TOR1 and TOR2 Have Distinct Locations in Live Cells

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TOR is a structurally and functionally conserved Ser/Thr kinase found in two multiprotein complexes that regulate many cellular processes to control cell growth. Although extensively studied, the localization of TOR is still ambiguous, possibly because endogenous TOR in live cells has not been examined. Here, we examined the localization of green fluorescent protein (GFP) tagged, endogenous TOR1 and TOR2 in live S. cerevisiae cells. A DNA cassette encoding three copies of green fluorescent protein (3XGFP) was inserted in the TOR1 gene (at codon D330) or the TOR2 gene (at codon N321). The TORs were tagged internally because TOR1 or TOR2 tagged at the N or C terminus was not functional. The TOR1D330-3XGFP strain was not hypersensitive to rapamycin, was not cold sensitive, and was not resistant to manganese toxicity caused by the loss of Pmr1, all indications that TOR1-3XGFP was expressed and functional. TOR2-3XGFP was functional, as TOR2 is an essential gene and TOR2N321-3XGFP haploid cells were viable. Thus, TOR1 and TOR2 retain function after the insertion of 748 amino acids in a variable region of their noncatalytic domain. The localization patterns of TOR1-3XGFP and TOR2-3XGFP were documented by imaging of live cells. TOR1-3XGFP was diffusely distributed throughout the cytoplasm and concentrated near the vacuolar membrane, whereas the TOR2-3XGFP signal was cytoplasmic but predominately in dots at the plasma membrane. Thus, TOR1 and TOR2 have distinct localization patterns, consistent with the regulation of cellular processes as part of two different complexes.

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MATERIALS AND METHODS

**Yeast media.** Rich medium (YPD) or synthetic medium (SD or SC) was prepared as described previously (35). Rapamycin was added to YPD medium from dilutions of a 1-mg/ml stock in 90% ethanol-10% Tween 20 just before plates were poured.

**Strains with 3XGFP inserted into TOR1 or TOR2.** A sequence for 3XGFP with the Ste5G mutation and optimized for expression in yeast was amplified from pBS-3XGFP-TRP1 (22) with primers to TOR1 sequences outside the cassette. PCR with primers TOR1/Fwd/H11001 and TOR2/Rev/H11002—tor1/rev1 (for TOR1) and diploid TB50 (for TOR2) strains by a high-efficiency, simplified method, and the transformants were selected on SD-uracil (SD-ura) plates (secondary colonies). Plasmid pSH47 introduces Gal-inducible Cre on a 5-fluoroorotic acid removable plasmid. Tiny amounts of single colonies were inoculated into 4 ml of SC-ura containing 1% galactose and 1% raffinose in 15-ml disposable tubes. After induction for 5 h (30°C), the cells were collected by centrifugation, resuspended in a small residual volume, and streaked so as to obtain single colonies on YPD plates. These tertiary colonies were identified, circled, and labeled, and streaked on YPD plates and YPD plates containing G418 to test for excision of kanMX6 by Gal-induced Cre. A convention, for example, TOR1N-15-6-1, was used to indicate the gene and site followed by three single colony numbers (for primary [G418], secondary [SD-ura], and tertiary [YPD] colonies after the introduction of Cre). The tertiary colonies that had lost the kanMX6 marker were studied further.

**Validation of TOR1-3XGFP strains.** TOR2-3XGFP strains were verified as described later in the text. TOR1-3XGFP strains were verified as follows. Recombination to integrate the full 3XGFP cassette was confirmed by colony PCR with primers to TOR1 sequences outside the cassette. PCR with primers TOR1/Fwd/H11032—tor1/rev1 and TOR1/Rev/H11032 (Table 2) gave the 2,599-nt band diagnostic for kanMX6/Rev 3XGFP cassette 5′ to 3′.

