RNF8 Promotes Epithelial–Mesenchymal Transition in Lung Cancer Cells via Stabilization of Slug

Jingyu Kuang1, Lu Min1, Chuanyang Liu1, Si Chen2, Changsong Gao1, Jiaxin Ma1, Xiaomin Wu1, Wenyi Li1, Lei Wu1,3, and Lingyun Zhu1

ABSTRACT

RNF8 (ring finger protein 8), a RING finger E3 ligase best characterized for its role in DNA repair and sperm formation via ubiquitination, has been found to promote tumor metastasis in breast cancer recently. However, whether RNF8 also plays a role in other types of cancer, especially in lung cancer, remains unknown. We show here that RNF8 expression levels are markedly increased in human lung cancer tissues and negatively correlated with the survival time of patients. Overexpression of RNF8 promotes the EMT process and migration ability of lung cancer cells, while knockdown of RNF8 demonstrates the opposite effects. In addition, overexpression of RNF8 activates the PI3K/Akt signaling pathway, knockdown of RNF8 by siRNA inhibits this activation, and pharmacologic inhibition of PI3K/Akt in RNF8-overexpressing cells also reduces the expression of EMT markers and the ability of migration. Furthermore, RNF8 is found to directly interact with Slug and promoted the K63-Ub of Slug, and knockdown of Slug disrupts RNF8-dependent EMT in A549 cells, whereas overexpression of Slug rescues RNF8-dependent MET in H1299 cells, and depletion of RNF8 expression by shRNA inhibits metastasis of lung cancer cells in vivo. Taken together, these results indicate that RNF8 is a key regulator of EMT process in lung cancer and suggest that inhibition of RNF8 could be a useful strategy for lung cancer treatment.

Implications: This study provides a new mechanistic insight into the novel role of RNF8 and identifies RNF8 as a potential new therapeutic target for the treatment of lung cancer.

Introduction

Lung cancer is the malignancy with the highest lethality worldwide (1). Approximately 80% of lung cancers are non–small cell lung carcinomas (NSCLC), which include adenocarcinomas, squamous cell carcinomas, adenosquamous cell carcinomas, and large cell carcinomas. Because of the metastatic behavior of these tumors, the prognosis of patients with locally advanced or metastatic disease is ominous, with a 5-year survival rate of <10% (2). Previous studies have suggested that the epithelial–mesenchymal transition (EMT) is a key step in the progression of tumors toward metastasis and invasion. Loss expression of E-cadherin, a key epithelial marker, is the hallmark of the EMT, which is coincident with the upregulation of E-cadherin transcriptional repressors, such as Slug, Snail, Zeb1, Zeb2, and Twist (3–7). In lung cancer, among these transcriptional factors, Slug is the most thoroughly investigated EMT regulator (8). High Slug expression is associated with lung cancer invasion and inversely correlated with patient outcome in metastatic non–small cell lung cancer (8, 9). In addition, the EMT is regulated through various signaling networks, including the MAPK, PI3K/Akt, Wnt/β-catenin, TGFβ, and Notch pathway (10–12). Although there have been significant advances in lung cancer treatments owing to our growing knowledge of the corresponding mechanisms of tumorigenesis, our understanding of the underlying molecular mechanisms of NSCLC metastasis remains limited. To address the problems associated with lung cancer, we should develop an in-depth understanding of the molecular mechanisms of lung cancer progression and metastasis.

Protein ubiquitylation is sequentially catalyzed by enzyme E1, which activates Ubiquitin, E2, which transfers Ubiquitin to E3, and E3, which ligates Ubiquitin to specific lysine residues of target proteins (13, 14). Proteins can be monoubiquitylated that alters their functions nonproteolytically. However, the nature of the enzyme interaction landscape but will not affect the protein stability. More extensively studied, the substrate protein attached ubiquitin could be further modified by sequential attachment of further ubiquitin molecules to K11- or K48-residues to form polymeric chains, which will target the modified proteins to the 26S proteasome for degradation. On the contrary, K63-linked polyubiquitylation specifies nonproteolytic fates for the substrate (14, 15). It was estimated that there are about 600 E3 ligases in human, which expand the diversity of ubiquitin signals but restrict the substrates for each E3 ligase. RNF8 is a ubiquitin E3 ligase with two conserved domains: the N-terminal FHA (ForkHead-Associated), which can specifically bind phospho-peptides motif (pTXFX) in target proteins (16), and the C-terminal RING (Really Interesting New Gene) domain which is responsible for the E3 ligase activity.

RNF8 was originally identified to catalyze K63-polyubiquitylation on histones (17, 18), resulting in the recruitment of key DNA damage repair proteins, such as 53BP1 and BRCA1, to DNA damage site (19, 20). RNF8 was also reported to catalyze K48-polyubiquitylation on Ku80 and JMj2A, promoting their degradation and thus...
facilitating nonhomologous end joining (NHEJ) repair (19, 21). In addition to DNA damage response (DDR; ref. 22), RNF8 also play important roles in mitosis, spermatogenesis, telomere end protection, and apoptosis (23–25).

