Supplemental material

Locke and Thorner, https://doi.org/10.1083/jcb.201807154

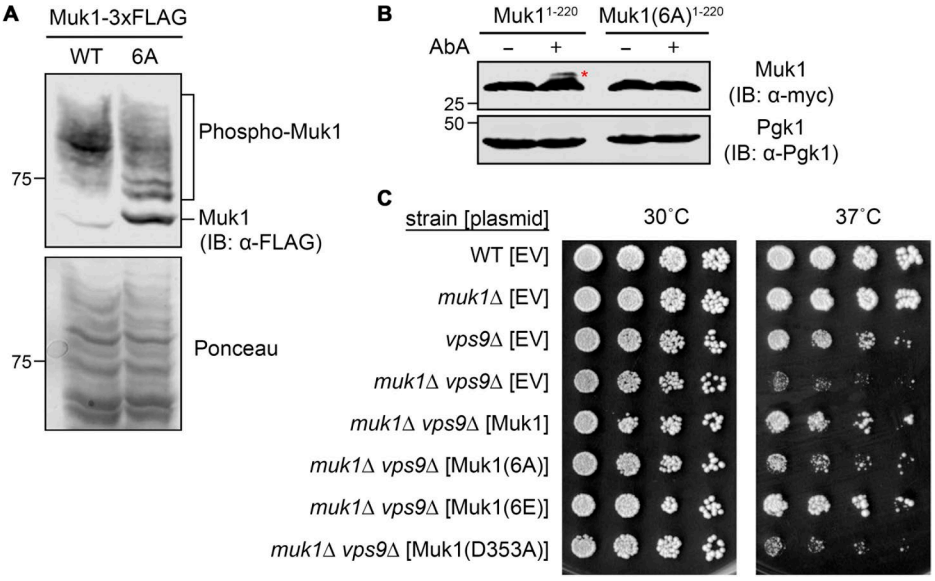

Figure S1. **Muk1 is phosphorylated at its Ypk1 consensus phospho-acceptor site motifs. (A)** WT (BY4741) cells expressing from the native *MUK1* promoter on a low-copy (*CEN*) vector either Muk1-3xFLAG (pMLT83) or Muk1(6A)-3xFLAG (pMLT84) were grown to mid-exponential phase, harvested, and lysed, and equivalent amounts of protein from the resulting extracts were resolved by Phos-tag SDS-PAGE and analyzed by immunoblotting (IB; top) and by staining with Ponceau S dye (bottom) to confirm equal sample loading, all as described in Materials and methods. **(B)** WT (BY4741) cells expressing either Muk1(1–220; pMLT56) or Muk1(1–220 6A; pMLT57) were grown to mid-exponential phase, treated with 1.8 µM AbA or vehicle for 2 h, harvested, and lysed, and the resulting extracts were analyzed by SDS-PAGE on a 75:1 12% acrylamide gel. Pgk1 served as a loading control. Asterisk (red) indicates phosphorylated species. **(C)** Ypk1-mediated phosphorylation stimulates the ability of Muk1 to support growth under heat stress. Samples of exponentially growing cultures of otherwise isogenic strains of the indicated genotype were plated in fivefold serial dilutions on appropriate selective growth medium and incubated at either 30° (left) or 37°C (right) for 3 d and then imaged.

