Cullin7 Is Required for Lung Cancer Cell Proliferation and Is Overexpressed in Lung Cancer

Xuelin Men,*† Lingcheng Wang,† Wenfei Yu,‡ and Yuanrong Ju*

*Department of Respiratory, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong, P.R. China
†Department of Respiratory Medicine, Jinan No. 4 People’s Hospital, Jinan, Shandong, P.R. China
‡Shandong University School of Medicine, Jinan, Shandong, P.R. China

Ubiquitin ligase Cullin7 has been identified as an oncogene in some malignant diseases such as choriocarcinoma and neuroblastoma. However, the role of Cullin7 in lung cancer carcinogenesis remains unclear. In this study, we explored the functional role of Cullin7 in lung cancer cell proliferation and tumorigenesis and determined its expression profile in lung cancer. Knocking down Cullin7 expression by small interfering RNA (siRNA) in lung cancer cells inhibited cell proliferation and elevated the expression of p53, p27, and p21 proteins. The enhanced p53 expression resulted from activation of the DNA damage response pathway. Cullin7 knockdown markedly suppressed xenograft tumor growth in vivo in mice. Moreover, Cullin7 expression was increased in primary lung cancer tissues of humans. Thus, Cullin7 is required for sustained proliferation and survival of tumor cells in vitro and in vivo, and its aberrant expression may contribute to the pathogenesis of lung cancer. Thus, our study provided evidence that Cullin7 functions as a novel oncogene in lung cancer and may be a potential therapeutic target for lung cancer management.

Key words: Cullin7; Lung cancer; Proliferation

INTRODUCTION

Lung cancer is one of the most common malignancies worldwide. However, the progression into invasive cancer is a multistep process with numerous alterations, with the underlying molecular mechanism poorly understood (1,2). In addition, because of a lack of reliable early diagnostic markers, the prognosis of lung cancer remains poor, with a low 5-year survival rate (3–6). Therefore, better defining the pathogenesis of lung cancer and exploring novel therapeutic targets is urgent.

Cullin7 assembles a SCF-like E3 complex containing Skp1, the Fbw8F-box protein, and ROC1 (7–9). Cullin7 selectively interacts with the Skp1•Fbw8 heterodimer (7,10,11). Recently, Cormier-Daire and colleagues have linked the 3-M syndrome, which is characterized by preand postnatal growth retardation, to Cullin7 germline mutations, a majority of which have been implicated for loss of the functional cullin domain (12). In addition to the genetic evidence in mice, Cullin7 mutations have also been identified in 3-M syndrome and the Yakuts short stature syndrome, both of which are characterized by pre- and postnatal growth retardation but with relatively normal mental and endocrine functions (12,13). Cullin7 may have additional functions that include transformation mediated by simian virus-40 (SV40) large T antigen, apoptosis, p53 regulation, and the degradation of cyclin D1 (7,14–16). Taken together, the emerging genetic evidence has strongly suggested a pivotal role for the Cullin7 E3 ligase in growth control. However, whether or not Cullin7 functions in lung cancer development remains unknown.

In the present study, we explored the potential oncogenic activities of Cullin7 and its expression profile in human lung cancer.

MATERIALS AND METHODS

Cell Culture and Reagents

The lung cancer cell lines (H1299, A427, H460, and A549) were obtained from the American Type Culture Collection (Rockville, MD, USA). Antibodies that had been raised against Cullin7, p53, p27, and p21 were purchased from Cell Signaling Technology (Beverly, MA, USA), and a mouse anti-γ-H2AX antibody was obtained from Abcam (Cambridge, MA, USA). All of the remaining reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.
Small Interfering RNA (siRNA) Treatment

Chemically modified Stealth™ siRNA targeting Cullin7 and control siRNA were from Invitrogen. siRNA for p53 was from Santa Cruz Biotechnology (sc-29435). Cells were transfected with siRNA by the Lipofectamine 2000 method (Invitrogen).

RNA Extraction, RT-PCR, and Real-Time Quantitative PCR (qRT-PCR)

RNA was extracted using TRIzol reagent, according to the manufacturer’s recommended protocol (Invitrogen). qRT-PCR was performed using Applied Biosystems (Foster City, CA, USA) Step One and Step One Plus Real-Time PCR Systems. GAPDH was used as a loading control. The experiments were repeated a minimum of three times to confirm the results.

