The WASp homologue Las17p functions with the WIP homologue End5p/verprolin and is essential for endocytosis in yeast

Suniti N. Naqvi*, Regina Zahn*, David A. Mitchell†, Brian J. Stevenson‡§ and Alan L. Munn*

Several *end* mutations that block the internalisation step of endocytosis in *Saccharomyces cerevisiae* also affect the cortical actin cytoskeleton [1]. *END5* encodes a proline-rich protein (End5p or verprolin) required for a polarised cortical actin cytoskeleton and endocytosis [2,3]. End5p interacts with actin [4], but its exact function is not yet known. To help elucidate End5p function, we sought other End5p-interacting proteins and identified the *LAS17*/*BEE1* gene (encoding the yeast homologue of the human Wiskott–Aldrich Syndrome protein, WASp) as a high-copy-number suppressor of the temperature-sensitive growth and endocytic defects of *end5-1* cells (carrying a frameshift mutation affecting the last 213 residues of End5p). *LAS17* is unable to suppress a full deletion of *END5* (*end5Δ*), however, suggesting that the defective End5-1p in *end5-1* mutants may be stabilised by Las17p. The amino terminus of Las17p interacts with the carboxyl terminus of End5p in the yeast two-hybrid system and similar interactions have been shown between WASp and a mammalian End5p homologue, WASp-interacting protein (WIP) [5]. As *las17Δ* deletion mutants are blocked in endocytosis, we conclude that Las17p and End5p interact and are essential for endocytosis.

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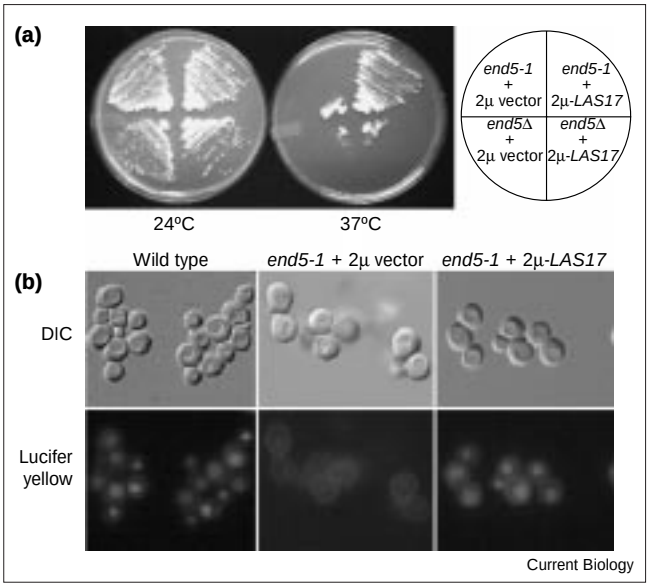
Results and discussion

To identify genes that can, in high copy number, suppress the temperature-sensitivity of *end5-1* cells, we transformed these cells (AMY66) with a high copy number (2μ) yeast genomic library and selected for loss of temperature-sensitivity. All of the suppressor plasmids contained either *END5* or *LAS17*/*BEE1* (Figure 1a). The smallest *LAS17* insert to have suppressor activity contained no other open reading frame. *LAS17* encodes the yeast homologue of a human protein defective in patients with Wiskott–Aldrich Syndrome [6]. In these patients, haematopoietic cells have a defective cortical actin cytoskeleton [7]. WASp is a proline-rich protein that interacts with Cdc42p, a low molecular weight GTPase important in regulating the cortical actin cytoskeleton [8–10].

