Supplemental Information

Supplemental Materials and Methods

Strains and Plasmids

Standard methods were used for growth, sporulation, tetrad dissection and DNA manipulations and transformations of yeast. The yeast strains used in this study are listed in Table 1. The plasmids used in this study are listed in Table 2.

TCA precipitation

Mid-log phase cells were harvest and resuspended in 1 ml of 10% trichloroacetic acid (TCA) with 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, a protease inhibitor), incubated on ice for 30 minutes, and precipitates were centrifuged at 14,000 rpm for 10 min at 4°C. Precipitates were washed twice with cold acetone, resuspended by sonication, and air-dried. Protein samples were processed for SDS-PAGE separation by adding 2X protein sample buffer with 0.1 mM AEBSF and solubilized by standard bead disruption.

SDS-PAGE and Immunoblotting

Proteins were separated on polyacrylamide mini gels (7.5-15%) at 18-25 mA in SDS running buffer (3mM SDS, 25 mM Tris base, 192 mM glycine) and then transferred onto nitrocellulose membranes at 80 V for 90 minutes in cold transfer buffer (20% methanol, 0.0375% SDS, 48 mM Tris base, 39 mM glycine). The membranes are blocked in 5% milk in TBST (10mM Tris, pH 7.5, 0.25 M NaCl, 0.025% Tween-20). Blots were incubated in the specified primary antibody, washed 3 times in TBST, incubated with secondary antibodies conjugated to HRP (Pierce, Rockford, IL) diluted 1:5000 in milk solution for 60 min. Blots were washed again 3 times in TBST, and then developed with Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) for 5 minutes at room temperature. The chemiluninescence was visualized on a Fluorochem 8000 Chemiluminescence System (Alpha Innotech Corp., San Leandro, CA).

Ste3-GFP Localization Analysis

Cells were transformed with a STE3-GFP (URA) plasmid (pBW0639) (Urbanowski and Piper, 2001). For Ste3-GFP visualization, the pH of the culture was adjusted with 10 mM Tris, pH 7.5, 5 minutes before observation. The cells were then pelleted and resuspended in the same media at 2-5 OD/ml density and imaged. Ste3-GFP images were acquired with a Zeiss LSM 510 META confocal microscope equipped with the appropriate lasers and filter set.

GFP-Ent1C-term Truncations Live-cell Imaging

GFP-Ent1C-term truncations images were collected with a 3i Marianas microscope (Intelligent Imaging Innovations, Denver, CO) equipped with an alpha Plan-Fluar 100x 1.46 NA objective and a Zeiss TIRF slider (Carl Zeiss, Thornwood, NY). Single color widefield GFP images were acquired using 488 nm laser excitation and a GFP dichroic and emission filter (Semrock, Rochester, NY) Slide-Book 4.2® software (Intelligent Imaging Innovations, Denver, CO) was used for image acquisition and dual channel image registration.

For live cell imaging, cells were grown to early log phase on rich medium plates at 30°C. Cells were placed in 200 μl minimal media on Concavalin A-coated 8 well Lab-Tek coverglass bottom dishes (Nalge Nunc, Rochester, NY). All imaging was at room temperature.

Image Analysis

Image analysis was performed using the National Institute of Health ImageJ (http://rsb.info.nih.gov/ij/) or Slide-Book 4.2® software.

Table1 Yeast Strains used in this study

Strain	*Genotype*	Source
SEY6210	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Laboratory strain
BWY2497	*Mat**a** leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 bar1 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW0768 (ENT1, CEN)*	This study
BWY2503	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 PAN1-GFP-KAN*	This study
BWY2506	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 PAN1-GFP-KAN + pBW0768 (ENT1, CEN)*	This study
BWY2567	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 yap1801::HIS3 yap1802::LEU2*	Laboratory strain
BWY2571	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 ede1::KAN + pBW0053 (ENT2, CEN)*	This study
BWY2572	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 PAN1-GFP-KAN + pBW0768 (ENT1, CEN)*	This study
BWY2574	*Mat**a** leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 bar1 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 ede1::KAN + pBW0768 (ENT1, CEN)*	This study
BWY2594	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 + pBW0768 (ENT1, CEN)*	Laboratory strain
BWY2595	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 + pBW0778 (ent1ENTH domain, CEN)*	Laboratory strain
BWY2596	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW0768 (ENT1, CEN)*	This study
BWY2597	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW0778 (ent1ENTH domain, CEN)*	This study
BWY2598	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901*	This study

	lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW0387 (ent1^{CBM-}, CEN)	
BWY2599	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW1375 (ent1^{UIM-}, CEN)*	This study
BWY2600	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW1376 (ent1^{NPF-}, CEN)*	This study
BWY2601	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW1381 (ent1$^{UIM-/NPF-}$, CEN)*	This study
BWY2602	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW1382 (ent1$^{UIM-/CBM-}$, CEN)*	This study
BWY2603	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW1383 (ent1$^{NPF-/CBM-}$, CEN)*	This study
BWY2604	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW1359 (ent1ENTH/UIMs, CEN)*	This study
BWY2605	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW1360 (ent1ΔNPFs, CEN)*	This study
BWY2606	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 PAN1-GFP-KAN + pBW0778 (ent1ENTH domain, CEN)*	This study
BWY2607	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 PAN1-GFP-KAN + pBW1375 (ent1^{UIM-}, CEN)*	This study
BWY2608	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 PAN1-GFP-KAN + pBW1376 (ent1^{NPF-}, CEN)*	This study
BWY2609	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3*	This study

