Table S3.

2.9. Statistical analysis

All statistical data values are presented as the means ± standard deviations (SDs) derived from a minimum of three independent experiments. Probability values (*P* values) were calculated by one-way analysis of variance (ANOVA). Apparent differences were assessed for significance using GraphPad Prism 5.0 (GraphPad software, San Diego, CA). Probability values less than 0.05 ($P < 0.05$) were considered significant.

3. Results

3.1. THG-1 knockdown decreases tumorsphere growth of TE13 cells with downregulated SALL4 and its target stemness gene expression

To investigate the role of THG-1 in TE13 cells, we first established THG-1 knockdown (KD) TE13 cells with shTHG-1 (Fig. 1A). Then, we performed tumorsphere-forming assay to analyze anchorage-independent growth potential of the THG-1 KD cells. The results show that tumorspheres from THG-1 KD cells could not grow to form large spheres (Fig. 1B and C). We also analyzed the population ratio of each size of tumorspheres (Supplementary Fig. S1). In our experimental conditions, tumorspheres could grow and survive until 15 days, and more than half of the tumorspheres of the parental TE13 cells had over 100 μm of sphere diameter (60.86%), THG-1 KD cells, however, showed a smaller population of large tumorspheres; 13.40% in shTHG-1#2 cells and 27.52% in shTHG-1#3 cells (Supplementary Fig. S1B).

Tumorsphere culture is widely used to evaluate stem cell function. Recent studies suggested that SALL4, which is known as a key stemness regulator, is highly expressed in various cancer. Therefore, we examined SALL4 expression in THG-1 KD cells using Western blot analysis and immunofluorescent staining (IF) (Fig. 1D and E) and found that SALL4 expression was downregulated in THG-1 KD cells. We further examined expression of SALL4 target genes using real-time quantitative PCR (qPCR). Consistently, expression of *NANOG* and *OCT4* was significantly downregulated in THG-1 KD cells under tumorsphere culture conditions.

3.2. Exogenous expression of SALL4 retrieved anchorage independent growth of THG-1 KD TE13 cells

To clarify whether SALL4 is critical for sphere formation of TE13 cells, we established exogenous SALL4 expression in THG-1 KD TE13 cells, which was confirmed using Western blot analysis (Fig. 2A). Then, we performed tumorsphere forming assay with these cells. The results show that exogenous SALL4 partially but significantly recovers the tumorsphere-forming ability of THG-1 KD TE13 cells (Fig. 2B and C, Supplementary Fig. S2). Exogenous SALL4 expression increased the number and the ratio of tumorspheres over 100 μm of diameter (Fig. 2C and Supplementary Figs. S2A and S2B). We further investigated stemness gene expression and found that expression of SALL4 target genes; *NANOG* and *OCT4* was significantly increased in exogenous SALL4 expressing THG-1 KD cells, suggesting that exogenous SALL4 promotes stemness gene expression, and boosts tumorsphere formation in THG-1 KD cells.

3.3. THG-1 rescues SALL4 from proteasomal degradation by NRBP1

To identify the molecular mechanism by which THG-1 regulates SALL4, we focused on NRBP1 which was reported to bind THG-1 and to downregulates SALL4 protein (17). To confirm the function of NRBP1 as a regulator of SALL4, we expressed NRBP1 with SALL4 in human embryonic kidney (HEK) 293T cells. Following 4 h cycloheximide (CHX) treatment, we could detect the reduced amount of SALL4 protein in the presence of NRBP1 (Fig. 3A). NRBP1 is known as an adaptor for Cullin 5-type E3 ubiquitin ligase (19), so we further examined the amount of SALL4 protein in the presence of a proteasomal inhibitor MG132. As a result, MG132 treatment rescued SALL4 protein that had been ubiquitinated by NRBP1, suggesting that NRBP1 downregulates SALL4 through proteasomal degradation (Fig. 3B). Additionally, we investigated the effect of THG-1 overexpression on the downregulation of SALL4 by NRBP1. We expressed SALL4, NRBP1 and THG-1 in HEK293T cells, and analyzed these protein amounts by Western blot analysis. The result showed that THG-1 recovered SALL4 protein from its downregulation by NRBP1 (Fig. 3C and D).

