Target of Rapamycin Complex 2 Regulates Actin Polarization and Endocytosis via Multiple Pathways

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Abstract

Target of rapamycin is a Ser/Thr kinase that operates in two conserved multiprotein complexes, TORC1 and TORC2. Unlike TORC1, TORC2 is insensitive to rapamycin, and its functional characterization is less advanced. Previous genetic studies demonstrated that TORC2 depletion leads to loss of actin polarization and loss of endocytosis. To determine how TORC2 regulates these readouts, we engineered a yeast strain in which TORC2 can be specifically and acutely inhibited by the imidazoquinoline NVP-BHS345. Kinetic analyses following inhibition of TORC2, supported with quantitative phosphoproteomics, revealed that TORC2 regulates these readouts via distinct pathways as follows: rapidly through direct protein phosphorylation cascades and slowly through indirect changes in the tensile properties of the plasma membrane. The rapid signaling events are mediated in large part through the phospholipid flippase kinases Fpk1 and Fpk2, whereas the slow signaling pathway involves increased plasma membrane tension resulting from a gradual depletion of sphingolipids. Additional hits in our phosphoproteomic screens highlight the intricate [...]
TORC2 regulates actin and endocytosis via multiple pathways

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Background: TORC2/Ypk1 regulates actin polarization and endocytosis via unknown effectors.

Results: Pharmacological inhibition of TORC2 reveals that flippase kinases and biophysical properties of the plasma membrane are major effectors of TORC2.

Conclusion: TORC2 regulates actin and endocytosis via multiple pathways, each with different signaling kinetics.

Significance: Elucidation of TORC2 effector pathways in yeast will inform future studies in higher eukaryotes.

SUMMARY
Target Of Rapamycin (TOR) is a Ser/Thr kinase that operates in two conserved, multiprotein complexes, TORC1 and TORC2. Unlike TORC1, TORC2 is insensitive to rapamycin and its functional characterization is less advanced. Previous genetic studies demonstrated that TORC2 depletion leads to loss of actin polarization and endocytosis. To determine how TORC2 regulates these readouts, we engineered a yeast strain in which TORC2 can be specifically and acutely inhibited by the imidazoquinoline NVP-BHS345. Kinetic analyses following inhibition of TORC2, supported with quantitative phosphoproteomics revealed that TORC2 regulates these readouts via distinct pathways: rapidly through direct protein phosphorylation cascades and slowly through indirect changes in the tensile properties of the plasma membrane. The rapid signaling events are mediated in large part through the phospholipid flippase kinases Fpk1 and Fpk2 while the slow signaling pathway involves increased plasma membrane tension resulting from a gradual depletion of sphingolipids. Additional hits in our phosphoproteomics screens highlight the
intricate control TORC2 exerts over diverse aspects of eukaryote cell physiology.

The Target Of Rapamycin (TOR) is a serine/threonine (Ser/Thr) kinase conserved in nearly all eukaryotes (1). Missense mutations in TOR can confer resistance to the growth-inhibitory properties of the natural product macrolide rapamycin and this property led to the discovery of TOR in a selection of rapamycin-resistant S. cerevisiae mutants (2-5). Metazoans typically encode a single TOR gene (mTOR in mammals) while the yeast S.cerevisiae encodes two TOR genes (TOR1 and TOR2).

TOR proteins form at least two distinct multiprotein complexes known as TOR Complex 1 (TORC1) and TOR Complex 2 (TORC2), and each perform one or more essential functions in both S. cerevisiae and mammals (6). In S. cerevisiae, there are two variants of TORC1; the main variant, TORC1-A, contains Tor1 as the catalytic subunit, while the minor variant, TORC1-B, contains Tor2 as the catalytic subunit (7) (Figure 1B). The kinase activities of TORC1-A, TORC1-B and mammalian TORC1 (mTORC1) are all acutely sensitive to rapamycin. In contrast, neither TORC2 nor mTORC2 are sensitive to acute exposure to rapamycin (7-9) although prolonged exposure can inhibit mTORC2, at least in certain cell types (10).

Rapamycin has been an incredibly valuable tool to dissect TORC1 functions, particularly in S. cerevisiae where large-scale phosphoproteomics studies have identified dozens of proximal and distal effectors downstream of this kinase complex (11,12). These data establish the groundwork for a molecular understanding of how TORC1 regulates many diverse aspects of eukaryote physiology including anabolism, autophagy and longevity (13). Lacking a specific small molecule inhibitor, it has been more challenging to characterize the effectors downstream of TORC2. Recently, the phosphoproteomes of mammalian cells treated with ATP-competitive small-molecules that inhibit both mTORC1 and mTORC2 have been obtained (14-16). Although these studies represent a significant increase in our understanding of the mTOR-dependent phosphoproteome, coverage is an issue and it remains a challenge to partition these phosphorylation events downstream of mTORC1 vs. mTORC2.

Dissection of TORC2 function in S. cerevisiae has relied heavily on the use of temperature sensitive alleles of TOR2 or genes encoding other essential components of TORC2. These tools led to the discovery that Ypk1 and Ypk2, a pair of AGC-family Ser/Thr kinases, are the essential substrates of TORC2 (17,18). Indeed, alleles of YPK1 or YPK2 that encode hyperactive kinases rescue the inviability of tor2 mutants (17,18). Through Ypk1 (the more prominent of these two Ypk)s, TORC2 regulates polarization of the actin cytoskeleton (17,19-21), endocytosis (22,23) and sphingolipid biosynthesis (24). TORC2/Ypk1 signals additionally regulate the activity of the protein phosphatase calcineurin (18,25), and the Protein Kinase C/Cell Wall Integrity MAP Kinase (CWI) pathway (26). However, the use of temperature sensitive and other conditional alleles (e.g. gene expression driven by glucose-repressible promoters) are not optimal tools to probe TORC2 functions as environmental stresses, including temperature shifts and changes in carbon source, per se, affect TORC2 activity and thus complicate interpretation of observations made from these experimental paradigms [(27); Loewith lab unpublished observations]. Moreover, an additional shortcoming of using these conditional alleles is the long time (often hours) required to inactivate TORC2. Thus, with the exception of the regulation of sphingolipid biosynthesis, a now well characterized TORC2-effector pathway (24,28), it is not known whether TORC2 regulates its other described readouts directly or indirectly.

The biochemistry of sphingolipid biosynthesis is relatively well understood and the mechanism by which TORC2 impinges upon this pathway has recently been identified (Figure 3A) (29,30). De novo synthesis begins with the condensation of L-serine with fatty acyl-CoA to produce long chain bases. This reaction is catalyzed by Serine Palmitoyltransferase (SPT), a multiprotein complex consisting of Lcb1, Lcb2, and Tsc3 (31,32). SPT is negatively regulated by the evolutionarily conserved, transmembrane, Orm proteins (Orm1 and Orm2 in S. cerevisiae) (33,34). TORC2 regulates SPT activity via phosphorylation
of threonine 662 embedded in a hydrophobic motif at the C-terminus of Ypk1 (18) which promotes Ypk1 kinase activity and additionally serves as a convenient proxy to monitor TORC2 activity (28). Ypk1 in turn regulates SPT by phosphorylating Orm1 (on serines 51, 52 and 53) and Orm2 (on serines 46, 47 and 48) that antagonizes their ability to inhibit SPT (24,33). In addition to being structural components of membranes, sphingolipids are thought to function as signal transducers (35). Indeed, there is considerable literature describing roles for these lipids in the regulation of various cellular functions in yeast including actin organization and endocytosis (36,37), particularly in response to acute heat stress. Given the role of TORC2 upstream of sphingolipid biosynthesis, and the proposed functions of sphingolipids in actin organization and endocytosis, one might hypothesize that TORC2 regulates at least some of its distal effectors via its influence on sphingolipid levels.

Like sphingolipids, phospholipids also affect many functions in a cell [reviewed in (38)] and are downstream targets of TORC2/Ypk1. Specifically, Ypk1 was recently shown to phosphorylate and inhibit the flippase protein kinase Fpk1 and its paralog Fpk2 (39). Fpks are activators of aminophospholipid flippases Dnf1 and Dnf2 (Drs2 Neo1 Family) that regulate phospholipid asymmetry in the plasma membrane. Additional targets of Fpks are suspected but have not yet been reported (40).

