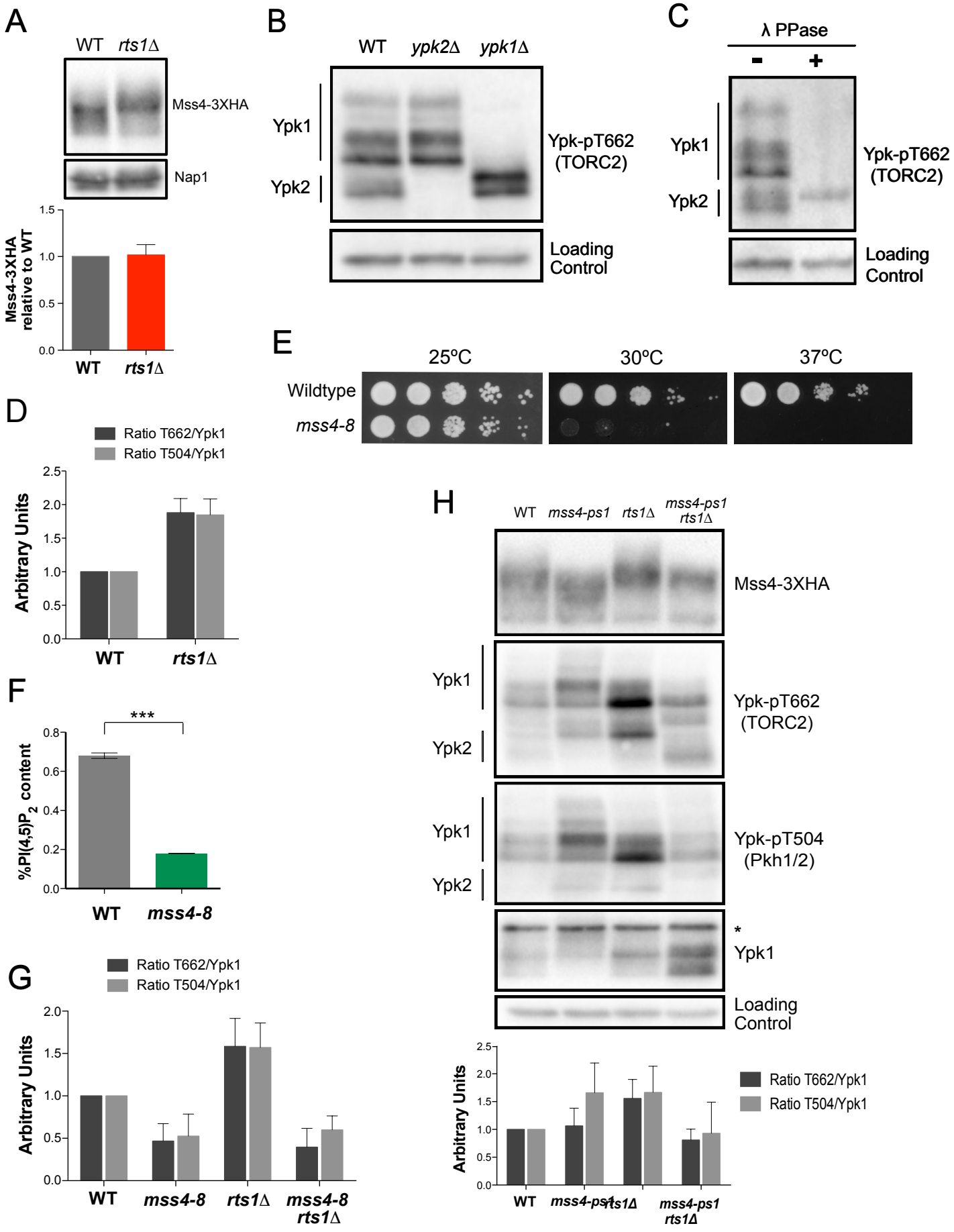

A
WT
rts1Δ
Mss4-3XHA
Nap1
Mss4-3XHA relative to WT
1.5
1.0
0.5
0.0
WT
rts1Δ
B
WT
ypk2Δ
ypk1Δ
Ypk1
Ypk2
Ypk-pT662 (TORC2)
Loading Control
C
λ PPase
-
+
Ypk1
Ypk2
Ypk-pT662 (TORC2)
Loading Control
D
Ratio T662/Ypk1
Ratio T504/Ypk1
Arbitrary Units
2.5
2.0
1.5
1.0
0.5
0.0
WT
rts1Δ
E
25ºC
30ºC
37ºC
Wildtype
mss4-8
F
***
%PI(4,5)P2 content
0.8
0.6
0.4
0.2
0.0
WT
mss4-8
G
Ratio T662/Ypk1
Ratio T504/Ypk1
Arbitrary Units
2.0
1.5
1.0
0.5
0.0
WT
mss4-8
rts1Δ
mss4-8 rts1Δ
H
WT
mss4-ps1
rts1Δ
mss4-ps1 rts1Δ
Mss4-3XHA
Ypk1
Ypk2
Ypk-pT662 (TORC2)
Ypk1
Ypk2
Ypk-pT504 (Pkh1/2)
*
Ypk1
Loading Control
Arbitrary Units
2.5
2.0
1.5
1.0
0.5
0.0
WT
mss4-ps1
rts1Δ
mss4-ps1 rts1Δ
Ratio T662/Ypk1
Ratio T504/Ypk1