	yap1801::HIS3 yap1802::LEU2 PAN1-GFP-KAN + pBW0387 (ent1^{CBM-}, CEN)	
BWY2613	*Mat**a** leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 bar1 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW1376 (ent1^{NPF-}, CEN)*	This study
BWY2619	*pan1::HIS3 (Longtine PCR vector) + pBW513 (PAN1 URA3 CEN)*	Laboratory strain
BWY2738	*Mat**a** leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 bar1 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW0781 (high copy ent1C-term^{WT})*	This study
BWY2739	*Mat**a** leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 bar1 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW1364 (high copy ent1C-term^{NPF-})*	This study
BWY2740	*Mat**a** leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 bar1 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW1375 (ent1^{UIM-}, CEN)*	This study
BWY2741	*Mat**a** leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 bar1 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW1376 (ent1^{NPF-}, CEN)*	This study
BWY2742	*Mat**a** leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 bar1 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW0387 (ent1^{CBM-}, CEN)*	This study
BWY2743	*Mat**a** leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 bar1 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW1381 (ent1$^{UIM-/NPF-}$, CEN)*	This study
BWY2744	*Mat**a** leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 bar1 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW1382 (ent1$^{UIM-/CBM-}$, CEN)*	This study
BWY2745	*Mat**a** leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 bar1 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW1383 (ent1$^{NPF-/CBM-}$, CEN)*	This study
BWY2746	*Mat**a** leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 bar1 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW1448 (ent1^{lipid-}, CEN)*	This study
BWY2747	*Mat**a** leu2-3 ura3-52 his3-Δ200 trp1-Δ901*	This study

	lys2-801 suc2-Δ9 bar1 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 + pBW1449 (ent1$^{lipid-/NPF-}$, CEN)	
BWY2748	*pan1::HIS3 [parent = BWY2619] + pBW1443 (Pan1-GFP, TRP1 CEN), 5FOA cured*	This study
BWY2749	*pan1::HIS3 [parent = BWY2619] + pBW1444 (Pan1^{EH-}-GFP, TRP1 CEN,) 5FOA cured*	This study
BWY2750	*Matα leu2-3 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ent1::LEU2 ent2::HIS3 yap1801::HIS3 yap1802::LEU2 STE3-GFP::KAN + pBW0053 (ENT2, CEN)*	This study

Table 2 Plasmids used in this study

Plasmid	Description	Details	Source
pBW0053	*pENT2(URA3)*	pRS416::ENT2 (URA3, CEN)	Laboratory plasmid
pBW0054			
pBW0387	*pent1⊿CBM(TRP1)*	pRS414::ent1 (aa1-450) (TRP1, CEN)	(Baggett et al, 2003)
pBW513	*PAN1(URA3)*	pRS416::PAN1	Laboratory plasmid
pBW0639	*pSTE3-GFP (URA3)*	pRS316::STE3-GFP (URA, CEN)	(Urbanowski and Piper, 2001)
pBW0768	*pENT1(TRP1)*	pRS414::ENT1 (TRP1, CEN)	Laboratory plasmid
pBW0778	*pent1ENTH $^{domain\ only}$ (TRP1)*	pRS414::ent1 (aa1-151) (TRP1, CEN)	Laboratory plasmid
pBW0781	*pMET25 ent1C-term^{WT} (URA)*	pMET25.426::ent1 (aa154-454) (URA3, 2μ)	This study
pBW0985	*pEDE1-RFP*	pRS416::EDE1-RFP (URA, CEN)	Laboratory plasmid
pBW1015	*pMET25 YAP1802 (URA3)*	pMET25.426::YAP1802 (URA3, 2μ)	This study
pBW1016	*pMET25 yap1802^{ANTH2} (URA3)*	pMET25.426::yap1802 (aa 1-277) (URA3, 2μ)	This study
pBW1019	*pMET25 yap1802Cterm^{WT} (URA3)*	pMET25.426::yap1802 (aa278-568) (URA3, 2μ)	This study
pBW1020	*pMET25 yap1802$^{ANTH+62aa}$ (URA3)*	pMET25.426::yap1802 (aa1-339) (URA3, 2μ)	This study
pBW1344	*pYAP1801(URA3)*	pRS416::YAP1801 (URA3, CEN)	(Wendland and Emr 1998)
pBW1345	*pYAP1802(URA3)*	pRS416::YAP1802 (URA3, CEN)	(Wendland and Emr 1998)
pBW1359	*pent1ENTH/UIMs (URA)*	pRS414::ent1 (aa1-229) (URA3, CEN)	Laboratory plasmid
pBW1360	*pent1⊿NPFs (URA)*	pRS414::ent1 (aa1-305) (URA3, CEN)	Laboratory plasmid
pBW1361	*pent1C-term^{WT}(URA)*	pRS316::ent1 (aa153-454) (URA, CEN)	This study
pBW1362	*pMET25 ent1C-term^{NPF1-} (URA)*	pMET25.426::ent1(aa154-454) P307A and F308A mutations introduced by site directed mutagenesis in	This study