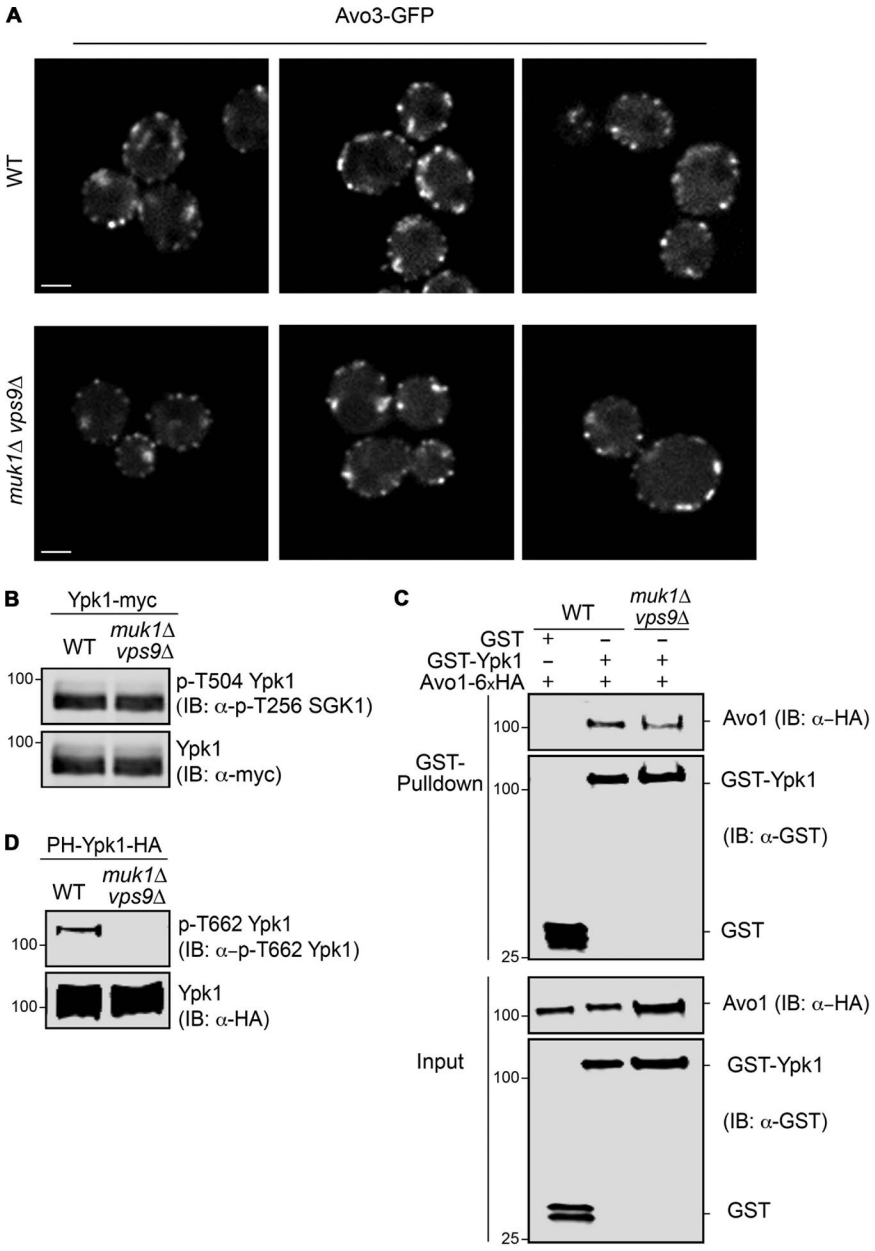

Figure S2. **Absence of Rab5 GEFs does not alter TORC2 localization at the cell cortex. (A)** Avo3 is an essential and very tightly bound subunit of TORC2. Hence, to visualize TORC2, either WT cells (yMLT64) or an otherwise isogenic strain lacking both Muk1 and Vps9 (yMLT66), as indicated, expressing Avo3-GFP from its native promoter at its endogenous locus on chromosome V, were examined. After growing the cultures to mid-exponential phase, samples were mounted on agarose pads and imaged using a confocal fluorescence microscope, as described in Materials and methods. Scale bar, 2 µm. **(B)** Absence of Rab5 GEFs does not impair activation loop phosphorylation of Ypk1 by eisosome-associated Pkh1/2. Either WT (BY4741) cells or otherwise isogenic *muk1Δ vps9Δ* (yMLT9) cells were transformed with Ypk1-myc (pAM20; Roelants et al., 2011), grown to mid-exponential phase, harvested, and lysed, and equivalent amounts of protein in the resulting extracts were resolved by SDS-PAGE and analyzed by immunoblotting (IB) with the indicated antibodies. We have documented before (Roelants et al., 2010) that commercial antibodies raised against the activation loop residue in mammalian SGK1 (anti-pT256 SGK1, sc-16744; Santa Cruz Biotechnology) phosphorylated