Oncogene (2006) 25, 7225–7234

www.nature.com/onc

ORIGINAL ARTICLE

Ras transformation requires metabolic control by 6-phosphofructo-2-kinase

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Neoplastic cells transport large amounts of glucose in order to produce anabolic precursors and energy within the inhospitable environment of a tumor. The *ras* signaling pathway is activated in several cancers and has been found to stimulate glycolytic flux to lactate. Glycolysis is regulated by *ras* via the activity of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFK2/FBPase), which modulate the intracellular concentration of the allosteric glycolytic activator, fructose-2,6-bisphosphate (F2,6BP). We report herein that sequential immortalization and *ras*-transformation of mouse fibroblasts or human bronchial epithelial cells paradoxically decreases the intracellular concentration of F2,6BP. This marked reduction in the intracellular concentration of F2,6BP sensitizes transformed cells to the antimetabolic effects of PFK2/FBPase inhibition. Moreover, despite co-expression of all four mRNA species (PFKFB1-4), heterozygotic genomic deletion of the inducible PFKFB3 gene in *ras*-transformed mouse lung fibroblasts suppresses F2,6BP production, glycolytic flux to lactate, and growth as soft agar colonies or tumors in athymic mice. These data indicate that the PFKFB3 protein product may serve as an essential downstream metabolic mediator of oncogenic *ras*, and we propose that pharmacologic inhibition of this enzyme should selectively suppress the high rate of glycolysis and growth by cancer cells.
Oncogene (2006) **25,** 7225–7234. doi:10.1038/sj.onc.1209709; published online 22 May 2006

Keywords: *ras*; metabolism; phosphofructokinase

Introduction

In the early 20th century, Otto Warburg discovered that rat tumor tissues, but not normal tissues, exhibit a high rate of glucose catabolism even in the presence of oxygen (i.e., aerobic glycolysis or the Warburg effect) (Warburg and Negelein, 1924). This phenomenon provides the basis for the most sensitive and specific imaging technique available for the diagnosis and staging of cancer: positron emission tomography of 2-[^{18}F]fluoro-2-deoxy-glucose uptake (Wechalekar *et al*., 2005). Sustaining the energy demands of the cell with glycolysis alone is an inefficient process; the catabolism of 1 mol of glucose to lactate yields only 2 mol of adenosine triphosphate (ATP). By contrast, 38 mol of ATP are generated in the presence of oxygen by complete combustion of glucose to CO_2 and H_2O. However, neoplastic cells require not only glycolytic ATP during hypoxia but also biosynthetic precursors in order to proliferate and invade. For example, glycolytic flux to lactate provides a ready supply of fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate for shunting into *de novo* nucleic acid synthesis (Boros *et al*., 2000; Boren *et al*., 2001; Boros *et al*., 2001).

The oncogene *ras* has recently emerged as a central regulator of enhanced glucose uptake and glycolysis in neoplastic cells (Mazurek *et al*., 2001; Blum *et al*., 2005; Ramanathan *et al*., 2005; Vizan *et al*., 2005). Stable transfection of oncogenic H-*ras*^{V12} into Rat-1 immortalized fibroblasts causes increased glucose uptake and lactate production (Racker *et al*., 1985; Kole *et al*., 1991). Moreover, recent studies by Ramanathan *et al*. (2005) have demonstrated that the introduction of an oncogenic allele of H-*ras* into human fibroblasts already immortalized with the telomerase catalytic subunit, SV40 large T antigen (LT) and small T antigen causes: (i) increased glucose uptake and lactate production; (ii) increased ribose-5-phosphate, a glucose-derived anabolic precursor of DNA and RNA; and (iii) increased sensitivity to the glycolytic inhibitors, 2-deoxyglucose and oxamate. Taken together, these data indicate that, under aerobic conditions, oncogenic *ras* causes a global shift in the metabolic source of energy from the citric acid cycle and electron transport toward glycolytic flux to lactate (i.e., the Warburg effect).

Transfection of immortalized Rat-1 fibroblasts with oncogenic *ras* has been observed to cause an increase in the activity of 6-phosphofructo-1-kinase (PFK-1), the first irreversible, committed step of glycolysis and thus a key control point in glycolytic flux (Kole *et al*., 1991). Flux at PFK-1 is suppressed by several allosteric effectors, including ATP (i.e., the Pasteur effect), H^+ ions and citrate, which exert negative feedback when energy is abundant (Van Schaftingen *et al*., 1981). Oncogenic *ras* has not been found to decrease these intrinsic inhibitors, but rather to increase the steady-state concentration of an allosteric activator of PFK-1, fructose-2,6-bisphosphate (F2,6BP) (Kole *et al*., 1991).

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Received 28 February 2006; revised 5 April 2006; accepted 5 April 2006; published online 22 May 2006

F2,6BP activates PFK-1 by shifting the conformational equilibrium of PFK-1 from a low to a high-affinity state for its substrate, F6P. This increase in intracellular F2,6BP relieves the tonic inhibition of PFK-1 by ATP, allowing untethered glycolytic flux at the PFK-1 checkpoint to proceed (Van Schaftingen *et al*., 1981).

The steady-state concentration of F2,6BP depends on the activity of the homodimeric bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2/FBPase), which is encoded by four genes (PFKFB1-4) and is capable of phosphorylating F6P to F2,6BP and dephosphorylating F2,6BP to F6P (Okar and Lange, 1999). The enzymes derived from PFKFB1, PFKFB2 and PFKFB4 were originally found to be expressed by cells of the liver/muscle, kidney/heart and testes, respectively, and all display nearly equal kinase: phosphatase ratios (Okar and Lange, 1999). The PFKFB3 gene encodes for the inducible PFK2/FBPase (Chesney *et al*., 1999; Atsumi *et al*., 2002; Mahlknecht *et al*., 2003), which is rapidly induced by inflammatory stimuli (Chesney *et al*., 1999) and hypoxia (Minchenko *et al*., 2002; Minchenko *et al*., 2004) and has also been termed placental PFK2 (Sakai *et al*., 1996; Sakakibara *et al*., 1999), ubiquitous PFK2 (Manzano *et al*., 1998; Kessler and Eschrich, 2001; Navarro-Sabate *et al*., 2001) and PGR1 (Hamilton *et al*., 1997)). The PFKFB3 protein product was recently documented to be over-expressed in the neoplastic cells of human solid tumors, including lung, breast, prostate and colon tumors and to display minimal phosphatase activity (kinase:phosphatase ratio 740:1), suggesting a constitutively pro-glycolytic activity (Sakakibara *et al*., 1997; Chesney *et al*., 1999; Chesney and Bucala, 2001; Atsumi *et al*., 2002).

