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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.
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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 RAP1, are geranylgeranylated by protein geranylgeranyltransferase 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 carbonyl 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 α 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.
Also, inhibiting GGTase-I in *C. albicans* 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).

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 (PGGT1B), and defined the impact of PGGT1B deficiency on cell viability, cell proliferation, and K-RAS–induced oncogenic transformation.

Results

Generation and validation of a conditional GGTase-I knockout allele. The strategy for creating PGGT1B mice is illustrated in Figure 1A. In the mutant allele (PGGT1B<sup>fl</sup>), loxP sites flank exon 7, which is critical for enzymatic activity (29). PGGT1B<sup>fl/+</sup> and PGGT1B<sup>fl/fl</sup> mice were healthy and fertile. To test the conditional allele, we treated PGGT1B<sup>fl/fl</sup> embryonic fibroblasts with a Cre-recombinase adenovirus, which produced a transcript lacking exon 7 (which yields a frameshift) (Figure 1B). As expected, inactivation of PGGT1B eliminated GGTase-I activity but did not affect FTase activity (Figure 1C). The inactivation of PGGT1B also resulted in a 2- to 4-fold accumulation of RHOA within cells (Figure 1D).

PGGT1B deficiency blocks proliferation and migration of normal fibroblasts and K-RAS<sup>G12D</sup>–expressing fibroblasts but does not affect cell viability. Inactivating a single PGGT1B allele in PGGT1B<sup>fl/fl</sup> fibroblasts did not affect cell proliferation (Figure 2A). Inactivating both alleles in PGGT1B<sup>fl/fl</sup> cells arrested proliferation (Figure 2A) but had little or no
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The effect on cell viability, as the cells remained viable for more than 3 weeks after the Cre-adenovirus treatment. The proliferation arrest in Pgtt1b<sup>Δ/Δ</sup> cells appeared to be irreversible, as we were unable to generate stable Pgtt1b<sup>Δ/Δ</sup> cell lines. In contrast, we had no difficulty.

**Figure 2**

Inactivation of Pgtt1b results in proliferation arrest in normal and K-RAS<sup>G12D</sup>-expressing fibroblasts. (A) Effect of Pgtt1b deficiency on the proliferation of primary mouse fibroblasts. Equal numbers of β-gal- and Cre-adenovirus–treated primary Pgtt1b<sup>fl/+</sup> and Pgtt1b<sup>fl/fl</sup> fibroblasts were seeded on 12-well plates. On days 0, 1, 2, and 5, the cells were trypsinized and counted. Values are mean ± SEM of 2 cell lines assayed in triplicate. (B) Upper panels: β-gal– and Cre-adenovirus–treated Pgtt1b<sup>fl/fl</sup> 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, ×10 (top); ×63 (bottom). (C) Migration of β-gal– and Cre-adenovirus–treated fibroblasts. Values are mean ± SEM of 2 cell lines assayed in triplicate. (D) Western blot of extracts from β-gal– and Cre-adenovirus–treated primary Pgtt1b<sup>fl/+</sup>, Pgtt1b<sup>fl/fl</sup> K<sup>LSL</sup>, and Pgtt1b<sup>Δ/Δ</sup> K<sup>LSL</sup> fibroblasts. (E) Effect of Pgtt1b deficiency on the proliferation of K-RAS<sup>G12D</sup>-expressing primary fibroblasts. Equal numbers of β-gal– and Cre-adenovirus–treated primary Pgtt1b<sup>fl/+</sup><sup>K<sup>LSL</sup></sup> and Pgtt1b<sup>fl/fl</sup><sup>K<sup>LSL</sup></sup> fibroblasts were seeded in 12-well plates. On days 0, 1, 2, 4, and 6, the cells were trypsinized and counted. Insert shows genotypes of cells assessed by PCR amplification of genomic DNA (lane 1, Pgtt1b<sup>fl/+</sup><sup>K<sup>LSL</sup></sup>; lane 2, Pgtt1b<sup>Δ/Δ</sup><sup>K<sup>LSL</sup></sup>; lane 3, Pgtt1b<sup>fl/fl</sup><sup>K<sup>LSL</sup></sup>; lane 4, Pgtt1b<sup>Δ/Δ</sup><sup>K<sup>LSL</sup></sup>). 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, ×10. (G) Cell-cycle analysis of β-gal– and Cre-adenovirus–treated Pgtt1b<sup>fl/fl</sup><sup>K<sup>LSL</sup></sup> fibroblasts. The experiment was repeated twice with similar results. PI, propidium iodide.
isolating Pggt1bΔ/Δ fibroblast cell lines from littermate embryos. The Pggt1bΔ/Δ 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 Pggt1bΔ/Δ cells impaired cellular migration, we performed a standard wound closure assay in cultured cells. The ability of Pggt1bΔ/Δ cells to migrate was reduced by 60%–80% (Figure 2C). Also, Western blots revealed increased levels of p21\(^{\text{ED}}\) and decreased levels of phosphorylated ERK1/2 in Pggt1bΔ/Δ cells than in the parental Pggt1bβ/β cells (Figure 2D).

