Supplementary Information

G protein-coupled receptor kinase 2 (GRK2) positively regulates epithelial cell migration

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Supplementary Materials and Methods

Materials. The cDNAs encoding GRK2-wt, the kinase-dead GRK2-K220R mutant, GRK2-D110A, GRK2-gg, GRK2-Δct and the phosphorylation mutants GRK2-S670D, GRK2-S670A, GRK2-Y86,92D (GRK2-Y2D) and GRK2-Y13,86,92F (GRK2-Y3F) have been previously described (Penela et al, 2001; Elorza et al., 2003). The cDNAs encoding the constitutively active c-Src-Y527F and the catalytically inactive c-Src-K295R mutants were generously provided by Dr. J.S. Gutkind (NIH, Bethesda, MD, USA). HEK-293, Cos7 and Hela cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Culture media and Lipofectamine were from Life Technologies Inc. (Gaithersburg, MD, USA). Affinity-purified rabbit polyclonal antibodies raised against GRK2 (C-19) or GIT1 (H-170), the affinity-purified goat polyclonal antibody raised against actin (I-19), the antiphosphotyrosine monoclonal antibody conjugated to horseradish peroxidase (PY99-HRP), the anti-MEK1 polyclonal

antibody and the polyclonal C-16 and C-14 antibodies that recognize ERK1 and ERK2 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The mouse monoclonal antibody raised against GRK2/3 was purchased from Upstate Biotechnology (Lake Placid, NY, USA). The anti-Paxillin monoclonal antibody was obtained from BD Transduction Laboratories. Anti-pSer$_{670}$-GRK2 polyclonal antibody was from Biosource International (Camarillo, CA). Anti-pThr$_{423}$-PAK1/pThr$_{402}$-PAK2 and anti-PAK1/2 polyclonal antibodies were provided by Chemikon International (Temecula, CA). The anti-Rac monoclonal antibody, the anti-pSer$_{217/221}$-MEK1 and anti-pSer$_{298}$-MEK1 polyclonal antibodies and the anti-phospho-ERK1/2 polyclonal antibody were purchased from Cell Signaling Technologies (Beverly, MA, USA). The anti-S1P$_1$ receptor polyclonal antibody was from Cayman Chemical. Fibronectin and sphingosine 1 phosphate (S1P) were obtained from Sigma (St. Louis, MO, USA). The Src inhibitor PP2, the ROCK inhibitor Y27632, the sphingosine kinase inhibitor SKI and the MEK kinase inhibitor PD98059 were obtained from Calbiochem (San Diego, CA, USA). The S1P receptor antagonist VPC23019 was purchased from Avanti Polar Lipids (Alabaster, AL). Glutathione-Sepharose 4B beads were obtained from Pharmacia Amersham Biotech and G-protein sepharose from Invitrogen (Carlsbad, CA, USA). Pertussis toxin was obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). All other reagents were of the highest commercially-available grades.

Cell transfection. Hela or COS7 cells (70-80% confluent monolayers in 60- or 100-mm dishes) were transiently transfected with the chosen combinations of cDNA or shRNAi constructs using the Lipofectamine/Plus method, following manufacturer's instructions and, when desired, co-transfected with cDNA encoding the CD8 antigen for subsequent cell selection by using polystyrene microbeads precoated with anti-CD8 antibody (Dynabeads M450, Dynal Biotech, Oslo, Norway). For the generation of stably-

transfected cell lines, these were transfected with the appropriate constructs using lipofectamine or FuGene HD (for βarr1/2-KO MEFs) followed by selection with the appropriate antibiotic for 2–4 weeks (200 μg/ml G418 for cells expressing GRK2-K220R or GRK2-S670D constructs and 250 μg/ml hygromycin for those expressing GRK2-wt or GRK2-S670A vectors). Individual clones were isolated and characterized in HEK-293 cells or collected as pooled positive transfectants cells in the case of COS7, βarr1/2-KO MEFs and Hela cells.

Adenoviral expression of GRK2-shRNA and cell infection. GRK2 knock-down was achieved by RNA interference using an adenovirus vector-medited shRNA delivery. The target GRK2 shRNA sequence (5'- GCAAGAAAGCCAAGAACAAGC-3') was designed using Invitrogen's RNAi Designer. Recombinant adenoviruses expressing the GRK2 shRNA were produced using the BLOCK-iT™ Adenoviral RNAi Expression System (Invitrogen) according to the manufacturer's instructions. Briefly, doubled stranded oligonucleotides encoding GRK2 shRNA were generated and cloned into the entry vector pENTR$_{TM}$/U6. The resulting U6GRK2 shRNA cassette, which contains a U6 promoter and Pol III terminator, was transferred into the adenoviral pAd/BLOCK-iT-DEST vector and transfected into 293A cells to produce a crude viral stock. A control adenovirus vector expressing lamin shRNA was also constructed with a similar approach. Finally, GRK2-shRNA and control adenovirus stocks were amplified and their titers determined by using the 293A cell line. Cells were infected either with AV.GRK2-shRNA and AV. LAM shRNA (AV.Ctrl) at 50 pfu/cell and 72 hours post-infection cells were analyzed for migration and signalling. As a control for infection efficiency total GRK2 protein was routinely measured by immunoblotting in cell lystes.

Immunoprecipitation and Western blot analysis. To obtain total cell lysates, cells were washed with ice-cold PBS-buffer and subsequently solubilized in lysis buffer A

(50mM Tris-HCl pH 7.5, 300mM NaCl, 1% Triton-X100, 0.1% SDS, 0.5% sodium deoxycholate and 1mM NaF, supplemented with 1 mM sodium orthovanadate plus a mixture of protease inhibitors). For immunoprecipitation of either GRK2 or GIT1 proteins, cells were scrapped and washed twice with ice-cold phosphate-buffered saline and solubilized in 500 µl/100-mm dish of lysis buffer A or B (20mM Tris-HCl, 100mM NaCl, 0.2 % Triton X-100, 1mM EDTA), respectively. Upon centrifugation, supernatants were incubated overnight at 4 ^{o}C with 2 µg of anti-GIT polyclonal antibody or 1 µg of anti-GRK2 monoclonal antibody, followed by re-incubation with protein G-Sepharose for 1h as previously reported (Mariggio et al., 2006). Immunoprecipitates or cellular lysates were resolved by 8-10% SDS-PAGE and immunoblotted with the indicated antibodies as described (see references Elorza et al., 2003; Mariggio et al., 2006)). Blots were developed using the chemiluminescence method (ECL, Amersham Pharmacia Biotech, UK). When required, bands were quantified by laser-scanner densitometry and the amount of co-precipitated protein normalized to the amount of the immunoprecipitated protein, as assessed by the specific antibodies.

Determination of ERK1/2, MEK and PAK activity ERK1/2 activation was determined in cell lysates by using an anti-phospho-ERK1/2 antibody. The immunoblot was then stripped and the total cellular ERK1/2 (ERK) was detected with specific antibodies. Phospho-ERK2 band densities were normalized to cognate total ERK2 densities. MEK1 and PAK activation were determined and quantified in a similar way, using their respective phospho-specific and total antibodies.

