TOR Complex 1 Includes a Novel Component, Tco89p (YPL180w), and Cooperates with Ssd1p to Maintain Cellular Integrity in Saccharomyces cerevisiae*

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The Tor1p and Tor2p kinases, targets of the therapeutically important antibiotic rapamycin, function as components of two distinct protein complexes in yeast, termed TOR complex 1 (TORC1) and TORC2. TORC1 is responsible for a wide range of rapamycin-sensitive cellular activities and contains, in addition to Tor1p or Tor2p, two highly conserved proteins, Lst8p and Kog1p. By identifying proteins that co-purify with Tor1p, Tor2p, Lst8p, and Kog1p, we have characterized a comprehensive set of protein-protein interactions that define further the composition of TORC1 as well as TORC2. In particular, we have identified Tco89p (YPL180w) and Bti61p (YJL058c) as novel components of TORC1 and TORC2, respectively. Deletion of TOR1 or TCO89 results in two specific and distinct phenotypes, (i) rapamycin-hypersensitivity and (ii) decreased cellular integrity, both of which correlate with the presence of SSD1-d, an allele of SSD1 previously associated with defects in cellular integrity. Furthermore, we link Ssd1p to Tap42p, a component of the TOR pathway that is believed to act uniquely downstream of TORC1. Together, these results define a novel connection between TORC1 and Ssd1p-mediated maintenance of cellular integrity.

Rapamycin is an antifungal antibiotic that inhibits the action of the Tor (target of rapamycin) kinase, a phosphatidylinositol 3-kinase-related kinase present in all eukaryotes examined to date, from yeast to humans (1–5). Two highly homologous Tor proteins exist in yeast, Tor1p and Tor2p, and both are inhibited by rapamycin (5). Treating yeast cells with rapamycin elicits a number of responses that mimic nutrient deprivation, including cessation of protein synthesis and ribosome biogenesis, induction of autophagy, cell cycle arrest at the G1/S boundary, and entry into G0 (4, 5). Rapamycin also affects some biogenesis, induction of autophagy, cell cycle arrest at the starvation, including cessation of protein synthesis and ribosome biogenesis, induction of autophagy, cell cycle arrest at the G1/S boundary, and entry into G0 (4, 5). Rapamycin also affects some biogenesis, induction of autophagy, cell cycle arrest at the G1/S boundary, and entry into G0 (4, 5). Rapamycin also affects some biogenesis, induction of autophagy, cell cycle arrest at the G1/S boundary, and entry into G0 (4, 5).

Together, these observations suggest that the Tor proteins act within an intracellular signaling network that controls cell growth according to nutrient availability. Downstream of Tor1p and Tor2p, many rapamycin-sensitive events are mediated by the type 2A phosphatases Pph21p and Pph22, the type 2A-related phosphatase Sit4p, and two regulatory proteins, Tap42p and Tip4lp (6–9). In particular, these proteins have been shown to regulate the phosphorylation state and activity of distinct transcriptional regulators whose target genes are controlled by Tor (10, 11). The precise mechanism by which these phosphatases and their regulators respond to Tor remains poorly understood, however, and is likely to be quite complex (9).

In addition to its rapamycin-sensitive function, Tor2p also regulates actin cytoskeletal dynamics during polarized cell growth in yeast (2, 5, 12–14). This Tor2p-unique function is not inhibited by rapamycin and involves signaling to components required for proper remodeling of actin at the site of bud emergence, including the Rho1p GTPase and its regulatory partners Rom2p and Sac7p (5, 13, 15). This Rho1p GTPase switch functions in part by signaling to Pck1p, the yeast homologue of mammalian protein kinase C, an upstream regulator of a mitogen-activated protein kinase (MAPK) cascade that includes Mpk1, one of five MAPKs in yeast (16). Interestingly, there is an emerging connection between this Tor2p-unique function involving Pck1p and what has been termed the "cell integrity pathway," whereby cell envelope and/or cell wall stability is monitored in response to osmotic and/or thermal stress (16–18). One explanation for this convergence is that the Pck1p/MAPK cascade is important not only for actin remodeling but also for regulated synthesis of cell wall structural components, activities essential for both cell cycle-dependent polarized cell growth as well as for maintenance of cellular integrity (15–17). Intriguingly, Tor1p and components downstream of rapamycin-sensitive Tor signaling, including Sit4p, have also been implicated recently in Pck1p/MAPK-mediated maintenance of cell integrity (19, 20).

Together, these findings raise the important question concerning the extent of potential overlap and/or cross-talk between Tor1p/Tor2p shared and Tor2p-unique activities.

In addition to Pck1p, a number of other components have been shown to influence cell integrity. In particular, results upon gene expression at several levels, including transcription, translation, and protein trafficking and stability (4, 5). Together, these observations suggest that the Tor proteins act within an intracellular signaling network that controls cell growth according to nutrient availability. Downstream of Tor1p and Tor2p, many rapamycin-sensitive events are mediated by the type 2A phosphatases Pph21p and Pph22, the type 2A-related phosphatase Sit4p, and two regulatory proteins, Tap42p and Tip4lp (6–9). In particular, these proteins have been shown to regulate the phosphorylation state and activity of distinct transcriptional regulators whose target genes are controlled by Tor (10, 11). The precise mechanism by which these phosphatases and their regulators respond to Tor remains poorly understood, however, and is likely to be quite complex (9).

