Supplemental Materials

Materials and methods

Cycloheximide chase assay

Cells expressing HA-tagged proteins were grown in indicated medium to OD_{600} ~0.6. Cycloheximide (50 μg/ml final concentration) was added to start the chase. Every 15 min, a 1 ml aliquot of the cell culture was withdrawn and total cellular proteins were prepared as described (1).

Figures and figure legends

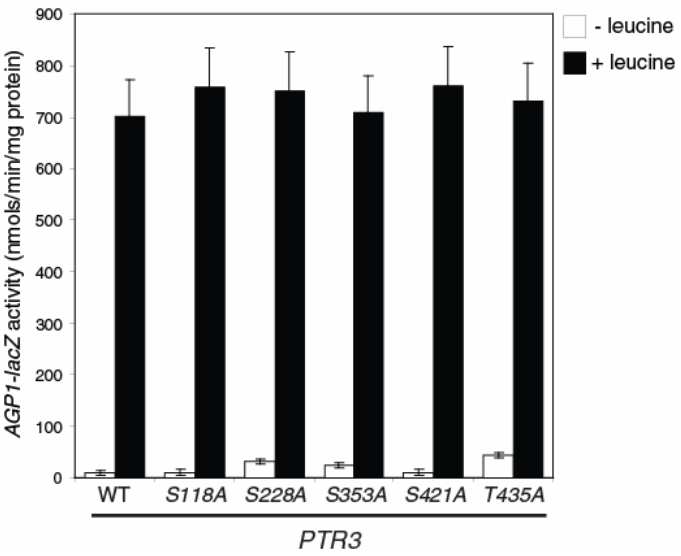

Figure S1. Mutations in five CKI consensus sites of *PTR3* do not significantly affect *AGP1-lacZ* reporter gene expression. *ptr3Δ* cells (strain ZLY1917) were transformed with plasmids carrying various *PTR3* constructs as indicated. Transformants were grown in SD medium with or without 0.02% leucine and β-galactosidase assays were conducted

as described in Materials and methods. Plasmids used: WT, pZL1949; *S118A*, pZL2157; *S228A*, pZL2147; *S353A*, pZL2265; *S421A*, pZL2261; *T435A*, pZL2269.

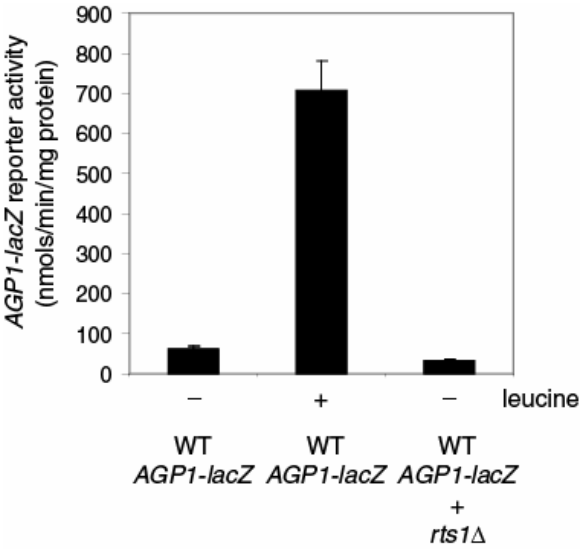

Figure S2. An *rts1Δ* mutation does not activate *AGP1-lacZ* expression in co-cultured wild type cells carrying an *AGP1-lacZ* reporter gene. Indicated cells were grown in SD medium with or without 0.02% lecuine as indicated and β-galactosidase activities were determined as described. ZLY044, WT *AGP1-lacZ*; ZLY2535, *rts1Δ*. In the co-culture experiment, equal amount of ZLY044 and ZLY2525 cells were inoculated and grew to a combined OD of 0.6-0.7. Based on growth rates of independent cultures, the ratio of the amount of ZLY044 to ZLY2535 cells in the collected co-culture for β-galactosidase assay is 1:1 when grown in SD medium without leucine.

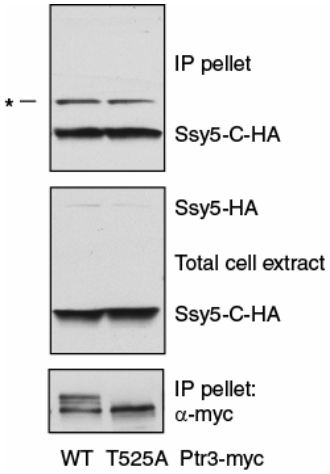

Figure S3. The threonine 525 to alanine mutation in Ptr3 does not affect its interaction with Ssy5-C. *ptr3Δ ssy5Δ* cells (RBY875) carrying centromeric plasmids expressing Ssy5-HA (pZL2340) and wild type Ptr3-myc (pZL1949) or the mutant Ptr3(T525A)-myc (pZL2122) were grown in SD medium with 0.02% leucine. Ptr3-myc and Ptr3(T525A)-myc were immunoprecipitated with anti-myc antibody and myc- and HA-tagged proteins were probed with anti-myc and anti-HA antibody, respectively. Ssy5 and Ssy5-C-HA indicate full-length and the C-terminal fragment of Ssy5. "*" indicates the heavy chain of anti-myc antibody.

