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Conditionally controlling nuclear trafficking in yeast by chemical-induced protein dimerization

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Abstract

We present here a protocol to conditionally control the nuclear trafficking of target proteins in yeast. In this system, rapamycin is used to heterodimerize two chimeric proteins. one chimera consists of a FK506-binding protein (FKBp12) fused to a cellular ‘address’ (nuclear localization signal or nuclear export sequence). the second chimera consists of a target protein fused to a fluorescent protein and the FKBp12-rapamycin-binding (FrB) domain from FKBp-12-rapamycin associated protein 1 (Frap1, also known as mtor). rapamycin induces dimerization of the FKBp12- and FrB-containing chimeras; these interactions selectively place the target protein under control of the cell address, thereby directing the protein into or out of the nucleus. By chemical-induced dimerization, protein mislocalization is reversible and enables the identification of conditional loss-of-function and gain-of-function phenotypes, in contrast to other systems that require permanent modification of the targeted protein. Yeast strains for this analysis can be constructed in 1 week, and the technique allows protein mislocalization within 15 min after drug treatment.

Introduction

In eukaryotes, protein activity is modulated through a variety of regulatory mechanisms controlling the abundance, conformational state and subcellular localization of a given gene product. With regard to the latter, regulated nuclear import and export is an important means of compartmentalizing key transcription factors and signaling proteins during cell growth and development. Examples of regulated nuclear trafficking are evident throughout the eukaryota; for instance, the vertebrate transcription factor NFAT and the related yeast protein Crz1p are dephosphorylated through calcineurin-dependent signaling events, enabling selective translocation into the nucleus for regulation of target promoters under conditions of

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elevated calcium^{1–4}. Numerous additional examples exist of proteins regulated endogenously by nucleo-cytoplasmic shuttling^{5–7}, underscoring both the biological importance of this regulatory mechanism and the potential utility in developing a method by which nuclear trafficking can be controlled exogenously.

Our platform for directed nuclear trafficking is adapted from a system developed jointly in the Crabtree and Schreiber laboratories^{8–10}. This system uses a small molecule that is capable of inducing the heterodimerization of two otherwise noninteracting proteins. The platform consists of three components: (i) the drug rapamycin, (ii) the 12-kDa protein FKBP12 and (iii) the 89-amino acid FRB domain from the protein mTor (or FRAP1). FK506-binding protein (FKBP12) and mTor do not interact in the absence of rapamycin; however, on introduction of the drug, rapamycin binds with high affinity to FKBP12, and this drug-protein complex gains high affinity for the FRB domain^{11–14}. Thus, rapamycin induces heterodimerization of FKBP12 and FRB (Fig. 1a). FKBP12 and FRB are incorporated in two chimeras: the FRB domain is fused to the carboxy (C) terminus of a target protein and the FKBP12 is fused at its C terminus to a functional tag (reviewed in Gestwicki and Marinec¹⁵). In our system, this tag is a type of cellular ‘address’—a nuclear localization signal (NLS)^{16,17} or nuclear export sequence (NES)¹⁸. Thus, rapamycin induces selective heterodimerization of FKBP12 and FRB; this reversibly pairs the target protein with the cellular address, thereby directing nuclear import or export of the chosen protein.

For these studies, we use a strain of yeast resistant to the antifungal effects of rapamycin and deleted for *FPR1*, the yeast ortholog of FKBP12 (Fig. 1a). The *fpr1*-null mutant exhibits no observable phenotypes on rapamycin treatment, and FKBP12s are nonessential in yeast; thus, this strain serves as an effective background for applications of this system. Researchers concerned about the use of this strain for a particular study may wish to validate any observable phenotypes using a non-chemical-induced protein dimerization (CID)-based method for permanent target protein mislocalization (see below for further discussion on such alternative approaches).

By drug-induced dimerization in yeast, we have directed the nuclear import or export of a test set of proteins^{19,20} and have successfully identified phenotypes associated with directed target protein mislocalization¹⁹. In our hands, rapamycin-driven nuclear trafficking is rapid, with steady-state localization of target yeast proteins evident after 15 min of drug treatment. We have also demonstrated the reversibility of this system; removal of rapamycin restores native localization of the protein in ~15 min¹⁹. In these experiments, reversibility was achieved by simply washing the cells repeatedly in drug-free media. The reversibility of this system was somewhat surprising, as *in vitro* studies suggest that the FRB-rapamycin-FKBP12 interaction is very stable. It should be noted, however, that we use a single copy of FKBP12 in our system, as opposed to the FKBP12x3 concatemer, which is expected to reduce affinity⁸. In addition, the affinity of other FKBP12 fusions is influenced by the fusion partner²¹; accordingly, the affinity of our system may be decreased from that of the free system. We consider the improved reversibility of this system to be advantageous in studying dynamic cellular processes. As needed, the reversibility of this system can be improved by adding FK506 or a synthetic ligand of FKBP12 (SLF, Cayman Chemical) to compete for FKBP12 binding, thereby accelerating reversibility.

Applications of small molecule-directed nuclear trafficking

In yeast, this system for drug-directed nuclear trafficking can be used in at least three types of studies. First, target proteins incorporated in our system can be mislocalized for the analysis of loss-of-function phenotypes. It is important to note that target protein mislocalization by this method is strictly dependent on the presence of drug; consequently, conditional loss-of-function mutants can be generated through this approach, with particular utility for the phenotypic analysis of essential genes. As proof-of-principle, in Geda *et al.*¹⁹, we directed the nuclear export of the essential heat-shock transcription factor Hsf1p, and its rapamycin-based mislocalization phenocopied a temperature-sensitive mutant under restrictive conditions. Relative to the generation of temperature-sensitive alleles, this platform for directed nuclear trafficking is less labor intensive and more amenable to systematic application. Second, target proteins can be directed to sites of activity, enabling analysis of whether a given localization shift is sufficient for wild-type protein activity in the absence of endogenous cell signaling. We have previously used this approach to direct nuclear import of the calcineurin-regulated transcription factor Crz1p in the absence of elevated calcium, identifying whether nuclear localization is itself sufficient for Crz1p transcriptional activity¹⁹. Third, if protein localization is sufficient for target protein activity, rapamycin-induced localization can be used to assess gain-of-function phenotypes in a conditional manner. Although this has not been attempted to date in *Saccharomyces cerevisiae*, chemical-induced nuclear import and export may provide a means to generate precise gain-of-function phenotypes without the confounding metabolic effects associated with changes in carbon source and/or other side effects from commonly used methods to control target gene expression. Thus, in total, this platform for drug-inducible nuclear trafficking enables a variety of phenotypic studies, with strong potential utility as a systematic method for large-scale analyses.

The methods presented here focus on directed nuclear trafficking in yeast; however, systems for chemical-induced dimerization hold broader applications for the localization of target proteins at additional subcellular sites. Although these approaches have not been implemented in yeast, drug-induced dimerization may be used to drive interactions between target proteins and a protein of interest, thereby regulating the subcellular distribution of the target. For example, a target protein may be dimerized with a polarity protein, possibly forcing the target protein to sites of polarized growth (e.g., the bud tip). Similar systems can be envisioned, enabling forced localization to the mitochondria, plasma membrane or Golgi apparatus. The protocols presented here provide a general template for the application of any such system, with much of the experimental design relevant to related studies in a broad range of eukaryotes and metazoans.