**TABLE 1. Primers for 3XGFP tagging of TOR1 and TOR2 by using pOM3**

| Primer          | Target  | Sequence (5′ to 3′) |
|-----------------|---------|---------------------|
| TOR1-N-Fwd      | N terminus | GGTTAAAGTCAAACTACAAGGCTAGCGGGTGTGGTTGACATGAT |
| TOR1-N-Rev      | N terminus | ATG TGC AGG TCG ACA ACC CTT AAT |
| TOR1-D67-Fwd    | D67     | TGACTTCTAAGGTTTGATGGAGTGGATGAGTTGCCGTAATGGG |
| TOR1-D67-Rev    | D67     | TGC AGG TCG ACA ACC CTT AAT |
| TOR1-D330-Fwd   | D330    | ATACATGCTAGTTTGGTATTTAAATAGGAAAGATCTTGTGTTGATT |
| TOR1-D330-Rev   | D330    | TGC AGG TCG ACA ACC CTT AAT |
| TOR2-N-Fwd      | N terminus | GCG GCC GCA TAG GCC ACT |
| TOR2-N-Rev      | N terminus | TCTTCTCAAAGAGATGTTGATTTCCATCCCATAATGAA |
| TOR2-N321-Fwd   | N321    | AGACAAATTTAATGGGCGTGTTAATGTAATGTTTAT |
| TOR2-N321-Rev   | N321    | GCG GCC GCA TAG GCC ACT |

**TABLE 2. Primers used in characterization of strains**

| Primer          | Locationa | Sequence |
|-----------------|------------|----------|
| TOR1/Fwd−500    | Promoter, −500 to −480 | 5′-AGACACAGCAAAACACCACATCGTGTCG-3′ |
| TOR1/Fwd−72     | Promoter, −72 to −53   | 5′-CCATATGAGTCGACGAG-3′ |
| TOR1/Fwd+801    | ORF, 801 to 821     | 5′-GGTGACGAGAATGATGTCG-3′ |
| TOR1/Rev−280    | ORF, 258 to 280     | 5′-TACGGCGGCAATTTTGGTGCC-3′ |
| TOR1/Rev+1101   | ORF, 1080 to 1101   | 5′-TGCTAATAGGGGAACACATCGG-3′ |
| TOR2/Fwd−486    | Promoter, −486 to −464 | 5′-CGTCAATTTTTTACATCTGCTGCG-3′ |
| TOR2/Fwd+711    | ORF, 711 to 730     | 5′-ATACAGGAGACATGTCGGC-3′ |
| TOR2/Fwd+931    | ORF, 931 to 951     | 5′-TCTTCAAGGTTTGACACAGG-3′ |
| kanMX6/Rev      | 3XGFP cassette  | 5′-TAGAGTAGCTGACGATGCAATCG-3′ |
| GFP/Rev         | 3XGFP cassette, 165 to 185 (after Cre) | 5′-GCTACATCACCACCCTACACC-3′ |

*aLocation is nt from ATG start codon where specified.*
TABLE 3. Yeast strains and plasmids

| Strain or plasmid | Genotype or description | Reference or source |
|------------------|-------------------------|---------------------|
| 3XGFP for colony TOR1/D67-1-1-1, which became strain VA38 (Table 3). PCR with the same set gave the expected 2,602-nt band expected for 3XGFP for colony TOR1/D330-1-1-6-1, strain VA41 (Table 3). PCR with TOR1/Fwd/+801 and TOR1/Rev/+1101 gave the expected 2,548-nt band diagnostic for 3XGFP with the same set gave the expected 2,602-nt band expected for 3XGFP for strain VA33.

