Yeast
Yeast 2007; **24**: 761–766.
Published online 28 June 2007 in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/yea.1511

Research Article

Yeast vectors for the integration/expression of any sequence at the *TYR1* locus

Mario G. Mirisola*, Leonarda Colomba, Alessia Gallo, Roberta Amodeo and Giacomo De Leo
Dipartimento di Biopatologia e Metodologie Biomediche, Università degli Studi di Palermo, Italy

**Correspondence to:*
Mario G. Mirisola,
Via Divisi 83,
Palermo 90133,
Italy.
E-mail: mirisola@unipa.it

Abstract

We have constructed new yeast vectors for targeted integration and conditional expression of any sequence at the *Saccharomyces cerevisiae TYR1* locus which becomes disrupted. We show that vector integration is not neutral, causing prototrophy for tyrosine and auxotrophy for the vector's selectable marker (uracil or leucine, depending on the vector used). This feature allows a double screening of transformed yeast cells, improving the identification of colonies with the desired chromosomal structure. The *GAL10* gene promoter has been added to drive conditional expression of cloned sequences. Using these vectors, chromosomal structure verification of recombinant clones is no longer necessary, since the noise of non-homologous recombination, as well as spontaneous reversion of the selected phenotype, can easily be identified. The ability of the vector to conditionally control gene expression has been confirmed using the gene for the green fluorescent protein (GFP) as a reporter. GenBank Accession Nos EF202083–202086. Copyright © 2007 John Wiley & Sons, Ltd.

Received: 22 January 2007
Accepted: 14 May 2007

Keywords: yeast integration vector; *TYR1* locus; expression vectors; heterologous expression; ends-out gene targeting

Introduction

The yeast *Saccharomyces cerevisiae* is a widely used eukaryotic model organism. It has been demonstrated to be one of the most valuable model systems for the understanding, among other things, of cell cycle control (Hartwell, 2002), protein trafficking (Bowens and Stevens, 2005; Nakano, 2004), protein folding and misfolding (Coughlan *et al*., 2005; Lindquist, 2002) and, more recently, ageing and neurosciences (Longo, 2004; Kaeberlein *et al*., 2001; Tissenbaum *et al*., 2002; Sherman and Muchowsky, 2003). Most genetic screens, in all these research fields, manipulate the yeast genome by removing or introducing DNA sequences. A Sequence Of Interest (SOI) may be introduced in different ways, but chromosomal gene targeting is surely one of the preferred choices. Stable integration does not need selective media and copy number cannot fluctuate either during growth or in comparing different experiments (Schneider and Guarente, 1991). In addition, stable recombinant yeast cells with different relevant genotypes may be crossed and the resulting new genetic combinations studied, e.g. for synthetic lethality. Integration of SOI is often accomplished by cloning the SOI into one of the yeast integrative plasmid (YIp) families and using a procedure known as 'ends-in' gene targeting (Langston and Symington, 2004; Hastings *et al*., 1993). This procedure, based on recombination between the vector selectable marker and its homologous chromosomal sequence, generates a duplication of the marker, whose wild-type copy restores the auxotrophic phenotype. Unfortunately, these repeated sequences may eject the integrated SOI by homologous intrachromosomal recombination. The uninterrupted selective pressure for the marker may help to solve the problem, but there is still the possibility of a recombination event that eliminates the

SOI but leaves a wild-type copy of the marker. Furthermore, the chromosomal structure of the target locus must be confirmed by Southern blotting or PCR, since random integration of the SOI within the yeast genome is always a possible unintended consequence.

However, gene replacement has been improved with a procedure called 'ends-out'. This is a three-step method. First, the target sequence is cloned in a bacterial vector. Second, the main internal part of the cloned sequence is substituted by a selectable marker, generating a vector containing the selectable marker flanked on both sides by two small fragments of the target sequence. Finally, the vector is cut in order to release and purify the selectable marker flanked by at least 40–50 bp of the target sequences. This fragment is called a recombination cassette, since it can direct the integration of the selectable marker within the chromosomal sequence homologous to the vector's target sequences. This procedure (Langston and Symington, 2004; Hastings *et al*., 1993) generates the integration of the marker within the chromosomal target sequence but does not create sequence duplications, and thus increases the chromosomal stability of integrants.

