Analysis of RhoA and Rho GEF activity in whole cells and the cell nucleus

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We have recently shown that a fraction of the total cellular pool of the small GTPase RhoA resides in the nucleus, and that the nuclear guanine nucleotide exchange factor (GEF) Net1 has a role in the regulation of its activity. In this protocol, we describe a method to measure both the activities of the nuclear pools of RhoA and Rho GEFs. This process required the development of a nuclear isolation protocol that is both fast and virtually free of cytosolic and membrane contaminants, as well as a redesign of existing RhoA and Rho GEF activity assays so that they work in nuclear samples. This protocol can be also used for other Rho GTPases and Rho GEFs, which have also been found in the nucleus. Completion of the procedure, including nuclear isolation and RhoA or Rho GEF activity assay, takes 1 h 40 min. We also include details of how to perform a basic assay of whole-cell extracts.

INTRODUCTION

The Rho family of small GTPases

Rho GTPases control many aspects of cell behavior, such as the organization of the cytoskeleton, cell migration, cell-cell and cell-matrix adhesion, cell cycle progression, gene expression and cell polarity[1,2]. Rho proteins act as molecular switches by cycling between an active (GTP bound) and an inactive (GDP bound) state. The activation of Rho proteins is mediated by specific GEFs, that catalyze the exchange of GDP for GTP. In the active state, GTPases interact with one of several downstream effectors to modulate their activity and localization. The regulation of these effector proteins ultimately leads to changes in cell behavior. The signal is terminated by hydrolysis of GTP to GDP, a reaction that is stimulated by GTPase-activating proteins (GAPs). Humans have more than 70 Rho GEFs and ~60 Rho GAPs, allowing cells to regulate the activity of 22 Rho GTPases through multiple pathways[3,4].

Methods for monitoring RhoA activity

The original method for measuring the activity of Rho GTPases was developed (initially for RhoA) by Ren *et al.*[5]. The assay takes advantage of the fact that effector proteins interact only with GTP-bound Rho GTPases. The authors used the Rho-binding domain (RBD) of the RhoA effector rhotekin to affinity-precipitate endogenous GTP-bound RhoA[5]. The amount of precipitated RhoA was then quantified by western blotting using specific antibodies. The binding of glutathione *S*-transferase (GST)-RBD to GTP-RhoA inhibits it conversion to GDP, so the assay provides a reliable measure of the total amount of active RhoA present in the cell at any given time. The same principle was later adapted to other GTPases of the Rho family, such as Rac, Cdc42 and RhoG, by using effector domains specific for these proteins (**Table 1**)[6,7]. These assays are now considered standard and have been used extensively to identify the Rho GTPases that are activated in response to specific signals or cellular processes. An assay was later developed to measure the activity of the direct upstream regulators of Rho GTPases, the GEFs and the GAPs[8]. These affinity-precipitation assays use constitutively active or dominant-negative mutants of the Rho GTPases to specifically precipitate Rho GAPs and Rho GEFs, respectively. These mutants were originally designed on the basis of their homology to the previously characterized constitutively active and nucleotide-free Ras mutants (Q61LRas and G15A, respectively)[9–12]. The constitutively active mutants have lost their capacity to hydrolyze GTP and they bind with high affinity to GAPs and effectors[13,14]. There are two different types of dominant-negative mutants. The 'traditional' dominant-negatives (T17N in Ras) are mutants that can bind GDP normally but that have a very low affinity for GTP, so they exist almost exclusively in a GDP state[11,15]. A second type of dominant-negative mutant has low affinity for both GDP and GTP, so it is found mostly in a nucleotide-free state[9]. It has been previously shown that, in the nucleotide exchange reaction catalyzed by GEFs, a nucleotide-free GTPase is one of the intermediate states, and it forms a high-affinity complex with a GEF[16]. This intermediate is short-lived and does not accumulate in cells. The GEF pull-down assay takes advantage of the properties of this mutant (G17A for RhoA) and uses it to pull down the active pool of Rho GEFs from cell lysates. **Table 1** lists the equivalent mutations used in the Rho GEF and GAP pull-down assays.

Rho GTPases and Rho GEFs in the cell nucleus

Development of the method. Despite their conceptual simplicity, the protocols described above for measuring RhoA activity are time-sensitive and require substantial optimization, mainly because the active GTP-bound form of the Rho GTPases is extremely short-lived and labile. We have now adapted these techniques to measure RhoA and Rho GEF activities from isolated nuclei[17]. Using these techniques, we have recently characterized a functional RhoA pathway in the cell nucleus that responds to DNA damage[17]. In this study, we show that a fraction of the total RhoA cellular pool resides in the nucleus, and that the nuclear RhoA-GEF Net1 has a role in the regulation of its activity. In addition, DNA damage signals, such as ionizing radiation (IR), specifically promote the activation of the nuclear pool of RhoA in a Net1-dependent manner[17]. We also include details of the basic whole-cell extract assay (**Box 1**).

TABLE 1 | Mutants used in Rho GTPase, Rho GEF and Rho GAP activity assays.

	Type of assay		
Target	**GTPase**	**GEF**	**GAPs/effectors**
RhoA, B, C	RBD	^{G17A}Rho*	^{Q63L}Rho*
Rac1, 2, 3	PBD	^{G15A}Rac†	^{Q61L}Rac†
Cdc42	PBD	^{G15A}Cdc42	^{Q61L}Cdc42
RhoG	ELMO	^{G15A}RhoG	^{Q61L}RhoG

ELMO, engulfment and cell motility.

The same mutation can be engineered in RhoA, B or C (*) or in Rac1, 2 or 3 (†) to identify specific GEFs and GAPs for each of these isoforms.

All mutants are expressed in bacteria and purified as GST-fusion proteins.

Applications of the method. This method can be used to monitor the activity of RhoA and RhoA-GEFs in the cell nucleus in response to different stimuli or other manipulations, such as inhibitors of gene silencing or overexpression of proteins of interest.

This method can be easily adapted to measure the activity of other nuclear GTPases. Other Rho GTPases, such as Rac1 and RhoC, have also been shown to reside in the nucleus[17,18]. Active nuclear RhoC can be detected by precipitation with GST-RBD and blotting with a RhoC antibody. To measure the activity of nuclear Rac1, GST–p21 binding domain (PBD) can be used instead of GST-RBD in the affinity pull-down in combination with specific antibodies against the GTPase of interest (**Table 1**).

Using the nuclear GEF pull-down, it should be possible to monitor the spatiotemporal changes in the activity of a particular Rho GEF when it shuttles between the nucleus and the cytosol. Aside from Net1 and Ect2, which are targeted to the nucleus at steady state, other Rho GEFs have been shown to shuttle between the nucleus and the cytosol, including leukemia-associated Rho GEF[19].

