Supplemental Data

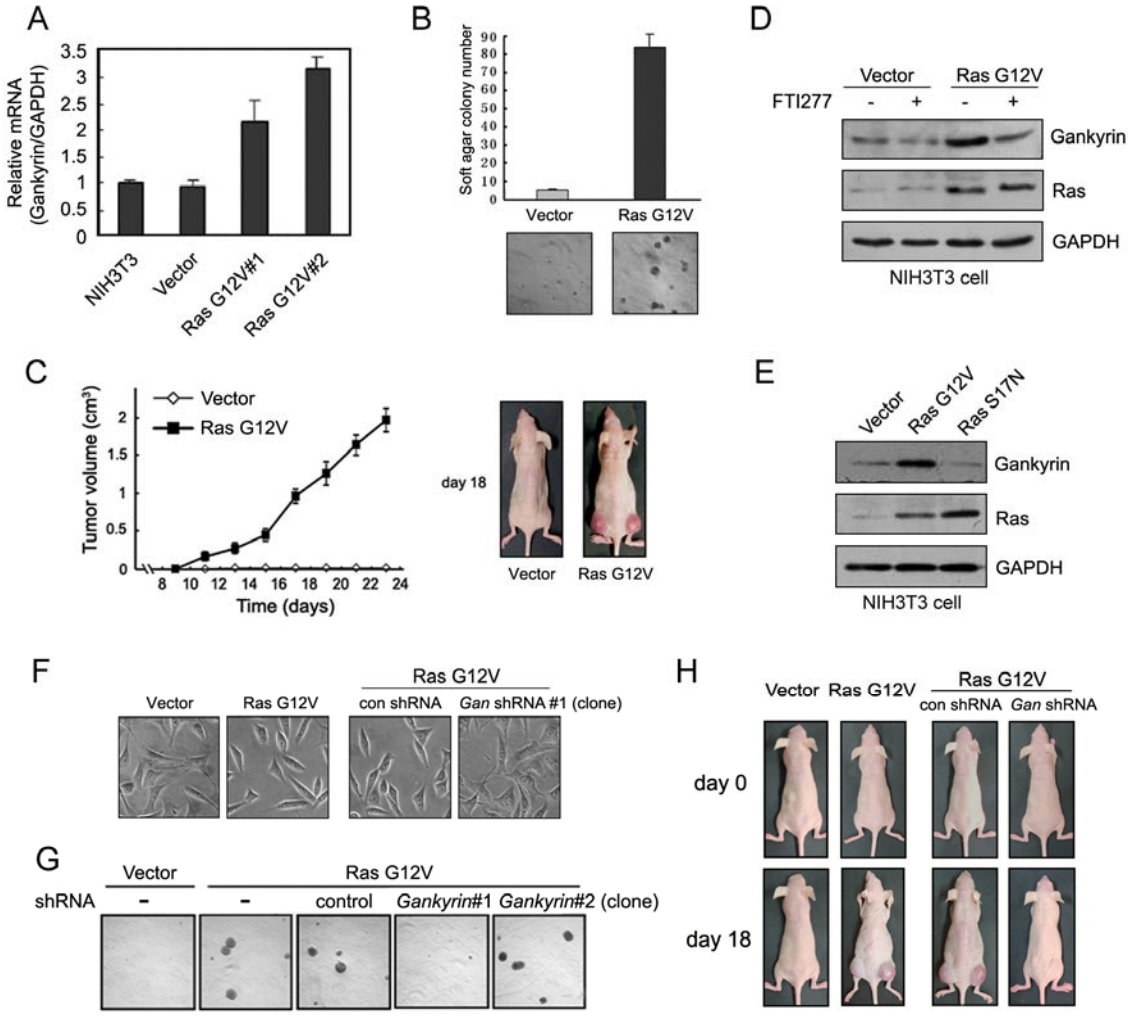

Figure 1 H-Ras G12V transformation increases Gankyrin expression and Gankyrin is essential for Ras mediate tumorigenesis.

(**A**) NIH3T3 cells were stably transfected with H-Ras G12V or vector. Total RNA from these same cells were harvested and subjected to Real-Time RT-PCR analysis.

(**B**) Analysis of the levels of cell growth in soft agar. Vector or Ras G12V transformed NIH3T3 cells were mixed with soft agar and seeded into six-well

plates, and then the number of foci was determined 2-3 weeks later. Data are presented as the total number of colonies per plate (up) and the representative images was shown (down). Data are shown as mean ± s.d. and are representative of three independent experiments.

