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Histone Demethylase RBP2 Promotes Lung Tumorigenesis and Cancer Metastasis

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Abstract

The retinoblastoma binding protein RBP2 (KDM5A) is a histone demethylase that promotes gastric cancer cell growth and is enriched in drug-resistant lung cancer cells. In tumor-prone mice lacking the tumor suppressor gene *RB* or *MEN1*, genetic ablation of RBP2 can suppress tumor initiation, but the pathogenic breadth and mechanistic aspects of this effect relative to human tumors have not been defined. Here, we approached this question in the context of lung cancer. RBP2 was overexpressed in human lung cancer tissues where its depletion impaired cell proliferation, motility, migration, invasion, and metastasis. RBP2 oncogenicity relied on its demethylase and DNA-binding activities. RBP2 upregulated expression of cyclins D1 and E1 while suppressing the expression of cyclin-dependent kinase inhibitor p27 (CDKN1B), each contributing to RBP2-mediated cell proliferation. Expression microarray analyses revealed that RBP2 promoted expression of integrin-β1 (ITGB1), which is implicated in lung cancer metastasis. Mechanistic investigations established that RBP2 bound directly to the *p27*, *cyclin D1*, and *ITGB1* promoters and that exogenous expression of cyclin D1, cyclin E1, or ITGB1 was sufficient to rescue proliferation or migration/invasion, respectively. Taken together, our results establish an oncogenic role for RBP2 in lung tumorigenesis and progression and uncover novel RBP2 targets mediating this role. *Cancer Res; 73(15); 4711–21. ©2013 AACR.*

Introduction

Retinoblastoma binding protein-2 (RBP2) was originally identified as a retinoblastoma protein (Rb)-binding partner (1). Subsequently, it was shown to participate in transcription by interacting with TBP, p107 (2), nuclear receptors (3), Myc (4), Sin3/HDAC (5), Mad1 (6), and RBP-J (7). In 2007, RBP2 was identified as a histone demethylase belonging to the JARID family of histone demethylases, which are able to remove di- and trimethyl groups from lysine 4 of histone 3 (H3K4) depend-

ing on its JmjC domain (8, 9). Therefore, RBP2 is also called KDM5A or JARID1A. The human JARID1 protein family consists of four members including RBP2/JARID1A, PLU1/JARID1B, SMCX/JARID1C, and SMCY/JARID1D (10). These four proteins are highly conserved and redundant in demethylation activities. As trimethylation of H3K4 (H3K4me3) is often associated with promoters of actively transcribed genes (11), RBP2 is believed to exert its function partly through repressing transcription of its target genes. Nevertheless, RBP2 also activates genes with less defined mechanisms (4, 8, 9, 12, 13). Lid, a *Drosophila* homolog of RBP2, interacts with dMyc and acts as a dMyc coactivator on its target genes by dMyc-mediated masking of RBP2 demethylase domain (4). In addition, Lid potentiates an Rpd3 target gene expression by inhibiting the histone deacetylase activity of Rpd3 (14).

The link of RBP2 to abnormal cell growth is first implicated by its binding to the tumor suppressor Rb (1). It is reported that Rb promotes cell senescence and differentiation by sequestering RBP2 (15, 16). Studies using RBP2 knockout mice show that loss of RBP2 prevents tumorigenesis of Rb- or multiple endocrine neoplasia type 1 (MEN1)-deficient mice (17). The following two findings further support that RBP2 is oncogenic. First, RBP2 is found overexpressed in gastric cancer specimens and RBP2 depletion leads to cell senescence and activation of its target genes CDKIs (18). Second, RBP2 is required for the establishment of drug-tolerant lung cancer–initiating cells (19). These studies highly suggest that RBP2 plays a role in tumorigenesis. However, the direct evidence is still lacking. It is not clear whether RBP2 is required for the development of

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tumor malignancy. Nor do we understand whether other downstream target genes than CDKIs contribute to the oncogenic function of RBP2.

Combined with structural and cell-based studies, in 2008, we identified that the AT-rich interaction domain (ARID) of RBP2 can recognize specific DNA sequence CCGCCC and that the K152 is the critical residue for RBP2 to contact DNA (20). Given that reduction in global H3K4 methylation is linked to poor prognosis in patients with lung cancer (21, 22), we set up to study the role of RBP2 in lung tumorigenesis and cancer development. In this study, we show that RBP2 was overexpressed in human lung cancer tissues and required for lung cancer cell proliferation, motility, migration, invasion, and metastasis. These abilities were further shown to be regulated by the demethylase and DNA-binding activities of RBP2. Novel RBP2 downstream targets were explored in this study.

Materials and Methods

Western, Plasmids, reverse transcription and quantitative PCR (RT-qPCR), chromatin immunoprecipitation (ChIP), and DNA primers, please see Supplementary Materials and Methods.

Lung cancer patients

Twenty cancerous and the adjacent normal specimens of human non–small cell lung cancer (NSCLC) were collected from patients in Taipei Veterans General Hospital from 2003 to 2004 for RBP2 gene expression analysis. Fifteen of them are adenocarcinoma, three are large cell carcinoma, and two are either squamous cell carcinoma or pleomorphic carcinoma.

Cell culture, gene knockdown, and rescue

Human NSCLC cell line CL1-5 was established and characterized for its invasiveness by Chu and colleagues (23). Human NSCLC cell lines H1299 and A549, and human lung fibroblast cell lines HEL299, WI-38, and MRC-5 were obtained from the American Type Culture Collection. All three NSCLC and lung fibroblast cell lines were grown at 37°C with 5% CO_2 in RPMI-1640 (Sigma-Aldrich) or α-MEM (Gibco) supplemented with 10% FBS (Biological Industries), respectively. For gene knockdown, short hairpin RNA (shRNA) clones that target to RBP2 (shRBP2 KD1 and shRBP2 KD2) were delivered by lentiviral infection and siRNA that targets to p27 was transfected by Lipofectamine 2000 (Invitrogen). Cells were seeded at culture plates 24 hours before addition of lentivirus and 8 μg/mL polybrene to growth media, and then cultured for another 72 hours. For rescue experiments, 2 μg of shRNA-resistant wild-type or mutant RBP2 plasmid, integrin β1 (ITGB1)-V5, CCND1-V5 or CCNE1-HA plasmid, or 40 nmol/L of p27 siRNA was transfected into RBP2-knocked down H1299 cells for 48 hours using Lipofectamine 2000.

