
Product Manual

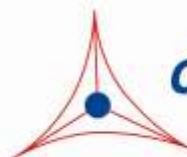
RhoA/Rac1/Cdc42 Activation Assay Combo Kit

Catalog Number

STA-405

3 x 10 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures



CELL BIOLABS, INC.
Creating Solutions for Life Science Research

Introduction

Small GTP-binding proteins (or GTPases) are a family of proteins that serve as molecular regulators in signaling transduction pathways. RhoA, Rac1 and Cdc42, 21 kDa proteins, belong to the family of Rho GTPases regulates a variety of biological response pathways that include cell motility, cell division, gene transcription, and cell transformation. Like other small GTPases, RhoA, Rac1 and Cdc42 regulate molecular events by cycling between an inactive GDP-bound form and an active GTP-bound form. In its active (GTP-bound) state, RhoA binds specifically to the Rho-binding domain (RBD) of Rhotekin, and Rac1 or Cdc42 binds specifically to the p21-binding domain (PBD) of p21-activated protein kinase (PAK) to control downstream signaling cascades.

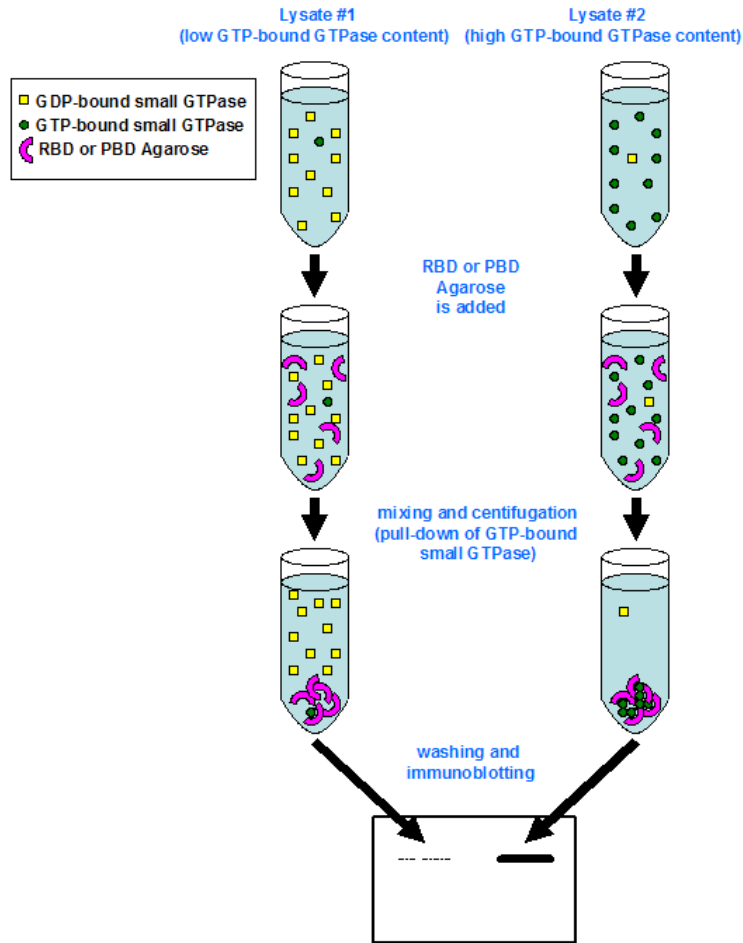
Cell Biolabs' RhoA/Rac1/Cdc42 Activation Assay utilizes Rhotekin RBD and PAK PBD Agarose beads to selectively isolate and pull-down the active form of Rho/Rac/Cdc42 from purified samples or endogenous lysates. Subsequently, the precipitated GTP-Rho or Rac or Cdc42 is detected by western blot analysis using an anti-RhoA or Rac1 or Cdc42 antibody (see Figure 3, Figure 5, Figure 7 and Assay Principle).

Cell Biolabs' RhoA/Rac1/Cdc42 Activation Assay Kit provides a simple and fast tool to monitor the activation of RhoA, Rac1 or Cdc42. The kit includes easily identifiable Rhotekin RBD and PAK1 PBD Agarose beads (see Figure 1), pink in color, along with RhoA, Rac1 and Cdc42 Immunoblot Positive Controls for quick Rho small GTPase identification. Each kit provides sufficient quantities to perform 10 assays for each small GTPase.



