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RYBP predicts survival of patients with non-small cell lung cancer and regulates tumor cell growth and the response to chemotherapy

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Abstract

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Conflict of interest

We do not have any conflict of interest.

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Ring1 and YY1 binding protein (RYBP) is a member of Polycomb group (PcG) proteins and regulates cell growth through both PcG-dependent and -independent mechanisms. Our initial study indicated that RYBP is down-regulated in human non-small cell lung cancer (NSCLC) tissues. The present study determined the molecular role of RYBP in the development of NSCLC. We systemically investigated the association between the RYBP expression and the survival of patients with NSCLC. We also carried out *in vitro* and *in vivo* studies to explore the molecular basis for the tumor suppressing role of RYBP in NSCLC. Our clinical results demonstrated that the RYBP mRNA and protein expressions were significantly down-regulated in NSCLC and significantly linked to the poor prognosis in NSCLC patients. The enforced expression of RYBP inhibited cell survival, induced apoptosis, and increase chemosensitivity in NSCLC cells; knockdown of RYBP showed the opposite effects. Moreover, adenoviral delivery of RYBP sensitized the NSCLC cells to chemotherapy *in vivo*. In addition, RYBP expression was induced by paclitaxel, the first-line chemotherapeutic agent for NSCLC. Our results reveal that RYBP inhibits the aggressiveness of NSCLC cells and downregulation of RYBP is associated with poor prognosis, suggesting that RYBP could be developed as a biomarker and a novel target for therapy in patients with lung cancer.

Keywords

RYBP; Apoptosis; Chemotherapy; Non-small cell lung cancer; Patient survival

1. Introduction

Lung cancer is one of the most diagnosed human malignancies and leading cause of cancer-related deaths worldwide [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers, responds poorly to therapy, and resists chemotherapy [2]. Despite recent progress made in the diagnosis and treatment of NSCLC, the 5-year survival rate remains poor (~15%) [1]. Therefore, there is an urgent need for a better understanding the pathogenesis of NSCLC, identifying prognostic factors, and exploring novel therapeutic targets.

The polycomb group (PcG) genes, originally identified as developmental regulators of body segmentation in *Drosophila melanogaster*, have been shown to be crucial for many biological processes, including development and differentiation in mammals [3–5]. PcG proteins assemble in multi-subunit nuclear complexes that have various biochemical functions, including the recognition and modification of histone posttranslational modifications and chromatin compaction [3–5]. There are two major polycomb repressive complexes, PRC1 and PRC2 [4, 5]. RING1 and YY1-binding protein (RYBP) is a newly identified member of PRC1 [6]; it binds to PcG members RING1 [7] and YY1 [8, 9] and represses gene transcription in both *Drosophila* [10] and mammalian cells [9].

Recently, RYBP has been found to have PcG-independent functions. It binds to several apoptotic mediators and induces apoptosis [11–13]. Enforced expression of RYBP inhibits the growth of cancer cells, but has minimal effects on non-transformed cells [14]. Additionally, RYBP has been suggested to act as a negative regulator of cell invasion [15]. More recently, we have demonstrated that RYBP activates tumor suppressor p53 through

blocking MDM2-mediated p53 ubiquitination and degradation [16]. Overexpression of RYBP inhibits cancer cell growth and enhances the sensitivity of cells to chemotherapy [17]. These results implicate that RYBP may be critical in cancer development, progression and therapy.

There is an increasing interest in exploring PcG in human cancer diagnosis and treatment. Aberrant expression, missense mutation, and chromosomal translocation of multiple PcG genes have been identified in various human cancer types [3–5, 18], suggesting that PcG deregulation may promote cancer development and progression through genetic and epigenetic mechanisms. In our initial study, we found that the RYBP level is reduced in human lung and liver cancer tissues compared to the paired non-cancerous tissues [16, 17]. However, the potential role of RYBP in NSCLC prognosis is unknown. The present study was designed to demonstrate the relationship between the RYBP expressions at both mRNA and protein levels and the survival of patients with NSCLC. To explore the molecular mechanisms by which RYBP regulates cancer cell survival and sensitivity to chemotherapy, we used *in vitro* and *in vivo* human NSCLC models to determine the effects of RYBP expression and knockdown on cancer cell growth and progression. It is hoped that these investigations would provide a basis for further development of RYBP as in prognostic biomarker and a valid therapeutic target in NSCLC.

2. Materials and Methods

2.1. Patients and tissue specimens

Archived tissue samples were obtained from NSCLC patients who underwent pulmonary surgery between April 1, 2006 and June 30, 2011 at the Tianjin Medical University Cancer Institute and Hospital, Tianjin, China. The study was approved by the Medical Ethics Committee of Tianjin Medical University and a written informed consent was obtained from each of the patients. Patient follow-up information was extracted by professional census enumerator according to a uniform guideline. The clinicopathological variables used in the present study are listed in Table 1. Further details can be found in the Supplementary Methods.

2.2. Tissue microarray and immunohistochemistry (IHC)

Tissue microarrays were produced as described previously [17]. All NSCLC cases were histologically reviewed by HE staining, and representative tumor areas were premarked in the paraffin blocks, away from necrotic and hemorrhagic materials. The immunohistochemical staining of serial TMAs was carried out as described previously [17]. The details and quantification of the RYBP expression are provided in the Supplementary Methods.

