Supplementary Information

A luminescent oxygen channeling biosensor that measures small GTPase activation

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**Methods**

**In vitro GTPγS or GDP Treatment.** 500 µl precleared cell lysate was incubated with 10 µl of 0.5 M EDTA pH 8.0 before the addition of 5 µl of 10 mM GTPγS (Jena Biosciences) or 5 µl of 100 mM GDP (Sigma). The mixture was incubated at 30°C for 15 minutes before terminating the loading process with 32 µl of 1 M MgCl2 on ice.

**Biotinylation of the effector proteins.** Proteins were first brought in a primary-amine-free buffer (e.g. HEPES), concentrated to 1 mg/ml and thereafter incubated with a 3-5 fold molar excess of freshly dissolved NHS-Sulfo-Biotin (Pierce protein science) in 1x PBS buffer. The reaction was terminated after 1h at room temperature with excess Tris buffer. Unreacted NHS-Sulfo-Biotin was removed by gel filtration on NAP-10 columns. In order to verify the coupling 1-3 ul of biotinylated and non-biotinylated protein was spotted on a nitrocellulose membrane, allowed to dry and after repetitive washing steps with 1x PBS buffer incubated with an anti-Biotin-FITC-labeled-antibody (BN-34, Sigma Aldrich) for 40 min at room temperature under subdued. Eventually the biotinylation was visualized on a fluorescent image analyzer (FujiFilm, FLA-3000).

**Luminescent oxygen channeling.** All measurements were taken on the plate reader LB940 Mithras from Berthold Instruments equipped with the AlphaScreen module. Both excitation and emission was set to 0.5 s. Pipetting of the bead reagents was done under subdued light conditions to prevent bleaching. The basic protocol includes the preparation of 2 mixtures:

1.) Bead/antibody mix: Streptavidin donor and protein A acceptor beads are diluted 1/50 (100 ug/ml) and mixed with a 1/100 dilution of the anti-Rac1-antibody (Santa Cruz Biotechnology) in 1x PBS, 3mM MgCl2 and preincubated for 90 min at 4 °C in the dark.

2.) GTPase/effector mix: in vitro loaded GTPase (400 nM) is incubated with biotinylated PAK1-effector (100 nM) in effector binding buffer (25 mM Tris/HCl, 30 mM MgCl2, 40 mM NaCl, 0,5 % Igepal, 1mM DTT, pH 8) for 90 min at 4 °C.

3.) Equal amounts (7 ul) of both mixtures are given into the wells of a 384 ProxiPlate (Perkin Elmer) and incubated for 90 min at 4 °C before the read out at 25 °C.
**Cell lysis and pulldown experiments**

Cells were harvested and lysed in lysis buffer (50mM Tris pH 7.5, 200mM NaCl, 10mM MgCl₂, 1% Igepal, 5% glycerol, 2mM DTT, 10µg/ml Aprotinin, 5µg/ml Leupeptin, 1mM PMSF) for 30 min on ice. Lysate was cleared and directly used for subsequent experiments. For pulldown analysis the indicated amount of lysate was incubated with 0.8 µM biotinylated GST-Pak1 and Strepavidin-agarose for 1h at 4°C. Subsequently beads were washed twice with Wash buffer (25mM Tris pH 7.5, 30mM MgCl₂, 40mM NaCl, 1mM DTT), resuspended in SDS loading buffer. Active Rac1 was separated on a 12 % SDS-PAGE gel, blotted on a nitrocellulose membrane and detected using anti-Rac1 antibody.

**Pilot screen**

For the pilot screen 200 nM GTPγS-loaded Rac1 and 50 nM biotinylated Pak170-117 were incubated with 80 model compounds including the 2 known Rac1 inhibitors EHT1864 and NSC23766. B) EHT1864 was tested for interference with the assay system at 10 µM (3,3 % DMSO). Biotinylated IgG was used as bridging ligand for streptavidin donor and protein A acceptor beads to exclude compounds that interfere either with donor- or acceptor bead conjugation. The TruHits kit (PerkinElmer) which consists of Streptavidin donor beads and biotinylated acceptor beads can be used to detect singlet oxygen quencher or light scatterer. Finally we tested the hit compound EHT1864 in an LOC-independent pull-down experiment. Again, the interaction of the GTPase with the effector Pak1 is abrogated in the presence of EHT1864 (Suppl. Fig. 6).