by PDK1 specifically detect the homologous site (pT504) in the activation loop of Ypk1 phosphorylated by Pkh1 (or Pkh2; Roelants et al., 2002). **(C)** Absence of Rab5 GEFs does not impair Ypk1-Avo1 association. Otherwise WT (yMLT69) or isogenic *muk1Δ vps9Δ* (yMLT70) cells expressing Avo1-6xHA expressed from its endogenous locus and coexpressing from the *GAL* promoter on a plasmid either GST alone (pMLT115) or GST-Ypk1 (pMLT116) were grown to mid-exponential phase, induced with galactose (2% final concentration) for 3 h, harvested, and lysed; the GST proteins in the extracts were isolated by adsorption to glutathione-agarose (see Materials and methods); and the bound proteins were resolved by SDS-PAGE and analyzed by immunoblotting. **(D)** Forced PM association mediated by a PtdIns4,5P_2-specific PH domain does not restore TORC2-dependent phosphorylation of the Ypk1 C-terminal regulatory tail in cells deficient in Rab5 GEFs. As in A, except the cells were transformed with a plasmid (pPL495) expressing PH^{Slm1}-Ypk1-3xHA (pPL495; Niles et al., 2012), treated with AbA (1.8 µM), and blotted with an antiserum that detects phosphorylation at one of the primary C-terminal sites (T662) in Ypk1 that is phosphorylated specifically by TORC2 (Niles et al., 2012; Leskoske et al., 2017).