Immunohistochemistry of Tissue

Immunohistochemical analyses were as described (9). Slides of tissue sections underwent deparaffinization and antigen unmasking, then were incubated with the antibody against Cullin7 (1:100, Bethyl Laboratories, USA) at 4°C overnight. Rabbit immunoglobulin was used as a negative control. Then slides were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG, and color was developed with use of the DAB Horseradish Peroxidase Color Development Kit (P0202, Beyotime). Slides were counterstained with hematoxylin.

Western Blot Assay

Equal amounts of protein were separated using SDS polyacrylamide gels and were electrotransferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were immunoblotted overnight at 4°C with primary antibodies, followed by their respective secondary antibodies. β-Actin was used as the loading control.

Colonies Formation Assay

The cells were seeded in 6-cm dishes at a density of 300 cells per dish. After incubation for 14 days, the colonies were fixed with methanol for 10 min and stained with crystal violet for 15 min, after which point the number of colonies containing more than 50 cells was scored.

Immunofluorescence Staining

The cells were grown on the sterile coverslips, and the cells were fixed with 4% paraformaldehyde and permeabilized using 0.1% Triton X-100. Cells were blocked with rabbit anti-γ-H2AX antibody followed by rhodamine-conjugated anti-rabbit secondary antibody. Finally, the cells were further stained with 4,6-diamidino-2-phenylindole (DAPI).

Tumor Formation Assay In Vivo

The in vivo tumorigenesis and metastasis assays were performed as previously described (9). Briefly, 1 x 10^6 cells were injected subcutaneously into the right flanks of severe combined immunodeficient mice. Tumor length (L) and width (W) were measured every 3 days, and tumor volume was calculated using the equation: volume = (W^2 x L)/2.

Figure 1. Impaired clonogenesis of lung cancer cells coupled with elevated expressions of p53, p21, and p27 proteins with Cullin7 siRNA knockdown. (A) RT-PCR analysis of Cullin7 expression in lung cancer cells with and without Cullin7 siRNA knockdown. Efficient depletion of Cullin7 expression was verified. (B) Clonogenic potential of lung cancer cells with Cullin7 siRNA knockdown. **p < 0.01 (foci numbers in Cullin7 siRNA vs. Control siRNA). (C) Western blot analysis of protein expression of Cullin7, p53, p27, and p21 in lung cancer cells with and without Cullin7 siRNA knockdown. GAPDH and β-actin were used as the internal controls for RT-PCR and Western blot, respectively.
After 6 weeks, the mice were killed, and the tumor volume and weight were measured. All of the animal experiments were performed with the approval of the Shandong University Animal Care and Use Committee.

Patients and Tissue Samples
A total of 39 lung cancer tissue samples, along with matched normal tissues, were used in this study. All of the samples were obtained from the Department of Respiratory, Shandong Provincial Hospital Affiliated to Shandong University between 2010 and 2013. For all of the patients who participated in this study, written informed consent was obtained, which was approved by the Ethical Committee of Shandong University.

Statistical Analysis
The results were analyzed using SPSS18.0 software (Chicago, IL, USA). Each experiment was repeated a minimum of three times. A two-tailed *t*-test was used to determine statistical significance. The results were presented as the means±SD. Values of *p*<0.05 were considered to be statistically significant.

RESULTS
Impaired Clonogenic Potentials of Tumor Cells Treated With Cullin7 siRNA
We knocked down the expression of Cullin7 using its specific siRNA in the lung cancer cells A549, H460, A427, and H1299. Efficient silencing of Cullin7 expression in all these cells was verified by qRT-PCR (Fig. 1A). Knockdown of Cullin7 led to significantly reduced foci numbers and sizes of all cell lines compared with the control (Fig. 1B).

Induction of p53, p27, and p21 Expression in Cancer Cells With Cullin7 siRNA Knockdown
To determine the underlying mechanism of Cullin7 inhibition, we examined the expression of the tumor suppressor p53, its target CDK inhibitor p27 and p21 in cells with Cullin7 siRNA knockdown. The expression of p53 and its downstream molecule p27 and p21 was increased in all cells with Cullin7 siRNA knockdown compared with control cells (Fig. 1C). To further define a causal relationship between p53 induction and defective foci

![Figure 2](image-url)

Figure 2. Induction of p53 by Cullin7 siRNA knockdown is via activation of the DNA damage response pathway. (A) Partial recovery of foci formation of A549 cells with Cullin7 and p53 siRNA knockdown. **+p<0.01 (foci numbers in Cullin7 siRNA only vs. Ctr siRNA), #p<0.01 (foci numbers in Cullin7 siRNA only vs. Cullin7 siRNA+p53 siRNA). (B) Western blot analysis of protein expression of the Cullin7, p53, p27, p21, and γ-H2AX with Cullin7 and p53 siRNA knockdown.
formation, we transfected A549 cells with both Cullin7 siRNA and p53 siRNA. p53 inhibition significantly restored the clonogenic potential of A549 cells treated with Cullin7 siRNA (Fig. 2A). Thus, Cullin7 siRNA knockdown impaired the clonogenic potential of tumor cells at least in part through p53 induction.

