LOSS OF P130 ACCELERATES TUMOR DEVELOPMENT IN A MOUSE MODEL FOR HUMAN SMALL CELL LUNG CARCINOMA

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Abstract

Small cell lung carcinoma (SCLC) is a neuroendocrine subtype of lung cancer. While SCLC patients often initially respond to therapy, tumors nearly always recur, resulting in a 5-year survival rate of less than 10%. A mouse model has been developed based on the fact that the *RB* and *p53* tumor suppressor genes are mutated in more than 90% of human SCLCs. Emerging evidence in patients and mouse models suggests that *p130*, a gene related to *RB*, may act as a tumor suppressor in SCLC cells. To test this idea, we used conditional mutant mice to delete *p130* in combination with *Rb* and *p53* in adult lung epithelial cells. We found that loss of *p130* resulted in increased proliferation and significant acceleration of SCLC development in this triple knockout mouse model. The histopathological features of the triple mutant mouse tumors closely resembled that of human SCLC. Genome-wide expression profiling experiments further showed that *Rb/p53/p130* mutant mouse tumors were similar to human SCLC. These findings indicate that p130 plays a key tumor suppressor role in SCLC. *Rb/p53/p130* mutant mice provide a novel pre-clinical mouse model to identify novel therapeutic targets against SCLC.

Keywords

p53; Rb; p130; mouse model; small cell lung carcinoma

INTRODUCTION

The prognosis for patients suffering from small cell lung carcinoma (SCLC) has not improved in the past 25 years. Despite advances in chemotherapy and radiation therapy regimens, 5-year survival rates for SCLC remain only 5-10%. At the time of diagnosis, SCLC has often already metastasized, and patients almost always relapse following treatment (1).

Based on the observation that the *RB* and *p53* tumor suppressor genes are both mutated in more than 90% of human SCLC, a mouse model for human SCLC has been generated. In this model, Cre-mediated deletion of *Rb* and *p53* conditional alleles in the lungs of adult mice results in

the development of tumors that share many characteristics with human SCLC, including their histopathology, the expression of neuroendocrine markers, and their ability to metastasize (2). This mouse model provides a system to identify genetic and epigenetic changes that may contribute to the development of SCLC in addition to loss of *Rb* and *p53* (2,3). In particular, emerging evidence suggests that p130, a cell cycle inhibitor related to RB (4), may normally suppress SCLC development. The first report describing loss of *p130* in a human cancer was in SCLC (5). In addition, low levels of the p130 protein have been associated with a higher histological grade, increased proliferation, and a trend toward poorer patient survival (6-8). Recent evidence further indicates that *p130* mRNA levels may be downregulated in lung cancer cells by members of the miR-17-92 microRNA cluster, which is often overexpressed in human SCLC and may play a role in the expansion of neuroendocrine cells (9,10). The possibility that p130 is a *bona fide* tumor suppressor has gained further support in mice where loss of *p130* cooperates with loss of *Rb* in retinoblastoma development (11) and in the development of small neuroendocrine lung lesions (12). Similarly, loss of E2F4, a major partner for p130, partially suppresses lung neuroendocrine hyperplasias in *Rb* mutant mice (13). Loss of *p130* also results in the acceleration of tumorigenesis in a mouse model for human lung adenocarcinoma (14). Interestingly, while p130 share many characteristics with p107, the third member of the RB family, there is no evidence that p107 may be involved in the suppression of SCLC (5,12). Here we tested the tumor suppressor role of *p130* in SCLC using a mouse genetics approach. We found that loss of *p130* accelerates the development of SCLC in *Rb/p53* mutant mice and that *Rb/p53/p130* mutant mice provide an improved mouse model of SCLC.

MATERIAL AND METHODS

Mice and adenoviral infections

Conditional mutant *Rb* and *p53* mice were bred to mice with a conditional allele for *p130* (2, 15) (Supplementary Fig. S1 and Supplementary Methods). Adenoviral infections were performed as described (14). Mice were maintained at the Stanford's Research Animal Facility accredited by the AAALAC.

DNA, RNA, and microarray analysis

Genomic DNA was prepared using lysis buffer containing Proteinase K (Sigma Aldrich). RNA was isolated from tumors or control lungs using TRIzol (Invitrogen). The Dynamo cDNA Synthesis Kit (New England Biolabs) was used to prepare cDNA from RNA. Real-time quantitative PCR was performed on an ABI Prism 7900HT Sequence Detection System with the SYBR GreenER qPCR mix (Invitrogen). The Ct value of each sample was normalized using the values for the TATA Binding Protein gene (*TBP*). Primer sequences are listed in Supplementary Tables S1 and S2. Affymetrix 430-2.0 arrays were hybridized and analyzed at the Stanford Microarray facility as described (15) (Supplementary Methods).

