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Rapid regulation of nuclear proteins by rapamycin-induced translocation in fission yeast

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Abstract

Genetic analysis of protein function requires a rapid means of inactivating the gene under study. Typically this exploits temperature sensitive mutations, or promoter shut-off techniques. We report the adaptation to *Schizosaccharomyces pombe* of the Anchor Away technique, originally designed in budding yeast (Haruki et al., 2008a). This method relies on a rapamycin-mediated interaction between the FRB and FKBP12 binding domains, to relocalize nuclear proteins of interest to the cytoplasm. We demonstrate a rapid nuclear depletion of abundant proteins as proof-of-principle.

Introduction

Analysis of gene function in yeasts typically requires an effective way to inactivate the protein of interest. The classic way to do this relies on temperature sensitive mutations (Bonatti et al., 1972; Hartwell, 1967). These protein products have attenuated activity at higher or lower temperature. However, temperature-sensitive mutants can be hard to isolate, slow to inactivate, and residual activity can give ambiguous results. A modification of this method uses a temperature-sensitive degron that leads to ubiquitylation and protein turnover by the proteasome in response to temperature (e.g, (Lindner et al., 2002)). An auxin-inducible degron system was developed to deplete protein level without introducing temperature stress (Kanke et al., 2011). However, not all proteins have proven amenable to these modifications.

Another option is to control gene expression. The challenge is identifying a promoter that can be rapidly repressed. In fission yeast, the *nmt1*⁺ promoter (Maundrell, 1990) and its derivatives (Basi et al., 1993) are frequently used. However, the lag in induction and leakiness upon repression have made these promoters useful primarily for already unstable proteins (Forsburg, 1993). The *urg1*⁺ promoter is capable of fast induction (>2 hours) in the

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presence of uracil and a 10-fold reduction of protein level was achieved 360 min after uracil removal (Watson et al., 2011). Genes regulated by a tetracycline-regulated promoter show even shorter induction time (~30 min) and reach maximum level around 4 hours (Zilio et al., 2012), but repression has not been characterized. Alternatively, DAmP (decreased abundance by mRNA perturbation) alleles were used to generate hypomorphic alleles by reducing the transcription level (Schuldiner et al., 2005). Again, although the mRNAs are subjected to fast turnovers, the proteins may still linger in the cells.

A third strategy is to sequester the protein. The hormone-binding domain (HBD) of the estrogen receptor attracts the Hsp90 complex. Proteins tagged with HBD form a complex with Hsp90, therefore are inactivated by steric hindrance (Boe et al., 2008). Upon addition of beta-estradiol, the HBD ligand, fusion proteins are released from the complex and activated. However, many HBD-fused nuclear proteins do not respond to beta-estradiol (Pai et al., 2012).

The anchor away (AA) system was developed in *Saccharomyces cerevisiae* to target nuclear proteins (Haruki et al., 2008a). This system exploits the rapamycin-driven interaction that occurs between two binding domains: the FRB domain, found in the TOR1/Tor1 protein, and the FKBP12 domain, found in the FRP1/Fkh1 protein (Otsubo and Yamamoto, 2008; Weisman, 2004). The system places the FKBP12 domain on a highly expressed cytoplasmic protein (anchor), and the FRB domain on a target protein of interest. In the presence of rapamycin, the target protein is depleted from the nucleus and anchored in the cytoplasm, allowing analysis of its depletion phenotype. We engineered the anchor-away (AA) system in the fission yeast *Schizosaccharomyces pombe* to target nuclear proteins to the cytoplasm. To eliminate competition from the endogenous TOR system, we used an allele *tor1-SE* that specifically blocks rapamycin binding to Tor1, and a strain deleted for *fkh1*⁺ (FKBP12) (Weisman et al., 2001). We applied this approach to two abundant nuclear proteins, Rad11 and Mcm4. Our results suggest that while the AA system will be a useful tool to study protein function, it has some limitations for use in fission yeast.

Materials and methods

Strains, growth conditions and basic manipulations

Strains are listed in Table 1. FY7128 was constructed by integrating NruI linearized pLD141 into the *leu1-32* locus in FY6653. Media and manipulations were performed as described in (Forsburg and Rhind, 2006).