In the present study, we have examined the metabolic and neoplastic requirements of the PFK2/FBPase encoded by PFKFB3 for *ras* transformation using a combination of siRNA and heterozygote genomic deletion technologies. We find that the expression of the inducible PFK2/FBPase is selectively necessary for the control of glycolytic flux in cells transformed with *ras* and propose that pharmacologic inhibition of this enzyme may affect the survival and growth of neoplastic cells.

Results

PFKFB3 activity sets the intracellular F2,6BP concentration

Whereas homozygotic deletion of PFKFB3 is embryonic lethal, PFKFB3$^{+/-}$ mice develop, grow and age normally (Chesney *et al*., 2005). In order to examine the role of the PFKFB3 protein product in neoplastic transformation, we introduced the immortalizing SV40 LT and then the V12 oncogenic allele of H-*ras* into primary adult lung fibroblasts isolated from PFKFB3$^{+/+}$ and PFKFB3$^{+/-}$ mice. Figure 1a demonstrates comparable ectopic protein expression of LT and then H-*ras*^{V12} among the lung fibroblast cell lines isolated from PFKFB3$^{+/+}$ and PFKFB3$^{+/-}$ mice. We examined primary lung fibroblasts and the two resultant cell lines from each strain for the presence of PFKFB1-4 mRNA species by multiplex polymerase chain reaction (PCR) and observed simultaneous expression of all four PFKFB mRNAs (PFKFB1-4) in each of the cell populations (Figure 1b and c). This phenomenon has been previously described in certain transformed cells and whole organs (Minchenko *et al*., 2003) and these data suggest that all four isozymes may be coordinating to set F2,6BP production. We then examined PFKFB3 protein product expression by Western blot analysis and observed an increase in this enzyme with the introduction of LT and a small increase after H-*ras*^{V12} introduction (Figure 1d and e). The steady-state intracellular F2,6BP concentration was measured in the primary lung fibroblasts, and the four cell lines isolated from the PFKFB3$^{+/+}$ and PFKFB3$^{+/-}$ mice. Unexpectedly, immortalization of the lung fibroblasts with LT in PFKFB3$^{+/+}$ fibroblasts caused a marked reduction of F2,6BP (Figure 1f; P-value <0.001). Conversely, the introduction of H-*ras*^{V12} into the LT-immortalized fibroblasts increased the intracellular F2,6BP, as previously reported in immortalized Rat-1 fibroblasts (P-value <0.001). Despite the simultaneous expression of four PFKFB mRNA species, heterozygotic genomic deletion of the PFKFB3 gene resulted in a marked decrease in F2,6BP concentration in the primary lung fibroblasts (P-value <0.001) and abolished the increase of F2,6BP in the *ras*-transformed cells (Figure 1f). These data indicate that the activity of the PFKFB3 protein product is essential for setting the intracellular F2,6BP concentration in primary, immortalized and transformed fibroblasts.

*PFKFB3 is selectively required by H-*ras^{V12}*-transformed cells for glycolysis*

If PFK2/FBPase activity derived from the PFKFB3 protein product is required for the control of glycolytic flux to lactate, then we expect a relative decrease in glycolysis in cells isolated from PFKFB3$^{+/-}$ mice. The purified fibroblast cell lines were cultured for 24–72 h and the glucose consumed and lactate secreted were measured. Whereas the introduction of LT caused a large increase in glucose consumption and lactate secretion (72 h; P-value <0.001), the addition of H-*ras*^{V12} did not further affect the consumption or secretion of these metabolites (Figure 2a and c). Surprisingly, glucose consumption and lactate secretion were identical in primary PFKFB3$^{+/+}$ and PFKFB3$^{+/-}$ fibroblasts and only moderately decreased in the LT-immortalized PFKFB3$^{+/-}$ lung fibroblasts (Figure 2a–d). However, we found that both glucose consumption and lactate secretion were markedly decreased in H-*ras*^{V12}-transformed PFKFB3$^{+/-}$ fibroblasts relative to PFKFB3$^{+/+}$ wild-type fibroblasts (Figure 2a–d; P-value <0.001). We also examined 2-deoxy-D-[^{14}C]glucose uptake in the six cell preparations and found that the glucose uptake mirrored glucose consumption (PFKFB3$^{+/+}$: 1° 231 $\pm$ 21 c.p.m., LT 1755 $\pm$ 221 c.p.m., LT/*ras* 1663 $\pm$ 323 c.p.m.; PFKFB3$^{+/-}$: 1° 197 $\pm$ 29 c.p.m, LT 1771 $\pm$ 191 c.p.m., LT/*ras* 388 $\pm$ 59 c.p.m.). In order to

