Chapter 24

Methods to Study the Ras2 Protein Activation State and the Subcellular Localization of Ras-GTP in *Saccharomyces cerevisiae*

Sonia Colombo and Enzo Martegani

Abstract

Ras proteins were highly conserved during evolution. They function as a point of convergence for different signalling pathways in eukaryotes and are involved in a wide range of cellular responses (shift from gluconeogenic to fermentative growth, breakdown of storage carbohydrates, stress resistance, growth control and determination of life span, morphogenesis and development, and others). These proteins are members of the small GTPase superfamily, which are active in the GTP-bound form and inactive in the GDP-bound form. Given the importance of studies on the Ras protein activation state to understand the detailed mechanism of Ras-mediated signal transduction, we provide here a simple, sensitive, and reliable method, based on the high affinity interaction of Ras-GTP with the Ras binding domain (RBD) of Raf1, to measure the level of Ras2-GTP on total Ras2 in *Saccharomyces cerevisiae*. Moreover, to study the localization of Ras-GTP in vivo in single *S. cerevisiae* cells, we expressed a probe consisting of a GFP fusion with a trimeric Ras Binding Domain of Raf1 (eGFP-RBD3), which was proven to be a useful live-cell biosensor for Ras-GTP in mammalian cells.

Key words Yeast, *Saccharomyces cerevisiae*, Nutrient, cAMP/PKA pathway, Small G proteins, Active Ras, Fluorescence microscopy

1 Introduction

Two different assays have been used to analyze quantitatively the guanine nucleotides bound to the Ras proteins in vivo. The first assay was developed by Gibbs et al. [1] and used by Colombo et al. [2]. After labeling the cells in vivo with [32P] orthophosphate, the Ras proteins were immunoprecipitated with antibodies against the human Ras protein (v-Hras 259). Guanine nucleotides were extracted, separated by TLC, and quantitated by phosphorimager technology. With this assay, the GTP/GDP ratio on the yeast Ras proteins could be measured only after overexpression of Ras2 protein, indicating that the sensitivity of this assay was probably not very high. A more recent assay to analyze quantitatively the guanine

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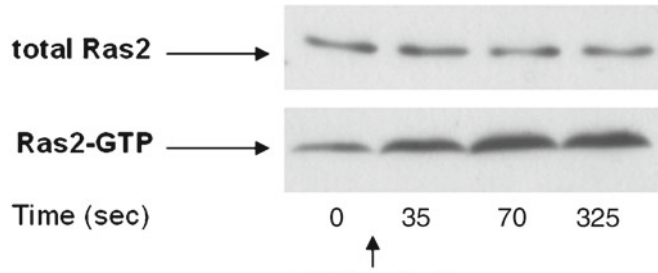

Time (sec)	Ras2-GTP/total Ras2 (%)
0	7.0
35	11.3
70	19.8
325	18.2

Fig. 1 Total Ras2 and Ras2-GTP content, before and after addition of 100 mM glucose to starved *tpk1*^{w1} *gpa2*Δ cells. Immunoblots of Ras2 in cell lysate (total Ras2) and Ras2-GTP bound to GST-RBD eluted with SDS-PAGE sample buffer

nucleotides bound to the Ras proteins in vivo was described by Taylor and Shalloway [3] and exploits the known specificity of the interaction between Ras-GTP and the Ras-binding domain (RBD) of Raf-1 to detect activated Ras. Since there is a high degree of homology between yeast and mammalian Ras proteins, the yeast Ras proteins are able to interact with the RBD of Raf-1. Consequently, we used this non-radioisotopic pull-down assay to measure the yeast Ras2-GTP loading without overexpression of this protein [4, 5]. We also set up conditions to specifically evaluate the reliability of the assay and we could clearly show that only the activated form of Ras2 was able to bind to the GST-RBD pre-bound to Glutathione Sepharose 4B, indicating that the assay was specific for Ras2-GTP [5]. Using this assay we showed that the increase in the level of cAMP after addition of glucose to glucose-starved cells is accompanied by a rapid increase in the level of Ras2-GTP/total Ras2 (an example is shown in Fig. 1). We also showed that this increase is dependent on Cdc25, glucose transport and phosphorylation and that deletion of the Ira proteins causes constitutively high GTP loading. Finally, we also provided evidence that the Ras proteins might be a target for the stringent feedback inhibition of PKA on cAMP synthesis [5]. This assay measures only the activation state of the Ras2 protein, but does not give any information about the localization of the active form of this GTPase. Spatial regulation of signalling events at the subcellular level is an important aspect of signal transduction; indeed, quite some evidence points to Ras playing distinct signalling roles depending on its subcellular

