



Published in final edited form as:

Oncogene. 2013 February 14; 32(7): 903–909. doi:10.1038/onc.2012.99.

The Rac1 splice form Rac1b promotes K-ras-induced lung tumorigenesis

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Abstract

Rac1b, an alternative splice form of Rac1, has been previously shown to be upregulated in colon and breast cancer cells, suggesting an oncogenic role for Rac1b in these cancers. Our analysis of NSCLC tumor and matched normal tissue samples indicates Rac1b is upregulated in a significant fraction of lung tumors in correlation with mutational status of K-ras. To directly assess the oncogenic potential of Rac1b *in vivo*, we employed a mouse model of lung adenocarcinoma, in which the expression of Rac1b can be conditionally activated specifically in the lung. While expression of Rac1b alone is insufficient to drive tumor initiation, the expression of Rac1b synergizes with an oncogenic allele of *K-ras* resulting in increased cellular proliferation and accelerated tumor growth. Finally, we show that in contrast to our previous findings demonstrating a requirement for Rac1 in K-ras-driven cell proliferation, Rac1b is not required in this context. Given the partially overlapping spectrum of downstream effectors regulated by Rac1 and Rac1b, our findings further delineate the signaling pathways downstream of Rac1 that are required for K-ras driven tumorigenesis.

Introduction

The Rac proteins are small G-proteins that harbor a GTPase-like domain and bind to guanine nucleotides. They function as molecular switches that cycle between an “ON” state

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Conflict of interest

The authors declare no conflict of interests.

when bound to GTP and an “OFF” state when bound to GDP. The Rac proteins are tightly regulated by various groups of proteins (1), including Rho-GEFs (Guanine Exchange Factors), which promote binding to GTP and Rho-GAPs (GTPase activating protein) that promote the hydrolysis of GTP to GDP by the Rac proteins. In addition, Rho-GDI (GDP-dissociation inhibitor) sequesters Rac-GDP in the cytoplasm and prevents exchange of GDP to GTP. The Rac proteins are master regulators of diverse signaling pathways that control the shape, motility and growth of cells. These are processes that often go awry in cancer. Therefore, there is considerable interest in establishing whether the deregulation of these Rac-controlled pathways plays an initiating and promoting role in tumorigenesis (2, 3). While many studies have implicated these pathways in various forms of human malignancies, in the vast majority of cases direct evidence has been elusive.

An alternatively spliced form of Rac1 has been identified and termed Rac1b. Rac1b is characterized by insertion of 19 residues immediately C-terminal to the “switch II” domain (residues 60–76) and this insertion greatly reduces the intrinsic GTPase activity of Rac1b and impairs its binding to RhoGDI. Thus Rac1b is preferentially in a GTP-bound, active, form (4). Rac1b has been shown to induce cyclin D1 transcription and transform cells, via NF- κ B, by inducing the phosphorylation of the NF- κ B inhibitor I κ B (5). Importantly, when compared to Rac1, Rac1b only poorly activates the p21-activated kinases (PAKs) or Jun N-terminal Kinase (JNK) (4). The expression of Rac1b in fibroblasts stimulated cell-cycle progression and survival under conditions of serum starvation (5). Interestingly, it has been recently shown that Rac1b mediates an MMP-3-epithelial to mesenchymal transition (EMT) in cultured cells, through the induction of Reactive Oxygen Species (ROS) (6). It has also been recently reported that Rac1b promotes canonical Wnt signaling, a pathway often deregulated in colon cancer (7). Finally, Rac1b levels were recently found to be upregulated in breast and colon cancer, suggesting an oncogenic role for Rac1b (8, 9).

To assess the role of Rac1b in lung tumorigenesis we examined a panel of NSCLC tumors and determined that Rac1b is upregulated in a significant number of tumors. Moreover, employing an endogenous mouse model of K-ras-driven lung adenocarcinoma in which expression of Rac1b is conditionally activated demonstrated that expression of Rac1b at physiological relevant levels promotes tumor progression with accelerated kinetics, further supporting an oncogenic role for Rac1b in NSCLC.

Results

Rac1b is upregulated in human lung adenocarcinoma

Previous reports have indicated Rac1b expression is upregulated in human breast and colon cancers (8, 9). We therefore sought to determine whether Rac1b is also upregulated in lung cancer. Total protein from six human NSCLC cell lines were analyzed by western blotting using a monoclonal antibody to Rac1. Using this Rac1-specific antibody, we were unable to detect endogenous Rac1b expression, unless Rac1b is overexpressed by introduction of an exogenous expression vector (Figure 4B). We therefore generated polyclonal antibodies that specifically recognize the human Rac1b splice form (Figure S1). Using this antibody we were able to detect Rac1b protein in six NSCLC cell lines, at varying degrees of expression (Figure 1A). To assess the expression of Rac1b in primary tumor samples, twenty-two

matched pairs of normal and tumor samples from human lung adenocarcinoma were analyzed by western blot using the same polyclonal anti-Rac1b antibody. We find that Rac1b is significantly upregulated, although at variable rates (a range of 1.8 to 34.5 fold change), when compared to Rac1, in more than 60% of the samples examined (14/22 samples; Representative examples shown in Figure 1B). To determine whether Rac1b expression is correlated with mutational status of the K-ras, we assessed the status of the K-ras allele in the tumor samples by direct sequencing. Of the 22 tumor samples examined, 19 were informative and 7 of these displayed activating mutations in K-ras codon 12. All seven tumors with mutated K-ras also displayed upregulated expression of Rac1b, indicating a significant correlation between the two. This high ratio of Rac1b to Rac1 expression correlates with the expression patterns previously determined for Rac1b in other tumor types, implying elevated expression of Rac1b might provide an advantage to tumor cells.

