Identification of defined structural elements within TOR2 kinase required for TOR complex 2 assembly and function in Saccharomyces cerevisiae

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ABSTRACT The mammalian target of rapamycin (mTOR) is a large protein kinase that assembles into two multisubunit protein complexes, mTORC1 and mTORC2, to regulate cell growth in eukaryotic cells. While significant progress has been made in our understanding of the composition and structure of these complexes, important questions remain regarding the role of specific sequences within mTOR important for complex formation and activity. To address these issues, we have used a molecular genetic approach to explore TOR complex assembly in budding yeast, where two closely related TOR paralogues, TOR1 and TOR2, partition preferentially into TORC1 versus TORC2, respectively. We previously identified an ∼500-amino-acid segment within the N-terminal half of each protein, termed the major assembly specificity (MAS) domain, which can govern specificity in formation of each complex. In this study, we have extended the use of chimeric TOR1-TOR2 genes as a “sensitized” genetic system to identify specific subdomains rendered essential for TORC2 function, using synthetic lethal interaction analyses. Our findings reveal important design principles underlying the dimeric assembly of TORC2 as well as identifying specific segments within the MAS domain critical for TORC2 function, to a level approaching single-amino-acid resolution. Together these findings highlight the complex and cooperative nature of TOR complex assembly and function.

INTRODUCTION
The mammalian target of rapamycin (mTOR) network is a conserved regulator of eukaryotic cell growth and cellular homeostasis, contributing to aging, and is dysregulated in many human diseases, including cancer (Wullschleger et al., 2006; Saxton and Sabatini, 2017; Mossmann et al., 2018; Magaway et al., 2019). The central component in this network is the large (∼280 kDa) mTOR protein, a Ser/Thr protein kinase and member of the conserved PIKK-related family of protein kinases (Bosotti et al., 2000; Wullschleger et al., 2006). In addition to a conserved C-terminal kinase domain and adjacent FRB domain that interacts with rapamycin, mTOR is composed almost entirely of helical repeats, including N-terminal and middle HEAT (N-HEAT and M-HEAT, respectively) and FAT/TPR repeat domains (Andrade and Bork, 1995; Andrade et al., 2001; Knutson, 2010; Tafur et al., 2020). mTOR functions as part of two distinct protein complexes, mTORC1 and mTORC2, where mTORC1 is uniquely inhibited by the macrolide antibiotic rapamycin (Kim et al., 2002, 2003; Sarbassov et al., 2004; Eltschinger and Loewith, 2016; Tafur et al., 2020). mTOR functions as part of two distinct protein complexes, mTORC1 and mTORC2, where mTORC1 is uniquely inhibited by the macrolide antibiotic rapamycin (Kim et al., 2002; Loewth et al., 2002; Jacinto et al., 2004; Sarbasov et al., 2004; Wullschleger et al., 2006; Eltschinger and Loewith, 2016; Tafur et al., 2020). In addition to mTOR, mTORC1 includes as core components Raptor and mLST8/GβL, while mTORC2 includes Rictor, SIN1, and mLST8/GβL (Hara et al., 2002; Kim et al., 2002, 2003; Sarbassov et al., 2004; Eltschinger and Loewith, 2016). Association with complex-specific partners and regulators is proposed to control their intracellular localization as well as governing substrate selection and regulating mTOR kinase activity (Eltschinger and Loewith, 2016; Yang et al., 2017).
In this context, we observed that many predicted sites of sequence flexibility exist within TOR2 to govern TORC2 assembly. Specifically, TORC1 contains either TOR1 or TOR2, as well as KOG1 (orthologue of Raptor), whereas TORC2 contains TOR2, AVO1 (orthologue of SIN1), and AVO3 (orthologue of Rictor). Both complexes contain LST8 (orthologue of mLST8/G35L) as a common subunit, as well as a number of nonconserved, complex-specific proteins, including AVO2 in TORC2 (Loewith et al., 2002; Wedaman et al., 2003; Reinke et al., 2004; Tafur et al., 2020). Models derived from biochemical and ultrastructural studies of both yeast and mammalian complexes indicate that they likely function as dimers and possess an overall rhomboid-like, symmetrical shape, determined primarily by the superhelical topology of the large N-terminal domain of TOR/mTOR (Wullschleger et al., 2005; Yang et al., 2013, 2016, 2017; Gaubitz et al., 2015; Aylett et al., 2016; BaretiL et al., 2016; Karuppasamy et al., 2017; Chen et al., 2018; Stuttfeld et al., 2018; Scaiola et al., 2020). High-resolution (∼3.0 Å) Cryogenic electron microscopy (Cryo-EM) models have emerged recently for both mTORC1 and mTORC2 (Yang et al., 2017; Scaiola et al., 2020), revealing key architectural details as well as insights into their regulation, for example evidence for conformation changes within the mTORC1 active site following interaction with the upstream activator Rheb (Yang et al., 2017). Despite these advances, many important structural features within these models remain unresolved, particularly for mTORC2 (Scaiola et al., 2020). Moreover, the role of specific amino acids within TOR/mTOR for complex assembly and function remains almost completely unknown. Finally, while structural models provide important information about macromolecular organization, to be useful they must be paired with other methods to examine structure–function relationships involved in complex assembly and activity, as well as test predictions regarding the significance of observed protein–protein contacts.

