Supplemental Table 1 Strains used in this study

Yeast Strain	Genotype
PY5218	*MATα his3 leu2 met15 ura3 bnr1::BNR1-GFP-HIS3 myo1::MYO1-CFP::KANR*
PY6596	*MATa his3 leu2 met15 ura3 bnr1::BNR1-GFP-HIS3 myo1::MYO1-CFP::KANR elm1Δ::KANR*
PY6595	*MATa his3 leu2 met15 ura3 bnr1::BNR1-GFP-HIS3 myo1::MYO1-CFP::KANR gin4Δ::KANR*
PY6597	*MATa his3 leu2 met15 ura3 bnr1::BNR1-GFP-HIS3 myo1::MYO1-CFP::KANR nap1Δ::KANR*
PY6598	*MATa his3 leu2 met15 ura3 bnr1::BNR1-GFP-HIS3 myo1::MYO1-CFP::KANR shs1Δ::KANR*
PY6581	*MATx his3 leu2 met15 ura3 bnr1::BNR1-GFP-HIS3*
PY6582	*MATa his3 leu2 met15 ura3 bnr1::BNR1-GFP-HIS3 gin4Δ::KANR*
PY6583	*MATa his3 leu2 met15 ura3 bnr1::BNR1-GFP-HIS3 elm1Δ::KANR*
PY6823	*MATx his3 leu2 met15 ura3 bnr1::BNR1-GFP-HIS3 gin4Δ::LEU2 elm1Δ::KANR*
PY6590	*MATa his3 leu2 met15 ura3 bnr1::BNR1-GFP-HIS3 cdc10-1*
PY6591	*MATa his3 leu2 met15 ura3 bnr1::BNR1-GFP-HIS3 cdc11-1*
PY6592	*MATa his3 leu2 met15 ura3 bnr1::BNR1-GFP-HIS3 cdc12-6*
PY3295	*MATa his3 leu2 met15 ura3*
PY3505	*MATa his3 leu2 met15 ura3 bnr1Δ::KANR*
PY6617	*MATx his3 leu2 met15 ura3 bni1-1::HIS3*
PY3744	*MATa his3 leu2 met15 ura3 bnr1Δ::KANR bni1-1::HIS3*
PY6623	*MATa his3 leu2 met15 ura3 elm1Δ::KANR bni1-1::HIS3*
PY6622	*MATa his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::HIS3*
PY6619	*MATx his3 leu2 met15 ura3 elm1Δ::KANR*
PY6618	*MATx his3 leu2 met15 ura3 gin4Δ::KANR*
PY6624	*MATa his3 leu2 met15 ura3 nap1Δ::KANR bni1-1::HIS3*
PY6625	*MATa his3 leu2 met15 ura3 shs1Δ::KANR bni1-1::HIS3*
PY6621	*MATa his3 leu2 met15 ura3 nap1Δ::KANR*
PY6620	*MATx his3 leu2 met15 ura3 shs1Δ::KANR*
PY6604	*MATx his3 leu2 met15 ura3 myo2::MYO2-GFP::HIS3*
PY3505	*MATa his3 leu2 met15 ura3 bnr1Δ::KANR myo2::MYO2-GFP::HIS3*
PY6614	*MATa his3 leu2 met15 ura3 bni1-1::HIS3 myo2::MYO2-GFP::HIS3*
PY6615	*MATa his3 leu2 met15 ura3 bnr1Δ::KANR bni1-1::HIS3 myo2::MYO2-GFP::HIS3*
PY6608	*MATa his3 leu2 met15 ura3 elm1Δ::KANR bni1-1::HIS3 myo2::MYO2-GFP::HIS3*
PY6606	*MATa his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::HIS3 myo2::MYO2-GFP::HIS3*