Yeast integrative vectors for general use have so far been based on the ends-in strategy, with all the limitations mentioned above. Voth *et al*. (2001) have reported the construction of some general yeast vectors capable of driving ends-out integration at the *HO* locus. Unfortunately, since these vectors lack a yeast promoter, they can only be used to integrate yeast genes along with their own promoter. In addition, integration at the *HO* locus is neutral and correct integrants must be identified by Southern blotting or PCR.

We report here the construction of new yeast vectors capable of targeting any SOI onto the *TYR1* locus. The integration procedure is based on the ends-out strategy and does not generate tandem duplication. In addition, since integration provokes deletion of the *TYR1* coding sequence (tyrosine prototrophy), correct integrants may be easily identified by replica-plating with no need to further verify their chromosomal structure. Finally, the presence of the *GAL10* yeast promoter drives the conditional expression of homologous/heterologous genes, allowing easy detection of toxic or lethal effects.

Materials and methods

Plasmids construction

The *TYR1* locus was obtained by polymerase chain reaction (PCR) of yeast genomic DNA, using oligonucleotides F100 and R100 (for DNA sequences, see Table 1). Both oligonucleotides contain an *Eco*RV restriction site to facilitate cloning (bold letters in Table 1). G nucleotide was added to both the forward and reverse oligos to create a *Sal*I restriction site necessary for subsequent manipulation (italic letters in Table 1).

Plasmid *pTyr* was constructed by replacing the 322 bp *Pvu*II fragment of the *pUC19* vector, containing the whole polylinker, with the 2023 bp PCR fragment containing the *TYR1* locus previously digested by *Eco*RV.

Plasmid *pINTyrA* was obtained by substituting the 1515 bp *Stu*I fragment of the *pTYR1* vector with the *SUP16* selectable marker.

The *GAL10* gene promoter was obtained by two subsequent PCRs. In the first reaction, 100 ng DNA of the *YEp51* shuttle vector (Rose and Broach, 1991) was amplified using the oligonucleotides F101 and R101 (for DNA sequence, see Table 1). In the second reaction, 100 ng of the first amplification product was amplified using the oligonucleotides F101 and R101b to insert a polylinker (for DNA sequence, see Table 1). A C nucleotide, non-homologous to the target sequence (for DNA sequence, see Table 1), was added to the F101 oligo to create, after ligation with the *Stu*I-digested *pTYR1* vector, a *Bam*HI site necessary for the subsequent manipulations. Bold letters in Table 1

Table 1. List and DNA sequence of oligonucleotides used

F100	5′-ggc**gatatc***g*TCGACATGGAAAGTGATGTT
R100	5′-ggc**gatatc***g*TCGACTTTATGACCAAAAACT
F101	5′-gc**gatatc***c*TCAAAAATCATCGCTTCGC
R101	5′-**gaattctcgaG**CAAAAATTCTTACTTTTTTTTTGGA
R101b	5′-ggatatc**aagcttcccgggGAATTCTCGAG**CAAAAA
GFP–RT	5′-TGGGTGCTCAGGTAGTGGTTGTCG
GFP–Fw	5′-CCACCTACGGCAAGCTGACCCT
GFP–Rev	5′-TGCCGTCCTCCTTGAAGTCGAT

For all oligonucleotides, capital letters indicate the homologous region, while lower case letters indicate a sequence inserted by the oligonucleotides. Bold letters indicate restriction sites inserted by the oligonucleotide. *g* in primers F100 and R100 fused to TCGAC homologous sequence creates a *Sal*I restriction site; *c* in primer F101 creates a *Bam*HI restriction site after *Eco*RV digestion and ligation to a *TyrL–StuI* fragment.

DOI: 10.1002/yea

indicate nucleotides inserted to create an *Eco*RV restriction site.

