Regulator of Cullins-1 (ROC1) Negatively Regulates the Gli2 Regulator SUFU to Activate the Hedgehog Pathway in Bladder Cancer

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Abstract

Background: The regulator of cullins-1 (ROC1) is an essential subunit in the Cullin-RING ligase (CRL) protein complex and was shown to be critical in bladder cancer cell survival and malignant progression. This study aimed to explore the regulatory mechanism of ROC1 in bladder cancer malignant progression.

Methods: This study explored the underlying mechanisms using both in vitro and in vivo experiments. The expression of the components of Sonic Hedgehog (SHH) pathway was determined by western blotting analysis. ROC1 expression in human tumours was evaluated by immunohistochemical analysis.

Results: The data showed that ROC1 overexpression promoted growth of bladder cancer cells, whereas knockdown of ROC1 expression had an opposite effect in bladder cancer cells. Mechanistically, ROC1 was able to target SUFU for ubiquitin-dependent degradation, allowing the Gli2 release from the SUFU complex to activate SHH pathway. Furthermore, knockdown of SUFU expression partially rescue the ROC1 knockdown-suppressed SHH activity as well as cancer cell growth inhibition. At ex vivo, tissue microarray analysis of human bladder cancer specimens revealed an positive association of ROC1 expression with the SHH pathway activity.

Conclusion: The current study demonstrated the dysregulation of ROC1-SUFU-GLI2 axis played an important role in bladder cancer progression and targeting of ROC1 expression is warranted further investigation as a novel strategy for future control of bladder cancer.

Background

Bladder cancer is a common malignancy, accounting for the 4th most commonly diagnosed cancer globally and the 8th leading cause of cancer-related deaths in male worldwide [1, 2]. Approximately 75% to 80% bladder cancer patients are initially diagnosed as non-muscle invasive bladder cancer (NMIBC) and a half of such patients will recur within five years, and up to 30% of NMIBC patients will progress to muscle-invasive bladder cancer (MIBC), and the latter initially occurs in approximately 25% of bladder cancer patients and has a poor prognosis [3]. Treatment of MIBC patients is usually to surgical tumor resection and then cisplatin-based chemotherapy [3, 4]. Surgery alone is difficult to effectively control tumor recurrence, while the cisplatin-based chemotherapy leads to the severe toxicity and relatively low anticancer efficiency; for example, only 40–65% of patients with metastatic bladder cancer showed clinical response [3, 4]. Thus, better understanding of the molecular mechanisms underlying bladder cancer progression and recurrence, will lead to identification of novel anticancer targets for bladder cancer therapy.

The Cullin-RING ligases (CRL) are the enzymes to target proteins for ubiquitin-mediated degradation and altered CRL activity contributed to development and progression of human cancers, including bladder cancer [5]. CRL, also known as Skp1, Cullin, or the F-box protein, belongs to the largest family of the E3 ubiquitin ligases which mediate the proteasome-targeted degradation of 20% of ubiquitinated protein substrates, like the cell cycle-related, DNA replication, and signal transduction proteins as well as

transcription factors [5]. Experimentally, the small molecule inhibitors, like MLN4924 were able to suppress CRL activation by inhibition of the Cullin activity, and in turn to effectively reduce growth of various human cancer cells *in vitro* [6]. Furthermore, the regulator of cullins–1 (ROC1), or namely as the RING box protein–1 (RBX1), is a key CRL subunit and heterodimerizes with other Cullins to form the CRL catalytic cores [5]. ROC1 contains a small zinc-binding domain (the RING finger), which is evolutionarily conserved and is essential in embryonic development, while aberrant ROC1 expression led to CRL dysfunction and embryonic lethality [7, 8]. ROC1 is also essential in maintenance of the genome integrity and cancer development [8–12]. Our previous studies demonstrated that ROC1 protein was overexpressed in bladder cancer tissues, while knockdown of ROC1 expression reduced the CRL activity, triggered accumulation of its specific substrates (such as p21, p27, and DEPTOR), leading to suppression of tumor progression [13, 14]. In this study, we further investigated and explored the underlying molecular mechanisms, by which ROC1 regulates the Sonic Hedgehog (SHH) pathway in bladder cancer using our *in vitro* and *in vivo* experiments. We expected to elucidate the molecular basis for the future development of a novel ROC1-based targeted therapy against bladder cancer.

Materials And Methods

Cell culture and reagents

Human bladder cancer 5637 and T24 cell lines were obtained from Chinese Academy of Science (Shanghai, China) and maintained in Roswell Park Memorial Institute medium–1640 (RPMI–1640; Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and1% penicillin-streptomycin in a humidified 5% CO2 environment at 37°C. Cycloheximide (CHX), the SHH signaling activator SAG (an SMO agonist), the SMO antagonist GDC0449, and the NAE (Nedd8-activating enzyme) inhibitor MLN4924 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored at –20°C as stock solutions. G418 was also from Sigma-Aldrich. The recombinant plasmid carrying HA-ubiquitin cDNA was purchased from Invitrogen (Shanghai, China).

Establishment of stable ROC1 overexpressed or silenced bladder cancer cell lines

To establish a stable ROC1 overexpressed bladder cancer cell sublines, we subcloned the full-length wild-type human ROC1 cDNA into the pcDNA3.1 vector (Invitrogen, Shanghai, China), named it as pcDNA3.1-ROC1. After DNA sequence confirmation, this recombinant plasmid or pcDNA3.1 vector-only plasmid was transfected into bladder cancer cells using Lipofectamine 2000 (Invitrogen) for 48 h and the cells were then cultured in G418-selecting cell culture medium at 100 μg/ml for 14 days. After that, individual of the G418-resistant monoclonal cells were selected and expanded under the 100 μg/ml G418-selective medium. The stable cell sublines were named as p-ROC1 or p-CONT. Furthermore, to knockdown ROC1 or SUFU expression, we purchased siRNA oligonucleotides targeting ROC1 or SUFU from Invitrogen

(Shanghai, China) and transfected them into bladder cancer cells according to the manufacturer's instructions. ROC1 siRNA sequences were 5'-GACTTTCCCTGCTGTTACCTAA–3'; SUFU siRNA sequences were 5'-GCCATGACAATCGGAAATCTA–3'; and scrambled control siRNA sequences were 5'-ACGTGACACGTTCGGAGAA–3'.

Cell viability and colonogenic assays

Changed cell viability was assessed by using the Cell Counting Kit–8 kit (Beyotime, China) and carried out as described previously [13]. For the colonogenic assay, tumor cells were seeded in triplicate into 35-mm culture dishes at a density of 400 cells (for 5637 tumor cells) or 1000 cells (for T24 cells) per well and cultured for 9 days. The cells were fixed and stained with crystal violet in 50% methanol and the number of cell colonies with more than 50 cells was counted.

