Title
Phosphorylation by the stress-activated MAPK Slt2 down-regulates the yeast TOR complex 2.

Permalink
https://escholarship.org/uc/item/5d3132ff

Journal
Genes & development, 32(23-24)

ISSN
0890-9369

Authors
Leskoske, Kristin L
Roelants, Françoise M
Emmerstorfer-Augustin, Anita
et al.

Publication Date
2018-12-01

DOI
10.1101/gad.318709.118

Peer reviewed
Phosphorylation by the stress-activated MAPK Slt2 down-regulates the yeast TOR complex 2

Kristin L. Leskoske, François M. Roelants, Anita Emmerstorfer-Augustin, Christoph M. Augustin, Edward P. Si, Jennifer M. Hill, and Jeremy Thorner

1Department of Molecular and Cell Biology, 2Department of Mechanical Engineering, University of California at Berkeley, Berkeley, California 94720, USA

Saccharomyces cerevisiae target of rapamycin (TOR) complex 2 (TORC2) is an essential regulator of plasma membrane lipid and protein homeostasis. How TORC2 activity is modulated in response to changes in the status of the cell envelope is unclear. Here we document that TORC2 subunit Avo2 is a direct target of Slt2, the mitogen-activated protein kinase (MAPK) of the cell wall integrity pathway. Activation of Slt2 by overexpression of a constitutively active allele of an upstream Slt2 activator (Pkc1) or by auxin-induced degradation of a negative Slt2 regulator (Sln1) caused hyperphosphorylation of Avo2 at its MAPK phosophoacceptor sites in a Slt2-dependent manner and diminished TORC2-mediated phosphorylation of its major downstream effector, protein kinase Ypk1. Deletion of Avo2 or expression of a phosphomimetic Avo2 allele rendered cells sensitive to two stresses [myriocin treatment and elevated exogenous acetic acid] that the cell requires Ypk1 activation by TORC2 to survive. Thus, Avo2 is necessary for optimal TORC2 activity, and Slt2-mediated phosphorylation of Avo2 down-regulates TORC2 signaling. Compared with wild-type Avo2, phosphomimetic Avo2 shows significant displacement from the plasma membrane, suggesting that Slt2 inhibits TORC2 by promoting Avo2 dissociation. Our findings are the first demonstration that TORC2 function is regulated by MAPK-mediated phosphorylation.

Keywords: signal transduction; protein kinase; regulation; yeast; mutants

Supplemental material is available for this article.

Received July 6, 2018; revised version accepted October 10, 2018.

Cellular homeostasis requires careful coordination of metabolic growth processes and adaptive stress responses. Multiple signaling networks coordinate expansion at the cell boundary with the accumulation of intracellular mass and progression through the cell cycle and also play critical roles in responding to environmental fluctuations that compromise the integrity of the plasma membrane (PM) and, in yeast, the cell wall (Levin 2011). Like all eukaryotes, yeast contains two evolutionarily conserved multicomponent protein kinase complexes in which a TOR [target of rapamycin] polypeptide is the catalytic subunit: TOR complex 1 (TORC1) and TORC2. TORC1 and TORC2 are essential regulators of growth and homeostasis but control different aspects of yeast cell physiology (González and Hall 2017; Tatebe and Shiozaki 2017). TORC2 itself localizes to the PM (Berchtold and Walther 2009). TORC2 is thought to monitor the condition of the cell envelope [PM and cell wall] and control, through downstream effectors, processes that maintain the integrity and function of these vital barriers. The primary target of TORC2 is the AGC family protein kinase Ypk1 (and its paralog, Ypk2) (Kamada et al. 2005; Roelants et al. 2011; Berchtold et al. 2012; Niles et al. 2012). TORC2 activates Ypk1 by phosphorylating its C-terminal segment at multiple sites, two of which are highly conserved [the so-called “turn” and “hydrophobic” motifs], but all of which are required for maximal Ypk1 catalytic activity and for Ypk1 stability (Leskoske et al. 2017). Ypk1 in turn phosphorylates multiple substrates that modulate PM lipid and protein composition (for review, see Gaubitz et al. 2016; Roelants et al. 2017a). It also has been reported [Nomura and Inoue 2015] that TORC2 phosphorylates C-terminal turn-like and hydrophobic-like motifs in Pkc1, an AGC protein kinase that controls
the cell wall integrity (CWI) pathway (Levin 2011); howev-
er, whether TORC2-mediated phosphorylation of Pkc1 af-
fects its localization, stability, or activity has not been
demonstrated.

Conditions that perturb the PM stimulate TORC2-me-
diated phosphorylation of Ypk1, including sphingolipid depl-
elion [Roelants et al. 2011], hypotonic stress [Berk-
told et al. 2012; Niles et al. 2012], heat shock [Sun et al.
2012], and elevated exogenous acetic acid [Guerreiro et
al. 2016], whereas hypotonic stress inhibits TORC2-
mediated phosphorylation of Ypk1 [Lee et al. 2012; Muir
et al. 2015]. How TORC2 “senses” different stresses could,
in some cases, involve direct feedback control. For exam-
ple, sphingolipids seem to negatively regulate TORC2
because treatment of cells with a potent inhibitor [myrio-
cin/ISP-1] of L-serine:palmitoyl-CoA C-palmitoyltrans-
ferase, the enzyme that catalyzes the first committed
step in sphingolipid biosynthesis, causes up-regulation
of TORC2-dependent and Ypk1-mediated phosphoryla-
tion of proteins whose modification markedly stimulates
the rate of sphingolipid production [Roelants et al. 2011;
Muir et al. 2014].

In addition, however, other pathways that sense PM or
cell wall stress may exert some of their effects via reg-
ulation of TORC2 function. In this regard, two mitogen-
activated protein kinase (MAPK) pathways in yeast sense
and respond to stresses that challenge the cell envelope:
the high-osmolarity glycerol (HOG) pathway, whose ter-

4331al MAPK is Hog1 [Saito and Posas 2012], and the
CWI pathway, whose terminal MAPK is Slt2/Mpk1
[Levin 2011]. Hog1 activation, which can occur via a branch
dependent on PM sensor Snl1 or a branch dependent
on PM sensor Sho1, is required for cell survival under hyper-
tonic conditions [Saito and Posas 2012] but can also be
evoked by other environmental insults, including both
heat shock [Winkler et al. 2002] and cold shock [Panadero
et al. 2006], citric acid stress [Lawrence et al. 2004], hypox-
ia [Hickman et al. 2011], glucose starvation [Vallejo
and Mayinger 2015], and, most notably from the perspec-
tive of this study, sphingolipid depletion [Tanigawa et
al. 2012]. Slt2 activation, which is initiated via stretch sen-
sors anchored in both the PM and the cell wall [Wsc1,
Mid2, and others] [Levin 2011], is required for cell survival
under hypotonic conditions [Davenport et al. 1995] but
can also be elicited by heat shock [Kamada et al. 1995]
and agents that directly damage the cell wall, such as
zymolyase, Calcofluor White, or Congo Red [de Nobel et
al. 2000; García et al. 2004; Rodríguez-Peña et al. 2013].
When activated, the stretch sensors recruit to the PM
and activate Rom2 and/or Tus1—guanine nucleotide ex-
change factors (GEFs) for the Rho1 GTPase. GTP-bound
Rho1 then binds to and activates Pkc1, which in turn ac-
tivates the CWI MAPK cascade [Levin 2011]. Rho1 and its
GEFs also localize at sites of polarized growth to promote
cell wall glucan synthesis, actin cytoskeleton organiza-
tion, and targeting of secretory vesicles to growth sites
[Perez and Rincón 2010].

Significant cross-talk exists between the HOG and CWI
pathways [Hahn and Thiele 2002; García-Rodríguez et
al. 2005; Fuchs and Mylonakis 2009; García et al. 2009] and
between these MAPK pathways and TORC2 function.
For example, down-regulation of TORC2–Ypk1 signaling
and activation of Hog1 act in concert to maintain cell vi-
ability during hyperosmotic stress [Muir et al. 2015]. In ad-
dition, one of the first functions reported for Tor2 (the
TOR subunit unique to TORC2) was proper polarization
of the actin cytoskeleton [Schmidt et al. 1996], linking
Tor2 to Rho1 and the CWI pathway. Indeed, various
means to stimulate CWI pathway output rescue the invi-
ability and actin polarization defects associated with loss
of TORC2 or Ypk1 function [Helliwell et al. 1998; Roe-
lants et al. 2002]. In addition, TORC2–Ypk1 signaling,
through its role in regulating lipid composition and or-
ganization, influences PM localization of Rho1 and Rom2
[Niles and Powers 2014; Hatakeyama et al. 2017].

In this study, we sought to establish whether the CWI
MAPK pathway directly modulates TORC2–Ypk1 signal-
ing, given the cumulative evidence for the interconnec-
tions between them. As documented here, we found
that activation of the CWI pathway MAPK results in di-
rect Slt2-mediated phosphorylation of TORC2 subunits
and that these modifications down-regulate TORC2–
Ypk1 signaling. By pinpointing the molecular basis of
this negative regulation, our results provide new mecha-
nistic insight into how TORC2 senses cell envelope
stress.