Despite of the positive biological functions of RNF8, we previously provided the first evidence that RNF8 promotes the EMT of breast cancer cells by inactivating GSK-3β and increasing the accumulation of β-catenin and thus affects the metastasis and prognosis of breast cancer (26). Lee and colleagues also proved that RNF8 facilitates breast cancer chemoresistance and progression through Twist activation by RNF8-mediated K63-linked polyubiquitin (27). Clearing RNF8-induced K63 ubiquitination of Twist is also the mechanism for Trabid to inhibit hepatocellular carcinoma growth and metastasis (28). In 2011, the Cancer Genome Project (Sanger Institute) reported high-level amplification of RNF8 in lung cancer and leukemia cells (29), but the role of RNF8 in tumorigenesis, especially in lung cancer metastasis, and the underlying downstream molecular pathways, remains poorly defined.

In our study, we demonstrated that RNF8 promoted lung tumorigenesis by regulating EMT and uncovered the RNF8-mediated stabilization of Slug and PI3K/AKT signaling were probably the underlying mechanisms in regulation of EMT by RNF8. Thus, we provided a more comprehensive understanding of lung cancer and a potential molecular target for lung cancer therapies.

Materials and Methods

Cell lines and culture

Human embryonic kidney HEK-293T and human lung cancer cells H1299, H1395, Calu-1, and A549 were purchased from BeNa Culture Collection (in 2017) and National Infrastructure of Cell Lines and Culture Collection (in 2017). All these cell lines were tested free using Mycoplasma PCR with 5% CO2 in a humid atmosphere.

Plasmid construction, siRNA, and antibodies

TG006 and CONO77 are both a lentiviral empty vector with GFP, and TG006-RNF8, 26620 (shRN8) were constructed as described previously. pLV-GFP, pLV-GFP-hRNF8, pLV-mcherry, and pLV-mcherry-His-Slug were purchased from VectorBuilder. The following antibodies were used: mouse anti-RNF8 (sc-271462; Santa Cruz Biotechnology, Inc.); rabbit anti-β-catenin (Ser33/37/thr47; #9561, Cell Signaling Technology); rabbit anti-β-catenin (#8480, Cell Signaling Technology); rabbit anti-β-actin (sc-1616-R; Santa Cruz Biotechnology, Inc.); rabbit anti-β-actin (sc-6248; Santa Cruz Biotechnology, Inc.); rabbit anti-β-actin (Ser37/40; Cell Signaling Technology); rabbit anti-Akt (#4691, Cell Signaling Technology); Secondary antibodies were horseradish peroxidase (HRP)-conjugated antimouse IgG (ZB-2305, ZSGB-Bio) or anti-Rabbit IgG(Fc) (ZB-2301, ZSGB-Bio). PI3K inhibitor LY294002(A8250) was purchased from APEXbio.

Transfection and infection

Lipofectamine RNAi MAX (Invitrogen) were used for transient knockdown by siRNA and Lipofectamine 2000 reagent (Invitrogen) were used for transient overexpression. All experiments were performed according to the manufacturer’s instructions. Lentiviral particles were produced by transfecting HEK293T cells with the pLV-GFP Vector or pLV-GFP-RNF8 or TG006 vector or TG006-RNF8 or TG006-RNF8-C406S or pLV-mcherry or pLV-mcherry-His-Slug, as well as the psPAX2 and pMD2.G packaging vectors. Polybrene (8 µg/mL) was used for lentivirus infection of A549 cells and H1299 cells.

Protein extraction and Western blotting

As described previously (26), immunoblotting was carried out to analyze the protein expression. Cells were lysed in RIPA buffer containing protease inhibitor cocktail and phospho-stop (Roche). Protein samples were separated in SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) filter membranes (Millipore). After blocking in PBS containing 0.05% Tween-20 and 5% non-fat milk powder, the membranes were then probed with the indicated antibodies, and the immunoreactive bands were developed using an ECL detection system (Millipore). All experiments were repeated for three times. For quantification, the gray density of the target bands in the immunoblot was analyzed by ImageJ software (NIH, Bethesda, MD) and normalized to the gray density of β-actin.

Commmunoprecipitation assays

HEK293T cells were transfected with corresponding plasmid, after 48 hours, adapted to suspension conditions and lysed in NETEN buffer (150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.40), 0.1% Nonidet P-40, 0.5 mmol/L EDTA, 1.5 mmol/L MgCl2, 10% glycerol) containing 1 mmol/L PMSF, 1 mmol/L DTT, and protease inhibitor cocktail. The supernatants were incubated with anti-HA-Tag-Agarose beads (Abmart, M20013M) or Ni-NTA beads (BBI Life Sciences, C600033-0025) for 2–4 hours at 4°C and the bound proteins were analyzed via immunoblotting.