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In addition to Pck1p, a number of other components have been shown to influence cell integrity. In particular, results
from a study by Kaeberlein and Guarente (21) suggest that there are at least two independent pathways that operate in parallel with Pkc1p to maintain cell viability in response to challenges to cellular integrity. One pathway is defined by Mpt5p, a protein implicated previously in a variety of cellular activities, including mating pheromone responsiveness, life span extension, and resistance to environmental stress (21). The second pathway is defined by Ssd1p, which has also been implicated in several distinct processes, including intracellular signaling, membrane trafficking, Ca2+ homeostasis, and cell growth and morphogenesis (21). How these three different pathways function to maintain cellular integrity is presently unknown, although it has been suggested that they may collaborate to control the transcription of genes required for proper plasma membrane and/or cell wall biosynthesis, potentially by converging on the MAPK cascade, in particular Mpk1p (21). An important unanswered question raised by this study is whether Tor signaling is involved in these distinct Pkc1p-independent branches of cell integrity maintenance.

In addition to downstream components involved in Tor signaling, results of recent studies demonstrate that Tor1p and Tor2p act in collaboration with a number of specific interacting proteins. In particular, Hall and co-workers (22) have described two distinct Tor complexes, termed TORC1 and TORC2, that are responsible for carrying out the rapamycin-sensitive Tor1p/Tor2p or the rapamycin-insensitive Tor2p-unique activities, respectively. TORC1 contains, in addition to either Tor1p or Tor2p, two highly conserved proteins, Kog1p and Lst8p, where mammalian homologues of these proteins have been shown to interact with and regulate the activity of mammalian Tor (23–25). TORC2 contains Tor2p, Lst8p, and three additional proteins, Avo1p–Avo3p (22). Independently, we have identified Tor2p-interacting proteins and characterized what most likely corresponds to TORC2 (26). We also demonstrated that Lst8p is an interacting partner of Tor1p and, moreover, that all three proteins localize to an internal, membranous compartment that is distinct from both the plasma membrane and the vacuole (26). Kaiser and co-workers (27) have also independently demonstrated that the Tor proteins interact physically with Lst8p. Intriguingly, these authors made the additional observation that several mutations in LST8 confer defects in cellular integrity, as defined by temperature-sensitive growth that is rescued by inclusion of the osmotic stabilizer sorbitol in the growth medium (27). Whether these lst8 alleles perturb a Tor1p/Tor2p-shared and/or a Tor2p-unique activity, however, was not addressed in this study.

Here we report a number of results that bear on the relationship between Tor signaling and maintenance of cellular integrity. By extending our previous biochemical approach of identifying proteins that associate with the Tor kinases, we have characterized two novel proteins, Tco89p and Bit61p, that independently demonstrated that the Tor proteins interact using PCR to amplify the gene using W303a or JK9-3da genomic DNA as a template. Primers were designed such the entire ORF as well as 400 bp upstream and downstream were amplified. The forward primer also contained a SacII site and the reverse primer contained an XmaI site to facilitate introduction of the PCR product into plasmids pRS315, pRS316, or pRS416 (33), as described in Table II, using standard recombinant DNA techniques. Plasmids expressing Myc1p were constructed in a similar manner except that primers were designed such that the entire ORF plus 600 bp upstream and downstream were amplified. In addition, the forward primer contained a BamHI site to facilitate the introduction of PCR fragments into different plasmids as described in Table II.

**Materials and Methods**

**Strains, Media, and General Methods**—Strains and plasmids used in this study are listed in Tables I and II, respectively. Genetic crosses of haploid strains, sporulation of diploid strains, and tetrad analysis were carried out as described (28). 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 (29). CFW was provided either as Fluostain I (Sigma) and used in SCD agar plates at 15 mg/ml (final concentration) or, alternatively, as fluorescent brightener 28 (Sigma) and used in YPD agar plates at 5 mg/ml (final concentration). Rapamycin (Sigma) was dissolved in Me2SO and added to SCD and YPD agar plates at the final concentrations indicated. Sorbitol (Sigma) was added to media at a final concentration of 1.0 M. Yeast transformations were performed using a lithium acetate procedure (30).

**Construction of Yeast Strains**—All deletion strains were constructed by replacing an entire open reading frame (ORF) with a selectable marker following transformation of a linear fragment of DNA constructed by PCR. Deletion strains using TRP1 or His3MX6 as selectable markers were constructed using forward and reverse primers that contained 50 base pairs (bp) corresponding to the 5′- or 3′-ends of the target ORF, followed by 20 bp corresponding to the 5′- or 3′-ends of the marker gene. TRP1 and His3MX6 were amplified from plasmids pFA6a-TRP1 and pFA6a-His3MX6, respectively (31, 32). Deletion strains using KanMX6 as a selectable marker were constructed using genomic DNA that was obtained from an appropriate deletion strain from Research Genetics (Invitrogen). Here PCR primers contained 200 bp of sequence corresponding to the 5′- or 3′-ends of the target ORF and were identified by PCR. Target ORFs were tagged at their carboxyl termini with multiple copies of the Myc epitope and Kog1p and Lst8p were tagged at their carboxyl termini with three copies of the HA epitope using a PCR-based gene tagging method, as described previously (26).