The *GAL10* gene promoter obtained by the above-mentioned PCRs was TA-cloned onto the *PCR2.1* vector (Invitrogen, Carlsbad, CA, USA), generating the *PCR2.1GAL* vector. Positive colonies were identified by colony PCR and confirmed by restriction analysis and automated DNA sequencing.

Plasmid *pINTyr* was constructed by substituting the 1515 bp *Stu*I fragment of the *pTYR1* vector with the 543 bp *Eco*RV fragment of the *PCR2.1GAL* vector containing the *GAL10* gene promoter. Plasmid *pINTyrB* was obtained by inserting the 2216 bp *LEU2 Xho*I/*Sal*I DNA fragment from the *Yep13* vector (Rose and Broach, 1991) within the *Bam*HI site of the *pGALTYR* vector. The *LEU2* fragment and the *pGALTYR* vector were partially filled before ligating in the presence of C/T and G/A nucleotides, respectively, to obtain compatible cohesive ends.

Plasmid *pINTyrC* was obtained inserting the 1355 bp *URA3 Xba*I DNA fragment from the *pUC19URA3* vector within the *Bam*HI site of the *pINTyr* vector. The *URA3* fragment and the *pGALTYR* vector were partially filled before ligation in the presence of C/T/A and G/T/A nucleotides, respectively, to obtain compatible cohesive ends.

Plasmid *pINTyr*D was obtained inserting the 772 bp *Xba*I/*Sal*I DNA fragment from the vector *pEGFP-N1* (Clontech Laboratories, Mountain View, USA), containing the complete coding sequence of the *GFP* gene, within the *Hin*dIII/*Xho*I site of the *pINTyrB* vector. *Xba*I and *Hin*dIII restriction sites had been previously filled in with the C/T and G/A nucleotides, respectively.

Yeast relevant genotypes and manipulation

AAT3B: a ade2 can1-100 CRI4 his3 leu2-3,112 lys1-1 ras1Δ̇ ras2::URA3 URA3-52 (Mirisola *et al*., 1994). *F4A2U6: a ade2 can1-100 CRI4 his3 leu2-3,112 lys1-1 ras1Δ̇ ras2::URA3 URA3-52* (Mirisola *et al*., 1994). Rich and selective media were as described by Sherman (2002). Standard media with a carbon source different from glucose were obtained by using 2% galactose in place of glucose. Genetic manipulation of yeasts was as described by Mortimer and Schild (1981). Yeast transformations were carried out as described by Ito *et al*. (1983).

Yeast genomic DNA extraction

The yeast strain of interest was incubated on the appropriate medium until saturation (usually 24 h). Cells, harvested by centrifugation, were washed twice with water and the pellet, resuspended in 0.05 volumes of lysis buffer [0.1 M Tris–Cl, pH 8.0, 0.05 M ethylenediaminotetracetate (EDTA)–1% sodium dodecyl sulphate (SDS)], was added to 0.05 volumes of acid-washed glass beads (212–300 μm mesh; Sigma–Aldrich, Milan, Italy). The mixture was vortexed for 30 s and NaCl was added to a final concentration of 0.250 M. After vortexing, the phases were separated by centrifugation at $9000 \times g$ for 8 min. The aqueous phase was phenol : chloroform-extracted and the resulting supernatant was ethanol-precipitated. After a 70% ethanol wash, the pellet was finally resuspended in 20 μl water. Genomic DNA was further purified using a commercial kit (GFX PCR DNA and gel band purification, GE Healthcare, Little Chalfont, UK) before being subjected to further manipulations.