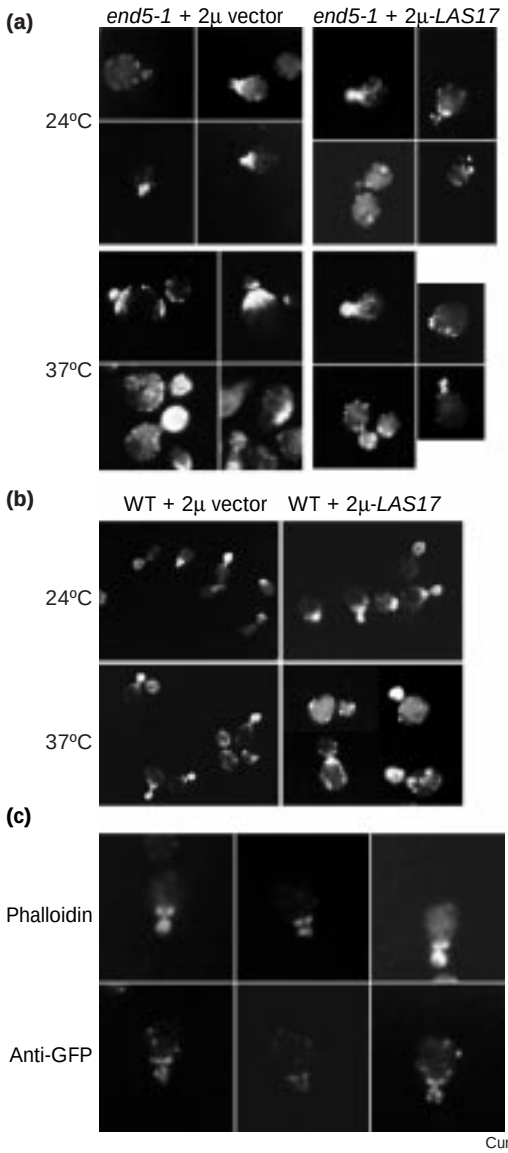
The 2μ-*LAS17* plasmid (pAM155) suppressed the temperature-sensitivity of *end5-1* cells (RH2075), but a centromeric plasmid containing *LAS17* (cen-*LAS17*, pAM161) did not have this ability. We tested the ability of 2μ-*LAS17* to suppress the temperature-sensitivity of mutants defective in actin (*act1-1*, RH2150), fimbrin

Figure 1



(a) The 2μ-*LAS17* plasmid suppresses the temperature-sensitivity of *end5-1*, but not *end5Δ*, cells. Ability to form colonies at 37°C was tested for *end5-1* cells (RH2075) or *end5Δ* cells (RH2892) transformed with the 2μ vector (YEplac181) or with the same plasmid containing *LAS17* (2μ-*LAS17*, pAM155). (b) The 2μ-*LAS17* plasmid suppresses the fluid-phase endocytosis defect of *end5-1* cells. Accumulation of the endocytic marker dye lucifer yellow in the vacuole at 37°C was assayed in *end5-1* cells transformed with the 2μ vector or with 2μ-*LAS17*. The same field of cells is shown with differential interference contrast (DIC) optics to highlight the vacuole (appears as an indentation) and with fluorescein isothiocyanate (FITC) fluorescence optics to visualise lucifer yellow.

Figure 2



(a) The 2μ-*LAS17* plasmid does not restore a polarised distribution of cortical actin patches to *end5-1* cells. The distribution of F actin in *end5-1* cells transformed with the 2μ vector or with 2μ-*LAS17* was visualised by staining fixed cells with rhodamine-labelled phalloidin. Cells were kept at 24°C or shifted to 37°C for 2 h before fixation. Note that the abnormal cell size seen for *end5-1* cells after shift to 37°C is corrected by 2μ-*LAS17*, even though a polarised distribution of cortical actin patches is not restored. (b) The 2μ-*LAS17* plasmid disrupts the polarised distribution of cortical actin patches in wild-type (WT) cells. The distribution of F actin in wild-type cells (RH1800) carrying the 2μ vector or carrying 2μ-*LAS17* was examined as in (a). **(c)** End5p–GFP localises to some, but not all, cortical actin patches. Shown are *end5-1* cells expressing a partially functional End5p–GFP fusion protein from a centromeric plasmid (pAM162) double-stained with rhodamine-conjugated phalloidin and with rabbit anti-GFP antibodies and FITC-conjugated goat anti-rabbit IgG secondary antibodies. Cells were visualised using rhodamine-specific (phalloidin, upper panels) or FITC-specific (anti-GFP, lower panels) light filters, respectively. A few of the End5p–GFP patches do not colocalise with cortical actin patches.