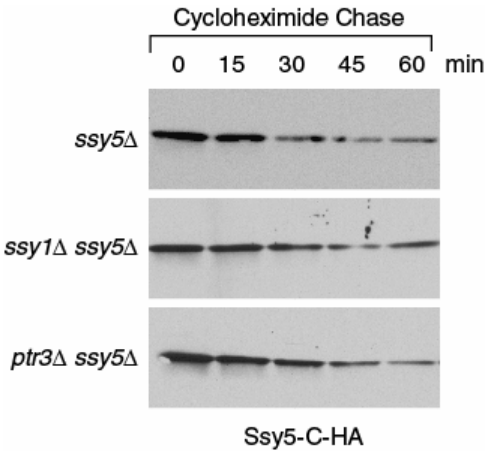

Figure S4. Mutations in *SSY1* and *PTR3* do not increase instability of Ssy5-C, the C-terminal fragment of Ssy5. *ssy5Δ* (RBY909), *ssy1Δ ssy5Δ* (RBY873), *ptr3Δ ssy5Δ* (RBY875) cells transformed with a centromeric plasmid expressing Ssy5-HA (pZL1668) were grown in SD medium with 0.02% leucine to mid-log phase. Stability of Ssy5-C-HA was examined by cycloheximide chase assay as described in Figure S1. Ssy5-C-HA was probed with anti-HA antibody.

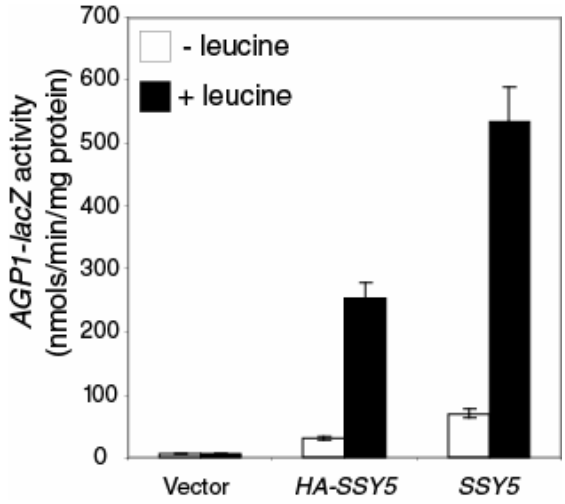

Figure S5. An N-terminal 6xHA tagged Ssy5 is not constitutively active. *ssy5Δ* cells carrying a *AGP1-lacZ* reporter gene (strain ZLY1939) were transformed with control vector pRS416, *HA-SSY5* (pZL840), or non-tagged *SSY5* plasmid (pZL736) and grown in SD medium with or without 0.02% leucine. β-galactosidase activity assay was carried out as described in Materials and methods

Tables

Strains used.

Strain	Genotype	Source/Reference
ZLY2879	*MATa ura3 PTR3-myc::kanMX4*	This study
ZLY2902	*MATa ura3 STP1-HA::kanMX4*	This study
ZLY1917	*MATa ura3-52 ptr3-Δ15 AGP1::AGP1-lacZ::kanMX4*	This study
ZLY044	*MATa ura3-52 AGP1::AGP1-lacZ::kanMX4*	This study
ZLY2535	*MATa ura3-52 rts1Δ::kanMX4*	This study
RBY875	*MATa ura3-52 lys2-Δ201 ptr3-Δ15 ssy5Δ::kanMX4*	This study
RBY909	*MATa ura3-52 lys2-Δ201 ssy5Δ::kanMX4*	This study
RBY873	*MATa ura3-52 lys2-Δ201 ssy1-Δ13 ssy5Δ::kanMX4*	This study
ZLY1939	*MATa ura3-52 ssy5Δ::kanMX4 AGP1::AGP1-lacZ::kanMX4*	This study

Plasmids used.

Plasmid	Description	Source/Reference
pZL1949	pRS416 containing *HIS6-PTR3-myc*, expressing Ptr3 with a 6xHis tag at the N-	This study

	terminal end and a 9xmyc tag inserted between residues 157 and 158 of Ptr3.	
pZL2157	pRS416 containing *HIS6-PTR3(S118A)-myc*.	This study
pZL2147	pRS416 containing *HIS6-PTR3(S228A)-myc*.	This study
pZL2265	pRS416 containing *HIS6-PTR3(S353A)-myc*.	This study
pZL2261	pRS416 containing *HIS6-PTR3(S421A)-myc*.	This study
pZL2269	pRS416 containing *HIS6-PTR3(T435A)-myc*.	This study
pZL1668	pRS416 containing *SSY5-HA*, expressing Ssy5 with a 3x HA tag at the C-terminal end.	This study
pZL2340	pRS417 (*CEN LYS2*) containing *SSY5-HA*, expressing Ssy5 with a 3x HA tag at the C-terminal end.	This study
pZL1949	pRS416 containing *HIS6-PTR3-myc*, expressing Ptr3 with a 9xmyc tag inserted between residues 157 and 158 of Ptr3 and a 6x HIS tag between residues 1 and 2.	This study
pZL2122	pRS416 containing *HIS6-PTR3(T525A)-myc9*.	This study
pZL840	pRS416 containing *HA-SSY5*, expressing Ssy5 with a 6x HA tag at the N-terminal end.	This study
pZL736	pRS416 containing *SSY5*.	This study

Supplemental References

1. **Liu, Z., M. Spirek, J. Thornton, and R. A. Butow.** 2005. A novel degron-mediated degradation of the RTG pathway regulator, Mks1p, by SCFGrr1. Mol Biol Cell **16:**4893-904.