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The stability and oncogenic function of LIN28A are regulated by USP28

Saba Haq[a,1], Soumyadip Das[b,1], Dong-Ho Kim[b], Arun Pandian Chandrasekaran[b], Seok-Ho Hong[c], Kye-Seong Kim[b,d], Suresh Ramakrishna[b,d,*]

[a] Department of Life Science, College of Natural Sciences, Hanyang University, Seoul, South Korea
[b] Graduate School of Biomedical Science and Engineering, Hanyang University, Seoul, South Korea
[c] Department of Internal Medicine, School of Medicine, Kangwon National University, Chuncheon, South Korea
[d] College of Medicine, Hanyang University, Seoul 04763, South Korea

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ABSTRACT

RNA-binding protein LIN28A is often highly expressed in human malignant tumors and is involved in tumor metastasis and poor prognosis. Knowledge about post-translational regulatory mechanisms governing LIN28A protein stability and function is scarce. Here, we investigated the role of ubiquitination and deubiquitination on LIN28A protein stability and report that LIN28A protein undergoes ubiquitination. Ubiquitin-specific protease 28 (USP28), a deubiquitinating enzyme, interacts with and stabilizes LIN28A protein to extend its half-life. USP28, through its deubiquitinating activity, antagonizes LIN28A protein turnover by reversing its proteasomal degradation. Our study describes the consequential impacts of USP28-mediated stabilization of LIN28A protein on enhancing cancer cell viability, migration and ultimately augmenting LIN28A-mediated tumor progression. Overall, our data suggest that a synergistic, combinatorial approach of targeting LIN28A with USP28 would contribute to effective cancer therapeutics.

1. Introduction

LIN28 was first discovered in the nematode *Caenorhabditis elegans* as a heterochronic gene involved in regulation of developmental timing [1]. Since its initial discovery, LIN28 has been identified as a highly conserved RNA binding protein, implicated in cancers, stem cell pluripotency and differentiation, reprogramming, normal development, and glucose metabolism [2]. LIN28 consists of two homologs, LIN28A and LIN28B, that have similar structural and functional characteristics and few differences [3]. Both LIN28A and LIN28B selectively block expression of let-7 microRNAs and function as oncogenes by promoting tumorigenesis and invasiveness in cancers such as prostate, ovarian, gastric, glioblastoma, and breast cancer through individual modes of action [4]. LIN28A recruits TUTase (zcchc11/TUTase4) to block the processing of let-7 in the cytoplasm while, LIN28B represses let-7 by inhibiting their processing by the Microprocessor [5].

LIN28 exhibits high expression in embryonic stem cells (ESCs) and its expression level is reduced upon differentiation [6,7]. Accordingly, LIN28 is a stem cell core factor and participates in cellular reprogramming [8–10]. Moreover, LIN28-expressing cancers demonstrate a less-differentiated, more aggressive profile [11]. Thus, LIN28-based control of cell proliferation is a pivotal factor in cancer aggressiveness [12–14]. Cancer stem cells (CSCs) are involved in tumor relapse and confer resistance to anticancer therapies [15,16]. A growing body of evidence indicates that LIN28 is a biomarker for CSCs [17]. The elucidation of factors that contribute to CSC-mediated aggressive pathology is necessary for designing effective therapeutic measures against CSCs [4,18]. Upstream transcriptional factors or loss of transcriptional repressors triggers activation of LIN28 expression in tumor cells [4]. For instance, depletion of c-Myc causes a reduction in LIN28 transcript and protein levels, while overexpressed c-Myc restores LIN28 expression level in breast carcinoma [19,20].

LIN28 undergoes post-translational modifications that affect stability and its protein level. A study demonstrated that ubiquitin ligase human TRIM71 polyubiquitinates and degrades LIN28B protein [21]; however, to the best of our knowledge, no study has reported on the ubiquitination and stability of LIN28A protein. Protein ubiquitination events are widely reported to be reversed by counteracting enzymes known as deubiquitinating enzymes (DUBs). Approximately 100 DUBs have been found in the human proteome, and they are divided into five major families. Ubiquitin-specific protease (USP) is one of the largest families, comprising approximately 55 DUB proteins [22]. To date, no

* Corresponding author at: Hanyang University, FTC 12th floor Rm1209-12, 222, Wangsimini-ro, Seongdong-gu, Seoul 04763, South Korea.
E-mail address: suri28@hanyang.ac.kr (S. Ramakrishna).
[1] These authors contributed equally to this work.

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DUB(s) have been identified for LIN28A protein. Therefore, the impact and functional mechanisms of LIN28A protein regulation by DUBs remain unknown.

In this study, we demonstrated that the oncogenic protein LIN28A undergoes ubiquitination by the 26S proteasomal degradation pathway. We further report that ubiquitin-specific protease 28 (USP28) interacts with and deubiquitinates LIN28A. We found that USP28 stabilizes LIN28A protein and extends its half-life by circumventing LIN28A protein turnover. Additionally, our study demonstrates the role of USP28 in promoting LIN28A-mediated cancer cell viability, invasion and migration.

2. Materials and methods

2.1. Cell culture and transfection

Human embryonic kidney cell line (HEK293T), human colon cancer cells (HCT116), human adenocarcinoma cell line (HT-29), human Caucasian colon adenocarcinoma cell line (SW620) and human erythroleukemic cell line (K562) were cultured in DMEM (GIBCO BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (GIBCO BRL, Rockville, MD, USA) and 1% penicillin and streptomycin (GIBCO BRL, Rockville, MD, USA). Human breast cancer cells (T47D), human embryonic carcinoma cells (NCCIT), *cervical* carcinoma cell line (HeLa), and *breast cancer* cell line (MCF-7) were grown in RPMI-1640 medium (GIBCO BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (GIBCO BRL, Rockville, MD, USA) and 1% penicillin and streptomycin (GIBCO BRL, Rockville, MD, USA) at 37 °C in a humidified atmosphere with 5% CO_2. Human embryonic stem cell line (H9) was maintained undifferentiated by culturing on a mouse embryonic fibroblast (MEF) feeder layer pretreated with mitomycin C in DMEM/F12 containing 20% KnockOut SR (serum replacement), 1% non-essential amino acids, 200 mM L-glutamine, 55 mM β-mercaptoethanol, antibiotics, and 4 ng/ml basic fibroblast growth factor (bFGF). The cells were usually passaged every 5–6 days depending on cell confluency after detaching Embryonic Stem cell colonies with dispase II (Sigma) (1 U/ml). HEK293T cells were used for exogenous experiments, while T47D and NCCIT cells were used for endogenous experiments. HeLa cells were used for anchorage-independent growth assay (soft agar assay). For transfection of HEK293T, HeLa and NCCIT cells, polyethyleneimine was used (Polysciences, Warrington, PA, USA). T47D transfection electroporation was performed via Neon® Transfection System (Thermo Fisher).

2.2. Construction of expression plasmids

A mammalian expression vector encoding Flag-tagged LIN28A was kindly provided by Dr. Yong-Mahn Han (Korea Advanced Institute of Science and Technology, South Korea), and Flag-tagged USP28 was purchased from Addgene (Addgene #15665). To obtain Myc-tagged LIN28A, Flag-tagged LIN28A was subcloned into pcDNA3.1-Myc and pcDNA3-6Myc vector. To generate a catalytic mutant of USP28, the active site residue Cysteine at position 171 was substituted with Alanine, producing USP28C171A by site-directed mutagenesis.

2.3. Antibodies

We used antibodies against c-Myc (SC-40, 1:1000), Ubiquitin (SC-8017, 1:1000), HA (SC-7392, 1:1000), LIN28 (SC-374460, 1:1000) (Santa Cruz Biotech), LIN28A (#657802, 1:1000) (Biolegend), LIN28A antibody (A177, #3978, 1:500) (Cell signaling Technology), Flag (Anti-DDDDK-tag, M185-3L, 1:1000) (MBL Life Science), USP28 (17707-1-AP, 1:1000) (Protein Tech), β-actin (CST, #4967, 1:1000) and GAPDH (CST, #2118, 1:1000) (Cell Signaling Technology).