3.4. THG-1 interrupts ubiquitination of SALL4 through antagonizing NRBP1

We next clarified the interaction between THG-1 and NRBP1 as well as NRBP1 and SALL4 by immunoprecipitation (IP)-Western blot analysis (Fig. 4A and B). Furthermore, we examined the effect of THG-1 on the interaction between NRBP1 and SALL4. The result showed that NRBP1-SALL4 interaction was interrupted by THG-1 in dose-dependent manner (Fig. 4C). THG-1 deletion mutant (THG-$1^{\Delta 81-92}$) has lower interaction with NRBP1 (Supplementary Fig. S3), so that we tested the effect of THG-$1^{\Delta 81-92}$ on the NRBP1-SALL4 interaction. As a result, THG-$1^{\Delta 81-92}$ did not interrupt the interaction between NRBP1 and SALL4 (Fig. 4D), indicating that THG-1 interrupts the NRBP1-SALL4 interaction via antagonizing NRBP1 binding to SALL4. Additionally, we performed ubiquitination assay to clarify the mechanism of proteasomal degradation of SALL4 by NRBP1. The result displayed increased ubiquitination of SALL4 by NRBP1 and that it was interrupted by THG-1 (Fig. 4E).

4. Discussion

Acquisition of the stemness is an important mechanism of cancer cells that enables them to be resistant to