pBW1363	*pMET25 ent1C-term^{NPF2-} (URA)*	pBW0781 (URA3, 2μ) pMET25.426::ent1(aa154-454) P408A and F409A mutations introduced by site directed mutagenesis in	This study
pBW1364	*pMET25 ent1C-term^{NPF-} (URA)*	pBW0781 (URA3, 2μ) pMET25.426::ent1(aa154-454) P408A and F409A mutations introduced by site directed mutagenesis in	This study
pBW1366	*pMET25 ent1C-term^{UIM1-} (URA)*	pBW1362 (URA3, 2μ) pMET25.426::ent1(aa154-454) S175D mutation introduced by site directed mutagenesis in pBW0781 (URA3, 2μ)	This study
pBW1367	*pMET25 ent1C-term^{UIM2-} (URA)*	pMET25.426::ent1(aa154-454) S201D mutation introduced by site directed mutagenesis in pBW0781 (URA3, 2μ)	This study
pBW1368	*pMET25 ent1C-term^{UIM-} (URA)*	pMET25.426::ent1(aa154-454) S175D mutation introduced by site directed mutagenesis in pBW1367 (URA3, 2μ)	This study
pBW1375	*pENT1^{UIM-} (TRP1)*	pRS414::ent1 S175D and S201D mutations introduced by site directed mutagenesis in pBW0768	This study
pBW1376	*pENT1^{NPF-} (TRP1)*	pRS414::ent1 P307A, F308A, P408A, and F409A mutations introduced by site directed mutagenesis in pBW0768	This study
pBW1381	*pENT1$^{UIM-/NPF-}$ (TRP1)*	pRS414::ent1 P307A, F308A, P408A, and F409A mutations introduced by site directed mutagenesis in pBW1375	This study
pBW1382	*pENT1$^{UIM-/CBM-}$ (TRP1)*	pRS414::ent1 S175D and S201D mutations introduced by site directed mutagenesis in pBW0387	This study
pBW1383	*pENT1$^{NPF-/CBM-}$ (TRP1)*	pRS414::ent1 P307A, F308A, P408A, and F409A	This study

		mutations introduced by site directed mutagenesis in pBW0387	
pBW1429	*pede1^{EH-}-RFP (URA3)*	pRS414::ede1 W56A, W176A, and W319A mutations introduced by site directed mutagenesis in pBW985	This study
pBW1443	*pPAN1-GFP (TRP1)*	C-terminal PAN1-GFP fragment from Longtine PCR plasmid subcloned into pRS414::PAN1 plasmid (XmnI-DraI genomic fragment)	This study
pBW1444	*ppan1^{EH-}-GFP (TRP1)*	pRS414::pan1 W312A and W642A mutations introduced by site directed mutagenesis into pBW1443	This study
pBW1448	*pENT1^{lipid-} (TRP1)*	pRS414::ent1 R62L and H72L mutations introduced by site-directed mutagenesis	This study
pBW1449	*pENT1$^{lipid-/NPF-}$ (TRP1)*	pRS414::ent1 P307A, F308A, P408A, and F409A mutations introduced by ligating a BstEII fragment from pBW1376 into pBW1448	This study
pBW1453	*pEnt1C-term^{WT} (TRP1)*	pMET25.4266::ent1(aa154-454)	This study
pBW1454	*pEnt1C-term^{UIM-} (TRP1)*	pMET25.4266::ent1(aa154-454) S175D, S201D	This study
pBW1455	*pEnt1C-term^{NPF-} (TRP1)*	pMET25.4266::ent1(aa154-454) P307A, F308A, P408A, and F409A	This study
pBW1466	*GFP-Ent1 (TRP1)*	pGOGFP(414)::ENT1	This study
pBW1468	*GFP-Ent1$^{NPF-/CBM-}$ (TRP1)*	pGOGFP(414)::ent1 P307A, F308A, P408A, and F409A; fragment subcloned from pBW1383 in place of similar fragment from pBW1466	This study

Supplemental Figure Legends

Supplemental Figure S1. The *yap1801Δyap1802Δ* cells exhibit normal rates of endocytosis and growth at high temperature. (A) *ent1*Δ*ent2*Δ containing the Ent1 ENTH domain (ENTH1) (BWY2595) and *yap1801*Δ*yap1802*Δ cells (BWY2567) were transformed with a plasmid encoding for Ste3-GFP (pBW0639). Cells were grown at 30°C on selective media and visualized using live-cell imaging and confocal microscopy. (B) *ent1*Δ*ent2*Δ containing either full length *ENT1* (BWY2594) or the ENTH1 domain (BWY2595) and *yap1801*Δ*yap1802*Δ cells (BWY2567) were grown on rich medium at 30°C or 37°C for three days.