To assess the effect of Pggt1b deficiency on K-RAS-induced cell transformation, we generated primary fibroblasts from Pggt1bβ/βKras2fl/fl (Pggt1bβ/βK-G12D) and Pggt1bβ/βKLSL embryos. The K\(^{\text{LSL}}\) 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 Pggt1bβ/βK\(^{\text{LSL}}\) 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 Pggt1bβ/βK\(^{\text{LSL}}\) 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\(^{\text{G1}}\) levels (Figure 2D). The proliferation arrest in Cre-adenovirus–treated Pggt1bβ/βK\(^{\text{G12D}}\) 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 Pggt1bβ/βK\(^{\text{LSL}}\) cells. To test this hypothesis, we transfected primary Pggt1bβ/βK\(^{\text{LSL}}\) fibroblasts with plasmids encoding fRHOA (Myo-tagged at the amino terminus), fCDC42 (HA-tagged), or both and treated the cells with Cre-adenovirus. Both fRHOA and fCDC42 were expressed in transiently transfected cells (Figure 3A). The expression of fRHOA and fCDC42 (but neither construct alone) allowed Pggt1bβ/βK\(^{\text{LSL}}\) 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, Pggt1bβ/βK\(^{\text{LSL}}\) 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 GGTase-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 Pggt1bβ/β mice with lysozyme M–Cre–transgenic mice (LC mice; mice expressing Cre under the control of a myeloid-specific lysozyme M promoter [ref. 31]). Pggt1bβ/βLC and Pggt1bβ/βLC 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 Pggt1bβ/βLC 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 mononuclear cells were positive for the macrophage cell surface markers galectin-3 (MAC-2) and sialoadhesin (MAC-3), as judged by immunofluorescence microscopy. In genomic DNA from circulating white blood cells of the same mice was 25% ± 0.5%, suggesting approximately 80% efficiency of Cre recombination in circulating myeloid cells.

A knockout of Pgst1b ameliorates phenotypes and extends the lifespan of mice expressing K-RAS<sup>G12D</sup>. To determine the effect of inactivating Pgst1b during K-RAS–induced transformation in vivo, we produced mice that were heterozygous for the K<sup>G12D</sup> allele (30) and the LC allele (31) on a background of the conditional Pgst1b 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<sup>G12D</sup>LC mice would develop a K-RAS<sup>G12D</sup>–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<sup>G12D</sup>LC mice was lung cancer (described further below).

