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ORIGINAL PAPER

Conditional Nuclear Import and Export of Yeast Proteins Using a Chemical Inducer of Dimerization

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Abstract In eukaryotes, reversible shuttling between the nucleus and cytoplasm is an important regulatory mechanism, particularly for many kinases and transcription factors. Inspired by the natural system, we recently developed a technology to control protein position in budding yeast using a chemical inducer of dimerization (CID). In this method, a nuclear export or localization signal is reversibly appended to a protein of interest by the CID, which effectively places its subcellular location under direct control of the chemical stimulus. Here, we explicitly tested the ability of this system to direct the nucleocytoplasmic transport of a panel of 16 representative kinases and transcription factors. From this set, we found that 12 targets (75%) are susceptible to re-positioning, suggesting that this method might be applicable to a range of targets. Interestingly, the four proteins that resisted mislocalization (Fun20p, Hcm1p, Pho4p, and Ste12p) are known to engage in a large number of protein–protein contacts. We suspect that, for these highly connected targets, the strength of the chemical signal may be insufficient to drive mislocalization and that proteins with relatively few partners might be most amenable to this approach. Collectively, these studies provide a necessary framework for the design of large-scale applications.

Keywords Rapamycin · Nuclear localization signal · Nuclear export signal · FK506 binding protein · Budding yeast · Conditional alleles

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Introduction

In eukaryotic cells, the subcellular compartmentalization of proteins is achieved either through specific protein–protein interactions or by virtue of short address sequences, such as a nuclear export signal (NES) or nuclear localization signal (NLS) [1]. These cellular addresses provide requisite information for determining the protein's position within the cell and, for certain proteins, these signals can be dynamically regulated [2]; for example, some transcription factors, such as NFAT and NFκB, are held in inactive, cytoplasmic pools until they are activated for transport into the nucleus [3]. In these systems, a typical method for studying the impact of subcellular trafficking involves permanently appending or deleting the address sequence and discerning the effect of this manipulation on protein function. Although this type of genetic experiment can uncover the absolute importance of an address, it does not permit insight into acute or dynamic changes.

Inspired by the reversibility of natural trafficking pathways, we recently developed a synthetic way to control protein position in the budding yeast, *Saccharomyces cerevisae* [4]. This method was adapted from work in mammalian models [5–7] and pioneering efforts by the Crabtree and Schreiber groups on chemical inducers of dimerization (CIDs; reviewed in [8–10]). CIDs are small, membrane-permeable molecules that bind to two proteins

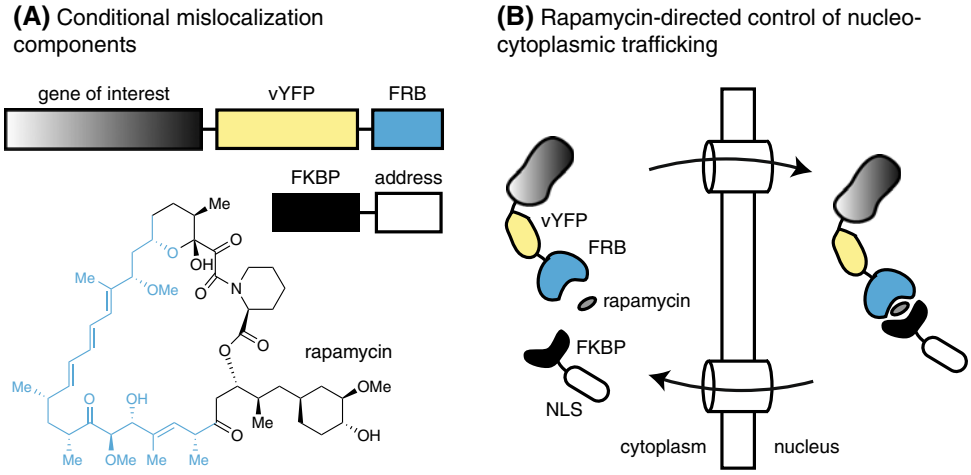

Fig. 1 Conditional mislocalization system. **a** Genetic and chemical components of the mislocalization system. The chemical structure of rapamycin is shown with each side of the molecule highlighted; the blue region interacts with the FRB domain and the other face binds FKBP. **b** Schematic of the rapamycin-mediated heterodimerization of FKBP-NLS and vYFP-FRB, reconstitution of the ternary complex and conditional protein import into the nucleus

at the same time. For example, one face of the naturally occurring CID, rapamycin, binds the FK506-binding protein (FKBP) [11, 12] and the other has affinity for the FKBP-rapamycin binding (FRB) domain of the target of rapamycin (Tor) [13, 14]. Thus, if two proteins are expressed as chimeric fusions with FRB and FKBP domains, rapamycin will trigger their heterodimerization (Fig. 1a). Moreover, if the FKBP component is fused to either an NES or NLS, then rapamycin will reversibly and conditionally reconstitute a FRB-tagged protein with the cellular address signal (Fig. 1b) [5].