Rapamycin (Sigma, R0395) was dissolved in DMSO. Final concentration is 2.5µg/mL. Strains with the Rpl13-2FKBP12 anchor were maintained in EMM–LEU+thiamine (15µM = 5µg/mL). Experiments (serial dilution assay and microscopy) were done in YES media. Electroporation was used to transform cells (Forsburg and Rhind, 2006).

LiOAc-SDS-based colony PCR was optimized for *S. pombe* from Looke et al. (2011). Single colony was resuspended in 100µL LiOAc-SDS buffer (200mM LiOAc, 1% SDS) in a 1.5mL eppendorf tube and incubated at 70°C. Three volumes (300µL) of 96% ethanol were added after 15 minutes. The sample was mixed by vortexing. The mixture was centrifuged at

15,000g for 3 minutes. The supernatant was removed by aspiration. After the pellet was completely air-dried, it was resuspended in 100µL TE and centrifuged at 15,000g for 3 minutes. The entire tube can be stored at 4°C for months without separating the supernatant (contains genomic DNA) and the pellet. The refrigerated sample was vortexed and centrifuged again before setting up the PCR experiments. One microliter of supernatant is sufficient for a 20µL PCR reaction.

Crosses were performed on SPAS plates (Forsburg and Rhind, 2006). Mating type was determined by colony PCR using primers: MT1: AGAAGAGAGAGTAGTTGAAG, MP: ACGGTAGTCATCGGTCTTCC and MM: TACGTTCACTAGACGTAGTG (Roguev et al., 2007).

Plasmids

Plasmids are listed in Table 2. Restriction maps are in Figure S1. pFA6a-FRB-KanMX6, pFA6a-FRB-GFP-KanMX6, and pFA6a-2xFKBP12-His3MX6 were purchased from Euroscarf (P30578, P30580, and P30583).

pLD103 is a C-terminus 2FKBP12 tagging plasmid. It was made by replacing the His3MX6 cassette between BglII and PmeI from pFA6a-2xFKBP12-His3MX6 (Haruki et al., 2008a) (EUROSCARF: P30583) with the HphMX6 cassette from pFA6a-hph (Hentges et al., 2005). pLD133 is an episomal expression vector. It was made by replacing the C-terminus 3HA tagging fragment between NotI and SalI in pSGP72 with C-terminus 2FKBP12 tagging fragment amplified from pLD103. pLD134 was made by inserting *rpl13⁺* ORF (lacking the stop codon) cloned from the *S. pombe* genomic DNA into pLD133 between XhoI and NotI sites. pLD134 is capable of expressing *rpl13-2fkbp12* from the *nmt1* promoter on an episomal plasmid. We also created a tagging integration vector, pLD138 by inserting the *2fkbp12-c* flanked by *nmt1* promoter (*Pnmt1*) and terminator (*Tnmt1*) released from pLD133 with PstI and SacI into pJK148. Finally, we made pLD141 by inserting a fragment of *Pnmt1-rpl13⁺* released from pLD134 with PstI and NotI into pLD138. pLD115 was made by replacing the KanMX6 cassette between BglII and PmeI in pFA6a-FRB-KanMX6 (EUROSCARF: P30578) with NatMX6 from pFA6a-NatMX6 (Hentges et al., 2005). pLD116 was made by replacing the KanMX6 cassette between BglII and PmeI in pFA6a-FRB-GFP-KanMX6 (EUROSCARF: P30580) with NatMX6 from pFA6a-NatMX6 (Hentges et al., 2005).

pLD114 is an episome that tags a protein of interest at the C terminus with FRB-GFP and expresses it under the *nmt1⁺* promoter. pLD133 provides the 2FKBP12 tag in a similar *nmt1* episomal vector, and can be used to screen anchors.

Generating anchor away strains

Amplification of C-FRB or C-FRB-GFP module—pLD115 can be used as template to fuse FRB to the carboxy terminus of your favorite protein. pLD116 can be used as template to fuse FRB-GFP to the carboxy terminus of your favorite protein. Both plasmids are pFA6a derivatives. The PCR primers used from the templates are Forward Primer: 5'-(gene-specific sequence)- CGGATCCCCGGGTAAATTAA-3' and Reverse Primer: 5'-(gene-specific

sequence)-GAATTCGAGCTCGTTTAAAC-3' (Bahler et al., 1998). The procedure of amplify the transformation module is described in (Bahler et al., 1998).

