Gankyrin plays an essential role in Ras-induced tumorigenesis through regulation of the RhoA/ROCK pathway in mammalian cells

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Activating mutations in Ras proteins are present in about 30% of human cancers. Despite tremendous progress in the study of *Ras* oncogenes, many aspects of the molecular mechanisms underlying Ras-induced tumorigenesis remain unknown. Through proteomics analysis, we previously found that the protein Gankyrin, a known oncoprotein in hepatocellular carcinoma, was upregulated during Ras-mediated transformation, although the functional consequences of this were not clear. Here we present evidence that Gankyrin plays an essential role in Ras-initiated tumorigenesis in mouse and human cells. We found that the increased Gankyrin present following Ras activation increased the interaction between the RhoA GTPase and its GDP dissociation inhibitor RhoGDI, which resulted in inhibition of the RhoA effector kinase Rho-associated coiled coil–containing protein kinase (ROCK). Importantly, Gankyrin-mediated ROCK inhibition led to prolonged Akt activation, a critical step in activated Ras–induced transformation and tumorigenesis. In addition, we found that Gankyrin is highly expressed in human lung cancers that have *Ras* mutations and that increased Gankyrin expression is required for the constitutive activation of Akt and tumorigenesis in these lung cancers. Our findings suggest that Gankyrin is a key regulator of Ras-mediated activation of Akt through inhibition of the downstream RhoA/ROCK pathway and thus plays an essential role in Ras-induced tumorigenesis.

Introduction

Carcinogenesis involves sequential mutations in genes that play key roles in the control of cell growth and proliferation (1). These mutations usually lead to either the loss of tumor suppressor function or the gain of function in oncogenes, making tumor cells autonomously proliferate and survive (1). Among the oncogenes, the small GTPases of the *Ras* family are the most frequently altered in human cancers and have been found to be mutated in about 30% of human cancers, such as colorectal, pancreatic, and lung cancers (2, 3). Ras transformation is mediated by numerous downstream effectors linked to diversified pathways, which have been evaluated primarily in mouse fibroblast model systems (4–6). Among them, 3 different effectors, Raf, PI3K, and Ral guanine nucleotide exchange factors (RalGEFs), which lead to distinct pathways, have been long appreciated to be minimally necessary, since inhibition of any 1 of these 3 pathways abolishes Ras-mediated transformation and tumorigenesis (7–9). Although much attention has been drawn to the Raf/MEK/ERK pathway, recent research efforts have expanded the diversity of the effectors and have identified a continually growing pool of proteins with diverse functions. PI3K is the next-best-characterized effector of Ras and has an important role in mediating Ras-driven carcinogenesis through the frequent activation of Akt. Akt, also known as protein kinase B, is an evolutionarily conserved serine/threonine kinase, which contributes to tumorigenesis by inhibiting apoptosis (10). Akt is frequently hyperactivated in human cancers through multiple mechanisms. Inactivating mutations or deletions of the tumor suppressor phosphatase and tensin homolog deleted from chromosome 10 (*PTEN*), which lead to Akt activation, occur frequently in human cancers (11–13). PTEN is a 3-phosphoinositide phosphatase that negatively regulates Akt activity by reducing the intracellular level of PIP3 produced by PI3K (14).

Recently, a role for RhoA and its effector kinase Rho-associated coiled coil–containing protein kinase (ROCK) in the regulation of PTEN activity has been reported (15–17). It was found that RhoA/ROCK activation is essential for PTEN function (15). RhoA is a member of Rho GTPase family and is highly regulated by 3 classes of proteins: GEFs, GTPase-activating proteins (GAPs), and GDP dissociation inhibitors (GDIs). GEFs and GAPs are important determinants of Rho GTPase activity by catalyzing nucleotide exchange or stimulating GTP hydrolysis. GDIs are pivotal regulators of RhoA function, which controls the access of RhoA to GEFs and GAPs, to effector targets, and to membranes in which such effectors reside (18, 19). Previous studies indicated that several proteins bind to RhoGDIs and might thereby regulate their association with Rho proteins, which is a potent regulatory mechanism of Rho proteins activity (20, 21). Therefore, exploring the mechanism that regulates the formation of the RhoA-RhoGDI complex is critical to understand the regulation of RhoA activation.

We have previously reported that the protein levels of Gankyrin are elevated in Ras-transformed cells (22). Gankyrin was initially characterized as the p28 protein, a component of the 26S proteasome, and was recently identified as an oncoprotein with repeated ankyrin domains (23–26). Although the protein levels of Gankyrin

Authorship note: Jiang-Hong Man and Bing Liang contributed equally to this work.
Conflict of interest: The authors have declared that no conflict of interest exists.
Citation for this article: *J Clin Invest.* 2010;120(8):2829–2841. doi:10.1172/JCI42542.

are high in hepatocellular carcinomas and Gankyrin functions as an oncoprotein (24, 25), the exact mechanism of Gankyrin oncogenic effect in cellular transformation remains elusive. Here, we report that Gankyrin plays an essential role in Ras-initiated transformation and tumorigenesis. We demonstrate that Gankyrin is a key regulator of Ras-mediated activation of Akt through inhibition of the RhoA/ROCK/PTEN pathway. In addition, we found that Gankyrin is highly expressed in human lung cancers that have *Ras* mutations and is involved in human tumorigenesis. Therefore, our findings suggest that Gankyrin is a critical mediator in Ras-induced tumorigenesis and a potential therapeutic target in tumors caused by *Ras* mutations.

Results

Induction of Gankyrin by RasG12V. To investigate the involvement of Gankyrin in Ras transformation, we established stable cell lines expressing the vector or H-Ras G12V, a constitutively active mutant of *Ras*, in NIH3T3 cells. As reported in our previous study (22), Gankyrin protein levels in H-Ras G12V–transformed NIH3T3 cells were elevated as compared with those in vector-transfected NIH3T3 cells or the parental cells (Figure 1A). The Ras-induced expression of Gankyrin is mostly regulated at transcriptional level, since Gankyrin mRNA levels were dramatically increased in the Ras-transformed cells compared with those in the vector-transfected cells or the parental NIH3T3 cells (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI42542DS1). The Ras-transformed NIH3T3 cells used in the rest experiments in this study are from clone 2 cells. Additionally, our results confirm that Ras G12V expression led to transformation of these stably transfected NIH3T3 cells (Supplemental Figure 1, B and C). To further confirm that Ras G12V is responsible for the induction of Gankyrin in these cells, we treated Ras-transformed NIH3T3 cells with a farnesyltransferase inhibitor (FTI-277), which blocks the posttranslational maturation of Ras family proteins (27). The Gankyrin protein level was significantly reduced in FTI-277–treated Ras-transformed cells, whereas FTI-277 almost did not affect the Gankyrin level in control cells (Supplemental Figure 1D). Consistent with these results, the expression of the nontransforming, dominant-negative mutant of *Ras*, H-Ras S17N (28), had no effect on Gankyrin expression (Supplemental Figure 1E). These results indicated that Gankyrin expression is upregulated by activated Ras.

Gankyrin is essential for Ras-mediated transformation and tumorigenesis. Since Gankyrin was recently identified as an oncogene (24) and we found that Gankyrin is upregulated by activated Ras, we then investigated whether Gankyrin plays a role in Ras-induced transformation by knocking down Gankyrin expression in Ras G12V cells. To do so, we transfected the Ras G12V stable cells with a pool of 3 shRNAs of Gankyrin in pSilencer-hygro vector, and multiple Gankyrin shRNA stably transfected cell clones were screened for the efficiency of Gankyrin knockdown by Western blotting. Two of these clones were selected for further study: clone 1, with high efficiency of Gankyrin knockdown, and clone 2, with poor efficiency of Gankyrin knockdown (Figure 1B). A shRNA with the scrambled sequence was introduced into the same Ras G12V stable cells as a control (Figure 1B). Then, these different cell clones, with different levels of Gankyrin expression, were used to study their morphologies and transformation activity. The stable Ras G12V NIH3T3 cells were spindle shaped and refractile, while the vector-transfected cells were flattened and firmly attached, as previously described by Repasky (29) (Supplemental Figure 1F). Notably, knocking down of Gankyrin reversed the "transformed" morphology of Ras G12V cells to the normal morphology of NIH3T3 cells (Supplemental Figure 1F). The control shRNA had no effect on the morphology of Ras G12V cells. We then assessed the ability of 2 clones of Ras G12V cells with Gankyrin shRNAs (Figure 1B) to form colonies in soft agar, using a transformation assay. We found that the loss of Gankyrin expression (clone 1 cells) abolished the ability of Ras G12V cells to form colonies in soft agar (Figure 1C and Supplemental Figure 1G). Similar effects of Gankyrin were also observed in other cell clones with efficient Gankyrin knockdown (data not shown).

Given the importance of Gankyrin in Ras-driven transformation in vitro, we next addressed the role of Gankyrin in tumorigenesis with a tumorigenicity assay in nude mice. The 4 cell lines (Figure 1B) were injected subcutaneously into nude mice, and tumor growth following each injection was monitored over time. Tumor growth could be detected in all mice that received the parental stable Ras G12V cells, but not the vector-transfected cells, 12 days after cell injection (Figure 1D). However, cells from clone 1, in which Gankyrin was knocked down, failed to develop any detectable tumor, even after 24 days after injection, while cells with control shRNA behaved similarly to the parental Ras G12V cells and caused massive tumor growth in nude mice 12 days after injection. Representative tumors were observed in mice at day 18 (Supplemental Figure 1H). Importantly, when the human Gankyrin was ectopically expressed in the Gankyrin knockdown cells (clone 1 cells), the presence of the human Gankyrin almost completely rescued the ability of these cells to grow in soft agar (Figure 1E) and develop tumors in nude mice, compared with the parental Ras G12V cells (Figure 1F). Thus, our results suggest that Gankyrin is essential for Ras-induced transformation and tumorigenesis.

