

Supplementary Materials

Construction of plasmids.

YEp352-PKC1^{T1125A} and YEp352-PKC1^{T1125A/S1143A} were constructed using a QuikChange site-directed mutagenesis kit (Stratagene) with pFR22 (YEp352-PKC1) or pFR74 (YEp352-PKC1^{S1143A}) (44) as a template, and the following oligonucleotides: 5'-GCGCCACCCACACTTGCTCCTCTGCCCTCTG-3' (T1125A1) and 5'-CAGAGGGCAGAGGAGCAAGTGTGGGTGGCGC-3' (T1125A2), which includes the codon for Thr¹¹²⁵ of *PKC1* (the underlined areas indicate mismatches for the substitution of Thr¹¹²⁵ to Ala). pFR22, YEp352-PKC1^{T1125A}, pFR74, and YEp352-PKC1^{T1125A/S1143A} were digested with SphI, and each DNA fragment containing a *PKC1* variant was cloned into the SphI site of YCp50 to yield YCp50-PKC1, YCp50-PKC1^{T1125A}, YCp50-PKC1^{S1143A}, and YCp50-PKC1^{T1125A/S1143A}, respectively.

To construct the pRS304-3HA-Tor2 plasmid, the fragment containing the N-terminal region of *3HA-TOR2* in pRS314-3HA-TOR2 (61) was amplified by PCR with HA-TOR2-F-BamHI and HA-TOR2-R-XhoI. The amplified DNA fragment was digested with BamHI and XhoI, and the resultant fragment was introduced into the BamHI and XhoI sites of pRS304. To construct the pRS306-Avo3-13myc plasmid, the fragment containing the C-terminal region of *AVO3-13myc* in YMY123 (62) was amplified by PCR with AVO3-GFP-F-SacII and AVO3-myc-R-XhoI. The amplified DNA fragment was digested with HindIII and XhoI, and the resultant fragment was introduced into the HindIII and XhoI sites of pRS306. To construct the genes fused with 3xFLAG tag, we constructed pRS306-3FLAG and pRS305-3FLAG plasmids. 3xFLAG tag was amplified by PCR with 3xFLAG-F-XhoI and 3xFLAG-R-XhoI using p3xFLAG-CMV-14 (Sigma-Aldrich) as the template. The resultant fragment was digested with XhoI, followed by cloning into the XhoI site of pRS306 to yield pRS306-3FLAG. To construct pRS305-3FLAG, pRS306-3FLAG was digested with PvuII, and the fragment containing 3xFLAG tag was cloned into the PvuII site of pRS305 to yield pRS305-3FLAG. To construct the pRS305-BIT61-3FLAG plasmid, the C-terminal region of *BIT61* was amplified with the primers BIT61-3FLAG-F-BglII and BIT61-3FLAG-R-BglII, and the PCR product was digested with BglII. The resultant fragment was introduced into the BamHI site of pRS305-3FLAG. The plasmids constructed (pRS304-3HA-TOR2, pRS306-AVO3-13myc, and pRS305-BIT61-3FLAG) were digested with SacI, XbaI, and BamHI, respectively, and each linearized DNA was introduced into the loci of *TOR2*, *AVO3*, and *BIT61*, respectively.

To construct the pRS306-PKC1-3FLAG, pRS306-PKC1^{T1125A}-3FLAG, and pRS306-PKC1^{S1143A}-3FLAG plasmids, the C-terminal region of *PKC1*, *PKC1*^{T1125A}, and *PKC1*^{S1143A} were amplified with the primers PKC1-3FLAGF and PKC1-3FLAGR using pFR22 (*PKC1*^{WT}) (44), YEp352-PKC1^{T1125A} (this study), and pFR74 (*PKC1*^{S1143A}) (44) as templates, respectively, and the PCR products were digested with BamHI. The resultant fragment was introduced into the BamHI site of pRS306-3FLAG. The plasmids were digested with EcoRI, and each linearized DNA was introduced into the locus of *PKC1*.