To address these gaps and to complement ongoing structural studies, we have employed a molecular genetic approach to interrogate the importance of specific elements within TOR1 and TOR2, taking advantage of their unique behavior in yeast, where TOR1 assemblies exclusively into TORC1 and TOR2 assemblies preferentially into TORC2 (Loewith et al., 2002; Wedaman et al., 2003; Reinke et al., 2004). By constructing a set of TOR1-TOR2 chimeras, we previously identified an ∼500-amino-acid domain corresponding to the N-HEAT domain, termed the major assembly specificity (MAS) domain, that can direct assembly of both TOR proteins into TORC1 versus TORC2 (Hill et al., 2018) (Figure 1A). In our present study, we have extended this approach to probe the importance of predicted quaternary interactions involving specific TORC2 partners as well as exploring the role of specific features within the TOR2 MAS domain for TORC2 function. Our findings reveal unanticipated complexity for TORC2 assembly as well as identifying specific subdomains within the TOR2 MAS critical for TORC2 activity.

RESULTS

Strategy and experimental approach

Our prior study revealed that distinct regions of TOR2, in addition to the MAS/N-HEAT domain (for simplicity referred to here as TOR2 MAS), are sufficient but not necessary to confer TORC2 function (Hill et al., 2018). These findings suggest that significant sequence flexibility exists within TOR2 to govern TORC2 assembly. In this context, we observed that many predicted sites of interaction between TOR2 and its TORC2-specific partners AVO2 and AVO3, based on a medium-resolution Cryo-EM structural model and protein–protein cross-linking data, map to discontinuous segments of TOR2 within several TOR1-TOR2 chimeras (Gaubitz et al., 2015; Karuppasamy et al., 2017; Hill et al., 2018) (Figure 1B). We therefore sought to use these chimeras to interrogate the importance of predicted quaternary interactions for TORC2 assembly and function, using a synthetic sick/lethal (SSL) genetic interaction approach (Kaiser and Schekman, 1990; Guarente, 1993). This approach takes advantage of the fact that two mutations in interacting gene products, including within multiprotein complexes, often yield exacerbated phenotypes compared with either single mutation alone. Moreover, using genetic approaches, including synthetic lethality, to explore the function of paralogous gene products can yield information about both the architecture and the evolution of protein complexes (Mirmy and Gelfand, 2002; Varga et al., 2021). Our general strategy, adapted from our prior study
To extend these results, we next tested the ability of these chimeras to provide TOR2 function in a strain that harbored a truncated allele of AVO3 lacking 147 amino acids at its C-terminus (termed avo3-ΔCT). Previous studies demonstrated that this allele is functional yet causes TORC2 to become sensitive to rapamycin, where it is proposed to increase access of the FRB-rapamycin complex to the FRB domain (Gaubitz et al., 2015). Given that AVO3 is encoded by an essential gene and, therefore, we could not analyze a null allele, we used avo3-ΔCT as a possible hypomorphic mutant. As expected, full-length TOR2 (pPL632) supported viability in tor2Δ avo3-ΔCT cells (Figure 2C). By contrast, as was observed in avo2Δ cells, both the TOR2 MAS domain (pPL626) and TOR1 MAS domain (pPL655) chimeras displayed synthetic lethal interactions with the avo3-ΔCT allele (Figure 2C). Interestingly, we observed that the chimera containing the entire N-terminus of TOR2 (pPL630) failed to support the growth of tor2Δ avo2Δ cells (Figure 2B). We conclude from these results that TOR2-specific sequences throughout the N-terminal region of TOR2 are required for proper TORC2 function in the absence of AVO2.

