SUPPLEMENTAL MATERIALS AND METHODS

Yeast strains and media

S. cerevisiae strains list in Table S3. Strains grown at 24ºC in YEPD (yeast extract/peptone/glucose) or SC (synthetic complete) media supplemented with the appropriate amino acids. Yeast plasmids derived from the pRS400 series of vectors.

Chromosomal integrants of Fab1p, Vac7p, Fig4p, Vac14p and Atg18p with a C-terminal tag of 3XGFP, the YFP variant, Venus or TAP were generated. The corresponding tagged versions of Fab1p, Fig4p, Vac14p and Atg18p were functional. However, Vac7p-Venus could not be detected (data not shown). Venus inserted between amino acids 84-85 of Vac7p produced a functional construct (pcVenus-Vac7).

Identification of *fab1-2* mutation site

The *FAB1* gene was cut with NdeI-XmaI. The resulting three *FAB1* fragments were cloned into linearized pRS416. Each plasmid was transformed into yeast strain *fab1-2* (EMY119) (Gary et al., 1998) to determine which fragment had the ability to undergo homologous recombination and replace the mutated region of DNA. Integration of wild-type DNA into the mutation site was inferred from the appearance of sporadic colonies at 37ºC. Homologous replacement of the mutation was also achieved with a Bsu36-MluI fragment. Isolation of this region of chromosomal DNA was achieved through homologous recombination using Bsu36-MluI gapped pRS416_*FAB1*. A single mutation of guanine to adenine at position 2591, resulted in a glycine to glutamic acid (G864E) substitution. Site directed mutagenesis of wild-type *FAB1* revealed that this single missense mutation caused phenotypes identical to the *fab1-2* strain.

Screen for the *vac14-2* mutant

VAC14 was PCR amplified from pRS416-*VAC14* using a primer from the *VAC14* 5' untranslated region, GCAGGATTACACCGTGATTTG and reverse primer (in pRS416) AGCGGGCAGTGAGCGCAACGC and standard Taq polymerase conditions (Boehringer Mannheim, Germany), to produce a 3.9 kb product, which included the full-length open reading frame of *VAC14*. PCR product was transformed into a *vac7*/Δ*vac14*Δ strain along with pRS416-*VAC14* linearized with XbaI, and transformants selected on SC-URA plates. Transformants were replica plated onto SC-URA plates supplemented with 100 μM bathophenanthroline disulfonic acid (Fluka - Switzerland). Wild-type colonies are white while *vac7*Δ cells are red and temperature sensitive for growth (Duex et al., 2006). Following incubation at 37°C for 24 hours, large white colonies were selected as potential candidates. Colonies were grown in SC-Ura liquid media, and vacuole morphology of the candidates was assessed with FM4-64. Plasmids isolated from positive candidates were confirmed by retransformation into *vac7*Δ / *vac14*Δ.

TAP tagged protein purification

S13 faction was prepared as described above. IgG Sepharose beads (GE Healthcare) were added to the S13 fraction and incubated at 4°C; 1 hour. Protein complexes bound to the beads were washed 7 times with lysis buffer containing 0.5% octyl-glucoside, followed by elution in sample buffer at 80°C, 5 min.

In vitro kinase assay

in vitro kinase assay adapted from (Okada et al., 1996). Fab1p-TAP protein from 5 OD cells bound to IgG beads, was used for one reaction. Assays performed with 10 μl beads, a liposome mixture 34.7 μl including 0.02 mg phosphatidylethanolamine (Sigma)

and 0.006 mg PI3P (Echelon), in a final volume of 65 μl of 25 mM HEPES pH 7.4, 120 mM NaCl, 1.5 mM $MgCl_2$, 5 mM 2-glycerophosphate and 1 mM DTT. Samples incubated at 30°C for the times indicated. Kinase reactions terminated by the addition of 243 μl Me-OH/$CHCl_3$ (2:1). Lipids were extracted with 58 μl 2.4 M HCl, 245 μl $CHCl_3$, and the lipid phase extracted a second time with 239 μl 1M HCl/methanol/chloroform (47:48:3). Reaction products were analyzed on K6 silica Gel 60A 20 x 20 cm glass-backed TLC plates (Whatman Inc) chromatographed with the solvent (chloroform/acetone/methanol/acetic acid/H_2O; 70:20:50:20:20). Labeled phosphoinositides were visualized by autoradiography.

^{32}P labeled lipid standards

^{32}P labeled PI3P and PI(3,5)P_2 generated as described (Rameh et al., 1995). PI and PI5P were phosphorylated using phosphoinositide 3-kinase p110γ human (Sigma). A liposome mixture, 20 μl, including 0.018 mg PE and 0.002 mg PI or 0.002 mg PI5P (Echelon) in 30 mM HEPES pH 7.0, 1 mM EGTA, ATP mixture 9 μl (0.5 mM ATP, 0.13 M $MgCl_2$, 0.04 M HEPES pH 7.0), and 1μl [^{32}P]ATP (GE Healthcare), final volume of 100 μl in 20 mM HEPES pH 7.0. After 30 min at 30°C, kinase reactions were terminated and products were analyzed by TLC.

Supplemental References

Duex, J.E., Tang, F. and Weisman, L.S. (2006) The Vac14p-Fig4p complex acts independently of Vac7p and couples PI3,5P2 synthesis and turnover. *J Cell Biol*, **172**, 693-704.

Gary, J.D., Wurmser, A.E., Bonangelino, C.J., Weisman, L.S. and Emr, S.D. (1998) Fab1p is essential for PtdIns(3)P 5-kinase activity and the maintenance of vacuolar size and membrane homeostasis. *J Cell Biol*, **143**, 65-79.