**RESULTS**

Identification of a region in TOR1 for internal 3XGFP tagging. Visualization of low-abundance proteins, such as TORs, often requires the incorporation of multiple tags. The incor-
poration of multiple copies can be either at the N terminus or at the C terminus of the protein in question. However, in our experience (data not shown), neither TOR1 nor TOR2 can tolerate the fusion of a tag to either the N or C terminus. Consistent with these previous observations, TOR1 or TOR2 with 3XGFP at the N terminus (encoded by TOR1\textsuperscript{N-3XGFP} or TOR2\textsuperscript{N-3XGFP}, respectively) was nonfunctional (see Fig. 5 and 8). TOR contains multiple subdomains, some of which have not been fully characterized but are important for interactions with conserved components of TOR complexes. We reasoned that an internal region permissive for the insertion of 3XGFP might be revealed by a comparison of evolutionary divergent
TOR proteins. To identify a candidate region for the insertion of 3XGFP, we aligned six TOR proteins (viz., human mTOR \[mammalian\] TOR, \textit{Saccharomyces cerevisiae} TOR1 and TOR2, \textit{Schizosaccharomyces pombe} Tor2, \textit{Caenorhabditis elegans} TOR, and \textit{Cryptococcus neoformans} TOR) (Fig. 1). We found a variable region near D330 in the noncatalytic domain of \textit{S. cerevisiae} TOR1. This region in some of the TORs contains natural insertions, and D330 in TOR1 was thus viewed as potentially permissive for the insertion of 3XGFP. To test this hypothesis, we generated three independent strains (VA33, VA34, and VA35), all of which contain an in-frame cassette encoding 3XGFP replacing D330 in \textit{TOR1} (see Materials and Methods and Table 3). The 3XGFP insertion is shown by the alignments in Fig. S1 in the supplemental material. We used three different phenotypic tests to determine the functionality of the new \textit{TOR1}D330-3XGFP allele encoding TOR1-3XGFP, as described directly below.

\textbf{TOR1-3XGFP encoded by \textit{TOR1}D330-3XGFP is functional.} TOR1-3XGFP was expressed as an intact protein with an apparent mass greater than 250 kDa (Fig. 2A). A phenotype for the loss of TOR1 is rapamycin hypersensitivity. Rapamycin hypersensitivity of \textit{tor1Δ} cells is due to a specific loss of TOR1 function because point mutations that inactivate TOR1 kinase activity cause hypersensitivity to a low concentration (1 nM) of rapamycin (33). The three independent strains of \textit{TOR1}D330-3XGFP were not hypersensitive to 1 nM or 2 nM rapamycin (Fig. 2B and C) and grew equivalently in the absence of rapamycin (Fig. 2A), indicating that TOR1-3XGFP was expressed and functional. A higher concentration of rapamycin (5 nM) nearly completely inhibited the growth of the \textit{TOR1}D330-3XGFP and \textit{TOR1} strains as expected (data not shown).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{TOR2-3XGFP is expressed, and \textit{TOR1}D330-3XGFP strains have normal sensitivity to rapamycin. (A) Western blot with anti-GFP antibodies of 40 μg of total protein (see Materials and Methods) of the control (Ctl), TB50a, and VA34 strains. The indicated strains were streaked onto YPD (B), YPD plus 1 nM rapamycin (Rap) (C), and YPD plus 2 nM rapamycin (D). Cells were grown for 2 days at 30°C after streaking and then scanned.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{\textit{TOR1}D330-3XGFP strains adapt to cold stress. The indicated strains were streaked onto YPD (A) or YPD plus 2 nM rapamycin (Rap) (B) Cells were grown for 4 days at 15°C (15 deg C) after streaking and then scanned.}
\end{figure}
The loss of TOR1 causes a cold-sensitive growth defect (Fig. 3). Adaptation to cold stress is not rescued by a kinase-defective TOR1 (data not shown). TOR1\textsuperscript{D330-3XGFP} strains were not cold sensitive (Fig. 3A). In fact, TOR1\textsuperscript{D330-3XGFP} strains grew somewhat better at 15°C than the wild type. This enhanced growth potential of TOR1\textsuperscript{D330-3XGFP} strains at 15°C was also observed in the presence of 2 nM rapamycin (Fig. 3B). This result shows as well that Tor1D330-3XGFP is expressed and functional because adaptation to cold stress requires active TOR1 kinase activity.

Pmr1 is a P-type ATPase that localizes predominantly to Golgi bodies and transports Ca\textsuperscript{2+} or Mn\textsuperscript{2+} into the lumen from the cytoplasm (13). Pmr1 function is important for Ca\textsuperscript{2+} growth potential of TOR1 somewhat better at 15°C than the wild type. This enhanced TOR1 pmr1 phenotypic tests, TOR1-3XGFP encoded by pmr1\Delta, is functional with 2XMyc but not higher numbers of Myc proteins, placed between residues 86 and 87 in this N-terminal segment (27; A. Lorberg, personal communication). We found that TOR1\textsuperscript{D67-3XGFP} was only partially functional because it conferred only partial resistance to 1 nM rapamycin (Fig. 5A). The function of TOR1\textsuperscript{D67-3XGFP} was greater than that of TOR1\textsuperscript{N-3XGFP}, as reflected in the rapamycin or cold sensitivity of the corresponding strains. The TOR1\textsuperscript{D330-3XGFP} strain was more functional for growth in the presence of rapamycin or at 15°C than either the TOR1\textsuperscript{N-3XGFP} or TOR1\textsuperscript{D67-3XGFP} strain (Fig. 5B).