Figure 1: Rhotekin RBD or PAK RBD Agarose beads, in color, are easy to visualize, minimizing potential loss during washes and aspirations.

Assay Principle



Related Products

1. STA-400: Pan-Ras Activation Assay Kit
2. STA-400-H: H-Ras Activation Assay Kit
3. STA-400-K: K-Ras Activation Assay Kit
4. STA-400-N: N-Ras Activation Assay Kit
5. STA-401-1: Rac1 Activation Assay
6. STA-401-2: Rac2 Activation Assay
7. STA-403-A: RhoA Activation Assay
8. STA-403-B: RhoB Activation Assay
9. STA-403-C: RhoC Activation Assay
10. STA-404: Rac1/Cdc42 Activation Assay Combo Kit

11. STA-410: Raf1 RBD Agarose Beads

Kit Components

1. Rhotekin RBD Agarose (Part No 240501): One vial – 400 μ L of 50% slurry, 200 μ g Rhotekin RBD in PBS containing 50% glycerol.
Note: Agarose bead appears pink in color for easy identification, washing, and aspiration.
2. PAK1 PBD Agarose (Part No STA-411): One vial – 800 μ L of 50% slurry, 400 μ g PAK1 PBD in PBS containing 50% glycerol.
Note: Agarose bead appears pink in color for easy identification, washing, and aspiration.
3. 100X GTP γ S (Part No. 240103): Two vials – 2 x 50 μ L of 10 mM GTP γ S dissolved in sterile water.
4. 100X GDP (Part No. 240104): Two vials – 2 x 50 μ L of 100 mM GDP dissolved in sterile water.
5. 5X Assay/Lysis Buffer (Part No. 240102): Two bottles – 2 x 30 mL of 125 mM HEPES, pH 7.5, 750 mM NaCl, 5% NP-40, 50 mM MgCl₂, 5 mM EDTA, 10% Glycerol.
6. Anti-RhoA, Mouse Monoclonal (Part No. 240302): One vial – 40 μ L in PBS, pH 7.4, 0.05% NaN₃, 0.1% BSA.
7. Anti-Rac1, Mouse Monoclonal (Part No. 240106): One vial – 40 μ L in PBS, pH 7.4, 0.05% NaN₃, 0.1% BSA.
Note: This monoclonal reacts with human, mouse, and rat Rac1; it also has slight cross-reactivity with Rac2. Additional unknown higher MW proteins may be detected in some preparations.
8. Anti-Cdc42, Mouse Monoclonal (Part No. 240201): One vial – 40 μ L in PBS, pH 7.4, 0.05% NaN₃, 0.1% BSA.
9. RhoA Immunoblot Positive Control (Part No. 240310): One vial – 100 μ L of partially purified, recombinant RhoA from *E. coli* (provided ready-to-use in 1X reducing SDS-PAGE Sample Buffer, pre-boiled).
10. Rac1 Immunoblot Positive Control (Part No. 240110): One vial – 100 μ L of partially purified, recombinant Rac1 from *E. coli* (provided ready-to-use in 1X reducing SDS-PAGE Sample Buffer, pre-boiled).
11. Cdc42 Immunoblot Positive Control (Part No. 240210): One vial – 100 μ L of partially purified, recombinant Cdc42 from *E. coli* (provided ready-to-use in 1X reducing SDS-PAGE Sample Buffer, pre-boiled).

Materials Not Supplied

1. Stimulated and non-stimulated cell lysates
2. RhoA, Rac1 and/or Cdc42 activators
3. Protease inhibitors
4. 0.5 M EDTA in water
5. 1 M MgCl₂
6. 30°C incubator or water bath
7. 4°C tube rocker or shaker
8. 2X reducing SDS-PAGE sample buffer
9. Electrophoresis and immunoblotting systems
10. Immunoblotting wash buffer such as TBST (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20)
11. Immunoblotting blocking buffer (TBST containing 5% Non-fat Dry Milk)
12. PVDF or nitrocellulose membrane
13. Secondary Antibody
14. ECL Detection Reagents

Storage

Store all kit components at -20°C. The 5X Assay/Lysis Buffer may be stored at either -20°C or 4°C. Avoid multiple freeze/thaw cycles.