Wound-healing scratch assay and cell migration assay

As described previously (26), one day before scratch, A549/ RNAi cells or H1299/RNAi cells were trypsinized and seeded equally into 6-well tissue culture plates and grew to reach almost 100% confluence in 16 hours. An artificial homogenous wound was created onto the monolayer with a sterile 10-μl tip. After scratching, the cells were washed with PBS and cultured with 1.5%-serum medium. Images of the A549 cells migrating into the wound were captured at time points of 0, 24, and 41 hours by inverted microscope (5×), and Images of the H1299 cells migrating into the wound were captured at time points of 0, 24, and 36 hours by inverted microscope (5×). All the experiments were repeated for at least three times.

Cell migration was measured according to the ability of the cells to migrate across a transwell filter (8-μm pores, Costar). A total of 0.5 × 10⁶ H1299 cells or 0.5 × 10⁵ A549 cells or 2.2 × 10⁴ A549 cells suspended in serum-free DMEM were added to the top chamber, and DMEM medium containing 10% FBS was added to the bottom chamber. After a 24-hour (for the siRNA-transfected H1299 cells) or 24-hour (for the lentivirus-infected A549 cells) incubation at 37°C in a 5% CO₂ humidified atmosphere, cotton swab scraped off the non-migrated cells of the filter and the cells that migrated to the bottom side of the top chamber, were fixed with 4% paraformaldehyde and stained with hematoxylin. The cells per microscopic field were taken pictures.
and counted in 10 randomly chosen fields. Triplicate wells were performed in each assay and the assay was repeated at least three times.

**In vivo metastasis**

H1299 cells that stably express control shRNA (CONO77) or 26620-2 (shRNF8), respectively. These cells were injected into the lateral tail vein (1 × 10^6 cells) of 6- to 7-week-old male BALB/c mice (n = 5). After 8 weeks, mice were sacrificed, and lungs were fixed in Bouin solution to see lung foci. Lungs then were fixed in formalin and embedded in paraffin blocks for slicing into thin sections. The paraffinized sections were stained with hematoxylin and eosin (H&E) according to standard protocols.

**Ethics approval and consent to participate**

Informed consent was obtained from each individual before surgery with supervision and approval of Ethical Board of the Second Xiangya Hospital of Central South University in Changsha, China. The Institutional Ethical Board of the College of Art and Science, National University of Defense Technology in Changsha, China approved the study protocol. This investigation was done following the principles outlined in the Declaration of Helsinki.

**Statistical analysis**

SPSS 19.0 (SPSS) and GraphPad Prism (version 5.0) were used for statistical analysis. As described previously (26), the differences between two independent groups were analyzed using Student t test. P < 0.05 was considered to be a statistically significant difference. Kaplan–Meier survival analysis for the relationship between survival time and RNF8 signature in breast cancer was performed using the online tool (http://kmplot.com/analysis/). Association of RNF8 with clinicopathologic factors using K-independent samples Kruskal-Wallis test and original data from https://xenabrowser.net/heatmap/.

**Data availability statement**

Publicly available datasets were analyzed in this study. These data can be found here: https://cancergenome.nih.gov/, https://xenabrowser.net/heatmap/, http://kmplot.com/analysis/. The detailed information can be found in figure legends.

**Results**

**RNF8 expression is upregulated in advanced lung cancer and is positively correlated with poor patient outcomes**

To explore the role of RNF8 in lung cancer, we analyzed the Cancer Genome Atlas (TCGA) database and found that RNF8 is more highly expressed in LC (lung cancer, n = 105) than in pared normal lung tissues (P = 0.0068; Fig. 1A). To confirm the bioinformatics analysis results, we examined RNF8 expression in 14 pairs of lung cancer tissues and corresponding noncancerous tissues by Western blotting. As shown in Fig. 1B and C, the expression levels of the RNF8 protein in 13 (92.86%) of the lung cancer tissues were clearly higher than those in matched adjacent normal lung tissues. Furthermore, with new TCGA data, we analyzed the expression of RNF8 in different pathologic types of lung cancers. Compared with normal lung tissue and adenocarcinoma, RNF8 expression is significantly higher in squamous cell carcinoma, while there is no significance between normal lung tissue and adenocarcinoma (Supplementary Fig. S1). These results suggest that RNF8 may play a role in the occurrence of lung cancer.

To reveal the clinical relevance between RNF8 and lung cancer, we performed survival analysis with Kaplan–Meier plotter (http://kmplot.com/analysis/). We found that higher RNF8 expression was associated with worse overall survival (OS, P = 0.014) and worse free progression (FP, P = 0.012) as shown in Fig. 1D and F. However, postprogression survival of patients with lung cancer (PFS, P = 0.86) was not significant (Fig. 1E) when the influences of systemic treatment, endocrine therapy, and chemotherapy were excluded. Above all, RNF8 may be used as a potential predictor of survival.