Kog1 is an essential protein that binds TOR1 or TOR2 in TORC1 (27). Strains containing Kog1 with 3XGFP or 8XGFP incorporated at the C terminus as genomic tags are viable (2, 37). We confirmed that KOG1\textsuperscript{C-8XGFP}, as reflected in the rapamycin or cold sensitivity of the corresponding strains. The TOR1\textsuperscript{D330-3XGFP} strain was more functional for growth in the presence of rapamycin or at 15°C than either the TOR1\textsuperscript{N-3XGFP} or TOR1\textsuperscript{D67-3XGFP} strain (Fig. 5B).

For comparison to the insertion at D330, we also constructed 3XGFP strains that targeted residue D67, nearer to the N terminus of TOR1, where there is significantly more divergence in sequence than at D330 (Fig. 1). Notably, TOR1 is functional with 2XMyc but not higher numbers of Myc proteins, placed between residues 86 and 87 in this N-terminal segment (27; A. Lorberg, personal communication). We found that TOR1\textsuperscript{D67-3XGFP} was only partially functional because it conferred only partial resistance to 1 nM rapamycin (Fig. 5A). The function of TOR1\textsuperscript{D67-3XGFP} was greater than that of TOR1\textsuperscript{N-3XGFP}, as reflected in the rapamycin or cold sensitivity of the corresponding strains. The TOR1\textsuperscript{D330-3XGFP} strain was more functional for growth in the presence of rapamycin or at 15°C than either the TOR1\textsuperscript{N-3XGFP} or TOR1\textsuperscript{D67-3XGFP} strain (Fig. 5B).

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TOR1-3XGFP is partially functional. (A) Growth on YPD containing 1 nM rapamycin. The indicated strains (Table 3) were streaked, and after 3 days of growth, the plate was scanned. Growth patterns on YPD from streaks in the same experiment were similar (data not shown). (B) Growth at 15°C (15 deg) on YPD or YPD plus 1 nM rapamycin (Rap). The indicated strains were assayed by a 10-fold dilution and pinning; TOR1\textsuperscript{D67-3XGFP} (VA38) was used. The plates were scanned after 4 days.

FIG. 5. TOR1\textsuperscript{D67-3XGFP} is partially functional. (A) Growth on YPD containing 1 nM rapamycin. The indicated strains (Table 3) were streaked, and after 3 days of growth, the plate was scanned. Growth patterns on YPD from streaks in the same experiment were similar (data not shown). (B) Growth at 15°C (15 deg) on YPD or YPD plus 1 nM rapamycin (Rap). The indicated strains were assayed by a 10-fold dilution and pinning; TOR1\textsuperscript{D67-3XGFP} (VA38) was used. The plates were scanned after 4 days.

FIG. 4. TOR1\textsuperscript{D330-3XGFP}, like TOR1, is sensitive to Mn\textsuperscript{2+} in a pmr1\Delta strain. The indicated strains were streaked onto YPD (A) or YPD containing 2 mM Mn\textsuperscript{2+} (final concentration) (B). Cells were grown for 2 days at 30°C, and then plates were scanned. The TOR1 PMR1 (TB50a), tor1\Delta PMR1 (AN9-2a), tor1\Delta pmr1\Delta (YGD25), TOR1 pmr1\Delta (LJ25-1A), TOR1\textsuperscript{D330-3XGFP} pmr1\Delta (VA68-9c), and TOR1\textsuperscript{D330-3XGFP} PMR1 (VA34) strains were used (Table 3).