Induction of p53 by Cullin7 siRNA Knockdown Is via Activation of the DNA Damage Response Pathway

A change in chromatin configuration mimics DNA damage and contributes to the activation of the DNA damage response pathway with increased p53 protein expression. DNA damage results in rapid phosphorylation of histone H2AX at Ser139 by ATM, and the phosphorylated H2AX (γ-H2AX) then localizes to sites of DNA damage at subnuclear foci. Therefore, we examined γ-H2AX activity in cells with Cullin7 siRNA knockdown. Inhibition of Cullin7 led to increased γ-H2AX foci formation in A549 cells, as revealed by Western blot assay. To further define a causal relationship between γ-H2AX and p53 induction, we transfected A549 cells with both Cullin7 siRNA and p53 siRNA. p53 inhibition significantly restored the γ-H2AX induction of A549 cells treated with p53 siRNA (Fig. 2B). We further tested the role of p53 in Cullin7 siRNA-induced p27 and p21 expression by Western blot. As shown in Figure 2B, the p27 and p21 expressions that were induced by Cullin7 siRNA were obviously attenuated following p53 knockdown using siRNA. Thus, Cullin7 siRNA knockdown impaired the γ-H2AX, p27, and p21 expression in lung cancer cells at least in part through p53 induction.

Impaired In Vivo Tumorigenesis With Cullin7 siRNA Knockdown

To explore the effects of Cullin7 on tumorigenesis in vivo, different cells were injected subcutaneously into the flanks of nude mice. We found that the tumor volumes were much smaller in those that were formed from the knockdown cells than in those that were formed from the control cells (Fig. 3A). We also analyzed the weight of the tumors (Fig. 3B). Therefore, Cullin7 may be required for tumor growth in mice.

Overexpression of Cullin7 in Human Cancerous Lung Tissues

Immunohistochemistry revealed normal lung mucosa negative for Cullin7 protein level. Most cells were negative for Cullin7, but a small proportion of epithelial cells showed weak Cullin7 signals in cytoplasm (Fig. 4A, B). However, in tumors, most cells were Cullin7 positive, with much stronger staining than in matched normal tissues. Cullin7 signal intensity was significantly heterogeneous within the same tumor. Cullin7 protein was present in both cytoplasmic and nuclear compartments of tumor cells, but a much stronger signal was seen in the nucleus (Fig. 4A, B).

DISCUSSION

Lung cancer is one of the most common cancers in the world. Despite the decline in lung cancer mortality, a number of lung cancer patients develop metastatic tumors even after surgical removal of the primary tumors (17,18). Its early diagnosis is difficult. Lung cancer can be diagnosed effectively in some cases with serological assessment, but the molecular mechanism of cell proliferation and effective biological markers for diagnosis are unclear (19–21). In the present article, we identified Cullin7 as a candidate target gene for the promotion of lung cancer growth.

The involvement of Cullin7 in tumorigenesis has been supported by recent findings of high expression of Cullin7 in ER-positive primary breast cancers and regulated by both Era and HIF1α in normoxia and hypoxia (7,22). However, the mechanisms underlying Cullin7-involved carcinogenesis and the roles of Cullin7 in other tumors...
are largely unknown. In the present study, we knocked down Cullin7 with its siRNA and found that the clonogenic potential was substantially impaired in all tested cells with Cullin7 siRNA knockdown. In addition, even transient Cullin7 knockdown could significantly inhibit tumor formation in mice injected with tumorigenic A549 cells. Therefore, Cullin7 may be essential for proliferation, survival, and tumorigenesis of cancer cells. Moreover, overexpression and aberrant distribution of Cullin7 was observed in primary tumor cells from patients with lung cancer. Cullin7 may be an important factor contributing to the pathogenesis of lung cancer.