To determine the relationship between RNF8 and clinical characteristics of lung cancer, we performed data mining from database (https://xenabrowser.net/heatmap/). As shown in Table 1, high RNF8 expression was positively correlated to older age (higher RNF8 expression percentage 29.8% vs. 27.9%, P = 0.039). Gender is associated with RNF8 expression, there are higher percentage elevated RNF8 in male (33.7%) than in female (22.9%) (P = 0.000). There was no significant correlation between RNF8 expression and pathologic stage and TNM (Tumor Node Metastasis), despite the higher percentage elevated RNF8 in (T2 + T3 + T4) than in T1 (31.7% vs. 23.4%) indicating a slight trend (P = 0.077).

**RNF8 regulates the EMT in lung cancer cell lines**

We further evaluated RNF8 expression in various lung cancer cell lines (A549, H1299, H1395, and Calu-1). The data showed that the protein expression levels of RNF8 were higher in lung cancer cell lines A549, H1299 and Calu-1, than in H1395 cells (Fig. 2A). We could not explain the lower expression of RNF8 in H1395 than in the other cell lines so far, but a reasonable speculation is that its degree of malignancy is mildly compared with Calu-1 (grade III) and H1299 (derived from metastatic site: lymph node). The EMT is considered to be one of the critical steps involved in cancer metastasis. To investigate the biological function of RNF8 in lung cancer cells, we first analysed the morphology change of A549 cell with exogenous RNF8. As shown in Fig. 2B, RNF8 overexpression led to a loss of cell-to-cell contacts, the morphology was more variable and the cells were more dispersed, compared with original A549 cells. Consistently, Western blot assay demonstrated that the expression of E-cadherin and ZO-1, two epithelial markers, were reduced while the mesenchymal markers Slug, β-catenin, Zeb1, and Snail were increased (Fig. 2B right). To further confirm that RNF8 affects EMT process, we performed RNAi assay in LUAD cell line H1299 with two individual siRNAs against RNF8 (siRNF8-1 and siRNF8-2). As shown in Fig. 2C (left), RNF8-depleted cells displayed a cobblestone-like morphology, compared with control cells, which exhibited a typical spindle-like fibroblastic morphology. Consistent with the morphology change, the protein levels of Vimentin, N-cadherin, Slug, Zeb1, and Snail were decreased, while the expression of E-cadherin was increased (Fig. 2C, right). We also interfered the expression of RNF8 in A549 cells. As shown in Fig. 2D, there are tighter cell-to-cell contacts, meanwhile, the expression of the epithelial markers E-cadherin and ZO-1 were increased, and the protein level of the mesenchymal markers Slug and Snail were decreased. Taken together, these results indicate that overexpression of RNF8 induces mesenchymal phenotypes and that depletion of RNF8 induces epithelial-like phenotypes in lung cancer cells, suggesting that RNF8 plays an important role in the progression of the EMT in lung cancer.

**RNF8 enhances the cell migration potential of lung cancer cells**

During cancer development, EMT is the crucial step which promote the migration and invasion of tumor cells. To further dissect the its function, manipulated RNF8 protein expression in A549 and H1299 cells by either overexpression or RNAi and investigated the migration changes by wound-healing and transwell assays. As shown in Fig. 3A and B, compared with control cells,
RNF8-silenced cells exhibited a decrease in wound closure. In addition, the cell migration potential was significantly elevated by 6.72-fold in RNF8-overexpressed A549 cells (Fig. 3C), while the migration potential was dramatically decreased to nearly one-fourth of the control levels in RNF8-depleted A549 cells (Fig. 3D), and to 1/9–1/10 of the control levels in RNF8-depleted H1299 cells, respectively (Fig. 3E). Taken together, these data demonstrate that RNF8 promotes cell migration in lung cancer cells.

RNF8 activates the PI3K/Akt signaling pathways in lung cancer cells

Activation of PI3K/Akt has been shown to regulate cancer cell migration and invasion through distinct pathways by promoting the transcriptional activation of various transcription factors (8, 30–32). Our previous work has shown that activation of β-catenin signaling, which can be regulated by PI3K/Akt, is involved in the induction of the EMT by RNF8 (26). The translocation of β-catenin to the nucleus can activate LEF/TCF (lymphoid enhancer factor/T cell factor)-mediated transcription and induce the expression of Snail, Slug, and Twist (33), thereby contributing to the progression of the EMT. To further explore the molecular mechanisms of RNF8, we evaluated the possibility that RNF8 contributes to the EMT by regulating PI3K/Akt signaling. As shown in Fig. 4A, the phosphorylation of Akt (Ser473) was increased.