Expression of Rac1b promotes K-ras-induced lung adenocarcinoma

To assess directly whether Rac1b can promote tumor initiation, we developed a mouse model in which expression of Rac1b can be conditionally activated under the control of a constitutive promoter (*Rosa26-LSL-Rac1b*). Importantly, the Rac1b cDNA used in these mice was of human origin to allow distinction of endogenous versus exogenous Rac1b, employing antibodies specific to either mouse or human proteins, respectively (Figure S1). The *Rosa26-LSL-Rac1b* mice were infected with an adenovirus expressing Cre-recombinase (Ad-Cre) by intranasal instillation, which leads to removal of a transcriptional stop element and activation of the Rosa26 promoter in infected cells in the lung. Mice were aged for up to 30 weeks post infection, however no evidence of hyperplasia or tumors was detected in the lungs of these mice (not shown). To assess whether Rac1b can cooperate in the initiation and/or progression of K-ras-induced lung tumors, we employed the *LSL-K-ras^{G12D}* mouse model of lung adenocarcinoma. In this model, infection of the lungs with an adenovirus expressing Cre-recombinase (Ad-Cre) results in the removal of a transcriptional stop element and activation of an oncogenic allele of *K-ras^{G12D}* under physiological control. Infected animals will develop numerous areas of hyperplasia, followed by adenoma and adenocarcinoma formation (10). Mice with a conditional activated allele of Rac1b, under the control of the Rosa26 promoter (*Rosa26-LSL-Rac1b*) were crossed to the *LSL-K-ras^{G12D}* animals to generate *LSL-K-ras^{G12D}; Rosa26-LSL-Rac1b* mice. In these mice, Cre-mediated recombination should result in the activation of both the *K-ras^{G12D}* and *Rac1b* alleles.

Following intranasal administration of Ad-Cre, mice from the *LSL-K-ras^{G12D}* and *LSL-K-ras^{G12D}; Rosa26-LSL-Rac1b* groups were sacrificed at 12, 18 and 24 weeks post-infection. Both tumor number and volume were assessed by histological examination. As shown in Figure 2A, the *LSL-K-ras^{G12D}; Rosa26-LSL-Rac1b* and *LSL-K-ras^{G12D}* mice displayed similar tumor numbers at 12 and 18-weeks post tumor initiation, with tumor numbers averaging between 4–5 tumors at 12 weeks and 9–10 tumors at 18 week per animal. At 24 weeks the tumors were too large and numerous in both groups that individual tumors could not be scored.

In addition to assessment of tumor initiation, we examined the ratio of tumor volume to lung volume (T/L ratio) at the various time points, as an indicator of overall tumor burden. In the

LSL-K-ras^{G12D} mice the tumor to lung volume ratio was at 3.0 % at 12 weeks post infection and T/L ratio was 4.0% and 7.0% at the 18 and 24-week time points, respectively (Fig. 2B). In the *LSL-K-ras^{G12D};Rosa26-LSL-Rac1b* mice the T/L ratio was at about 3.0% at 12 weeks and rose to approximately 9.0% and 17.0% at the 18 and 24-week time points, respectively. Thus, while no differences in T/L ratio were observed at the 12-week time point, differences began to emerge at 18-weeks and are fully significant at 24-weeks post tumor initiation ($p < 0.05$). These data indicate that expression of Rac1b synergized with oncogenic K-ras to further promote tumor progression.

To assess the status of Rac1b expression in the tumors, several tumors from the various groups at 18 weeks post infection were dissected and protein was extracted. Rac1b expression was evaluated by Western-blotting using the antibody specific to human Rac1b. All tumors examined from the *LSL-K-ras^{G12D};Rosa26-LSL-Rac1b* mice group displayed Rac1b expression. As expected, tumors from the *LSL-K-ras^{G12D}* mice group did not display human Rac1b expression (Figure 2C).

Histological examination of the tumors and pre-cancerous lesions that arose in the different groups indicates that they were similar. In both groups three distinct types of lesions were found. Atypical adenomatous hyperplasia (AAH) was present at 12-week post infection in both groups of mice, as were small papillary adenomas. At the 18 and 24 week time points adenocarcinomas were observed in both genotypes, although tumor sizes were significantly larger in the *LSL-K-ras^{G12D};Rosa26-LSL-Rac1b* mice (Figs. 2B and 3).