Okada, T., Hazeki, O., Ui, M. and Katada, T. (1996) Synergistic activation of PtdIns 3-kinase by tyrosine-phosphorylated peptide and beta gamma-subunits of GTP-binding proteins. *Biochem J*, **317 (Pt 2)**, 475-480.

Rameh, L.E., Chen, C.-S. and Cantley, L.C. (1995) Phosphatidylinositol (3,4,5)P_3 interacts with SH2 domains and modulates PI 3-kinase association with tyrosine-phosphorylated proteins. *Cell*, **83**, 821-830.

Table S1. Yeast mutants Vac14-L>R, *fab1-2* and *vac14-2* are defective in hyperosmotic shock induced synthesis and turnover of $PI(3,5)P_2$.

$PI(3,5)P_2$ / PI3P %				
		After hyper-osmotic shock		
	basal	10 min	20 min	30 min
WT	0.043 ± 0.006	0.49 ± 0.023	0.27 ± 0.018	0.089 ±0.016
L>R	0.026 ± 0.0009	0.064 ± 0.0053	0.064 ±0.0091	0.063 ± 0.0025

$PI(3,5)P_2$ / PI3P %				
		After hyper-osmotic shock		
	basal	10 min	20 min	30 min
WT	0.056 ± 0.0038	1.15 ± 0.086	0.32 ± 0.034	0.11 ± 0.0031
fab1-2	0.033 ± 0.0064	0.076 ± 0.0049	0.035 ± 0.0036	0.023 ± 0.0049

$PI(3,5)P_2$ / PI3P %				
		After hyper-osmotic shock		
	basal	10 min	20 min	30 min
WT	0.046 ± 0.0016	0.38 ± 0.0011	0.13 ± 0.0056	0.041 ± 0.0093
14-2	0.11 ± 0.0051	0.19 ± 0.0012	0.15 ± 0.0051	0.085 ± 0.010

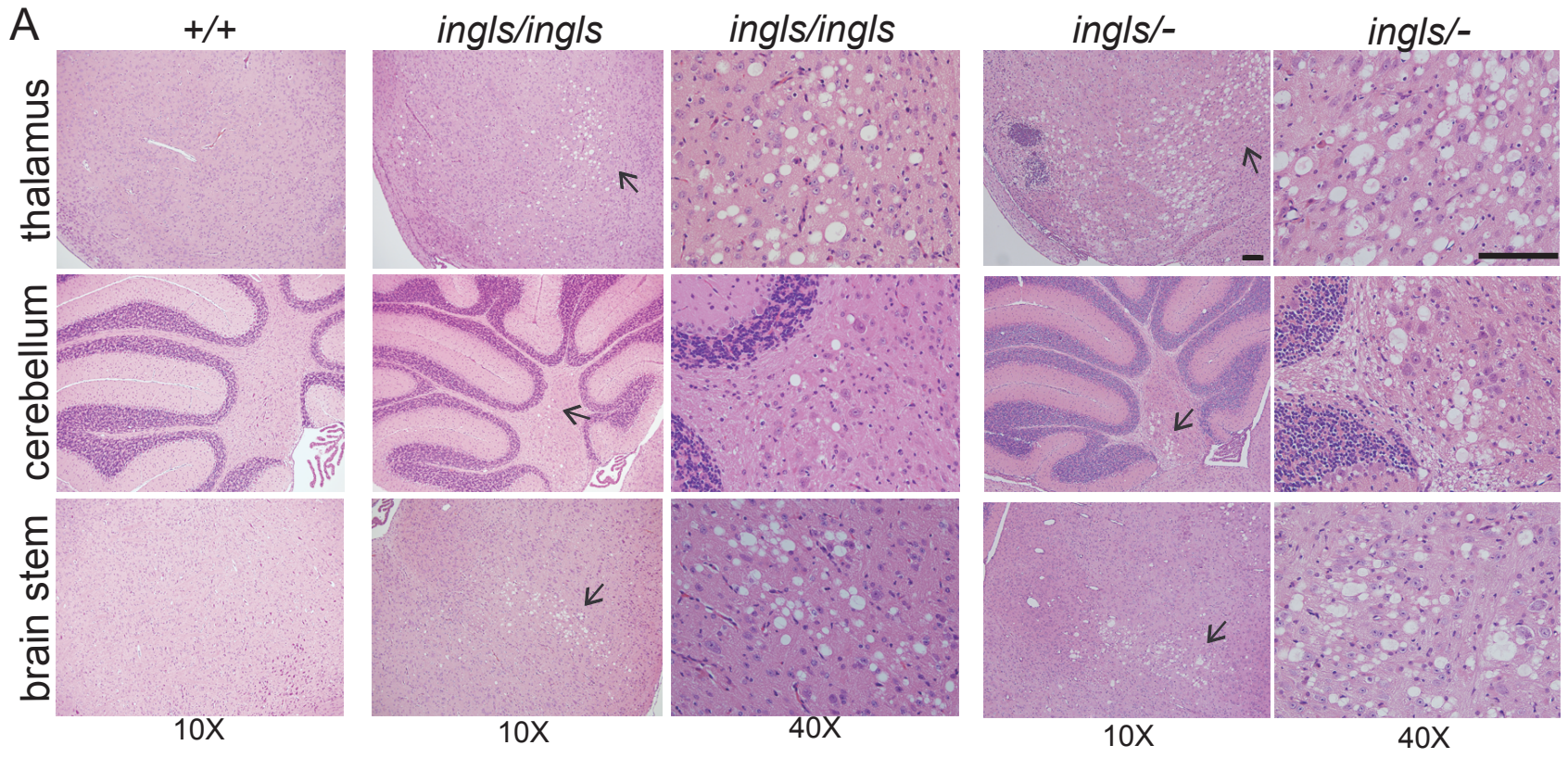
Table S2. Comparison of the *ingls* mouse with two other mutants in the PI(3,5)P_2 pathway.
n.a., not applicable because mice do not survive long enough to evaluate pigmentation.
References: ingls, this paper; *β-geo*, (Zhang et al., 2007)*; pale tremor*, (Chow et al., 2007).