2.4. siRNA transfection

siRNA targeting USP28 (CUGCAUUCACCUUAUCAUU) [23] and negative control/scramble-siRNA control (CCUACGCCACCAAUUU CGU) were purchased from Bioneer (Korea). All siRNAs transfection was done by electroporation in T47D cells with 15 μM siRNA, according to manufacturer's protocol.

2.5. In vitro co-immunoprecipitation assays

Myc-tagged LIN28A and Flag-tagged USP28 were cotransfected into HEK293T cells. Cells were lysed after 48 h and immunoprecipitated with anti-Myc or anti-Flag and immunoblotted with indicated antibodies to assess binding interactions. For ubiquitination of LIN28A, cells were transfected with Myc-LIN28A and HA-ubiquitin. After 48 h, cells were harvested and lysed in buffer B (50 mM Tris pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF) for 20 mins. Cell lysates were incubated with indicated antibodies at 4 °C overnight and next day those lysates were incubated with 20 μl of protein A/G Sepharose beads (Santa Cruz Biotech) at 4 °C for 2 h. Beads were washed with lysis buffer and eluted with SDS sample buffer. Co-immunoprecipitated proteins were detected by Western Blot analysis. For exogenous deubiquitination assay, HEK293T cells were transfected with Myc-LIN28A, Flag-USP28, Flag-USP28C171A and HA-ubiquitin constructs. For endogenous deubiquitination assay, NCCIT cells were transfected with HA-ubiquitin and Flag-USP28 constructs. The cells lysates were subjected to immunoprecipitation assay as described above.

2.6. Real-time PCR

Total RNA was isolated using Trizol reagent (Favorgen, Kaohsiung, Taiwan). RNA pellets were suspended in 30 μl nuclease-free water, after which the RNA concentration was determined. Total mRNAs were reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Life Technologies) with an oligo dT primer. Quantitative PCR was performed using Fast SYBR Green master mix (Life Technologies) and StepOnePlus Real-Time PCR System (Life Technologies, USA) with GAPDH-targeting primers (5′CATGTTCGTCA TGGGTGTGAACCA3′, 5′AGTGATGGCATGGACTGTGGTCAT3′) and LIN28A-targeting primers (5′TCCGTGTCCAACCAGCAGTT3′, 5′GTCAT GGACAGGAAGCCGAA3′).

For stem loop real-time PCR, we used TaqMan miRNA assays as previously described [24]. Reverse transcription (RT) reactions were run in a GeneAmp PCR 9700 Thermocycler (Applied Biosystems, CA). Gene expression levels were quantified using C1000 thermal cycler (BioRAD). Comparative real-time PCR were performed in triplicate using each specific primer for hsa-let-7a (ID: 000377), hsa-let-7b (ID: 000378), and U6 (ID: 001093) using Taqman PCR mix (Applied Biosciences, UK). All primer sets for specific miRNAs were purchased from Applied Biosystems. The reaction underwent 40 cycles of denaturation for 15 s at 95 °C and annealing and prolongation at 60 °C for 30 s. U6 was used as the endogenous control for relative quantification analyses.

2.7. CRISPR/Cas9 gene editing-related constructs

Two sgRNAs separately targeting USP28 for Cas9-mediated genome editing were designed using the sgRNA designer website (https://www.broadinstitute.org). The sgRNA sequences are illustrated in Fig. 4A. The dCas9-VP64 was used to target the promoter region of LIN28A gene for effective endogenous transcriptional activation. The primer sequences for cloning the sgRNA construct are FP: 5′ CACCGGAAGTCCGAAGGC AAAGGGT 3′; RP: 5′ AAACACCCTTTGCCTTCGGACTTCC 3′.

2.8. T7E1 assay

T7E1 assays were performed as previously described [25,26]. After isolation of genomic DNA using DNeasy Blood & Tissue kits (Qiagen) according to the manufacturer's instructions, the region of DNA containing the nuclease target site was PCR-amplified using hemi-nested primers (First PCR FP: 5′ TCTTGGAGCTTGTTGGTTCTT 3′, RP: 5′ CAGGGTTACCCGAACCACAA 3′; Second PCR FP1: 5′ GCCTCAGGCCT GTTTATCCAAG 3′, RP: 5′ CAGGGTTACCCGAACCACAA 3′). Amplicons were denatured by heating and annealed to form heteroduplex DNA, which was treated with 5 units of T7 endonuclease 1 (New England Biolabs) for 15 to 20 mins at 37 °C and analyzed using 2% agarose gel electrophoresis. Mutation frequencies were calculated based on band intensity using ImageJ software and the equation: mutation frequency (%) = 100 × (1 − [1 − fraction cleaved] $^{1/2}$), where fraction cleaved was the total relative density of the cleavage bands divided by the sum of the relative density of cleavage and uncut bands.

2.9. USP28 knockout cell lines using CRISPR/Cas9

NCCIT cells were transfected with Cas9 and sgRNA1 targeting USP28. After 3 days, NCCIT cells were seeded into 96-well plates at an average density of 0.25 cells/well. At 16 days after seeding, wells were microscopically evaluated, and single cell-derived round colonies were selected. Selected colonies were individually trypsinized and replated into 24-well plates. Four days after subculturing, genomic DNA was isolated from clones and used for T7E1 assay. T7E1-positive clones were expanded and stored in liquid nitrogen tank.

2.10. Cell viability assays

T47D and HEK293T cells were transfected in a 24-well plate with the indicated constructs. Post 48 h. of transfection, cells were equally seeded into 96-well plates. Next day, the cells were subjected to Cell TiterGlo luminescent cell viability assay (Promega). All experiments were performed in triplicate on 3 separate occasions. For cell viability, luminescent intensity was read using a microplate luminometer (Panomics, USA) according to the manufacturer's instructions.

2.11. Colony formation assay

Transfected NCCIT cells with indicated constructs, were equally seeded and maintained in G418 (400 μg/ml) containing culture medium. Two weeks after selection, colonies were fixed in ice cold methanol for 10 mins and stained with 1% crystal violet in methanol for 15 mins. Colonies were rinsed with water and counted. The USP28 knockout cell line was transfected with LIN28A and compared with Mock and LIN28A transfected NCCIT cells, followed by colony formation assay as described above.

2.12. Wound healing assay

Migration and proliferation rates mediated by LIN28A were analyzed by wound healing assay. NCCIT cells were transfected with Flag-USP28, Myc-LIN28A, Myc-LIN28A + Flag-USP28, Myc-LIN28A + Flag-USP28C171A, and Myc-LIN28A + sgRNA2-USP28 constructs. Transiently transfected NCCIT cells were cultured to near 90% confluency. Scratches were made on NCCIT monolayers with a sterile pipette tip in a definite array. The wounded cell layer was washed with PBS and incubated with complete medium. Wound closure was captured at 0 and 24 h using a fluorescence microscope (IX71, Olympus, Tokyo, Japan). Migration rates in wild-type and USP28 knockout NCCIT cell lines were measured similarly, the area of the wound was compared at 0 h and 24 h by ImageJ software and the percentage of wound-healing is calculated as previously described [27].

2.13. Matrigel invasion assay

NCCIT cells expressing endogenous LIN28A were transfected with Flag-USP28 or sgRNA2 targeting USP28. Cell invasion was assessed using 0.8 μm transwell chambers (Falcon), coated with matrigel (Corning, USA) according to the manufacturer's protocol. Briefly, 2.5×10^4 cells suspended in 500 μl serum-free RPMI medium were placed into the chamber. Then, 750 μl of complete medium was added to the 24-well chamber. Cells were incubated in a 5% CO_2 atmosphere at 37° C. After 24 h, the cells on the upper surface of the insert were scraped off, and the cells on the lower surface were fixed with ice-cold methanol and stained with crystal violet. Under light microscopy, the average cell number was counted and compared between untransfected cells, USP28 transfected and sgRNA2 targeting USP28 (transient knockout) cells. (Scale bar: 100 μm). The invaded cells were represented graphically.

2.14. Soft agar assay

HeLa cells were transfected with expression constructs of pcDNA6-Myc (Control), Myc-LIN28A, Myc-LIN28A + Flag-USP28 and Myc-LIN28A + sgRNA2-USP28. For this, 1 ml of 0.75% agarose gel with 1 × DMEM complete medium (equal ratio) was placed into 6 well-plates for 45 min at room temperature. At 48 h after transfection, cells were harvested individually, and 1×10^4 cells were suspended in 500 μl of DMEM medium containing 500 μl of 0.75% agarose and inoculated on the surface of the solidified gel. These cells were cultured for 15 days at 37° C in a humidified atmosphere containing 5% CO_2. Under light microscopy, the anchorage-independent colony numbers were counted after staining with crystal violet. (Scale bar: 200 μm). The numbers of colonies were represented graphically.

