GGTase-I deficiency reduces tumor formation and improves survival in mice with K-RAS–induced lung cancer

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Protein geranylgeranyltransferase type I (GGTase-I) is responsible for the posttranslational lipidation of CAAX proteins such as RHOA, RAC1, and cell division cycle 42 (CDC42). Inhibition of GGTase-I has been suggested as a strategy to treat cancer and a host of other diseases. Although several GGTase-I inhibitors (GGTIs) have been synthesized, they have very different properties, and the effects of GGTIs and GGTase-I deficiency are unclear. One concern is that inhibiting GGTase-I might lead to severe toxicity. In this study, we determined the effects of GGTase-I deficiency on cell viability and K-RAS–induced cancer development in mice. Inactivating the gene for the critical β subunit of GGTase-I eliminated GGTase-I activity, disrupted the actin cytoskeleton, reduced cell migration, and blocked the proliferation of fibroblasts expressing oncogenic K-RAS. Moreover, the absence of GGTase-I activity reduced lung tumor formation, eliminated myeloproliferative phenotypes, and increased survival of mice in which expression of oncogenic K-RAS was switched on in lung cells and myeloid cells. Interestingly, several cell types remained viable in the absence of GGTase-I, and myelopoiesis appeared to function normally. These findings suggest that inhibiting GGTase-I may be a useful strategy to treat K-RAS–induced malignancies.

Introduction

More than 100 intracellular proteins contain a CAAX motif that directs isoprenylation at a carboxyterminal cysteine (the “C” of the CAAX motif) (1). Some CAAX proteins, such as RHOA, cell division cycle 42 (CDC42), and RAPI, are geranylgeranylated by protein geranylgeranyl-transferase type I (GGTase-I). Others, such as K-RAS and N-RAS, are farnesylated by protein farnesyltransferase (FTase) (2). If the “X” of the CAAX motif is a leucine, the protein is generally geranylgeranylated; otherwise, it is farnesylated (3). Isoprenylation renders the carboxyl terminus of the CAAX proteins more hydrophobic, enhancing their ability to bind to membranes within cells, and also regulates protein–protein interactions. GGTase-I and FTase share a common subunit of GGTase-I (FTase) (2). If the “X” subunit but have unique β subunits that dictate their substrate specificities (2).

In some eukaryotic cells, GGTase-I is an essential enzyme. Null mutations in the β subunit of GGTase-I are lethal in both Drosophila melanogaster (4) and Saccharomyces cerevisiae (5). The lethality of GGTase-I deficiency in S. cerevisiae was likely due to the failure to geranylgeranylate Rho1p and Cdc42p, as the lethality could be overcome by expressing mutant Rho1p and Cdc42p proteins engineered to undergo farnesylation by FTase (6). Interestingly, Candida albicans and Arabidopsis thaliana are viable in the absence of GGTase-I (7, 8). Remarkably, the impact of GGTase-I deficiency in mammalian cells has never been studied.

The realization that the RAS proteins are farnesylated (9) has fueled interest in protein isoprenylation. Farnesylation is important for the proper membrane targeting of RAS proteins and for their transforming ability (10). In mouse models, farnesyltransferase inhibitors (FTIs) have significant antitumor activity and minimal toxicity (11). In human clinical trials, however, FTIs have been disappointing, at least for the treatment of solid tumors (12), likely because K-RAS and N-RAS—the RAS isoforms most often implicated in human cancer—can be geranylgeranylated in the presence of an FTI (13).

The realization that K-RAS and N-RAS can be geranylgeranylated in the setting of FTI therapy prompted efforts to develop GGTase-I inhibitors (GGTIs). GGTIs could be used in combination with FTIs to block the isoprenylation of K-RAS and N-RAS. The rationale for using GGTIs in cancer therapy is further underscored by the fact that geranylgeranylated proteins, such as RHOA, RHOC, and RALA, are intimately involved in tumor development and metastasis (14–16). Several GGTIs inhibit the growth of tumor cell lines, including cell lines with K-RAS mutations (17–19). Interest in developing GGTIs actually extends beyond cancer therapy (20). Inhibition of GGTase-I ameliorated disease phenotypes in a mouse model of multiple sclerosis (21) and inhibited hepatitis C viral replication in hepatoma cells.

Nonstandard abbreviations used: AAH, atypical adenomatous hyperplasia; CDC42, cell division cycle 42; CDC42, farnesylated CDC42; RHOA, farnesylated RHOA; FTase, farnesyltransferase; FTI, farnesyl transferase inhibitor; GGTase-I, geranylgeranyltransferase type I; GGTI, GGTase-I inhibitor; Kras2LSL, Kras2 knock-in mice; Rho1p, Cdc42p, yeast proteins engineered to undergo farnesylation by FTase for the β subunit of GGTase-I.

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is being evaluated.