PY6607	*MATa his3 leu2 met15 ura3 elm1Δ::KANR myo2::MYO2-GFP::HIS3*
PY6605	*MATa his3 leu2 met15 ura3 gin4Δ::KANR myo2::MYO2-GFP::HIS3*
PY6610	*MATa his3 leu2 met15 ura3 nap1Δ::KANR bni1-1::HIS3 myo2::MYO2-GFP::HIS3*
PY6612	*MATa his3 leu2 met15 ura3 shs1Δ::KANR bni1-1::HIS3 myo2::MYO2-GFP::HIS3*
PY6609	*MATa his3 leu2 met15 ura3 nap1Δ::KANR myo2::MYO2-GFP::HIS3*
PY6611	*MATa his3 leu2 met15 ura3 shs1Δ::KANR myo2::MYO2-GFP::HIS3*
PY6633	*MATx his3 leu2 met15 ura3 bni1Δ::HIS3 [BNI1::URA3]*
PY6631	*MATa his3 leu2 met15 ura3 elm1Δ::KANR bni1Δ::HIS3 [BNI1::URA3]*
PY6630	*MATa his3 leu2 met15 ura3 gin4Δ::KANR bni1Δ::HIS3 [BNI1::URA3]*
PY6632	*MATa his3 leu2 met15 ura3 nap1Δ::KANR bni1Δ::HIS3 [BNI1::URA3]*
PY6634	*MATx his3 leu2 met15 ura3 shs1Δ::KANR bni1Δ::HIS3 [BNI1::URA3]*
PY6626	*MATa his3 leu2 met15 ura3 swe1Δ::KANR bni1-1::HIS3*
PY6908	*MATa his3 leu2 met15 ura3 elm1Δ::KANR bni1-1::HIS3 swe1Δ::KANR*
PY6627	*MATa his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::HIS3 swe1Δ::KANR*
PY6628	*MATx his3 leu2 met15 ura3 shs1Δ::KANR bni1-1::HIS3 swe1Δ::KANR*
PY6650	*MATa his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::HIS3 [URA3]*
PY6651	*MATa his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::HIS3 [pGIN4-GIN4-2HA::URA3]*
PY6652	*MATa his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::HIS3 [pGIN4-GIN4-Δkinase-2HA::URA3]*
PY6908	*MATa his3 leu2 met15 ura3 bnr1::BNR1-GFP-HIS3 gin4Δ::KANR [URA3]*
PY6910	*MATa his3 leu2 met15 ura3 bnr1::BNR1-GFP-HIS3 gin4Δ::KANR [pGIN4-GIN4-2HA::URA3]*
PY6911	*MATa his3 leu2 met15 ura3 bnr1::BNR1-GFP-HIS3 gin4Δ::KANR [pGIN4-GIN4-Δkinase-2HA::URA3]*
PY6979	*MATa his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::HIS3 myo2::MYO2-GFP::HIS3 [URA3]*
PY6984	*MATa his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::HIS3 myo2::MYO2-GFP::HIS3 [pGIN4-GIN4::leu2::URA3]*
PY6985	*MATa his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::HIS3 myo2::MYO2-GFP::HIS3 [pGIN4-GIN4-2HA::URA3]*

PY6980	*MATa his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::HIS3 myo2::MYO2-GFP::HIS3 [pGIN4-GIN4-Δkinase-2HA::URA3]*
PY6868	*MATa his3 leu2 met15 ura3 bnr1::bnr1ΔDAD-3HA::HIS3*
PY6960	*MATa his3 leu2 met15 ura3 bni1-1::HIS3 bnr1::bnr1ΔDAD-3HA::HIS3*
PY6974	*MATa his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::HIS3 bnr1::bnr1ΔDAD-3HA::HIS3*
PY6975	*MATa his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::HIS3 bnr1::bnr1ΔDAD-3HA::HIS3*
PY6667	*MATa his3 leu2 met15 ura3 bni1-1::his3::LEU2*
PY7001	*MATa his3 leu2 met15 ura3 bni1-1::HIS3 bnr1::bnr1ΔDAD-3HA::HIS3 myo2::MYO2-GFP::HIS3*
PY6995	*MATx his3 leu2 met15 ura3 bnr1::bnr1ΔDAD-3HA::HIS3 myo2::MYO2-GFP::HIS3*
PY6999	*MATa his3 leu2 met15 ura3 bni1-1::his3::LEU2 gin4Δ::KANR bnr1::bnr1ΔDAD-3HA::HIS3 myo2::MYO2-GFP::HIS3*
PY7000	*MATa his3 leu2 met15 ura3 bni1-1::his3::LEU2 gin4Δ::KANR bnr1::bnr1ΔDAD-3HA::HIS3 myo2::MYO2-GFP::HIS3*
PY6636	*MATa his3 leu2 met15 ura3 bnr1::BNR1-758-GFP-HIS3*
PY6953	*MATa his3 leu2 met15 ura3 bnr1::BNR1-660-GFP-HIS3*
PY6637	*MATa his3 leu2 met15 ura3 bnr1::BNR1-596-GFP-HIS3*
PY6991	*MATα his3 leu2 met15 ura3 bnr1::BNR1-660-GFP-HIS3 gin4Δ::KANR*
PY6888	*MATa his3 leu2 met15 ura3 KANR::bnr1::pGAL1::BNR1-L1(35-501)-GFP-HIS3*
PY6915	*MATx his3 leu2 met15 ura3 KANR::bnr1::pGAL1::BNR1-L2(596-758)-GFP-HIS3*
PY6899	*MATx his3 leu2 met15 ura3 KANR::bnr1::pGAL1::BNR1-ΔL1(596-C)-GFP-HIS3*
PY6947	*MATx his3 leu2 met15 ura3 KANR::bnr1::pGAL1::BNR1-L1(35-501)-GFP-HIS3 gin4Δ::kanr:LEU2*
PY6936	*MATx his3 leu2 met15 ura3 KANR::bnr1::pGAL1::BNR1-L2(596-758)-GFP-HIS3 gin4Δ::kanr:LEU2*
PY6948	*MATa his3 leu2 met15 ura3 KANR::bnr1::pGAL1::BNR1-ΔL1(596-C)-GFP-HIS3 gin4Δ::kanr:LEU2*
PY7012	*MATa his3 leu2 met15 ura3 bnr1::KANR p[3GFP-BNR1-URA3]*
PY7010	*MATa his3 leu2 met15 ura3 bnr1::KANR p[3GFP-BNR1-ΔL1-HIS3]*
PY7011	*MATa his3 leu2 met15 ura3 bnr1::KANR p[3GFP-BNR1-ΔL2-HIS3]*
PY7019	*MATx his3 leu2 met15 ura3 gin4::KANR p[3GFP-BNR1-URA3]*
PY7015	*MATx his3 leu2 met15 ura3 gin4::KANR p[3GFP-BNR1-ΔL1-HIS3]*
PY7016	*MATx his3 leu2 met15 ura3 gin4::KANR p[3GFP-BNR1-ΔL2-HIS3]*