RNA preparation

The desired yeast strain was grown at 30 °C on the appropriate selective glucose-based medium to $OD_{560nm} = 0.8/1$. The cells were collected by centrifugation and resuspended in the same medium, except for induced cultures, in which galactose replaced glucose at the same concentration and which were cultured at 30 °C for an additional 24 h. After centrifugation, the pellet, resuspended in acetate buffer (50 mM sodium acetate, 10 mM EDTA, pH 5), was vortexed for 5 min at 65 °C in the presence of one volume of acid-washed glass beads (212–300 μm mesh) and one volume of phenol, pH 5. The aqueous phase was extracted twice with chloroform and RNA was finally precipitated with 2.5 volumes of absolute ethanol, 0.3 M sodium acetate, pH 5.5. After centrifugation, the pellet was resuspended in 10 mM Tris. C1, 1 mM EDTA, pH8 (TE 10 : 1). The RNA quality was verified by evaluating the ribosomal RNA appearance after gel electrophoresis in denaturing conditions. Poly(A)$^+$ RNA was obtained using a commercially available kit (QuickPrep™ Micro mRNA Purification Kit, GE Healthcare), according to the manufacturer's instructions.

DOI: 10.1002/yea

RT–PCR

1 μg poly(A)$^+$ RNA was subjected to GFP-specific retrotranscription (RT) with the GFP–RT primer (for DNA sequence, see Table 1) in the presence of 1 μl Improm reverse transcriptase (Promega Corp., Madison, WI, USA) at 42 °C for 1 h, according to the manufacturer's instructions. Different amounts of cDNA were used for the subsequent PCR. PCR of cDNA was performed using the GFP–Fw and GFP–Rev primers indicated in Table 1 under the following conditions: 94 °C for 5 min, followed by 30 cycles of: 94 °C for 30 s; 58 °C for 30 s; 72 °C for 1 s.

Results and discussion

Disrupting the *TYR1* locus: resulting phenotype

As a first step, we engineered a disruption vector targeted to the *TYR1* locus. The chromosomal *TYR1* gene was obtained by PCR cloning (for details, see above and Table 1). The *TYR1* amplicon contained a *Sal*I restriction site at both extremities, created by adding, during PCR, a G nucleotide to a TCGAC sequence occurring within the natural *TYR1* sequence (see primers F100 and R100 of Table 1). The amplicon was cloned onto a modified *Escherichia coli pUC19* vector lacking the whole polylinker to generate the *pTYR1* vector (not shown). After cloning, the described *Sal*I restriction sites were used to recover the *TYR1* DNA fragment (recombination cassette), leaving at both extremities only naturally occurring, and thus highly recombinogenic, *TYR1* sequences. In order to use this construct for targeted disruption of the *TYR1* locus, the vector's *Stu*I fragment, containing the whole coding sequence of the cloned *TYR1*, was substituted with the SUP16 selectable marker to generate the *pINTyr*A vector shown in Figure 1. This disruption vector contained two small (278 and 234 bp) *TYR1* homologous regions (respectively TL and TR in Figure 1) surrounding a selectable marker (SUP16) and was capable of directing ends-out gene disruption of the *TYR1* locus.

To confirm the previously identified tyrosine prototrophy subsequent to *TYR1* mutations (Mannhaupt *et al.*, 1989) using our *pINTyr*A disruption vector, competent cells of the yeast strains AAT3B and F4A2U6 (with the same relevant genotype but obtained by different crossings; Mirisola *et al.*, 1994) were transformed using the purified *INTyrA* recombination cassette (see Figure 1) obtained after *Sal*I restriction of the *pINTyr*A vector and agarose gel purification of the fragment. Genomic DNA extracted from selected transformants were analysed to check the chromosomal structure of the *TYR1* locus. A mixture of 10 individual clones with the correct chromosomal structure of both strains were replica-plated onto the same medium but lacking tyrosine. As shown in Figure 2, *TYR1* deletion provokes tyrosine prototrophy in both yeast strains, confirming the possibility of using *TYR1* as a selectable marker. The recombinant strains were also assayed in different growth conditions and in the presence of different carbon sources without showing any additional phenotype (results not shown).