Immunoblot analysis and immunostaining

For immunoblot analysis (14), the antibodies used were: p130 (BD Biosciences, 610261), p107 (Santa Cruz, SC-318), Synaptophysin (Neomarkers, RB-1461-P1), Karyopherin beta 1 (Santa Cruz, SC-1919), and α-Tubulin (Sigma, T9026).

Paraffin sections were rehydrated in Trilogy reagent (Cell Marque). Sections were mounted in ProLong® Gold Antifade reagent (Invitrogen) after immunostaining (14). The primary antibodies used were: Phospho-Histone 3 Ser10 (PH3) (Millipore, 06-570), BrdU (Becton-Dickinson, 347580), Ki67 (BD Biosciences, 55069), MCM6 (Santa Cruz Biotechnology, sc-9843), PCNA (Santa Cruz Biotechnology, sc-56), Surfactant protein C (SP-C) (Dr. Jeff Whitsett, University of Cincinnati), Clara cell secretory protein (CCSP) (Santa Cruz Biotechnology, sc-9772), Synaptophysin (SYP) (Neomarkers, RB-1461-P1). Alexa Fluor®

secondary antibodies (Invitrogen) were used for antibody detection. Quantification was performed using the Bio-Quant image analysis software.

Statistics

Statistical significance was assayed by the Student's t-test with the Prism Graphpad software.

RESULTS AND DISCUSSION

To test the possibility that *p130* may act as a tumor suppressor gene in SCLC, we performed intranasal instillation of adenovirus expressing the Cre recombinase (Ad-Cre) in *Rb/p53/ p130* triple conditional mutant mice (Fig. 1*A*). After six months, primary lung tumors and liver metastases were detected at the surface of these organs; no other tumor types were identified in the mutant mice at this time point (Fig. 1*B* and data not shown, see below). PCR analysis on genomic DNA showed deletion of the three genes in the tumor cells but not in tail DNA in all cases examined (Fig. 1*C*). Quantitative RT-PCR analysis confirmed the decreased expression of these three genes in the triple mutant tumors (Fig. 1*D*). Finally, immunoblot analysis showed depletion of the p130 protein in all the triple mutant tumors analyzed (Supplementary Fig. S2). *p107* levels increased similarly in double and triple mutant tumors compared to wild-type lungs (Fig. 1*D* and Supplementary Fig. S2), indicating that loss of p130 does not induce *p107* expression in SCLC cells. Together, these experiments suggested that loss of *p130* was not counter-selected during tumorigenesis, allowing us to test the role of p130 in SCLC development. Recent observations indicate that the combined loss of *Rb* and *p130* is not sufficient for SCLC development in mice (16).The fact that we did not find tumors that were only *p53/p130* mutant is also suggestive that loss of *p130* does not alleviate the need for loss of *Rb* in SCLC, but this idea remains to be directly tested in *p53/p130* mutant mice.

To determine the functional role of p130 in SCLC development, we compared tumorigenesis in the lungs of *Rb/p53* and *Rb/p53/p130* mutant mice. We found that six months after Ad-Cre infection, few tumors were present at the surface of the lungs of *Rb/p53* mutant mice, as reported (2). In contrast, a number of tumors were visible in the lungs of *Rb/p53/p130* mutant mice (Fig. 1*B*). Histological examination confirmed that tumors were rarely found at this time point in *Rb/p53* mutant lung while tumors of various sizes were present in *Rb/p53/p130* mutant lungs (Fig. 2*A*). Even three months after Ad-Cre infection, numerous lesions were readily identified in sections of *Rb/p53/p130* mutant lungs while similar size lesions were observed six months after infection of *Rb/p53* mutant mice (Fig. 2*A*). Tumor burden was measured in the lungs of *Rb/p53/p130* mutant mice 6 months after Ad-Cre infection and was similar to that of *Rb/p53* mutant mice 9-12 months after infection (Fig. 2*B*). Not only did the triple mutant mice developed tumors more rapidly, they also developed significantly more tumors than the double mutant animals (Fig. 2*C*). We found that very few double mutant mice died before 28 weeks (data not shown), similar to what was described before (2). In contrast, one triple mutant mouse died at 17 weeks, another at 19 weeks, and two at 26 weeks; in addition, all the *Rb/p53/ p130* mutant mice analyzed at the 6 months time point were moribund. While we have not performed a complete survival analysis, these observations suggest that *Rb/p53/p130* mutant mice may die faster than *Rb/p53* mutant mice with the dose of Ad-Cre used in these studies. Thus, p130 is a potent suppressor of lung cancer initiated by loss of *Rb* and *p53* function in mice.