**Plasmid Construction**—Plasmids containing Ssd1Δ were constructed using PCR to amplify the gene using W303a or JK9-3da genomic DNA as a template. Primers were designed such the entire ORF as well as 400 bp upstream and downstream were amplified. The forward primer also contained a SacII site and the reverse primer contained an XmaI site to facilitate introduction of the PCR product into plasmids pRS315, pRS316, or pRS416 (33), as described in Table II, using standard recombinant DNA techniques. Plasmids expressing Myc1p were constructed in a similar manner except that primers were designed such that the entire ORF plus 600 bp upstream and downstream were amplified. In addition, the forward primer contained a BamHI site to facilitate the introduction of PCR fragments into different plasmids as described in Table II.

**Immunoffinity Purification of HA3-tagged Proteins**—The following procedure is an adaptation of the purification strategy described previously for HA–anti-HA antibodies produced by Washington Biotechnology, Inc. (Baltimore, MD). For this, the following dipeptide HA sequence was synthesized: CYPY-DVPDYAGYPYDPYVPYG. This dipeptide sequence consists of 10 amino acids of HA represented twice and contains an N-terminal cysteine for conjugation. The synthesized peptide was conjugated to the carrier protein KLH, after which immunization of two New Zealand white rabbits was performed. Boosts and bleeds were carried out on one of the two rabbits, and then 60 ml of serum was affinity-purified against the dipeptide to yield ~5 ml of purified polyclonal HA antibody at a concentration of ~2 mg/ml. The enzyme-linked immunosorbent assay titer of the serum was 5.0 × 105, and that of the affinity-purified antibody was 1.0 × 107.

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prechilled mortar and pestle containing liquid nitrogen and ground into a fine powder (~150 strokes followed by the addition of liquid nitrogen, repeated three times). After the liquid nitrogen had boiled off, the powder that remained was transferred to 1.5-ml microcentrifuge tubes and thawed. All of the following steps were performed at 4°C unless otherwise indicated. Extracts were then centrifuged twice at 20,000 × g for 20 min, transferring the supernatant to a new tube each time. From 4 liters of cells, we typically obtained ~4 ml of clarified whole cell extract. The extract was then divided into 10 tubes containing ~400 μl each and preclotted by binding to 25 μl of protein A-coupled beads (Amersham Biosciences) for 30 min. Each tube of clarified extract was then incubated with 5 μl of affinity-purified anti-HA polyclonal anti-HA antibody (9E10) (Covance; Berkeley, CA), followed by overnight incubation as from previously described strains that expressed N-terminal HA3 epitope did not interfere with the function of either protein as a control, an extract was also prepared from an untagged parental control strain in rich media (YPD), indicating that the HA3 epitope sequence was not sufficient to bind to the resin, and the HA3 epitope did not interfere with the function of either protein or target proteins.

\[ \text{TORC1 cooperates with Ssd1p to maintain cellular integrity} \]

\[ \text{Identification of novel Tor1p- and Tor2p-interacting proteins—To investigate further the composition of Tor1p-} \]

\[ \text{and Tor2p-containing protein complexes and to identify new potential interacting partners, we extended our previously successful approach of using three copies of the HA epitope (HA3) as an immunopurification tool to characterize these complexes (26). Accordingly, we constructed yeast strains that expressed C-} \]

\[ \text{terminally tagged versions of Lst8p (LST8-HA3) or Kog1p (KOG1-HA3). Both strains grew as well as the untagged parent} \]

\[ \text{al control strain in rich media (YPD), indicating that the HA epitope did not interfere with the function of either protein (data not shown). In addition, Western blot analysis} \]

\[ \text{of extracts demonstrated that a single protein of the expected} \]

\[ \text{molecular weight was recognized by anti-HA antibody in each strain (data not shown).} \]

\[ \text{Whole cell extracts were prepared from these strains as well as from previously described strains that expressed N-terminally} \]

\[ \text{tagged versions of Tor1p (HA3-TOR1) or Tor2p (HA3-TOR2) (26). As a control, an extract was also prepared from an} \]

\[ \text{untagged parental strain. These extracts were incubated with immobilized affinity-purified polyclonal anti-HA antibody and} \]

\[ \text{washed, and epitope-tagged proteins were eluted by the addition of competing HA dipeptide, as described under "Materials} \]

\[ \text{Immunogold electron microscopy (IEM)—IEM was performed on ultrathin cryosections as described previously (34).} \]
When analyzed by SDS-PAGE, specific co-eluting proteins could be detected (Fig. 1 and data not shown). The identities of co-eluting proteins were determined by trypsin digestion followed by tandem mass spectrometric analysis (35, 36). In addition to the tagged protein present in each sample, a predominant number of peptides corresponding to a characteristic set of proteins were identified, of which none were present in samples prepared from the untagged control strain (Table III). These co-purifying proteins included those identified in recent biochemical studies as specific interacting partners of Tor1p and/or Tor2p (22, 26). Two additional proteins were identified, encoded by ORFs YPL180W and YJL058C, which, for reasons explained below, we have termed Tco89p (for 89-kDa subunit of Tor complex one) and Bit61p (for 61-kDa binding partner of Tor2p), respectively.