Cell proliferation, soft agar, cell-cycle analysis, and luciferase assay

Cells were seeded onto culture plates and assayed with a CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega) according to the manufacturer's protocols. Cell proliferation was also measured using the Real-Time

Cell Analyzer (RTCA) Dual Plate (DP) system (xCELLigence, Roche Diagnostics GmbH) and shown by cell index as defined in Roche website. The system monitors cell status using proprietary microelectronic sensor technology. Briefly, cells were seeded onto E-plate containing RPMI-1640 supplemented with 10% FBS and monitored every 30 minutes. Soft agar assay was conducted as reported previously (24). For cell-cycle analysis, cells were trypsinized, fixed with 70% ethanol, stained with 4 μg/mL propidium iodide for 30 minutes and analyzed using a flow cytometer (Canto II, BD Biosciences). Luciferase assay was conducted as reported previously (20).

Xenografic tumor formation

Seven-week-old male nonobese diabetic-severe combined immunodeficient (NOD-SCID) mice were injected subcutaneously with 1×10^6 of CL1-5 cells carrying scramble, RBP2 KD1, or RBP2 KD2 shRNA. Tumor growth and body weight were monitored twice per week. Tumor volumes were calculated by the formula $l^*w^*h/2$ (mm^3). At day 16 postimplantation, complete autopsy was conducted on each mouse.

Cell tracking in time-lapse video microscopy, wound healing, cell migration, and invasion

For cell tracking in time-lapse video microscopy, 1×10^3 CL1-5 cells mock or lentiviral infected with scramble shRNA, shRBP2 KD1, or shRBP2 KD2 were seeded onto 6-well plates for 24-hour incubation. Plates were then placed into a culture chamber with 5% CO_2 at 37°C and cells were photographed under Leica life Image System every 10 minutes for 12 hours. Cell motility was measured after collection of sequential time-lapse images. Five cells under each field were selected randomly for tracking and analysis by MetaMorph software (MDS Analytical Technologies). Wound-healing assay and Matrigel-based invasion assay were conducted as reported previously (25). Cell migration and invasion were also measured using the RTCA DP system (xCELLigence) as reported previously (26).

Tail vein injection for *in vivo* invasion

The CL1-5 luc2 cell line was developed by infecting CL1-5 cells with lentivirus carrying EF1 promoter-driven firefly luciferase gene, followed by blasticidin selection. Seven-week-old female nude mice were injected with CL1-5 luc2 carrying scramble, RBP2 KD1, or RBP2 KD2 shRNA. A total of 5×10^5 cells in 100 μL of PBS were injected intravenously via the lateral tail vein. At week 6 postinjection, all mice were euthanized and their lungs were removed. Luciferase activity of lung was measured in photos by an IVIS Spectrum Imaging System (Caliper Life Sciences). The number of surface metastases per lung was determined under dissecting microscope.

cDNA microarray

Total RNAs isolated from H1299 cells carrying scramble shRNA or shRNA targeting RBP2 (RBP2 KD1) were subjected to reverse transcription, labeling, and hybridization to Affymetrix human U133 2.0$^+$ chips according to the manufacturer's protocols. Data were analyzed as reported previously (26). Genes

up- or downregulated at least 1.5-fold (log 2) in RBP2-knocked down cells were selected and classified by GO ontology. Array data have been posted at the NCBI GEO database (GEO accession: GSE41443).

Statistical analysis

Statistical analysis was indicated in each corresponding figure legend. All data are mean ± SD from 3 independent assays. All analyses were examined using SAS program version 9.1 (SAS Institute Inc.) and SPSS. P values were calculated from two-tailed statistical tests. A difference is considered statistically significant when $P < 0.05$.

Results

RBP2 is overexpressed in human lung cancer tissues and cell lines

To investigate whether RBP2 has an oncogenic potential in lung cancer, we first analyzed its mRNA and protein levels in human lung cancer tissues by RT-qPCR and immunohistochemistry, respectively. RBP2 mRNA was elevated in 17 of 20 human lung cancerous specimens with 11 showing more than 2 fold increase of RBP2 mRNA level compared with normal controls (Fig. 1A). Immunohistochemical staining of 5 human lung cancer specimens further indicated that the level of RBP2 protein was increased in the tumor region compared with its adjacent normal tissue (Supplementary Fig. S1). Note

that the samples were diagnosed using hematoxylin and eosin (H&E) staining. These results are consistent with the oncogenic implication of RBP2 in gastric cancer (18) and in other cancers analyzed by Oncomine. In agreement with the above observations, the protein level of RBP2 was upregulated in lung cancer cell lines CL1-5, H1299, and A549, compared with normal lung fibroblasts HEL299, WI-38, and MRC-5 (Fig. 1B). Together, it is believed that RBP2 is overexpressed in human lung cancer.

RBP2 is required for lung cancer cell proliferation and xenograft tumor formation

Next, we determined whether RBP2 is necessary for the proliferation of lung cancer cells by RBP2 knockdown strategy. Figure 2A shows that RBP2 protein level was greatly reduced by 2 independent shRNA sequences RBP2 KD1 and RBP2 KD2 in lung cancer cell CL1-5. Importantly, CL1-5 cells transfected with these two RBP2 shRNAs grew significantly slower (Fig. 2B), had impaired ability to form colony in soft agar assays (Fig. 2C), and generated smaller xenograft tumors in NOD-SCID mice (Fig. 2D), indicating that RBP2 is required for proliferation, the anchorage-independent growth, and tumorigenesis potential of CL1-5 cells. Note that another lung cancer cell A549 depleted of RBP2 also showed decreased proliferation rate (Supplementary Fig. S2). Collectively, our *in vitro* and *in vivo* observations support that RBP2 functions as an oncoprotein in lung cancer.