p130 is a known regulator of cell cycle progression (4), which led us to examine the proliferative status of double and triple mutant tumor cells *in vivo*. Immunostaining for Ki67, a marker of cycling cells, and PCNA and MCM6, two markers of S phase, showed that both double and triple mutant cells were actively proliferating (Supplementary Fig. S3). Quantitative analysis of the mitotic index of tumors using immunostaining for phospho-histone 3 (PH3) further indicated that triple mutant tumors had more cells in G2/M than double mutant tumors of similar

sizes (Fig. 2*D*). These data suggest that one mechanism of tumor suppression by p130 in SCLC is to limit the proliferation of *Rb/p53* mutant cells (see below), without excluding the possibility that p130 loss may accelerate SCLC development by other mechanisms. In particular, *p130* loss could change the type of lung cancer developing in the mutant mice. While histopathological analysis showed that a few lesions had some characteristic of lung adenocarcinomas, adenocarcinomas with neuroendocrine features, large cell neuroendocrine carcinomas, and large cell carcinomas, all these were present in mice from both genotypes. Furthermore, the large majority of tumors developing in *Rb/p53* and *Rb/p53/p130* mutant mice had clear architectural, cytological, and clinical features of human SCLC (Fig. 2*A*,Supplementary Fig. S4, and data not shown). Furthermore, although the number of mice analyzed thus far remains limited, another striking phenotype of the *Rb/p53/p130* mutant model is a high incidence of metastasis to the liver, one of the primary organs to which SCLC is known to metastasize in patients. Three out of five *Rb/p53/p130* mice had several liver metastases 6 months after Ad-Cre infection, while no lesions were identified in *Rb/p53* mice at the same time point and a lower incidence of liver metastasis was observed or previously reported at 9 months or later time points (Supplementary Fig. S4 and data not shown)(2). In addition, *Rb/p53p130 mice* developed metastases to pulmonary lymph nodes and kidney, two common sites of metastasis of human SCLC (Supplementary Fig. S4).

We next examined the expression of lung cancer markers in *Rb/p53/p130* mutant tumors. Immunostaining analysis showed that triple mutant lesions expressed Synaptophysin (SYP), a synaptic vesicle protein used as a marker for human SCLC, but were largely negative for SP-C and CCSP, two markers of lung alveolar and bronchiolar epithelial cells, respectively, similar to *Rb/p53* mutant tumors (Fig. 3*A*). Even one month after Ad-Cre infection, at a time when no lesions are visible in *Rb/p53* mutant mice, small triple mutant lesions were evident. Cells in these lesions were dividing as assessed by BrdU incorporation and expressed SYP (Fig. 3*B*). Moreover, quantitative RT-PCR showed that all *Rb/p53/p130* mutant tumors examined expressed typical markers of human SCLC such as *Cgrp*, *Syp, Ncam1*, and *Ascl1* (17) (Fig. 3*C*). Immunoblot analysis further indicated that the triple mutant tumor cells expressed the SYP protein (Fig. 3*D*). Cell lines derived from triple and double knock-out mutant tumors grew in floating clusters, similar to human SCLC cells (18); the mouse tumor cells also retained the expression of SYP, but, as expected, *Rb/p53/p130* mutant tumor cells did not express p130 (Supplementary Fig. S5).