We tested association of Tor1p and Tor2p with Tco89p and Bit61p by co-immunoprecipitation experiments. For this, we constructed strains that expressed both HA3-Tor2p and versions of Tco89p or Bit61p that carried at their C termini multiple copies of the Myc epitope. Extracts were prepared from these strains as well as from a control strain that expressed HA3-Tor2p alone. Immunoprecipitations were performed using anti-Myc antibody followed by Western blotting using a monoclonal anti-HA antibody to detect HA3-Tor2p and a commercially available polyclonal antibody to detect Tor1p. The results showed that HA3-Tor2p but not Tor1p co-precipitated with Bit61p-Myc (Fig. 1C). By contrast, Tor1p but not HA3-Tor2p co-precipitated with Tco89p-Myc (Fig. 1D). These results agree with our mass spectrometry analysis and demonstrate that Bit61p interacts specifically with Tor2p, whereas Tco89p interacts specifically with Tor1p.

Inspection of Table III reveals remarkable self-consistency in terms of observed protein-protein interactions from immunopurified, HA3-tagged versions of Tor1p, Tor2p, Kog1p, and Lst8p. To a first approximation, these results agree well with the model proposed by Hall and co-workers wherein Tor1p, Lst8p, and Kog1p associate to form TORC1, and Tor2p, Lst8p, and Avo1p-Avo3p associate to form TORC2 (22) (Fig. 1B). In addition, we conclude that Tco89p and Bit61p represent novel components of TORC1 and TORC2, respectively (Fig. 1B). The observation that Bit61p is a component of TORC2 is also consistent with the fact that this protein was identified in a genome-wide two-hybrid analysis as an interacting partner of Avo3p, another TORC2 component (37). Where our findings continue to differ with Loewith et al. (22) is our inability to detect Tor2p as a significant and stable component of TORC1 in wild type cells; thus, we do not observe significant association of Tor2p with either Kog1p or Tco89p (Table III) (26). Nevertheless, we present genetic evidence below that is consistent with Tor2p being able to substitute for Tor1p in TORC1.

tor1Δ and tco89Δ Cells Are Hypersensitive to Rapamycin and Display Cell Integrity Defects—Tco89p and Bit61p are each encoded by nonessential genes. To examine their contribution to Tor signaling, we disrupted each gene individually. For
A defect was detected in an avo2 mutant strain associated with TORC1 in the ability to detect any differences in several transcriptional readouts, including Ribosomal proteins being a component of TORC1. However, we were unable to detect any differences in several transcriptional readouts associated with TORC1 in the avo2 mutant strain, in comparison with wild type cells, including ribosomal protein as well as Rpg1p/Rtg3p and Gln3p-dependent target genes (10, 11, 38-40). Thus, we conclude that Tco89p is dispensable for these specific TORC1-dependent functions.

A second significant phenotype we found associated with both tor1Δ and tco89Δ cells was temperature-sensitive (ts) growth at 37 °C that could be rescued by inclusion of the osmotic stabilizer sorbitol in the growth medium (Fig. 2B). This phenotype is a hallmark of a defect in cellular integrity involving stress at the plasma membrane and/or cell wall (41, 42). Moreover, we determined that prolonged growth at the nonpermissive temperature correlated with decreased cell viability of tor1Δ cells, suggesting that cell lysis had occurred, a characteristic of cell integrity defects (21) (Fig. 2C).

**Fig. 2. Phenotypic characterization of strains deleted for specific components of TORC1 or TORC2.** A, wild type (WT) (W303a), tco89Δ (PLY32), tor1Δ (PLY330), avo2Δ (PLY362), bit61Δ (PLY364), and avo2Δ bit61Δ (PLY365) cells were grown to midlog phase (A600nm = 0.5), washed with sterile water, serially diluted, and plated onto a YPD agar plate or YPD agar plates that contained 1.0 M sorbitol or 5 µg/ml CFW and incubated at 30 or 37 °C, as indicated, for 3 days. C, comparing the viability of tor1Δ cells under different growth conditions. Wild type and tor1Δ cells were grown in YPD media to midlog phase at 30 °C, shifted to the media conditions for the time indicated, and then serially diluted and plated onto YPD agar plates for 3 days at 25 °C.
reduction in viability of tco89Δ cells was detected, however, which correlated with the less severe ts phenotype for this strain (Fig. 2B and data not shown). Finally, we observed that CFW, an independent agent that induces cell integrity defects (43), severely reduced the growth of both tor1Δ and tco89Δ cells (Fig. 2B). Together, these data demonstrate that loss of either Tor1p or its interacting partner Tco89p results in cells becoming susceptible to challenges to cellular integrity.

tor1Δ and tco89Δ Cell Integrity Phenotypes Are Strain-dependent—The ts behavior of the tor1Δ strain described above was surprising given that this phenotype had not been reported in an original characterization of this gene in studies by Hall and co-workers (14). We reasoned that this was possibly due to differences in parental strain backgrounds, in that we used strain W303a, whereas the Hall laboratory used JK9-3da. To test this possibility directly, we disrupted TOR1 in both parental strain backgrounds and examined their behavior at 37 °C. We observed that W303a-derived tor1Δ cells were ts at 37 °C, whereas JK9-3da-derived tor1Δ cells displayed essentially wild type growth (Fig. 3A). To gain insight into the genetic basis for these differences, we mated the two tor1Δ haploid strains to yield a homozygous tor1Δ/tor1Δ diploid that was of a mixed genetic background. This strain grew as well as the haploid JK9-3da-derived tor1Δ strain, indicating that the ts behavior of the W303a-derived tor1Δ strain was a recessive phenotype (Fig. 3B).