Generation and validation of a conditional GGTase-I knockout allele

Results

Fig. 1

Generation of a conditional knockout allele for the β subunit of GGTase-I (Pgg1tb). (A) A sequence-replacement gene-targeting vector in which exon 7 of Pgg1tb is flanked by loxP sites (arrowheads). Expression of Cre recombinase results in the excision of exon 7, creating a frameshift mutation and a null allele. The locations of primers for genotyping are indicated. neo, neomycin phosphotransferase cassette; tk, thymidine kinase. (B) PCR and RT-PCR analyses demonstrating the Cre-induced inactivation of Pgg1tb. Heterozygous Pgg1tb<sup>β+/−</sup> and homozygous Pgg1tb<sup>β−/−</sup> fibroblasts were treated with either a β-gal- or a Cre-adenovirus, and genomic DNA and total RNA were isolated 2 days later. (C) GGTase-I and FTase activity in extracts of β-gal- and Cre-adenovirus–treated Pgg1tb<sup>β−/−</sup> fibroblasts. Values are mean ± SEM of 2 different cell lines in 2 independent experiments. (D) Western blots of extracts from 2 different Pgg1tb<sup>β−/−</sup> fibroblast cell lines treated with β-gal- and Cre-adenovirus. The blots were incubated with antibodies recognizing nonprenylated RAP1, total RAP1, and RHOA. Total ERK1/2 was used as a loading control.

We reasoned that our understanding of the physiologic importance of protein geranylgeranylation could be improved substantially with genetic studies in mice. In this study, we created mice with a conditional knockout allele for the β subunit of GGTase-I (Pgg1tb<sup>β−/−</sup>) and defined the impact of Pgg1tb deficiency on cell viability, cell proliferation, and K-RAS–induced oncogenic transformation.

Also, inhibiting GGTase-I in <i>C. albicans</i> is being evaluated as a strategy to treat nosocomial infections (23).

Despite indications that inhibiting GGTase-I might be useful therapeutically, there is concern regarding the potential toxicity of this approach, in part because geranylgeranylated proteins are more numerous than farnesylated proteins (1). Another reason for concern is that GGTIs have been shown to induce apoptosis of cultured cells and cause toxicity in mouse models (24–26). Other studies, however, have suggested that some GGTIs might not be particularly toxic (27, 28).

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The strategy for creating Pgg1tb<sup>β−/−</sup> mice is illustrated in Figure 1A. In the mutant allele (Pgg1tb<sup>β−/−</sup>), loxP sites flank exon 7, which is critical for enzymatic activity (29). Pgg1tb<sup>β+/−</sup> and Pgg1tb<sup>β−/−</sup> mice were healthy and fertile. To test the conditional allele, we treated Pgg1tb<sup>β+/−</sup> embryonic fibroblasts with a Cre-adenovirus. Cre recombination converted the Pgg1tb<sup>β0</sup> allele to a null allele (Pgg1tb<sup>β−/−</sup>), which produced a transcript lacking exon 7 (which yields a frameshift) (Figure 1B). As expected, inactivation of Pgg1tb eliminated GGTase-I activity but did not affect FTase activity (Figure 1C). In heterozygous knockout cells (Pgg1tb<sup>β−/−</sup>), GGTase-I activity was reduced by approximately 50% (data not shown).

To assess the impact of Pgg1tb deficiency on the processing of 2 GGTase-I substrates, RAP1 and RHOA, we subjected fibroblast extracts to Western blot analysis with an antibody specific for nonprenylated RAP1. Nonprenylated RAP1 accumulated in both the soluble and membrane fractions of β-gal– or Cre-adenovirus–treated Pgg1tb<sup>β−/−</sup> fibroblasts. T, total extract.

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The proliferation arrest in normal and K-RAS\(^{G12D}\)–expressing fibroblasts. (A) Effect of \(Pgg1b\) deficiency on the proliferation of primary mouse fibroblasts. Equal numbers of \(\beta\)-gal– and Cre-adenovirus–treated primary \(Pgg1b^{fl/+}\) and \(Pgg1b^{fl/fl}\) fibroblasts were seeded on 12-well plates. On days 0, 1, 2, and 5, the cells were trypsinized and counted. Values are mean of 1 cell line assayed in duplicate. Similar results were obtained in 2 experiments with 2 different \(Pgg1b^{fl/+}\) and \(Pgg1b^{fl/fl}\) cell lines. (B) Upper panels: \(\beta\)-gal– and Cre-adenovirus–treated \(Pgg1b^{fl/fl}\) cells from the experiment described in A. Lower panels: cells from the experiment in A stained with phalloidin (red) to visualize polymerized actin and with DAPI (blue) to visualize DNA. Original magnification, \(\times 10\) (top); \(\times 63\) (bottom). (C) Migration of \(\beta\)-gal– and Cre-adenovirus–treated fibroblasts. Values are mean ± SEM of 2 cell lines assayed in triplicate. \(P < 0.01\) at 8 and 24 hours, \(P < 0.0001\) at 48 hours compared with \(\beta\)-gal. (D) Western blot of extracts from \(\beta\)-gal– and Cre-adenovirus–treated primary \(Pgg1b^{fl/+}\), \(Pgg1b^{fl/fl}\), and \(Pgg1b^{fl/fl}KLSL\) fibroblasts. (E) Effect of \(Pgg1b\) deficiency on the proliferation of K-RAS\(^{G12D}\)–expressing primary fibroblasts. Equal numbers of \(\beta\)-gal– and Cre-adenovirus–treated primary \(Pgg1b^{fl/+}KLSL\) and \(Pgg1b^{fl/fl}KLSL\) fibroblasts were seeded in 12-well plates. On days 0, 1, 2, 4, and 6, the cells were trypsinized and counted. Inset shows genotypes of cells assessed by PCR amplification of genomic DNA (lane 1, \(Pgg1b^{fl/+}KLSL\); lane 2, \(Pgg1b^{fl/+}KLSL\); lane 3, \(Pgg1b^{fl/fl}KLSL\); lane 4, \(Pgg1b^{fl/fl}KLSL\)). Values are mean of 1 cell line assayed in duplicate. Similar results were obtained in 2 experiments with 2 cell lines per genotype. (F) Photographs of fibroblasts from the experiment described in E. Original magnification, \(\times 10\). (G) Cell-cycle analysis of \(\beta\)-gal– and Cre-adenovirus–treated \(Pgg1b^{fl/+}KLSL\) fibroblasts. The experiment was repeated twice with similar results. PI, propidium iodide.
isolating $Pggt1b^{fl/fl}$ fibroblast cell lines from littermate embryos. The $Pggt1b^{fl/fl}$ cells were small and spindle shaped, and the amount of polymerized actin was dramatically reduced (Figure 2B). To determine whether the reduced levels of polymerized actin in $Pggt1b^{fl/fl}$ cells impaired cellular migration, we performed a standard wound closure assay in cultured cells. The ability of $Pggt1b^{fl/fl}$ cells to migrate was reduced by 60%–80% (Figure 2C). Also, Western blots revealed increased levels of p21$^{CIP1}$ and phosphorylated AKT$^{S473}$ and decreased levels of phosphorylated ERK1/2 in $Pggt1b^{fl/fl}$ cells than in the parental $Pggt1b^{LSL}$ cells (Figure 2D).