(**C**) Ras G12V transformed NIH3T3 cells have the ability to form tumors in the nude mice. 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 (left). The photographs are representative tumors at day 18 after injection (right).

(**D**) Immunoblot analysis of NIH3T3 cells treated with 3 μM FTI-277 for 24 hours for Gankyrin and Ras. FTI-277, farnesyltransferase inhibitor.

(**E**) Immunoblot analysis of NIH3T3 cells stably transfected with vector or Ras S17N. Ras S17N, a non-transformed dominant-negative mutant of *H-Ras*.

(**F**) Representative microscopic images of the vector transfected or Ras G12V transformed NIH3T3 cells (left two panels), and Ras G12V transformed NIH3T3 cells stably expressing *Gankyrin* shRNA or a scramble control (right two panels).

(**G**) Cells described in Figure 1B were mixed with soft agar and seeded into six-well plates, and then the number of foci was analyzed 2-3 weeks later. Data are presented as the representative images colonies per plate.

(**H**) 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.

The photographs are representative tumors at day 0 and 18 after injection.

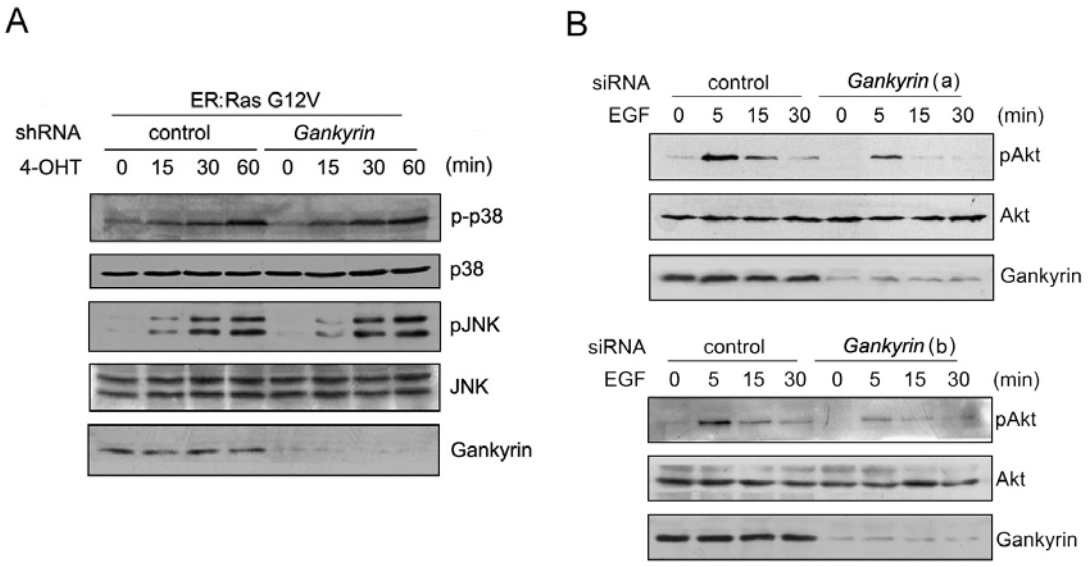

Figure 2 Knockdown of Gankyrin impedes Akt activation.

(**A**) NIH3T3 cells stably expressing control or *Gankyrin* shRNA were infected with a retroviral construct, ER:Ras G12V, expressing inducible activatable Ras, was induced by 4-OHT as indicated times. The cells lysates as described in Figure 2A were analyzed the phosphorylation of p38 and JNK by immunoblot.

(**B**) HEK293 cells transfected with control or *Gankyrin*-specific siRNA (a or b) were serum starved overnight, then treated with EGF (100 ng/ml) for the indicated times. The cells were then harvested and probed with the indicated antibodies by immunoblot.