Via direct transformation—Base strain (FY7128 or FY7129) was grown at 32°C in EMM-LEU+thiamine liquid to mid-log phase. Cells were harvested and electroporated with the transformation module. Transformed cells were plated on EMM-LEU+thiamine plates and incubated overnight at 32°C. The plates were replica plated on YES+G418+Nat plates and incubated at 32°C for 2-4 days (colonies usually appear in 2 days). Transformants were restreaked onto EMM-LEU+thiamine plates immediately after visible and incubated at 32°C for 3-4 days, then replica plated on YES+G418+Nat plates. The G418+Nat resistant transformants were tested by colony PCR and confirmed by sequencing. Proteins tagged with FRB-GFP were also confirmed by Western blotting.

Via genetic crosses— *fkh1* (FY6609) was electroporated with the transformation module. Transformants were isolated as Nat resistant candidates and isolated using the same protocol as the *via direct transformation* section above. The correct transformant was crossed with *tor1SE Pnmt1-rpl13-2FKBP12* strain (FY6792) on SPAS or ME. Random spore analysis (RSA) procedure was used to select the final assay strain. The spores were germinated and grow on EMM-LEU+thiamine plate then replica plated onto YES +G418+Nat and –URA plate.

Western blotting

The procedure was the same as in Ding and Forsburg (2011). Fifty micrograms of total protein were separated by SDS-PAGE in a 8% acrylamide gel. JL8 (Clontech; 1:1000) was used to detect GFP. PC10 (Delta Biolabs; 1:1000) was used to detect PCNA. Anti-mouse-HRP (Sigma; 1:2000) was used to recognize the primary antibodies. The signal was captured by the ChemiDoc system (Bio-Rad) under ChemHi Sensitivity setting. The exposure time was 999 seconds. The images were taken with Quantity One software (Bio-Rad).

Microscopy

A single colony was grown at 32°C in 3mL YES overnight to ~OD=0.8. One half milliliter of culture was mixed with 0.5mL YES+ 5µg/mL rapamycin (1µL 2000X stock) or 0.5mL YES+ 1µL DMSO in 15mL culture tubes. The culture tubes were shaken vigorously in a 32°C shaker during the time-course. One hundred microliters of culture was collected in 1.5mL eppendorf tube and centrifuged at 3000 rpm for 30 seconds. Ninety microliters of supernatant was removed and the pellet was resuspended in the rest of the media. Three microliters of the suspension was mounted on a 2% SPAS agar pad.

All images were taken on a DeltaVision core microscope (Applied Precision/GE) using a GFP/mCherry polychroic mirror and oil-immersion Olympus 60X lens with Numerical Aperture of 1.4. Eighteen z-sections at 0.3µm were taken to reconstruct the projected images in softWoRx 5.5. Cell length was measured using the distance tool in softWoRx 5.5 (Applied Precision/GE).

Digital image manipulation

All the plates and films were electronically scanned using a ScanJet IIcx scanner (Hewlett-Packard). Digitized pictures/photos were analyzed and contrast-enhanced by NIH ImageJ software and assembled in Canvas software (ACD Systems).

Results

Construction of the anchor-away strains

The TOR kinases are part of a conserved eukaryotic signaling system associated with stress response and metabolism (reviewed in Otsubo and Yamamoto (2008)). *S. pombe* Tor1 and Tor2 each contain a conserved FRB (FKBP12-rapamycin binding) domain. Tor1 is not essential, but is required for proper response to stress, for amino acid uptake, and for mating (Kawai et al., 2001; Weisman and Choder, 2001; Weisman et al., 2005). Tor2 is essential for viability (Alvarez and Moreno, 2006; Matsuo et al., 2007; Uritani et al., 2006; Weisman and Choder, 2001; Weisman et al., 2007). Rapamycin binds to the FKBP12 domain in Fkh1, and results in formation of a stable complex with the FRB domain in TOR (reviewed in Otsubo and Yamamoto (2008)). The Anchor-Away system exploits this interaction by fusing the FKBP12 domain to an abundant cytoplasmic protein and the FRB domain to a target nuclear protein (Haruki et al., 2008a). The cytoplasmic anchor traps the target outside of the nucleus.

