Oncogene (2013) **32,** 4921–4931

www.nature.com/onc

ORIGINAL ARTICLE

Endoplasmic reticulum ribosome-binding protein 1 (RRBP1) overexpression is frequently found in lung cancer patients and alleviates intracellular stress-induced apoptosis through the enhancement of GRP78

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Lung cancer is the leading cause of cancer deaths and is the most occurring malignancy worldwide. Unraveling the molecular mechanisms involved in lung tumorigenesis will greatly improve therapy. During early tumorigenesis, rapid proliferating tumor cells require increased activity of endoplasmic reticulum (ER) for protein synthesis, folding and secretion, thereby are subjected to ER stress. Ribosome-binding protein 1 (RRBP1) was originally identified as a ribosome-binding protein located on the rough ER and associated with unfolding protein response (UPR). In this report, we investigated the role of RRBP1 in lung cancer. RRBP1 was highly expressed in lung cancer tissue, as compared with adjacent normal tissues as assessed by immunohistochemistry (IHC) using lung cancer tissue array ($n = 87$). Knockdown of RRBP1 by short-hairpin RNAs caused ER stress and significantly reduced cell viability and tumorigenicity. This effect was associated with a significant reduction in the expression of glucose-regulated protein 78 (GRP78). UPR regulator GRP78, an anti-apoptotic protein that is widely upregulated in cancer, has a critical role in chemotherapy resistance in some cancers. According to our results, cells with a higher level of RRBP1 were more resistant to ER stress. Ectopic expression of RRBP1 alleviated apoptosis that was induced by the ER-stress agent tunicamycin, 2-deoxy-D-glucose (2DG) or doxorubicin via enhancing GRP78 protein expression. A strong correlation was observed between the expression of RRBP1 and GRP78 in tumor biopsies using the database GSE10072. Our results also indicated that RRBP1 may involve in the regulation of mRNA stability of UPR components including ATF6 and GRP78. Taken together, RRBP1 could alleviate ER stress and help cancer cell survive. RRBP1 is critical for tumor cell survival, which may make it a useful target in lung cancer treatment and a candidate for the development of new targeted therapeutics.

Oncogene (2013) **32,** 4921–4931; doi:10.1038/onc.2012.514; published online 14 January 2013

Keywords: RRBP1; GRP78; UPR; apoptosis; lung cancer

INTRODUCTION

Lung cancer is the leading cause of cancer deaths worldwide in both men and women. The high incidence of lung cancer is considered to be a consequence of smoking, environmental exposures, genotoxic and family history. Long-term expose to carcinogen may induce tumorigenesis. Non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancers worldwide.[1] Adenocarcinoma is one of the main types of NSCLC and accounts for 40% of all lung cancers.[2] Patients with stage IA disease are treated with surgical resection resulting in a 5-year survival of 70%,[3] whereas in stage IV, the 5-year survival is $<1\%$.[4] The achievement of early diagnosis and treatment has improved the patients' outcome. During the early stage of tumor development, cancer cells with high proliferation rates require the increased activity of endoplasmic reticulum (ER) protein folding, assembly, transport and subjected to ER stress. During tumorigenesis, adaptive stress response promotes the unfolded protein response (UPR) and initiates the activation of survival cascade mechanisms.[5] The activation of the UPR would affect tumorigenesis and protect tumor cell from ER stress. There is inconclusive evidence of UPR activation mechanisms in human lung tumorigenesis until now. Thus, unraveling the molecular mechanisms involved in lung tumorigenesis will provide novel insight to improve therapy.

The ER protein RRBP1 (ribosome-binding protein 1) was originally identified as a ribosome-binding protein on the rough ER, which is one the crucial step in the transportation and secretion of intracellular proteins in mammalian cells.[6] RRBP1 has been studied in yeast, where it is a member of the ER stress response and associated UPR. In yeast, expression of RRBP1 results

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Received 15 May 2012; revised 5 September 2012; accepted 20 September 2012; published online 14 January 2013

in an increase in the levels of *KAR2* mRNA, whose sequence shares homology with the mammalian protein BiP/GRP78 (glucose-regulated protein 78).[7] In a recent report, RRBP1 was recognized as a potential protein marker for colorectal cancer.[8] In lung cancer database, RRBP1 is overexpressed in lung cancer compared with adjacent normal tissues. However, there are limited reports of RRBP1 involved in UPR pathway related to cancer development until now.

UPR is involved in signaling pathways emanating from ER. There are three ER stress sensors: IRE-1 (inositol-requiring protein 1), PERK (PKR-like ER kinase) and ATF6 (activating transcription factor). The activation of these sensors is dependent on their dissociation from the ER chaperone BIP/GRP78.[9,10] GRP78 is a master regulator of the UPR, resides primarily in the ER of mammalian cells and has been implicated in promoting tumor proliferation, metastasis, drug resistance and apoptosis.[11,12] GRP78 is frequently overexpressed in lung cancer but not in normal lung tissue.[13,14] GRP78 expression can be induced by treatment with either 2DG (a hypoglycemia-inducing agent) or tunicamycin (an inhibitor of protein glycosylation).[15] These two agents have been shown to induce antitumor effects *in vitro* and *in vivo* in combination with chemotherapy.[16,17] Knockdown of GRP78 enhanced the tunicamycin- and 2DG-induced increase in apoptosis, as compared with a luciferase short-hairpin RNA (shRNA) control.[18–20]

In this study, we aimed to investigate the role of the RRBP1 in lung cancer. We detected the expression of RRBP1 proteins in lung cancer and its adjacent normal lung tissue. Knockdown of RRBP1 by shRNAs caused ER stress and significantly reduced cell viability. Ectopic expression of RRBP1 alleviated ER stress and protected tumor survival *in vitro*. We investigated the relationship between the expression of RRBP1 and GRP78 in order to explore the role of RRBP1 in tumorigenesis of lung cancer.

RESULTS

Elevated RRBP1 expression in lung adenocarcinomas cells and patients

To investigate whether RRBP1 has a role in NSCLC, we first examined the expression of RRBP1 using a tissue array, which contained 87 clinically annotated NSCLC samples. The staining signal of RRBP1 was mainly distributed in the cytoplasm and showed a wide range of expression levels that varied between NSCLC samples (Figures 1a and b). To elucidate the specificity of RRBP1 antiserum, we used rabbit IgG as isotype control for IHC analysis. No signals were detected in the tumor regions using rabbit IgG (Supplementary Figure S3A). Based on the expression of RRBP1, the NSCLC specimens were classified into four groups: level 0 (negative staining), level 1 (0–20% of tumor cells stained), level 2 (20–50% of tumor cells stained) and level 3 ($>50\%$ of tumor cells stained). Statistical analysis showed no significant correlation between RRBP1 expression and tumor stages. However, there was a trend of higher expression of RRBP1 in the early tumor stages (stage I, II and III), which could be observed from the tissue array analysis (Figure 1c). In addition, the tissue array analysis demonstrated that RRBP1 was commonly expressed in the tumor regions but was rare in normal lung tissues (Figure 1a). This result was confirmed by quantitative PCR (qPCR) analysis on 12 paired normal-tumor specimens, which demonstrated that RRBP1 mRNA was high expression in lung tumors compared with normal tissues (Figure 1d). Overexpression of RRBP1 in lung tumors was further supported by data analysis from the GEO database (GSE7670; GSE10072), which showed higher RRBP1 mRNA levels in lung adenocarcinoma than in normal lung tissue (Supplementary Figures S1 and S2). In four normal-tumor paired specimens, RRBP1 was overexpressed in 75% (3/4) of lung tumor samples (Figure 1e). The expression profiles of RRBP1 mRNA and protein were examined by qPCR and western blotting in eight lung cancer cell lines (Figures 1f and g). Consistent with the tissue array data, RRBP1 expression was highly variable in lung cancer cell lines. We then treated five lung cancer cell lines with 2 μM tunicamycin and found that high endogenous RRBP1 expression displayed resistance to ER stress agent tunicamycin (Figure 1h). We concluded that overexpression of RRBP1 might suggest its important role for the survival and maintenance of tumor cells during tumorigenesis.