Determination of Rac activity. A pulldown assay method was used to determine GTP-bound active form of Rac. Cells detached and kept in suspension for 3 h in serum-free DMEM (cells in suspension, S) or upon plating on dishes coated with 20 µg/ml

fibronectin for the indicated times were lysed in buffer containing 0.5% NP-40, 50 mM Tris pH 7.4, 100 mM NaCl, 10% glycerol, 2mM MgCl2, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1µg/ml aprotinin, and 1µg/ml leupeptin. Cell extracts were incubated overnight at 4ºC with 10 µg GST-PAK CRIB domain immobilized to glutathione-Sepharose 4B beads. Precipitated complexes were washed three times in lysis buffer and eluted with SDS sample buffer. Bound Rac was analyzed by Western blotting using a monoclonal anti-Rac antibody.

Immunofluorescence. Cells kept or not in suspension were seeded onto collagen IV (10µg/ml)- or fibronectin (10 µg/ml)-coated coverslips, fixed with 4% paraformaldhehyde in PBS and permeabilized with 0.1% Triton X-100. Upon blocking with 3% bovine serum albumin or 2% foetal calf serum in PBS, coverslips were incubated with different primary antibodies diluted in blocking medium (monoclonal paxillin antibody, dilution 1/500; monoclonal GRK2 antibody, dilution 1/350; polyclonal GIT1 antibody, dilution 1/500, polyclonal $S1P_1$ antibody, dilution 1/100) followed by incubation with the appropriate Alexa 488 or 594-conjugated secondary antibodies. F-actin was stained with phalloidin-Alexa594 (dilution 1/150). Serial sections (0.4 µm, 63x objective) were taken on a confocal MicroRadiance (BioRad) coupled to an Axioskop-2 Zeiss microscope and analyzed with the Confocal Assistant 4.02 program and the Image J program. For quantification of $S1P_1$ subcellular distribution, cells positive for membrane receptor presence were counted in different fields (objective x63) by analyzing 150-200 cells per condition in each one of three independent experiments.

Cell detachment experiments under defined laminar flow. For detachment experiments, control Hela cells or clones stably expressing GRK2-wt or the GRK2-S670A mutant were allowed to adhere onto uncoated glass-bottomed Petri dishes for 2 h

at 37°C. Then, shear stress was applied over the cells by pulling assay buffer (Hanks balanced salt solution [HBSS] buffer with 2% fetal calf serum [FCS]) through a parallel plate flow chamber with a programmable syringe pump (Harvard Apparatus Inc, Holliston MA) starting at 0.5 dyn/cm^2 and increasing up to 30 dyn/cm^2 at 1-minute intervals. Eight different fields were recorded after each shear stress interval using an x20 phase-contrast objective. Cell detachment was obtained from the analysis of the area free of cells in each field at first and last time points. Digitization was performed with Sonic My DVD Software and area analysis with Image J Software.

Determination of S1P levels in Hela cells. Hela cells stably overexpressing GRK2-wt or control Hela cells were plated on 150-mm dishes and cultured at confluence for 2-3 days. Conditioned media (15ml) were collected, lyophilized and resuspended in 1ml of water. Cells were harvested and lysed in 1ml of 50mM Tris-HCl pH 7.5, 300mM NaCl, 1% Triton-X100, 0.1% SDS, 0.5% sodium deoxycholate and 1mM NaF, supplemented with 1 mM sodium orthovanadate plus a mixture of protease inhibitors. Cellular extracts were centrifuged at 1,200xg for 5 min and post-nuclear supernatants were then clarified by ultra-centrifugation (200,000xg, 60 min at 4ºC). Presence of S1P in both cytosolic fractions and conditioned media was quantified by using a Sphingosine 1 Phosphate ELISA KIT (Echelon Biosciences Inc., USA) following the manufacturer's instructions.

In vitro wound-healing assays. COS7 cells infected with adenovirus expressing a GRK2-shRNA or a control construct were grown to 90% confluence on fibronectin-coated 24-well plates. Cell monolayers were scrapped with a 2-20µl pipette tip and washed with PBS. Wound closure in the presence of 10% serum was monitored by taking phase contrast images at 0h and 72h post-wounding.

Analysis of cell proliferation in wounded tissue. Proliferating cells in the wounded tissue of GRK2 +/- and GRK2+/+ mice were detected by BrdU labelling. At day 2 post-

wounding, mice were injected with BrdU and killed 2 hours later. Tissue was isolated and fixed in 4% paraformaldehyde in PBS, embedded in paraffin wax and sectioned. Sections from the middle of the wound were incubated with a peroxidase-conjugated anti-BrdU antibody and stained with a diaminobenzidine-peroxidase substrate kit.

Statistics. Statistical analyses for in vivo wound healing assays were performed with SPSS 12.0 by repeated measures using ANOVA. Time (days 2-6) was treated a within group factor, and phenotype as between group factors. Post-hoc analyses, to test daily effects, were performed with an independent sample t-test. All analyses were two-tailed and used α=0.05 to determine significance. For the remaining experimental procedures statistical analyses were performed using the two-tailed Student's t-test. The data are presented as means ± S.E.M.

Supplementary References

Elorza, A, Penela, P Sarnago, S and F. Mayor, jr. (2003) MAPK-dependent degradation of G protein-coupled receptor kinase 2. *J.Biol. Chem*. 278: 29164- 29173

Mariggio, S, Garcia-Hoz, C, Sarnago, S, De Blasi, A, Mayor, F, Jr. and Ribas, C. (2006) Tyrosine phosphorylation of G-protein-coupled-receptor kinase 2 (GRK2) by c-Src modulates its interaction with Galphaq. *Cell Signal* 18: 2004-2012.

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Supplementary Figure Legends

Figure S1. Increased GRK2 expression alters morphology and motility in HEK-293 cells. (A) HEK-293 cells over-expressing or not GRK2 wt were analyzed by phase-contrast microscopy (objective x20). Increased expression of GRK2 induces cell elongation and extended filopodia-like projections. **(B)** GRK2 positively modulates migration of HEK-293 towards FN in a kinase-independent manner. Parental and HEK-293 cells stably expressing GRK2wt (either mass- or clonally- selected) or the kinase-dead mutant GRK2-K220R were seeded on transwell filters coated with 20 μg/ml FN and their migration rates determined as described in Methods. **(C)** Hela cells were transiently co-transfected with empty vector, GRK2wt ,the kinase mutants GRK2-D110A (defective in binding to Gαq subunits), GRK2-Δct (GRK2 $_{1\text{-}546}$, lacking the Gβγ-interacting region) or GRK2gg (a geranyl-geranylated form targeted to the membrane) as indicated and the CD-8 antigen. After cell selection by using microbeads precoated with anti-CD8 antibody, cellular migration towards fibronectin was assessed. GRK2 expression levels were analysed by western blot (inserts). The antibody used in panel C, AbFP2, shows some cross-reaction with a protein that co-migrates with GRK2$_{1\text{-}546}$. Data are mean±S.E.M of 4 independent experiments. *, $p< 0.05$, ** $p< 0.01$ compared to parental cells. Values for control HEK-293 and Hela migrating cells were 175±59 (B) and 35±10 (C) cells/field, respectively (mean±SD). The data indicate that GRK2-induced migration to fibronectin does not depend on its interaction with Gαq subunits, but requires adequate Gβγ-binding-dependent recruitment to specific membrane locations.