These findings indicate that while Rac1b alone is insufficient to drive lung tumorigenesis it cooperates with K-ras to accelerate tumor growth.

Expression of Rac1b accelerates lung cancer cell proliferation in vivo

To assess how expression of Rac1b might promote tumorigenesis *in vivo*, we compared the rates of cell proliferation and apoptosis between tumors in the *LSL-K-ras^{G12D}* and *LSL-K-ras^{G12D};Rosa26-LSL-Rac1b* mice. To determine rates of apoptosis and proliferation, lung sections from 24 weeks post infection were stained with an antibody against cleaved caspase 3 or ki-67, respectively. Similar rates of cleaved caspase 3 staining were observed in similar grade tumors from the *LSL-K-ras^{G12D}* and *LSL-K-ras^{G12D};Rosa26-LSL-Rac1b* mice, indicating no significant difference in apoptosis rates between the two genotypes (Not shown). Staining for ki-67 revealed a significant although subtle increase in rate of proliferating cells in the *LSL-K-ras^{G12D};Rosa26-LSL-Rac1b* tumors compared to *LSL-K-ras^{G12D}* tumors, as shown in Figure 3B–C.

To investigate how Rac1b affects lung cancer cell functions and biological behaviors *in vitro*, A549 human lung cancer cells, which carry an activating mutation of K-ras and express low levels of Rac1b (Figure 1A), were infected with a retroviral vector expressing mouse Rac1b (A549-Rac1b) or empty vector control (A549-pBabe). The A549-Rac1b cells exhibited the same growth rate as the A549-pBabe cells, indicating that exogenous Rac1b expression does not accelerate cell proliferation (Figure 4A). Western-blotting analysis with an anti-Rac1 antibody confirmed the overexpression of Rac1b in the A549-Rac1b cells (Figure 4B). Similar results were obtained with LKR13, a mice derived lung

adenocarcinoma cell line with an oncogenic K-ras mutation which express low levels of endogenous Rac1b (Figure 5B). As shown in Figure 5A overexpression of Rac1b in LKR13 cells did not accelerate cell proliferation.

Expression of Rac1b is not required for proliferation of K-ras driven lung adenocarcinoma cells

We have previously shown that Rac1 is required for K-ras induced transformation (11). We therefore wished to assess whether Rac1b is likewise required. Towards this aim we ablated expression of both or either one of the two isoforms in LKR13 cells. To suppress Rac1b only while sparing Rac1, we designed two siRNA oligos that target the unique sequences of Rac1b splice form (Figure 5C). Using these siRNA we are able to specifically knockdown Rac1b without effecting Rac1 levels (Figure 5D). Likewise, to target Rac1 only without impairing Rac1b expression we developed an siRNA duplex against the sequences flanking the Rac1b specific coding sequences (Figure 5C). Using these oligos we were able to knockdown Rac1 only, without effecting Rac1b levels (Figure 5D).

We next examined the effects of Rac1 and Rac1b knockdown on the proliferation of LKR13 cells. Consistent with our previous findings, knockdown of Rac1 alone (Rac1-SI#1) or of Rac1 and Rac1b together (Rac1-Pool) was incompatible with cell proliferation. In contrast, the knockdown of Rac1b only in LKR13 cells did not have an effect on cell proliferation (Figure 5E). One of the previously reported differences between Rac1b and Rac1 is the inability of Rac1b to activate the p21-activated kinases (PAKs) (4, 12, 13). To assess this in our system we examined the phosphorylation state of PAK1 and 2 (p-PAK1/2), which correlates with the activation state of the kinases. Consistent with previous reports, we find that the overexpression of Rac1b did not result in activation of Pak1 or Pak2 (Figure 5B). Likewise, the knockdown of Rac1b fails to recapitulate the reduction in activated p-PAK1/2 levels that are observed when Rac1 is knocked down (Figure 5D). These findings indicate that expression of Rac1b is not required for proliferation of lung cancer cells carrying an oncogenic K-ras allele.

Discussion

Previous studies have demonstrated the elevated expression of Rac1b in human breast and colorectal tumors. Similarly, we find that Rac1b is upregulated in a significant fraction of human lung adenocarcinomas examined. These findings suggest a tumor-promoting role for Rac1b in these cancers. Corroborating evidence to support this notion comes from in vitro studies in which the overexpression of Rac1b was shown to promote malignant transformation in a variety of cancer cell lines. In the current study, we focused on the tumor-promoting function of Rac1b in lung adenocarcinoma, in vivo. Towards this aim, we developed a mouse model with conditional activation of Rac1b expression. Using this model, we find that Rac1b alone is not sufficient to drive lung tumor initiation.

Analysis of K-ras status in the NSCLC lung adenocarcinomas indicated that activating K-ras mutations correlate significantly with elevated Rac1b expression. However, a significant number of tumors demonstrating elevated Rac1b expression were wild type for K-ras, suggesting that other mutations, perhaps in EGFR, might drive elevated Rac1b levels.