Ectopic RRBP1 expression enhances growth of human lung cancer xenografts

The result described above suggested that RRBP1 may have effects on the maintenance of lung cancer growth and survival *in vivo*. To test this hypothesis, we generated stable PC13 cell lines that expressed either pCDNA as a control or pCDNA/RRBP1 (Figure 2a). These cell lines were then injected subcutaneously into NSG mice and allowed to grow as xenografts, and the tumor growth was monitored weekly. The tumor size and growth curves of RRBP1-overexpressing tumors were comparable with control tumors (Figures 2b and c). There was approximately a 2-fold increase in tumor size in RRBP1-overexpressing cells. The weight of the xenografts was measured and the RRBP1-overexpressing tumors showed a significant increase (2-fold) compared with control tumors (Figure 2d). In addition, *in vitro* tumorigenicity assays were performed on these two cell lines using a soft agar assay. RRBP1-overexpressing cells showed more colonies as compared with the control cells (Figure 2e). These data support the findings above and show that RRBP1 has an important role during tumor development and helps tumor cells to survive.

Knockdown of RRBP1 expression in high-expressing NSCLC cells significantly reduces cell viability and tumorigenicity *in vitro*

Base on our screening results using tissue array and xenograft assay *in vivo*, our data demonstrated that RRBP1 expression is increased in human lung cancer, as compared with normal alveoli. Therefore, we assessed whether RRBP1 expression may influence the growth characteristics and cell viability of human lung cancer cells. To assess this possibility, we used shRNA lentiviruses to specifically knock down RRBP1 expression in A549 and CL1-5 cells. A549 and CL1-5 cells were infected with either RRBP1 shRNA-containing lentiviruses or luciferase shRNA-containing lentiviruses as a control. The RRBP1 knockdown efficiency of each individual shRNA clones was analyzed by western blotting (Figures 2f and g). shRNA clones No. 2 and 5 inhibited RRBP1 expression by 70% at both RNA and protein levels, as compared with the control group. Cell growth and cell viability were analyzed by CellTiter-Glo. Cell viability was significantly decreased in the RRBP1-knockdown cells, as compared with the cells with the luciferase shRNA (Figures 2h and i). In addition, RRBP1 silencing significantly decreased tumorigenicity, as measured by a soft agar assay (Figure 2j). Taken together, the loss of RRBP1 reduced cell viability and tumorigenicity in lung cancer cells. Therefore, RRBP1 is essential for tumor survival and maintenance of tumor growth.

RRBP1-knockdown significantly reduces tumorigenesis *in vivo*

To investigate the impact of RRBP1-knockdown on tumorigenesis, we subcutaneously injected the cells expressing RRBP1 shRNA or luciferase shRNA into NSG mice. The tumor size and growth curves of RRBP1-knockdown tumors were monitored and compared with control (Supplementary Figures S4B and C). Knockdown of RRBP1 significantly reduced tumor growth by 75% compared with control tumors (Supplementary Figure S4D). For orthtopic lung model, we established A549-Luc (luciferase) stable clone. A549-Luc-scramble and A549-Luc-shRRBP1 cells were injected into the left lateral thorax of each mouse. Luminescence image was monitored and quantified by noninvasive bioluminesence system. The total luminescence counts were almost equal in

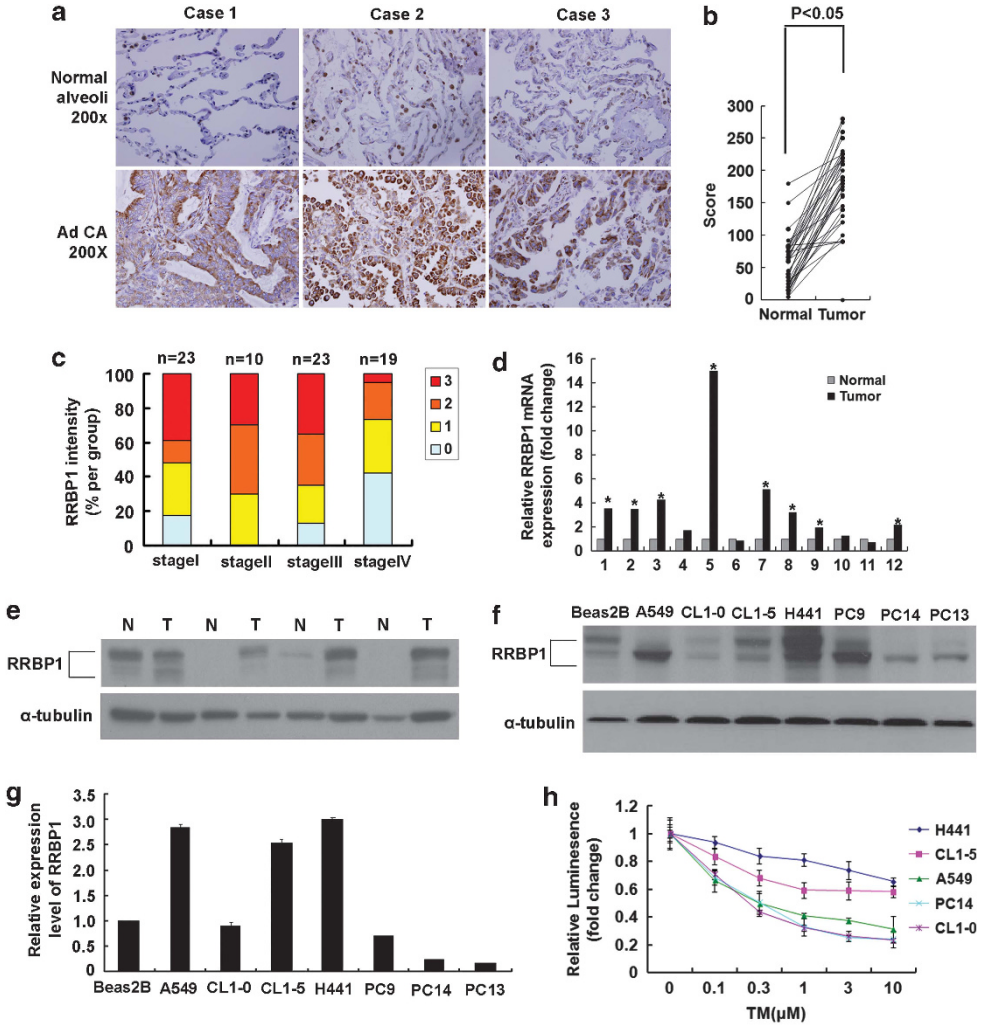

Figure 1. RRBP1 is highly expressed in NSCLCs and patients. (**a**) RRBP1 IHC staining in lung cancer specimens. Protein overexpression was judged by comparing the staining intensity of NSCLC tumor cells and the adjacent normal Noveli cells. RRBP1 was overexpressed in the cytoplasm (see arrow). (**b**) Plot representation of scores according to cytoplasmic IHC expression of RRBP1 in primary lung tumors related to paired normal tissue. The scores are calculated by intensity × percentage of stained cells. (**c**) Quantification of RRBP1 expression by IHC analysis of lung cancer specimens. The number (*n*) of samples for each stage is indicated on the top. (**d**) RRBP1 transcript levels in 12 clinical samples were analyzed by qPCR. RRBP1 transcripts were high in the NSCLC tumor compared with normal tissue. Statistical analysis of RRBP1 transcripts in 12 clinical samples was performed. (**e**) RRBP1 expression profiles in human lung specimens were analyzed by western blotting. (**f**, **g**) Endogenous RRBP1 protein and mRNA transcript expression profiles in NSCLC were analyzed by western blotting and qPCR. (**h**) H441, CL1-5, A549, CL1-0 and PC14 cells were treated with 0, 0.1, 0.3, 1, 3 and 10 μM tunicamycin for 48 h. The cell viability was detected by CellTiter-Glo. N, Normal; T, Tumor; AdCA, adenocarcinoma. Asterisks indicate statistically significant.