To assess the effect of $Pggt1b$ deficiency on K-RAS-induced cell transformation, we generated primary fibroblasts from $Pggt1b^{fl/fl}$ Kras$^{G12D}$ ($Pggt1b^{fl/fl}$ KLSL) and $Pggt1b^{LSL}$ embryos. The $K^{LSL}$ allele is normally silent; expression of Cre removes a floxed transcriptional terminator sequence (stop cassette) and activates the expression of K-RAS$^{G12D}$ (30). Cre-adenovirus treatment of $Pggt1b^{fl/fl}$ KLSL fibroblasts activated K-RAS$^{G12D}$ expression and resulted in rapid cell proliferation, immortalization, focus formation, increased expression of cyclin D1, and reduced expression of phosphorylated ERK1/2 (Figure 2, D–F). In contrast, Cre-adenovirus treatment of $Pggt1b^{LSL}$ cells caused cell rounding and proliferation arrest (Figure 2, E and F) but not apoptosis or cell death. Inactivation of $Pggt1b$ also prevented the K-RAS$^{G12D}$-induced increase in cyclin D1 and resulted in increased levels of phosphorylated AKT but did not affect p21$^{CIP1}$ levels (Figure 2D). The proliferation arrest in Cre-adenovirus–treated $Pggt1b^{fl/fl}$ KLSL cells was associated with an increased fraction of cells in the G1 phase of the cell cycle (Figure 2G).

Farnesylation of RHOA and CDC42 prevents cell rounding and temporarily restores proliferation of $Pggt1b$-deficient fibroblasts expressing K-RAS$^{G12D}$. In S. cerevisiae, expression of farnesylated versions of RHOA and CDC42 (fRHOA and fCDC42, respectively) overcame the lethality associated with a deficiency of the yeast ortholog of $Pggt1b$ (also known as CAL1) (6). Accordingly, we hypothesized that fRHOA and fCDC42 might overcome the proliferation arrest in Cre-adenovirus–treated $Pggt1b^{fl/fl}$ KLSL cells. To test this hypothesis, we transfection primary $Pggt1b^{fl/fl}$ KLSL fibroblasts with plasmids encoding RHOA (Myc-tagged at the amino terminus), fCDC42 (HA-tagged), or both and treated the cells with Cre-adenovirus. Both RHOA and CDC42 were expressed in transiently transfected cells (Figure 3A). The expression of fRHOA and fCDC42 (but neither construct alone) allowed $Pggt1b^{fl/fl}$ KLSL cells to escape, at least temporarily, the proliferation arrest and form colonies (Figure 3, B and C). Treatment of the colonies with an FTI promptly induced cell rounding and proliferation arrest (compare Figure 3C with the lower-right panel in Figure 2F). However, even in the absence of an FTI, $Pggt1b^{LSL}$ KLSL colonies eventually stopped growing and could not be developed into stable cell lines, presumably because the farnesylation of these proteins was inadequate for growth or because other GGTagase-I substrates are also important for cell growth.

Myeloid cells are viable in the absence of $Pggt1b$. To further explore the effects of $Pggt1b$ deficiency in mammalian cells, we bred $Pggt1b^{fl/}$ mice with lysozyme M–Cre–transgenic mice (LC mice; mice expressing Cre under the control of a myeloid-specific lysozyme M promoter [ref. 31]). $Pggt1b^{fl/}$ LC and $Pggt1b^{fl/}$ LC mice were healthy and fertile, with normal white blood cell counts and normal histology of the spleen, liver, and bone marrow.