Interestingly, previous studies in colorectal tumors demonstrate a correlation between B-Raf mutations and Rac1b upregulation, but no correlation with K-ras mutations (14). These observed differences might be a reflection of the different tumor types examined.

Thus, while Rac1b overexpression alone did not promote tumorigenesis, the correlation with K-ras mutations suggests Rac1b could cooperate with K-ras activation. Indeed, by combining the *Rosa26-LSL-Rac1b* mice with a conditionally-activated oncogenic allele of K-ras, we find that Rac1b synergizes with oncogenic K-ras to facilitate lung cancer progression, *in vivo*. Comparative analysis of similar stage tumors from the different genotypes demonstrated no differences in rates of cell death, but did show a statistically significant increase in rates of cell proliferation in the tumors in *LSL-K-ras^{G12D};Rosa26-LSL-Rac1b* mice compared to the *LSL-K-ras^{G12D}* mice. While the difference is small, over time it compounds to manifest as a significant increase in the tumor growth observed *in vivo*. Of note, our cell based studies in both human and mouse lung tumor cells that carry oncogenic K-ras mutations did not reveal a growth advantage when Rac1b is overexpressed. Given the subtle growth advantage conferred by Rac1b overexpression *in vivo*, the effects on proliferation in cultured cells might be below the level of detection possibly due to these cells being already at a maximal proliferative rate through the activity of other pathways. Further *in vivo* analysis will be required to unveil these mechanisms.

Our finding that Rac1b is not required for K-ras induced lung tumorigenesis stands in contrast to our previous findings demonstrating a requirement for Rac1 (11). As an alternative splice variant of Rac1, Rac1b exhibits different features from Rac1. The extra 19 amino acid insertion in Rac1b accelerates GDP/GTP exchange and decreases hydrolysis of GTP. Rac1b is impaired in interaction with RhoGDI and the p21-activated kinases (PAKs) and fails to stimulate Rac1 signaling effectors such as c-Jun-NH₂-kinase and p38 mitogen-activated protein kinase (4, 15). Our studies in which we specifically knockdown either Rac1 or Rac1b demonstrate the specific requirement for Rac1 for cell proliferation. This suggests that signaling pathways regulated by Rac1, but not Rac1b, are likely to be required for K-ras induced transformation. Previous studies have indicated that Rac1b shows a diminished ability to activate the PAKs (4, 15). In support of this we find that overexpression of Rac1b did not enhance Pak1/2 activity. Likewise, in contrast to the knockdown of Rac1, the knockdown of Rac1b does not impact the activation state of the PAKs, which have been previously shown to be required for K-ras induced transformation in multiple tumor types (16). Finally, recent studies have also suggested Rac1b can interact with downstream effectors that are not engaged by Rac1, such as p120 catenin (17). Our findings therefore imply that these Rac1b-specific functions are not required for cell proliferation in the context of oncogenic K-ras.

Materials and methods

Antibodies

A polyclonal antibody specific to human Rac1b was produced by immunization of rabbits with the Ac-VGETYGGKDI¹SRGKDKPIAC-amide peptide. A polyclonal antibody specific to mouse Rac1b was produced by immunization of rabbits with the Ac-VGDTGKDRPSRGKDKPIAC-amide peptide. The specificity of the antibodies was

validated in cells by western blotting, employing HEK293 cell, expressing a murine Rac1b-YFP fusion protein or human Rac1b (Figure S1). Other antibodies employed are commercially available: Rac1 (Millipore, cat. no.: 05-389), p-Pak1/2 (Cell signaling technology cat # 2606), α -Tubulin (Sigma, cat. no.: T5168).

Tumor Samples

After obtaining informed consent from patients with adenocarcinomas, tumor samples and samples from adjacent non-tumor-involved lung were snap-frozen in liquid nitrogen. All patients had clinically resectable, early stage (Stage 1 or 2) tumors. Samples were homogenized and extracted with RIPA lysis buffer (50 mM TRIS-HCl, pH 7.5, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, and 1 mM NaF) and analyzed by SDS-PAGE followed by western blotting. Relative levels of Rac1b expression were calculated using ImageJ software to calculate the density of the relevant signal in each lane and adjusted to the relative density of Rac1 in the same lane.

Analysis of K-ras status

Genomic DNA was extracted from tumor samples using the GenElute Mammalian Genomic DNA Miniprep kit, according to the manufacturer's instructions (Sigma-Aldrich). 100ng of genomic DNA were used as template in a PCR reaction using the HotStarTaq polymerase and buffer (Qiagen) and primers to amplify K-ras exon 2: 5'-AAGGCCTGCTGAAAATGACTG-3' and 5'-CAAAGAATGGTCCTGCACCAG-3'. PCR products were run on a gel to verify a single amplification product (173bp) and purified using the MinElute PCR purification Kit (Qiagen). The purified DNA product was sequenced using 5'-CAAAGAATGGTCCTGCACCAG-3' as a primer and chromatograms were analyzed with 4peaks software package for mutations.