Figure S1 PP2A^{Rts1} controls the TORC2 signaling network via Mss4, a PI(4)P kinase. Related to Figure 1 and Table S1.

(A) Quantification of Mss4-3XHA levels in WT and *rts1*Δ cells. Mss4-3XHA was quantified relative to a loading control. **(B)** Characterization of a phosphospecific antibody that recognizes TORC2-dependent phosphorylation of Ypk1 and Ypk2. Extracts from wildtype, *ypk1*Δ and *ypk2*Δ cells were analyzed by western blot to detect a TORC2-dependent phosphorylation site on Ypk1 and Ypk2. The antibody detects phosphorylated T662 in Ypk1 and a related site in Ypk2. **(C)** Extracts from wildtype cells were treated with lambda-phosphatase and Ypk-pT662 phosphorylation was assayed by western blot. **(D)** Quantification of TORC2- and Pkh1/2-dependent phosphorylation of Ypk1/2 in wild type and *rts1*Δ cells. All phosphorylation forms of Ypk1/2 were included in the quantification. Quantification represents the average of 3 biological replicates. Error bars represent the standard deviation of the mean. **(E)** Characterization of a new temperature-sensitive allele of *MSS4.* A series of 10-fold dilutions of wild type and *mss4-8* cells were grown at 25°C, 30ºC and 37ºC on YPD. **(F)** Bar plots showing levels of PI(4,5)P_2 in wildtype and *mss4-8* cells grown to early log phase in YPD medium at 25°C, which is a semi-restrictive temperature. PI(4,5)P_2 was quantified as the percentage of anionic phospholipids. Error bars represent the standard deviation of the mean of two biological replicates. *** denotes p= 0.0002 in Student's t-test. **(G)** Quantification of TORC2- and Pkh1/2-dependent phosphorylation of Ypk1/2 in wild type, *mss4-8*, *rts1*Δ and *mss4-8 rts1*Δ cells. All phosphorylation forms of Ypk1/2 were included in the quantification. Quantification represents the average of 3 biological replicates. Error bars represent the standard deviation of the mean. **(H)** Cells of the indicated genotypes were grown overnight to early log phase at 22°C in YPD medium. Western blotting was used to detect Mss4-3XHA, as well as TORC2- and Pkh1/2-dependent phosphorylation of Ypk1/2. Total Ypk1 was detected using an anti-Ypk1 antibody. The asterisk indicates a non-specific band.

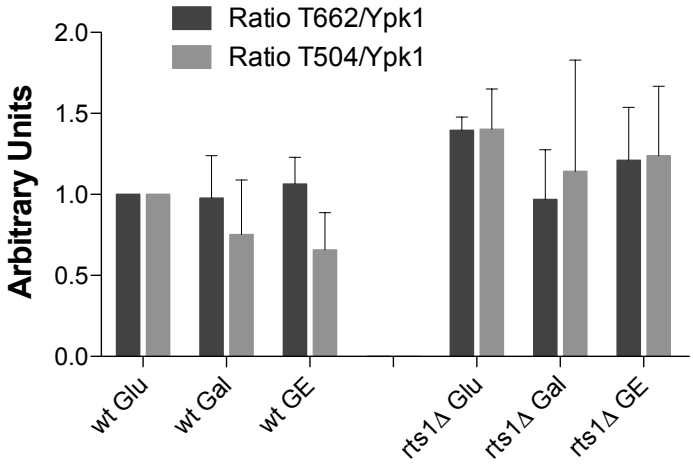

Ratio T662/Ypk1
Ratio T504/Ypk1
Arbitrary Units
2.0
1.5
1.0
0.5
0.0
wt Glu
wt Gal
wt GE
rts1Δ Glu
rts1Δ Gal
rts1Δ GE

Figure S2: The TORC2 network is modulated by nutrients. Related to Figure 2 and Table S1.

Quantification of TORC2- and Pkh1/2-dependent phosphorylation of Ypk1/2 in wild type and *rts1*Δ cells growing in different carbon sources. All phosphorylation forms of Ypk1/2 were included in the quantification. Quantification represents the average of 3 biological replicates.