Figure 1. RBP2 overexpression in human lung cancer tissues and cell lines. A, mRNA level of RBP2 in lung cancerous specimens and surrounding nonneoplastic stroma tissues after normalization to actin. B, Western blot analysis indicates that three lung cancer cell lines CL1-5, H1299, and A549 show increased protein level of RBP2 compared with normal lung fibroblasts HEL299, WI-38, and MRC-5. α-Tubulin served as a loading control.
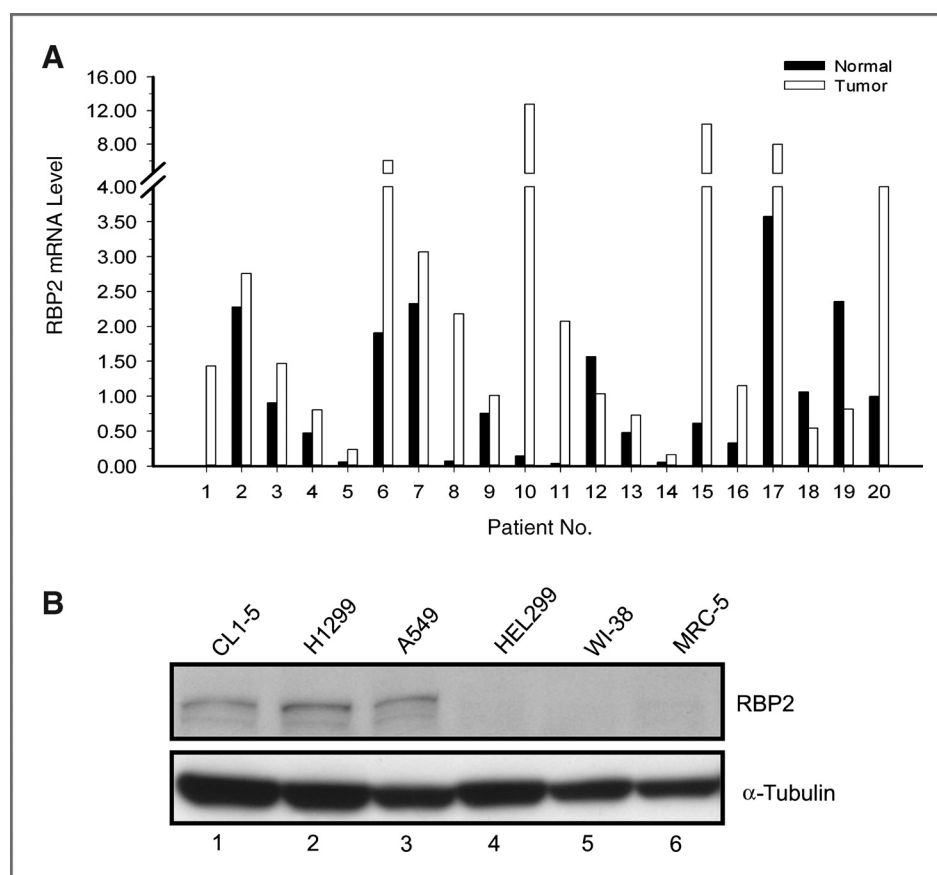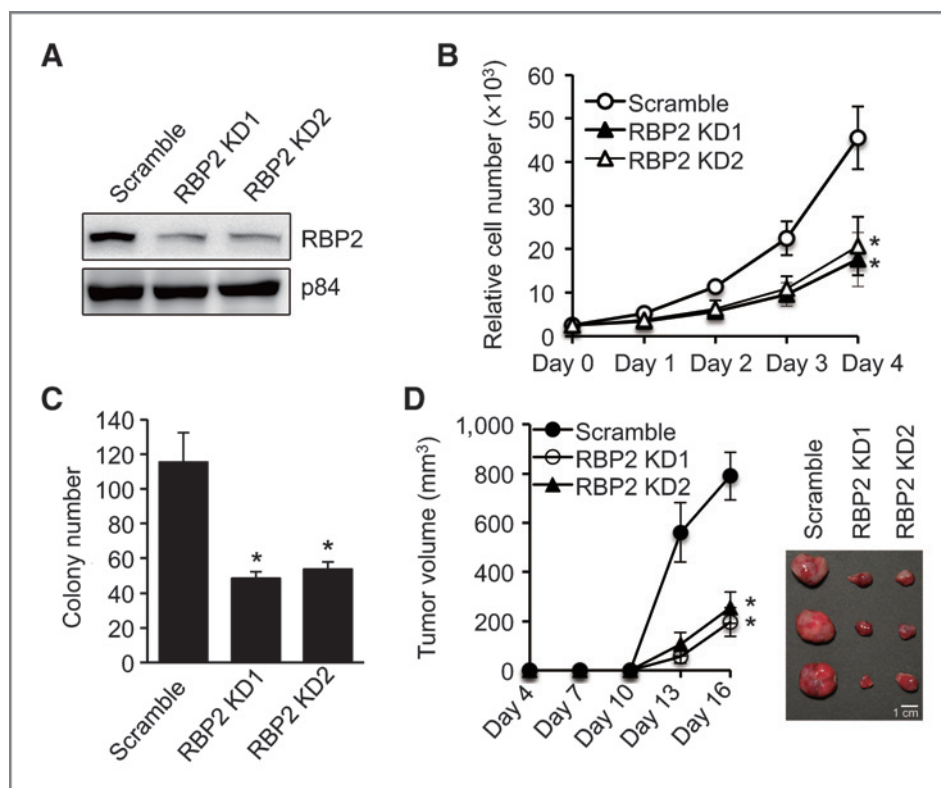
Figure 2. RBP2 depletion inhibits growth and tumorigenesis of lung cancer cell CL1-5. A, Western blot analysis showing RBP2 protein level in cells with scramble shRNA or shRBP2 KD1 or KD2. p84 served as a loading control. B, RBP2 depletion decreases cell proliferation. C, RBP2 knockdown impairs the ability of cells to form colonies on soft agar. D, cells depleted of RBP2 generate smaller tumors in NOD-SCID mice. The tumor volume was measured at the indicated time points after tumor cell implantation. Right, the image of representative tumor mass isolated at day 16 after cell implantation. A total of 5 mice were analyzed in each group, *, $P < 0.05$ versus the scramble control. The statistics were determined by Student t test in B and C and Wilcoxon rank-sum test in D.

RBP2 is necessary for lung cancer cell migration and invasion

The potential role of RBP2 in lung cancer metastasis was explored. First, we investigated whether RBP2 regulates cell motility. The cell motility/movement from single cell with or without RBP2 depletion was monitored. The result clearly showed that the total moving distance per cell was significantly shorter in cells with RBP2 knockdown (Fig. 3A, video in Supplementary Material). Consistently, wound-healing assays indicated that fewer cells migrated from the wound edge to the center space in cells with reduced levels of RBP2 (Fig. 3B). Importantly, Matrigel-based Transwell assays showed that RBP2 silencing inhibited cell invasion (Fig. 3C). As we have shown in Fig. 3A that RBP2 positively regulated cell motility, it is unlikely that the reduced migration and invasion of RBP2-depleted cells was simply due to decreased proliferation of these cells. Furthermore, we showed by tail vein injection that the number of surface lung nodules caused by CL1-5 cells metastasized to lung was decreased when RBP2 was depleted from cells (Fig. 3D and Supplementary Fig. S3A and S3B). Together, these data indicate that RBP2 likely plays an important role in lung cancer metastasis by facilitating cell motility, migration, and invasion.