The HA-tagged *PKC1* plasmids pFL39-PKC1-3HA, pFL39-PKC1^{T1125A}-3HA,

YCplac111-PKC1-3HA, and YCplac111-PKC1^{S1143A}-3HA were constructed as follows: the *PKC1* genes containing the promoter region and just before the stop codon of *PKC1*^{WT}, *PKC1*^{T1125A}, and *PKC1*^{S1143A} were amplified with the primers PKC1-HA-F-PstI and PKC1-HA-R-BglII using pFR22 (*PKC1*^{WT}) (44), YEp352-PKC1^{T1125A} (this study), and pFR74 (*PKC1*^{S1143A}) (44) as templates, respectively. Each amplified DNA fragment was digested with PstI and BglII, and the resultant fragments were cloned into the PstI and BglII sites of pSLF172 (63), which was designed to add a 3xHA tag at the C terminus of the respective protein. Since the plasmids obtained (pSLF172-PKC1, pSLF172-PKC1^{T1125A}, and pSLF172-PKC1^{S1143A}) lacked the terminator region of *PKC1*, each HA-tagged *PKC1* gene amplified with the primers PKC1-HA-F-PstI and PKC1-HA-R-PstI, followed by digestion with PstI, was cloned into the PstI site of pRS424-PKC1term, which contained the terminator region (approximately 500 bp of the *PKC1* 3' untranslated region) of *PKC1* that was amplified by PCR with PKC1t-F-NotI and PKC1t-R-SacII. The resultant plasmids were named pRS424-PKC1-3HA, pRS424-PKC1^{T1125A}-3HA, and pRS424-PKC1^{S1143A}-3HA. Each plasmid was digested with SphI, and the DNA fragment containing the *PKC1* promoter—*PKC1* ORF-3xHA—*PKC1* terminator was cloned into the SphI site of pFL39 (64) or YCplac111 to yield pFL39-PKC1-3HA, pFL39-PKC1^{T1125A}-3HA, YCplac111-PKC1-3HA, and YCplac111-PKC1^{S1143A}-3HA.

To construct pFL39-PKC1^{4C/S}-3HA, pRS424-PKC1^{4C/S}-3HA was firstly constructed. The 1.2 kb fragment containing the C1 region of *PKC1*^{4C/S} in pHPS29 (49) was amplified with the primers PKC1-ClaI-F and PKC1-ClaI-R, and the PCR product was digested with ClaI. The resultant fragment was introduced into the ClaI site of pSLF172-PKC1, as constructed above, to replace the C1 domain to yield pSLF172-PKC1^{4C/S}. To add the *PKC1* terminator, *PKC1*^{4C/S}-3HA, which had been amplified by PCR with the primers PKC1-HA-F-PstI and PKC1-HA-R-PstI using pSLF172-PKC1^{4C/S} as a template followed by digestion with PstI, was cloned into the PstI site of pRS424-PKC1term (laboratory stock). Finally, the plasmid obtained (pRS424-PKC1^{4C/S}-3HA) was digested with SphI, and the resultant fragment containing the *PKC1* promoter—*PKC1*^{4C/S} ORF-3xHA—*PKC1* terminator was cloned into the SphI site of pFL39 to yield pFL39-PKC1^{4C/S}-3HA.

To construct pFL39-PKC1^{AHR1}-3HA, the *pkc1* gene, which had been deleted for the HR1 domain that had been cloned in pVD71 (60), was amplified by PCR with PKC1-HA-F-SalI and PKC1-HA-R-BglII. The amplified DNA fragment was digested with SalI and BglII, and the resultant fragment was introduced into the SalI and BglII sites of pRS424-PKC1-3HA. The resultant plasmid (pRS424-PKC1^{AHR1}-3HA) was digested with SphI, and the fragment containing *PKC1*^{4C/S}-3HA was cloned into the SphI site of pFL39.

To construct ADH1p-PKC1C1 and ADH1p-PKC1C1^{4C/S}, the C1 domain of *PKC1* in pVD81 (60) and pHPS29 (49) was amplified with the primers pVD81-F-HindIII and pVD81-R-XhoI, followed by digestion with HindIII and XhoI, and each fragment was introduced into the HindIII and XhoI sites of pKW430 (65).