To determine the efficiency of Cre-induced inactivation of $Pggt1b$, we used quantitative PCR analysis of genomic DNA and cDNA. In bone marrow–derived, in vitro–differentiated macrophages from $Pggt1b^{fl/}$ LC mice, the efficiency of Cre recombination was 90% (genomic DNA, 90% ± 2.8%; cDNA, 90% ± 3.1%, n = 2).
In unsorted thioglycollate-elicited peritoneal macrophages, the efficiency was somewhat lower (77%–89%). The yield of macrophages from different groups of mice did not differ, and more than 90% of monolayer cells were positive for the macrophage cell surface markers galectin-3 (MAC-2) and sialoadhesin (MAC-3), suggesting approximately 80% efficiency of Cre recombination in circulating myeloid cells.

A knockout of Pgtt1b ameliorates phenotype and extends the lifespan of mice expressing K-RAS\textsuperscript{G12D}. To determine the effect of inactivating Pgtt1b during K-RAS–induced transformation in vivo, we produced mice that were heterozygous for the K\textsuperscript{LSL} allele (30) and the LC allele (31) on a background of the conditional Pgtt1b allele (Figure 4A). Because LC mice have primarily been used in studies of conditional allele inactivation in myeloid cells (32–34), we suspected that K\textsuperscript{LSL}LC mice would develop a K-RAS\textsuperscript{G12D}–induced myeloid malignancy. However, lysozyme M is also known to be expressed in type II pneumocytes in the lung (35, 36). Indeed, the dominant phenotype of the K\textsuperscript{LSL} LC mice was lung cancer (described further below).

The K\textsuperscript{LSL} LC mice was a 10-fold increase in lung weight at days 18–22 (Figure 4F) that resulted from lung tumors (see below). The K\textsuperscript{LSL} LC allele (30) and LC allele (31) on a background of the conditional Pgtt1b allele (Figure 4A). Because LC mice have primarily been used in studies of conditional allele inactivation in myeloid cells (32–34), we suspected that K\textsuperscript{LSL} LC mice would develop a K-RAS\textsuperscript{G12D}–induced myeloid malignancy. However, lysozyme M is also known to be expressed in type II pneumocytes in the lung (35, 36). Indeed, the dominant phenotype of the K\textsuperscript{LSL} LC mice was lung cancer (described further below).

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The survival of mice was monitored for 14 weeks; on day 98, all surviving mice were euthanized. Pgg1b<sup>fl/fl</sup>-K<sub>LSL</sub>-LC mice (K<sub>LSL</sub>-LC mice that were homozygous for the Pgg1b conditional allele) survived far longer than Pgg1b<sup>fl/fl</sup>-K<sub>LSL</sub>-LC mice, some for the entire 98 days (Figure 4C; P < 0.0001). Pgg1b<sup>fl/fl</sup>-K<sub>LSL</sub>-LC mice gained weight at the same rate as control mice until day 40 and then grew more slowly (Figure 4G). At days 18–22, the percentages of neutrophils and lymphocytes in Pgg1b<sup>fl/fl</sup>-K<sub>LSL</sub>-LC mice were entirely normal histology (Figure 4E), and the white blood cell counts remained low throughout the experiment. The lung weight was lower in Pgg1b<sup>fl/fl</sup>-K<sub>LSL</sub>-LC than in Pgg1b<sup>fl/fl</sup>-K<sub>LSL</sub>-LC mice but was still higher than in control mice (Figure 4F).

Inactivation of Pgg1b delays the onset and reduces severity of K-RAS-induced lung cancer. The Pgg1b<sup>fl/+</sup>-K<sub>LSL</sub>-LC mice developed lung tumors that ranged from atypical adenomatous hyperplasia (AAH), adenoma, and adenocarcinoma at day 11 (Figure 5A) to diffuse adenocarcinoma that obliterated alveolar spaces by day 20 (Figure 5B), undoubtedly explaining the dyspnea and premature death. In contrast, the Pgg1b<sup>fl/+</sup>-K<sub>LSL</sub>-LC mice exhibited normal lung histology with only very mild AAH at day 11. At day 20 (Figure 5, C and D) and day 62, the lungs of Pgg1b<sup>fl/+</sup>-K<sub>LSL</sub>-LC mice contained a few AAH lesions, but many segments of the lungs retained entirely normal histology. By day 98, the AAH had progressed and scattered adenomas were shown (Figure 5, E and F). Lung histology in the control mice is shown in Figure 5, G and H.