Gankyrin is required for Ras-mediated Akt activation. To investigate the underlying mechanism of the function of Gankyrin in Ras-induced tumorigenesis, we first examined whether Gankyrin is involved in Ras signaling. It is well documented that activated Ras regulates many different signaling pathways, including the Raf/MEK/ERK and the PI3K/Akt pathways, both of which are critical for Ras-induced tumorigenesis (4–6, 28, 30). Therefore, we tested whether knockdown of Gankyrin affects Ras-induced activation of ERK and Akt. To detect Ras-induced activation of ERK and Akt more efficiently, we used the ER:Ras G12V stable cells, a post-translationally inducible system of activated Ras (8). As shown in Figure 2A, the addition of the ligand 4-hydroxytamoxifen (4-OHT) led to rapid phosphorylation of ERK and Akt. However, when Gankyrin was knocked down, Ras-induced phosphorylation of Akt was dramatically decreased while ERK activation was not affected. In addition, Ras-induced activation of JNK and p38 was not affected in Gankyrin knockdown cells (Supplemental Figure 2A). Previous observations have demonstrated that blocking PI3K/Akt pathway suppressed Ras-induced tumorigenesis (8, 31, 32). To further test whether the loss of Akt activation accounts for the loss of transformation caused by Gankyrin knockdown, we expressed constitutively activated Akt, Myr-Akt (33), in Ras G12V cells with Gankyrin knockdown. We found that colony formation was dramatically decreased in Ras-transformed NIH3T3 cells when Gankyrin was knocked down, but Myr-Akt expression rendered the cells insensitive to the loss of Gankyrin expression (Figure 2B). These results suggest a specific involvement of Gankyrin in Ras-induced activation of Akt.

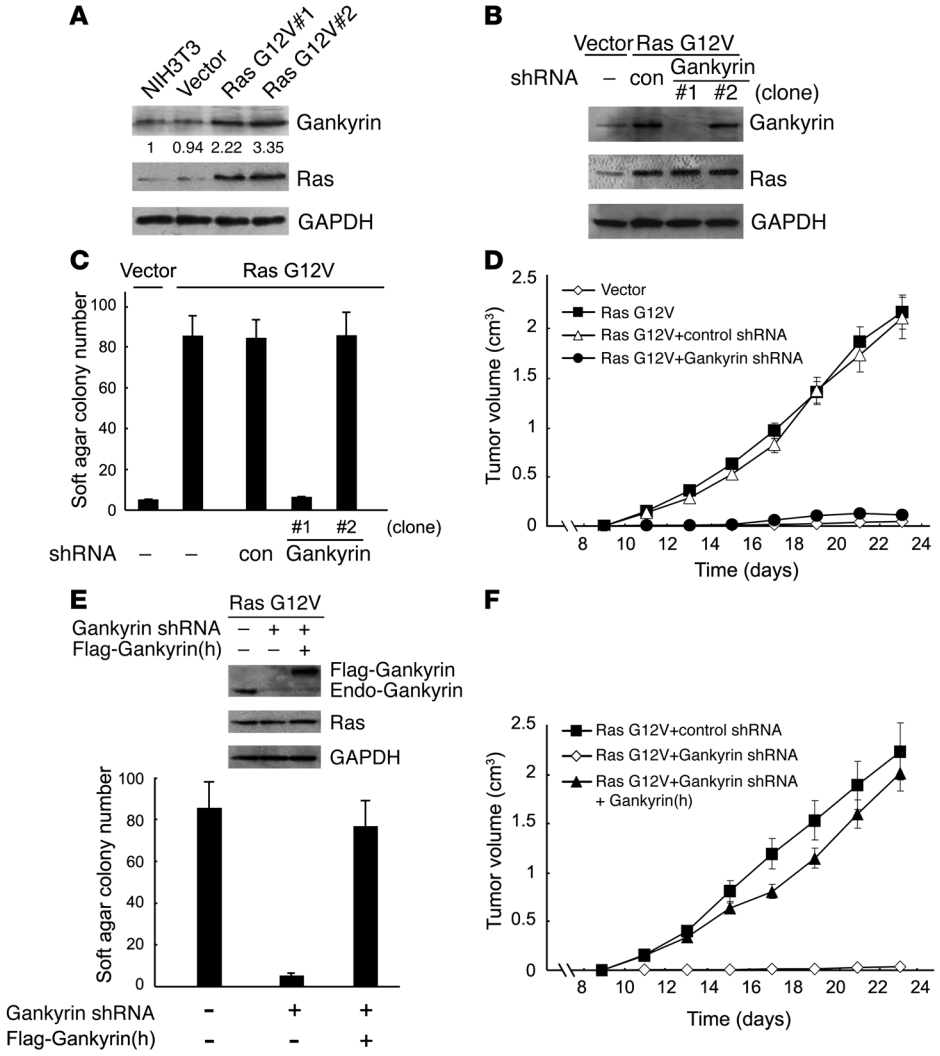


Figure 1

Knockdown of Gankyrin blocks Ras-induced transformation and tumorigenesis. (**A**) NIH3T3 cells were stably transfected with H-Ras G12V or vector. The cell lysates were subjected to immunoblot analysis with the indicated antibody. The relative amount of Gankyrin normalized to GAPDH in NIH3T3 WT cells is designated as 1.0, and the numbers under the first row indicate the relative amount of Gankyrin for other cell lines. (**B**) Analysis of endogenous levels of Gankyrin by immunoblot in Ras-transformed NIH3T3 cells stably expressing Gankyrin shRNA or a scramble control (con). Two cell clones (#1 and #2) were detected following hygromycin selection. Total GAPDH serves as the loading control. (**C**) Analysis of the levels of cell growth in soft agar. Cells described in **B** were mixed with soft agar and seeded into 6-well plates, and then the number of foci was determined 2–3 weeks later. Data are expressed as the total number of colonies per plate. (**D**) The knocking down of Gankyrin expression reduces the ability of Ras G12V–transformed NIH3T3 cells to form tumors in the nude mice tumor growth assay. Nude mice (n = 8) were injected subcutaneously in each flank with 1 × 10^6 cells, and the tumor growth was monitored for 3–4 weeks by caliper measurements. (**E** and **F**) Reexpression of Gankyrin (human [h]) in Ras G12V–transformed NIH3T3 cells stably expressing Gankyrin shRNA by infection with a retroviral vector encoding human Gankyrin restored the tumorigenesis capability of the cells in the (**E**) soft agar assay and the (**F**) nude mice tumor growth assay. endo, endogenous. Data are shown as mean ± SD and are representative of 3 independent experiments.

Since Ras signalling is frequently activated in many tumors with growth factor receptor tyrosine kinase overexpression (34), and the overexpressed EGFR elicits growth-promoting signals primarily through activation of Ras proteins (35), we next examined the effect of Gankyrin on the PI3K/Akt pathway under EGFR activation conditions. Consistent with our results above, silencing of Gankyrin expression also reduced EGF-induced phosphorylation of Akt but not ERK (Figure 2C). To rule out off-target effects caused by the siRNA, relevant data were also confirmed with 2 additional RNAi sequences (a and b) that deplete Gankyrin with a similar efficiency (Supplemental Figure 2B). More importantly, when the mouse Gankyrin was ectopically expressed in Gankyrin knockdown cells, the EGF-induced phosphorylation of Akt was restored to a level comparable with that in control cells (Figure 2D). These results indicate that Gankyrin is a key regulator of Akt activation.

Gankyrin regulates Akt activation through RhoGDI-intervened inhibition of the RhoA/ROCK/PTEN pathway. To gain understanding of the molecular mechanism whereby Gankyrin regulates Akt activation, we performed yeast 2-hybrid screening with Gankyrin as the bait and