In this study we wished to understand better how TORC2/Ypk1 regulates actin polarization and endocytosis. Specifically, we wished to determine if these processes are directly targeted through phosphorylation events or are indirectly regulated through lipid messengers. To address this problem we employed a reverse chemical genetic approach where we engineered TORC1 to be resistant to the ATP-competitive small molecule TOR inhibitor NVP-BHS345. Addition of NVP-BHS345 to these cells triggered a specific and acute inhibition of TORC2. Our subsequent observations indicate that TORC2 regulates actin polarization and endocytosis via distinct pathways involving (i) the Fpks, (ii) Fpk-independent phosphorylation of proteins involved in endocytosis, and (iii) slower effects due to changes in membrane tension.

EXPERIMENTAL PROCEDURES

Yeast strains and plasmids
Plasmids and strains used in this study are summarized in Tables 1 and 2. Yeast strains were generated through crosses or by transformation with indicated plasmids or PCR fragments for homologous recombination. Tagged proteins are expressed from their own promoters. Unless indicated otherwise, yeast cultures were grown in synthetic complete (SC) medium lacking appropriate amino acids required for plasmid selection.

Drugs and chemicals
Rapamycin (LC Laboratories) was dissolved in 90% ethanol 10% Tween®20 at 1 mg/mL and used at a final concentration of 200 nM. BHS345 (Novartis) was dissolved in DMSO at 10 mM and used at final concentrations ranging from 10 to 50 μM. Myriocin (Sigma) was dissolved in methanol at 2.5 mM and used at final concentration of 2.5 μM. Aureobasidin A (Takara) stocks were prepared in ethanol at 5 mM and used at 2.5 μM. 1NM-PP1 (Calbiochem®) was dissolved in DMSO at 1 mM and used at a final concentration of 500 nM. Chlorpromazine-hydrochloride was used at 500 μM (Sigma) and dissolved in water.

Antibodies
The following antibodies were used in this study: goat anti-Ypk1 1:1000 (Cell Signaling), rabbit anti-phospho-Ypk1T662 1:500 (28), rabbit anti-Phospho-Sch9S737 1:250 (41), mouse Anti-HA 1:20’000 (Sigma-Aldrich), rabbit anti-Phospho-Akt Substrate (RXXS/T) 1:2000 (Cell Signaling), mouse anti-GFP 1:700 (Santa Cruz Biotechnology), rabbit anti-Hog1 1:10’000 (Santa Cruz Biotechnology) and the appropriate infrared dye coupled secondary antibodies (all Alexa Fluor 680 conjugated secondary antibodies from Invitrogen Life Technologies, all IRDye® 800 conjugated secondary antibodies from Rockland Bioconcept).

Protein extraction and western blotting
Yeast cells were grown at 30 °C to an OD_{600} of 0.6-0.8, followed by drug or mock treatment. After treatment, protein extracts for immunodetection were prepared essentially as described in (42).
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For immunoprecipitation experiments cells in urea buffer (25 mM Tris pH 6.8, 6 M Urea, 1 % SDS) were lysed using a FastPrep bead beater (Lucerna Chem.) and glass beads (Fisher scientific, Retsch). Extracts were diluted in IP buffer (PBS, 10 % (v/v) glycerol, 0.5 % Tween®20 supplemented with Roche complete protease inhibitor cocktail containing EDTA (Roche), 1 mM PMSF) and the soluble fraction was incubated with mouse monoclonal Anti-HA (clone HA-7, Sigma-Aldrich)-coupled Protein G Dynabeads® (Invitrogen Life Technologies) for 3 h at 4 °C. After incubation beads were washed twice with IP buffer and bound proteins were released with addition of 1x SDS sample buffer containing 5 % β-mercaptoethanol and incubation for 5 min at 95 °C. SDS-PAGE was performed and separated proteins were transferred to nitrocellulose membranes (Axon Lab) and immunodetection was performed using the antibodies indicated. Primary antibodies were detected with secondary antibodies coupled to infrared dyes. Proteins were visualized and the amount quantified using the Odyssey® IR imaging system (Li-Cor Biosciences).

Sample preparation for label-free phosphoproteomics

Samples for label-free phosphoproteomics were prepared as described previously (11). Briefly, all strains were grown to saturation in synthetic media. After dilution to OD 0.2 at 600 nm, cells were grown to exponential phase and treated as indicated. 100 % TCA was added to a final concentration of 6 % and the mixture was incubated on ice for 30 min. After centrifugation, cells were washed twice with acetone.

Phospho-proteomics sample preparation and analysis by Label-free Quantification

For each of the 3 sample replicates, 3 mg of protein was digested with trypsin (1:125 w/w) and cleaned by reverse phase chromatography. Phosphopeptide enrichment was performed with titanium dioxide resin (1.25 mg resin for each sample). Isolated phosphopeptides were analyzed by an LTQ Orbitrap XL (Thermo) (43). The mass spectrometry data were searched against an SGD decoy database for yeast proteins using Sequest (44). OpenMS version 1.8 (45) was used both to detect MS1 features and to align them between the different mass spectrometry runs. By using a decoy database (46), a Peptide Prophet’s probability threshold was computed in order to achieve a false discovery rate below 1%, and was used to filter OpenMS results. Phosphopeptides features with identical sequence and phosphorylation state but different charge states were merged together. Statistical analysis was performed by MSstat R package (47), which fits the data to an ANOVA model.

Analysis of MS data Normalization

The MS intensity of each peptide in each condition is recorded; data was log2 transformed to comply with linear regression requirements. In situations where the recorded values are null, we will assume that those corresponds to situations where the peptide was not identified.

QC and PCA

Sample quality was assessed using Principal Component Analysis: based on data from all peptides, sample labeled Area_6 was removed from Exp1 and samples labeled Cond2 and Cond3.2 were removed from experiment 2. We then removed all peptides that add any missing value in the control group of interest to the analysis (DMSO), or treated groups of interest (WT|BHS, WT|Rapamycin). Peptides that had all values below the 2.5% quantile in one group of samples, but no missing values in the other one were rescued for analysis. Once bad samples and missing values are removed, remaining samples are well separated according to treatment for both Exp1 and Exp2.

Model building and Analysis

For both experiments the intensity of the remaining peptides was modeled after the interaction of treatment (BHS345 at doses noted, or rapamycin at doses noted). The contrasts were calculated to provide a log fold change (logFC) and a p-value – and data are available in the two supplementary Excel files.

Microscopy

Cells were grown in synthetic media at 30 °C to an OD600 of 0.4 at which point drug/vehicle was added and cells were incubated for an additional 90 min. After treatment, 40 % formaldehyde was added to 3.6% final concentration for fixation and cells were incubated for 30 min at room temperature with constant tumbling. After collection, cells were washed twice in 1x PBS and resuspended in 1x PBS.
containing 0.1 % Triton X-100 and incubated for 10 min, followed by two washes with 1x PBS. Actin was stained using Rhodamine phalloidin for 1 h at 4 °C in the dark. After washing twice with 1x PBS, cells were resuspended in PBS containing 60 % glycerol and imaged using a Zeiss Axio Z1 fluorescent microscope at 100 x magnification. Budding yeast cells were assessed for actin distribution as described previously by Helliwell et al., 1998 (19). In brief, actin was considered as polarized if less than five patches were observed in the mother cell. Around 100 budding cells were counted per experiment; two biological replicates were performed for all experiments. Lucifer Yellow treatment to assess endocytosis was performed essentially as described (48). Quantification of LY uptake was assessed on 30 cells per condition: using ImageJ, a 10-pixel-thick line that bisected the vacuole was drawn across each cell image. The peak value of the signal intensity along the line (corresponding to the vacuole) was then divided by the minimum signal intensity (corresponding to the cytoplasm) to yield the vacuolar:cytoplasmic signal ratio (Figure 2A). Aberrantly stained cells (those yielding a ratio lying outside 1.96 standard deviations from the mean) were excluded from these analyses.

