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-ß1 (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.
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 (HDMs) which are able to remove di- and tri-methyl groups from lysine 4 of histone 3 (H3K4) depending 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. Since tri-methylation 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 co-activator 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 demonstrate 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 tumor malignancy. Nor do we understand if 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 over-expressed in human lung cancer tissues and required for lung cancer cell proliferation, motility, migration, invasion and metastasis. These abilities were further demonstrated 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, RT-qPCR, 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 et al (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% CO₂ in RPMI-1640 (Sigma-Aldrich) or α-MEM (Gibco) supplemented with 10% FBS (Biological Industries), respectively. For gene knockdown, 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 h before addition of lentivirus and 8 μg/ml polybrene to growth media, and then cultured for another 72 h. For rescue experiments, 2 μg of shRNA-resistant wild-type or mutant RBP2 plasmids, ITGB1- V5, CCND1-V5 or CCNE1-HA plasmid, or 40 nM of p27 siRNA was transfected into RBP2-knockdown H1299 cells for 48 h 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 min. Soft agar assay was performed as reported previously (24). For cell cycle analysis, cells were trypsinized, fixed with 70% ethanol, stained with 4 μg/ml propidium iodide for 30 min and analyzed using a flow cytometer (Canto II, BD Biosciences). Luciferase assay was performed as reported previously (20).

Xenografic tumor formation. Seven week-old male 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 post implantation, complete autopsy was performed 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 h incubation. Plates were then placed into a culture chamber with 5% CO₂ at 37 °C and cells were photographed under Leica life Image.
System every 10 minutes for 12 h. 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 performed 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. 5 × 10^5 cells in 100 µl of PBS were injected intravenously via the lateral tail vein. At week 6 post injection, 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 to 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 down-regulated 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 IHC, respectively. RBP2 mRNA was elevated in seventeen out of twenty human lung cancerous specimens with 11 showing greater than 2 fold increase of RBP2 mRNA level compared to normal controls (Fig. 1A). IHC staining of 5 human lung cancer specimens further indicated that the level of RBP2 protein was increased in the tumor region compared to its adjacent normal tissue (Fig. S1). Note that the samples were diagnosed using 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 up-regulated in lung cancer cell lines CL1-5, H1299 and A549, compared to normal lung fibroblasts HEL299, WI38 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 two 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 (Fig. S2). Collectively, our in vitro and in vivo observations support that RBP2 functions as an oncoprotein in lung cancer.

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 demonstrated that RBP2 silencing inhibited cell invasion (Fig. 3C). Since we have shown in Figure 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 Figs. 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 if these two 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 Fig. S4). H1299 was used here for two 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 demonstrate the effect observed previously in figures 2 and 3 is universal but not CL1-5-specific. As shown in Figure 4A, the equal expression of the shRNA-resistant RBP2 variants was confirmed by western using both RBP2 and HA Abs. 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 demonstrate 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 Figure 5A, RBP2 knockdown increased cell population in G1 stage, suggesting that RBP2-deficient cells arrested at G1. To explore the underlying mechanism, we examined if cell cycle genes, which are involved in G1-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 (Fig. S5). This suggests that RBP2 activates p21 expression in lung cancer cells. Since currently it is 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 G1 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 (CDKs), 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 UTR (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 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 (Fig. S6B) did not rescue RBP2-depletion effect is likely because p27 is an upstream regulator of cyclins D1 and E1. Therefore, it’s not easy to reveal the rescue phenotype of p27 depletion when cyclins D1 and E1 are both downregulated in RBP2-deficient cells. Since 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 might promote cell proliferation by directly repressing p27 and activating cyclin D1 and indirectly stimulating cyclin E1 expression.

RBP2 activates integrin β1 (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 performed and the differential gene expression in H1299 with or without expressing RBP2 shRNA was compared. 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 integrin β1 showed the biggest 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 demonstrated that, consistent to the microarray data, RBP2 depletion led to ITGB1 reduction in both mRNA 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 demonstrating that the ITGB1 promoter-driven luciferase activity was reduced by RBP2 depletion (Fig. 6C). Importantly, re-expression of ITGB1 significantly restored migration and invasion of RBP2-depleted H1299 cells (Fig. 6D and 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 (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. Since 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, while 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 performed 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 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 which indicate that RBP2 plays an important role in differentiation (10, 13, 15).

Our data in Figures 5C and 6B demonstrate 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). Analysis of 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 -1500 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 (-1134 to +1146 bp) except proximal regions to TSS (-436 to +805 bp). However we demonstrate 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, ex, different Ab 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, since H3K4me3 is an activation epigenetic mark (11), RBP2 is believed to exert its function as a transcriptional repressor partly through removing the tri-methyl 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 homologue 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 which consist of α- and β-subunit, and their receptors mediate the alternation of 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 early NSCLC patients (32). Importantly, integrin β1 is up-regulated 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 (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 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 anti-cancer drug EGFR TKI gefitinib (19). However this cannot explain RBP2 overexpression in the lung cancer patients 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 up-regulated in lung cancer analyzed in Oncomine. Whether ELK1 is the key factor leading to RBP2 overexpression in lung cancer is currently under investigation.

Increasing evidences 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, mis-regulation 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 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 two activities of RBP2 may offer a novel strategy to combat the disease. Importantly, compromising RBP2 activity will unlikely induce normal cell death as Klose et al. has demonstrated that RBP2 knockout mice appear to be grossly normal (9). Together
these studies indicate that RBP2 is a potential anti-lung cancer target.

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Figure legends

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 indicates that three lung cancer cell lines CL1-5, H1299, and A549 show increased protein level of RBP2 compared to normal lung fibroblasts HEL299, WI-38, and MRC-5. α-tubulin serves as a loading control.