Flow cytometry cell cycle distribution assay

Both ROC1-overexpressed and siRNA-transfected bladder cancer cells were detached from cell culture dishes and fixed in ice-cold 70% ethanol overnight. One day after, cells were washed twice with ice-cold phosphate buffered saline (PBS) and then stained with propidium iodide (PI; Sigma-Aldrich) solution (20 mg/ml) for 5 min and then analyzed by using a BD FACScan flow cytometer (San Diego, CA, USA).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was isolated with the Trizol reagent (Invitrogen) and reversely transcribed into cDNA with a PrimeScript Reverse Transcription kit (Takara, China) according to the manufacturers' protocols. The resulted cDNA samples were then amplified in the 7300 Real Time PCR system (Applied Biosystems, Foster City, CA) with the SYBR Green master mix kit (Takara, Dalian, China) for detection of different genes using gene-specific primers (the detailed DNA sequences of each primer used in this study are available upon request). All measurements were performed in triplicate and quantified using the $2^{-\Delta\Delta}$Ct method.

Western blotting and co-immunoprecipitation

After cells were subjected gene transfection or drug treatments, cell lysates were prepared and quantified according to a previous study [14]. The Western blot was carried out as described previously [13], while the co-immunoprecipitation (Co-IP) Kit (Cat. #26419) from Thermo Scientific (Waltham, MA, USA) was used according to the manufacturer's instructions with the following antibodies, i.e., an anti-ROC1

(Abcam, Cambridge, MA, USA), anti-cyclinD1, anti- Cdc25c, anti-SUFU, anti-Gli1, anti-GAPDH, anti-HA (all from Abcam, Hangzhou, China), or anti-Gli2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody.

Immunofluorescence staining

Immunofluorescence staining was used to assess Gli2 expression in cells as described previously [13]. Briefly, cells were grown on coverslips, fixed, and permeabilized, and then incubated with a primary antibody against Gli2 (Santa Cruz Biotechnology) followed by incubation with the Alexa 548-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA). Subsequently, the cells were counterstained by using 4, 6-diamidino–2-phenylindole (DAPI; Sigma) and analyzed under a Zeiss LSM500 confocal microscope (Zeiss International, Oberkochen, Germany).

In vivo tumor cell xenograft assay

An orthotopic tumor model of bladder cancer was used. In particularly, tumor cells were cultured to reach 70% to 80% confluent, harvested and resuspended in PBS, then mixed with Matrigel (Invitrogen) at 1:1 vol/vol ratio. Next, mice (6-week-old, male, athymic, BALB/C nu/nu; n = 10 per group) were anesthetized by using 40 mg/kg of sodium pentobarbital, and a small lower abdominal incision was made to expose the bladder for tumor cell injection. Tumor cells were then injected into the bladder wall using a 28-gauge needle; thereafter, the injection site was pressed with a cotton swab for 30 s and the skin incision was then closed with the absorbable line. Tumor cell xenograftformation and growth were assessed by using the whole-body fluorescence imaging system weekly, with an IVIS Spectrum imaging system (Promega, Madison, WI, USA) with 470 nm excitation from an MT–20 light source. The emitted fluorescence signal was collected by using appropriate filters on a DP70 CCD camera, and processed for contrast and brightness with Paint Shop Pro 8 (Corel, Ottawa, ON, Canada). Three months after nude mice were inoculated with the pROC1 or pCONT tumor cells, the mice were sacrificed and xenografts tissues were resected. This study protocol was approved by the Animal Care and Use Committee of Yancheng First people's Hospital (Jiangsu, China) and carried out following the Guidelines of the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research.

Human bladder tissue samples and immunohistochemistry

Bladder cancer tissue specimens were retrospectively collected from 93 bladder cancer patients who were cared in Yancheng First People's Hospital (Jiangsu, China) between January 2010 and May 2015. The patients were 79 males and 14 females with median age of 67 years (range between 45 to 87 years old), while 43 of them underwent a transurethral resection, 12 underwent a partial cystectomy, and 38 underwent a radical cystectomy. Their tumor grade and stage were classified according to the World Health Organization (WHO) 1973 criteria and the American Joint Committee on Cancer (AJCC) 2002 TNM system. This study of human subjects was approved by the Medical Ethics Committee of Yancheng First

People's Hospital with a Permit Number of 2013KY004 and informed consent was obtained from each patient before enrolling into this study.

Paraffin embedded tissue blocks were retrieved from Pathology Department and used for preparation of tissue microarray and then immunostained with a primary antibody against ROC1 (Abcam), SUFU (Abcam), Ki67 (Boster, Wuhan, China) or Gli2 (Boster) according to our previous study [13].

Statistical analysis.

The Western blot band intensities were quantified by using the Image J software (National Institute of Heath, Bethesda, MD, USA). The data were expressed as means ± standard error of the mean (SEM) and statistically analyzed by using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) for the Bonferroni t-test after one-way analysis of variance (ANOVA) for multi-group comparison, while two-group comparison was analyzed by using Student's *t*-tests and the correlation between ROC1 or SUFU and Gli2 expression was assessed by using the Pearson's $\chi 2$ test. A $p < 0.05$ was considered statistically significant.

Results

ROC1 induction of bladder cancer cell growth *in vitro* and *in vivo*

This study first assessed the ROC1 oncogenic activity in bladder cancer 5637 and T24 cells by stable transfection of ROC1 cDNA (p-ROC1) or small interfering RNA (siRNA) (siROC1), while the empty vector (p-CONT) and the negative control siRNA (siCONT) were used as their controls, respectively (Fig. S1). In these two cell lines, knockdown of ROC1 expression reduced tumor cell growth (Fig. 1A and 1B) and their colony forming potential (Fig. 1C and 1D). In contrast, ectopic overexpression of ROC1 significantly induced growth and colony forming capacity of both cell lines (Fig. 1A-D) *in vitro.*

Furthermore, our *in vivo* orthotopic bladder cancer cell xenograft model in nude mice also showed that overexpression of ROC1 resulted in a significantly acceleration of tumor cell xenograft growth (Figure 1E and F), while our Western blot analysis of the tumor cell xenografts confirmed ROC1 upregulation in the pROC1 group of mice compared with the vector-control group of mice (Fig. 1G). Moreover, immunohistochemical staining of the Ki67 antibody also indicated that ROC1 overexpression enhanced the percentage of proliferating xenografted cells (Fig. 1H).

ROC1 upregulation of cell cycle progression of bladder cancer cells

Our flow cytometric analysis of the cell-cycle distribution showed that knockdown of ROC1 expression in bladder cancer 5637 and T24 cells increased the number of cells in the G2/M phase of the cell cycle (Fig.

S2). Moreover, levels of cell cycle regulated proteins were also changed, i.e., expression of CyclinD1 and Cdc25c was markedly downregulated after knockdown of ROC1 expression in both 5637 and T24 cells, whereas ROC1 overexpression upregulated the levels of CyclinD1 and Cdc25c proteins (Fig. 2A and 2B).