Results

TORC2 subunits are targets of the Slt2 MAPK

TORC2 is a 1.4-MDa dimeric complex in which each pro-
tomer is a hetero-oligomer comprising Tor2, Lst8, Avol,
Avo2, Avo3, and either Bit2 or Bit61 [Wullschleger et al.
2005; Karuppasamy et al. 2017]. Phosphoproteomic analy-
ses [for compilations, see the Saccharomyces Genome
Database, https://www.yeastgenome.org] have detected
in vivo phosphorylation of -SP- and -TP- sites in four of
the six TORC2 subunits [Fig. 1A]. Most strikingly, nine
such sites are present in Avo2, and four confirmed sites
cluster at its C-terminal end; 11 sites are present in
Avo3, and five confirmed sites cluster at its N-terminal
end [Fig. 1A]. Both Hog1 and Slt2 phosphorylate only
-SP- or -TP- motifs [Mok et al. 2010], just like the pro-
line-directed Ser- or Thr-specific MAPKs in other eukary-
otes [Roux and Blenis 2004].

To determine whether any -SP- or -TP- sites in TORC2
subunits are direct targets of the Slt2 MAPK, we focused
initially on Avo2. As expected for a phosphoprotein,
Avo2 exists, even under basal conditions, in a series of
isoforms of distinct mobility, as resolved by Phos-tag
SDS-PAGE [Fig. 1B], a technique in which the degree
of retardation of a protein reflects the extent of its phos-
phorylation [Kinoshita et al. 2009]. A convenient method to ac-
tivate Slt2 in the absence of an external stimulus is
to introduce a mutation at the critical for the inhibitory
function of its pseudosubstrate sequence have been mutated. After induction of PKC1∗
expression, Avo2 became hyperphosphorylated, and the increase in Avo2 phosphorylation coincided with the kinetics of appearance of active Slt2 [Fig. 1B]. Overexpression of a catalytically inactive (“kinase-dead” [KD]) derivative, Pkc1∗(D949A), to the same level as Pkc1∗ [Fig. 1C] did not activate Slt2 or alter Avo2 phosphorylation [Fig. 1B].

In addition to robust Slt2 activation, we noted that overexpression of Pkc1∗ also evoked Hog1 activation, in agreement with other observations demonstrating cross-talk between these two MAPK pathways [Hahn and Thiele 2002, García-Rodríguez et al. 2005; Fuchs and Mylonakis 2009, García et al. 2009]. Therefore, to distinguish which between these two MAPK pathways (Hahn and Thiele 2002, García-Rodríguez et al. 2005; Fuchs and Mylonakis 2009, García et al. 2009). Therefore, to distinguish which MAPK was primarily responsible for the Avo2 hyperphosphorylation induced by Pkc1∗ overexpression, we repeated these experiments in both slt2Δ and hog1Δ mutants. We found that the Pkc1∗-induced mobility shift was markedly reduced in slt2Δ cells but not in hog1Δ cells [Fig. 1D]. Moreover, to confirm that the Pkc1∗-induced Slt2-mediated modifications were occurring on the -SP- and -TP- sites, the same experiments were repeated with an Avo2 allele (Avo2∗A) in which all nine sites were mutated to Ala. Indeed, absence of these sites prevented Pkc1∗-induced hyperphosphorylation of Avo2 [Fig. 1D]. Although Slt2 appears to be the major MAPK responsible for Pkc1∗-induced hyperphosphorylation of Avo2, these modifications were not completely abrogated in slt2Δ cells and were modestly reduced in hog1Δ cells compared with wild-type cells, suggesting that Hog1 makes a minor contribution to Pkc1∗-induced hyperphosphorylation of Avo2.

In further support of the capacity of Slt2 to modify Avo2, wild-type Slt2 immuno-enriched from cells overexpressing Pkc1∗—but not a catalytically deficient derivative, Slt2(K54R), purified in the same manner—robustly phosphorylated recombinant wild-type Avo2 in vitro, as described in the Materials and Methods. Reaction products were resolved by SDS-PAGE and analyzed by Coomassie blue staining.  

Figure 1. MAPK Slt2 phosphorylates TORC2 subunits Avo2 and Avo3. (A) The primary structure of each indicated TORC2 subunit is depicted schematically, with domains labeled as in Gaubitz et al. [2016]. (Black asterisk) An -SP- or -TP- site; (red asterisk) an -SP- and -TP- detectably phosphorylated in vivo in various phosphoproteomic analyses, as cataloged in the Saccharomyces Genome Database (http://www.yeastgenome.org). (B) Wild-type cells [JTY5363] carrying both a CEN plasmid expressing Avo2-3xFlag [pKL16] or GST-Avo2 in vitro, and Pkc1 was the loading control. (C) As in B except the cells carried plasmids expressing either Pkc1∗-myc [pAEA376] or Pkc1∗-KD-myc [pJEN12], and the extracts were resolved by standard SDS-PAGE prior to immunoblotting. (D) Wild-type cells [JTY5363] or otherwise isogenic slt2Δ [YFR536-A] or hog1Δ [YFR549-A] derivatives carrying plasmids expressing either Avo2-3xFlag [pKL1] or Avo2∗A-3xFlag [pKL2], as indicated, well as either empty vector [pEPL12] or the same vector expressing Pkc1∗ from the GAL1 promoter [pT5660], as shown, were grown to mid-exponential phase in selective minimal medium containing 2% raffinose and 0.2% sucrose. Expression of Pkc1∗ or Pkc1∗KD was induced by addition of galactose [2% final concentration]. Cell samples were removed at the indicated times and lysed, the proteins in the resulting extracts were resolved by Phos-tag SDS-PAGE, and the indicated proteins were analyzed by immunoblotting with the appropriate antibodies, all as described in the Materials and Methods. Pgc1 was the loading control. (E) As in B except the cells carried plasmids expressing either Pkc1∗-myc [pAEA376] or Pkc1∗-KD-myc [pJEN13], and the extracts were resolved by standard SDS-PAGE prior to immunoblotting. (F) Wild-type cells [JTY5363] or otherwise isogenic slt2Δ [YFR536-A] derivatives carrying plasmids expressing either Avo2-3xFlag [pKL1] or Avo2∗A-3xFlag [pKL2], as indicated, well as either empty vector [pEPL12] or the same vector expressing Pkc1∗ from the GAL1 promoter [pT5660], as shown, were grown to mid-exponential phase in selective minimal medium containing 2% raffinose and 0.2% sucrose. After addition of galactose [2% final concentration], the cells were cultured for 3 h, harvested, and lysed, and the indicated proteins were analyzed as in B. (E) Activated wild-type Slt2 [pKL63] and kinase-dead [KD] Slt2 [pKL64] were purified from Saccharomyces cerevisiae as described in the Materials and Methods, and incubated with [γ-32P]ATP and either GST-Avo3∗WT [pKL16] or GST-Avo3∗A [pKL17] purified from Escherichia coli as described in the Materials and Methods. Reaction products were resolved by SDS-PAGE and analyzed by Coomassie blue staining [left] and autoradiography [right]. As in B, the substrates were purified recombinant GST-Avo3[1-100] [pKL81] or GST-Avo3[1-100]A [pKL82]. (C) A strain [YFR617] expressing Avo3-3xFlag and carrying a plasmid expressing either Pkc1∗-myc [pJEN12] or Pkc1∗-KD [pJEN12] from the GAL1 promoter [pT5660], as shown, was grown to mid-exponential phase in selective minimal medium containing 2% raffinose and 0.2% sucrose. Expression of Pkc1∗ or Pkc1∗KD was induced by addition of galactose [2% final concentration]. Cell samples were removed at the indicated times and lysed, the proteins in the resulting extracts were resolved by Phos-tag SDS-PAGE, and the indicated proteins were analyzed by immunoblotting with the appropriate antibodies, all as described in the Materials and Methods. Pgc1 was the loading control. (E) As in B except the cells carried plasmids expressing either Pkc1∗-myc [pAEA376] or Pkc1∗-KD-myc [pJEN13], and the extracts were resolved by standard SDS-PAGE prior to immunoblotting.

Downloaded from genesdev.cshlp.org on November 27, 2018 - Published by Cold Spring Harbor Laboratory Press
and this incorporation was eliminated in an Avo2\(^{9A}\) mutant [Fig. 1E]. Likewise, Avo3\([1–100]\), an N-terminal fragment containing six of the 11 \(-SP-\) and \(-TP-\) sites in Avo3 (five of which have been detected as phosphorylated in vivo), was robustly phosphorylated in vitro by wild-type Stt2 but not by the catalytically deficient Stt2 mutant, and incorporation was markedly reduced by mutation of the six sites to Ala [Fig. 1F]. Moreover, as judged by its mobility shift, intact Avo3-3xFlag was phosphorylated in vivo upon overexpression of Pkc1\(^{*}\) but not upon overexpression of Pkc1\(^{*}\)KD [Fig. 1G]. Collectively, these results show that at least two core subunits of TORC2 are physiologically relevant substrates of the Stt2 MAPK.