To compare genome-wide expression profiles in *Rb/p53 and Rb/p53/p130* mutant tumors, we performed a microarray analysis with double mutant tumors 9 months after Ad-Cre infection and triple mutant tumors 6 months after Ad-Cre infection. While this difference in time could affect tumor evolution in the two models, the histology and the size of double and triple mutant tumors at these time points are similar (Figs. 2*A* and 2*B*). Significant Analysis of Microarrays (SAM) showed that only 151 genes were differentially expressed between the two genotypes out of total 14712 genes with a minimal 1.5 fold change of expression level and a false discovery rate of 10% or lower (Supplementary Fig. S6). This observation strongly indicates that tumors from the two genotypes are very similar at the level of global gene expression. Using DAVID bioinformatics tools, we found that a few of the differentially expressed genes were implicated in the control of cell cycle progression (Supplementary Fig. S6*A*).Quantitative RT-PCR analysis confirmed increased levels of the tyrosine kinase gene c-*Yes* and the DNA repair gene *Rad54l* in *Rb/p53/p130* mutant tumors compared to *Rb/p53* mutant tumors; in contrast, the expression of classical E2F targets such as genes coding for B-Myb, Cyclin E and PCNA was not significantly different (Supplementary Figure S7). The functional role of the genes whose expression is significantly different in triple knock-out tumors and the mechanisms underlying the increased proliferation of triple mutant cells versus double mutant cells will be investigated in future experiments.

We next compared the datasets from the mouse models to datasets from human SCLC and NSCLC (Non-Small Cell Lung Cancer); because SCLC patients rarely undergo surgery, less than 50 genome-wide transcription profiling microarray data for primary human SCLC samples are currently publicly available(19-21); the 13 datasets from double and triple mutant mouse tumors increase the number of primary tumors analyzed by more than 25%. A number of genes overexpressed in human SCLC were readily identified in the list of overexpressed genes in *Rb/p53/p130* and *Rb/p53* mutant tumors (Fig. 4*A*). Gene Set Enrichment Analysis (GSEA) further showed that the genes overexpressed in human SCLC were significantly enriched in *Rb/p53/p130* mutant tumors (Supplementary Fig. S8). Importantly, cross-specific clustering analysis showed that the two mouse models clustered with human SCLC rather than with normal lung or NSCLC (Fig. 4*B*). Taken together, these findings extend the similarity between the *Rb/p53* and the *Rb/p53/p130* mutant lung tumors and human SCLC from the histopathological level to global gene expression, further validating the *Rb/p53/p130* mutant mouse model.

In conclusion, the ablation of *p130* in the context of *Rb* and *p53* loss accelerates tumor development while maintaining the overall histopathological and molecular features of SCLC. Our data directly demonstrate that p130 serves a potent tumor suppression function in murine SCLC. Loss of p130 function results in increased proliferation, providing an explanation for why reduced p130 levels may be selected in human tumors. Compared to *Rb/p53* mutant mice, *Rb/p53/p130* mutant mice develop more tumors at a faster rate and will be instrumental in exploring the mechanisms of SCLC initiation, progression, and metastasis. Future studies will employ different doses of virus to change tumor burden and explore the long-term survival and phenotypes of the triple mutant mice. This novel mouse model for human SCLC will also serve to identify novel diagnostic markers and therapeutic targets against this deadly cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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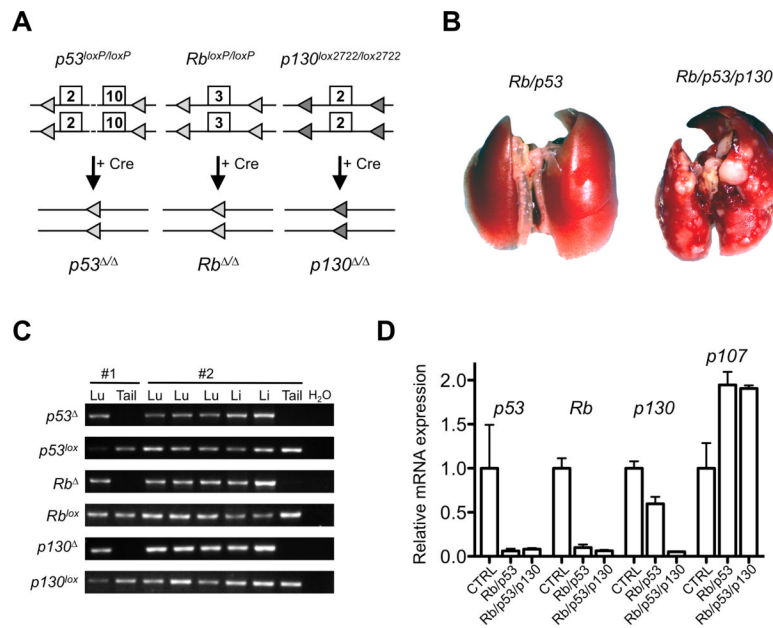
**Figure 1. Deletion of p53, Rb, and p130 in the lungs of adult mice leads to the development of lung tumors**