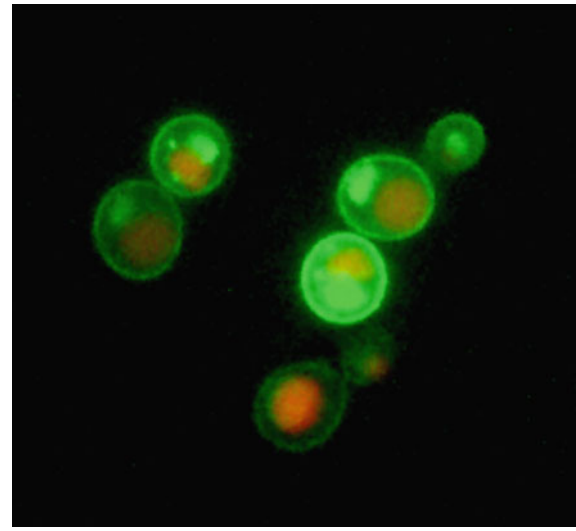

Fig. 2 Nuclear and plasma membrane localization of active Ras in the W303-1A wild type strain. Cells were transformed with pYX212-eGFP-RBD3, grown in 2 % glucose medium at 30 °C until exponential phase and then eGFP was photographed on living cells with a Nikon fluorescence microscope. In red, the autofluorescence of the vacuole is visible

location [6]. Consequently, the scientific question we addressed was the following: where are active Ras proteins localized and when are they localized in a certain cellular compartment? Augsten et al. [7] and Rubio et al. [8] successfully performed Ras-GTP imaging in mammalian cells, using a probe consisting of a GFP fusion with a trimeric Ras Binding Domain of Raf1 (eGFP-RBD3) as a reporter for Ras activation. In order to investigate the localization of active Ras in *Saccharomyces cerevisiae*, we inserted the sequence coding for the eGFP-RBD3 fusion in multicopy plasmids for yeast and we expressed the fusion protein in wild type and different mutant strains in the cAMP/PKA pathway. Our results showed that the eGFP-RBD3 probe is a useful live-cell biosensor to report Ras activation in *S. cerevisiae*. In particular, our data showed that the probe accumulates mainly at the plasma membrane and in the nucleus in wild type cells growing on medium containing glucose (Fig. 2), while it accumulates mainly in mitochondria in glucose-starved cells and relocalizes to the plasma membrane and to the nucleus upon addition of this sugar. We also showed that Gpa2 and Hxk2 play a role in the localization of active Ras [9]. Moreover, Leadsham et al. [10] showed that cells lacking Whi2p exhibit an aberrant accumulation of activated Ras2 at the mitochondria in response to nutritional depletion. In this mutant, the failure to address Ras2 to the vacuole and consequently the failure to shut down Ras signalling would lead to mitochondrial dysfunction, the accumulation of damaging ROS and cell death.

2 Materials

Prepare all solutions using deionized water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). We do not add sodium azide to the reagents.

2.1 Determination of the Ras2-GTP/Total Ras2 Ratio In Vivo

2.1.1 E. coli [pGEX2T-RBD] Culture Growth

1. *E.coli* strain transformed with the expression vector pGEX2T-RBD (*E. coli* [pGEX2T-RBD]), which encodes amino acids 1–149 of cRaf-1 fused to GST [3].
2. LB medium: 1 % (w/v) peptone, 0.5 % (w/v) yeast extract, 1 % (w/v) NaCl, 0.1 % (w/v) glucose.
3. Ampicillin (500×): 50 mg/ml in deionized water (store in small aliquots at −20 °C).
4. Isopropyl 1-thio-β-D-galactopyranoside (IPTG) (1000×): 100 mM in deionized water (store in small aliquots at −20 °C).

2.1.2 Preparation of Cleared E. coli [pGEX2T-RBD] Lysates

1. Phosphate buffered saline (PBS; 10×): 1.37 M NaCl, 0.027 M KCl, 0.043 M $Na_2HPO_4 \cdot 2H_2O$, 0.014 M KH_2PO_4, pH 7.4.
2. Lysis buffer A: PBS 1×, 1 mM EDTA, 0.5 mM dithiothreitol, one tablet of Protease Inhibitor Mixture (Roche Applied Science) in 50 ml of lysis buffer (*see* **Note 1**).
3. Glass beads 425–600 μm.
4. 10 % (w/v) Triton X-100 in PBS 1×.
5. Glycerol (better 87 %, which is easier to pipet).
6. Bio-Rad protein Assay (Bio-Rad).