MAPK phosphorylation of Avo2 impairs TORC2 activity

Unlike Avo3 [1430 residues], which is essential for TORC2 function because it has an important structural role [Wullschleger et al. 2005; Karuppasamy et al. 2017], Avo2 is a relatively small [426 residues] peripherally located nonessential subunit of TORC2, with the highest overall density of \(-SP-\) and \(-TP-\) sites [Fig. 1A], suggesting that its Stt2-mediated phosphorylation might have a readily detectable regulatory role. To examine this possibility, in addition to wild-type Avo2 and the MAPK site-deficient Avo2\(^{9A}\) mutant, we also generated a phosphomimetic allele, Avo2\(^{9E}\). First, we found that all three proteins were expressed at comparable steady-state levels [Fig. 2A]; thus, the absence or presence of MAPK phosphorylation does not affect Avo2 stability. Strikingly, however, even though Avo2 is nonessential for cell viability under nonstress conditions, we found that Avo2 is essential for cell survival during myriocin-induced sphingolipid depletion [Fig. 2B, top row], a condition in which cells require full TORC2-mediated activation of Ypk1 to survive [Roelants et al. 2011; Leskoske et al. 2017]. Furthermore, compared with cells expressing wild-type Avo2 or Avo2\(^{9A}\), cells expressing Avo2\(^{9E}\) were much more sensitive to the growth inhibitory effect of myriocin [Fig. 2B], suggesting that, like avo2\(^{Δ}\) cells, they are deficient in TORC2-dependent Ypk1 activation. In direct support of that conclusion, the myriocin sensitivity of both the avo2\(^{Δ}\) mutant and Avo2\(^{9E}\)-expressing cells was rescued by coexpression of a Ypk1 allele, Ypk1[D242A], that is fully active in the absence of TORC2 phosphorylation [Fig. 2C; Kamada et al. 2005; Roelants et al. 2011; Leskoske et al. 2017]. These findings indicate that Avo2 is required for optimal TORC2 function and that phosphorylation interferes with Avo2 function, resulting in down-regulation of TORC2 activity.

These conclusions were confirmed by monitoring TORC2 activity in vivo. We documented previously that TORC2 phosphorylates Ypk1 at multiple sites that lie within 40 residues of its C terminus and that full phosphorylation of these sites is necessary for both maximal Ypk1 activity and Ypk1 stability [Leskoske et al. 2017]. Therefore, to assess TORC2 activity in vivo, we examined phosphorylation of Ypk1 at four of its C-terminal TORC2-dependent phosphorylation sites using Phos-tag SDS-PAGE. Treatment with myriocin, a condition that activates TORC2 activity in wild-type cells, failed to stimulate TORC2 phosphorylation of Ypk1 in Avo2\(^{9E}\)-expressing cells [Fig. 2D]. As presented further below, Avo2 has also been found in association with PM

Figure 2. Phosphorylation of Avo2 at its MAPK sites impairs TORC2 activity. (A) Otherwise isogenic strains JTY7318 (avo2\(^{Δ}\)), yKL32 (avo2\(^{9A}\)-3xFlag), yKL34 (avo2\(^{9E}\)-3xFlag), and yKL33 (avo2\(^{9A}\)-3xFlag) were grown to mid-exponential phase in rich medium, harvested, and lysed, and the resulting extracts were resolved by SDS-PAGE and analyzed by immunoblotting, as described in the Materials and Methods. Pgk1 was the loading control. (B) Overnight cultures of the strains in A were adjusted to \(A_{600}\) nm = 0.1, spotted undiluted and in a series of fivefold serial dilutions on YPD plates either lacking or containing 0.8 \(μM\) myriocin, incubated for 3 d at 30°C, and then imaged. (C) Strains JTY7318 (avo2\(^{Δ}\)), yKL32 (avo2\(^{9E}\)-3xFlag), or yKL34 (avo2\(^{9E}\)-3xFlag) expressing either empty vector (pRS315) or Ypk1[D242A]-myc from the same vector (pFR234) were adjusted to \(A_{600}\) nm = 0.1, spotted as in B but on SCD-L-T plates lacking or containing 0.8 \(μM\) myriocin, incubated for 3 d at 30°C, and then imaged. (D) Wild-type (BY4742), Avo2\(^{9E}\)-3xFlag expressing either empty vector (pRS315) or Ypk1[D242A]-myc from its native promoter on a CEN plasmid (pFR234) were adjusted to \(A_{600}\) nm = 0.1, spotted as in B but on SCD-L-T plates lacking or containing myriocin at the indicated concentrations, and imaged after incubation for 3 d at 30°C. (E) Wild-type (BY4742), Avo2\(^{9A}\)-3xFlag, or Avo2\(^{9E}\)-3xFlag expressing either empty vector (pRS315) or Ypk1[D242A]-myc from its native promoter on a CEN plasmid (pFR246) were cultured to mid-exponential phase in selective minimal medium and then treated with vehicle (methanol) or 1.25 \(μM\) myriocin for 2 h. After harvesting, whole-cell lysates were prepared, resolved by SDS-PAGE, and analyzed as described in the Materials and Methods. A representative of three independent experiments is shown. (F) Overnight cultures of the strains in A were adjusted to \(A_{600}\) nm = 0.1, spotted undiluted and in a series of 10-fold serial dilutions on SCD plates either lacking or containing acetic acid at the indicated concentrations, and imaged after incubation for 3 d at 30°C.
structures called eisosomes [Douglas and Konopka 2014], and Pil1, a major eisosome component, has been shown to be another target of Slt2 [Mascaraque et al. 2013]. Hence, we also examined Ypk1 phosphorylation in cells expressing both Avo2<sup>9E</sup> and Pil1<sup>3D</sup> and again found that, unlike in wild type or cells expressing Pil1<sup>3D</sup> alone [Supplemental Fig. S1], myriocin treatment failed to stimulate TORC2 phosphorylation of Ypk1 in the cells expressing both Avo2<sup>9E</sup> and Pil1<sup>3D</sup> [Fig. 2E]. These additional observations verify that it is phosphorylation of Avo2 that makes a major contribution to interfering with TORC2 function.

Another stress that cells require TORC2-mediated Ypk1 activation to survive is exposure to elevated exogenous acetic acid [Guerreiro et al. 2016]. As with myriocin treatment, avo2Δ cells were markedly more sensitive than cells expressing either wild-type Avo2 or especially Avo2<sup>9E</sup> [Fig. 2E]. These results again show that Avo2 is required for full TORC2 activity and that MAPK phosphorylation of Avo2 impairs TORC2 function.

### Figure 3. Activation of MAPK Slt2 down-regulates TORC2-mediated Ypk1 phosphorylation.

#### A

**Ypk1<sup>14A-myc</sup>**

<table>
<thead>
<tr>
<th>Time + Gal. (min):</th>
<th>Pkc1&lt;sup&gt;*Δ&lt;/sup&gt;KD</th>
<th>Pkc1&lt;sup&gt;**&lt;/sup&gt;</th>
<th>Wild-type</th>
<th>slt2Δ</th>
<th>hog1Δ</th>
<th>kss1Δ</th>
<th>slt2Δ kss1Δ</th>
<th>hog1Δ kss1Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
</tbody>
</table>

**I: myc**

<table>
<thead>
<tr>
<th>pYpk1</th>
<th>pSt2</th>
<th>pKss1</th>
<th>pHog1</th>
<th>Pgk1</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### B

**Ypk1<sup>14A-myc</sup>**

<table>
<thead>
<tr>
<th>Time + Gal. (min):</th>
<th>Pkc1&lt;sup&gt;*Δ&lt;/sup&gt;KD</th>
<th>Pkc1&lt;sup&gt;**&lt;/sup&gt;</th>
<th>Wild-type</th>
<th>slt2Δ</th>
<th>hog1Δ</th>
<th>kss1Δ</th>
<th>slt2Δ kss1Δ</th>
<th>hog1Δ kss1Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

**I: myc**

<table>
<thead>
<tr>
<th>pYpk1</th>
<th>pSt2</th>
<th>pKss1</th>
<th>pHog1</th>
<th>Pgk1</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### C

**Time + Gal. (min):**

<table>
<thead>
<tr>
<th>WT</th>
<th>slt2Δ</th>
<th>hog1Δ</th>
<th>kss1Δ</th>
<th>slt2Δ kss1Δ</th>
<th>hog1Δ kss1Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
</tbody>
</table>

**I: myc**

<table>
<thead>
<tr>
<th>pYpk1</th>
<th>pSt2</th>
<th>pKss1</th>
<th>pKog1</th>
<th>Pgk1</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### D

**Time + Gal. (min):**

<table>
<thead>
<tr>
<th>WT</th>
<th>slt2Δ</th>
<th>hog1Δ</th>
<th>kss1Δ</th>
<th>slt2Δ kss1Δ</th>
<th>hog1Δ kss1Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
</tbody>
</table>