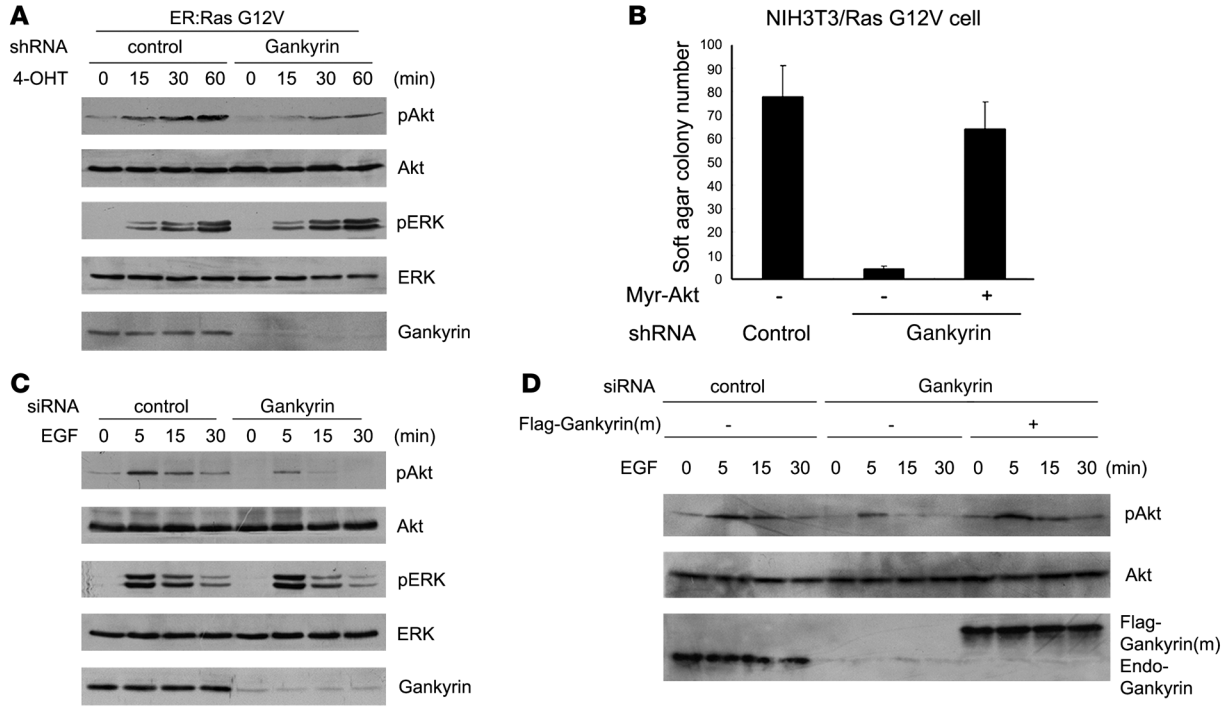


Figure 2

Knockdown of Gankyrin impedes Akt activation. (**A**) NIH3T3 cells stably expressing Gankyrin shRNA or a scramble control were infected with a retroviral construct encoding ER:Ras G12V. Cells were serum starved overnight followed by induction with 100 nM 4-OHT for the indicated times. Cells were then harvested and probed with the indicated antibodies by immunoblot. ER:Ras G12V, expressing inducible activated Ras, was induced by 4-OHT. (**B**) Constitutively activated Akt rescues tumorigenicity in Ras-transformed NIH3T3 cells with Gankyrin knocked down. Overexpression of Myr-Akt in Ras-transformed NIH3T3 cells stably expressing Gankyrin shRNA restored the tumorigenesis capability of the cells in the soft agar assay. Data are shown as mean ± SD and are representative of 3 independent experiments. (**C**) HEK293 cells transfected with control or Gankyrin-specific siRNA were serum starved overnight and then treated with EGF (100 ng/ml) for the indicated times. The cells were then harvested and probed with the indicated antibodies by immunoblot. (**D**) Complementation of Flag-Gankyrin in knockdown HEK293 cells rescues Akt activation. HEK293 cells cotransfected with Gankyrin-specific siRNAs and the expression construct Flag-Gankyrin (mouse [m]) were serum starved overnight, treated with EGF, and tested for the phosphorylation of Akt.

identified several positive clones, and one of them encodes the protein of Rho guanine nucleotide dissociation inhibitor 1 (RhoGDI1) (36). To verify this finding, we examined the interaction between Gankyrin and RhoGDI1 by coimmunoprecipitation and GST pull-down assays. RhoGDI1 was coprecipitated with Flag-Gankyrin and was pulled down by GST-Gankyrin in these experiments (Figure 3, A and B). More importantly, when the endogenous RhoGDI1 was immunoprecipitated with an anti-RhoGDI1 antibody, Gankyrin was detected in the precipitants (Figure 3C). These results suggest that Gankyrin does interact with RhoGDI1 under physiological conditions. Because RhoGDI functions as a RhoA inhibitor and is known to interact with RhoA, we also probed the same blot with an anti-RhoA antibody and found that RhoA was coprecipitated with RhoGDI1 as well (Figure 3C). This observation raised the question of whether Gankyrin is in the same complex with RhoGDI/RhoA. We addressed this issue with coimmunoprecipitation experiments by transfecting Flag-Gankyrin, Myc-RhoA, and Myc-RhoGDI1 in HEK293 cells. Myc-RhoA was barely coprecipitated with Flag-Gankyrin without the presence of Myc-RhoGDI1, but Myc-RhoA was easily detected in the precipitant of Flag-Gankyrin when Myc-RhoGDI1 was cotransfected (Figure 3D). More importantly, when the endogenous Gankyrin was immunoprecipitated with an anti-Gankyrin antibody, both RhoA and RhoGDI1 were detected in the precipitant (Figure 3E). These results indicate that Gankyrin, RhoA, and RhoGDI1 are present in the same complex. We next tested whether Gankyrin binds to these components directly. The recombinant RhoA and RhoGDI proteins were prepared by in vitro translation from an insect cell system. Then, these 2 proteins were incubated with GST-Gankyrin protein-linked glutathione-Sepharose beads. The RhoGDI protein, but not the RhoA protein, was pulled down by Gankyrin (Supplemental Figure 3A). To assess the effect of Gankyrin on the interaction between RhoGDI1 and RhoA, we performed immunoprecipitation experiments with an anti-RhoGDI1 antibody in the presence of different amounts of transfected Flag-Gankyrin. When the expression of Flag-Gankyrin was increased, more RhoA was coprecipitated with RhoGDI1 (Figure 3F), while no obvious increases of the 2 other members of Rho family, Rac1 and Cdc42 proteins, were observed (Supplemental Figure 3B). These experiments suggest that the existence of Gankyrin in RhoGDI1 immunoprecipitates increased the presence of the RhoGDI1 and RhoA complex. This facilitating effect of Gankyrin on the interaction between RhoGDI1 and RhoA is further confirmed by knocking down of Gankyrin expression. Increasing amounts of RhoA and Gankyrin were observed in RhoGDI1 immunoprecipitates following EGF stimulation (Figure 4A). Notably, when the expression of Gankyrin was knocked down, the inter-