ROC1 induced bladder cancer cell proliferation via the Hedgehog pathway

Accumulating evidence suggests an essential role of the Hedgehog signaling in tumor cell proliferation; thus, we first assessed levels of the key molecules of Gli1 and PTCH1 mRNA and found that knockdown of ROC1 expression was able to downregulate expression of Gli1 and PTCH1, whereas ROC1 overexpression could upregulate expression of Gli1 and PTCH1, compared with those of the corresponding controls (Fig. 2C and D). Moreover, knockdown ROC1 expression reduced expression of Gli2, but not Gli1 in 5637 cell line (Fig. 2E and Fig S3). In addition, the Hedgehog signaling activator SAG (an SMO agonist) was able to rescue such downregulation (Fig. 2E and Fig. S3). In contrast, ROC1 overexpression upregulated Gli2 expression in T24 cells was attenuated by the SMO antagonist GDC0449 (Fig. 2F and Fig. S3).

ROC1 activation of Hedgehog signaling through SUFU (Suppressor of fused) deregulation

To explore the molecules that were primarily responsible for Hedgehog activation, we assessed SUFU expression in ROC1-knockdown or overexpression bladder cancer cells. SUFU protein act as a CRL substrate and the naturally occurring Hedgehog inhibitor [15, 16]. Our data showed that expression of SUFU protein was significantly elevated in the ROC1-knockdown 5637 cancer cells compared with that of the control cells (Fig. 3A). In contrast, ROC1 overexpression reduced SUFU levels in T24 cell line (Fig. 3B).

We then performed experiments using the simultaneous siRNA knockdown of both SUFU and ROC1 expression in 5637 cells, and found that knockdown of SUFU expression markedly attenuated ROC1 knockdown-inhibited Hedgehog pathway, as evident by reduction of Gli1 and PTCH1 mRNA levels (Figure 3C). Our cell viability assay also demonstrated that knockdown of SUFU expression was able to partially restore tumor cell growth upon ROC1 knockdown in 5637 and T24 cells (Fig. S4). These data suggest that SUFU played a key role in transactivation of the Hedgehog pathway triggered by the ROC1.

The ROC1-SUFU-Gli2 axis regulation of the Hedgehog pathway in bladder cancer cells

SUFU, the key subunit of the Hedgehog pathway, can bind to Gli1/Gli2 to form the SUFU-Gli1/Gli2 complex and functions to prevent it from nuclear translocation, resulting in the cytoplasmic retention. We

thus, first performed the co-immunoprecipitation assay to assess which Gli family member interacts with SUFU, and found the interaction between SUFU and Gli1/Gli2 in 5637 and T24 cells (Figure S5).

To assessed whether ROC1-regulation of Gli2 expression or activity was through the ROC1 interaction with SUFU, we performed SUFU immunoprecipitation assay upon ROC1 transfected with either siROC1 or pROC1. Our data showed that knockdown of ROC1 expression suppressed the SUFU-mediated Gli2 expression, whereas ROC1 overexpression promoted the SUFU-mediated Gli2 expression.(Fig. 3E and F). Our data indicated that ROC1-regulated Hedgehog activation is through the SUFU-Gli2 axis.

ROC1 regulation of SUFU degradation via the ubiquitination-dependent manner

Since knockdown of ROC1 expression using siROC1 enhanced level of SUFU protein (Fig. 3A), while mRNA level of SUFU remained unchanged or even slightly deceased after knockdown of ROC1 expression (Fig. 3C), indicating that ROC1-downregulated SUFU expression could be through a protein synthesis perturbation or a protein post-translational mechanism. Thus, we treated tumor cells with a protein translation blocker Cycloheximide and then assessed the SUFU turnover rate at the same setting. As shown in Fig. 4A–4B, we found that knockdown of ROC1 expression significantly delayed SUFU turnover and prolonged the half-life of SUFU protein in both 5637 and T24 cells. We then postulated that the ubiquitination modification may regulate ROC1-related SUFU degradation by checked the level of ubiquitination. As shown in Fig. 4C and D, exogenous overexpression of HA-Ub in transfected cells did interact with SUFU, while ROC1 overexpression significantly promoted polyubiquitination of SUFU. However, knockdown of ROC1 expression using ROC1 siRNA strongly suppressed the SUFU polyubiquitination in both 5637 and T24 cells. These findings indicate that ROC1 mediates and targets SUFU for ubiquitination and degradation in bladder cancer cells.

CRL inactivity inhibition of Hedgehog signaling in bladder cancer cells

The selective inhibitor of NAE (Nedd8-activating enzyme) MLN4924 [6] was able to block the cullin neddylation required for the CRL activity in cells [17]. Our data showed that treatment with MLN4924 significantly reduced viability (Fig. S6A and B) and colony formation of 5637 and T24 cells in a dose dependent manner (Fig. S6C and D). Furthermore, 72 h-MLN4924 treatment also significantly reduced expression of cyclinD1 and Cdc25c in both cell lines, while the Hedgehog pathway activity was also inhibited, as demonstrated by decrease in Gli2 expression (Fig. 4E-F). These results suggested that inhibition of CRL activity had the similar effect as ROC1 in inhibition of Hedgehog signaling in bladder cancer cells.

Associations of ROC1 expression with SUFU and Gli2 in human bladder cancer tissues

To explore association of the ROC1, SUFU, and Gli2 expression with tumor clinicopathological grade, we analyzed their expression immunohistochemically in human bladder cancer tissues. We then divided their expression levels into two categories (low vs. high) and found that ROC1 and Gli2 expression was low, but SUFU expression was high in the low-grade bladder cancer tissues compared with those in high-grade tumors (Fig. 5A). Moreover, levels of ROC1 and SUFU expression were associated with tumor pathological grade (Fig. 5B), whereas ROC1 expression was inversely associated with SUFU expression ($P = 0.014$), but positively associated with Gli2 expression ($P = 0.001$). Collectively, bladder cancer tissues with low ROC1 expression had the high SUFU and low Gli2 expression (Table 1).

		ROC1 expression ($n = 93$)		*P* value
		Low	High	
SUFU	Low	5	39	0.000
	High	36	13	
Gli2	Low	35	12	0.000
	High	6	40	
Correlations between ROC1, SUFU and Gli2 expression were analysed by Pearson's χ^2 test. ROC1 expression exhibited negative correlations with SUFU ($p = 0.000$, R = -0.625) and positive correlations with Gli2 ($p = 0.000$, R = 0.619).				

Table 1
ROC1, SUFU and Gli2 expression and correlations

Discussion

Our present study demonstrated that ROC1 overexpression promoted growth of bladder cancer 5637 and T24 cell lines *in vitro,* and enhanced growth of mouse orthotopic xenografts in nude mice, whereas knockdown of ROC1 expression has such opposite effects. Mechanistically, ROC1 targeted SUFU for ubiquitin-dependent degradation to release Gli2 from the SUFU complex, to in turn activate the Hedgehog pathway. These findings suggested that ROC1 played an important role in bladder cancer progression, while targeting of ROC1 expression may facilitate to control bladder cancer progression or recurrence in future.