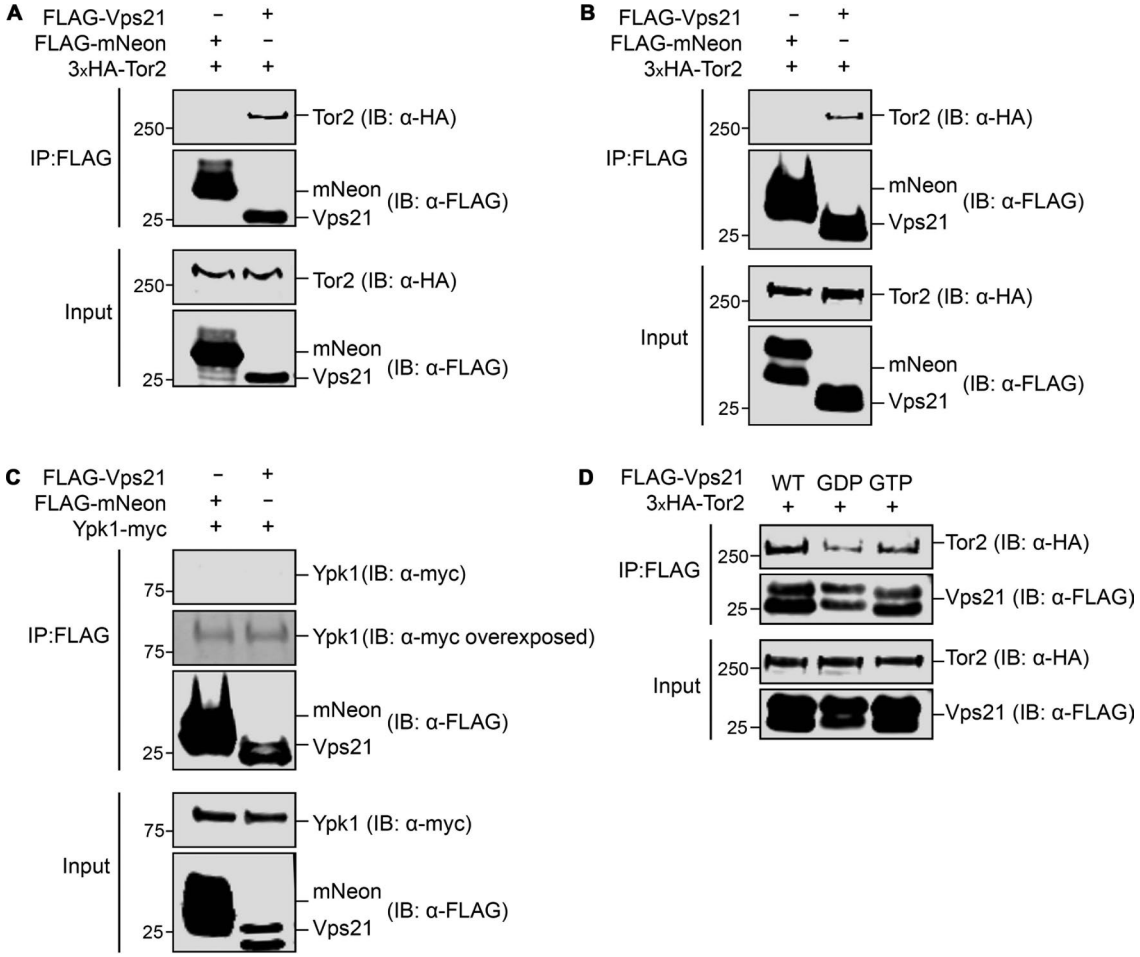

Figure S3. **Vps21 physically interacts with TOR2. (A)** Strain y2470 (*tor1Δ tor2Δ*) expressing 3xHA-Tor2 from a CEN plasmid was transformed with plasmids expressing either FLAG-mNG (pMLT114) or FLAG-Vps21 (pMLT101). The resulting transformants were grown to mid-exponential phase, and expression of the FLAG-tagged proteins was induced with 2% galactose for 4 h. The induced cells were harvested and lysed, and FLAG-tagged proteins were immuno-isolated from the extracts using resin coated with anti-FLAG antibodies as described in Materials and methods. Samples of the bound proteins were resolved by SDS-PAGE on 8% gels and analyzed by immunoblotting (IB) with anti-HA antibodies and on 13% gels and analyzed by immunoblotting with anti-FLAG antibodies. IP, immunoprecipitation. **(B)** Strain yMLT78 expressing 3xHA-Tor2 from its endogenous locus was transformed with the same plasmids and treated as in A, except that proteins bound to the anti-FLAG resin were resolved by SDS-PAGE on a 4–20% gradient gel. **(C)** WT cells (BY4741) expressing Ypk1-myc (pAM20) from a *LEU2*-marked *CEN* plasmid and coexpressing either FLAG-Vps21 (pMLT101) or FLAG-mNEON (pMLT114) from a *URA3*-marked 2 µm vector were examined as in A. **(D)** Strain yMLT78 was transformed with plasmids expressing either FLAG-Vps21 (pMLT101), FLAG-Vps21(S21L; pMLT102), or FLAG-Vps21(Q66L; pMLT103), and proteins were immunoprecipitated, resolved, and analyzed as in B.