Alternative approaches

Two general methods exist by which the nuclear-cytoplasmic ratio of a target protein can be controlled: (i) a cellular address or tag can be permanently appended to the target protein; or (ii) CID systems can be used to direct target protein localization, as presented here. Using the former approach, coding sequence for the target protein can be modified to encode an in-frame cellular address (e.g., NLS or palmitoylation tag). These small tags can be inserted at the 3' end of the gene, at the 5' end of the gene or internally within the coding sequence,

directing the targeted protein to a given subcellular compartment. The simplicity of this method is advantageous; however, it does not allow for conditional control of protein localization. CID-based systems do enable conditional regulation of target proteins and have been used for a variety of studies in many eukaryotes^{22–26}. In addition to our method for directed nuclear import and export of targeted proteins in *S. cerevisiae*, Haruki *et al.*²⁷ present a closely related CID-based system, termed ‘Anchor-Away’, for the depletion of yeast nuclear proteins. The Anchor-Away approach uses rapamycin-induced dimerization to tether nuclear proteins to an abundant ribosomal protein, thereby depleting the nucleus of the targeted protein. As Anchor-Away is also driven by rapamycin-induced protein dimerization, its strengths and weaknesses closely parallel those presented here. As reported, Anchor-Away yields a high success rate in depleting targeted nuclear proteins when a ribosomal protein of the large subunit serves as the ‘anchor’, but does not allow directed nuclear import. Recently, Busch *et al.*²⁸ have implemented a CID system with NLS- and NES-containing chimeras to measure quantitative efficiencies of nuclear import and export in cultured human HeLa cells, underscoring the utility of CID systems for conditional control of nucleocytoplasmic trafficking in eukaryotes. The approach used by Busch *et al.* is similar to the one presented here, except that it is not directly applicable to yeast.

Limitations

From our experience, this drug-directed system for regulated nuclear trafficking is effective in controlling the subcellular distribution of most target proteins; however, empirical evidence suggests that the approach is sometimes ineffective in regulating the localization of proteins that engage in large numbers of protein-protein interactions²⁰. In previous studies from our groups, the majority of targets that were resistant to this methodology were reported to participate in ~20 or more protein-protein interactions, whereas successfully mislocalized proteins were associated with five interactors or fewer on average²⁰. For example, Prp45p is a protein component of the spliceosome, associated with ~70 proteins by virtue of reported affinity capture–mass spectrometry studies (data drawn from *Saccharomyces* Genome Database (SGD) at <http://www.yeastgenome.org/>). From our analysis, we were unable to direct nuclear export of Prp45p by rapamycin-induced dimerization, with no statistically significant change in nuclear protein levels evident on rapamycin treatment. This observed trend must be interpreted with caution, however, for two reasons. First, our sample size is small, encompassing ~17 yeast proteins tested for drug-directed nuclear import and export. Second, our assessment of protein-protein interactions is drawn strictly from published work deposited in the yeast genome database SGD²⁹ and does not draw on original or comprehensive data. Consequently, we cannot foretell with certainty the success of this approach in regulating the localization of any given protein, but caution should be exercised in targeting proteins that are known components of large complexes.

In addition, by this method—and possibly by any method—it is unlikely that targeted proteins can be exhaustively mislocalized such that absolutely no residual protein remains at its native localization. When analyzing cell phenotypes, this can be a complication; however, we have successfully identified mutant phenotypes on directed mislocalization of targeted proteins and expect, in general, that CID-based approaches are highly amenable to

systematic application, with relevance for the generation of conditional mutants and directed mislocalization screens.

Experimental design

Small molecule selection and dosage—In designing applications of small molecule-induced nuclear trafficking in yeast, several considerations need to be addressed. In particular, a choice must be made as to the specific small molecule used. Rapamycin is an antifungal agent that arrests yeast cell growth by inhibiting Tor1p^{11,30}. Consequently, if rapamycin is used to induce heterodimerization between FKBP12 and FRB, a rapamycin-resistant strain of yeast must be used. The *TOR1-1* point mutation (S1972R) confers resistance to rapamycin^{31–33}; *TOR1-1* encodes a protein with reduced of this strain is increased affinity for rapamycin, such that the LD₅₀ by at least 1,000-fold relative to wild type on rapamycin treatment¹⁹. Coupled with deletion of the yeast FKBP12 ortholog *FPR1*, the *TOR1-1* strain provides an effective genetic background for the application of rapamycin-induced dimerization systems (Fig. 1a). The necessity for this *TOR1-1 fpr1* background, however, can be limiting, particularly as the generation of an integrated point mutation without an associated marker is typically time consuming. As an alternative, chemical-induced FKBP12-FRB dimerization can be driven with a modified form of rapamycin that exhibits essentially no affinity for the wild-type FRB domain. We have previously used the C20-methallylrapamycin (MaRap) analog to induce drug-dependent protein dimerization in yeast. C20-MaRap binds a FRB mutant (FRB^{PLF}) in which lysine 2095, threonine 2098 and tryptophan 2101 are replaced with proline, leucine and phenylalanine, respectively^{10,24,34,35}. For these studies, we synthesized C20-MaRap in-house according to the strategy presented in Figure 1b and in Box 1. As an alternative, Ariad Pharmaceuticals makes available rapamycin analogs (e.g., rapamycin analog 21967) for the dimerization of FKBP and FRB, as well as plasmid sources of these proteins. The use of any such rapamycin analog obviates the need for the *TOR1-1* mutation, but the background yeast strain must still be deleted for *FPR1* to achieve maximum efficiency. In our hands, we have not observed any off-target effects resulting from treatment with rapamycin analogs; thus, both strategies are viable for implementation in *S. cerevisiae*.

Box 1

SYNTHESIS OF THE C20-METHALLYLLRAPAMYCIN (C20-MaRap) ANALOG

1. C20-MaRap is synthesized by an adaptation of the method of Luengo *et al.*⁴⁰. For this procedure, dissolve rapamycin (30 mg; 0.033 mmol) and trimethylmethallylsilane (0.08 ml) in dichloromethane (3 ml) and cool to – 40 °C. Briefly, trimethylmethallylsilane is obtained by adding chlorotrimethylsilane (1.87 g, 1 eq), 3-chloro-2-methyl-propene (3.12 g, 2 eq) and magnesium (1.14 g, 2.75 eq) to 50 ml THF and stirring at 60 °C for 5 h (ref. 41).
2. Quickly add boron trifluoride diethyl etherate (0.025 ml) to the solution of rapamycin and trimethylmethallylsilane, and stir the reaction at – 40 °C under argon atmosphere.

3. After 5 h, quench the reaction with three volumes of aqueous saturated sodium bicarbonate and slowly warm to room temperature.
4. Extract the organic products into dichloromethane and subject to silica-gel chromatography²⁴ (1:3, hexanes:ethyl acetate). This process yields ~15 mg of MaRap as a mixture of three isomers: C20-(*R*)-methallylrapamycin, C20-(*S*)-methallylrapamycin and C16-(*R*)-methallylrapamycin. Both the *R* and *S* forms have identical activity in biological assays²⁴.
5. Co-purify the C20-MaRap diastereomers (hereafter called C20-MaRap) by reverse-phase HPLC²⁴. Preheat the solvents and column (Waters Xterra M8 phenyl 5 mm, 19 mm × 100 mm column) to 50 °C. The mobile phase consisted of an isocratic mixture of nine parts of 80% CH₃OH/H₂O (vol/vol) and one part of 20% CH₃CN/H₂O (vol/vol).
6. At a flow rate of 17 ml min⁻¹, separate the desired product, C20-MaRap (*t_r* = 10.5–11.7 min) from the unreacted rapamycin (*t_r* = 8.5 min) and C16-(*R*)-MaRap (*t_r* = 14.5 min).
7. Characterize the purity of the product by measuring UV absorbance. Briefly, unreacted rapamycin was found to be the major contaminant in some samples, but C20-MaRap lacks the characteristic triene of this compound, so the absorbance at 278 nm can be used to monitor purity. If needed, repurify the C20-MaRap sample by HPLC until the purity is > 99%. Further characterize the product (4.7 mg, 16% purified yield) using ¹H NMR (proton nuclear magnetic resonance) and mass spectrometry (ESI-MS (electrospray ionization mass spectrometry) [C₅₄H₈₃NO₁₂ + Na⁺] expected *m/z* 960, observed *m/z* 960.5).

