Cell Reports

Supplemental Information

Amino Acids Stimulate TORC1 through Lst4-Lst7, a GTPase-Activating Protein Complex for the Rag Family GTPase Gtr2

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Supplemental Figures

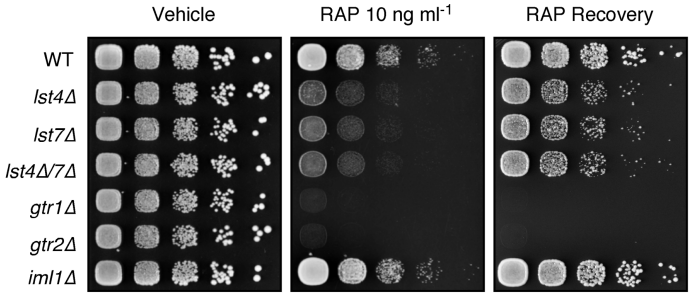

Figure S1. Loss of Lst4 and/or Lst7 Causes Rapamycin-Sensitivity and a Mild Defect in Recovery from a Rapamycin-Induced Growth Arrest, Related to Figure 1

Wild-type and isogenic mutant cells (genotypes indicated) were grown exponentially in YPD (standard rich medium with 2% glucose) and spotted as 10-fold serial dilutions on YPD plates containing no rapamycin (vehicle) or 10 ng ml^{-1} rapamycin (RAP). To assay the ability of cells to recover from a rapamycin-induced growth arrest, exponentially growing cells were treated for 6 h with rapamycin (200 ng ml^{-1}), washed twice, and then spotted as 10-fold serial dilutions on YPD plates (RAP Recovery).

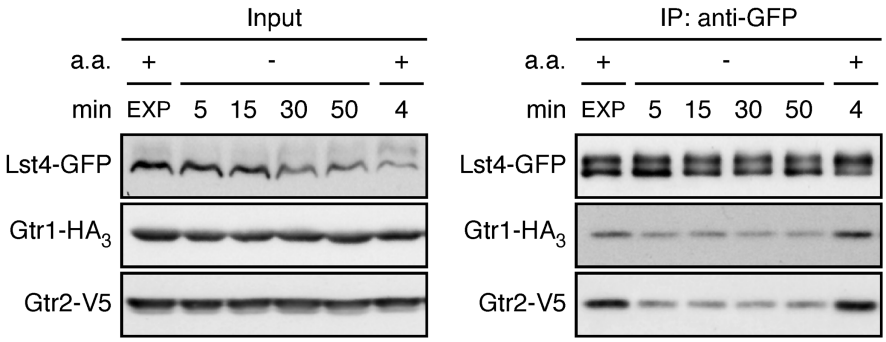

Figure S2. Short or Prolonged Amino Acid Starvation and Subsequent Refeeding Reduces and Re-Stimulates, Respectively, the Interaction Between Lst4 and Gtr1-Gtr2, Related to Figure 2

Lst4-GFP was IP-ed in extracts from cells that co-expressed Gtr1-HA_3 and Gtr2-V5. Cells were grown as in Figure 2E, but were harvested following 5, 15, 30, and 50 min of amino acid starvation (a.a.; -), and after 4 min of amino acid refeeding (a.a.; +; 4 min) to 50 min-starved cells.