Table 1. The correlation of RNF8 expression with clinico-pathologic characteristics of patients with lung cancer.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases (n)</th>
<th>High</th>
<th>Low</th>
<th>RNF8</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤55</td>
<td>136</td>
<td>38</td>
<td>(27.9%)</td>
<td>98</td>
<td>(72.1%)</td>
</tr>
<tr>
<td>&gt;55</td>
<td>964</td>
<td>287</td>
<td>(29.8%)</td>
<td>677</td>
<td>(70.2%)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>455</td>
<td>104</td>
<td>(22.9%)</td>
<td>351</td>
<td>(77.1%)</td>
</tr>
<tr>
<td>Male</td>
<td>673</td>
<td>227</td>
<td>(33.7%)</td>
<td>446</td>
<td>(66.3%)</td>
</tr>
<tr>
<td>Pathologic stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>578</td>
<td>159</td>
<td>(27.5%)</td>
<td>419</td>
<td>(72.5%)</td>
</tr>
<tr>
<td>II + III + IV</td>
<td>537</td>
<td>168</td>
<td>(31.3%)</td>
<td>369</td>
<td>(68.7%)</td>
</tr>
<tr>
<td>Pathologic_T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>312</td>
<td>73</td>
<td>(23.4%)</td>
<td>239</td>
<td>(76.6%)</td>
</tr>
<tr>
<td>T2 + T3 + T4</td>
<td>813</td>
<td>258</td>
<td>(31.7%)</td>
<td>555</td>
<td>(68.3%)</td>
</tr>
<tr>
<td>Pathologic_N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>716</td>
<td>211</td>
<td>(29.5%)</td>
<td>505</td>
<td>(70.5%)</td>
</tr>
<tr>
<td>N1 + N2 + N3</td>
<td>389</td>
<td>117</td>
<td>(30.1%)</td>
<td>272</td>
<td>(69.9%)</td>
</tr>
<tr>
<td>Pathologic_M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>831</td>
<td>263</td>
<td>(31.6%)</td>
<td>568</td>
<td>(68.4%)</td>
</tr>
<tr>
<td>M1</td>
<td>35</td>
<td>9</td>
<td>(25.7%)</td>
<td>26</td>
<td>(74.3%)</td>
</tr>
</tbody>
</table>

RNF8 expression is upregulated in advanced lung cancer and indicates poor patient outcomes. A, The mRNA expression of RNF8 is higher in lung cancer tissues than in normal lung tissues; the original data were exported from the TCGA database (P = 0.0068). B and C, RNF8 protein expression is higher in lung cancer tissues than in noncancerous tissues as determined by Western blotting. C depicts a graphical representation of protein quantification by densitometry. D–F, Higher RNF8 expression was associated with a worse overall survival (OS, P = 0.014), free progression (FP, P = 0.012), and no significant difference in post-progression survival (PPS, P = 0.86) in patients with lung cancer. Kaplan-Meier survival analysis was performed by using an online tool (http://kmplot.com/analysis/). A P < 0.05 was considered to be a statistically significant difference (*, P < 0.05; **, P < 0.01; *** P < 0.005).
in RNF8-overexpressing A549 cells. Phosphorylated Akt (Ser473) can phosphorylate the Ser9 site of GSK-3β, producing an inactive form. Consistently, the phosphorylation of β-catenin at Ser33/37/Thr47, which is catalyzed by GSK-3β, was decreased, whereas the total β-catenin level was increased, in A549 cells overexpressing RNF8. In line with this finding, the phosphorylation of Akt (Ser473) was decreased and the phosphorylation of β-catenin was increased in RNF8-depleted H1299 cells (Fig. 4B). These data show that RNF8 activates Akt and β-catenin. Furthermore, LY294002 is a PI3K inhibitor, which can inhibit the phosphorylation of Akt. In RNF8-overexpressing A549 cells, LY294002 reduced the Snail and Slug expression but increased the ZO-1 expression (Fig. 4C), indicating impairment of the EMT, and accordingly, LY294002 also reduced the migration ability of RNF8 overexpression A549 cells (Fig. 4D). These results indicate that the EMT promotion effect of RNF8 is mediated, at least partially, by activating the PI3K/Akt signaling pathways.
RNF8 Promotes EMT in Lung Cancer

Figure 3.
RNF8 regulates the migration of lung cancer cells. A and B, Wound healing scratch assay was performed to detect the migration of RNF8-depleted A549 cells and RNF8-depleted H1299 cells (magnification, 50 ×; left), the relative migration rate was calculated by dividing the change in the distance between the scratch edges by the initial distance (right), ***, P < 0.001. C, The migration of A549 cells infected with TG006 or TG006-RNF8 virus were examined by transwell migration assay (2.2 × 10⁴ cells per chamber). Photos of representative fields are on the left (magnification, 200 ×). Overexpression of RNF8 enhances the migration of A549 cells. D, The migration of A549 cells transfected by siControl or siRNF8-1 were examined by transwell migration assay (5 × 10⁴ cells per chamber). Photos of representative fields are on the left (magnification, 200 ×). RNF8-depleted A549 cells showed decreased migration abilities. E, The migration of H1299 cells transfected by siControl or siRNF8-1 were examined by transwell migration assay (5 × 10⁴ cells per chamber). Photos of representative fields are on the left (magnification, 200 ×). Knockdown of RNF8 inhibits the migration of H1299 cells. Migrated cell number is shown from the results of three independent experiments. ***, P < 0.005.