A sensitized genetic system to identify TOR2 elements essential for TORC2 function

Our above findings revealed novel genetic interactions between distinct TOR1-TOR2 chimeras and TORC2-specific partners. We reasoned that we could employ a similar approach to identify synthetic interactions within TOR2 (i.e., intragenic interactions), as an approach to refine our understanding of structural elements within TOR2 important for TORC2 function. We focused on the TOR2 MAS domain, where we showed previously that approximately 500 amino acids of TOR2 are sufficient to confer TORC2 function within the context of an otherwise full-length TOR1 protein (Hill et al., 2018).
We constructed several new chimeras using the TOR2 MAS domain construct (pPL626) as a starting platform (see Materials and Methods). Viability was determined based on the number of tor2Δ avo3-Δ CT haploid progeny that carried a plasmid compared with total tor2Δ progeny that carried a plasmid. The relative colony size was assessed as in B. (D) Western blot analysis of TOR1-TOR2 chimeras. The indicated plasmids were introduced into a double heterozygous TOR2/tor2Δ AVO3/avo3-Δ CT strain, followed by sporulation and tetrad dissection. Viable haploid progeny were genotyped by growth on selective media (see Materials and Methods). Viability was determined based on the number of tor2Δ avo3-Δ CT haploid progeny that carried a plasmid compared with total tor2Δ progeny that carried a plasmid. The relative colony size was assessed as in B. (D) Western blot analysis of TOR1-TOR2 chimeras. 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TOR2 sequences required for TORC2

Methods. TOR2 activity was assessed by transformation of a TOR2/tor2Δ diploid strain, followed by sporulation and tetrad dissection. Here we scored both the frequency of finding haploid strains that harbored both a tor2Δ deletion and a chimera plasmid (described as percent viability) and their relative colony size. In parallel, we assessed the rapamycin resistance of each strain as a measure of TORC1 function as well as monitoring steady state levels of TOR protein by Western blot analysis.

In a first approach, we constructed “Minimal” (pPL628) and “Sub-Minimal” (pPL629) TOR2 MAS domain chimeras (Figure 4A). The TOR1/2 boundaries of these chimeras encompassed a central core of predicted TOR2 contacts for AVO2 and AVO3 (pPL628) or, alternatively, for AVO3 alone (pPL629) (Figure 1B) (Gaubitz et al., 2015; Hill et al., 2018). Surprisingly, the Minimal TOR2 MAS domain (pPL628) afforded only very limited TOR2 function, and the Sub-Minimal TOR2 MAS domain (pPL629) was completely non-functional as TOR2 (Figure 4A). Both chimeras produced normal levels of protein and conferred rapamycin resistance, consistent with an observed defect in TORC2 activity alone (Figure 4, B and C). To test whether the impaired phenotype of the Minimal TOR2 MAS domain was due to loss of TOR2-specific sequences at either end of this domain, we constructed two additional chimeras that restored TOR2 sequences at either the N-terminal side (Minimal MAS+N; pPL635) or the C-terminal side (Minimal MAS+C; pPL636), respectively. Each construct displayed only minor improvement in TOR2 phenotypes (Figure 4A). We conclude from this analysis that sequences outside the central core of the TOR2 MAS domain are required for proper TORC2 function.

In a second approach, we tested whether the TOR2 MAS domain would tolerate the introduction of short stretches of TOR1 sequence interspersed throughout its length and retain TOR2 function. Accordingly, we constructed 10 chimeras (TOR2 MAS-A through TOR2 MAS-J), each representing a contiguous series of ∼50-amino-acid segments of TOR1-specific sequences that replaced corresponding TOR2 sequences (Figure 5A) (see Materials and Methods). To maximize the likelihood of proper protein folding, chimeras were designed to maximize two criteria where possible: 1) TOR1-TOR2 boundaries (“switch points”) were placed where TOR1 and TOR2 sequences were identical; and 2) each construct encompassed complete predicted α-helical repeat units (Ferry and Kleckner, 2003; Knutson, 2010; Karuppasamy et al., 2017). Chimeras were introduced into a TOR2/tor2Δ strain and analyzed as above. Here we observed surprising phenotypic diversity with respect to the level of rescue of tor2Δ lethality (Figure 5A). The most deleterious phenotypes were observed for chimeras that swapped sequences within the central region (chimeras E and F) as well as at the N- and C-termini (chimeras A, B, and J) (Figure 5A). In particular, chimeras MAS-B and MAS-E were most impaired in their ability...
to rescue the lethality of a tor2Δ strain (Figure 5A). Together these results both confirm that outer sequences of the TOR2 MAS domain are important for TORC2 function and highlight the importance of the central core of this domain.