In contrast to *S. cerevisiae*, wild type *S. pombe* is not sensitive to rapamycin (Otsubo and Yamamoto 2008; Figure 2A). However, to eliminate competition from the endogenous TOR signaling system, we used a strain with a deletion of *fkh1*⁺ (eliminating the native FKBP12 domain), and combined this with *tor1-S1834E* (*tor1SE*) (Laor et al., 2014; Weisman et al., 2005). Mutation of the conserved serine (S) residue in the FRB domain of Tor1 to glutamic acid (E) residue dramatically reduces the interaction with Fkh1 in the presence of rapamycin (Weisman et al., 2005). In analogy to its paralog in the *S. cerevisiae* anchor away system (Haruki et al., 2008b), and because the *tor1SE fkh1* strain already had a mating phenotype, we left the essential *tor2*⁺ unchanged.

Two abundant non-nuclear proteins ScPma1 (a plasma membrane proton pump/ATPase) and ScRpl13A (a cytoplasmic ribosomal 60S subunit protein) were used as anchors in *S. cerevisiae* (Haruki et al., 2008a). We therefore constructed anchors using their *S. pombe* orthologs, SpPma1 (*pma1*⁺: SPAC1071.10c) and SpRpl13 (*rpl13*⁺: SPAC664.05).

We generated a tagging plasmid, pLD103, from pFA6a-2xFKBP12-His3MX6 (EUROSCARF: P30583). The new tagging plasmid is marked by HphMX6 and used to tag potential anchors at the C-terminus. We tagged *pma1*⁺ at its native locus with two copies of FKBP12 (FY6554 and FY6555). However, the tagged strains had a slow growth and sterile phenotype (data not shown). Therefore, we focused on Rpl13, which was a better anchor in budding yeast (Haruki et al., 2008a). Unlike *Sc RPL13*, *Sp rpl13*⁺ is an essential gene (Hayles et al., 2013; Kim et al., 2010). We were not able to insert a C-terminal 2FKBP12 tag at its native locus. To solve this problem, we created pLD141, a *leu1* integration plasmid that expresses *rpl13-2FKBP12* from the *nmt1* promoter. pLD141 was linearized with NruI and integrated at *leu1-32* in a *tor1SE* strain (FY6641). This maintains the wild type *rpl13*⁺ in

addition to the integrated, tagged version. The resulting strains (FY6792 and FY6793) were unable to mate with h^+ wildtype cells on SPAS but mated successfully on ME. Interestingly, *tor1-SE* alone was mating competent (data not shown). This is consistent with a slightly decreased mating efficiency of *tor1-SE* (Weisman et al., 2005). *S. pombe* Tor1 is known to play a role in sexual development and nitrogen starvation responses (Kawai et al., 2001; Weisman and Choder, 2001; Weisman et al., 2007); this suggests some impairment of *tor1-SE* that is background dependent. Similarly, we observed that *tor1-SE fkh1* in the absence of the anchor was also partially sterile on SPAS, although the mating efficiency was slightly better on ME (data not shown).

Despite the mating defects, none of the strains show any growth defects or sensitivity to DNA damaging agents (Figure 2A). To investigate whether the anchor away technique works in *S. pombe*, we conducted proof-of-principle experiments using three well-studied essential nuclear proteins: Rad11 (Rpa1; Parker et al., 1997), Mcm4 (Coxon et al., 1992), and Mcb1 (Ding and Forsburg, 2011; Li et al., 2011; Santosa et al., 2013). We tagged each gene with FRB-GFP domain using the tagging plasmid pFA6a-FRB-GFP-KanMX6 (EUROSCARF: P30580) in a wildtype strain. All three proteins were successfully tagged as assessed by Western blot (Figure S2). We constructed the assay strains as follows (Figure S3): we crossed each tagged candidate into a *fkh1* strain (FY6609), then crossed it to *tor1SE* strain (FY6641). Since both our target genes and *tor1SE* were marked by Kan, we pulled tetrads and isolated nonparental ditypes (NPD) (we later made Nat marked pLD116 and pLD115 to avoid pulling tetrads). Finally, since *tor1-SE fkh1* is partially sterile, we integrated the NruI-linearized pLD141 at the *leu1-32* locus to provide the anchor.