**MM4-64 staining**

Cells were grown over night in synthetic media at 30 °C, diluted to OD₆₀₀ of 0.15, grown to OD₆₀₀ 0.4-0.5 and treated with drug for 30 min or 90 min. Gently pelleted cells were resuspended in cold YPD supplemented with 50 µM MM4-64 from a 1 mM stock in DMSO as well as the appropriate drug/vehicle and incubated in ice water for 10-15 min. Next, cells were incubated in a 30 °C water bath for 30 min. After incubation, cells were gently pelleted and washed 2-3 times with ice cold YNB lacking a carbon source. The cell pellet was resuspended in YNB lacking a carbon source to an optimal cell density and cells were immediately imaged using a Rhodamine filter of a Zeiss Axio Z1 fluorescent microscope at 100 x magnification. Around 100 cells were imaged and all experiments were performed in duplicates or triplicates. Cells were considered as wild-type when MM4-64 accumulated at the vacuolar membrane, while cells presenting stained cytoplasmic structures were assumed to have internalization defects.

**RESULTS**

BHS345 specifically inhibits TORC1 and TORC2 in yeast

Several, ATP-competitive mTOR inhibitors, that inhibit both mTORC1 and mTORC2 in mammalian cells, have been developed (49). These compounds, however, are generally not efficient inhibitors of TOR in S. cerevisiae [(50); and data not shown]. Recently, we identified BHS345 as a specific inhibitor of TORC1 and TORC2 both in vitro and in yeast (51). BHS345 is structurally similar to BEZ235 (Figure 1A), a PI3K / mTOR inhibitor in several advanced oncology clinical trials (Clinicaltrials.gov). Expression of TOR2M2286T, containing a missense mutation in the kinase domain, confers partial resistance to BHS345 (51) owing to the fact that Tor2 can assemble into both TORC2 and also TORC1-B (Figure 1B). As TORC1-A is still inhibited by BHS345 in cells expressing TOR2M2286T we wondered if further resistance could be procured by co-expressing the equivalent TOR1 allele, TOR1M2282T. Indeed, although expression of TOR1M2282T did not confer resistance to BHS345 on its own (as Tor1 functions only in TORC1-A and not TORC2), it did improve growth in the presence of compound when co-expressed together with TOR2M2286T (Figure 1C). By monitoring the phosphorylation status of Sch9 and Ypk1 respectively, we observed that expression of TOR1M2282T partially restored TORC1 kinase activity, while expression of TOR2M2286T partially restored TORC1 and robustly restored TORC2 kinase activity in BHS345-treated cells; co-expression of TOR1M2282T and TOR2M2286T robustly restored both TORC1 and TORC2 kinase activities in BHS345-treated cells. (Figure 1D). These results confirm that both TORC1 and TORC2 activities are inhibited by BHS345 and that this compound inhibits no other essential function(s) in yeast when used at concentrations ranging from 10 to 15 µM. Importantly, these results additionally demonstrate that we can specifically inhibit Tor2 activity by

**Supplementary Information**

Supplemental Information includes two files.

- Rispal2014_PProteomics_Exp1_contrasts.xlsx
- Rispal2014_PProteomics_Exp2_contrasts.xlsx
treating \(TOR1^{M2282T}\) cells with 15 \(\mu\)M BHS345, a strategy that we exploit as described below.

**Acute inhibition of TORC2 rapidly arrests endocytosis and triggers actin depolarization**

Previous genetic studies demonstrated that gradual depletion of TORC2 eventually (after several hours) triggers inhibition of endocytosis and depolarization of the actin cytoskeleton. To begin to characterize the signaling outputs of TORC2 that impinge upon these distal readouts we first determined the time required for BHS345-induced TORC2 inhibition to block endocytosis and depolarize actin.

Fluid-phase endocytosis is conveniently monitored by the internalization of the fluorescent compound Lucifer Yellow (52). Inhibition of TORC2 with BHS345 arrested endocytosis within 1h (Figure 2A). Consistent results were obtained using the lipophilic styryl dye MM4-64 which in \(\text{wt}\) cells, but not endocytosis-deficient cells, accumulates in the vacuolar membrane (53): inhibition of TORC2 abrogated the vacuolar membrane accumulation of MM4-64 which instead partially accumulated in cytoplasmic structures (Figure 2B). These results confirm that, like depletion of TORC2 activity through conditional genetic methods (22,23), chemical inhibition of TORC2 leads to an endocytosis defect, however, with much faster kinetics.

Yeast cells possess three types of actin filament networks which can be visualized by staining with rhodamine-phalloidin (54): cables, which are linear bundles of short actin filaments involved in vesicle trafficking and maintenance of cell polarity; a contractile ring composed of short linear actin filaments required for cytokinesis; and, cortical patches, which are meshes of branched actin filaments found at the cell periphery involved in clathrin-mediated endocytosis. Inhibition of TORC2 with BHS345 led to a rapid (half maximal effect by 30 minutes) depolarization of the actin cytoskeleton manifested as a loss of both actin cables and the contractile ring found at the bud neck, and a redistribution of cortical patches from the bud to the mother cell (Figure 2C), entirely consistent with previous observations using conditional genetic methods of inactivating Tor2 (21) but again with faster kinetics.

The effect on endocytosis and depolarization of actin structures observed upon TORC2 inhibition was suppressed in cells expressing hyperactive \(Ypk1^{D242A}\) (Figure 2D-G, (18)). These phenomena were also suppressed in cells expressing a BHS345-resistant allele of \(TOR2\) (data not shown) and recapitulated upon inhibition of analog-sensitive \(Ypk1\) with 1NM-PP1 (data not shown (18)). Together, these observations confirm that regulation of endocytosis and actin polarization are \(Ypk\)-dependent outputs of TORC2 and exclude the possibility of off-target effects of BHS345. The relatively fast kinetics suggests that TORC2 signals to these effectors directly and we next set out to characterize the relevant TORC2 effector pathways.

**The essential function of TORC2 is to maintain sphingolipid biosynthesis**

TORC2, via \(Ypk1\), stimulates sphingolipid synthesis by inactivating the SPT inhibitors Orm1 and Orm2, (Figure 3A) and we wondered to which extent sphingolipids influence TORC2 readouts. Initially, we asked if the lethality of \(TOR2\) deletion is due to insufficient levels of sphingolipids. This appears to be the case as the growth of \(tor2\) cells can be rescued by double deletion of \(ORM1\) and \(ORM2\), (Figure 3B and C). However, further experiments with \(orm1\ orm2\) cells were not possible, as the slow growth phenotype of these cells was rapidly suppressed. Instead, we made use of two low molecular weight compounds known to specifically reduce sphingolipid biosynthesis; myriocin inhibits SPT, and aureobasidin A blocks conversion of PHC into IPC. We treated \(\text{wt}\) cells to assess whether these treatments mimic BHS345 induced readouts. Indeed, consistent with previous work (37,55,56), we found that block of complex sphingolipid biosynthesis with myriocin and aureobasidin A treatments decreased the uptake of LY (Figure 3D), led to aberrant vacuolar staining with MM4-64 (Figure 3E) and triggered actin depolarization (Figure 3F).

The above observations are consistent with the idea that TORC2 regulates these readouts via sphingolipid intermediates. However, several observations suggest that this is not the primary mechanism by which TORC2-mediated signals impinge upon these readouts. Both myriocin and aureobasidin A inhibited endocytosis and
depolarization of the actin cytoskeleton, but fewer cells within the population were affected as compared to BHS345 treatment. Furthermore, the time required to achieve actin depolarization in 50% of the cells was 90 minutes for myriocin / aureobasidin A but only 30 minutes for BHS345 (Figure 2C and 3F). These significant penetrance and temporal differences suggest that despite being essential targets of TORC2 (Figure 3B and C) sphingolipids play only a partial and slow role in coupling TORC2 signals to the actin polarization and the endocytic machinery.

We wished to determine the mechanism by which sphingolipid depletion inhibits endocytosis and depolarizes actin. Sphingolipids are a major component of the yeast plasma membrane and we have previously suggested that their depletion could increase the line tension of the plasma membrane (28). Prior studies have linked plasma membrane stress to actin depolarization (57). We confirmed in our strain background that treatments affecting the tensile properties of the plasma membrane, such as hypo-osmotic shock, heat shock, and addition of chlorpromazine (an antipsychotic compound known for its propensity to intercalate into lipid bilayers (58)), all elicit actin depolarization and presumably plasma membrane tension as readout by increased phosphorylation of Ypk1 T662 (Figure 4A and B). These observations support the notion that sphingolipid depletion blocks endocytosis and depolarizes actin by altering the physical properties of the plasma membrane. To test this further, we asked if inclusion of the osmotic stabilizer sorbitol in the growth medium would suppress actin depolarization normally induced by myriocin and aureobasidin A treatments. Inclusion of sorbitol in the media prevented actin depolarization and presumably plasma membrane tension as readout by increased phosphorylation of Ypk1 T662 (Figure 4C) but did not block actin depolarization induced by BHS345-mediated TORC2 inhibition (Figure 4D). From these observations we conclude that sphingolipid depletion upon TORC2 inhibition gradually increases membrane tension that subsequently blocks actin polarization and endocytosis. The observation that sorbitol does not suppress actin depolarization induced by TORC2 inhibition confirms that effectors other than sphingolipids must couple TORC2 to the actin cytoskeleton.