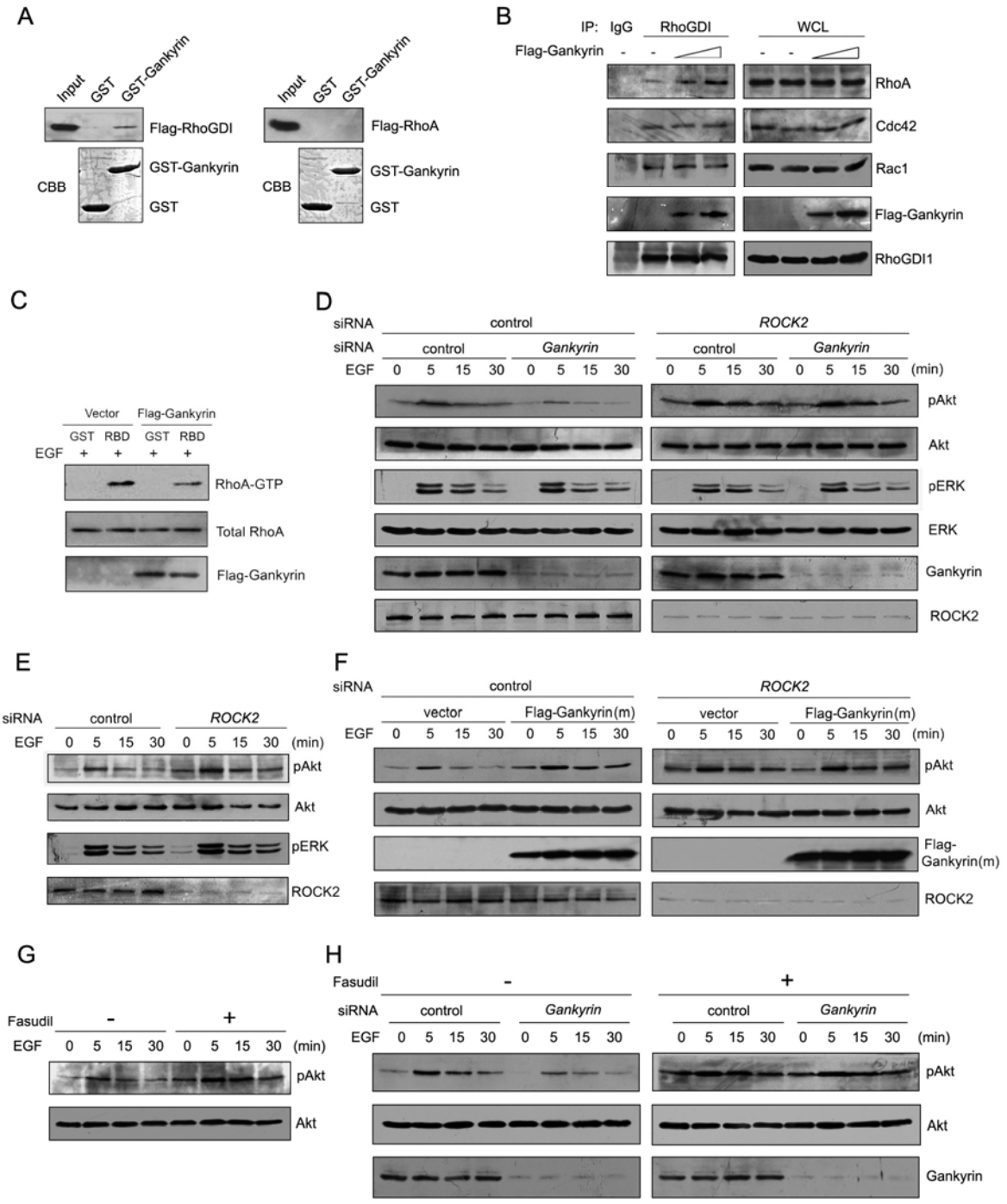

Figure 3 Gankyrin activates Akt through RhoA/ROCK/PTEN signaling pathway.

(**A**) GST or GST-Gankyrin purified from *E. coli* were incubated with recombinant Flag-RhoGDI or Flag-RhoA. Bound proteins were analyzed by immunoblot using anti-FLAG antibody.

(**B**) Immunoprecipitation was performed as descript in Figure 3F, Bound proteins were analyzed by immunoblot using indicated antibodies.

(**C**) NIH3T3 cells stably transfected with vector and Flag-Gankyrin were serum starved overnight, after stimulation with EGF (100ng/ml) for 5 minutes, cells were lysed and incubated with beads bound to a GST-RBD fusion protein or GST alone, as indicated. The amount of activated RhoA (RhoA-GTP) associated with the beads was tested by anti-RhoA immunoblotting.

(**D**) HEK293 cells were transfected with control or *ROCK2* siRNA. After 24 hours, these cells were subsequencely transfected with the same siRNA plus control or *Gankyrin* siRNA respectively. Before harvest, 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.