It has also been shown that several Rho GAPs localize to the nucleus or cycle between the nucleus and the cytosol, such as p190RhoGAP, DLC1 and srGAPs[17,20–22]. Similarly to the nuclear Rho GEF assay, a previously developed Rho GAP pull-down assay[8] could be optimized to analyze nuclear Rho GAP activity (**Table 1**).

Experimental design

The protocol consists of a rapid nuclear isolation step using an iodixanol cushion, followed by extraction of the nuclear proteins. Active RhoA and RhoA-specific GEFs are then precipitated from the nuclear extracts using GST-fusion proteins of the RBD of rhotekin, or of a nucleotide-free mutant of RhoA (G17A-RhoA), respectively. The results of these assays are monitored by western blotting using antibodies against RhoA or the GEFs of interest.

A key element in this method is the development of a nuclear isolation protocol that is both fast and virtually free of cytosolic and membrane contaminants. The speed of the nuclear isolation is essential, in particular for the RhoA pull-down, as the activity decreases rapidly after the cells are lysed. Considering that nuclear RhoA accounts for only 5% of the total RhoA in the cell, it is also essential to prevent contamination with extranuclear RhoA. Even a small percentage of contamination from cytosolic or plasma membrane-bound RhoA could cause gross overestimation of the amount of active nuclear RhoA and hinder the procedure's ability

Box 1 | Analysis of RhoA and Rho GEF activity from whole-cell lysates

The original RhoA and Rho GEF activity assays were designed to measure RhoA[5] or Rho GEF[8] activity from whole-cell lysates. Here we describe these protocols in detail.

1| Use a near-confluent monolayer of cells. Usually a confluent 10-cm plate is sufficient for a pull-down assay (the number of plates used per assay may need to be adjusted).

▲ **CRITICAL STEP** A good starting point when characterizing a new cell line is 1 mg of total nuclear or cytosolic protein per condition.

2| Aspirate the medium and rinse the monolayer once with ice-cold PBS + 1 mM $MgCl_2$.

RhoA activity assay ● **TIMING 1 h**

3| Add ice-cold RBD buffer to the dish (500 µl for a 10-cm dish) and scrape the cells into the medium with a cell scraper.

4| Centrifuge at maximum speed (14,000*g*) for 5 min at 4 °C in a tabletop centrifuge. Transfer the supernatant to a fresh tube.

5| Transfer an aliquot of the total cell lysate (TCL) to a fresh tube for SDS-PAGE analysis (usually 100 µl).

▲ **CRITICAL STEP** The amount of protein loaded in the gel will be determined after the protein concentration is estimated.

6| Determine protein concentration from the TCL fraction using the Bio-Rad DC protein assay kit. Protein concentration can be measured using different commercially available methods. However, we favor this kit because it is specially formulated to accurately measure protein concentration in the presence of high detergent concentration.

▲ **CRITICAL STEP** Accurately measuring the protein concentration in the different fractions is essential for the success of the subsequent steps, as the same amount of protein is used in each condition for the different pull-down assays.

7| Equalize both the total amount of protein and the volume used for each sample using RBD lysis buffer. Ideally, 1 mg or more of total protein should be used in 0.5–1 ml of total volume.

8| Add 50 µg of fusion protein on beads to each sample and rotate the samples for 30 min at 18 r.p.m. at 4 °C.

▲ **CRITICAL STEP** The glycerol present in the bead buffer may introduce errors when transferring the beads to fresh tubes. Use wide-bore tips or cut the end of the tips and resuspend the fusion protein-bead slurry thoroughly by careful pipetting to ensure the same volume of slurry is used for each condition.

9| Wash the beads three times with RBD wash buffer, aspirate off the supernatant and resuspend the beads in 30–40 µl of SDS-PAGE sample buffer.

▲ **CRITICAL STEP** To prevent unintended aspiration of the beads, a 27.5-gauge needle can be connected to the vacuum aspirator line and used to remove the wash buffer. The needle slows the liquid flow and allows a more precise handling of the beads. This size needle

(continued)

Box 1 | (CONTINUED)

is small enough that it can be safely inserted into the beads without aspirating them. However, we recommend using the needle on the side of the tube and aspirating as much liquid as possible without touching the beads, and only inserting the needle carefully into the beads after the last wash to remove the remaining buffer.

10| Boil for 10 min and centrifuge briefly to recover the sample.

■ **PAUSE POINT** After resuspending the beads in sample buffer, the sample can be stored at −20 °C for several days before proceeding with SDS-PAGE separation and western blotting. All other samples, including total lysates and loading controls, can also be stored at −20 °C.

Rho GEF activity assay ● **TIMING 1 h**

11| Add ice-cold Rho GEF buffer to the dish (500 µl for a 10-cm dish) and scrape the cells into the medium with a cell scraper.

12| Centrifuge at max speed (14,000*g*) for 5 min at 4 °C in a tabletop centrifuge. Transfer the supernatant to a fresh tube.

13| Transfer an aliquot of the TCL to a fresh tube for SDS-PAGE analysis (usually 100 µl).

▲ **CRITICAL STEP** The amount of protein loaded in the gel will be determined after the protein concentration is estimated.

14| Determine the protein concentration from the TCL fractions using the Bio-Rad DC protein assay kit.

▲ **CRITICAL STEP** Measuring accurately the protein concentration in the different fractions is essential for the success of the subsequent steps, as the same amount of protein is used in each condition for the different pull-down assays.

15| Equalize both the total amount of protein and also the volume used for each condition using Rho GEF buffer. Ideally, 1 mg or more of total protein should be used in 0.5–1 ml of total volume.

16| Add 30 µg of GST-RhoA^{G17A} beads to each sample and rotate the tubes at 18 r.p.m. for 45 min at 4 °C.

▲ **CRITICAL STEP** The glycerol present in the bead buffer may introduce errors when transferring the beads to fresh tubes. Use wide-bore tips or cut the end of the tips and resuspend the fusion protein-bead slurry thoroughly by careful pipetting to ensure the same volume of slurry is used for each condition.

17| Wash the beads three times with Rho GEF buffer, aspirate the supernatant and resuspend the beads in 40 µl of SDS-PAGE sample buffer.

▲ **CRITICAL STEP** To prevent unintended aspiration of the beads, a 27.5-gauge needle can be connected to the vacuum aspirator line and used to remove the wash buffer. The needle slows the liquid flow and allows a more precise handling of the beads. This size needle is small enough that it can be safely inserted into the beads without aspirating them. However, we recommend using the needle on the side of the tube and aspirating as much liquid as possible without touching the beads, and only inserting the needle carefully into the beads after the last wash to remove the remaining buffer.

18| Boil for 10 min and centrifuge briefly to recover the sample.