To simplify the system, we generated base strains (FY7139 and FY7128) that contain *fkh1 tor1-SE* and the integrated cytoplasmic anchor Rpl13-2FKBP12. These strains have neither growth defects nor drug sensitivity (Figure 2A), though they were partially sterile (data not shown). We converted the drug marker in the tagging plasmids from KanMX6 to NatMX6 (pLD115 and pLD116) to facilitate tagging the target protein with FRB or FRB-GFP, respectively. The assay strain of a gene of interest can be constructed simply by a direct transformation method (Figure 1A) or via a genetic cross (Figure 1B).

Proof of concept

Rad11-FRB-GFP—RPA, replication protein A, is a nuclear protein complex composed of Rpa1, Rpa2, and Rpa3. The heterotrimeric complex coats the single-stranded DNA (ssDNA) and plays important roles in DNA replication, DNA repair, and homologous recombination (Wold, 1997). The accumulation of Rpa1/Rad11, the largest subunit, has been used as a measure of ssDNA level (Sabatinos et al., 2012). Since *rad11*⁺ is an essential gene, no full deletion can be made, but a temperature sensitive allele *rad11A* has been characterized (Parker et al., 1997), and a hypomorphic allele shows DNA damage sensitivity (Ono et al., 2003). We investigated whether the anchor-away system generates a reliable conditional *rad11* with an appropriate phenotype. First, we followed the outline in Figure S3 to make an assay strain expressing *rad11-FRB-GFP* (FY6962). It has no growth defect or drug sensitivity, suggesting it is fully functional. As expected, viability was reduced in the presence of rapamycin. Importantly, expressing *rad11-FRB-GFP* in a wild type *tor1*⁺ *fkh1*⁺

strain with anchor (FY7189) was not sensitive to rapamycin (Figure 2B), indicating that the anchor-away system requires neutralization of the Tor1-Fkh1 interaction.

Next, we explored the dynamics of the nuclear depletion of Rad11 by visualizing the GFP signal every hour over a 5-hour time-course (Figure 3). Rad11-FRB-GFP localized in the nucleus in asynchronous cells at the beginning of the time-course (T=0). Following the addition of rapamycin, we observed translocation of Rad11-FRB-GFP from the nucleus to cytoplasm within an hour (Figure 3A). One hour after the treatment, 65% of the population exhibited GFP signal in the cytoplasm, and 35% showed a nuclear void (Figure 3B). Although binucleate cells were still observed, the population showed cell elongation, reminiscent of the *cdc* phenotype observed in *rad11A* mutants (Figure 3C, D; Parker et al. 1997). In two hours, the nuclear void population was predominant. There were about 5% of cells with punctate nuclear and cytoplasmic GFP signals, accompanied by a dramatic increase of cell length. Three hours into the treatment, 47% of cells had nuclear GFP puncta. By the end of the time-course, 89% of treated cells showed a punctate nuclear GFP signal, the nuclear GFP signal remained the same in untreated cells and mock cells. A large population of the treated cells were highly elongated (38% >15µm), compared to untreated (6% >15µm) and mock cells (12% >15µm).

We also generated another assay strain of *rad11-FRB-GFP* (FY7222) using the base strain and following the protocol showed in Figure 1A. We found a similar response to rapamycin with FY7222 compared to FY6962 (Figure 2B, Figure S4), again, with evidence for nuclear puncta particularly by 5h. There was no significant difference in the effect observed using rich media (YES) compared to EMM (data not shown), so further experiments were performed in YES.