(**E**) HEK293 cells were transfected with control or *ROCK2* siRNA. Before harvest, the cells were serum starved overnight and then treated with EGF (100 ng/ml) for the indicated times. The lysates of these cells were analyzed by immunoblot using indicated antibodies.

(**F**) HEK293 cells were transfected with control or *ROCK2* siRNA. After 24 hours, these cells were subsequencely transfected with same *ROCK2* siRNA plus vector or Flag-Gankyrin (mouse) respectively for additional 48 hours, and then treated with EGF (100 ng/ml) for the indicated times after serum starved overnight. The lysates of these cells were analyzed by immunoblot using indicated antibodies.

(**G** and **H**) HEK293 cells or HEK293 cells transfected with control or *Gankyrin* siRNA, were treated with or without Fasudil (20μM). Cells were then lysed at the indicated time after EGF treatment and analyzed by immunoblot using indicated antibodies.

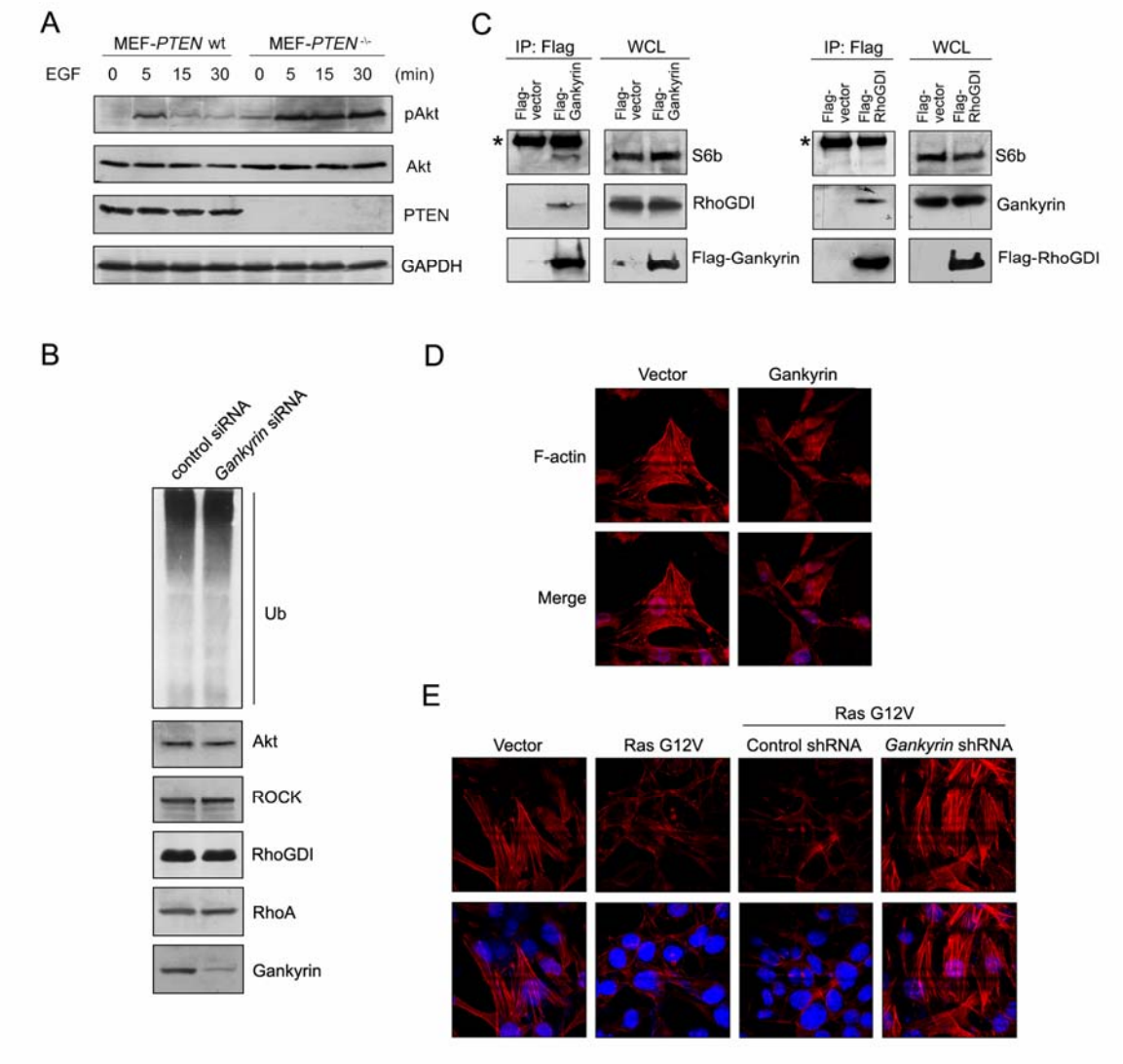

Figure 4 The effect of Gankyrin on Akt activation is independent of protesome.