■ **PAUSE POINT** After resuspending the beads in sample buffer, the sample can be stored at −20 °C for several days before proceeding with SDS-PAGE separation and western blotting. All other samples, including total lysates and loading controls, can also be stored at −20 °C.

SDS-PAGE, transfer and western blotting

19| Run the lysates (TCL) from the different conditions and the corresponding pull-down samples on SDS-PAGE gels. Run RBD pull-down samples in 15% (wt/vol) gels and Rho GEF pull-down samples in 7.5–10% (wt/vol) gels, depending on the GEF molecular weight. Use samples saved from Steps 5 and 10 for the Rho assay, and samples saved from Steps 13 and 18 for the GEF assay. Load equal amounts of protein for the lysates (TCL) based on the protein concentration measurement in Steps 6 and 14 (typically 20 µg of total protein). Transfer and western blot for RhoA in the Rho assay, and for Net1 or the GEF of interest in the GEF assay.

to investigate signaling pathways that specifically target only the nuclear pool of RhoA.

The nuclear isolation method we developed is based on previously described protocols that use an iodixanol (OptiPrep) gradient to isolate nuclei based on their density[23–25]. However, the length of these nuclear isolation protocols negated the ability to successfully precipitate active nuclear RhoA because of the labile nature of GTP-bound RhoA. We therefore introduced modifications that substantially reduced the time required (by eliminating both the need of a discontinuous gradient and the use of an ultracentrifuge) while maintaining the purity of the nuclear fraction (NUC) obtained.

In theory, any nuclear purification method could be coupled to RhoA and Rho GEF activity assays, as long as it does not rely on the use of detergents. Most of the traditional nuclear purification methods use a combination of hypotonic swelling and lysis, using detergents such as Igepal CA-630 (NP-40). Although they are quite efficient and fast, these methods would not be of use for Rho GTPases because they would solubilize the active nuclear Rho GTPases, which are associated with the nuclear membrane into the cytosolic fraction.

After nuclear isolation, the nuclear proteins are extracted and can be used in either the RhoA or Rho GEF pull-down assay. A postnuclear supernatant (PNS) fraction assay for RhoA and Rho GEFs can be performed in parallel as well, thereby allowing for the simultaneous detection of nuclear and non-nuclear (cytosolic and membrane-bound) RhoA or Rho GEF activity. To ensure that all the components of the assay are working properly, positive controls are recommended for the first-time user (see TROUBLESHOOTING).

Limitations

One of the main limitations of this type of assay is its sensitivity. Typically, it requires a substantial amount of protein to be able to precipitate a detectable amount of active GTPase or GEF, and to reliably detect differences between treatments.

Compared with total cell lysates (TCLs), in which the Rho GTPases and GEFs are fairly abundant, nuclear assays require a markedly higher amount of starting material (see PROCEDURE for estimate amounts). In recent years, an ELISA protocol has been developed by Cytoskeleton to monitor the activity of Rho GTPases from TCLs. This assay uses only a fraction of the protein required in the standard pull-down assay, and it should be possible to adapt the protocol for nuclear pull-downs. This may allow the use of this assay in experiments where the cell number is limiting.

MATERIALS

REAGENTS

- OptiPrep (60% (vol/vol) solution of iodixanol in H_2O, Sigma, cat. no. D1556) ▲ **CRITICAL** OptiPrep is light sensitive and should be stored in a dark container at 4 °C.
- Sodium chloride (NaCl, Fisher, cat. no. BP358-2120)
- Tris base (Tris(hydroxymethyl)-aminomethane; Fisher, cat. no. BP152-5)
- HEPES (Sigma, cat. no. H3375)
- Hydrochloric acid (HCl; Fisher, cat. no. A144-500) ! **CAUTION** HCl is extremely corrosive and toxic and readily produces vapors. Use a fume hood, face mask, eye protection and gloves while handling.
- Methanol (Fisher, cat. no. A411-20) ! **CAUTION** It is toxic by inhalation, in contact with skin and if swallowed. Use a fume hood, face mask, eye protection and gloves while handling.
- Sodium hydroxide (NaOH; Fisher, cat. no. S318) ! **CAUTION** It is extremely corrosive and toxic. Use a fume hood, face mask, eye protection and gloves while handling.
- Sucrose (Sigma, cat. no. S0389)
- Potassium chloride (KCl; Fisher, cat. no. P217-500)
- $MgCl_2{\cdot}6H_2O$ (Mallinckrodt, cat. no. 5958-04)
- Glycine (Fisher, cat. no. BP381-5)
- Bio-Rad DC protein assay kit (Bio-Rad, cat. no. 500-0112)
- Coomassie Plus protein reagent (Pierce, Thermo Scientific, cat. no. 23236)
- DMEM (Invitrogen, cat. no. 31053-028)
- FBS (Sigma, cat. no. F2442)
- Penicillin-streptomycin solution (pen-strep, 100×; Invitrogen, cat. no. 15140)
- Aprotinin (Sigma, cat. no. A1153)
- Leupeptin (Sigma, cat. no. L2884)
- Pepstatin A (Sigma, cat. no. P4265)
- PMSF (Sigma, cat. no. P7626)
- Protease inhibitor cocktail (Sigma, cat. no. P8340)
- Hoechst 33342 (20 mM in H_2O; AnaSpec, cat. no. 83218)
- Triton X-100 (Sigma, cat. no. X100-1L)
- Tween-20 (Fisher, cat. no. BP337-500)
- Sodium deoxycholate (Sigma, cat. no. D6750)
- SDS (Fisher, cat. no. BP166-500)
- PAGE gels
- Dithiothreitol (DTT; Fisher, cat. no. BP172) ! **CAUTION** DTT is a reducing agent that may be harmful by inhalation, ingestion or skin absorption. Wear gloves and use in a chemical fume hood when handling concentrated stocks.
- *E. coli* TOP10 chemically competent cells (Invitrogen, cat. no. C4040)
- pGEX4T-1 prokaryotic expression constructs encoding GST-$RhoA^{G17A}$ or GST-RBD (available upon request from R.G.-M.)
- Ampicillin (Sigma, cat. no. A0166)
- IPTG (isopropyl β-D-1-thiogalactopyranoside; Gold Biotechnology, cat. no. I2481C5)
- BactoYeast (BD, cat. no. 288620)
- Tryptone (Fisher, cat. no. BP1421)
- Glutathione-Sepharose 4B beads (GE, cat. no. 17-0756-010)
- Nocodazole (Sigma, cat no. N1404)
- See Blue prestained molecular weight markers (Invitrogen, cat. no. LC5625)
- Nonfat dry milk (Member's Mark)
- N_3Na (Sigma, cat. no. S2002) ! **CAUTION** It can be fatal if swallowed or if it comes in contact with skin. Wash hands thoroughly after handling. Avoid releasing it into the environment. Wear protective gloves/protective clothing.
- Immobilon western blot reagent (Millipore, cat. no. WBKL50500)
- Ponceau S solution (Sigma, cat. no. P7170)
- Immobilon-P transfer membrane (PVDF) (Millipore, cat. no. IPVH00010)
- Anti-tubulin antibody (Sigma, cat. no. T9026)
- Anti-lamin A antibody (Sigma, cat. no. L1293)
- Anti-Na/K-ATPase (Abcam, cat. no. ab7671)
- Anti-EEA1 (BD Transduction Laboratories, cat. no. 610457)
- Anti-RhoA antibody (clone 26C4; Santa Cruz, cat. no. sc-418)
- Anti-p115 Rho GEF (Cell Signaling, cat. no. 3669)
- Anti-GEF-H1 (Cell Signaling, cat. no. 4076)
- Anti-Net1 (Rabbit polyclonal antibody against Net1 generated using a peptide containing the last 18 amino acids of human Net1, C-RRARDKALSGGKRKETLV[17])
- Donkey anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (Jackson ImmunoResearch, cat. no. 305-005-003)
- Donkey anti-mouse HRP-conjugated antibody (Jackson ImmunoResearch, cat. no. 715-005-150)