2.15. Statistics

The results are representative of multiple experiments using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Statistical analysis was performed with analysis of variance (ANOVA), Student's *t*-test and the Newman Keuls method. *P*-values < 0.05 denoted statistically significant differences.

3. Results

3.1. LIN28A undergoes ubiquitination

To investigate if LIN28A protein is degraded by the ubiquitin proteasomal pathway (UPP), we examined endogenous LIN28A protein level in the presence of the proteasomal inhibitor MG132. We treated LIN28A-expressing T47D and NCCIT cells with increasing concentrations of MG132 for 8 h. Similarly, HEK293T cells transfected with LIN28A construct were also treated with MG132 for indicated hours. The result showed that MG132 dose-dependently increased LIN28A protein level (Fig. 1A, Supplementary Fig. S1A and C). To exclude any trivial alterations in LIN28A mRNA upon MG132 treatment, we performed qPCR in both T47D and NCCIT cells, which revealed no significant changes at the transcriptional level (Fig. 1B and Supplementary Fig. S1B). The increase in LIN28A protein only upon treatment with a proteasome inhibitor led us to hypothesize that the UPP was involved in regulating LIN28A protein turnover. To test our hypothesis, we determined interactions between ubiquitin and LIN28A. For this purpose, we cotransfected HEK293T cells with HA-tagged ubiquitin and Myc-tagged LIN28A and harvested the cells after 48 h. Cell lysates were subjected to immunoprecipitation assay (IP) using anti-Myc, followed by immunoblotting using anti-HA (Fig. 1C). Our data showed a characteristic high molecular weight smear of polyubiquitin molecules conjugated to LIN28A protein in cotransfected cells (Fig. 1C, upper panel). We also carried out immunoprecipitation assay using anti-HA,

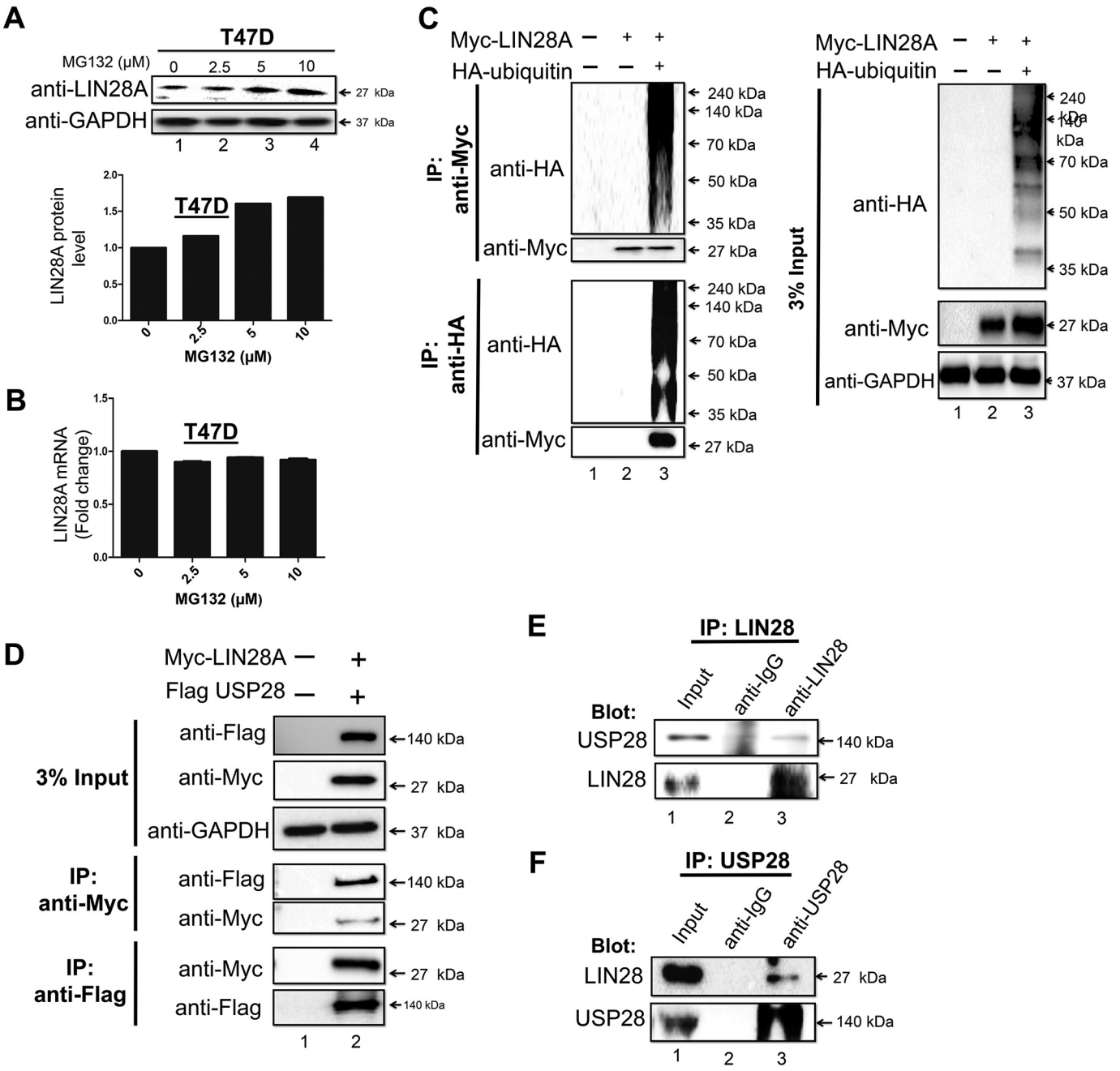

Fig. 1. LIN28A undergoes 26S proteasomal degradation and interacts with USP28. A, Endogenous LIN28A expression upon dose-dependent treatment of MG132 was determined in T47D cells. The LIN28A band intensity was estimated by ImageJ software and normalized with GAPDH to represent graphically. B, Real-time PCR was conducted to assess the effect of MG132 treatment (doses indicated) on mRNA levels of endogenous LIN28A in T47D cells. C, Myc-LIN28A was transfected individually or with HA-ubiquitin into HEK293T cells for immunoprecipitation with Myc antibody, followed by immunoblotting with HA or Myc antibody. Reciprocal immunoprecipitation was performed with HA antibody, followed by immunoblot with HA or Myc antibody. D, Myc-tagged LIN28A and Flag-tagged USP28 were co-transfected into HEK293T cells. Samples were immunoprecipitated using anti-Myc or anti-Flag antibodies and immunoblotted using indicated antibodies. GAPDH was used as a loading control. E and F, Endogenous interaction between USP28 and LIN28A proteins in NCCIT cells. Cell lysates from NCCIT cells were immunoprecipitated with specific USP28 or LIN28 antibodies and immunoblotted with LIN28 and USP28 antibodies.

followed by immunoblotting with anti-Myc (Fig. 1C, lower panel). The results suggest that LIN28A protein interacts with ubiquitin molecules and undergoes polyubiquitination through the ubiquitin proteasomal pathway.

3.2. LIN28A interacts with USP28

Ubiquitination is one of the most important post-translational modifications for the stability and function of proteins. The process of ubiquitination can be counteracted by the functions of DUBs [28]. Thus, we aimed to investigate DUBs that could stabilize LIN28A protein. A previous study on breast cancer cells reported that USP28 is a possible candidate associated with LIN28, based on immunoprecipitation assays combined with mass spectrometry (MS) analyses [29].