2.3. Chemicals, plasmids, siRNA, reagents, and cell lines

All chemicals and solvents were of analytical grade. Antibodies, plasmids, and siRNAs were obtained commercially or were generated by our lab. Human NSCLC cell lines A549, H1299, H358 and H838 were obtained from American Type Culture Collection (Manassas, VA). Normal human lung epithelial cell line NL-20 was a gift from Dr. Yi Sun (University

of Michigan Health System, Ann Arbor, MI). Further details can be found in the Supplementary Methods.

2.4. Adenovirus construction, generation, purification and viral titers determination

The pAd-Easy1 adenovirus system was kindly provided by Dr. T-C He (University of Chicago). RYBP was subcloned into the pAd-track vector and then recombined with BJ5183/pAd-Easy1 competent cells by using calcium transformation method [17, 19]. The dilution which have 10–20% GFP-positive cell was used for titer calculation [17]. More details can be found in the Supplementary Methods.

2.5. Cell viability, colony formation, and apoptosis assays

Cells were transfected plasmids or infected with AdRYBP or exposed to various concentrations of Paclitaxel, and cell viability, colony formation, and apoptosis assays were performed as described previously [17, 20–23].

2.6. Western blotting analysis and Real-time reverse transcription PCR

The protein and mRNA expression of RYBP and related molecules was analyzed by Western blotting and real-time quantitative PCR, respectively [16, 17, 20–23]. Further details can be found in the Supplementary Methods.

2.7. Mouse xenograft model of NSCLC and treatment

The animal protocol was approved by the Institutional Animal Use and Care Committee (IACUC) of the Texas Tech University Health Sciences Center. The A549 and H1299 xenograft models were established as described previously [17, 20–23]. The treatment of the animals with AdRYBP and conventional chemotherapy, the evaluation of their tumor growth and clinical status, and the tissue pathology studies are detailed in the Supplemental Methods.

2.8. Hematoxylin and Eosin (H&E) Staining and TUNEL assay

The HE staining was performed as described previously [17, 22, 23]. *In vivo* apoptosis was measured by TUNEL staining [17, 22, 23]. All sections were analyzed under a phase-contrast Olympus microscope (Olympus America Inc, Central Valley, PA). Further details can be found in the Supplementary Methods.

2.9. Statistical analyses

All clinical statistical analyses were performed with the SPSS 18.0 software program for Windows (IBM). The value for mRNA expression level were calculated based on the formula of $\log_{10} 2^{-Ct}$. Paired-Samples T-Test or Wilcoxon rank test was used to examine the differences in RYBP mRNA or protein levels. Kaplan-Meier method and log-rank test were used for survival analysis. Cox's Proportional Hazard Model was used to estimate the relationship between mRNA or protein expression value and patients survival in univariate and multivariate analyses. All experimental data were analyzed using Prism software version 6 (Graph Pad software Inc., San Diego, CA, USA). The Student *t* test was used for comparisons between two groups. The quantitative data are reported as means \pm SEM from

at least three independent experiments. Differences were considered statistically significant at $P < 0.05$. All statistical tests were two-sided.

3. Results

3.1. RYBP is downregulated in NSCLC tissues

To determine the association of RYBP expression in NSCLC with disease outcome, we examined the relative *RYBP* mRNA levels in 100 pairs of human NSCLC tissue samples by quantitative real-time PCR. Our results showed that *RYBP* mRNA expression was significantly down-regulated (5.06 ± 0.44) in NSCLC specimens, as compared to that of corresponding adjacent normal lung tissues (5.58 ± 0.31) ($P < 0.001$; Figure 1A). To further investigate if the protein level of RYBP is associated with NSCLC, 58 pairs of human NSCLC tissue samples were examined by Western blot. We found that 38 of 58 (65.5 %) tumor samples had lower level than that in adjacent normal lung tissues ($p < 0.05$, Figure 1B). In addition, TMAs from 64 patients with NSCLC were also examined by IHC. As shown in Figure 1C, RYBP protein was positive in 41 cases (64 %) of tumor tissues, and 60 cases (94 %) of corresponding adjacent normal lung tissues. The RYBP protein level was significantly decreased in NSCLC compared with their corresponding adjacent normal lung tissues ($P < 0.001$). Moreover, Spearman's rank correlation analysis showed that the *RYBP* mRNA expression was positively associated with RYBP protein level detected by Western blotting ($R = 0.274$, $P = 0.038$, Figure 1D left) and IHC ($R = 0.191$, $P = 0.008$, Figure 1D right), samples with down-regulated *RYBP* mRNA expression was presented with low RYBP protein expression.

3.2. Low level of RYBP predicts poor prognosis in NSCLC patients

To confirm the results, the expression of *RYBP* mRNA was further evaluated in 441 human NSCLC tissue samples. Of these cases, 53 % (232/441) of which showed high *RYBP* expression, whereas 47 % (209/441) showed low *RYBP* expression. In addition, downregulation of *RYBP* was significantly associated with gender ($P < 0.001$), smoking history ($P < 0.001$), histological subtype ($P < 0.001$), and tumor infiltration ($P = 0.005$) (Supplementary Table 1). The value of RYBP protein level as a clinical predictor of NSCLC prognosis was also subsequently evaluated in 190 human NSCLC tissue samples. We found that 114 (60.0%) tumor samples were shown negative RYBP expression, while 76 (40.0%) tumor samples were shown positive. Chi-square test showed that the RYBP protein expression were associated with gender ($P = 0.002$), smoking history ($P = 0.003$), histology subtype ($P < 0.001$), and TNM stage ($P = 0.020$) (Supplementary Table 2). Log-rank test showed that NSCLC patients with low level of RYBP (both mRNA and protein) had shorter overall survival (OS) than those with high RYBP expression (Figures 1E and 1F). In Univariate and multivariate analysis, the tumor RYBP (both mRNA and protein) status was defined as an independent prognostic factor for OS (Supplementary Tables 1 and 2).