Preparation of Reagents

- 1X Assay/Lysis Buffer: Mix the 5X Stock briefly and dilute to 1X in deionized water. Just prior to usage, add protease inhibitors such as 1 mM PMSF, 10 µg/mL leupeptin, and 10 µg/mL aprotinin.

Preparation of Samples

Note: It is advisable to use fresh cell lysates because GTP-small GTPase is quickly hydrolyzed to GDP-small GTPase; frozen lysates stored at -70°C may be used. Performing steps at 4°C or on ice may reduce hydrolysis. Avoid multiple freeze/thaw cycles of lysates.

I. Adherent Cells

1. Culture cells to approximately 80-90% confluence. Stimulate cells with RhoA, Rac1 or Cdc42 activator(s) as desired.
2. Aspirate the culture media and wash twice with ice-cold PBS.
3. Completely remove the final PBS wash and add ice-cold 1X Assay/Lysis Buffer to the cells (0.5 - 1 mL per 100 mm tissue culture plate).
4. Place the culture plates on ice for 10-20 minutes.
5. Detach the cells from the plates by scraping with a cell scraper.
6. Transfer the lysates to appropriate size tubes and place on ice.

7. If nuclear lysis occurs, the cell lysates may become very viscous and difficult to pipette. If this occurs, lysates can be passed through a 27½-gauge syringe needle 3-4 times to shear the genomic DNA.
8. Clear the lysates by centrifugation for 10 minutes (14,000 x g at 4°C).
9. Collect the supernatant and store samples on ice for immediate use, or snap freeze and store at -70°C for future use.
10. Proceed to GTPγS/GDP Loading for positive and negative controls, or Pull-Down Assay.

II. Suspension Cells

1. Culture cells and stimulate with RhoA, Rac1 or Cdc42 activator(s) as desired.
2. Perform a cell count, and then pellet the cells by centrifugation.
3. Aspirate the culture media and wash twice with ice-cold PBS.
4. Completely remove the final PBS wash and add ice-cold 1X Assay/Lysis Buffer to the cell pellet (0.5 – 1 mL per 1×10^7 cells).
5. Lyse the cells by repeated pipetting.
6. Transfer the lysates to appropriate size tubes and place on ice.
7. If nuclear lysis occurs, the cell lysates may become very viscous and difficult to pipette. If this occurs, lysates can be passed through a 27½-gauge syringe needle 3-4 times to shear the genomic DNA.
8. Clear the lysates by centrifugation for 10 minutes (14,000 x g at 4°C).
9. Collect the supernatant and store samples on ice for immediate use, or snap freeze and store at -70°C for future use.
10. Proceed to GTPγS/GDP Loading for positive and negative controls, or Pull-Down Assay.

Assay Protocol

Important Note: Before running any Small GTPase pulldown assay, it is always a good practice to run a Western Blot directly on the cell lysate using the antibody provided in this kit. For example: load 5 μg, 10 μg and 20 μg of lysate onto an SDS-PAGE gel, transfer and blot. When proceeding with the pulldown assay, use 100-times the amount of lysate that gave you a clear band of your desired small GTPase in the direct Western blot. For example: if the 5 μg band was faint but the 10 μg band was clear and strong, use $100 \times 10 \mu\text{g} = 1 \text{ mg}$ of lysate in the assay. Using sufficient lysate in the pulldown assay is critical to success.

I. GTPγS/GDP Loading (Positive and Negative Controls)

Note: Samples that will not be GTPγS/GDP loaded may be kept on ice during the loading of controls.

1. Aliquot 0.5 – 1 mL of each cell lysate to two microcentrifuge tubes.
Note: Typical protein content/sample is > 0.5 mg.
2. Adjust the volume of each sample to 1 mL with 1X Assay Lysis Buffer.

3. Add 20 μL of 0.5 M EDTA to each sample.
4. Add 10 μL of 100X GTP γ S to one tube (positive control) and 10 μL of 100X GDP to the other tube (negative control). Mix and label each tube appropriately.
5. Incubate the tubes for 30 minutes at 30°C with agitation.
6. Stop the loading by adding 65 μL of 1 M MgCl₂ to each tube. Mix and place tubes on ice.
7. Continue with Pull-Down assay.