The K<sup>G12D</sup> allele in K<sup>S32</sup>LC mice was activated in bone marrow and spleen, as expected, but it was also activated in lung (Figure 4B). The phenotypes of Pgst1b<sup>+/−</sup>LC and Pgst1b<sup>−/−</sup>LC mice were indistinguishable from each other. Both appeared normal until they were 17 days old. At that point, however, their health deteriorated rapidly, and more than 95% of the mice died or had to be euthanized due to severe dyspnea and reduced body weight (median survival, day 20; maximum survival, day 24 and day 35, respectively) (Figure 4C). Although K-RAS<sup>G12D</sup> was expressed in myeloid cells, white blood cell counts were not increased in the Pgst1b<sup>−/−</sup>LC mice (Figure 4D); however, the percentage of neutrophils in the peripheral blood was higher and the percentage of lymphocytes was lower than in littermate controls expressing K-RAS<sup>G12D</sup>-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<sup>S32</sup>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. \textit{Ppgt1b}^{fl/fl} K\textsuperscript{LSL} LC mice (K\textsuperscript{LSL} LC mice that were homozygous for the \textit{Ppgt1b} conditional allele) survived far longer than \textit{Ppgt1b}^{fl/+} K\textsuperscript{LSL} LC mice, some for the entire 98 days (Figure 4C; \( P < 0.0001 \)). \textit{Ppgt1b}^{fl/0}/K\textsuperscript{LSL} 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 \textit{Ppgt1b}^{fl/0}/K\textsuperscript{LSL} LC mice were entirely normal (Figure 4E), and the white blood cell counts remained low throughout the experiment. The lung weight was lower in \textit{Ppgt1b}^{fl/0}/K\textsuperscript{LSL} LC than in \textit{Ppgt1b}^{fl/0} K\textsuperscript{LSL} LC mice but was still higher than in control mice (Figure 4F).

\textit{Inactivation of Ppgt1b delays the onset and reduces severity of K-RAS–induced lung cancer.} The \textit{Ppgt1b}\textsuperscript{fl/0}/K\textsuperscript{LSL} LC mice developed lung tumors that ranged from atypical adenomatous hyperplasia (AAH), adenoma, and adenocarcinoma at day 11 (Figure 5A) to a diffuse adenocarcinoma that obliterated alveolar spaces by day 20 (Figure 5B), undoubtedly explaining the dyspnea and premature death. In contrast, the \textit{Ppgt1b}^{fl/0}/K\textsuperscript{LSL} 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 \textit{Ppgt1b}^{fl/0}/K\textsuperscript{LSL} 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 evident (Figure 5, E and F). Lung histology in the control mice is shown in Figure 5, G and H. \textit{Ppgt1b}\textsuperscript{fl/0} K\textsuperscript{LSL} LC mice had an infiltration of myeloid cells in the liver (Figure 5I). In contrast, \textit{Ppgt1b}^{fl/0}/K\textsuperscript{LSL} LC mice had entirely normal livers at days 20, 62 (Figure 5J), and 98, that were indistinguishable from those of control mice (Figure 5K). The lung tumors of \textit{Ppgt1b}^{fl/0}/K\textsuperscript{LSL} LC mice (lanes 1–4) and Cre-adenovirus–treated \textit{Ppgt1b}^{fl/0} fibroblasts (lane 13) and lower levels in lung tissue from \textit{Ppgt1b}^{fl/fl} LC mice (lanes 9 and 10). Nonprenylated RAP1 was undetectable in lung tumors from \textit{Ppgt1b}^{fl/0}/K\textsuperscript{LSL} LC mice (lanes 5–8), normal lung tissue of \textit{Ppgt1b}^{fl/0} LC mice (lane 11), β-gal–adenovirus–treated \textit{Ppgt1b}^{fl/0} fibroblasts (lane 12) and \textit{Ppgt1b}^{fl/0} fibroblasts (lane 14) fibroblasts, and Cre-adenovirus–treated \textit{Ppgt1b}^{fl/0} 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 \textit{Ppgt1b}^{fl/0}/K\textsuperscript{LSL} LC mice and 2 tumors from \textit{Ppgt1b}^{fl/0}/K\textsuperscript{LSL} LC mice were analyzed with similar results.
2.4-fold higher in lung tumors of \textit{Pggt1b}^{+/K^{LSL}} \textit{LC} mice than in the lungs from \textit{Pggt1b}^{fl/fl} \textit{LC} mice, as judged by densitometric analyses of Western blots (\(P < 0.01, n = 4\); Figure 5L and data not shown). These data strongly suggest that lung tumors in \textit{Pggt1b}^{+/K^{LSL}} \textit{LC} mice arose from cells that accumulate nonprenylated \textit{RAP1} (i.e., cells in which both \textit{Pggt1b} alleles had been inactivated).