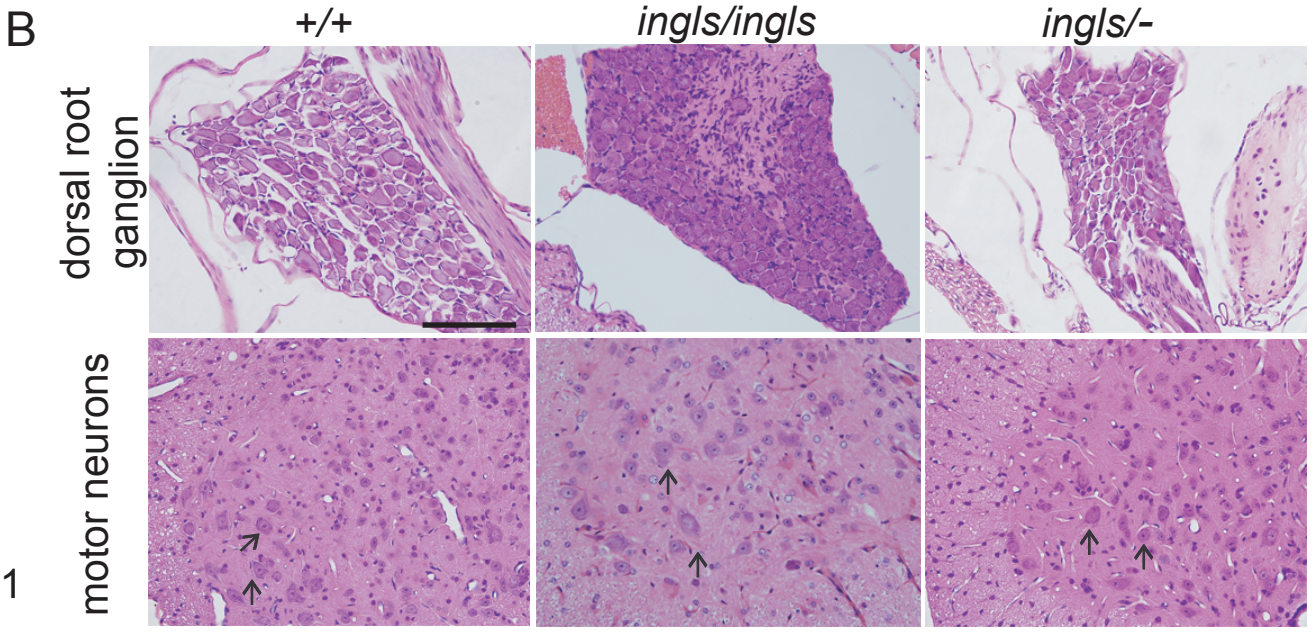
mutant	*ingls*	*β-geo*	*pale tremor*
gene	***Vac14***	***Vac14***	***Fig4***
mutation	L156R	null	null
protein level	normal	none	none
Maximal survival	P21	P1	P42
pigmentation dilution	mild	n.a.	severe
fibroblast vacuoles	yes	yes	yes
fibroblast PI(3,5)P_2	low	low	low
CNS vacuolization	moderate	moderate	severe
enlarged ventricles	severe	moderate	moderate
astrocytosis	severe	moderate	moderate
DRG neurons	normal	degenerated	degenerated
spleen	normal	normal	degenerated

Table S3. Strains used in this paper.