**I: myc**

<table>
<thead>
<tr>
<th>pYpk1</th>
<th>pSt2</th>
<th>pKss1</th>
<th>pKog1</th>
<th>Pgk1</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### E

**Congo Red**

<table>
<thead>
<tr>
<th>pYpk1</th>
<th>pSt2</th>
<th>pKog1</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### F

**Congo Red**

<table>
<thead>
<tr>
<th>pYpk1</th>
<th>pSt2</th>
<th>pKog1</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The loading control. [Red dots] TORC2-dependent isoforms of Ypk1 that persist. [D] As in C, except the cells coexpressed Pkc1<sup>*Δ</sup> KD from the GAL1 promoter on the plasmid [pEN12]. [E] Wild-type [BY4741] cells expressing Ypk1<sup>14A-myc</sup> from its native promoter on a CEN plasmid [pFR246] were cultured to mid-exponential phase and then treated with increasing amounts of Congo Red [0, 10, 20, or 40 µg/mL final concentration] for 2 h. After harvesting, whole-cell lysates were prepared, resolved by SDS-PAGE, and analyzed as described in the Materials and Methods. [F] Same as in E, except that wild-type [JTY5336] and slt2Δ [YFR549] strains were used, and, when added, Congo Red was added at 20 µg/mL final concentration.
phosphorylation or Ypk1 protein were not further enhanced in hog1Δ slt2Δ or kss1Δ slt2Δ double mutants (Fig. 3C). Most revealingly, in every case where Slt2 is absent, two of the most prominent and highly TORC2 phosphorylated Ypk1 isoforms persisted [Fig. 3C, red dots]. Thus, Slt2 is the MAPK primarily responsible for inhibiting TORC2-mediated Ypk1 phosphorylation.

It has been reported that Pkc1 is a substrate of TORC2 [Nomura and Imoue 2015], hence, it was possible that overexpression of even Pkc1-YKΔ KD might titrate away a sufficient amount of TORC2 to interfere with its efficient phosphorylation of Ypk1. However, this was not the case because, in either wild-type cells or all of these same mutants, induction of Pkc1-YKΔ KD had little or no effect on the pattern of the Ypk1 isoforms [Fig. 3D]. Furthermore, treatment of cells expressing normal Pkc1 and Slt2 at their endogenous levels with the cell wall-perturbing dye Congo Red, a physiological condition that has been demonstrated to elicit Pkc1-dependent Slt2 activation [de Nobel et al. 2000], resulted in down-regulation of TORC2 function [Fig. 3E], and the down-regulation was dependent on Slt2 [Fig. 3F].

**TORC2 is down-regulated during sustained Sln1 inactivation**

Sln1 is a histidine kinase similar to those in bacterial two-component signaling systems, and its inactivation, as occurs during hyperosmotic shock, leads to activation of the Hog1 MAPK [Saito and Posas 2012]. In a global screen of the *Saccharomyces cerevisiae* kinome, we found that, like a tor2Δ mutant [Leskoske et al. 2017], when a sln1Δ mutant was shifted to the restrictive temperature, there was a dramatic loss of TORC2-dependent Ypk1 phosphorylation [Supplemental Fig. S2A] that was accompanied by hyperphosphorylation of Avo2 [Supplemental Fig. S2B], indicating that, like activation of the CWI MAPK pathway, activation of the HOG MAPK pathway down-regulates TORC2 function and does so via a similar mechanism.

To confirm this conclusion and inactivate Sln1 independently of any external temperature or hypertonic stress, we constructed a strain expressing the plant F-box protein TIR1 and an allele of Sln1 tagged with a monoblotting as described in the Materials and Methods.

Consistent with the cross-talk by which activation of Hog1 leads to activation of Slt2 [García-Rodríguez et al. 2005], the drastic reduction in Ypk1 C-terminal phosphorylation and protein level caused by Sln1 degradation was ameliorated in either a hog1Δ mutant or a slt2Δ mutant and was not further enhanced in a hog1Δ slt2Δ double mutant [Fig. 4B]. Moreover, similar to what we observed upon Pkc1-YKΔ induced CWI pathway activation, activation of the HOG pathway by Sln1 degradation markedly stimulated phosphorylation of Avo2, as judged by the shift to a spectrum of slower mobility isoforms in the Phos-tag gel [Fig. 4C, left two lanes], and no such shift was observed upon starvation or deletion of the promoter of Avo2.
for the Avo2^{9A} mutant, indicating that these modifications were occurring on its -SP- and -TP- sites [Fig. 4C, right two lanes]. Furthermore, there was no change in the pattern of Avo2 isoforms upon Sln1 degradation in cells lacking either Hog1 or Slt2 [or both] [Fig. 4C, middle lanes], indicating that Avo2 phosphorylation was MAPK-dependent. Importantly, activation of Hog1 alone [upon degradation of Sln1 in slt2Δ cells] was not sufficient to down-regulate TORC2-mediated Ypk1 phosphorylation [Fig. 4B] or stimulate Avo2 phosphorylation [Fig. 4C], indicating that Slt2 is the MAPK responsible for these effects and that, in this case, the primary role of activated Hog1 is to stimulate production of activated Slt2.

Interestingly, we documented previously that when the HOG pathway is activated by exposure of cells to 1 M sorbitol, a cell-impermeable osmolyte that robustly but transiently activates Hog1 [Saito and Posas 2012], there is rapid and transient collapse of the TORC2-dependent Ypk1 isoforms, but that response still occurs even in hog1Δ cells [Lee et al. 2012; Muir et al. 2015]. Although treatment with 1 M sorbitol activates Hog1, it does not activate Slt2, and the collapse of the TORC2-dependent Ypk1 isoforms still occurs in cells lacking either Slt2 or Hog1 [or both] [Supplemental Fig. S3]. Thus, in contrast to the Slt2-dependent down-regulation of TORC2-mediated Ypk1 phosphorylation that occurs upon prolonged inactivation of Sln1, the loss of the TORC2-dependent Ypk1 isoforms caused by exposure to 1 M sorbitol occurs in a MAPK-independent manner. Possibly, the hypertonic shock caused by 1 M sorbitol activates an as yet to be identified phosphatase. In any event, both acute and chronic stimulation of the HOG pathway leads to down-regulation of TORC2–Ypk1 signaling, albeit by different mechanisms.

MAPK phosphorylation of Avo2 alters its subcellular localization

Studies of various integral and peripheral PM proteins in yeast have shown that different components often reside in discrete microdomains [Spira et al. 2012]. Many permeases, such as the arginine transporter Can1, colocalize in a membrane compartment called the “MCC” that is congruent with eisosomes [Strádalová et al. 2009], protein-coated PM invaginations whose major constituents include Pil1, an F-BAR [FCH/bin-amphiphysin-rvs] domain-containing protein [Karotki et al. 2011]. Initially, it was reported that all of the components of TORC2, including Avo2, reside in a PM microdomain dubbed the “MCT” that is distinct from eisosomes [Berchtold and Walther 2009]. However, subsequent work demonstrated that Avo2 partitions between TORC2 and eisosomes [Bartlett et al. 2015]. For these reasons, and having established that MAPK phosphorylation does not affect Avo2 stability [Figs. 2A, 5C], to explain how MAPK phosphorylation of Avo2 contributes to down-modulation of TORC2 activity, we examined whether MAPK phosphorylation of Avo2 affected its subcellular localization.

Toward that end, we expressed either Avo2, Avo2^{9A}, or Avo2^{9E} [each tagged at its C terminus with mNeon Green [mNG]] [Shaner et al. 2013] from its endogenous locus on chromosome XIII in cells coexpressing the eisosome marker Pil1 [tagged at its C terminus with red fluorescent protein [RFP]] [Fradkov et al. 2000] from its endogenous locus on chromosome VII. We document [1] that Avo2-mNG is fully functional [Supplemental Fig. S4] and [2] that both Avo2-mNG and Pil1-RFP displayed a punctate distribution at the PM and that these puncta were often, but not always, congruent [Fig. 5A, left], as we resolved and quantified using CellProfiler and appropriate masking [Supplemental Fig. S5]. The same was observed for Avo2^{9A}-mNG and Pil1-RFP [Fig. 5A, middle]. However, there were significant differences found in the cells expressing Avo2^{9E}-mNG and Pil1-RFP. Generally, the Avo2^{9E}-containing puncta were smaller and less prominent [Fig. 5A, right], and their overall mean pixel intensity was significantly lower than in cells expressing Avo2 or Avo2^{9A} [Fig. 5B] despite the fact that the total amount of Avo2, Avo2^{9A}, and Avo2^{9E} in each of these cells was very similar [Fig. 5C], and the intensity of the Pil1-RFP foci remained essentially unchanged [Fig. 5A,B]. Moreover, the decrease in the intensity of the Avo2^{9E}-containing PM puncta was the same regardless of whether the Avo2^{9E} colocalized with Pil1 [Fig. 5D] or was located at distinct PM sites, presumably with TORC2 [Fig. 5E]. Taken together, these findings suggest that MAPK phosphorylation promotes dissociation of Avo2 from either of its two PM sites: eisosomes and TORC2.