(**A**) Knockout of *PTEN* enhanced EGF-activated phosphor-Akt levels. MEF-*PTEN* wt and MEF-*PTEN* knockout 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.

(**B**) The lysates of HEK293 cells (10% FBS) transfected with control siRNA or *Gankyrin* siRNA were analyzed by immunoblot using indicated antibodies.

(**C**) Immunoassay of HEK293 cells transfected with vectors expressing

Flag-vector, Flag-Gankyrin or Flag-GDI1; lysates immunoprecipitated (IP) with anti-Flag and analyzed by immunoblot (IB) with anti-S6b, anti-RhoGDI1, anti-Gankyrin and anti-Flag antibodies. ***** , heavy chain.

(**D** and **E**) NIH3T3 cells, described as indicated, were fixed and phalloidin stained to show the stress fibres. The nucleus was stained with DAPI.

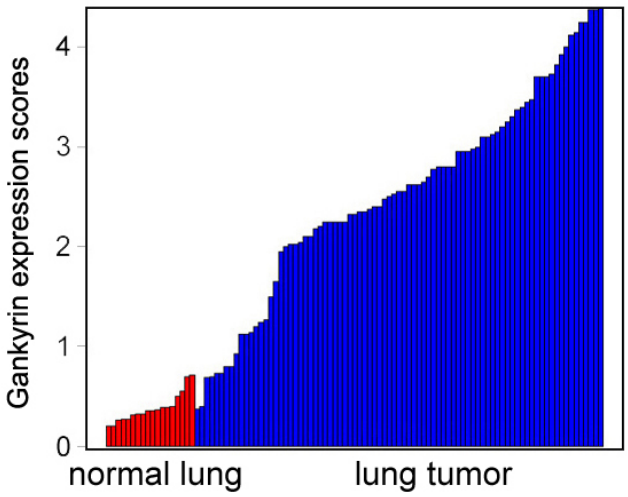

Figure 5 Gankyrin is highly expressed in human lung cancers.

The tissue array containing normal lung and lung tumor were performed by immunohistochemistry with Gankyrin antibody. Immunohistochemical expression scores of Gankyrin are shown. P< 0.001.