To construct pET-15b-Ypk2, an open reading frame of YPK2 was amplified with primers HIS-YPK2-F-BamHI and HIS-YPK2-R-BamHI, followed by digestion with BamHI, and the resultant fragment was introduced into the BamHI site of pET-15b.

Effect of MG on TORC2 activity *in vitro*.

TORC2 was immunopurified using HA-tagged Tor2 (pRS314-3HA-TOR2) or kinase dead HA-Tor2 (pRS314-3HA-TOR2^{D2298E}) (61). Immunopurified TORC2 was incubated with 5 µg of Pkc1 peptide (APPTLTPLPSVLTTTSQQEEFRGFSFMPDDL). The reaction was initiated by adding 100 µM ATP in the presence (50 µM) or absence of methylglyoxal. After being incubated for 30 min at 30°C, the reaction was terminated by the addition of SDS-PAGE sample buffer, and samples were then incubated for 5 min at 65°C. Samples were subjected to Tricine-SDS-PAGE (33) or SDS-PAGE, and phosphorylated peptide was detected by anti-phospho-Pkc1 at Ser¹¹⁴³ (p-S1143) antibodies.

SUPPLEMENTAL REFERENCES

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Table S1. *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype/description	Source/Reference
YPH250	<i>MATa trp1-Δ1 his3-Δ200 leu2-Δ1 lys2-801 ade2-101 ura3-52</i>	Yeast Genetic Stock Center
DL100	<i>MATa leu2-3, 112 trp1-1 ura3-52 his4 can1</i>	66
5JK9-3da	<i>MATa leu2-3, 112 ura3-52 rme1 trp1 his4 GAL⁺</i>	43
TB50a	<i>MATa his3 leu2-3, 112 ura3-52 rme1 trp1</i>	45
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Invitrogen
<i>bck1Δ</i>	YPH250, <i>bck1Δ::LEU2</i>	This study
<i>mkk1Δ</i> <i>mkk2Δ</i>	YPH250, <i>mkk1Δ::LEU2 mkk2Δ::HIS3</i>	This study
<i>mpk1Δ</i>	YPH250, <i>mpk1Δ::HIS3</i>	This study
<i>wsc1Δ</i>	YPH250, <i>wsc1Δ::LEU2</i>	This study
<i>mid2Δ</i>	YPH250, <i>mid2Δ::KanMX4</i>	This study
<i>wsc1Δ</i> <i>mid2Δ</i>	YPH250, <i>wsc1Δ::LEU2 mid2Δ::KanMX4</i>	This study
DL376	DL100, <i>pkc1Δ::LEU2</i>	66
SH121	JK9-3da, <i>tor2Δ::ADE2-3 YCplac111[tor2-21^{ts}]</i>	43
JM340	TB50a, [<i>HIS3MX6</i>]- <i>GAL1p-TOR2</i> <i>CRZ1-13myc-[KanMX6]</i>	67
RL25-1C	TB50a, [<i>KanMX4</i>]- <i>GAL1p-3HA-AVO1</i>	45
RS61-5b	TB50a, [<i>KanMX4</i>]- <i>GAL1p-3HA-AVO3</i>	45
YOC2573	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 wsc1::cgLEU2</i>	30
TNP46	<i>MATa trp1 leu2 ade2 ura3 his3 can1-100 Δmpk1::HIS3</i>	31
YOC788	<i>MATα aro7 can1 leu2 trp1 ura3 fks1::URA3</i>	59
YOC764	<i>MATα ade2 his3 leu2 lys2 trp1 ura3 rho1::HIS3</i> <i>ade3::(pRH01-RHO1::LEU2)</i>	68
YOC752	<i>MATα ade2 his3 leu2 lys2 trp1 ura3 rho1::HIS3</i> <i>ade3::(pRH01-rho1-2::LEU2)</i>	68
YOC729	<i>MATα ade2 his3 leu2 lys2 trp1 ura3 rho1::LYS2</i> <i>ade3::(pRH01-rho1-3::HIS3)</i>	68
YOC755	<i>MATα ade2 his3 leu2 lys2 trp1 ura3 rho1::HIS3</i> <i>ade3::(pRH01-rho1-5::LEU2)</i>	68
<i>wsc2Δ</i>	BY4741, <i>wsc2Δ::KanMX4</i>	Invitrogen
<i>mtl1Δ</i>	BY4741, <i>mtl1Δ::KanMX4</i>	Invitrogen