**Figure 5**

Pgg1b<sup>fl/+</sup>-K<sub>LSL</sub>-LC mice develop lung cancer and hepatic leukocyte infiltration, and Pgg1b deficiency ameliorates these phenotypes. (A–K) H&E-stained sections of lung and liver. (A) Advanced adenoma in lung of a day 11 Pgg1b<sup>fl/+</sup>-K<sub>LSL</sub>-LC mouse. (B) Diffuse adenocarcinoma that obliterated the majority of alveolar spaces in lung of a day 20 Pgg1b<sup>fl/+</sup>-K<sub>LSL</sub>-LC mouse; inset shows K<sub>67</sub> immunostaining. (C) AAH lesions (arrows) in lung of a day 20 Pgg1b<sup>fl/+</sup>-K<sub>LSL</sub>-LC mouse. (D) Magnification of AAH lesion indicated by left arrow in C. (E) Papillary adenoma in lung of a day 98 Pgg1b<sup>fl/+</sup>-K<sub>LSL</sub>-LC mouse. (F) Magnification of E. (G) Normal lung of a day 11 control mouse. (H) Magnification of G. (I–L) Leukocyte infiltration in liver of a Pgg1b<sup>fl/+</sup>-K<sub>LSL</sub>-LC mouse; arrows indicate clusters of leukocytes. (J) Normal appearance of liver from a day 62 Pgg1b<sup>fl/+</sup>-K<sub>LSL</sub>-LC mouse. (K) Normal liver from a day 17 control mouse. Scale bars: 50 μm (A, inset in B, D, F, H, and I–K); 100 μm (B, C, E, and G). (L) Western blot showing high levels of nonprenylated RAP1 in lung tumors from Pgg1b<sup>fl/+</sup>-K<sub>LSL</sub>-LC mice (lanes 1–4) and Cre-adenovirus–treated Pgg1b<sup>fl/+</sup> fibroblasts (lane 13) and lower levels in lung tissue from Pgg1b<sup>fl/+</sup>-LC mice (lanes 9 and 10). Nonprenylated RAP1 was undetectable in lung tumors from Pgg1b<sup>fl/+</sup>-K<sub>LSL</sub>-LC mice (lanes 5–8), normal lung tissue of Pgg1b<sup>fl/+</sup>-LC mouse (lane 11), β-gal-adenovirus–treated Pgg1b<sup>fl/+</sup> fibroblasts (lane 12) and Pgg1b<sup>fl/+</sup>-LC mice (lane 14) fibroblasts, and Cre-adenovirus–treated Pgg1b<sup>fl/+</sup> fibroblasts (lane 15). Total ERK1/2 expression was analyzed on the same blot as a loading control. Protein extracts from an additional 4 tumors of Pgg1b<sup>fl/+</sup>-K<sub>LSL</sub>-LC mice and 2 tumors from Pgg1b<sup>fl/+</sup>-K<sub>LSL</sub>-LC mice were analyzed with similar results.

Both alleles of Pgg1b are inactivated in lung tumors of Pgg1b<sup>fl/fl</sup>-K<sub>LSL</sub>-LC mice. To determine whether both alleles of Pgg1b had been inactivated in the lung tumors of Pgg1b<sup>fl/fl</sup>-K<sub>LSL</sub>-LC mice, we performed quantitative PCR and Western blots. The tumor cells expressed K-RAS<sup>G12D</sup> and lacked the stop cassette in the promoter of the K<sub>LSL</sub> allele. By quantitative PCR of genomic DNA (n = 10 tumors from 5 mice), the amount of the stop cassette was reduced by 68% ± 1.7% in the tumors of Pgg1b<sup>fl/+</sup>-K<sub>LSL</sub>-LC mice, indicating that 68% of the cells in the tumor cells were tumor cells. Theoretically, if both alleles of Pgg1b had been inactivated in all of the tumor cells, there would be approximately a 68% reduction in Pgg1b. Indeed, Pgg1b levels were reduced by 65% ± 1.7% (the reductions in the stop cassette and Pgg1b were not different; P = 0.13). In addition, nonprenylated RAP1 was present at high levels in tumor extracts from Pgg1b<sup>fl/+</sup>-K<sub>LSL</sub>-LC mice but was never detected in tumors from Pgg1b<sup>fl+/+</sup>-K<sub>LSL</sub>-LC mice or in Cre-adenovirus–treated Pgg1b<sup>fl/+</sup> fibroblasts, as shown by Western blots (Figure 5L). Moreover, the levels of nonprenylated RAP1 were...
Inactivation of Pggt1b inhibits proliferation and colony growth of K-RASG12D-expressing hematopoietic cells. Spleens from Pggt1bfl/flKLSL LC mice contained an increased proportion of GR-1-, CD11b+, and CD117+ cells, consistent with expansion of a pool of immature myeloid cells (Figure 7A). We hypothesized that these splenocytes would grow autonomously in vitro. Indeed, splenocytes from Pggt1bfl/flKLSL LC mice formed colonies in the absence of growth factors, whereas splenocytes from control and Pggt1bfl/flKLSL LC mice did not (Figure 7B). Similar results were obtained with bone marrow cells (Figure 7C). In the presence of growth factors (SCF, IL-3, IL-6, erythropoietin), bone marrow cells from Pggt1bfl/flKLSL LC, Pggt1bfl/flKLSL LC, and control mice formed similar numbers of colonies (Figure 7D). However, there were more GM-CFUs in the bone marrow of Pggt1bfl/flKLSL LC mice than in Pggt1bfl/flKLSL LC or control mice (Figure 7D; P < 0.01). Genotyping of the GM-CFU colonies shown in Figure 7D demonstrated that the KLSL allele was activated and that both Pggt1b alleles were inactivated (Figure 7E).