Figure S2. Effects of ROCK, MEK1 or c-Src inhibitors on GRK2-induced cell migration to fibronectin. Migration rates of control Cos7 cells or cells stably

expressing GRK2wt were determined in the presence of vehicle or of 30 μM Y27632, an inhibitor of ROCK activity **(A**), 50μM PD98059, a specific MEK inhibitor **(B)** or 5 μM PP2, a c-Src inhibitor **(C**) as detailed in Methods. Despite the fact that some body retraction of Cos7 cells over-expressing GRK2 is noted (see Fig.1), ROCK is not involved in such phenotype, since its inhibition does not affect GRK2-induced migration or cell morphology. In contrast, both MEK and c-Src activities appear to be instrumental for the GRK2-dependent increase in migration. Data are mean±S.E.M of 3-4 independent experiments performed in duplicate. **, $p< 0.01$, compared to vehicle-treated Cos7 cells; † $p < 0.05$, †† $p< 0.01$ comparison between vehicle- and inhibitor-treated Cos7-GRK2wt cells. Number of control cells per field (mean±SD) migrating to fibronectin was 28±7 (A), 97±36 (B) and 73±21 (C)

Figure S3. Exogenous S1P rescues SK inhibitor-dependent inhibition of cell migration to fibronectin. Cell migration in response to FN **(A, B)** was assessed as in Fig.1C in parental Cos7 and Hela cells or in Cos7-GRK2wt and Hela-GRK2wt cells as indicated in presence or absence of different combinations of 10 μM SKI added to the upper chamber and 1μM S1P added to the lower chamber. **(C)** S1P-promoted cell motility in Cos7 cells with endogenous GRK2 levels or stably overexpressing wt-GRK2 or GRK2-K220R was determined as in Fig. 2E. **(D)** Hela cells were infected with a control adenovirus (AV-Ctrl) or a virus harbouring a specific GRK2-shRNA sequence (AV-GRK2-shRNA) as detailed in Material and Methods, and resulting GRK2 levels analyzed by immunoblot analysis (inset). Cell migration to 1μM S1P was determined 48h post-infection and compared to control cells. Values for control cells (mean±SD) were 83±16 migrating cells/field (Hela, panel A), 26±3 (Hela, panel B), 44±2 (Hela-GRK2wt, panel B), 48±12 (Cos7, panel C) and 28±2 (Hela, panel D). Data are mean±S.E.M of 3-4 independent experiments. *, $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

Figure S4. The effect of GRK2 on S1P-tiggered ERK1/2 activation and fibronectin-induced cell motility is not mediated by β-arrestins. As ligand-dependent phosphorylation of $S1P_1$ by GRK2 can promote binding of β-arrestins to the receptor complex and such proteins can act as GPCR signal agonist-dependent transducers to ERK (reviewed in DeWire, S. M. et al. (2007). *Annual Review of Physiology* 69: 483-510), ERK1/2 activation in reponse to S1P (panel **A**) was measured in both βarr1/2-KO MEFs and wt MEFs cells as in Fig. 3A. FN-directed migration of βarr1/2-KO MEFs was compared to that of wt MEFs cells **(** panel **B)** or βarr1/2-KO MEFs stably expressing (panel **C**) increased GRK2 levels (as determined by immunoblot analysis, see inserts) as detailed in Material and Methods. Number of control wt MEFs and βarr1/2-KO MEFs cells migrating to fibronectin was 47±6 **(B)** and 105±27 **(C)** cells per field (mean±SD), respectively. **(D)** ERK stimulation upon 1 μM S1P challenge was determined in Cos7 cells expressing endogenous kinase levels or stably over-expressing wt-GRK2 or the inactive GRK2-K220R mutant as in Fig.3A and panel A. In all panels, data are mean±S.E.M from 3-4 independent experiments. *, $p<0.05$, **, $p<0.01$ compared to control or untreated cells. Our data indicate that in the absence of β-arrestins neither S1P-mediated ERK1/2 activation nor migration to fibronectin is impaired but in fact clearly enhanced compared to wt cells. . Interestingly, we had previously reported that GRK2 levels are higher in β-arrestin 1 and 2-KO MEFs compared to control MEFs (Salcedo et al., 2006). Therefore, these data would be in agreement with our notion that increased GRK2 levels promote enhanced migration and S1P/ERK signaling. Moreover, cell migration of βarr1/2-KO MEFs was further increased by stably overexpression of exogenous GRK2 protein thus suggesting that GRK2 modulates ERK signaling and motility in a β-arrestin independent manner.

Figure S5. Cell migration to fibronectin involves PI3K but not Akt activity in Hela and Cos7 cells. It has been reported that ERK1/2 activation by $S1P_1$ receptors is dependent on PI3K/Akt activity in some cellular contexts, while in others it depends on c-Src tyrosine kinase activation (Waters CM et al., 2005, *Cell Signal*.17: 263-277; Kranenburg O and Moolenaar WH (2001) *Oncogene* 20: 1540-1546). Thus, the involvement of PI3K and Akt function on GRK2-induced motility was examined. We did not detect Akt stimulation either upon S1P challenge in Hela cells and COs7 cells (panel A) or during adhesion to fibronectin in Hela cells (not shown) displaying endogenous or increased GRK2 protein levels, while other stimuli such as IGF1 did trigger Akt activation. However, migration of such cells was affected by inhibition of PI3K activity with 10μM LY294002 (B), but not of AKT by addition of 10μM ML9 (not shown), suggesting the involvement of other PI3K-dependent targets. Number of Hela control cells (mean±SD) per field migrating to fibronectin was 38±7 (B). Data are mean±S.E.M of 2 independent experiments.

Figure S6. S1P or adhesion to fibronectin promotes phosphorylation of GRK2 at tyrosine and serine residues. Hela cells stably expressing GRK2wt were serum-starved and challenged with 1μM S1P **(A)** or kept in suspension (S) for 2h and then allowed to adhere on plates coated with 10 μg/ml FN **(B)** for the indicated times. Immunoprecipitates of GRK2 or cellular lysates were analyzed with an anti-phosphotyrosine monoclonal antibody and with a phospho-specific anti-pS670 GRK2 antibody, respectively, as detailed in Methods. After stripping, the presence of total GRK2 was immunodetected in the same gels. GRK2 displays a rapid and robust rise in its phosphotyrosine content, which peaks after 2-5 min of S1P challenge or 10 min of adhesion and rapidly declines thereafter. A marked phosphorylation of GRK2 at S670 in

response to both cell adhesion and S1P challenge is also detected at later time points. Gels representative of 2 independent experiments are shown.