EQUIPMENT

- Dounce homogenizer (7 ml capacity; Bellco, cat. no. 1984-10007)
- Cell scraper (Sarstedt, cat. no. 83-1830)
- Conical tubes (15 ml)
- Tissue culture plates (10 cm)
- Inverted phase-contrast microscope
- Needle, 27.5 gauge
- Refrigerated tabletop centrifuge (Eppendorf, model no. 5415R)
- Swinging bucket centrifuge (Eppendorf, model no. 5804) ▲ **CRITICAL** Keep at 4 °C in a cold room; alternatively, use a refrigerated model such as model no. 5804R or similar.
- Ultrasonic processor with microtip probe (Sonics VibraCell, Sonics & Materials, model no. VCX130).
- UV-visible spectrophotometer (Eppendorf BioPhotometer Plus)
- SDS-PAGE electrophoresis system (Bio-Rad Mini Protean Tetra Cell, Bio-Rad)
- Tube rotator
- Electrophoresis power supply
- Transfer power supply

REAGENT SETUP

HEK293 or other cell lines Grow cells in DMEM medium supplemented with 10% (vol/vol) FBS and antibiotics (pen-strep) at 37 °C in 5% CO_2. Other cell lines can be used but the nuclear isolation protocol may need to be adjusted (see PROCEDURE).

500 mM Tris-HCl pH 7.8 Dissolve 6.06 g of Tris base in 90 ml of H_2O, adjust to pH 7.8 with 1 M HCl and make up to 100 ml. It can be stored at 4 °C for several months.

1 M KCl Dissolve 7.45 g of KCl base in 100 ml of H_2O. It can be stored at 4 °C for several months.

1 M $MgCl_2{\cdot}6H_2O$ Dissolve 20.3 g of $MgCl_2{\cdot}6H_2O$ in 100 ml of H_2O. It can be stored at 4 °C for several months.

Solution A OptiPrep (60% (wt/vol) iodixanol in H_2O). It can be stored at 4 °C for several months.

Solution B (diluent) Combine 150 mM KCl, 30 mM $MgCl_2$, and 120 mM Tris-HCl (pH 7.8). It is stable for several months if stored at 4 °C.

Solution C (working solution) 50% (wt/vol) iodixanol; mix 5 volumes of solution A with 1 volume of solution B. It is stable for several months if stored at 4 °C.

Solution D (homogenization medium) Combine 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$ and 20 mM Tris-HCl (pH 7.8). It is stable at 4 °C for about 1 month.

Solution E (gradient solution) 30% (wt/vol) iodixanol: prepare by diluting solution C with solution D (6 volumes + 4 volumes). ▲ **CRITICAL** It should be freshly prepared before use.

Solution F (hypotonic solution) Combine 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT (freshly added before use). It is stable for several months if stored at 4 °C.

RBD buffer Mix 50 mM Tris-HCl (pH 7.6), 500 mM NaCl, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) SDS, 0.5% (wt/vol) deoxycholate and 10 mM $MgCl_2$ (freshly added before use). The stock solution without Mg^{2+} can be stored at 4 °C for several months.

3× RBD buffer Combine 150 mM Tris-HCl (pH 7.6), 1,500 mM NaCl,

3% (vol/vol) Triton X-100, 0.3% (wt/vol) SDS, 1.5% (wt/vol) deoxycholate and 30 mM $MgCl_2$ (freshly added before use). It is stable for several months if stored at 4 °C.

RBD wash buffer Combine 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% (vol/vol) Triton X-100 and 10 mM $MgCl_2$ (freshly added before use). The stock solution without Mg^{2+} can be stored at 4 °C for several months.

Rho GEF buffer Mix 20 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 1% (vol/vol) Triton X-100, 1 mM DTT and protease inhibitor cocktail. DTT and protease inhibitor should be freshly added before use. It is stable for several months if stored at 4 °C.

3× Rho GEF buffer Combine 60 mM HEPES (pH 7.5), 450 mM NaCl, 15 mM $MgCl_2$, 3% (vol/vol) Triton X-100, 3 mM DTT and protease inhibitor cocktail. DTT and protease inhibitor should be freshly added before use. It is stable for several months if stored at 4 °C

HEPES-buffered saline (HBS buffer) Combine 20 mM HEPES (pH 7.5) and 150 mM NaCl. It is stable for several months if stored at 4 °C.

LB medium Combine 5 g of BactoYeast, 10 g of tryptone and 10 g of NaCl; dissolve in 900 ml of deionized water, adjust pH to 7.4 with NaOH, and then bring the total volume up to 1,000 ml with deionized water and autoclave. It is stable for several months at room temperature (25 °C).

Lysis buffer Combine 20 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM MgCl2, 1% (vol/vol) Triton X-100, 1 mM DTT and protease inhibitors (1 mM PMSF, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} pepstatin A). It can be stored at 4 °C for several months. DTT and protease inhibitors should be freshly added before use.

Laemmli buffer Combine 62.5 mM Tris-HCl (pH 6.8), 25% (vol/vol) glycerol, 2% (wt/vol) SDS and 0.01% (wt/vol) bromophenol blue. Add 50 μl of β-mercaptoethanol per 950 μl of sample buffer for a final concentration of 5% (vol/vol) β-mercaptoethanol (710 mM). β-mercaptoethanol should be freshly added before use. The stock solution without β-mercaptoethanol can be stored for several months at room temperature.

Running buffer To prepare 1 liter of 10× electrode (running) buffer (pH 8.3), dissolve 30.3 g of Tris base, 144.0 g of glycine and 10.0 g of SDS and bring the total volume up to 1,000 ml with deionized water. Do not adjust pH with acid or base. It can be stored at room temperature for several months.