\textbf{Inactivation of \textit{Pggt1b} inhibits proliferation and colony growth of K-RAS\textsuperscript{G12D}-expressing hematopoietic cells.} Spleens from \textit{Pggt1b}^{+/K^{LSL}} \textit{LC} mice contained an increased proportion of GR-1\textsuperscript{+}, CD11b\textsuperscript{+}, and CD117\textsuperscript{+} 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 \textit{Pggt1b}^{+/K^{LSL}} \textit{LC} mice formed colonies in the absence of growth factors, whereas splenocytes from control and \textit{Pggt1b}^{fl/fl} \textit{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 \textit{Pggt1b}^{+/K^{LSL}} \textit{LC}, \textit{Pggt1b}^{+/K^{LSL}} \textit{LC}, and control mice formed similar numbers of colonies (Figure 7D). However, there were more GM-CFUs in the bone marrow of \textit{Pggt1b}^{Fl/K^{LSL}} \textit{LC} mice than in \textit{Pggt1b}^{+/K^{LSL}} \textit{LC} or control mice (Figure 7D; \(P < 0.01\)). Genotyping of the GM-CFU colonies shown in Figure 7D demonstrated that the \textit{K^{LSL}} allele was activated and that both \textit{Pggt1b} alleles were inactivated (Figure 7E).

\textbf{Discussion}

In this study, we created mice with a conditional \textit{Pggt1b} knockout allele and show that the inactivation of \textit{Pggt1b} arrested proliferation and reduced migration of cultured fibroblasts; it also improved survival and reduced lung tumor formation and myeloproliferation in \textit{K^{LSL}} \textit{LC} mice. Moreover, several cell types were viable in the absence of \textit{Pggt1b}, including fibroblasts, macrophages, lung tumor cells, and cells within GM-CFU colonies.

In normal fibroblasts, inactivation of \textit{Pggt1b} arrested proliferation, disrupted the actin cytoskeleton, increased the levels of \textit{p21\textsuperscript{CIP1}}, and reduced cell migration. In fibroblasts expressing K-RAS\textsuperscript{G12D}, the absence of \textit{Pggt1b} did not increase \textit{p21\textsuperscript{CIP1}} levels and resulted in cell rounding. A potential mechanism behind the cytoskeletal disruption and proliferation arrest in \textit{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 \textit{Pggt1b}-deficient K-RAS\textsuperscript{G12D}-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 \textit{Pggt1b} significantly reduced lung tumor growth and improved survival in \textit{K^{LSL}} \textit{LC} mice. However, tumors eventually developed in the \textit{Pggt1b}^{+/K^{LSL}} \textit{LC} mice. Theoretically, these tumors might have arisen from K-RAS\textsuperscript{G12D}-expressing cells in which \textit{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 \textit{Rac1} deficiency on K-RAS-induced lung cancer (37). However, in the current study, we provide strong evidence that both alleles of \textit{Pggt1b} were inactivated in lung tumor cells expressing K-RAS\textsuperscript{G12D}.