FIG. 5. 1824 STURGILL ET AL. EUKARYOT. CELL

A. YPD

B. YPD + 2 mM Mn\textsuperscript{2+}
We confirmed the perivacuolar localization of the TOR1-3XGFP signal by comparing it to that of FM4-64, which specifically stains the vacuolar membrane at steady state (Fig. 6B) (38). A portion of TOR1-3XGFP overlapped with the expected ring-like staining of FM4-64 marking the vacuolar membrane.

The predominant localization of KOG1-GFP to the vacuolar membrane has been reported previously (2, 37). We compared TOR1-3XGFP and Kog1-GFP strains to a strain containing both GFP fusions (VA121) (see Fig. S2 in the supplemental material). The perivacuolar signal for GFP in the double-GFP strain was dramatically increased (see Fig. S2 in the supplemental material) compared to that in either single-GFP strain. TOR2-3XGFP, studied for comparison, did not show perivacuolar localization (see Fig. S2 in the supplemental material).

The localization of TOR1-3XGFP was compared to that of Sec7-dsRed or FYVE-dsRed (Fig. 7) (31). Cells were grown in selective synthetic medium to maintain the plasmid encoding the dsRed marker. Sec7 is a high-molecular-weight protein that contains a guanine-nucleotide exchange activity for Arf proteins involved in Golgi function (8). Sec7-dsRed is a marker for the trans-Golgi (28). The localization of TOR1-3XGFP was qualitatively different from that of Sec7-dsRed. First, the Sec7-dsRed vesicles were more numerous than the dots of TOR1-3XGFP, and second, the punctate signals for TOR1-3XGFP (Fig. 7) did not exactly correspond. A closer correspondence was observed between TOR1-3XGFP and the FYVE-dsRed marker.

The FYVE-dsRed fusion protein binds phosphatidylinositol-3-phosphate (for a review, see reference 23) and is generally a marker for early endosomes but is also found near the vacuole. FYVE-dsRed localizes “to punctate structures adjacent to the vacuole, weakly on the vacuole-limiting membrane, and in some cases within the vacuole” (18). We emphasize this description because TOR1-3XGFP localization was similar to this description with regard to the vacuole. TOR1-3XGFP also appeared to localize very near to, if not within, the FYVE-dsRed punctate structures near the vacuolar membrane.

FIG. 6. TOR1D330-3XGFP is predominantly cytoplasmic and concentrated as dots near the vacuolar membrane. (A) Localization of TOR1-3XGFP in cells grown in YDP. Merged GFP (green; GFP channel) and autofluorescence images for TB50 (control) lacking a GFP cassette and TOR1-3XGFP (VA34) strains are shown. Strains were grown overnight in YPD, diluted, and imaged while still in log phase after centrifugation and suspension in synthetic medium. The arrow indicates a dot near the vacule, and the feathered arrow indicates a dot near the vacuolar membrane. (B) The TOR1-3XGFP signal overlaps FM4-64 staining (see Materials and Methods) of the vacuolar membrane. Control, strain TB50a lacking a GFP cassette; TOR1-3XGFP, strain VA102. The exposure settings used were as follows: DIC, 300 ms; GFP, 10,000 ms; and Fm4-64 (Cy3), 10,000 ms.
However, TOR1-3XGFP localization was distinct from FYVE-dsRed localization in that TOR1-3XGFP was also diffusely cytoplasmic and not all the dot-like concentrations of TOR1-3XGFP were found near the vacuole. TOR2 is functional when 3XGFP is inserted to replace N321.

Our success with TOR1D330-3XGFP encouraged us to generate GFP fusion alleles of TOR2 (Fig. 8). We targeted the N terminus and residue N321 of TOR2. Like D330 in TOR1, N321 in TOR2 corresponds to a variable region in the noncatalytic domain of TOR2. The TOR2-targeted 3XGFP cassette was introduced into diploid strains because TOR2 is essential. To assess the functionality of the GFP fusion proteins, diploids containing the desired TOR2N-3XGFP or TOR2N321-3XGFP allele were sporulated, dissected, and germinated. The TOR2N-3XGFP allele was nonfunctional because only two spores were viable in 13/13 tetrads dissected (see Fig. S3 in the supplemental material). In contrast, TOR2N321-3XGFP was functional (see Fig. S3 in the supplemental material). Nineteen of 23 dissected tetrads for TOR2N321-3XGFP produced four viable spores. An expected diagnostic PCR product (441 nt) was observed with 2:2 segregation for all tetrads analyzed, and the product was absent in control cells (Fig. 8A).