II. Small GTPase Pull-Down Assay

1. Aliquot 0.5 – 1 mL of cell lysate (treated with small GTPase activators or untreated) to a microcentrifuge tube.
2. Adjust the volume of each sample to 1 mL with 1X Assay Lysis Buffer.
3. Thoroughly resuspend the Rhotekin RBD or PAK PBD Agarose bead slurry by vortexing or titrating.
4. Quickly add 40 μL of resuspended bead slurry to each tube (including GTP γ S/GDP controls).
5. Incubate the tubes at 4°C for 1 hour with gentle agitation.
6. Pellet the beads by centrifugation for 10 seconds at 14,000 x g.
7. Aspirate and discard the supernatant, making sure not to disturb/remove the bead pellet.
8. Wash the bead 3 times with 0.5 mL of 1X Assay Buffer, centrifuging and aspirating each time.
9. After the last wash, pellet the beads and carefully remove all the supernatant.
10. Resuspend the bead pellet in 40 μL of 2X reducing SDS-PAGE sample buffer.
11. Boil each sample for 5 minutes.
12. Centrifuge each sample for 10 seconds at 14,000 x g.

III. Electrophoresis and Transfer

1. Load 20 μL /well of pull-down supernatant to a polyacrylamide gel. Also, it's recommended to include a pre-stained MW standard (as an indicator of a successful transfer in step 3).
Note: If desired, 10 μL /well of RhoA, Rac1 or Cdc42 Immunoblot Positive Control (provided ready-to-use, pre-boiled) can be added as an immunoblot positive control.
2. Perform SDS-PAGE as per the manufacturer's instructions.
3. Transfer the gel proteins to a PVDF or nitrocellulose membrane as per the manufacturer's instructions.

IV. Immunoblotting and Detection (all steps are at room temperature, with agitation)

1. Following the electroblotting step, immerse the PVDF membrane in 100% Methanol for 15 seconds, and then allow it to dry at room temperature for 5 minutes.
Note: If Nitrocellulose is used instead of PVDF, this step should be skipped.

2. Block the membrane with 5% non-fat dry milk in TBST for 1 hr at room temperature with constant agitation.

Incubate the membrane with Anti-RhoA, Rac1 or Cdc42 Antibody, freshly diluted 1:200 to 1:1000 in 5% non-fat dry milk/TBST, for 1-2 hr at room temperature with constant agitation.

Note: To conserve antibody, incubations should be performed in a plastic bag.

3. Wash the blotted membrane three times with TBST, 5 minutes each time.
4. Incubate the membrane with a secondary antibody (e.g. Goat Anti-Mouse IgG, HRP-conjugate), freshly diluted in 5% non-fat dry milk/TBST, for 1 hr at room temperature with constant agitation.
5. Wash the blotted membrane three times with TBST, 5 minutes each time.
6. Use the detection method of your choice. We recommend enhanced chemiluminescence reagents from Pierce.

Example of Results

The following figure demonstrates typical results seen with Cell Biolabs' RhoA/Rac1/Cdc42 Activation Assay Kit. One should use the data below for reference only.

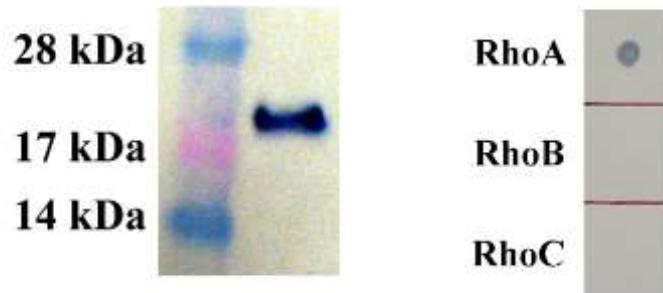


Figure 2: RhoA Activation Assay. *Left Image:* RhoA Immunoblot Positive Control. *Right Image:* Demonstrates Anti-RhoA monoclonal antibody specificity by dot blot.

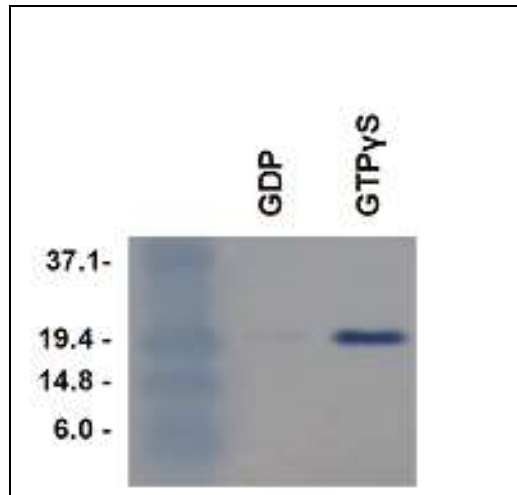


Figure 3: RhoA Activation Assay. *Lane 1:* MW Standard. *Lane 2:* MDA-231 cell lysate loaded with GDP and incubated with Rhotekin RBD Agarose beads. *Lane 3:* MDA-231 cell lysate loaded with GTP γ S and incubated with Rhotekin RBD Agarose beads.