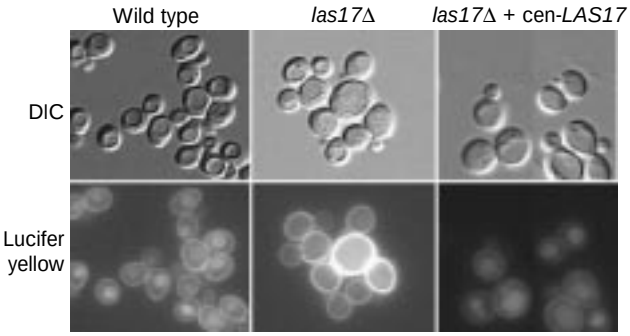
(*sac6Δ*, RH2651), and the products of the *SLA1* (*sla1Δ*, RH2546), *END3* (*end3-1*, RH1623; *end3Δ*, RH1995), and *END4/SLA2* (*end4-1*, RH1597; *end4Δ*, RH1965) genes, all of which are required for endocytosis ([11–14]; A. Wesp, H. Riezman, unpublished data). In addition, we tested for suppression of the temperature-sensitivity of *cdc42-1* (WAY299) [15], because mammalian WASp binds mammalian Cdc42p. Suppression of temperature-sensitivity was complete for *end5-1*, weak for *end3-1*, and absent for the other mutants tested; 2μ-*END5* (pAM160) was also found to be unable to suppress temperature-sensitivity for any of the above mutants except *end5-1* (and *end5Δ*, see below). Using the endocytic marker dye lucifer yellow, 2μ-*LAS17* was shown to be able to restore endocytosis to *end5-1* cells at 37°C (Figure 1b), but not at 24°C (data not shown).

To test whether the cytoskeletal defect of *end5-1* cells could be corrected by *LAS17*, *end5-1* cells bearing 2μ-*LAS17* were grown at 24°C and then either left at 24°C or shifted to 37°C. F actin was visualised by staining with rhodamine-labelled phalloidin (Figure 2a). Although the abnormal size of *end5-1* cells at 37°C was corrected by 2μ-*LAS17*, there was no obvious change in cortical actin patch distribution at 24°C or 37°C. Some abnormalities within cortical actin patches in *end5-1* mutants may have been corrected without a restoration of normal localisation. When the same experiment was carried out on wild-type cells carrying 2μ-*LAS17* (Figure 2b), the distribution of cortical actin patches was found to be perturbed in a temperature-dependent manner, and this effect of *LAS17* may explain the lack of suppression seen in Figure 2a.

As End5p and Las17p are both unusually proline-rich, we tested whether overexpression of Las17p can functionally replace End5p. Neither temperature-sensitivity nor endocytosis were corrected in *end5Δ* cells transformed with 2μ-*LAS17* (Figure 1a and data not shown), suggesting that the correction of defects in *end5-1* cells relies on residual activity of mutant End5-1p. Characterisation of the *end5-1* gene showed that it has a frameshift mutation after codon 604 (of 818), leading to mistranslation for 146 codons before termination.

Las17p has been shown to localise to cortical actin patches [6]. To determine the localisation of End5p, we used the gene for green fluorescent protein (GFP) fused to the last codon of intact *END5*. When carried on a centromeric plasmid this *END5–GFP* construct is partially functional (see Materials and methods; data not shown). End5p–GFP can be seen in live cells in patches that are concentrated in small buds and putative bud sites like cortical actin patches (data not shown). In double-labelling immunofluorescence experiments, colocalisation of End5p–GFP and F actin to some, but not all, cortical patches was observed (Figure 2c).

Figure 3



LAS17 is essential for fluid-phase endocytosis in yeast. Lucifer yellow accumulation assays were performed at 24°C on wild-type cells (lDY19) and on an isogenic *las17Δ* strain (lDY166) transformed with a centromeric vector (YCplac111) or with the same plasmid containing *LAS17* (cen-*LAS17*, pAM161). The same fields of cells are shown with DIC optics to visualise the vacuoles (upper panels) and with FITC fluorescence optics to visualise lucifer yellow (lower panels).