A second consideration affecting the design of drug-induced nuclear trafficking studies in yeast revolves around the dosage and frequency of drug application. In our studies, we routinely incubate yeast cells in 10 μM rapamycin. From our experience, this concentration of rapamycin/C20-MaRap is sufficient to induce strong levels of targeted nuclear import and export; increased concentrations of drug do not result in any detectable increases in nuclear trafficking, as visualized by fluorescence microscopy of a target protein-fluorescent protein chimera¹⁹. For some applications, however, it may be beneficial to maintain lower levels of directed nuclear import and export, in which case, optimal drug doses will need to be determined empirically. As a starting point, we recommend applying a strong dose of rapamycin (10 μM) and subsequently testing lower doses as needed, ranging from 0.1 nM to 5 μM. We also find that frequent dosing of yeast with rapamycin is unnecessary. As presented in Geda *et al.*¹⁹, rapamycin is degraded in yeast at a modest rate, with ~75% of the drug present 24 h after treatment. Thus, daily doses are sufficient to maintain steady-state levels of FKBP12-FRB dimerization, facilitating extended phenotypic studies of target protein nuclear import or export. As previously described, other rapamycin analogs exhibit shorter lifetimes *in vivo* and may require more frequent dosing²⁴.

Expression levels of the FKBP12- and FRB-containing chimeras

To achieve maximal levels of drug-directed nuclear trafficking, it is advisable to maintain a molar excess of the FKBP12-NLS/NES construct relative to the target protein-FP-FRB chimera. We typically maintain the target protein chimera at endogenous, or near endogenous, levels by integrating a cassette consisting of the coding sequence for a desired fluorescent protein, the FRB domain and a drug-resistance marker at the 3' end of the target gene sequence. Alternatively, the target gene may be cloned on a low-copy centromeric vector as an FP-FRB fusion gene for introduction into a strain deleted of the endogenous target gene. By these approaches, endogenous or moderately overexpressed levels of the target gene may be maintained. In contrast, expression of the FKBP12-cell address fusion is driven from the constitutive *GPD1* promoter on a high-copy yeast shuttle vector. This strategy ensures that the availability of the cell tag will not be limiting. As needed, the FKBP12 chimera can be expressed from a variety of promoters (constitutive and inducible) on yeast vectors of varying copy numbers, provided that the FKBP12 fusion is still expected to be in molar excess of the targeted gene product. From our experience, we have not detected any cell growth phenotypes resulting from overexpression of the FKBP12-NLS/NES fusion. It should also be noted that the NLS or NES does not localize FKBP12 to the nucleus or cytoplasm in the absence of interaction with the FRB fusion; the FKBP12-NES and -NLS proteins are distributed evenly over the cell before rapamycin treatment and FRB interaction, possibly because the small size of FKBP12-NLS/NES may allow for nonspecific translocation across the nuclear pore.

Phenotypic readout—As the approach presented here centers on a cellular response to drug treatment, cell-to-cell variability in rapamycin-directed nuclear trafficking is both expected and observed. We will discuss the degree of this variability in ANTICIPATED RESULTS; however, before using this system, it is worthwhile to consider the feasibility of designing an optimized reporter or assay that accurately measures the phenotypic readout of target protein mislocalization. For this purpose, single-cell reporters are ideal; cells that exhibit the desired localization of the target protein can be selected for phenotypic analysis, eliminating contributions from any background of nonresponsive cells. Easily measurable cell phenotypes can be effectively determined by this approach. For example, cell morphology and cell cycle stage can be conveniently identified on a single-cell level in cells that exhibit mislocalized target protein. This is not to say that rapamycin-induced nuclear trafficking systems cannot be used to identify the phenotypic consequences of mislocalization over a population of cells as a whole. Typically, the degree of drug-directed mislocalization achieved through this system is sufficient to identify such population-wide phenotypes. We find, in fact, that phenotypes generated by rapamycin-directed mislocalization largely mimic phenotypes obtained using corresponding temperature-sensitive alleles or repressible and/or inducible promoter constructs¹⁹. Thus, a wide variety of readouts may be used to assess the phenotypic consequences of drug-induced protein mislocalization, but single-cell measurements are expected to provide the most accurate results.

Experimental controls—To accurately interpret results from these studies, several important controls must be implemented. The cassettes and constructs that we have built for

this nuclear trafficking system are designed to generate fusions at the C terminus of a targeted protein. As C-terminal modification can perturb endogenous protein localization, it is necessary to confirm that the subcellular localization and activity of the targeted protein is unaffected in the absence of the drug. The particular background strain containing the integrated FRB fusion should also be treated with the drug in the absence of the plasmid-based FKBP12-NLS/NES fusion to ensure that no off-target effects are observed. This is particularly important if C20-MaRap is used, as any contaminating rapamycin in the preparation will arrest yeast cell growth. Details regarding live-cell microscopy are included in the protocol; however, to gain familiarity with this system and the subtleties of imaging yeast, it may be beneficial to begin with a simplified study in which rapamycin is simply used to direct the nuclear import or export of a fluorescent protein, rather than a yeast protein of interest. This trial run can be used to identify optimal growth conditions and drug dosage for subsequent experiments. In total, the procedure itself is rapid and simple, with detailed protocols given below. The major steps of the procedure are outlined in Figure 2.

MATERIALS

REAGENTS

- Yeast strain. For our studies, we use the strain KT2270 (*MAT α leu2 ura3 his3 TOR1-1*) (gift from K. Tatchell, Louisiana State University) with the *fpr1 Σ* kanMX replacement.
- *fpr1 TOR1-1* yeast strain—available on request.
- DNA cassette consisting of sequence encoding the Venus variant of yellow fluorescent protein (vYFP)³⁶, FRB^{PLF} domain and hygromycin B resistance gene (Fig. 2)—available on request.
- Plasmid derivatives of pRS425 with the FKBP12-NLS and FKBP12-NES fusions under transcriptional control of the constitutive *GPDI* promoter (Fig. 2)—available on request.
- Rapamycin (Sigma-Aldrich, cat. no. R0395)
- C20-MaRap (made in-house, see Box 1)
- G418 sulfate (Sigma-Aldrich, cat. no. A1720)
- Hygromycin B (Sigma-Aldrich, cat. no. H3274)
- SC-Leu dropout mix (Sigma-Aldrich, cat. no. Y 1376)
- Yeast nitrogen base without amino acids (BD Biosciences, cat. no. 291920)
- Dextrose (Sigma-Aldrich, cat. no. 7528)
- Bacto agar (BD Biosciences, cat. no. 214030)
- Yeast extract (BD Biosciences, cat. no. 212750))
- Peptone (BD Biosciences, cat. no. 211677)

