Roelants et al., http://www.jcb.org/cgi/content/full/jcb.201410076/DC1

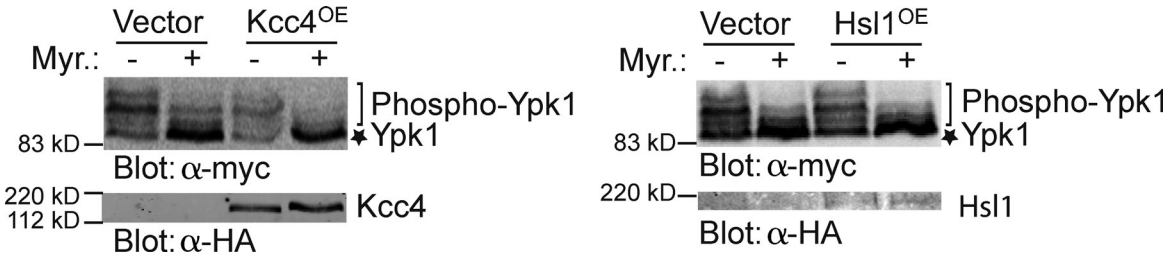
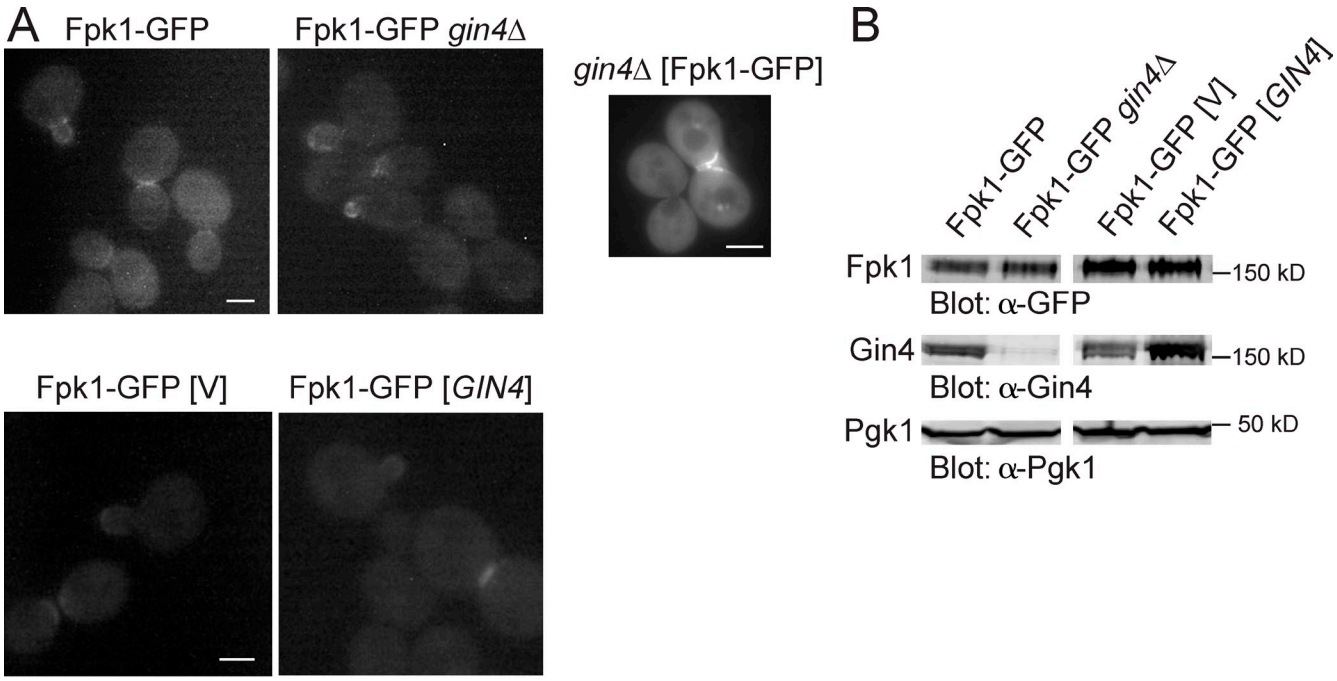