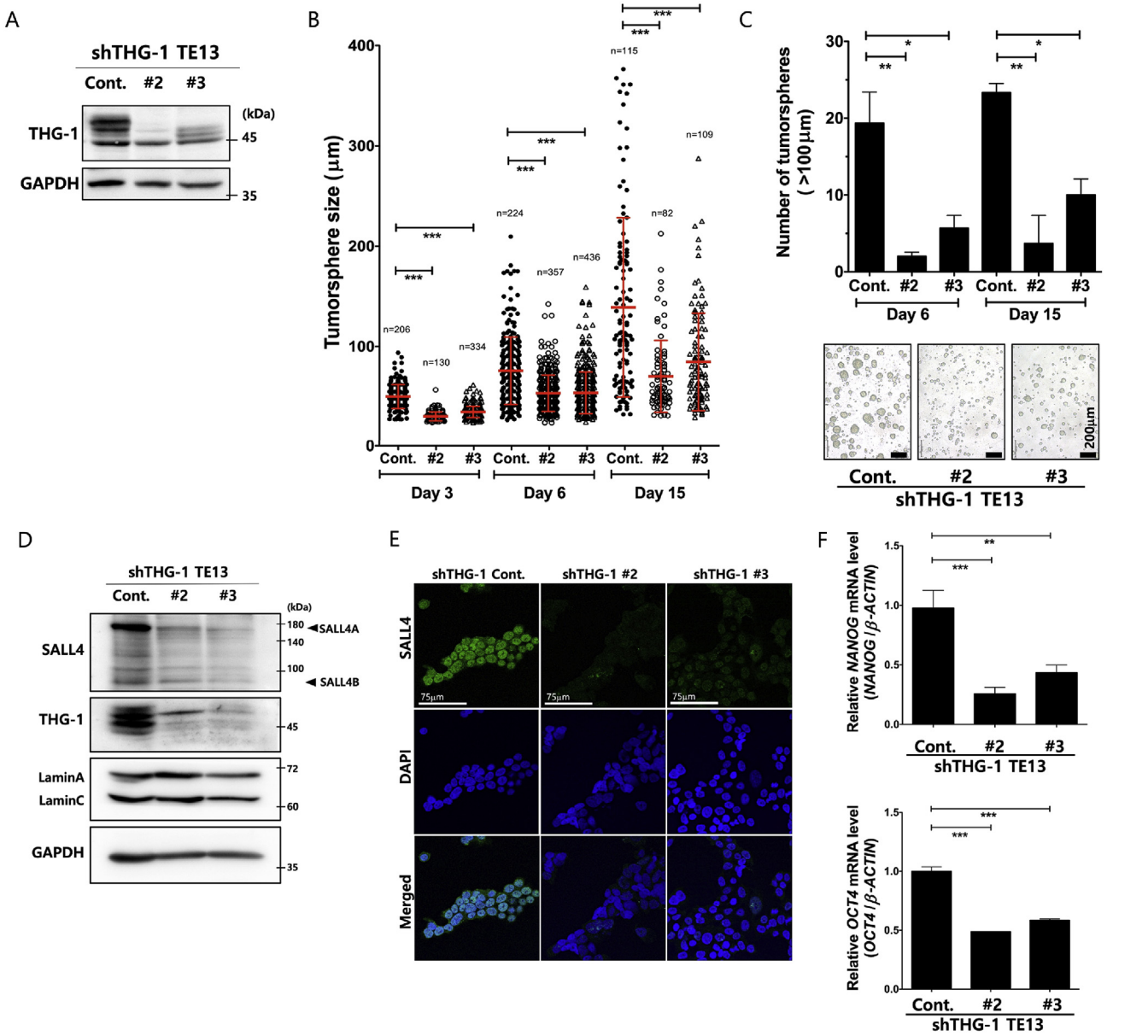

Fig. 1. THG-1 knockdown (KD) decreases tumorsphere growth of TE13 cells with lower expression of SALL4 and its target genes. (A) Confirmation of THG-1 KD (shown as shTHG-1 #2 and #3) in TE13 cells by Western blot analysis. (B, C) Tumorsphere forming assay with THG-1 KD TE13 cells. (B) Tumorspheres over 25 μm of diameter were protted in each day. (C) Number of tumorspheres over 100 μm of diameter were counted in each day. All scale bars indicates 200 μm. (D) Endogenous protein expression of SALL4 in THG-1 KD TE13 cells by Western blot analysis. (E) Immunofluorescence staining of THG-1 KD TE13 cells with anti-SALL4 antibody. All scale bars indicate 75 μm (F) Stemness gene expression (*NANOG* and *OCT4*) in THG-1 KD TE13 cells. Relative gene expression levels were quantified and normalized by *β-ACTIN*. (E) Statistical data are presented as the means ± SDs derived from 3 independent experiments (n = 3), ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