The expression of LIN28A protein is not ubiquitously found in all cell lines. Previous research showed that the endogenous expression of LIN28A is detected mostly in embryonic stem cells. In contrast, USP28 expression is readily detected in both cancer and normal cell lines,

including stem cells [30,31]. Thus, we used public database to assess the mRNA expression level of both LIN28A and USP28 in several cell lines. The expression level of LIN28A was found to be high in human embryonic carcinoma cells, while low or undetectable levels were found in most of the cell lines (Supplementary Fig. S2A) whereas, USP28 is moderately or highly expressed in normal and cancer cell lines including stem cells (Supplementary Fig. S2B). Furthermore, we used cell lysates from several normal and cancer cell lines to study the LIN28A and USP28 endogenous protein expression via Western Blot (WB) analysis. The result showed expression of USP28 protein in all normal and cancer cell lines, especially expressing high in embryonic stem cells (H9 cells) and human embryonic carcinoma (NCCIT) cells (Supplementary Fig. S3A). In contrast, the expression of LIN28A was confined to only H9 cells and NCCIT cells (Supplementary Fig. S3A). Furthermore, previous studies have shown that NCCIT cells express both LIN28 homologs LIN28A and LIN28B [32,33], whereas T47D cells express only LIN28A [5,34]. Thus, we checked LIN28A expression in T47D cells (Supplementary Fig. S3B) and used these cells in our experiments.

We determined whether USP28 was a potential DUB for LIN28A by immunoprecipitation assay. Flag-tagged USP28 was cotransfected with Myc-tagged LIN28A, followed by immunoprecipitation with Myc or Flag antibodies and Western Blots with reciprocal antibodies. Myc-LIN28A was found to coprecipitate Flag-USP28, and vice versa (Fig. 1D). To confirm the endogenous interaction between LIN28A and USP28 proteins, NCCIT cells endogenously expressing both LIN28A and USP28 were subjected to immunoprecipitation with both LIN28- and USP28-specific antibodies, followed by Western Blots with respective antibodies (Fig. 1E and F). Our results suggest that LIN28A and USP28 interact with each other at exogenous and endogenous levels.

3.3. USP28 stabilizes LIN28A protein and extends its half-life

As, we found that USP28 interacts with LIN28A protein, we explored if USP28 regulates LIN28A at a transcriptional or translational level. We investigated the effects of dose-dependent overexpression of USP28 on mRNA level of LIN28A. For positive controls, we used a dead Cas9-VP64 activator (dCas9-VP64) [35], targeting the promoter region of the LIN28A gene for effective endogenous transcriptional activation. Endogenous transcriptional activation of LIN28A by dCas9-VP64 resulted in up to a 7-fold increase in the mRNA level of LIN28A, while dose-dependent overexpression of USP28 did not show any significant changes at transcriptional levels (Supplementary Fig. S4). This result suggests that USP28 does not have any transcriptional regulatory role on LIN28A.

Next, we determined the effect of transient overexpression of USP28 (wild type), USP28C171A with a mutation at the core enzymatic domain, and siRNA targeting USP28 on the endogenous expression level of LIN28A protein in T47D cells. USP28 overexpression led to LIN28A protein stability (Fig. 2A, lane 2, left panel), whereas USP28C171A failed to do so (Fig. 2A, lane 3, left panel). Furthermore, knockdown of USP28 mediated by siRNA showed reduction in LIN28A protein level (Fig. 2A, lane 2, right panel) compared to the negative/scramble-siRNA control (Fig. 2A, lane 1, right panel), indicating USP28 stabilizes LIN28A at post-translation level (Fig. 2A and B).

Deubiquitinating enzymes have major effects on protein half-life via their ability to remove polyubiquitin molecules from target proteins undergoing 26S proteasomal degradation [36]. Moreover, previous research demonstrated that USP28 stabilizes proto-oncogenic factors such as MYC and increases its protein half-life [31]. To assess the functional consequence of interaction between USP28 and LIN28A, we examined whether USP28 has a similar positive impact on LIN28A protein stabilization as on MYC protein. To this end, we cotransfected HEK293T cells with a constant concentration of Myc-LIN28A along with a dose-dependent increased concentration of Flag-USP28 or Flag-USP28C171A catalytic mutant construct. Data showed that Flag-USP28 could stabilize LIN28A protein in a dose-dependent manner (Fig. 2C and D), in contrast to catalytic mutant Flag-USP28C171A (Fig. 2E and F). Similarly, a dose dependent increase of Flag-USP28 stabilized the endogenous LIN28 expression in NCCIT cells (Fig. 2G and H), depicting a positive regulatory role of USP28 on LIN28A protein stability.

To further confirm the effect of USP28-mediated LIN28A protein stability, we measured the half-life of Myc-LIN28A in the presence or absence of Flag-USP28. We transfected Myc-LIN28A alone or co-transfected with Flag-USP28 constructs into HEK293T cells. At 48 h post transfection, cycloheximide (CHX, 100 μg/ml) was added for the indicated time, and cells were harvested periodically. In line with the previous reports [37], our data also showed that the half-life of LIN28A protein was about 12 h (Fig. 2I). Co-transfection of Flag-USP28 and Myc-LIN28A resulted in extension of the half-life of Myc-LIN28A protein (Fig. 2J). Taken together, our results indicate that USP28 stabilizes LIN28A protein.

3.4. USP28 deubiquitinates LIN28A

To investigate if USP28 has deubiquitinating activity on LIN28A, we performed deubiquitination assay. Myc-LIN28A was cotransfected into HEK293T cells with HA-Ubiquitin, Flag-USP28, and Flag-USP28C171A. Ubiquitination smear of LIN28A was significantly reduced upon co-expression of Flag-USP28 (Fig. 3A, lane 4), while USP28C171A could not reduce LIN28A ubiquitination smear (Fig. 3A, lane 5). We also performed endogenous ubiquitination and deubiquitination of LIN28A protein. For this purpose, NCCIT cells were transfected with HA-Ubiquitin alone or together with Flag-USP28. The endogenous LIN28A protein was immunoprecipitated using LIN28 antibody and immunoblotted with anti-HA; the results showed reduction in ubiquitination smear in the presence of USP28 (Fig. 3B, lane 3) compared to the ubiquitination smear observed in absence of USP28 (Fig. 3B, lane 2). These data suggest that USP28 deubiquitinates LIN28A protein by preventing its proteolysis.

3.5. USP28 enhances the LIN28A-mediated inhibition of let-7 miRNAs

To explore whether USP28 can enhance the LIN28A-mediated inhibition of endogenous let-7 miRNA processing, we measured the levels of mature let-7a and let7b in endogenously expressing LIN28A cell lines such as T47D (Fig. 3C and D). Our results showed that the expression levels of let-7a and let-7b miRNA were significantly lower in cells transfected with USP28 and higher in cells transfected with siRNA targeting USP28 as compared with respective controls (Fig. 3C and D). This indicates that USP28 enhances the LIN28A-mediated inhibition of endogenous let-7 miRNA.

3.6. CRISPR/Cas9-mediated genome editing of USP28 and its impact on LIN28A protein

Next, we used CRISPR/Cas9 to disrupt the USP28 gene by designing two sgRNAs specific to exon 2 of USP28 (Fig. 4A). The gene disruption efficiency of sgRNA1 and sgRNA2 was estimated by T7E1 assay. We first assessed the knockout efficiency in NCCIT cells with sgRNA1 and sgRNA2 with or without antibiotic (puromycin) selection (Fig. 4B). The effect of sgRNA1 and sgRNA2 gene disruption in the presence or absence of puromycin was also investigated at the protein level, and the results demonstrated a significant decrease in protein expression upon knockout of USP28 (Fig. 4C). Additionally, we generated a single-cell clone of USP28-knockout in NCCIT cells using sgRNA1 and validated the gene disruption by T7E1 assay and western blot (Fig. 4D and E). The result showed that the expression of USP28 protein was completely abolished in USP28 knockout cell lines compared to control NCCIT cells (Fig. 4E, upper panel), while the expression of LIN28A was reduced (Fig. 4E, middle panel). Loading controls were confirmed by the expression of anti-β actin (Fig. 4E, lower panel).