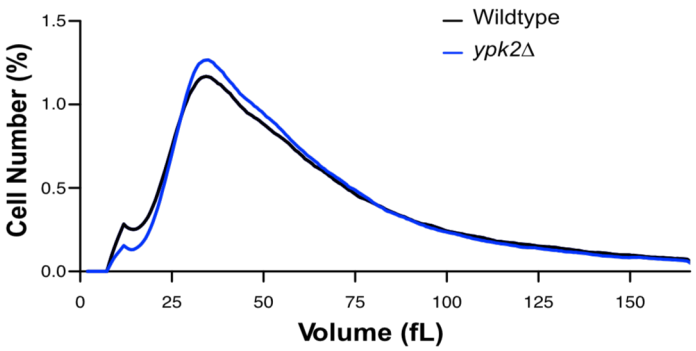
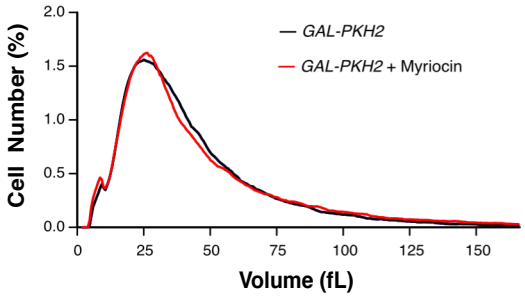
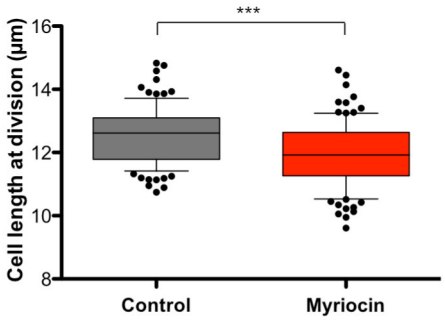

Wildtype
ypk2Δ
Cell Number (%)
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Volume (fL)

Figure S3: *ypk2*Δ has no effect on cell size. Related to Figure 3 and Table S1.
Cells of the indicated genotypes were grown to log phase at 22°C in YPD and cell size distributions were determined using a Coulter counter.

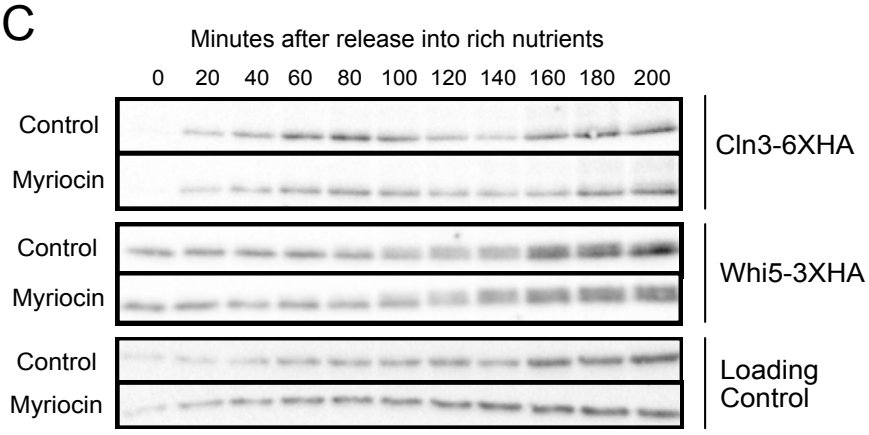
A


Cell Number (%)
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Volume (fL)
GAL-PKH2
GAL-PKH2 + Myriocin

B


***
Cell length at division (µm)
16
14
12
10
8
Control
Myriocin

C


Minutes after release into rich nutrients
0 20 40 60 80 100 120 140 160 180 200
Control
Myriocin
Cln3-6XHA
Control
Myriocin
Whi5-3XHA
Control
Myriocin
Loading Control