Plasmids and RNAi

The expression vector for human Rac1b was purchased from Open Biosystems (pCMV-XL5-hRac1b). The expression vector for the mouse Rac1b-YFP fusion was a Gift from Dr. Radisky (Mayo Clinic). The mouse Rac1b cDNA was cloned into the pBabe vector using the Sal I and EcoR I cloning sites. The siRNA used to knock-down mouse Rac1 and Rac1b simultaneously was the Rac1 Smartpool from Dharmacon (cat. no.: 100041170). The following siRNA sequences were used to knockdown Rac1 only (AGACAGACGUGUUCUAAUUU) or Rac1b only (GACACAUGUGGUAAGAUAAU and UGGAGACACAUGUGGUAAGAUAAU).

Mice

The *LSL-K-ras^{G12D}* mice were generated as previously described (10, 18). The Rosa26-LSL-Rac1b mice were generated by knocking-in the human Rac1b cDNA into the Rosa26-Lox-Stop-lox locus, using the pBigT and pRosa26-PA vectors, as previously described (19). Analysis of the recombined Rac1b allele in the LSL-Rac1b mice was performed by PCR using the following primers: P1 = 5'-CAGTAAGGGAGCTGCAGTGG-3', P2 = 5'-

GCGAAGAGTTTGTCTCAACC-3. The PCR program was set as 95°C for 5' followed by 30 cycles of 95°C for 1'; 52°C for 1'; 72°C for 90".

Tumor model and Histological analysis

Animals were sacrificed at the indicated time points. Tissue harvesting and histological analysis were performed as previously described (20). The proliferative index of tumors was assessed by counting the ratio of ki-67 positive cells to total cells in a given microscopic field. For each genotype, 6 animals from the 24 week time point were examined and multiple similar grade tumors were scored.

Statistics

Statistical significance of tumor to lung volume ratio was determined by Wilcoxon rank-sum (Mann-Whitney) test. For analysis of tumor proliferative index (ki-67 over total cell count), the effects of Kras;Rac1b vs. Kras alone were examined by a mixed-effect model with mouse as random-effect. The results show that Kras;Rac1b tumors have significantly higher ratio of ki-67 over total cell counts ($p=0.006$). Two-tailed unpaired student's t test was applied to all the other analysis. P values ≤ 0.05 were considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Derek Radisky for sharing the pEYFP-C1-mRac1b vector and members of the Kissil and Albelda labs for technical assistance. We thank the Wistar Transgenics and Animal core facilities. This work was supported by grant CA124495 (J.L.K).

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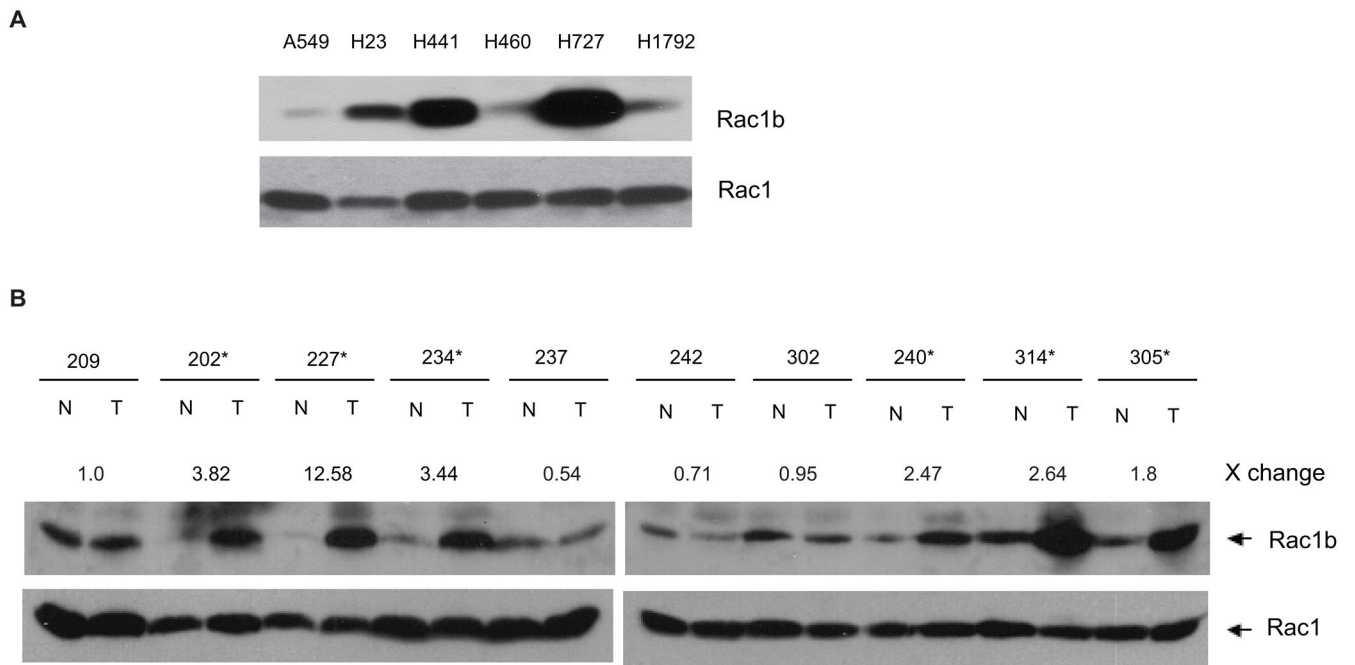