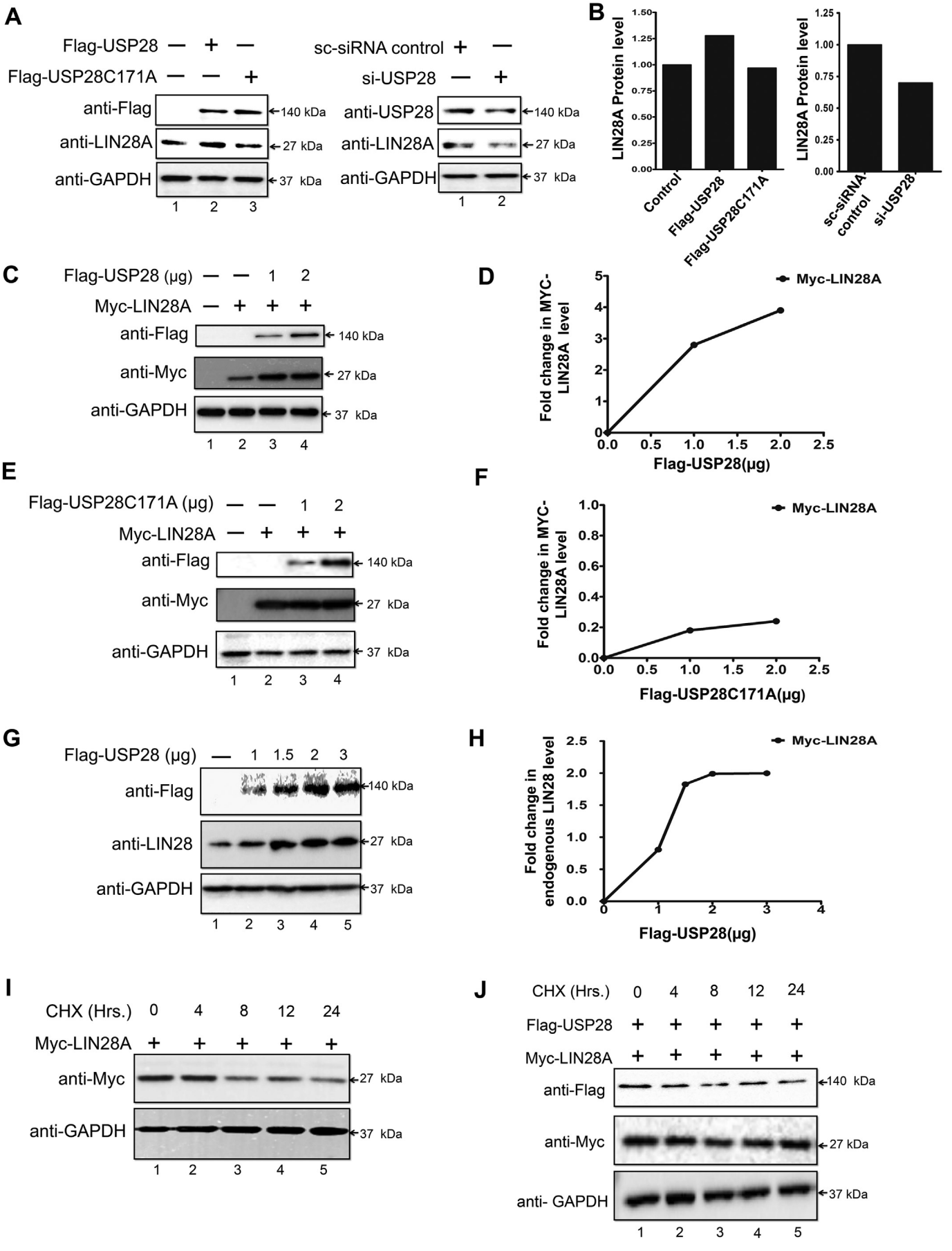

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Fig. 2. USP28 stabilizes and extends the half-life of LIN28A protein. A and B, The effects of exogenous overexpression of USP28 (left panel) and transient knockdown of USP28 via USP28 siRNA (right panel) on endogenous LIN28A levels in T47D cells. After 48 h, cell lysates were prepared and immunoblotted with indicated antibodies. C and D, USP28 stabilizes LIN28A protein. HEK293T cells were co-transfected with constant amount of Myc-LIN28A along with Flag-USP28 at an increasing concentration (1–2 μg). E and F, HEK293T cells were co-transfected with constant amounts of Myc-LIN28A along with an increasing concentration (1–2 μg) of Flag-USP28C171A. G and H, Endogenous LIN28A stabilization by USP28 in a dose-dependent manner. NCCIT cells were transfected with Flag-USP28 at an increasing concentration (1.0–3 μg). Band intensity was estimated using the ImageJ program, normalized with GAPDH and graphically represented. I, HEK293T cells were transfected with Myc-LIN28A alone or J in combination with Flag-USP28. 48 h after transfection, cells were incubated with cycloheximide (CHX, 100 μg/ml) and harvested at different time points and analyzed by Western Blot analysis with the indicated antibodies.

3.7. USP28 enhances LIN28A-mediated cell viability and colony formation

To determine if the deubiquitinating activity of USP28 influences LIN28A-mediated cell viability, we performed the viability assay in T47D cells. As compared to scramble-siRNA control, cells transfected with USP28 siRNA significantly reduced number of viable cells (Fig. 5A). Additionally, cells transfected with USP28, resulted in enhancement of cell viability whereas USP28C171A and sgRNAs against USP28 failed to do so (Fig. 5B). Cumulatively, our data indicates that USP28 is pivotal in promoting LIN28A-mediated cell viability.

Next, we investigated the effect of USP28 on LIN28A-mediated cell growth by performing the colony formation assay. To this end, we overexpressed LIN28A alone or with USP28 into NCCIT cells and analyzed colony formation. The number of colonies that appeared in cells co-transfected with LIN28A and USP28 was significantly higher than in LIN28A only transfected cells (Fig. 5C and D). Additionally, we performed same experiment by transfecting LIN28A alone in NCCIT wild type cells as well as USP28 knockout cell line and analyzed colony formation. As a result, fewer colonies were found in the USP28 knockout NCCIT cell line than in wild type NCCIT cells (Fig. 5E and F). Our data suggests that USP28 enhances LIN28A-mediated proliferative behavior and thus augments the tumorigenic characteristics of LIN28A protein in cancer cells.

3.8. USP28 enhances LIN28A-mediated cancer progression

LIN28A protein has oncogenic characteristics and promotes cellular migration [19]. Thus, we hypothesized that USP28 has a positive influence on the cell migration ability of LIN28A. To evaluate the effect of USP28 on LIN28A-mediated cellular migration, wound healing assay was performed on NCCIT cells by exogenously transfecting LIN28A and USP28-related constructs. LIN28A overexpressed with USP28 resulted in a significantly increased wound healing rate compared to mock control or cell transfected with LIN28A or USP28 alone, whereas transient knockout of USP28 showed reduced wound closure (Fig. 6A). Furthermore, we conducted a wound healing assay on NCCIT wild type and USP28 knockout NCCIT cell lines. Our results showed that the wound healing rate observed in NCCIT wild type was faster than that observed in the USP28 knockout NCCIT cell line (Fig. 6B and C).

In order to further confirm that USP28 enhances the oncogenic function of LIN28A, we performed matrigel cell invasion assay in NCCIT cells transfected with only Flag-USP28 or sgRNA2 targeting USP28. Our results showed a significant increase of invasive capacity in USP28-transfected NCCIT cells in contrast with un-transfected cells or those transfected with sgRNA2 targeting USP28 construct (Fig. 6D).

We next examined the formation of cancer cell colonies on soft agar to investigate the cell anchorage-independent proliferation potential of LIN28A in the presence or absence of USP28 in vivo. HeLa cells co-transfected with USP28 and LIN28A showed an increased number of colonies, whereas the cells transfected with sgRNA2-targeting USP28 showed a decrease in colony number compared with control cells (Fig. 6E and F). Altogether, the data suggests that USP28 has critical regulatory effects on the proliferation, invasion, and migratory characteristics of LIN28A, indicating that USP28 enhances the tumorigenic behavior of LIN28A by its deubiquitinating activity.