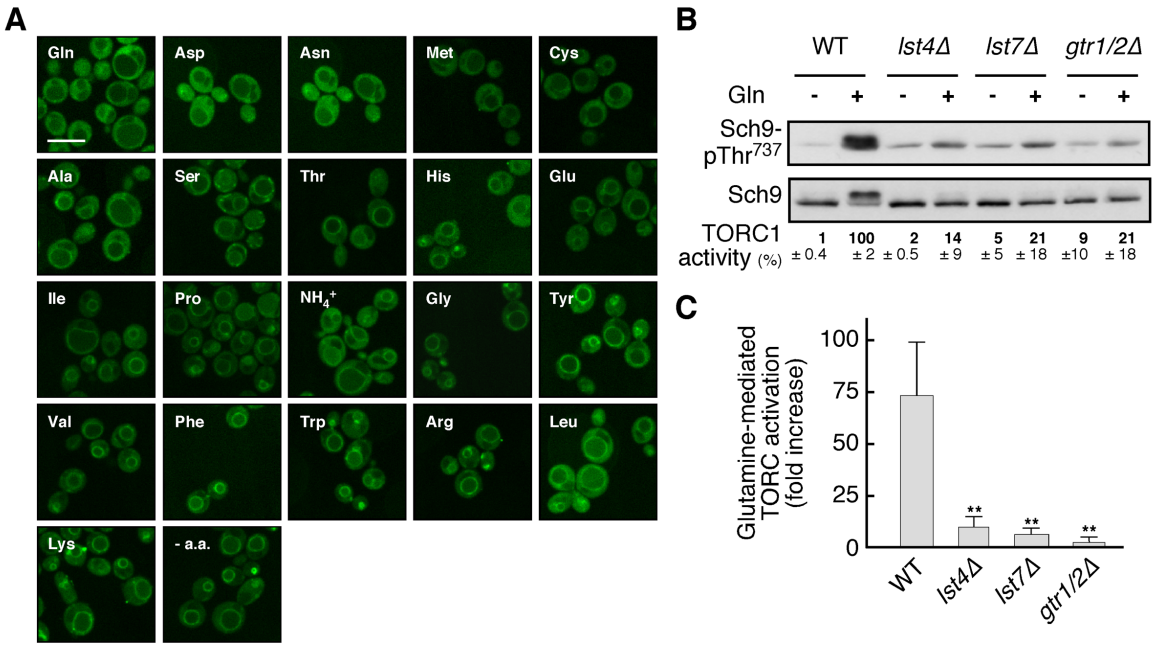

Figure S3. Amino Acids Such as Glutamine Stimulate TORC1 in an Lst4-, Lst7-, and Gtr1/2-Dependent Manner, Related to Figure 4

(A) Glutamine (Gln), aspartate (Asp), asparagine (Asn), methionine (Met), and cysteine (Cys) are highly competent in displacing Lst4-GFP from the vacuolar membrane. Lst4-GFP-expressing cells were starved for 5 min for all amino acids, re-stimulated for 12 min with individual amino acids (3 mM) or NH_4^+ (37.7 mM), and then analyzed for Lst4-GFP localization. One representative image is shown for each condition and the quantification data are presented in Figure 4D. The scale bar (white; top left panel) represents 5 µm and applies to all panels.

(B and C) Glutamine stimulates TORC1 activity in amino acid-starved cells in an Lst4-, Lst7- and Gtr1/2-dependent manner. In (B), TORC1 activity (*i.e.* Sch9-pThr^{737}/total Sch9) was assayed in exponentially growing cells with the indicated genotypes that were deprived for 5 min of all amino acids (Gln; -) and subsequently stimulated for 12 min with 3 mM glutamine (Gln; +). TORC1 activities (means ± SD from three independent experiments) were normalized to the value of glutamine-stimulated WT cells (set to 100%). The respective glutamine (Gln) -mediated fold-increase in TORC1 activity (*i.e.* the ratio of the TORC1 values between glutamine-stimulated and amino acid-starved cells) for each strain is shown in (C). Significance was estimated by Student's t-test (**$P < 0.01$).

Supplemental Tables

Table S1. Strains Used in This Study

Strain	Genotype	Source	Figure
YL515	[BY4741/2] *MAT*α; *his3*Δ*1, leu2*Δ*0, ura3*Δ*0*	(Binda et al., 2009)	1A-D; S1
MP347-4A	[YL515] *MAT*α*; lst4*Δ*::KanMX*	This study	1A-D; S1
MP348-3C	[YL515] *MAT*α*; lst7*Δ*::KanMX*	This study	1A-D; S1
MP354-9A	[YL515] *MAT*α*; lst4*Δ*::KanMX, lst7*Δ*::KanMX*	This study	1A-C; S1
MB36-4B	[YL515] *MAT*α*; gtr1*Δ*::kanMX*	This study	1A-C; S1
MB33	[YL515] *MATa; gtr2*Δ*::kanMX*	(Binda et al., 2009)	1A-C; S1
NP04-C4	[YL515] *MAT*α*; iml1*Δ*::KanMX*	(Panchaud et al., 2013b)	1A-C; S1
MP06-8B	[YL515] *MAT*α*; gtr1*Δ*::kanMX, gtr2*Δ*::kanMX*	(Binda et al., 2009)	1C
MB27	[YL515] *MAT*α*; gtr1*Δ*::HIS3*	(Binda et al., 2009)	1D
MP359-5A	[YL515] *MAT*α*; lst4*Δ*::KanMX gtr1*Δ*::HIS3*	This study	1D
MP360-2C	[YL515] *MAT*α*; lst7*Δ*::KanMX gtr1*Δ*::HIS3*	This study	1D
MB28	[YL515] *MAT*α*; gtr2*Δ*::HIS3*	(Binda et al., 2009)	1D
MP361-7D	[YL515] *MAT*α*; lst4*Δ*::KanMX gtr2*Δ*::HIS3*	This study	1D
MP362-4A	[YL515] *MAT*α*; lst7*Δ*::KanMX gtr2*Δ*::HIS3*	This study	1D
KT1961	*MATa*; *his3, leu2, ura3-52, trp1*	(Pedruzzi et al., 2003)	S3B, C
KP09	[KT1961] *MATa; lst4*Δ*::KanMX*	This study	S3B, C
KP10	[KT1961] *MATa; lst7*Δ*::KanMX*	This study	2A; S3B, C
MP409-2A	[KT1961] *MATa; LST4-GFP::HIS3MX*	This study	2B, D; 4D, E; S3A
MP410-5B	[KT1961] *MATa; LST7-GFP::HIS3MX*	This study	2B
MP374-1C	[KT1961] *MATa; LST4-GFP::HIS3MX, lst7*Δ*::KanMX*	This study	2A, C
MP372-2D	[KT1961] *MATa*; *LST7-GFP::HIS3MX, lst4*Δ*::KanMX*	This study	2C
MP406-8A	[KT1961] *MATa; LST4-GFP::HIS3MX, gtr1*Δ*::natMX, gtr2*Δ*::natMX*	This study	2E; 4A-C, F; S2
MP405-3D	[KT1961] *MATa; LST7-GFP::HIS3MX, gtr1*Δ*::natMX, gtr2*Δ*::natMX*	This study	2F; 4F
MP268-2B	[KT1961] *MATa; gtr1*Δ*::natMX, gtr2*Δ*::natMX*	This study	S3B, C