Sporulation of the resulting diploid followed by tetrad dissection resulted in a complex growth pattern of spore clones at 37 °C, with many tetrads giving rise to two or three viable spores of different relative sizes (Fig. 3C). Of 138 tetrads examined, a total of 67 (48%) produced three viable spores (data not shown). We concluded that this complexity was directly related to the cell integrity defects described above as inclusion of sorbitol in the growth medium resulted in all cases in production of four viable spore clones (Fig. 3C). Whereas a number of possible models could explain these results, one potential explanation is that there are at least two unlinked recessive genetic factors in the W303a background that must combine with each other as well as with the tor1 deletion in order to produce a ts phenotype. A similar genetic analysis of tco89Δ mutants derived from the two strain backgrounds gave similar results, suggesting that both TOR1 and TCO89 interact with the same genetic factor(s) required for temperature-resistant growth (data not shown).

SSD1 Rescues tor1Δ and tco89Δ Cell Integrity Phenotypes—Based on our genetic analyses, we postulated that introduction of a single allele from the JK9-3da background into the W303a-derived tor1Δ and tco89Δ strains should be sufficient to restore temperature-resistant growth. Accordingly, we took a candidate-based approach and introduced into these strains a number of genes reported to be involved in distinct branches of the cell integrity pathway, including MPK1, MPT5, PKC1, and SSD1 (21). Each gene was cloned from JK9-3da and introduced into the W303a-derived strains on low copy (CEN/ARS) as well as on high copy (2μ) plasmids, under control of their native promoters, as described under “Materials and Methods.” Cells were then examined for temperature-resistant growth and for CFW resistance.
We found a single gene, *SSD1*, that when expressed at low copy restored both temperature-resistant growth and CFW resistance to *tor1Δ* as well as *tco89Δ* cells (Fig. 4, A and C, and Table IV). *SSD1* is a polyphenotypic factor that has been implicated in a variety of cellular functions, including maintenance of cellular integrity (21). To test directly the possibility that *SSD1* represented one of the genetic differences between W303a and JK9-3da, we introduced a plasmid that carried the W303a-derived *SSD1* allele into *tor1Δ* and *tco89Δ* cells. This plasmid was unable to rescue any of the mutant phenotypes of either strain (Fig. 4, B and C, and Table IV). Based on these results, we conclude that distinct *SSD1* alleles contribute to the observed strain-specific differences of *tor1Δ* and *tco89Δ* cells.

This conclusion agrees well with previous findings that the W303a strain background contains an allele of *SSD1*, termed *SSD1*-d, which renders cells susceptible to challenges to cellular integrity (21).

We also observed that expression of *TOR2* on a low copy plasmid specifically rescued the *tor1Δ* mutant phenotypes (Fig. 4A and Table IV). *TOR2* did not rescue the *tco89Δ* mutant phenotypes, however, suggesting that the additional Tor2p provided by this plasmid suppressed *tor1Δ* mutant phenotypes by substituting directly for the absence of Tor1p function, rather than representing an allelic difference between W303a and JK9-3da. We also found that overexpression of MPK1, encoding the MAPK associated with maintenance of cellular integrity (16), provided weak rescue of the ts phenotype of *tor1Δ* cells (Fig. 4A and Table IV). Overexpression of MPK1 did not rescue the CFW sensitivity of *tor1Δ* cells, however, nor did it rescue any of the *tco89Δ* mutant phenotypes (Fig. 4A and Table IV). Thus, whereas these results support the recently reported link between Tor1p and Mpk1p (20), we conclude that MPK1 is also unlikely to represent an allelic difference between W303a and JK9-3da, at least in terms of the phenotypes reported here.

To determine the scope of influence of *SSD1* on the observed *tor1Δ* and *tco89Δ* mutant phenotypes, we asked whether expression of JK9-3da-derived *SSD1* affected the rapamycin hypersensitivity of these strains. Indeed, we found that expression of this gene restored the ability of both mutants to grow on plates containing 1.0 ng/ml rapamycin, in contrast to cells expressing either a control plasmid or a plasmid expressing W303a-derived *SSD1* (Fig. 5 and data not shown).

**TAP42 Is Functionally Linked to SSD1 and to Cell Integrity Maintenance**—The above results are consistent with a model wherein defects in two components of TORC1, Tor1p and Tco89p, render cells defective in maintenance of cellular integrity in the presence of the W303a-derived *SSD1* allele. A prediction of this model is that components downstream of TORC1 might also be influenced by the allelic nature of *SSD1*. In this regard, it is significant that *SSD1* was originally identified as a gene required for viability when cells are compromised for Sit4p function (44). In addition, *SSD1* function has also been linked to Pph21p and Pph22p (45). To date, however, a relationship has not been established between TAP42 and *SSD1*. Interestingly, Broach and co-workers (9) characterized recently a set of temperature-sensitive *tap42* alleles in the W303a background, which prompted us to consider the relationship between the temperature sensitivity of these mutants and defects in cellular integrity and, more specifically, whether Sit1p was involved.