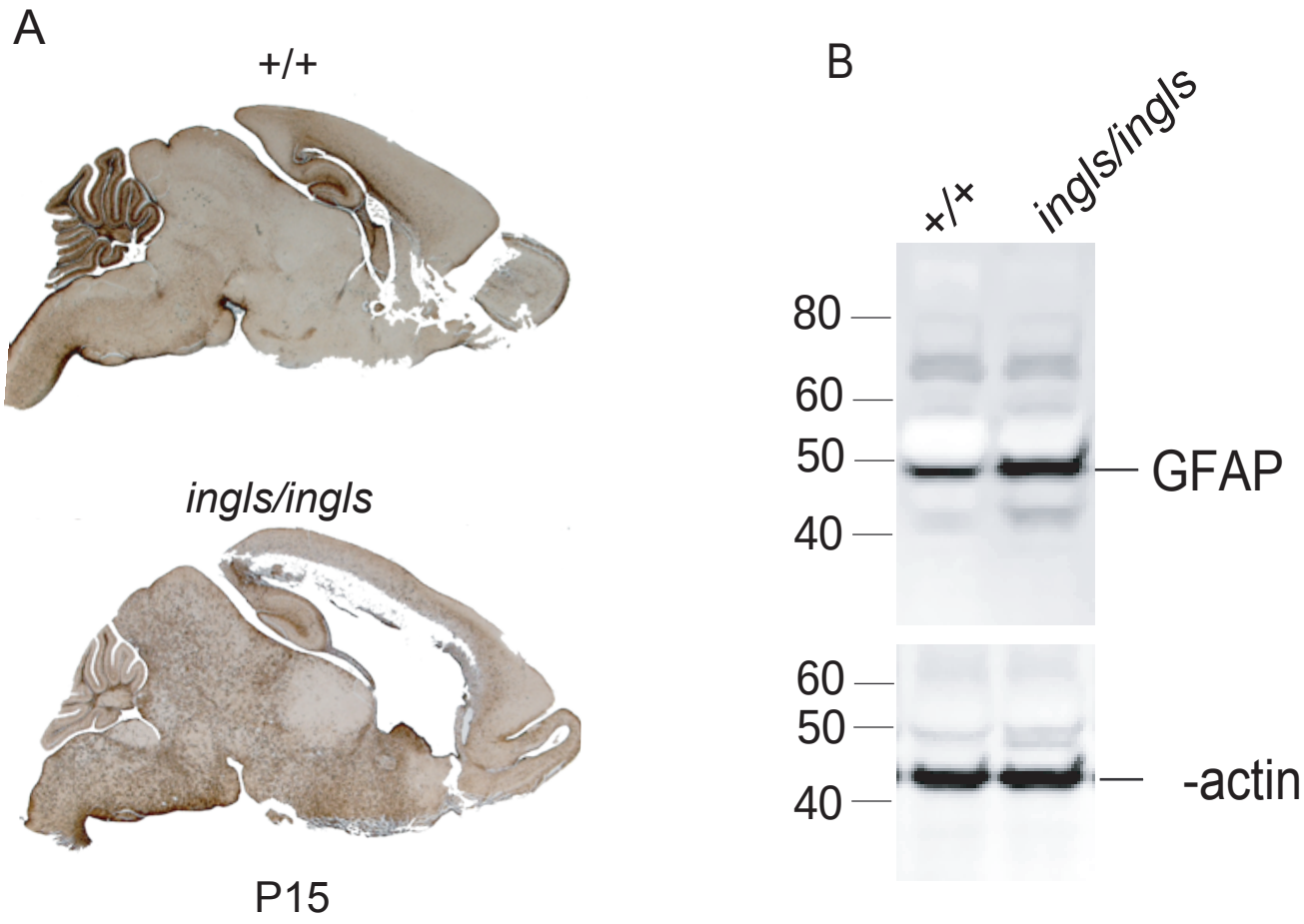
Strains	Genotype	Source
PJ69-4A	*MATa,leu2,3-112, ura3-5, his-Δ200, trp1-901, gal4Δ, gal80Δ, GAL2-ADE2,met::GAL7-lacZ*	(James et al., 1996)
LWY7235	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9*	(Bonangelino et al., 1997)
LWY2055	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, fab1Δ::LEU2*	(Bonangelino et al., 1997)
LWY5177	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9,vac14Δ::TRP1*	(Bonangelino et al., 1997)
LWY6474	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, fig4Δ::TRP1*	(Duex et al., 2006a)
LWY6538	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, fig4Δ::TRP1, vac14Δ::TRP1*	(Duex et al., 2006b)
LWY8792	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, FIG4-3xGFP::KAN*	This study
LWY8014	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, VAC14-Venus::KAN*	This study
LWY8953	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, FAB1-Tdtomato::HIS3, VAC14-Venus::KAN*	This study
LWY8251	*MATα, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, vac14Δ::TRP1, fig4Δ::TRP1, ATG18-Venus::KAN*	This study
LWY8429	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, vac14Δ::TRP1, FAB1-TAP::KAN*	This study
LWY8436	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, fig4Δ::TRP1, FAB1-TAP::KAN*	This study
LWY8789	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, vac14Δ::TRP1, FAB1-3xGFP::KAN*	This study
LWY8798	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ, vac14Δ::TRP1, FIG4-3xGFP::KAN*	This study
LWY8812	*MATα, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, fig4Δ::TRP1, FAB1-3xGFP::KAN*	This study
LWY8640	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, fig4Δ::TRP1, VAC14-Venus::KAN*	This study
LWY8818	*LWY2055, FIG4-3xGFP::KAN*	This study
LWY8257	*LWY2055, VAC14-Venus::KAN*	This study
LWY8958	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, FAB1-TAP::KAN, VAC14-Venus::KAN*	This study
LWY8964	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, fig4Δ::TRP1, FAB1-TAP::KAN, VAC14-Venus::KAN*	This study
LWY8966	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801,*	This study

	suc2-Δ9, FAB1-TAP::KAN, FIG4-Venus::KAN	
LWY8967	*MATα, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, FAB1-Tap::KAN, FIG4-Venus::KAN, vac14Δ::TRP1*	This study
LWY8274	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, VAC14-Mcherry::HIS3, FIG4-Venus::KAN*	This study
LWY8538	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, VAC14-Mcherry::HIS3, vac7Δ::KAN*	This study
LWY8279	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, VAC14-Mcherry::HIS3, ATG18-Venus::KAN*	This study
LWY8816	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, VAC14-Venus::KAN, vac7Δ::KAN*	This study
LWY8923	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, FAB1-3xGFP::KAN, vac7Δ::KAN*	This study
LWY8834	*MATα, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, FIG4-4xGFP::KAN, vac7Δ::KAN*	This study
LWY8287	*MATa, leu2,3-112, ura3-52, his-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, vac14Δ::TRP1, vac7Δ::KAN*	This study