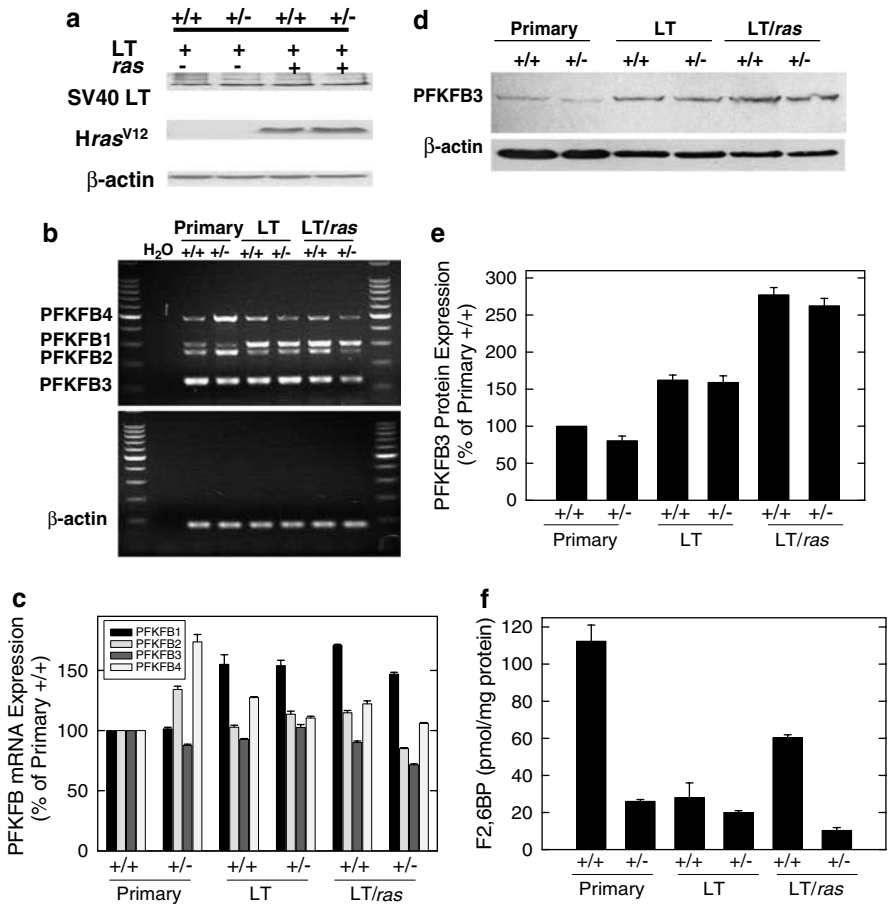

Figure 1 Heterozygotic genomic deletion of PFKFB3 reduces the intracellular F2,6BP concentration and suppresses the induction of F2,6BP by *ras* in immortalized cells. Data are expressed as the mean ± s.d. of three experiments. (**a**) Western blot analyses of SV40 LT, H*ras*^{V12} and *β*-actin in lung fibroblasts isolated from PFKFB3$^{+/+}$ and PFKFB3$^{+/-}$ mice. (**b**) Multiplex RT–PCR analysis for the simultaneous expression of all four PFKFB isozymes in primary, LT immortalized and LT/*ras* transformed lung fibroblasts isolated from PFKFB3$^{+/+}$ and PFKFB3$^{+/-}$ mice. (**c**) Densitometric analysis of multiplex RT–PCR. (**d**) Western blot analysis of PFKFB3 protein product expression by the indicated lung fibroblasts. (**e**) Densitometric analysis of Western blotting. (**f**) F2,6BP measurements in the indicated lung fibroblasts.

further characterize this metabolic distinction between PFKFB3$^{+/+}$ and PFKFB3$^{+/-}$ LT/*ras*-transformed fibroblasts, we examined the direct channeling of fully labeled $^{13}C_6$-glucose to lactate using one-dimensional (1D) nuclear magnetic resonance spectroscopy. We confirmed that the PFKFB3$^{+/-}$ LT/*ras*-transformed fibroblasts converted significantly less glucose to lactate than the PFKFB3$^{+/+}$ LT/*ras*-transformed fibroblasts (Figure 2e). That the heterozygotic genomic deletion of PFKFB3 especially affects glycolysis in the LT/*ras*-transformed cells suggests that *ras* signaling may cause metabolic alterations that selectively require the activity of the enzyme encoded by PFKFB3.

*PFKFB3 expression is required for anchorage-independent growth by H-*ras^{V12}*-transformed lung fibroblasts*

Lactate production has been correlated previously with growth and invasiveness of neoplastic cells (Wang *et al*., 1976; Walenta *et al*., 2004) and we postulated that the observed decrease in lactate secretion in PFKFB3$^{+/-}$ fibroblasts may limit growth. We found that LT/*ras* lung fibroblasts isolated from PFKFB3$^{+/-}$ did display a slight reduction in growth relative to wild-type cells in hypoxic and normoxic conditions (Figure 3a). We hypothesize that PFKFB$^{+/-}$ lung fibroblasts proliferate at a lower rate than PFKFB$^{+/+}$ lung fibroblasts owing to decreased glycolytic flux into anabolic pathways such as the nonoxidative pentose shunt. Anchorage-independent growth in soft agar by PFKFB3$^{+/-}$ lung fibroblasts after introduction of both LT and *ras*^{V12} was markedly reduced relative to PFKFB3$^{+/+}$ fibroblasts (Figures 3b; *P*-value <0.001), and the few colonies that did form demonstrated decreased diameter (Figure 3c). These data support the hypothesis that transformed cells grown in spheres with limited diffusion potential may be especially dependent on increased aerobic glycolysis.

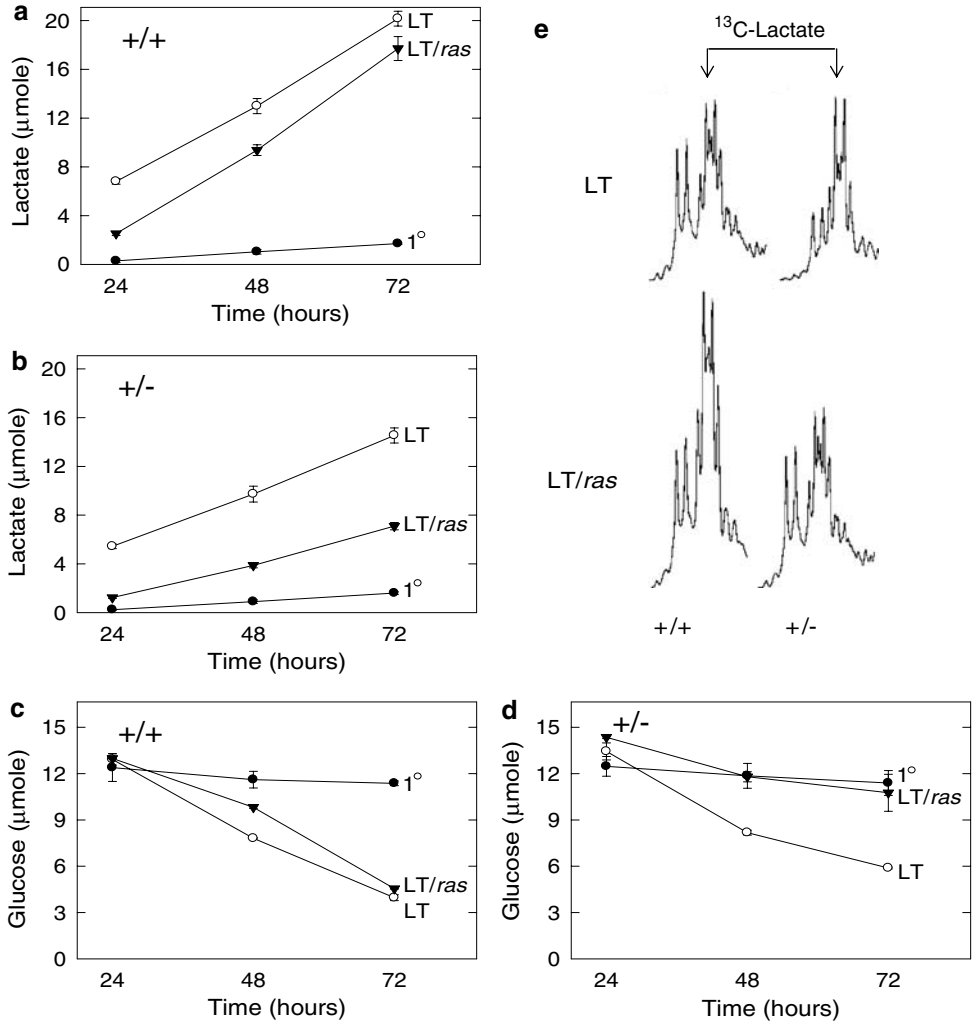

Figure 2 Heterozygotic genomic deletion of PFKFB3 selectively reduces glycolytic flux to lactate in *ras*-transformed fibroblasts. Data are expressed as the mean ± s.d. of three experiments. (**a** and **b**) Measurement of lactate secretion after 24–72 h by primary (•), LT immortalized (○) and LT/*ras* transformed (▼) lung fibroblasts isolated from PFKFB3$^{+/+}$ and PFKFB3$^{+/-}$ mice. (**c** and **d**) Measurement of unconsumed glucose in the supernatant of cultures of primary (•), LT immortalized (○) and LT/*ras* transformed (▼) lung fibroblasts isolated from PFKFB3$^{+/+}$ and PFKFB3$^{+/-}$ mice. (**e**) 1D NMR spectroscopy of secreted ^{13}C-lactate after 48 h exposure of PFKFB3$^{+/+}$ and PFKFB3$^{+/-}$ LT immortalized and LT/*ras* transformed lung fibroblasts to fully labeled (1 gm/l) $^{13}C_6$ glucose.