To adapt the CID-based import/export components for use in yeast, we generated Gateway cassettes [15] for the rapid creation of FRB and FKBP fusions; the FRB vector allows installation of the Venus variant of yellow fluorescent protein (vYFP) and FRB to the C-terminus of the gene-of-interest, while the FKBP vector can be used to produce FKBP-NES and FKBP-NLS chimeras [4]. Using these reagents, we showed that vYFP-FRB could be conditionally imported or exported from the nucleus and that the kinetics is rapid, achieving completion within 10–15 min [4]. Importantly, we also generated a strain that is resistant to rapamycin, which permits use of this inexpensive, commercially available CID at high concentrations (at least 10 μM). These studies provided evidence that CID-mediated protein mislocalization could be accomplished in yeast and, further, provided an initial characterization of the kinetics and potential uses.

Despite these promising efforts, the suitability of this system for use in large-scale studies remains unexplored. For example, only two yeast proteins have been conditionally imported/exported [4] and, thus, the potential influence of molecular weight, shape, size, and other parameters on the efficiency of mislocalization are not yet clear. To address this issue, we generate a pilot panel of 16 vYFP-FRB fusions and report that most (12 of 16) of these targets were efficiently mislocalized upon addition of rapamycin. Interestingly, a subset of the proteins seemed more resistant, and preliminary analyses suggest that these targets engage in plentiful protein–protein contacts. Together, these findings provide an enabling framework for designing large-scale studies.

Materials and Methods

Yeast Strains and Mislocalization Components

The rapamycin-resistant yeast strain, which contains both a deletion of the endogenous FKBP (*fpr1Δ*) and a point mutation in Tor that blocks rapamycin binding (*tor1-1*), was reported [4]. For target genes, we integrated a cassette consisting of vYFP [16], the FRB domain, and a yeast selectable marker encoding resistance to hygromycin B [17] at the 3′-end of a target gene, forming a translational fusion between the vYFP-FRB coding sequence and the carboxy terminus of the target protein.

Drug Treatments and Fluorescence Microscopy

Overnight cultures (OD_{600} ~ 0.8) were diluted 1:5 with the appropriate selection media and grown for 3 h at 30°C to an OD_{600} ~ 0.6. Rapamycin or the solvent control (dimethylsulfoxide; DMSO) was added to the selection media, such that the final drug concentration was 10 μM and the DMSO level would be less than 0.1%. Except where noted, rapamycin treatments were 30 min. For the experiments to determine native localization, cells were fixed in ethanol, treated with 4,6-diamidino-2-phenylindole (DAPI) at 0.5 μg/ml for 30 min and washed with PBS, as previously described [4]. All microscopy was performed on an upright Nikon Eclipse 80i microscope with a CoolSNAP ES^2 CCD (Photometrics, Tucson, AZ); images were acquired using the MetaMorph software package.

Analysis of Rapamycin-Mediated Control of Target Protein Localization

Using v1.38 ImageJ software, the enrichment or depletion of nuclear fluorescence was measured relative to the overall cell background. If the average change in fluorescence intensity in the nucleus was at least five-fold across at least 60% of the population, then the response was defined as significant; note that this is a somewhat conservative measure of efficiency, but our goal is to characterize this system in anticipation of large-scale studies that require robust methodology. Using these data, we determined the success of the rapamycin treatment in controlling target protein localization. To understand the possible mechanisms affecting this success rate, protein interaction data were extracted from the *Saccharomyces* Genome Database (SGD; www.yeastgenome.org). Specifically, protein interactions in yeast have been analyzed through several large-scale studies utilizing a combination of co-immunoprecipitation approaches coupled with mass spectrometry [18–20]. We selected these data sets rather than two-hybrid results because of the high rate of false-positives encountered in two-hybrid studies [21]. Importantly, for the proteins in our pilot set, the interaction data did not resemble a scale-free distribution; thus, the averages presented are representative of the interactions per bin.