Test compounds were diluted to 30 uM in 1x PBS, 0,003 % Triton X100, 10 % DMSO and 7 uL were given into the well of a Proxiplate 384 (Perkin Elmer). The GTPase/effector and the Bead/antibody mix were preincubated for 90 min at 4 °C and given into the wells. After additional 90 min at 4 °C the plate was measured at 25 °C. To validate the performance of the screen, z’-values were calculated using the formula:

\[ z' = 1 - \frac{3\sigma_p + 3\sigma_n}{|\mu_p - \mu_n|} \]

\( \sigma \) and \( \mu \) correspond to the means and the standard deviation of the positive (p) and negative (n) controls. The positive control included DMSO, whereas in the negative control GDP-loaded Rac1 was used. 8 replicates for negative and positive controls were tested, respectively.
Supplementary Figure 1. Determination of the concentration range of Rac1-antibody and the effector Pak1. (A) Schematic for the sensor assembly. PA: protein A coupled to acceptor beads; Rac1 mAB: anti-Rac1 rabbit IgG; Yn: GST fused to Pak1 (n=1), or biotin-GST-Pak1 (n=2); Xn: glutathione (n=1) or streptavidin (n=2) coupled to donor beads. (B) Signal dependence on Rac1 mAB concentration on GSH donor beads (X1); (GTPγS-loaded Rac1: 200 nM; biotin-GST-Pak1: 100 nM). black: GTPγS; white: GDP. (C) Excess of effector protein results in loss of signal intensity. Rac1 mAB: 7.6 nM; GTPγS-loaded Rac1: 200 nM. GSH-donor and protein A acceptor beads: 40 µg/ml. (D) Signal dependence on Rac1 mAB concentration on streptavidine donor beads (X2); (GTPγS-loaded Rac1: 200 nM; biotin-GST-Pak1: 100 nM). black: GTPγS; white: GDP.
Supplementary Figure 2. Dynamic range of Rac1-GTP determination.

(A) Dynamic range for Rac1-GTP measurement. The anti-Rac1 antibody concentration was set to 7.6 nM, Pak1 to 100 nM and both GSH-Donor and protein A acceptor beads at 40 µg/ml. Black bars: GTP\(_\gamma\)S loaded Rac1; white bars: GDP. (B) Dynamic range for Rac1-GTP measurement for biotinylated Pak1. The Rac1 antibody concentration was set to 7.6 nM, biotin-Pak1 to 50 nM and both streptavidin-donor and protein A acceptor beads at 40 µg/ml.
Supplementary Figure 3. LOC experiment to determine the dynamic range for Rac1-GTP using GTP instead of GTPγS.

Purified Rac1 was preloaded with GTP or GDP respectively and subjected to the LOC based assay. The signals obtained were similar to those shown in Figure 2A for GTPγS indicating that endogenous GTP hydrolysis is not significantly altering the results in the assay. The anti-Rac1 antibody concentration was set to 7.6 nM, Pak1 to 100 nM and both GSH-Donor and protein A acceptor beads at 40 µg/ml. Black bars: GTP; white bars: GDP.
Supplementary Figure 4. The LOC based determination is approximately one order of magnitude more sensitive than conventional pull-down assays

NIH 3T3 cells were serum starved overnight and lysed in lysis buffer (50mM Tris pH 7.5, 200mM NaCl, 10mM MgCl₂, 1% Igepal, 5% glycerol, 2mM DTT, 10µg/ml Aprotinin, 5µg/ml Leupeptin, 1mM PMSF) for 30 min on ice. Lysate was cleared and preloaded with GDP or GTPγS.

Subsequently the indicated amounts of lysate were subjected to a conventional pulldown assay. While 4µg lysate already yield a solid signal in the LOC assay (see Figure 2), hardly any signal was detected in the pulldown using 40µg lysate. (*) unspecific band recognized by anti-Rac1 antibody.
Supplementary Figure 5. Calculation of the fraction of GTP-bound Rac1

In parallel to the experiment shown in Fig. 3 Rac1 pre-loaded with GTPγS or GDP respectively was titrated in a LOC assay in the presence of 0.2 µg/µl cell lysate. Data points were fitted using a 4 parameter model (\( Y = \frac{A*X^h}{Kd^h + X^h} + c \)). The hill slope obtained (\( h = 2.14 \)) is most likely due to some cooperative effect caused by the multivalent nature of the bead based Alpha screen assay. Using this equation the concentration of Rac1 in the assay depicted in Figure 3A (4 µg lysate) was calculated to be 3.8 nM (~ 0.082 ng/µl). Therefore the fraction of active Rac1 in the lysate is determined as 0.000082 µg/µl ÷ 0.2 µg/µl = 0.4‰.
Supplementary Figure 6. Scheme illustrating the principle of the assay setup used in Figure 4C
Supplementary Figure 7. Pull-down of Rac1 in the presence of the Rac1 inhibitor EHT1864

HEK 293 cells overexpressing Rac1 were harvested, lysed and in vitro loaded with GTPγS. 400 µg cell lysate was incubated with 20 µg biotinylated Pak1 70-117, 20 µL monomeric Avidin agarose slurry (Pierce Protein Science) and 30 µM EHT1864 in 3.3 % DMSO. Rac1 was separated on a 12 % SDS PAGE-gel and probed with an anti-Rac1 antibody.