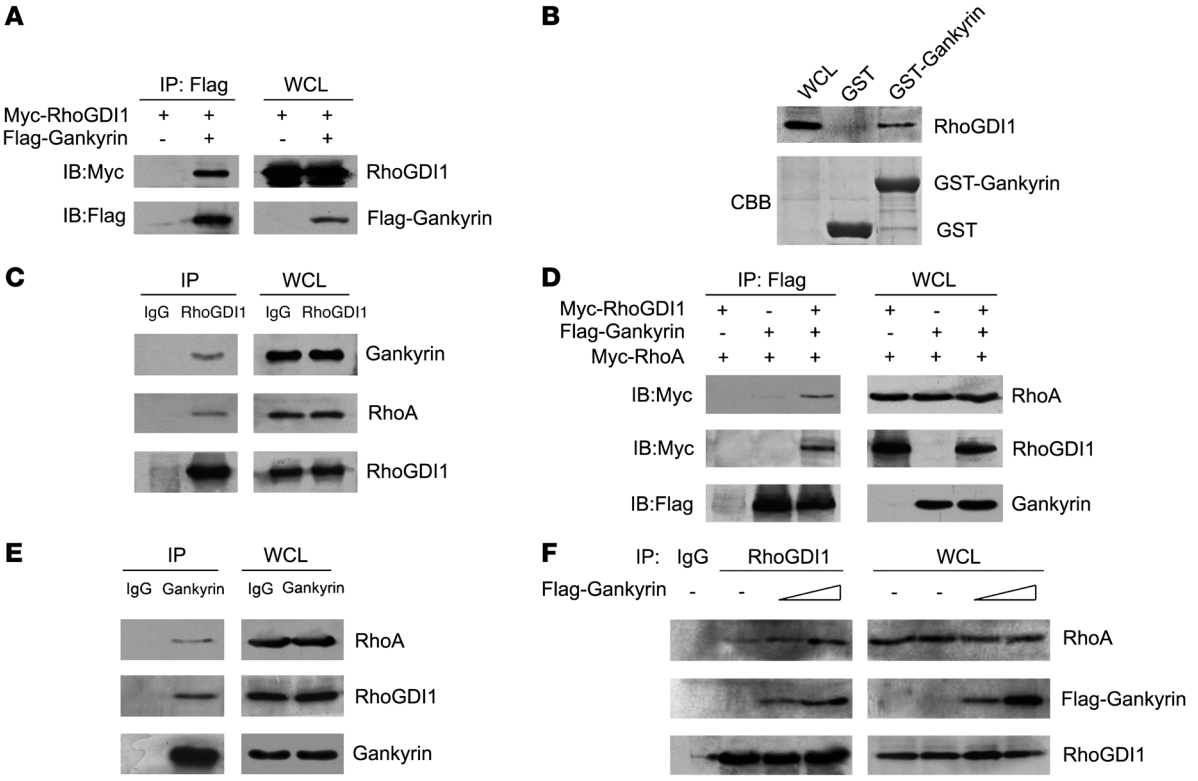


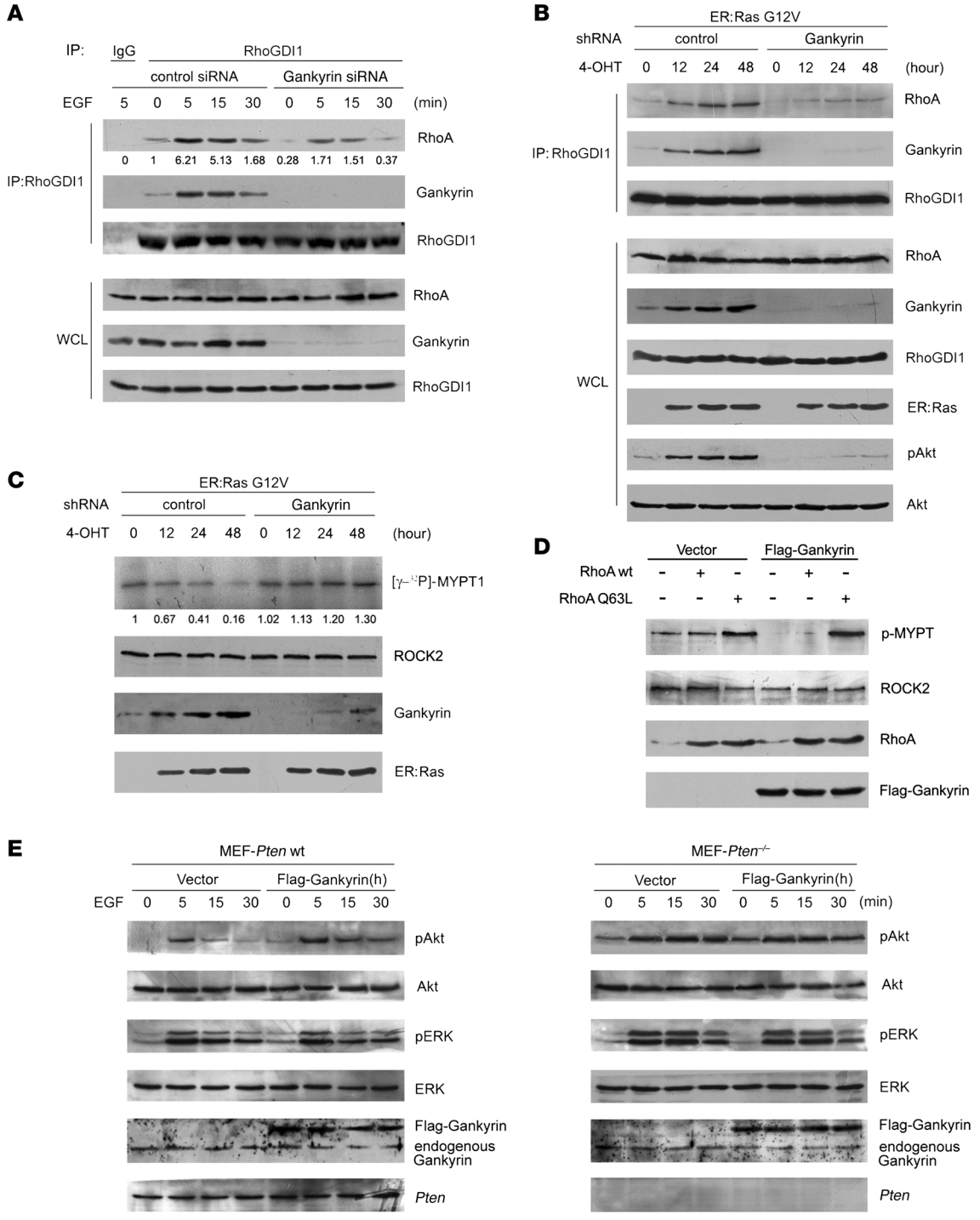
Figure 3

Gankyrin interacts with RhoGDI1 and increases the association of RhoGDI1 and RhoA. (**A**) Immunoassay of HEK293 cells cotransfected with vectors expressing Flag-Gankyrin and Myc-RhoGDI1. Lysates were immunoprecipitated with anti-Flag and then analyzed by immunoblot with anti-Myc and anti-Flag antibodies. WCLs, whole cell lysates. (**B**) Immunoblot analysis of bound proteins in lysates of HEK293 cells expressing Myc-RhoGDI1, incubated with Sepharose beads coupled to GST alone or a fusion protein of GST and Gankyrin (GST-Gankyrin). CBB, Coomassie brilliant blue staining. (**C**) Immunoblot analysis of the interaction among endogenous Gankyrin, RhoA, and RhoGDI1 in lysates of HEK293 cells after immunoprecipitation with rabbit IgG or anti-RhoGDI1 (left) and whole cell lysates (right). (**D**) Immunoassay of HEK293 cells cotransfected with vectors expressing Flag-Gankyrin, Myc-RhoA, and Myc-RhoGDI1. Lysates were immunoprecipitated with anti-Flag and analyzed by immunoblot with anti-Myc and anti-Flag antibodies. (**E**) Immunoblot analysis of the interaction among endogenous Gankyrin, RhoA, and RhoGDI1 in lysates of HEK293 cells after immunoprecipitation with rabbit IgG or anti-Gankyrin (left) and whole cell lysates (right). (**F**) Immunoassay of HEK293 cells transfected with increasing amounts of Flag-Gankyrin. RhoGDI1 in lysates was immunoprecipitated with anti-RhoGDI1, followed by immunoblot analysis of immunoprecipitates (left) and whole cell lysates (right).

action between RhoGDI1 and RhoA was dramatically decreased, supporting the conclusion that Gankyrin facilitates the presence of the RhoA and RhoGDI complex. In addition, the knocking down of Gankyrin did not affect the expression of RhoGDI1 and RhoA (Figure 4A). Similarly, Gankyrin also plays a critical role in facilitating the interaction between RhoGDI and RhoA in the inducible Ras G12V system. As shown in Figure 4B, the induction of activated Ras resulted in the elevated interaction between RhoGDI1 and RhoA in the presence of Gankyrin, but when Gankyrin was knocked down, the amount of RhoA that coprecipitated with RhoGDI1 was dramatically decreased. Importantly, the induction of Ras led to the increase of the protein level of Gankyrin and the prolonged activation of Akt (Figure 4B). Taken together, the results suggest that Gankyrin interacts with RhoGDI and increases the association of RhoGDI and RhoA.

It has been reported that RhoA and its effector kinase ROCK play a role in activating lipid phosphatase PTEN, which inhibits PI3K-Akt signaling pathway (15–17). Therefore, based on our findings that Gankyrin upregulates Ras-induced Akt activation and that Gankyrin enhances RhoGDI and RhoA interaction, we postulate that Gankyrin inhibits RhoA and ROCK activity through RhoGDI. We then examined the effect of Gankyrin on the activity of RhoA and its downstream kinase ROCK. To test the RhoA activity, we used the GST-RBD pull-down assay that only captures the active GTP-bound form of RhoA. EGF treatment caused an increase in the amount of RhoA-GTP, but when Flag-Gankyrin was overexpressed, the amount of RhoA-GTP was decreased, whereas the total amount of RhoA was not affected by the overexpression of Gankyrin (Supplemental Figure 3C). These results indicate that the increased expression of Gankyrin reduced the RhoA-GTP level.

To further demonstrate the inhibitory effect of Gankyrin on the RhoA/ROCK pathway, we next examined the effect of knocking down Gankyrin on ROCK activity in the inducible Ras G12V system. To measure ROCK activity, we performed in vitro kinase assays by immunoprecipitating ROCK2 protein. In these assays, the recombinant myosin-binding subunit (MYPT1, also known as MBS) of myosin light-chain phosphatase was used as the substrate (37). In our results, under serum-starved conditions, the phosphorylation of MYPT1 was decreased when the level of Gankyrin was increased following the induction of Ras G12V (Figure 4C).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IgG
RhoGDI1
control siRNA
Gankyrin siRNA
EGF
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IP:RhoGDI1
RhoA
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Gankyrin
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[γ-32P]-MYPT1
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ROCK2
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RhoA wt
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Figure 4
Gankyrin mediates the interaction of RhoA and RhoGDI1 and regulates Akt phosphorylation through the RhoA/ROCK/PTEN signaling pathway. (**A**) Serum-starved HEK293 cells, transfected with control or Gankyrin siRNA, were treated with EGF for the indicated times. The lysates of these cells were subjected to immunoprecipitation with indicated antibodies. The relative amount of the coimmunoprecipitated RhoA normalized to the immunoprecipitated RhoGDI, in 0 minutes, is designated as 1.0, and the numbers under the first row indicate the relative amount of RhoA for other time points. (**B**) As described in Figure 2A, NIH3T3 cells were induced with 4-OHT for the indicated times. The lysates of these cells were subjected to immunoprecipitation with anti-RhoGDI1 antibody and immunoblotting with indicated antibodies. (**C**) SDS-PAGE and autoradiography of NIH3T3-ER:Ras-G12V-control shRNA and Gankyrin shRNA cells, which were serum starved overnight and treated for 0–48 hours with 4-OHT. Extracts immunoprecipitated with anti-ROCK2 were incubated with a substrate of a fusion protein of GST-MYPT1 (amino acids 654–880). Phosphorylation of MYPT1 was then detected by autoradiography. (**D**) *RhoA* WT or *RhoA Q63L* mutant were overexpressed with Flag-Gankyrin or vector through infection with retroviral vectors in NIH3T3 cells. Extracts immunoprecipitated with anti-ROCK2 were incubated with a substrate of a fusion protein of GST-MYPT1. We then detected the phosphorylation of MYPT1 with the indicated phospho-specific antibodies. (**E**) MEF-*Pten* WT and MEF-*Pten* knockout cells stably expressed Gankyrin through infection with a retroviral vector encoding human Gankyrin. After that, the cells were serum starved overnight and then treated with EGF for the indicated times. The lysates of these cells were analyzed by immunoblot using indicated antibodies.