Growth Assays

Stock cultures (5 ml SC-Leu-Ura) were prepared from single colonies and incubated overnight at 30°C with continuous shaking. After determining the OD_{600} ($\sim$0.8), the stock cultures were diluted to $\sim$0.3 and 50 µl of this sample added to 96-well microtiter plates (ThermoFisher, Sci.). Rapamycin was diluted in culture media and then added to the wells to achieve the desired concentration. The plate was then incubated at 30°C with continuous shaking. After 20 h, the culture density was monitored by OD_{600} on a Spectra Max M5 plate reader (Molecular Devices, Sunnyvale, CA).

Results and Discusssion

Appending the vYFP-FRB Tag Does Not Disrupt Normal Protein Localization

To gauge the potential of the CID-based platform for large-scale studies, we prepared an exploratory panel of yeast open reading frames (yORFs) fused to vYFP-FRB. Specifically, we selected a test set of 16 proteins, consisting largely of kinases and transcription factors, because these proteins seem most likely to be regulated by compartmentalization (Table 1) [22]. In addition,

Table 1 Select panel of yeast kinases and transcription factors

Gene	Description/function	Normal subcellular localization	Localization of FRB-vYFP fusion	Tested cell address	Conditional localization[a]
ATG1	Kinase required for autophagy	PAS/vacuole/cytoplasm	Cytoplasm	FKBP-NLS	+
ELF1	Transcription elongation factor	Nuclear	Nuclear	FKBP-NES	+
FAR1	Cyclin-dependent kinase inhibitor	Cytoplasm/nucleus/bud tip	Cytoplasm/ nucleus	FKBP-NLS, FKBP-NES	+, +
FUN20	Pre-mRNA splicing protein	Nucleus	Nucleus	FKBP-NES	−
HCM1	Forkhead transcription factor	Nucleus	Nucleus	FKBP-NES	−
NPR1	Kinase stabilizing AA transporters	Cytoplasm	Cytoplasm/ nucleus	FKBP-NLS	+
PHD1	PHG transcription factor	Nucleus	Nucleus	FKBP-NES	+
PHO4	Phosphate-regulated transcription factor	Cytoplasm/nucleus	Cytoplasm/ nucleus	FKBP-NLS	−
SCH9	Kinase regulating G1 progression	Cytoplasm/nucleus	Cytoplasm/ nucleus	FKBP-NLS	+
SKY1	SRPK1-like protein kinase	Cytoplasm	Cytoplasm	FKBP-NLS	+
STE12	Mating/PHG transcription factor	Nucleus	Nucleus	FKBP-NES	−
SRB6	RNA pol II mediator subunit	Nucleus	Nucleus	FKBP-NES	+
TEC1	Transcription factor for Ty1 expression and PHG	Nucleus	Nucleus	FKBP-NES	+
TFB3	Transcription initiation factor	Nucleus	Nucleus	FKBP-NES	+
UGA3	Transcriptional activator of GABA gene expression	Nucleus	Nucleus	FKBP-NES	+
YGR251W	Essential protein of unknown function	Nucleus	Nucleus	FKBP-NES	+

[a] − <5-fold change in nuclear localization; + ≥5-fold change in nuclear localization

these proteins were chosen to span a range of expected molecular weights (from 14 to 101 kDa), in an attempt to reveal whether this parameter would impact mislocalization. For this study, we integrated a vYFP-FRB cassette at the 3′-end of each gene, yielding a yORF-vYFP-FRB chimera suitable for interaction with the complementary FKBP-NES or FKBP-NLS fusion. Although the FRB domain is relatively small (~90 amino acids), one concern is that appending vYFP-FRB tags might disrupt normal localization. To address this question, we examined the resting localization of the chimeric proteins in the absence of rapamycin and compared these findings to the expected patterns compiled in the protein localization database Organelle DB [23]. Satisfyingly, we found that the vYFP-FRB chimeras had normal localization, suggesting that the appended domains do not mask inherent address information in the select targets (Table 1 and Fig. 2). Moreover, the cells expressing vYFP-FRB fusions to essential genes, such as SRB6, TFB3, and YGR251W, were phenotypically normal, which suggests that they retain function.