TORC2 does not signal to the actin cytoskeleton via the CWI pathway

Previous work has demonstrated that hyperactivation of the Cell Wall Integrity (CWI) pathway restores actin organization and growth to cells possessing reduced TORC2 function (19,20,59). The CWI pathway is composed of the cell-surface sensors Wsc1, -2, -3, Mid2, and Mtl1, which activate the Rho1 GTPase via the guanine-nucleotide exchange factors Rom1 and Rom2. One of Rho1’s many targets is Pkc1, which in turn activates the MAP Kinase cascade consisting of Bck1, Mkk1/2 and Slt2. It is believed that TORC2 acts upstream of the CWI pathway by stimulating the exchange activity of Rom2 (26). Based on these prior reports it seemed a likely possibility that TORC2 regulates actin polarization via activation of the CWI pathway. This, however, appears not to be the case as acute inhibition of TORC2 with BHS345 results in activation of the CWI pathway as readout by increased phosphorylation of Slt2 (Figure 5A). From these results we conclude that actin depolarization induced by TORC2 inhibition is not caused by inactivation of the CWI pathway.

Fpk1/2 hyperactivation upon TORC2 inhibition is a major contributor to actin depolarization and inhibition of endocytosis

Like Orm1 and Orm2, Fpk1 and Fpk2 are inhibited through direct phosphorylation by Ypk1; and, like orm1 orm2 mutants, fpk1 fpk2 mutants also restore growth of ypk1 hypomorphic strains and ypk1 Δypk2 (39). Consistently, FPK1/2 deletion improves the growth of cells in which TORC2 is inhibited with BHS345 (Figure 5B), but unlike ORM1 ORM2 deletion does not rescue growth of TOR2 deletion (data not shown). Building on these observations, we found that actin polarization and endocytosis were largely unaffected by TORC2 inhibition in fpk1 fpk2 cells (Figure 5C, D and E). This suppression is not due to decreased uptake of the drug in fpk1 fpk2 cells as Ypk1 dephosphorylation is still observed (data not shown).

Fpk1/2 regulate phospholipid asymmetry via activation of the phospholipid translocases including Dnf1 and Dnf2, which belong to the type 4 P-type ATPases (P4-ATPases) (39,40,60). We wondered if these could be the relevant targets that couple Fpk-mediated signals to actin and
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endocytosis. To test this, we assessed the extent of actin polarization after TORC2 inhibition in dnf1 dnf2 dnf3 cells and found weak to no suppression of the actin depolarization and endocytosis arrest (data not shown; Dnf3 is a related flippase localized to the trans-golgi network (61)). Similar results (i.e. only slight suppression) were obtained with lem3, dnf1 dnf2, dnf1 dnf3, dnf1 dnf2 lem3, dnf1 dnf2 dnf3 and dnf3 dnf2 cells (data not shown). A quadruple deletion of drs2 dnf1 dnf2 dnf3 is lethal (61) and therefore we cannot exclude the possibility that Fpks indeed regulate actin polarization and endocytosis redundantly through the activity of these phospholipid translocases. However, it seems more likely that other, yet-to-be described effectors of Fpk1/2 serve to couple TORC2 to actin and endocytosis.

Quantitative mass spectrometry identifies new effectors downstream of TORC2

To better understand how TORC2 signals impinge upon its distal readouts in general and actin polarization and endocytosis in particular we performed quantitative phosphoproteomics using BHS345. We previously used quantitative mass spectrometry to define the rapamycin-sensitive phosphoproteome of S. cerevisiae cells and identified many novel TORC1 effectors (11,62). Having a yeast-permeant TORC2 inhibitor allowed us to similarly ascertain the TORC2-dependent phosphoproteome by comparing the rapamycin-sensitive phosphoproteome to the BHS345-sensitive phosphoproteome. As a prelude to this experiment, we first determined the length of time required for rapamycin and BHS345 to efficiently inhibit TORC1 as assessed by loss of phosphorylation on T737 in the hydrophobic motif of Sch9. We found that the effect of BHS345 on TORC1 was detectable more rapidly than the effect due to rapamycin treatment: Sch9 was dephosphorylated after 1 minute of BHS345 treatment (51) compared to 8 minutes required for rapamycin treatment (data not shown). Based on this observation we designed experiments with short compound exposure time (<30 minutes) to capture relatively early events downstream of TORC1 and TORC2 inhibition. We performed two separate experiments according to the flowchart in (Figure 6A), to cover different time points and inhibitor concentrations. Each experimental dataset was analyzed independently to retain phosphopeptides with a greater than two-fold change and with a p-value of ≤0.05 for compound versus vehicle treatment (Supplementary Files 1 and 2). In the first experiment, 123 peptides were found to be dephosphorylated and 44 hyperphosphorylated by BHS345 whilst remaining unaffected by rapamycin. In the second experiment, 58 and 36 peptides were similarly affected respectively. These peptides are presumably regulated downstream of TORC2 and not TORC1. Enriched amongst these peptides were “RXXS” and “SP” motifs (where S is the phosphorylated residue). RXXS is a minimal recognition sequence for AGC kinases and its enrichment could suggest that many of the TORC2-dependent phosphoproteins that we identified are direct targets of Ypk1.

The union of affected genes from the two experiments provided us with a working list of 117 proteins that are hypophosphorylated (Table 3) and 47 proteins that are hyperphosphorylated (Table 4) upon inhibition of TORC2. Having identified these putative novel effectors downstream of TORC2 we attempted to validate a subset of them.

Molecular links between TORC2, actin organization and endocytosis

Our phosphoproteomic experiments identified several proteins implicated in the regulation of actin organization and endocytosis (Tables 3 and 4 and (63,64)). Highly conserved, clathrin-mediated endocytosis involves internalization and pinching off of plasma membrane to generate an endocytic vesicle (cartooned in Figure 6B). Strikingly, we observed that BHS345, but not rapamycin, induced changes in phosphorylation of both coat proteins as well as regulators of actin polymerization (Figure 6B, supplementary Files 1 and 2). Coat disassembly is mediated by the Ark family proteins, which, in S. cerevisiae, is made up of Ark1, Prk1 and the lesser studied Akl1 (65). Suggestively, we found Prk1 and Akl1 to be hypophosphorylated upon BHS345 treatment. Known substrates of these kinases include the coat proteins Pan1, Sla1, End3, Ent1 and Ent2, and indeed, these were all found to be hypophosphorylated upon BHS345 treatment. In the cases of Pan1, and Ent2, the residues found to be hypophosphorylated included residues in Ark-consensus motifs ([L/V][XXQ/N/T]XTG). In low throughput assays, we confirmed that Ent1 and
Pan1 are dephosphorylated after BHS345 or 1NM-PP1-mediated inhibition of Ypk1, but not rapamycin treatment (Figures 6C and D and data not shown), demonstrating that they are TORC2/Ypk1 effectors. BHS345-induced dephosphorylation was suppressed in cells expressing Ypk1D242A (Figures 6C and D), and by TOR2MT (data not shown) again demonstrating that these are Ypk-dependent outputs of TORC2 and excluding the possibility of off-target effects of BHS345.

We asked if the dephosphorylation of Ent1 or Pan1 was recapitulated by myriocin or aureobasidin A or suppressed by FPK1/2 deletion. Neither myriocin nor aureobasidin A treatments triggered Ent1 or Pan1 dephosphorylation (Figures 6C and D), despite the fact that actin was depolarized and endocytosis was blocked at the chosen treatment time points (Figure 3D-F). In contrast, Ent1 and Pan1 became hyperphosphorylated upon myriocin and aureobasidin A treatments, likely due to hyperactivation of TORC2 elicited by these treatments (28). Furthermore, Ent1 and Pan1 were dephosphorylated upon TORC2 inhibition in fpk1Δfpk2 cells similarly to wt cells (Figure 6D and E) meaning that phosphoregulation of Ent1 and Pan1 occurs independently of these kinases. These observations demonstrate that there are multiple pathways, operating with different kinetics, by which TORC2 regulates actin polarization and endocytosis.