- T4 DNA ligase (New England BioLabs, cat. no. M0202)
- Zymolyase (Zymo Research, cat. no. E1004)
- DMSO (Sigma-Aldrich, cat. no. D2650) ! **CAUTION** DMSO is toxic and readily absorbed through skin. Wear gloves to avoid direct contact with the solution.
- 4',6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich, cat. no. D9542)
- Triton X-100 (Sigma-Aldrich, cat. no. T8787)
- Concanavalin A (ConA; Sigma-Aldrich, cat. no. C9017)
- α-YFP antibody (rabbit polyclonal IgG; Santa Cruz Biotechnology, cat. no. sc-32897)
- Dichloromethane (Sigma-Aldrich, cat. no. 154792)
- Chlorotrimethylsilane (Sigma-Aldrich, cat. no. C72854)
- 3-Chloro-2-methyl propene (Sigma-Aldrich, cat. no. 64230)
- Boron trifluoride diethyl etherate (Sigma-Aldrich, cat. no. 15719)
- Sodium bicarbonate (aqueous solution; Sigma-Aldrich, cat. no. S9276)
- Restriction endonucleases SmaI, XbaI, HindIII, BamHI and SacII (New England BioLabs, cat. nos. R0141S, R0145S, R0140S, R0136S and R0157S, respectively)
- Salmon sperm DNA (Sigma-Aldrich, cat. no. 31149)
- Lithium acetate (Sigma-Aldrich, cat. no. 517992)
- PEG (average M_n 3,350; Sigma-Aldrich, cat. no. 202444)
- Phenol red (Sigma-Aldrich, cat. no. P0290)
- Distilled, deionized water
- Magnesium (Sigma-Aldrich, cat. no. 254118)
- Tetrahydrofuran (THF) (Sigma-Aldrich, cat. no. 186562)
- Methanol (CH_3OH) (Sigma-Aldrich, cat. no. 322415)
- Acetonitrile (CH_3CN) (Sigma-Aldrich, cat. no. 271004)
- High-fidelity DNA polymerase
- Taq polymerase and polymerase buffer
- dNTPs
- Gel purification kit (Qiagen, cat. no. 28704)
- Sorbitol (Sigma-Aldrich, cat. no. S1876)
- Tris-Cl (Sigma-Aldrich, cat. no. T5941)

- EDTA (Sigma-Aldrich, cat. no. E5134)
- Sodium chloride (Sigma-Aldrich, cat. no. S6191)
- β -Mercaptoethanol (Sigma-Aldrich, cat. no. M7522)
- SDS (Sigma-Aldrich, cat. no. L3771)
- Glycerol (Sigma-Aldrich, cat. no. G8773)

EQUIPMENT

- Standard equipment for molecular/cellular biology: refrigerated benchtop microcentrifuge (Eppendorf 5417R (Eppendorf) or comparable unit), refrigerated clinical tabletop centrifuge (Sorvall Legend RT or comparable unit), floor model shaking incubators for growth of yeast and bacteria (New Brunswick 12500 series incubator shaker, New Brunswick), apparatuses for agarose gel electrophoresis and western blotting (Owl Scientific and Hoefer), thermal cycler (MJ Research PTC-200 or equivalent unit)
- 96-well flat-bottomed microtiter plate (Corning, cat. no. 3650)
- Microscope slide (ThermoFisher, cat. no. 12-544-1)
- Glass cover slips (Corning, cat. no. 2940–243)
- Epifluorescence microscope with $\times 60$ water (numerical aperture 1.2) and $\times 100$ oil immersion (numerical aperture 1.3) objectives; cooled charge-coupled device camera; filter modules for DAPI, fluorescein isothiocyanate, cyan fluorescent protein, yellow/green fluorescent protein (YFP/GFP) and tetramethyl rhodamine isothiocyanate. We use an upright Nikon Eclipse 80i microscope (Nikon) with a CoolSNAP ES² charge-coupled device (Photometrics). Images were acquired using the MetaMorph software package (Molecular Devices).
- Plate reader or spectrophotometer. We use the Spectra Max M5 plate reader (Molecular Devices)
- Waters Xterra M8 (Waters)
- GE Healthcare Micro-Spin S300-HR column (GE Healthcare)
- Equipment for silica-gel chromatography

REAGENT SETUP

SC-Leu medium—For liquid medium, dissolve 6.7 g of yeast nitrogen base without amino acids, 20 g of dextrose and 2g of SC-Leu dropout mix in distilled and deionized water to a final volume of 1 liter, and then autoclave. For plates, add 20 g of bacto agar before autoclaving. Plates can be stored for 2–3 months at 4 °C. Liquid medium can be stored 2–3 months at room temperature (20–25 °C).

YPD medium—For liquid medium, dissolve 10 g of yeast extract, 20 g of peptone and 20 g of dextrose in distilled, deionized water to a final volume of 1 liter, and then autoclave. For plates, add 20 g of bacto agar before autoclaving. Liquid medium can be stored at room temperature (20–25 °C) for 2–3 months and plates can be stored for the same length of time at 4 °C.

DAPI stock—Dissolve sufficient DAPI in distilled, deionized water to prepare a 1 mg ml^{−1} (1,000×) solution. This stock solution can be stored in the dark for up to 1 year at −20 °C.

Concanavalin A stock—Prepare a 2 mg ml^{−1} stock solution of concanavalin A in water. This stock can be stored up to 1 year at −20 °C.

Geneticin G418 stock (1,000×)—Dissolve 200 mg G418 sulfate in a total volume of 1 ml ddH₂O. Filter-sterilize and aliquot as required (e.g., 50-μl aliquots). Use at a working concentration of 200 mg l^{−1}. Store at 4 °C. **▲ CRITICAL** G418 is temperature sensitive and is stable for at least 2 months in solution at 4 °C.

PROCEDURE

Selecting an appropriate yeast background strain

- 1| Select a starting strain of yeast that contains mutations supporting appropriate auxotrophies; at minimum, the strain should be deleted for *LEU2*, as the FKBP12-containing plasmid carries the *LEU2* marker. The background strain should also contain a deletion of the endogenous FKBP12 gene (*fpr1*). If rapamycin is to be used, the background strain must contain a mutation substituting serine for arginine at position 1972 in the endogenous TOR1 gene (*TOR1-1*). The *fpr1 TOR1-1* strain is available on request.

▲ CRITICAL STEP If C20-MaRap is used, the *TOR1-1* mutation is unnecessary and any *fpr1 leu2* strain will be sufficient.

Generation of the FrB fusion to a target gene ● TIMING 6–7 d

- 2| We have constructed a DNA cassette consisting of sequence encoding the vYFP³⁶, FRB^{PLF} domain and hygromycin B resistance gene (Fig. 2). This cassette is designed for integration at the 3' end of a target gene and is available on request. The DNA sequence of the FRB^{PLF} domain is presented in Figure 3a and the method by which this cassette was constructed is presented in Box 2.
- 3| To integrate vYFP-FRB^{PLF} sequence at a selected genomic locus, first amplify the integration cassette by PCR using the following primers: forward primer, 5'-(gene-specific sequence) AGTAAAGGAGAAGAAGCTTTTCACTGGAGTTGTCC-3'; reverse primer, 5'-(gene-specific sequence) TCGACACTGGATGGCGGCGTTAGTATCGAATCGAC-3'. In the forward

primer, AGT represents the second codon in vYFP, and this codon must be in frame with the target gene sequence. Incorporate at least 40 nucleotides of gene-specific sequence in each primer. Be sure to exclude the stop codon of the target gene in designing the forward primer; this will allow an in-frame fusion between the 3' end of the gene coding sequence and the sequence encoding the vYFP-FRB^{PLF} cassette. Mix the reagents for PCR as follows: 10 ng plasmid template containing the integration cassette, polymerase buffer made up to a 1× final concentration, 0.2 mM of each dNTP, 0.5 μM concentrations of the forward and reverse primers, 1 U of DNA polymerase; and water to obtain a final volume of 50 μl. Perform PCR according to the following cycling conditions: initial denaturation at 98 °C for 30 s; 35 cycles of incubation at 98 °C for 30 s, 60 °C for 30 s and 72 °C for 30s; and final extension at 72 °C for 10 min. Annealing temperatures may need to be modified if the primer sequences are modified from those presented above.