3.3. RYBP affects NSCLC cell growth and apoptosis *in vitro*

We next evaluated the expression of RYBP in normal lung epithelial cells (NL20 and BEAS-2B) and NSCLC cells (A549, H838, H358, H1299). The results showed that both *RYBP* mRNA expression and protein level were lower in most NSCLC cells than that in the

normal lung epithelial cells (Supplemental Figures S1A and B). To investigate the potential role of RYBP in NSCLC cells, A549 (p53 wild-type) and H1299 (p53 null) cells were transfected with the Myc-RYBP or RYBP siRNA, and the cell viability was performed by using MTT and colony formation assays. As shown in Figure 2A and 2B, the enforced expression of RYBP resulted in reduced cell viability and inhibited cell colony formation in both A549 and H1299 cells, regardless of the p53 status. We then examined the apoptotic rate of control and RYBP-transfected, the results showed that RYBP overexpression induced high apoptosis in a dose-dependent manner (Figure 2C). Compared with the vehicle control, transfection of 5 μ g RYBP increased the apoptotic index by 2.6-fold ($P < 0.01$) and 2.5-fold ($P < 0.01$) in the A549 and H1299 cells, respectively. In addition, RYBP overexpression also led to increased Bax, PARP cleavage, caspase-8 and -10 cleavages. The p53 levels were upregulated in the treated A549 cells (Figure 2D). In contrast, downregulation of RYBP markedly improved the cell viability and colony formation, inhibited apoptosis, and decreased the expression of proteins involved in apoptosis (Figures 2A–D).

3.4. RYBP affects the chemosensitivity of NSCLC cells to Paclitaxel *in vitro*

One of the strategies to enhance the cytotoxic effects of gene therapy is combinatory use of chemotherapeutic agents [24]. We tested the combined effects of RYBP and chemotherapy (Paclitaxel, the first-line anti-NSCLC agent used in clinic) in the treatment of NSCLC cells. As shown in Figure 3A, Paclitaxel inhibited the cell growth with an IC_{50} value 6.4 nM, enforced RYBP cooperated with Paclitaxel reduced the IC_{50} value to 0.1 nM. H1299 cells showed a similar sensitivity to combination treatment compared with Paclitaxel treated alone (from 13.1 nM to 0.6 nM). Contrastingly, reduced RYBP expression was associated with decreased sensitivity to Paclitaxel, the IC_{50} being 17.4- and 8.7-fold lower than that in A549- and H1299-RYBP-knockdown cells, respectively. Similar results were observed in colony formation assay (Figure 3B). To further explore whether the enhanced chemosensitivity by RYBP is linked to its role in regulating apoptosis, we determined the apoptosis in NSCLC cells in the presence or absence of chemotherapy. As shown in Figure 3C, 100 nM Paclitaxel alone increased the apoptotic index by about 3.2- and 4.1-fold, and combined with RYBP, enhanced the apoptotic index by about 7.0- and 7.1-fold in A549 and H1299 cells, respectively, compared to vehicle-treated cells. In contrast, RYBP knockdown resulted in less apoptotic cells, suggesting RYBP is critically involved in chemotherapy-induced apoptosis in NSCLC cells. We next wanted to know by which mechanism that RYBP mediates chemotherapy-induced cell apoptosis. As shown in Figure 3D, in both A549 and H1299 cells, Paclitaxel treatment induced RYBP expression. Paclitaxel or RYBP treatment activated Bax, Cleaved-PARP, Cleaved-Caspase 8 and -Caspase 10. In addition, RYBP overexpression enhanced, while RYBP knockdown inhibited the Paclitaxel-induced protein levels, indicating that RYBP may be involved in Paclitaxel-induced cell death through the regulation of pro-apoptotic protein expression.

3.5. AdRYBP suppresses the malignant properties of NSCLC cells and sensitizes NSCLC cells to chemotherapy *in vitro*

Gene therapy represents a novel treatment approach for cancer and adenoviral vectors have been used extensively in cancer gene therapy [25]. We generated a replication-deficient adenovirus driving the expression of RYBP (AdRYBP) to determine whether the virus will

suppress or eradicate the tumor. Consistent with the results obtained by plasmid transfection method, AdRYBP infection, but not AdGFP, decreased the cell viability, inhibited cell colony formation, induced apoptosis and modulated protein expression in both cell lines, regardless of the p53 status (Figure 4A–E). Similarly, combining AdRYBP with conventional therapies significantly decreased cell viability and dramatically increased cell apoptosis and related protein levels compared to Paclitaxel treatment alone (all $P < 0.01$; Figure 4A–E). The results indicated that the combination of AdRYBP and Paclitaxel was more efficacious than AdRYBP or Paclitaxel alone and RYBP could sensitize the NSCLC cells to the chemotherapeutic agent.