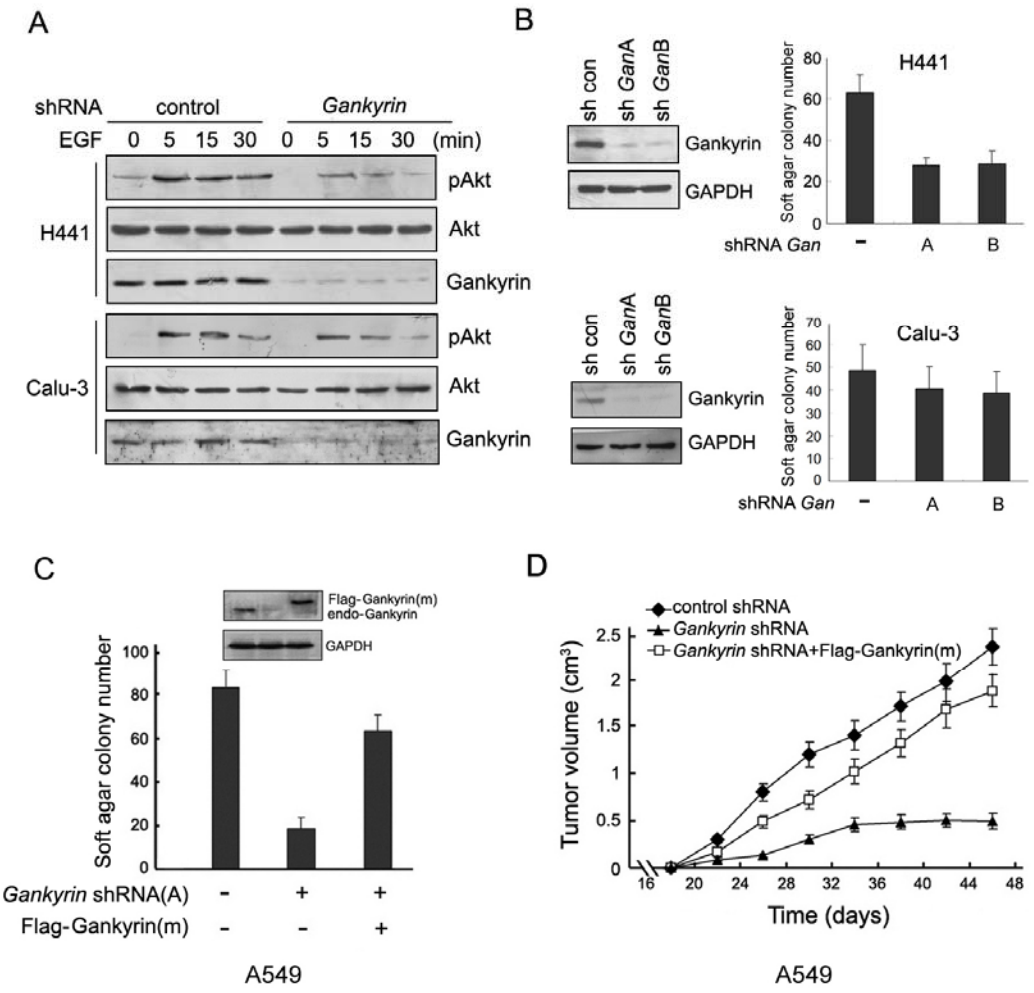

Figure 6 Gankyrin is required for the tumorigenesis of human lung cancer cells with *K-Ras* mutation.

(**A**) Cells stably transfected with control or *Gankyrin*-specific shRNA were serum starved overnight and stimulated with EGF (100 ng/ml) for the indicated times, cells were then harvested and probed with the indicated antibodies by immunoblot.

(**B**) Cells stably infected with control or *Gankyrin*-specific shRNA were used in soft agar assay as described in Figure 1. Knockdown efficacy of two independent shRNAs (A or B) of *Gankyrin* was measured by western blot (left) and colony formation in soft agar was enumerated (right).

(**C** and **D**) A549 cells stably infected with control or *Gankyrin*-specific shRNA with/out Flag-Gankyrin (mouse) were used in soft agar assay (**C**) and nude mice tumor growth assay (**D**) as described in Figure 1. Up panel (**C**), immunoblot analysis of the silencing and restored expression of Gankyrin.