Unpaired Student t test was used to analyze the differences between two independent groups. P < 0.05 was considered to be a statistically significant difference.

Slug is a direct target of RNF8
Slug, Snail, Zeb1, Zeb2, and Twist were commonly accepted as the key transcriptional factor, which promoted EMT (3–7), and RNF8 was reported to directly target Twist through K63-linked ubiquitination and, as a result, leading to Twist activation (3–7). In lung cancer, Slug is the most thoroughly investigated EMT regulator (8). Because Slug expression was found to be changed upon the manipulation of RNF8 expression in our work (Fig. 2B–D) and its crucial role in cancer progression, we sought to determine whether RNF8 could also directly target Slug. Using coimmunoprecipitation assays, we proved that exogenous RNF8 directly interact with endogenous Slug (Fig. 5A), meanwhile, we also proved that exogenous Slug interact with exogenous RNF8 (Fig. 5B). Because RNF8 is an E3 ligase, we also detected whether its E3 ligase activity is necessary to promote EMT in lung cancer cells. We constructed TG006-RNF8-C406S to express an inactive form of RNF8 in A549 cells. As shown in Fig. 5C, compared with overexpression of RNF8 in A549 cells, overexpression of TG006-RNF8-C406S resulted in a reduction in the mesenchymal markers Claudin-1. In addition, RNF8 can promote phospho-AKT, while the RNF8-mutant C406S devoid of E3 ligase activity cannot. Furthermore, we investigated the effect of RNF8 mutation on cell migration using transwell assay, these results indicate C406S cannot improve the migration potential of A549 cells (Supplementary Fig. S2). These indicated C406S impaired the process of EMT, and the ligase activity of RNF8 is necessary to promote EMT in lung cancer cells. To further explore whether RNF8 regulates the ubiquitination of Slug, subsequent in vivo ubiquitination assay was carried out. The results revealed that RNF8 induces the ubiquitination of Slug (Fig. 5D), we knockdown RNF8 using siRNF8-1 in HEK293T cells, despite Slug was still binding to the remaining endogenous RNF8, was shown to partially abolished the ubiquitination of Slug. This incomplete downregulation of the ubiquitination of Slug may attribute to the activity of endogenous RNF8, which was not knocked down in this experiment. These data demonstrate that the RNF8-mediated ubiquitination of Slug was dependent on the E3 ligase activity of RNF8. Furthermore, we use K63-Ub instead of Myc-Ub to detect the kind of ubiquitination that RNF8 performed to Slug. As shown in Fig. 5E, knockdown of RNF8 significantly downregulated the K63-Ub of Slug. Despite RNF8 regulates K63-Ub of Slug, as K63-linked polyubiquitylation, it is different from K48-Ub commits the substrate to degradation by the 26S proteasome, specifies nonproteolytic fates for the substrate, and whole protein for Slug IP is enough, we can see the Slug total level in the last lane is not obviously affected. According to the above results, Slug is a direct target of RNF8, and RNF8 regulates the K63-Ub of Slug.