PY6982	*MATx his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::his3::LEU2 bnr1::KANR [URA]*
PY6983	*MATx his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::his3::LEU2 bnr1::KANR [3GFP-BNR1-URA3]*
PY6986	*MATx his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::his3::LEU2 bnr1::KANR [3GFP-BNR1-ΔL1-URA3]*
PY7062	*MATx his3 leu2 met15 ura3 shs1Δ::KANR bni1-1::his3::LEU2 bnr1::KANR [URA]*
PY7064	*MATx his3 leu2 met15 ura3 shs1Δ::KANR bni1-1::his3::LEU2 bnr1::KANR [3GFP-BNR1-URA3]*
PY7066	*MATx his3 leu2 met15 ura3 shs1Δ::KANR bni1-1::his3::LEU2 bnr1::KANR [3GFP-BNR1-ΔL1-URA3]*
PY6987	*MATx his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::his3::LEU2 bnr1::KANR [HIS3]*
PY6988	*MATx his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::his3::LEU2 bnr1::KANR [3GFP-BNR1-ΔL1-HIS3]*
PY6989	*MATx his3 leu2 met15 ura3 gin4Δ::KANR bni1-1::his3::LEU2 bnr1::KANR [3GFP-BNR1-ΔL2-HIS3]*
PY6845	*MATa his3 leu2 met15 ura3 bnr1::BNR1-758-13myc-HIS3 GIN4::Gin4-TAP::HIS3*
PY6844	*MATa his3 leu2 met15 ura3 bnr1::BNR1-758-13myc-HIS3 GIN4::Elm1-TAP::HIS3*
PY6907	*MATa his3 leu2 met15 ura3 bnr1::BNR1-758-13myc-HIS3 gin4Δ::KANR p[URA3]*
PY6909	*MATa his3 leu2 met15 ura3 bnr1::BNR1-758-13myc-HIS3 gin4Δ::KANR p[pGIN4-GIN4-2HA::URA3]*
PY7020	*MATa his3 leu2 met15 ura3 bnr1::BNR1-758-13myc-HIS3 gin4Δ::KANR p[pGIN4-GIN4-Δkinase-2HA::URA3]*
PY6998	*MATa ura3 leu2 trp1-1 prb1-122 pep44 pre1-451 bnr1::KANR p[pGAL-6HIS-BNR1-758::HIS3::URA3 2μ]*
PY6952	*MATa ura3 leu2 trp1-1 prb1-122 pep44 pre1-451 bnr1::KANR p[pGAL-6HIS-BNR1::URA3 2μ]*