Supplemental Figure S2. The NPF motif-containing region of Ent1 is required for growth at high temperature in ΔΔΔΔ cells. ent1Δ*ent2*Δ (ΔΔ*)* or ΔΔΔΔ cells containing either full length Ent1 (BWY2594*),* an Ent1 C-terminal truncation (BWY2598, BWY2604 and BWY2605), or the ENTH1 domain (BWY2597) were grown on rich medium at 30°C, 33°C, 35°C or 37°C for three days.

Supplemental Figure S3. NPF motifs are essential for the endocytosis function of the Ent1 C-terminus. (A) Overexpression of the Ent1 C-terminus *in trans* restores viability at high temperature of *ΔΔΔΔ*+ENTH1 cells. *ΔΔΔΔ*+ENTH1 cells coexpressing the Ent1C-term^{WT} at endogenous levels (endog Ent1C-term^{WT}) (pBW1361) or overexpressed from a MET25 promoter (METpEnt1C-term^{WT}) (pBW0781) were grown on rich media at 30°C or 37°C for 3 days. Endogenous levels of the Ent1 C-terminus were controlled using the endogenous *ENT1* promoter. (B) NPF motifs are required for viability of ΔΔΔΔ+ENTH1 cells at high temperatures. *ΔΔΔΔ*+ENTH1 cells (BWY2597) coexpressing the Ent1C-term^{WT} (pBW0781), Ent1C-term^{UIM1-} (pBW1366), Ent1C-term^{UIM2-} (pBW1367), Ent1C-term^{UIM-} (pBW1368), Ent1C-term^{NPF1-} (pBW1362), Ent1C-term^{NPF2-} (pBW1363), and the Ent1C-term^{NPF-}(pBW1364) were tested for viability at high temperature. Cells were grown on rich media at 30°C and 37°C for 3 days. (C)

An immunoblot showing the Ent1 C-terminal point mutant constructs described above as detected by an antibody against the Ent1 C-terminus. (D) Wild type and mutant Ent1 C-terminal truncations localize to the plasma membrane in *ΔΔΔΔ*+ENTH1 cells. GFP N-terminal tagged Ent1 C-terminal fragments [(GFP-Ent1C-term^{WT} (pBW1453), GFP-Ent1C-term^{UIM-} (pBW1454), and GFP-Ent1C-term^{NPF-} (pBW1455)] were expressed in *ΔΔΔΔ*+ENTH1 cells (BWY2597). Cells were grown on selective media and visualized using live-cell fluorescence microscopy. GFP-Ent1 was used as a localization control (pBW0054).

Supplemental Figure S4. Ent1 NPF motifs are critical for endocytosis. (A) *ΔΔΔΔ* cells expressing full length Ent1 (BWY2596) or the following mutants: Ent1 UIM motif (Ent1^{UIM-}) (BWY2599), Ent1 NPF motif (Ent1^{NPF-}) (BWY2600), Ent1 CBM (Ent1^{CBM-}) (BWY2598), Ent1 UIM and NPF motif (Ent1$^{UIM-/NPF-}$) (BWY2601), Ent1 UIM and CBM (Ent1$^{UIM-/CBM-}$) (BWY2602), and Ent1 NPF and CBM (Ent1$^{NPF-/CBM-}$) (BWY2603) were transformed with a Ste3-GFP plasmid (pBW0639), grown at 30°C and assessed for Ste3-GFP internalization by live-cell confocal microscopy. (B) Localization of GFP-Ent1^{WT} (pBW1466) and GFP-Ent1$^{NPF-/CBM-}$ (pBW1468) in SEY6210 cells was visualized using the Marianas wide field epifluorescence microscope.

Supplemental Figure S5. The NPF motifs are necessary for normal dynamics of the late scaffold protein Pan1. (A) Pan1-GFP patches exhibit cortical localization in *ΔΔΔΔ* cells expressing Ent1 mutants. Single frames of a movie showing Pan1-GFP in ΔΔΔΔ cells expressing Ent1 (BWY2572) or the following mutants: Ent1 UIM motif (Ent1^{UIM-}) (BWY2599) and Ent1 CBM motif (Ent1^{CBM-}) (BWY2598). Pan1-GFP cortical patches in ΔΔΔΔ cells expressing Ent1 mutants localize to the plasma membrane and show the same type of movement as Pan1-GFP cortical patches in ΔΔΔΔ cells expressing wild type Ent1. Images were collected at one-second intervals and viewed for 2 min. (B) Moving Pan1-GFP patches in ΔΔΔΔ+ Ent1^{NPF-} cells exhibit a similar behavior to Pan1-GFP cortical patches in ΔΔΔΔ+Ent1 cells. Average lifetime ± S.D. of dynamic Pan1-

GFP cortical patches in ΔΔΔΔ+Ent1, ΔΔΔΔ+Ent1^{UIM-}, ΔΔΔΔ+Ent1^{NPF-}, and ΔΔΔΔ+Ent1^{CBM-} cells (n=15 patches, except for Ent1^{CBM-}, n=12).