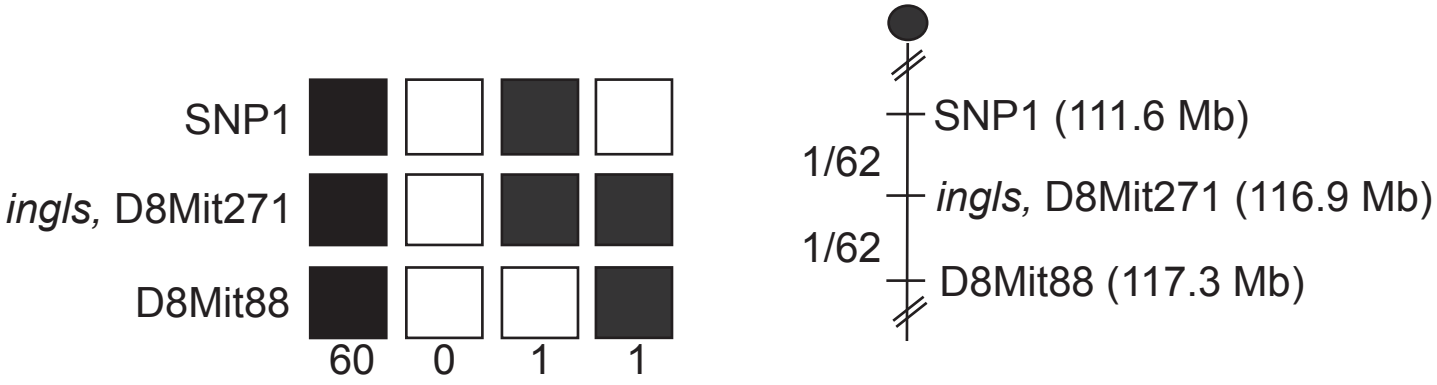


Jin et al., Supp Fig 1



Jin et al., Supp Fig 2

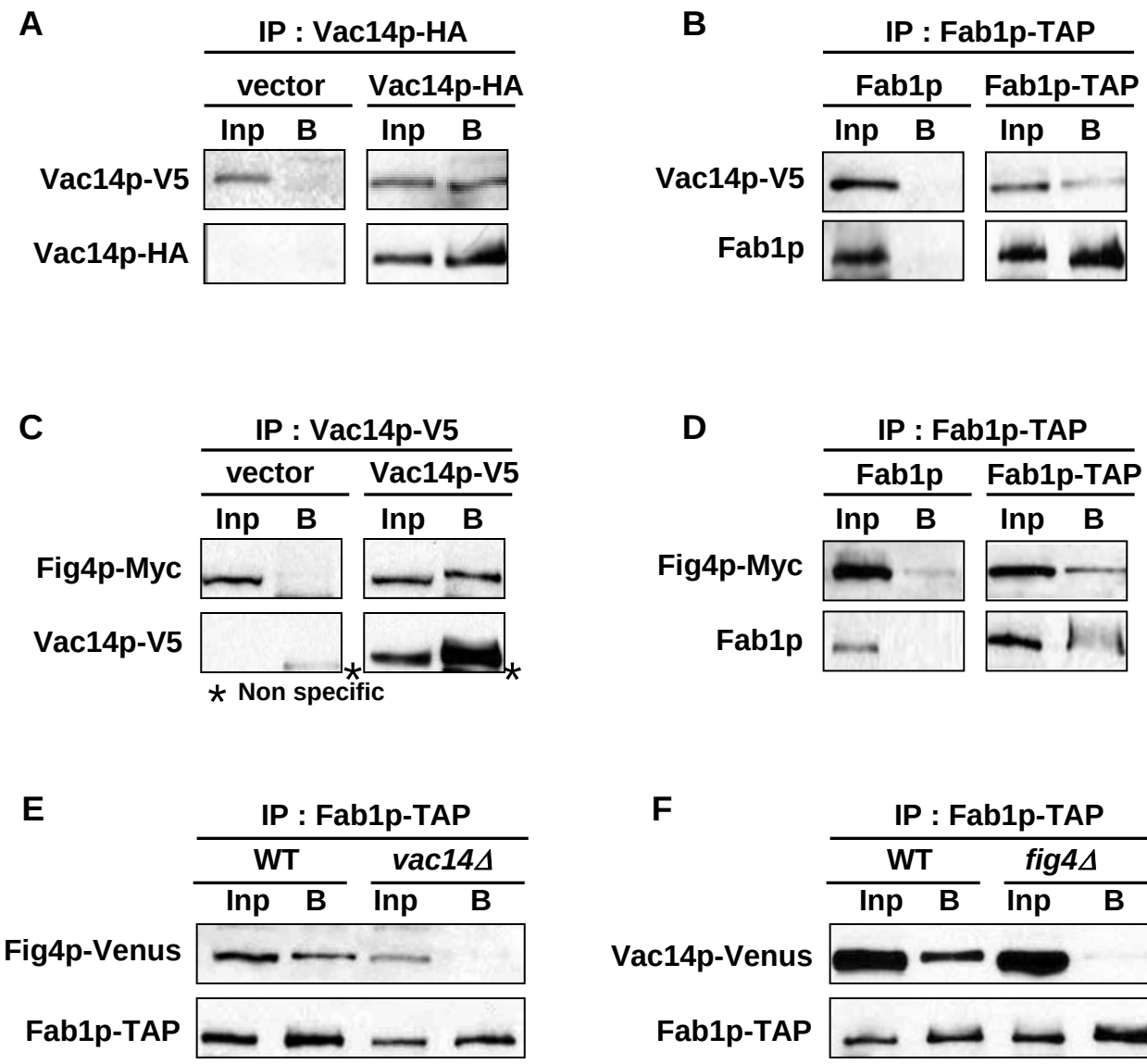
A



B

# of F2 offspring	*ingls/-*	*ingls/+*	+/-	+/+
at P0	5	7	6	6
at P14	0	7	6	6

Jin et al., Supp Fig 3