Dysregulation of cell proliferation is a landmark during tumorigenesis and progression, while induction of cell cycle arrest is an important strategy to develop anticancer drugs to effectively control of human cancers[18]. Our previous studies and studies from others demonstrated that knockdown of ROC1 expression was able to induce cancer cell G2/M arrest, autophagy and senescence [10, 13, 19]. In our

current study, we revealed that exogenous overexpression of ROC1 promoted bladder cancer cell proliferation, whereas knockdown of ROC1 expression inhibited tumor growth through induction of the G2/M arrest. Consistent with our current finding and further supporting the importance of CRL in the regulation of tumor cell proliferation, the suppression of the CRL activity using the FBXO22 silencing inhibited cancer progression through targeting of HDM2 for degradation in breast cancer [12]. Mechanistically, DNA damage is the most common cause to induce cell G2/M arrest[18]. Indeed, ROC1 silencing was able to trigger the DNA damage response (DDR) as a result of DNA re-replication [8]. Furthermore, a recent study showed that ROC1 silence led to deficiency in DNA double strand break repair by EXO1 excessive degradation [20]. Taken together, suppression of CRL activity inhibited cancer cell proliferation by induction of cell cycle G2 arrest due to causing DNA damage.

The Hedgehog pathway is important in cell differentiation and embryo development[21]. A variety of cancer types have also been linked to the aberrantly activity of the Hedgehog signaling pathway[21, 22]. Gli1 and Gli2 are the two important transcriptional factors in the Hedgehog pathway, i.e., Gli1 acts as a transcriptional activator and is regulated by Gli2, while Gli2 protein acts as a strong activator of the Hedgehog signaling[21]. Upon stabilization, Gli2 translocates into the cell nucleus to promote transcription of both Gli1 and Gli2 and other target genes [23]. In general, Gli2 could play a more important role in bladder cancer [24]. In our current study, we demonstrated that knockdown of ROC1 expression inhibited Gli2 expression but not Gli1 expression, whereas ROC1 overexpression promoted Gli2 expression in bladder cancer cells. Notably, another study revealed that the SPOP-CUL3-RBX1, another CRL E3 ubiquitin ligase, could activate the Hedgehog pathway through ZBTB3 degradation[25]. Overall, CRL inactivation could suppress Hedgehog pathway via different signaling. However, the precise underlying molecular mechanisms need further investigation.

SUFU, a key negative regulator of the Hedgehog pathway, can bind to Gli to inhibit the Hedgehog pathway activity [15], while SUFU protein can be degraded by the CRL^{Fbxl17} E3 ubiquitin ligase [16]. In the current study, we demonstrated that SUFU accumulation inactivated the Hedgehog signaling upon ROC1 knockdown, while blockage of SUFU expression restored the Hedgehog signaling suppression triggered by the ROC1 knockdown, indicating that ROC1-regulated the Hedgehog signaling in bladder cancer was dependent on SUFU degradation. One conceivable explanation may be that with the cooperation with ROC1, the F-box protein Fbxl17 binds to SUFU and promotes degradation in the CRL complex. Moreover, we also observed that silencing of both ROC1 and SUFU expression only partially restored the Hedgehog pathway activity, indicating that the SUFU-Gli2 axis is necessary but not sufficient for regulation of Hedgehog activity and that other regulatory pathways may also be involved.

In addition, our current ex vivo data further revealed an association of ROC1 expression with the Hedgehog pathway proteins, i.e., ROC1 expression was inversely associated with SUFU ($P = 0.000$), but positively associated with Gli2 expression ($P = 0.000$) in bladder cancer tissues. We speculate that ROC1 could be a promising prognostic biomarker for prediction of bladder cancer progression. However, our sample size in the current study was small, and the assosition of ROC1 expression with patient prognosis

was not investigated; therefore, future study with a larger sample size with follow-up data is needed to verify our current data.

The small molecule MLN4924 (Pevonedistat), abrogated neddylation of cullin subunit of CRLs, was identified as a promising anti-cancer drug[6]. A recent study showed that MLN4924 was able to synergistically enhance cisplatin cytotoxicity to urothelial carcinoma [26]. Our current study revealed that knockdown of ROC1 expression induced the same effect as MLN4924 does and we therefore, hypothesized that combination of cisplatin with the Hedgehog inhibition by ROC1 silencing would provide a novel strategy in control of bladder cancer in future.

Conclusion

Our current data demonstrate a novel mechanism by which ROC1 played a pivotal role in bladder cancer progression to regulate the Hedgehog signaling pathway. This indicates that ROC1 could be a novel anticancer target for bladder cancer therapy, although the precise underlying molecular mechanisms need to further investigation.

Declarations

Ethics approval and consent to participate

This study of human subjects was approved by the Medical Ethics Committee of Yancheng First People's Hospital with a Permit Number of 2013KY004 and informed consent was obtained from each patient before enrolling into this study. This study protocol was approved by the Animal Care and Use Committee of Yancheng First people's Hospital (Jiangsu, China) and carried out following the Guidelines of the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author up on request.

Competing interests

The authors declared that there is no conflict of interest in this work.

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Authors' contributions

W. W, M. G conceived and designed the experiments; W. W and M. G prepared the manuscript; H. C, J. L, H. C, and L. L performed the experiments; J. Q, and P. Q analyzed the data.

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References

1. Siegel RL, Miller KD, Jemal A: Cancer statistics, 2020. CA: a cancer journal for clinicians 2020, 70(1):7-30.
2. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al: Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. International journal of cancer 2015, 136(5):E359-386.
3. Kamat AM, Hahn NM, Efstathiou JA, Lerner SP, Malmstrom PU, Choi W, et al: Bladder cancer. Lancet 2016, 388(10061):2796-2810.
4. Felsenstein KM, Theodorescu D: Precision medicine for urothelial bladder cancer: update on tumour genomics and immunotherapy. Nature reviews Urology 2018, 15(2):92-111.
5. Fouad S, Wells OS, Hill MA, D'Angiolella V: Cullin Ring Ubiquitin Ligases (CRLs) in Cancer: Responses to Ionizing Radiation (IR) Treatment. Frontiers in physiology 2019, 10:1144.
6. Soucy TA, Smith PG, Milhollen MA, Berger AJ, Gavin JM, Adhikari S, et al: An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. Nature 2009, 458(7239):732-736.
7. Wei D, Sun Y: Small RING Finger Proteins RBX1 and RBX2 of SCF E3 Ubiquitin Ligases: The Role in Cancer and as Cancer Targets. Genes Cancer 2010, 1(7):700-707.
8. Jia L, Bickel JS, Wu J, Morgan MA, Li H, Yang J, et al: RBX1 (RING box protein 1) E3 ubiquitin ligase is required for genomic integrity by modulating DNA replication licensing proteins. The Journal of biological chemistry 2011, 286(5):3379-3386.
9. Li Y, Liu Y, Xu H, Jiang G, Van der Jeught K, Fang Y, et al: Heterozygous deletion of chromosome 17p renders prostate cancer vulnerable to inhibition of RNA polymerase II. Nat Commun 2018, 9(1):4394.