Figure S1. **Kcc4 and Hsl1 do not affect Fpk1-dependent phosphorylation of Ypk1.** Wild-type cells (Y258) carrying a plasmid expressing Ypk1-myc from the *GAL1* promoter (pAM54) and either an empty vector (BG1805) or the same vector expressing Kcc4 (pKcc4-zz; left) or Hsl1-$(HA)_3$ (pMJS109; right) from the *GAL1* promoter were lysed, and the resulting extracts were resolved by SDS-PAGE and analyzed by immunoblotting with anti–c-myc mAb 9E10 and anti-HA antibodies, as indicated.



C

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  1 MAGHHHEHEQ ERDHEQEHEH DLSQRPTTGS ERTRSISFSK LLTRSWKRNA SSSNNMSVSS
 61 VNLYSDPENS RESDHNNSGS EGQSSRFSKL KSMFQSGNSS KNASAHNSSQ SSLEGDSASS
121 SSKLRYVKPM TSVANASPAS PPLSPTIPET DVLQTPKMVH IDQHEHEREH SNCGSPIMLS
181 SSSFSPTVAR TGTGRRRSPS TPIMPSQNSN NSSSTSAIRP NNYRHHSGSQ GFSSNNPFRE
241 RAGTVRSSNP YFAYQGLPTH AMSSHDLDEG FQPYANGSGI HFLSTPTSKT NSLTNIKNLS
301 NLSLNEIKEN EEVQEFNNED FFFHDIPKDL SLKDTLNGSP SRGSSKSPTI TQTFPSIIVG
361 FDNEYEEDNN NDKHDEKEEQ QTTTDNKTRN LSPTKQNGKA THPRIKIPLR RAASEPNGLQ
421 LASATSPTSS SARKTSGSSN INDKIPGQSV PPPNSFFPQE PSPKISDFPE PR
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Figure S2. **Gin4 does not affect Fpk1 localization or level.** (A) Wild-type cells (YFR221) or isogenic *gin4Δ* cells (YFR224) expressing Fpk1-GFP (top left) from its chromosomal locus, as well as *gin4Δ* cells (YAT100) expressing Fpk1-GFP from the *TPI1* promoter on a *CEN* plasmid (pFR150; top right), and wild-type cells (YFR221) expressing Fpk1-GFP from its endogenous promoter and carrying an empty vector [V] (YCpUG) or the same vector overexpressing *GIN4* (pMVB115) from the *GAL1* promoter (bottom) were grown to mid-exponential phase and viewed with fluorescence microscopy. Bars, 2 µm. (B) The same cells as in A were lysed and the resulting extracts were resolved by SDS-PAGE and analyzed by immunoblotting with anti-GFP and anti-Gin4 antibodies. (C) Sites in the N-terminal regulatory domain of Fpk1 phosphorylated by Gin4 were determined by mass spectrometry, as described in the Materials and methods. Sequence coverage is indicated by bold blue letters. Ser and Thr residues phosphorylated in the presence of Gin4 are indicated in red. Occurrences of the apparent Gin4 consensus sequence (-R/K-x-x-S-) derived from this work and from analysis of synthetic peptide arrays (Mok et al., 2010), with Arg (purple) and Lys (green) highlighted, are boxed. Asterisks indicate the 10 Ser and 1 Thr mutated to Ala in Fpk1^{11A}.