Figure 1. Analysis of Rac1b expression in human lung adenocarcinomas
 Protein was extracted from (A) human lung adenocarcinoma cell lines and (B) matched pairs of normal and tumor samples from human lung tumors. Protein levels of Rac1 and Rac1b were analyzed by western-blotting, using either an anti-Rac1 antibody or anti-human Rac1b specific antibody, as indicated. Ten out of twenty two pairs analyzed are shown. Samples shown upregulation of Rac1b are indicate by “*” and fold change levels are indicated below. N= normal adjacent tissue, T= tumor tissue.

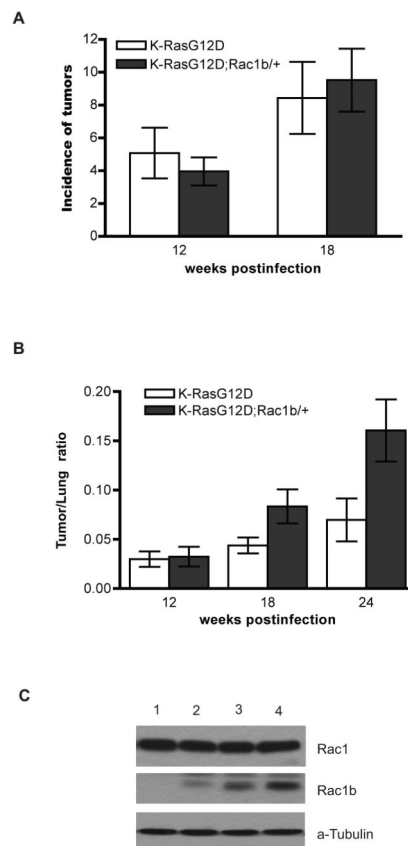


Figure 2. Analysis of lung tumorigenesis in *LSL-K-ras^{G12D}* and *LSL-K-ras^{G12D}; Rosa26-LSL-Rac1b/+* mice

Mice were treated with Ad-Cre and sacrificed at the indicated time points. **(A)** average incidence of tumors at the 12- and 18-week time points. The difference in tumor incidence is not statistically significant at both time points. Bars, SD; $p > 0.05$. **(B)**, average tumor to lung volume ratios at the different time points. Wilcoxon rank-sum (Mann-Whitney) test was used to compare the tumor to lung ratios between the two mouse groups. No significant differences were observed at weeks 12 ($p=0.81$) or 18 ($p=0.08$). However a significant difference was observed at week 24 ($p=0.02$). Bar represents mean volume ratio \pm SD. Numbers of animals analyzed are 5 *LSL-K-ras^{G12D}* and 10 *LSL-K-ras^{G12D};Rosa26-LSL-Rac1b/+* at each time point. **(C)** Rac1b expression in tumors. Tumors were excised from the *LSL-K-ras^{G12D}* mice at 18-week (lane 1) and *LSL-K-ras^{G12D};Rosa26-LSL-Rac1b/+* mice at 18-week (lanes 2–4). Protein was extracted and probed with an anti human Rac1b antibody by Western-blotting.