**Functional consequences of Ent1 and Pan1 phosphorylation**

The TORC2-regulated Pan1 phosphorylation sites were found in Ark-consensus motifs. Previous work demonstrated that alanine replacement of threonines within the 15 [L/V]XX[Q/N/T]XTG motifs in Pan1 (Pan1-15TA) causes severe defects in actin organization and uptake of LY (66). TORC2 inhibition also affects phosphorylation of Ark-consensus motifs in Ent1. It has been proposed that phosphorylation at these sites alters the actin-binding properties of Ent1 (67). It is possible that TORC2 regulates actin organization and endocytosis via regulation of Ark1/Prk1 activity. Alternatively, Ark1/Prk1 activity may be affected indirectly as a consequence of actin depolarization.

We additionally found that TORC2 inhibition affects Ent1 phosphorylation within RXXS/T motifs favored by ACG family kinases (Figure 6) suggesting that these sites may be directly targeted by Ypk1. Indeed, Ent1 dephosphorylation upon TORC2 inhibition was very fast, with a significant loss of phosphorylation after 7.5 min of Ypk1 inhibition (data not shown). However, we were unable to observe direct phosphorylation of Ent1 by Ypk1 in *in vitro* kinase assays (data not shown). Thus it remains unclear if Ent1 is directly phosphorylated by Ypk1.

To our knowledge, a function for the RXXS/T phosphorylation sites in Ent1 has not been investigated. Ent1 has six RXXS/T motifs, two of which, T160 and S175, are known to be phosphorylated (*S. cerevisiae* genome database (SGD)). T160 was also identified as a TORC2 effector in our present phosphoproteomics study. Conversion of T160 and S175 to alanine reduced, but did not eliminate TORC2-dependent phosphorylation on RXXS/T motifs (Figure 7A). Therefore, we mutated the serines and threonines within all six RXXS/T motifs into alanine or aspartate. In these mutants (Ent1-6A and Ent1-6D) phosphorylation within RXXS/T motifs was no longer detectable (Figure 7A).

To assess the functional importance of Ent1 RXXS/T phosphorylation we expressed Ent1-2A (data not shown), Ent1-6A and Ent1-6D in *ent1Δent2* cells. With the exception of Ent1-6D which slightly reduced basal LY uptake, expression of none of these constructs affected bulk endocytosis, or altered the BHS345-induced endocytic arrest (Figure 7B, C and D).

Interestingly, actin was moderately depolarized in cells expressing Ent1-6D but not the Ent1-2A (data not shown) or Ent1-6A constructs (Figure 7E). Thus it appears that Ent1 phosphorylation within RXXS/T motifs indeed plays some role in TORC2-dependent polarization of the actin cytoskeleton.

**DISCUSSION**

Using BHS345, a novel, small molecule inhibitor of TOR signaling in yeast, we have investigated the readouts downstream of TORC2. In particular, we have exploited the ability to acutely inhibit TORC2
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kinase activity to temporally dissect these readouts, something that was not previously possible using chronic genetic approaches that slowly deplete TORC2 from cells. Although the minor TORC1-B containing Tor2 is also inhibited in our experimental setup, we could confirm that the BHS345-induced phenomena were due to TORC2 inhibition by showing that they 1) are not induced by rapamycin; 2) are rescued in cells expressing a hyperactive allele of Ypk1 (Ypk1D242); and/or 3) are recapitulated upon inhibition of analog sensitive Ypk1. This work complements a recent study by the Shokat group (50) using a related mTOR inhibitor BEZ235 (Figure 1A), and an ‘analog-sensitive’ allele of TOR2 to globally characterize TORC2 signaling by systematic genetic means in yeast. In our present study, we have also begun to systemically define TORC2 signaling by using quantitative phosphoproteomics. Focusing on known distal effectors, we found that TORC2 regulates actin polarization and endocytosis through at least three distinct signaling pathways (Figure 8): a slow pathway involving a plasma membrane stress induced by depletion of complex sphingolipids, and two fast pathways, one of which is mediated by the flippase kinases.

The slow pathway
Sphingolipids, particularly phosphorylated long chain bases, have been suggested to signal to the endocytosis and actin polarization machineries (68). Stimulation of sphingolipid production through direct phosphorylation of the Orm proteins by Ypk1 appears to be the essential function of TORC2 (Figure 3). Thus, we explored the possibility that TORC2 regulates actin and endocytosis via its control of sphingolipid synthesis. This hypothesis appears to be correct, albeit not in the manner we anticipated. Blocking sphingolipid synthesis with either myriocin or aureobasidin A depolarizes actin and blocks endocytosis and thus mimics TORC2 inhibition (Figure 3). However, a specific role for long chain bases, or their phosphorylated derivatives, in this signaling is negated by the observation that both myriocin and aureobasidin A trigger these readouts equally. Also curious is the striking difference in time required for myriocin/aureobasidin A vs. BHS345-induced TORC2 inhibition to trigger these readouts. Based on this difference, we speculated that sphingolipid species per se are not acting as signaling molecules as we had envisioned, but that chronic depletion of sphingolipids could be causing a plasma membrane stress that consequently triggers signaling events that impinge upon actin polarization and endocytosis. A prediction of this model is that manipulations that cause plasma membrane stress should also trigger depolarization of the actin cytoskeleton. Indeed, three orthogonal treatments, each targeting the tensile properties of the plasma membrane, similarly caused depolarization of the actin cytoskeleton with relatively rapid kinetics. How plasma membrane stress triggers actin depolarization remains to be determined.

The fast pathways
There is substantial, previously published genetic data suggesting that the CWI pathway functions downstream of TORC2 (20,26,56). Therefore, we were surprised to see that acute inhibition of TORC2 triggers hyperphosphorylation of Slt2 and thus presumably hyperactivation of the CWI pathway. This hyperactivation of the CWI pathway may be due to residual inhibition of TORC1 (TORC1-B) in our experimental setup (69,70). Regardless, it is clear from these results that the CWI pathway is not inhibited upon BHS345 treatment and thus inactivation of this pathway cannot be what leads to actin depolarization and cessation of endocytosis.

To better understand the mechanism(s) leading to actin depolarization and endocytosis arrest, we set out to identify the TORC2-dependent phosphoproteome (Tables 3 and 4). Our phosphoproteomic dataset demonstrates that TORC2, like TORC1, influences many aspects of cellular physiology. To our knowledge, this is the first time that a TORC2-specific phosphoproteome has been described. Future characterization of these TORC2 effectors promises to bring insight into the regulation of many important processes ranging from DNA repair and transcription to RNA and amino acid metabolism. Amongst the TORC2-regulated phosphoproteins were many components of the endocytosis machinery (Figure 6B) whose phosphorylation status can be observed to change in less than 15 minutes following TORC2 inhibition. The critical targets in terms of linking TORC2 to actin polarization and endocytosis remain to be identified and characterized. We studied the regulation of two proteins, Pan1 and
Ent1 and we confirmed a TORC2-dependent phosphoregulation in low throughput assays. In addition, we showed that RXXS/T sites in Ent1 are linked to the organization of actin. Future studies will be aimed at defining the nature of this link. Both Pan1 and Ent1/2 are essential for endocytosis and as such are prime candidates to serve as key regulatory nodes.

Finally we also found that Fpk1 and Fpk2 are important players in the fast coupling of TORC2 signals to actin and endocytosis (Figure 5). While this work was in progress, the Powers group, using an ATP-analog-sensitive Ypk1, also reported that Fpks perform a major function in coupling Ypk1 signals to the actin cytoskeleton (56). This observation fits well with our present work. However deletion of FPK1/2 did not completely suppress actin depolarization induced by TORC2 inhibition suggesting that additional effectors downstream of Ypk1 could be involved. Consistent with this notion is our observation that deletion of FPK1 and FPK2 does not prevent BHS345-induced Ent1 and Pan1 dephosphorylation.