Deletion of the *LAS17* gene was shown to block fluid-phase endocytosis (Figure 3). Like *end5* cells, *las17Δ* cells are temperature-sensitive (data not shown) and the 2μ-*END5* construct did not suppress their temperature-sensitivity. To determine the phenotype of *end5Δ las17Δ* cells, we crossed a *las17Δ* strain to an *end5Δ* strain and isolated recombinant haploid spores. Whereas *las17Δ* and *end5Δ* spores formed colonies at 24°C, the *las17Δ end5Δ* spores germinated but formed only microcolonies (see Supplementary material published with this article on the internet), showing that some functions of End5p and Las17p in growth are redundant.

Recently, a human gene encoding a proline-rich WASp-interacting protein (WIP) was isolated using a yeast two-hybrid screen [5]. WIP and End5p share a short stretch of high homology in their amino-terminal domains (in the putative actin-binding motif) and some homology in the remainder of the proteins [5] (see Supplementary material). Testing WIP deletions for interaction with WASp revealed that the carboxy-terminal 126 residues of WIP are required for binding to WASp [5]. WIP could be a human homologue of End5p. If so, it is interesting that the region missing in *end5-1* shows some homology to that region in WIP which is required for interaction with WASp. It is possible that the mutant protein encoded by *end5-1* interacts poorly with Las17p and overexpression of Las17p is necessary to produce sufficient interaction. We tested whether full-length Las17p and End5p interact in the yeast two-hybrid system [16] and found that they interact strongly. We then tested interactions between different parts of Las17p and End5p and showed that, in an analogous manner to WIP and WASp, the amino terminus of Las17p interacts with the carboxyl terminus of End5p (Table 1).

Table 1

The carboxyl terminus of End5p interacts with the amino terminus of Las17p.

Activation domain fusion	Binding domain fusion	β-gal activity in Miller units
End5p 1–816	–	0.13
Las17p 2–266	–	0.079
–	End5p 480–816	0.11
–	Las17p 2–633	0.15
End5p 1–816	Las17p 2–633	80.08
Las17p 2–266	End5p 480–816	146.05

Interaction between End5p and Las17p was tested using the yeast two-hybrid system. β-galactosidase (β-gal) activity was assayed to assess the strength of the interaction. Data shown here represent an average of three independent assays.

We have identified *LAS17* as a gene which, in high copy number, can suppress the growth defects and endocytosis defect of *end5-1* mutants. We suggest that End5p and Las17p form a complex that plays a crucial role in endocytosis based on the following observations: Las17p and End5p both localise to cortical actin patches; Las17p and End5p are each required for endocytosis; suppression by 2μ-*LAS17* relies on residual End5-1p function; and Las17p interacts with End5p in the yeast two-hybrid system. By analogy to the interaction of human WASp with WIP, interaction of Las17p and End5p may normally involve interaction of the amino-terminal domain of Las17p with the carboxy-terminal domain of End5p.

Materials and methods

Strains, growth conditions, genetic methods, and manipulation of DNA

Standard methods and media for yeast growth, sporulation and tetrad analysis were used [17]. All DNA manipulations were as described [18]. AMY66 is a haploid derived from a cross of RH2075 to the wild-type *ade2* strain RH2878. The high-copy-number suppressor screen involved transformation of a yeast genomic DNA library (a gift of M.I. Geli, A. Wesp and H. Riezman, University of Basel) carried in the *ADE2* 2μ vector pASZ12 (a gift of P. Linder, University of Geneva) into AMY66 and selection for growth at 37°C on SD selective medium. Plasmids present in the resulting colonies were isolated, amplified in *Escherichia coli* and retransformed into AMY66 to confirm that suppression is conferred by the plasmid.