Mislocalization of a Panel of Transcription Factors and Kinases

Using the panel of 16 chimeras, we assayed each for its response to rapamycin; the results of this study are summarized in Table 1 and representative images shown in Fig. 3. In general, most of the targets were directed into the expected subcellular compartment. For example, the nuclear proteins Elf1p, Phd1p, Srb6p, Tec1p, Tfb3p, Uga3p, and the nuclear protein product from YGR251W were directed from the nucleus to the cytoplasm (Fig. 3a). Likewise, the primarily cytoplasmic proteins Atg1p, Npr1p, Sch9p, and Sky1p were directed to the nucleus (Fig. 3a); thus, both import and export could be achieved. One interesting target was the cyclin-dependent kinase inhibitor Far1p, which translocates from the nucleus to the cytoplasm and mating projection tip in response to mating pheromone [24]. Far1p-vYFP-FRB was largely nuclear under standard growth conditions but with some cytoplasmic fluorescence as well; so, we attempted to direct its localization to the nucleus and cytoplasm in separate trials, using the

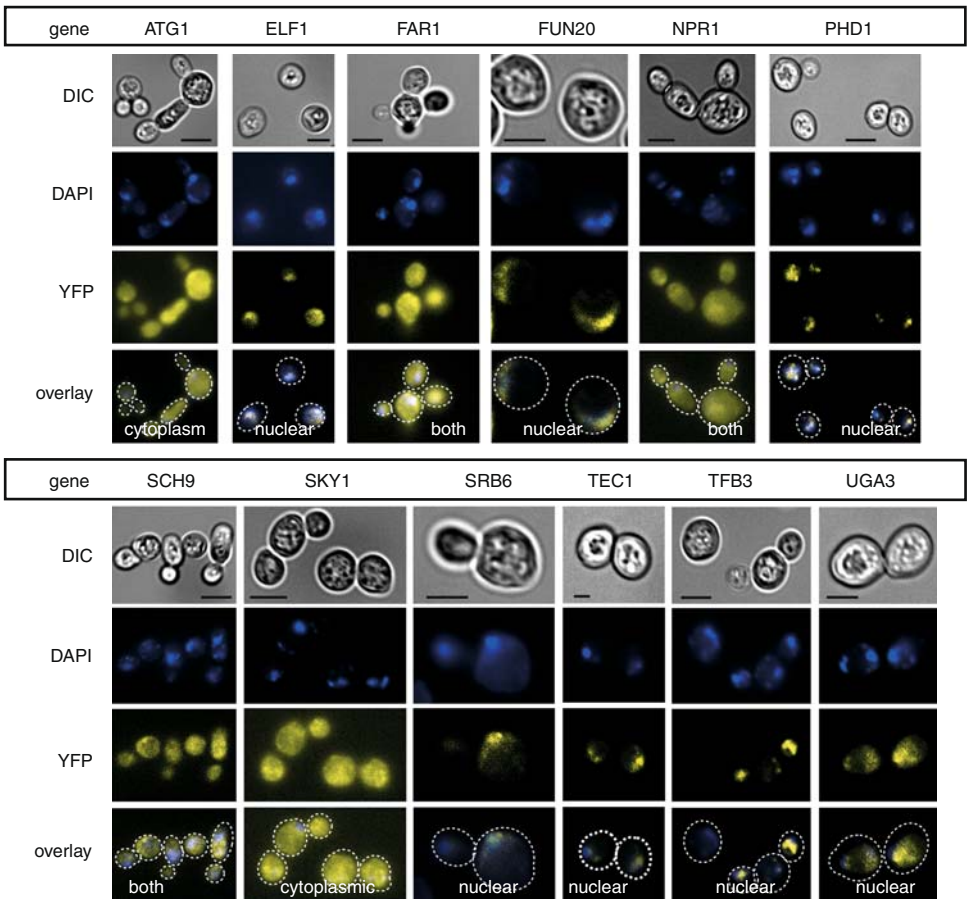

Fig. 2 Appending a panel of yeast kinases and transcription factors to vYFP-FRB does not disrupt normal localization. DAPI was used to reveal the nuclei. Size bars are 3 μm