The 441-nt product is that expected from the reverse primer amplifying at the closest site in the first GFP sequence. To further assess this, we performed a colony PCR with primers chosen close to and flanking the N321 site (Fig. 8B). One candidate had the complete 3XGFP cassette because the principal product was an ~2.3-kb band and was chosen for further study (becoming VA102). The other candidates were 2XGFP or 1XGFP. TOR2-3XGFP in VA102 was expressed as a >250-kDa protein by Western blotting with anti-GFP antibodies (Fig. 8C). These results together demonstrate that TOR2 remains functional despite the insertion of 3XGFP at N321 whereas TOR2 with 3XGFP at the N terminus is nonfunctional.

TOR2-3XGFP localizes to punctate structures near the plasma membrane. The localization of TOR2-3XGFP (encoded by TOR2N321-3XGFP in VA102) was compared with that of Sec7-dsRed or FYVE-dsRed under conditions similar to those used for TOR1-3XGFP (Fig. 9). TOR2-3XGFP did not colocalize with either Sec7-dsRed or FYVE-dsRed. Instead, TOR2-3XGFP was detectable above the background in punctate structures. These structures were most apparent beneath the plasma membrane.

To better resolve these structures, we used confocal microscopy (Fig. 10; see also Fig. S4 in the supplemental material). The TOR2-3XGFP signal was cytoplasmic and concentrated in punctate structures at or very near the plasma membrane. The identities of the TOR structures near the plasma membrane are unknown, although they resemble eisosomes in location and appearance but are less numerous than eiso...
DISCUSSION

We constructed functional TOR1-3XGFP and TOR2-3XGFP proteins to localize TOR in living cells. Importantly, this required the insertion of GFP within the TOR1 and TOR2 ORFs rather than at the N or C terminus. TOR1-3XGFP was cytoplasmic and concentrated at a prevacuolar compartment and at the vacuolar membrane. TOR2-3XGFP was also cytoplasmic and, most strikingly, concentrated at the plasma membrane. We did not detectably observe TOR1-3XGFP in the nucleus, and the nucleus qualitatively often correlated with decreased staining versus the surrounding cytoplasm (data not shown). More studies will be required to address this. Imaging functional tagged proteins should be complementary to imaging proteins in fixed cells with antibodies. Fixation may cause a loss of vesicular localization of proteins, for example, Rheb (34). The various localization patterns of TORs may provide a molecular basis for the large number of processes controlled by TOR and may also explain why different studies have observed TOR in different cellular locations. The localization of TOR in mammals has also been ambiguous, possibly because mTOR (also known in literature as FRAP [FKBP12-rapamycin-associated protein]) is also found in different locations. mTOR has been reported to be principally cytoplasmic and to shuttle between the cytoplasm and nucleus (20), to be principally nuclear in some cancer cell lines (42), to be cytoplasmic and associated with Golgi bodies and the ER (26), or to be associated with a regulatory subunit of protein kinase A (RIta) on late endosomes and autophagosomes (30). Only the last study used GFP-tagged mTOR, but GFP-tagged mTOR was over-

somes. Eisosomes have recently been defined and characterized as punctate structures at the plasma membrane involved in the early steps of endocytosis (36, 39).