A, schematic representation of the conditional mutant alleles for *p53, Rb*, and *p130. B*, representative photographs of the lungs from double and triple mutant mice 6 months after Ad-Cre infection. *C*, PCR analysis for the deletion of the conditional alleles on genomic DNA obtained from lung tumors (Lu), liver metastasis (Li), and the tails of two representative mice (#1 and #2). *D*, RT-qPCR analysis of *p53*, *Rb*, p130, and *p107* expression levels in control lungs (CTRL) (n=3) and double (n=6) and triple mutant tumors (n=3).
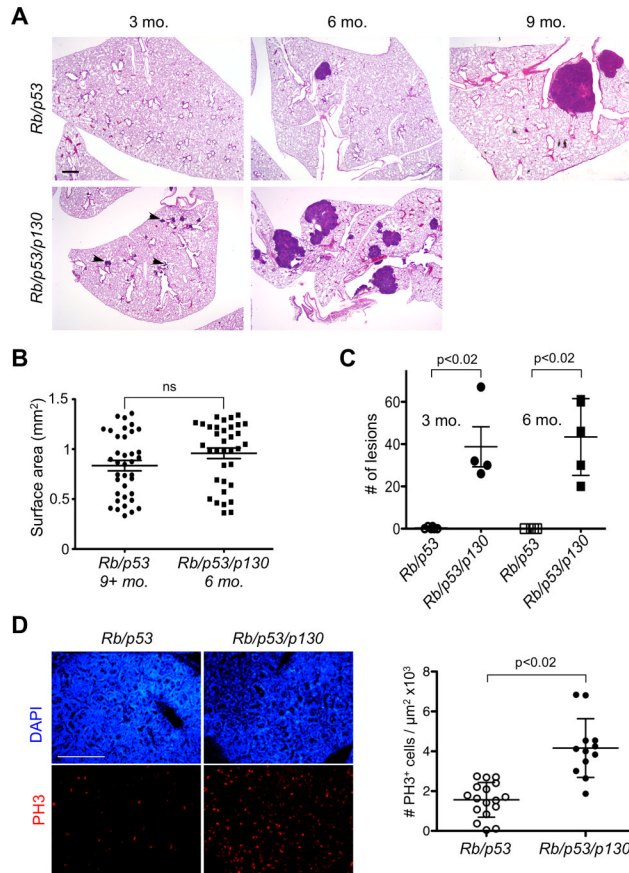
Figure 2. Loss of p130 results in accelerated lung cancer development in Rb/p53 mutant mice
A, hematoxylin and eosin (H&E) staining of sections from the lungs of double and triple mutant mice at three time points post-Ad-Cre injection – no triple mutant mice were alive at the 9 months time point. Bar: 400 μm. Arrowheads show small lesions. *B*, quantification of tumor surface area in triple mutant mice 6 months after Ad-Cre injection compared to double mutant mice 9-12 months after Ad-Cre infection. *C*, quantification of tumor numbers in double and triple mutant mice 3 and 6 months after Ad-Cre injection. *D*, analysis of proliferation in double (~9 months) and triple (~6 months) mutant tumors of similar size by immunostaining for phospho-histone 3 (PH3). Left: representative immunostaining (PH3, red – DAPI, blue). Right: quantification. ns, not significant. Bar: 50 μm.
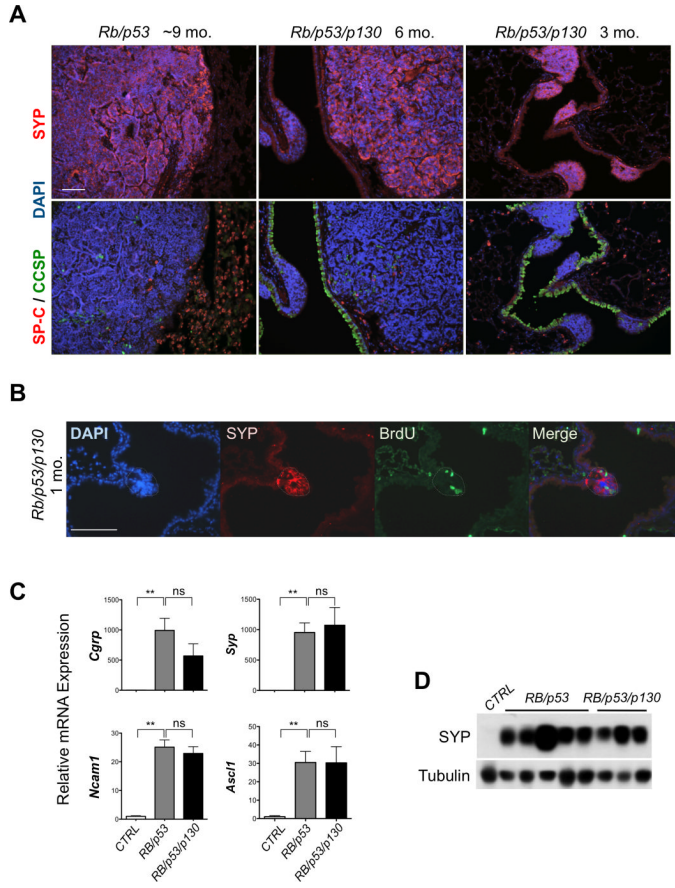
Figure 3. Rb/p53/p130 triple mutant tumors have features of neuroendocrine SCLC