3.6. AdRYBP suppresses the NSCLC tumor growth and sensitizes NSCLC cells to chemotherapy *in vivo*

The anticancer effects of RYBP were then evaluated *in vivo*. As shown in Figure 5, AdRYBP treatment, not AdGFP, decreased the A549 and H1299 xenograft tumor growth by about 62% ($P < 0.01$) on Day 33 (Figure 5A), and 70% ($P < 0.01$) on Day 21 (Figure 5B), respectively. Tumor growth was inhibited by 63% and 66% in Paclitaxel treatment group (Figure 5A and B), respectively. When AdRYBP combined with Paclitaxel, the tumor growths were almost regressed in both models (Figure 5A and B). There were no remarkable differences in the body weight (as a surrogate marker of toxicity) (Figure 5C and D), and gross necropsy revealed no internal abnormalities in different tissues (Liver, lung, kidney, spleen, heart and brain) in all treatment groups (Supplementary Figure 2), indicating that there were no significant side effects of the treatments. We then compared the protein levels from different treatment groups to investigate the mechanism(s) by which RYBP inhibited tumor growth and sensitized chemotherapy. As shown in Figure 5E, *in situ* TUNEL assays showed substantially increased apoptosis in the tumors of AdRYBP- and Paclitaxel-treated mice, whereas the combination of AdRYBP and Paclitaxel dramatically enhanced the apoptotic cells, supporting the notion that RYBP performs anti-proliferative role through the induction of apoptosis in NSCLC cells. These observations were further confirmed by western blotting analyses, and similar results were obtained in both xenograft models. Paclitaxel and AdRYBP treatment induced RYBP and Bax protein levels, and AdRYBP enhanced the Paclitaxel-induced expression of p53 and Bax (Figure 5F).

4. Discussion

In this study, we used an extensive collection of NSCLC tumors and *in vitro* and *in vivo* experiments, and described a novel role of RYBP in the progression of NSCLC and the correlation between RYBP expression and survival in NSCLC patients. Herein, we have demonstrated at least four novel points: First, both RYBP mRNA expression and protein level were downregulated in NSCLC tissues, the low level of RYBP predicted poor patient survival in NSCLC; Second, RYBP overexpression decreased cell viability, inhibited colony formation and induced apoptosis in NSCLC cells, and promotion of apoptosis was one of the main mechanism of RYBP-mediated cell growth inhibition in NSCLC; Third, successful AdRYBP infection led to a decreased NSCLC tumor xenograft growth; Forth, chemotherapeutic agent induced RYBP expression, and RYBP sensitized NSCLC cells to conventional chemotherapeutic agent by apoptosis induction both *in vitro* and *in vivo*. These

results demonstrated that RYBP may be a novel prognostic factor in patients with NSCLC and be a target for cancer therapy.

Although the previous studies from the other researchers and our lab have implicated *RYBP* as a potential tumor suppressor gene [16, 17, 26–28], the physiological and pathological role(s) of RYBP are still poorly understood. Integrative genomic profiling is shown the loss of *RYBP* gene in prostate and cervical cancers and the decrease of *RYBP* transcript has been linked to poor prognosis [27, 28]. We demonstrate that the low RYBP expression is an independent predictor of a poor prognosis in patients with HCC [17]. In contrary, *RYBP* is suggested to be overexpressed in primary classic Hodgkin's lymphoma and adult T-cell leukemia/lymphoma cases [29, 30]. Although the discrepancy between solid tumors and lymphoma has not been addressed, it seems that RYBP participates in carcinogenesis and cancer progression via distinct mechanisms. In the present study, to our best knowledge, we presented the first large-scale study to examine the prognostic value of RYBP expression in NSCLC patients. The results highlighted RYBP as a signature molecule correlated with the prognosis of NSCLC patients. We found that NSCLC patients with low levels of RYBP (both mRNA and protein) were at significantly high risk of overall survival and RYBP was an independent prognostic factor.

In a recent study, we have observed that RYBP positively regulates the levels and function of p53 and involves in cell cycle progression and cellular response to DNA damage [16]. These findings suggest that RYBP may have an essential role in regulating the p53 pathway. In the present study, we demonstrated that enforced RYBP significantly reduced the cell viability and induced cell apoptosis in NSCLC cells regardless of p53 status. Intriguingly, we also observed the effect of RYBP on cell cycle progression, consistent with our previous study, our results showed that enforced RYBP arrested cells in G1 phase in A549 cells, while no significant effects were observed in H1299 cells (data not shown). Although our previous studies show that RYBP inhibits MDM2-mediated ubiquitination of p53, we cannot exclude other possibilities. Since MDM2 has E3 ligase activity and RYBP can be monoubiquitinated [31], it is possible that MDM2 promotes RYBP ubiquitination, thus modulating the ability of RYBP to induce apoptosis or regulate transcription. Actually, MDM2 is capable of repressing transcription when tethered to a reporter gene promoter in the absence of p53 [32]. Therefore, it is possible that RYBP and MDM2 (with or without other PcG members) may form a protein complex in which RYBP recruits MDM2 to its target gene promoter(s). Alternatively, MDM2 may ubiquitinate RYBP or other members of the PcG family to directly regulate the trans-repressional activities of the PcG complex. In p53 non-functional cells, it is also possible that RYBP directly binds to Bax or other targets. As examples of the potential p53-independent effects of RYBP, RYBP physically binds to several apoptotic mediators, such as caspases-8 and -10, and Fas-associated protein with death domain (FADD) through their cytoplasmic death effector domains to form the death inducing signaling complex (DISC), resulting in the proteolytic cleavage and activation of these caspases, and subsequent induction of apoptosis [11]. Based on our observation, it is possible that RYBP regulates cell cycle arrest and apoptosis induction by different mechanisms. It is also possible that the apoptosis induction by RYBP is a process independent of p53 and needs other partners. In the future, the possible p53-independent

functions of RYBP need to be explored, which will provide a complete picture for RYBP's role in carcinogenesis and cancer development.