Discussion

In this study, we created mice with a conditional Pggt1b knock-out allele and show that the inactivation of Pggt1b arrested proliferation and reduced migration of cultured fibroblasts; it also improved survival and reduced lung tumor formation and myelo-proliferation in KLSL LC mice. Moreover, several cell types were viable in the absence of Pggt1b, including fibroblasts, macrophages, lung tumor cells, and cells within GM-CFU colonies.

In normal fibroblasts, inactivation of Pggt1b arrested proliferation, disrupted the actin cytoskeleton, increased the levels of p21CIP1, and reduced cell migration. In fibroblasts expressing K-RASG12D, the absence of Pggt1b did not increase p21CIP1 levels and resulted in cell rounding. A potential mechanism behind the cytoskeletal disruption and proliferation arrest in Pggt1b-deficient cells is inhibition of geranylgeranylation of the RHO family proteins such as RHOA and CDC42. In keeping with this idea, expressing RHOA and ICDC42 prevented cell rounding in Pggt1b-deficient K-RASG12D-expressing fibroblasts. However, the RHOA and ICDC42 did not fully restore cell proliferation, suggesting that additional GGTase-I substrates are required for normal levels of cell proliferation.

The inactivation of Pggt1b significantly reduced lung tumor growth and improved survival in KLSL LC mice. However, tumors eventually developed in the Pggt1bfl/flKLSL LC mice. Theoretically, these tumors might have arisen from K-RASG12D-expressing cells in which Pggt1b was not completely inactivated. In fact, such a scenario was recently proposed to explain the results of experiments designed to assess the impact of Rac1 deficiency on K-RAS-induced lung cancer (37). However, in the current study, we provide strong evidence that both alleles of Pggt1b were inactivated in lung tumor cells expressing K-RASG12D.

The finding that both alleles of Pggt1b were inactivated in K-RASG12D-expressing lung tumors suggests that mammalian cells can proliferate in the absence of GGTase-I activity. This notion is further supported by the finding that both Pggt1b alleles were inactivated in individual GM-CFU colonies from bone marrow of Pggt1bfl/flKLSL LC mice. Why would the tumor cells and bone marrow cells be different from the cultured fibroblasts, which never proliferated in the absence of GGTase-I? One possibility is that the tumor cells accumulated additional mutations that allowed them to overcome the effect of Pggt1b deficiency. Another possibility is that alternate prenylation of key substrates by FTase was somehow more efficient in Pggt1b-deficient tumor cells. Some CAAX proteins, such as RHOB, KRAS, and Nras, can be prenylated by both FTase and GGTase-I (38, 39). Perhaps the tumor cells, but not the fibroblasts, eventually accumulated sufficient amounts of farnesylated proteins to permit cell growth.

In addition to lung cancer, activation of the KLSL allele by the LC allele resulted in a myeloproliferative phenotype, with infiltration of leukocytes in the liver and growth factor–independent colony growth of hematopoietic cells. Both of those phenotypes were eliminated in the absence of GGTase-I in Pggt1bfl/flKLSL LC mice, and the white blood cell counts in the Pggt1bfl/flKLSL LC mice remained significantly reduced lung tumor cells. In fibroblasts expressing K-RASG12D, the absence of Pggt1b did not increase p21CIP1 levels and resulted in cell rounding. A potential mechanism behind the cytoskeletal disruption and proliferation arrest in Pggt1b-deficient cells is inhibition of geranylgeranylation of the RHO family proteins such as RHOA and CDC42. In keeping with this idea, expressing RHOA and ICDC42 prevented cell rounding in Pggt1b-deficient K-RASG12D-expressing fibroblasts. However, the RHOA and ICDC42 did not fully restore cell proliferation, suggesting that additional GGTase-I substrates are required for normal levels of cell proliferation.

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low for the entire length of their lifespan (up to 98 days). In the future, it would be interesting to define the impact of GGTase-I deficiency on other experimental myeloproliferative disease syndromes, for example the one elicited when the K\textsubscript{LSL} allele is activated by the Mx1-\textsubscript{Cre} transgene (40, 41).

In a recent study, inactivating the β subunit of FTase (\textit{Fntb}) did not alter the development of K-RAS–induced lung cancer and had only a minor effect on H-RAS–induced skin cancer (42). Also, \textit{Fntb}–deficient fibroblasts grew in cell culture, albeit at a reduced rate. The current study indicates that GGTase-I deficiency has a greater impact than FTase deficiency on cell proliferation, migration, and K-RAS–induced transformation. Multiple factors likely contribute to this difference. One is that there are simply more geranylgeranylated than farnesylated proteins in cells (1). It is also possible that the lipid modification is more important for geranylgeranylated proteins. A geranylgeranyl lipid is significantly more hydrophobic than a farnesyl lipid (43), so it seems plausible that the loss of protein geranylgeranylation could have a greater effect on the avidity of proteins for membrane surfaces, and hence greater functional consequences, than loss of protein farnesylation.