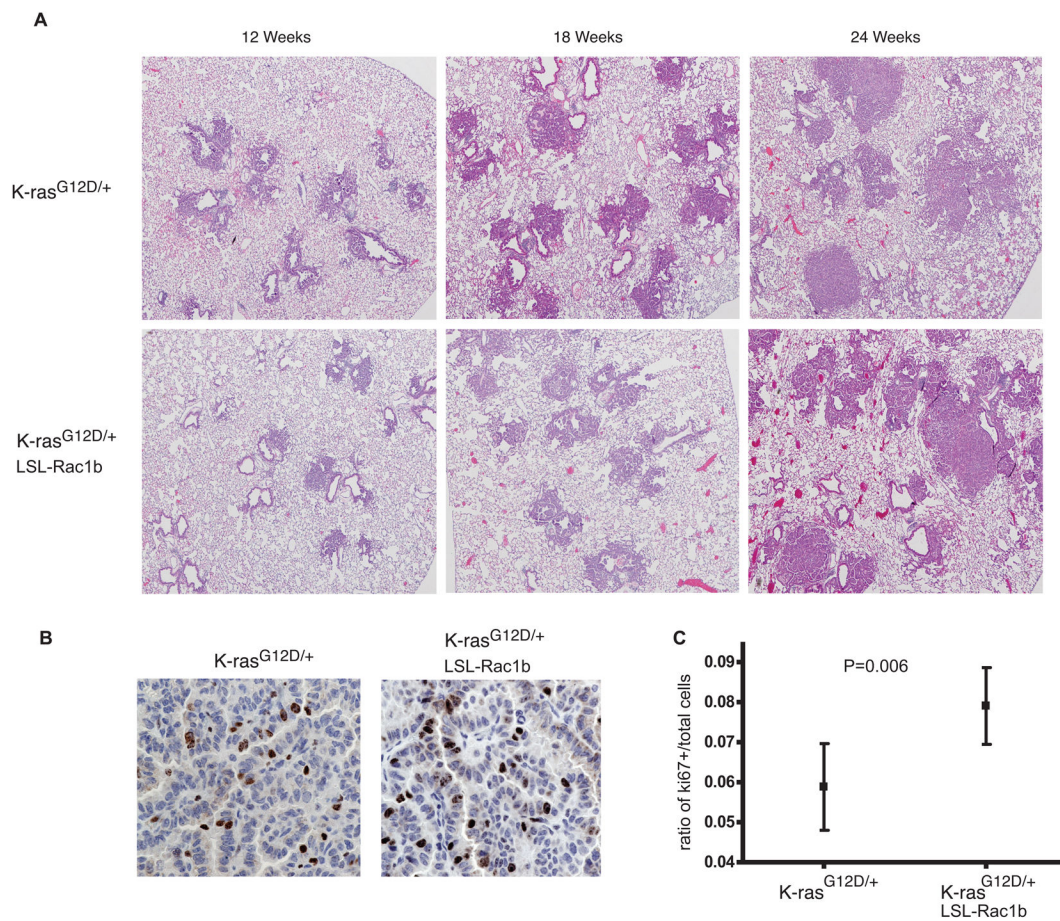


Figure 3. Time course progression of tumors in *LSL-K-ras^{G12D}* and *LSL-K-ras^{G12D}; Rosa26-LSL-Rac1b/+* mice

Mice were sacrificed at 12, 18, and 24 weeks post infection. **(A)** Representative H&E stained histological images from both animal groups at these time points are shown. The two groups displayed similar lesions at all time points, however, tumor sizes were significantly larger in the *LSL-K-ras^{G12D}; Rosa26-LSL-Rac1b/+* mice. Magnification, $\times 4$. **(B)** Ki-67 expression by immunohistochemistry in lung tumors of *LSL-K-ras^{G12D}* and *LSL-K-ras^{G12D}; Rosa26-LSL-Rac1b/+* mice. Representative images of Ki-67-positive staining in tumors of the same grade from both groups of mice at 24-week time point. Magnification, $\times 60$. **(C)** The estimated mean ratio of ki-67 positive cells over the total cells per field. Multiple similar grade tumors from six *LSL-K-ras^{G12D}* and six *LSL-K-ras^{G12D}; Rosa26-LSL-Rac1b/+* mice were analyzed from each genotype. P=0.006. Bars represent 95% confidence interval of the estimated ratio.