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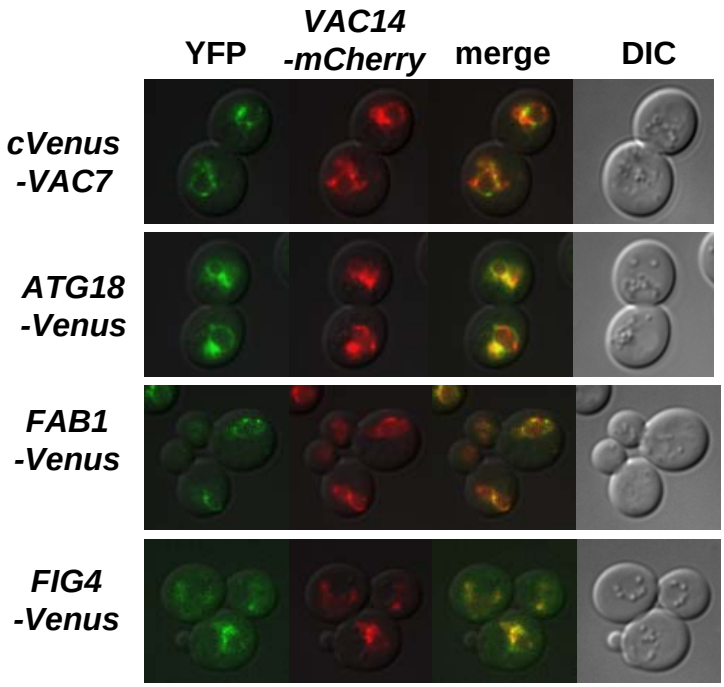
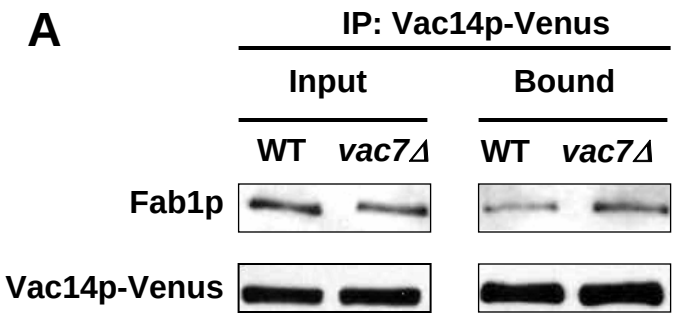
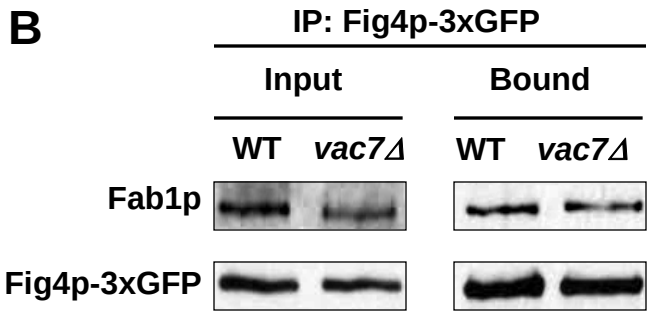
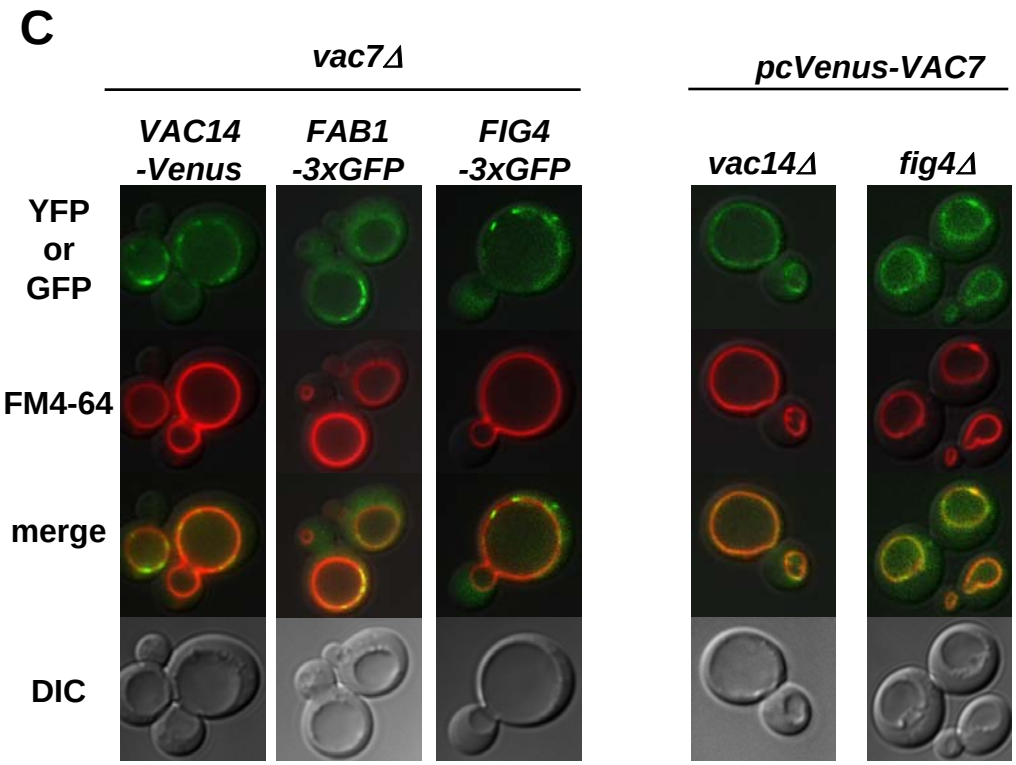