Mcm4-FRB-GFP—Mcm4 is a component of the heterohexameric MCM complex that is the primary replicative helicase (reviewed in Forsburg (2004)). An essential gene, *mcm4*⁺ has been studied using a number of conditional alleles (Kanke et al., 2011; Lindner et al., 2002; Nitani et al., 2008). We compared these phenotypes to an anchor away version of Mcm4. The anchor away strain of Mcm4-FRB-GFP exhibited a slow growth phenotype even in the absence of rapamycin, and was sensitive to a wide range of DNA damaging drugs (Figure 2B), suggesting that this protein function is attenuated in this background. Consistent with this, we observed substantial elongation of the cells in the starting strain.

Mcm4 is an abundant nuclear protein (Kearsey et al., 2000). Prior to treatment, Mcm4-FRB-GFP localized in the nucleus, but the signal was not strong (Figure 4A). In the presence of rapamycin we observed rapid disappearance (~ 1 hour) from the nucleus. Unlike Rad11-FRB-GFP, Mcm4-FRB-GFP remained in the cytoplasm at later time-points, and the cells ceased dividing (Figure 4B). The treated cells were dramatically elongated by the end of the time-course (Figure 4D).

Mcb1-FRB-GFP and Mcb1-FRB—Mcb1, an abundant essential protein, is localized in nucleus and cytoplasm (Ding and Forsburg, 2011; Li et al., 2011). We tagged the Mcb1 with FRB-GFP in a wildtype strain. Similar to Mcb1-GFP (Ding and Forsburg, 2011), we were not able to visualize it, although it is detectable by Western blot (Figure S2). We crossed the

mcb1-FRB-GFP (FY6549) strain with *fkh1* (FY6609), then crossed the resulting strain with FY6792 to make the assay strains of *mcb1-FRB-GFP* (FY7194 and FY7195). Neither assay strain is rapamycin sensitive (data not shown). To rule out the possibility that the GFP in FRB-GFP tag hindered rapamycin access, we constructed assay strains of *mcb1-FRB* (FY7196 and FY7197) followed the outline in Figure 1A using both base strains. Again, we found Mcb1 did not respond to rapamycin (Figure 2B).

Discussion

The anchor-away system depletes nuclear proteins by exploiting a rapamycin-dependent interaction between two protein domains, FKBP12 and FRB, derived from the TOR signaling network (Haruki et al., 2008a). We have adapted this system to fission yeast, with mixed results. We used a test strain *tor1-SE fkh1* that eliminates Tor1-Fkh1 interactions in the presence of rapamycin, and expresses an ectopic copy of *rpl13-2FKBP12* from the *nmt1* promoter. However, this strain is sterile, so any genetic manipulations must be performed on its precursors.

We examined three abundant nuclear proteins required for DNA replication. We observed the most success with Rad11-FRB-GFP. The strain was sensitive to rapamycin only in the presence of *tor1-SE fkh1*, but showed no other significant drug sensitivities. The protein was depleted rapidly from the nucleus accompanied by cell elongation upon treatment with rapamycin. However, at later time-points, discrete GFP puncta appeared in the nucleus. Since RPA foci are a metric for DNA damage and single stranded DNA (ssDNA) accumulation (Sabatinos et al., 2012), this suggests that the protein can re-enter into the nucleus and bind ssDNA. Importantly, we observed no effect on growth or sensitivity to DNA damaging agents in the absence of rapamycin.

Success was mixed with the Mcm4-FRB-GFP strain. In the absence of *tor1-SE fkh1*, the strain was healthy. However, in the *tor1-SE fkh1* background, even without rapamycin, the strain had a slow-growth phenotype and sensitivity to DNA damage. The cells were already elongated prior to rapamycin treatment. Following treatment, the GFP signal disappeared from the nucleus and the phenotype worsened. However, the growth defects without rapamycin make this of limited utility. It is not clear why the *mcm4-FRB-GFP tor1-SE fkh1 rpl13-2FKBP12* strain shows evidence for damage without rapamycin treatment, when the individual components do not. These data suggest that Mcm4 function is attenuated. Cells lacking *tor1* are also DNA damage sensitive (Schonbrun et al., 2009); the *tor1-SE fkh1* mutant may be sufficiently attenuated to cause a synthetic phenotype. Previous studies have suggested that while tagged MCM proteins are functional, they may be prone to genetic interactions in combination with other mutations (Gómez et al., 2002).