The oncogenic potential of RBP2 depends on its demethylase and DNA contact activities

So far, our data support a positive role of RBP2 in lung cancer formation and progression. Given that RBP2 regulates gene expression through histone demethylation (6, 8, 9, 12, 13, 18) and DNA contact (20), we asked whether these 2 activities

contribute to the oncogenic function of RBP2. To this end, the cDNA encoding HA-tagged wild-type RBP2 or RBP2-mutant defective in demethylase activity (H483A) or DNA contact (K152E) was introduced back to H1299 cells depleted of RBP2 by siRNA (Fig. 4A and Supplementary Fig. S4). H1299 was used here for 2 reasons. First, H1299 is a lung cancer cell line with much better transfection efficiency and thus using this cell line can increase the expression of shRNA-resistant constructs of RBP2. Second, we need to show the effect observed previously in Figs. 2 and 3 is universal but not CL1-5–specific. As shown in Fig. 4A, the equal expression of the shRNA-resistant RBP2 variants was confirmed by Western blot analysis using both RBP2 and HA antibodies. The effect of the RBP2 mutations on cell proliferation (Fig. 4B), anchorage-independent growth (Fig. 4C), and invasion (Fig. 4D) was analyzed. In these three assays, wild-type RBP2 greatly restored the cancer phenotypes reduced by RBP2 depletion. Importantly, neither demethylase-dead nor DNA-binding mutant achieved the same effect. These results not only show that the RBP2 knockdown phenotypes are not off-target effects, but also indicate that the enzymatic activity and the DNA interaction ability of RBP2 are indispensable for RBP2 to function as an oncoprotein.

RBP2 promotes cell-cycle progression through activation of cyclins D1 and E1 and repression of p27

To understand how RBP2 promotes cell proliferation, we analyzed cell-cycle progression of cells with or without RBP2 depletion. As shown in Fig. 5A, RBP2 knockdown increased cell population in G_1 stage, suggesting that RBP2-deficient cells arrested at G_1. To explore the underlying mechanism, we
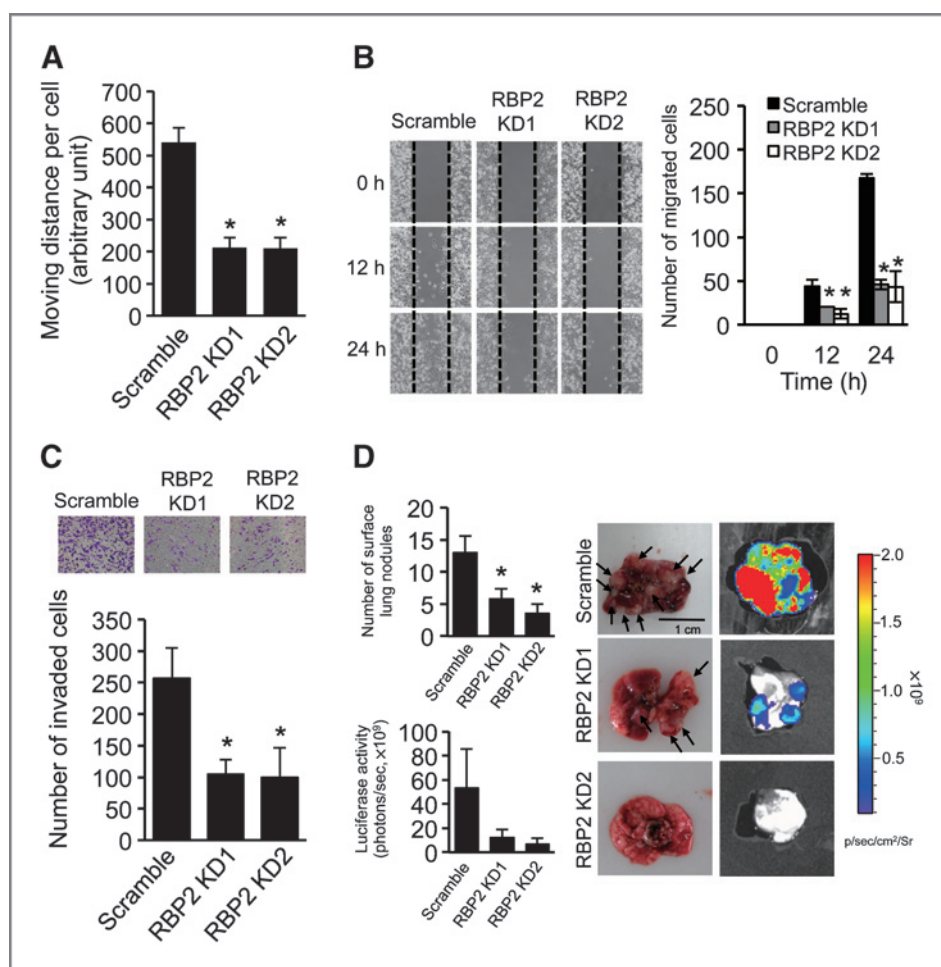
Figure 3. RBP2 knockdown inhibits CL1-5 cell motility, migration, invasion, and cancer metastasis. A, RBP2 knockdown impairs cell movement. Movement rate of individual cells was monitored using time-lapse microscopy. Median speed was calculated by tracking five cells per group. B, RBP2 knockdown reduces cell migration. The results of wound healing assays at indicated time were photographed under microscopy with ×40 magnification after the scratch (left). The numbers of migrating cells are shown in a bar chart (right panel). *, $P < 0.05$. C, RBP2 depletion in CL1-5 cells inhibits cell invasion. Cells migrating through the Matrigel-coated filter membrane were counted in three fields under a 200-fold high power field of microscopy after 24 hours of incubation (top). The numbers of invading cells are shown in a bar chart (bottom). D, RBP2 knockdown suppresses cancer metastasis. Number of surface nodules in lungs of nude mice ($n = 5$/group) 6 weeks after tail vein injection of CL1-5 cells tagged with luciferase reporter gene and with scramble shRNA or shRBP2 KD1 and KD2 is shown (left: top). *, $P < 0.05$. Luciferase activity of the lungs was quantified in the mice (left, bottom). Right, representative photographs and luciferase activity image of lungs taken 42 days after tail vein injection of cells into mice. Black arrows, nodules. Statistics were determined by Student t test.