2.1.3 Preparation of GST-RBD Pre-bound to Glutathione Sepharose 4B

1. Glutathione Sepharose 4B (GE Healthcare).
2. Phosphate buffered saline (PBS; 10×): as Subheading 2.1.2, **item 1**.
3. Phosphate buffered saline 1×, containing 1 mM EDTA (PBSE).
4. Complex phosphate buffered saline buffer (CPBS): PBS 1×, 1 % (w/v) Triton X-100, 10 % (w/v) glycerol, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM sodium vanadate, one tablet of Protease Inhibitor Mixture in 50 ml of this solution (*see* **Note 2**).
5. Lysis buffer B: 25 mM HEPES, pH 7.5, 150 mM NaCl, 1 % (w/v) Nonidet P-40, 0.25 % (w/v) sodium deoxycholate, 10 % (w/v) glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium vanadate, one tablet of Protease Inhibitor Mixture in 50 ml of extraction medium (*see* **Note 3**).

2.1.4 Yeast Growth and Yeast Crude Extract Preparation

1. Medium as appropriate for the cell strain under investigation.
2. Nitrocellulose filters (Whatman Protran pure nitrocellulose transfer and immobilization membrane, PerkinElmer or a similar product).

3. Sterile tooth sticks.
4. FastPrep tubes.
5. Lysis buffer B: as in Subheading 2.1.3, **item 5**.
6. Dry ice-ethanol.
7. Glass beads 425–600 μm.
8. FastPrep instrument (Savant).
9. Bio-Rad protein assay.

2.1.5 Purification of Ras2-GTP

1. Lysis buffer B: as in Subheading 2.1.3, **item 5**.
2. SDS-sample buffer, 2×: 100 mM Tris–HCl, pH 6.8, 2 % (w/v) β-mercaptoethanol, 4 % (w/v) SDS, 0.2 % (w/v) bromophenol blue, 20 % (w/v) glycerol.

2.1.6 SDS-PAGE Components

1. Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8, 0.4 % SDS.
2. Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8, 0.4 % SDS.
3. 30 % acrylamide–bis-acrylamide solution (29.2:0.8 acrylamide–bis-acrylamide). Store at 4 °C.
4. Ammonium persulfate: 10 % solution in deionized water (*see* **Note 4**).
5. *N*,*N*,*N*,*N*′-tetramethyl-ethylenediamine (TEMED). Store at 4 °C.
6. SDS-PAGE running buffer, 5×: 0.125 M Tris, 1.25 M glycine, 0.5 % SDS (it is not necessary to adjust the pH, which should automatically reach a value of about 8.6).

2.1.7 Immunoblotting, Immunodetection, and Densitometric Analysis

1. Nitrocellulose membrane (Whatman Protran pure nitrocellulose transfer and immobilization membrane, PerkinElmer or a similar product).
2. Western blot transfer buffer: SDS-PAGE running buffer 1×, 20 % methanol.
3. Tris buffered saline (TBS; 5×): 0.75 M NaCl, 0.05 M Tris–HCl, pH 7.4.
4. TBS 1× containing 0.05%Tween-20 (TBST).
5. Blocking solution: 5 % low fat dried milk in TBST. Store at 4 °C.
6. Anti-Ras2 polyclonal antibodies (Santa Cruz Biotechnology).
7. Donkey anti-Goat IgG-HRP (Santa Cruz Biotechnology).
8. ECL western blotting detection reagent.
9. BioMax Light Film.
10. X-Ray fixer.
11. X-Ray developer.
12. NIH-Image software (freeware http://www.nist.gov/lispix/imlab/prelim/dnld.html), or similar.

2.2 Localization of Active Ras

2.2.1 Equipment and Instrumentation

1. Nikon Eclipse E600 microscope equipped with a 60×, 1.4 oil Plan-Apochromat objective and a standard FITC filter set (Nikon, EX 450–490, DM 505, BA 520) for GFP fluorescence and a Cy3 filter set for Rhodamine fluorescence. Images were recorded digitally using a Leica DC 350 F camera.
2. Leica TCS SP2 confocal microscope equipped with an inverted Leica DMIRE2 microscope and a PL APO 63× oil immersion objective (numerical aperture: 1.4). For GFP fluorescence, λ excitation 488 nm (Ar laser), λ emission 500–550. For Rhodamine fluorescence, λ excitation 540 nm (Ar laser), λ emission 600–650.
3. Thoma chamber.
4. Adobe Photoshop (Adobe Systems, Inc.) and ImageJ software (freeware http://imagej.en.softonic.com/).