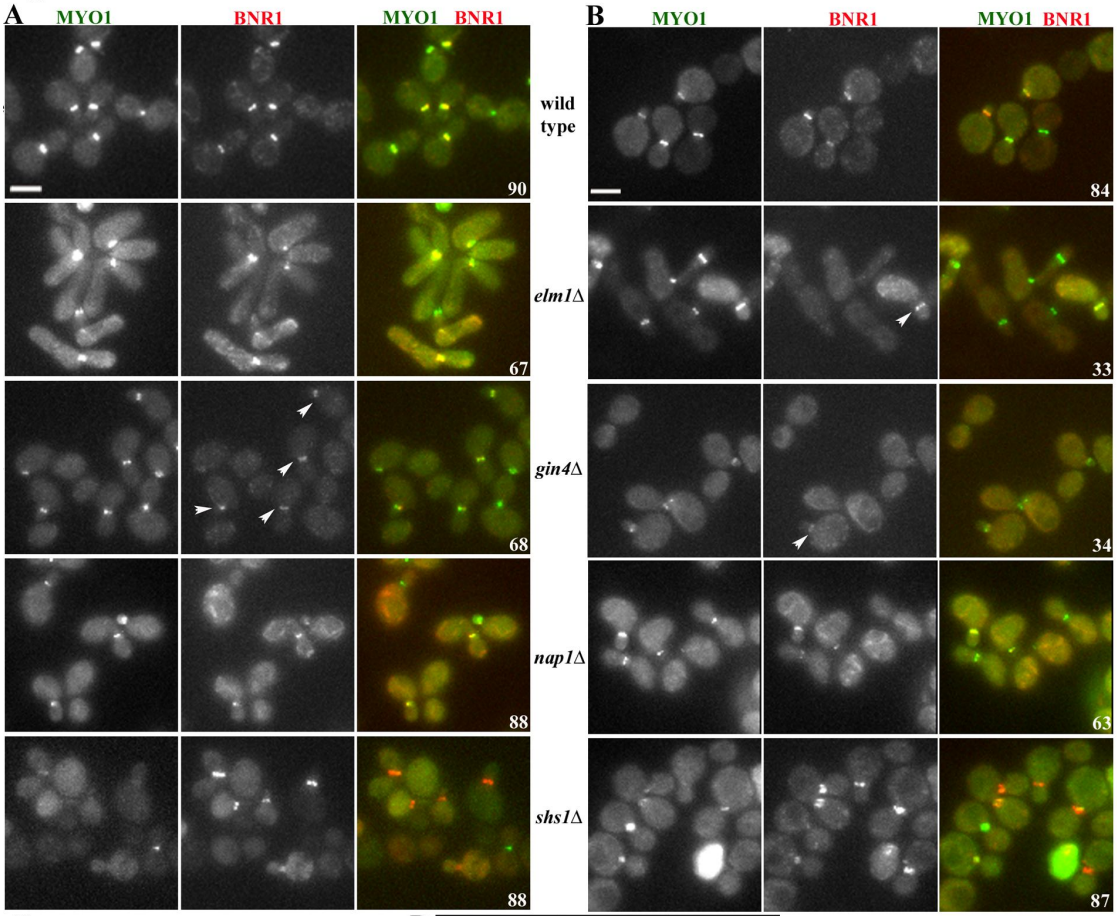
Supplemental Table 2: Plasmid List

Name	GENE (ORF)	Marker	Other*	Source
PB1025	*BNI1*	*URA3*	CEN	C. Boone
PB59	empty	*URA3*	CEN	this study
PB2921	*3GFP-BNR1*	*URA3*	CEN	this study
PB2961	*3GFP-BNR1ΔL1*	*HIS3*	CEN	A. Bretscher
PB2962	*3GFP-BNR1ΔL2*	*HIS3*	CEN	A. Bretscher
PB2966	*GIN4*	*LEU2*	CEN	J. Pringle
PB2987	*GIN4*	*URA3*	CEN	this study
PB2977	*GIN4-2HA*	*URA3*	CEN	M. Iwase
PB2978	*GIN4Δkinase-2HA*	*URA3*	CEN	M. Iwase
PB2939	GST	AMPR		pGEX 4T
PB2974	GST-GIN4	AMPR		J. Thorner
PB2984	GST-GIN4Δkinase	AMPR		this study
PB2812	pCAL-n-FLAG-Gin4-C	AMPR		this study
PB2952	*pGAL-6HIS-BNR1*	URA3	2μ	this study
PB2955	*pGAL-6HIS-BNR1*-758	URA3	2μ	this study

* CEN- Yeast Centromeric Plasmid

Supplementary Figure Legends

Figure S1



D

	24°C	37°C 4 hr
wild type	90.5 (279)	85.2 (214)
elm1Δ	46.0 (559)	18.7 (475)
gin4Δ	37.7 (889)	17.1 (235)
elm1Δ gin4Δ	30.0 (566)	21.4 (384)