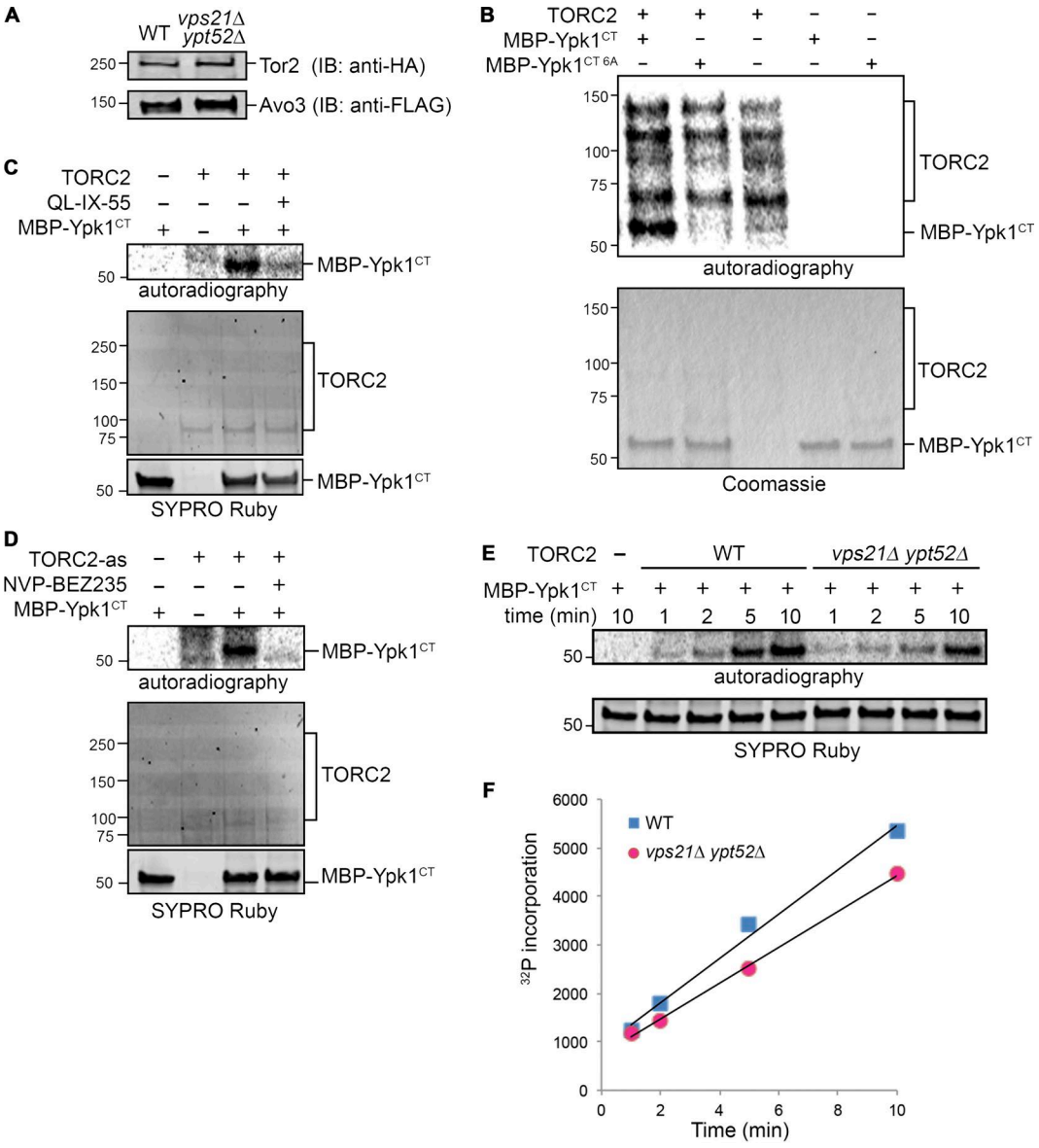

Figure S4. **A Rab5 GTPase stimulates TORC2 activity in vitro. (A)** WT (BY4741) and yMLT85 (*vps21Δ ypt52Δ*) strains expressing both Avo3-3xFLAG and 3xHA-Tor2 from their endogenous loci were grown to mid-exponential phase, harvested, and lysed, and TORC2 was immuno-enriched from the resulting extracts as described in Materials and methods. The immuno-isolated TORC2 was analyzed by SDS-PAGE and immunoblotting (IB). **(B)** TORC2 isolated from WT cells was incubated with [γ-^{32}P]ATP and recombinant MBP-Ypk1^{CT}(603–680) containing its TORC2 phosphorylation sites or MBP-Ypk1^{CT} 6A(603–680) with the TORC2 sites mutated to Ala. Products were separated on SDS-PAGE and analyzed by Coomassie blue staining and autoradiography. **(C)** TORC2 isolated from WT cells was incubated with [γ-^{32}P]ATP and recombinant MBP-Ypk1^{CT} in the presence or absence of the TORC2 inhibitor QL-IX-55 (500 µM) or vehicle (DMSO). **(D)** TORC2 isolated from TORC2-as (yKL7) cells was incubated with [γ-^{32}P]ATP and recombinant MBP-Ypk1^{CT} in the presence or absence of the NVP-BEZ235 (500 µM) or vehicle (dimethyl formamide). **(E)** Equivalent amounts of TORC2 immunopurified from WT (BY4741) or yMLT85 (*vps21Δ ypt52Δ*) strains were incubated with [γ-^{32}P]ATP and recombinant MBP-Ypk1^{CT}. Reactions were terminated at the indicated time points. **(F)** Quantitation of the ^{32}P autoradiogram in E.