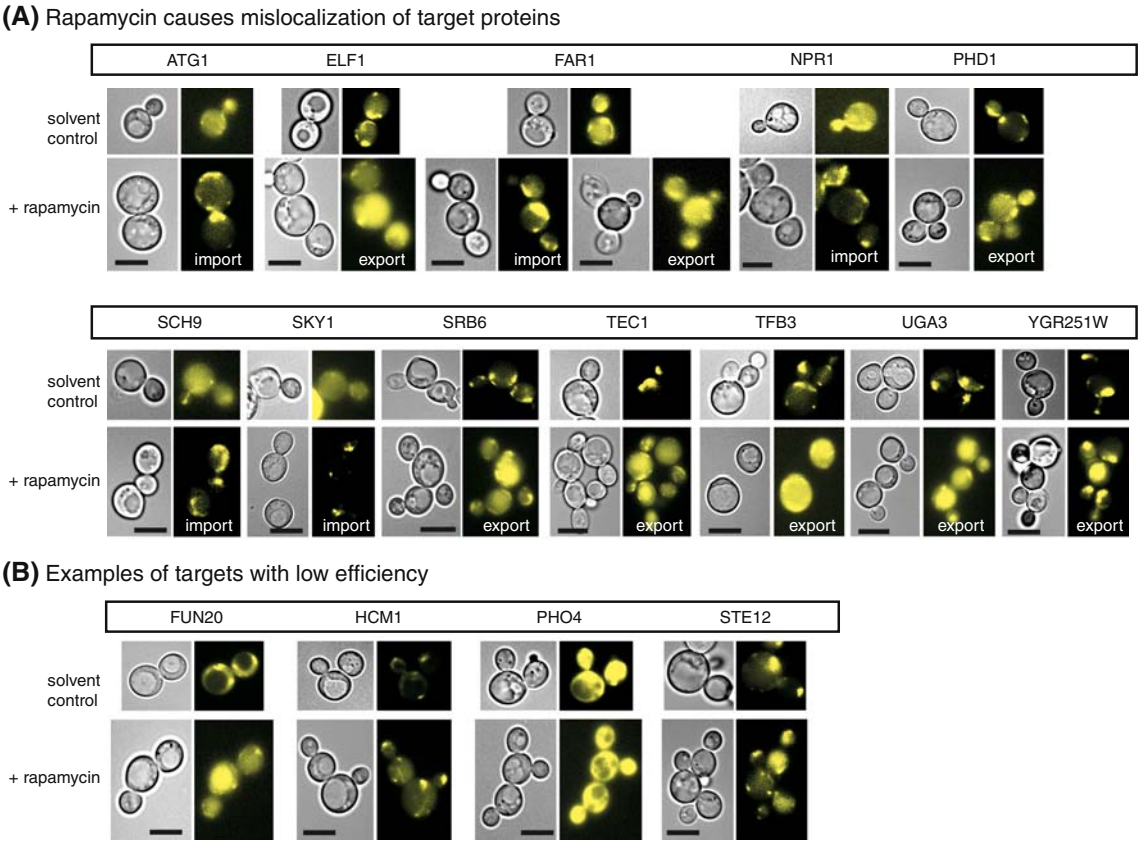

Fig. 3 Rapamycin induces nucleocytoplasmic trafficking of many targets. **a** Representative images of DIC and vYFP fluorescence are shown following 30-min treatment with rapamycin (10 μM) or a solvent control (0.1% DMSO). Scale bar is 3 μm. **b** Rapamycin-directed localization was inefficient (<5-fold change) for four targets

FKBP-NLS and FKBP-NES constructs, respectively. Interestingly, both options were successful and this factor could be directed to reside principally in the nucleus or cytoplasm, depending on the FKBP chimera used (Fig. 3a).

Efficiency of Transport Might be Influenced by the Target's Protein–Protein Contacts

Rapamycin treatment successfully controlled the subcellular distribution of most targets; however, the nuclear proteins Fun20p, Hcm1p, and Ste12p were partially resistant to rapamycin treatment, as was the phosphate-regulated transcription factor Pho4p (Fig. 3b). To explore the origins of this differential efficiency, we calculated the expected molecular mass of the targets because we reasoned that large proteins might encounter transport barriers. However, we found no correlation between predicted molecular mass and transport efficiency; for example, Sch9p (92 kDa) was a better target than Fun20p (42 kDa), despite being two-fold larger. Next, we reasoned that the number of protein–protein contacts between the target and its cellular partners might influence efficiency. Using co-immunoprecipitation/mass spectrometry data deposited in SGD [18–20], we calculated the predicted number of protein interactions for each target and, from this analysis, it was striking that the four proteins least accessible to mislocalization possessed an average of ~45 known protein–protein interactions, while the 12 most amenable targets averaged only 3.4 (Fig. 4). This analysis is highly limited by the low sample number but the results suggest that proteins in multi-component ensembles might be more resistant. It is important to note that other factors likely contribute because the low efficiency target, Hcm1, is predicted to have only three interactors. Despite the preliminary nature of this assignment, the empirical correlation is intriguing and future experiments will discern whether protein connectivity might be predictive of "good" and "bad" targets.