Plasmid construction

A 3 kb *Bam*HI–*Sph*I fragment (derived from the smallest library clone, pAM150) which contains the promoter and coding region of *LAS17* was subcloned into the centromeric vector YCplac111 [19] and the 2μ-based vector YEplac181 [19] to create plasmids pAM161 and pAM155, respectively. The *las17Δ::LEU2* and *las17Δ::URA3* alleles have the DNA sequence between base pairs 42 and 1863 of the *LAS17* open reading frame replaced by *LEU2* or *URA3*, respectively. The *end5Δ::URA3* allele has the entire open reading frame of *END5* from the upstream *Not*I site to the downstream *Nco*I site replaced by *URA3*.

We constructed an *END5–GFP* gene fusion as follows: the S65T mutant *GFP* gene [20] (provided by R. Tsien, HHMI, UC San Diego) was cloned into YCplac111 to yield pAM158. The *END5* gene (lacking its termination codon) was then ligated in-frame and upstream of the *GFP* gene in pAM158 to obtain pAM162, which complements the temperature-sensitivity of *end5-1* (RH2075) but not *end5Δ* (RH2892), while pAM158 complements neither.

2μ-*END5* (pAM160) was made by subcloning a 3.4 kb *Hin*dIII–*Bam*HI fragment containing the promoter and entire coding region of *END5* from pEND5.1 into YEplac181.

Microscopy

Fluorescence microscopy was performed using a Leica DMLB fluorescence microscope and images were captured either digitally, using an Optronix DEI-470T cooled charge-coupled device camera (Leica Pte. Ltd, Singapore), or on Kodak TMAX-400 film, using a 35 mm film camera. Cells were fixed with formaldehyde for 30 min at the temperature of growth before staining. F actin was visualised by staining with rhodamine–phalloidin (Sigma) as described [21].

For immunofluorescence, *end5-1* cells (RH2075) transformed with pAM162 were grown in SD medium at 24°C, fixed, and stained with rabbit polyclonal anti-GFP antibodies (a gift from J. Kahana and P. Silver, Dana Farber Cancer Institute, Boston, MA) followed by FITC-conjugated donkey anti-rabbit IgG antibodies (Jackson Immunoresearch Laboratories) and rhodamine–phalloidin, using standard procedures. Stained cells were visualised using FITC- and rhodamine-specific light filters.

Lucifer yellow uptake assay

Assays for measuring fluid-phase endocytosis of Lucifer yellow were performed at 24°C or 37°C as described [22].

Yeast two-hybrid interaction test

Yeast two-hybrid interactions [16] were measured using the method and kit supplied by Clontech Laboratories.

Supplementary material

Additional materials and methods, the tables of yeast strains and plasmids used in this study, the genetic data showing synthetic lethality of the *end5Δ* and *las17Δ* mutations, and the amino-acid sequence comparison of End5p and WIP are published with this article on the internet.

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Supplementary material

The WASp homologue Las17p functions with the WIP homologue End5p/verprolin and is essential for endocytosis in yeast

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Materials and methods

Construction of the END5–GFP *fusion*

The primers used for amplifying the *GFP* gene were 5′GFPC-*Bam*HI (5′-GATAGGATCCATGAGTAAAGGAG-3′) and 3′GFP-*Sma*I (5′-GCGCGCCCCGGGTCATTTGTATAGTTCATCCAT-3′). Amplification with these primers creates a *Bam*HI site immediately before the GFP start codon. The *Bam*HI–*Sma*I fragment was ligated into YCplac111 to make pAM158. A *Hin*dIII–*Sal*I fragment derived from pEND5.1 provided the 5′ upstream regulatory sequences and amino-terminus-encoding half of the *END5* open reading frame. The primers used for amplifying the carboxyl-terminus-encoding half of the *END5* open reading frame were E5F2 (5′-GCTCCGCCTCCACCACCAC-3′) and E5C *Bam*HI (5′-ATGGAGGGATCCTCGCGTAAAT-3′). Amplification using these primers removes the termination codon of *END5* and adds a *Bam*HI site at this position. The fragment encoding the carboxyl terminus of *END5* was digested with *Sal*I and *Bam*HI and ligated with a *Hin*dIII–*Sal*I fragment (derived from pEND5.1) encoding the amino terminus of *END5* to reconstruct a complete *END5* gene lacking only the termination codon. This was then ligated with pAM158 cut with *Hin*dIII and *Bam*HI to place *END5* upstream of, and in frame with, the *GFP* gene.