2.2.2 Cloning of eGFP-RBD3 and Yeast Transformation

1. General molecular biology reagents for restriction cloning procedures.
2. The peGFP-C2 plasmid expressing the fusion eGFP-RBD3 [7]: it may be provided by I. Rubio University of Jena, Germany.
3. The pYX expression vectors, pYX212 and pYX242 (*see* **Note 5**).
4. Restriction enzymes: NheI, SalI, and EcoRI.
5. The Klenow fragment of DNA polymerase I and the DNA ligase.
6. JETSORB Gel Extraction Kit (genomed GmbH).

2.2.3 Yeast Transformation

1. YPD medium: 1 % (w/v) yeast extract, 2 % (w/v) bacto-peptone, 2 % (w/v) glucose.
2. YPDA medium: YPD with 0.005 % (w/v) adenine hemisulfate.
3. Synthetic medium (SD): 6.7 g/l YNB w/o amino acids, the proper selective drop-out CSM (Complete Synthetic Medium) as appropriate for the cell strain under investigation, 2 % (w/v) glucose, 2.5 %(w/v) agar.

   TE (10×): 0.1 M Tris–HCl, 10 mM EDTA, pH 7.5.

   Lithium acetate: prepare a 100 mM and a 1 M solution in TE 1×, sterilize by filtration.

   Polyethyleneglycol 6000 (PEG 6000): prepare a 50 % (w/v) in water, sterilize by filtration.

   Salmon Sperm DNA: 2 mg/ml Stock solution in water denatured at 100 °C for 5 min and chilled in ice.

2.2.4 Mitochondria Labeling with Vital Mitochondrial Marker

1. Rhodamine B hexyl ester perchlorate: prepare a 1 mM stock solution in DMSO, aliquot, and store at –20 °C.
2. Staining solution: dilute the Rhodamine stock solution with water to make a 10 μM staining solution.

2.2.5 Imaging of Ras-GTP

1. Selective medium as appropriate for the cell strain under investigation.
2. Optionally: Starvation buffer: 25 mM MES, pH 6.
3. Cover glass.
4. Concanavalin A: prepare 100 μg/ml stock in deionized water, aliquot, and store at −20 °C.
5. Thoma chamber.

3 Methods

3.1 Determination of the Ras2-GTP/Total Ras2 Ratio In Vivo

3.1.1 E. coli [pGEX2T-RBD] Culture Growth (1 l)

1. Inoculate 40 ml of LB broth containing ampicillin (100 μg/ml) and grow the culture at 37 °C overnight with vigorous shaking.
2. Inoculate 1 l of LB broth containing ampicillin (100 μg/ml) 1:25 with the noninduced overnight culture and grow at 37 °C with vigorous shaking until an OD_{600} of 0.5 is reached (about 2–3 h).
3. Induce expression by adding IPTG to a final concentration of 0.1 mM.
4. Incubate the culture for an additional 4–5 h, at 37 °C with vigorous shaking.
5. Harvest the cells by centrifugation at 4,000 × *g* for 20 min at 4 °C.
6. Store cell pellet at −80 °C or proceed immediately to protocol for preparation of cleared *E. coli* lysates (next **step**).

3.1.2 Preparation of E. coli [pGEX2T-RBD] Lysates Under Native Conditions

1. Thaw the cell pellet and resuspend in 40 ml ice-cold Lysis buffer A.
2. Separate the cell suspension into two 50 ml tubes.
3. Put the two 50 ml tubes at −80 °C for 15 min and defrost the cell suspensions in H_2O at room temperature.
4. Separate the cell suspension into four 50 ml tubes (about 10 ml cell suspension each tube) and add 4 ml glass beads to each tube.
5. Break the cells using a vortex: five cycles, 1 min each, with a 1 min cooling period between each cycle.
6. Add TritonX-100 to a final concentration of 1 % (use 10 % TritonX-100 in PBS 1×).
7. Incubate at 4 °C for 30 min, mixing gently on a rotary shaker.
8. Centrifuge lysate at 10,000 × *g* for 20 min at 4 °C to pellet the cellular debris. Save supernatant.
9. Add glycerol to a final concentration of 10 % (*see* **Note 6**).

10. Store in aliquots at −80 °C or proceed immediately to protocol for preparation of GST-RBD fusion protein pre-bound to Glutathione Sepharose 4B (next **step**).