- 4| Introduce the PCR-amplified vYFP-FRB-Hyg cassette into *S. cerevisiae* according to the standard lithium acetate transformation protocol of Ito *et al.*³⁷ (Box 3).
- 5| Select for transformants on YPD plates supplemented with hygromycin B (300 μg ml⁻¹). After incubation at 30 °C for 3–4 d, multiple yeast colonies should be observed.
- 6| Restreak at least four colonies on a YPD plate supplemented with hygromycin B (300 μg ml⁻¹) and G418 (200 μg ml⁻¹); incubate at 30 °C overnight. Growth in the presence of G418 ensures that the *fpr1* mutation (kanMX cassette) in the background strain has been maintained.
- 7| Confirm proper chromosomal integration by colony PCR (Box 4).

▲ **CRITICAL STEP** Although the FRB^{PLF} domain itself is relatively small (89 amino acids), addition of the FP-FRB tag could disrupt normal protein localization and function. To address this concern, examine the subcellular localization and function of the chimeric protein in the absence of rapamycin or its derivatives by fluorescence microscopy according to standard protocols^{17,19,38}. If the target protein exhibits a known mislocalization or null phenotype, the gene-FP-FRB fusion can be introduced into a strain deleted for the endogenous gene to check for complementation of the phenotypic defect.

■ **PAUSE POINT** Store the yeast strains as glycerol stocks; mix the yeast culture with glycerol such that the final concentration of glycerol is 15% (vol/vol) and store at – 80 °C. The yeast strains will be viable for many years.

Box 2

CONSTRUCTION OF A FRB-CONTAINING CASSETTE FOR GENOMIC DNA INTEGRATION

1. Here, we present protocols for the construction of a vYFP-FRB^{PLF}-Hyg^R integration cassette. Amplify the vYFP sequence from the plas-

mid pBS7 (Yeast Resource Center, University of Washington, Seattle, WA, USA) with Phusion DNA polymerase (or other high-fidelity DNA polymerase) following the manufacturer's protocol using the forward primer 5'-GAGTTCCCGGGAGTAAAGGAGAAGAAGTCTTTTCAC-3' and the reverse primer 5'-

GGTATTCTAGATTTGTATAGTTCATCCATGCC-3'. Perform PCR as follows: denature initially at 98 °C for 30 s; followed by 35 cycles of incubation at 98 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s; and extend at 72 °C for 10 min. Load the PCR reaction onto a 1.0% (wt/vol) agarose gel, electrophorese and visualize the products. Excise a band containing the desired PCR product (~700 bp) and purify the DNA from the agarose gel slice using any commercially available gel purification kit. Digest the purified product with SmaI and XbaI in a standard 50-μl reaction that is 1× with respect to the suggested manufacturer enzyme buffer (e.g., New England BioLabs buffer 4) for 90 min at 25 °C and subsequently for 90 min at 37 °C. Purify the digested PCR product by spin-column chromatography according to suggested protocols (GE Healthcare Micro-Spin S300-HR column). Store at 4 °C up to 1 week as needed.

2. Prepare the yeast shuttle vector pRS305 plasmid using any standard alkaline lysis method^{42,43} or commercially available plasmid preparation kit. Digest ~2 μg of the plasmid preparation with SmaI and XbaI using the volumes and incubation conditions indicated in Step 1. Load the digestion reaction onto a 1% (wt/vol) agarose gel, excise the band consisting of the 5.4-kbp vector fragment and purify the DNA from the agarose gel slice using any commercially available gel purification kit, as indicated in Step 1. Store at 4 °C up to 1 week as needed.
3. Ligate ~100 ng of the vYFP fragment (from Step 1) and 200 ng of the pRS305 fragment (from Step 2) with T4 DNA ligase in a 100 μl volume that is 1× with respect to T4 ligation buffer with 1 Weiss unit of T4 DNA ligase; incubate overnight at 16 °C. Subsequently, introduce the ligation mixture into *E. coli* by standard methods of DNA transformation⁴³. Confirm the construct by DNA sequencing using a primer that contains 21 nt of sequence ~50 bp upstream of the SmaI recognition site in pRS305 (e.g., 5'-AGGGAACAAAAGCTGGG TACC-3'). This construct now consists of the sequence for vYFP in pRS305 (pRS305-vYFP).
4. Amplify the FRB^{PLF} sequence by PCR from plasmid ps-FRB (available from the authors on request) with Phusion DNA polymerase (or other high-fidelity DNA polymerase) following the manufacturer's protocol using the forward primer 5'-GTGTATCTAGAATGCTCGAGATGTGGCATG-3' and the reverse primer 5'-GAAGACCGCGGGTTCGACCTGCTTTGAGATTTCG-3'.

- Perform PCR as follows: denature initially at 98 °C for 30 s; followed by 35 cycles of incubation at 98 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s; and extend at 72 °C for 10 min. Analyze the PCR reaction by agarose gel electrophoresis as described in Step 1, and excise the band containing the PCR product (~300 bp). Recover the DNA from the agarose gel slice using any commercially available gel purification kit, and digest the PCR product with XbaI and SacII in a standard 50-μl reaction that is 1× with respect to the suggested manufacturer enzyme buffer (e.g., New England BioLabs buffer 4) at 37 °C for 2 h. Purify the digested PCR product by spin-column chromatography, as indicated in Step 1, and store at 4 °C up to 1 week as needed.
5. Digest the pRS305-vYFP plasmid (from Step 3) with XbaI and SacII using the conditions described above in Step 4. Load the digestion reaction onto a 1% (wt/vol) agarose gel, excise the band consisting of the 6.2-kbp vector fragment and purify the DNA from the agarose gel slice using any commercially available gel purification kit, as indicated in Step 1. Store at 4 °C up to 1 week as needed.
 6. Ligate the FRB^{PLF} fragment from Step 4 and the pRS305-vYFP fragment (Step 5) with T4 DNA ligase according to the reaction conditions indicated in Step 3. Transform into competent *E. coli* as indicated in Step 3. Confirm the construct by DNA sequencing using a primer that contains 21 nt of sequence corresponding to the reverse complement of a region ~50 bp downstream of the SacII recognition site in pRS305 (e.g., 5'-CACGACGTTGTAAAACGACGG-3'). This construct now contains sequence encoding the vYFP-FRB^{PLF} fusion in pRS305 (pRS305-vYFP-FRB).
 7. Amplify the vYFP-FRB^{PLF} sequence from pRS305-vYFP-FRB (generated in Step 6) by PCR as described in Step 1 using the forward primer 5'-GAGTTAAGCTTAGTAAAGGAGAAGAACTTTTCAC-3' and the reverse primer 5'-GAAGAGGATCCGTCGACCTGCTTTGAGATTCG-3'. Analyze the PCR reaction by agarose gel electrophoresis as described in Step 1, and excise the band containing the PCR product (~1 kbp). Recover the DNA from the agarose gel slice using any commercially available gel purification kit, and digest the PCR product with HindIII and BamHI in a standard 50-μl reaction that is 1× with respect to the suggested manufacturer enzyme buffer (e.g., New England BioLabs buffer 2) at 37 °C for 2 h. Purify the digested PCR product by spin-column chromatography as indicated in Step 1, and store at 4 °C up to 1 week as needed.
 8. Prepare the plasmid pAG32 (ref. 44) as described in Step 2. Digest ~2 μg of the plasmid preparation with HindIII and BamHI using the volumes and incubation conditions indicated in Step 7. Load the

- digestion reaction onto a 1% (wt/vol) agarose gel, excise the band consisting of the 4.1-kbp vector fragment and purify the DNA from the agarose gel slice using any commercially available gel purification kit, as indicated in Step 1. Store at 4 °C up to 1 week as needed.
9. Ligate the vYFP-FRB^{PLF} fragment (from Step 7) and the pAG32 fragment (from Step 8) using T4 DNA ligase in a standard reaction as described in Step 3. Transform the ligation mixture into *E. coli* by standard methods of DNA transformation⁴³. Confirm the construct by DNA sequencing using the primer 5'-CCTTATGTATCATAACATAC-3', which reads into the HindIII site of pAG32. This construct now contains the final vYFP-FRB^{PLF}-Hyg^R cassette in the pAG32 vector backbone.
- **PAUSE POINT** Store the confirmed constructs in *E. coli* as glycerol stocks at – 80 °C.