Tris-buffered saline/Tween-20 (TBST) Mix 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.5% (vol/vol) Tween-20. It can be stored at room temperature for several weeks.

Blocking buffer Blocking buffer is 5% (wt/vol) nonfat dry milk in TBST. It can be stored at 4 °C for several weeks in 0.01% (wt/vol) N_3Na.

Transfer buffer To prepare 1 liter of 10× transfer buffer (pH 8.3), dissolve 30.3 g of Tris base, 144.0 g of glycine, 10.0 g of SDS and bring the total volume up to 1,000 ml with deionized water. Do not adjust pH with acid or base. For 1× solution, mix 100 ml of the 10× solution with 200 ml (20% (vol/vol)) of methanol and deionized water to 1 liter. It can be stored at room temperature for several weeks.

PROCEDURE

Expression and purification of GST-RBD and GST-Rho constructs ● TIMING 3–4 d

1| Transform *E. coli* with the appropriate construct and spread onto LB-agar plates supplemented with 100 μg ml^{-1} ampicillin. Incubate overnight at 37 °C. For RhoA activity assays, use GST-RBD and for RhoA-GEF pull-downs use GST-$RhoA^{G17A}$. Cloning of these constructs has been described previously[8,26].

■ **PAUSE POINT** The plates can be stored for several weeks at 4 °C. Alternatively, glycerol stocks of each culture can be stored at −80 °C and used when needed to start a liquid culture.

2| Pick a single colony from each plate and grow a 50-ml culture overnight at 37 °C to full density in 50 ml of LB with 50 μg ml^{-1} ampicillin (optical density (OD) > 1.0).

3| Dilute the culture into 450 ml of LB-ampicillin and let it grow for 30 min at 37 °C.

4| Induce protein expression by adding IPTG to a final concentration of 100 μM; shake the culture at room temperature for 20–24 h.

5| Centrifuge the bacteria at 4,000*g* for 15 min and resuspend in 10 ml of lysis buffer.

▲ **CRITICAL STEP** All purification steps must be carried out at 4 °C.

6| Sonicate the resuspended bacteria on ice for 1 min using a 3-mm tip (sonicator set at 40%).

▲ **CRITICAL STEP** Avoid foaming.

7| Clarify the lysate by centrifugation at 27,000*g* for 15 min in a prechilled rotor.

8| Transfer the supernatant to a fresh 15-ml conical tube and add 100–500 μl of 50% (vol/vol) glutathione-Sepharose slurry pre-equilibrated in lysis buffer. The amount of beads used depends on the yield of the bacterial culture. Typically, GST-RBD expresses at a much higher level than GST-$RhoA^{G17A}$, so as a general rule we use more beads for GST-RBD.

9| Rotate the tube at 4 °C, 18 r.p.m. for 60 min.

10| Centrifuge the glutathione-Sepharose beads at 1,500*g* for 1 min at 4 °C in swinging-bucket centrifuge.

11| Wash twice with 10 ml of lysis buffer and two more times with 10 ml of HBS, 5 mM $MgCl_2$ and 1 mM DTT.

12| Aspirate sufficient wash buffer to get the initial Sepharose volume (i.e., ~50% slurry). Add 0.5 volumes of glycerol.

13| Estimate the protein concentration of the GST-fusion protein slurry. The amount of purified protein can be estimated without the need for eluting the proteins from the beads. We use the Coomassie Plus reagent according to the manufacturer's directions, and we use a small aliquot of the purified GST-beads (2.5 µl of slurry is usually enough) to measure protein concentration. BSA can be used as a standard, and empty glutathione-Sepharose beads can be used in the blank and in the standards to account for the effects of the beads on the measurements. However, in our hands, the amount of beads used for the protein concentration measurement has a negligible effect on the absorbance reading. Alternatively, to estimate the concentration of the purified GST proteins, a small fraction of the purified beads can be boiled in SDS sample buffer, run in a 12% (wt/vol) SDS-PAGE gel at 120 V alongside known concentrations of a reference protein such as BSA and stained with Coomassie blue. Running the proteins in a gel also allows the visualization of the purity and the integrity of the purified GSTs.

■ **PAUSE POINT** Store the beads at 4 °C. Typically, both GST-RBD and GST-RhoA^{G17A} beads are stable for at least 2 weeks. For longer storage, the GST-RBD beads can be aliquotted, snap frozen and stored at −80 °C in 50% (vol/vol) glycerol. However, we usually do not recommend freezing the GST beads, as it may decrease the efficiency of the pull-down assay. This is particularly important for GST-RhoA^{G17A} beads, which are even less stable than GST-RBD beads.

Homogenization ● TIMING 20 min

14| Use a near-confluent monolayer of cells. This assay was optimized using HEK293 cells; a confluent 10-cm plate is usually sufficient for a pull-down assay. However, the yield (total protein content) for other cells is usually lower and the number of plates used per assay may need to be adjusted.

▲ **CRITICAL STEP** A good starting point when characterizing a different cell line is 1 mg of total nuclear or cytosolic protein per condition.

15| Aspirate the medium and rinse the monolayer at least three times with ice-cold PBS + 1 mM $MgCl_2$.

16| Add ice-cold Solution F (hypotonic) to the dish (1 ml for a 10-cm dish) and scrape the cells into the medium with a cell scraper. Do not try to produce a single-cell suspension.

▲ **CRITICAL STEP** When using more than one 10-cm dish per condition, the amount of buffer F used can be reduced proportionally to prevent unnecessary dilution of the cytosolic fraction. The volume of buffer F used is not as critical when only nuclear pull-downs are performed, as the first centrifugation step concentrates the nuclei. If you are working with a small number of cells, or when maximum recovery is necessary, there is the option of washing the plate with 1 ml of Solution F to recover any remaining cells.

17| Transfer the crudely resuspended monolayer to a 15-ml conical tube on ice. Incubate for 5 min on ice to let the cells swell.

? TROUBLESHOOTING

18| Homogenize the cells using 20 strokes of the pestle of a tight-fitting Dounce homogenizer.

▲ **CRITICAL STEP** Depending on the homogenizer and cell type used, the number of strokes required to obtain complete homogenization may vary. The process can be monitored using an inverted phase-contrast microscope similar to those used for tissue culture inverted microscopy (×20 objective). After 10 strokes, place a drop of homogenate on a glass slide, cover with a cover slip and observe the cells under the phase-contrast microscope. Continue homogenization until >90% of the cells are broken.

? TROUBLESHOOTING

19| Transfer an aliquot of TCL to a fresh tube for SDS-PAGE analysis (usually 100 µl).

▲ **CRITICAL STEP** The amount of protein loaded in the gel will be determined after the protein concentration is estimated in Step 32.