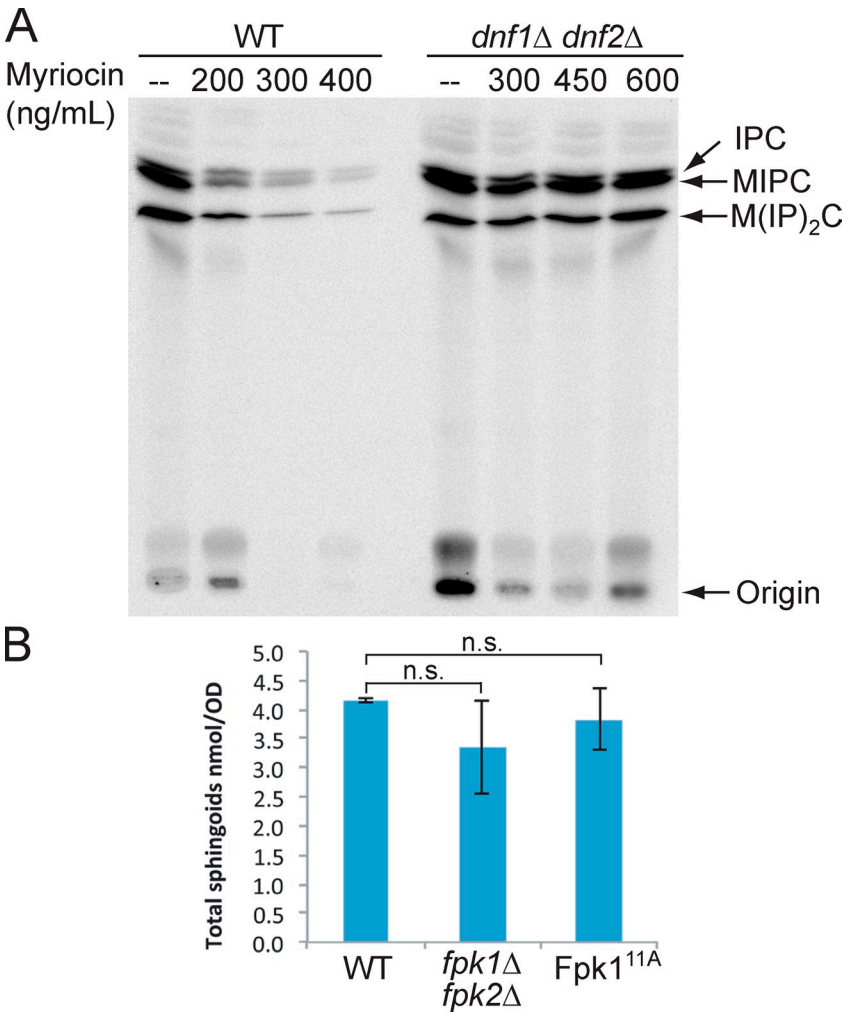

Figure S3. **PM flippase activity is required for susceptibility to Myr, but does not affect sphingolipid levels.** (A) Wild-type (BY4741, WT) and isogenic *dnf1Δ dnf2Δ* (YFR313) cells were grown at 30°C to mid-exponential phase, then treated with the indicated amount of Myr and cultivated for an additional 8 h. An equivalent number of cells from each culture (2 ml of A_{600nm} = 1.0) were then labeled with 100 µCi of [^{32}P]H_2PO_4 for 3 h. Complex (inositol-P–containing) sphingolipids were then extracted and analyzed by ascending TLC and autoradiography as described in the Materials and methods. The identity of the indicated complex sphingolipid species was assigned on the basis of either pharmacological inhibition or mutational ablation of the enzymes responsible for the production of IPC (Aur1), MIPC (Sur1-Csg2 and Csh1-Csg2), and $M(IP)_2C$ (Ipt1) in control cultures (not depicted). (B) Total sphingoid base (PHS) was generated from equivalent numbers of wild-type (BY4741, WT), isogenic *fpk1Δ fpk2Δ* (YFR205), and Fpk1^{11A} (YJW2) cells and quantified by HPLC, as described in the Materials and methods. Values represent the mean and SD (error bars) of five independent experiments. n.s., not statistically significant.