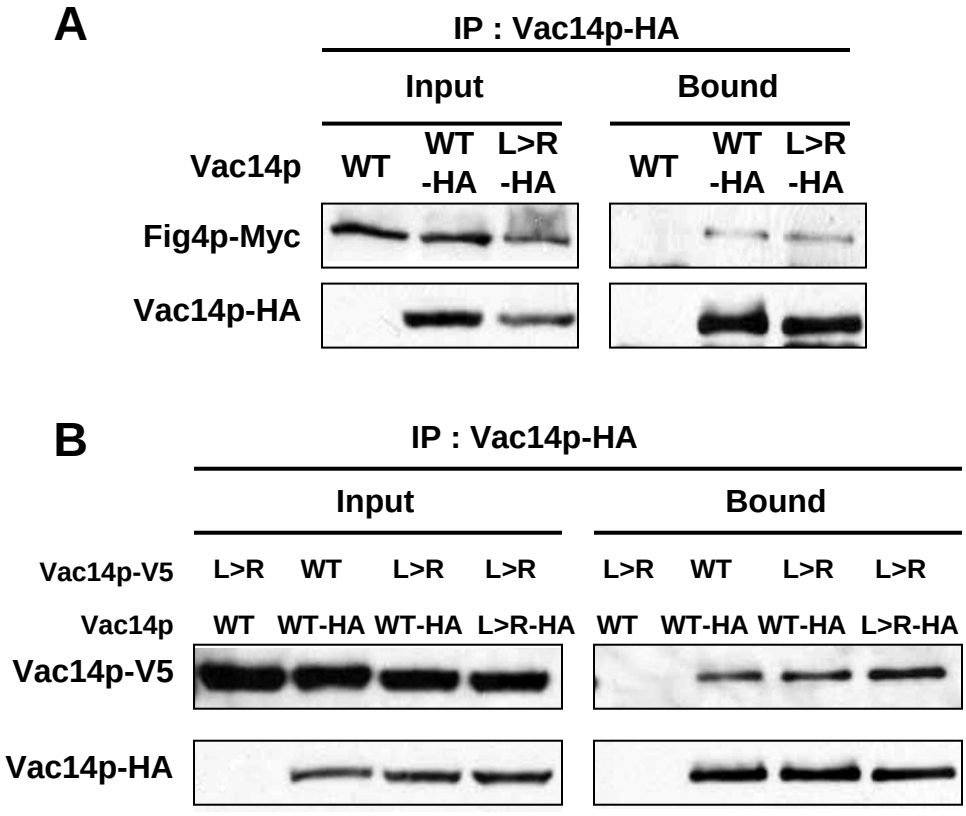
Jin et al., Supp Fig 5







Jin et al., Supp Fig 6



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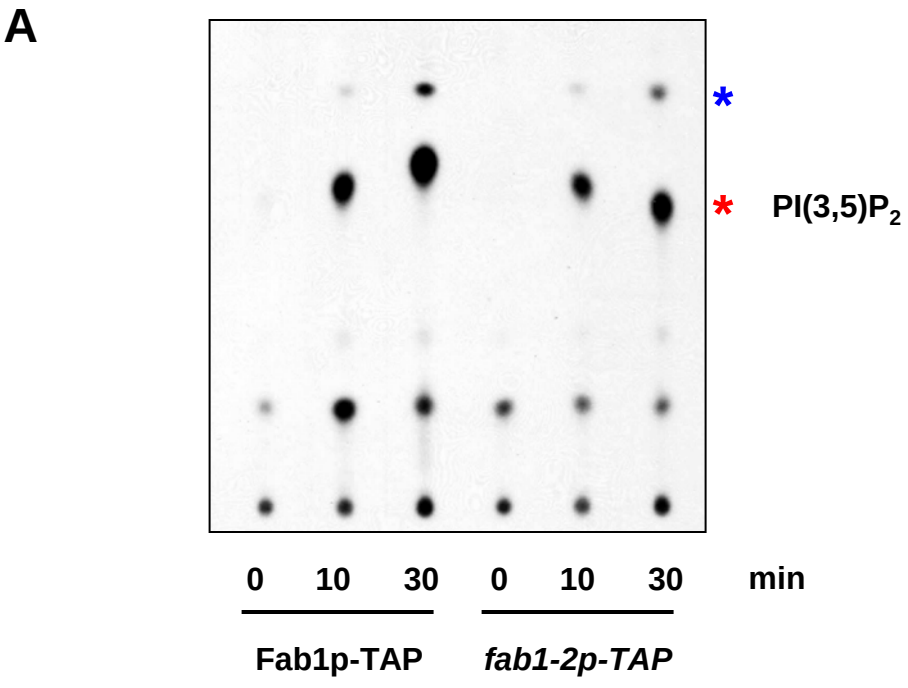

Jin et al., Supp Fig 8

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Video. Movement disorder of the ingls mouse. Shown are a compound heterozygous *Vac14*^{ingls}/*Vac14*$^{\beta\text{-}geo}$ mutant and corresponding wild-type littermate.

Supplementary Figure 1. **Morphology of the *ingls* nervous system**. A. Brain sections from wildtype (C57BL/6J), *ingls* (L156R/L156R) and compound heterozygous (L156R/-) mice. B. DRG and spinal cord sections. H&E stained.