4. Discussion

A plethora of information is available on LIN28 transcriptional modulations [1,6,38]. However, knowledge about post-translational modifications on LIN28A such as acetylation, phosphorylation, methylation, and ubiquitination and their effects on activity, stability and protein-protein interactions are not fully characterized. P300/CBP-associated factor (PCAF) acetyltransferase directly interacts with and acetylates LIN28, thereby reducing LIN28 protein level. This process is reversible by the deacetylase Sirtuin 1 [39]. Thus, the balance of post-translational modifications such as acetylation and deacetylation substantially affect regulation of LIN28 protein stability and activity. In pluripotent stem cells, LIN28 undergoes phosphorylation by MAPK/ERK, leading to protein stability [37]. In this study, we found that the highly conserved RNA-binding protein LIN28A undergoes proteasomal degradation by the 26S proteasome pathway. Furthermore, we report that USP28 is a deubiquitinating enzyme for LIN28A that reverses LIN28A ubiquitination to stabilize LIN28A protein. LIN28A, a chief regulator of let-7 miRNA processing [40], is overexpressed in human malignancies and promotes proliferation of cancer cells and tumorigenesis [18,41,42]. LIN28 has emerged as a factor that can influence the stemness and self-renewal of mammalian embryonic stem cells [3]. Although LIN28A is a widely known potential target for cancers, no studies have been conducted on the ubiquitin-mediated post-translational modification of LIN28A.

In 2014, Lee et al., reported regulation of LIN28B protein level via ubiquitin-mediated proteasomal degradation and identified ubiquitin ligase TRIM71 as a negative regulator of LIN28B [21]. Mass spectrometry data followed by IP assays suggested a possible interaction between USP28 and LIN28A protein [29]. Ubiquitination is one of the most important post-translational modifications for the stability and function of proteins, and is reversed by DUBs [28]. Thus, we tested a possible relationship between LIN28A and USP28 proteins and if their interaction regulated the function of LIN28A in cancer. Our results showed ubiquitin-mediated proteasomal degradation of LIN28A and confirmed an interaction between LIN28A and USP28 proteins (Fig. 1). We also reveal that USP28 stabilizes LIN28A only at post translational level and not on mRNA level (Supplementary Fig. S4). The functional interaction of USP28 with LIN28A contributed to LIN28A protein stabilization and prolonged its half-life (Fig. 2). Our data demonstrated a significant decrease in LIN28A ubiquitination in the presence of USP28 (Fig. 3). The expression levels of let-7a and let-7b miRNA were significantly lower in the LIN28A-expressing endogenous T47D cell line transfected with USP28 compared to the control, suggesting that USP28 enhances LIN28A-mediated inhibition of let-7 miRNAs (Fig. 3C and D). USP28 knockout NCCIT cells demonstrated less stabilized endogenous LIN28A level (Fig. 4E).

Previous studies showed that USP28 is overexpressed in many tumors, and the endogenous overexpression of USP28 promotes growth of cancer cells [30,43,44]. USP28 stabilizes proto-oncogenic factors such as MYC by its deubiquitinating activity in human tumor cells, and depletion of USP28 mimics the effects of MYC depletion in those cells [31]. LIN28A activates the androgen receptor via regulation of c-Myc and promotes malignancy of breast cancer cells [19]. In concordance with the stabilization of MYC protein by USP28 and its influence on tumor cell proliferation, we hypothesized that USP28 also influences

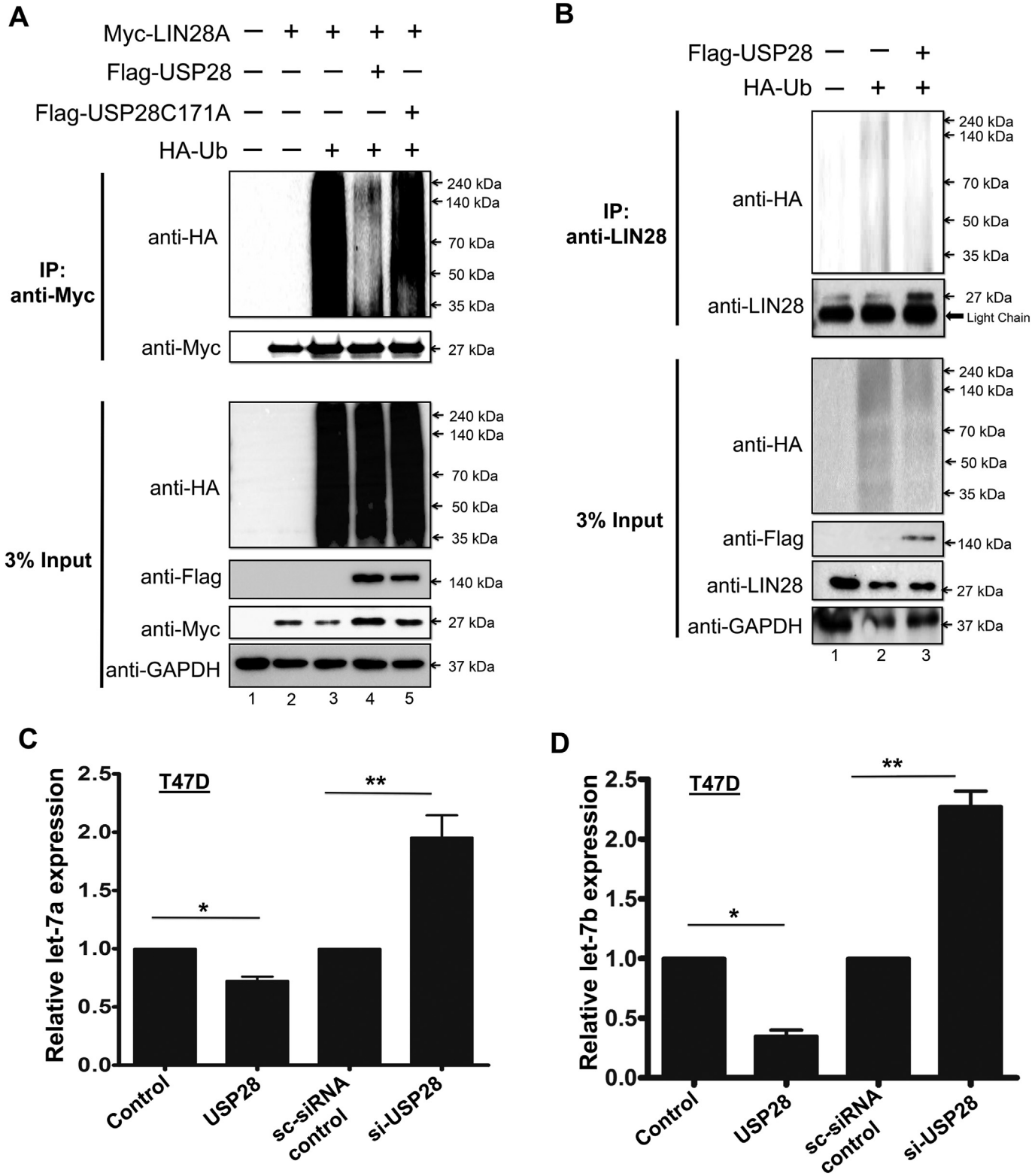

Fig. 3. USP28 deubiquitinates LIN28A and enhances LIN28A-mediated inhibition of let-7-miRNA processing. A, Deubiquitination of LIN28A by USP28. HEK293T cells were transfected with Myc-LIN28A alone or with HA-ubiquitin, Flag-USP28, or Flag-USP28C171A. Deubiquitination of LIN28A was confirmed by coimmunoprecipitation with an anti-Myc antibody and immunoblotted with an anti-HA antibody. B, Endogenous ubiquitination and deubiquitination of LIN28 were analyzed by transfecting NCCIT cells with HA-ubiquitin, in presence or absence of Flag-USP28, followed by immunoprecipitation with an anti-LIN28 antibody and immunoblotting with an anti-HA antibody. C and D. Mature let-7a and let-7b expression levels were quantified by stem loop real-time PCR in T47D cells in presence or absence of USP28 as well as siRNA against USP28.