However, when Gankyrin was knocked down, we did not observe the reduction of the phosphorylation of MYPT1 after 4-OHT treatment, even though the levels of Ras G12V were comparable with those without Gankyrin knockdown (Figure 4C). These results suggested that Gankyrin has an inhibitory effect on ROCK kinase activity. To further demonstrate that Gankyrin works by promoting RhoGDI1 inactivation of RhoA and ROCK, we examined whether the *RhoA Q63L* mutant, which is insensitive to RhoGDI inhibition (38), could overcome the inhibitory effect of Gankyrin on ROCK activity. Overexpressed Gankyrin abolished WT RhoA-mediated ROCK activity but had no effect on RhoA Q63L–induced ROCK activity (Figure 4D). These results further confirmed our conclusion that Gankyrin increases the interaction of RhoA and RhoGDI to inhibit ROCK.

Our conclusion is also supported by experiments with knockdown of ROCK2 protein. When Gankyrin was knocked down, EGF-induced Akt activation was dramatically decreased. However, when ROCK2 expression was also knocked down, Gankyrin was not required for Akt activation anymore (Supplemental Figure 3D). In addition, knocking down the expression of ROCK2 enhanced EGF-induced Akt activation (Supplemental Figure 3E) and abolished the regulatory effect of Gankyrin on Akt activation (Supplemental Figure 3F). A similar effect was also observed with the ROCK inhibitor, Fasudil (39) (Supplemental Figure 3, G and H).

Since RhoA/ROCK inhibits Akt activation through PTEN, we then tested the effect of Gankyrin on Akt activation in *Pten*-null murine embryonic fibroblasts (MEFs), with WT MEFs as the control. As expected, the EGF-induced Akt activation was dramatically increased in *Pten*$^{-/-}$ MEFs compared with WT MEFs (Supplemental Figure 4A). Importantly, when Gankyrin was overexpressed in WT MEFs, EGF-induced Akt activation was dramatically enhanced. However, overexpression of Gankyrin had no effect on Akt activation in *Pten*$^{-/-}$ MEFs (Figure 4E). Taken together, these results suggest that Gankyrin regulates Akt activation through inhibition of the RhoA/ROCK/PTEN pathway. It is known that Gankyrin is a chaperone for the assembly of the 19S cap structure of the 26S proteasome, but the loss of Gankyrin alone had little effect on the 26S proteasome assembly and its function (40–43). To test whether knockdown of Gankyrin disturbs the function of the 26S proteasome, we examined the accumulation of polyubiquitinated proteins in Gankyrin knockdown cells. Neither the overall levels of polyubiquitinated proteins, nor the protein levels of RhoGDI, RhoA, ROCK, and Akt were changed when Gankyrin was knocked down (Supplemental Figure 4B). In addition, coimmunoprecipitation experiments showed that Gankyrin, but not S6b ATPase, was found in the immunoprecipitates of RhoGDI (Supplemental Figure 4C). These results indicated that the function of Gankyrin on Akt activation is independent of the proteasome. We further found that Gankyrin expression levels correlated with morphological changes in the actin cytoskeleton (Supplemental Figure 4, D and E).

Deregulation of Gankyrin expression is associated with human cancers. Since Ras activation leads to the increase of Gankyrin protein levels in NIH3T3 cells and Gankyrin is required for Ras-driven transformation and tumorigenesis, we investigated whether deregulation of Gankyrin expression is associated with human cancers. We first checked Gankyrin expression in normal human and cancer tissues with the SAGE Anatomic Viewer (http://cgap.nci.nih.gov/SAGE) and found that the expression level of Gankyrin mRNA in human lung adenocarcinomas is much higher than that in normal lung. To confirm this finding, we next examined Gankyrin expression by immunohistochemically analyzing tissue microarrays of human lung cancer. The results revealed that the levels of Gankyrin expression were significantly upregulated in the majority of lung tumor tissues compared with those in normal lung tissues (Figure 5A and Supplemental Figure 5; $P < 0.001$). Moreover, Gankyrin expression levels were higher in adenocarcinomas than those in squamous cell carcinomas (Figure 5B; $P = 0.007$). Importantly, *Ras* mutations are often associated with adenocarcinomas but rarely associated with squamous cell carcinomas (2). Representative images stained with the anti-Gankyrin antibody from lung cancer tissue arrays are shown in Figure 5C. In addition, we checked the Gankyrin protein levels in some tumor samples by Western blotting with a polyclonal antibody against human Gankyrin. The tumor samples were compared with their adjacent normal tissues, and the level of GAPDH was used as the protein loading control. Consistent with the results of tissue microarray analysis, the protein levels of Gankyrin were higher in tumor samples than in their adjacent normal tissues. Furthermore, we observed that Gankyrin expression was higher in adenocarcinomas than in squamous carcinomas (Figure 5D).

Gankyrin is involved in tumorigenesis of human lung tumor cells. To further investigate the involvement of Gankyrin in transformation and tumorigenesis of human lung tumor cells, we performed immunoblot analysis on lung cancer cell lines with or without *K-Ras* mutation. The gankyrin protein level was much higher in the *K-Ras* mutant cell lines (A549, Calu-1, H358, H441, and H820) (44–46) than in cell lines without *K-Ras* mutation (SK-Mes-1, H661, Calu-3, and HCC827) (47–51) (Figure 6A). In normal lung cell lines, MRC-5 and WI-38, the Gankyrin level was much lower than that in lung cancer cell lines. Our data also showed that the level of Akt phosphorylation in *K-Ras* mutant cancer cell lines was

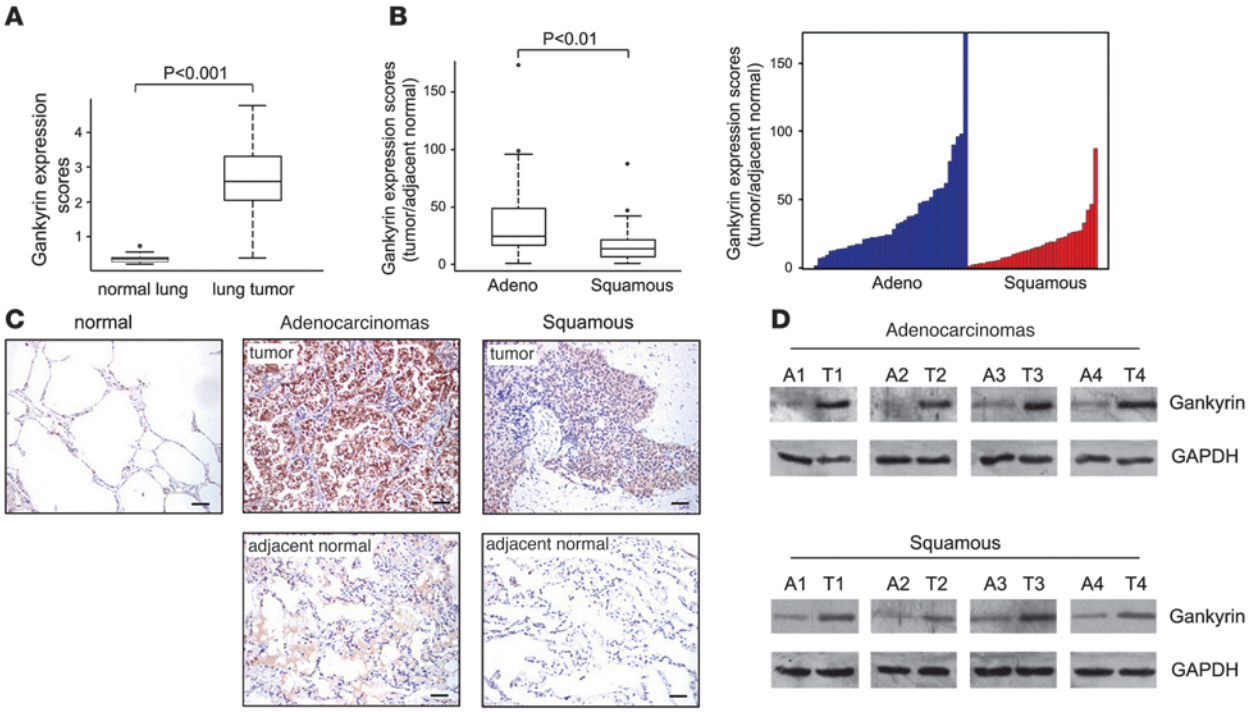

Figure 5

The expression of Gankyrin is increased in human lung cancers with *Ras* mutation. (**A**) IHC was performed on the tissue array containing normal lung and lung tumor samples with anti-Gankyrin antibody. (**B**) The Gankyrin expression levels in adenocarcinoma (adeno) and squamous cell carcinoma were defined as the ratio of the quantitation in each tumor tissue to that of its adjacent normal tissue. $P = 0.007$. (**A** and **B**) Immunohistochemical Gankyrin expression scores are shown as box plots, with the horizontal lines representing the median, the bottom and top of the boxes representing the 25th and 75th percentiles, respectively, and the vertical bars representing the range of data. $P < 0.001$ (*t* test). (**C**) Representative images from lung tissue microarrays stained with anti-Gankyrin antibody and hematoxylin. Scale bar: 100 μm. (**D**) Protein samples of different human tumors (T) and their adjacent normal tissues (A) were probed with anti-Gankyrin antibody, and anti-GAPDH antibody was used as a loading control.

higher than that in *K-Ras* WT cancer cells and normal lung cells. These results indicated that the level of Akt activation correlates with the level of Gankyrin expression. We then tested whether Gankyrin expression is controlled by Ras in the human cancer cells. We observed that the Gankyrin protein level was significantly reduced in A549 and Calu-1 cells when Ras was knocked down by a *K-Ras* siRNA, whereas Gankyrin protein was almost unchanged in SK-Mes-1 cells that have a WT Ras (Figure 6B). These results indicated that the Gankyrin expression is regulated by activated Ras in human tumor cells. Consistent with the results above, Gankyrin knockdown also significantly reduced Akt activation in lung cancer cell lines with *K-Ras* mutation (A549, Calu-1, and H441), while the level of phosphorylated Akt changed slightly in lung cancer cells with WT *K-Ras* (SK-Mes-1 and Calu-3) when Gankyrin was knocked down (Figure 6C and Supplemental Figure 6A).