Slug is a key transcriptional factor in EMT and is required for RNF8-mediated EMT progression in lung cancer cells
To identify whether Slug is required for the function of RNF8 in the EMT, we knocked down Slug in RNF8-overexpressing A549 cells by
Figure 4.
RNF8 activates the PI3K/Akt signaling pathways in lung cancer cells. A549 cells were transfected with pLV or pLV-RNF8 (A), and H1299 cells were transfected with siControl, siRNF8-1 or siRNF8-2 (B) and subjected to immunoblotting for p-Akt (Ser473), Akt, p-β-catenin (Ser33/37/Thr47), and β-catenin; β-actin was used as an internal control. Quantification of each indicated protein expression was plotted in the right panel. All data are shown as the means ± S.D. of at least three independent experiments. The P values were calculated using unpaired Student t tests. No, no significance; *, P < 0.05; **, P < 0.01; ***, P < 0.005. C, A549 cells were infected with TG006 or TG006-RNF8, and A549 TG006-RNF8 cells were treated with/without LY294002 at the concentration of 20 μmol/L for 24 hours. Western blot analysis of the protein expressions in the indicated cells. D, The migration of A549 cells described in C was examined by transwell migration assay (LY294002 was added to the top chamber, 2 × 10⁵ cells per chamber). Photos of representative fields are on the left (magnification, 200×). Migrated cell number is shown from the results of three independent experiments. Unpaired Student t test was used to analyze the differences between two independent groups (*, P < 0.05; ***, P < 0.005).
RNF8 directly targets Slug and mediates the K63-Ub of Slug. **A**, HEK-293T cells were infected with TG006 (control) or TG006-HA-RNF8 and harvested after 36 hours of infection. HEK-293T cell lysates were incubated with HA-Sepharose beads, and the bound proteins were analyzed via immunoblotting with HA and Slug antibodies. **B**, HEK-293T cells were infected with TG006 or TG006-FH-RNF8 and pLV-His-Slug. At 48 hours posttransfection, the cells were harvested and communoprecipitated with anti-His-tag agarose beads, and the Slug protein complexes were subjected to immunoblotting with anti-Slug and anti-RNF8 antibodies. **C**, A549 cells were, respectively, infected with TG006, TG006-HA-RNF8, TG006-HA-RNF8-C406S viruses for 5 days. Cells were harvested to detect EMT markers (Slug, Claudin-1, Snail, and Zeb1) and signal pathway molecules (p-AKT and AKT) by Western blotting; β-actin was used as an internal control. **D**, HEK-293T cells were serially transfected with siControl or siRNF8-1, pcDNA3.1, or pcDNA3.1-myc-ub in combination with pLV-His-Slug. At 48 hours posttransfection, the cells were harvested and communoprecipitated with anti-His-tag agarose beads, and the Slug protein complexes were subjected to immunoblotting with anti-RNF8, anti-Slug and anti-myc antibodies. **E**, pcDNA3.1-myc-Ub in D was replaced by pcDNA-HA-K63Ub, and the cells were also harvested and communoprecipitated with anti-His-tag agarose beads, and the Slug protein complexes were subjected to immunoblotting with anti-RNF8, anti-Slug, and anti-HA antibodies.
siRNA. As shown in Fig. 6A, the decreased Slug level was associated with the increased expression of epithelial markers (e.g., E-cadherin, ZO-1, and Claudin-1), indicating impairment of the EMT, and accordingly, knockdown of Slug also reduced the migration ability of RNF8-overexpressing A549 cells (Fig. 6B). In contrast, Slug overexpression in RNF8-knockdown H1299 cells decreased the expression...
of E-cadherin and Claudin-1, and increased Snail, indicating partially recovery of the EMT (Fig. 6C). Correspondingly, Slug overexpression reverted the migration activity of RNF8 knockdown H1299 cells (Fig. 6D). These findings indicate that Slug is required for RNF8-mediated regulation of the EMT in lung cancer.

Depletion of RNF8 expression by shRNA inhibits lung cancer cell metastasis in vivo

To further investigate the role of RNF8 in lung cancer metastasis in vivo, H1299 cells were transfected with control or RNF8 shRNA (26620) to generate stable cell lines. We selected different monoclonal

Figure 7.
shRNA-mediated downregulation of RNF8 inhibits tumor metastasis in vivo. A, Before injection, the expression of RNF8 and EMT marker in H1299-Control, H1299-26620-1, and H1299-26620-2 cells were examined by Western blotting to pick the best monoclonal cell line. B, An in vivo lung metastasis analysis. The indicated H1299 cell lines were injected into the mice (n = 5/group) through the tail vein. The metastatic lung foci were detected using Bouin solution. Representative images of mice lungs staining are shown. C, A graph with quantification of lung foci between the two groups. Unpaired Student t test was used to analyze the differences between two independent groups (*, P < 0.05). D, Representative images of mice lungs H&E staining are shown (magnification, 4× left, 40× right).
Kuang et al.

cell lines and chose 26620-2 for further study (Fig. 7A). We have also assessed the effect of RNF8 on tumor metastasis in immunocompromised male BALB/c mice (n = 5) through tail vein injection of H1299-Control and H1299-shRNF8 (26620-2) cells. Compared with those in the control group, H1299-shRNF8 cells had less and smaller lung metastases nodules or cancerous area, which was further confirmed by histologic analyses (Fig. 7B–D). These data provided further evidences that depletion of RNF8 expression by shRNA inhibits lung cancer cell metastasis in vivo.

Discussion

The epithelial–mesenchymal transition plays a critical role in cancer invasion and metastasis, and tumor metastasis is the major cause of lung cancer–related death. In a previous study, we reported that RNF8 played play an oncogenic role in breast cancer. In this study, using multiple lung cancer cell lines and validation with human lung cancer tissues, we demonstrated that RNF8 may promote lung cancer metastasis by stabilizing Slug, thereby inducing EMT phenomenon. We also revealed that RNF8 activate PI3K/Akt signaling pathway in lung cancer cells. These results extend our understanding of the role of RNF8 in tumorigenesis.

The two Snail family zinc-finger transcriptional repressors, Slug (SNAI2) and Snail (SNAI1) are well characterized for their function of repress E-cadherin expression and trigger the EMT. Higher expression of Slug and Snail is associated with aggressiveness, chemotherapy resistance and poor survival in patients with lung cancer. There for, the abundance and stability of Slug/Snail are essential for lung cancer metastasis. The human homolog of Ppa, FBXL14, was shown to target Slug for UPS-mediated degradation in tumor cells (34). In NSCLC, wild-type p53 upregulates MDM2 (murine double minute 2), a ubiquitin ligase, which target Slug for ubiquitylation and degradation, and subsequently inhibits cancer cell invasion (35). E3 ligase CHIP can target slug proteins, which were posttranslationally modified by GSK3β, for proteasomal degradation in lung cancer. Dysregulation of the GSK3β–CHIP–Slug pathway may promote cancer metastasis (36). Pellino-1 contributes to lung tumorigenesis by promoting the EMT through Snail and Slug K63-polyubiquitination and subsequent stabilization (30). The finding the E3 ligases that regulate Slug stability is important for the development of cancer therapies targeting Slug.