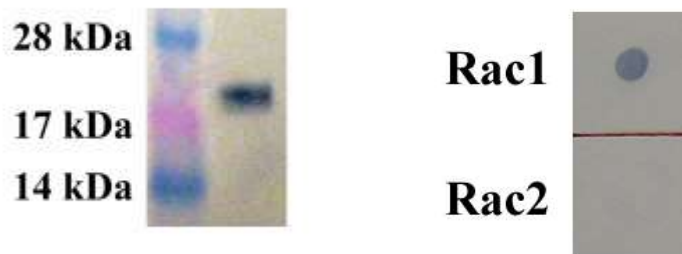


Figure 4: Rac1 Activation Assay. *Left Image:* Rac1 Immunoblot Positive Control. *Right Image:* Demonstrates Anti-Rac1 monoclonal antibody specificity by dot blot.

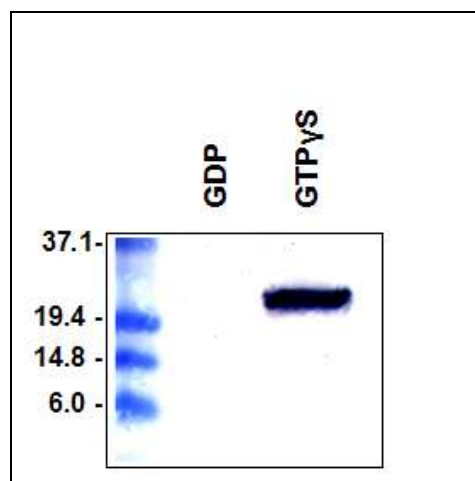


Figure 5: Rac1 Activation Assay. Lane 1: MW Standard. Lane 2: 293 cell lysate loaded with GDP and incubated with PAK PBD Agarose beads. Lane 3: 293 cell lysate loaded with GTP γ S and incubated with PAK-1 PBD Agarose beads.

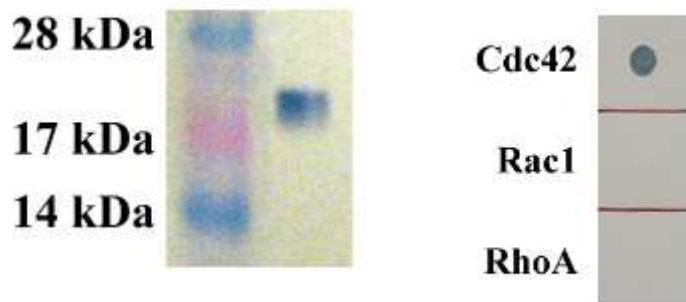


Figure 6: Cdc42 Activation Assay. Left Image: Cdc42 Immunoblot Positive Control. Right Image: Demonstrates Anti-Cdc42 monoclonal antibody specificity by dot blot.