All chimeras tested in this second approach produced full-length TOR protein and conferred resistance to rapamycin, an indication that they all assemble and function within TORC1 (Figure 5, B and C). However, three chimeras, MAS-A (pPL637), MAS-E (pPL641), and MAS-F (pPL642), resulted in significantly reduced steady state levels of protein and conferred weaker rapamycin resistance (Figure 5, B and C). We note that chimeras MAS-A (pPL637) and Minimal MAS+C (pPL636) share overlapping TOR1-specific sequences at the N-terminus of the TOR2 MAS domain, highlighting the sensitivity of this region to changes in TOR2-specific sequences (compare Figures 4 and 5). In addition, our findings that chimeras MAS-E and MAS-F result in reduced levels of TOR protein and impaired rapamycin resistance indicate that the central core of the TOR2 MAS domain is also sensitive to the precise composition of TOR1 versus TOR2 sequences and impact TOR complex assembly and/or TOR protein stability.

Fine dissection of TOR2 elements crucial for TORC2 function

To begin to understand, at the single-amino-acid level, the importance of elements within the TOR2 MAS domain, we focused on chimera TOR2 MAS-B, as introduction of TOR1-specific sequences into this relatively discrete region severely disrupted TORC2 function without affecting overall protein levels or TORC1 activity. We subdivided TOR2 MAS-B into three separate chimeras, MAS-B1 through MAS-B3, each representing approximately 6–10 amino acids of the TOR1 sequence within the context of an otherwise complete TOR2 MAS domain (Figure 6A). Each construct was introduced into a TOR2/tor2Δ strain and analyzed as above.

We observed that chimera MAS-B1 (pPL652) was severely impaired in its ability to rescue the lethality of a tor2Δ strain (Figure 6A). By contrast, chimeras MAS-B2 (pPL653) and MAS-B3 (pPL654) both provided near-wild-type rescue (Figure 6A). All three constructs produced normal levels of TOR protein and conferred strong rapamycin resistance (Figure 6, B and C). Remarkably, chimera MAS-B1 contains only 10 amino acids that differ between TOR1 and TOR2, with respect to the 498 amino acids that constitute the TOR2 MAS domain, affirming the utility of this approach to reveal amino acids crucial for TORC2 function (Figure 6A). We observed that chimera MAS-B2 includes two predicted amino acid contacts for AVO2 within MAS-B (Figure 6A). However, these amino acids are identical in TOR1 and TOR2; thus, it is unlikely that they contribute directly to complex specificity, a conclusion consistent with our findings that this chimera supports full TOR2 activity.

We used a similar approach to dissect chimera MAS-E, which is significantly impaired in TORC2 activity but showed defects in
TORC1 as well (Figure 5). We subdivided chimera MAS-E into four separate constructs, MAS-E1 through MAS-E4, where each chimera possessed only four- to six-amino-acid differences between TOR1 and TOR2 with respect to the TOR2 MAS domain (Figure 7A). Interestingly, in contrast to the complete MAS-E chimera, all four constructs rescued a tor2Δ allele, produced normal levels of TOR protein, and provided strong rapamycin resistance (Figure 7, A–C). We note that two constructs, MAS-E3 (pPL649) and MAS-E4 (pPL650), displayed modest growth defects in tor2Δ cells, where MAS-E3 is the location of predicted sites of interaction between TOR2 and AVO3 (Figure 7A). We conclude from these findings that the TOR2 MAS domain can tolerate smaller clusters of TOR1 sequences within its central core and maintain adequate TORC2 activity, produce stable TOR protein, and function within TORC1.

DISCUSSION

In this study, we have used chimeric TOR1-TOR2 proteins to extend our understanding of specific determinants within TOR2 that are critical for TORC2 assembly and function. For example, the pattern of synthetic genetic interactions between TOR chimeras and mutant alleles of TORC2-specific components AVO2 and AVO3, examined within the context of a structural model for TORC2, suggests important design features for assembly of TORC2. In particular, our observation that proper TORC2 activity requires interactions between TOR2-specific elements within the context of the same TOR2 chain and both copies of AVO3 suggests that stable assembly of TORC2 requires complex quaternary interactions across the dimer interface. Because this arrangement of physical interactions is conserved between mTOR and Rictor, we predict that interactions between monomers will also turn out to be crucial for mTORC2 assembly and/or stability. Interestingly, an inspection of the structure of mTORC1 reveals that Raptor also makes contacts with both copies of mTOR within the mTORC1 dimer (Yang et al., 2017), raising the interesting possibility that both mTORC1 and mTORC2 assemble as obligate dimers.