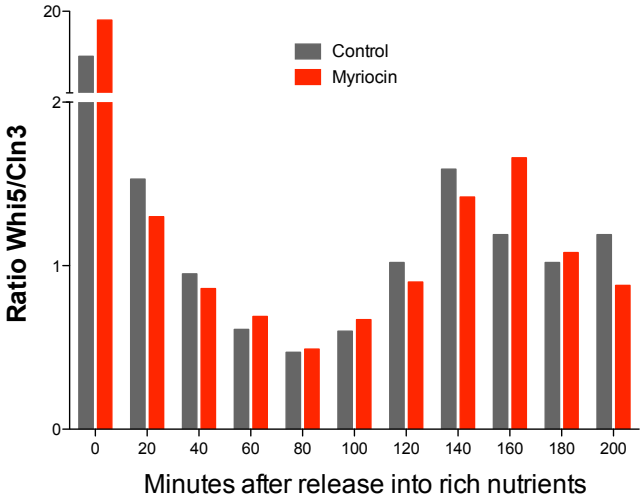
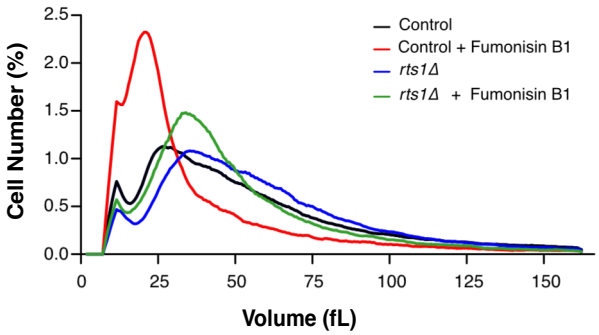

Ratio Whi5/Cln3
20
2
1
0
Control
Myriocin
0 20 40 60 80 100 120 140 160 180 200
Minutes after release into rich nutrients

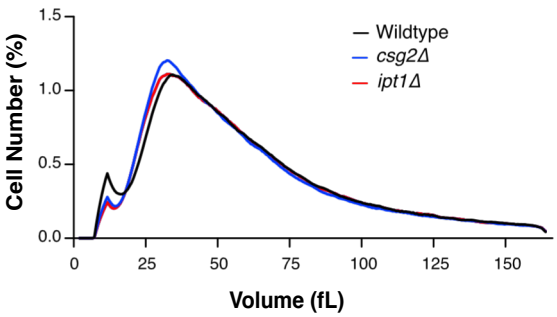
Figure S4: Ceramides are required for normal control of cell size. Related to Figure 4 and Table S1.

(A) Overexpression of PKH2 suppresses effects of myriocin on cell size. *GAL1-PKH2* cells were grown in YPGal medium at 22°C for 16 hours to early log phase with or without 0.4 µg/ml myriocin. Cell size distributions were determined using a Coulter counter. **(B)** Myriocin causes reduced cell size in fission yeast. Cell length at division of wildtype fission yeast cells grown for 16 hours at 22°C in the presence or absence of 0.3 µg/ml myriocin. Boxes are delimited by the 25-75% of the data and the central lines indicate the median. Whiskers mark maximum and minimum values within a 10-90% range of the data; individual dots represent cells outside the range. *** indicates a p-value of 0.0001 in Student's t-test **(C)** Myriocin does not cause changes in Whi5 or Cln3 protein levels during G1 phase. Cells were grown to early log phase in YPG/E medium. Small unbudded cells were isolated by centrifugal elutriation and were released into YPD medium with or without 1 µg/ml myriocin at 25°C. Samples were taken at 20 minute intervals and Cln3-6XHA and Whi5-3XHA were assayed by western blot. We consistently observed that cells carrying Cln3-6XHA and Whi5-3XHA budded prematurely at a reduced volume, which suggests that the tags cause a slight perturbation of function. The lower panel shows quantification of the ratio of the Whi5 signal over the Cln3 signal.

A


Cell Number (%)
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Volume (fL)
Control
Control + Fumonisin B1
rts1Δ
rts1Δ + Fumonisin B1

B


Cell Number (%)
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Volume (fL)
Wildtype
csg2Δ
ipt1Δ

C

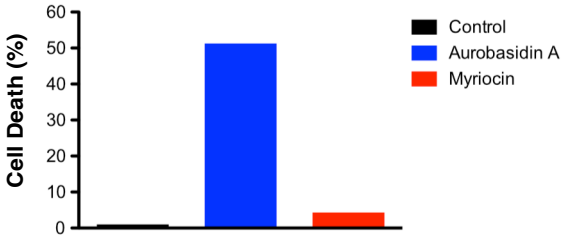

Cell Death (%)
60
50
40
30
20
10
0
Control
Aurobasidin A
Myriocin

Figure S5: Blocking synthesis of complex sphingolipids does not cause effects on cell size. Related to Figure 5 and Table S1.