Decreased tumor growth by H-ras^{V12}-transformed lung fibroblasts isolated from PFKFB3$^{+/-}$ mice

Based on the selective inhibitory effect of PFKFB3 reduction on *ras*^{V12}-mediated anchorage-independent growth, we speculated that the LT/*ras* lung fibroblasts isolated from PFKFB3$^{+/-}$ mice would grow poorly in athymic mice. We subcutaneously (s.c.) injected LT/*ras* lung fibroblasts isolated from PFKFB3$^{+/+}$ and PFKFB3$^{+/-}$ mice into the flanks of athymic mice and measured tumor establishment and growth using micro-calipers. These lung fibroblasts were highly transformed in that they grew to measurable tumors within 3 days of implantation (Figure 4a). Although LT/*ras* lung fibroblasts isolated from PFKFB3$^{+/-}$ mice grew as solid tumors, they exhibited a clear reduction in growth relative to PFKFB3$^{+/+}$ fibroblasts that persisted throughout the study period (days 5–13, *P*-value <0.001). The relative disparity between these modest results and those observed in soft agar may be due to the presence of signals that enable compensatory increases in either the activity of the residual PFKFB3 protein or other signaling molecules or proteins involved in the regulation of glycolysis. We next examined a subset of mice using micro-positron emission tomography (microPET) and found that tumors established from PFKFB3$^{+/-}$ cells displayed a relative decrease in 2-[^{18}F]fluoro-2-deoxy-glucose uptake (Figure 4b).

Immortalization and transformation of normal human bronchial epithelial cells is associated with reduced intracellular F2,6BP

In order to correlate the results obtained from mouse lung fibroblasts with human cancer, we examined

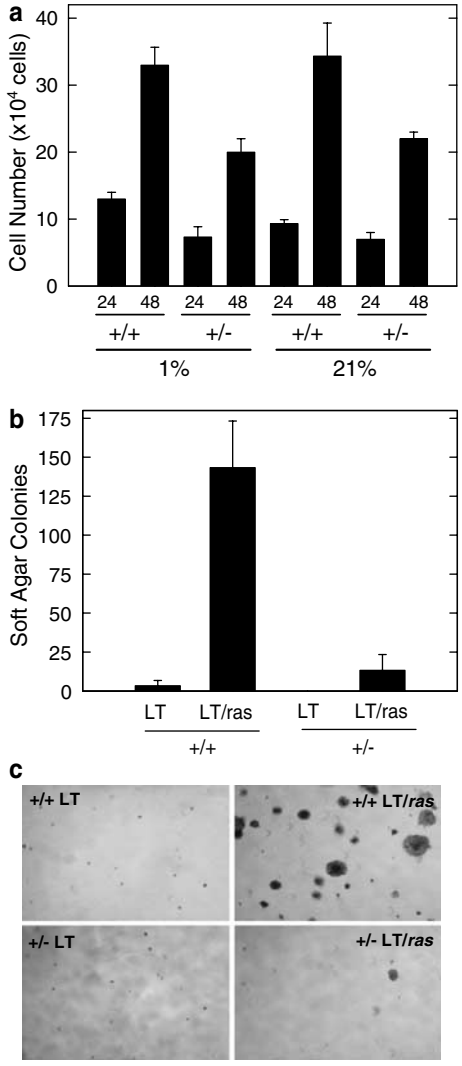

Figure 3 Heterozygotic genomic deletion of PFKFB3 suppresses anchorage-independent growth of LT/*ras*-transformed lung fibroblasts. Data are expressed as the mean ± s.d. of three experiments. (**a**) Live cell number of LT/*ras*-transformed lung fibroblasts isolated from PFKFB3$^{+/+}$ and PFKFB3$^{+/-}$ mice after 24 and 48 h exposure to 1 and 21% oxygen. (**b**) and (**c**). Soft agar colony growth by LT immortalized and LT/*ras*-transformed lung fibroblasts isolated from PFKFB3$^{+/+}$ and PFKFB3$^{+/-}$ mice.

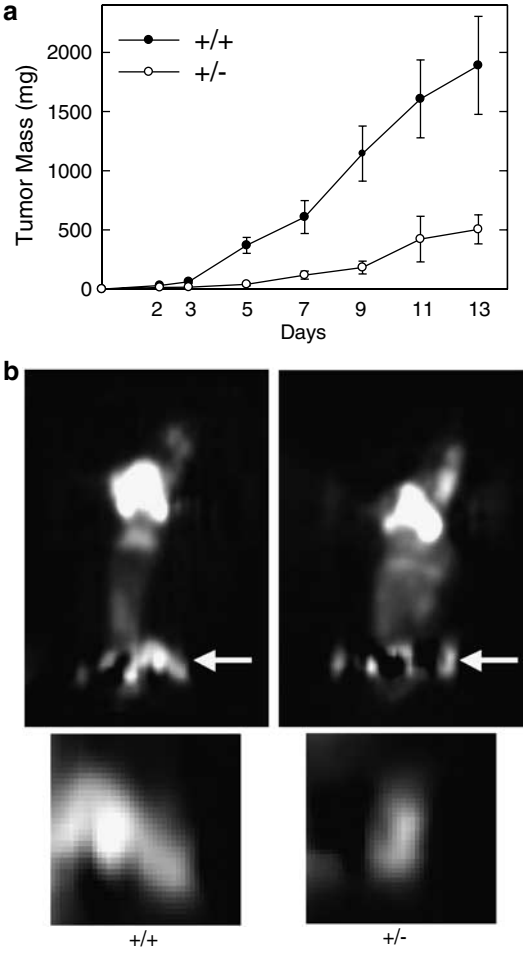

Figure 4 Heterozygotic genomic deletion of PFKFB3 suppresses growth of LT/*ras*-transformed lung fibroblasts in athymic mice. Data are expressed as the mean ± s.d. of three experiments. (**a**) LT/*ras*-transformed lung fibroblasts isolated from PFKFB3$^{+/-}$ and PFKFB3$^{+/+}$ mice were injected s.c. into athymic mice and established tumors were measured at the indicated days using microcalipers ($n = 10$ mice per group). (**b**) FDG-micro-PET of established tumors after 11 days of growth.