examined whether cell-cycle genes, which are involved in G_1–S progression, are affected by RBP2. Consistent to a previous report analyzing the role of RBP2 in gastric cancer (18), p27 mRNA and protein levels were increased in lung cancer cell line upon RBP2 depletion (Fig. 5B). Thus, it is likely that p27 is a common target of RBP2 in different cancer. In sharp contrast to the same report in which p21 level is upregulated in RBP2-depleted gastric cancer cell AGS (18), p21 expression was downregulated upon RBP2 deletion in two lung cancer cell lines H1299 and CL1-5 (Supplementary Fig. S5). This suggests that RBP2 activates p21 expression in lung cancer cells. Because it is currently not clear how activation of p21, a putative tumor suppressor in cell-cycle arrest, contributes to oncogenesis, we decided to temporarily focus on p27 for the oncogenic function of RBP2. Subsequently, we found that the

mRNA and protein levels of cyclins D1 and E1 were decreased in RBP2-depleted cell H1299 (Fig. 5B), A549 and CL1-5 (Fig. S6A), suggesting that RBP2 is a positive regulator for G_1 cyclin expression. Regulation of p27, cyclins D1 and E1 by RBP2 was likely specific as RBP2 depletion did not alter the expression of cyclin-dependent kinases (CDK), such as CDK2, CDK4, and CDK6 (Fig. 5B).

To determine whether p27, cyclins D1 and E1 are direct or indirect targets of RBP2, the chromatin immunoprecipitation (ChIP) assay was applied. The results indicate that RBP2 directly associated with the p27 and cyclin D1 promoters and the association was enriched in the transcription start site (TSS) and the 5′-untranslated region (Fig. 5C, left and middle). Please note that the ChIP signals of RBP2 binding to p27 and cyclin D1 promoters were specific as the signals were reduced
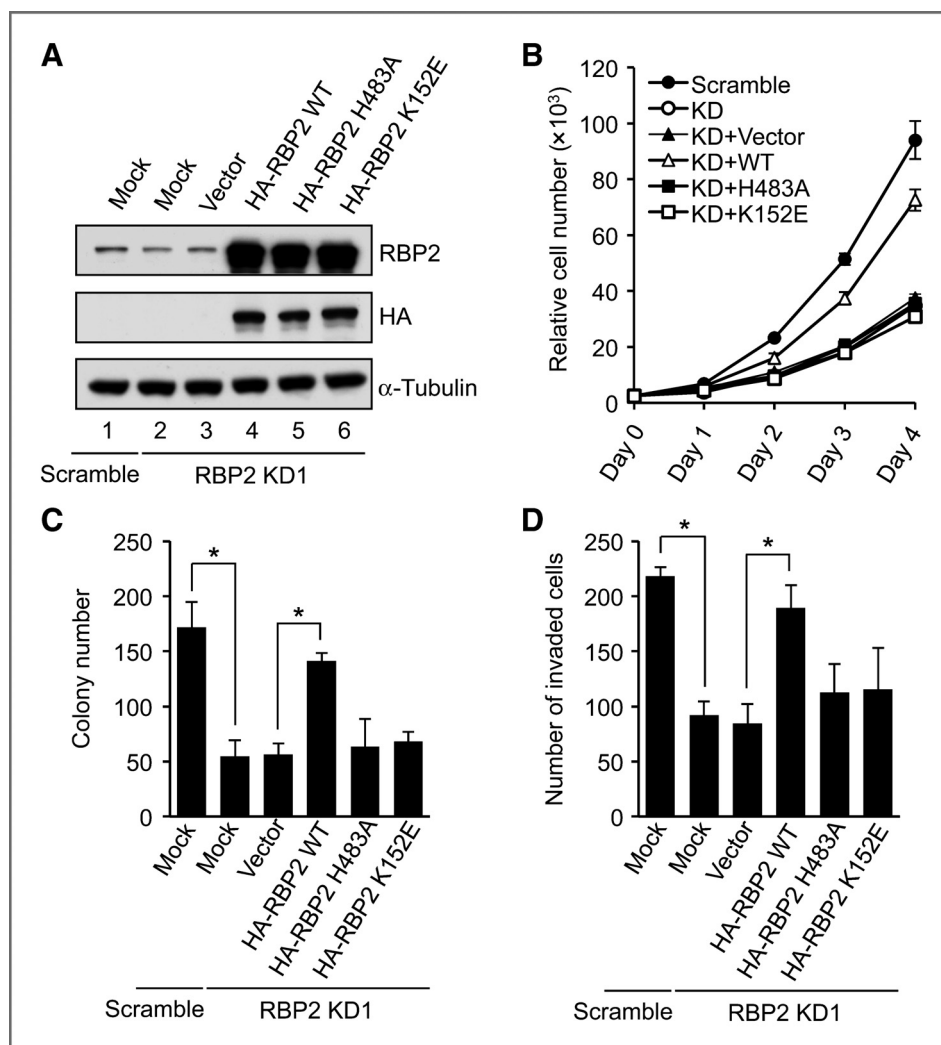
Figure 4. The oncogenic potential of RBP2 depends on the enzymatic and DNA contact activities of RBP2. A, Western blot analysis showing successful expression of shRNA-resistant HA-tagged wt and mutant RBP2 in H1299 cells depleted of RBP2 by shRNA transfection. α-Tubulin served as a loading control. B–D, shRNA-resistant wild-type, but not mutant, HA-RBP2 partially rescues cell proliferation (B), colony formation (C), and cell invasion (D). *, $P < 0.05$. The statistics were determined by Student t test.

in cells depleted of RBP2. In contrast to *p27* and *cyclin D1*, *cyclin E1* promoter did not seem to be bound by RBP2 (Fig. 5C, right), suggesting that *cyclin E1* might be a secondary target of RBP2. Importantly, the rescue experiments show that adding back cyclin D1 or E1, but not knocking down p27, partially regained the growth ability of RBP2-depleted lung cancer cells (Fig. 5D). It should be noted that neither p27 depletion nor cyclin D1 or E1 expression altered the proliferation rate of cells with scramble RNA or vector alone to a degree comparable to that in RBP2-knocked down cells (data not shown), suggesting that cyclins D1 and E1 are specific for RBP2 function. Sufficient p27 knockdown (Supplementary Fig. S6B) did not rescue RBP2 depletion effect is likely because p27 is an upstream regulator of cyclins D1 and E1. Therefore, it is not easy to reveal the rescue phenotype of p27 depletion when cyclins D1 and E1 are both downregulated in RBP2-deficient cells. As RBP2 is known to regulate promoter activity and we observed that RBP2 bound to the TSS proximal region of *p27* gene, a region with potential *p27* gene regulatory function (27), and that RBP2 depletion significantly increased the mRNA and protein levels of p27, we believe that RBP2 very likely binds to and regulates

p27 promoter. Together these results suggest that RBP2 may promote cell proliferation by directly repressing p27 and activating cyclin D1 and indirectly stimulating cyclin E1 expression.