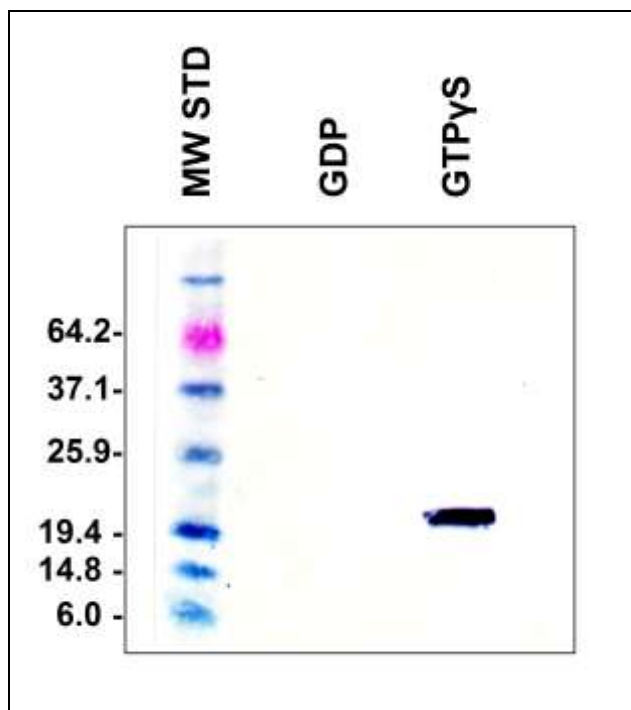


Figure 7: Cdc42 Activation Assay. Lane 1: MW Standard. Lane 2: 293 cell lysate loaded with GDP and incubated with PAK PBD Agarose beads. Lane 3: 293 cell lysate loaded with GTP γ S and incubated with PAK-1 PBD Agarose beads.

References

1. Ren X.D. and Schwartz M. A. (2000) *Methods Enzymol.* **325**:264-72.

2. Raftopoulou M., and Hall A. (2004) *Dev Biol.* **265**:23-32.
3. Bar-Sagi D., and Hall A. (2000) *Cell* **103**:227-38.
4. Benard, V., Bohl, B. P., and Bokoch, G. M. (1999) *J. Biol. Chem.* **274**:13198-13204.

Recent Product Citations

1. Jaafar, L. et al. (2021). StarD13 differentially regulates migration and invasion in prostate cancer cells. *Hum Cell.* doi: 10.1007/s13577-020-00479-8.
2. Ghassibe-Sabbagh, M. et al. (2021). Altered regulation of cell migration in IRF6-mutated orofacial cleft patients-derived primary cells reveals a novel role of Rho GTPases in cleft/lip palate development. *Cells Dev.* doi: 10.1016/j.cdev.2021.203674.
3. Li, M.J. et al. (2021). Ezrin Promotes the Proliferation, Migration, and Invasion of Ovarian Cancer Cells. *Biomed. Environ. Sci.* **34**(2):139-151. doi: 10.3967/bes2021.020.
4. Matarrese, P. et al. (2021). Physical Interaction between HPV16E7 and the Actin-Binding Protein Gelsolin Regulates Epithelial-Mesenchymal Transition via HIPPO-YAP Axis. *Cancers (Basel).* **13**(2):353. doi: 10.3390/cancers13020353.
5. El-Mais, N. et al. (2020). Human recombinant arginase I [HuArgI (Co)-PEG5000]-induced arginine depletion inhibits ovarian cancer cell adhesion and migration through autophagy-mediated inhibition of RhoA. *J Ovarian Res.* **14**(1):13. doi: 10.1186/s13048-021-00767-3.
6. El-Chami, D. et al. (2021). Recombinant anthrax lethal toxin inhibits cell motility and invasion in breast cancer cells through the dysregulation of Rho GTPases. *Oncol Lett.* **21**(2):1-7. doi: 10.3892/ol.2020.12424.
7. Al Haddad, M. et al. (2020). Differential regulation of rho GTPases during lung adenocarcinoma migration and invasion reveals a novel role of the tumor suppressor StarD13 in invadopodia regulation. *Cell Commun Signal.* **18**(1):144. doi: 10.1186/s12964-020-00635-5.
8. Badaoui, M. et al. (2020). Vav3 Mediates Pseudomonas aeruginosa Adhesion to the Cystic Fibrosis Airway Epithelium. *Cell Rep.* **32**(1):107842. doi: 10.1016/j.celrep.2020.107842.
9. Rajakylä, E.K. et al. (2020). Assembly of Peripheral Actomyosin Bundles in Epithelial Cells Is Dependent on the CaMKK2/AMPK Pathway. *Cell Rep.* **30**(12):4266-4280.e4. doi: 10.1016/j.celrep.2020.02.096.
10. Hirano, T. et al. (2020). FARP1 boosts CDC42 activity from integrin $\alpha\beta 5$ signaling and correlates with poor prognosis of advanced gastric cancer. *Oncogenesis.* **9**(2):13. doi: 10.1038/s41389-020-0190-7.
11. Nicolas, S. et al. (2019). Hypoxia and EGF Stimulation Regulate VEGF Expression in Human Glioblastoma Multiforme (GBM) Cells by Differential Regulation of the PI3K/Rho-GTPase and MAPK Pathways. *Cells.* **8**(11). pii: E1397. doi: 10.3390/cells8111397.
12. Fostok, S. et al. (2019). Connexin 43 Loss Triggers Cell Cycle Entry and Invasion in Non-Neoplastic Breast Epithelium: A Role for Noncanonical Wnt Signaling. *Cancers (Basel).* **11**(3). pii: E339. doi: 10.3390/cancers11030339.
13. El Atat, O. et al. (2019). RHOG Activates RAC1 through CDC42 Leading to Tube Formation in Vascular Endothelial Cells. *Cells.* **8**(2). pii: E171. doi: 10.3390/cells8020171.
14. Kim, D. et al. (2019). Lysophosphatidic acid increases mesangial cell proliferation in models of diabetic nephropathy via Rac1/MAPK/KLF5 signaling. *Exp Mol Med.* **51**(2):18. doi: 10.1038/s12276-019-0217-3.
15. Zhang, F. et al. (2018). GAP43, a novel metastasis promoter in non-small cell lung cancer. *J Transl Med.* **16**(1):310. doi: 10.1186/s12967-018-1682-5.