To confirm that conclusion for TORC2 specifically by an independent biochemical approach, we immunooisolated TORC2 [in which the Avo3 subunit carried a 3xFlag epitope tag] from the cells expressing Avo2, Avo2^{9A}, or Avo2^{9E} and analyzed the amount of Avo2 present in these complexes. In the immunoprecipitates obtained using anti-Flag antibodies, equivalent amounts of both Avo3-3xFlag and the Tor2 catalytic subunit were found regardless of whether the cells expressed Avo2, Avo2^{9A}, or Avo2^{9E} [Fig. 5F]. Likewise, in cells expressing Tor2-mNG and Avo2, Avo2^{9A}, or Avo2^{9E} there was little or no effect on the amount of Tor2 present in PM-associated TORC2 [Supplemental Fig. S6]. However, quantification of the immunoprecipitates showed that, compared with the amount of Avo2 or Avo2^{9A} bound to TORC2, the amount of Avo2^{9E} was always reduced by 30%–40%. Thus, in agreement with the observations made by fluorescence imaging, these results suggest that MAPK phosphorylation of Avo2 promotes its dissociation from TORC2.

Discussion

It has been unclear whether TORC2 adjusts its activity by serving itself as a direct sensor of perturbations to the cell envelope or responding to inputs from other stress-sensing pathways [or both]. We demonstrated here, for the first time, that Slt2/Mpk1 [mammalian ortholog ERK5/MAPK7] [Truman et al. 2006], the MAPK of the CWI pathway, phosphorylates two subunits of TORC2 (Avo2 and Avo3) and, as a result, down-regulates TORC2.
phosphorylation of its primary downstream effector, AGC
family kinase Ypk1 (mammalian ortholog SGK1) (Casa-
mayor et al. 1999). We also found that modification of
Avo2 was sufficient to explain Slt2-mediated inhibition
of TORC2, but it is possible that Slt2 phosphorylation of
Avo3 also contributes to TORC2 down-modulation. Like-
wise, all of the other subunits of TORC2 contain potential
MAPK phosphorylation sites; however, unlike for Avo2
and Avo3, phosphorylation at very few of these other can-
didate sites has been detected in global phosphoproteomic
studies.

In any event, the control circuit that we discovered
makes physiological sense because under conditions
that interfere with cell wall synthesis or damaged cell
wall structure, activation of the CWI pathway will
exert feedback that will negatively regulate the growth-
promoting functions of TORC2.
wall structure, activation of the CWI pathway will exert feedback that will negatively regulate the growth-promoting functions of TORC2 (Fig. 5G). For yeast cell growth, enlargement of the cell wall needs to be tightly coupled to both an increase in cell mass and expansion of the PM. TORC2–Ypk1 signaling controls the processes that maintain adequate PM levels of all the lipid classes (sphingolipids, glycerolipids, and sterols) needed to sustain growth and viability [Roelants et al. 2017a, 2018]. Thus, down-regulation of TORC2 activity upon CWI pathway activation provides a mechanism by which the status of the cell wall can be sensed by TORC2 and thereby the rates of the reactions necessary for PM homeostasis can be adjusted accordingly.

The need for regulatory circuitry in which the state of the cell wall can feed in and influence TORC2 function perhaps explains why a recognizable Avo2 homolog is present throughout the vast majority of the fungal clade—from *S. cerevisiae* and its sensu stricto relatives to divergent yeast species (e.g., *Debaromyces hansenii* and *Yarrowia lipolytica*) to filamentous fungi (e.g., *Aspergillus nidulans* and *Neurospora crassa*) [see https://portals.broadinstitute.org/cgi-bin/regio/orthogroups/show_orthogroup.cgi?orf=YMR068W]—but is not conserved in multicellular organisms that lack a cell wall. In this same regard, evidence genetic indicates that the Slt2 ortholog (Pmk1) in fission yeast (*Schizosaccharomyces pombe*) has a role in negatively regulating TORC2-mediated activation of the fission yeast Ptk1 ortholog (Gad8) [Cohen et al. 2014], providing further support for conservation of the overall mechanism that we describe here.

Our results show that Avo2 is not only an important site of Slt2-mediated phosphorylation but also necessary for optimal TORC2 activity, especially under stressful conditions. However, the function of Avo2 in TORC2 is not currently known. Within its N-terminal half, Avo2 contains at least five ankyrin repeats [Gaubitz et al. 2016], which are known to mediate protein–protein interactions but, in some cases, also interactions with other classes of biomolecules. Initial dissection of the subunit contacts in yeast TORC2 by coimmunoprecipitation and pull-down assays suggested that Avo2 associates only with Avo1 and Avo3 [Wullschleger et al. 2005]. However, recent modeling of a predicted Avo2 structure into the cryo-electron microscopy [cryo-EM] density of yeast TORC2, if interpreted correctly, indicates intimate contact of Avo2 with the HEAT repeat and FAT domain regions of Tor2, much less extensive interaction with Avo3, and no contact with Avo1 [Karuppasamy et al. 2017]. Nonetheless, this placement is surface-exposed and quite distant from the active site of the kinase domain in Tor2 and hence does not readily suggest an obvious function for Avo2 [Karuppasamy et al. 2017].

As first detected in global two-hybrid screens, Avo2 also interacts with two other paralogous proteins, Slm1 and Slm2, and the ability of Avo2 to associate with Slm1 and Slm2 has been observed by both coimmunoprecipitation [Audhya et al. 2004] and in vitro binding assays [Fadri et al. 2005]. Slm1 and Slm2 are components of the furrrow-like PM invaginations called eisosomes that are coated with the PtdIns4,5P2-binding F-BAR domain-containing proteins Pil1 and Lsp1 [Karotti et al. 2011]. Similarly, the central ~190 residues of Slm1 [686 residues] and Slm2 [656 residues] contain a predicted I-BAR [inverse BAR] domain [which is required for their targeting to eisosomes] [Oliveira-Couto et al. 2011] and a demonstrated ~110-residue PtdIns4,5P2-binding PH domain near their C-terminal ends [Audhya et al. 2004; Gallego et al. 2010]. Absence of Slm1 and Slm2 reportedly disrupts eisosome structure [Kamble et al. 2011].

It has been observed before that each component subunit of TORC2 localizes around the cell periphery in multiple PM-associated “dots” and that these puncta are quite dynamic [Berchtold and Walther 2009], whereas the eisosomes are considered rather static structures [Brach et al. 2011]. Moreover, it had been suggested that these two compartments are discrete [Berchtold and Walther 2009]. However, as we found and document here, a substantial fraction of Avo2 is readily found in association with the diagnostic eisosome marker Pil1, in agreement with the observations of at least one other group [Bartlett et al. 2015]. Furthermore, there is convincing evidence that the Avo2-interacting proteins and eisosome components Slm1 and Slm2 are required for TORC2-mediated phosphorylation of Ypk1 [Berchtold et al. 2012; Niles et al. 2012]. However, the model proposed was that, under appropriate stress conditions (including hypotonic shock and sphingolipid depletion), Slm1/2 dissociate from eisosomes, bind Ypk1, and deliver this substrate to the TORC2 compartment [Berchtold et al. 2012; Niles et al. 2012]. On the other hand, equally compelling data demonstrate that it is the CRIM element present in Avo1 and its orthologs in fission yeast (Sin1) and mammalian cells [mSin1] that binds and presents substrates to the catalytic center in TORC2 [Liao and Chen 2012; Tatebe and Shiozaki 2017].

Hence, the findings that we describe here suggest an obverse model; namely, that the role of Avo2 (assuming, as its gene name indicates, that it “associates voraciously” with Tor2 and the other components of TORC2) is to act as an adaptor that, through its association with Slm1/Slm2, is responsible for the dynamic docking of TORC2 at eisosomes. What, then, is the function of the Avo2–Slm1/2 interaction in promoting TORC2 function? As demonstrated by multiple groups [Bultynck et al. 2006; Tabuchi et al. 2006], Slm1/2 also bind tightly Ca2+/calmodulin-activated phosphoprotein phosphatase 2B [also known as calcineurin] and do so via canonical so-called PxxIT motifs located very near their C termini [673–680 PNIYIQ676 in Slm1 and 640–645 PEFYIE644 in Slm2]. Hence, it is possible that the calcineurin bound to Slm1/2 acts to remove the inhibitory phosphorylations present on Avo2 and other TORC2 subunits, thereby alleviating this negative regulation. Consistent with this model, we found that Avo2<sup>W62E</sup>, an allele that mimics the permanently phosphorylated state and hence would be immune to calcineurin action, was inhibitory to optimal TORC2 function in vivo, exhibited enhanced dissociation from PM sites, and reduced association with TORC2. Thus, based on the behavior of this phosphomimetic allele, a primary
effect of Slt2-mediated phosphorylation is to impede its integration into TORC2 and interfere with Avo2-mediated association of TORC2 with the cell cortex. Whether Slt2 phosphorylation of Avo3 (or other TORC2 subunits) further promotes this dissociation and other ramifications of this model provide fertile ground for further study.