We previously identified the TOR2 MAS domain as a contiguous ∼500-amino-acid segment within the N-terminus of TOR2 that is sufficient to confer TORC2 activity in the context of a TOR1-TOR2 chimera. We were surprised that we were unable to narrow this domain further, even though most predicted interactions between TOR2 and both AVO2 and AVO3 are located within a central ∼200-amino-acid core of this domain. Instead, we systematically exchanged discrete segments within the TOR2 MAS domain, by introducing a “moving window” of TOR1 sequence, to identify specific clusters of essential TOR2-specific sequences. This approach employed a
TOR2 MAS domain chimera as a “sensitized” genetic background to reveal the importance of specific TOR2 sequences. Remarkably, for one segment, TOR2 MAS-B1, we were able to resolve this element to near-amino-acid resolution.

At a structural level, chimera MAS-B1 corresponds to the N-terminal helix of HEAT repeat 10, where it is positioned at the exterior of the MAS domain (Karuppasamy et al., 2017) (Figure 6D). Within this segment are four nonconservative amino acid differences between TOR1 and TOR2, all predicted to lie on an external-facing edge of the helix (Figure 6D). At present, there are no predicted interactions involving these residues. Thus, one possibility is that this helix interacts with portions of AVO2 or AVO3 that have yet to be identified at the ultrastructural level or, alternatively, with a different component essential for TORC2 assembly and/or function, for example, the TTT-R2TP cochaperone complex required for proper (m)TOR folding (Takai et al., 2007, 2010; Kim et al., 2013). Examination of a structural model for mTORC2 reveals that this same helix within mTOR is similarly positioned at the outer surface of mTORC2 (Figure 6D) (Scaiola et al., 2020). In this context, we note that, as for AVO3, a substantial portion of Rictor (approximately 35%) remains unidentified within the mTORC2 structure (Scaiola et al., 2020).

The segment of TOR2 corresponding to MAS-E is predicted to be involved in interactions with both AVO3 and the other TOR2 chain within the TORC2 dimer interface (Karuppasamy et al., 2017; Hill et al., 2018) (Figure 7D). This arrangement of interactions is conserved in mTORC2, where corresponding sequences in mTOR are located adjacent to Rictor as well as the other mTOR chain (Scaiola et al., 2020) (Figure 7D). Interestingly, for both AVO3 and Rictor, in the present structural models significant portions of both proteins are missing directly adjacent to MAS-E, suggesting that there may be additional associations with this region of TOR (Karuppasamy et al., 2017; Scaiola et al., 2020). Moreover, in a recent structural model for mTORC1, Raptor displays extensive interactions with sequences corresponding to MAS-E (Yang et al., 2017) (Figure 7D), providing a possible rationale for why changes in this region affect both TORC1 and TORC2 activity. Alternatively, this region includes intermolecular contacts between the two mTOR proteins within the dimeric structures of mTORC1 and mTORC2 (Yang et al., 2017; Scaiola et al., 2020), as well as for TOR2 within TORC2 (Karuppasamy et al., 2017).
We were surprised to find that several chimeras we constructed resulted in reduced steady state levels of protein, likely contributing to impaired TORC1 as well as TORC2 behavior. One possibility is that the introduction of shorter TOR1-specific sequences within these regions, for example in chimeras MAS-A (pPL637) and Minimal MAS+C (pPL636), impairs localized folding, despite these sequences functioning normally within the context of a complete TOR1 protein. A more interesting possibility is that such a chimera may juxtapose sequences specific for TORC1 versus TORC2 assembly, therefore causing localized disruptions and/or competition for crucial quaternary interactions for complex-specific partners or chaperones, resulting in destabilized chimeras susceptible to degradation. As described above, consistent with the latter possibility is the observation that chimera TORC2 MAS-E involves sequences predicted to be involved in multiple protein–protein interactions within both complexes. The MAS domain is also predicted to interact with the TTT-R2TP cochaperone complex (Takai et al., 2007, 2010).