20| Centrifuge the cells (228*g* (~1,000 r.p.m.) for 5 min at 4 °C) to produce a crude nuclear pellet (swinging bucket centrifuge). Transfer the supernatant to a fresh tube (PNS fraction).

21| Transfer an aliquot of the PNS fraction to a fresh tube for SDS-PAGE analysis (100 µl).

▲ **CRITICAL STEP** The amount of protein loaded in the gel will be determined after the protein concentration is estimated in Step 32.

Nuclear isolation ● **TIMING 20 min**

22| Resuspend the pellet in 1.5 ml of a 30% (wt/vol) iodixanol solution carefully pipetting up and down with a 1-ml tip (Solution E) and transfer it to a 1.5-ml tube.

23| Centrifuge at 10,000*g* for 10 min at 4 °C in a tabletop centrifuge.
? TROUBLESHOOTING

24| Discard the supernatant carefully by aspiration. A gel-loading tip can be used to avoid accidental aspiration of the pellet. Make sure to aspirate the lipid layer that may accumulate at the surface as well as on the wall of the tube. The pellet should look white and fluffy.

25| Repeat Steps 22–24 once. This second centrifugation can be shortened to 5 min at 10,000*g* at 4 °C. The obtained pellet contains the isolated nuclei.
? TROUBLESHOOTING

RhoA activity assay ● **TIMING 1 h**

26| Resuspend the nuclear pellet by pipetting it up and down in 300 µl of RBD buffer.

27| Sonicate for 10 s on ice (sonicator set at 40%).
▲ **CRITICAL STEP** Avoid foaming.
? TROUBLESHOOTING

28| Centrifuge at maximum speed (14,000*g*) for 5 min at 4 °C in a tabletop centrifuge. Transfer the supernatant to a fresh tube. This is the NUC.

29| Transfer an aliquot of the NUC to a fresh tube for SDS-PAGE analysis (usually 50 µl).
▲ **CRITICAL STEP** The amount of protein loaded in the gel will be determined after the protein concentration is estimated in Step 32.

30| Dilute the PNS fraction obtained from Step 20 with a one-third volume of 3× RBD buffer.

31| Centrifuge at maximum speed (14,000*g*) for 5 min at 4 °C in a tabletop centrifuge. Transfer the supernatant to a fresh tube.

32| Determine the protein concentration from the PNS and NUCs using the Bio-Rad DC protein assay kit. Protein concentration can be measured using different commercially available methods; however, we favor this kit because it is specially formulated to accurately measure protein concentration in the presence of a high detergent concentration.
▲ **CRITICAL STEP** Accurately measuring the protein concentration in the different fractions is essential for the success of the subsequent steps, as the same amount of protein is used in each condition for the different pull-down assays.

33| Equalize both the total amount of protein and also the volume used for each condition using RBD lysis buffer. Ideally 1 mg or more of total protein should be used in 0.5–1 ml of total volume.

34| Add 50 µg of fusion protein on beads to each lysate (PNS and NUC) and rotate the samples for 30 min at 18 r.p.m. at 4 °C.
▲ **CRITICAL STEP** The glycerol present in the bead buffer may introduce errors when transferring the beads to fresh tubes. Use wide-bore tips or cut the end of the pipette tips and resuspend the fusion protein-bead slurry thoroughly by careful pipetting to ensure that the same volume of slurry is used for each condition.

35| Wash the beads three times with RBD wash buffer, aspirate off the supernatant and resuspend the beads in 30–40 µl of SDS-PAGE sample buffer.
▲ **CRITICAL STEP** To prevent unintended aspiration of the beads, a 27.5-gauge needle can be connected to the vacuum aspirator line and used to remove the wash buffer. The needle slows the liquid flow and allows a more precise handling of the beads. This size needle is small enough that it can be safely inserted into the beads without aspirating them. However, we recommend using the needle on the side of the tube and aspirating as much liquid as possible without touching the beads, and only inserting the needle carefully into the beads after the last wash to remove the remaining buffer.

36| Boil for 10 min and centrifuge briefly to recover the sample.
■ **PAUSE POINT** After resuspending the beads in sample buffer, the sample can be stored at −20 °C for several days before proceeding with SDS-PAGE separation and western blotting. All other samples, including total lysates and loading controls can also be stored at −20 °C.

Rho GEF activity pull-down assay ● TIMING 1 h

37| Resuspend the nuclear pellet (Step 25) by pipetting it up and down in 300 µl of Rho GEF buffer.

38| Sonicate for 10 s on ice (sonicator set at 40%).
▲ **CRITICAL STEP** Avoid foaming.
? TROUBLESHOOTING

39| Centrifuge at maximum speed (14,000*g*) for 5 min at 4 °C in a tabletop centrifuge. Transfer the supernatant to a fresh tube. This is the NUC.

40| Transfer an aliquot of the NUC to a fresh tube for SDS-PAGE analysis (usually 50 µl).
▲ **CRITICAL STEP** The amount of protein loaded in the gel will be determined after the protein concentration is estimated in Step 43.

41| Dilute the PNS fraction obtained from Step 20 with a one-third volume of 3× Rho GEF buffer.

42| Centrifuge at maximum speed (14,000*g*) for 5 min at 4 °C in a tabletop centrifuge. Transfer the supernatant to a fresh tube.

43| Determine the protein concentration from the PNS and NUCs using the Bio-Rad DC protein assay kit.
▲ **CRITICAL STEP** Accurately measuring the protein concentration in the different fractions is essential for the success of the subsequent steps, as the same amount of protein is used in each condition for the different pull-down assays.

44| Equalize both the total amount of protein and also the volume used for each condition using Rho GEF buffer. Ideally 1 mg or more of total protein should be used in 0.5–1 ml of total volume.

45| Add 30 µg of GST- $RhoA^{G17A}$ beads to each lysate and rotate the samples at 18 r.p.m. for 45 min at 4 °C.
▲ **CRITICAL STEP** The glycerol present in the bead buffer may introduce errors when transferring the beads to fresh tubes. Use wide-bore tips or cut the end of the pipette tips and resuspend the fusion protein-bead slurry thoroughly by careful pipetting to ensure that the same volume of slurry is used for each condition.

46| Wash the beads three times with Rho GEF buffer, aspirate the supernatant and resuspend the beads in 40 µl of SDS-PAGE sample buffer.
▲ **CRITICAL STEP** To prevent unintended aspiration of the beads, a 27.5-gauge needle can be connected to the vacuum aspirator line and used to remove the wash buffer. The needle slows the liquid flow and allows a more precise handling of the beads. This size needle is small enough that it can be safely inserted into the beads without aspirating them. However, we recommend using the needle on the side of the tube and aspirating as much liquid as possible without touching the beads, and only inserting the needle carefully into the beads after the last wash to remove the remaining buffer.