To this end, we first asked whether inclusion of sorbitol in the growth medium affected the ability of two of the reported *tap42* mutants, *tap42-106* and *tap42-109*, to grow at elevated temperatures. Indeed, we observed that sorbitol restored the ability of both mutants to grow at 33 °C (Fig. 6A). Similarly, expression of JK9-3da-derived *SSD1* on a low copy plasmid specifically restored the ability of these mutants to grow at 33 °C (Fig. 6B). Together, these results affirm a functional connection between *SSD1* and the TAP42 with respect to maintenance of cellular integrity. However, we found that neither sorbitol nor JK9-3da-derived *SSD1* rescued the ts phenotypes of *tap42-106* or *tap42-109* strains at 37 °C, suggesting that these alleles remain defective in other Tor-dependent and/or Tor-independent functions at this temperature (Fig. 6, A and B).

**tor1Δ and tco89Δ Deletions Are Synthetically Lethal in Combination**—In the course of our genetic analyses of *tor1Δ* and *tco89Δ* cells, we were unable to obtain a *tor1Δ tco89Δ* double mutant. For example, sporulation of a diploid that was heterozygous for *tor1*:TRP1 and *tco89*:HIS3MX6 never gave rise to Trp+ His+ haploid spore clones, even when sorbitol was included in the growth medium (data not shown). We were, however, able to obtain such a double mutant when the diploid was first transformed with a plasmid containing an additional copy of *TOR2* prior to sporulation and tetradi dissection (data not shown). One likely interpretation of this result was that the additional Tor2p provided by the plasmid was sufficient to compensate for the loss of Tor1p, as described above for the *tor1Δ* and *tco89Δ* strains by expression of specific genes. A, *tor1Δ* cells were transformed with a control plasmid (pRS316) or plasmids that expressed JK9-3da-derived alleles of *TOR2* (pNB100), *SSD1* (pPL092), and *MPK1* (pPL101 (CEN/ARS) or pPL103 (2μ)). Cells were grown in SCD media lacking uracil at 30 °C to midlog phase, serial dilutions were made, and cells were plated out onto SCD agar plates that lacked uracil and were incubated at the temperatures indicated. B, *tor1Δ* cells were transformed with a control plasmid (pRS316) or plasmids that expressed either the JK9-3da-derived or the W303a-derived alleles of *SSD1* (pPL092 or pPL093, respectively). C, *tco89Δ* cells were transformed with a control plasmid (pRS316), plasmids that expressed JK9-3da-derived alleles of *TOR2* (pNB100) or *SSD1* (pPL092), or a plasmid expressing the W303a-derived allele of *SSD1* (pPL093). In B and C, cells were grown in SCD media lacking uracil at 30 °C to midlog phase, serial dilutions were made, and cells were plated out onto SCD agar plates that lacked uracil and were incubated at the temperatures indicated. In A–C, agar plates contained 15 μg/ml CFW where indicated. Photographs of plates were taken following ~3 days of growth. In A and B, *tor1Δ* corresponds to strain PLY254, and in C, *tco89Δ* corresponds to strain PLY332.
single deletion. A corollary to this conclusion is that Tor2p and Tco89 interact functionally (e.g. by the ability of Tor2p to substitute for Tor1p within TORC1).

We confirmed the synthetic lethality of tor1 and tco89 mutations using the following approach. First, tor1Δ tco89Δ double mutant cells carrying a TOR2 URA3 plasmid were transformed with either a second TOR2 plasmid that possessed LEU2 as a selectable marker or, alternatively, an empty LEU2 control vector. Next, cells were plated onto media containing 5-fluororotic acid to counterselect the TOR2 URA3 plasmid. We found that viable cells were obtained on 5-fluororotic acid-containing plates if they carried the TOR2 LEU2 plasmid but not the empty LEU2 plasmid (Fig. 7).

In Situ Localization of Tco89p—To gain further insight into the cellular function of Tco89p, we examined the intracellular localization of this protein. Here we used IEM to visualize Tco89p-Myc in ultrathin cryosections prepared from fixed cells, a technique we employed previously to detect both of the Tor proteins as well as Lst8p (26). Sections were incubated with anti-Myc antibody and by 5-nm gold-decorated secondary antibody, followed by visualization of gold particles by electron microscopy. Gold particles were found clustered in regions that were proximal to the plasma membrane as well as within the cell interior, often in juxtaposition to characteristic membranous tracks (Fig. 8, A and B, arrows). This pattern agreed very well with the localization patterns we observed previously for Tor1p, Tor2p, and Lst8p (26) and is consistent with our biochemical and proteomic studies demonstrating that these proteins interact functionally (Fig. 1 and Table III). Strikingly, however, we also observed significant labeling of vacuolar structures, where gold particles were often clustered directly at membranes surrounding the vacuole (Fig. 8, A, C, and D, arrowheads). Importantly, such close juxtaposition of gold particles to the vacuole was not observed previously with Tor1p, Tor2p, or Lst8p (26). Together, these results demonstrate that Tco89p localizes to regions within the cell that are both overlapping and distinct from the Tor proteins as well as one of their interacting partners. These findings are consistent with our genetic observations suggesting that Tco89p may have an additional, TORC1-independent cellular role.

**FIG. 5.** SSD1 rescues the rapamycin hypersensitivity of tor1Δ and tco89Δ strains. tor1Δ (PLY254) and tco89Δ (PLY332) cells were transformed with either a control plasmid (pRS116) or a plasmid that expressed the JK9-3da-derived allele of SSD1 (pPL092) and were streaked out onto SCD agar plates lacking uracil. Agar plates contained 1.0 μg/ml rapamycin where indicated. Plates were incubated at 30 °C for 3 days and then photographed.