Supplemental Figure S6. Ede1 requires its EH-domains for normal spatiotemporal dynamics. (A) Ede1$^{\mathbf{EH-}}$-RFP patches localize to the cell cortex in WT and ΔΔΔΔ+Ent1 cells. Single frames of a movie showing Ede1-RFP (pBW0985) and Ede1^{EH-}-RFP (pBW1429) cortical patches in WT (SEY6210) and ΔΔΔΔ+Ent1 (BWY2596) cells. (B) Ede1^{EH-}-RFP patches exhibit higher maximum fluorescence intensity than Ede1-RFP cortical patches in wild type cells. The maximum fluorescence intensity of individual Ede1-RFP and Ede1$^{\mathbf{EH-}}$-RFP patches in WT and ΔΔΔΔ+Ent1 *cells* was analyzed using *Slide-Book* 4.2® software (n=100 patches for each strain). **(**D) Ede1 is required for endocytosis at 30°C in ΔΔΔΔ cells. ΔΔΔΔ+Ent1 (BWY2596) and *5*Δ+Ent1 (BWY2572) cells were transformed with a Ste3-GFP plasmid (pBW0639). Cells were grown on selective media at 30°C and assessed for Ste3-GFP internalization using live-cell confocal microscopy.

Supporting Movies

MovieS1. Real-time fluorescence microscopy of Pan1-GFP cortical patches in Wild Type cells. 750ms exposures taken every sec for 2 min, as described in Materials and Methods.

MovieS2. Real-time fluorescence microscopy of Pan1-GFP cortical patches in ΔΔΔΔ+Ent1 cells. 750ms exposures taken every sec for 2 min, as described in Materials and Methods.

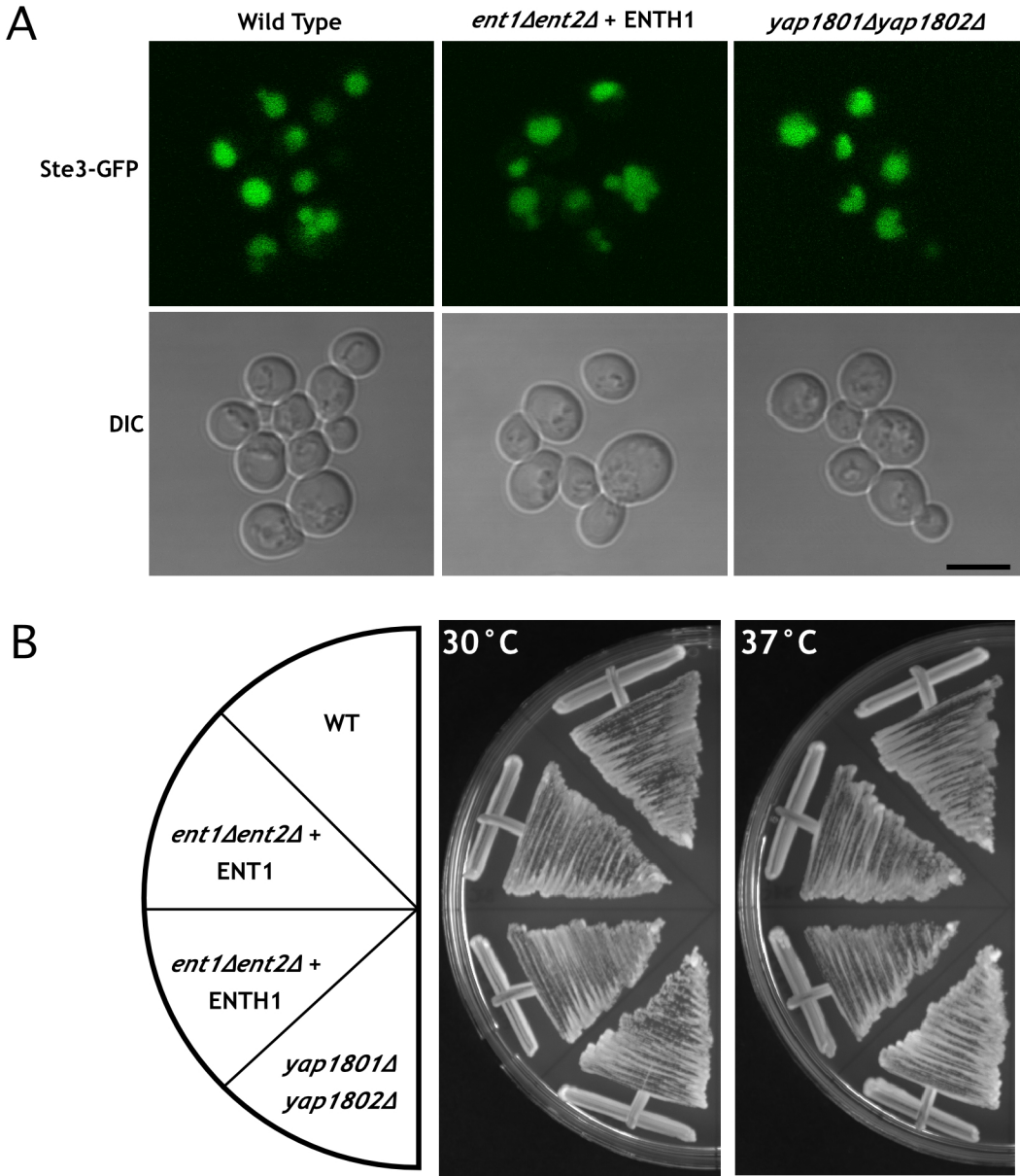
MovieS3. Real-time fluorescence microscopy of Pan1-GFP cortical patches in ΔΔΔΔ+ENTH1 cells. 750ms exposures taken every sec for 2 min, as described in Materials and Methods.