What could be the role of Fpk1 and Fpk2 in actin polarization and endocytosis? It has been reported that the phospholipid translocases are the relevant targets of the Fpks in signaling to actin – a result that is seemingly discordant with our data. We do not presently understand why our two studies arrive at these different conclusions. Niles and Powers subsequently present compelling data that reactive oxygen species generated downstream of hyperactive phospholipid translocases trigger actin depolarization (56). Future work will be needed to determine if these ROS species are generated by flippase activity or other Fpk targets, as suggested by our present work.

ACKNOWLEDGEMENTS
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FIGURE LEGENDS

FIGURE 1. BHS345, an ATP-competitive TOR inhibitor as a tool to study TORC2 function.

A Structures of BHS345, used here, and the related PI3K/mTOR inhibitor BEZ235.

B Cartoon of the TOR complexes in *S. cerevisiae*. Rapamycin-sensitive TORC1 is found in two variants, one with Tor1 as the catalytic component (TORC1-A) and the other with Tor2 as the catalytic component (TORC1-B). Tor2, but not Tor1, functions in rapamycin-insensitive TORC2. TORC1-A, TORC1-B and TORC2 are all inhibited by BHS345 (BHS).

C BHS345 dose response curves of WT, TOR1<sup>MT</sup> (TOR1<sup>M2282T</sup>), TOR2<sup>MT</sup> (TOR2<sup>M2286T</sup>) and TOR1<sup>MT</sup> TOR2<sup>MT</sup> strains grown in SC. OD<sub>600</sub> was determined after 30 hours incubation at 30°C.

D TORC1 and TORC2 activity status, respectively assayed by western blot detecting Sch9<sup>T737</sup> and Ypk1<sup>T662</sup> phosphorylation, in the indicated strains before and after treatment with rapamycin (200 nM, 20 minutes) or BHS345 (10 µM, 12 minutes).

FIGURE 2. TORC2 regulates actin polarization and endocytosis via Ypk1.

A Left panel, and D Micrographs of TOR1<sup>MT</sup> cells treated for 90 minutes with Lucifer Yellow and either BHS345 or drug vehicle as indicated.

A Center panel: Cartoon demonstrating how Lucifer Yellow accumulation in the vacuole was quantified. Signal intensity of LY was scored along a line that bisected the vacuole. The peak vacuolar signal (V) was divided by the minimum cytoplasmic signal (C) to generate the vacuolar/cytoplasmic signal ratio.

A Right panel and E: Quantification of the vacuolar vs. cytoplasmic accumulation of Lucifer Yellow in cells of the indicated genotypes after the indicated treatments. Two independent experiments were performed. Error bars represent standard error of the mean (SEM). Statistically significant differences are indicated (Student’s t-test) with a p-value p<0.001 (***)

B and F Micrographs showing MM4-64 accumulation in cells with the indicated genotype and after the indicated treatments. Cells were drug treated in SC-media for 30 min, followed by 30 min incubation with 50 µM MM4-64 in YPD containing the drug. The values represent the percentage of cells with vacuolar staining +/- SEM.

C and G Micrographs of rhodamine phalloidin stained TOR1<sup>MT</sup> cells after the indicated treatments. The percentage of cells presenting polarized actin is indicated. Expression of hyperactive Ypk1<sup>D242A</sup>, but not an empty vector (EV) restores endocytosis (D, E and F) and actin organization to BHS345 treated cells (C and G).

A-G BHS345 was used at 15 µM.

FIGURE 3. Sphingolipids are major mediators of TORC2 signaling.


B and C. tor2 lethality is suppressed by deletion of ORM1 and ORM2. Spore colonies obtained upon dissection of TOR2/tor2 (DRY49) and TOR2/tor2 ORM1/orm1 ORM2/orm2 (DRY50) diploids. No viable tor2 cells were obtained from 10 dissected asci obtained from TOR2/tor2 diploids (left panel). In contrast, viable tor2 cells (marked with red circles, right panel) were readily observed from dissected ascis obtained from TOR2/tor2 ORM1/orm1 ORM2/orm2 diploids. These tor2 haploids all additionally harbored the orm1 and orm2 deletions. C Orm double deletion suppresses lethality of tor2, demonstrated by the ability of tor2 ORM1/orm1 cells to lose a URA3-marked plasmid containing YPK2<sup>D239A</sup> on 5-FOA medium.

D Micrographs of TOR1<sup>MT</sup> cells treated for 90 minutes with Lucifer Yellow and either Myriocin (Myr) or Aureobasidin A (AbA) as indicated. D Right panel: Quantification of the vacuolar vs. cytoplasmic accumulation of Lucifer Yellow after the indicated treatments. Statistically significant differences are indicated (Student’s t-test) with a p-value p<0.001 (***)

E Micrographs showing MM4-64 accumulation in TOR1<sup>MT</sup> cells after the indicated treatments. Numbers represent the percentage of cells with vacuolar labelling.
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D-F Cells were treated with 2.5 µM myriocin or 2.5 µM aureobasidin A.

FIGURE 4. Plasma-membrane-stress-induced actin depolarization is rescued by sorbitol addition.
A Micrographs of rhodamine phalloidin stained TOR1MT cells after hypo-osmotic shock (SC Sorb 1M → SC), heat shock (22°C → 37°C, in SC medium) or chlorpromazine (CPZ 500 µM, in SC medium) treatments. Numbers in the micrographs indicated the percentage of cells with polarized actin +/- SEM.
B Western blots showing the extent of phosphorylation of Ypk1 at T662 following the treatments as indicated in A.
C–D Cells growing exponentially in SC medium, SC + 1M sorbitol or SC + 2M sorbitol were treated with myriocin, aureobasidin A or BHS345 for the indicated times before processing with rhodamine phalloidin to visualize actin structures.

FIGURE 5. TORC2 regulates endocytosis and actin polarization not via the cell wall integrity pathway but via the flippase kinases Fpk1 and Fpk2.
A TOR1MT cells were treated with BHS345 for the indicated times before aliquots were removed to assess Slt2T190/Y192 and Ypk1T662 phosphorylation by western blotting. Hog1 was used as a loading control.
B Deletion of FPK1 and FPK2 partially rescues the growth of TOR1MT cells in the presence of 10 µM BHS345.
C Micrographs of TOR1MT and TOR1MT fpk1 fpk2 cells treated for 90 minutes with Lucifer Yellow and either BHS345 or drug vehicle with quantifications (right panel).
D Micrographs of TOR1MT and TOR1MT fpk1 fpk2 cells showing MM4-64 accumulation after the indicated treatments. Numbers indicate the percentage of cells with vacuolar staining.
E Micrographs of rhodamine phalloidin stained TOR1MT and TOR1MT fpk1 fpk2 cells after the indicated treatments with BHS345.
A,C-E BHS345 was used at 15 µM.

FIGURE 6. TORC2 regulates phosphorylation of endocytic proteins independently of sphingolipids and Fpk proteins.
A Experimental flowchart for the two independent phosphoproteomic experiments, each performed with biological triplicates.
B Cartoon of a budding vesicle with endocytic proteins. Red “P”s demark proteins that become hypophosphorylated upon TORC2 inhibition.
C TOR1MT cells expressing Ent1-5HA and either an empty vector or a vector expressing hyperactive Ypk1D242A were treated as indicated. Denatured protein extracts were subsequently made and RXXS/T motif phosphorylation of immunoprecipitated Ent1-5HA was assessed by western blotting.
D TOR1MT cells expressing Pan1-GFP and either an empty vector, or a vector expressing hyperactive Ypk1D242A, were treated as indicated. The extent of Pan1-GFP phosphorylation was assessed by SDS-PAGE migration shifts.
D and E. Deletion of FPK1 and FPK2 does not block BHS345-induced Ent1 and Pan1 hypophosphorylation.
C-E BHS345 was used at 15 µM, myriocin at 2.5 µM and aureobasidin A at 2.5 µM.

FIGURE 7. Ent1 RXXS/T phosphorylation antagonizes actin polarization.
A HA-tagged wt or phosphosite mutant Ent1 was immunoprecipitated from TOR1MT ent1 ent2 cell extracts obtained from SC-grown cells that had been treated for 30 minutes with BHS345 or drug vehicle. RXXS/T motif phosphorylation of immunoprecipitated Ent1-5HA variants was assessed by western blotting.
B and C: Micrographs and quantification of TOR1MT ent1 ent2 cells expressing Ent1-wt, Ent1-6A or Ent1-6D treated for 60 minutes with Lucifer Yellow and either BHS345 or drug vehicle as indicated.
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Quantification of the vacuolar vs. cytoplasmic accumulation of Lucifer Yellow after the indicated treatments. Statistically significant differences are indicated (Student’s t-test) with a p-value p<0.001 (***) and p<0.05 (*).