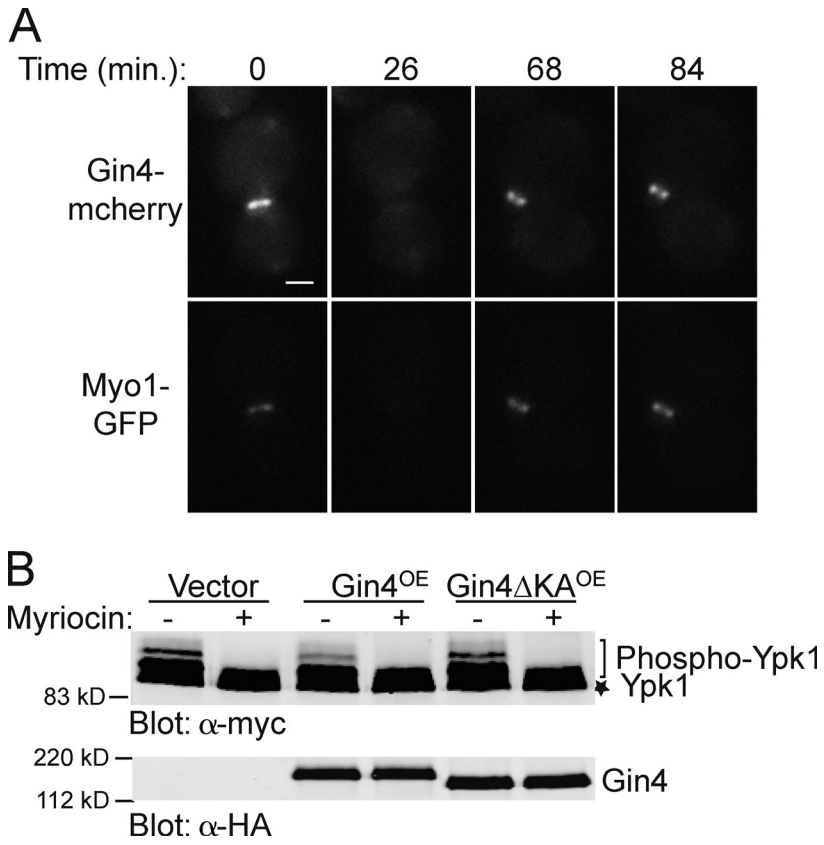

Figure S4. **KA1 domain–mediated PM targeting of Gin4 is required for its negative regulation of Fpk1 activity.** (A) Cells coexpressing Gin4-mCherry and Myo1-GFP (YFR388) were observed by time-lapse microscopy at the indicated times. Bar, 2 µm. (B) Wild-type strain (Y258) expressing Ypk1-myc from the *GAL1* promoter (pAM54) and also carrying either an empty vector (BG1805) or the same vector expressing Gin4 (pGin4-zz) or Gin4ΔKA-zz (pFR305) from the *GAL1* promoter, were grown to mid-exponential phase, not treated or treated with Myr (1.25 µM), and lysed. The resulting extracts were resolved by SDS-PAGE and analyzed by immunoblotting with anti–c-myc mAb 9E10 and anti-HA antibodies.

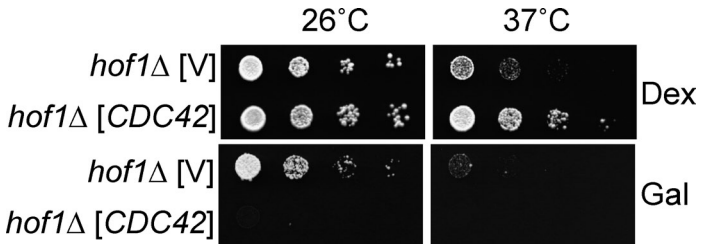

Figure S5. **Modest elevation of Cdc42 rescues *hof1Δ* cells, whereas galactose-driven overexpression is toxic.** Cultures of *hof1Δ* cells (YFR386) carrying both an empty vector (YCpLG, V), or the same vector expressing *CDC42* (PB3050) and pRS316-*HOF1*, were streaked onto plates containing 5-FOA medium to select against the presence of the *URA3*-marked pRS316-*HOF1* vector. Freshly appearing colonies were then streaked on a second set of 5-FOA plates. The resulting colonies were grown to mid-exponential phase in SCD-L and serial 10-fold dilutions were spotted on SCD-L (Dex, low/leaky expression of GAL_{prom}-*CDC42*) or SCG-L (Gal, high expression of GAL_{prom}-*CDC42*) plates. After incubation for 2 d (Dex) or 5 d (Gal) at either 26°C or 37°C, as indicated, the plates were photographed.