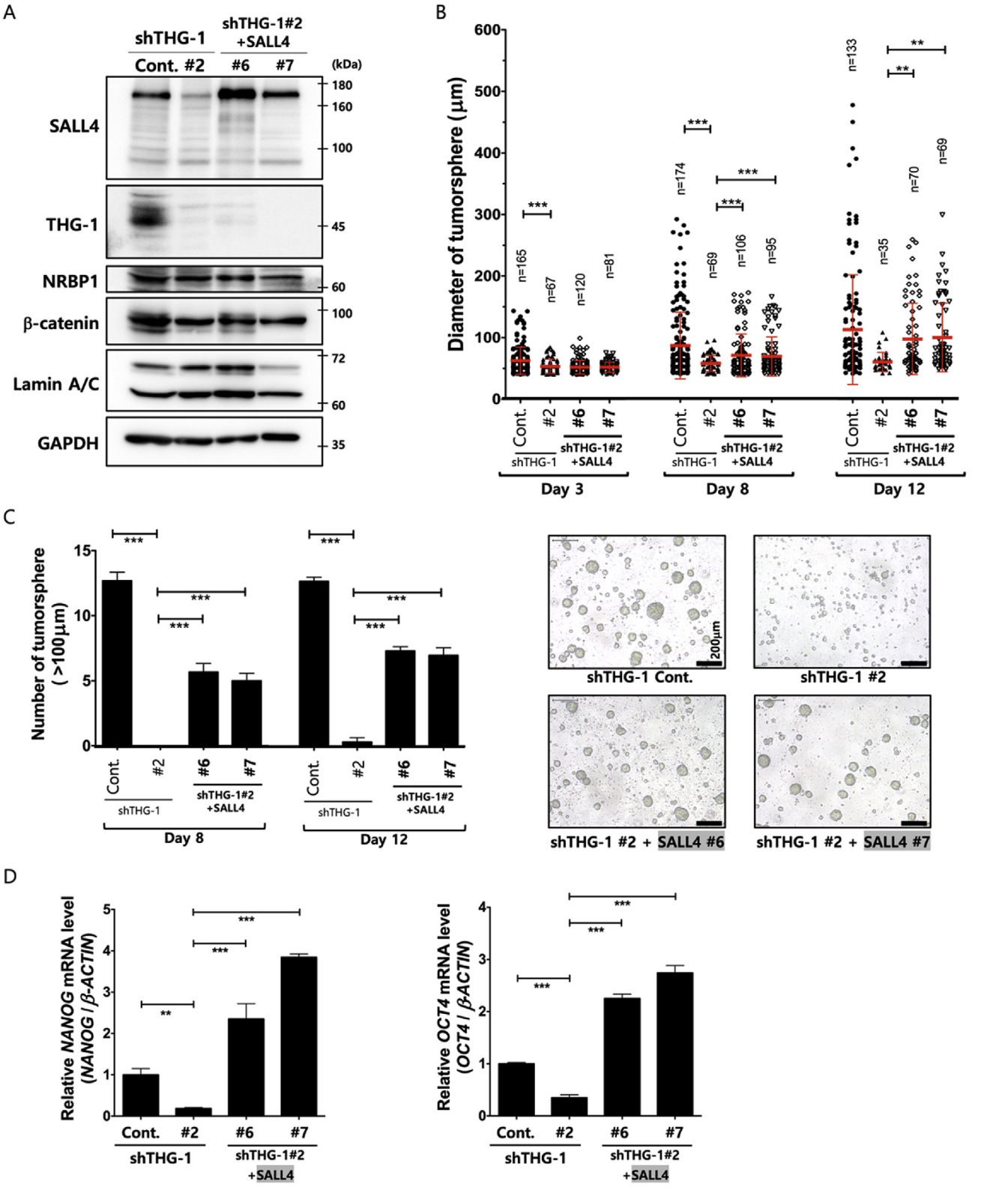

Fig. 2. Exogenous SALL4 expression recovered tumorsphere growth abilitly in THG-1 KD TE13 cell. THG-1 KD TE13 cells (shTHG-1 #2) were transfected with pyCAG-IB-FLAG-human SALL4 plasmids or empty vector (for control of SALL4 overexpressing THG-1 KD TE13 cells) and selected by blasticidin in DMEM culture medium. Several single clones were selected and #6 and #7 clones were selected as exogenous SALL4 expressing THG-1 KD TE13 cells. Control means the TE13 cells transfected with non-targeting shRNA (shControl). (A) Confirmation of exogenous SALL4 expression in THG-1 KD cell (shTHG-1 #2) by Western blot analysis. (B, C) Tumorsphere forming assay with exogenous SALL4 expressing THG-1 KD TE13 cells. (B) Tumor spheres over 50 μm in diameter of exogenous SALL4 expressing THG-1 KD TE13 cells were plotted in each day. (C) Numbers of tumor spheres over 100 μm in diameter of exogenous SALL4 expressing THG-1 KD TE13 cells were counted in each day. All scale bars indicates 200 μm. (D) Stemness gene expression (*NANOG* and *OCT4*) in exogenous SALL4 expressing THG-1 KD TE13 cells. Relative gene expression were quantified and normalized by *β-ACTIN*. Statistical data are presented as the means ± SDs derived from 3 independent experiments (n = 3), (*P < 0.05, **P < 0.01, ***P < 0.001).