Finally, what we reveal here is one molecular mechanism by which a stress signal restrains a growth-promoting signal. As we document here for yeast, it seems likely that MAPK pathways activated by stress will impede TORC2 function in mammalian cells in a similar manner because continued growth and response to stress are invariably incompatible cellular states.

Materials and methods

Construction of yeast strains and growth conditions

Unless indicated otherwise in Table 1, *S. cerevisiae* strains used in this work were all constructed using conventional yeast genetic methods (Sherman 2002). Unless stated otherwise, yeast cultures were grown in standard rich (YPD) medium or defined minimal (SC) medium (Sherman 2002) containing 2% glucose/dextrose and were supplemented with the appropriate nutrients to permit growth of auxotrophs and/or select for plasmids. Cultures were propagated at 30°C, unless indicated otherwise. Induction of genes under the control of the GAL promoters was carried out either by adding 2% galactose to strains pregrown in the appropriate SC medium with 2% raffinose and 0.2% sucrose followed by incubation over the course of 3 h or, for strains containing the Gal4 human estrogen receptor herpes simplex virus transactivator VP16 fusion protein [Gal4-ER-VP16 (GEV)] (Quintero et al. 2007), by addition of 20 µM β-estradiol (final concentration) followed by incubation over the course of 3 h. For strains containing a protein tagged with an AID, the cells were cultured in YPD or the appropriate SC medium buffered with 50 mM potassium phosphate (pH 6.2), and degradation was induced by addition of a synthetic auxin [1-NAA] (Sigma-Aldrich) for the indicated time periods.

Plasmids and recombinant DNA methods

Plasmids used in this work [Table 2] were constructed using standard procedures in *Escherichia coli* strain DH5α (Green and Sambrook 2012). All PCR reactions were performed with Phusion high-fidelity DNA polymerase (New England Biolabs, Inc.). Site-directed mutagenesis was performed by using the appropriate mismatch oligonucleotide primers with the QuikChange method (Agilent Technologies, Inc.) according to the manufacturer’s instructions. The fidelity of all constructs was verified by nucleotide sequence analysis.

Cell extract preparation and immunoblotting

Samples of exponentially-growing cells were harvested by brief centrifugation and stored at –80°C. Cell pellets were thawed on ice and lysed in 150 µL of 1.85 M NaOH and 7.4% β-mercaptoethanol. Proteins were precipitated by the addition of 150 µL of 50% trichloroacetic acid for 10 min on ice. Precipitated proteins were pelleted by centrifugation and washed twice with ice-cold acetone. Protein pellets were solubilized in 5% SDS in 0.1 M Tris base, adjusted to a final concentration of 0.025 A_{600} per microlitre, mixed with one-fifth volume of 5× SDS sample buffer, boiled for 10 min, and then resolved by SDS-PAGE. Phosphoproteins were resolved by Phos-tag SDS-PAGE [Kinoshita et al. 2009]: Phosphorylated Ypk1-myc was resolved in 8% acrylamide gels containing 35 µM Phos-tag affinity reagent [Wako Chemicals USA, Inc.] and 70 µM MnCl₂, phosphorylated Avo2-3xFlag was resolved by in 8% acrylamide gels containing 17.5 µM Phos-tag reagent and 35 µM MnCl₂, and phosphorylated Avo3-3xFlag was resolved in 8% acrylamide gels containing 20 µM Phos-tag reagent and 40 µM MnCl₂.

For immunoblotting, proteins in gels were transferred electrophoretically to a nitrocellulose membrane, incubated with Odyssey blocking buffer (LI-COR Biosciences, Inc.) diluted 1:1 with PBS or TBS, and then probed by addition of the appropriate primary antibody at the indicated dilution: mouse anti-myc mAb 9E10 [1:100], Monoclonal Antibody Facility, Cancer Research Laboratory, University of California at Berkeley, mouse anti-Flag M2 mAb (1:10,000, Sigma-Aldrich), mouse anti-HA.11 mAb [1:1000, BioLegend], rabbit polyclonal anti-RFP [1:5000, Rockland Immunochemicals], rabbit polyclonal anti-hrFP [1:1000 to detect mKate, Evrogen], or rabbit polyclonal anti-Pglk1 [1:30,000, this laboratory, prepared as described in Baum et al. 1978]. Activated [dual phosphorylated] Hog1 was detected using rabbit anti-p38 MAPK phospho-Thr180/phospho-Tyr182 mAb [1:1000, Cell Signaling Technology], and activated [dual phosphorylated] Slt2 was detected using rabbit anti-p44/42 [Erk1/2] MAPK phospho-Thr202/Thr204 mAb [1:1000, Cell Signaling Technology]. After rinsing the membranes, filter-bound immune complexes were detected with an appropriate infrared dye-labeled secondary antibody—CF770-conjugated goat anti-mouse IgG (Biotium), IRDye800CW-conjugated goat anti-rabbit IgG (LI-COR), or IRDye680IRD-conjugated goat anti-mouse IgG (Li-Cor)—that was diluted 1:10,000 in 1:1 Odyssey blocking buffer:PBS (or TBS) containing 0.1% Tween-20 and 0.02% SDS and visualized using an infrared imaging system [Odyssey CLx, Li-Cor].

Slt2 in vitro kinase assay

To purify activated Slt2, 1 L each of yeast strain CGA54 cotransformed with *P* _GAL1-CALP* and either pKL63 (*P* _GAL1-Slt2^KD^, TAP) or pKL64 (*P* _GAL1-Slt2^WT^, TAP) was grown to mid-exponential phase at 30°C. Expression was induced by addition of 20 µM β-estradiol followed by incubation for 3 h. The cells were harvested, washed once in ice-cold TAP-B buffer containing phosphatase and protease inhibitors [200 mM NaCl, 1.5 mM MgOAc, 1 mM DTT, 2 mM NaVO₃, 10 mM NaF, 10 mM Na-PPI, 10 mM β-glycerophosphate, 1× complete protease inhibitor [Roche], 50 mM Tris-HCl at pH 7.5], resuspended in 4 mL of TAP-B buffer, and flash-frozen as droplets in liquid N₂. The cells were ruptured cryogenically in a Mixer Mill MM301 (Retsch). The resulting lysate was thawed on ice, diluted with 8 µL of TAP-B buffer, and clarified by centrifugation at 15,000g for 20 min. The resulting clarified extract was subjected to centrifugation at 78,000g for 1 h, and the resulting supernatant fraction was adjusted to a final concentration of 0.15% NP-40 using an appropriate volume of a 10% NP-40 stock. Slt2-TAP in the detergent-containing soluble fraction was captured by binding to IgG-agarose resin [GE Healthcare]. After washing the resin extensively with TAP wash buffer [200 mM NaCl, 1.5 mM MgOAc, 1 mM DTT, 0.01% NP-40, 2 mM NaVO₃, 10 mM NaF, 10 mM Na-PPI, 10 mM β-glycerophosphate, 50 mM Tris-HCl at pH 7.5], it was washed with Protease 3C buffer [200 mM NaCl, 1.5 mM MgOAc, 1 mM DTT, 0.01% NP-40, 10% glycerol, 2 mM NaVO₃, 10 mM NaF, 10 mM Na-PPI, 10 mM β-glycerophosphate, 50 mM Tris-HCl at pH 7.5]. To elute the bound Slt2-TAP, the washed resin was resuspended in 500 µL of Protease 3C buffer containing 60 U of PreScission protease [GE Healthcare]. After incubation for 5 h at 4°C, the resin was
removed by centrifugation, and the Protease 3C in the resulting superna-}


tant fraction was removed by incubation with a slurry of glutathione-Sepharose beads (GE Healthcare) followed by centrifugation. The affinity-purified Slt2 in the resulting final supernatant solution was diluted into kinase assay buffer (125 mM potassium acetate, 5% glycerol, 12 mM MgCl₂, 0.5 mM EGTA, 10 mM Hepes, pH 7.5). The affinity-purified Slt2 was incubated with Avo2 in the kinase assay buffer for 10 min at 30°C. The kinase assay was initiated by the addition of GSK3 kinase substrate [32P]GSK3-

\[ \text{Slt2 phosphorylates and down-regulates TORC2} \]