Figure S1: Localization of Bnr1 to the bud neck requires the septin-associated proteins Gin4 and Elm1 (Associated with Figure 1.) (A, B) Bnr1 localization to the bud neck is disrupted in *gin4Δ* and *elm1Δ*, but not *nap1Δ* or *shs1Δ* strains. In contrast, *shs1Δ* compromises the localization of Myo1, but not Bnr1. These representative images are normalized, maximum projections of Myo1-CFP (green) Bnr1-GFP (red) (co-localization in yellow) in strains of the indicated genotype. Cells were fixed after incubation at room temperature (A) or 4 hr at 37°C (B) and imaged in three dimensions with five 0.5-μm stacks. Arrowheads indicate cells with slight Bnr1-GFP localization to the bud neck.

Numbers in the lower right corner indicate the percentage of cells with Bnr1-GFP localization at the bud neck. Scale bar: 5 μm. (C) Bnr1 protein expression is modestly decreased in *elm1Δ* and *gin4Δ* strains. Western blots with anti-GFP and anti-α-tubulin to compare the protein expression of Bnr1 in the strains of the indicated genotype. Cells were grown at 24°C prior to lysate preparation. (D) Deletion of both Elm1 and Gin4 did not increase the localization defect of Bnr1-GFP. Data shown are the percent localization of Bnr1-GFP in strains of the indicated genotype following incubation at 24ºC or a 4-hr shift to 37°C. Entries are the mean percentage of cells with Bnr1 localization at the bud neck for the number of cells counted (indicated in parentheses). Experiments were performed twice with at least 100 cells scored for each sample.

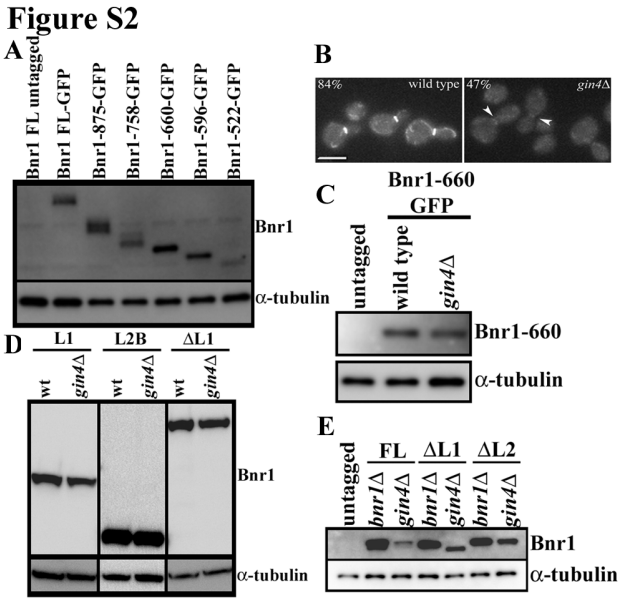


Figure S2: Bnr1 localization requires two distinct localization signals, L1 and L2; L2 localization depends on Gin4. (Associated with Figure 2.) (A) Steady-state protein levels of the indicated Bnr1-GFP truncation constructs. The comparison of the data with that in Figure 2C demonstrates that the expression level of the constructs is independent of the fluorescence intensity at the bud neck. (B) The localization of Bnr1-660-GFP requires Gin4. Images are normalized, maximum projections of Bnr1-660-GFP in either wild-type (left) or *gin4Δ* (right) strains. Cells were fixed and imaged in three dimensions with 0.5-μm stacks. Scale bar: 5 μm. The number in the upper left indicates the mean percentage of cells with GFP signal at the bud neck. Experiments were repeated two times with at least 100 cells scored for each sample. (C) Western blots to detect the GFP-fusion proteins in the strains imaged in B. (D) The steady-state levels of Bnr1-L1, Bnr1-L2, and Bnr1-ΔL1-GFP in wild-type and *gin4Δ* strains after galactose-induced expression indicate that the loss of localization of Bnr1-L2-GFP and Bnr1-ΔL1-GFP in *gin4Δ* strains was not due to differences in protein expression. These samples were prepared as for the imaging experiments in Figure 2C. Thus, the GAL induction time is different for L1 and ΔL1 (4 hr) and L2 (1 hr), and we used two separate western blots with separate loading controls. (E) Western blots of endogenous level 3GFP-Bnr1 constructs in *bnr1Δ* and *gin4Δ* from the strains imaged in Figure 2D.

