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 GA; 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.

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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 muk1\Delta vps9\Delta (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 muk1A vps9A (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 PtdIns4,5P₂-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^{SIm1}-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 (*tor1*Δ *tor2*Δ) 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.







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		Switch I			Switch II	
HSRHEBL	MPLVRYRKVVILGYRCVGKTSLAHQFVEGEFS	EGYDPTVE	NTY-SKIVTLGKD	EFHLHLVDTAGQDE	YSILPYSFIIG	V <mark>H</mark> GYVL
HSRHEB	MP <mark>QSK</mark> SRKIAILGYRSVGKSSLTIQFVEGQFV	DSYDPTIE	NTF-TKLITVNGQ	<mark>E</mark> YHLQLVDTAGQDE	YSIFPQ T YSID	INGYIL
ScRhb1	MEYATMSSSNSTHNFQRKIALIGARNVGKTTLTVRFVESRFV	ESYYPTIE	NEE-TRIIPYKSH	DCTLEILDTAGQDE	VSLLNIKSLTG	VRGIIL
SpRyh1	MSENYSFSLRKFKLVFLGEQSVGKTSLITRFMYDQFD	N TYQ ATIG	IDFLSKTMYLEDR	TVRLQLWDTAGQER	F <mark>R</mark> SL <mark>I</mark> PSYIRD	SS 7AII
ScVps21	MNTSVTSIKLVLLGEAAVGKSSIVLRFVSNDFA	ENKEPTIG.	AAFLTQRVTIN <mark>E</mark> H	TVKFEIWDTAGQER	FASLAPMYYRN	AQ \ ALV
HsRab5B	MT <mark>SR</mark> STA <mark>RPNGQ</mark> P QASKICQFKLVLLGESAVGKSSLVLRFVKGQFH	EYQESTIG.	AAFLTQSVCLDDT	TVKFEIWDTA <mark>GQ</mark> ER	YHSLAPMYYRG	AQ \AIV
ScYpt53	MDKHTAAIPTLTITIKVVLLGESAVGKSSIVLRFVSDDFK	ESKEPTIG.	AAFLTKRIT <mark>R</mark> DGK	VIKFEIWDTAGQER	FA <mark>P</mark> LAPMYYRN	AQAALV
HsRab5A	M <mark>ASRGATRPNGPNTGNKICQFKLVLLGESAVGKSSLVLRFVKGQ</mark> FH	E <mark>FQ</mark> ESTIG.	AAFLTQTVCLDDT	TVKFEIWDTAGQEG	YHSLAPMYYRG	AQAAIV
ScYpt52	MLQFKLVLLGDSSVGKSSIVHRFVKDTFD	ELRESTIG.	AAFLSQSITI <mark>HPN</mark> DGNETI	KDV <mark>VIKFEIWDTAGQER</mark>	Y <mark>K</mark> SLAPMYYRN	ANAALV
HsRab5C	M(37)GAARPNGPAAGNKICQFKLVLLGESAVGKSSLVLRFVKGQFH	EYQESTIG.	AAFLTQTVCLDDT	TVKFEIWDTAGQER	YHSLAPMYYRG	AQAAIV
ScRas1	MQ <mark>G</mark> NKS <mark>T</mark> IREYKIVVVGGGGVGKSALTIQF <mark>I</mark> QSYFV	DEYDPTIE	DSYRKQ- <mark>VVIDDK</mark>	VSILDILDTAGQEE	YSAMREQYMR <mark>T</mark>	GEGFLL
ScRas2	MPLNKSNIREYKLVVVGGGGVGKSALTIQLTQSHFV	DEYDPTIE	DSYRKQ-VVIDDB	VSILDILDTAGQEE	YSAMREQYMRN	GEGFLL
			Hypervariable			
HSRHEBL	VYSVTSLHSFOVTESTVOK-THEGHGKTRVPVVLVGNKADI	SPER	EVOAVEGKKLAESWG	ATEMESSARE	NOL TOGTET	KV
HSRHEB	VYSVTSIKSFEVIKVIHGK-LLDMVGKVOIPIMLVGNKKDL	HMER	VISYEEGKALAESWN	AAFLESSAKE	NOTAVDVFR	
ScRhb1	CYSIINRASEDLIPILWDK-LWDOLGKDNLPVILVGTKADLGRSTK-		CVTKAEGEKLASTIG-SO	DKRNOAAFTECSAEL	DYNVEETEM	
CoDuch 1	WYDTENUNSE WNEEKETEDYD AEDCDDYT TYL WCNKEDT	ADER	OVTOFECER	TMUMERCAKA		
Soupe 21	VIDIININSF VNIJNW EDVNADNODDIIIVGVNIDDI	CCER	VIOLEGERIAREIR	ILFEFTSAKT	GENVNDVET GT	
HeRab5B	VIDVINIOSI – IRAKIWVKELOBOASODII – IALVGNADODOE – – –					AKKT.D-
ScVnt53	VIDIINOHI AKAKIWYKELOKOASIBIY IALAGNAADD	para nere		NLCERENILIVEE ASAKT	GENTYOTEOTL	CEKVDC
HsRab5A	VYDTTNEESF-ARAKNWVKELOROASPNTV-TALSGNKADLAN		KRAVDFOEAO	SYADDNSLLEMETSAKT	SMNVNETFMAT	AKKT.PA
ScYpt52	VYDITOEDSI-OKARNWVDELKNKVGDDDIVIYLUGNKVDLCOETPS	TETSPOSN	EGGDEEOKVRAISTEEAK	OYAOEOGLILFREVSAKT	GEGVKEIFODI	GEKLYD
HsRab5C	VYDITNTDTF-ARAKNWVKELOROASPNIV-IALAGNKADLAS		KRAVEFOEAO	AVADDNSLLFMETSAKT	AMNVNEIFMAI	AKKLP
ScRas1	VYSVTSRNSFD-ELISYYOOIORVKDSDYIPVVVVGNKIIDLEN		OVSYEDGLELAKOLN	APFLETSAKO	AINVDEAFYSL	RLVRD
ScRas2	VYSITSKSSLD-ELMTYYQQILRVKDTDYVPIVVVGNKSDLEN		OVSYODGLNMAKOMN	APFLETSAKO	AINVEEAFYTL	ARLVRD
HeRHERT.	OPTARMENSVEORBRCHIM. 183 residues					
HSRHEB	TLEARKMDCAASOCKSSCSVM. 184 residues					
ScRhb1	IKOMERVEGTLGIDAENNNKCSIM. 209 residues					
SpRvh1	CMENNETOSTOMIDUS TOPN_ENE_SSONC	201 res	idues			
ScVps21	IKTAEEONSASNERESNNORVDINAANDGTSAN-SACSC.	210 res	idues			
HsRab5B	KSEPONLGCAAGRSRGVDLHEOSOO-NK-SOCCSN.	215 res	idues			
ScYpt53	PEONTROSSTHORTITONORIDLESTTVESTRETGGCNC.	220 res	idues			
HsRab5A	KNEPONPCANSARGRGVDLTEPTO-PTRN-OCCSN.	215 res	idues			
ScYpt52	LKKDEILSKONROIGCGNNGOVDINLORP-STNDPTSCCS.	234 res	idues			
HsRab5C	KNEPONATGAPGRNRGVDLOENNP-ASRS-OCCSN.	249 res	idues			
ScRas1	DGGKYNSMNRO(119)GCCIIC.	309 res	idues			
ScRas2	EGGKYNKTLTE(132)GCCIIS.	322 res	idues			

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.

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