10. Wang Y, Tan M, Li H, Li H, Sun Y: Inactivation of SAG or ROC1 E3 Ligase Inhibits Growth and Survival of Renal Cell Carcinoma Cells: Effect of BIM. Translational oncology 2019, 12(6):810-818.
11. Zhang J, Li S, Shang Z, Lin S, Gao P, Zhang Y, et al: Targeting the overexpressed ROC1 induces G2 cell cycle arrest and apoptosis in esophageal cancer cells. Oncotarget 2017, 8(17):29125-29137.
12. Bai J, Wu K, Cao MH, Yang Y, Pan Y, Liu H, et al: SCF(FBXO22) targets HDM2 for degradation and modulates breast cancer cell invasion and metastasis. Proceedings of the National Academy of Sciences of the United States of America 2019, 116(24):11754-11763.
13. Wang W, Liu Z, Qu P, Zhou Z, Zeng Y, Fan J, et al: Knockdown of regulator of cullins-1 (ROC1) expression induces bladder cancer cell cycle arrest at the G2 phase and senescence. PLoS One 2013, 8(5):e62734.
14. Wang W, Chen H, Liu Z, Qu P, Lan J, Chen H, et al: Regulator of cullins-1 expression knockdown suppresses the malignant progression of muscle-invasive transitional cell carcinoma by regulating mTOR/DEPTOR pathway. British journal of cancer 2016, 114(3):305-313.
15. Huang D, Wang Y, Tang J, Luo S: Molecular mechanisms of suppressor of fused in regulating the hedgehog signalling pathway. Oncol Lett 2018, 15(5):6077-6086.
16. Raducu M, Fung E, Serres S, Infante P, Barberis A, Fischer R, et al: SCF (Fbxl17) ubiquitylation of Sufu regulates Hedgehog signaling and medulloblastoma development. The EMBO journal 2016, 35(13):1400-1416.
17. Ohh M, Kim WY, Moslehi JJ, Chen Y, Chau V, Read MA, et al: An intact NEDD8 pathway is required for Cullin-dependent ubiquitylation in mammalian cells. EMBO Rep 2002, 3(2):177-182.
18. Hanahan D, Weinberg RA: Hallmarks of cancer: the next generation. Cell 2011, 144(5):646-674.
19. Yang D, Li L, Liu H, Wu L, Luo Z, Li H, et al: Induction of autophagy and senescence by knockdown of ROC1 E3 ubiquitin ligase to suppress the growth of liver cancer cells. Cell death and differentiation 2013, 20(2):235-247.
20. Xie Y, Liu YK, Guo ZP, Guan H, Liu XD, Xie DF, et al: RBX1 prompts degradation of EXO1 to limit the homologous recombination pathway of DNA double-strand break repair in G1 phase. Cell Death Differ 2019.
21. Jeng KS, Chang CF, Lin SS: Sonic Hedgehog Signaling in Organogenesis, Tumors, and Tumor Microenvironments. International journal of molecular sciences 2020, 21(3).
22. Riobo-Del Galdo NA, Lara Montero A, Wertheimer EV: Role of Hedgehog Signaling in Breast Cancer: Pathogenesis and Therapeutics. Cells 2019, 8(4).
23. Maiti S, Mondal S, Satyavarapu EM, Mandal C: mTORC2 regulates hedgehog pathway activity by promoting stability to Gli2 protein and its nuclear translocation. Cell Death Dis 2017, 8(7):e2926.
24. Mechlin CW, Tanner MJ, Chen M, Buttyan R, Levin RM, Mian BM: Gli2 expression and human bladder transitional carcinoma cell invasiveness. The Journal of urology 2010, 184(1):344-351.
25. Jin X, Wang J, Li Q, Zhuang H, Yang J, Lin Z, et al: SPOP targets oncogenic protein ZBTB3 for destruction to suppress endometrial cancer. American journal of cancer research 2019, 9(12):2797-

2812.

26. Ho IL, Kuo KL, Liu SH, Chang HC, Hsieh JT, Wu JT, et al: MLN4924 Synergistically Enhances Cisplatin-induced Cytotoxicity via JNK and Bcl-xL Pathways in Human Urothelial Carcinoma. Sci Rep 2015, 5:16948.