3.1.3 Preparation of GST-RBD Pre-bound to Glutathione Sepharose 4B (Ten Samples)

1. Sediment 300 μl of Glutathione Sepharose 4B bed volume by centrifugation at 500 × *g* for 5 min at 4 °C and discard the supernatant (*see* **Note 7**).
2. Wash the matrix with ten bed volumes of ice-cold PBSE.
3. Centrifuge the suspension at 500 × *g* for 5 min at 4 °C to sediment the matrix. Discard the wash.
4. Repeat the wash twice for a total of three washes.
5. Wash the matrix with ten bed volumes of ice-cold CPBS.
6. Repeat the wash once for a total of two washes.
7. Completely remove the wash (*see* **Note 8**).
8. Add 600 μl of cleared *E. coli* [pGEX2T-RBD] lysate each 20 μl of bed volume (each sample) and mix gently on a rotary shaker at 4 °C for 60 min (*see* **Note 9**).
9. Centrifuge the suspension at 500 × *g* for 5 min at 4 °C to sediment the matrix. Remove the supernatant.
10. Wash the pellet with ten bed volumes of ice-cold CPBS.
11. Centrifuge the suspension at 500 × *g* for 5 min at 4 °C to sediment the matrix. Discard the wash.
12. Repeat the wash once for a total of two washes.
13. Wash the pellet with ten bed volumes of ice-cold Lysis buffer B.
14. Centrifuge the suspension at 500 × *g* for 5 min at 4 °C to sediment the matrix. Discard the wash.
15. Repeat the wash twice for a total of three washes and completely remove the wash (*see* **Note 8**).
16. Resuspend the GST-RBD fusion protein pre-bound to Glutathione Sepharose 4B in ice-cold Lysis buffer B to a final concentration of 20 %.
17. Transfer 100 μl of the 20 % GST-RBD pre-bound to Glutathione Sepharose 4B slurry into each 1.5 ml tube (*see* **Note 10**).
18. Store the 20 % GST-RBD pre-bound to Glutathione Sepharose 4B slurry aliquots in ice until use (until the purification of Ras2-GTP step, Subheading 3.1.5).

3.1.4 Harvesting of the Yeast Cells and Crude Extract Preparation

1. Harvest about 3×10^8 yeast cells per treatment (each sample), either by centrifugation (3,000 × *g* for 5 min at 4 °C) or by fast filtration on nitrocellulose filters in a refrigerated environment (4 °C, cold room) (*see* **Note 11**).

2. If cells are harvested by centrifugation, transfer them to a 1.5 ml FastPrep tube and add 300 µl ice-cold Lysis buffer B. If cells are harvested by filtration, using a sterile toothpick, transfer them to a 1.5 ml FastPrep tube containing 300 µl ice-cold Lysis buffer B.
3. Immediately freeze the cells in dry ice-ethanol (cell pellet can be stored at −80 °C until use).
4. Add 300 µl glass beads to each tube without defrosting the cells.
5. Disrupt the cells, using a FastPrep instrument, set on speed 6: three cycles, 20 s each, with a 1 min cooling period between each cycle.
6. Centrifuge lysate at 13,000 × *g* for 1 min at 4 °C to pellet the cellular debris. Transfer the supernatant to a clean tube.
7. Quickly wash the glass beads with 600 µl of ice-cold Lysis buffer B and add it to the supernatant of the previous step.
8. Centrifuge at 13,000 × *g* for 10 min at 4 °C and transfer the supernatant to a clean tube.
9. Centrifuge at 13,000 × *g* for 20 min at 4 °C and transfer the supernatant to a clean tube.
10. Measure the protein concentration of the cleared yeast crude extract preparation.
11. Transfer a volume of yeast crude extract containing 240 µg of total proteins to a clean tube and add ice-cold Lysis buffer B to a final volume of 600 µl (consequently, the protein concentration of the sample will be 0.4 µg/µl).
12. Store in ice until use (until next step, the purification of Ras2-GTP step).

*3.1.5 Purification of Ras2-GTP (see **Note 11**)*

1. Add 500 µl of yeast crude extract preparation (Subheading 3.1.4, **step 11**) to 100 µl of the 20 % GST-RBD fusion protein pre-bound to Glutathione Sepharose 4B slurry (Subheading 3.1.3, **step 18**) and incubate at 4 °C for 1 h, mixing gently on a rotary shaker.