Box 3

YEAST TRANSFORMATION

- 1 We describe here the standard lithium acetate transformation protocol of Ito *et al.*³⁷ used for yeast transformation; start a yeast culture from a single colony in 5 ml of appropriate medium, and incubate overnight at 30 °C with continuous shaking at 200 r.p.m.
- 2 After overnight incubation, dilute 100 µl of culture by tenfold in water and measure optical density at 600 nm.
- 3 Dilute the overnight culture into 50 ml appropriate medium to an OD₆₀₀ of 0.2. Incubate the culture at 30 °C with continuous shaking at 200 r.p.m. for 3–5 h, until the OD₆₀₀ of the cell culture reaches 0.6–0.8.
- **CRITICAL STEP** Do not use overgrown cells for transformation, as the efficiency of the process will decrease significantly.
- 4 Spin down cells at 2,500 r.p.m. for 5 min and then wash with 30 ml of sterile water. These cells are enough for five transformations.
- 5 Spin down cells at 2,500 r.p.m. for 5 min and pour off H₂O. Resuspend pellet in 500 µl of 0.1 M lithium acetate.
- 6 Aliquot 100 µl cells to 1.5-ml microcentrifuge tubes.
- 7 Spin tubes for 1 min at 15,000g, and collect the supernatant.
- 8 To cell pellet, add (in order):
 - 74 µl DNA plus ddH₂O
 - 10 µl of 10 mg ml^{–1} salmon sperm DNA
 - 36 µl of 1 M lithium acetate

240 μ l of 50% PEG (average molecular weight 3,350 Da).

- 9** Resuspend pellet in transformation mix by vortexing vigorously.
- 10** Incubate cell mixture at 30 °C for 30 min and then place in a 42 °C water bath for 25 min.
- 11** Spin down cells at 15,000*g* for 1 min and collect the supernatant. If transforming a strain of yeast, wherein selection is based on an amino acid auxotrophy, follow option A; if using drug resistance as a selectable marker, follow option B.

A. Selection by amino acid auxotrophy

- i.** Spin down the cells at 15,000*g* for 30 s and discard the supernatant.
- ii.** Add 100 μ l of appropriate medium into each tube and resuspend the pellet with a pipette tip.
- iii.** Plate appropriated dilution of the cell suspension on selection medium.

B. Selection by drug resistance

- i.** Transfer cells to culture tube containing 1 ml appropriate medium.
- ii.** Incubate the cultures at 30 °C for 3–4 h without shaking.
- iii.** Spin the cultures at 3,000*g* for 2 min.
- iv.** Pour off all but ~100 μ l of medium.
- v.** Resuspend cell pellet in remaining medium.
- vi.** Plate entire suspension on selective drug plates.
- 12** Incubate the plates at 30 °C for 2–3 d.

Box 4

COLONY PCR IN YEAST

- 1.** Use a sterile pipette tip to scrape a small amount of a yeast colony and resuspend the scraped cells in a PCR tube containing 5 μ l of 3 mg ml⁻¹ zymolyase.
- 2.** Incubate cell suspension at 37 °C for 30 min.
- 3.** Incubate cell suspension at 95 °C for 10 min.
- 4.** Use this zymolyase cell suspension as a template in a 25- μ l PCR reaction with regular Taq polymerase. Add polymerase buffer to a 1 $\times$ final concentration, 0.2 mM of each dNTP, 0.5 μ M concentrations of the forward and reverse primers, and 1 U of DNA polymerase, and add water to obtain a final volume of 25 μ l. The forward primer will be gene specific, corresponding to a 22-bp sequence ~300 bp upstream of the start codon. The reverse primer (5'-

- GTGTTGGCCATGGAACAGGTAG-3') is the reverse complement of sequence from the YFP gene ~180 bp internal from the start codon of the gene.
5. Perform PCR as follows: denature initially at 95 °C for 5 min; follow by 29 cycles of incubation at 95 °C for 30 s, 54 °C for 30 s and 68 °C for 2 min; and extend at 68 °C for 10 min.

Introduction of the FKBP12-nls or -nes plasmid into the background strain ● TIMING ~3 d

- 8| Once the vYFP-FRB^{PLF} fusion has been confirmed, introduce a plasmid carrying the FKBP12-NLS or FKBP12-NES sequence into the strain from Step 7 by standard lithium acetate transformation (Box 3). We have constructed plasmid derivatives of pRS425 with the FKBP12-NLS and FKBP12-NES fusions under transcriptional control of the constitutive *GPD1* promoter (Fig. 2); these plasmids are available on request. The sequences of FKBP12-NES and of FKBP12-NLS are provided in Figure 3b.
- 9| Select transformants on SC-Leu medium. Yeast colonies should be visible after 2 d of growth at 30 °C.

■ **PAUSE POINT** Store the yeast strain as a glycerol stock at – 80 °C.

Growth assays to identify proper drug dosage ● TIMING ~2 d

- 10| Prepare twofold serial dilutions of rapamycin in 100 µl DMSO from a 5 mM rapamycin stock solution in DMSO; prepare eight twofold dilutions from 5 mM to 39.1 µM. Further dilute the DMSO dilutions by tenfold in SC-Leu medium (e.g., 500 µM to 3.91 µM) in a total volume of 100 µl.
- ▲ **CRITICAL STEP** For this procedure, ethanol works equally well in place of DMSO. These serial dilutions can be performed in a 96-well plate if desired.
- ▲ **CRITICAL STEP** Store the serial dilutions of rapamycin stock solution as 100-µl aliquots at - 20 °C. Avoid repeated freezing and thawing.
- 11| Inoculate 10 ml of appropriate medium with a single colony of the strain prepared in Step 9 and incubate overnight at 30 °C with continuous shaking at 250 r.p.m.
- 12| After overnight incubation, dilute 100 µl of the culture by tenfold in the same yeast growth medium as used in Step 11 and measure optical density (OD) at 600 nm.
- 13| Dilute the overnight culture to an optical density of 0.4, and add 90 µl of the diluted culture to each well of a 96-well flat-bottomed microplate. Withdraw 10 µl of each twofold serial dilution of rapamycin prepared in Step 10. Add 10 µl rapamycin solution to each well of the 96-well plate containing diluted culture. The final concentration of rapamycin should be 50 µM (for the 500 µM

dilution or 25 μ M for the 250 μ M dilution from Step 10, for example), and the final concentration of DMSO restricted to 1%.

- 14| Incubate the plate at 30 °C with continuous shaking at 250 r.p.m. Monitor growth by measuring the optical density of the cultures hourly for 24 h. Generate an approximate estimation of yeast doubling time, and identify a preferred concentration of drug that does not significantly retard yeast cell growth. For strains carrying the *TOR1-1* and *fpr1* mutations, doubling times of 2–3 h are typical. We routinely use rapamycin at a final concentration of 10 μ M.

? TROUBLESHOOTING

Analysis of regulated nuclear trafficking ● TIMING 1–4 d

- 15| Start a yeast culture from a single colony (of the strain from Step 9) in 10 ml of appropriate medium (e.g., SC-Leu) and incubate overnight at 30 °C with continuous shaking at 250 r.p.m. Be sure that this yeast strain grows well; doubling time should not be greater than 3 h, as indicated in Step 14.