Figures

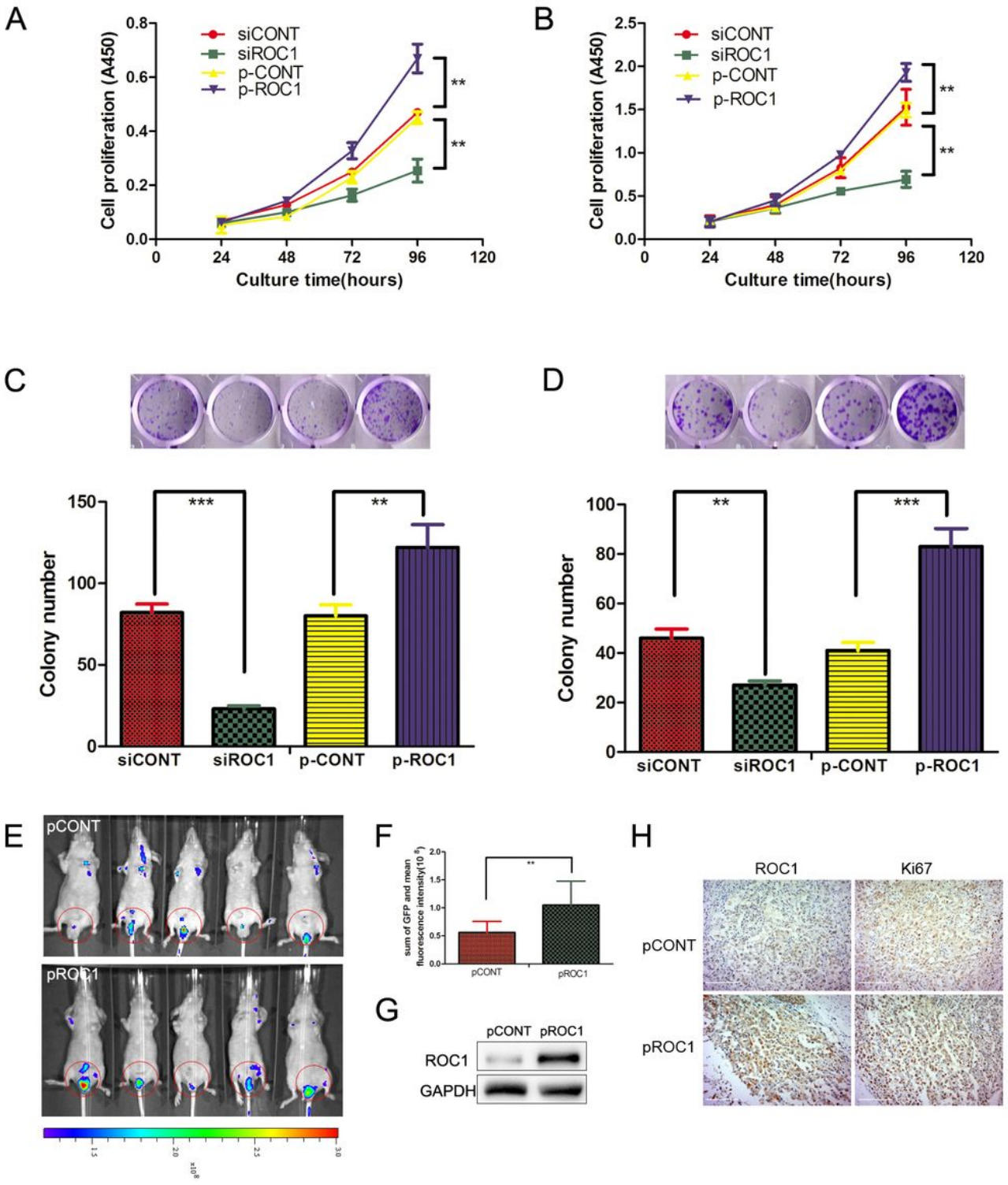


Figure 1

ROC1 induction of bladder cancer cell proliferation in vitro and in vivo. (A and B) Cell viability CCK8 assay. Stable ROC1 overexpressed bladder cancer 5637 (A) and T24 (B) cells and transient ROC1 siRNA-transfected 5637 (A) and T24 (B) were grown and subjected to cell viability assay. (C and D) Colony formation assay. Stable ROC1 overexpressed bladder cancer 5637 (C) and T24 (D) cells and transient ROC1 siRNA-transfected 5637 (C) and T24 (D) cells were grown and subjected to the tumor cell colony formation assay. (E) Nude mouse orthotopic tumor cell xenograft assay. Mice were inoculated with the pROC1 or pCONT transfected bladder cancer T24 cells and monitored with the IVIS imaging (the blue to red represents the low to high intensity of tumor burden) over the period of experimental time. (F) Quantitation of the fluorescence intensity in mice after injected with the pROC1 or pCONT transfected cells. (G) Western blot. Tumor xenografts were taken and subjected to Western blot analysis of ROC1 protein. (H) Immunohistochemistry. Tumor xenografts were taken and subjected to immunohistochemistry. Cells with brown color were considered immune-positive. Representative results of three independent experiments are shown as means ± s.e.m. ** $P < 0.01$, *** $P < 0.001$. Scale bar, 50 μm.

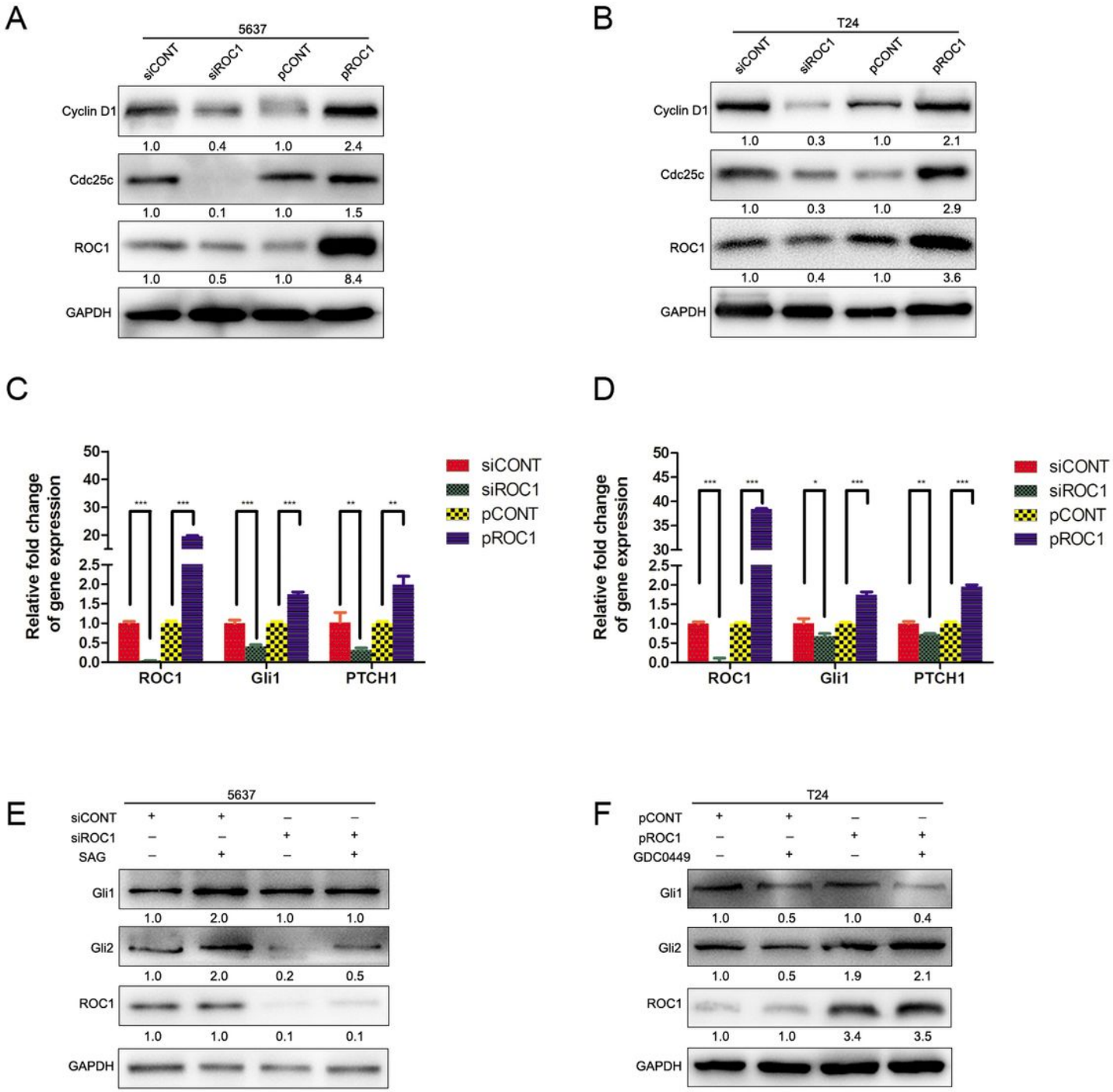


Figure 2

ROC1 regulation of tumor cell growth through the Hedgehog signaling. (A and B) Western blot. Stable ROC1 overexpressed and transient ROC1 siRNA-transfected 5637 (A) and T24 (B) were grown and subjected to Western blot analysis of CyclinD1 and Cdc25c expression. (C and D) qRT-PCR. Stable ROC1 overexpressed and transient ROC1 siRNA-transfected 5637 and T24 cells were grown and subjected to qRT-PCR analysis of Gli1 and PTCH1. (E and F) Western blot. Transient ROC1 siRNA-transfected 5637

cells treated with SAG (E), and stable ROC1 overexpressed T24 cells treated with the Hedgehog signaling pathway inhibitor GDC0449 (F), and then subjected to Western blot analysis of Gli1 and Gli2 in 5637 cells (E) and T24 cells (F). bars, s.e.m. * P < 0.05, ** P < 0.01, *** P < 0.001.