   Take 40 µl of yeast crude extract preparation (Subheading 3.1.4, **step 11**), add 40 µl of 2× SDS-sample buffer and boil for 10 min. This is the "total Ras2 protein sample." Freeze until use (until the SDS-PAGE will be performed).
2. Centrifuge at 500 × *g* for 5 min at 4 °C to sediment the matrix. Discard the supernatant.
3. Wash the matrix with 1 ml of ice-cold Lysis buffer B. Be careful not to lose the matrix.
4. Centrifuge at 500 × *g* for 5 min at 4 °C to sediment the matrix. Discard the wash.

5. Repeat the wash twice for a total of three washes.
6. Completely remove the wash (*see* **Note 8**).
7. Add 80 μl of 1.25× SDS-sample buffer and boil for 10 min. This is the "Ras2-GTP protein sample." Freeze until use (until the SDS-PAGE will be performed).

3.1.6 SDS-PAGE and Western Blotting

1. Perform a 10 % SDS-PAGE. Load 7.5 μl of "total Ras2 protein sample" (corresponding to 1.5 μg of total proteins) (Subheading 3.1.5, **step 1**) and 12 μl of "Ras2-GTP protein sample" (Subheading 3.1.5, **step 7**) (*see* **Note 12**).
2. Blot for 1 h and a half at 100 V.

3.1.7 Immunodetection and Densitometric Analysis

1. Block the membrane with blocking solution for 1 h at room temperature with gentle shaking.
2. Add Anti-Ras2 polyclonal antibodies diluted 1:200 with blocking solution to the membrane and incubate overnight at 4 °C with gentle shaking (*see* **Note 13**).
3. Briefly rinse the membrane once with TBST and then wash three times for 10 min with fresh changes of TBST at room temperature with gentle shaking.
4. Add Donkey anti-Goat IgG-HRP diluted at 1:5,000 with blocking solution to the membrane and incubate 1 h at room temperature with gentle shaking.
5. Briefly rinse the membrane once with TBST and then wash three times for 10 min with fresh changes of TBST at room temperature with gentle shaking.
6. Incubate the membrane in the ECL western blotting detection reagent, exactly as described by the manufacturer.
7. Place a sheet of autoradiography film on top of the membrane, close the cassette and expose for a proper time (*see* **Note 14**).
8. Quantify the bands on the film by densitometric analysis (NIH-Image software or similar) and determine the ratios of Ras2-GTP/total Ras2 taking in account the amounts of proteins loaded. A representative example is shown in Fig. 1.

3.2 Localization of Active Ras

3.2.1 Cloning of eGFP-RBD3 and Yeast Transformation

1. Digest the peGFP-C2 plasmid with *Nhe*I to recover the fragment encoding the fusion eGFP-RBD3.
2. Fill in the 3′ recessing ends with the Klenow fragment of DNA polymerase I.
3. Purify the blunted DNA with the JETSORB Gel Extraction Kit (Genomed GmbH) and digest again with *Sal*I.
4. Digest both the pYX212 and pYX242 with *Eco*RI.
5. Fill in the 3′ recessing ends with the Klenow fragment of DNA polymerase I.

6. Purify the DNA with the JETSORB Gel Extraction Kit (Genomed GmbH) and digest with *SalI*.
7. Ligate the fragment containing the fusion eGFP-RBD3 (**step 3**) with the linearized vectors (**step 6**): pYX212-eGFP-RBD3 and pYX242-eGFP-RBD3 are obtained.

3.2.2 Yeast Transformation

Transform yeast cells with a modified lithium acetate method [11] (*see* **Note 15**):

1. Inoculate cells in 5 ml of YPDA medium and grow overnight at 30 °C. Dilute in 25 ml of fresh YPDA at OD_{600nm} of about 0.4 and regrow at 30 °C till OD_{600nm} of about 1.5.
2. Harvest cells by centrifugation at 2,000 × *g* for 10 min at 4 °C and wash by suspending the cells in 12.5 ml of sterile water and subsequent centrifugation as above.
3. Suspend the cell pellet in 0.5 ml of 100 mM lithium acetate, transfer the suspension in a microfuge tube and centrifuge at 1,000 × *g* for 15 s.
4. Suspend the cells in 0.25 ml of 100 mM lithium acetate and divide in 50 µl aliquots in microfuge tubes. Centrifuge at 1,000 × *g* for 15 s and then add to each tube the following solutions in the given order: 240 µl of 50 % PEG 6000, 36 µl of 1 M lithium acetate, 25 µl of 2 mg/ml denatured salmon sperm DNA, 50 µl of sterile water containing 0.1 µg of plasmid DNA.
5. Vortex briefly and incubate at 30 °C for 30 min, then at 42 °C for 20 min.
6. Spin down for 15 s, wash the cells in 1 ml of sterile water, centrifuge and suspend the cell in 0.1 ml of sterile water and then plate on selective solid medium (SD medium, 2 % agar, without uracil or leucine).
7. Incubate the plates at 30 °C. Colonies of transformed yeast will grow in 2–3 days.