RBP2 activates ITGB1 for increased lung cancer cell migration and invasion

To determine the downstream genes responsible for the role of RBP2 in lung tumorigenesis and metastasis, cDNA microarray analysis was conducted and the differential gene expression in H1299 with or without expressing RBP2 shRNA was compared. Supplementary Table S1 shows the RBP2-regulated genes in cell cycle/cell growth/DNA replication, apoptotic process, cell motility/cell migration/cell–matrix adhesion/cell–cell adhesion, cytoskeleton organization, and Wnt receptor signaling pathway. Among these genes, the *ITGB1* showed a great fold change and so was analyzed further for its role in RBP2-mediated cell migration and invasion. ITGB1 has been reported to mediate cell–matrix interaction (28, 29). First, we showed that, consistent to the microarray data, RBP2 depletion led to ITGB1 reduction in both mRNA
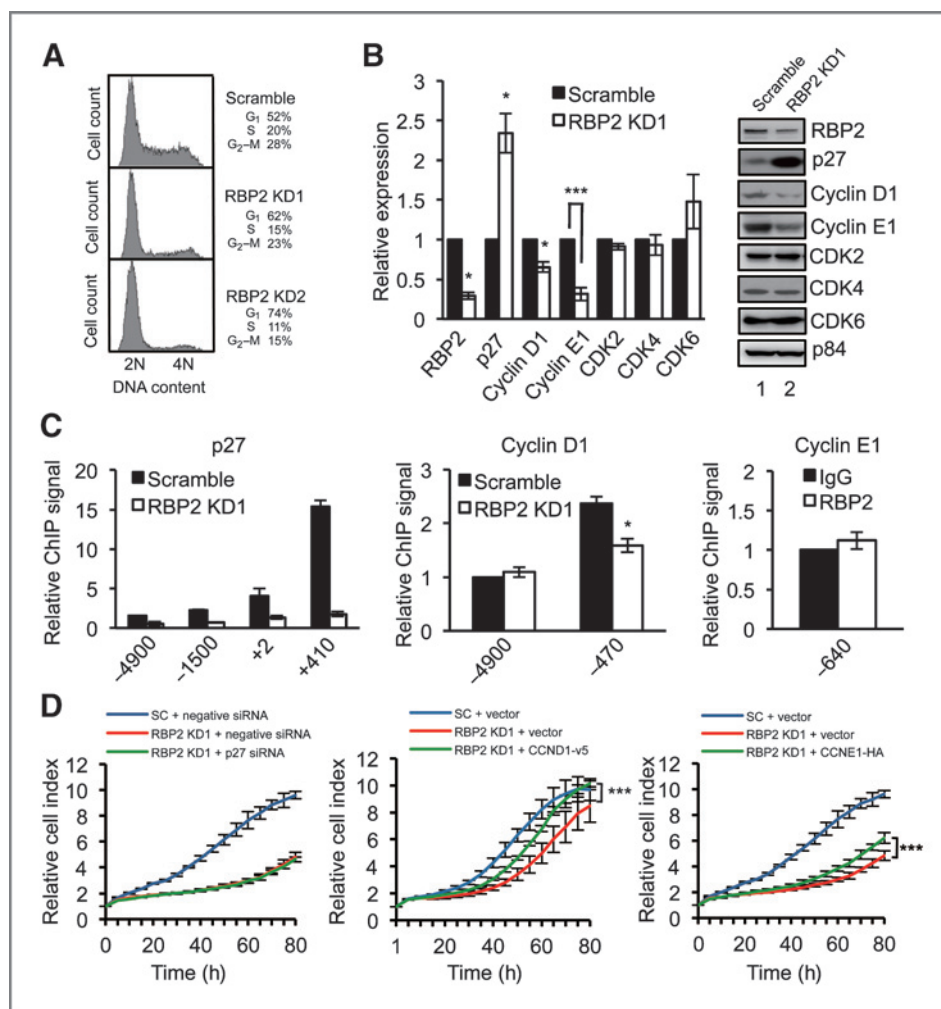
Figure 5. RBP2 knockdown increases G_1 population in lung cancer cells and alters expression of cell-cycle genes. A, RBP2-depleted CL1-5 cells exhibit an increased entry into G_1 phase of cell cycle when compared with scramble control. B, relative mRNA level of genes was analyzed by RT-qPCR and normalized to the level of actin. *, $P < 0.05$; ***, $P < 0.001$ versus the scramble control. Protein levels of indicated genes in H1299 cells with scramble shRNA or RBP2 shRNA were measured by Western blot analysis. p84 served as a loading control. C, ChIP assays show that RBP2 binds to p27 and cyclin D1, but not cyclin E1, promoters *in vivo*. H1299 cells with scramble or RBP2 knockdown were subjected to ChIP assays with RBP2 antibody (Bethyl) or rabbit IgG (Upstate) for chromatin precipitation. The numbers indicate the positions of the first 5′ nucleotide of each PCR primer used, relative to the transcription start site +1. *, $P < 0.05$ versus scramble control. D, reexpression of cyclins D1 (CCND1) and E1 (CCNE1), but not p27 knockdown, partially rescues cell proliferation decreased by RBP2 deficiency. RBP2-depleted H1299 cells were transfected with scramble RNA or p27 siRNA (left), vector or v5-tagged CCND1 (middle), or HA-tagged CCNE1 (right), followed by analysis of cell proliferation by RTCA DP (xCELLigence). The statistics were determined by Student *t* test in B and C and repeated-measure ANOVA in D.

and protein levels (Fig. 6A), indicating that RBP2 is a positive regulator for ITGB1 expression. Moreover, ChIP assays showed the enriched binding of RBP2 to the proximal promoter of *ITGB1*, but not *hepatocyte nuclear factor 4α* or *GATA1* (Fig. 6B), suggesting that RBP2 might facilitate *ITGB1* gene expression by directly binding to and regulating *ITGB1* promoter. This hypothesis was further supported by the reporter assay showing that the *ITGB1* promoter-driven luciferase activity was reduced by RBP2 depletion (Fig. 6C). Importantly, reexpression of ITGB1 significantly restored migration and invasion of RBP2-depleted H1299 cells (Fig. 6D and Supplementary Fig. S7A), but did not cause any effect in cells with scrambled control construct (data not shown). ITGB1 failed to rescue the proliferation defect of RBP2-

deficient cells either (Supplementary Fig. S7B). These results indicate that ITGB1 is a direct and specific downstream target of RBP2, likely playing a critical role in RBP2-mediated cancer metastasis.