Table S2. Primers used in this study

Primer	Sequence
mid2-F	5'-CAATCTTTACCCGTTATTTTTGCCGTTTTG-3'
mid2-R	5'-AGGGAGAAATTCACAGAACTCGGTAAGTTT-3'
WSC1F	5'-GCCCCCAAAGTCGCCTTCTGCAAAATAATG-3'
WSC1R	5'-CATTGGGCCACGAGGAACAGATCTTGAGAA-3'
MPK1FSalI	5'-GCACTGTCCATTCATAAGGCACAGGGTCAA-3'
MPK1REcoRI	5'-AAGAATTCAAGAGGCGATAACAACTTCCG-3'
HA-TOR2-F-BamHI	5'-TCGGATCCCCTGACTGAAAATTCATGAATC-3'
HA-TOR2-R-XhoI	5'-ATCCTCGAGATAAGGTGCTTTCAAGCTGAG-3'
AVO3-GFP-F-SacII	5'-TAGTCCGCGGAGCAGACCAAGCTTTATATG-3'
AVO3-myc-R-XhoI	5'-CGCTCGAGAAGACTGTCAAGGAGGGTATTC-3'
BIT61-3FLAG-F-BglII	5'-ATCAGATCTTGCCTCCTTTTTGACAGATTC-3'
BIT61-3FLAG-R-BglII	5'-TGCAGATCTTATACTCATTCTAATGCCTGC-3'
PKC1-HA-F-PstI	5'-ACCCTGCAGTGCATGCCGGTCACGCTGAAG-3'
PKC1-HA-R-PstI	5'-AAACTGCAGCTAAGCAGCGTAATCTGGAAC-3'
pVD81-F-HindIII	5'-TTTAAGCTTATGGGGGATCTCTCCAGCGGGCAGATT TTGTAAACACTAG-3'
pVD81-R-XhoI	5'-CCTCTCGAGCTACTCTTGATTACGTTTTGT-3'
PKC1-HA-F-SalI	5'-ACCGTCGACTGCATGCCGGTCACGCTGAAG-3'
PKC1-HA-R-BglII	5'-CCTAGATCTCCTAAATCCAAATCATCTGGC-3'
PKC1-3FLAGF	5'-CACCAGAGAAAACACTGGATCCAACGTCGA-3'
PKC1-3FLAGR	5'-TTTGGATCCTAAATCCAAATCATCTGGCAT-3'
PKC1-ClaI-F	5'-CAGAGAGCTACGTTACTATA-3'
PKC1-ClaI-R	5'-GGTTTGTGTGCACCTTCTG-3'
HIS-YPK2-F-BamHI	5'-GAGGATCCGATGCATTCTGGCGAATATCC-3'
HIS-YPK2-R-BamHI	5'-TTTGGATCCCTAACTAATGCTTCTCCCCTG-3'
3xFLAG-F-XhoI	5'-ATTCTCGAGAGTACATGACCTTACGGGACT-3'
3xFLAG-R-XhoI	5'-TCACAGGGATGCCACTCGAGATCACTACTT-3'
PKC1t-F-NotI	5'-TGATTTGGATTTAGCGGCCGCAGGTCATGCCATGA-3'
PKC1t-R-SacII	5'-GAGTATAGTCGACCGCGTAACAGTGATTC-3'