RRBP1-knockdown and scramble control cells at first day (Figures 3b and c). At day 49 post-injection, total luminescence counts in scramble control mice were 20-fold higher than in RRBP1-knockdown tumor in mice (Figures 3d and e), and RRBP1-knockdown tumor size were significantly reduced as compared with scramble controls (Figure 3f).

Downregulation of RRBP1 activates the p38 and JNK pathway and exacerbates tunicamycin-induced apoptosis by downregulating GRP78 expression in human NSCLC cells

ER stress can activate the MAPK (mitogen-activated protein kinase) family signaling pathway. Therefore, we investigated if RRBP1-knockdown in human NSCLC could trigger JNK (c-Jun N-terminal kinase) and p38 MAPK activation (Figure 4a). RRBP1-knockdown would cause cellular ER stress. We also found that RRBP1-knockdown reduced ATF6 and GRP78 mRNA expression but not IRE1 and PERK (Figure 4b). Next, we investigated how ATF6 and GRP78 were regulated by RRBP1. We used actinomycin D (transcription inhibitor) to treat RRBP1-knockdown cell. The ATF6, GRP78 and RRBP1 levels were analyzed by qPCR. GRP78 and ATF6 were significantly reduced in RRBP1-knockdown cells compared with shluc controls (Figure 4c). We suggest that RRBP1 may involve in ATF6 and GRP78 mRNA stability. However, the molecular mechanism is still unclear.

Furthermore, RRBP1-knockdown and shluc control cells were treated with tunicamycin. The subG1 population was increased in RRBP1-knockdown cells, as compared with the controls. Apoptotic cell death was increased from 7.8% to 27.7% and 5.1% to 16.2% in the tunicamycin-treated A549 and CL1-5 cells, respectively (Figures 4d and e). Western blotting analysis showed that GRP78 protein was significantly decreased in the RRBP1-knockdown cells when compared with the control cells. Cleaved PARP (poly ADP-ribose polymerase) was also increased in the tunicamycin-treated, RRBP1-knockdown cells (Figures 4f and g). Furthermore, downregulation of GRP78 by GRP78 shRNA enhanced

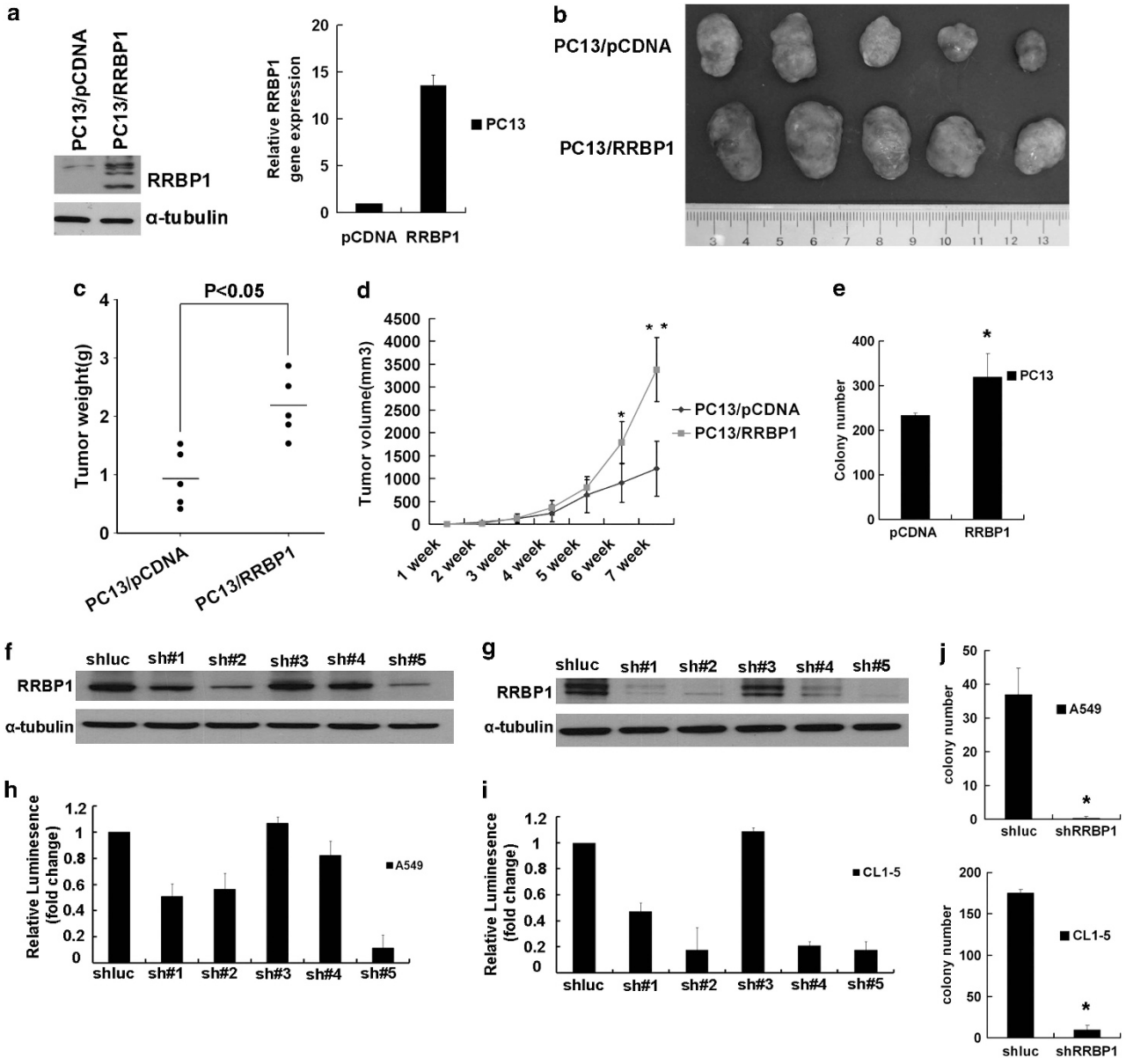

Figure 2. RRBP1 is essential to maintain lung cancer survival and enhance tumorigenicity *in vivo*. (**a**) Immunoblot and qPCR analysis of RRBP1 overexpression or endogenous RRBP1 in PC13 cells. (**b**) Tumors from a mouse injected with 5×10^6 cells/100ul of PC13/pCDNA (upper) or PC13/RRBP1 cells (bottom) ($N = 5$) after 7 weeks. (**c**) The tumor weight of PC13/pCDNA and PC13/RRBP1 tumors was measured when mice were killed. (**d**) The tumor growth curves of PC13/pCDNA and PC13/RRBP1 tumors were monitored over a period of 49 days. (**e**) Soft agar assays in which pCDNA control or RRBP1-overexpressing PC13 cells were seeded at a density of 1000 cells per six-well dish and cultured in 0.35% soft agar in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum at 37 °C for 14–21 days. (**f**, **g**) After delivery of RRBP1 and luciferase shRNA lentiviruses into A549 and CL1-5 cells, RRBP1 knockdown efficiency of the individual shRNA clones was analyzed by western blotting. (**h**, **i**) The cell viability of RRBP1 and luciferase knockdown cells were detected by CellTiter-Glo. (**j**) Soft agar assays for A549 and CL1-5 cells after introduction of shRNAs against RRBP1 or luciferase. The data are expressed as means ± s.e. A Student's *t*-test was performed, and $^*P < 0.05$ was considered to be statistically significant. shluc, shluciferase; sh#1,2,3,4,5, shRRBP1#1, shRRBP1#2, shRRBP1#3, shRRBP1#4, shRRBP1#5.

tunicamycin-induced apoptosis compared with controls (Figures 4h and i). We suggest that RRBP1-knockdown exacerbates tunicamycin-induced apoptosis mediated through GRP78. However, the mechanism is still unclear until now.