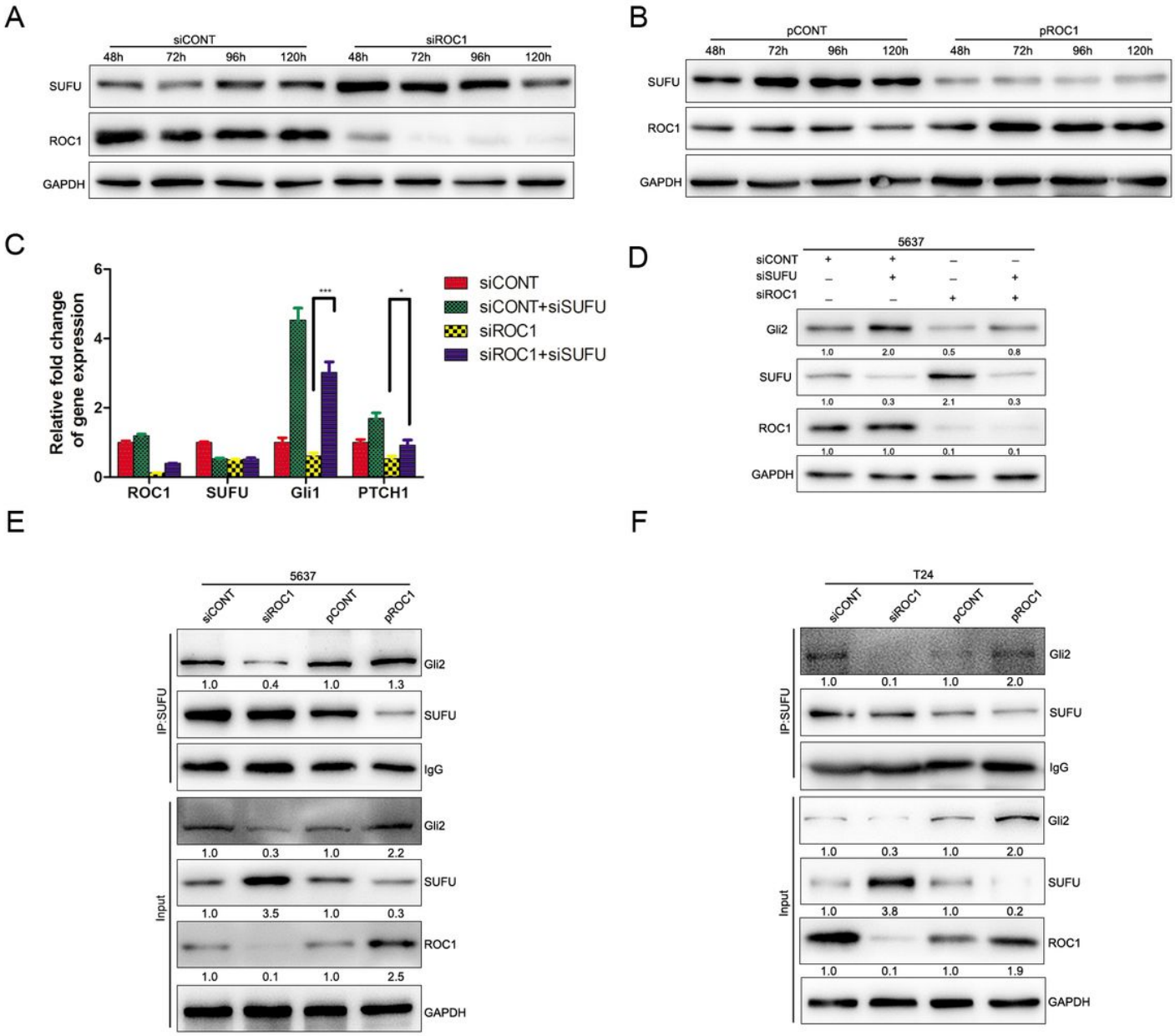


Figure 3

ROC1 modulation of SUFU protein levels. (A and B) Western blot. Expression of SUFU protein in 5637 (A) and T24 cells (B) after transfected with siROC1 or plasmid-ROC1 for 48, 72, 96, and 120 h. (C) qPCR. Bladder cancer 5637 cells were co-transfected with siRNA targeting ROC1 and SUFU and then subjected to qRT-PCR analysis of Gli1 and PTCH1 mRNA. (D) Western blot. Bladder cancer 5637 cells were co-transfected with siRNA targeting ROC1 and SUFU and then subjected to Western blot analysis of Gli2 and SUFU protein. (E and F) Immunoprecipitation. Immunoprecipitation of SUFU from 5637 cells (E) and T24

cells (F) that was transfected with either siROC1 or pROC1. Nonspecific rabbit immunoglobulin G (IgG) was used as a negative control. Cell lysates were subject to western blot analysis. bars, s.e.m. * P < 0.05, *** P < 0.001.