The multifaceted function of RYBP in inducing apoptosis and regulating the p53 tumor suppressor provides an ideal target for cancer therapy, and may be useful either alone or in combination with conventional chemotherapeutics. In this study, we also demonstrated that chemotherapeutic agent can induce RYBP expression and gene delivery of RYBP combined with chemotherapy may provide a better efficacy for NSCLC therapy. However, the mechanisms underlying RYBP-mediated chemosensitization are still not fully understood. Further studies are needed to explore the molecular mechanisms by which RYBP inhibits cancer progression and increases chemosensitization, and whether other members are capable of regulating RYBP.

In conclusion, we demonstrate the downregulation of RYBP and an inverse correlation between RYBP expression levels and prognosis in NSCLC patients. Based on the observed specific effects of RYBP on NSCLC cells, reactivating RYBP in cancer cells that are RYBP-deficient or express low levels of RYBP may provide an effective and safe therapeutic approach to NSCLC therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Both RYBP mRNA expression and protein level were downregulated in NSCLC tissues, the low level of RYBP predicted poor patient survival in NSCLC.
- Enforced RYBP inhibits cell viability and induces apoptosis in NSCLC cell.
- The one of the main mechanism of RYBP-mediated cell growth inhibition is promotion of apoptosis.
- Successful AdRYBP infection inhibits NSCLC tumor xenograft growth.
- Chemotherapeutic agent induces the expression of RYBP.
- RYBP sensitizes NSCLC cells to conventional chemotherapy both *in vitro* and *in vivo*.

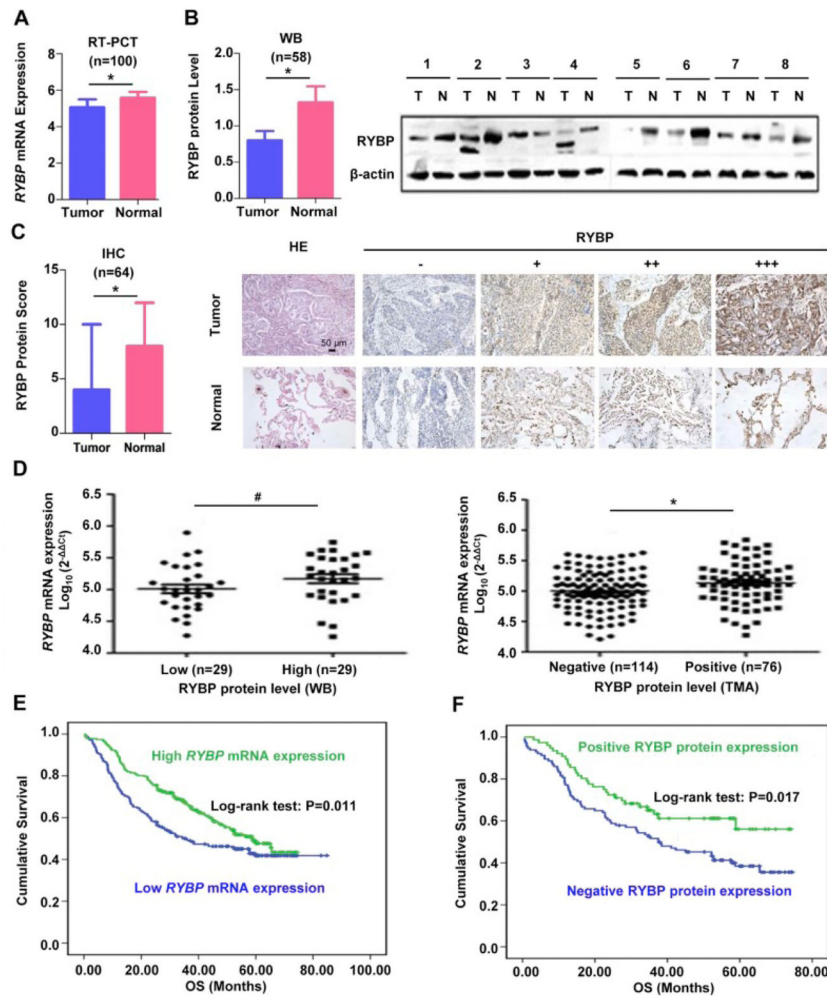


Figure 1. Down-regulation of RYBP expression is common in primary NSCLC tissues
 (A) The quantitative real-time analyses of the RYBP mRNA level in training cohort of NSCLC patients and adjacent nonmalignant lung tissues (n = 100); (B) Western blot (WB) analyses were performed to determine the RYBP protein level in NSCLC patients and corresponding non-malignant adjacent tissue samples (n = 58). The graphs on the left showed the quantification of the Western blotting data; the graphs on the right showed the representative images of RYBP expression in malignant and adjacent normal lung tissues; (C) The immunohistochemical (IHC) analysis of the RYBP expression in tumor and normal adjacent tissue samples (n = 64). The graphs on the left showed the quantification of the IHC analysis; the graphs showed the representative images of RYBP expression with H&E and IHC staining on the right panel, in malignant and adjacent normal lung tissues ((-) no signal, (+) weak, (++) moderate, (+++) marked) (scale bar, 50 μ m); (D) The correlation analyses of RYBP mRNA expression and RYBP protein expression levels detected by WB and IHC; (E) The overall survival (OS) rates of 441 patients with NSCLC were compared between the low or high RYBP mRNA level groups using the Kaplan–Meier method; (F) The correlation between RYBP protein expression level and survival in validation cohort of NSCLC patients (n=190). (# P < 0.05; *P < 0.01)