We were unable to deplete the essential Mcb1 protein. In a previous study, we showed that Mcb1-GFP, despite being stable and abundant, could be detected immunologically but could not be observed by fluorescence (Ding and Forsburg, 2011). Similarly, we were unable to observe Mcb1-FRB-GFP and found no effect of rapamycin treatment on Mcb1-FRB-GFP or Mcb1-FRB. Together, these observations suggest that the C-terminal fusions on Mcb1 are

inaccessible or otherwise structurally hidden. Therefore, not all proteins will be successfully tagged.

In addition to the bulky tag and relative lack of markers, the system may be affected by the mutations *tor1-SE* and *fkhl*, which are required to see the depletion, but also cause sterility and potentially other defects. Importantly, Tor2 is still intact and contains an FRB domain and potentially also could contribute to the phenotypes. It may be possible to fine-tune the system by adjusting the *tor* alleles used, including use of a *tor2-SE* allele. Alternatively, it might be sufficient to use *fkhl* and wild type *tor1*⁺. The effectiveness of the system may also be influenced by the anchor or target protein shuttling in and out of the nucleus. We conclude that this system may be of use on a case-by-case basis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Outlines of anchor-away assay strain construction

(A) Construction of an assay strain via direct transformation. (B) Construction of an assay strain via genetic crosses. See Materials and methods section for details.

Figure 2. Drug sensitivity tests of indicated strains
(A) FY11, FY7128, FY7139, FY6792, FY7135, FY1107, FY6641, FY6609. (B) FY7189, FY6962, FY7222, FY7190, FY6992, FY7188, FY7196, FY7197. 1:5 serial dilution on YES plates containing indicated drugs. Plates were scanned 2 days after incubated at 32°C.

Figure 3. Rad11-FRB-GFP is translocated from nucleus to cytoplasm in the presence of Rapamycin

(A) Representative pictures of re-localization of Rad11-FRB-GFP. Left, no change in localization was observed in the absence of Rapamycin at 100 or 300 minute. DMSO is the solvent control. Right, Rad11-FRB-GFP was removed from the nucleus until approximately 300min after Rapamycin treatment. Bar = 15 μ m. (B) Cells with different patterns of GFP signal were counted, $n>50$. At later time-points, speckles of Rad11, distinct from the original nuclear distribution, were observed. (C) Cells were counted and categorized as mononucleated, binucleated, and binucleated with septa, $n>50$. (D) Cell length was measured and categorized into 5-12 μ m, 12-15 μ m, and >15 μ m, $n=50$.

Figure 4. Mcm4-FRB-GFP is translocated from nucleus to cytoplasm in the presence of Rapamycin

(A) Representative pictures of re-localization of Mcm4-FRB-GFP. Left, no change in localization was observed in the absence of Rapamycin at 100 or 300 minute. DMSO is the solvent control. Right, Mcm4-FRB-GFP was removed from the nucleus until approximately 300min after Rapamycin treatment. Bar = 15 μ m. (B) Cells with different patterns of GFP signal were counted, $n>50$. (C) Cells were counted and categorized as mononucleated, binucleated, and binucleated with septa, $n>50$. (D) Cell length was measured and categorized into 5-12 μ m, 12-15 μ m, and >15 μ m, $n=50$.