Table S2. Plasmids Used in This Study

Plasmid	Genotype	Source	Figure
pRS413	CEN, ARS, *HIS3*	(Brachmann et al., 1998)	1A-D; 2A, E, F; S3B, C
pRS414	CEN, ARS, *TRP1*	(Brachmann et al., 1998)	2A-D; 4A, C-E; S2; S3A-C
pRS415	CEN, ARS, *LEU2*	(Brachmann et al., 1998)	1A-D; 2A-C, E, F; 4A, D-F; S3A-C
pRS416	CEN, ARS, *URA3*	(Brachmann et al., 1998)	1D; 2A-D; 4A, D, E; S3A-C
pJU1030	[pRS416] *SCH9p-SCH9^{T570A}-HA$_5$*	(Urban et al., 2007)	1A, C, D
pJU793	[pRS416] *SCH9p-GFP-SCH9*	(Urban et al., 2007)	1B
pJU1058	[pRS415] *SCH9p-SCH9^{T570A}-HA$_5$*	(Urban et al., 2007)	1D
YCplac33	CEN, ARS, *URA3*	(Gietz and Sugino, 1988)	
pMB1393	[YCplac33] *TetON-GTR1*	(Binda et al., 2009)	1D
pMB1394	[YCplac33] *TetON-GTR1^{Q65L}*	(Binda et al., 2009)	1D
pMB1395	[YCplac33] *TetON-GTR1^{S20L}*	(Binda et al., 2009)	1D
YCplac111	CEN, ARS, *LEU2*	(Gietz and Sugino, 1988)	
pPM1621	[YCplac111] *TetON-GTR2*	(Binda et al., 2009)	1D
pPM1622	[YCplac111] *TetON-GTR2^{Q66L}*	(Binda et al., 2009)	1D
pPM1623	[YCplac111] *TetON-GTR2^{S23L}*	(Binda et al., 2009)	1D
pMP2562	[pRS414] *LST7p-LST7-V5-HIS$_6$*	This study	2A
pYM2847	[YCplac111] *VAC8p-VAC8-Cherry*	This study	2D
pMPG2177	[pRS414] *GTR2p-GTR2-V5-HIS$_6$*	This study	2E, F; 4F
pNP2055	[YCplac111] *ADH1p-IML1- HIS$_6$-TEV-ProtA*	(Panchaud et al., 2013b)	3B
pNP2035	[pET-24d] *GST-TEV-GTR1*	(Panchaud et al., 2013b)	3B
pNP2038	[pET-24d] *GST-TEV-GTR2*	(Panchaud et al., 2013b)	3A-E
pJU1046	[pGEX-6P] *GST-TEV-GTR1^{Q65L}-HIS$_6$*	R. Loewith	3A-E
pJU1048	[pGEX-6P] *GST-TEV-GTR2^{Q66L}-HIS$_6$*	(Panchaud et al., 2013b)	3B
pMP2101	[pGEX-4T] *GST-CDC42*	(Panchaud et al., 2013b)	3B
pAS2570	[pET28b$^+$] *HIS$_6$-LST4*	This study	3A-E
pAS2571	[pET15b$^+$] *HIS$_6$-LST7*	This study	3A-E
pJU650	[pRS416] *GTR1p-GTR1*	R. Loewith	4A
pJU652	[pRS416] *GTR1p-GTR1^{S20L}*	R. Loewith	4A, B
pJU653	[pRS416] *GTR1p-GTR1^{Q65L}*	R. Loewith	4A
pMP2337	[pRS416] *GTR1p-GTR1-HA$_3$*	This study	2E, F; 4A, C, F; S2
pMP2338	[pRS416] *GTR1p-GTR1^{S20L}-HA$_3$*	This study	4C
pMP2339	[pRS416] *GTR1p-GTR1^{Q65L}-HA$_3$*	This study	4C
pJU661	[pRS415] *GTR2p-GTR2*	R. Loewith	4A
pJU658	[pRS415] *GTR2p-GTR2^{S23L}*	R. Loewith	4A
pJU659	[pRS415] *GTR2p-GTR2^{Q66L}*	R. Loewith	4A
pMP2136	[pRS415] *GTR2p-GTR2-V5-HIS$_6$*	This study	4C, S2
pMP2777	[pRS415] *GTR2p-GTR2^{S23L}-V5-HIS$_6$*	This study	4C
pMP2778	[pRS415] *GTR2p-GTR2^{Q66L}-V5-HIS$_6$*	This study	4C
pMP2782	[pRS414] *GTR2p-GTR2^{Q66L}-V5-HIS$_6$*	This study	4B
pPL132	CEN, ARS, *LEU2*, *HA$_3$-TOR1*	(Reinke et al., 2006)	4B
pPL155	CEN, ARS, *LEU2*, *HA$_3$-TOR1^{A1957V}*	(Reinke et al., 2006)	4B
pPL156	CEN, ARS, *LEU2*, *HA$_3$-TOR1^{I1954V}*	(Reinke et al., 2006)	4B