Figure S7. Effect of GRK2 on cell adherence under laminar flow. In order to dissect whether the defect in spreading could be due to a decreased integrin adhesion activity, a detachment experiment was performed. Control and Hela cells stably expressing GRK2 wt (Hela wt) or the GRK2-S670A mutant (Hela A1) were plated onto uncoated glass-bottomed Petri dishes and shear stress was applied with increasing flow rates (ranging from 0.5 to 30 dyn/cm2) followed by cell observation as detailed in Methods. The percentage of detachment was inferior to 5% in any analyzed clone, indicating that the adhesion strength was unaltered. Therefore, the reduction in spreading cannot be explained as a result of defective adhesion, suggesting that a signaling process downstream integrin activity is involved instead. Results are representative of 2 independent experiments.

Figure S8. Impairment of serine670 phosphorylation of GRK2 interferes with normal PAK activation in response to S1P. Hela cells stably expressing GRK2wt or GRK2-S670A were serum-starved and challenged with 1μM S1P for the indicated times. The activation sate of PAK was determined in cellular lysates by using a specific antibody raised against the auto-phosphorylation site of PAK1 at residue T423. After stripping, gels were blotted with a general PAK antibody detecting both PAK1 and PAK2 isoforms. Representative gels from 2 independent experiments are shown.

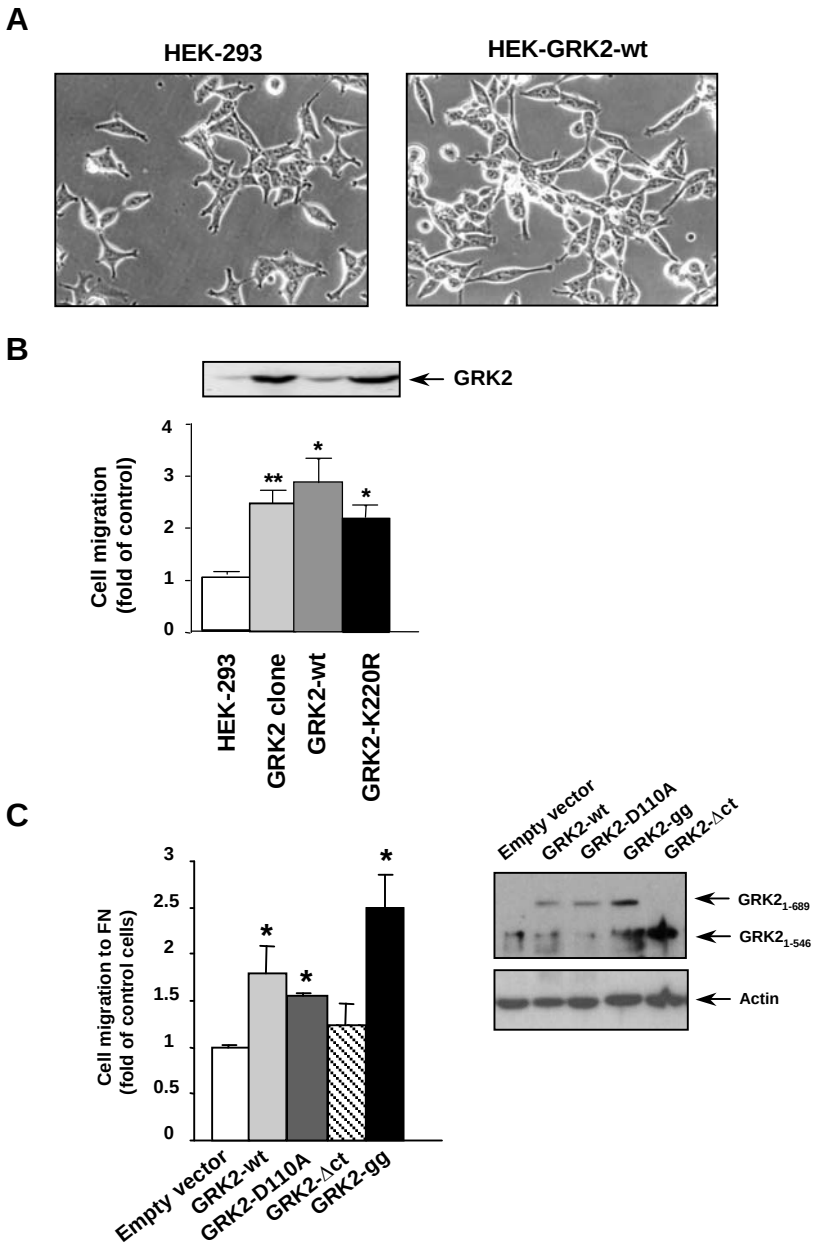
Figure S9. The MEK-scaffolding function of GIT1 is required for GRK2-dependent ERK activation in response to S1P. Control Hela cells or cells stably overexpressing GRK2wt were transiently co-transfected with HA-ERK1 and GIT1-ΔSHD or empty vector. Stimulation of tagged ERK1 in response to S1P challenge was determined in cellular lysates by using an anti-phospho-ERK1/2 antibody (P-ERK). The

immunoblot was then stripped and the total cellular ERK1/2 (ERK) was detected with specific antibodies. Cell lysates were also analyzed using anti-GIT1 antibodies. A representative blot is shown. Data are mean±S.E.M from 4-5 independent experiments. * p< 0. 05 and † p < 0.05 when compared to vehicle-treated cells and to untreated parental Hela cells, respectively.

Figure S10. Membrane localization of GRK2 is required for GRK2/GIT1 complex functionality but not for GRK2/GIT1 association. (a) HEK-293 cells were transiently co-transfected with GIT1 and wt GRK2 or the mutants GRK2-Δct or GRK2-gg,that display impaired or increased membrane localization, respectively. The GRK2-Δct displays enhanced association probably as a result of conformational changes leading to unmasking of interaction domains GIT1 immunoprecipitates were analyzed as detailed in Material and Methods. Data are mean±S.E.M of 3 experiments *, p< 0.05 compared to GRK2wt/GIT1 association. The asterisk denotes a cross-reacting unspecific band. Please see Fig S1A for the effects of these constructs on cell migration to Fibronectin. **(b) GIT1 protein levels are not altered by increased GRK2 expression**. Cellular lysates of control Hela cells or cells stably expressing GRK2wt, GRK2-S670A or GRK2-S670D were resolved by SDS-PAGE and analyzed for GIT1 expression with a specific antibody as detailed in Methods. Equal protein loading was confirmed by determining actin expression in the same gels.

Figure S11. Reduced GRK2 expression causes defective wound closure *in vitro* and *in vivo*. **(a)** Cos7 cells were infected with an adenovirus harbouring a specific GRK2-shRNA or a control construct as described in Material and Methods. 24h post-infection, near -confluent cell monolayers were wounded with a pipette tip and allowed to migrate for 72 h in the presence of serum. Control cells completely filled the wounded area 72h following scratch wounding whereas GRK2-shRNA-infected cells did not. Dotted lines

indicate the wound borders **(b)** Two days after wounding, sections of wounds from control mice and GRK2+/- mice were stained with an anti-BrdU antibody. The number of dividing cells is similar in both animal groups, despite the fact that GRK2+/- mice show a thinner hyperproliferative epithelium, consistent with a primary defect in keratinocyte migration. Arrowheads and arrows indicate BrdU positive nuclei and the epithelial wound edge, respectively. G,granulation tissue; HE, hyperproliferative epithelium; F, fatty tissue.