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

Strain	Genotype[a]	Source/reference
BY4741	*MATa his3-Δ1*[b] *leu2Δ0 met15Δ0 ura3Δ0*	Research Genetics, Inc.
YAT100	BY4741 *gin4Δ::* KanMX4	This study
YFR326	BY4741 *gin4Δ::* KanMX4 *sap190Δ::*KanMX4 *lys2Δ0*	This study
YFR313	BY4741 *dnf1Δ*::KanMX4 *dnf2Δ*::KanMX4 *lys2Δ0*	This study
YFR191	BY4741 *fpk1Δ*::KanMX4	This study
YFR205	BY4741 *fpk1Δ*::KanMX4 *fpk2Δ*::KanMX4 *lys2Δ0*	Roelants et al., 2010
YFR221	BY4741 *FPK1-GFP(S65T)::HIS3MX6*	Roelants et al., 2010
YFR224	BY4741 *FPK1-GFP(S65T)::HIS3MX6 gin4Δ:: KanMX4*	This study
YFR328	BY4741 *FPK1-GFP(S65T)::HIS3MX6 sap190Δ::KanMX4*	This study
YJW2	BY4741 *FPK1*^{11A}*::HIS3*[c]	This study
YFR320	BY4741 *sap190Δ*::KanMX4	This study
YFR323	BY4741 *FPK1*^{11A}*::HIS3 sap190Δ::KanMX4*	This study
YFR355	BY4741 *GIN4-mCherry::caURA3 lys2Δ0*	This study
YFR385	BY4741 *MYO1-mCherry::caURA3*	This study
YFR388	BY4741 *GIN4-mCherry::caURA3 MYO1-GFP(S65T)::HIS3MX6*	This study
YFR386	BY4741 *hof1Δ::KanMX4* [pRS316-*HOF1*]	This study
YFR396	BY4741 *FPK1*^{11A}*::HIS3 hof1Δ*::KanMX4 [pRS316-*HOF1*]	This study
YFR398	BY4741 *fpk1Δ*::KanMX4 *fpk2Δ::HIS3 hof1Δ*::KanMX4 [pRS316-*HOF1*]	This study
YFR425	BY4741 *fpk1Δ*::KanMX4 *fpk2Δ*::KanMX4 *cyk3Δ*::KanMX4 *hof1Δ*::KanMX4 [pRS316-*HOF1*]	This study
BY4742	*MATα his3-Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Research Genetics, Inc.
YFR307	BY4742 *FPK1*^{11A}*::HIS3*	This study
YFR308	BY4742 *FPK1*^{11A}*::HIS3 dnf1Δ::*Kan*MX4*	This study
YFR312	BY4742 *FPK1*^{11A}*::HIS3 dnf1Δ::*Kan*MX4 dnf2Δ::*Kan*MX4*	This study
JTY6581	BY4742 *fpk1Δ*::KanMX4	Research Genetics, Inc.
YFR278	BY4742 *fpk1Δ::*KanMX4 *fpk2Δ::HIS3 gin4Δ::* KanMX4 *LYS2 met15Δ0*	This study
sap190Δ	BY4742 *sap190Δ*::KanMX4	Research Genetics, Inc.
YFR387	BY4742 *hof1Δ*::KanMX4 [pRS316-*HOF1*]	This study
YFR422	BY4742 *cyk3Δ*::KanMX4 *hof1Δ*::KanMX4 [pRS316-*HOF1*]	This study
YFR424	BY4742 *fpk1Δ*::KanMX4 *fpk2Δ*::KanMX4 *hof1Δ*::KanMX4 [pRS316-*HOF1*]	This study
YFR438	BY4742 *myo1Δ::LEU2* [pRS316-*MYO1*] *LYS2 met15Δ0*	This study
YFR439	BY4742 *myo1Δ::LEU2 FPK1*^{11A}*::HIS3* [pRS316-*MYO1*] *LYS2*	This study
YFR440	BY4742 *myo1Δ::LEU2 fpk1Δ*::KanMX4 *fpk2Δ*::KanMX4 [pRS316-*MYO1*] *met15Δ0*	This study
YFR448	BY4742 *myo1Δ::leu2*::KanMX *FPK1*^{11A}*::HIS3* [pRS316-*MYO1*] [YCpLG] *MATa*	This study
YFR449	BY4742 *myo1Δ::leu2*::KanMX *FPK1*^{11A}*::HIS3* [pRS316-*MYO1*] [YCpLG-*CDC42*] *LYS2 met15Δ0*	This study
YFR451	BY4742 *myo1Δ::leu2*::KanMX *FPK1*^{11A}*::HIS3* [pRS316-*MYO1*] [pRS315]	This study
YFR452	BY4742 *myo1Δ::leu2*::KanMX *FPK1*^{11A}*::HIS3* [pRS316-*MYO1*] [p*METprom-CDC24*] *LYS2*	This study
Y258	*MATa his4-580 ura3-52 leu2-3,112 pep4-3*[d]	GE Healthcare
BY4743	*his3-Δ1/his3-Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/MET15 lys2Δ0/LYS2*	Research Genetics, Inc.
YFR420	BY4743 *myo1Δ::LEU2/MYO1*	This study
YFR412	BY4743 *myo1Δ::LEU2/MYO1 FPK1*^{11A}*::HIS3/FPK1*^{11A}*::HIS3*	This study
YFR443	BY4743 *myo1Δ::leu2*::KanMX*/MYO1 FPK1*^{11A}*::HIS3/FPK1*^{11A}*::HIS3*	This study
YFR413	BY4743 *myo1Δ::LEU2/MYO1 fpk1Δ*::KanMX4*/fpk1Δ*::KanMX4 *fpk2Δ*::KanMX4*/fpk2Δ*::KanMX4	This study
YFR421	BY4743 *inn1Δ::LEU2/INN1*	This study
YFR419	BY4743 *inn1Δ::LEU2/INN1 FPK1*^{11A}*::HIS3/FPK1*^{11A}*::HIS3*	This study
YFR414	BY4743 *inn1Δ::LEU2/INN1 fpk1Δ*::KanMX4*/fpk1Δ*::KanMX4 *fpk2Δ*::KanMX4*/fpk2Δ*::KanMX4	This study
YFR433	BY4743 *dnf1Δ*::KanMX4*/dnf1Δ*::KanMX4 *dnf2Δ*::KanMX4*/dnf2Δ*::KanMX4	This study
YFR434	BY4743 *inn1Δ::LEU2/INN1 dnf1Δ*::KanMX4*/dnf1Δ*::KanMX4 *dnf2Δ*::KanMX4*/dnf2Δ*::KanMX4	This study