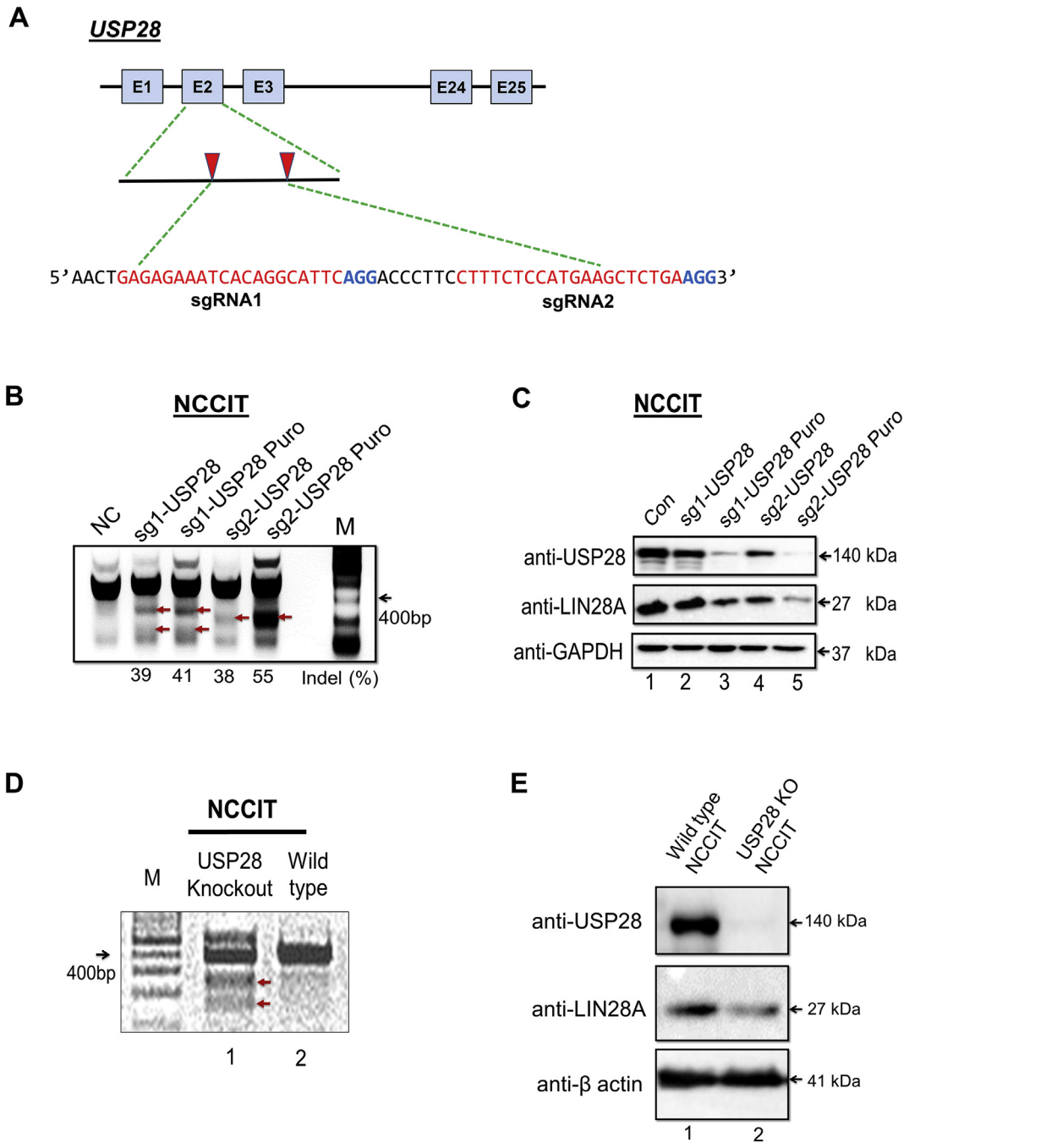

Fig. 4. CRISPR/Cas9-mediated genome editing of the USP28 gene. A, Schematic of the RNA-guided engineered nuclease (RGEN) targeting the human USP28 gene with designed sgRNA1 and sgRNA2 that target sequences in exon 2. Blue boxes represent exons. Red arrowheads indicate the positions of sgRNAs targeting the top strand. PAM sequences are marked by bold blue font. B, The cleavage efficiency of sgRNA1 and sgRNA2 was determined by the T7E1 assay in NCCIT cells after transfection with plasmids encoding Cas9 and sgRNAs (with or without puromycin selection, 1 μg/ml). The size marker (M) are shown. Arrows indicate the expected positions the cleaved DNA bands. The numbers at the bottom of the gel indicate mutation percentages measured by band intensity using ImageJ software. Untransfected cells were used as the negative control (NC). C, The knockout efficiency of sgRNAs targeting USP28 was determined by Western Blot analysis in NCCIT cells (with or without puromycin selection, 1 μg/ml), with specific USP28 and LIN28A antibodies D, T7E1-based clonal analysis in NCCIT cells. The genomic DNA isolated from each clone was subjected to PCR followed by T7E1 assay. Arrows indicate the expected positions of cleaved DNA bands. Untransfected cells were used as the wild type NCCIT cells. E, Estimation of USP28 and LIN28A protein expression in wild type NCCIT cells and USP28 knockout NCCIT cells using indicated antibodies.

the proliferation of tumors mediated by LIN28A. Thus, the tumorigenic function of LIN28A was examined in the presence or absence of USP28. Our data suggests that USP28 induced the tumorigenic function of LIN28A by increasing cell viability, migration, invasion, and anchorage-independent growth of mammalian cancer cells (Figs. 5–6).

To fully elucidate the functions of LIN28A in cancers, in-depth characterization of its physiological functions is required. The precise manner by which LIN28A protein actively contributes to carcinogenesis presents a compelling avenue for exploration. We demonstrated that LIN28A ubiquitination was reversed upon USP28-mediated deubiquitination, resulting in protein stability and higher cellular viability and migration rates. The identification of USP28 deubiquitinating enzyme