Figure S3

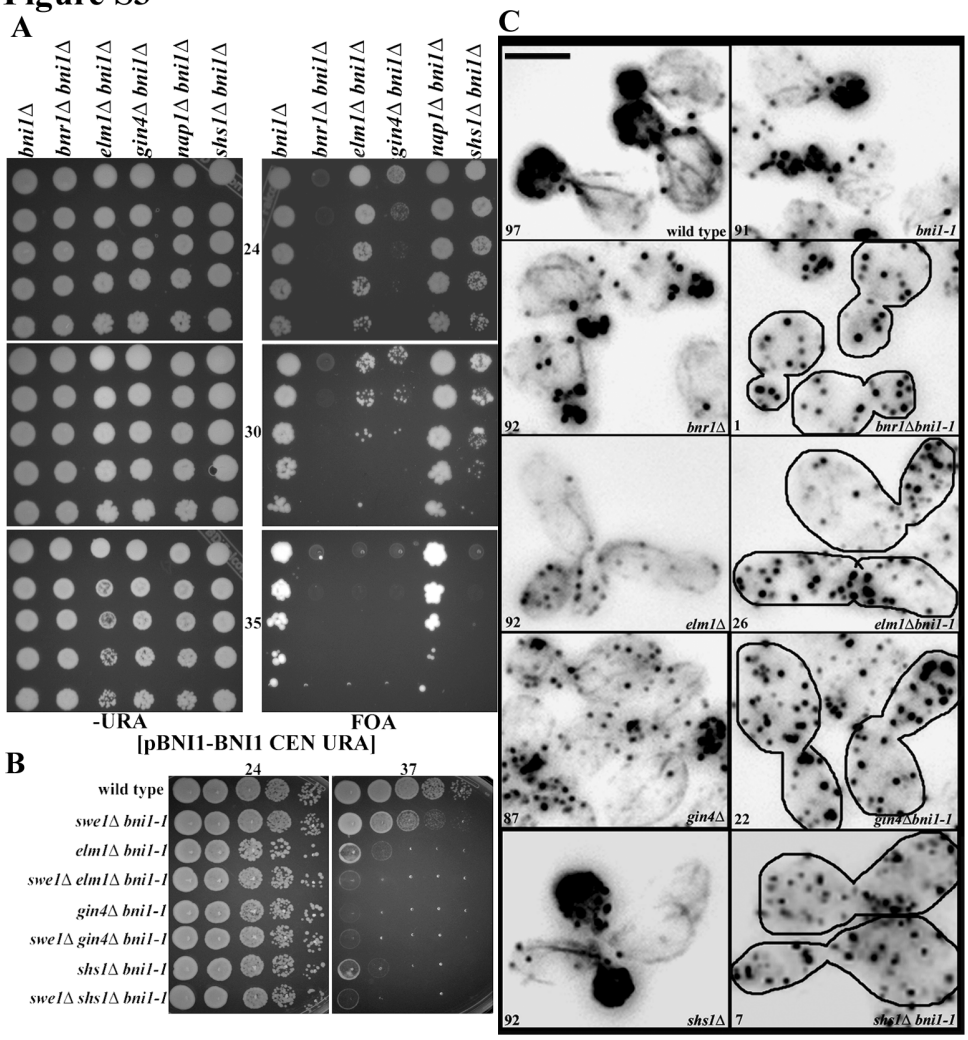
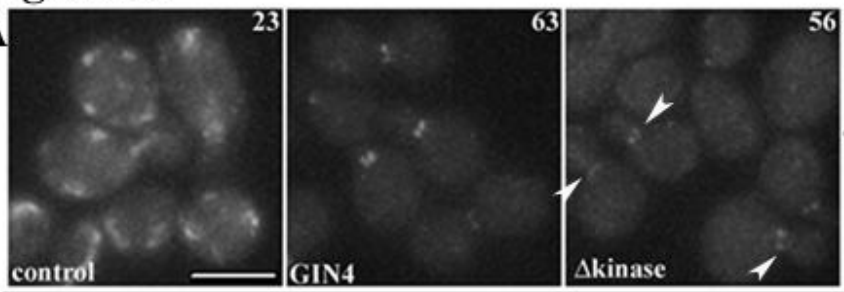