Table S3. Plasmids used in this study

Plasmid	Description	Source/Reference
ADH1p-PKC1C1	pKW430 (2 μ -type, <i>URA3</i> marker) backbone, for the expression of the C1 domain (373-553 a. a.) of <i>PKC1</i> driven by the <i>ADH1</i> promoter	This study
ADH1p-PKC1C1 ^{4C/S}	pKW430 (2 μ -type, <i>URA3</i> marker) backbone, for the expression of the C1 domain (373-553 a. a.) of <i>PKC1</i> ^{4C/S} driven by the <i>ADH1</i> promoter	This study
pFR22	YEp352 (2 μ -type, <i>URA3</i> marker) harboring <i>PKC1</i>	44
pFR74	YEp352 (2 μ -type, <i>URA3</i> marker) harboring <i>PKC1</i> ^{S1143A}	44
YEp352-PKC1 ^{T1125A}	YEp352 (2 μ -type, <i>URA3</i> marker) harboring <i>PKC1</i> ^{T1125A}	This study
YEp352-PKC1 ^{T1125A/S1143A}	YEp352 (2 μ -type, <i>URA3</i> marker) harboring <i>PKC1</i> ^{T1125A/S1143A}	This study
pRS306-AVO3-13myc	pRS306 (integrate-type, <i>URA3</i> marker) backbone, for replacing endogenous <i>AVO3</i> with <i>AVO3-13MYC</i>	This study
pRS304-3HA-TOR2	pRS304 (integrate-type, <i>TRP1</i> marker) backbone, for replacing endogenous <i>TOR2</i> with <i>3HA-TOR2</i>	This study
pRS305-BIT61-3FLAG	pRS305 (integrate-type, <i>LEU2</i> marker) backbone, for replacing endogenous <i>BIT61</i> with <i>BIT61-3FLAG</i>	This study
pRS306-PKC1-3FLAG	pRS306 (integrate-type, <i>URA3</i> marker) backbone, for replacing endogenous <i>PKC1</i> with <i>PKC1-3FLAG</i>	This study
pRS306-PKC1 ^{T1125A} -3FLAG	pRS306 (integrate-type, <i>URA3</i> marker) backbone, for replacing endogenous <i>PKC1</i> with <i>PKC1</i> ^{T1125A} -3FLAG	This study
pRS306-PKC1 ^{S1143A} -3FLAG	pRS306 (integrate-type, <i>URA3</i> marker) backbone, for replacing endogenous <i>PKC1</i> with <i>PKC1</i> ^{S1143A} -3FLAG	This study
YCp50-PKC1 ^{R398P}	YCp50 (<i>CEN</i> -type, <i>URA3</i> marker) harboring <i>PKC1</i> ^{R398P}	23
YCp50-PKC1	YCp50 (<i>CEN</i> -type, <i>URA3</i> marker) harboring <i>PKC1</i>	This study
YCp50-PKC1 ^{T1125A}	YCp50 (<i>CEN</i> -type, <i>URA3</i> marker) harboring <i>PKC1</i> ^{T1125A}	This study
YCp50-PKC1 ^{S1143A}	YCp50 (<i>CEN</i> -type, <i>URA3</i> marker) harboring <i>PKC1</i> ^{S1143A}	This study
YCp50-PKC1 ^{T1125A/S1143A}	YCp50 (<i>CEN</i> -type, <i>URA3</i> marker) harboring <i>PKC1</i> ^{T1125A/S1143A}	This study
pFL39-PKC1-3HA	pFL39 (<i>CEN</i> -type, <i>TRP1</i> marker) harboring <i>PKC1-3HA</i>	This study

pFL39-PKC1 ^{ΔHR1} -3HA	pFL39 (<i>CEN</i> -type, <i>TRP1</i> marker) harboring <i>PKC1</i> ^{ΔHR1} -3HA	This study
pFL39-PKC1 ^{4CS} -3HA	pFL39 (<i>CEN</i> -type, <i>TRP1</i> marker) harboring <i>PKC1</i> ^{4CS} -3HA	This study
pFL39-PKC1 ^{T1125A} -3HA	pFL39 (<i>CEN</i> -type, <i>TRP1</i> marker) harboring <i>PKC1</i> ^{T1125A} -3HA	This study
YCplac111-PKC1-3HA	YCplac111 (<i>CEN</i> -type, <i>LEU2</i> marker) harboring <i>PKC1</i> -3HA	This study
YCplac111-PKC1 ^{S1143A} -3HA	YCplac111 (<i>CEN</i> -type, <i>LEU2</i> marker) harboring <i>PKC1</i> ^{S1143A} -3HA	This study
pET-15b-YPK2	pET-15b backbone, for overexpression of <i>YPK2</i> in <i>E. coli</i>	This study
pRS314-3HA-TOR2	pRS314 (<i>CEN</i> -type, <i>TRP1</i> marker) harboring <i>3HA-TOR2</i>	61
pRS314-3HA-TOR2 ^{D2298E}	pRS314 (<i>CEN</i> -type, <i>TRP1</i> marker) harboring <i>3HA-TOR2</i> ^{D2298E}	61