RRBP1 and GRP78 expression correlates in human primary lung tumors

Next, we investigated whether RRBP1 and GRP78 expression correlate in NSCLC. We performed IHC-based tissue array screening on 87 clinically annotated NSCLCs and found a wide range of GRP78 expression. GRP78 expression is mainly distributed in the cytoplasm of NSCLC. GRP78 overexpression was judged by comparing the staining intensity of NSCLC tumor cells and the adjacent normal alveolar cells (Figures 5a and b). Overexpression of GRP78 in lung tumors was further supported by data analysis from the GEO database GSE10072 (Supplementary Figure S2B). We examined whether there is an association between RRBP1 and GRP78 expression in human NSCLC tumors. We determined the expression of GRP78 by IHC in the same serial sections of patients used for the analysis of RRBP1 expression. Quantification of the immunostaining results revealed that RRBP1 expression strongly

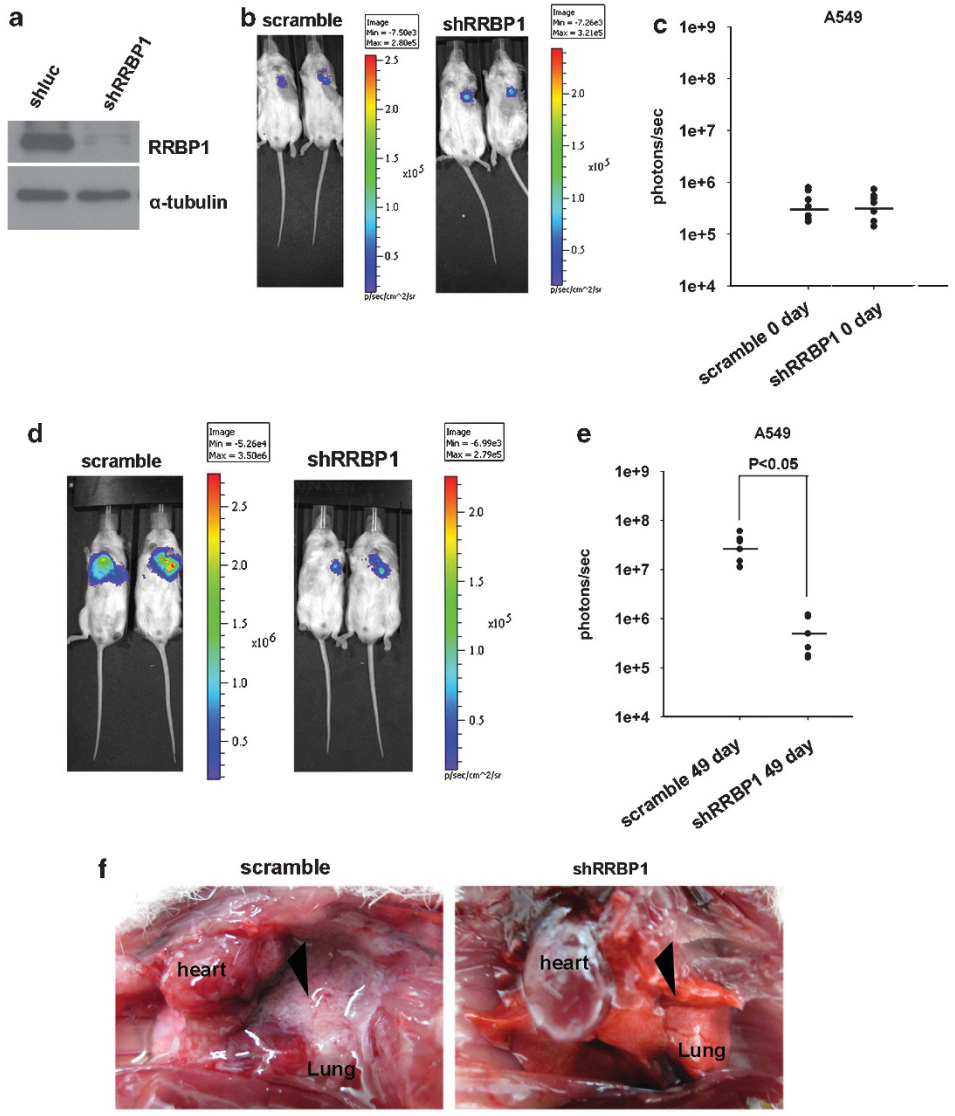

Figure 3. Knockdown of RRBP1 reduced tumorigenesis *in vivo*. (**a**) RRBP1 knockdown efficiency was confirmed by western blotting. (**b**–**e**) A549-scramble and A549-shRRBP1 cells were orthotopic injected into left lung. Luminesence image were monitored and quantitatively measured by noninvasive bioluminesence system (IVIS spectrum) at days 1 and 49. (**f**) Representative images of orthotopic tumor at day 49. Black arrowhead indicates orthotopic tumor.

correlated with GRP78 expression in human lung tumors (Figure 5c). Using the database GSE10072, statistical analysis showed that RRBP1 mRNA expression was highly correlated with GRP78 mRNA expression in the human normal lung and lung tumors (tested by Pearson's correlation test, correlation coefficient = 0.42, $P < 0.05$, Supplementary Table S2). The results were consistent with those obtained by IHC, showing a significant correlation between the expression levels of RRBP1 and GRP78 (tested by Pearson's correlation test, correlation coefficient = 0.281, $P < 0.05$, Figure 5d).

Ectopic RRBP1 overexpression alleviates apoptosis induced by the ER stress in lung cancer cells by enhancing GRP78 expression

In cancer cells, elevated glucose metabolism, increased glycolytic activity and poor vascularization in fast growing tumors can lead to ER stress. We assessed whether ectopic RRBP1 overexpression would make cells resistant to ER stress agents and support tumor cell survival. RRBP1-overexpressing PC13 and PC14 cells were treated with 2 μM tunicamycin or 0, 20 or 40 mM 2DG. In tunicamycin-treated cells, the subG1 population in either the PC13/pCDNA or the PC14/pCDNA vector control cells was 11.5% and 20.8%, respectively. However, the subG1 population was 3.5% and 12.5% in PC13 and PC14 RRBP1-overexpressing cells, respectively (Figure 6a). The subG1 population in RRBP1-overexpressing cells was significantly decreased when compared with vector control cells. The results were similar in cells treated with 2DG. The subG1 population in PC13 and PC14 vector control cells was 20.9% and 11.6%, while the subG1 population was 3.5% and 6.2% in RRBP1-overexpressing cells (Figure 6b and Supplementary Figure S5). According to the western blotting results, overexpression of RRBP1 decreased the cleaved-PARP in the tunicamycin- and 2DG-treated cells and enhanced GRP78 protein expression (Figures 6c–f). Furthermore, GRP78 was knocked down in RRBP1-overexpressing cells and treated with 2 μM TM or 40 mM 2DG. After GRP78 knockdown, RRBP1-overexpressing cells showed