Figure S3: Bnr1-mediated actin cable assembly requires Gin4 and Elm1.
(Associated with Figure 3.) (A) Loss of *GIN4*, *ELM1*, and *SHS1* show synthetic genetic interactions with loss of *BNI1*. The *gin4Δ bni1Δ*, *elm1Δ bni1Δ*, and *shs1Δ bni1Δ* strains are temperature-sensitive for growth, but *nap1Δ bni1Δ* is not. The growth defect of *gin4Δ bni1Δ* is slightly stronger than the growth defect of *elm1Δ bni1Δ* or *shs1Δ bni1Δ*. Five-fold dilutions of cells were spotted on medium lacking uracil (left) or medium containing 5-FOA (right). The plates were grown for one day at 30°C (middle) or 35°C (bottom) or two days at 24°C (top). (B) The deletion of *SWE1* does not rescue temperature sensitivity of *gin4Δ bni1-1*, *elm1Δ bni1-1*, or *shs1Δ bni1-1*. Five-fold dilutions of cells were spotted on YPD plates and grown for one day at 37°C (right) or two days at 24°C

(left). (C) Actin cables are diminished in *elm1Δ bni1-1, gin4Δ bni1-1, or shs1Δ bni1-1* at the restrictive temperature. Phalloidin actin staining in cells fixed after 15 min at 35°C. Shown are representative images of the indicated genotype; images are deconvolved, maximum projections generated from 0.3-μm stacks. The genotype is indicated in lower right corner. The percentage of cells with actin cable staining is in the lower left corner; cells were scored as positive for actin cables when any linear actin structures were detected. Arrowheads indicate cells with actin cables. Experiments were performed three times with at least 100 cells quantified for each experiment. For *gin4Δ bni1-1* and *elm1Δ bni1-1* strains, we analyzed two independent isolates, which both showed similar effects on growth and actin cable staining. Scale bar: 5 μm.

Figure S4

A



B

	24°C	37°C 4 hr
control	56.1 (857)	22.9 (701)
Gin4	78.2 (1118)	62.8 (699)
Gin4Δkinase	70.0 (693)	56.1 (928)

Figure S4: Localization of Bnr1 to the bud neck is independent of the kinase domain of Gin4. (Associated with Figure 4.) (A, B) The kinase domain of Gin4 is not required for Bnr1-GFP localization to the bud neck. Images of Bnr1-GFP are normalized, maximum projections in strains of the indicated genotype. The *gin4Δ Bnr1-GFP* strain was transformed with *URA CEN* (control) plasmids with [*pGIN4-GIN4-2HA*] or [*pGIN4-Δkinase-2HA*]. Cells were fixed after incubation at 24°C or 4 hr at 37°C and imaged with 0.5-μm stacks. The percentage of cells with Bnr1-GFP localized to the bud neck is indicated in the upper right corner. (B) The percentage of cells with bud neck localization of Bnr1-GFP in strains of the indicated genotype maintained at 24°C or after 4 hr at 37°C. The numbers shown are the percentage of cells with Bnr1 localization at the bud neck and the number of cells analyzed (indicated in parentheses). Experiments were performed three times (n > 200 cells per experiment).

Figure S5

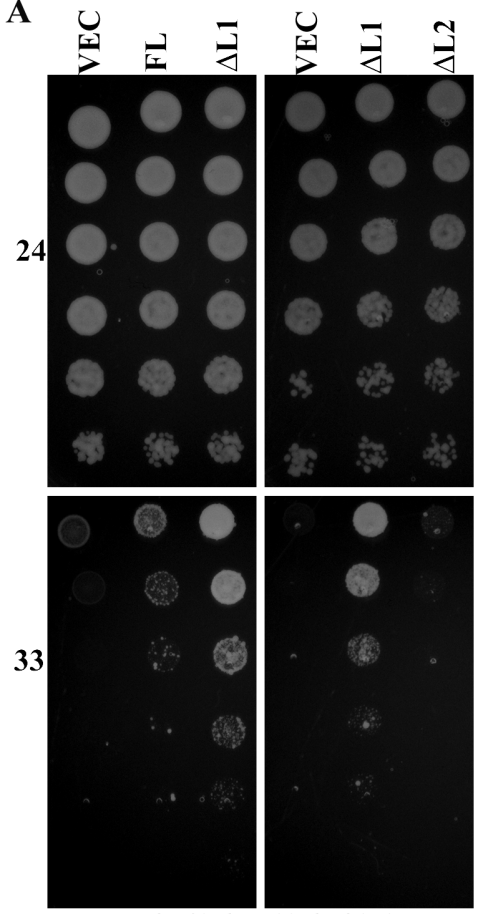


Figure S5: Expression of activated Bnr1 rescues the growth defect of a *gin4Δ bnr1Δ bni1-1*. (Associated with Figure 6.) (A) Expression of [*pBNR1-3GFP-BNR1*] and [*pBNR1-3GFP-BNR1ΔL2*] did not rescue the growth defect of *gin4Δ bnr1Δ bni1-1*, whereas [*pBNR1-3GFP-BNR1ΔL1*] did rescue growth. Ten-fold dilutions of cells were spotted on –URA plates and grown for two days at 33°C (bottom) or three days at 24°C (top).