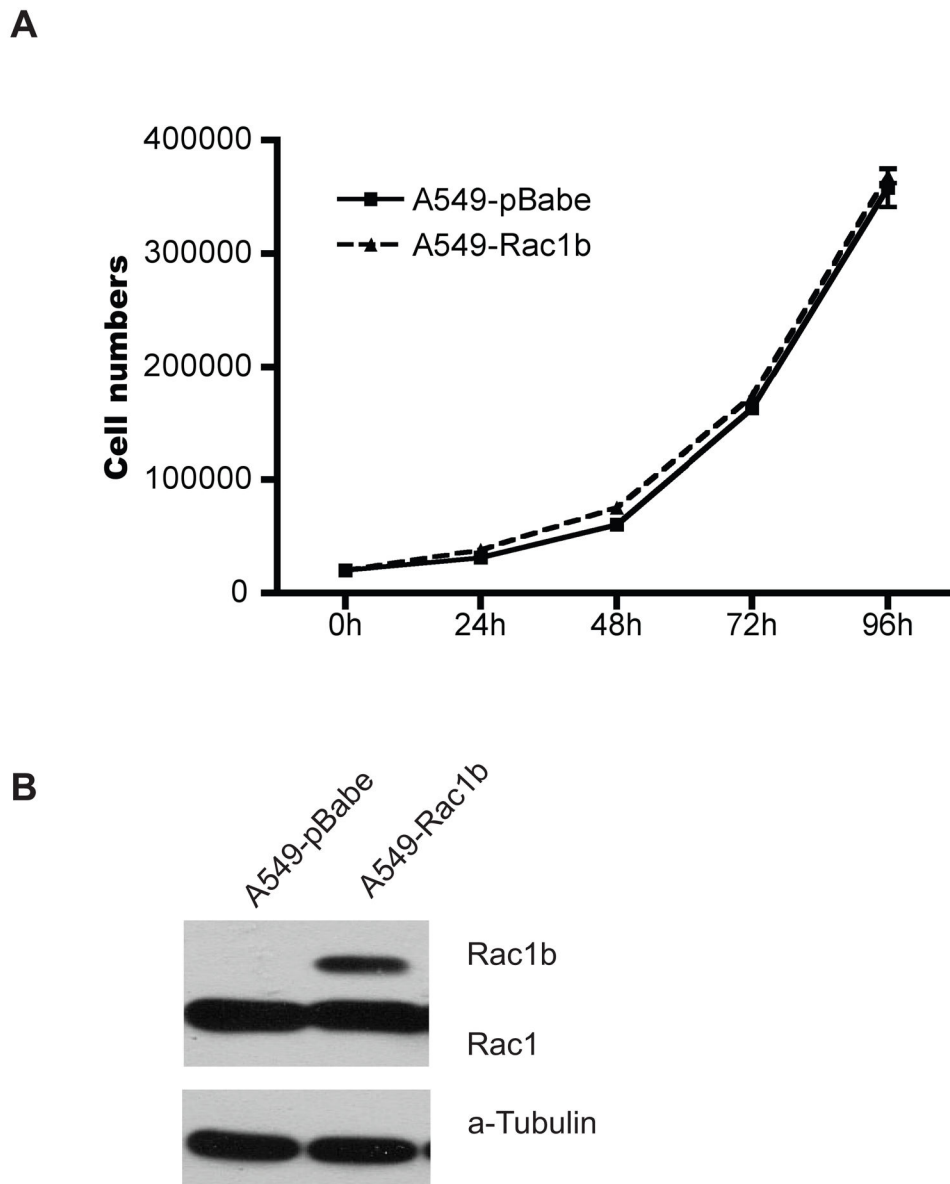


Figure 4. Expression of Rac1b does not affect cell proliferation

(A) A549 cells stably expressing Rac1b were generated by retroviral infection. Cells were plated in triplicates and were counted every 24 hours. The data shown are representative of three independent experiments. Bars, SD. (B) Western blot analysis of Rac1 and Rac1b expression in control A549 cells (A549-pBabe) or A549 stably infected with a mouse Rac1b expression vector (A549-Rac1b) detected by western-blotting with anti-Rac1 antibody.

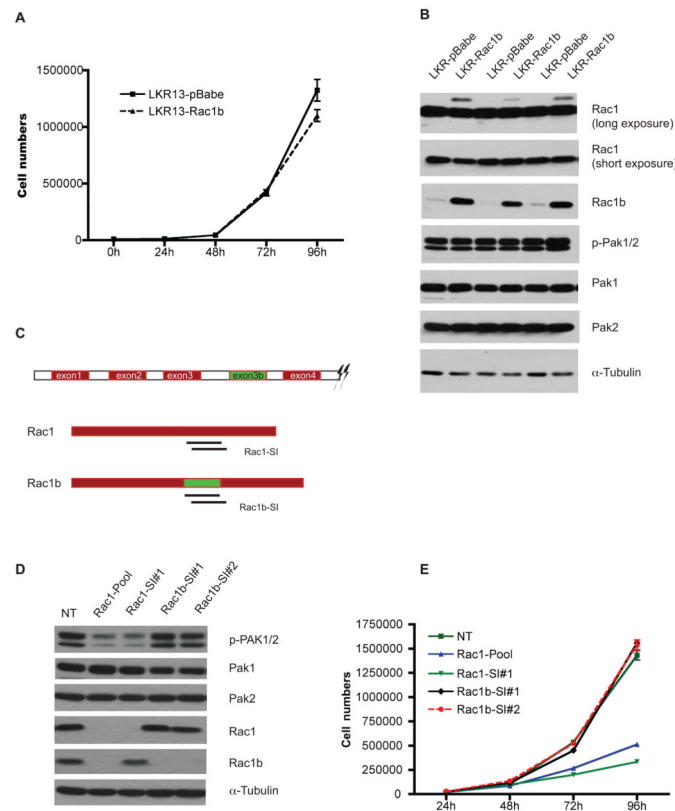


Figure 5. Rac1b is not required for cell proliferation of K-ras driven lung cancer cells
(A) LKR13 cells stably infected with retrovirus encoding mouse Rac1b (LKR-Rac1b) or empty vector control ((LKR-pBabe) were plated in triplicates and counted daily. The data shown are representative of three independent experiments. Bars, SD. **(B)**, Expression of Rac1, Rac1b, Pak1 and 2, phosphorylated Pak1/2 in 3 independent LKR13-pBabe and LKR13-Rac1b lines as detected by western blotting with anti-Rac1, anti-mouse Rac1b, anti-p141/144 Pak1/2, Pak1 and Pak2 antibodies. α -tubulin was used as a loading control. **(C)** Alternative splicing of the Rac1 allele results in Rac1 or Rac1b splice forms. Locations of siRNA specific to each splice form are indicated. **(D)** LKR13-cells were transfected with siRNA targeting Rac1 (Rac1-SI#1), Rac1b (Rac1b-SI#1 and Rac1b-SI#2) or both (Rac1-Pool). Levels of Rac1 and Rac1b and phosphorylated Pak1 and 2 (p-Pak1/2) as determined by western blot analysis. α -tubulin was used as a loading control. **(E)** LKR13 cells transfected with siRNA for the different Rac1 isoforms were plated in triplicate and counted daily. The data shown are representative of three independent experiments. Bars, SD.