(A) Cells of the indicated genotypes were grown for 16 hours in complete synthetic media with 200 μM fumonisin B1 or methanol control and cell size distributions were determined using a Coulter counter. All strains included *yor1*Δ, which increases sensitivity to the drug by reducing transporter-mediated drug efflux. **(B)** Cells of the indicated genotypes were grown to log phase in YPD at 22°C and cell size distributions were determined using a Coulter counter. Csg2 is required for production of normal levels of MIPC and Ipt1 is required for production of $M(IP)_2C$. **(B)** Wildtype cells were grown to log phase at 22ºC in YPD medium or YPD medium containing 12.5 ng/ml Aureobasidin A or 1 μg/ml myriocin. Cell viability was determined by trypan blue exclusion assay**.**

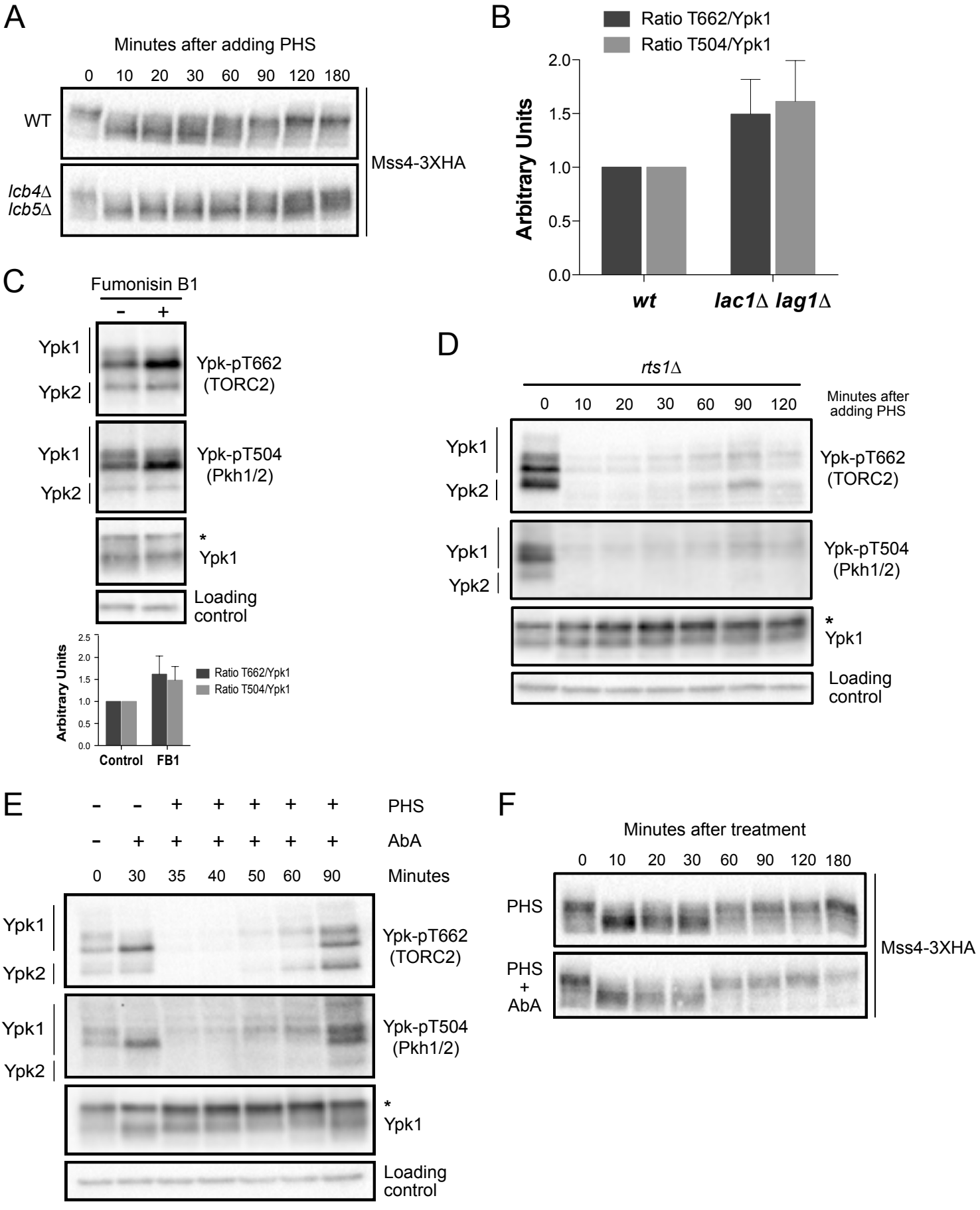
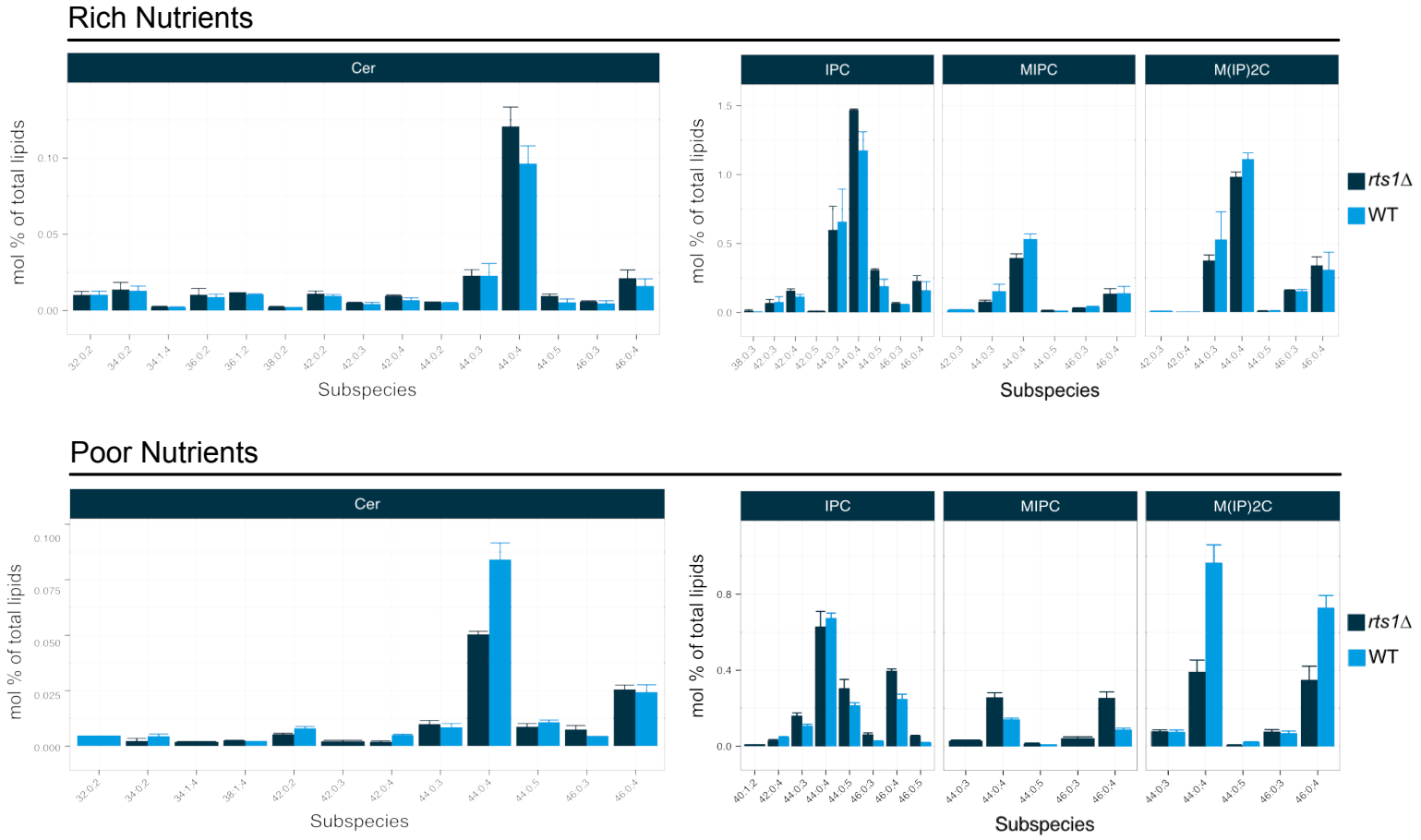