? TROUBLESHOOTING

- 16| After overnight incubation, dilute 100 μ l of culture by tenfold in medium and measure the optical density of the culture at 600 nm.
- 17| Adjust the cell density of the culture with medium to achieve an optical density of 0.2. Grow the cells for an additional 3–4 h at 30 °C, with shaking at 250 r.p.m., to achieve approximately two cell doublings.
- 18| Aliquot 1 ml of the cell culture to a microcentrifuge tube or microtiter plate. Add DAPI (1 mg ml⁻¹ stock) to the aliquot of cell culture at a final concentration of 1.0 μ g ml⁻¹ and grow the cells for an additional 30 min (1.0 μ l DAPI stock to 1 ml cell culture). DAPI will enable visualization of the nucleus.

? TROUBLESHOOTING

- 19| At this point, the cell culture aliquot is ready for treatment with rapamycin and subsequent visualization of regulated nuclear trafficking in one of two ways:

(A) Imaging the immediate response to rapamycin

- i. Pellet the cells by centrifugation at 3,000g for 1 min at room temperature, and remove approximately 50–75% of the supernatant to increase cell density for microscopy. Avoid this step to minimize cell stress if the culture is already sufficiently concentrated; for microscopy, at least 10–20 yeast cells should be visible in the field of view at $\times 60$ magnification. Do not pellet the cells and resuspend in water before imaging or analysis, as this amounts to a cell stress that can substantially affect the subcellular distribution of yeast proteins.

- ii. Place 4 μ l of yeast cell culture on the center of a glass slide. Create a small chamber for the cell culture on the slide by placing a cover slip to each side of the spotted culture; place a cover slip on top such that it is slightly elevated from the glass slide by the thickness of the flanking cover slips. This technique is only necessary on an upright microscope; for an inverted microscope, an imaging chamber allowing for medium exchange can be used.

- iii. In bright-field mode, isolate a field of view containing healthy yeast cells.

? TROUBLESHOOTING

- iv. Add rapamycin to one edge of the chamber such that the final concentration when mixed with the cell culture aliquot is $\sim 10 \mu\text{M}$ and the DMSO concentration is $\sim 1\%$.

- v. Draw the rapamycin across the chamber by placing a thin absorbent lab wipe at the opposite edge of the chamber. By capillary action, the rapamycin solution will be drawn over the spotted cell culture through the chamber formed by the three cover slips. In this manner, the flow of drug and solution over the cells is gentle and will not displace the yeast cells excessively. An imaging chamber may be used as an alternative; however, a custom imaging chamber will likely need to be designed to accommodate an upright microscope.

? TROUBLESHOOTING

- vi. Collect bright-field and fluorescence images every 1–3 min according to protocols described in the user manual for your microscope. Do not image at shorter time intervals, as you may quench the fluorescent signal and/or damage the yeast cells by oxidative stress.

? TROUBLESHOOTING

(B) Imaging the steady-state response to rapamycin

- i. Add rapamycin to the cell culture aliquot at a final concentration of $10 \mu\text{M}$ and a DMSO concentration of no more than 1% .
- ii. Incubate at 30°C with shaking at 250 r.p.m. for 30 min. As needed, a sterile glass bead or two can be added to the tube or microtiter plate well to improve aeration and mixing.
- iii. Place 2–4 μ l of yeast cell culture on the center of a glass microscope slide and cover with a cover slip.
- iv. In bright-field mode, isolate a field of view containing healthy yeast cells. Collect bright-field and fluorescence images as above (Step 19A(vi)).

? TROUBLESHOOTING

- 20| Images can be quantified for percent nuclear localization using any appropriate software; we use the MetaMorph software package for this purpose. Using

images that indicate DAPI staining and vYFP fluorescence, outline the whole cell (I_{total}) and nucleus (I_{nuc}); calculate the average pixel intensity (I) of each area according to protocols described for the software system used in conjunction with your microscope. The nuclear localization can then be determined using the following formula:

$$\text{Nuclear Enrichment}(\%) = [(I_{\text{nuc}} - I_{\text{total}}) / I_{\text{total}}] \times 100$$

- 21| If desired, the nuclear concentration of the target protein can also be determined by western blotting of protein preparations isolated from yeast nuclei before and after rapamycin treatment. Antibody directed against vYFP can be used for these blots. A protocol for the preparation of yeast nuclei is provided in Box 5; western blotting is carried out as described in Xu *et al.*³⁹.

Box 5

PREPARATION OF YEAST NUCLEI FOR WESTERN BLOTTING

1. This procedure is a variation of the protocol used in Keogh *et al.*⁴⁵, and several reagent names ('SB', 'PSB', 'EBX' and 'NIB') are as referred to in that protocol. Grow 100 ml of cells in appropriate medium to an OD of < 0.6–0.8 at 600 nm. All steps below are carried out with prechilled solutions on ice unless stated otherwise.
2. Collect cells by centrifugation for 5 min at 4,000g at 4 °C.
3. Discard supernatant and add 30 ml water to wash in a 50-ml U-bottomed tube. Collect cells by centrifugation for 5 min at 4,000g at 4 °C, discard supernatant and add 30 ml SB (combine 1 M sorbitol and 20 mM Tris-Cl (pH 7.4); store at 4 °C for 1 month or less) to wash. Collect cells by centrifugation for 5 min at 4,000g at 4 °C and discard supernatant. Store pellets at – 80 °C.
4. Thaw pellets on ice. Add 1.5 ml PSB (combine 20 mM Tris-Cl (pH 7.4), 2 mM EDTA, 100 mM NaCl and 10 mM β-mercaptoethanol; store at 4 °C for 1 month or less) and resuspend the pellet. Transfer to a 2-ml microcentrifuge tube. Centrifuge the suspension at 2,000g for 5 min at 4 °C in a benchtop microcentrifuge and discard supernatant. Add 1.5 ml SB, mix to generate a cell suspension, centrifuge at 2,000g for 5 min at 4 °C and discard the supernatant.
5. Resuspend the pellets completely in 1 ml SB. Add 125 μl zymolyase 20T (10 mg ml^{–1} in SB). Incubate at room temperature with rotation.
6. Determine OD at 600 nm every 30 min, and continue incubation until it drops to 80% (should take ~1 h). To measure the OD₆₀₀, remove 10 μl and add to 1 ml 1% SDS (wt/vol); determine OD at 600 nm and compare against a blank sample of 10 μl SB in 1 ml of 1% SDS.▲
CRITICAL STEP Some mutants may be more zymolyase resistant

- than wild-type strains; if digestion is suspiciously slow after 1 h, add another 75 μ l zymolyase 20T and continue incubation.
7. Once the digestion is complete, add 1 ml SB and collect by centrifugation for 5 min at 2,000*g* at 4 °C. Subsequently, repeat the wash with SB.
▲ CRITICAL STEP Spheroplasts are more fragile than cell-walled yeast, so gentle centrifugation is suggested.
8. Resuspend spheroplasts gently with 500 μ l EBX (combine 20 mM Tris-Cl (pH 7.4), 100 mM NaCl and 0.25% Triton X-100, and add Phenol red to a final concentration of 0.005% (vol/vol); store at 4 °C for 1 month or less).
9. Accounting for the pellet volume, add Triton X-100 to a final concentration of 0.5% to lyse the outer cell membrane.
10. Place on ice for 10 min with gentle mixing every few minutes (aliquot as total cell preparation for western blot analysis).
11. Layer over 1 ml NIB (combine 20 mM Tris-Cl (pH 7.4), 100 mM NaCl, 1.2 M sucrose and 15 mM β -mercaptoethanol; store at 4 °C for 1 month or less) and centrifuge at 12,000*g* for 15 min at 4 °C.
12. Three layers should be evident: (i) a cloudy reddish (because of the phenol red) upper layer (aliquot as cytoplasmic fraction for western blot analysis), (ii) possibly a white lipid interphase (it may be on the side of the tube) and (iii) a glassy, white nuclear pellet.
13. Remove the supernatant and gently resuspend the nuclei in 500 μ l of EBX. Accounting for the pellet volume, add Triton X-100 to a final concentration of 1% to lyse the nuclear membrane.
14. Place on ice for 10 min with gentle mixing every few minutes (aliquot as nuclear preparation for western blot analysis).