Supplementary Figures

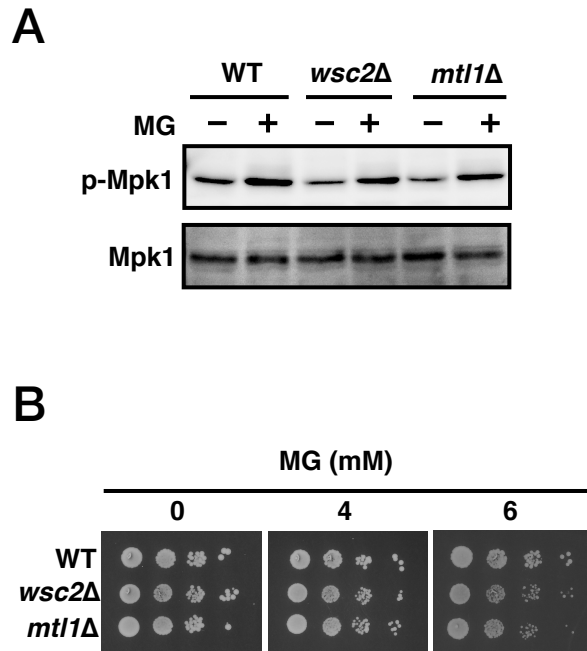


FIG S1 Role of Wsc2 and Mtl1 in the MG-induced Mpk1 MAP kinase signaling. (A) Wild-type (BY4741), *wsc2Δ*, and *mtl1Δ* cells were cultured in SD medium until $A_{610}=0.3-0.5$, treated with 10 mM MG for 30 min, and the phosphorylation of Mpk1 was then determined. (B) Wild-type (BY4741), *wsc2Δ*, and *mtl1Δ* cells were cultured in SD medium until the log phase of growth, diluted serially (1:10) with a 0.85% NaCl solution, and 4 μ l of each cell suspension was then spotted onto SD agar plates containing MG.

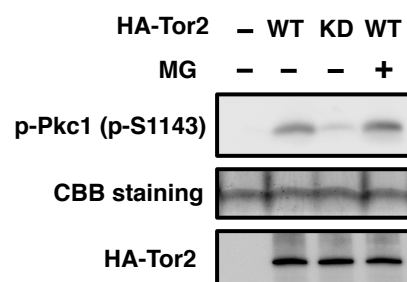


FIG S2 *In vitro* kinase assay. The Pkc1^{WT} peptide was incubated with immunopurified TORC2 from HA-Tor2 (WT) or kinase dead mutant HA-Tor2 (KD) and ATP. After Tricine-SDS-PAGE, the phosphorylation of Pkc1 peptide was detected by anti-phospho-Pkc1^{Ser1143} antibodies (pS1143). To examine whether MG directly affects TORC2 activity, 50 μ M MG was added in the reaction mixture.

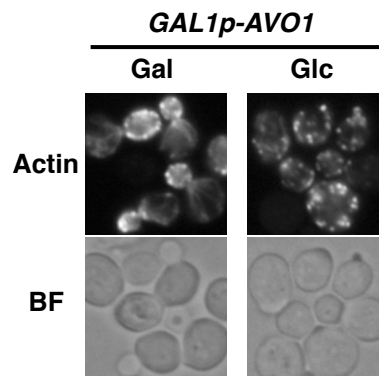


FIG S3 Impairment of TORC2 function by decreasing *AVO1* expression. Wild-type (TB50a) and *GAL1p-AVO1* (RL25-1c) cells carrying pFL39-PKC1-3HA were cultured overnight in SC/Gal medium (Gal). After being transferred to SD medium (Glc), cells were cultured for 17 h. Cells were stained for actin with rhodamine-phalloidin, and were then observed using a fluorescence microscope.