The finding that both alleles of \textit{Pggt1b} were inactivated in K-RAS\textsuperscript{G12D}-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 \textit{Pggt1b} alleles were inactivated in individual GM-CFU colonies from bone marrow of \textit{Pggt1b}^{+/K^{LSL}} \textit{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 \textit{Pggt1b} deficiency. Another possibility is that alternate prenylation of key substrates by FTase was somehow more efficient in \textit{Pggt1b}-deficient tumor cells. Some CAAX proteins, such as RHOB, KRAS, and NTRAS, 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 \textit{K^{LSL}} 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 \textit{Pggt1b}^{+/K^{LSL}} \textit{LC} mice, and the white blood cell counts in the \textit{Pggt1b}^{+/K^{LSL}} \textit{LC} mice remained...
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In the future, it would be interesting to define the impact of GGTase-I deficiency on other experimental myeloproliferative diseases, for example the one elicited when the K_{LSL} allele is activated by the Mx1-Cre transgene (40, 41).

In a recent study, inactivating the β subunit of FTase (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, 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 Pgt1b 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 p21_{CIP1} and induce apoptosis in cultured cells (27, 28, 44, 45). However, in the current study, p21_{CIP1} levels were not increased in K-RAS\_G12D–expressing Pgt1b\_deficient cells. The explanation for this difference is not known. However, now that a genetic model of GGTase-I deficiency is in hand, it should be possible to define which effects of GGTIs are due to inhibition of GGTase-I and which are likely to be due to off-target effects of the GGTI.

Figure 7

Pgt1b deficiency eliminates K-RAS–induced myeloproliferation. (A) Flow cytometry showing an increased percentage of CD11b/Gr-1 (upper panels) and CD11b/CD117 (lower panels) double-positive cells in the spleens of Pgt1b\^{fl/}_{\alpha}K_{LSL}LC mice compared with control and Pgt1b\^{fl/}_{\alpha}K_{LSL}LC mice (n = 3 mice of each genotype). Shown are representative scatter plots of data from 1 mouse of each genotype and the mean percentage of double-positive cells. The increase in double-positive cells in spleens of Pgt1b\^{fl/}_{\alpha}K_{LSL}LC mice was statistically significant (P < 0.001 versus control and Pgt1b\^{fl/}_{\alpha}K_{LSL}LC mice). (B) Growth factor–independent colony growth of splenocytes from control (n = 4), Pgt1b\^{fl/}_{\alpha}K_{LSL}LC (n = 5), and Pgt1b\^{fl/}_{\alpha}K_{LSL}LC (n = 5) mice. Splenocytes were seeded in methylcellulose medium, and the colonies were counted after 10 days. Values are mean ± SEM. (C) Growth factor–independent colony growth of bone marrow cells from control (n = 4), Pgt1b\^{fl/}_{\alpha}K_{LSL}LC (n = 6), and Pgt1b\^{fl/}_{\alpha}K_{LSL}LC (n = 5) mice. Bone marrow cells were seeded in methylcellulose medium, and colonies were counted after 10 days. Values are mean ± SEM. (D) Colony growth of bone marrow cells from control, Pgt1b\^{fl/}_{\alpha}K_{LSL}LC, and Pgt1b\^{fl/}_{\alpha}K_{LSL}LC 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′-GTTCGGTCTTTCTCTATACATGGAAGAGCT-3′ and 5′-GTTCGGTCTTTCTCTATACATGGAAGAGCT-3′ using genomic DNA from 129/OlaHsd ES cells as a template. The fragment was cloned into the EorI site of pNB1, which contains a polynuclein flanked by xpoI sites. The flxed exon 7 fragment was cloned into the SalI site of pKSloB/PTMD, a commonly used plasmid for gene-constructing constructs (46). In this vector, the xpoI sites flanking the neo cassette had been replaced with Fl site. A 2.4-kb 5′ arm was ampliﬁed with primers 5′-AGAGATATGTTGGAGTAGTTGATTCTCTATTGATTGGG-3′ and 5′-AGCAGGCGATATCCTCTACACCCAGAAC-TAACCAACCCC-3′ and cloned into the BamHI site of the targeting vector. Finally, a 5.3-kb 3′ arm was ampliﬁed with primers 5′-AGCAGGCCGGCCTATTATCATCAGGTGGAGCATGGCAGTGC-3′ and 5′-GTCTGCTCTTTAATAGCTAAAGAGAGCTGTTGCTCGTCTCTAGTTGTCCAGGATCC-3′ and cloned into the PacI and Asel sites of the vector. The vector was linearized with XhoI and electroporated into 129/OlaHsd ES cells. After selection in G418 for 10 days, 306 colonies were screened by Southern blotting of Spl-digested genomic DNA with a 3′-flanking probe. Two targeted clones (Pggt1bΔlox) were used to generate chimeric mice, which transmitted the mutation to their offspring.