PFKFB mRNA expression and F2,6BP in normal human bronchial epithelial (NHBE) cells that had been sequentially immortalized and transformed using the telomerase catalytic subunit (hT), SV40 LT and an oncogenic allele of *ras* (Soejima *et al*., 2003). We observed simultaneous expression of three isozymes, PFKFB2-4, but not PFKFB1 (i.e., the liver isozyme), as well as a modest increase in PFKFB3 protein product expression and a large decrease in intracellular F2,6BP with immortalization (Figure 5a–e), which was similar to our findings with the mouse lung fibroblasts. We next sought to correlate the expression and activities of PFKFB3 with glycolytic flux to lactate. Surprisingly, we found that both the normal and the *ras*-transformed bronchial epithelial cells equally consumed more glucose and secreted more lactate than the immortalized cells (Figure 5d; *P*-value <0.001). We also examined 2-deoxy-D-[^{14}C]glucose uptake in the four cell preparations and found that the glucose uptake mirrored glucose consumption (NHBE, 829 ± 82 c.p.m.; hT, 139 ± 21 c.p.m.; hT/LT, 153 ± 43 c.p.m.; hT/LT/*ras*, 776 ± 62 c.p.m.). Although these data confirm that oncogenic *ras* expression increases lactate secretion in immortalized epithelial cells, the finding that primary cells also secrete increased lactate indicates that high lactate secretion in and of itself may not be a distinct characteristic of neoplastic transformation. We next screened four siRNA species for their ability to silence PFKFB3 mRNA and protein product expression. Unfortunately, we did not observe any change in PFKFB3 protein product expression after transient transfection into these cells, which was likely due to poor transfection efficiency (*data not shown*).

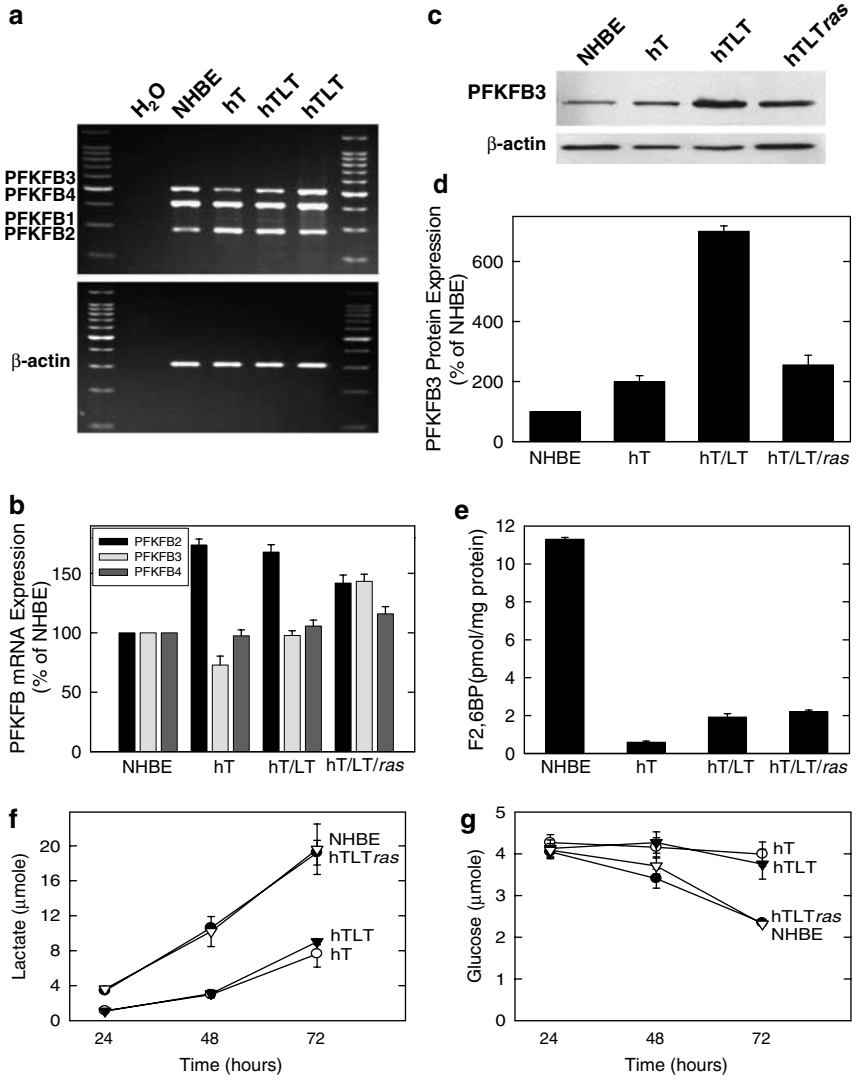

Figure 5 Transformation of human bronchial epithelial cells with oncogenic *ras* is associated with reduced intracellular F2,6BP and normalization of glucose consumption and lactate secretion. Data are expressed as the mean ± s.d. of three experiments. NHBE cells sequentially immortalized and transformed using the telomerase catalytic subunit (hT), SV40 LT and H-*ras*^{V12} were analysed by: (**a**) Multiplex RT–PCR for PFKFB1-4. (**b**) Densitometric analysis of multiplex RT–PCR. (**c**) Western blot analysis for PFKFB3 protein product expression. (**d**) Densitometric analysis of PFKFB3 Western blotting. (**e**) Measurement of intracellular F2,6BP. (**f**) Measurement of lactate secretion after 24–72 h. (**g**) Measurement of residual glucose after 24–72 h.

siRNA silencing of PFKFB3 in A549 human lung adenocarcinoma cells decreases anchorage-independent growth

A549 cells are easily transfectable with siRNA species, express oncogenic K-*ras*^{V12} and display anchorage-independent growth, a hallmark of neoplastic transformation (Valenzuela and Groffen, 1986). We examined A549 cells (and three other transformed cell lines) for PFKFB family mRNA expression using multiplex reverse transcription (RT)–PCR and found that, like the hT/LT/*ras* human bronchial epithelial cells, three mRNAs (PFKFB2-4) were co-expressed (Figure 6a). We then found that two PFKFB3-specific siRNA species reduced PFKFB3 protein product expression (Figure 6b and c; siRNA1, 56% of lamin control; siRNA2, 44% of lamin control; $^{**}P$-value <0.001), F2,6BP (Figure 6d; siRNA1, 74% of lamin control; siRNA2, 65% of lamin control), and lactate secretion (siRNA1: 77% of lamin control; siRNA2: 73% of lamin control) and that siRNA silencing of PFKFB3 disproportionately decreased anchorage-independent growth more than cell proliferation (Figure 6e–g; $^{*}P$-value <0.05, $^{**}P$-value <0.001). These data thus indicate that the protein product of PFKFB3 also may be required for *ras* transformation in cells derived from human epithelia.