**TABLE IV**

| Strains | tor1Δ | 30 °C | 36 °C | 37 °C | 30 °C + CFW |
|---------|-------|-------|-------|-------|-------------|
| pRS116  | + +   | -     | -     | -     | -           |
| pRS426  | + +   | + /−  | -     | -     | -           |
| pNB100  | + +   | + /−  | + /−  | + /−  | +           |
| pPL093  | + +   | + /−  | -     | -     | -           |
| pPL092  | + +   | + /−  | +     | +     | +           |
| pPL094  | + +   | + /−  | +     | +     | +           |
| pPL102  | + +   | + /−  | -     | -     | -           |
| pPL101  | + +   | + /−  | -     | -     | -           |
| pPL103  | + +   | +     | -     | -     | -           |

**DISCUSSION**

Our findings presented here extend previous conclusions that Tor1p and Tor2p associate with a distinct yet overlapping set of interacting partners (22, 26). In particular, we find that Tor1p co-purifies with Kog1p, Lst8p, and Tco89p, whereas Tor2p co-purifies with Avo1p-Avo3p, Lst8p, and Bit61p. In agreement with these results, we find that Kog1p co-purifies with Tor1p, Lst8p, and Tco89, suggesting that all four proteins exist within the same protein complex. By contrast, Lst8p co-purifies with Tor1p and Tor2p as well as each of their interacting partners, consistent with previous conclusions that Lst8p is a common component of distinct Tor1p- and Tor2p-containing protein complexes (22, 26, 27). A key advance presented here is the identification of co-purifying partners for four different proteins in parallel, leading to an internally consistent set of protein-protein interactions that define further the composition of Tor1p and Tor2p protein complexes.

In general, our results are in excellent agreement with the model proposed by Hall and co-workers wherein Tor1p, Lst8p, and Kog1p associate to form TORC1, and Tor2p, Lst8p, and...
Avo1p-Avo3p associate to form TORC2 (22). Based on this model, we conclude that Tco89p and Bit61p represent novel components of TORC1 and TORC2, respectively (Fig. 1B). By contrast, however, we remain unable to detect Tor2p as a significant and stable component of TORC1 in wild type cells. In this context, it is notable that only relatively weak co-immunoprecipitation between Tor2p and Kog1p was observed in the study by Loewith et al. (22) and, moreover, only in cells that lacked Tor1p (22). One possible explanation for these observations is that the majority of Tor2p is normally associated with Avo1p-Avo3p and Bit61p in the form of TORC2. A corollary to this conclusion is that substoichiometric association between Tor2p and its partners may be sufficient for Tor2p to carry out essential TORC1-dependent activities. An alternative explanation is that significant differences exist in the relative stability of interactions between Kog1p, Tco89p, and Tor1p versus Kog1p, Tco89p, and Tor2p that are detectable by these assays. This latter explanation may account for our inability to detect a significant interaction between Tor2p and Kog1p even in a tor1Δ mutant. Nevertheless, we believe that our observation that an additional copy of TOR2 rescues the synthetic lethality of a tor1Δ tco89Δ double mutant provides at least genetic evidence that Tor2p is likely to substitute for Tor1p within TORC1.

A second important finding reported here is that TORC1 activity is required for normal maintenance of cellular integrity. Thus, tor1Δ and tco89Δ strains display temperature-sensitive growth that is suppressible by the osmotic stabilizer sorbitol and, in addition, are hypersensitive to calcofluor white, both of which are hallmarks of cells possessing increased sensitivity to plasma membrane and/or cell wall stress. Subsequent genetic analysis revealed that these phenotypes are strain-specific and correlate with the SSD1-d allele present within the W303a strain background, as evidenced by the fact that introduction of the JK9-3da-derived allele of SSD1 rescues these mutant phenotypes. A previous study has concluded that W303a-derived SSD1-d behaves as a null allele (21), suggesting that the JK9-3da-derived allele of this gene provides an essential function in the absence of Tor1p or Tco89p activity when cellular integrity is challenged. In addition to temperature sensitivity and CFY hypersensitivity, we have found that both tor1Δ and tco89Δ strains are also hypersensitive to caffeine, an agent that also results in cell integrity defects. However, the relationship between this latter phenotype and the presence of the ssd1-d allele is not straightforward, and we believe that caffeine may act via a different mechanism to result in lethality in these mutants. We are presently exploring the molecular basis of these caffeine-dependent phenotypes.

Two additional observations support our conclusion that SSD1 influences TORC1-specifically. First, introduction of JK9-3da-derived SSD1 rescues the rapamycin hypersensitivity associated with both tor1Δ and tco89Δ cells. Second, this allele of SSD1 also provides partial rescue of the temperature sensitivity conferred by two tap42 mutant alleles, tap42-106 and tap42-109, that were identified by Broach and co-workers in the W303a background (9). This latter result is important, since it links further SSD1 to the phosphatase regulatory network that controls several rapamycin-sensitive events downstream of TORC1. In addition to Tap42p, this network includes Pph21, Pph22, and Sit4p, all of which display genetic interactions with SSD1 (9, 44, 45). Also in agreement with our present findings, Heitman and co-workers (46, 47) have previously reported a genetic interaction between TOR1 and SSD1 (note that in Ref. 46, SSD1 is referred to as SRK1). However, a link between Ssd1p or Tor1p and cell integrity was not established in these earlier studies.