47| Boil for 10 min and centrifuge briefly to recover the sample.
■ **PAUSE POINT** After resuspending the beads in sample buffer, the sample can be stored at −20 °C for several days before proceeding with SDS-PAGE separation and western blotting. All other samples, including total lysates and loading controls can also be stored at −20 °C.

SDS-PAGE, transfer and western blotting ● TIMING 8 h

48| Run the lysates from the different fractions (TCL, PNS, NUC) and the corresponding pull-down samples on SDS-PAGE gels. Run RBD pull-down samples in 15% (wt/vol) gels and Rho GEF pull-down samples in 7.5–10% (wt/vol) gels, depending on the GEF molecular weight. Use samples saved from Steps 19, 21, 29 and 36 for the Rho assay, and samples saved from Steps 19, 21, 40 and 47 for the the GEF assay. Load equal protein amounts for the lysates (TCL, PNS and NUC) on the basis of the protein concentration measurement in Steps 32 and 43 (typically 20 µg of total protein). Transfer and blot for RhoA in the Rho assay and for Net1 or the GEF of interest in the GEF assay.
▲ **CRITICAL STEP** Run a separate set of lysates (TCL, PNS and NUC) to determine the efficiency of the fractionation. Transfer and immunoblot for tubulin (soluble marker) and lamin A (nuclear marker). Alternatively, the membrane used to blot for total RhoA can be reblotted for tubulin and lamin A.
? TROUBLESHOOTING

? TROUBLESHOOTING

A list of common problems, possible causes and solutions associated with these assays is shown in **Table 2**. Another problem associated with these pull-down assays is the quality of the GST beads. The beads will lose their efficiency over time and we usually use them within 1–2 weeks of preparation. When testing new conditions in which the outcome of RhoA or Rho GEF activation is not known, we recommend including a positive control for RhoA and RhoA GEFs activation so the sensitivity of the assay can be monitored. There are several known stimuli for RhoA-GEFs and RhoA activation, including LPA, thrombin and nocodazole (see refs. 5,27–29). We recommend nocodazole (treat cells with 10 μM nocodazole for 30 min–1 h prior to homogenization), as it produces a robust activation of RhoA and its upstream GEF, GEF-H1. Regarding positive controls for nuclear RhoA and Rho GEF activity, the only known stimulus is IR. We typically irradiate the cells with a dose of 10 Gy in a cesium irradiator and then return them to the incubator for at least 1 h at 37 °C before performing the assays.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible cause	Solution
17,18	Cytosolic contamination	Incomplete homogenization	Incubate longer in hypotonic buffer
			Increase the number of strokes in the Dounce homogenizer step
23	Nuclei did not pellet	Cell line–specific differences	Decrease the iodixanol concentration gradually until nuclei pellet; 28.5% (wt/vol) is a good starting point
25	Cytosolic contamination	Iodixanol concentration is inaccurate	Make sure the gradient solution is exactly 30% (wt/vol) iodixanol
27,38	Nuclear extract too viscous	Incomplete sonication	Increase sonicator power or sonicate for an extra 10 s
48	No RhoA activity/no Rho GEF activity	Total protein concentration is too low	Increase the number of cells used for each assay
		Activity is lost during handling	Make sure $MgCl_2$ was added to the lysis buffer
			Make sure all steps are carried out at 4 °C. Minimize the waiting intervals
		Fusion protein is degraded	Make sure the beads are not too old. Stain the PVDF membranes after transfer with Ponceau S to check the size of the fusion proteins

● TIMING

Steps 1–13, Preparation of GST-RBD or GDT-17A RhoA: 3–4 d
Steps 14–25, Homogenization and nuclear isolation: 40 min
Steps 26–47, RhoA or Rho GEF pull-down: 1 h
Step 48, SDS-PAGE, transfer and western blotting: 8 h
Box 1, Steps 3–10, RhoA activity assay: 1 d
Box 1, Steps 11–18, Rho GEF activity assay: 1 d

ANTICIPATED RESULTS

Nuclear isolation

Figure 1a shows a typical nuclear preparation as seen by phase-contrast and fluorescence microscopy. Nuclei are intact, as seen in the phase image and by staining the DNA with Hoechst. There are no signs of intact or partially broken cells and of extranuclear material at this magnification. Electron microscopy analysis also shows intact nuclei with no visible contamination (**Fig. 1b**). In addition, the nuclear membrane was intact and even structures such as nuclear pores could be clearly visualized at higher magnification (data not shown). Analysis of the different fractions by SDS-PAGE shows a NUC with a very distinct protein pattern when compared with the total (TCL) and PNS lysates (**Fig. 1c**). The purity of our isolated NUCs was routinely tested by blotting with different proteins commonly used as markers for various cellular fractions. Plasma membrane (Na^+/K^+ ATPase), endosomal (EEA1) and cytosolic (tubulin) contaminants were not observed in the nuclear (NUC) fraction within the detectable limit of these marker antibodies (**Fig. 1d**). **Figure 1** shows that, although the majority of endogenous RhoA is

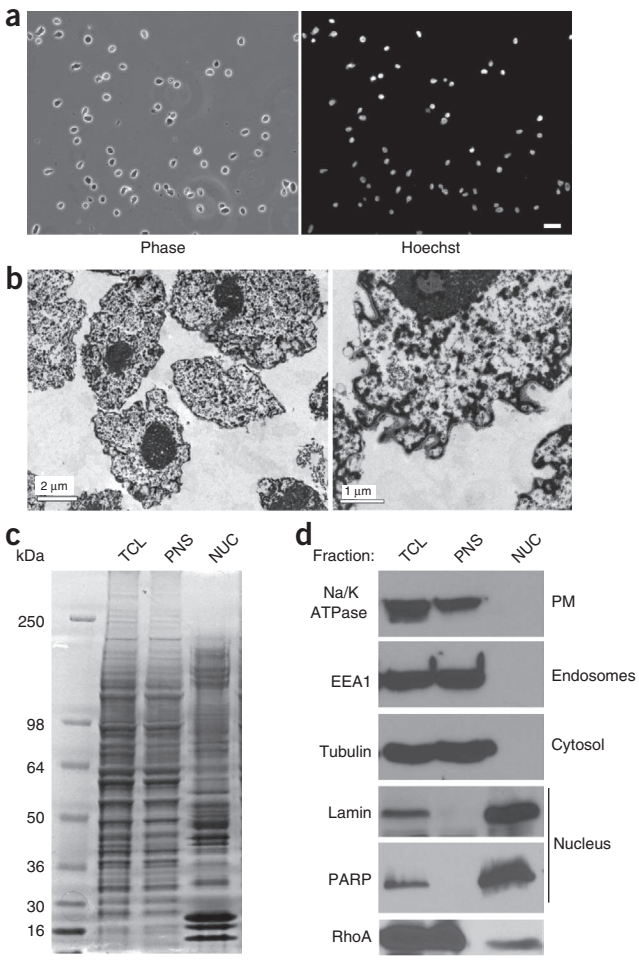

Figure 1 | Characterization of nuclear isolation protocol. (**a**) Isolated nuclei were analyzed by phase-contrast and by fluorescence microscopy. DNA was stained using Hoechst by resuspending the nuclear pellet from Step 26 in mounting solution containing Hoechst 33342 (10 µM final). Scale bar, 25 µm. (**b**) Ultrastructural analysis of nuclei. Isolated nuclei were fixed and processed for transmission electron microscope analysis as described previously[33]. (**c**,**d**) Fractions were separated by SDS-PAGE and stained by Coomassie blue (**c**), or transferred to PVDF membranes and analyzed by western blot using different subcellular markers as indicated (**d**). Panel **d** is reprinted from Dubash *et al.*[17].