Discussion

The rationale for simultaneous expression of different PFKFB mRNA species by a homogeneous cell population may be related to differential regulation of the

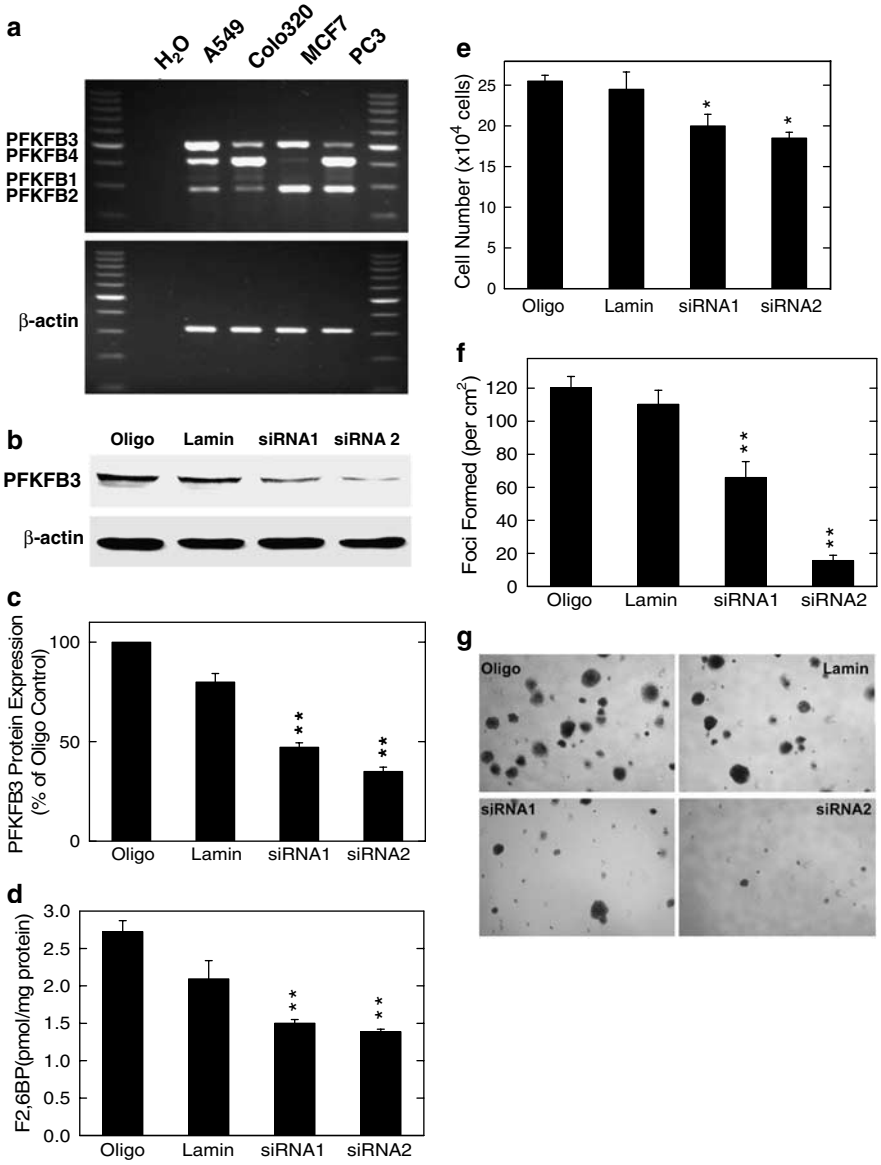

Figure 6 siRNA silencing of PFKFB3 expression in A549 alveolar adenocarcinoma cells reduces F2,6BP concentration and anchorage-independent growth in soft agar. Data are expressed as the mean ± s.d. of three experiments. Statistical significance was assessed by the two-sample *t*-test (**P*-value <0.05, ***P*-value <0.001). (**a**) Multiplex RT–PCR analysis for PFKFB1-4 mRNA expression in A549 alveolar adenocarcinoma cells, Colo320 colon adenocarcinoma cells, MCF7 breast adenocarcinoma cells and PC3 prostate adenocarcinoma cells. A549 cells were cultured in the presence of transfection reagent (oligo) +/− control Lamin siRNA or the PFKFB3-specific siRNA species and analysed for: (**b**) PFKFB3 protein product expression by Western blot analysis. (**c**) Densitometric analysis of PFKFB3 Western blotting. (**d**) F2,6BP concentration. (**e**) Cell viability. (**f**) Soft agar colony growth enumeration. (**g**) Photographs of soft agar growth.

enzymes or to the existence of unknown nonglycolytic functions. Regardless, the high kinase:phosphatase ratio of the PFKFB3 protein product apparently trumps the activities of the other three PFK2/FBPases in terms of enabling increased intracellular F2,6BP as both heterozygote genomic deletion and siRNA silencing of PFKFB3 strikingly reduced the steady-state intracellular concentration of F2,6BP.

The initial rationale for examining the requirement of PFKFB3 for *ras* transformation was based on past studies that found that introduction of oncogenic *ras* caused an increase in F2,6BP and PFK-1 activity. In wild-type LT-immortalized mouse lung fibroblasts and hT/LT-immortalized NHBE cells, the introduction of an oncogenic allele of *ras* caused a small increase in F2,6BP as previously reported in Rat-1 immortalized fibroblasts. However, this effect was minuscule relative to the large decrease in the F2,6BP concentration observed with immortalization by LT or hT/LT in the primary mouse fibroblasts or human epithelial cells. Most previous studies on the metabolic effects of oncogenic *ras* have compared immortalized to transformed cells and have not included analyses of glucose utilization and F2,6BP in primary cells. This surprising reduction

of F2,6BP during immortalization may reflect a negative feedback compensation in response to increased flux at PFK-1, changes in F2,6BP stability *in situ* (e.g., low intracellular pH) or increased conversion of F2,6BP into F6P for glycolytic utilization. Nevertheless, these data run counter to the widely held assumption that the intracellular F2,6BP concentration directly corresponds to and possibly sets the proliferative rate (Van Schaftingen *et al*., 1981; Hue and Rousseau, 1993; Chesney *et al*., 1999).