A
HEK-293
HEK-GRK2-wt
B
GRK2
Cell migration
(fold of control)
4
3
2
1
0
**
*
*
HEK-293
GRK2 clone
GRK2-wt
GRK2-K220R
C
Cell migration to FN
(fold of control cells)
3
2.5
2
1.5
1
0.5
0
*
*
*
Empty vector
GRK2-wt
GRK2-D110A
GRK2-Δct
GRK2-gg
Empty vector
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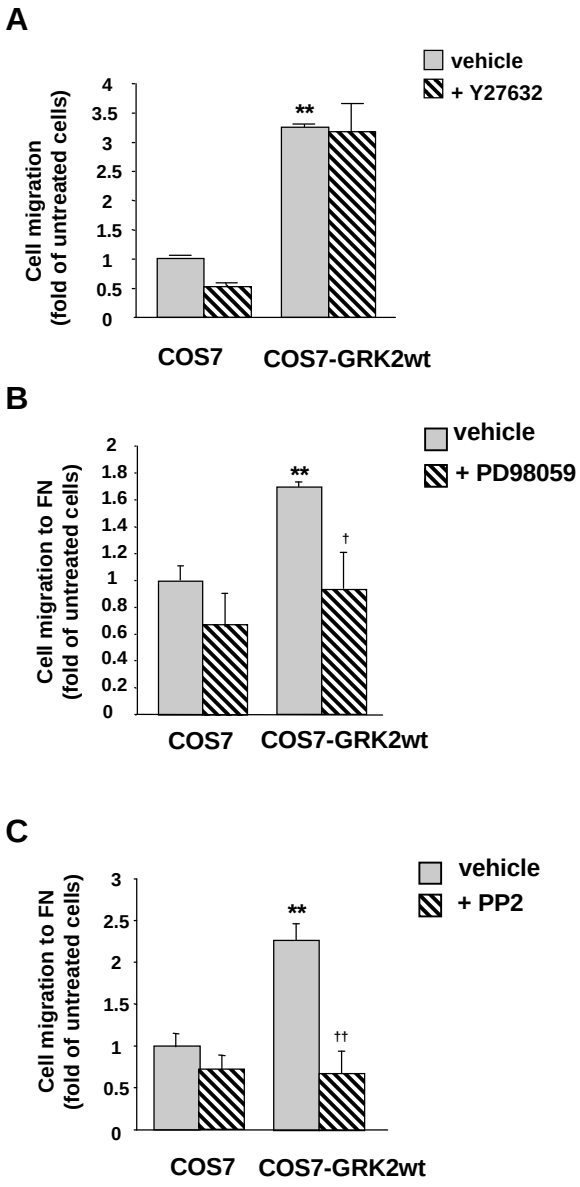
GRK2 1-689
GRK2 1-546
Actin

Figure S1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vehicle
+ Y27632
Cell migration
(fold of untreated cells)
4
3.5
3
2.5
2
1.5
1
0.5
0
**
COS7
COS7-GRK2wt
B
vehicle
+ PD98059
Cell migration to FN
(fold of untreated cells)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
†
COS7
COS7-GRK2wt
C
vehicle
+ PP2
Cell migration to FN
(fold of untreated cells)
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1
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0
**
††
COS7
COS7-GRK2wt

Figure S2

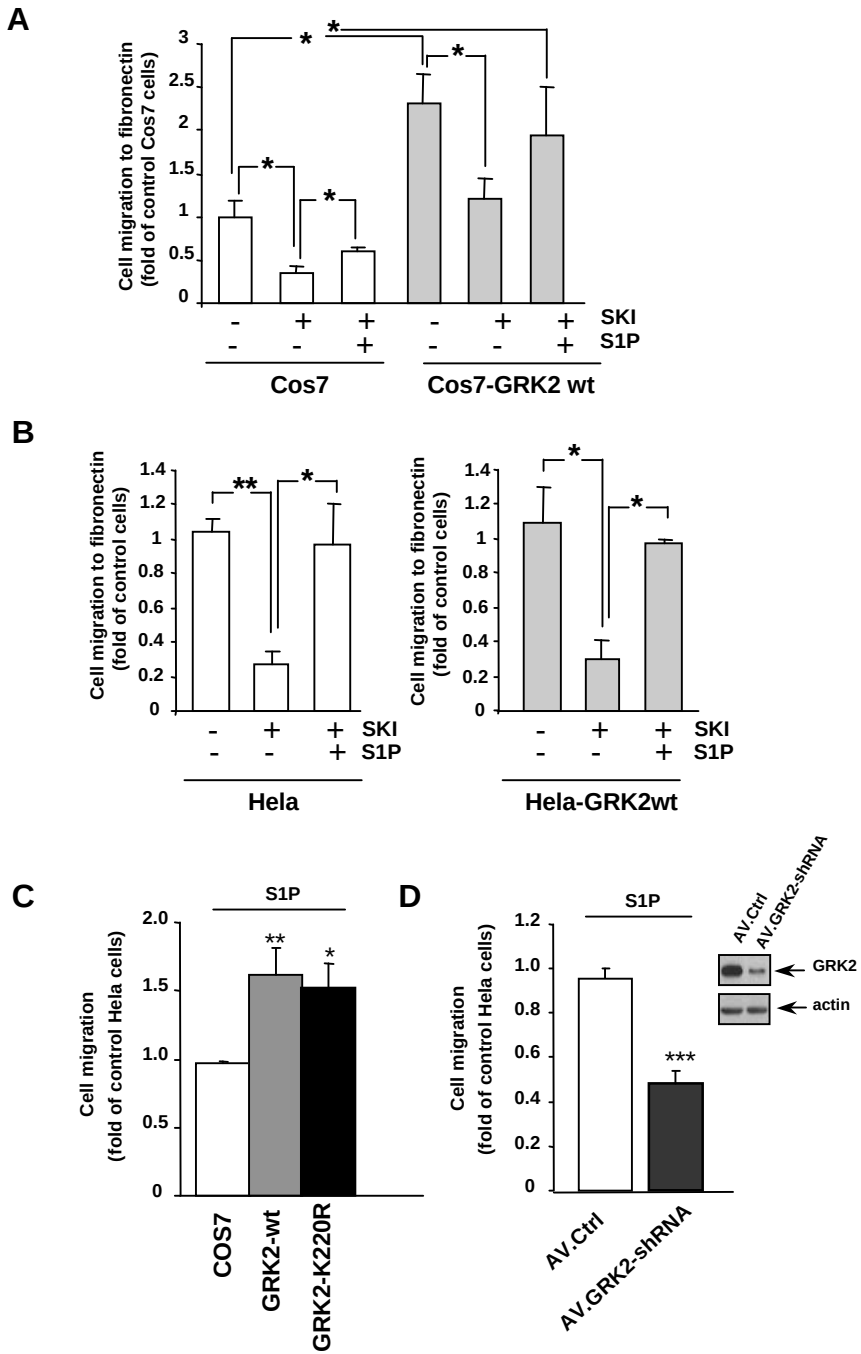
A
Cell migration to fibronectin (fold of control Cos7 cells)
SKI
S1P
Cos7
Cos7-GRK2 wt
B
Cell migration to fibronectin (fold of control cells)
Hela
Hela-GRK2wt
C
S1P
Cell migration (fold of control Hela cells)
COS7
GRK2-wt
GRK2-K220R
D
S1P
Cell migration (fold of control Hela cells)
AV.Ctrl
AV.GRK2-shRNA
GRK2
actin