Our functional studies highlight the cooperative nature of TOR complex assembly, where distinct domains distributed throughout TOR2 are normally sufficient for the formation of TORC2. By employing TOR1-TOR2 chimeras, we have been able to identify specific regions by making them essential for TORC2 identity. These findings now point the way for more detailed analyses of their role in TORC2 assembly and function. Our approach underscores the utility of analyzing paralogues to understand the molecular basis of assembly, as well as evolution, of large protein complexes (Miry and Gelfand, 2002; Capra et al., 2012; de Juan et al., 2013; Schick et al., 2019; Varga et al., 2021). Owing to the conservation of several important elements within mTOR, including the MAS domain, our findings point to the likely importance of these regions for both mTORC1 and mTORC2 assembly. We anticipate that applying a similar structure–function approach to mTOR will be a successful avenue for testing the importance of amino acid contacts identified in both mammalian complexes. This includes testing the importance of defined interactions between mTOR and complex-specific partners during dimer assembly as well as determining whether essential elements within the MAS domain interact with currently unidentified interacting components essential for mTOR complex assembly and signaling.

**FIGURE 7:** Identification of functional elements within the TOR2 MAS-E chimera. (A) MAS-E plasmid pPL641 containing the S1972R (RapR) TOR1-1 allele was modified to create the indicated TOR1-TOR2 chimeras. Each construct, E1 through E4, contains approximately 6–10 amino acids of TOR1 sequence within an otherwise TOR2 MAS domain, as depicted in the indicated sequence alignments for TOR1 and TOR2. We note that chimera E1 includes four additional TOR1-specific amino acids that are not present in MAS-E. Predicted amino acids contacts between TOR2 and AVO3 are boxed in blue in E3. Plasmids were introduced into heterozygous TOR2/tor2Δ cells and analyzed as described in the legend to Figure 3A. (B) Western blot analysis of TOR1-TOR2 chimeras. TOR2/tor2Δ cells carrying the indicated plasmids were prepared and analyzed as described in the legend to Figure 2D. (C) TOR2/tor2Δ cells carrying the indicated plasmids were tested for rapamycin resistance as described in the legend to Figure 3B. (D) Sequences corresponding to MAS-E (black) are highlighted in Cryo-EM structures for TORC2, mTORC2, and mTORC1, as indicated. Also shown are the indicated partners within each complex (red) as well as portions of TOR2 or mTOR (blue) corresponding to the other protein chain within each structure. Regions of AVO3 or Rictor absent within these structures are indicated by dashed red lines in each model. Protein chains are labeled according to nomenclature described in Karuppasamy et al. (2017).
MATERIALS AND METHODS

Request a protocol through Bio-protocol.

Strains, media, and general methods

Strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Cells were cultured in YPD (2% yeast extract, 1% peptone, and 2% dextrose) or synthetic complete dextrose (SCD) medium (0.8% yeast nitrogen base without amino acids, pH 5.5, 2% dextrose) supplemented with amino acids as described (Sherman, 1991). Rapamycin (Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) and added to SCD medium and agar plates to a final concentration of 0.2 µg/ml. Hygromycin B (Sigma-Aldrich, St. Louis, MO) was dissolved in water and added to agar plates at a final concentration of 200 µM. Yeast transformations were performed using a lithium acetate procedure (Gietz and Woods, 2002).

Construction and analysis of yeast strains for synthetic genetic interactions

Double heterozygous TOR2/tor2Δ AVO2/avo2Δ diploid strains carrying individual control or chimera plasmids were constructed by mating tor2Δ haploid strains carrying a plasmid to an avo2Δ haploid strain. Diploids were selected by growth on SCD minus leucine and tryptophan media. Following sporulation, haploid progeny were analyzed by scoring the presence or absence of markers for tor2Δ.

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TABLE 1: Saccharomyces cerevisiae strains used in this study.