Clonogenic assay revealed that cells with Cullin7 knockdown showed highly diminished ability to form foci. We further found the level of the tumor suppressor p53 increased substantially in p53-carrying cancer cells with Cullin7 siRNA knockdown. Consistent with the enhanced expression of p53, that of p27 and p21, the downstream targets of p53, was upregulated in the same cells. Given the key role of p53 in inhibiting proliferation, increased expression of p53, p27, and p21 is likely responsible for growth arrest in Cullin7-inhibited cells. In support of this finding, abolishing p53 upregulation attenuated the clonogenic decrease in cancer cells with Cullin7 knockdown. We observed a significantly increased phosphorylated γ-H2AX signal in those cells, which indicates activation of the DNA damage response pathway. Structural alteration of chromatin mimics DNA damage and triggers DNA damage response from ATM phosphorylation to p53 stabilization (23,24). Therefore, Cullin7 inhibition may lead to consequent activation of the DNA damage response pathway. Increased p53 level would result from its stabilization in cancer cells with Cullin7 inhibition.

Furthermore, the severely impaired clonogenicity with Cullin7 knockdown contrasted with the low degree of growth arrest in lung cancer cells. Because a clonogenic assay reflects the repopulation potential of cancer stem/progenitor cells (25), these findings strongly indicate that such cells are vulnerable to Cullin7 inhibition. This result may be implicated in cancer therapy. Further investigations are required to elucidate the effect of Cullin7 on cancer stem and progenitor cells and to test Cullin7 as a therapeutic target for cancer stem/progenitor cell clearing.

The Cullin7 expression profile differed significantly in normal and cancerous human lung tissues. We observed overexpression of Cullin7 in most lung cancer specimens, and its nuclear accumulation increased with less differentiated tumors, which indicates a clinical relevance of Cullin7 in lung cancer. These observations, together with those from in vitro and in vivo experiments, suggest that Cullin7 may be an important factor contributing to lung tumorigenesis, and targeting this molecule may be a novel therapeutic strategy for human lung malignancies.

ACKNOWLEDGMENT: The authors declare no conflict of interest.