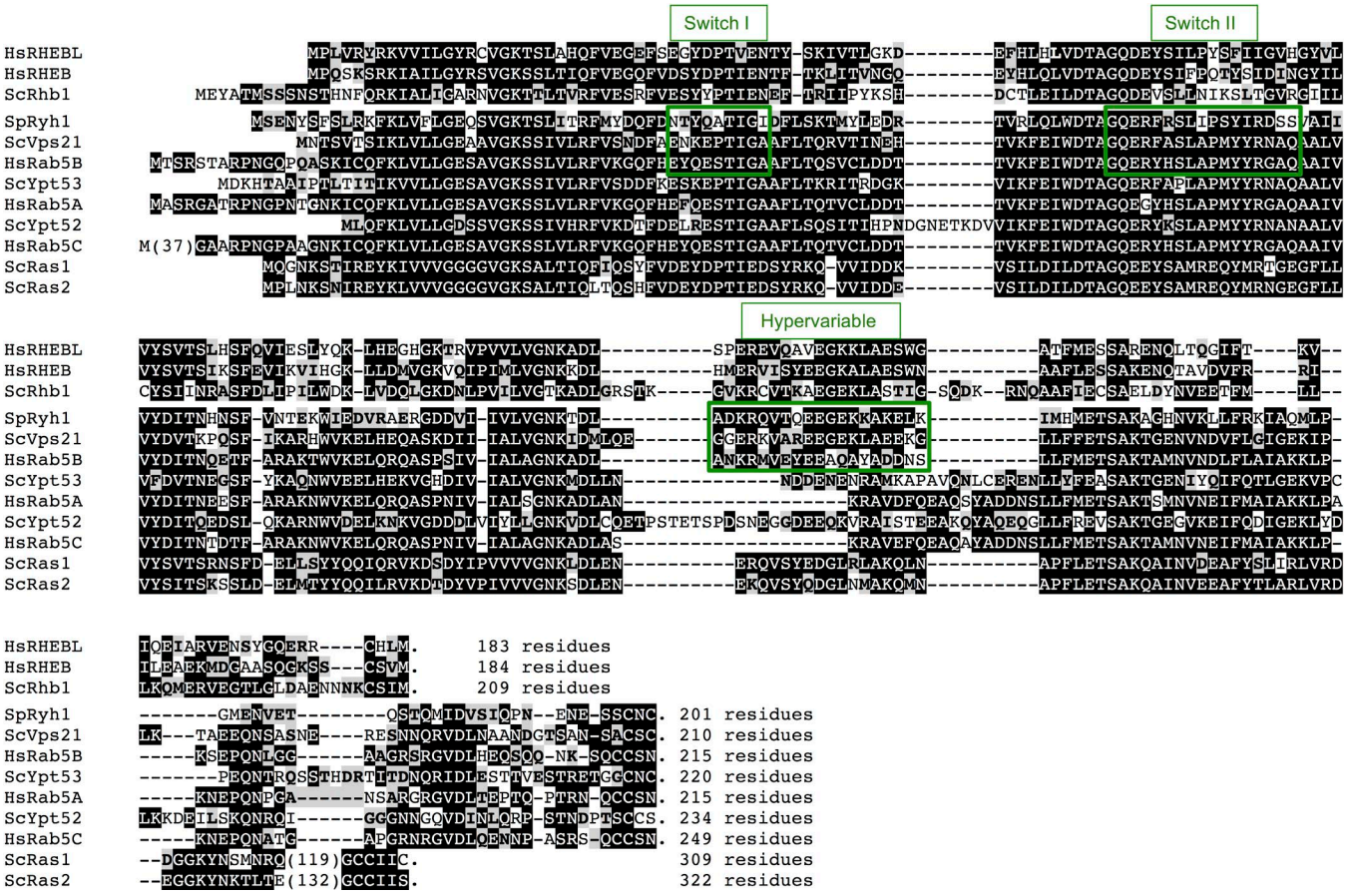

Figure S5. **Sequence alignment of the indicated small GTPases.** Sequence identities shared among related classes of GTPase are indicated by a white letter on a black box, and standard conservative substitutions are indicated by a bold letter on a gray box. Hs, *Homo sapiens*; Sc, *S. cerevisiae*; Sp, *S. pombe*. The similarities in the Switch I, Switch II, and hypervariable loop regions of Sp Ryh1 and ScVps21 and HsRab5B are indicated by green boxes.

References

Leskoske, K.L., F.M. Roelants, M.N.M. Marshall, J.M. Hill, and J. Thorner. 2017. The stress-sensing TORC2 complex activates yeast AGC-family protein kinase Ypk1 at multiple novel sites. *Genetics*. 207:179–195. https://doi.org/10.1534/genetics.117.1124

Niles, B.J., H. Mogri, A. Hill, A. Vlahakis, and T. Powers. 2012. Plasma membrane recruitment and activation of the AGC kinase Ypk1 is mediated by target of rapamycin complex 2 (TORC2) and its effector proteins Slm1 and Slm2. *Proc. Natl. Acad. Sci. USA*. 109:1536–1541. https://doi.org/10.1073/pnas.1117563109

Roelants, F.M., P.D. Torrance, N. Bezman, and J. Thorner. 2002. Pkh1 and Pkh2 differentially phosphorylate and activate Ypk1 and Ykr2 and define protein kinase modules required for maintenance of cell wall integrity. *Mol. Biol. Cell*. 13:3005–3028. https://doi.org/10.1091/mbc.e02-04-0201

Roelants, F.M., A.G. Baltz, A.E. Trott, S. Fereres, and J. Thorner. 2010. A protein kinase network regulates the function of aminophospholipid flippases. *Proc. Natl. Acad. Sci. USA*. 107:34–39. https://doi.org/10.1073/pnas.0912497106

Roelants, F.M., D.K. Breslow, A. Muir, J.S. Weissman, and J. Thorner. 2011. Protein kinase Ypk1 phosphorylates regulatory proteins Orm1 and Orm2 to control sphingolipid homeostasis in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA*. 108:19222–19227. https://doi.org/10.1073/pnas.1116948108