A-B, immunostaining analysis of SCLC at different time points (1, 3, 6, and 9 months) for the expression of SYP (neuroendocrine), CCSP (bronchiolar), and SP-C (alveolar) and the incorporation of BrdU (S phase). DAPI stains the DNA in blue. The small lesion in a triple mutant mouse in *B* is circled by a dotted line. Bar: 50 μm. *C*, RT-qPCR analysis for the expression of four neuroendocrine genes in micro-dissected double (n=5) and triple (n=3) mutant tumors compared to control lungs (n=4). **, p<0.005. *D*, immunoblot analysis for SYP in double and triple mutant tumors compared to a control lung. Tubulin serves as a loading control.
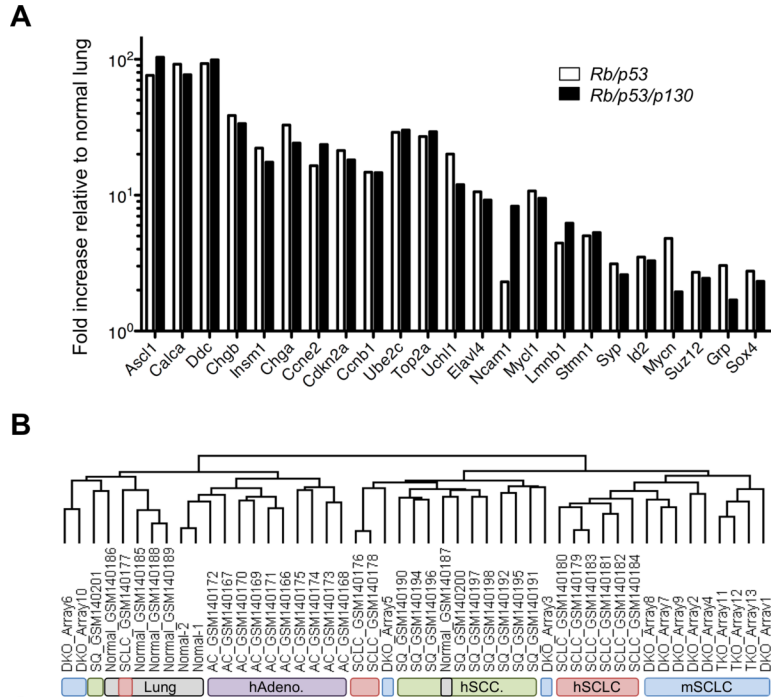
Figure 4. Rb/p53/p130 triple mutant mice provide a novel mouse model for human SCLC
A, Expression of selected genes previously found to be overexpressed and/or implicated in human SCLC is graphed as fold increase relative to control lungs. The values were obtained from SAM analysis of gene expression profiles from 3 *Rb/p53/p130* mutant tumors (dissected 6 months after Ad-Cre), 10 *Rb/p53* mutant tumors 9 months after Ad-Cre), and 5 control lungs. Noticeably, expression levels for most of the well-known genes are similar in both mouse models. The q-value for all the genes listed above is less than 0.05%. *B*, Expression profiles of genes in *Rb/p53/p130* and *Rb/p53* were compared to those of orthologous genes in human array datasets of lung cancers using hierarchical clustering. hAdeno: human adenocarcinoma; hSCC: human squamous cell carcinoma; hSCLC: human SCLC; mSCLC: mouse SCLC.