16. Chen, Z.S. et al. (2018). Planar cell polarity gene Fuz triggers apoptosis in neurodegenerative disease models. *EMBO Rep.* **19**(9). pii: e45409. doi: 10.15252/embr.201745409.
17. Schiapparelli, P. et al. (2017). NKCC1 regulates migration ability of glioblastoma cells by modulation of actin dynamics and interacting with cofilin. *EBioMedicine* doi: 10.1016/j.ebiom.2017.06.020.
18. Prudent, J. et al. (2016). Mitochondrial Ca²⁺ uptake controls actin cytoskeleton dynamics during cell migration. *Sci. Rep.* **6**:36570.
19. Shen, J. et al. (2016). NMDA receptors participate in the progression of diabetic kidney disease by decreasing Cdc42-GTP activation in podocytes. *J Pathol.* doi:10.1002/path.4764.
20. Le, L. T. et al. (2016). Loss of miR-203 regulates cell shape and matrix adhesion through ROBO1/Rac/FAK in response to stiffness. *J Cell Biol.* **212**:707-719.
21. Namachivayam, K. et al. (2015). All-Trans Retinoic Acid Induces TGF- β 2 in Intestinal Epithelial Cells via RhoA- and p38 α MAPK-Mediated Activation of the Transcription Factor ATF2. *PLoS One.* **10**(7):e0134003. doi: 10.1371/journal.pone.0134003.
22. Giralt, A. et al. (2015). Pyk2 is essential for astrocytes mobility following brain lesion. *Glia.* doi:10.1002/glia.22952.
23. Mori, H. et al. (2015). Smad3 deficiency leads to mandibular condyle degradation via the sphingosine 1-phosphate (S1P)/S1P 3 signaling axis. *Am J Pathol.* doi: 10.1016/j.ajpath.2015.06.015.
24. Toyoda, K. et al. (2015). Grp78 is critical for amelogenin-induced cell migration in a multipotent clonal human periodontal ligament cell line. *J Cell Physiol.* doi: 10.1002/jcp.25087.
25. Barbati, C. et al. (2015). THU0381 autoantibodies specific to D4GDI isolated from SLE patients “unlock” RHO small GTPases and affect actin remodeling in T lymphocytes. *Ann Rheum Dis.* **74**:334-334.
26. Barbati, C. et al. (2015). Autoantibodies specific to D4GDI modulate Rho GTPase mediated cytoskeleton remodeling and induce autophagy in T lymphocytes. *J Autoimmun.* doi: 10.1016/j.jaut.2015.01.005.
27. Nasrallah, A. et al. (2014). Effect of StarD13 on colorectal cancer proliferation, motility and invasion. *Oncol Rep.* **31**:505-515.
28. Luis-Ravelo, D. et al. (2014). RHOB influences lung adenocarcinoma metastasis and resistance in a host-sensitive manner. *Mol Oncol.* **8**:196-206.
29. Khalil, B. D. et al. (2014). The regulation of RhoA at focal adhesions by StarD13 is important for astrocytoma cell motility. *Exp Cell Res.* **321**:109-122.
30. Hanna, S. et al. (2014). StarD13 is a tumor suppressor in breast cancer that regulates cell motility and invasion. *Int J Oncol.* **44**:1499-1511.

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