It is presently unknown how SSD1 may influence cellular integrity. As described in the Introduction, Ssd1p has been implicated in a variety of cellular functions where defects in any one may result in diminished cellular integrity (21). Ssd1p
shows limited homology to 5'–3' exoribonucleases and binds RNA in vitro (48), suggesting that this protein may be involved in post-transcriptional regulation of gene expression by directly interacting with mRNAs (48). Moreover, Ssd1p appears to act within one of at least three distinct pathways that regulate cell wall structure and cellular integrity (21). Thus, one possibility is that when this Ssd1p-specific pathway is impaired, as occurs in strains carrying the SSD1-d allele, cells become susceptible to diminished TORC1 activity. Whether TORC1 functions as part of this Ssd1p-specific pathway (e.g. by regulating Ssd1p activity directly) or acts independently to influence cellular integrity remains to be determined.

Our findings are also in agreement with recent results by de la Torre-Ruiz and co-workers (20), who have reported a connection between rapamycin-sensitive Tor activity (e.g. TORC1) and maintenance of cellular integrity. Specifically, these investigators have linked Tor1p, as well as Tap42p and Sit4p, to a branch of the cell integrity pathway that involves Pkc1p and the MAPK cascade, in particular Mpk1p (19, 20). Independently, Krause and Gray (49) have also linked Tor to this MAPK cascade with respect to events related to cell integrity. Importantly, this MAPK cascade has also been linked to the TORC2-specific activity involving actin dynamics during polarized cell growth by regulating the Rho1p GTPase switch (16–18). Together, these results raise the possibility that significant cross-talk exists between TORC1 and TORC2 with respect to events related to maintenance of cellular integrity. Consistent with this view, earlier genetic analyses of different tor2 alleles provided evidence for a closer than expected juxtaposition of Tor1p/Tor2p shared and Tor2p-unique functions (14). An important question to address in the future is whether there is also a functional relationship between SSD1 and TORC2.

In addition to SSD1, our genetic analysis of the W303a and JK9-3da strain backgrounds indicates that there is likely to be at least one additional, presently undetermined, gene that contributes to the cell integrity defects associated with tor1Δ and tco89Δ cells. This gene does not appear to correspond to several other components linked to cell integrity, including MPT5, MPK1, and PKC1, since expression of JK9-3da-derived alleles of these genes on low copy plasmids failed to rescue the mutant
phenotypes of tor1Δ or tco89Δ cells. Similarly, we note that JK9-3da-derived alleles of several additional genes linked to Tor activity, including RHO2, PLC1, and MSS4 (14) also failed to rescue the mutant phenotypes of tor1Δ or tco89Δ cells.

An additional important finding reported here is that tor1 and tco89 deletions are synthetically lethal in combination in a manner that cannot be rescued by sorbitol or by JK9-3da-derived SSD1. Synthetic interactions between two mutants are often an indication that their gene products act in separate, parallel pathways to carry out an essential function, particularly when null mutations are examined, as is the case with gene deletions (50). We therefore conclude that it is likely that Tco89p has an additional cellular role that is independent of TORC1. An alternative view that we cannot presently exclude is that a functional redundancy exists between Tor1p and Tco89p with respect to a common TORC1 function and that therefore the synthetic lethality of the double mutant is the result of impairment of a common pathway and/or activity. Indeed, such a situation is expected primarily of proteins that interact physically (50), as we have shown for these proteins. However, we point out that this latter interpretation fails to account for our finding that expression of an additional copy of TOR2 rescues the lethality of the tor1Δ tco89Δ double mutant but does not suppress any of the tco89Δ single mutant phenotypes.

A final novel finding reported here is that Tco89p localizes in a pattern that is overlapping yet distinct from that of other TORC1 components we have examined by IEM, specifically Tor1p and Let82p (26). Specifically, strong labeling of vacuolar structures is observed with Tco89p, which has not been observed with the other components. In agreement with our results, a very recent proteome-wide screening of green fluorescent protein-tagged proteins in yeast also reported a vacuolar location for Tco89p (51). Remarkably, in this same study, Kog1p was also reported to display a vacuolar localization (51). At present, the significance of this vacuolar localization of Tco89p is unclear, as is the nature of its possible TORC1-independent function. This protein was identified originally in a genetic screen for components involved in glycerol uptake under conditions of osmotic stress (52). Thus, Tco89p could potentially be involved in a pathway required for maintenance of cellular integrity that is distinct from TORC1/SSD1. Alternatively, Tco89p may have a cellular role apart from cell integrity maintenance but that nevertheless depends upon an adequate level of functional TORC1.

The finding that Tco89p has a distinct cellular location pattern compared with Tor1p and Tor2p introduces the concept that the Tor proteins are unlikely to be involved in exclusive interactions with their identified partners. Such a view is consistent with results of a recent proteome-wide quantitation of interaction with their identified partners. Such a view is consistent with results of a recent proteome-wide quantitation of interaction with their identified partners. Such a view is consistent with results of a recent proteome-wide quantitation of interaction with their identified partners.

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TOR Complex 1 Includes a Novel Component, Tco89p (YPL180w), and Cooperates with Ssd1p to Maintain Cellular Integrity in *Saccharomyces cerevisiae*

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