Supplementary Figure 2. Enlarged ventricles and gliosis in *ingls* mutant mice. A. Saggital sections of control and mutant brain immunostained for GFAP, a marker of astrocytes. B. Western blot of cortical extracts stained for GFAP.

Supplementary Figure 3. Genetic mapping of *ingls* and noncomplementation of the lethality of the null allele *Vac14*$^{\beta\text{-}geo}$. A. Marker genotypes for 62 affected (DBA.ingls X B6)F2 mice localize *ingls* to a 5.7 Mb interval. B. Genotypes of 24 F2 mice from a cross between heterozygous ingls/+ mice (*Vac14*$^{L156R/+}$) and heterozygous null mice (*Vac14*$^{\beta\text{-}geo\,/+}$) mice. Compound heterzygotes were born in the predicted 25% proportion (P0) but were uniformly lethal by two weeks of age (P14).

Supplementary Figure 4.

(A) - (D) Immunoprecipitation of Fab1p and its regulators. (A) Vac14p-HA coimmunoprecipitated Vac14p-V5. *vac14Δ* cells co-expressing pRS413-*VAC14-HA* and pRS416-*VAC14-V5* were used. (B) Fab1p-TAP coprecipitated Vac14p-V5. *vac14Δ* or *vac14Δ* / *FAB1-TAP* cells expressing pRS416-*VAC14-V5* were used. (C) Vac14p-V5 coimmunoprecipitated Fig4p-Myc. *vac14Δ* / *fig4Δ* cells expressingpRS415-*FIG4-Myc* / mock or pRS415-*FIG4-Myc* / pRS413-*VAC14-V5* were used. (D) Fab1-TAP coprecipitated Fig4p-Myc. *fig4Δ* or *fig4Δ* / *FAB1-TAP* cells expressingpRS415-*FIG4-Myc* were used. (E) and (F) In the absence of Vac14p or Fig4p, Fab1p does not form a complex with Fig4p or Vac14p, respectively. Pull-down experiments (E) using *FAB1-TAP* in *FIG4-Venus /VAC14* or *FAB1-TAP* in *FIG4-Venus* /*vac14Δ* cells, (F) *FAB1-TAP* in *VAC14-Venus* / *FIG4* or *FAB1-TAP* in *VAC14-Venus* / *fig4Δ* cells.

Supplement Figure 5. Vac14p colocalizes with Fab1p, Fig4p, Vac7p and Atg18p. *VAC14-mCherry/ FAB1-Venus* cells, *VAC14-mCherry/ FIG4-Venus* cells, *VAC14-mCherry/vac7Δ* expressing *pcVenus-VAC7* cells and *VAC14-mCherry/ATG18-Venus* cells were used.

Supplement Figure 6. Vac7p is not required for the formation of the ternary complex. (A)-(B) The ternary complex forms in *vac7Δ* cells. Fig4p-3xGFP or Vac14p-Venus protein was immunoprecipitated from detergent solubilzed cell extracts using anti-GFP antibody. (A) *FIG4-3xGFP / VAC7* or *vac7Δ* cells. (B) *VAC14-Venus / VAC7* or *vac7Δ* cells. (C) Vac7p is not required for the localization of Fab1p, Fig4p and Vac14p.

Also Vac14p and Fig4p are not required for the localization of Vac7p. *VAC14-Venus*, *FIG4-3xGFP* or *FAB1-3xGFP* / *vac7Δ* cells were used. A *vac14Δ* / *vac7Δ* or *fig4Δ* / *vac7Δ* cell expressing *cVenus-VAC7* was used. Cells were labeled with FM4-64 to visualize the vacuole membrane.

Supplement Figure 7. The vac14-L149R mutant is partially functional. (A) The vac14 L149R mutant retains its ability to interact with Fig4p. pRS413-*VAC14*(WT), pRS413-*VAC14-HA* (WT-HA) or pRS413-*vac14-L149R-HA* (L>R-HA) were co-expressed with pRS415-*FIG4-Myc* in a *vac14Δ* / *fig4Δ* strain. The indicated Vac14 protein was immunoprecipitated from detergent solubilzed cell extracts using anti-HA antibody. (B) The vac14 L149R mutant retains its ability to interact with itself. pRS413-*VAC14*(WT), pRS413-*VAC14-HA*(WT-HA) or pRS413-*vac14-L149R-HA*(L>R-HA) were coexpressed with pRS416-*VAC14-V5*(WT) or pRS416-*vac14-L149R-V5*(L>R) in a *vac14Δ* strain. Immunoprecipitation of Vac14p-HA or vac14-L149R-HA protein coprecipitates Vac14p-V5 or vac14-L149R-V5 proitein using an anti-HA antibody.

Supplement Figure 8. When measured in vitro, isolated fab1-2 protein has same kinase activity as Fab1p. A *fab1Δ* strain expressing *FAB1-TAP* or *fab1-2-TAP* was used. Fab1p-TAP or fab1-2-TAP protein was bound to IgG beads, protein from 5 OD cells was used for each reaction. Liposomes contained phosphatidylethanolamine and PI3P (70:30). (A) Autoradiograph of a thin-layer chromatogram of the products of the *in vitro* kinase assay. The red asterisk indicates the migration of ^{32}P-labeled

PI(3,5)P_2standards. The blue asterisk indicates the migration of standard ^{32}P-labeled PI3P. (B) Western blot of Fab1-TAP or fab1-2-TAP pulled down for each reaction.