[a]All null alleles indicated with a Δ symbol are complete deletions of the ORF, unless otherwise indicated.
[b]This null allele is an internal deletion of a 187-bp HindIII–HindIII fragment (nucleotides 305–492) from the *HIS3* ORF (Scherer and Davis, 1979).
[c]The *FPK*^{11A} allele carries the following substitution mutations of *FPK1* (*S35A S51A S52A S53A S200A S227A S229A S300A S414A T435A S436A*), as described in this study.
[d]The mutations carried by this strain represent: *his4-580*, a strongly polar mutation that drastically reduces all three activities (phosphoribosyl-ATP pyrophosphatase, phosphoribosyl-AMP cyclohydrolase, and histidinol dehydrogenase) encoded in the *HIS4* gene product (Fink and Styles, 1974), but whose molecular identity has not yet been characterized (Fink, G.R., personal communication); *ura3-52*, an insertion of the endogenous yeast retrotransposon Ty1 at codon 121 in the *URA3* ORF (and transcribed in the same orientation as would the *URA3* mRNA; Rose and Winston, 1984); *leu2-3,112*, containing two frameshift mutations that destroy the *LEU2* ORF, a G insertion at nucleotide 249, and a G insertion at nucleotide 792, as well as a GTC-to-GTT silent change at codon 56, a GTT-to-GCT missense change at codon 69, a GTT-to-GTC silent change at codon 299, and a GAC-to-AAC missense change at codon 300 (Hinnen et al., 1978); and *pep4-3* (Hemmings et al., 1981), a nonsense mutation converting TGG (Trp) at codon 39 of the *PEP4* ORF to TGA (stop; Woolford et al., 1993).