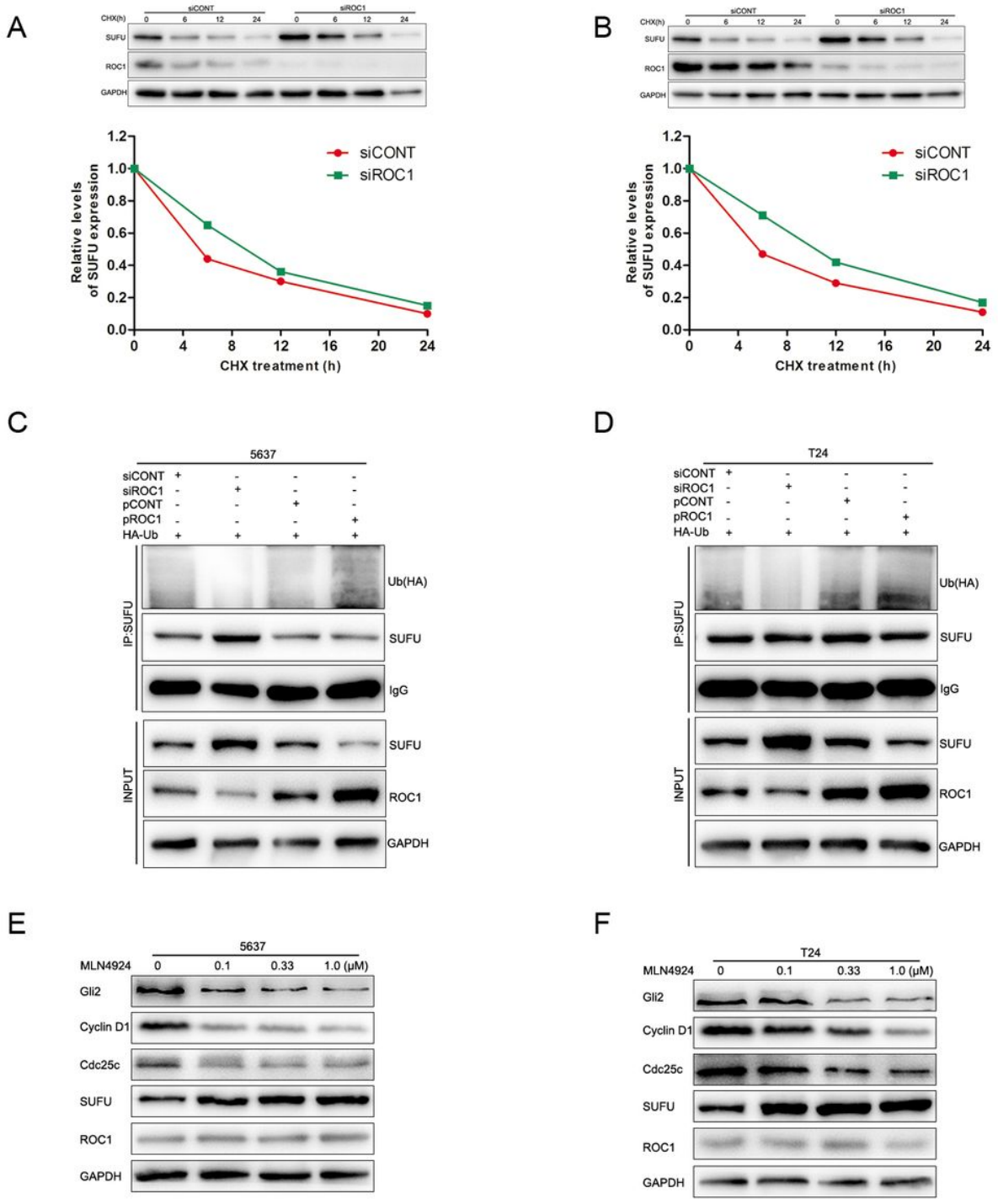
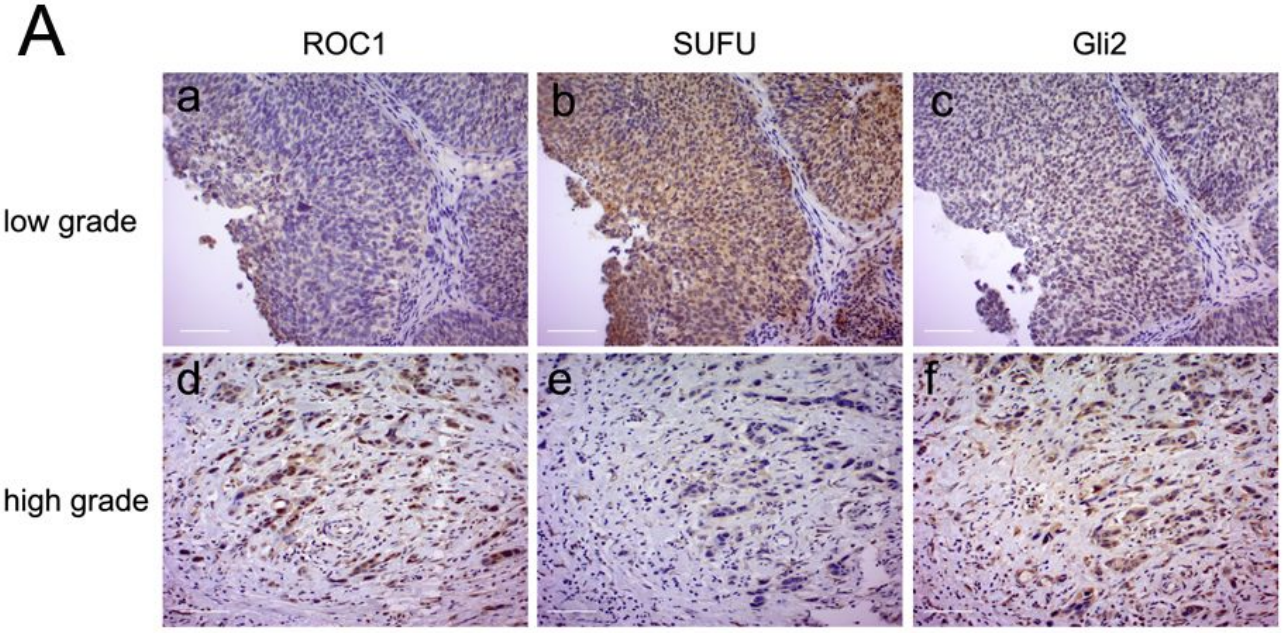


Figure 4

ROC1 regulation of SUFU ubiquitination for degradation. (A and B) Western blot. ROC1-knocked down and overexpressed 5637 (A) and T24 (B) cells were treated with Cycloheximide (CHX) for the indicated time points and then subjected to Western blot analysis of SUFU protein. The graph is the quantified data

of the Western blots showing in the bottom panel. (C and D) Co-Immunoprecipitation. Detection of ubiquitylated SUFU in 5637 (C) and T24 (D) cells co-transfected with HA tagged-ubiquitin (Ub) along with either siROC1 or pROC1, Immunoprecipitation of HA antibody of nonspecific rabbit immunoglobulin G (IgG) was used as a negative control, and cell lysates were subject to Western blot analysis. (E and F) Western blot. 5637 (E) and T24 (F) cells were treated with the CRL inhibitor MLN4924 at different concentrations and then subjected to Western blot analysis of Gli2, CyclinD1, Cdc25c, and SUFU proteins.

A



B

		Bladder cancer		*P* value
		Low-grade	High-grade	
ROC1	low	34	7	0.005
	high	29	23	
SUFU	low	25	19	0.033
	high	38	11	
Gli2	low	37	10	0.022
	high	26	20	

Figure 5

ROC1 expression in human bladder cancer tissues. (A) Expression of ROC1, SUFU and Gli2 proteins were immunohistochemically analyzed in human bladder cancer tissues. Representative immunohistochemical images of pathological low-grade or high-grade cancer are shown. (B) Association of ROC1, SUFU, and Gli2 expression with clinicopathological grade. The expression levels were divided into two categories (low vs. high) according to their immunoreactivity scores and associate with cancer pathological grade (low-grade vs. high-grade) analyzed by using the $\chi 2$ test. Scale bar = 50 μm.

Supplementary Files

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