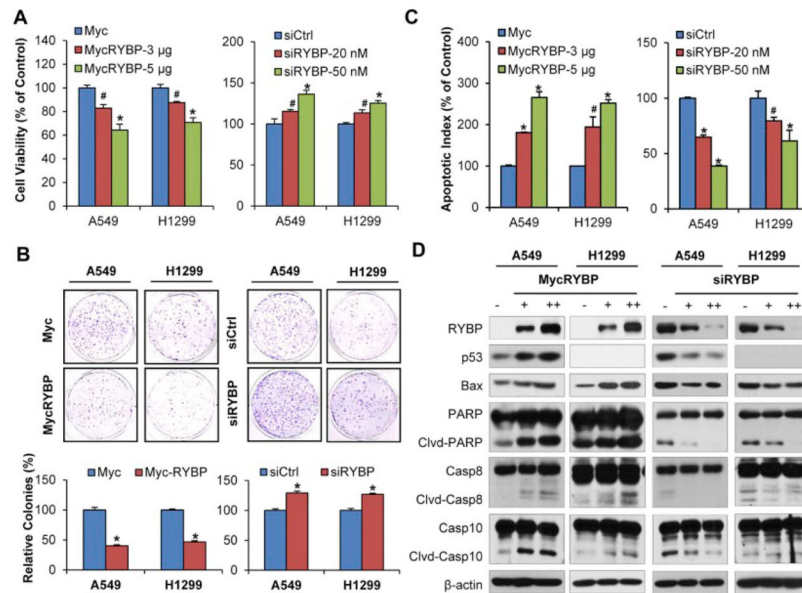


Figure 2. RYBP affects the cell growth and apoptosis in NSCLC cells

A549 and H1299 cells were transiently transfected with Myc-RYBP plasmid or corresponding empty vector for 24 h or with RYBP siRNA or a nontargeting negative control siRNA for 48 h. (A) The cell viability was performed by the MTT assay; (B) The cell survival was determined by the colony formation assay, graphs (lower panel) showed the quantification of the colonies; (C) The cell apoptosis was measured by the Annexin V-FITC method; and (D) RYBP and apoptosis-related proteins expression was detected by Western blot analysis. All assays were performed in triplicate, and all the experiments were repeated at least three times. (# $P < 0.05$; * $P < 0.01$).

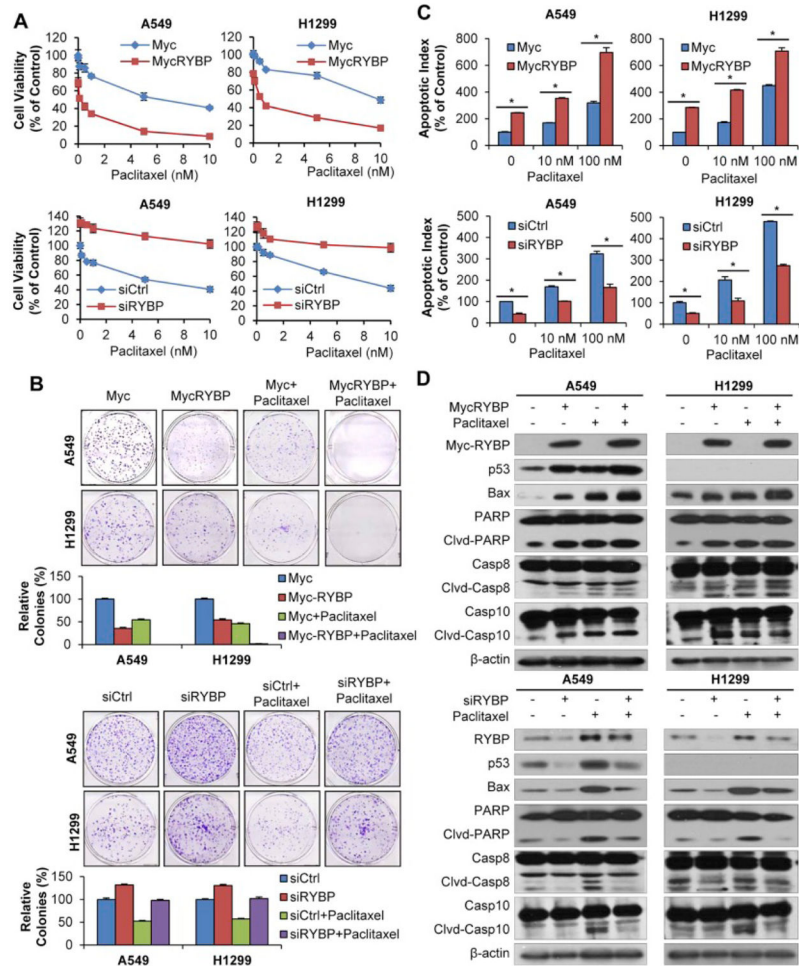


Figure 3. RYBP sensitizes NSCLC cells to Paclitaxel

A549 and H1299 cells were treated with Paclitaxel (0–100 nM for MTT assay, and 5 nM for other assays) and Myc-RYBP plasmid (5 μg, 24 h) or RYBP siRNA (50 nM, 48 h), then the cell viability was determined by the MTT assay (A); the cell survival was determined by the colony formation assay, graphs (lower panel) showed the quantification of the colonies (B); the cell apoptosis was determined by the Annexin V-FITC method (C); RYBP and apoptosis-related proteins expression was detected by Western blot analysis (D). All assays were performed in triplicate, and all the experiments were repeated at least three times. (# P < 0.05; *P < 0.01).