Construction of the integrative/expression vector and verification

We took advantage of the above-demonstrated property of the *pINTyr*A vector integration to display two independent phenotypes to create an easy-to-screen family of integration vectors for general usage. The *Stu*I fragment of the *pTYR1* vector, described above, was exchanged with the *Eco*RV fragment containing the GAL10 gene promoter, the latter obtained by two rounds of PCR (see above). Oligonucleotides used for PCR introduced a polylinker at the 3′ end of the *GAL10* gene promoter and, after ligation to the vector, created a unique *Bam*HI restriction site at the 5′ end of the promoter (*pINTyr* vector, not shown). *LEU2* or *URA3* markers were inserted as a *Xho*I/*Sal*I DNA fragment from the *YEp13* vector and *Xba*I DNA fragment from the *pUC19URA3* vector, respectively, and ligated, after partial fill, to the *Bam*HI restriction site to produce *pINTyrB* and *pINTyr*C vectors, respectively (for details, see above and Figure 1). After *Sal*I restriction, the gel-purified recombination cassette from *pINTyrB* and *pINTyr*C vectors were used to transform yeast competent cells of the AAT3B strain. After selection for leucine or uracil auxotrophy, depending on the vector used, transformed cells were replica-plated on a medium lacking tyrosine. In our experiments all clones showing tyrosine prototrophy had the correct chromosomal structure, demonstrating

DOI: 10.1002/yea

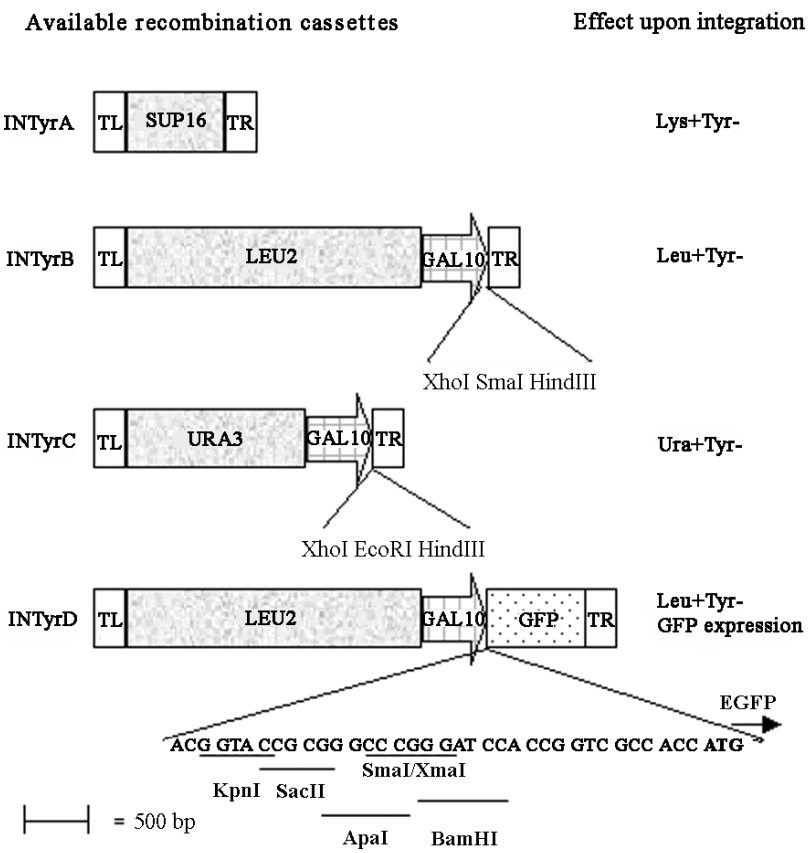

Figure 1. List of all recombination cassettes available. Each recombination cassette may be obtained from the corresponding circular vector by *Sal*I restriction and agarose gel purification

that this vector, thanks to the double selection it drives, facilitates the identification of transformed yeast cells with the desired chromosomal structure.