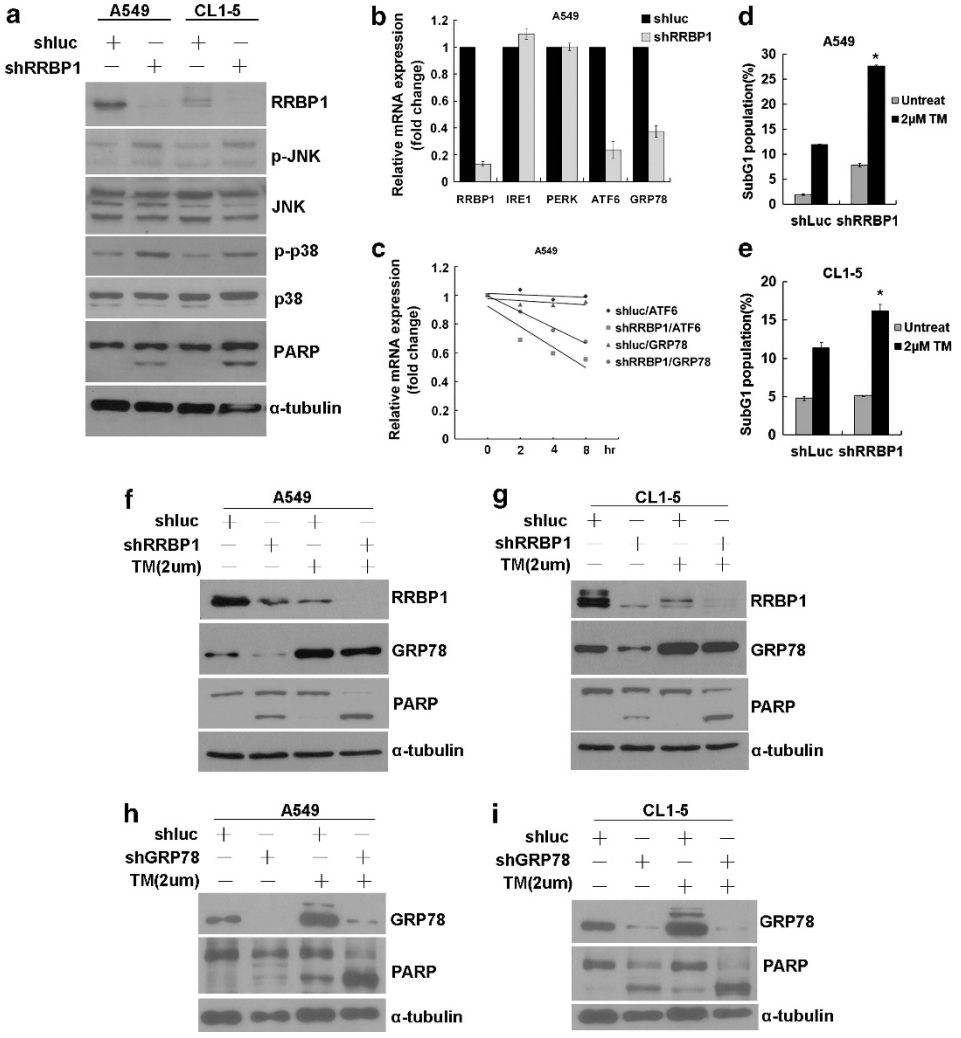

Figure 4. RRBP1-knockdown exacerbates tunicamycin (TM)-induced apoptotic cell death through the downregulation of GRP78 in human NSCLCs. (**a**) RRBP1-knockdown cell lysates were prepared, and the MAPK family was detected by specific antibodies (anti-p38 and anti-JNK). (**b**) RRBP1, IRE1, PERK, ATF6 and GRP78 mRNA levels were quantitated by qPCR in RRBP1-knockdown cells. (**c**) RRBP1-knockdown and shluc A549 cells were treated with 1 μg/ml actinomycin D for 0, 2, 4 and 8 h. Cell total RNA were extracted and reversed to cDNA. ATF6, GRP78 mRNA expression was analyzed by qPCR. (**d**, **e**) RRBP1-knockdown A549 and CL1-5 cells were treated with 2 μM TM for 24 h. The subG1 population was stained with propidium iodide and analyzed by flow cytometry. (**f**, **g**) A549 and CL1-5 cells were infected with RRBP1 shRNA and luciferase shRNA lentiviruses for 48 h, and cells were treated with or without 2 μM TM for 24 h. Cell lysates were prepared for detecting the cleaved form of PARP by western blotting. (**h**, **i**) A549 and CL1-5 cells were infected with GRP78 shRNA and luciferase shRNA lentiviruses for 48 h, and cells were treated with or without 2 μM TM for 24 h. Cell lysates were prepared for detecting the cleaved form of PARP by western blotting. Asterisks indicate statistically significant differences ($P<0.05$).

enhanced sensitivity to TM and 2DG treatment (Figures 6g and h). Our data showed RRBP1 would protect cancer cells from ER stress-induced apoptosis through GRP78. In addition, when cells were treated with 2 μg/ml actinomycin D for 0, 1, 2 or 4 h. GRP78 expression was significantly increased in RRBP1-overexpressing cells compared with controls (Supplementary Figure S6). We suggest that RRBP1 alleviates 2DG and tunicamycin-induced apoptosis by enhancing GRP78 expression. Taken together, our data suggest that RRBP1 may attenuate ER stress and help tumor survive during tumorigenesis.

Ectopic RRBP1 overexpression alleviates doxorubicin-induced apoptosis in lung cancer cells through the regulation of GRP78

A positive correlation was observed between RRBP1 and GRP78 expression in lung tumors. We suspect that RRBP1 could be an anti-apoptotic factor involved in the initiation of tumor development. We aimed to investigate the role of RRBP1 in clinical drug treatment. RRBP1-overexpressing PC13 and PC14 cells were treated with 0.1 μM doxorubicin. Cells were treated with doxorubicin, reactive oxygen species levels were detected by using DHE (dihydroethidium) dye (Figures 7a and b). Caspase 3/7 activity was also detected by using caspase 3/7 substrate. RRBP1 overexpression suppressed caspase 3/7 activity induced by doxorubicin treatment compared with vector controls (Figures 7c and d). Ectopic expression of RRBP1 increased resistance to doxorubicin, and cell lysates were prepared and analyzed by western blotting. The amount of the cleaved form of PARP was decreased significantly in the RRBP1-overexpressing cells compared with vector controls (Figures 7e and f). The subG1 population in PC13 and PC14 vector control cells was 46.45% and 36.35%, respectively. However, the subG1 population was 31.55% and 19.65% in RRBP1-overexpressing cells (Figure 7g). The subG1 population was significantly decreased in comparison to

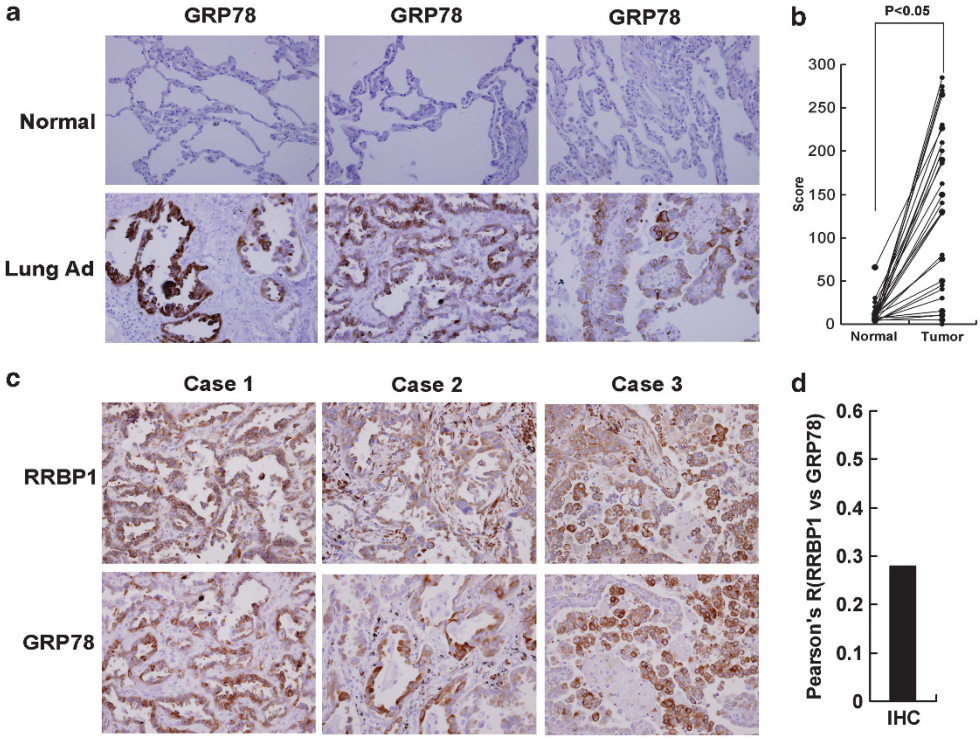

Figure 5. RRBP1 and GRP78 expression correlates in human primary lung tumors. (**a**) GRP78 IHC staining in lung cancer specimens. Protein overexpression was judged by comparing the staining intensity of NSCLC tumor cells and the adjacent normal Noveli cells. (**b**) Plot representation of scores according to the cytoplasmic IHC expression of RRBP1 in primary lung tumors related to paired normal tissue. The scores are calculated by intensity × percentage of stained cells. (**c**) IHC staining of RRBP1 and GRP78 in serial sections. (**d**) A positive correlation between RRBP1 and GRP78 expression was tested using Pearson's correlation test (correlation coefficient = 0.281, $P < 0.05$).