3.2.3 Labeling of Mitochondria with Vital Dye (Optional)

1. Add the staining solution to the cells at a final concentration of 100 nM.
2. Incubate in the dark for about 5–10 min before imaging.

3.2.4 Imaging of Ras-GTP Either by Fluorescence Microscope or by Confocal Microscope

1. Prepare in advance concanavalin A-coated cover glass as follows:
   (a) Cover the glass with 100 µg/ml concanavalin A solution and incubate 1 h at room temperature.
   (b) Remove concanavalin A solution (we recover and reuse the solution 2–3 times) and wash with deionized water. Glasses can be stored in deionized water in a sealed container for 1 day at 4 °C.
2. Grow cells at 30 °C in the appropriate medium until exponential phase.

3. Optional: if required, label organelles with vital dyes (*see* Subheading 3.2.3).
4. Seed about 50 µl of cells suspension on concanavalin A-coated cover glass for 10 min.
5. Wash the cover glass four times using about 1 ml of the proper medium each time and put on top of a Thoma chamber (*see* **Note 16**).
6. Pipet, between the cover glass and the Thoma Chamber, 5 µl of the proper medium on each side.
7. Focus the cell layer using transmission light and select a field for imaging Ras-GTP (optionally, acquire an image).
8. Switch to GFP fluorescence setting and acquire an image. If mitochondria have been labeled, switch to red fluorescence setting and acquire an image (*see* **Note 17**).
9. Process the images using either Adobe Photoshop (pictures acquired by fluorescence microscope) or ImageJ software (pictures acquired by confocal microscope). A representative example of a picture taken using a fluorescence microscope is shown in Fig. 2.

3.2.5 Glucose Induction Imaging of Ras-GTP Using a Fluorescence Microscope

1. Prepare in advance concanavalin A-coated cover glass as described above (*see* Subheading 3.2.4).
2. Grow cells at 30 °C in the appropriate medium until exponential phase.
3. Collect cells by centrifugation (max 3,000 × *g*, 3 min), resuspend in 25 mM MES buffer, pH 6 (5×10^7 cells/ml, final concentration) and incubate for at least 1 h at 30 °C.
4. Optional: if required, label organelles with vital dyes (*see* above Subheading 3.2.3).
5. Seed about 50 µl of cells suspension on concanavalin A-coated cover glass for 10 min.
6. Wash the cover glass four times with 25 mM MES buffer (about 1 ml each time) and put on top of a Thoma chamber (*see* **Note 16**).
7. Pipet, between the cover glass and the Thoma Chamber, 5 µl of 25 mM MES buffer on each side.
8. Focus the cell layer using transmission light, select a field for imaging Ras-GTP and acquire an image.
9. Switch to GFP fluorescence setting, acquire an image and switch back to transmission light. This is the control image before stimulation.
10. Add 20 µl of 3 % glucose dissolved in starvation buffer by pipetting between the cover glass and the Thoma Chamber. Be careful not to touch the Thoma chamber. Immediately,

switch to green fluorescence setting and acquire images. To avoid bleaching, acquire the fluorescence images for 1 s every minute for at least 10 min and keep the shutter off in the meantime (*see* **Note 18**).