A
Minutes after adding PHS
0 10 20 30 60 90 120 180
WT
lcb4Δ
lcb5Δ
Mss4-3XHA
B
Ratio T662/Ypk1
Ratio T504/Ypk1
Arbitrary Units
2.0
1.5
1.0
0.5
0.0
wt
lac1Δ lag1Δ
C
Fumonisin B1
- +
Ypk1
Ypk2
Ypk-pT662
(TORC2)
Ypk-pT504
(Pkh1/2)
*
Ypk1
Loading
control
Arbitrary Units
Control
FB1
D
rts1Δ
0 10 20 30 60 90 120
Minutes after
adding PHS
E
PHS
AbA
0 30 35 40 50 60 90
Minutes
F
Minutes after treatment
0 10 20 30 60 90 120 180
PHS
PHS
+
AbA

Figure S6: Phytosphingosine must be converted to ceramides to influence TORC2 network signaling. Related to Figure 6 and Table S1.

(A) Wildtype and *lcb4*Δ *lcb5*Δ cells were grown to early log phase at 22ºC in YPD medium. 20 µM phytosphingosine (PHS) was added to each culture followed by incubation at 25°C. Samples were taken at the indicated times and Mss4-3XHA was detected by western blot. Lcb4/5 are required for phosphorylation of long chain bases. **(B)** Quantification of TORC2- and Pkh1/2-dependent phosphorylation of Ypk1/2 in wild type and *lac1*Δ *lag1*Δ cells. All phosphorylation forms of Ypk1/2 were included in the quantification. Quantification represents the average of 3 biological replicates. Error bars represent the standard deviation of the mean. **(C)** *yor1*Δ cells were grown to early log phase in complete synthetic media at 22°C. Samples were taken 90 minutes after addition of 200 µM of fumonisin B1 or methanol control. **(D)** *rts1*Δ cells were grown to early log phase in YPD at 22°C. Samples were taken at the indicated times after addition of 20 µM phytosphingosine (PHS) and TORC2- and Pkh1/2-dependent phosphorylation of Ypk1/2 were assayed by western blot. Total Ypk1 protein levels were assayed with an anti-Ypk1 antibody. **(E)** Wildtype cells were grown to early log phase in YPD and 0.5 µg/ml Aureobasidin A (AbA) was added to the cultures followed by 30 min incubation at 25°C to allow time for depletion of IPC. After this time, 20 µM phytosphingosine (PHS) was added. Samples were taken at the indicated times and western blotting with phosphospecific antibodies was used to detect a TORC2-dependent phosphorylation site (Ypk-pT662) and a Pkh1/2-dependent site (Ypk-pT504) on Ypk1/2. Total Ypk1 was detected using an anti-Ypk1 antibody. **(F)** Wildtype cells were grown to early log phase in YPD medium. 20 µM phytosphingosine (PHS) or 20 µM phytosphingosine and 0.5 µg/ml Aureobasidin A (AbA) were added to the cultures, followed by incubation at 25°C. Samples were taken at the indicated times and Mss4-3XHA was detected by western blot.

A


Rich Nutrients
Cer
IPC
MIPC
M(IP)2C
mol % of total lipids
Subspecies
rts1Δ
WT
Poor Nutrients
Cer
IPC
MIPC
M(IP)2C
mol % of total lipids
Subspecies
rts1Δ
WT