D Micrographs showing MM4-64 accumulation in \( \text{TOR1}\text{MT} \) \( \text{ent1} \) \( \text{ent2} \) expressing Ent1-wt/6A/6D cells after the indicated treatments. Numbers represent the percentage of cells with vacuolar labelling.

E Micrographs of rhodamine phalloidin stained \( \text{TOR1}\text{MT} \) \( \text{ent1} \) \( \text{ent2} \) expressing Ent1-wt/6A/6D cells after 60 min with the indicated treatments.

A-E Cells were grown in SC medium and, where indicated, BHS345 was used at 15 µM.

FIGURE 8. Hierarchy of TORC2 distal effectors.
TORC2 regulates actin polarization and endocytosis via 3 distinct pathways.
1) A fast, “direct” pathway potentially involving direct phosphorylation of endocytic proteins by Ypk1;
2) A slow, “indirect” pathway involving the depletion of sphingolipids and a subsequent increase in plasma membrane tension or related stress; and,
3) A fast pathway involving undefined Fpk1/2 targets.

TABLES

Table 1: Yeast strains used in this study

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Table 3 TORC2-dependent hypophosphorylated proteins

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<td>CDC14, CLA4, FKH2, GI1, KAR3, MCK1, PAN1, PEA2, PIN4, PTK2, SIS2, SMI1, STB1, SW15, TOP1, VHS2, VRP1, WH15, ZDS1</td>
</tr>
<tr>
<td>protein phosphorylation</td>
<td>AKL1, CKB1, CLA4, GI1, HAL5, LRE1, MCK1, PRK1, PR1, PTK2, SKY1, YPK1</td>
</tr>
<tr>
<td>endocytosis</td>
<td>ABF1, BDF1, CB1, CYC8, MCK1, NUP60, PIN4, RFA1, RSC30, SPT5</td>
</tr>
<tr>
<td>cellular response to DNA damage stimulus</td>
<td>BDF1, BUD14, BZZ1, CDC14, CLA4, ENO1, KAR3, NAP1, PEA2, SAC7</td>
</tr>
<tr>
<td>regulation of organelle organization</td>
<td>CDC14, CLA4, GI1, KAR3, SIS2, SMI1, VRP1, ZDS1</td>
</tr>
<tr>
<td>signaling</td>
<td>AVO1, BOI2, GIS4, LCB3, MDS3, MSK1, PXL1, SAC7, SLM2, TIP41</td>
</tr>
<tr>
<td>regulation of cell cycle</td>
<td>CYC8, IST2, LAG1, LCB3, MSS4, PAH1, SUR1, YPK1</td>
</tr>
<tr>
<td>chromatin organization</td>
<td>CLA4, LTV1, NAP1, NUP2, NUP60, SKY1, SRP40, ZDS1</td>
</tr>
<tr>
<td>lipid metabolic process</td>
<td>CDC14, CLA4, KAR3, MCK1, MSC3, RFA1, SW15, TOP1</td>
</tr>
<tr>
<td>nuclear transport</td>
<td>CLA4, LTV1, MSN4, PEA2, PR1, RAS2, SKY1</td>
</tr>
<tr>
<td>regulation of DNA metabolic fission</td>
<td>CDC14, CLA4, KAR3, MCK1, MSC3, RFA1, SWI5, TOP1</td>
</tr>
<tr>
<td>response to chemical</td>
<td>CLA4, LTV1, MSN4, PEA2, PR1, RAS2, SKY1</td>
</tr>
<tr>
<td>DNA repair</td>
<td>AVO1, LRE1, SAC7, SLA1, SMI1, SSM1, ZEO1</td>
</tr>
<tr>
<td>protein targeting</td>
<td>APL6, ATG20, CLA4, NAP1, NUP2, NUP60, SKY1</td>
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<tr>
<td>protein complex biogenesis</td>
<td>BUD14, BZZ1, DCP2, GI1, NAP1, SEC16, SLA1</td>
</tr>
<tr>
<td>cell budding</td>
<td>BOI1, BOI2, GI1, NAP1, PAN1, PEA2, VRP1</td>
</tr>
<tr>
<td>meiotic cell cycle</td>
<td>CDC14, KAR3, MCK1, MSC3, MSS4, RAS2, RFA1</td>
</tr>
<tr>
<td>cell wall organization or biogenesis</td>
<td>AVO1, LRE1, SAC7, SLA1, SMI1, SSM1, ZEO1</td>
</tr>
<tr>
<td>cytokinesis</td>
<td>BOI1, BOI2, PAN1, PEA2, VHS2, VRP1, ZDS1</td>
</tr>
</tbody>
</table>

TORC2 regulates actin and endocytosis via multiple pathways.
**Table 4** TORC2-dependent hyperphosphorylated proteins

<table>
<thead>
<tr>
<th>BIOLOGICAL PROCESS</th>
<th>GENES</th>
</tr>
</thead>
<tbody>
<tr>
<td>response to chemical</td>
<td>MIG1, MLF3, RCK2, SCH9, SCP160, STE20, YCK2</td>
</tr>
<tr>
<td>protein phosphorylation</td>
<td>ALK1, HRK1, KSP1, RCK2, SCH9, STE20, YCK2</td>
</tr>
<tr>
<td>transcription from RNA polymerase II promoter</td>
<td>GIS1, HPC2, MIG1, REG1, RPH1, SCH9</td>
</tr>
<tr>
<td>carbohydrate metabolic process</td>
<td>NUS1, PFK2, REG1, TSL1, VAN1</td>
</tr>
<tr>
<td>mitotic cell cycle</td>
<td>ALK1, HPC2, REI1, STE20</td>
</tr>
<tr>
<td>ion transport</td>
<td>AVT1, CCC1, PFK2, YBT1</td>
</tr>
<tr>
<td>lipid metabolic process</td>
<td>GIS1, PCT1, PIK1, SCH9</td>
</tr>
<tr>
<td>organelle fission</td>
<td>ALK1, RCK2, SCP160, STE20</td>
</tr>
<tr>
<td>signaling</td>
<td>KSP1, RCK2, SCP160, STE20</td>
</tr>
<tr>
<td>response to starvation</td>
<td>GIS1, KSP1, MIG1, REG1</td>
</tr>
<tr>
<td>proteolysis involved in cellular protein catabolic</td>
<td>DIA2, RAD23, RPN8</td>
</tr>
<tr>
<td>chromatin organization</td>
<td>GIS1, HPC2, RPH1</td>
</tr>
<tr>
<td>DNA replication</td>
<td>DIA2, HEK2, ORC6</td>
</tr>
<tr>
<td>endocytosis</td>
<td>ALY2, SWH1, YCK2</td>
</tr>
<tr>
<td>ribosomal large subunit biogenesis</td>
<td>NOP8, REI1, RRP15</td>
</tr>
<tr>
<td>response to osmotic stress</td>
<td>RCK2, SCH9, STE20</td>
</tr>
<tr>
<td>other functions</td>
<td>BUL1, DED1, EAP1, FEN1, GLY1, JSN1, LYS21, NGR1, SEC31, SKG1, SMY2, SOL1, TIF3, YTA6</td>
</tr>
</tbody>
</table>
Figure 1

TORC2 regulates actin and endocytosis via multiple pathways

A

NVP-BEZ235
MW: 469.55

NVP-BHS345
MW: 420.48

B

TORC1-A

Tor1
Kog1
(Tco89)
Lst8

Rapamycin

BHS345

TORC1-B

Tor2
Kog1
(Tco89)
Lst8

TORC2

Tor2
Avo1
(Avo3)
Lst8

Avo2
(Bit61)

D

Sch9-P

Ypk1-P

Ypk1

DMSO

+ + + + - - - - -

RAP

- - - - + - - - -

BHS345

- - - - + + + + +

Minimal media 30 h

WT

TOR1

TOR2

TOR1+TOR2

0.1 0.2 0.4 0.6 1 2 4 6 10 20 40 60 100 200

Growth (OD600)

BHS345 conc (μM)