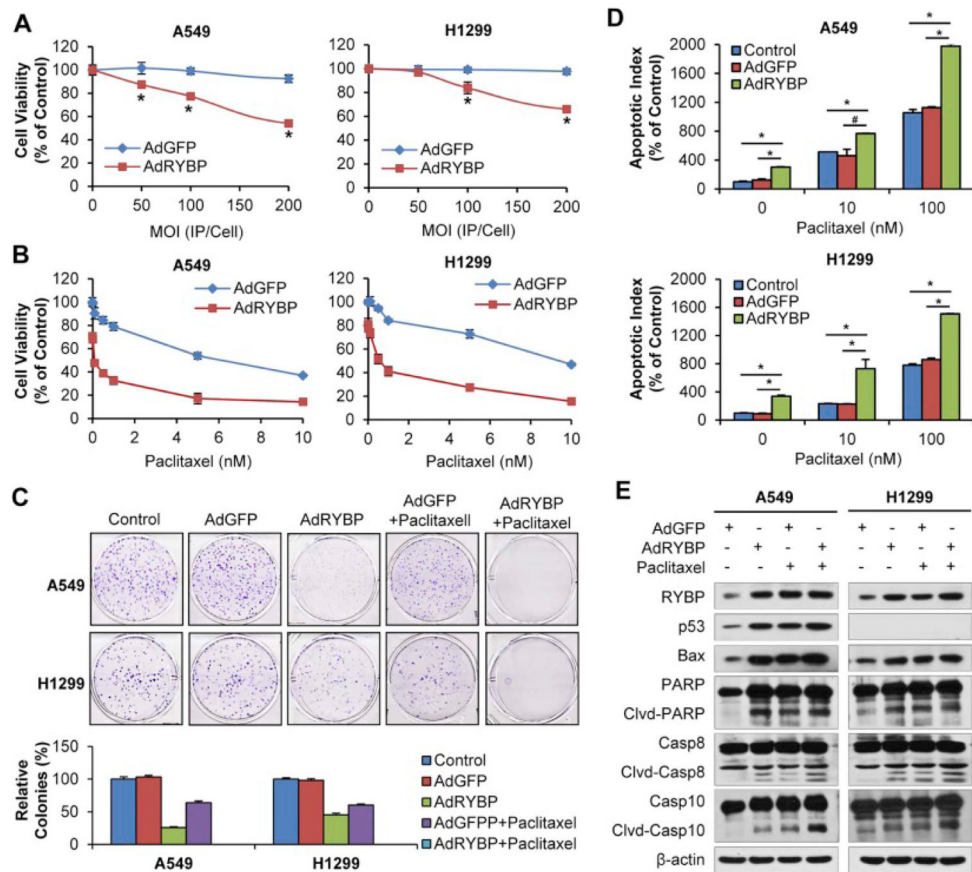


Figure 4. AdRYBP infection induces NSCLC cell growth arrest and apoptosis, and increases the chemosensitivity of NSCLC cells to Paclitaxel

(A) A549 and H1299 cells were infected with AdRYBP (0, 50, 100 and 200MOI) or AdGFP (0, 50, 100 and 200MOI) for 24 h, then the cell viability was determined by the MTT assay; (B) Cells were treated with Paclitaxel (0–10 nM) and infected AdRYBP (200 MOI, 24 h), then the cell viability were determined by the MTT assay; (C) Cells were treated with Paclitaxel (5 nM) or/and infected AdRYBP (200 MOI, 24 h), then maintained for additional 7–10 days. The cell survival was performed by the colony formation assay, graphs (lower panel) showed the quantification of the colonies; (D) Cells were treated with Paclitaxel (0, 10 and 100 nM) or/and infected AdRYBP (200 MOI, 24 h), the cell apoptosis was measured by the Annexin V-FITC method; (E) Cells were treated with Paclitaxel (5 nM) or/and infected AdRYBP (200 MOI, 24 h), RYBP and apoptosis-related proteins expression was detected by Western blot analysis. All assays were performed in triplicate, and all the experiments were repeated at least three times. (# $P < 0.05$; * $P < 0.01$).