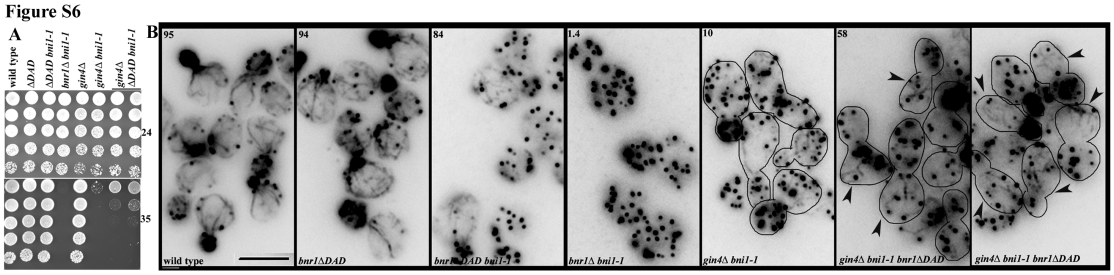


Figure S6: Expression of activated Bnr1 rescues the actin polarity defect of *gin4Δ bni1-1* cells. (Associated with Figure 7.) (A) Expression of *bnr1ΔDAD-3HA* partially rescues the temperature-sensitive phenotype of a *gin4Δ bni1-1* strain. Five-fold dilutions of cells were spotted on YPD plates and grown for one day at 35°C (bottom) or two days at 24°C (top). The experiment here represents an independent technical replicate of the spot plate growth from that shown in Figure 7. (B) A *bnr1ΔDAD-3HA* truncation partially rescues the actin polarity defect of *gin4Δ bni1-1* cells. Phalloidin actin staining in cells fixed after 15 min at 35°C. Images are normalized, maximum projections generated from 0.3-μm stacks. Genotype is indicated in lower left corner; percentage of cells with actin cable staining is in the upper left corner. We analyzed two independent isolates of *gin4Δ bni1-1 bnr1ΔDAD-3HA*, which both showed a moderate rescue of actin cable staining after a 15-min shift to 35°C. We performed experiments three times with at least 100 cells scored for each experiment. Scale bar: 5 μm.

Figure S7

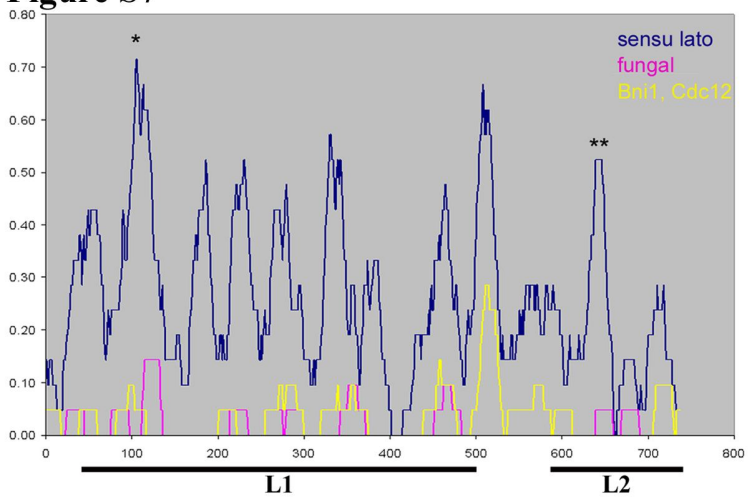


Figure S7: Conservation of the Bnr1 minimal localization domain. (A) Homology scanning of Bnr1 1-758 to analyze the percent identity of sequences of Bnr1 from sensu lato yeast (blue), fungi (pink), and Bni1 and Cdc12 (yellow). L2 contains a peak that is present in sensu lato and fungi, but not in Cdc12 or Bni1. Consistent with our localization data, this peak (denoted with **) includes amino acids 639−660. There is also a well-conserved peak (denoted with *) at (100−135), which could be important for localization via L1. Other peaks in the L1 region are also present in Bni1 and Cdc12; these peaks coincide with the position of the functional surfaces of the RBD/DID domain.