To further demonstrate the relevance of Gankyrin deregulation in human cancer, we investigated the role of Gankyrin in colony formation and tumorigenesis of the human lung cancer cell lines. Knocking down the expression of Gankyrin dramatically decreased colony formation and tumorigenesis of *K-Ras* mutant cell lines (A549, Calu-1, and H441), while having a minor effect on the lung cancer cells without *K-Ras* mutation (SK-Mes-1 and Calu-3) (Figure 6, D, E, and Supplemental Figure 6B). Ectopic expression of mouse Gankyrin in A549 cells with Gankyrin knockdown restored the abilities of these cells in colony formation and tumorigenesis (Supplemental Figure 6, C and D). Taken together, our results suggested that upregulated Gankyrin is required for tumorigenesis of human lung tumor cells with *Ras* mutation.

Since it is known that growth factor-induced activation of Akt is required for the survival of normal cells and tumor cells (52) and we found that Gankyrin is required for Ras or growth factor-induced Akt activation, we then measured cell proliferation when Gankyrin was knocked down in A549 cells and NIH3T3 cells. We found that Gankyrin knockdown indeed compromised the growth rate of both types of cells (Supplemental Figure 7, A and B). We then examined cell growth rate in Ras-transformed NIH3T3 cells with different levels of Gankyrin knockdown. Akt activation was dramatically decreased in cells with efficient knockdown of Gankyrin expression (clone 1 cells), and Akt phosphorylation was partially inhibited in cells with partial knockdown of Gankyrin (clone 3 cells) (Supplemental Figure 7C). These results indicated that the level of Akt activation correlates with the level of Gankyrin expression. We also performed a cell proliferation assay and a transformation assay with these 4 cell lines. The efficient knockdown of Gankyrin expression in Ras-transformed cells (clone 1 cells) abrogated the increased cell proliferation and transformation induced by Ras. However, when Gankyrin was partially knocked down, as seen in clone 3 cells, Ras-induced transformation was dramatically decreased, while cell proliferation was only marginally affected, compared with the parental Ras G12V NIH3T3 cells (Supplemental Figure 7, D and E).

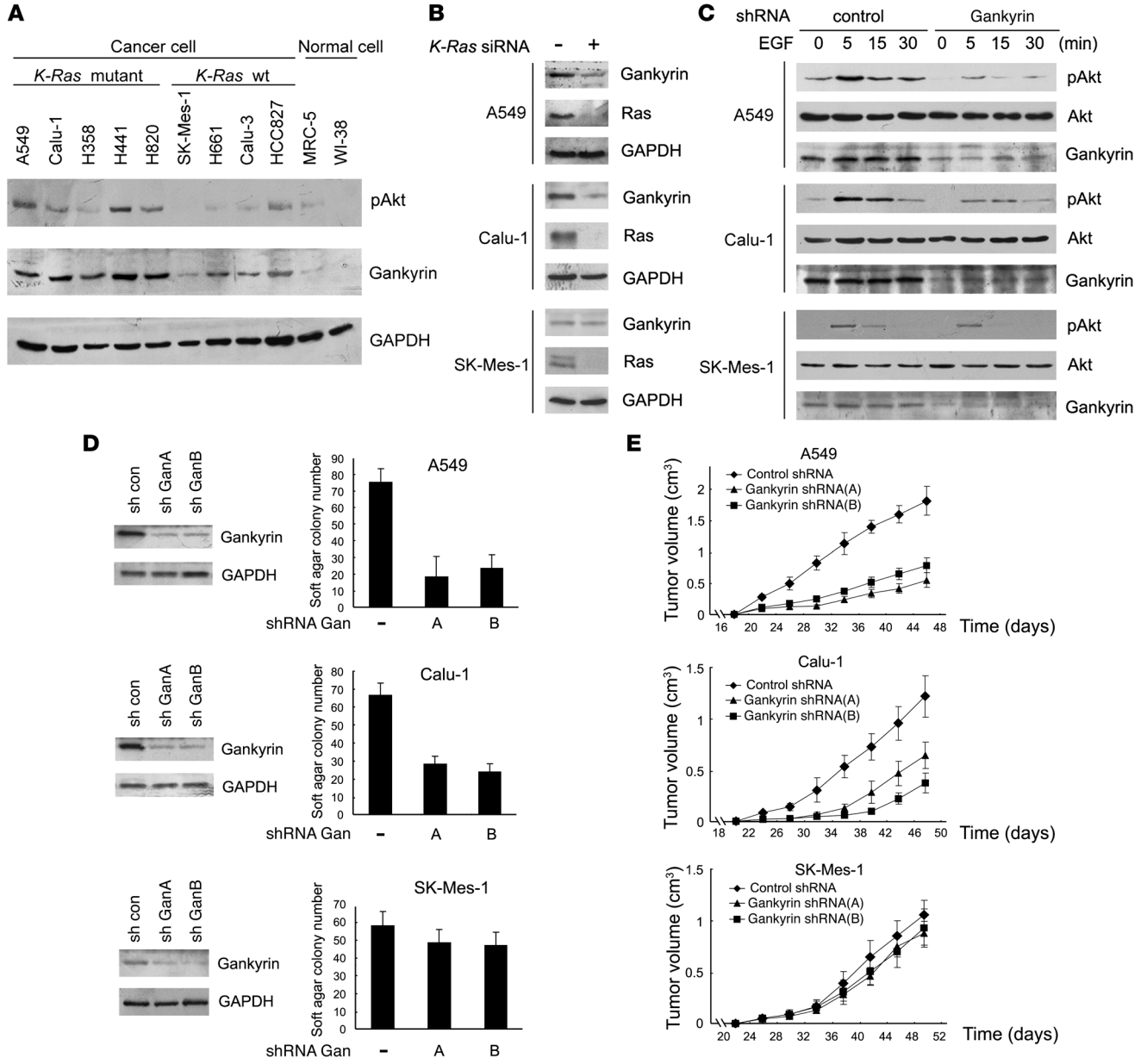

Figure 6

Gankyrin is required for the tumorigenesis of human lung cancer cells with *K-Ras* mutation. (**A**) Immunoblot analysis of the level of Gankyrin expression and Akt phosphorylation (pAkt) at Ser473 in *K-Ras* WT cell lines, *K-Ras* mutant lung cancer cell lines, and normal lung cell lines. (**B**) Cells were transfected with control or *K-Ras*–specific siRNA. After 72 hours of transfection, the cells were harvested and probed with the indicated antibodies by immunoblot. (**C**) Cells stably infected with control or Gankyrin-specific shRNA were serum starved overnight and treated with EGF for the indicated times. Cells were then harvested and probed with the indicated antibodies by immunoblot. (**D** and **E**) Gankyrin is required for the tumorigenesis of human lung cancer cells. Cells stably infected with control or Gankyrin-specific shRNA were used in soft agar assay (**D**) and nude mice tumor growth assay (**E**), as described in Figure 1. Knockdown efficacy of 2 independent shRNAs (sequence A [sh GanA] or B [sh GanB]) of Gankyrin was measured by Western blot (**D**, left), and colony formation in soft agar was enumerated (**D**, right). Data are shown as mean ± SD and are representative of 3 independent experiments.

To test whether the RhoGDI-mediated regulation of the RhoA/ROCK/PTEN pathway by Gankyrin is also important for Ras transformation in human tumor cells, we next examined the formation of Rho/RhoGDI complexes in the human lung cancer cells. In our results, the interaction between RhoGDI1 and RhoA was dramatically decreased, whereas the total amount of RhoA was not affected when Gankyrin was knocked down in A549 cells (Figure 7A). To further demonstrate that Gankyrin works by promoting RhoGDI1 inactivation of RhoA and ROCK in human cancer cells, we expressed WT or constitutively activated RhoA (RhoA Q63L) together with Gankyrin in A549 cells and found that Gankyrin abolished WT RhoA-mediated ROCK activity but had no effect