the vector control. In addition, RRBP1-overexpressing PC14 cell was also deprived of GRP78 and treated with 0.1 μM doxorubicin. Again, the deprivation of GRP78 in RRBP1-overexpressing cells showed more sensitive to doxorubicin treatment (Figure 7h), indicating that this is a GRP78-dependent effect.

DISCUSSION

In this report, we showed that RRBP1 is overexpressed in human NSCLC by both IHC and qPCR. We show that RRBP1 protein is upregulated in most lung cancer primary tumors, as compared with normal lung epithelial cells (Figures 1a and e). Most samples analyzed in our study were early-stage tumors (stages I and II), which suggests that RRBP1 overexpression is an early event in the progression of lung cancer. We have analyzed the contribution of RRBP1 overexpression to the maintenance of the transformed phenotype in NSCLC cells (Figure 2). Interestingly, the unique feature of RRBP1 primary structure is a highly conserved motif, which is repeat 54 times in tandem near the N terminus of the protein. Multiple splicing isoforms of human RRBP1 have been reported.[21] The multiple isoforms of RRBP1 are due to alternative splicing in the tandem repeat or intra–exonic splicing.[22–24] The biological functions of these isoforms remain unclear. To elucidate whether the multiple bands are all RRBP1 isoforms in our western blot result, we performed RRBP1 knockdown assay by different RRBP1 shRNA clones (Figure 2g, Supplementary Figures S3B and 3C). We also used rabbit IgG as isotype control for IHC analysis. No signals were detected in the tumor regions using rabbit IgG (Supplementary Figure S3A). We demonstrated that RRBP1 antiserum is specific to RRBP1 protein.

Previous studies have shown that RRBP1 is associated with the UPR. Inhibition of the UPR regulator GRP78 and PERK suppresses fibrosarcoma xenograft growth.[25,26] In our study, we suggest that RRBP1 is a key component for enhancing tumorigenicity both *in vitro* and *in vivo* (Figure 2). However, the subcutaneous microenvironment for human visceral tumors is different from their original environment. This difference may result in the lack of metastases, altered drug responses and tumor growth in the subcutaneous models. The orthotopic injection is an improvement for subcutaneous implantation.[27–30] In our data, knockdown of RRBP1 by shRNAs significantly reduced tumorigenicity in orthotopic lung model (Figure 3).

ER stress is coupled to the activation of stress-activated protein kinases.[31] These kinases, including JNK and p38 MAPK, are activated through a cascade of kinase activities. In our investigation, RRBP1-knockdown activated JNK and p38 (Figure 4a). Previous study, the UPR is an evolutionarily conserved mechanism that activates survival pathways to allow cells to adapt to ER stress.[32] Overexpression of GRP78 is prominent in a variety of tumors and protects tumor cells from ER stress.[33] The direct role of GRP78 in tumor formation has been shown in xenograft models in heterozygous GRP78 mice, in which reduced GRP78 protein expression results in the inhibition of tumor cell proliferation and increased apoptosis.[34] Knockdown of GRP78 induced cell death in human endothelial cells and enhanced tunicamycin-induced apoptosis, as compared with luciferase shRNA controls.[35] In our data, downregulation of RRBP1 expression significantly reduced GRP78 expression and sensitized cells to tunicamycin treatment (Figures 4f and g). These data suggest that RRBP1 has an important role in tumor cell survival, malignancy maintenance and adaptation to ER stress.

We also sought to determine whether RRBP1 expression is related to the expression of the UPR regulator GRP78. According to previous reports, three novel cell markers, AGR2, GRP78 and RRBP1, were also identified in the crypt.[36] In addition, several similarities exist between the induction of the UPR and the

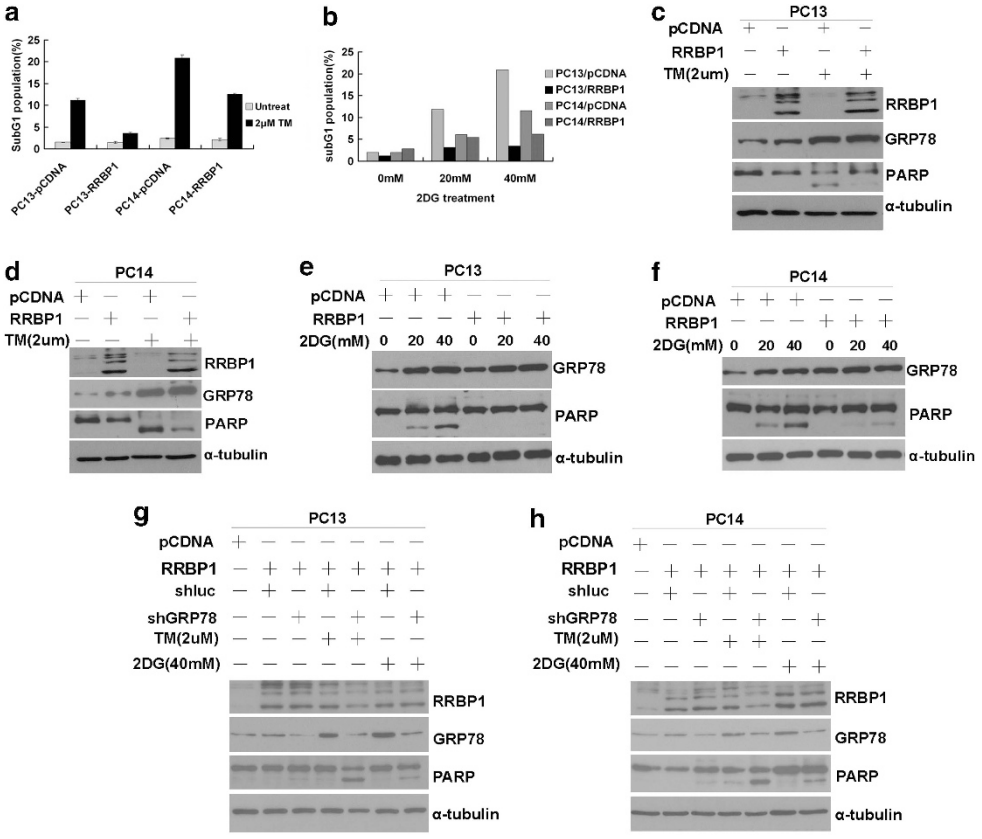

Figure 6. RRBP1 attenuated development of ER stress induced by tunicamycin (TM) and 2DG through GRP78 regulation. (**a**, **b**) RRBP1-overexpressing PC13 and PC14 cells were treated with 2 μM tunicamycin for 72 h and treated with 5, 20 or 40 mM 2DG for 48 h. The subG1 population was analyzed by flow cytometry. (**c**, **d**) PC13 and PC14 cell lysates were prepared from tunicamycin-treated, RRBP1-overexpressing cells, and antibodies were used to detect GRP78, cleaved PARP and tubulin. (**e**, **f**) PC13 and PC14 cell lysates were prepared from 2DG-treated, RRBP1-overexpressing cells and detected by anti-GRP78, anti-cleaved-PARP and anti-tubulin antibodies. (**g**, **h**) GRP78 was knocked down in RRBP1-overexpressing PC13 and PC14 cells. The cells were then treated with 2 μM tunicamycin and 40 mM 2DG for 48 h. GRP78 and PARP protein were detected by western blotting analysis.