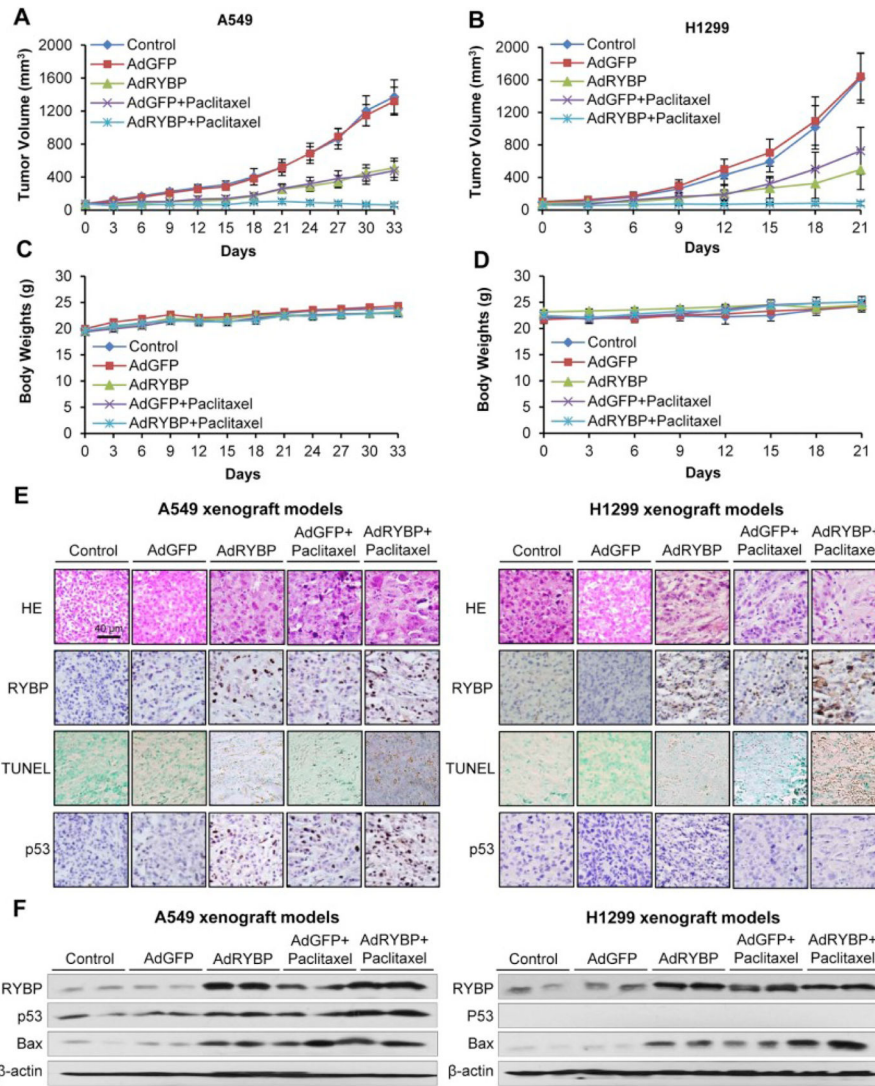


Figure 5. RYBP suppresses NSCLC tumor growth and sensitizes NSCLC cells to chemotherapy *in vivo*

AdGFP or AdRYBP (2×10^9 infectious particles (IP)) was administered by intratumoral injection every three days, and Paclitaxel (15 mg/kg) was administered by intraperitoneal injection every two weeks to nude mice bearing A549 (A) or H1299 (B) tumors. The animals were monitored for changes in body weight as a surrogate marker for toxicity in both the A549 (C) and H1299 (D) xenograft models. At the end of the experiment, xenograft tumors were exercised, fixed or homogenized and analyzed for protein expression by H&E, immunohistochemistry and TUNEL staining (scale bar, 40 μm) (E) and Western blot (F).

Table 1

Clinical characteristics and RYBP expression in NSCLC patients

Variables	RYBP mRNA expression (RT-PCR)			RYBP protein expression (TMA)						
	n (%)	Low	High	n (%)	-	+	P value			
Age at diagnosis										
60	199(45.1)	90	109	54.8	82(43.2)	44	38	46.3	0.12	
> 60	242(54.9)	119	123	50.8	108(56.8)	70	38	35.2		
Gender										
Male	267(60.5)	162	105	39.3	<0.001	122(64.2)	83	39	32	0.002
Female	174(39.5)	47	127	73		68(35.8)	31	37	54.4	
Family history of cancer ^d										
No	357(81.7)	166	191	53.5	0.715	153(80.5)	66	153	69.9	0.113
Yes	80(18.3)	39	41	51.3		35(18.4)	10	35	77.8	
Smoking history										
No	165(37.4)	39	126	76.4	<0.001	59(31.1)	26	33	55.9	0.003
Yes	276(62.6)	170	106	38.4		131(68.9)	88	43	32.8	
Histology subtype										
SCC	202(45.8)	142	60	29.7	<0.001	102(53.7)	27	102	79.1	<0.001
ADC	194(44.0)	44	150	77.3		71(37.4)	41	71	63.4	
others	45(10.2)	23	22	48.9		17(8.9)	8	17	68	
TNM stage										
I-II	284(64.4)	130	154	54.2	0.36	114(60.0)	83	31	24.6	0.02
III-IV	157(35.6)	79	78	49.7		76(40.0)	31	33	51.6	
Tumor infiltration										
T1T2	380(86.2)	170	210	55.3	0.005	164(86.3)	98	66	40.2	0.863
T3T4	61(13.8)	39	22	36.1		26(13.7)	16	10	38.5	
Lymph node metastasis ^d										
N0	241(55.5)	113	128	53.1	0.871	107(56.6)	71	36	33.6	0.053
N1-N3	193(44.5)	92	101	52.3		82(43.4)	43	39	47.6	
Distant metastasis										
M0	415(94.1)	196	219	52.8	0.784	183(96.3)	112	71	38.8	0.084

Variables	RYBP mRNA expression (RT-PCR)			RYBP protein expression (TMA)		
	n (%)	Low	High	n (%)	-	+
M1	26(5.9)	13	13	7(3.7)	2	5
			50			71.4
			P value			P value

^aNumber not equal to the total number due to missing data; SCC: Squamous cell carcinoma; ADC: Adenocarcinoma