Mouse breeding. Pggt1bΔlox mice were bred with mice that were heterozygous for a Cre-inducible Kεlox allele (30), generating Pggt1bεlox/εlox mice. The Kεlox allele contains an activating mutation (G12D) and a flxed transcriptional terminator sequence. Pggt1bεlox/εlox mice were also bred with LC mice (the Jackson Laboratory) to produce Pggt1bεlox/LCS/εlox mice (designated Pggt1bεlox/LC). The LC allele (31) yields expression of Cre recombinase in granulocytes, monocytes, macrophages, and type II pneumocytes. Finally, we bred Pggt1bεlox/LC mice with Pggt1bεlox/LCS/εlox mice to produce Pggt1bεloxε/εlox/LC, Pggt1bεlox/εlox/LC, and Pggt1bεloxε/εlox/LC mice, in which Cre simultaneously activates the expression of K-RASG12D and inactivates the expression of the Pggt1bεlox allele. Control mice inherited either the Kεlox or LC allele, but not both. The mice had a mixed genetic background (129/SV and C57BL/6). Mouse experiments were approved by the Animal Research Ethics Committee at Goteborg University.

Genotyping. The Pggt1bεlox allele was genotyped by PCR ampliﬁcation of genomic DNA from tail biopsies with forward primer 5′-CTCGAATGCCA-GATCTGTTGGA-3′ and reverse primer 5′-CTCATGAAAGTGCACCAGCA-CA-3′. The Pggt1bεlox and Pggt1bεloxΔalleles yielded 280- and 360-bp products, respectively. The LC allele was genotyped according to the distributor’s instructions (http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objt ype=protocol&protocol_id=747); the Kεlox and activated KεloxΔalleles were genotyped as described (30, 40).

Isolation of embryonic ﬁbroblasts and Cre recombinase infection. Experiments with primary ﬁbroblasts (isolated from Pggt1bεlox/εlox, Pggt1bεloxε/εlox, and Pggt1bεloxε/εloxΔembryos on day 15.5 post coitum) were performed with passage 2–4 cells. To inactivate Pggt1bεlox and activate K-RASG12D expression, 106 cells were seeded in 100-mm dishes and infected with 5 × 105 pfu/ml of Cre-adenovirus (AdRSVCre; University of Iowa, Iowa City, Iowa, USA) for 24 hours in 5 ml of medium. As a control, the same cell lines were treated with an adenovirus encoding β-gal (AdRSVβgal; University of Iowa).