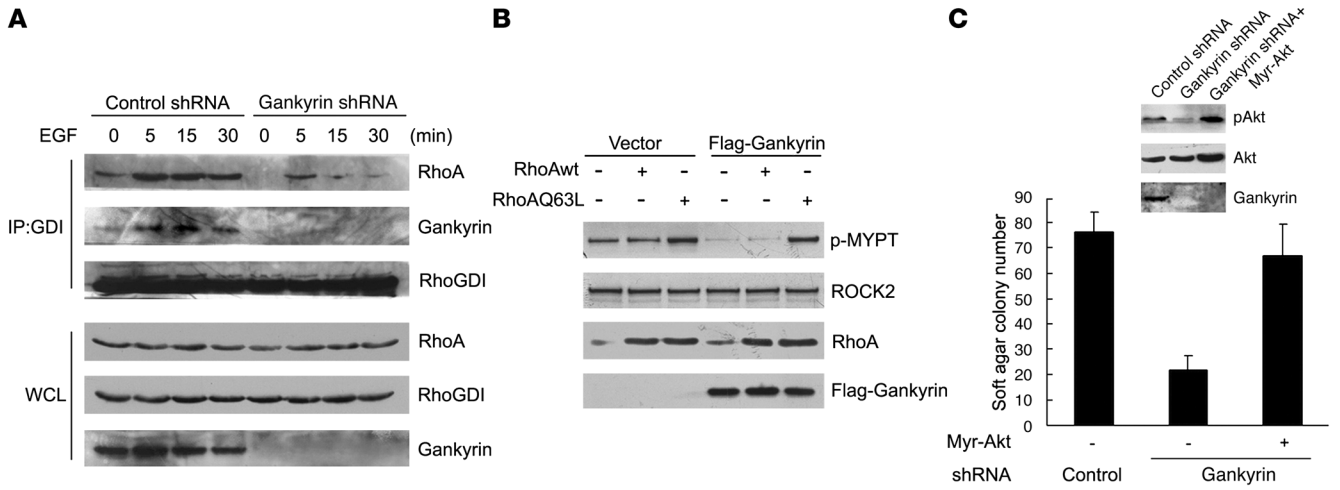

Figure 7

Gankyrin mediates the interaction of RhoA and RhoGDI1 and inhibits the RhoA/ROCK pathway in A549 cells. (**A**) Serum-starved A549 cells stably infected with control shRNA or Gankyrin shRNA were treated with EGF for the indicated times. The lysates of these cells were subjected to immunoprecipitation with anti-RhoGDI1 antibody and immunoblotting with indicated antibodies. (**B**) *RhoA* WT or *RhoA Q63L* mutant were overexpressed with Flag-Gankyrin or vector through infection with retroviral vectors in A549 cells. Extracts immunoprecipitated with anti-ROCK2 were incubated with a substrate of a fusion protein of GST-MYPT1. Phosphorylation of MYPT1 was then detected with the indicated phospho-specific antibody. The whole cell lysates were also analyzed by Western blotting for the expression of ROCK2, RhoA, and Flag-Gankyrin. (**C**) Constitutively activated Akt rescues tumorigenicity in A549 cells with Gankyrin knocked down. Overexpression of Myr-Akt in A549 cells stably expressing Gankyrin shRNA restored the tumorigenesis capability of the cells in the soft agar assay. Data are shown as mean ± SD and are representative of 3 independent experiments. The top panel shows a Western blot verifying the expression of Akt in A549 cells, as indicated.

on RhoA Q63L–induced ROCK activity (Figure 7B). In addition, we expressed constitutively activated Akt, Myr-Akt, in A549 cells with Gankyrin knockdown. We found that colony formation was dramatically decreased when Gankyrin was knocked down, but the cells became insensitive to the loss of Gankyrin when Myr-Akt was ectopically expressed (Figure 7C). Taken together, these results suggest that Gankyrin promotes Ras-mediated tumorigenesis through RhoGDI-mediated inhibition of RhoA/ROCK activity and subsequently, the activation of Akt in human lung tumor cells.

Discussion

Ras mutation is frequent in human cancers. Most effector pathways activated by *Ras* mutations promote cell growth and contribute to malignant transformation (9). However, many aspects of the underlying molecular mechanisms of Ras-mediated transformation and tumorigenesis are still not fully understood. Here, we demonstrated that Gankyrin plays an essential role in Ras-mediated transformation and tumorigenesis. We found that Gankyrin is a key regulator of Ras-mediated Akt activation through inhibition of RhoA/ROCK activity. We showed that Gankyrin is required for RhoGDI to interact with RhoA, a critical step to inhibit RhoA. More importantly, RhoA/ROCK inhibition by Ras-induced Gankyrin results in a prolonged Akt activation and the increased Gankyrin protein is essential for Ras-induced transformation and tumorigenesis (Figure 8). We also found that Gankyrin is highly expressed in some human lung cancers with *Ras* mutations and plays a similar role in human lung tumor cells.

Gankyrin was initially identified as a component of the 26S proteasome and later found to function as an oncoprotein when its expression level is high (23–26). Previously, we reported that Gankyrin expression was dramatically increased during Ras-induced transformation (22). In this study, we demonstrated that Gankyrin is essential for Ras-induced transformation and tumorigenesis. The MAPK/ERK pathway and PI3K/Akt pathway are 2 essential components in Ras-induced transformation and tumorigenesis (6–9). Recent studies with mouse models have revealed that the PI3K/Akt pathway is critical for cell growth and the development of activated Ras–induced tumors (31, 32, 52, 53). It was reported that RhoA and its effector kinase ROCK inhibit Akt activation by enhancing the activity of PTEN, which negatively regulates Akt signaling pathway (15–17). Our current study presented convincing evidence that Gankyrin specifically increases Ras-induced Akt activation but not ERK activation. Surprisingly, we found that Gankyrin regulates Akt activation by inhibiting the activity of RhoA/ROCK pathway. We showed that Gankyrin interacts with RhoGDI and facilitates the presence of the complex of RhoGDI with RhoA, which leads to the decreased level of RhoA-GTP and subsequently the inhibition of ROCK activity (Figures 4 and 7 and Supplemental Figure 3). Furthermore, our data suggest that Gankyrin-mediated inhibition of the RhoA/ROCK/PTEN pathway and prolonged Akt activation are a key regulatory steps in Ras-induced transformation and tumorigenesis. This conclusion was further supported by our findings that Gankyrin lost its regulatory effect on Akt activation when ROCK was knocked down or *PTEN* was deleted (Figure 4 and Supplemental Figures 3 and 4). Since it is known that inactivation of PTEN can result in Akt activation in the absence of any exogenous stimulus and lead to tumorigenesis (12, 54), our work suggests that the oncogenic effect of Gankyrin overexpression is at least partially due to its inhibitory regulation of the RhoA/ROCK/PTEN pathway.

Interestingly, we found that Gankyrin not only plays a key role in Ras-induced Akt activation but also is required for growth factor-induced activation, such as EGF-induced activation, of Akt (Figure 2). These findings raised the possibility that Gankyrin

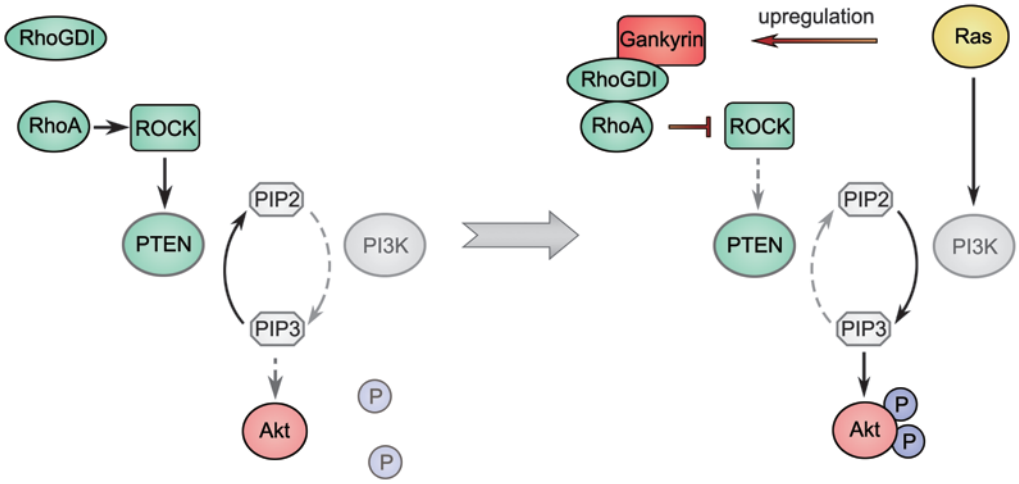

Figure 8
A model for the regulation of RhoGDI/RhoA/ROCK/PTEN signaling by Gankyrin downstream of Ras. PIP2, phosphatidylinositol-4,5-bisphosphate.

is a key mediator of growth factor-induced Akt activation under physiological conditions. Following EGF treatment, a transient increase of the interaction of Gankyrin, RhoGDI, and RhoA was observed (Figure 4A), and the kinetics of this transient increase of protein interaction correlates well with the activation course of Akt (Figure 2). However, under pathological conditions, such as Ras-induced transformation and tumorigenesis, the interactions among these proteins were prolonged and Akt activation was sustained (Figure 4B, Figure 6, and Figure 7). Importantly, one notable difference between these physiological and pathological conditions is the expression level of Gankyrin. The elevated Gankyrin expression most likely results in the consistent inhibition of RhoA and ROCK activity during Ras-induced transformation and tumorigenesis. Currently, it is not clear how the interactions of Gankyrin, RhoGDI, and RhoA are regulated, and further study is necessary to understand the underlying mechanism of the regulation of these protein interactions.

In Ras-transformed cells, it is known that RhoA-GTP levels are elevated, and yet, ROCK activity is suppressed, which is involved in Ras transformation (55–57). Therefore, it is proposed that RhoA-GTP loses its control on ROCK activity in Ras-transformed cells (58), but the underlying mechanism of this observation is unclear. In our study, the activation of ROCK was decreased when the level of Gankyrin was increased following the induction of Ras G12V. However, when Gankyrin was knocked down, we found that the activity of ROCK was dramatically increased (Figure 4C). Therefore, our current study suggested that the function of Gankyrin is to uncouple the signaling link from RhoA to ROCK and to reduce ROCK activity in response to Ras transformation. During the process, although the formation of the RhoA/RhoGDI/Gankyrin complex partially inhibits RhoA activation, RhoA-GTP levels are elevated due to Ras activation in Ras-transformed cells.