Supplemental Experimental Procedures

Coimmunoprecipitation

Yeast cells expressing the indicated fusion proteins were harvested by filtration. Filters were immediately snap-frozen in liquid nitrogen and stored at -80°C. Cells were then resuspended in lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 0.1% NP-40, and 1x protease and phosphatase inhibitor cocktails [Roche]) and lysed with glass beads using the Precellys cell disruptor. Lysates were clarified by two successive centrifugations for 10 min at 13'000 rpm. For input samples, aliquots of cleared lysates were concentrated by precipitation with ice-cold methanol, resuspended in 6x concentrated loading buffer, and denatured for 10 min at 65°C. For coimmunoprecipitations, cleared lysates were incubated for 2 hours at 4°C with prewashed GFP-Trap® Magnetic-Agarose beads (Chromotek). After three washes with the lysis buffer, beads were resuspended in 6x concentrated loading buffer and denatured for 10 min at 65°C. Inputs and pull-down samples were analyzed by SDS-PAGE immunoblot with anti-GFP (Roche), anti-HA (HA.11; SantaCruz), and anti-V5 (Lubio).

Fluorescence microscopy and image quantification

Images were captured with an inverted Spinning Disk Confocal Microscope (VisiScope CSU-W1) equipped with an Evolve 512 (Photometrics) EM-CCD camera and a 100x 1.3 NA oil immersion Nikon CFI series objective. Quantification of the signal intensity at the vacuolar membrane was performed using the ImageJ software as follows: For each cell the median intensity value of the total GFP-signal was measured and subtracted from the respective median intensity value of the GFP-signal at the vacuolar membrane. Quantifications were performed on three independent experiments (with at least 9 cells analyzed in each experiment).

Protein purification

Iml1-His_6 was purified from *Saccharomyces cerevisiae* as previously described (Panchaud et al., 2013b). GST-Gtr2, GST-Gtr1^{Q65L}-His_6/Gtr2-His_6, Gtr1-His_6/GST-Gtr2^{Q66L}-His_6, GST-Cdc42 and His_6-Lst4/His_6-Lst7 were produced in the *Escherichia coli* Rosetta strain (Novagen) after induction with 0.5 mM IPTG during 5 hours at 18°C (GST-Gtr2, GST-Gtr1^{Q65L}-His_6/Gtr2-His_6, Gtr1-His_6/GST-Gtr2^{Q66L}-His_6), or at 37°C (GST-Cdc42) or overnight at 16°C (His_6-Lst4/His_6-Lst7). Cells were collected by centrifugation and lysed with a microfluidizer in the appropriate buffer. Purification of GST-tagged proteins was done using Glutathione-Sepharose beads (GE Healthcare) in Buffer A (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1.5 mM $MgCl_2$, 5% glycerol, 1 mM DTT, 0.1% NP40, and 0.1 mM GDP) and proteins were finally eluted with Buffer A + 10 mM reduced glutathione. His_6 purification (His_6-Lst4/His_6-Lst7) was performed using Ni-NTA agarose beads (Qiagen) in Buffer B (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 0.1% NP40, and 50 mM imidazole) and elution was achieved in Buffer B + 250 mM imidazole. Glycerol was added to a final concentration of 20% and proteins were stored at -80°C.

Supplemental References

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