| Strain   | Genotype                          | Source                      |
|----------|-----------------------------------|-----------------------------|
| MPR1     | TB50a avo3-dCT(-157aa) [HpH]      | Gaubitz et al., 2015        |
| PLY 362  | avo2::TRP W303a                   | Reinke et al., 2004         |
| PLY 1760 | W303a/α tor2::His/TOR2 [pPL632]  | This study                  |
| PLY 1761 | W303a/α tor2::His/TOR2 [pPL655]  | This study                  |
| PLY 1762 | W303a/α tor2::His/TOR2 [pPL630]  | This study                  |
| PLY 1763 | W303a/α tor2::His/TOR2 [pPL626]  | This study                  |
| PLY 1764 | TOR2/tor2::His AVO2/avo2::TRP [pPL632] | This study                  |
| PLY 1765 | TOR2/tor2::His AVO2/avo2::TRP [pPL626] | This study                  |
| PLY 1766 | TOR2/tor2::His AVO2/avo2::TRP [pPL655] | This study                  |
| PLY 1767 | TOR2/tor2::His AVO2/avo2::TRP [pPL630] | This study                  |
| PLY 1768 | TOR2/tor2::His AVO3/avo3-dCT::HpH [pPL632] | This study                  |
| PLY 1769 | TOR2/tor2::His AVO3/avo3-dCT::HpH [pPL626] | This study                  |
| PLY 1770 | TOR2/tor2::His AVO3/avo3-dCT::HpH [pPL655] | This study                  |
| PLY 1771 | TOR2/tor2::His AVO3/avo3-dCT::HpH [pPL630] | This study                  |
| PLY 1772 | W303a/α tor2::His/TOR2 [pPL628]  | This study                  |
| PLY 1773 | W303a/α tor2::His/TOR2 [pPL629]  | This study                  |
| PLY 1774 | W303a/α tor2::His/TOR2 [pPL635]  | This study                  |
| PLY 1775 | W303a/α tor2::His/TOR2 [pPL636]  | This study                  |
| PLY 1776 | W303a/α tor2::His/TOR2 [pPL637]  | This study                  |
| PLY 1777 | W303a/α tor2::His/TOR2 [pPL638]  | This study                  |
| PLY 1778 | W303a/α tor2::His/TOR2 [pPL639]  | This study                  |
| PLY 1779 | W303a/α tor2::His/TOR2 [pPL640]  | This study                  |
| PLY 1780 | W303a/α tor2::His/TOR2 [pPL641]  | This study                  |
| PLY 1781 | W303a/α tor2::His/TOR2 [pPL642]  | This study                  |
| PLY 1782 | W303a/α tor2::His/TOR2 [pPL643]  | This study                  |
| PLY 1783 | W303a/α tor2::His/TOR2 [pPL644]  | This study                  |
| PLY 1784 | W303a/α tor2::His/TOR2 [pPL645]  | This study                  |
| PLY 1785 | W303a/α tor2::His/TOR2 [pPL646]  | This study                  |
| PLY 1786 | W303a/α tor2::His/TOR2 [pPL652]  | This study                  |
| PLY 1787 | W303a/α tor2::His/TOR2 [pPL653]  | This study                  |
| PLY 1788 | W303a/α tor2::His/TOR2 [pPL654]  | This study                  |
| PLY 1789 | W303a/α tor2::His/TOR2 [pPL647]  | This study                  |
| PLY 1790 | W303a/α tor2::His/TOR2 [pPL648]  | This study                  |
| PLY 1791 | W303a/α tor2::His/TOR2 [pPL649]  | This study                  |
| PLY 1792 | W303a/α tor2::His/TOR2 [pPL650]  | This study                  |
(growth on SCD minus histidine media), avo2Δ (growth on SCD minus tryptophan), and the relevant plasmid (growth on SCD minus leucine media). Double heterozygous TOR2/tor2Δ AVO3/avo3-Δ diploid strains carrying individual control or chimera plasmids were constructed by mating tor2Δ haploid strains carrying a plasmid to an avo3-Δ CT haploid strain. Diploids were identified following growth in SCD minus leucine media by their ability to form spores in sporulation media. Following sporulation, haploid progeny were analyzed by scoring the presence or absence of markers for tor2Δ (growth on SCD minus histidine media), avo3-Δ CT (growth in the presence of hygromycin), and the relevant plasmid (growth on SCD minus leucine media).

Plasmid construction
Plasmids used in this study are listed in Table 2. Plasmid construction was carried out in conjunction with Genscript (Piscataway, NJ). Plasmids pPL626, pPL630, and pPL632 were constructed using previously constructed parental plasmids (Hill et al., 2018) and used as templates for construction of new chimeras, as indicated in Table 2. The precise positions of switch points and specific chimeric sequences for each plasmid are listed in Supplemental Table S1, and all sequences are provided in Supplemental Table S2. Synthetic DNA sequences were subcloned into expression vectors by fragment exchange using two unique restriction sites or by modified Gibson Assembly (Casini et al., 2015) (see Supplemental Table S1). Plasmids were sequenced in their entirety to confirm the accuracy of construction.