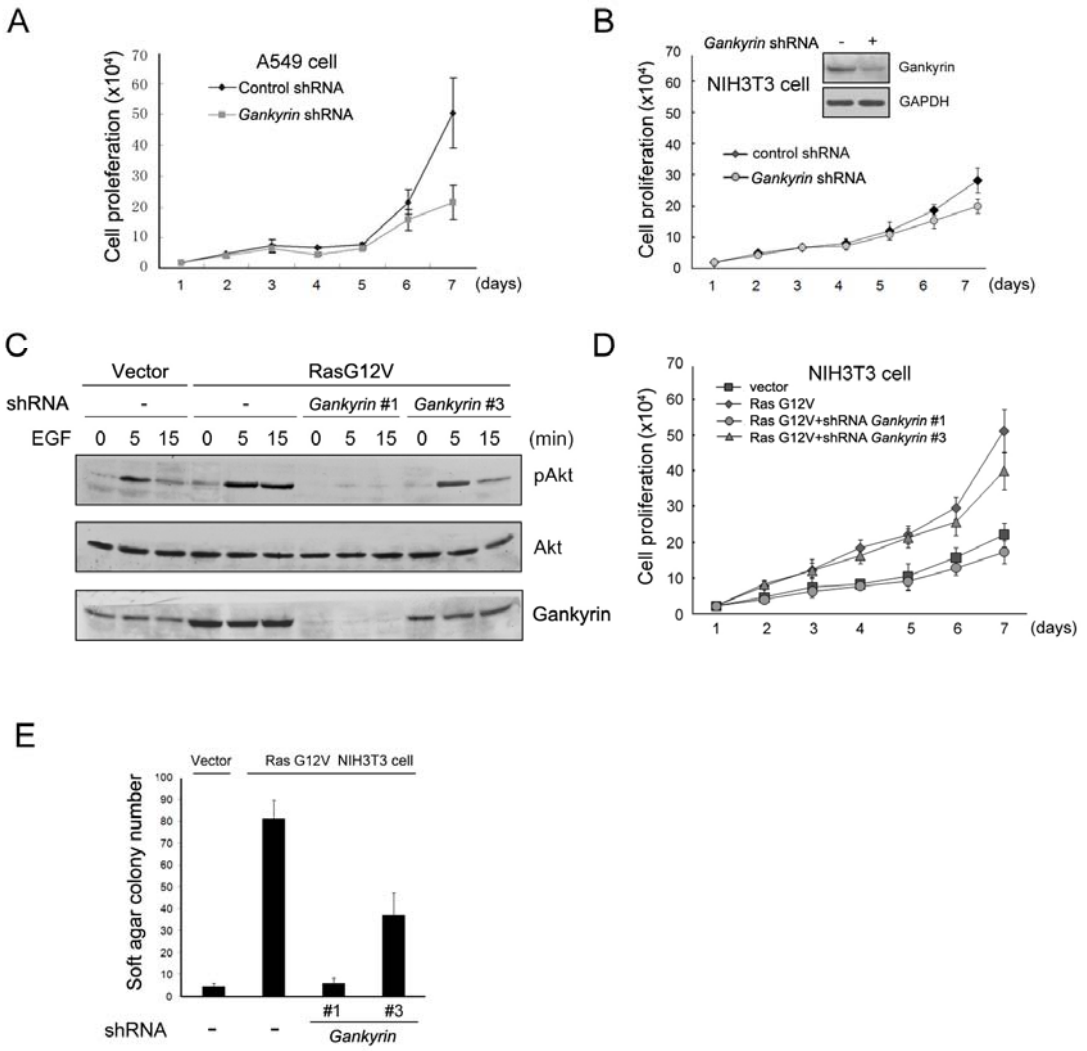

Figure 7 Effect of Gankyrin on cell proliferation and transformation.

(**A**) A549 cells stably expressed with control shRNA or *Gankyrin* shRNA were assayed for proliferation. The proliferation of control cells or *Gankyrin* knocked down cells was analyzed over a period of 7 days by counting cells every 24 hours following plating. Data are shown as mean ± s.d. and are representative of three independent experiments.

(**B**) NIH3T3 cells stably expressed with control shRNA or *Gankyrin* shRNA were assayed for proliferation. The proliferation of these cells was analyzed over a period of 7 days by counting cells every 24 hours following plating. Data

are shown as mean ± s.d. and are representative of three independent experiments.

(**C**) NIH3T3 cells, Ras transformed NIH3T3 cells, the #1 and #3 cell clones with different level of Gankyrin knocked down, 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.

(**D**) Cells described in (**C**) were assayed for proliferation. Data are shown as mean ± s.d. and are representative of three independent experiments.

(**E**) Cells described in (**C**) were assayed for the levels of cell growth in soft agar.

Supplemental Methods

Kinase assays

Kinase assays were performed by an adaptation of a published protocol (61). Samples of immunoprecipitates of ER:Ras G12V-NIH3T3 cells were analyzed by *in vitro* kinase assay. Kinase assays mixture was incubated for 30 min at 30 ^{o}C in kinase buffer [50mM Tris-HCl PH 7.4, 10mM $MgCl_2$, 5mM NaCl, 0.1mM EGTA, 100 mM cold ATP, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM p-nitrophenyl phosphate (PNPP), 1 mMNa_3VO_4, 1 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, aprotinin (10 mg/ml), leupeptin (1 mg/ml), pepstatin (1 mg/ml) and 1 mM dithiothreitol] in the presence of 10μCi [γ-^{32}P] ATP and substrate GST-MYPT1 (654–880aa, Upstate). Reactions were stopped by the addition of sample buffer and assessed by SDS-PAGE and visualized by autoradiography.

Real Time RT-PCR Analysis

Total RNAs were isolated from 1×10^6 cells by using Trizol Reagent (Invitrogen) and complementary DNA was synthesized according to the manufacturer's instructions. Primers used to amplify the gene fragments were available from the authors. The diluted RNA (1:100) was analyzed using real-time PCR (ABI) with SYBR Green JumpstartTM Taq ReadyMixTM (Sigma).