### Table 1. S. cerevisiae strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4741</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</td>
<td>Research Genetics, Inc.</td>
</tr>
<tr>
<td>YFR549</td>
<td>BY4741 trp1Δ::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>YFR538-A</td>
<td>BY4741 hog1Δ::KanMX trp1Δ::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>YFR560</td>
<td>BY4741 kss1Δ::CgHIS3 trp1Δ::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>YFR559</td>
<td>BY4741 slt2Δ::KanMX hog1Δ::KanMX trp1Δ::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>YFR567</td>
<td>BY4741 slt2Δ::KanMX kss1Δ::CgHIS3 trp1Δ::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>JTY5336</td>
<td>BY4741 Avo3-3xFlag::KanMX trp1Δ::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>JTY5473</td>
<td>BY4741 sln1ts-4::KanMX</td>
<td>Costanzo et al. 2010</td>
</tr>
<tr>
<td>yKL15</td>
<td>BY4741 Sln1-6xHA::HygR TIR1::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>yKL10</td>
<td>BY4741 Sln1-AID-6xHA::HygR TIR1::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>yKL20</td>
<td>BY4741 lys2Δ0 Sln1-AID-6xHA::HygR hog1Δ::KanMX TIR1::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>yKL16</td>
<td>BY4741 Sln1-AID-6xHA slt2Δ::URA3 TIR1::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>yKL22</td>
<td>BY4741 Sln1-AID-6xHA hog1Δ::KanMX slt2Δ::URA3 TIR1::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>JTY7318</td>
<td>BY4742 avo2Δ::KanMX</td>
<td>Research Genetics, Inc.</td>
</tr>
<tr>
<td>yKL32</td>
<td>BY4742 Avo2-3xFlag::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>YFR181</td>
<td>BY4742 Pil1-RFP LYS2 met15Δ0</td>
<td>This study</td>
</tr>
<tr>
<td>YFR528</td>
<td>BY4742 Avo2Δ[T144A T219A S233A S240A S249A T310A S315A T330A S333A]::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>yKL31</td>
<td>BY4742 Avo2Δ[T144E T219E S233E S240E S249E T310E S315E T330E S333E]::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>yKL33</td>
<td>BY4742 Avo2Δ[T144A T219A S233A S240A S249A T310A S315A T330A S333A]::3xFlag::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>yKL34</td>
<td>BY4742 Avo2Δ[T144E T219E S233E S240E S249E T310E S315E T330E S333E]::3xFlag::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>yEPS2</td>
<td>BY4741 Pil1Δ[S163D S230D T233D]::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>YFR641</td>
<td>BY4742 Avo2Δ[T144E T219E S233E S240E S249E T310E S315E T330E S333E]::URA3 Pil1Δ[S163D S230D T233D]::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>yAEA348</td>
<td>BY4741 Pil1-RFP Avo2Δ-mNG-3xHA::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>yAEA349</td>
<td>BY4741 Pil1-RFP Avo2Δ[T144A T219A S233A S240A S249A T310A S315A T330A S333A]::mNG-3xHA::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>yAEA350</td>
<td>BY4741 Pil1-RFP Avo2Δ[T144E T219E S233E S240E S249E T310E S315E T330E S333E]::mNG-3xHA::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>yAEA301-A</td>
<td>BY4742 Tor2-mNg-3xHA</td>
<td>This study</td>
</tr>
<tr>
<td>YFR589</td>
<td>BY4741 Avo2Δ-mKet::SpHis5 Tor2-mNg-3xHA</td>
<td>This study</td>
</tr>
<tr>
<td>YFR624</td>
<td>BY4741 Avo2Δ-mKet::SpHis5 Tor2-mNg-3xHA Avo3-3xFlag::KanMX</td>
<td>This study</td>
</tr>
<tr>
<td>YFR626</td>
<td>BY4741 Avo2Δ[T144A T219A S233A S240A S249A T310A S315A T330A S333A]::mKet::SpHis5 Tor2-mNg-3xHA Avo3-3xFlag::KanMX</td>
<td>This study</td>
</tr>
<tr>
<td>YFR628</td>
<td>BY4741 Avo2Δ[T144E T219E S233E S240E S249E T310E S315E T330E S333E]::mKet::SpHis5 Tor2-mNg-3xHA Avo3-3xFlag::KanMX</td>
<td>This study</td>
</tr>
<tr>
<td>CGA84</td>
<td>MATa leu2Δ::GEV::NATMX pep4Δ::His3 prb1Δ1.6 R ura3-52 trp1-ls 2-801a leu2Δ1 his3Δ200 can1 GAL</td>
<td>Alvaro et al. 2014</td>
</tr>
</tbody>
</table>

*For strain yAEA301-A, the coding sequence for an mNG-3xHA cassette was inserted in-frame between Ans321 and Thr322 of the TOR2 ORF on chromosome XI, constructed using a CRISPR-Cas9-based method. In brief, BY4742 was transformed with URA3-marked pRS316-GALpomp-Cas9 (pGF-V789) [Finnegan and Thorner 2016], expression of Cas9 was induced in a resulting transformant by addition of galactose [2% final concentration], and, after 6 h, the cells were cotransformed with HIS3-marked pRS423-sgRNA-Tor2 (pAEA272) and a TOR2-mNg-3xHA-TOR2 repair cassette generated and amplified by overlap extension PCR using appropriate DNA templates and synthetic oligonucleotide primers. The desired [viable] transformants were selected on SCΔ-Ura+His+ plates and then colony-purified by streaking on SD+Ura+His plates containing 5-FOA (to select for derivatives that lost pGF-V789 and pAEA272). The single-guide RNA [sgRNA] encoded by pRS423-sgRNA-Tor2 [pAEA272] was 5′-ATTTTGCGCCAGTGA TAAG-3′ (the PAM sequence is underlined) directed against the corresponding sequence within the TOR2 promoter [where +1 is the first base of the initiator ATG of the TOR2 ORF]. In the upstream primer used to generate the TOR2-mNg-3xHA-TOR2 repair cassette DNA, to prevent recognition by the sgRNA, the sequence in and around the PAM was mutated to 5′-AGAAGTCGAC-3′. The proper in-frame insertion of the mNG-3xHA-coding sequence in the TOR2 ORF was verified by colony PCR and DNA sequencing.*
2 mM DTT, 1 mM PMSF, 0.1 mM chymostatin, 4 mM AEBSE, 12.5 mM β-glycerol phosphate, 1 mM NaVO₄, 40 mM Tris-HCl at pH 7.5). Substrates to be tested were purified as recombinant proteins by fusion to the C terminus of GST in appropriate pGEX vectors and expressed in E. coli BL21(DE3)pLysS. One liter of cultures was grown at 30°C to mid-exponential phase and then induced with 0.5 mM IPTG for 3 h at 30°C. The bacterial cells were harvested by centrifugation and lysed, and the GST fusion protein of interest was adsorbed to glutathione-agarose beads (Prometheus Laboratories Inc.), which were washed and resuspended in kinase assay buffer. The same volume of either affinity-purified Slt2 subunit were collected by immunoabsorption to 40 µL of mouse anti-Flag affinity gel (Biotool) equilibrated in 1× TNEG buffer. The resin was washed four times with 1 mL of 1× TNEG buffer and resuspended in 2× urea buffer.

Fluorescence microscopy and image analysis

Fluorescence microscopy was performed using an Elyra PS.1 structured illumination microscope (Carl Zeiss AG) equipped with a 100× plan-apo 1.46NA TIRF objective, a main focus drive of the AxioObserver Z1 stand, and a WSB PiezoDrive 08 controlled by Zen, and images were recorded using a 512 × 512 (100-nm × 100-nm pixel size) EM-CCD camera (Andor Technology). To visualize Avo2 or Tor2 tagged with mNG (excitation λ₅06 nm, emission λ₅17 nm), cell samples were excited with an argon laser at 488 nm at 2.3% power (100 mW), and emission was captured in a 495- to 550-nm window using a bandpass filter; for Pil1 tagged with RFP/DsRed (excitation λ₅83 nm, emission λ₅83 nm), excitation was at 561 nm at 2.3% power (100 mW), and emission was monitored in a 570- to 620-nm window using a different bandpass filter. Images (average of eight scans; 300 msec/scan) were analyzed using Fiji (Schindelin et al. 2012). To avoid changes in image quality due to occasional fluctuations in laser intensity, all panels shown in any given figure represent experiments performed on the same day and scaled and adjusted identically for brightness using Fiji (Schindelin et al. 2012). For quantitative automated analysis of fluorescence intensity at the PM, CellProfiler was used (Carpenter et al. 2006). To train CellProfiler to apply the appropriate mask and quantify the signal, a corresponding pipeline was created, which was adapted from prior software (Bray et al. 2015; Chong et al. 2015). Prior to loading into the CellProfiler pipeline, cell images were segmented manually using Fiji (Schindelin et al. 2012). To avoid any selection bias, every cell visible in the bright-field image in a frame from any sample except those out of focus was chosen. All plots and statistical analyses in this study were performed with the R statistical analysis package (http://www.R-project.org).