REFERENCES
1. Wang, Q.; Wang, Y.; Zhang, Y.; Zhang, Y.; Xiao, W. The role of uPAR in epithelial-mesenchymal transition in small airway epithelium of patients with chronic obstructive pulmonary disease. Respir. Res. 14:67; 2013.
2. Wang, Q.; Wang, H.; Zhang, Y.; Zhang, Y.; Xiao, W. Activation of uPAR is required for cigarette smoke extract-induced epithelial-mesenchymal transition in lung epithelial cells. Oncol. Res. 21:295–305; 2013.
3. Shaw, M. G.; Ball, D. L. Treatment of brain metastases in lung cancer: Strategies to avoid/reduce late complications of whole brain radiation therapy. Curr. Treat. Options Oncol. 14:553–567; 2013.
4. Singh, S.; Chellappan, S. Lung cancer stem cells: Molecular features and therapeutic targets. Mol. Aspects Med. 39C: 50–60; 2014.
5. Pikor, L. A.; Ramnarine, V. R.; Lam, S.; Lam, W. L. Genetic alterations defining NSCLC subtypes and their therapeutic implications. Lung Cancer 82:179–189; 2013.
6. Lang-Lazdunski, L. Surgery for nonsmall cell lung cancer. Eur. Respir. Rev. 22:382–404; 2013.
7. Ponyeam, W.; Hagen, T. Characterization of the Cullin7 E3 ubiquitin ligase—Heterodimerization of cullin substrate receptors as a novel mechanism to regulate cullin E3 ligase activity. Cell. Signal. 24:290–295; 2012.
8. Xu, Y.; Wang, Y.; Ma, G.; Wang, Q.; Wei, G. CUL4A is overexpressed in human pituitary adenomas and regulates pituitary tumor cell proliferation. J. Neurooncol. 116:625–632; 2014.
9. Wang, Y.; Wen, M.; Kwon, Y.; Xu, Y.; Liu, Y.; Zhang, P.; He, X.; Wang, Q.; Huang, Y.; Jen, K. Y.; LaBarge, M. A.; You, L.; Kogan, S. C.; Gray, J. W.; Mao, J. H.; Wei, G. CUL4A induces epithelial-mesenchymal transition and promotes cancer metastasis by regulating ZEB1 expression. Cancer Res. 74:520–531; 2014.
10. Paradis, V.; Albuquerque, M.; Mebarki, M.; Hernandez, L.; Zalinski, S.; Quentin, S.; Belghiti, J.; Soulier, J.; Bedossa, P. Cullin7: A new gene involved in liver carcinogenesis related to metabolic syndrome. Gut 62:911–919; 2013.
11. Wang, Y.; Ma, G.; Wang, Q.; Wen, M.; Xu, Y.; He, X.; Zhang, P.; Wang, Y.; Yang, T.; Zhan, P.; Wei, G. Involvement of CUL4A in regulation of multidrug resistance to P-gp substrate drugs in breast cancer cells. Molecules 19:159–176; 2014.
12. Huber, C.; Dias-Santagata, D.; Glaser, A.; O’Sullivan, J.; Brauner, R.; Wu, K.; Xu, X.; Pearce, K.; Wang, R.; Uzielli, M. L.; Dagoneau, N.; Chemaitilly, W.; Superti-Furga, A.; DosSantos, H.; Megarbane, A.; Morin, G.; Gillessen-Kaesbach, G.; Hennekam, R.; Vander Burgt, I.; Black, G. C.; Clayton, P. E.; Reud, A.; Le Merrer, M.; Scambler, P. J.; Munnich, A.; Pan, Z. Q.; Winter, R.; Cormier-Daire, V. Identification of mutations in CUL7 in 3-M syndrome. Nat. Genet. 37:1119–1124; 2005.
13. Hu, H.; Yang, Y.; Ji, Q.; Zhao, W.; Jiang, B.; Liu, R.; Yuan, J.; Liu, Q.; Li, X.; Zou, Y.; Shao, C.; Shang, Y.; Wang, Y.; Gong, Y. CRL4B catalyzes H2AK119 monoubiquitination and coordinates with PRC2 to promote tumorigenesis. Cancer Cell 22:781–795; 2012.
14. Hartmann, T.; Xu, X.; Kronast, M.; Muehlich, S.; Meyer, K.; Zimmermann, W.; Hurwitz, J.; Pan, Z. Q.; Engelhardt, S.; Sarikas, A. Inhibition of Cullin-RINGE3 ubiquitin ligase 7 by simian virus 40 large T antigen. Proc. Natl. Acad. Sci. USA 111:3371–3376; 2014.
15. Fahlbusch, F. B.; Dawood, Y.; Hartner, A.; Menendez-Castro, C.; Noel, S. C.; Tszechoppe, A.; Schneider, H.; Strissel, P.; Beckmann, M. W.; Schleussner, E.; Ruebner, M.; Dorr, H. G.; Schild, R. L.; Rascher, W.; Dotsch, J. Cullin7 and Fbxw8 expression in trophoblastic cells is regulated via oxygen tension: Implications for intrauterine growth restriction? J. Matern. Fetal Neonat. Med. 25:2209–2215; 2012.
16. Fu, J.; Lv, X.; Lin, H.; Wu, L.; Wang, R.; Zhou, Z.; Zhang, B.; Wang, Y. L.; Tsang, B. K.; Zhu, C.; Wang, H. Ubiquitin ligase cullin7 induces epithelial-mesenchymal transition in human choriocarcinoma cells. J. Biol. Chem. 285:10870–10879; 2010.
17. Finigan, J. H.; Kern, J. A. Lung cancer screening: Past, present and future. Clin. Chest Med. 34:365–371; 2013.
18. Kim, E. S.; Pandya, K. J. Advances in personalized therapy for lung cancer. Expert Opin. Med. Diagn. 7:475–485; 2013.
19. Cagle, P. T.; Allen, T. C.; Olsen, R. J. Lung cancer biomarkers: Present status and future developments. Arch. Pathol. Lab. Med. 137:1191–1198; 2013.
20. Roy, M.; Luo, Y. H.; Ye, M.; Liu, J. Nonsmall cell lung cancer therapy: Insight into multitargeted small-molecule growth factor receptor inhibitors. Biomed. Res. Int. 2013:964743; 2013.
21. Minuti, G.; D’Incecco, A.; Cappuzzo, F. Targeted therapy for NSCLC with driver mutations. Expert Opin. Biol. Ther. 13:1401–1412; 2013.
22. Zhao, Y.; Sun, Y. Cullin-RING ligases as attractive anticancer targets. Curr. Pharm. Des. 19:3215–3225; 2013.
23. Sheu, J. J.; Guan, B.; Choi, J. H.; Lin, A.; Lee, C. H.; Hsiao, Y. T.; Wang, T. L.; Tsai, F. J.; Shih, I. M. Rsf-1, a chromatin remodeling protein, induces DNA damage and promotes genomic instability. J. Biol. Chem. 285:38260–38269; 2010.
24. Vidanes, G. M.; Bonilla, C. Y.; Toczyski, D. P. Complicated tails: Histone modifications and the DNA damage response. Cell 121:973–976; 2005.
25. Sun, Y.; Wang, Y.; Fan, C.; Gao, P.; Wang, X.; Wei, G.; Wei, J. Estrogen promotes stemness and invasiveness of ER-positive breast cancer cells through Gli1 activation. Mol. Cancer 13:137; 2014.