Table S2. **Plasmids used in this study**

Plasmid	Description	Source/reference
pGEX4T-1	GST tag, bacterial expression vector	GE Healthcare
pAB1	pGEX4T-1 *GIN4*	This study
pAT103	pGEX4T-1 *Gin4(K48A)*	This study
pFR143	pGEX4T-1 *FPK1*	Roelants et al., 2010
pFR144	pGEX4T-1 *fpk1(D621A)*	Roelants et al., 2010
pBS1	pGEX4T-1 *fpk1(1–472)*	This study
pBS2	pGEX4T-1 *fpk1(473–893) D621A*	This study
pJW2	pGEX4T-1 *fpk1(1–472) S35A S51A S52A S53A S200A S227A S229A S300A S414A T435A S436A*	This study
pRS303	*HIS3*; an integrative (YIp) vector	Sikorski and Hieter, 1989
pJW4	pRS303 *FPK1(S35A S51A S52A S53A S200A S227A S229A S300A S414A T435A S436A)*	This study
YEp352GAL	*2 µm*, *URA3*, $GAL1_{prom}$ vector	Benton et al., 1994
pAM76	YEp352*GAL YPK1-myc*	Roelants et al., 2002
YEp351GAL	*2 µm*, *LEU2*, $GAL1_{prom}$ vector	Benton et al., 1994
pAM54	YEp351*GAL YPK1-myc*	Casamayor et al., 1999
BG1805	*2 µm*, *URA3*, $GAL1_{prom}$, C-terminal tandem affinity (TAP) tag vector	GE Healthcare
pGin4-zz	BG1805 *GIN4-zz*	GE Healthcare
pFR305	BG1805 *gin4(Δ1026-1125)-zz*	This study
pKcc4-zz	BG1805 *KCC4*	GE Healthcare
YCpUG	*CEN*, *URA3*, $GAL1_{prom}$ vector	Bardwell et al., 1998
pMVB115	YCpUG *GIN4*	Versele and Thorner, 2004
YCpLG	*CEN*, *LEU2*, $GAL1_{prom}$ vector	Bardwell et al., 1998
pMJS109	YCpLG $HSL1\text{-}HA_3$	Shulewitz et al., 1999
pJT5241	YCpLG *GIN4-eGFP*[a]	G. Finnigan, Thorner laboratory
pRS415	*CEN*, *LEU2*, $GAL1_{prom}$ vector	New England Biolabs, Inc.
PB3050	pRS415 *HA-CDC42*	Atkins et al., 2013
pJT4350	pRS415 $MET15_{prom}$ *GFP(S65T)-*A_8*-CDC24*	Toenjes et al., 1999
pRS315	*CEN*, *LEU2*	Sikorski and Hieter, 1989
pRC181	pRS315-$TPI1_{prom}$ vector	R.E. Chen, Thorner laboratory
pFR150	pRC181-*FPK1-eGFP*	Roelants et al., 2010
pRS316	*CEN*, *URA3*	Sikorski and Hieter, 1989
pRS316-*HOF1*	pRS316 *HOF1*	Vallen et al., 2000
pRS316-*MYO1*	pRS316 *MYO1*	G. Finnigan, Thorner laboratory
pTS408	*CEN*, *URA3*, $GAL1_{prom}$ GFP vector	Carminati and Stearns, 1997
pES10	pTS408 *dnf1(1403-1571)-myc*	This study

[a]eGFP is GFP(F64L S65T).

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