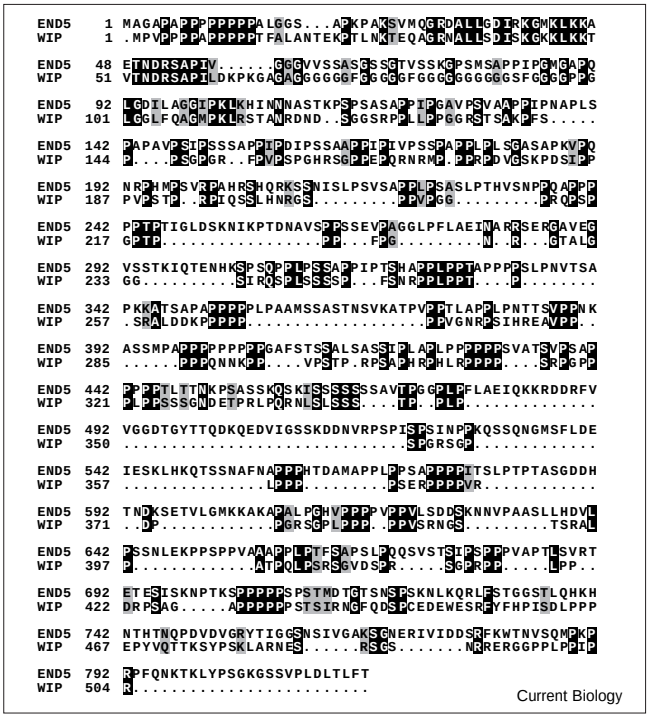
Sequence of end5-1

The *END5* and *end5-1* genes were amplified from wild-type (RH1800) and *end5-1* (RH2075) cells, respectively, using *Pfu* polymerase (Stratagene). Primers used to amplify the genes were END5 N (5′-AACCGTTTGTTGAAGAAGCCTACC-3′) and END5 C (5′-TCACAGCGAAAAGGACGGGAAGCT-3′). The PCR products were cloned into the pCR-script SK+ cloning vector (Stratagene). Several different clones of *END5* and *end5-1* were sequenced using an Omni base Sequencing Kit (Promega).

Yeast two-hybrid interaction test

Activation domain fusion constructs pAM173 (End5p 1–816 in pB42AD) and pAM174 (Las17p 2–266 in pB42AD) and LexA DNA-binding domain fusion constructs pAM175 (End5p 480–816 in pLexA) and pAM178 (Las17p 2–633 in pLexA) were used to transform EGY48 individually and in combinations. The DNA fragments contained in the two-hybrid fusion constructs were obtained by PCR amplification of the fragments using primers containing appropriate restriction sites.

Figure S1



End5p shows amino-acid sequence homology to WIP, a human WASp-interacting protein. An alignment is shown between the amino-acid sequences encoded by *S. cerevisiae END5*/*VRP1* (Genbank accession number X87806) and human *WIP* (AF031588). Identical residues are shaded in black and conservative substitutions in grey.

Table S1

The *end5Δ* and *las17Δ* mutations exhibit synthetic lethality.

Tetrad class	Parental ditype	Tetratype	Non-parental ditype	Total
Phenotype of spores	2 Leu^+ Ura^- 2 Leu^- Ura^+	1 Leu^+ Ura^- 1 Leu^- Ura^+ 1 Leu^- Ura^- 1 inviable	2 Leu^- Ura^- 2 inviable	
Genotype of spores	2 *las17Δ END5* 2 *LAS17 end5Δ*	1 *las17Δ END5* 1 *LAS17 end5Δ* 1 *LAS17 END5* 1 *las17Δ end5Δ* (inviable)	2 *LAS17 END5* 2 *las17Δ end5Δ* (inviable)	
Number of tetrads	9	20	9	38

An *end5Δ::URA3* strain (RH2893) was crossed with a *las17Δ::LEU2* strain (lDY223) and the resulting diploid (AMY25) was sporulated. A total of 38 tetrads were dissected and the genotypes of the spores scored.