Figure S3

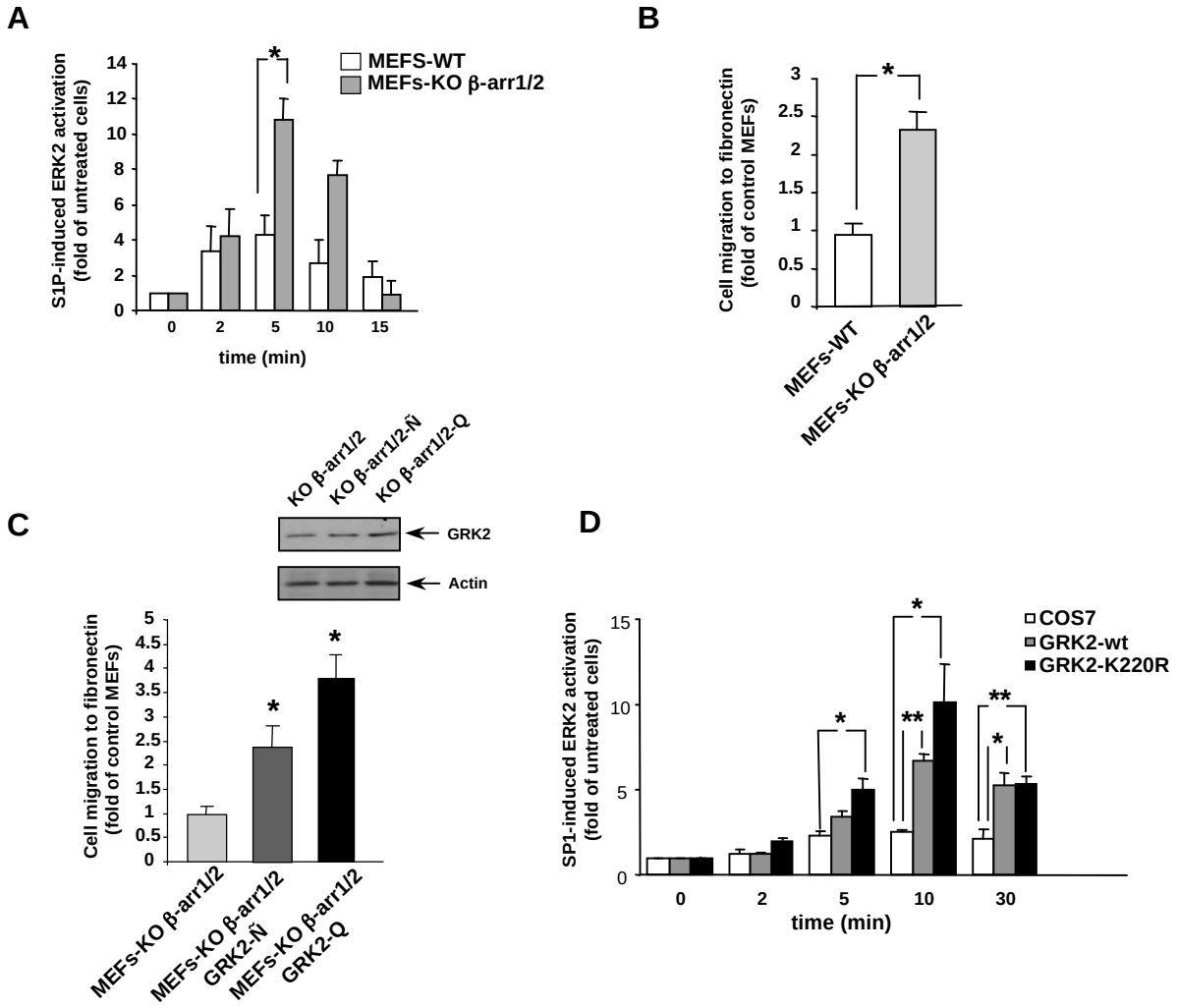
A
S1P-induced ERK2 activation (fold of untreated cells)
MEFS-WT
MEFs-KO β-arr1/2
time (min)
B
Cell migration to fibronectin (fold of control MEFs)
MEFs-WT
MEFs-KO β-arr1/2
C
KO β-arr1/2
KO β-arr1/2-N
KO β-arr1/2-Q
GRK2
Actin
Cell migration to fibronectin (fold of control MEFs)
MEFs-KO β-arr1/2
MEFs-KO β-arr1/2 GRK2-N
MEFs-KO β-arr1/2 GRK2-Q
D
SP1-induced ERK2 activation (fold of untreated cells)
COS7
GRK2-wt
GRK2-K220R
time (min)

Figure S4

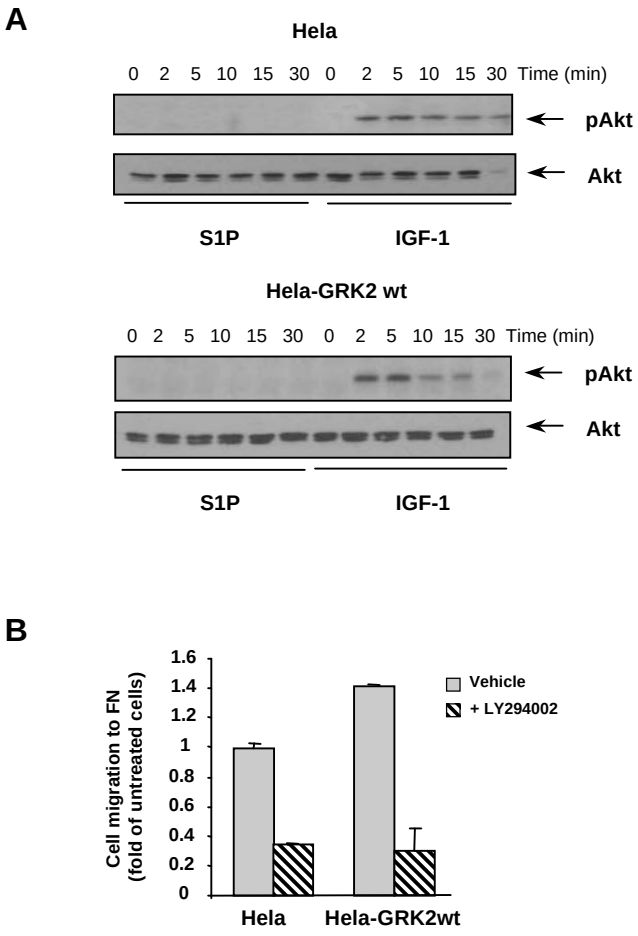
A
Hela
0 2 5 10 15 30 0 2 5 10 15 30 Time (min)
pAkt
Akt
S1P
IGF-1
Hela-GRK2 wt
0 2 5 10 15 30 0 2 5 10 15 30 Time (min)
pAkt
Akt
S1P
IGF-1
B
Cell migration to FN
(fold of untreated cells)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
+ LY294002
Hela
Hela-GRK2wt