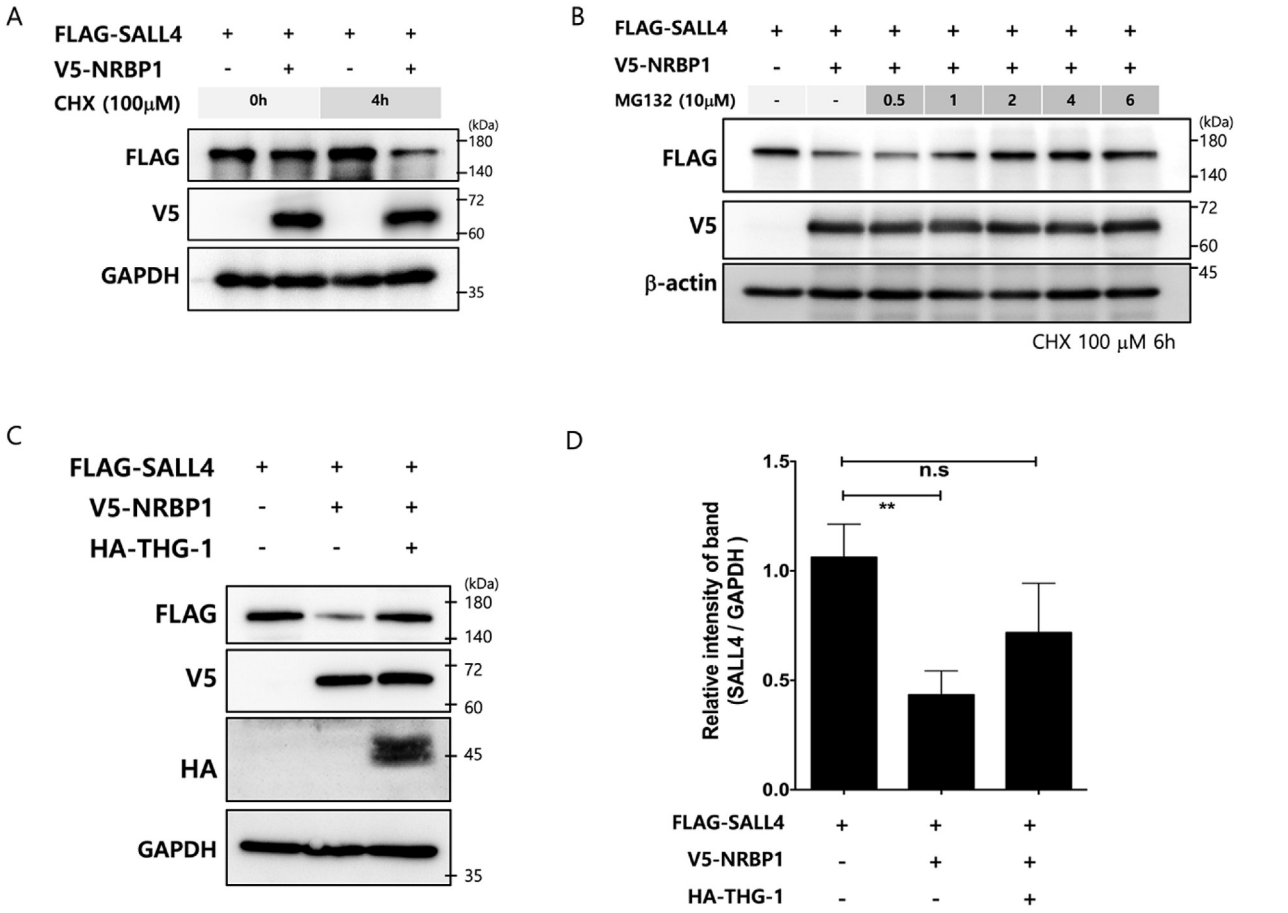

Fig. 3. THG-1 rescues SALL4 protein expression from proteasomal downregulation by NRBP1. (A) Downregulation of SALL4 by NRBP1 was confirmed using Western blot analysis with Cycloheximide (CHX) treatment. (B) Rescue of SALL4 protein expression from proteasomal degradation by NRBP1 was identified using Western blot analysis with MG132 and CHX treatment. (C, D) THG-1 rescued SALL4 protein expression from downregulation by NRBP1. (D) Intensities of SALL4 protein bands were quantified and normalized by GAPDH. Statistical data are presented as the means ± SDs derived from 3 independent experiments (n = 3), (**$P < 0.01$, n.s; no significance).