RNF8 is an E3 ubiquitin ligase mediating K48- and K63-linked polyubiquitination, which destines target proteins to a fate of proteasomal degradation or signal transduction. Until now, few RNF8 substrates have been identified. For example, RNF8 catalyzes the formation of lysine 63-linked polyubiquitin chains (K63-Ubs) on histones and catalyzes the formation of K48-linked ubiquitin chains on JM12A and KU80, which are involved in the DNA damage response (DDR; refs. 19, 21). RNF8 also catalyzes the formation of lysine 63-linked polyubiquitin chains (K63-Ubs) on twist, which facilitates breast cancer chemoresistance and progression (27). In this study, we found that overexpression of RNF8 promoted the EMT through the upregulation and knockdown of RNF8 inhibited the EMT through the downregulation of Slug, meanwhile changes in Slug expression can alter RNF8-induced EMT progression. Here, we demonstrated that RNF8 directly interacts with Slug and promotes its K63 ubiquitination.

It has been demonstrated that the PI3K/Akt and Erk1/2 signaling pathways induce migration and invasion mediated by the upregulation of Snail, Slug, ZEB1, and β-catlin in association with GSK3β inhibition in various cancers (8, 30, 36–38). We found that RNF8 activated PI3K/Akt signaling in A549 cells and H1299 cells and pharmacologic inhibition of PI3K/Akt in RNF8-overexpressing cells reduces the expression of EMT markers and migration changes. However, the detailed mechanism remains to be elucidated, especially as this is the first time that RNF8 has been revealed to regulate the phosphorylation of Akt.

Lung cancer is the leading cause of cancer-related death and worldwide and difficult to cure as the cancer cells are easily get metastasis and develop drug resistance. Some metastasis-associated proteins are also important DDR regulators and contribute to IR/anticancer drug resistance. RNF8 is established to play crucial roles in DDR. Loss of RNF8 function can sensitize cells to both IR and DNA damage-inducing agents, including anticancer drugs (22, 39–41). Given the important role of RNF8 in promoting the lung cancer cell EMT and its critical role in the DDR pathway, targeting RNF8 can provide two benefits simultaneously. Briefly, by targeting RNF8, we can not only suppress or eliminate the metastatic potential of cancer cells but also increase the sensitivity of cancer cells to anticancer drugs. Thus, our results further demonstrate that RNF8 is a promising target for anticancer therapy.

In summary, RNF8 promotes the EMT in lung cancer cells through the stabilization of Slug. The expression of RNF8 is upregulated in lung cancer tissues and is inversely correlated with the survival time of patients with lung cancer. These findings offer evidence for the potential of RNF8 as a therapeutic target in lung cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

J. Kuang: Conceptualization, resources, data curation, software, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing—original draft, project administration, writing—review and editing.
L. Min: Conceptualization, data curation, software, formal analysis, validation, visualization, methodology, project administration.
C. Liu: Conceptualization, data curation, software, formal analysis, investigation, visualization, writing—review and editing.
S. Chen: Resources, formal analysis, methodology.
G. Gao: Conceptualization, data curation, methodology.
J. Ma: Data curation, investigation, methodology.
W. Wu: Data curation.
L. Wu: Project administration, writing—review and editing.
L. Zhu: Conceptualization, resources, data curation, formal analysis, funding acquisition, validation, project administration, writing—review and editing.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (31870855, to L. Zhu was directly supported by the grant; 31900537, to J. Kuang was directly supported by the grant). We gratefully thanks Tao Hou who’s from department of oncology, the Second Xiangya Hospital, Central South University, for his work in collecting human lung cancer tissues.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 16, 2019; revised March 11, 2020; accepted July 22, 2020. Published first August 4, 2020.


11. De Craene B, Berx G. Regulatory networks de...
Molecular Cancer Research

RNF8 Promotes Epithelial–Mesenchymal Transition in Lung Cancer Cells via Stabilization of Slug

Jingyu Kuang, Lu Min, Chuanyang Liu, et al.


Updated version
Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-19-1211

Supplementary Material
Access the most recent supplemental material at: http://mcr.aacrjournals.org/content/suppl/2020/08/04/1541-7786.MCR-19-1211.DC1

Cited articles
This article cites 41 articles, 11 of which you can access for free at: http://mcr.aacrjournals.org/content/18/11/1638.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at: http://mcr.aacrjournals.org/content/18/11/1638.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://mcr.aacrjournals.org/content/18/11/1638. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.