Discussion

Our current data indicate that the histone demethylase RBP2 is an oncoprotein overexpressed in lung cancer to promote cell proliferation, motility, migration, invasion, and metastasis. Because the oncogenic function of RBP2 could be observed in three different lung cancer cell lines CL1-5, H1299, and A549, we believe that the effect is not cell type-specific. We also uncovered that RBP2 repressed *p27* gene expression,
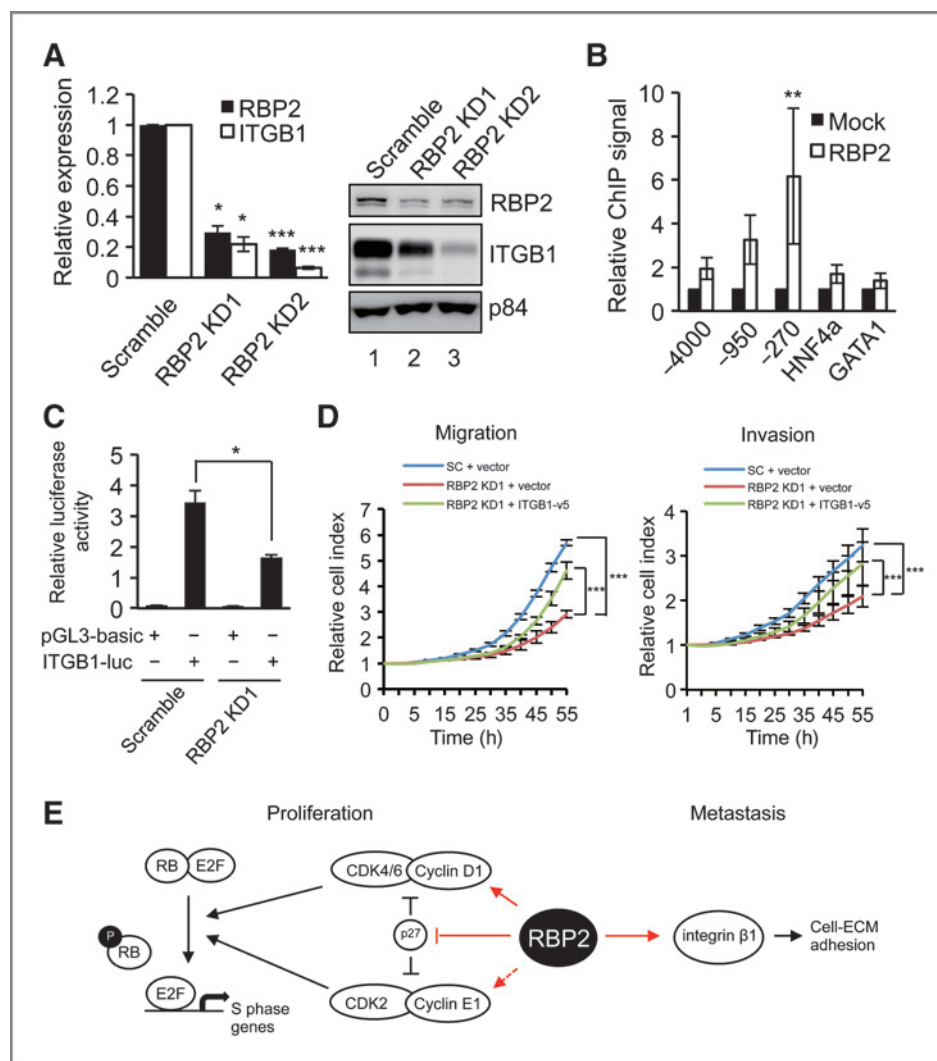
Figure 6. ITGB1 partially accounts for RBP2-mediated cell migration and invasion. A, RBP2 depletion reduces *ITGB1* gene expression. Relative mRNA (left) and protein (right) levels of RBP2 and ITGB1 were measured in H1299 cells with scramble shRNA or RBP2 shRNAs. The mRNA levels of RBP2 and ITGB1 were normalized to the level of actin. p84 served as a loading control for Western blot analysis. *, $P < 0.05$; ***, $P < 0.001$ versus scramble control. B, ChIP assays show that RBP2 binds to ITGB1 promoter. The chromatin from H1299 cells was incubated with RBP2 or HA antibody, followed by ChIP assays. RBP2 ChIP results were normalized to the level of HA antibody (Santa Cruz, sc-805). The numbers ($-4,000$, -950, and -270) indicate the positions of the first 5' nucleotide of each PCR primers used. **, $P < 0.01$. C, RBP2 deficiency reduces ITGB1 promoter activity. The ITGB1 promoter ($-979/+7$ bp) reporter and RL-SV40 plasmid were cotransfected into H1299 cells, followed by luciferase activity assay. The relative firefly luciferase activity was normalized to the *Renilla* activity. D, exogenous ITGB1 expression restores cell migration and invasion reduced by RBP2 depletion. RBP2-depleted H1299 cells were transfected with vector alone or plasmid expressing ITGB1-v5, followed by analysis of cell migration and invasion by RTCA DP. The statistics were determined by Student *t* test in A–C and repeated-measure ANOVA in D. E, a summary diagram of RBP2-regulated genes for lung tumorigenesis and metastasis. Previous studies have indicated that cyclins/CDKs form complexes that potentiate RB phosphorylation, freeing E2F to activate genes that promote S-phase progression. The cyclin-dependent kinase inhibitor p27 is able to block the activities of cyclin/CDK complexes and therefore stop cell-cycle progression. In our study, RBP2 was found to increase cyclin D1 but reduce p27 expression via direct binding to these two genes. Moreover, RBP2 indirectly upregulates cyclin E1 gene expression. In summary, RBP2 likely enhances cell growth through upregulation of specific cyclins and downregulation of specific CDK inhibitors. For metastasis, RBP2 directly binds to ITGB1 gene promoter and increases ITGB1 expression. ITGB1 is a membrane receptor that, together with specific integrin α subunit, mediates the interaction of cells and extracellular matrix (ECM). Solid and dotted red lines/arrows indicate that RBP2 directly or indirectly binds to these genes, respectively.

whereas activated cyclin D1/E1 and integrin β1 (ITGB1) mRNA synthesis in lung cancer cells. The former (p27/cyclins) and latter (ITGB1) potentially contribute to RBP2-mediated cell growth and migration/invasion/metastasis, respectively (Fig. 6E). Consistent with our finding that the oncogenic function of RBP2 requires its CCGCCC contact ability (Fig. 4), we found that *p27*, *cyclin D1*, and *ITGB1* promoters all contain CCGCCC and similar sequences (data not shown).