Figure S5


A
IP: GRK2
0 2 5 10 30 S1P (min)
IB
GRK2-pTyr
GRK2
0 2 5 10 15 30 SP1 (min)
pSer670-GRK2
GRK2
Hela-GRK2wt
B
IP: GRK2
S 10 60 Fn-adhered (min)
IB
GRK2-pTyr
GRK2
Fn-adhered
S 5 10 30 60 min
pSer670-GRK2
GRK2
Hela-GRK2wt

Figure S6

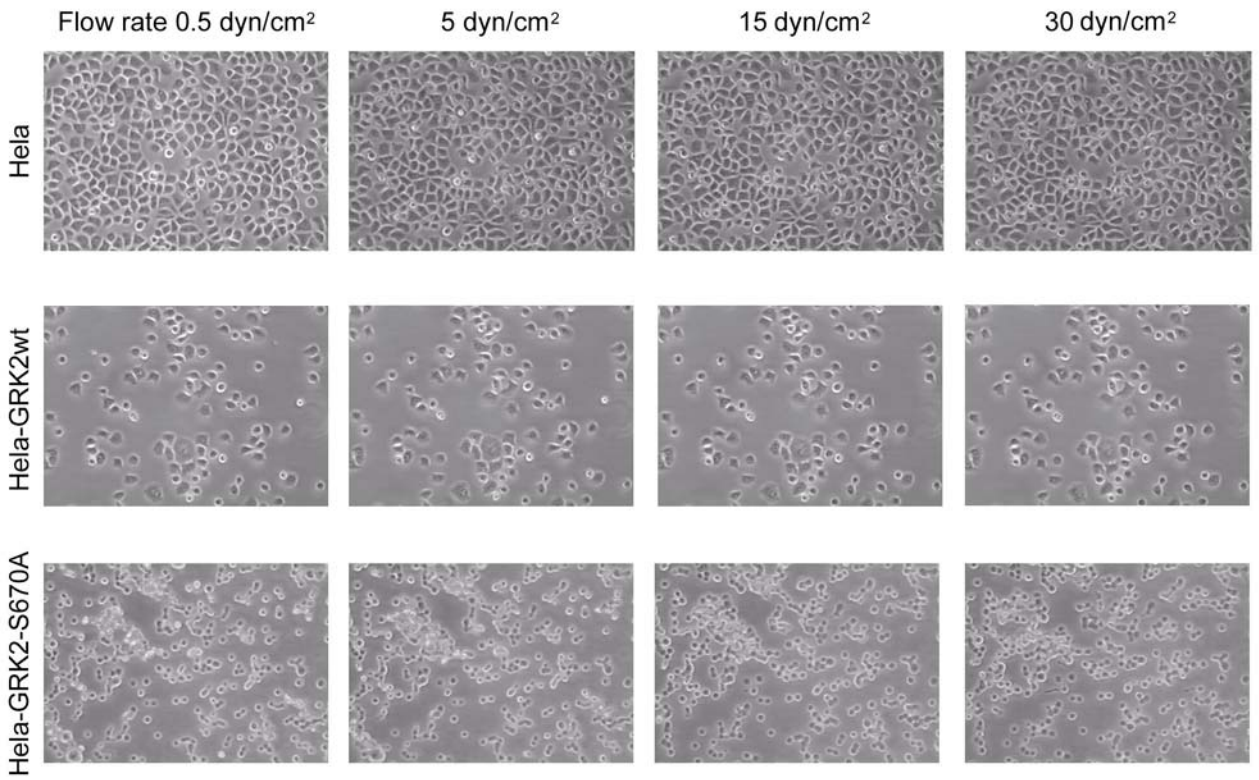
Flow rate 0.5 dyn/cm²
5 dyn/cm²
15 dyn/cm²
30 dyn/cm²
Hela
Hela-GRK2wt
Hela-GRK2-S670A

Figure S7

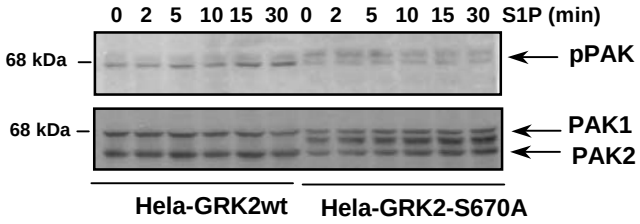
0 2 5 10 15 30 0 2 5 10 15 30 S1P (min)
68 kDa
pPAK
68 kDa
PAK1
PAK2
Hela-GRK2wt
Hela-GRK2-S670A

Figure S8

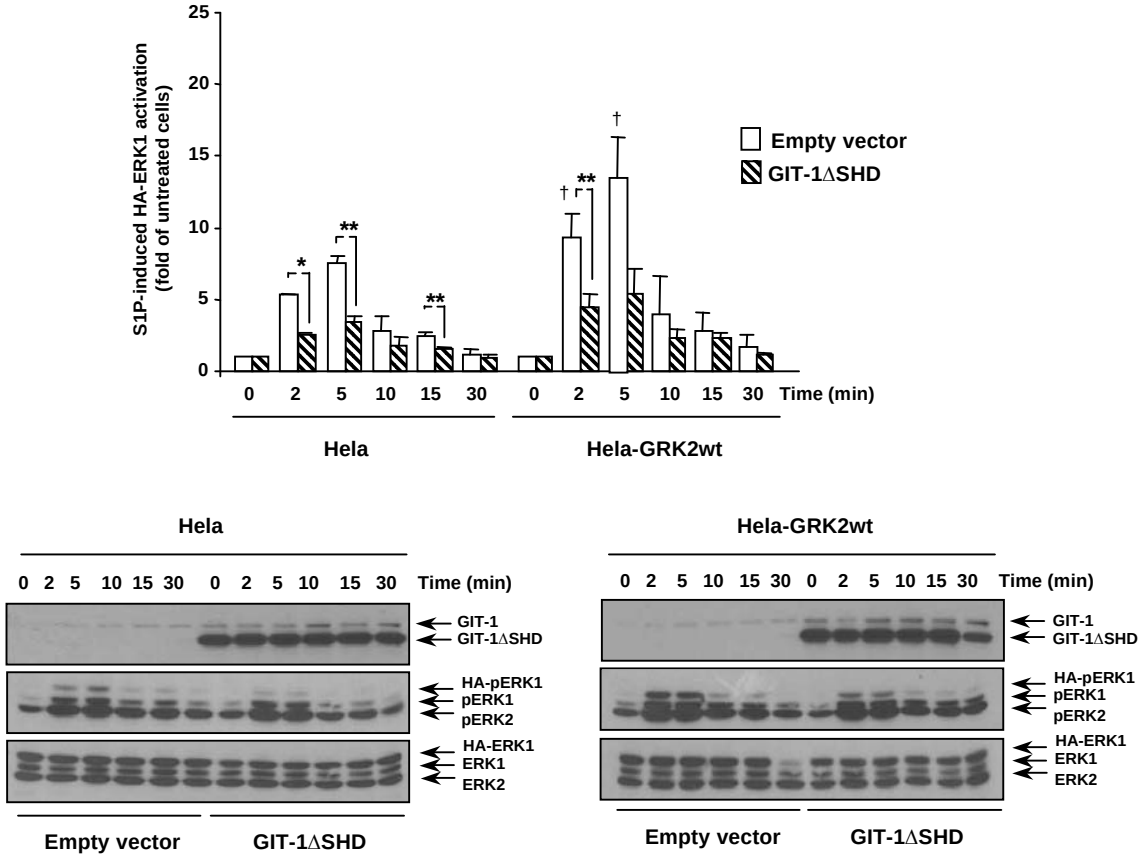
S1P-induced HA-ERK1 activation
(fold of untreated cells)
25
20
15
10
5
0
Empty vector
GIT-1ΔSHD
*
**
**
†
†
**
0 2 5 10 15 30 0 2 5 10 15 30 Time (min)
Hela
Hela-GRK2wt
Hela
Hela-GRK2wt
0 2 5 10 15 30 0 2 5 10 15 30 Time (min)
GIT-1
GIT-1ΔSHD
HA-pERK1
pERK1
pERK2
HA-ERK1
ERK1
ERK2
Empty vector
GIT-1ΔSHD
Empty vector
GIT-1ΔSHD