Table 1

Strains

Strain	Genotype	Source
FY11	<i>h- ade6-M210</i>	Our stock
FY1107	<i>h- rad3::ura4⁺ ura4-D18 leu1-32 ade6-M210</i>	Our stock
FY6609	<i>h- fkh1::ura⁺ ura4 -D18 leu1-32 ade6-M210</i>	From TA96 (Weisman et al., 2001)
FY6641	<i>h⁺ tor1SE:KanMX ura4 -D18 leu1-32</i>	From TA1463 (Laor et al., 2014)
FY6792	<i>h⁺ tor1SE:KanMX leu1-32::[nmt1-rpl13-2FKBP12-leu1⁺] ura4-D18</i>	This study
FY6962	<i>h- rad11-FRB-GFP::kan leu1-32::[nmt1-rpl13-2FKBP12-leu1⁺] tor1SE:KanMX fkh1::ura⁺ his3-D1 ura4 -D18 leu1-32</i>	This study
FY6992	<i>h⁺ mcm4-FRB-GFP::kan leu1-32::[nmt1-rpl13-2FKBP12-leu1⁺] tor1SE:KanMX fkh1::ura⁺ ura4-D18 leu1-32 ade6- M210</i>	This study
FY7128	<i>h- tor1SE:KanMX fkh1::ura⁺ leu1-32::[nmt1-rpl13-2FKBP12-leu1⁺] ura4 -D18 leu1-32 ade6-M210</i>	This study
FY7139	<i>h⁺ tor1SE:KanMX fkh1::ura⁺ leu1-32::[nmt1-rpl13-2FKBP12-leu1⁺] ura4 -D18 leu1-32 ade6-M210</i>	This study
FY7135	<i>h- fkh1::ura⁺ leu1-32::[nmt1-rpl13-2FKBP12-leu1⁺] ura4 -D18 leu1-32 ade6-M210</i>	This study
FY7189	<i>h⁺ rad11-FRB-GFP::kan leu1-32::[nmt1-rpl13-2FKBP12-leu1⁺] his3-D1 ura4-D18 leu1-32 ade6-M210</i>	This study
FY7222	<i>h⁺ rad11-FRB-GFP::nat tor1SE:KanMX fkh1::ura⁺ leu1-32::[nmt1-rpl13-2FKBP12-leu1⁺] ura4 -D18 leu1-32 ade6- M210</i>	This study
FY7190	<i>h⁺ mcm4-FRB-GFP::kan leu1-32::[nmt1-rpl13-2FKBP12-leu1⁺] his3-D1 ura4-D18 leu1-32 ade6-M210</i>	This study
FY7188	<i>h⁺ mcb1-FRB-GFP::kan leu1-32::[nmt1-rpl13-2FKBP12-leu1⁺] his3-D1 ura4-D18 leu1-32 ade6-M210</i>	This study
FY7196	<i>h- mcb1-FRB::Nat tor1SE:KanMX fkh1::ura⁺ leu1-32::[nmt1-rpl13-2FKBP12-leu1⁺] ura4 -D18 leu1-32 ade6-M210</i>	This study
FY7197	<i>h⁺ mcb1-FRB::Nat tor1SE:KanMX fkh1::ura⁺ leu1-32::[nmt1-rpl13-2FKBP12-leu1⁺] ura4 -D18 leu1-32 ade6-M210</i>	This study

Table 2

Plasmids

Plasmid	Purpose	Source
pFA6a-FRB-KanMX6	to tag gene of interest with FRB at endogenous locus; nourseothricin resistant	EUROSCARF: P30578
pFA6a-FRB-GFP-KanMX6	to tag gene of interest with FRB-GFP at endogenous locus; nourseothricin resistant one XhoI site between FRB and GFP (pFA6a-FRB-(XhoI)-GFP-kanMX6)	EUROSCARF: P30580
pFA6a-2xFKBP12-His3MX6	to tag gene of interest with FRB-GFP at endogenous locus; histidine prototroph	EUROSCARF: P30583
pLD103	pFA6a-2xFKBP12-hph: to tag gene of interest with 2xFKBP12 at endogenous locus; hygromycin B resistant	This study
pLD115	pFA6a-FRB-natMX6: to tag gene of interest with FRB at endogenous locus; nourseothricin resistant	This study
pLD116	pFA6a-FRB-(XhoI)-GFP-natMX6: to tag gene of interest with FRB-GFP at endogenous locus; nourseothricin resistant	This study
pLD133	Episomal expression vector that allows 2FKBP12 to be fused to C-terminus; gene of interest under full strength <i>nmt1</i> promoter, <i>LEU2</i>	This study
pLD134	Episomal expression vector of <i>rpl13-2FKBP12</i> , under full strength <i>nmt1</i> promoter, <i>LEU2</i>	This study
pLD138	<i>leu1-32</i> integration plasmid that allows 2FKBP12 to be fused to C-terminus; gene of interest under full strength <i>nmt1</i> promoter	This study
pLD141	<i>leu1-32</i> integration plasmid that expresses <i>rpl13-2FKBP12</i> under full strength <i>nmt1</i> promoter	This study