The conditional \textit{Pggt1b} allele we developed will make it feasible to compare the impact of genetic and pharmacologic inhibition of GGTase-I. Such studies could be enlightening because they might help to sort out mechanism-related versus compound-related effects of different GGTIs. Previously, GGTIs were reported to upregulate \textit{p21\textsuperscript{CIP1}} and induce apoptosis in cultured cells (27, 28, 44, 45). However, in the current study, \textit{p21\textsuperscript{CIP1}} levels were not increased in K-RAS\textsuperscript{G12D}–expressing \textit{Pggt1b\textsuperscript{fl/fl}} mice (n = 2 of each genotype) in the presence of growth factors. Cells were seeded in methylcellulose medium supplemented with recombinant SCF, IL-3, IL-6, and erythropoietin, and the colonies were counted and morphologically typed 10 days later. E-BFU, burst-forming unit-erythroid; GEMM, granulocyte, erythrocyte, macrophage, megakaryocyte. (E) PCR amplification of genomic DNA from individual GM-CFU bone marrow colonies from the experiment in D.
Methods
Generation of a conditional Pggt1b knockout allele. A 765-bp fragment containing exon 7 and parts of flanking introns from Pggt1b was amplified with primers 5′-GGCTGCTTTCATA-3′ and 5′-GGCTGCTTTCATA-3′ using genomic DNA from 129/OlaHsd ES cells as a template. The fragment was cloned into the EcoRI site of pNB1, which contains a polylinker flanked by loxP sites. The amplified exon 7 fragment was cloned into the SalI site of pKSloa/PNTmod, a commonly used plasmid for gene-targeting constructs (46). In this vector, the loxP sites flanking the neo cassette had been replaced with Frt sites. A 2.4-kb 5′ arm was amplified with primers 5'-AGGCAATTCTTGAGTGAAGTGTTATTCAGGGG-3′ and 5′-GTGCCATCTCTGATAAGGAGCTTG-3′. Cre recombination was also assessed by PCR of the Pggt1b allele (described above), the recombinant Pggt1b allele (genomic DNA), 5′-CTCTTTGCACTTGGTCCTGTA-3′ and 5′-CTCTTGCACTTGGTCCTGTA-3′; and cyclophilin cDNA (used as reference gene), 5′-TGGAGAGACACACAGAGACA-3′ and 5′-TGGCGAGTGTGGCAAATAT-3′. Cre recombination was also assessed by PCR of the Pggt1b allele (described above), the recombinant Pggt1b allele (genomic DNA), 5′-CTCTTTGCACTTGGTCCTGTA-3′ and 5′-CTCTTGCACTTGGTCCTGTA-3′; and cyclophilin cDNA (used as reference gene), 5′-TGGAGAGACACACAGAGACA-3′ and 5′-TGGCGAGTGTGGCAAATAT-3′.

Prenyltransferase activity measurements. To measure GGTagase activity, cytosolic preparations from total cellular extracts were incubated with [3H]-labeled geranylgeranylporphosphate and recombinant human H-Ras-Cys-Val-Leu-Leu; for FTase activity, the cytosolic preparations were incubated with [3H]-labeled farnesylpyrophosphate and recombinant human H-Ras-Cys-Val-Leu-Ser. These assays have been described (47).

Subcellular fractionation and Western blotting. Soluble and membrane fractions of cultured fibroblasts were prepared by ultracentrifugation at 100,000 g as described in ref. 48. For Western blotting, equal amounts of total protein from subcellular fractions or total protein extracts from fibroblasts and tissues were size fractionated on 10%–20% SDS-PAGE (Criterion; Bio-Rad). The proteins were transferred to nitrocellulose membranes and incubated with antibodies recognizing nonprenylated RAP1A (catalog no. sc-1482), total RAP1 (catalog no. sc-65), RHOA (catalog no. sc-418), p21 (catalog no. sc-6246), cycling D1 (catalog no. sc-450), and the HA tag (catalog no. sc-7392) (Santa Cruz Biotechnology Inc.), phosphorylated ERK1/2 (catalog no. 9106), phosphorylated AKT (catalog no. 9271), total AKT (catalog no. 9272), total ERK1/2 (catalog no. 9102), CDC42 (catalog no. 2462) (Cell Signaling Technology, Inc.), and the Myc tag (catalog no. R950-25). Protein bands were visualized with a horseradish peroxidase-conjugated secondary antibody (catalog nos. sc-2314, sc-2313, and sc-2354 [Santa Cruz Biotechnology Inc.] and NA931 [Amersham]) and the ECL Western Blotting System (Amersham Biosciences). Protein bands were analyzed by densitometry with Quantity One 4.4.0 software (Bio-Rad), and the data were normalized to total ERK1/2.

Cell proliferation. Cells (n = 20,000) infected with the Cre- or β-gal–adenovirus were seeded in duplicate 12-well plates and incubated in serum-free medium overnight. The medium was then replaced with normal medium (10% serum), and the cells were trypsinized and counted at defined intervals.

Staining of the actin cytoskeleton. Fibroblasts on chamber slides were fixed in 4% paraformaldehyde for 15 minutes and permeabilized with 0.1% Triton X-100 for 5 minutes at room temperature. F-actin was stained with Alexa Fluor 546–labeled phalloidin (Invitrogen) for 20 minutes at room temperature, and nuclei were stained with DAPI. The slides were photographed with a Zeiss Axioscram MRm digital camera mounted on a Zeiss Axioplan 2 fluorescence microscope.