The Kinetics and Reversibility of Rapamycin-Directed Nuclear Export

For this technology to be employed in a range of applications, it would be useful to understand how quickly a protein could be reversibly imported/exported. In previous studies, we explored the kinetics of a model chimera (vYFP-FRB) and found that it was rapidly exported or

gene	# of interactions *
ATG1	1
ELF1	5
FAR1	12
FUN20	31
HCM1	3
NPR1	1
PHD1	1
PHO4	125
SCH9	2
SKY1	1
STE12	20
TEC1	5
TFB3	8
UGA3	0
YGR251W	3

* see ref 18-20.

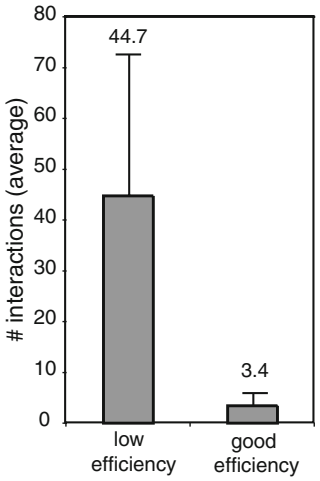

Fig. 4 Efficiency of nucleocytoplasmic trafficking is reduced for proteins with many binding partners. The number of predicted interactions for each gene is shown (values taken from references [18–20]). Targets with greater than five-fold change in nuclear localization had a significantly lower average number of interactors (3.4 ± 1.6), while the low efficiency targets had a higher number of interactors (44.7 ± 27.5; see Sect. Materials and Methods). Error is standard error measurement

imported within approximately 10 min [4]. To explicitly test whether a target protein shares this property, we examined the Uga3 fusion in the presence of FKBP-NES at various times after addition of rapamycin (Fig. 5). Starting at 5 min after treatment, the levels of the Uga3-vYFP-FRB protein were noticeably reduced in the nucleus. Within 10–15 min, the Uga3 was mostly exported and, by 20 min, less than 10% of the original signal remained in that compartment. This export was reversible; replacing the media reverted the system to a resting state within 20 min (Fig. 5).

Conditional Mimicry of the Growth Phenotype of Essential Genes

One potential use of conditional mislocalization is to generate conditional alleles for essential genes. To directly explore this idea, we measured the viability of cells expressing vYFP-FRB fusions to the essential targets, SRB6 or TFB3. Treatment with rapamycin impaired growth of the modified cells but not the parent strain (*fpr1Δ, tor1-1*) or the strains lacking the FKBP-NES partner (Fig. 6). These studies confirm that temporary inactivation of essential targets can partially phenocopy growth defects of the null mutant.

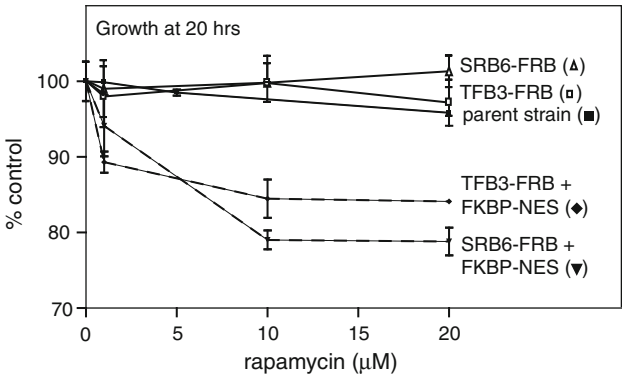

Fig. 6 Conditional export of essential targets partially phenocopies the null mutation. Rapamycin treatment caused slow growth in cells expressing FKBP-NES and the essential gene fusions to vYFP-FRB. This phenotype was dependent on nuclear mislocalization, because the same treatment had no effect on the parent strain (*tor1-1, fpr1Δ*) or the strains lacking the FKBP-NES partner. Experiments were performed in triplicate and error bars indicate standard deviation of the mean