Whole cell extraction, Western blot analysis, and quantification
Cells were grown overnight to mid–logarithmic phase (A\textsubscript{600} = 2.0) in SCD minus leucine media at 30°C. Protein extracts were prepared

| Plasmid  | Description | Chimera name | Source                      |
|----------|-------------|--------------|-----------------------------|
| pRS315   | LEU2 CEN/ARS |              | Sikorski and Hieter, 1989   |
| pPL130   | LEU2 CEN/ARS TOR1-1 | | Reinke et al., 2004 |
| pPL132   | LEU2 CEN/ARS TOR1 | | Reinke et al., 2004 |
| pPL172   | pPL132, TOR2 114-1770 | | Hill et al., 2018 |
| pPL273   | pPL130, TOR2 428-946 | | Hill et al., 2018 |
| pPL321   | pPL130, TOR2 | | Hill et al., 2018 |
| pPL630   | pPL172, with S1972R | N-term TOR2 | This study |
| pPL632   | pPL321, with S195R | Full-length TOR2 | This study |
| pPL651   | pPL632, TOR1 428-946, with S1975R | TOR1 MAS S1975R | This study |
| pPL655   | pPL632, TOR1 428-946 | TOR1 MAS | This study |
| pPL626   | pPL73 | TOR2 MAS | This study |
| pPL628   | pPL626, TOR2 462-814 | Minimal MAS | This study |
| pPL629   | pPL626, TOR2 644-766 | Sub-Minimal MAS | This study |
| pPL635   | pPL626, TOR2 462-926 | Minimal MAS + N | This study |
| pPL636   | pPL626, TOR2 428-814 | Minimal MAS + C | This study |
| pPL637   | pPL626, TOR1 423-472 | TOR2 MAS-A | This study |
| pPL638   | pPL626, TOR1 473-522 | TOR2 MAS-B | This study |
| pPL639   | pPL626, TOR1 523-572 | TOR2 MAS-C | This study |
| pPL640   | pPL626, TOR1 573-622 | TOR2 MAS-D | This study |
| pPL641   | pPL626, TOR1 623-672 | TOR2 MAS-E | This study |
| pPL642   | pPL626, TOR1 673-722 | TOR2 MAS-F | This study |
| pPL643   | pPL626, TOR1 723-772 | TOR2 MAS-G | This study |
| pPL644   | pPL626, TOR1 773-823 | TOR2 MAS-H | This study |
| pPL645   | pPL626, TOR1 824-873 | TOR2 MAS-I | This study |
| pPL646   | pPL626, TOR1 874-946 | TOR2 MAS-J | This study |
| pPL652   | pPL626, TOR1 473-489 | TOR2 MAS-B1 | This study |
| pPL653   | pPL626, TOR1 490-507 | TOR2 MAS-B2 | This study |
| pPL654   | pPL626, TOR1 508-522 | TOR2 MAS-B3 | This study |
| pPL647   | pPL626, TOR1 611-626 | TOR2 MAS-E1 | This study |
| pPL648   | pPL626, TOR1 627-641 | TOR2 MAS-E2 | This study |
| pPL649   | pPL626, TOR1 642-656 | TOR2 MAS-E3 | This study |
| pPL650   | pPL626, TOR1 657-672 | TOR2 MAS-E4 | This study |

TABLE 2: Plasmids used in this study.
using the NaOH cell lysis method (Dilova et al., 2004). Equivalent amounts of extract were loaded onto SDS–PAGE gels and then transferred to nitrocellulose membranes. Membranes were probed with α-HA (12CA5; 1:5000 dilution; Covance) and α-G6PDH (1:100,000 dilution; Sigma-Aldrich) primary antibodies. Secondary antibodies conjugated to IRDye (1:5000 dilution; LI-COR Biosciences) were used, and blots were imaged using either an Odyssey Infrared Imaging System (LI-COR Biosciences) or an Azure Sapphire Biomolecular Image. Images were quantified using LI-COR Image Studio Lite.

Averages of three independent biological replicates are presented with means ± SD. The p values were calculated using Student’s t test: *p < 0.05 and **p ≤ 0.01.

Molecular modeling

Molecular graphics and analyses were performed with the UCSF Chimera package (Petterson et al., 2004). The following Cryo-EM models were used for analysis: S. cerevisiae TORC2 (PDB accession number 6EMK) (Karuppasamy et al., 2017); human mTORC2 (PDB accession number 6ZWM) (Scaiola et al., 2020); and human mTORC1 (PDB accession number 6BCX) (Yang et al., 2017).

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