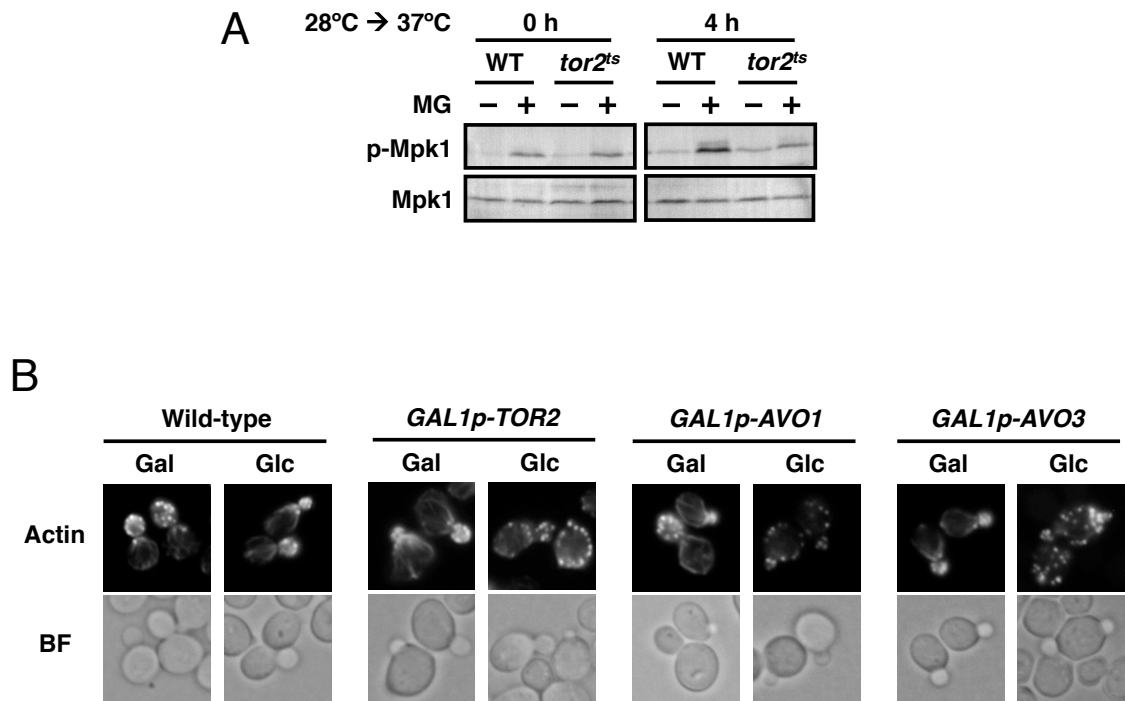


FIG S4 MG functions as an initiator of the TORC2-Pkc1 signaling pathway. (A) Wild-type (JK9-3da) and *tor2^{ts}* (SH121) cells were cultured in SD medium at 28°C until $A_{610}=0.3-0.5$, and shifted to 37°C. After an incubation for 4 h, cells were treated with 10 mM MG for 30 min, and the phosphorylation of Mpk1 was determined. (B) *GAL1p-TOR2* (JM340), *GAL1p-AVO1* (RL25-1c), and *GAL1p-AVO3* (RS61-5b) cells were cultured overnight in SC/Gal medium (Gal). After being transferred to SD medium (Glc), cells were cultured for 16 h (*GAL1p-TOR2*), 9 h (*GAL1p-AVO1*), and 10 h (*GAL1p-AVO3*), and were then stained for actin with rhodamine-phalloidin.

