TORC2-dependent phosphorylation of Ysp2 disrupts its association with *beta*-propeller proteins located at PM-ER contact sites

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The PM of *S. cerevisiae* is highly enriched in ergosterol, a sterol essential for maintaining fungal PM fluidity, rigidity and thickness. Ergosterol is synthesized in the ER, but is further transported to the PM mainly by mechanisms that require lipid transfer proteins (LTPs). Recently, a new family of sterol binding LTPs located at membrane contact sites (MCSs) between the ER and other organelles, which have been designated LAMs (for "lipid transfer protein anchored at membrane contact sites) or LTCs (for "lipid transfer at contact sites"), was identified. *S. cerevisiae* contains six members of this new LAM/LTC family. Two of these proteins, Ysp2/Lam2/Ltc4 and its paralog Lam4/Ltc3 are specifically located at ER-PM contact sites and appear to be responsible for the majority of the non-vesicular retrograde transport of ergosterol from the PM back to the ER [1]. Our prior work identified Ysp2 as a substrate of the TORC2-dependent protein kinase Ypk1 [2] and, in a more recent study, we demonstrated that Ypk1-mediated phosphorylation of Ysp2 and Lam4 inhibits their ability to promote retrograde transport of sterols from the PM to the ER [3]. Moreover, we found that the retention of sterol in the PM that results from this control mechanism promotes cell survival under membrane-perturbing conditions.

In the work presented here, we address the molecular mechanism by which phosphorylation impedes the function of Ysp2. Using an *in vitro* pull-down assay, we found that authentic Ypk1-mediated phosphorylation of Ysp2 (or a phospho-mimetic Ysp2 allele) exhibited a marked reduction in its ability to bind to the WD40 repeat-containing protein Ymr102c, a known Ysp2-associated protein and MCS component, and that Ysp2-Ymr102c interaction does not require the PH-like domain in Ysp2. Using fluorescence microscopy, we document that GFP-Ysp2 co-localizes with Ymr102c-mKate at ER-PM MCSs under normal growth conditions and in cells treated with myriocin (a stress that up-regulates TORC2-Ypk1 signaling). We show that Ysp2 still localizes to ER-PM MCSs in cells lacking both Ymr102c and its paralog Dgr2, whereas Ymr102c and Dgr2 do not localize to the ER-PM MCSs in the absence of Ysp2 and Lam4, indicating that Ysp2 and Lam4 are required for the recruitment of Ymr102c and Dgr2.

Taken together, our findings support the conclusion that one important effect of Ypk1-mediated phosphorylation of Ysp2 is to block its interaction with Ymr102c (and likely Dgr2) thereby impeding assembly of the MCS structures needed for efficient retrograde sterol transport.

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[2] Muir A., Ramachandran S., Roelants F.M., Timmons G., Thorner J. (2014) TORC2-dependent protein kinase Ypk1 phosphorylates ceramide synthase to stimulate synthesis of complex sphingolipids. *Elife* **3**: e03779.

[3] Roelants F.M., Chauhan N., Muir A., Davis J.C., Menon A.K., Levine T.P., Thorner J. (2018) TOR complex 2-regulated protein kinase Ypk1 controls sterol distribution by inhibiting StARkin domain-containing proteins located at plasma membrane-endoplasmic reticulum contact sites. *Mol. Biol. Cell* **29**: 2128-2136.