In this study, we conducted cDNA microarray analysis in H1299 cells with or without RBP2 depletion. The expression of a total of 81 genes was altered with at least 1.5-fold change (log 2) upon RBP2 knockdown. Among these genes, 30 genes were

upregulated and 51 downregulated. In Supplementary Table S1, we show the microarray-identified genes participating in tumorigenesis and metastasis. Indeed, most other genes regulated by RBP2 (not shown here) are involved in development and differentiation, consistent with previous studies that indicate that RBP2 plays an important role in differentiation (10, 13, 15).

Our data in Figs. 5C and 6B show that RBP2 preferentially bound to the proximal promoter regions of *p27, cyclin D1*, and *ITGB1*. Genome-wide location of RBP2 has been studied in several different cell types (5, 12, 13, 30). After analyzing these published data, it was found that, only in the mouse myoblast cell C2C12 (5), *p27* and *cyclin D1* are identified as RBP2 target genes and RBP2 binds to TSS region and the proximal downstream region to TSS of *p27* gene. In contrast, the study did not show the weak RBP2 binding to the −1,500 bp upstream distal region of *p27* gene as observed in our study (Fig. 5C). In addition, the pattern of RBP2 binding to cyclin D1 gene in C2C12 cells is very different from our observation in lung cancer cells. In C2C12 cells, RBP2 associates with almost all regions examined (−1,134–+1,146 bp) except proximal regions to TSS (−436–+805 bp). However, we show that RBP2 bound to −470 bp region (Fig. 5C). These discrepancies can be explained by several reasons including cell type specificity, sequence conservation of mouse and human genes and variation in methodology, for example, different antibody and chromatin fragmentation length, etc.

In our study, RBP2 was found to act as a repressor for p27 expression but an activator for cyclin D1 and ITGB1 through direct binding to promoters of these genes. As mentioned in Introduction, because H3K4me3 is an activation epigenetic mark (11), RBP2 is believed to exert its function as a transcriptional repressor partly through removing the trimethyl groups from H3K4 of its target genes. This mechanism very likely contributes to RBP2-mediated suppression of p27 expression. As for the activator role of RBP2, although still unclear, *Drosophila* homolog of RBP2 is reported to activate gene transcription via dMyc-mediated masking of RBP2 demethylase domain (4) or by inhibiting the histone deacetylase activity of Rpd3 (14). Interestingly, *cyclin D1* and *ITGB1* promoters both contain E-box elements, potential binding sites for Myc proteins. Whether similar mechanisms used by *Drosophila* RBP2 accounts for mammalian RBP2-mediated activation of cyclin D1 and ITGB1 is an intriguing question.

Integrins, a group of glycoproteins that consist of α- and β-subunits and their receptors, mediate the cell–extracellular matrix interaction, the first step of metastasis (31). It is reported that increased expression of integrins α5, β1, and β3 correlates with poor survival of patients with early NSCLC (32). Importantly, integrin β1 is upregulated in gefitinib-resistant NSCLC cell line PC9/AB2 for enhanced cell adhesion and migration and reduction of integrin β1 restores sensitivity of PC9/AB2 cells to gefitinib (33). Our finding that RBP2 directly activates integrin β1 expression further suggests that RBP2 is an important factor to mediate cancer metastasis and drug resistance. Although ITGB1 is also implicated in cell proliferation (34–36), in our study, ITGB1 could not restore

the proliferation defect of RBP2-deficient cells (Supplementary Fig. S7B). It suggests that ITGB1 does not play a role in RBP2-mediated H1299 cell growth. Indeed, a previous study indicated that integrin β1 is not required for the proliferation of vulval squamous cell carcinoma cells but is important for cell invasion (37).

How RBP2 is upregulated in lung cancer is an intriguing question. It should be noted that RBP2 has been shown enriched in the drug-tolerant lung cancer cells challenged by anticancer drug EGF receptor tyrosine kinase inhibitor gefitinib (19). However, this cannot explain RBP2 overexpression in the patients with lung cancer analyzed in the current study as these patients did not receive prior chemotherapy. Analysis of the RBP2 promoter indicates that the transcription factor ELK1 is likely involved in RBP2 overexpression in lung cancer cells. We generated reporter plasmids containing RBP2 promoter from upstream 1.5 Kb to TSS or its serially deleted mutants. It was found that the DNA region from −250 to −41 bp (relative to TSS) is the minimal element to activate RBP2 promoter activity. Using site-directed mutagenesis and reporter assay, our unpublished data indicate that ELK1-binding site is important for RBP2 transcriptional activity. Importantly, mRNA level of ELK1 is upregulated in lung cancer analyzed in Oncomine. Whether ELK1 is the key factor leading to RBP2 overexpression in lung cancer is currently under investigation.

Increasing evidence show that the expression of specific histone methyltransferases and demethylases is elevated in several human cancers and their expression participates in cell proliferation and senescence regulation (38–41). Indeed, misregulation of tumor suppressor genes and oncogenes by histone methyltransferases and demethylases has been shown to associate with various human cancers (42, 43). For example, PLU1/JARID1B is overexpressed in breast, prostate, and lung cancers (40, 41, 44, 45) and is involved in silencing tumor suppressor genes such as 14-3-3-σ, CAV1, HOXA5, and BRCA1 (41). These and our current study highly support the importance of epigenetic regulation in cancer.

Lung cancer, especially non–small cell lung cancer, remains to be a leading cause of cancer-related deaths worldwide. Development of efficient therapies against lung cancer is urgent. Our observation that the oncogenic function of RBP2 depends on the demethylase and DNA-binding activities of RBP2, suggesting that inhibiting these 2 activities of RBP2 may offer a novel strategy to combat the disease. Importantly, compromising RBP2 activity will unlikely induce normal cell death as Klose and colleagues have shown that RBP2 knockout mice appear to be grossly normal (9). Together, these studies indicate that RBP2 is a potential anti-lung cancer target.

Disclosure of Potential Conflicts of Interest

Authors' Contributions

Conception and design: Y.-C. Teng, M.-D. Tsai, L.-J. Juan
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