? TROUBLESHOOTING

Troubleshooting advice can be found in table 1.

● TIMING

Steps 1–7, Selection of yeast strain and generation of FRB fusion to target gene in appropriate background: ~1 week

Steps 8 and 9, Introduction of the FKBP12-containing plasmid into background strain: ~3 d

Steps 10–14, Growth assays to identify optimal drug dosage: ~2 d

Steps 15–17, Preparation of cell culture for drug treatment: ~14 h

Steps 18 and 19, Drug treatment and image capture: ~30 min–1 h

Steps 20 and 21, Data analysis and confirmation of mislocalization: 1–4 d

ANTICIPATED RESULTS

Cell-to-cell variability will be evident when visualizing target proteins after rapamycin treatment. With the vYFP fusion generated as an integrated allele, we routinely observe a background of approximately 20–30% of cells that exhibit little or no fluorescence. Presumably, this reflects endogenous variability in protein levels within a population of yeast cells and is consistent with our observations from large-scale studies of protein localization in yeast^{7,38}. Beyond this variability in protein abundance, individual cells within a population will respond differentially to the small-molecule treatment. In our hands, treatment with rapamycin typically yields the desired subcellular distribution of target protein in approximately 40–70% of cells in which fluorescence above background is observed. To assess the efficiency of drug-directed mislocalization, the percentage of nuclear fluorescence against total cell fluorescence can be calculated, as described above, for a sampling of 100 yeast cells before and after drug treatment. Mean nuclear and total cell fluorescence levels calculated for this sample size provide an accurate assessment of target protein distribution, with sufficient statistical strength to determine the significance of the observed results. From our experience, proteins amenable to this CID system show a greater than threefold change in nuclear levels on drug treatment, as assessed by fluorescence intensity. Proteins resistant to CID-based mislocalization typically yield a statistically insignificant change in the nuclear-cytoplasmic ratio of the targeted protein on drug treatment. Sample successful and unsuccessful applications of the rapamycin system are indicated in Figures 4 and 5. It is interesting to note that we have not yet encountered proteins that yield intermediate results: the drug-dependent mislocalization system is either successful or unsuccessful, as defined above, in directing target protein localization. For the majority of studies, we expect that the degree of directed mislocalization will be sufficient to enable detection of phenotypes over a population of cells, and this approach compares favorably with cell-cell variability observed using temperature-sensitive alleles under restrictive conditions.

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Figure 1. Overview of this approach using chemical-induced dimerization to modulate the nuclear import and/or export of target proteins in yeast. **(a)** Schematic representation of the components in the conditional mislocalization system. For simplicity, only drug-directed nuclear import is shown here. Genetic modifications necessary to adapt this procedure for yeast are indicated in the inset box. The underlying mutation in *TOR1-1* is indicated with a star in the diagram; chromosomal coordinates of the *TOR1* gene are shown. Replacement of the *FPR1* gene with the kanMX6 cassette is also indicated; the chromosomal sequence (ten nucleotides) immediately upstream of the kanMX replacement is shown. W, Watson strand; C, Crick strand. **(b)** Scheme for the synthesis of the rapamycin analog, C20-methallylrapamycin (C20-MaRap).

Figure 2. Workflow for application of the drug-directed nuclear trafficking system in yeast. Numbers correspond to the steps in the protocol as presented here. Steps 1–9 are used to generate a strain of yeast suitable for this analysis. Steps 10–14 enable identification of the optimal dosage of rapamycin or C20-MaRap to be used; rapamycin is shown in the upper right box, with the face of the molecule that interacts with FRB domain shown in blue. Steps 15–21 detail the small molecule treatment, cell imaging and basic data analysis. *yfg*, your favorite gene.

Figure 3. Coding sequence for the FRB^{PLF}, FKBP12-NES and FKBP12-NLS fusions. **(a)** DNA sequence of the FRB^{PLF} domain used in our studies. Mutated sequences encoding the P, L and F residues are indicated in lower case and shaded in orange. **(b)** DNA and amino acid sequences of the FKBP12-NES and FKBP12-NLS fusions are shown. As indicated, the NES is derived from the HIV-1 REV protein, and the NLS is derived from the SV40 large T-antigen. The HIV-1 REV NES is highlighted in blue. The SV40 NLS sequence is highlighted in red.

Figure 4. Sample quantified results from applications of chemical-induced mislocalization. (a) shows successful and (b) shows unsuccessful examples. Bar graphs indicate percent nuclear fluorescence. FKBP12 chimeras used in each experiment are indicated above the respective bar graphs. Sky1p is a SRPK1-like kinase, and Elf1p is a transcription elongation factor. Pho4p is a phosphate-regulated transcription factor and Fun20p is a pre-mRNA splicing protein. Rap, rapamycin.

Figure 5.

Sample microscope images indicating successful and unsuccessful applications of this system for drug-dependent control of nuclear protein localization in yeast. **(a)** Sample images of successful rapamycin-mediated nuclear import of vYFP. DAPI staining indicates the nucleus, and imaging by differential interference contrast (DIC) microscopy indicates the cell outline. Note the shift in vYFP fluorescence into the nucleus on rapamycin treatment. **(b)** Unsuccessful application of the rapamycin system to direct Pho4p into the nucleus under normal growth conditions. As shown, strong nuclear localization is not evident on rapamycin treatment.

TABLE 1

Troubleshooting table.

Step	Problem	Possible reason	Solution
14	Yeast culture grows very poorly on treatment with rapamycin or C20-MaRap	Contaminating rapamycin in the C20-MaRap preparation; intended <i>TOR1-1</i> point mutation incorrect	As evidence of rapamycin contamination, look for large and unbudded yeast cells with large vacuoles and 1nDNA content. If observed, synthesize a new preparation of C20-MaRap. If rapamycin was used, confirm the <i>TOR1-1</i> mutation by DNA sequencing and reconstruct the point mutation if necessary
15	The <i>TOR1-1 fpr1</i> strain grows slowly	Unknown, but possibly the combination of mutations confers a mild synthetic fitness defect	Grow the cells in 2× SC dropout medium (SC medium at twice the solute concentration)
18	Significant nonspecific cytoplasmic or cell body staining	Inability of DAPI to traverse cell wall and plasma membrane	Improve staining by permeabilizing the cell through the addition of 0.1% Triton X-100 to the medium
19A(iii)	Presence of large vacuoles and/or excessive vacuolar fluorescence	Nutritional/environmental stress from growth conditions and imaging steps; cell physiology perturbed	Manipulate the cells as little and as gently as possible before microscopy; image cells immediately after placing on slide (do not let the cells sit on the slide for a prolonged period of time before imaging). In our experience, this is very important for the effective application of this system
19A(v)	Slow rate of solvent flow	Small chamber does not allow for sufficient solvent flow across the slide	Place absorbent lab wipes on both sides of the cover slip chamber to improve flow through
19A(vi)	Cells are being carried out of field of view	Capillary action during rapamycin addition is displacing cells in field of view	Treat glass cover slips with ConA to increase cell adherence. On day of use, place 5 µl ConA on cover slip and spread with a clean pipette tip. Allow to air dry before use
19B(iv)	Target protein localization unaffected	The target may function as part of a large protein complex (see ANTICIPATED RESULTS); if a non-standard genetic background is used, it may be possible that rapamycin is being cleared from the cell more effectively than in a wild-type strain	Determine the number of reported protein-protein interactions for a target protein from an interaction database or from SGD; although we do not find this to be necessary routinely, mutation of the yeast multidrug transporter gene <i>PDR5</i> may improve rapamycin accessibility