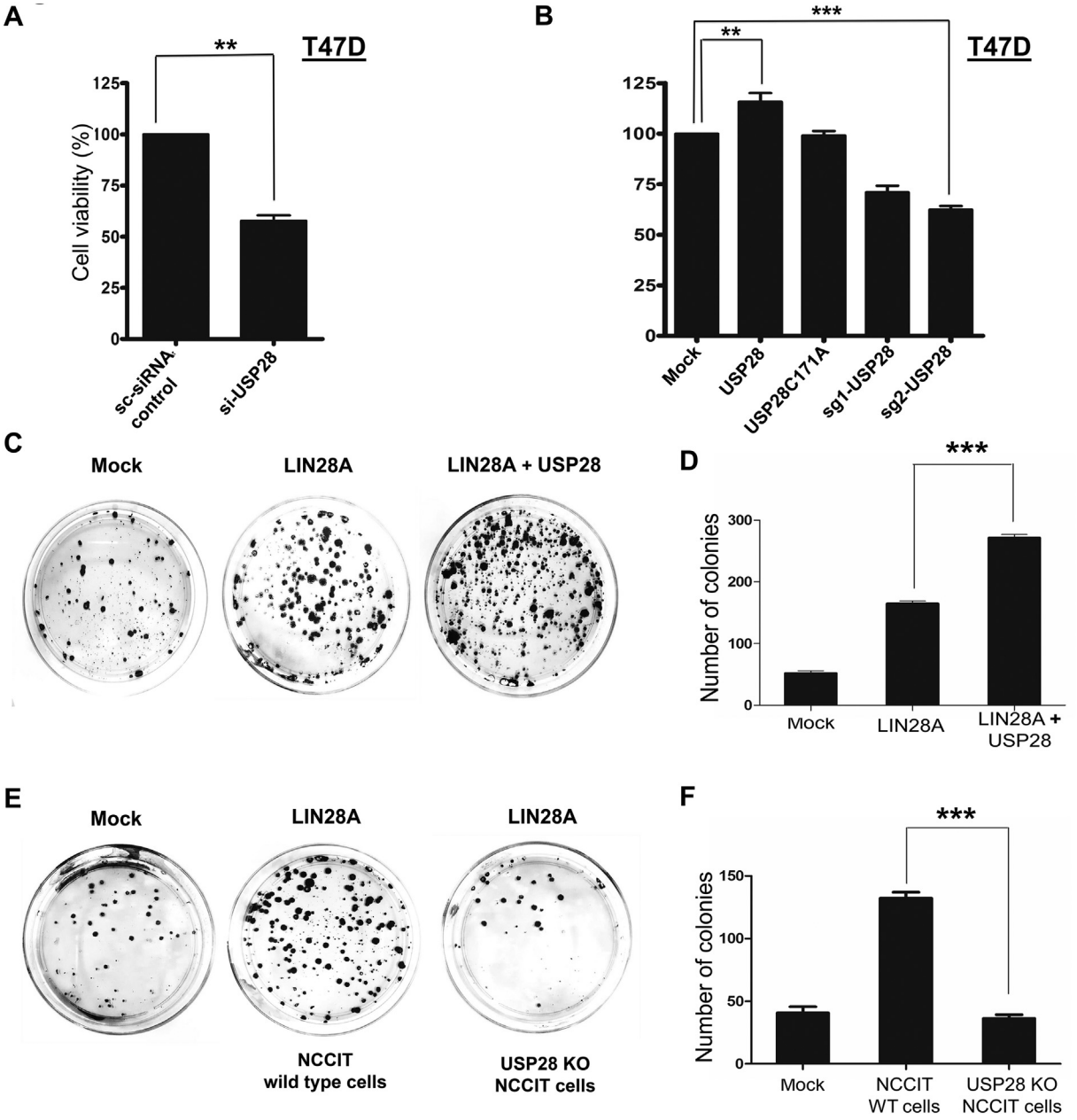

Fig. 5. USP28 enhances LIN28A-mediated cell viability and colony formation. A and B, Cell viability assay: T47D cells were transfected with the indicated constructs and cell viability assay was performed. C and D Colony formation assay: NCCIT cells were transfected with Myc-LIN28A alone or combined with Flag-USP28 and incubated with media containing G418 (400 μg/ml). After 14 days of selection, colonies were stained, and images were captured. The number of surviving colonies was counted and graphically represented. **E** and **F**, Wild type NCCIT cells and USP28 knockout NCCIT cells were transfected with Myc-LIN28A alone for colony formation assay. The number of colonies was counted and graphically represented. Error bars represent triplicate experiments, ($p < 0.05$ by *t*-test was considered significant).

as a LIN28A protein stabilizer demonstrates a novel result with implications of USP28-based regulatory mechanisms in cancer cell proliferation. Our research suggests a unique approach of concomitantly targeting LIN28A and USP28 proteins to reduce tumor progression. Such a therapeutic intervention to target tumors could lead to improved efficiency of tumor therapy and optimal clinical outcomes.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2018.12.006.

Abbreviations

CSC	Cancer stem cells
bFGF	basic fibroblast growth factor
CHX	Cycloheximide
DUB	Deubiquitinating enzymes
dCas9-VP64	Dead Cas9-VP64 activator
IP	Immunoprecipitation assay
MS	Mass spectrometry
NC	negative control
PCAF	P300/CBP-associated factor
RGEN	RNA-guided engineered nuclease
RT	Reverse transcription
USP	Ubiquitin-specific protease
USP28	Ubiquitin-specific protease 28
UPP	Ubiquitin proteasomal pathway
WB	Western Blot

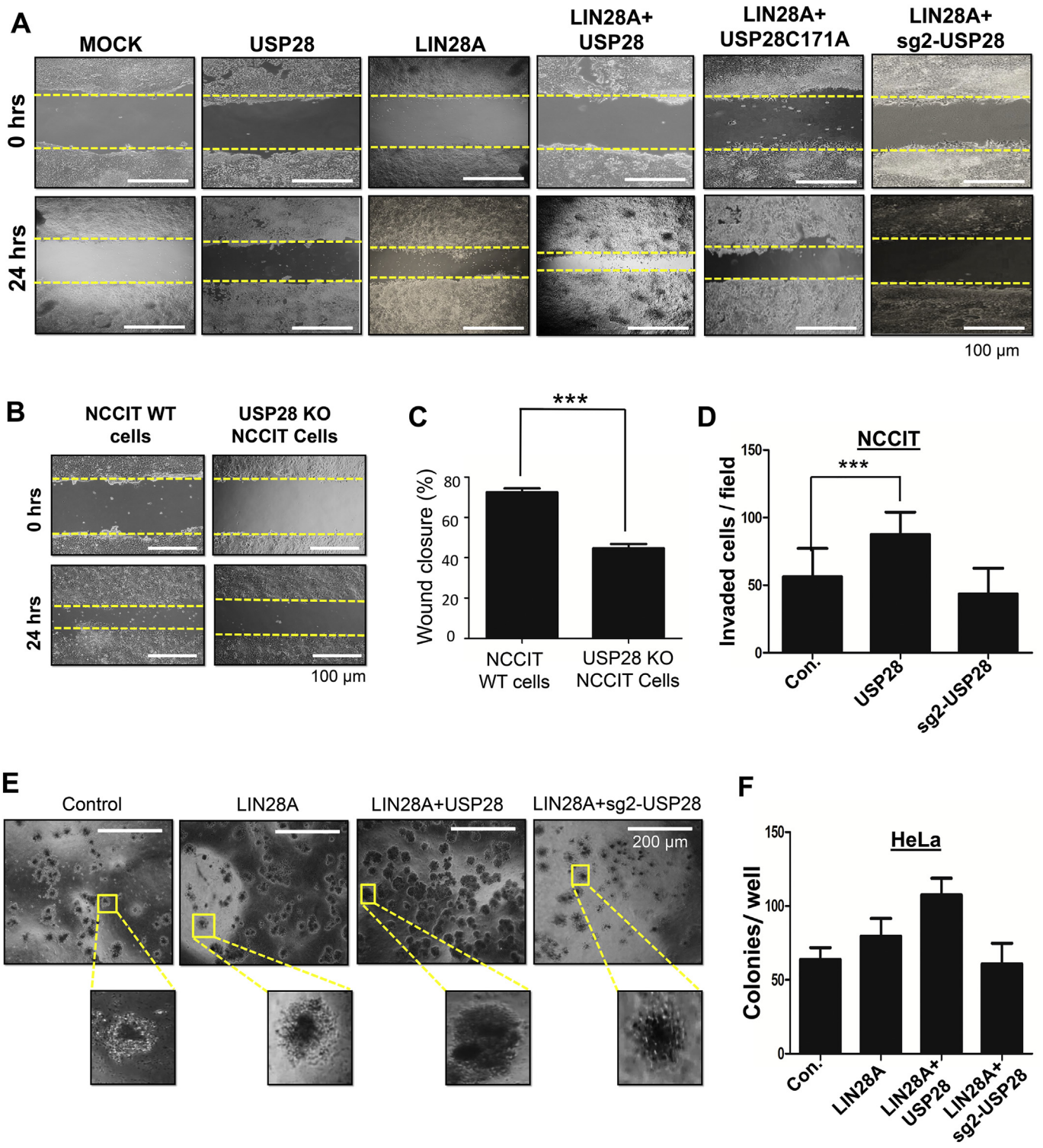

Fig. 6. USP28 enhances LIN28A-mediated cellular migration, cancer cell invasion and anchorage independent growth. A, NCCIT cells transiently transfected with the indicated constructs were assessed for LIN28A-mediated migratory potential by wound healing assay. Images were captured at 0 and 24 h. B and C, Wound healing was performed on NCCIT wild type and USP28 knockout cell lines. Images were captured at 0 and 24 h. Assays were performed in triplicate. Wound closure was shown as the space between two broken lines. The area of the wound at 0 h and 24 h were estimated by ImageJ software and the percentage of wound closure is represented graphically. (Scale bar: 100 µm). Error bars represent triplicate experiments, ($p < 0.05$ by *t*-test was considered significant). D, Matrigel cell invasion assay was performed in NCCIT cells transfected with only Flag-USP28 or sgRNA2 targeting USP28 in NCCIT cells. Cells were seeded in the upper chamber of the plate. After 24 h of incubation, cells on the bottom of the chamber membrane were fixed; stained and invaded cells were counted for graphical representation. E and F, HeLa cells transfected with Myc-LIN28A alone or cotransfected with either USP28 or sgRNA2-targeting USP28, were cultured on soft agar for three weeks. The colonies formed were stained with 0.01% crystal violet, counted and represented graphically. Three independent experiments were performed ($p < 0.05$ by *t*-test was considered significant).

Conflict of interest

The authors declare no conflict of interest.

Author contributions

S.R. directed the study; S.H., S.D., and S.H.K. performed the experiments; S.R., S.H., S.D., and K.S.K. wrote and edited the manuscript; D.K. performed real-time PCR; S.H., S.D. and A.P.C. performed gene editing.

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Transparency document

The Transparency document associated this article can be found, in online version.

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