Earlier studies suggested that Gankyrin renders its oncogenic effect by destabilizing pRb and p53 (23–25, 59). To test the possible inhibitory effect of Gankyrin on Rb and p53 during Ras-induced transformation, we examined Rb and p53 stability, while knocking down the expression of Gankyrin. Whereas knocking down Gankyrin strongly affected Akt activation, we only detected minor changes in the stability of Rb and p53 (data not shown).

We found that Gankyrin expression levels are high in human lung tumors, particularly adenocarcinomas, which are known to frequently have *Ras* mutations (Figure 5). More importantly, Gankyrin knockdown strongly inhibits tumorigenesis of human lung tumors cells that have *Ras* mutation. Therefore, our study suggests that Gankyrin-mediated activation of the PI3K/Akt pathway plays a critical role in tumorigenesis driven by oncogenic Ras. Interestingly, Gankyrin levels are high in most lung adenocarcinomas, whereas *Ras* mutations were detected in about 30% of adenocarcinomas. Similarly, Gankyrin expression is elevated in most hepatocellular carcinomas (23–25), which do not have high frequency of *Ras* mutation. It is known that Ras signalling pathways are frequently activated in tumors in which growth factor receptor tyrosine kinases have been overexpressed (34) and that EGFR overexpression elicits growth-promoting signals primarily through activation of Ras proteins (35). Thus, it is possible that Gankyrin is overexpressed and required in those tumors without *Ras* mutation but in which Ras is activated, such as those with EGFR overexpression.

Because the increased expression of Gankyrin is the key for its tumor-promoting effect, it is reasonable to speculate that siRNA knockdown of Gankyrin expression will eliminate the tumorigenic effect of Gankyrin in Ras-initiated cancers. Since knockdown of Gankyrin will not completely remove the protein, the residual amount of Gankyrin should be effective for its physiological function. In other words, siRNA knockdown of Gankyrin as a cancer therapy may have limited side effects on normal tissues. Therefore, Gankyrin is a potential therapeutic target that might be suitable for therapeutic intervention of Ras-driven tumors.

Methods

Cell lines, plasmids, and transfection. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in DMEM or 1640, respectively, supplemented with 10% FBS. Phoenix packaging cells were used to generate ecotropic retroviruses and the retroviral shRNA. Murine fibroblast NIH3T3 or NIH3T3 stably expressing Gankyrin shRNA cells were stably infected to generate polyclonal populations with the indicated retrovirus derived from pBabePuro with no insert or by subcloning into the vector cDNAs encoding human Gankyrin and ER:RasG12V (gifts of Christo-

pher M. Counter, Duke University Medical Center, Durham, North Carolina, USA). MEFs or MEF-*PTEN*$^{-/-}$ cells were stably infected to generate polyclonal populations with retrovirus derived from pBabePuro with Gankyrin. Myr-HA-Akt (33) was provided by Jin Q. Cheng (H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida, USA), and EGFP-*RhoA Q63L* was purchased from Addgene.

For shRNA experiments, NIH3T3 cells or Ras-transformed NIH3T3 cells and human lung cancer cell lines A549, Calu-1, H441, SK-Mes-1, and Calu-3 were stably transfected or infected with the pSilencer-hygro or indicated retroviruses derived from pSuper-retro-GFP/neo (Oligoengine), containing the shRNA mouse Gankyrin sequences 5′-GATGGTTCACATCCTTCTG-3′, 5′-GGACCATTACGATGCTACA-3′, and 5′-GAATAGGCATGAGATT-GCT-3′ and the shRNA human Gankyrin sequence A (25) 5′-GAGATC-GCTGTCATGTTAC-3′, sequence B 5′-CTGACCAGGACAGCAGAAC-3′, or the scrambled derivative 5′-AAGCCAGAGACGTTACGTA-3′. Forty-eight hours after transfection or infection, cells were selected with hygromycin or G418 for at least 7 days or for 2 weeks.

Transient siRNA-based silencing of the genes indicated below was achieved using the following siRNAs: human Gankyrin, 5′-GAGATCGCT-GTCATGTTAC-3′ (25), PSMD10 Stealth RNAi siRNA (catalog HSS108733 and HSS108734, Invitrogen), ROCK2 (HSS114107 and HSS114108, Invitrogen), and siRNA directed against specific *K-Ras* target (catalog sc-35731, Santa Cruz Biotechnology Inc.). The Lipofectamine Reagent (Invitrogen) was used for transfections, and the final concentration of siRNA was 0.2 nmol/l. The cells were transfected twice and were harvested 48–72 hours after the second transfection.

Immunoprecipitation and Western analysis. For immunoprecipitation experiments, cells were harvested in ice-cold E1A buffer (250 mM NaCl, 50 mM Hepes, pH 7.4, 0.1% NP-40, 5 mM EDTA), supplemented with a complete protease inhibitor cocktail (Boehringer Mannheim) and 1 mM DTT. Immunoprecipitate was performed with either mouse anti-Flag M2 agarose (Sigma-Aldrich) or rabbit polyclonal anti-RhoGDI1 antibody (Santa Cruz Biotechnology Inc.). Proteins were separated by SDS-PAGE and analyzed by Western blotting. Antibodies used in these studies were as follows: rabbit polyclonal anti–phospho-Akt (Ser473) antibody, rabbit polyclonal anti–phospho-ERK (Thr202/Tyr204) antibody, rabbit polyclonal anti-Akt antibody, and rabbit polyclonal anti-ERK2 antibody (all from Cell Signaling Technologies). Rabbit polyclonal anti-Gankyrin, anti-RhoA, and anti-Cdc42 antibodies were obtained from Santa Cruz Biotechnology Inc. Anti–phospho-MYPT, anti-19S regulator ATPase subunit S6b/Rpt3, and anti-Rac1 antibodies were obtained form Upstate.

Immunohistochemistry and statistical analysis. Routine formalin-fixed and paraffin-embedded excision biopsies were analyzed, which included 19 normal lung tissues, 86 human lung tumors (35 squamous cell carcinomas, 41 adenocarcinomas, and 10 other types of lung tumors), and their adjacent normal tissues (in collaboration with Shanghai Biochip Company Ltd., Shanghai, China). Immunohistochemistry (IHC) was performed based on the method described in ref. 60. Briefly, heat-induced antigen retrieval was performed using 10 mM sodium citrate and 2 mM EDTA buffer (pH 6.0) in a 95°C water bath for 20 minutes. After quenching of endogenous peroxidase and blocking in normal serum, tissues were incubated with primary polyclonal anti-Gankyrin antibody (1:50 dilution, overnight 4°C incubation), followed by secondary antibody and DAB disclosure.

To quantify the protein expression, photographs were obtained from a computerized image system composed of an Olympus CCD camera Dp72 that was connected to an Olympus BX51TRF microscope (Olympus Corporation) at a magnification of ×200. The obtained images were analyzed using Image-Pro Plus version 6.2 software (Media Cybernetics Inc.). Gankyrin expression levels were semiquantitatively assessed in tissue samples. Both the extent and intensity of Gankyrin immunostaining were taken into consideration when analyzing the data. The intensity of staining was scored from 0 to 5, and the extent of staining was from 0% to 100%. The final quantitation of each staining was obtained by multiplying the 2 scores. The slides were analyzed by 2 independent pathologists.

Lung tumor tissue samples and adjacent normal tissues were obtained from the 307 Hospital of People's Liberation Army of China, with the informed consent of patients and with approval for experiments from the Institute of Basic Medical Sciences.

Soft agar transformation and tumor growth assays. Animal studies were approved by the Institutional Animal Care Committee of the Institute of Basic Medical Sciences. To monitor the capacity of different cells to grow in semi-solid medium in vitro, cells were transferred to 2 ml complete DMEM or 1640 containing 0.3% low-gelling agar (Sigma-Aldrich). Then, 1×10^4 cells were seeded in duplicate into 6-well plates containing a 2-ml layer of solidified 0.5% agar in complete medium. The number of foci was determined 2–3 weeks later. For analysis of the tumorigenic capacity of different cells in vivo, nude mice were injected subcutaneously in each flank with 1×10^6 cells (NIH3T3) or 2×10^6 cells (A549, Calu-1, or SK-Mes-1). Tumor growth was monitored by caliper measurements.

Additional methods. Information on plasmid construction, kinase assays, real-time RT-PCR analysis, Rho activity pull-down assay, and protein purification is provided in the Supplemental Methods.

Statistics. Comparisons between groups were analyzed by *t* test. A *P* value of less than 0.01 was considered significant. Statistical calculations were performed using SPSS 13.0.

Acknowledgments

We thank Christopher M. Counter and Jin Q. Cheng for the gift of plasmids. This work was supported by grants from National Natural Science Foundation of China (no. 30830097, no. 30770471, no. 30872348, no. 30871234, and no. 30871275), National High Technology Research and Development Program of China (grant 2009AA02Z103), National Basic Research Program of China (grant 2010CB911900, 2006CB910802), and the Key State Science and Technology Projects (2009ZX09503-001 and 2008ZX10002-016).

Received for publication February 3, 2010, and accepted in revised form May 26, 2010.

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