B

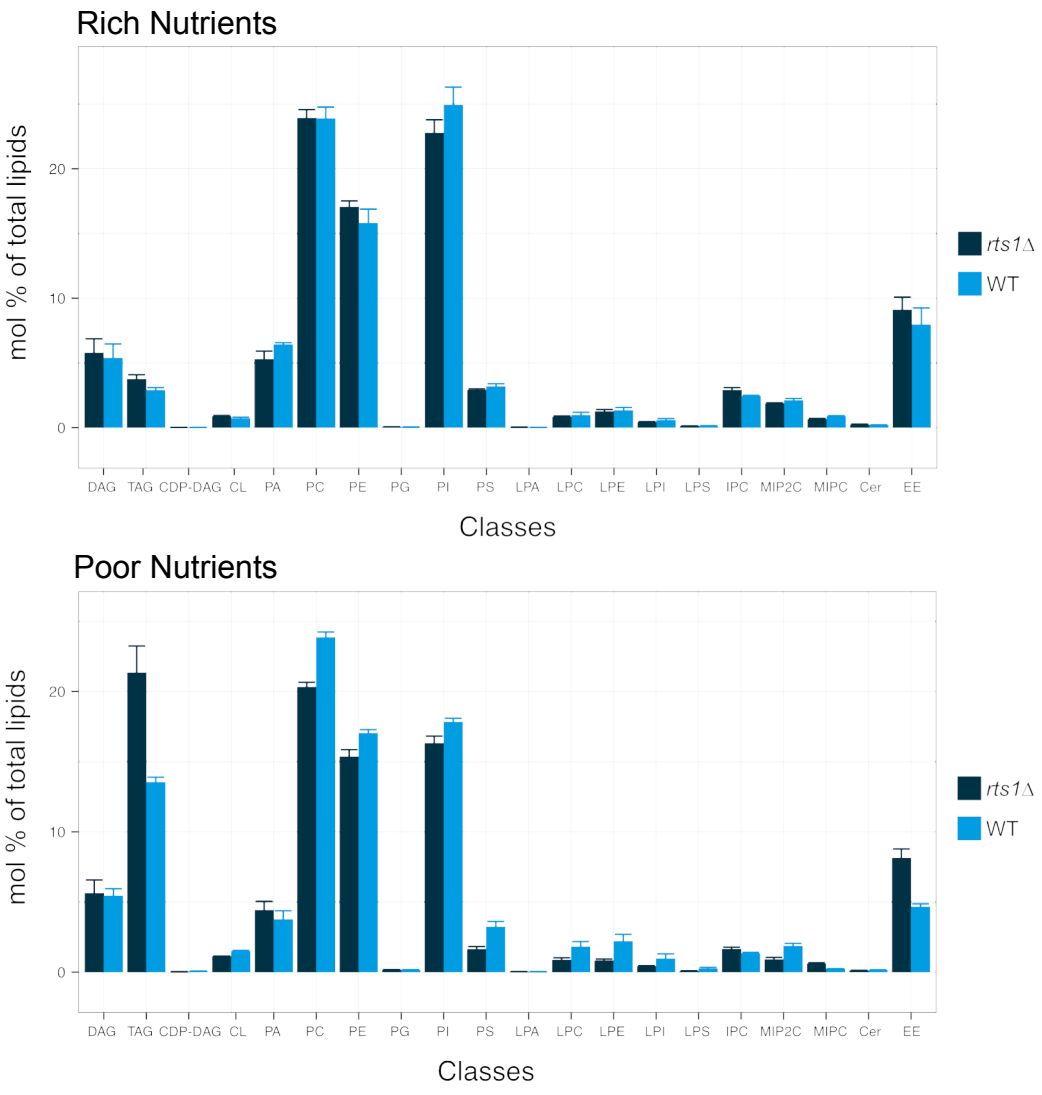

Rich Nutrients
mol % of total lipids
DAG TAG CDP-DAG CL PA PC PE PG PI PS LPA LPC LPE LPI LPS IPC MIP2C MIPC Cer EE
Classes
rts1Δ
WT
Poor Nutrients
mol % of total lipids
DAG TAG CDP-DAG CL PA PC PE PG PI PS LPA LPC LPE LPI LPS IPC MIP2C MIPC Cer EE
Classes
rts1Δ
WT

Figure S7: Levels of ceramides and complex sphingolipids are modulated by carbon source and PP2A^{Rts1}. Related to Figure 7, Table S1 and Data S1.
(A) Quantification of subspecies of ceramides, IPC, MIPC and $M(IP)_2C$ in wild type and *rts1*Δ cells growing in rich or poor carbon. **(B)** Quantification of the lipid classes in wild type and *rts1*Δ cells growing in rich or poor carbon.

Strain	MAT	Genotype	Source
DK186	a	*bar1*	[S1]
DK647	a	*bar1 rts1Δ::kanMX6*	[S2]
DK2326	a	*bar1 MSS4-3xHA::HisMX6*	This study
DK2342	a	*bar1 MSS4-3xHA::HisMX6 rts1Δ::kanMX6*	This study
DK3157	a	*bar1 MSS4-GFP::HisMX6 SPC42:mRuby2::KanMX6*	This study
DK2862	a	*ba1 MSS4-GFP::HisMX6 rts1Δ::KanMX6*	This study
DK2925	a	*bar1 mss4-8::HisMX6*	This study
DK2967	a	*bar1 mss4-8::HisMX6 rts1Δ::KanMX6*	This study
DK3116	a	*bar1 ypk1Δ::HisMX6*	This study
DK3158	a	*bar1 ypk1Δ::HisMX6 rts1Δ::KanMX6*	This study
DK1523	a	*bar1 pkh1-D398G pkh2Δ::LEU2 CLN2-3xHA::ADE2*	This study
DK1721	a	*bar1 pkh1-D398G pkh2Δ::LEU2 CLN2-3xHA::ADE2 rts1Δ::HIS*	This study
DK3092	a	*bar1 CLN3-6xHA::HIS WHI5-3xHA::hphNT1*	This study
DK3267	a	*bar1 lag1Δ::kanMX6 lac1Δ::HIS*	This study
DK3268	a	*bar1 lag1Δ::kanMX6 lac1Δ::HIS MSS4-3xHA::TRP*	This study
DK2546	a	*bar1 ypk2Δ::His3*	This study
DK2758	a	*bar1 GAL1-3xHA-PKH2::URA*	This study
DK2759	a	*bar1 GAL1-3xHA-PKH1::URA*	This study
DK1037	h-	*972*	Paul Nurse
DK3184	a	*bar1 csg2Δ::KanMX6*	This study
DK3186	a	*bar1 ipt1Δ::KanMX6*	This study
DK3216	a	*bar1 MSS4-3xHA::HisMX6 lcb4Δ::NatNT2 lcb5Δ::KanMX6*	This study
DK3108	a	*bar1 MSS4-ps1-3xHA::HisMX6*	This study
DK3127	a	*bar1 MSS4-ps1-3xHA::HisMX6 rts1Δ::kanMX6*	This study
DK3190	a	*bar1 yor1Δ::kanMX6*	This study
DK3476	a	*bar1 yor1Δ::kanMX6 rts1Δ::NatNT2*	This study

Table S1: Yeast strains used in this study. Related to Figures 1-7 and S1-7.

Supplemental References

S1. Altman, R., and Kellogg, D. (1997) Control of mitotic events by Nap1 and the Gin4 kinase. The Journal of Cell Biology. *138*, 119–130

S2. Artiles, K., Anastasia, S., McCusker, D., and Kellogg, D. R. (2009) The Rts1 regulatory subunit of protein phosphatase 2A is required for control of G1 cyclin transcription and nutrient modulation of cell size. PLoS Genet. *5*, e1000727