present in the PNS fraction, a low amount is consistently detected in the NUC (**Fig. 1d**). We have estimated that ~5% of total cellular RhoA is localized to the nucleus of HEK293 and other cell lines[17].

RhoA activity assay

Figure 2a–c is an example of a GST-RBD pull-down assay from the NUC of HEK293 cells. As can be seen, a fraction of the nuclear RhoA pool is efficiently precipitated with GST-RBD, but not with GST alone, showing that nuclear RhoA is present in an active GTP-bound form at steady state (**Fig. 2a**)[17]. **Figure 2b** shows a nuclear GST-RBD pull-down assay, demonstrating that overexpression of a predominantly nuclear Rho GEF such as Net1 promotes an increase in the nuclear activity of RhoA. The nuclear pool of RhoA can therefore respond to GEF-mediated nucleotide exchange, and the ability of other GEFs to modulate nuclear RhoA activity can be tested by overexpression or knockdown (**Fig. 2b**).

The main use for this type of pull-down assay is to monitor changes in RhoA activation state in response to different stimuli or treatments. **Figure 2c** demonstrates the ability to detect changes in RhoA activity in the NUC in response to IR, which has been previously shown to induce RhoA and Net1 activity[30]. We specifically wanted to determine whether this previously observed IR-induced increase in RhoA and Net1 activity occurred in the nucleus or in the cytoplasm. By simultaneously analyzing the nuclear and cytoplasmic (PNS) activity of RhoA after IR exposure, we were able to show that, whereas the activity of nuclear-localized RhoA increased significantly in response to IR exposure, the activity of cytoplasmic (PNS fraction) RhoA was not affected (**Fig. 2c**).

Rho GEF activity pull-down

To validate the nuclear GEF pull-down, we performed the assay from both nuclear and PNS fractions and blotted for two RhoA-specific GEFs: Net1 and p115 Rho GEF. Our results show that, as described previously, endogenous Net1 is highly enriched in the nucleus[31], whereas p115 Rho GEF is restricted to the PNS fraction (**Fig. 3a**)[32]. Importantly, a considerable amount of nuclear Net1 precipitated with GST-RhoA^{G17A}, indicating that Net1 is in an active form in the nucleus (**Fig. 3a**)[17]. In contrast, as predicted by its localization, the activity of p115 Rho GEF was detected exclusively in the PNS fraction (**Fig. 3a**).

Similarly to the results obtained for nuclear RhoA (**Fig. 2c**), Net1 activity can be compared between the nuclear and PNS fractions in response to a specific stimulus, such as IR. The results in **Figure 3b** show that Net1 activity in the NUC, but not the PNS fraction, is regulated by IR[17].

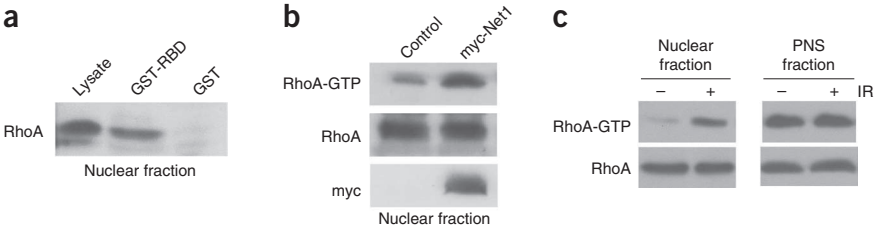

Figure 2 | Nuclear RhoA assay. (**a**) Nuclear lysates were incubated with GST-RBD (or GST alone as a control) to precipitate active RhoA from the nucleus of HEK293 cells. Samples were then subjected to SDS-PAGE and blotted with an anti-RhoA antibody. (**b**) HEK cells were transfected with wild-type myc-tagged Net1 or a control vector. Twenty-four hours after transfection, nuclear lysates were isolated and GST-RBD pull-downs performed. Samples were blotted for RhoA and exogenously expressed myc-Net1 (using a myc-specific antibody). (**c**) HEK293 cells were either left untreated or exposed to ionizing radiation (10 Gy). After incubation at 37 °C for 1 h, PNS and nuclear fractions were isolated and RhoA activity assays were performed. The samples were then blotted with antibodies for the indicated proteins. Figures are reprinted from Dubash *et al.*[17].

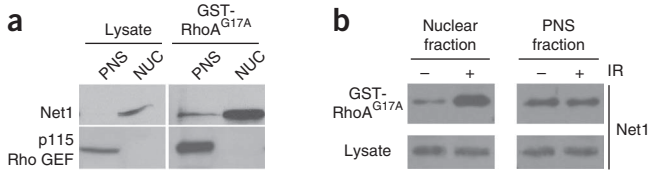

Figure 3 | Nuclear Rho GEF assay. (**a**) Active GEF pull-downs with GST-RhoA^{G17A} were performed from PNS and nuclear fractions of HEK293 cells, and the samples blotted with antibodies for the GEFs Net1 and p115 Rho GEF. (**b**) HEK293 cells were either left untreated or exposed to ionizing radiation (10 Gy). After incubation at 37 °C for 1 h, PNS and nuclear fractions were isolated, and GEF activity assays were performed. The samples were then blotted with antibodies for the indicated proteins. **a** is modified and **b** is reprinted from Dubash *et al.*[17].

Our results suggest that both the RhoA assay and the Rho GEF pull-down assay can be used to distinguish changes in the activity of Rho GTPases and GEFs that occur selectively in the cytosol or the nucleus in response to different biological stimuli.

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AUTHOR CONTRIBUTIONS R.G.-M. conceived and supervised the project, and devised and optimized the nuclear isolation strategy. R.G.-M., A.D.D. and C.G. devised and optimized the nuclear RhoA and Rho GEF activity pull-downs. R.G.-M. wrote the manuscript.

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