Immunoprecipitation of TORC2

One-hundred milliliters of cultures of yeast strains expressing Avo3-3xFlag Tor2-mNG-3xHA and either Avo2-mKate [YFR624], Avo2KD-mKate [YFR626], or Avo2DB-mKate [YFR628] was grown in YPD to mid-exponential phase, harvested by centrifugation, and frozen in liquid nitrogen. Cells pellets were resuspended in 500 µL of 2× TNEG buffer [100 mM Tris at pH 7.6, 300 mM NaCl, 20% glycerol, 0.24% Tergitol, 2 mM EDTA, 0.1 mM PMSF, 1× Roche complete protease inhibitor tablet (Roche)]. Glass beads were added to the meniscus of the cell suspension, and lysis was achieved by vigorous vortex mixing for 3 min. After lysis, 2× vol of 1× TNEG buffer was added, and then the lysate was clarified by centrifugation at 1000g for 5 min at 4°C followed by a second centrifugation at 2000g for 10 min. TORC2 complexes marked by the tightly bound Avo3-3xFlag

Table 2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS315</td>
<td>CEN, LEU2, vector</td>
<td>Sikorski and Hieter 1989</td>
</tr>
<tr>
<td>pFR246</td>
<td>pRS315 Ypk1[T51A T71A T504A S644A T662A]-myc</td>
<td>Leskoske et al. 2017</td>
</tr>
<tr>
<td>pKL1</td>
<td>pRS315 Avo2-3xFlag</td>
<td>This study</td>
</tr>
<tr>
<td>pKL2</td>
<td>pRS315 Avo2[T144A T219A S233A S240A S249A T310A S315A T330A S333A]-3xFlag</td>
<td>This study</td>
</tr>
<tr>
<td>pFR234</td>
<td>pRS315 Ypk1[D242A]-myc</td>
<td>Roeclants et al. 2017b</td>
</tr>
<tr>
<td>yePac112</td>
<td>2 µm, TRP1, vector</td>
<td>Gietz and Sugino 1988</td>
</tr>
<tr>
<td>pJEN12</td>
<td>YEpac112 pGALI-Pck1[R398A R405A R406A D949A]</td>
<td>This study</td>
</tr>
<tr>
<td>pEA376</td>
<td>YEpac112 pGALI-Pck1[R398A R405A R406A]-myc</td>
<td>This study</td>
</tr>
<tr>
<td>pJEN13</td>
<td>YEpac112 pGALI-Pck1[R398A R405A R406A D949A]-myc</td>
<td>This study</td>
</tr>
<tr>
<td>BG1805</td>
<td>2 µm, URA3, pGALI, C-terminal tandem affinity [TAP] tag vector</td>
<td>Open Biosystems, Inc.</td>
</tr>
<tr>
<td>pKL63</td>
<td>BG1805 Slt2</td>
<td>This study</td>
</tr>
<tr>
<td>pKL64</td>
<td>BG1805 Slt2[K54R]</td>
<td>This study</td>
</tr>
<tr>
<td>pGE6p-1</td>
<td>GST tag, bacterial expression vector</td>
<td>GE Healthcare, Inc.</td>
</tr>
<tr>
<td>pKL16</td>
<td>pGE6p-1 Avo2</td>
<td>This study</td>
</tr>
<tr>
<td>pKL17</td>
<td>pGE6p-1 Avo2[T144A T219A S233A S240A S249A T310A S315A T330A S333A]</td>
<td>This study</td>
</tr>
<tr>
<td>pKL81</td>
<td>pGE6p-1 Avo3[1-100]</td>
<td>This study</td>
</tr>
<tr>
<td>pKL82</td>
<td>pGE6p-1 Avo3[1-100][S11A T24A T28A S50A S84A S87A]</td>
<td>This study</td>
</tr>
</tbody>
</table>
Acknowledgments

We thank Victor J. Cid and Maria Molina (University of Madrid Complutense) for the Pke1 plasmid, Steve Ruzin and Denise Schichnes (Biological Imaging Facility, University of California at Berkeley) for their invaluable advice about fluorescence microscopy, and all members of the Thorner laboratory for helpful discussions. We apologize in advance to any investigator whose work was not cited due to the limitation on the total number of allowed references imposed by the editorial policy of this journal. This work was supported by National Institutes of Health [NIH] Predoctoral Traineeship GM07232 and a University of California at Berkeley MacArthur and Lakhan-Pal Graduate Fellowship to K.L.L., Erwin Schroedinger Fellowship F3787-R21 from the Austrian Science Fund to A.E.-A., Marie Sklodowska-Curie Individual Fellowship GA 750835 from the European Commission to C.M.A., and NIH R01 research grant GM21841 to J.T. This work was also aided in part by NIH S10 Equipment Grant OD018136 (to Steven E. Ruzin, Director, University of California at Berkeley Biological Imaging Facility) for a Zeiss Elyra S1 structured illumination microscope.

Author contributions: K.L.L. and F.M.R. designed, executed, and analyzed experiments and drafted the manuscript. A.E.-A. designed, executed, and analyzed experiments and conducted fluorescence microscopy. C.M.A. set up the specific CellProfiler pipeline to quantify the fluorescent intensities and performed all of the statistical analyses. E.P.S. and J.M.H. provided technical assistance with several experiments. J.T. designed and analyzed experiments and revised the manuscript.

References


Berchtold D, Walther TC. 2009. TORC2 plasma membrane localization is essential for cell viability and restricted to a distinct domain. Mol Biol Cell 20: 1565-1575. doi:10.1091/mbc.e08-10-1001


Leskoske et al.


Phosphorylation by the stress-activated MAPK Slt2 down-regulates the yeast TOR complex 2


*Genes Dev.* published online November 26, 2018
Access the most recent version at doi:10.1101/gad.318709.118
Fig. S1. TORC2 function is not affected by phospho-mimetic Pil1 allele. Wild-type (BY4742) or Pil1<sup>3D</sup> (yEPS2) strains were cultured to mid-exponential phase in selective minimal medium and then treated with vehicle (methanol) or 1.25 µM myriocin for 2 h. After harvesting, whole-cell lysates were prepared, resolved by SDS-PAGE, and analyzed as described in Materials and Methods.
Supplemental Figure 2

Fig. S2. **TORC2 function is down-regulated after Sln1 inactivation.** (A) Wild-type cells (BY4741) and an otherwise isogenic *sln1*ts derivative (JTY5473) expressing Ypk1⁵A-myc from its native promoter on a *CEN* plasmid (pFR246) were propagated at 26°C to mid-exponential phase and then a portion of the culture was maintained at 26°C and another portion shifted to 37°C. After 2 h at the respective temperatures, samples of the separate cultures were harvested, lysed and Ypk1 phosphorylation analyzed by Phos-tag SDS-PAGE and immunoblotting. Pgk1 was the loading control. (B) Strain JTY5473 (*sln1*ts) expressing either Avo2-3xFLAG (pKL1) or Avo2⁹A-3xFLAG (pKL2) were grown to mid-exponential phase and then either kept at 26°C or shifted to 37°C. After 2 h, Avo2 phosphorylation was analyzed as in (A).
Supplemental Figure 3

Fig. S3. **Challenge with a high exogenous sorbitol concentration rapidly diminished TORC2-mediated Ypk1 phosphorylation.** Strains JTY5336 (*HOG1* *SLT2*), YFR549 (*slt2Δ*), YFR538A (*hog1Δ*), and YFR559 (*slt2Δ hog1Δ*), each expressing Ypk1<sup>5A</sup>-myc from its native promoter on a *CEN* plasmid (pFR246), were grown to mid-exponential phase, then diluted into fresh SCD-Leu medium in the absence (-) or presence (+) of sorbitol (1 M final concentration). After 5 min, the cells were harvested, lysed and the resulting extracts subjected to Phos-tag SDS-PAGE to resolve Ypk1 phosphorylation and to standard SDS-PAGE to resolve the other proteins, and analyzed by immunoblotting, as described in Materials and Methods.
Supplemental Figure 4

Fig. S4. Avo2-mNG-3xHA is fully biologically functional. Overnight cultures of otherwise isogenic Pil1-RFP (yFR181) and Pil1-RFP Avo2-mNG-3xHA (yAEA348) strains were adjusted to $A_{600\text{nm}} = 0.1$, spotted undiluted and in a series of 10-fold serial dilutions on YPD plates either lacking or containing myriocin at the indicated concentrations, incubated at 30°C for 3 d, and then imaged.
Fig. S5. **Illustration of the masking used to quantify fluorescent images of the cell periphery with CellProfiler.** The images and other data shown in Fig. 5 were obtained using a custom CellProfiler pipeline, as described in Materials and Methods. The blob-like or thread-like cytosolic structures observed in the GFP channel are due to intrinsic autofluorescence of the mitochondria.
Fig. S6. The state of Avo2 phosphorylation does not affect Tor2 localization. (A) Strains YFR624 (Avo2-mKate Tor2-mNG-3xHA Avo3-3xFLAG), YFR626 (Avo2<sup>9A</sup>-mKate Tor2-mNG-3xHA Avo3-3xFLAG) and YFR628 (Avo2<sup>9E</sup>-mKate Tor2-mNG-3xHA Avo3-3xFLAG) were grown to mid-exponential phase and examined by fluorescence microscopy as described in Materials and Methods and processed using CellProfiler (as in Fig. S3). (B) Mean values of the relative pixel intensities of the Tor2-mNG foci in images, as in (A), were measured using CellProfiler, and those obtained for strain YFR624 (Avo2-mKate Tor2-mNG-3xHA Avo3-3xFLAG) were set to 100%, to which all the other values were normalized. Error bars represent 95% confidence intervals.