Rho activity pull-down assay

The expression and purification of glutathione S-transferase (GST)-Rho-binding domain (RBD) polypeptides (Rhotekin for RhoA) described in detail in Xia et al. (62). For the Rho-GTP pull-down assay, cells were washed with ice-cold Tris-buffered

saline and lysed in RIPA buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, complete protease inhibitor cocktail, and 1mM PMSF). Cell lysates were clarified by centrifugation at 10,000 g at 4°C for 10 min, and equal volumes of lysates were incubated with GST-RBD or GST beads (10 mg) at 4°C for 2 hours. The beads were washed four times with buffer B (Tris buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, complete protease inhibitor cocktail, and 0.1 mM PMSF). Bound Rho proteins were detected by Western blotting using a polyclonal antibody against RhoA.

Protein Purification and GST precipitation assay.

The recombinant Flag-RhoA and Flag-RhoGDI proteins were prepared with TNT® T7 Insect Cell Extract Protein Expression System (Promega). For purification, the recombinant proteins were bound to M2-agarose (Sigma) and then eluted with 0.1 mg/ml Flag-peptide (Sigma). The fusion protein of GST and GST-Gankyrin were prepared as described (61). Approximately 5 μg GST fusion protein bound to agarose beads was added to 500ng purified Flag-RhoA and Flag-RhoGDI, followed by incubation for 4 h with gentle rotation. Beads were washed three times and proteins were eluted with SDS sample buffer and analyzed by immunoblot.

Plasmids

The Ras expression vectors and Ras transformed NIH3T3 cells were described previously (22). The primer sequences of expression vectors used in the study is listed below.

pBabe-puro-Flag-Gankyrin(h)	Up:5’-CGGGATCCACCATGGACTACAAGGACGA-3’ Down: 5’-CGGAATTCTTAACCTTCCACCATTCTCTTG-3’
pBabe-puro-Flag-Gankyrin(m)	Up: 5’- CGGGATCCACCATGGACTACAAGGACGA-3’ Down:5’-CGGAATTCCTACATAGAAGCCTCTTCACTT-3’
pcDNA3-Flag-Gankyrin(h)	Up:5’-CCCAAGCTTACCATGGAGGGGTGTGTGTCTAAC-3’ Down: 5’-CGGGATCCTTAACCTTCCACCATTCTCTTG-3’
pcDNA3-Flag-Gankyrin(m)	Up:5’-CCCAAGCTTACCATGGAGGGGTGTGTGTCTAAC-3’ Down: 5’-CGGGATCCCTACATAGAAGCCTCTTCACTT-3’
pGEX-KG-Gankyrin(h)	Up:5’-CGGGATCCACCATGGAGGGGTGTGTGTCTAAC-3’ Down: 5’-CCCAAGCTTTTAACCTTCCACCATTCTCTTG-3’
pXJ40-Myc-RhoGDI	Up:5’-CGGGATCCACCATGGCTGAGCAGGAGCCC-3’ Down:5’-CCCAAGCTTTCAGTCCTTCCAGTCCTTC-3’
pXJ40-Myc-RhoA	Up:5’-CGGGATCCACCATGGCTGCCA TCCGGAAGA-3’ Down:5’-CCCAAGCTTTCACAAGACAAGGCACCCA-3’
pcDNA3-Flag-RhoGDI	Up:5’-CCCAAGCTTACCATGGCTGAGCAGGAGCCC-3’ Down:5’-CGGGATCCTCAGTCCTTCCAGTCCTTC-3’
pcDNA3-Flag-RhoA	Up:5’-CCCAAGCTTACCATGGCTGCCA TCCGGAAGA-3’ Down:5’-CGGGATCCTCACAAGACAAGGCACCCA-3’
pF25A-ICE-T7-Flag-RhoGDI	Up: 5’-GCGATCGCACCATGGACTACAAGGACGA-3’ Down: 5′-GTTTAAACTCAGTCCTTCCAGTCCTTC-3'
pF25A-ICE-T7-Flag-RhoA	Up: 5’-GCGATCGCACCATGGACTACAAGGACGA-3’ Down:5’-GTTTAAACTCACAAGACAAGGCACCCA-3'
pBabe-puro-RhoAQ63L	Up:5’-CGGGATCCACCATGGCTGCCA TCCGGAAGA-3’ Down:5’-CGGAATTCTCACAAGACAAGGCACCCA-3’

Reference

61. Li HY, et al. Deactivation of the kinase IKK by CUEDC2 through recruitment of the phosphatase PP1. *Nature Immunology*. 2008;9(5):533-541.

62. Xia M, Land H. Tumor suppressor p53 restricts Ras stimulation of RhoA and cancer cell motility. *Nat Struct Mol Biol*. 2007;14(3):215-223.