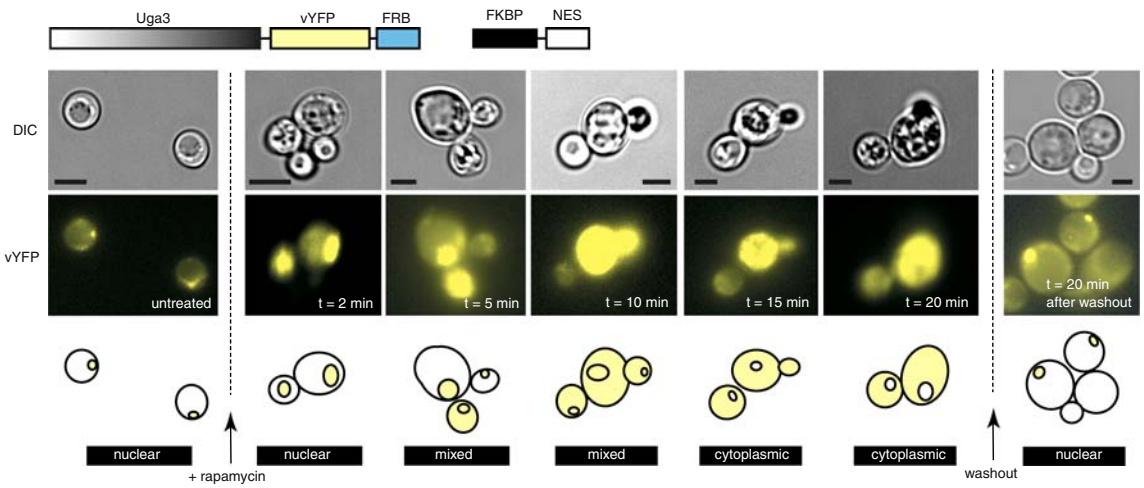

Fig. 5 Nuclear export of Uga3p is rapid. Addition of 10 μM rapamycin (time = 0) triggered nuclear export within approximately 10–15 min and, by 20 min, nuclear levels of Uga3p were decreased 20-fold. Reducing rapamycin levels by replacing the culture media restored original nuclear localization within 20 min. A schematic of the results are shown beneath the images. Scale bar is 3 μm

Conclusions

Signaling networks exploit nucleocytoplasmic shuttling to gain rapid and reversible regulatory control. We have generated a synthetic version of this regulatory mechanism that places protein localization under control of a membrane-permeable CID. The current study shows that this system can be used to dictate the position of a subset of different yeast proteins. From the set of 16 gene products, 75% were suitable for conditional mislocalization and both import and export were achieved with similar efficiency. Although we sampled a modest set of targets, the initial findings suggest that this system is most effective for cytoplasmic proteins exhibiting relatively few contacts with other cellular factors. For example, the 12 best targets are predicted to have an average of 3.4 partners and never more than 12, while the 4 worst targets have 3, 20, 31, and 125. Because the average number of interactions across the yeast proteome is approximately 5 [25, 26], we predict that a substantial subset of genes might be amenable to this technology. These findings provide insight into possible design criteria and expectations for large-scale studies of protein location.

In this report, we have explored the versatility of a CID-based system for controlling nucleocytoplasmic shuttling; however, replacing the NES or NLS with another address signal, such as a membrane localization sequence or an endoplasmic reticulum (ER) retention marker, could be used to expand this system [9, 27]. In support of this idea, a myristoylation domain has been used to conditionally target a protein to the plasma membrane in a mammalian system [28] and fusions to membrane-bound proteins have been used to mislocalize targets in a system termed "Anchors Away" [29]. Clearly, implementing these and similar methodologies will open a range of possibilities for studying the role(s) of dynamics in protein localization.

More generally, many techniques for systematically regulating protein function, such as temperature sensitive alleles and inducible transcription reporters (e.g., Tet-on/off systems), have been widely explored in yeast and other organisms [10]. How does CID-mediated, conditional mislocalization add to this battery of methods? In this report, we found that mislocalization to the cytosol was able to partially re-capitulate the growth defects of the essential transcription factors, Srb6p and Tfb3p (Fig. 6). This result highlights one potential use of this method: studying the proximal effects of acute disruption of essential functions. Other potential applications include testing whether subcellular location is sufficient for initiation or maintenance of a process [4] and exploring models of asymmetric inheritance during cell division. These applications require specific and reversible control over subcellular position and, thus, fall within the projected domain of this method. In this context, the findings presented here and others [29] begin to define some dominant features and limitations of the technology, which should aid future experimental design and interpretation.

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