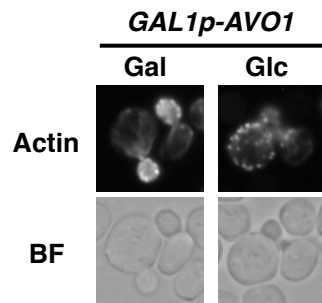


FIG S5 Impairment of TORC2 function by decreasing *AVO1* expression. Wild-type (TB50a) and *GAL1p-AVO1* (RL25-1c) cells carrying YCplac111-PKC1-3HA were cultured overnight in SC/Gal medium (Gal). After being transferred to SD medium (Glc), cells were cultured for 15 h, and were then stained for actin with rhodamine-phalloidin.

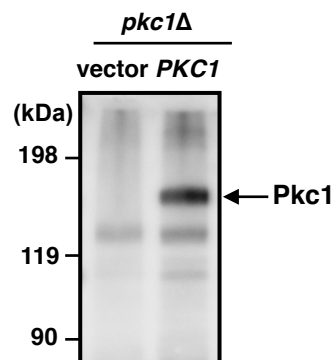


FIG S6 Verification of the specificity of anti-Pkc1 antibodies. *pkc1Δ* (DL376) cells carrying vector (YEp352) or YEp352-PKC1 (pFR22) were cultured in SD medium containing 1 M sorbitol until the log phase of growth. Cell extracts were subjected to SDS-PAGE followed by Western blotting to determine the protein levels of Pkc1 using anti-Pkc1 antibodies. Molecular weight of Pkc1 is approximately 131 kDa.

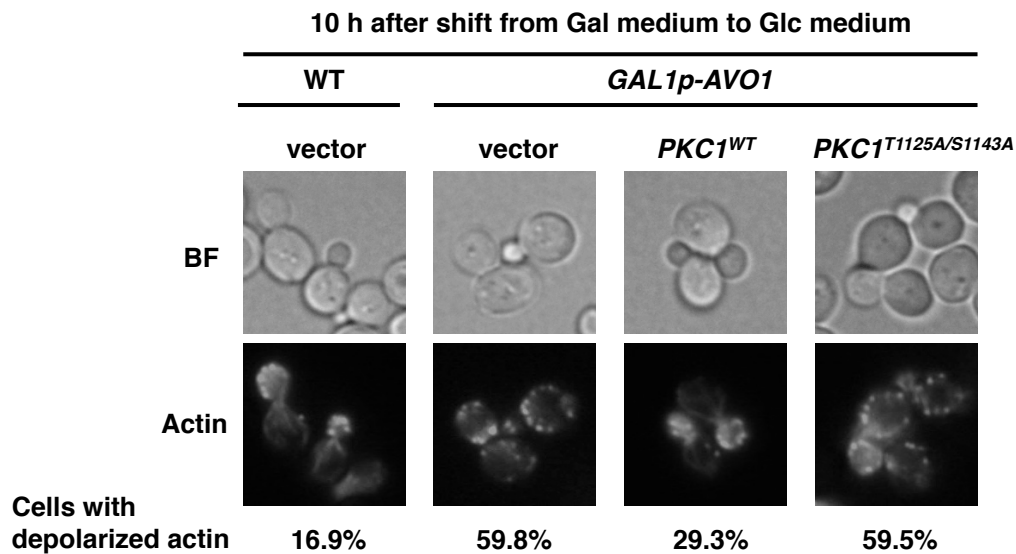


FIG S7 Physiological significance in phosphorylation states of Pkc1 at Thr¹¹²⁵ and Ser¹¹⁴³. Cells with *GAL1* promoter-driven *AVO1* (RL25-1C) carrying vector (YEpl352, 2 μ -type plasmids), *PKC1^{WT}*, or *PKC1^{T1125A/S1143A}* were cultured in overnight in SC/Gal medium (Gal). After being transferred to SD medium (Glc), cells were cultured for 10 h, and were then stained for actin with rhodamine-phalloidin. Proportion of cells with depolarized actin was determined by counting cells whose actin was not accumulated in the bud. Approximately >200 cells were counted in each experiment.