▲▲

■■

♦♦

●●
**Figure 2**

**A** Mock BHS345 Brightfield LY

**B** Mock BHS345

**C** Mock BHS345

**D**

**E**

**F**

**G**

TORC2 regulates actin and endocytosis via multiple pathways
TORC2 regulates actin and endocytosis via multiple pathways

A

Serine + palmitoyl-CoA → TORC2

Myriocin (Myr) → Om2 → Ypk1/2

LCBs (sphinganine, 4-OH sphinganine) → degradation

PHC → Aureobasidin A (AbA)

IPM → M(IP)C

B

TOR2/Δtor2
ORM1/Δorm1
ORM2/Δorm2

C

WT
Δtor2 pYpk2^D239A
Δorm1/2
Δorm1/2 Δtor2 pYpk2^D239A
Δorm1/2 Δtor2 pYpk2^D239A

D

Quantification

Vacuolar/cytoplasmic signal ratio

Myr AbA

E

TOR2/Δtor2
ORM1/Δorm1
ORM2/Δorm2

F

[Min] Mock Myr AbA
Figure 4

TORC2 regulates actin and endocytosis via multiple pathways

A

<table>
<thead>
<tr>
<th>[Min]</th>
<th>0</th>
<th>7.5</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>90</th>
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<tbody>
<tr>
<td>SC</td>
<td>98 +/- 1</td>
<td>60 +/- 5</td>
<td>47 +/- 9</td>
<td>44 +/- 9</td>
<td>77 +/- 8</td>
<td>86 +/- 9</td>
</tr>
<tr>
<td>22°C</td>
<td>100 +/- 0</td>
<td>79 +/- 9</td>
<td>28 +/- 10</td>
<td>13 +/- 8</td>
<td>14 +/- 4</td>
<td>76 +/- 3</td>
</tr>
<tr>
<td>37°C</td>
<td>97 +/- 1</td>
<td>69 +/- 9</td>
<td>41 +/- 4</td>
<td>43 +/- 4</td>
<td>53 +/- 0</td>
<td>58 +/- 8</td>
</tr>
<tr>
<td>CPZ (500 µM)</td>
<td>62 +/- 0</td>
<td>14 +/- 1</td>
<td>84 +/- 5</td>
<td>44 +/- 4</td>
<td></td>
<td></td>
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</tbody>
</table>

B

<table>
<thead>
<tr>
<th>[min]</th>
<th>0.1</th>
<th>7.5</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>90</th>
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</thead>
<tbody>
<tr>
<td>SC Sorb 1M</td>
<td>0.1</td>
<td>0.1</td>
<td>7.5</td>
<td>15</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>SC Sorb 1M</td>
<td>0.1</td>
<td>0.1</td>
<td>7.5</td>
<td>15</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>SC Sorb 1M</td>
<td>0.1</td>
<td>0.1</td>
<td>7.5</td>
<td>15</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>SC Sorb 1M</td>
<td>0.1</td>
<td>0.1</td>
<td>7.5</td>
<td>15</td>
<td>30</td>
<td>60</td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>[Min]</th>
<th>90</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC Sorb 1M</td>
<td>62 +/- 0</td>
<td>14 +/- 1</td>
</tr>
<tr>
<td>SC Sorb 1M</td>
<td>84 +/- 5</td>
<td>44 +/- 4</td>
</tr>
<tr>
<td>SC Sorb 2M</td>
<td>95 +/- 4</td>
<td>71 +/- 6</td>
</tr>
</tbody>
</table>

D

<table>
<thead>
<tr>
<th>[Min]</th>
<th>30</th>
<th>60</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td>41 +/- 1</td>
<td>11 +/- 3</td>
<td>4 +/- 3</td>
</tr>
<tr>
<td>SC Sorb 1M</td>
<td>56 +/- 3</td>
<td>10 +/- 3</td>
<td>11 +/- 1</td>
</tr>
<tr>
<td>SC Sorb 2M</td>
<td>65 +/- 2</td>
<td>24 +/- 2</td>
<td>11 +/- 1</td>
</tr>
</tbody>
</table>

Note: The images and tables show the regulation of Ypk1 and pYpk1 under different conditions.
Figure 5

TORC2 regulates actin and endocytosis via multiple pathways.
Figure 6

A

Phosphopeptide detection

Treatment versus DMSO, P-peptides >2-fold change, p-value <0.05

Rapamycin and/or BHS345-dependent de/phosphorylation

B

TORC2 regulates actin and endocytosis via multiple pathways

C

D

E

TOR1^MT

\( \Delta fpk1 \)

\( \Delta fpk2 \)

\( \Delta fpk1/2 \)

\( \text{Mock} \)

\( \text{BHS} \)

\( \text{Myr} \)

\( \text{AbA} \)

\( \text{Ypk1}^{D242A} \)

\( \text{Ent1-HA} \)

\( \text{pRXXS/T} \)

\( \text{Pan1-GFP} \)

\( \text{pRXXS/T} \)

\( \text{Ent1-HA} \)

\( \text{pRXXS/T} \)

\( \text{Ent1-HA} \)

\( \text{pRXXS/T} \)

\( \text{Ent1-HA} \)
Figure 7

TORC2 regulates actin and endocytosis via multiple pathways

A

\[
\begin{array}{cccc}
\Delta \text{ent1} & \Delta \text{ent2} & \text{TOR1}^{MT} \\
\text{pEnt1-wt} & \text{pEnt1-2A} & \text{pEnt1-6A} & \text{pEnt1-6D} \\
\text{Mock} & \text{BHS} & \text{Mock} & \text{BHS} & \text{Mock} & \text{BHS} \\
pRXXS/T & \text{Ent1-HA} \\
\end{array}
\]

B

\[
\begin{array}{cccc}
\Delta \text{ent1} & \Delta \text{ent2} & \text{TOR1}^{MT} \\
\text{pEnt1-wt} & \text{pEnt1-2A} & \text{pEnt1-6A} & \text{pEnt1-6D} \\
\text{Mock} & \text{BHS345} & \text{Mock} & \text{BHS345} & \text{Mock} & \text{BHS345} \\
\text{Brightfield} & \text{LY} & \text{Brightfield} & \text{LY} & \text{Brightfield} & \text{LY} \\
\end{array}
\]

C

\[
\begin{align*}
\text{Vacuolar/cytoplasmic signal ratio} & \\
\text{Mock} & \text{BHS} & \text{Mock} & \text{BHS} & \text{Mock} & \text{BHS} \\
pRXXS/T & \text{Ent1-HA} & \text{Mock} & \text{BHS} & \text{Mock} & \text{BHS} \\
\end{align*}
\]

D

\[
\begin{array}{cccc}
\Delta \text{ent1} & \Delta \text{ent2} & \text{TOR1}^{MT} \\
\text{pEnt1-wt} & \text{pEnt1-2A} & \text{pEnt1-6A} & \text{pEnt1-6D} \\
\text{Mock} & \text{BHS345} & \text{Mock} & \text{BHS345} & \text{Mock} & \text{BHS345} \\
93 +/- 0 & 8 +/- 0 & 91 +/- 0 & 13 +/- 4 & 92 +/- 1 & 6 +/- 2 \\
\end{array}
\]

E

\[
\begin{array}{cccc}
\Delta \text{ent1} & \Delta \text{ent2} & \text{TOR1}^{MT} \\
\text{pEnt1-wt} & \text{pEnt1-2A} & \text{pEnt1-6A} & \text{pEnt1-6D} \\
\text{Mock} & \text{BHS345} & \text{Mock} & \text{BHS345} & \text{Mock} & \text{BHS345} \\
92 +/- 6 & 40 +/- 4 & 94 +/- 1 & 36 +/- 8 & 72 +/- 4 & 13 +/- 2 \\
\end{array}
\]
TORC2 regulates actin and endocytosis via multiple pathways

1 fast, direct via Ypk1
2 slow, indirect "sphingolipid pathway"
3 "Fpk pathway"
Signal Transduction:
Target of rapamycin complex 2 regulates actin polarization and endocytosis via multiple pathways

Delphine Rispal, Sandra Eltschinger, Michael Stahl, Stefania Vaga, Bernd Bodenmiller, Yann Abraham, Ireos Filipuzzi, N. Rao Movva, Ruedi Aebersold, Stephen B. Hellwell and Robbie Loewith

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