We observed marked differences in glucose metabolism between primary mouse fibroblasts and human bronchial epithelial cells during *ras*-transformation. The main distinction is that immortalized and transformed mouse fibroblasts exhibit increased glucose metabolism relative to primary mouse fibroblasts, whereas primary and *ras*-transformed human bronchial epithelial cells exhibit increased glucose metabolism relative to immortalized human bronchial epithelial cells. It is important to note that few previous studies of glucose metabolism in cancer have examined the basal glucose uptake and lactate secretion of primary fibroblasts or epithelial cells that have been sequentially immortalized with LT ($+/-$telomerase) and then transformed with an oncogenic allele of *ras*. While we are quite confident of the observations, we are admittedly unsure of the rationale and mechanism for these metabolic differences.

Our previous observations that heterozygotic deletion of PFKFB3 does not affect normal embryogenesis, development, growth or aging suggest either that the reduced F2,6BP is physiologically permissive or that an unidentified metabolic compensation has occurred. We were surprised by the observation that the PFKFB3$^{+/-}$ fibroblasts expressed similar PFKFB3 protein product but markedly reduced F2,6BP and postulate that allosteric effectors, subcellular localization, substrate and product concentrations may all contribute to this phenomenon. We found that introduction of LT followed by an oncogenic allele of *ras* is permissive for anchorage-independent growth by primary lung fibroblasts isolated from PFKFB3$^{+/+}$ mice but not from PFKFB3$^{+/-}$ mice. Additionally, we observed that the PFKFB3$^{+/-}$ LT/*ras* cells grow in athymic mice at a markedly reduced rate relative to the PFKFB3$^{+/+}$ LT/*ras* cells. That reduced F2,6BP found in cells derived from PFKFB3$^{+/-}$ mice causes no overt pathology yet almost completely abrogates the capacity of oncogenic *ras* to confer the capacity for anchorage-independent growth suggests that PFKFB3 may be a highly selective target for the development of antineoplastic agents. Accordingly, this study should promote the development of PFK2/FBPase inhibitors for the treatment of neoplasms that have been found to require activation of the *ras* signaling pathway.

The observation that heterozygotic deletion of PFKFB3 especially affects both glycolysis and spheroid growth by LT/*ras* lung fibroblasts supports the hypothesis that the growth of cells in 3-dimensional masses requires enhanced glycolytic flux. We postulate that transformed cells may be sensitive to inhibition of glycolytic flux when oxygen and glucose diffusion limitations are imposed by surrounding cells present in such masses. Specifically, oxygen and glucose diffusion from the culture medium and vasculature may be limited by adjacent cells during soft agar growth and tumor growth, respectively, but remain relatively unlimited for cells grown as a monolayer *in vitro* (Mueller-Klieser *et al*., 1986). Accordingly, if glycolytic flux is inadequate, then such diffusion limitations may restrict the availability of energy and anabolic precursors, which then may hinder spheroid or tumor growth. Regardless, the increased dependency of *ras* for F2,6BP-activated glycolysis supports the previously reported shift by oncogenic *ras* away from oxidative phosphorylation and toward aerobic glycolytic flux to lactate (Mazurek *et al*., 2001; Ramanathan *et al*., 2005; Vizan *et al*., 2005). We postulate that the combination of reduced intracellular F2,6BP and an increased need for high PFK-1 flux may sensitize *ras*-transformed cells to the effects of PFKFB3 inhibition.

Metabolic adaptation to hypoxic exposure is mediated in part by hypoxia inducible factor-1 (HIF-1), which promotes the expression of several glycolytic enzymes and glucose transporter mRNAs (Zhong *et al*., 1999). Minchenko *et al*. (2003) have demonstrated that hypoxia induces PFKFB3 transcription in various cell types and mouse tissues and that this induction is completely abrogated in mouse embryonic fibroblasts conditionally nullizygous for HIF-1α (Minchenko *et al*., 2002, 2003). PFKFB3 thus may serve as an essential transcriptional target of HIF-1α and the adaptive response to hypoxia by transformed cells.

In summary, this study provides data that run counter to several widely held assumptions. These include our novel observations that: (i) intracellular F2,6BP is decreased in immortalized and transformed cells relative to primary cells; (ii) PFKFB3 expression is selectively required for glycolytic flux to lactate in immortalized and transformed cells and for the growth of transformed cells in soft agar and athymic mice; and (iii) primary and *ras*-transformed epithelial cells similarly secrete increased lactate relative to immortalized cells. Taken together, these observations support the conclusion that while the glycolytic activities of primary and *ras*-transformed epithelial cells are similar, both the metabolic needs for survival and growth and the regulatory requirements may be quite dissimilar. Understanding these distinctions should prove useful in the development of antimetabolic approaches for the development of novel chemotherapeutic agents.

Materials and methods

Cells lines and cell culture

Lung fibroblasts were isolated from PFKFB3$^{+/+}$ and PFKFB3$^{+/-}$ mice. After the lungs were resected, the tissue was minced under aseptic conditions and passed though a 40 μm mesh to separate the cells. These cells were grown in culture with Dulbecco's modified Eagle medium (DMEM) and 10% fetal calf serum (FCS) at 37°C in 5% CO_2 for 7–10 days.

A549 lung adenocarcinoma cells were obtained from ATCC (Manassas, VA, USA). NHBE cells were obtained from Cambrex (Walkersville, MD, USA) and NHBE cells expressing telomerase (hT), SV40 LT (hT/LT) and activated *ras* (hT/LT/*ras*) were gifts from Dr BJ Rollins, Dana Farber Cancer Institute. Fibroblasts and A549 cells were grown in DMEM containing 10% FCS. NHBE, hT, hT/LT and hT/LT/*ras* cells were grown in media formulated with bovine pituitary extract, recombinant human epidermal growth factor, hydrocortisone, insulin, epinephrine, tri-iodothyronine, transferrin, gentamicin, amphotericin B and retinoic acid (BEGM Bullet Kit, Cambrex, Walkersville, MD, USA). All cells were grown at 37°C in 5% CO_2.

Retroviral transductions
PFKFB3$^{+/-}$ and PFKFB3$^{+/+}$ murine lung fibroblasts were sequentially transduced with REBNA/IRESGFP retroviruses expressing SV40 LT and human H-*ras*^{V12} mutant (*ras*) (both gifts from Dr R Mitchell, University of Louisville, Louisville, KY, USA).

siRNA and transfection
siRNA sequences were designed against the PFKFB3 gene siRNA 1 (target sequence 5′-agcctcgcatcaacagc-3′) and siRNA 2 (target sequence 5′-acgaacttggacaggtgt-3′). These sequences are not present in the PFKFB1, PFKFB2 and PFKFB4 genes. Cells were transiently transfected with siRNA species using Oligofectamine (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions, and harvested at 24, 48 and 72 h after transfection.