11. Precess the time series of images using Adobe Photoshop.

4 Notes

1. We suggest to prepare Lyses buffer A fresh each time, starting from stocks solutions prepared in advance. Stock solutions: PBS 10× (*see* Subheading 2.1.2.), 0.5 M EDTA in deionized water, 0.5 M dithiothreitol in deionized water, one tablet of Protease Inhibitor Mixture in 2 ml of deionized water (25×). With the exception of PBS 10×, store the other stock solutions in small aliquots at −20 °C.
2. We suggest to prepare CPBS fresh each time, starting from stocks solutions prepared in advance, with the exception of sodium vanadate that it is best to prepare fresh each time. Stock solutions: PBS 10× (*see* Subheading 2.1.2.), 10 % (w/v) Triton X-100 in PBS 1×, glycerol (better 87 %, which is easier to pipet), 0.5 M EDTA in deionized water, 0.5 M dithiothreitol in deionized water, 100 mM sodium vanadate in deionized water, one tablet of Protease Inhibitor Mixture in 2 ml of deionized water (25×). Cut end of a blue tip to aspirate Triton X-100 and glycerol. Store 0.5 M EDTA, 0.5 M dithiothreitol, and Protease Inhibitor Mixture 25× in small aliquots at −20 °C.
3. We suggest to prepare Lysis buffer B fresh each time, starting from stocks solutions prepared in advance, with the exception of sodium vanadate and Nonidet P-40 that it is best to prepare fresh each time. Stock solutions: 200 mM HEPES, 5 M NaCl in deionized water, 10 % Nonidet P-40 in deionized water, 5 % sodium deoxycholate in deionized water, glycerol (better 87 %, which is easier to pipet), 0.5 M NaF, 75 mM $MgCl_2$, 0.5 M EDTA in deionized water, 100 mM sodium vanadate in deionized water, one tablet of Protease Inhibitor Mixture in 2 ml of water (25×). Cut end of a blue tip to aspirate Nonidet P-40 and glycerol. Store 0.5 M EDTA and Protease Inhibitor Mixture 25× in small aliquots at −20 °C.
4. We suggest to prepare this fresh each time.
5. Vectors pYX212 and pYX242 are 2μ vectors; they contain a TPI promoter and only differ for the yeast selectable marker, which are respectively *URA3* and *LEU2*.
6. Measure the protein concentration using the Bio-Rad protein Assay to make sure that it is about 3 μg/μl. If the protein concentration is less, *see* **Note 8**.

7. Use 20 μl of bed volume per sample. Start with at $N+5$ bed volumes, where N is the number of samples to be processed. The reason for this is that, due to the repeated washing, part of the resin will inevitably get lost.
8. We suggest to use a proper Hamilton syringe, but be careful not to aspirate the matrix with the needle.
9. Use $N+2$ bed volumes, where N is the number of samples to be processed. The reason for this is that, due to the repeated washing, part of the resin will inevitably get lost. 600 μl of cleared *E. coli* [pGEX2T-RBD] lysate should contain approximately 1.8 mg of total proteins. If the protein concentration of cleared *E. coli* [pGEX2T-RBD] lysate is less than 3 μg/μl, increase the volume proportionally.
10. Each tube corresponds to a treatment (sample). It is very important to verify, before continuing to the next step, that the amount of GST-RBD pre-bound to Glutathione Sepharose 4B is exactly the same, 20 μl bed volume, in each tube.
11. The fast filtration is particularly recommended when performing kinetics to study the Ras-GTP levels after addition of 100 mM glucose.
12. The amounts of "total Ras2 protein sample" and "Ras2-GTP proteins sample" to be loaded on the SDS-PAGE are indicative and may be varied depending either on the cell strain under investigation or the experimental conditions used.
13. Since both active Ras1 and Ras2 proteins bind to the GST-RBD pre-bound to Glutathione Sepharose 4B, theoretically this protocol gives the possibility to purify both Ras1-GTP and Ras2-GTP. Nevertheless, at the moment there are not specific yeast anti-Ras1 antibodies available.
14. Normally, if the amount loaded is in the range suggested at point 1, Subheading 3.1.6., an exposition of 1–5 min should be enough.
15. Importantly, with the only exception of the *gpa2*Δ mutant, the eGFP-RBD3 expression did not modify the growth rate in the wild type and in the mutant strains analyzed, indicating that this probe does not alter endogenous Ras signalling [9].
16. Drain the excess of medium from the cover glass before putting it on top of the Thoma chamber.
17. To test the specificity of the probe, we expressed eGFP in wild type cells and eGFP-RBD3 in GG104 *cdc25*Δ cells (in this strain almost all Ras proteins should be present in the inactive form). In both cases we observed a diffuse staining, indicating that the nuclear and plasma membrane staining, shown for the wild type strain growing exponentially on glucose medium and for other strains, occurs as a result of activated forms of Ras [9]. As a control, we used strain GG104 *cdc25*Δ since, for

unknown reason, the probe was poorly expressed in a Ras1,2 deletion mutant. Interestingly, in *S. cerevisiae* the nuclear accumulation is specific. This is in contrast with what happens in mammalian cells [7, 8, 12, 13], where RBD-based fluorescent reporter probes tend to accumulate aspecifically in the nucleus of many cell types.

18. We suggest to add glucose under transmission light to be able to check immediately for possible cell or focus drift as a consequence of these manipulations.

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