MovieS4. Real-time fluorescence microscopy of Pan1-GFP cortical patches in ΔΔΔΔ+Ent1^{NPF-} cells. 750ms exposures taken every sec for 2 min, as described in Materials and Methods.

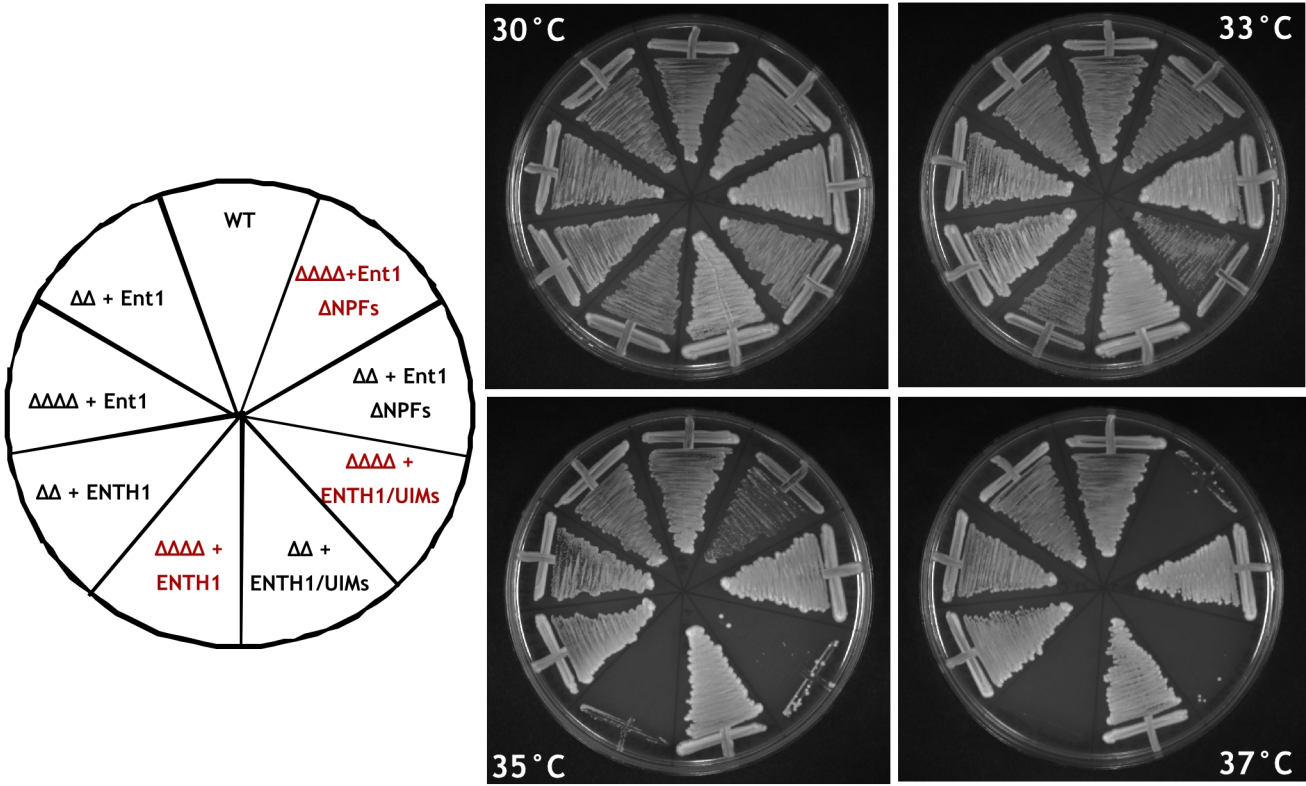
MovieS5. Real-time fluorescence microscopy of Ede1-RFP cortical patches in pan1Δ cells. 750ms exposures taken every sec for 2 min, as described in Materials and Methods.

MovieS6. Real-time fluorescence microscopy of Ede1^{EH-}-RFP cortical patches in *pan1*Δ cells. 750ms exposures taken every sec for 2 min, as described in Materials and Methods.