chemoradiotherapy, which leads to metastasis and relapse [1–3]. In this paper, we demonstrated that THG-1 is expressed in ESCC cells. We discovered that THG-1 binds to NRBP1 and stabilizes SALL4 through interrupting the proteasomal degradation of SALL4 by NRBP1. Knockdown of THG-1 suppressed the expression of SALL4 and its target genes such as *NANOG* and *OCT4* in TE13 cells. Exogenous SALL4 partially recovered tumorsphere growth in THG-1 KD TE13 cells, indicating that THG-1 enhances stemness gene expression and tumorsphere-forming activity at least partially through the regulation of SALL4.

Some studies have demonstrated the increase of SALL4 expression in various cancer cells and clinical samples [8–13]. Du et al. suggested that EGFR signaling induced SALL4 expression through ERK1/2 pathway in CD44-positive lung cancer cells [35], and Wilson et al. suggested NRBP1 as negative regulator of SALL4 [14], but the molecular mechanism by which SALL4 is regulated has been elusive. Our findings indicate that NRBP1 downregulates SALL4 via protein-protein interaction as a substrate of E3 ligase and drives ubiquitination of SALL4.

Human SALL4 has two isoforms (SALL4A and SALL4B) with different internal splicing patterns in exon 2 [32]. It is known that both isoforms form homodimers or heterodimers and play different roles in murine embryo development [32,33]. Wilson et al. showed that NRBP1 KO murine intestinal tissue expressed Sall4b [14]. Moreover, Chen et al. suggested that exogenous SALL4B expression induces growth of cervical squamous cell carcinoma cells and *in vivo* tumor formation [34]. On the other hand, we detected SALL4A as the major SALL4 in TE13 ESCC cells, and that exogenous SALL4A expression in THG-1 KD TE13 cells enhanced tumorsphere-forming ability. Together with the previous observation by Chen et al., both isoforms (SALL4A and B) are demonstrated to have tumorigenic activities in different organs.

Wei et al. discovered that NRBP1 downregulates β-catenin protein in breast cancer cells via an unknown mechanism [19]. As we discovered that NRBP1 suppresses SALL4 activity as an adaptor E3 ligase, there is a possibility that NRBP1 downregulates β-catenin through ubiquitination and following proteasomal degradation.

In this study, we investigated the function of exogenous SALL4 in tumorsphere formation of THG-1 KD TE13 cells. In addition to exogenous SALL4 expressing THG-1 KD TE13 cells, SALL4 knockdown in parental TE13 cells may provide supportive results regarding the role of SALL4 in tumorsphere formation of TE13 cells. In addition, our findings indicate THG-1 as a potential therapeutic target of SALL4 overexpressing ESCC cells. SALL4 increased anchorage-independent growth of ESCC cells is closely connected to CSC properties, so THG-1 targeting would be a possible strategy to target the cancer stem cells in THG-1 overexpressing ESCC.