proliferation of ER membranes upon expression of RRBP1. In both the processes, the levels of mRNAs encoding ER-localized chaperone genes are elevated.[37–39] In yeast, expression of RRBP1 resulted in an increase in the steady-state levels of the *KAR2* mRNAs but not an increase in transcription levels.[40] KAR2 protein sequence is homologous to mammalian BiP/GRP78.[41] In a recent report, RRBP1 acts as a general mRNA receptor on the ER, and its knockdown reduced the amount of ER-associated mRNA.[42] In our data, RRBP1-knockdown affected ATF6 and GRP78 mRNA stability (Figure 4c). Our data also demonstrated that RRBP1 and GRP78 expression was mainly distributed in the cytoplasm. Statistical analysis by Pearson's correlation test revealed that RRBP1 and GRP78 had a high correlation in lung cancer specimens in the microarray database GSE10072 (Supplementary Table S2).

During tumorigenesis, the maintenance of tumor cell survival is very important. In rapidly growing tumors, cancer cells suffer from microenvironments that are characterized by nutrient deprivation and acidosis. In our study, we mimicked ER stress by treating RRBP1-overexpressing cells with 2DG, tunicamycin or doxorubicin. Interestingly, we found that overexpression of RRBP1 can protect cells from ER stress. Doxorubicin is a quinone-containing anthracycline drug that is used widely as an anti-cancer chemotherapeutic agent. It is commonly used in the treatment for lung cancer, as well as some other types of cancer.[43,44] GRP78 is known to protect against various DNA-damaging agents, including adriamycin.[45,46] Some studies have shown that GRP78 conferred resistance against adriamycin-mediated apoptosis in cancer cells, at least in part through caspase-7 activation.[45,47]

In conclusion, our findings demonstrated that RRBP1 is associated with the UPR master regulator GRP78 in lung cancer. RRBP1 could be a key player in the maintenance of tumor cell survival under stress conditions. We believe that it is important to further elucidate the role that RRB1 has during lung cancer tumorigenesis.

MATERIALS AND METHODS

Cell lines and growth supplements

All lung cancer cell lines and the normal bronchial epithelial cell line, Beas2B, were obtained directly from the American Type Culture Collection. All lung cancer cell lines were cultured in either RPMI 1640 (H441, CL1-0 and CL1-5) or Dulbecco's modified Eagle's medium (A549, PC13 and PC14) supplemented with 0.1 mM non-essential amino acids, 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml). All of the cell lines were cultured in a humidified atmosphere with 5% CO_2. The ER stress agent 2DG and the tunicamycin were purchased from Sigma-Aldrich (Sigma-Aldrich, St Louis, MO, USA). Doxorubicin was purchased from Pfizer (Pfizer, New York, NY, USA). The vector pCDNA/RRBP1 was kindly provided by Dr Kiyoko, Japan Institute of Leather Research. All tissue culture reagents were obtained from Invitrogen (Carlsbad, CA, USA).

Patient samples and IHC analysis

The tissues used were obtained from Kaohsiung Medical University Hospital with IRB approval (KMUH-IRB-2011-0286). The surgical specimens had been fixed in formalin and embedded in paraffin before they were

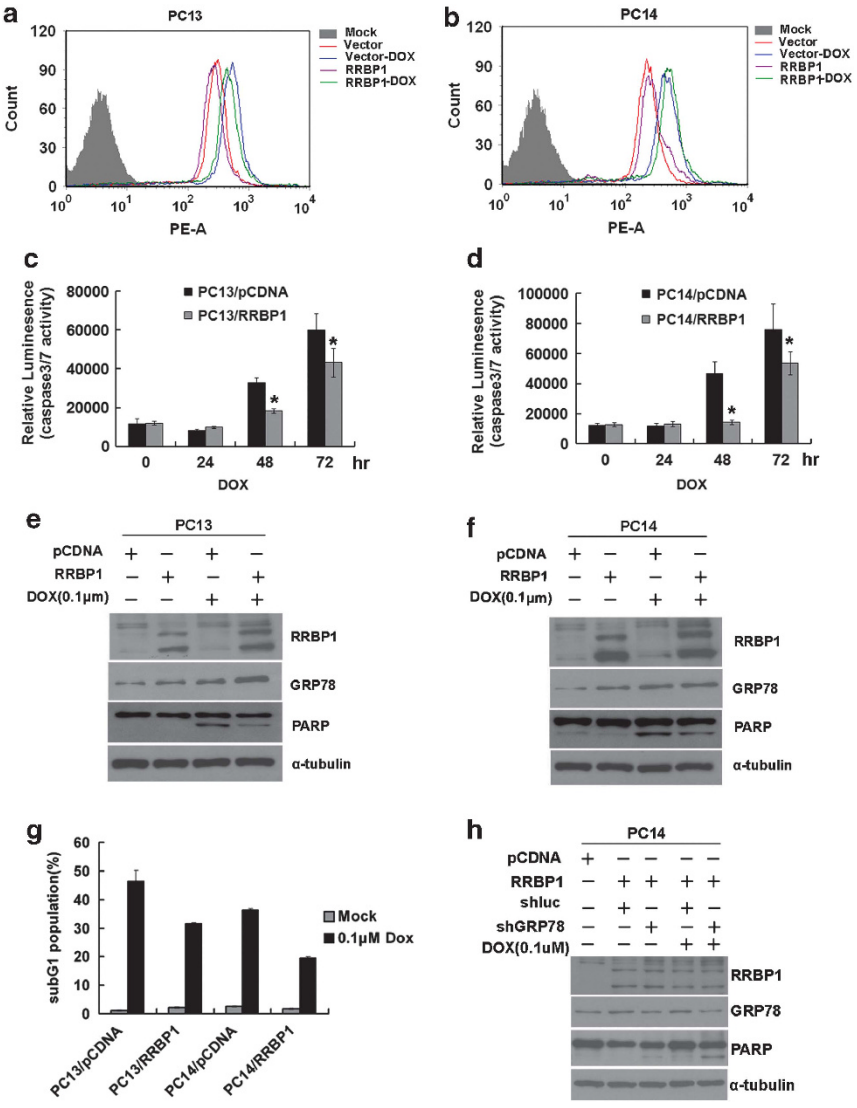

Figure 7. Ectopic expression of RRBP1 increases resistance to doxorubicin (DOX) treatment. RRBP1-overexpressing PC13, PC14 and pCDNA control cells that were treated with 0.1 μM doxorubicin for 72 h. (**a**, **b**) Reactive oxygen species was detected by adding 10 μM dihydroethidium into the tissue culture media for 30 min at 37 °C and analyzed by flow cytometry. (**c**, **d**) Caspase3/7 activity was detected by adding caspase3/7 substrate into the cell culture media and analyzed by luminometer. (**e**, **f**) Cell lysates were prepared and analyzed by western blotting. Cleaved PARP and GRP78 were detected by specific antibodies. (**g**) Cells were stained with propidium iodide and analyzed by flow cytometry. The subG1 population was analyzed between PC13/pCDNA, PC13/RRBP1, PC14/pCDNA and PC14/RRBP1 cells with doxorubicin or without doxorubicin treatment. (**h**) Western blot analysis of RRBP1, GRP78, cleaved PARP and α-tubulin in PC14/RRBP1 cells after GRP78 silencing and treated with 0.1 μM doxorubicin for 48 h.

archived. The histological diagnosis of the specific type of lung cancer was made according to the recommendations of WHO. Tumor size, local invasion, lymph node involvement, distal metastasis and final disease stage were determined according to the definitions from the TNM (tumor, lymph node, metastatis) classification of lung cancer.[48] Follow-up of patients was conducted up to 200 months. We used rabbit anti-RRBP1 (1:400, Sigma, St Louis, MO, USA), anti-GRP78 antibody (1:400, Cell Signaling, Boston, MA, USA), rabbit IgG antiserum (1:10 000, Southern Biotech, Birmingham, AL, USA) and anti-rabbit secondary antibody by Ventana IHC staining system (Ventana, Tucson, AZ, USA) to visualize the IHC staining signal. Sections were counterstained lightly with Mayer's hematoxylin and scored. The samples were considered to be 'negative' when the total score was 0 and 'positive' when the total score was $\geqslant 1$.