Figure 2. RBP2 depletion inhibits growth and tumorigenesis of lung cancer cell CL1-5. A, Western showing RBP2 protein level in cells with scramble shRNA or shRBP2 KD1 or KD2. p84 is 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. The image of representative tumor mass isolated at 16 days post cell implantation is shown at right panel. A total of 5 mice were analyzed in each group, p < 0.05 vs. the scramble control. The statistics was determined by student’s t-test in B and C and Wilcoxon rank-sum test in D.

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 X magnification after the scratch (left panel). The numbers
of migrating cells are shown with a bar chart at 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 h of incubation (upper panel). The numbers of
invading cells are shown with a bar chart (lower panel). D, RBP2 knockdown
suppresses cancer metastasis. Number of surface nodules in lungs of nude mice (n=5
per group) six 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:
upper panel). *, p < 0.05. Luciferase activity of the lungs was quantified in the mice
(left: bottom panel). Representative photographs and luciferase activity image of
lungs taken 42 days after tail vein injection of cells into mice are shown at the right
panel. Black arrows indicate nodules. Statistics were determined by student’s t-test.

Figure 4. The oncogenic potential of RBP2 depends on the enzymatic and DNA
contact activities of RBP2. A, Western blots showing successful expression of
shRNA-resistant HA-tagged wt and mutant RBP2 in H1299 cells depleted of RBP2
by shRNA transfection. α-tubulin serves as a loading control. B to 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’s t-test.

Figure 5. RBP2 knockdown increases G1 population in lung cancer cells and
alters expression of cell cycle genes. A, RBP2-depleted CL1-5 cells exhibit an
increased entry into G1 phase of cell cycle when compared to 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 vs. the scramble control. Protein levels of indicated genes in H1299 cells with scramble shRNA or RBP2 shRNA were measured by western. p84 serves 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 vs. scramble control. D, Re-expression 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’s t-test in B and C and repeated-measure ANOVA in D.

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 serves as a loading control for western. *, p < 0.05; ***, p < 0.001 vs. 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 Ab (Santa Cruz, sc-805). The numbers (-4000, -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 co-transfected into H1299 cells, followed by luciferase activity assay. The relative firefly luciferase activity was normalized to the Renilla activity. The statistics was determined by student’s t-test. 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’s t-test in A, B and 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 which potentiate RB phosphorylation, freeing E2F to activate genes which 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 up-regulates cyclin E1 gene expression. In summary, RBP2 likely enhances cell growth through up-regulation of specific cyclins and down-regulation of specific CDK inhibitors. For metastasis, RBP2 directly binds to integrin β1 (ITGB1) gene promoter and increases ITGB1 expression. ITGB1 is a membrane receptor which, 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.
Figure 1

A

![Graph showing RBP2 mRNA level compared to patient number, with bars for normal and tumor samples.]

B

![Western blot images showing RBP2 and α-tubulin expression in different cell lines.]

- CL1-5
- H1299
- A549
- HEL299
- WI-38
- MRC-5

RBP2
α-tubulin
Figure 2

A

Scramble  RBP2 KD1  RBP2 KD2  

RBP2  
p84  

B

Relative cell number (×10^3)

Scramble  Δ RBP2 KD1  Δ RBP2 KD2

Day 0  Day 1  Day 2  Day 3  Day 4

C

Colony number

Scramble  RBP2 KD1  RBP2 KD2  

*  *

D

Tumor volume (mm^3)

Scramble  Δ RBP2 KD1  Δ RBP2 KD2

Day 4  Day 7  Day 10  Day 13  Day 16

1 cm

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Figure 3

A

Moving distance per cell (arbitrary unit)

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B

Number of migrated cells

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C

Number of invaded cells

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D

Luciferase activity (photons/sec x 10^5)

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Figure 5

A

Cell count

DNA content

Cell count

2N  4N

Scramble
G1 52%
S 20%
G2/M 28%

RBP2 KD1
G1 62%
S 15%
G2/M 23%

RBP2 KD2
G1 74%
S 11%
G2/M 15%

B

Relative expression

Scramble
RBP2 KD1

*  
***  

RBP2
p27
Cyclin D1
Cyclin E1
CDK2
CDK4
CDK6
p84

1 2

C

Relative ChIP signal

-4900 -1500 +2 +410

Scramble
RBP2 KD1

p27

Cyclin D1

Cyclin E1

D

Relative cell index

Time (hour)

SC + negative siRNA
RBP2 KD1 + negative siRNA
RBP2 KD1 + p27 siRNA
SC + vector
RBP2 KD1 + vector
RBP2 KD1 + CCND1-v5
SC + vector
RBP2 KD1 + vector
RBP2 KD1 + CCNE1-HA

**  
***  

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Figure 6

A

Relative expression

Scramble RBP2 KD1 RBP2 KD2

RBP2 ITGB1

Scramble RBP2 KD1 RBP2 KD2

RBP2 ITGB1 p84

B

Relative ChIP signal

-4000 -950 270 HNF4a GATA1

Mock RBP2

C

Relative luciferase activity

pGL3-basic ITGB1-luc

Scramble RBP2 KD1

D

Migration

SC + vector RBP2 KD1 + vector RBP2 KD1 + ITGB1-v5

Time (hour)

Relative cell index

E

Proliferation Metastasis

RB E2F CDK4/6 Cyclin D1

RB p27 RBP2 integrin β1

E2F S phase genes CDK2 Cyclin E1

Cell-ECM adhesion

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Histone Demethylase RBP2 Promotes Lung Tumorigenesis and Cancer Metastasis

Yu-Ching Teng, Cheng-Feng Lee, Ying-Shiuan Li, et al.

Cancer Res  Published OnlineFirst May 30, 2013.