F 2,6BP measurements
An equal number of cells were trypsinized, washed twice with phosphate-buffered saline (PBS), and collected to measure total intracellular F2,6BP as previously described (Van Schaftingen *et al*., 1982). The F2,6BP concentration was normalized to total cellular protein as measured by the bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL, USA). All data are expressed as the mean ± s.d. of three experiments. Statistical significance was assessed by the two-sample *t*-test (independent variable).

Anchorage-independent proliferation
A feeder layer of 0.6% agarose (Agar Noble, Becton Dickinson, Sparks, MD, USA) in DMEM was plated in 6 cm plates. Cells (2.5×10^4) were resuspended in 0.3% agarose in DMEM and placed on top of the feeder layer. Cells were allowed to grow at 37°C in 5% CO_2 and media replenished once a week until colonies became visible. Colonies were stained with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] and counted in three random 1 cm^2 areas per plate under 40 × magnification. All data are expressed as the mean ± s.d. of three experiments. Statistical significance was assessed by the two-sample *t*-test (independent variable).

Lactate and glucose measurements
Lactate concentrations in the media were measured using a lactate oxidase-based assay read at 540 nm (Trinity, Wicklow, Ireland). Glucose concentrations were measured using a hexokinase-glucose-6-phosphate dehydrogenase enzymatic assay read at 340 nm (Sigma, St Louis, MO, USA). All lactate and glucose data were normalized to cell number. Cells were also subjected to an assay of 2-deoxy-D-[^{14}C]glucose (0.075 μCi/well) uptake as described previously (van den Berghe *et al*., 1994). All data are expressed as the mean ± s.d. of three experiments. Statistical significance was assessed by the two-sample *t*-test (independent variable).

Nuclear magnetic resonance
A 20% stock solution of [U-$^{13}C_6$]-glucose (98% ^{13}C) (Cambridge Isotopes Laboratories, Andover, MA, USA) was prepared in PBS, sterile filtered through a 0.2 μm filter and added to glucose-free DMEM containing 10% FCS. Equal numbers of cells were cultured in the presence of fully labeled (1 gm/l) ^{13}C glucose for 48 h. 1D nuclear magnetic resonance (NMR) spectra were recorded at 14.1 T on a Varian Inova NMR spectrometer at 20°C using a 90° excitation pulse with an acquisition time of 2 s and a relaxation delay of 3 s.

Protein extraction and Western blotting
Cells were treated with 0.25% trypsin-EDTA, washed in PBS, and lysed in 2 × RIPA buffer. Protein samples were resolved on a 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene fluoride membrane. Membranes were blocked in TBS–Tween-20 (1%) containing 5% milk. Either rabbit anti-PFKFB3 C-terminus polyclonal antibody (1:125, Abgent, San Diego, CA, USA), mouse anti-β-actin (1:5000, Sigma, St Louis, MO, USA) anti-pan *ras* (1:1000, Oncogene/Calbiochem, San Diego CA, USA) or anti-SV40 LT (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were resuspended in 10 ml of TBS–Tween-20, (5% milk) and incubated with the membrane for 1 h. Secondary antibodies were goat anti-rabbit or anti-mouse HRP conjugated (1:8000, Pierce Biotechnology). All Western blotting experiments were repeated for a total of four experiments.

Multiplex PCR analyses
The following multiplex mRNA primers were custom synthesized (IDT, Coralville, IA, USA) against mouse PFKFB1-4 and human PFKFB1-4. The PCR amplicons were purified from the agarose gel and each was then cloned using the TOPO TA Cloning® kit (Invitrogen), sequenced and aligned with PFKFB1-4 using MegAlign (DNASTAR). The multiplex potential of these primers was confirmed using cDNA species isolated from liver (PFKFB1), heart (PFKFB2), brain (PFKFB3) and testis (PFKFB4) (*data not shown*).

Mouse PFKFB1-4:

5′-GAAGAGGACCATTCAGACAG -3′
5′-CCAGGAAGTATGCTAGAAGG -3′
5′-GAGGCTAGAACAGGAAGTTA -3′
5′- CACATTAGGCAGATCTCCAG -3′
5′-AGGTCGGCATGTTGAAGAGT-3′
5′- AGAGAACAGAGCGTAGGAAG-3′
5′-GGTCCACATAGCAGAGTCAG-3′
5′- CATCCAGCCATTATCCACAG -3′

Human PFKFB1-4:

5′-GTCTGGAGCCAGTGATAATG-3′
5′-CGATGAGGACACAGGCAGTT-3′
5′-TGTGGAATCCGTCTGTGATG-3′
5′-TGCGAGGCTGGACGTGGATA-3′
5′-CCTCACTCGCAGCCACTTCT-3′
5′-CAGTTCCTACTCAATTCCAA-3′
5′-AGACAGGGCAGGAAGTTACC-3′
5′-GGCTGCCTCTCTGGGACCAA-3′

Densitometry
Scanned images were quantified by densitometric analysis using Quantiscan software Version 3.0 (Biosoft, UK). Values obtained were normalized to β-actin and expressed in

densitometric units as a percentage of the control samples (regarded as 100%). All values represent the mean ± s.d. of three independent experiments. Statistical significance was assessed by the two-sample *t*-test (independent variable).

In vivo *studies*

PFKFB3$^{+/+}$ and PFKFB3$^{+/-}$ fibroblasts were collected from exponential growth phase culture in DMEM supplemented with 10% FCS. Cells were washed twice and resuspended in PBS (1×10^7 cells/ml). Groups of CD1 nude female mice (20 gm) were injected s.c. with 0.10 ml of the cell suspension (1×10^6 cells). The tumors were followed from the time of appearance until they reached 10% of the animal weight. Tumor masses were determined in a blinded fashion with Vernier calipers according to the following formula: weight (mg) = (width, mm)^2 × (length, mm)/2 (Taetle *et al*., 1987). All data are expressed as the mean ± s.d. of three experiments. Statistical significance was assessed by the two-sample *t*-test (independent variable).

Micro-positron emission tomography

Mice were injected with 2-(^{18}F)-fluoro-2-deoxy-glucose (150 μCurie, 100 μl in H_2O) intraperitoneally using a 27-gauge needle and were anesthetized after 30 min with 2% isoflurane in oxygen. These mice were then transferred to an R-4 Rodent Scanner Micro-PET (CTI Concorde Microsystems Inc.) and data were collected for 75 min.

Acknowledgements

We thank Robert Mitchell for providing the *ras*V12 and LT retroviruses and Barrett Rollins for providing the hT, hT/Lt and hT/LT/*ras* bronchial epithelial cells. We gratefully acknowledge helpful discussions with Douglas Dean and Otto Grubraw. This work was supported by the following grants: NIH 1P20 RR18733 (JC), NIH 1 R01 CA11642801 (JC), and the P Morris External Research Program (JC and JWE).

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