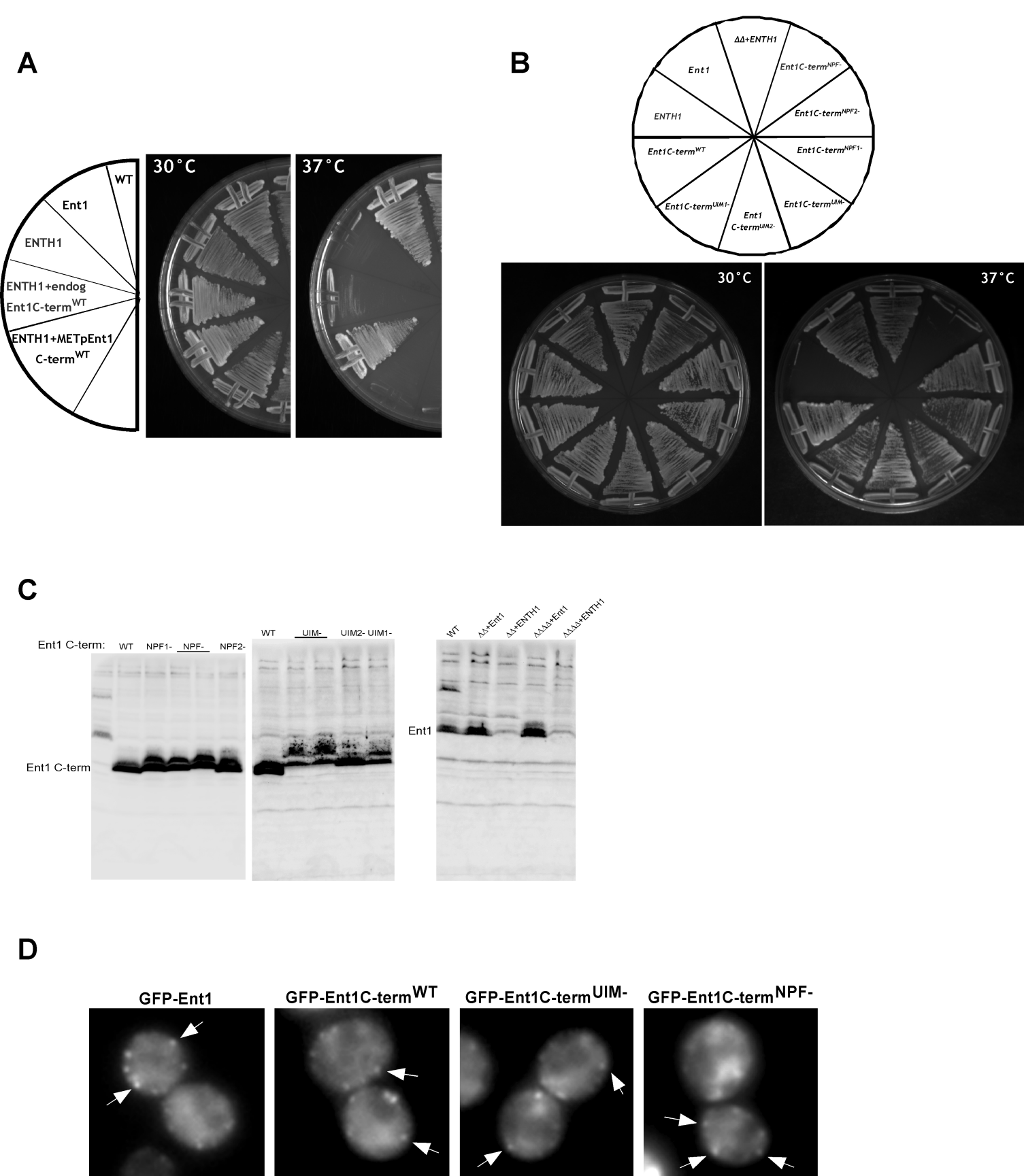
Supplemental Figure 1



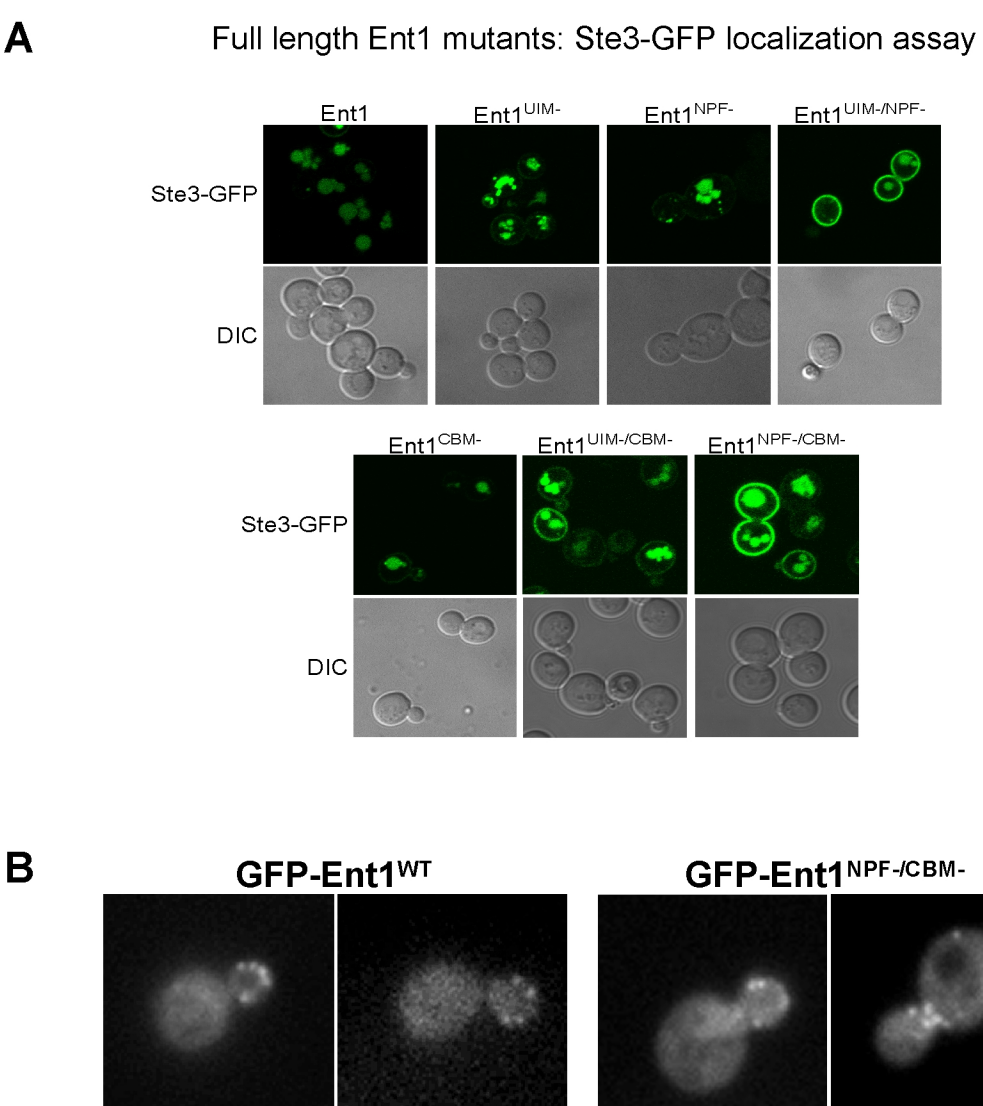
Supplemental Figure 2