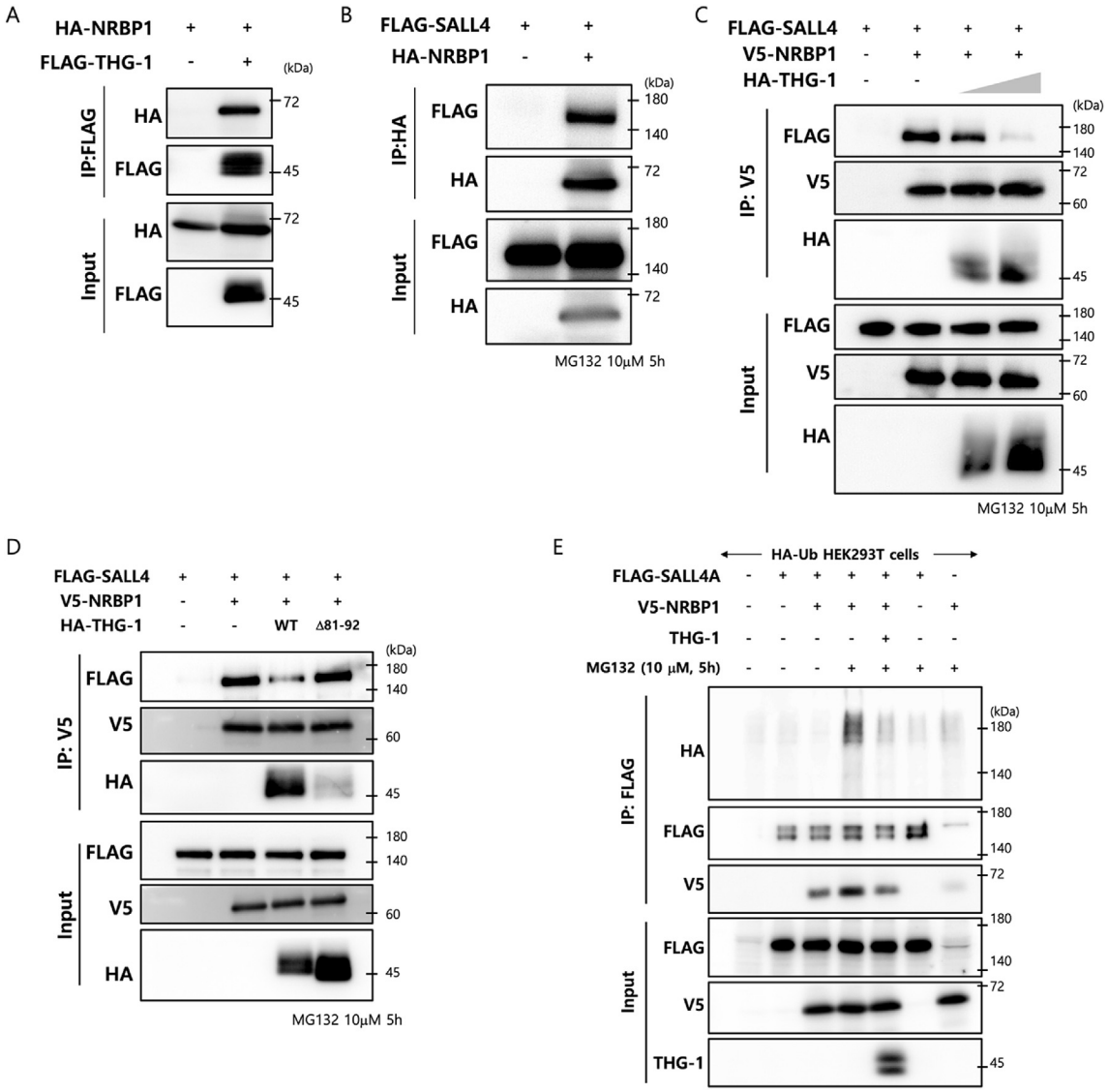

Fig. 4. THG-1 interrupts ubiquitination of SALL4 through antagonizing NRBP1. (A) Interaction between THG-1 and NRBP1 was confirmed by immunoprecipitation (IP)-Western blot analysis. (B) Interaction between SALL4 and NRBP1 was confirmed by IP-Western blot analysis. (C) Interruption of SALL4-NRBP1 interaction by THG-1 overexpression was identified by IP-Western blot analysis. (D) Decreased binding of THG-1 deletion mutant (THG-1$^{\Delta 81-92}$) to NRBP1 and loss of competition to SALL4-NRBP1 binding was confimed by IP-Western blot analysis in the presence of MG132. (E) IP-ubiquitination assay with SALL4, NRBP1 and THG-1 was performed. (B–D) To see the interaction between NRBP1 and SALL4, proteasomal inhibitor (MG132, 10 μM) was treated into cells for 5h.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.11.149.

Transparency document

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