Determining the efﬁciency of Cre recombination. DNA and total RNA were prepared from ﬁbroblasts, macrophages, tissues, and blood from mice. cDNA was synthesized from equal amounts of total RNA with the iScript cDNA Synthesis Kit (Bio-Rad). Genomic DNA and cDNA were used for real-time quantitative PCR with Power SYBR Green PCR Master Mix on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The following primer pairs were used: Pggt1b genomic DNA, 5′-CGATCTGC- GGTCTTCTCAT3′ and 5′-CATGGTTACATCCACCAGCACA-3′; STOP cas- sette in Kεlox allele genomic DNA, 5′-TCGAGGACCTAATAACTTTCTG-3′ and 5′-CATGATGCTATACCTGCTGGA-3′; RclI genomic DNA (used as a control), 5′-CAGTAAATCTGTTGGAGGAG-3′ and 5′-CGTTGGCAATA- ACTGTTTTC-3′, Pggt1b DNA, 5′-GGGAGACCCGCTGTGACT-3′ and 5′-GTGGTACCATCAGGCACAGTA-3′; and cyclophilin cDNA (used as reference gene), 5′-TGGGAGACCAACAAGACAGAC-3′ and 5′-TGGCGGAGTGCAGAACATGA-3′. Cre recombination was also assessed by PCR of the Pggt1bεlox allele (described above), the recombined Pggt1bεlox allele (genomic DNA, 5′-CCCTGACAGCTTGTGGGA-3′ and 5′-CCCTGACTGACAAGCGGTCCTCA-3′; product size, approximately 1,300 bp), exon 7–9 sequences (cDNA, 5′-CTCTTCTGTTGGAGCAGTA-3′ and 5′-AGCCCATGCTGAAATTTAG-3′; product size, 600 bp), and exon 4–9 sequences (cDNA, 5′-AGATGACTTTAGGCGTGTG-3′ and 5′-CGCGATCTCC-AAGTACCC-3′; product sizes, 575 bp [Pggt1bΔlox] and 390 bp [Pggt1bεlox]).

Prenyltransferase activity measurements. To measure GGTase-I activity, cytosolic preparations from total cellular extracts were incubated with 1H-labeled geranylgeranylated phosphomonoester and recombinant human H-Ras-Cys-Val-Leu-64; for FThase activity, the cytosolic preparations were incubated with 1H-labeled farnesylated phosphomonoester and recombinant human H-Ras-Cys-Val-Leu-64. 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 RalA (catalog no. sc-1482), total RalA (catalog no. sc-418), p21CIP1 (catalog no. sc-6246), cyclin D1 (catalog no. sc-450), and the Ha tag (catalog no. sc-7392) (Santa Cruz Biotechnology Inc.), phosphorylated ERK1/2 (catalog no. 9106), phosphorylated AKTSer473 (catalog no. 9271), total AKT (catalog no. 9272), total ERK1/2 (catalog no. 9102), CDC42 (catalog no. 2462) (Cell Signaling Technology), and the Myc tag (catalog no. R950-25; Invitrogen). 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 NA9311 [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 phallolin (Invitrogen) for 20 minutes at room temperature, and nuclei were stained with DAPI. The slides were photographed with a Zeiss Axiocam MRm digital camera mounted on a Zeiss Axioplan 2 fluorescence microscope.

In vitro cell migration assay. To assess cellular migration, Pggt1bεlox/εloxΔﬁbroblasts 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 RH0A and JDC42. A Myc-tagged RH0A construct (RH0A-CVL5) was generated by PCR ampliﬁcation of mouse ﬁbroblast cDNA with forward primer 5′-AGGATCCGGAATTCGAGCCAAAACCTCATCCT-
CAGAAGAGGATCTGGTGGTGCATCGAGAAGAAAACCTGG-3' and reverse primer 5'-AGGATCCCTGACAAACGCGCCCACTGCCTTCCCTTCCCC-3'. A HA-tagged CDC42 construct (CDC42-CVLS) was co-transfected with 2 μg of plasmid DNA per well. Cell cycle distribution of fibroblasts was determined by staining with propidium iodide and with flow cytometry. Apoptosis was measured with 1:200 dilution of Annexin V:FITC Apoptosis Detection Kit I (catalog no. 556547; 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 with flow cytometry. Apoptosis was measured with the Annexin V:FITC Apoptosis Detection Kit I (catalog no. 556547; BD Biosciences) and by RT-PCR to determine the relative levels of BAX and BCL-2 in a semiquantitative fashion (APC-PCR; Sigma-Aldrich).

Methylcellulose colony assays. Spleen cells (10^7) and bone marrow cells (2 x 10^7) from control, Pgg1fl/fl/K14::LC, and Pgg1fl/fl/K14::LC mice were seeded in duplicate 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 cellular migration, concentrations and percentages of white blood cells, lung weights, densitometry of protein bands on Western blots, colony-forming ability of hematopoietic cells, and FACs analyses were determined 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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