Figure S9

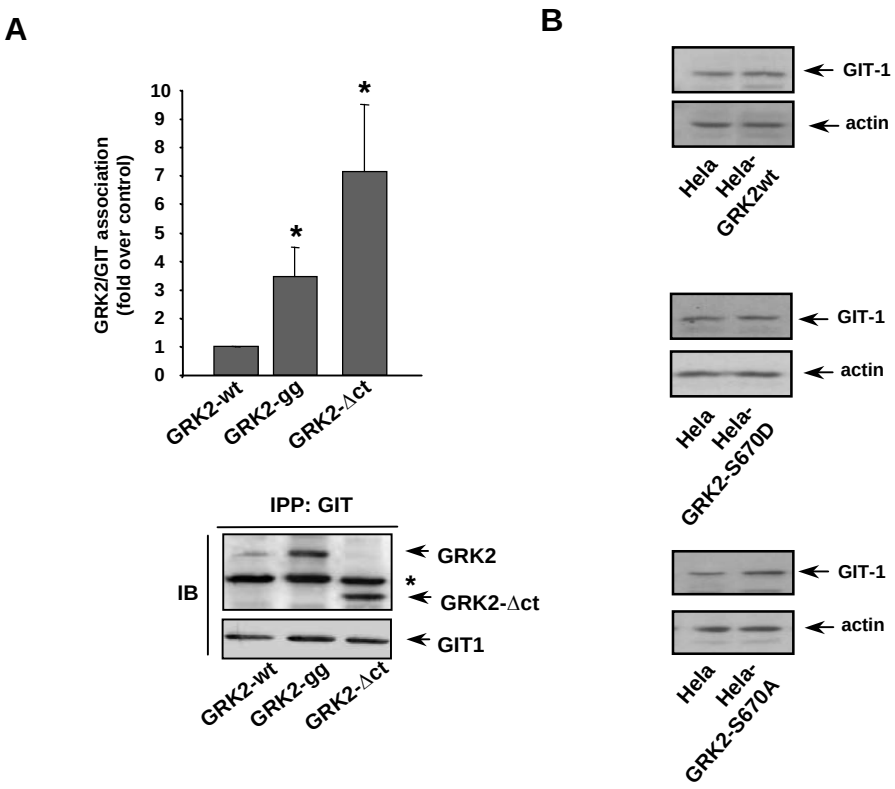
A
B
GRK2/GIT association (fold over control)
10
9
8
7
6
5
4
3
2
1
0
*
*
GRK2-wt
GRK2-gg
GRK2-Δct
IPP: GIT
IB
GRK2
*
GRK2-Δct
GIT1
GRK2-wt
GRK2-gg
GRK2-Δct
GIT-1
actin
Hela
Hela-GRK2wt
GIT-1
actin
Hela
Hela-GRK2-S670D
GIT-1
actin
Hela
Hela-GRK2-S670A

Figure S10

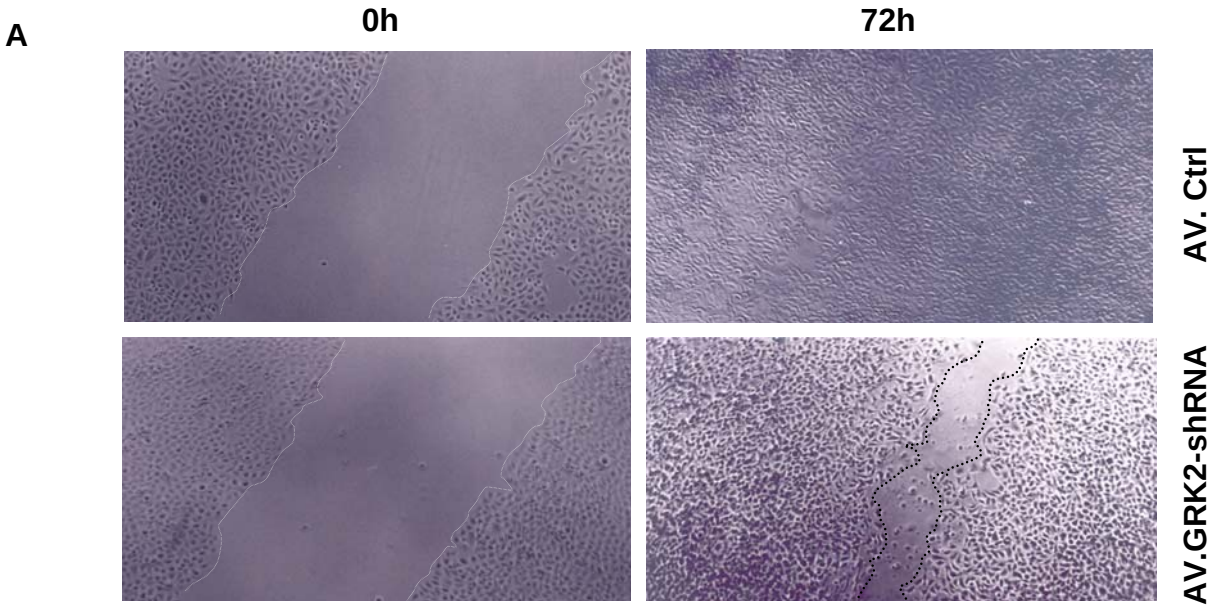

Figure S11