In vitro cell migration assay. To assess cellular migration, Pggt1bα/β fibroblasts were infected with Cre- or β-gal–adenovirus for 72 hours. Then 2 × 10⁶ cells were seeded to confluence in 60-mm dishes and allowed to adhere for 24 hours. The cells were washed with PBS, and an approximately 1-mm-wide gap was created by scraping the monolayer with a pipette tip. Photographs were taken at 0, 8, 24, and 48 hours, and cells that had migrated into the gap were counted.

Vectors expressing RHOA and CDC42. A Myc-tagged RHOA construct (RHOA-CVLS) was generated by PCR amplification of mouse fibroblast cDNA with forward primer 5′-AGGATCCGAAATGGAACAAAAACTCATC-
CAGAAGAGGATCTGTAGGCTGCAACTGAGAAGAAGCTG-3' and reverse primer 5'-AGGATCTTCTAGAACAAGCGCCACGGAGTGGTTCCGTTTCCTCCC-3'. A HA-tagged ICDC42 construct (CDCC42-CVLs) was generated by PCR amplification of cDNA with forward primer 5'-AGGATCCGAATGGAAATCCTTGACTGCTGATGGATGTTGGTTGCTG-3' and reverse primer 5'-AGGATCTTTGACGACACACACCTGCCTGTC-3'. The fragments were cloned into the double-expression vector pVTsR02-mcs (InvivoGen). Fibroblasts were transfected with 3 μg of the vector with FuGENE 6 reagent (Roche Diagnostics) before transfection.

Isolation of bone marrow-derived and peritoneal macrophages. Bone marrow cells were plated in conditioned DMEM from CMG14-12 cells (49), which overexpress mouse macrophage CSF. After 7–9 days, differentiated macrophages were harvested for preparation of DNA and total RNA. Peritoneal macrophages were collected 3 days after intraperitoneal injection of 2 ml of thioglycollate (3%) and cultured in RPMI-1640 medium. After 2–3 days, genomic DNA and total RNA were prepared.

Blood counts. Complete blood counts were measured with a Hemavet 950FS cell counter (Drew Scientific).

Histology and immunohistochemistry. For routine histology, sections (4 μm) of paraformaldehyde-fixed tissues were stained with H&E. For immunohistostaining, tissue pieces were embedded in Tissue-Tek (Miles Laboratory) and frozen at −80°C. Cryosections (5 μm) were incubated with antibodies recognizing CD11b (catalog no. 557394), CD4 (catalog no. 557277), CD8 (catalog no. 558733) (BD Biosciences—Pharmingen), Ki-67 (catalog no. M-7249; DakoCytomation), SP-C (catalog no. sc-7705; Santa Cruz Biotechnology Inc.), and CC10 (07-623-82; E-mail: E-mail: pvtrodeo@utsouthwestern.edu) overnight at 4°C. The slides were incubated with a biotin-labeled secondary antibody (Vector Laboratories) for 1 hour and then with StreptABComplex (DakoCytomation) and peroxidase substrate. DAPI was used to stain nuclei. Slides were photographed with an Axiocam HRc digital camera on a Zeiss Axiophot microscope.

Blood counts. Complete blood counts were measured with a Hemavet 950FS cell counter (Drew Scientific).

FACS and apoptosis detection. Spleen cells were incubated with antibodies recognizing the cell-surface markers CD3 (catalog no. 553066), CD11b (catalog no. 550993), CD13 (catalog no. 557845), CD14 (catalog no. 553727), CD19 (catalog no. 552854), CD34 (catalog no. 553733), CD45 (catalog no. 557659), CD117 (catalog no. 553355), CD45R (catalog no. 553129) (BD Biosciences—Pharmingen) and analyzed in a FACSAnova Flow Cytometer (BD Biosciences). Data were analyzed with FACSDiva software (version 5.0.1; BD Biosciences). Cell-cycle distribution of fibroblasts was determined by staining with propidium iodide and by flow cytometry. Apoptosis was detected with the Annexin V/FITC Apoptosis Detection Kit 1 (catalog no. 556547; BD Biosciences) and by RT-PCR to determine the relative levels of BAX and BCL-2 in a semiquantitative fashion (APO-PCR; Sigma-Aldrich).

Methylcellulose colony assays. Spleen cells (105) and bone marrow cells (2 x 105) from control, Pgkt1fl/fl-C32i, and Pgkt1fl/fl-C32i/LC mice were seeded into 30-mm dishes in methylcellulose medium in the absence of growth factors (MethoCult M3234; StemCell Technologies) or in the presence of recombinant SCF, IL-3, IL-6, and erythropoietin (MethoCult M3232; StemCell Technologies). Ten days later, the number and type of colonies were assessed with an inverted microscope.

Statistics. Data are plotted as mean ± SEM. Differences in cell surface specificity of protein prenylation, protein prenylation specificity, and protein prenylation specificity of protein prenylation were assessed with 2-tailed Student’s t-test or 1-way ANOVA with Tukey’s procedure. Survival was assessed with the log-rank test.

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