FIG. 8. TOR2 is functional if N321 is replaced by 1X-, 2X-, or 3XGFP. (A) A 441-nt product (asterisk) establishes the insertion of GFP in germinated spores with 2:2 segregation (see the text). Primers TOR2F/−711 and GFP/Rev and spores 2A to 2D (template) were used for colony no. 1 (TB50/α/α background). (B) Results of PCR consistent with 2XGFP replacing N321 after the recombination event in JK9 colony no. 1, 1XGFP in TB50 colony no. 2, and 3XGFP in TB50 colony no. 1 (see the text). Primers TOR2Fwd/−931 and TOR2Rev/−990 were used. The templates used were as follows: 2XGFP-JK9 lanes, 2A, 2B, none, and 20C; 1XGFP-TB50 lanes, 18A, 18B, 20A, and 20B (colony no. 2); and 3XGFP-TB50 lanes, 2A, 2B, 7C, and 7D (colony no. 1). Arrows indicate diagnostic bands for 1X-, 2X-, and 3XGFP (see the text). (C) Western blot with anti-GFP of 40 µg of lysate protein (see Materials and Methods) from the control (Ctl), TB50α, and VA102 strains.
expressed and N terminally tagged with 1XGFP and its functionality remains to be established.

The concentration of TOR1-3XGFP near the vacuolar membrane is consistent with some reports in the literature. The loss of TOR1 is synthetically lethal with loss of class C VPS (vacuolar protein sorting) genes (43). This genetic interaction is likely specific to TOR1 because the overexpression of TOR2 fails to rescue the synthetic lethality. As a second correlation, the TOR1 interactors Kog1 and Tco89 were detected near the vacuolar membrane by imaging of Kog1-GFP in live cells or by immunogold staining for Tco89 in fixed cells (2, 32, 37). Because strain differences can affect TOR1-related phenotypes (9, 32), we confirmed that Kog1-8XGFP was also concentrated at the vacuolar membrane in TB50 (see the supplemental material). As a third correlation, TOR1 interacts genetically and biochemically with Gtr2 in the Ego complex, found near the vacuolar membrane (12). Finally, the phosphorylation of Sch9 by TORC1 may occur near the vacuole because Sch9 localizes near this organelle and an artificial Sch9 substrate tethered to the vacuolar membrane is phosphorylated in a TORC1-dependent manner (37). If there is a connection of TORC1 to the vacuole, it may derive from the role of the vacuole in nutrient supply or regulation of autophagy (19). The physiological relevance of TOR1 in the cytoplasm or at the plasma membrane also remains to be determined.

The localization of TOR2-3XGFP at discrete sites near the plasma membrane is similar to that of eisosomes, recently described as protein complexes important for endocytosis (39). Interestingly, TOR2 is found mainly in TORC2, which is implicated in endocytosis and actin dynamics (14). Furthermore, TOR2 activates the AGC family kinase Ypk2 (17). Ypk2 phosphorylates Pil1 and Lsp1, proteins involved in eisosome formation and function (29). Moreover, TORC2 is required for sphingolipid biosynthesis (4) and Ypk2 is also activated by sphingolipids (for a review, see reference 25). These findings make eisosomes an interesting candidate for the location of TOR2 at the plasma membrane. We attempted to address this by introducing an mCherry tag at the C terminus of LSP1 or SUR7 in the TOR2-3XGFP strain and found that (i) the Lsp1 and Sur7 mCherry signals are very much brighter than the TOR2-3XGFP signal and (ii) the TOR2-3XGFP signal showed partial colocalization with these markers (data not shown). Avo3 shows partial colocalization with Pil1 (an eisosome marker) by immunofluorescence of fixed cells (R. Shioda, unpublished data). The specific localization of TOR2-3XGFP to eisosomes remains to be defined.

Our findings provide insight into the structure of TOR. It is remarkable that TOR, a strongly conserved protein of ~2,500 amino acids, retains at least partial function after the insertion of 748 amino acids (3XGFP cassette) in the noncatalytic domain. The functionality of the internally tagged TOR1-3XGFP and TOR2-3XGFP proteins may be due to the placement of 3XGFP between subdomains. The majority of the N terminus of TOR consists of repeated HEAT motifs (21). D330 and N321 are in a gap between HEAT repeats (see Fig. S5 in the supplemental material). Furthermore, a recent electron microscopy structure of TOR1 suggests that the noncatalytic domain forms an N-terminal head, a turn, and an arm (1). We
predict that D330 and N321 are near a gap between two of these subdomains. The preservation of function with internal tagging of TOR with GFP may also be due to the fact that the N terminus and the C terminus of GFP are near each other (see Protein Data Bank entry 1EMM), minimizing displacement at the point of insertion.

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