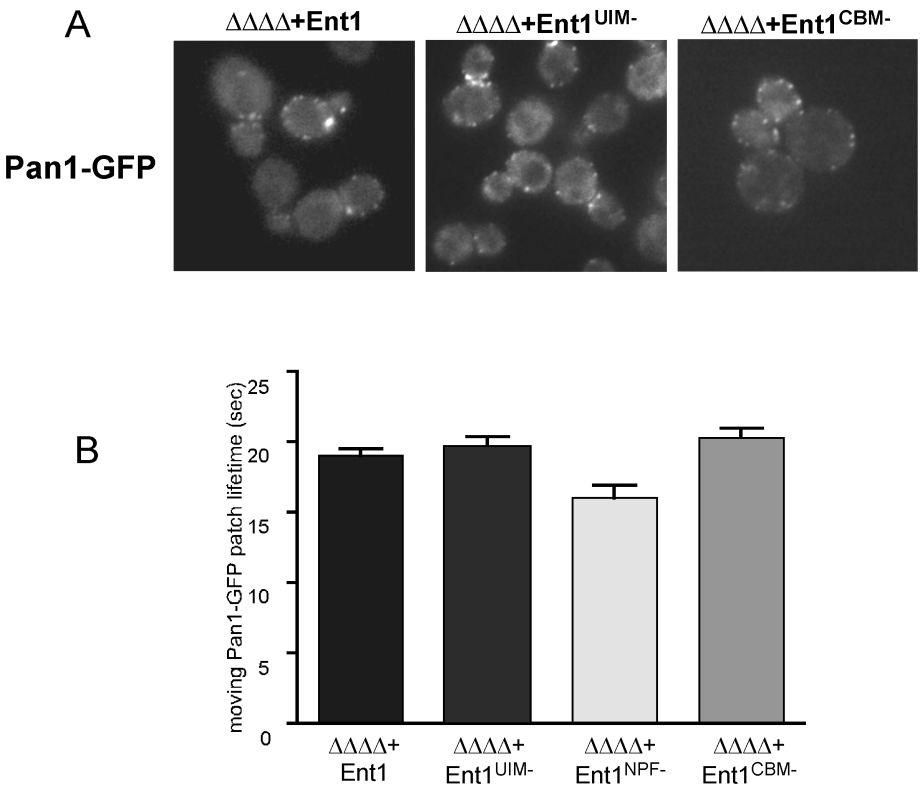
Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5



Supplemental Figure 6