Table S2

Yeast strains used in this study.

Name	Genotype	Source
AMY25	*MATα /MAT**a** his3/HIS3 his4/HIS4 leu2/leu2 ura3/ura3 lys2/LYS2 trp1/TRP1 las17Δ::LEU2/LAS17 end5Δ::URA3/END5 bar1/BAR1*	This study
AMY66	*MATα his3 his4 leu2 ura3 ade2 end5-1 bar1*	This study
RH1800	*MAT**a** his4 leu2 ura3 bar1*	H. Riezman
RH1597	*MAT**a** his4 leu2 ura3 end4-1 bar1*	H. Riezman
RH1623	*MAT**a** his4 leu2 ura3 end3-1 bar1*	H. Riezman
RH1965	*MAT**a** his4 leu2 ura3 lys2 end4Δ::LEU2 bar1*	H. Riezman
RH1995	*MAT**a** his4 leu2 ura3 end3Δ::URA3 bar1*	H. Riezman
RH2075	*MATα his4 leu2 ura3 end5-1 bar1*	H. Riezman
RH2076	*MAT**a** his4 leu2 ura3 trp1Δ::URA3 end5-1 bar1*	H. Riezman
RH2150	*MAT**a** his4 leu2 lys2 act1-1 bar1*	H. Riezman
RH2546	*MAT**a** ura3 lys2 sla1Δ::URA3 bar1*	H. Riezman
RH2651	*MATα his3 leu2 ura3 trp1 sac6Δ::URA3 (bar1?)*	H. Riezman
RH2878	*MAT**a** his3 leu2 ura3 lys2 ade2 bar1*	H. Riezman
RH2892	*MAT**a** his4 leu2 ura3 lys2 end5Δ::URA3 bar1*	H. Riezman
RH2893	*MATα his4 leu2 ura3 lys2 end5Δ::URA3 bar1*	H. Riezman
WAY299	*MATα his3 leu2 ura3 lys2 trp1 cdc42-1 bar1Δ::LYS2*	A. Wesp
lDY19	*MAT**a** his3 leu2 ura3 trp1 mfa2Δ:: FUS1:lacZ*	G. Sprague
lDY166	*MAT**a** his3 leu2 ura3 trp1 las17Δ::URA3*	This study
lDY223	*MAT**a** his3 leu2 ura3 trp1 las17Δ::LEU2*	This study
EGY48	*MATα his3 ura3 trp1 $LexA_{op(x6)}$-LEU2*	Clontech

Table S3

Plasmids used in this study.

Name	Description	Source
pAM150	Library clone: pASZ12 containing *LAS17*	This study
pASZ12	2μ-containing *ADE2* vector	P. Linder
YEplac181	2μ-containing *LEU2* vector	[19]
YCplac111	*ARS-CEN* containing *LEU2* vector	[19]
pEND5.1	*END5* in YCplac111	[3]
pCR-script SK+	Ap^R cloning vector	Stratagene
pEND5	*END5* carried in pCR-Script SK+	This study
pend5-1	*end5-1* carried in pCR-Script SK+	This study
pAM155	*LAS17* under its own promoter in YEplac181	This study
pAM158	*GFP* in YCplac111	This study
pAM160	*END5* under its own promoter in YEplac181	This study
pAM161	*LAS17* under its own promoter in YCplac111	This study
pAM162	*END5* fused with *GFP* in pAM158	This study
pLexA	Cloning vector for DNA-binding domain fusion constructs	Clontech
pB42AD	Cloning vector for activation domain fusion constructs	Clontech
pAM173	pB42AD expressing $End5p_{1–816}$	This study
pAM174	pB42AD expressing $Las17p_{2–266}$	This study
pAM175	pLexA expressing $End5p_{480–816}$	This study
pAM178	pLexA expressing $Las17p_{2–633}$	This study