In vivo tumorigenicity assay

Age-matched non-obese, diabetic, severe combined immunodeficient, male mice (6–8-weeks old) were used as tumor xenograft models. To measure *in vivo* tumorigenicity, 5×10^6 cells with A549-luc, A549-shRRBP1, PC13/pCDNA, PC13/RRBP1 were resuspended in 100 μl of phosphate-buffered saline and injected subcutaneously. Mice were monitored each week for the development of tumors. The tumor growth was monitored by measuring tumor length (*L*) and width (*W*), and tumor volume was calculated as $1/2\ LW^2$. For orthotopic growth assays, cells (1×10^6 A549-luc-scramble, A549-luc-shRRBP1 cells) were resuspended in a equal volume of mixture of phosphate-buffered saline and GFR-Matrigel (BD Labware, Franklin Lakes, NJ, USA). This mixture was then injected into the left lateral thorax of each mouse. Lung tumor growth was monitored and quantified using the noninvasive bioluminescence system (IVIS-Spectrum, Perkin-Elmer Life Sciences, Waltham, MA, USA).[49,50] All animal studies were performed in compliance with the Academia Sinica Institutional Animal Care and Use Committee (IACUC) guidelines.

In vitro tumorigenicity assay

For the soft agar colony formation assays (FMC, Philadelphia, PA, USA), cell lines were cultured in six-well plates with a 0.35% agar layer. Cell lines were

seeded at 1000 cells per six-well cell culture dish and cultured for 14–21 days at 37 °C under 5% CO_2. Colonies were counted from the entire dish using light microscopy. The two-tailed Student's *t*-test was used to determine the differences between the groups.

Lentivirus production and infection

The lentiviral RRBP1 and GRP78 shRNA constructs were purchased from the National RNAi Core Facility at Academia Sinica, Taipei, Taiwan. The target sequences of these shRNAs are described in Supplementary Table S1. Lentiviruses were produced by cotransfecting the shRNA-expressing plasmid (pLKO.1), the envelope plasmid (pMD2.G) and the packaging plasmid (pCMV-dR8.91) into 293T cells using calcium phosphate. All 293T cells were incubated in a humidified incubator at 37 °C with 5% CO_2 for 18 h. The culture medium was changed on day 2, and the viral supernatants were harvested and titered. Monlayer cells were infected with shRNA lentiviruses in the presence of 8 μg/ml polybrene.

Quantitative real-time reverse transcriptase–PCR analysis

Cellular mRNAs were extracted, and cDNA was synthesized using the Stratascript cDNA synthesis kit (Stratagene, La Jolla, CA, USA). The cDNA products were analyzed using SYBR green dye and quantitative real-time PCR machine (Bio-Rad, Hercules, CA, USA). The reaction cocktail contained primer cDNA and 2X SYBR green qPCR master mix. The primers sequences used were as follows: human RRBP1, 5′-:AGTTCGGACCAG GTGAGGGAG CAC-3′(sense) and 5′-GCGTCTTCAGCTGAACGGGGTC CT-3′(antisense), human GRP78, 5′-GTGGCCACTAATGGAGATACTC ATC-3′(sense) and 5′-GC CCGTTTGGC CTTTTCTAC-3′(antisense), and human S26, 5′-CCGTGCCT CCAAGATGACA AAG-3′(sense) and 5′-ACTCAGCTCCT TACATGGGCTT-3′ (antisense). The data were analyzed using the Bio-RadiQ5 software (Bio-Rad).

Cell viability assay

Lung adenocarcinoma cells were transduced with RRBP1 shRNA lentiviruses, selected with 2 μg/ml puromycin (Sigma) and incubated for 72 h. Cells were trypsinized and seeded 2000 cells per well of a 96-well plate and incubated for 72 h. A549, H441, CL1-5, CL1-0 and PC14 cells were seeded at 2000 cells per well of a 96-well plate and incubated for 24 h. Cells were treated with 0, 0.1, 0.3, 1, 3 and 10 μM tunicamycin for 48 h. An equal volume of culture media containing CellTiter-Glo Reagent (Promega, Madison, WI, USA) was added to the cells and incubated for 15 min. The cell viability was then measured using VICTOR^3 (PerkinElmer Life Sciences).

Flow cytometry analysis

Cells were prepared and stained with propidium iodide (light sensitive). The samples were placed in 12 × 75 Falcon tubes and analyzed using a fluorescence- activated cell sorter (BD Biosciences, La Jolla, CA, USA). For cell-cycle analysis, 10 000 stained cells were sorted by flow cytometery and data were analyzed using the Modfit software (BD Biosciences).

Caspase 3/7 substrate assay

Before beginning the assays, the Caspase-Glo 3/7 Reagent was prepared (Promega). In all, 100 μl of Caspase-Glo 3/7 Reagent was added to each well of a white-walled 96-well plate containing 100 μl of blank, negative control cells or treated cells in culture media. Gently the contents of the wells was mixed using a plate shaker at 300–500 r.p.m. for 30 s. The plate was incubated at room temperature for 30 min, depending upon the cell culture system. The luminescence of each sample was measured in a plate-reading luminometer as directed by the manufacturer (PerkinElmer Life Sciences).

Reactive oxygen species generation measurement

Intracellular peroxide and superoxide free radical levels were measured by adding 10 μM dihydroethidium (DHE) (Sigma) into the tissue culture media for 30 min in a 37 °C incubator. In the presence of superoxides, the dihydroethidium dye probe is oxidized to ethidium. The observation wavelength was 585 nm for red fluorescence and measured by flow cytometry.

Western blot analysis

The cells were lysed in RIPA buffer. Western blot analysis was performed using the following antibodies: anti-RRBP1 (1:5000), anti-tubulin (1:10 000) from Sigma. anti-PARP (1:1000), anti-GRP78 (1:20 000), anti-p38 (1:500), anti-phosphorylated p38 (1:1000), anti-JNK (1:1000) and anti- phosphorylated JNK (1:500) from Cell Signaling. Acrylamide gel was prepared according to the manufacturer's instructions (Bio-Rad). The membranes were immersed in a 5% milk-phosphate-buffered saline Tween 20 solution to block non-specific background staining. The second antibody was horseradish peroxidase-labeled for enhanced chemiluminescence detection (1:5000, Jackson Immuno Research Laboratories, Suffolk, UK). The enhanced chemiluminescentchemical reagents were added to the membrane, and the membrane was exposed on film (KODAK, Rochester, NY, USA) in a dark room for optimal exposures (approximately 3–5 min) before final development.

Statistical analysis

Statistical evaluation was performed using SPSS software (version 11.05, SPSS Inc., Chicago, IL, USA). For the *in vitro* studies, each experiment was performed independently at least twice with similar results. Continuous data were presented as means ± s.e.ms., and two-group comparisons were performed using the Student's unpaired *t*-test. Pearson's rank correlations were used for comparison of the RRBP1 and GRP78 immunostaining. All statistical tests were two-sided, and $P<0.05$ was considered to be significant. Calculated *P*-values of $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***) were as indicated.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Miss Tracy Tsai for assisting in the pathological staining analysis. RRBP1 plasmids were kindly provided by Dr Kiyoko, Japan Institute of Leather Research. The study was supported by grants from the National Science Council and Academia Sinica.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)