To test the conditional expression of sequences cloned within our integrative vector, the *Xba*I–*Sal*I fragment of the *pEGFP-N1* vector, containing the complete coding sequence of the green fluorescent protein (GFP) gene, was cloned within the *Hin*dIII–*Xho*I site of the *pINTyrB* vector, generating the *pINTyr*D vector (see Figure 1). After chromosomal integration of the construct, recombinant yeast cells were grown on a glucose-based medium and an aliquot of the culture was transferred to a galactose-based medium to induce the *GAL10*-driven expression of GFP. Total RNA was extracted from both glucose- and galactose-grown cells and subjected to RT–PCR, using oligonucleotides directed against the GFP coding sequence (see Materials and methods). The results, shown in Figure 3, confirm the conditional expression of the GFP cloned in our integrative vector. It is worth noting that *pINTyrD* vector may be used to generate SOI–GFP fusion proteins and to monitor their subcellular compartmentalization.

We therefore conclude that these vectors can improve the ends-out integrative procedure of DNA sequences onto the yeast genome. The presence of a yeast promoter within the recombination cassette allows expression of sequences from every organism. Furthermore, the possibility of conditionally expressing the SOI sequence can be helpful in cases where a toxic or lethal effect is possible. In addition, since the *TYR1* locus is not subjected to chromatin silencing by the known deacetylases Hda1 and Sir2 (Robyr *et al*., 2002), this vector may be useful to free yeast gene expression from this control level. The double selection based on tyrosine prototrophy and leucine or uracil auxotrophy

DOI: 10.1002/yea

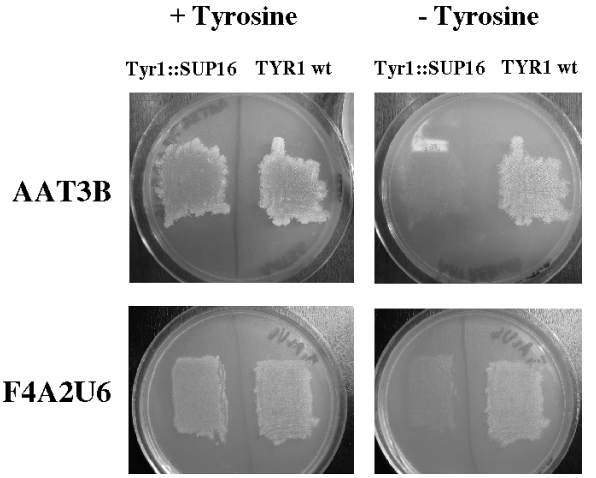

Figure 2. Growth properties of strains with *TYR1* deletion. Yeast competent cells of two different yeast strains (AAT3B and F4A2U6) were transfected with 10 μg *INTyrA* recombination cassette (*TYR1::SUP16*) or with the corresponding empty vector (*TYR1wt*). A pool of 10 selected integrants with the correct chromosomal structure were grown on complete medium (+ tyrosine) and replica-plated on a medium lacking tyrosine (− tyrosine). The plates were photographed after 48 h growth at 30 °C

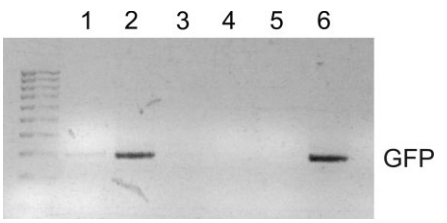

Figure 3. GFP-specific RT–PCR of RNA extracted from different yeast strains grown in different conditions. Total RNA from the strain AAT3B *TYR1::INTyrD* (1–4) grown on glucose (1, 3) or galactose (2, 4), and from the parental strain AAT3B grown on galactose (5), were subjected to RT–PCR (1, 2, 5) or used dierctly (3, 4) for PCR, using GFP-specific oligonucleotides. PCR using *pINTyr*D plasmid DNA as a template (6) was used as a positive control

guarantees an easy identification of correct integrants. It must be noted that, since the recombination cassette can only be released by *Sal*I restriction digestion, the *Sal*I restriction site must be absent within the SOI. Finally, the *pINTyr* vector may be used to clone, within the unique *Bam*HI restriction site, selectable markers different from those used in the present work.

References

Bowers K, Stevens TH. 2005. Protein transport from the late Golgi to the vacuole in the yeast *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **10**(3): 438–454.

Coughlan CM, Brodsky JL. 2005. Use of yeast as a model system to investigate protein conformational diseases. *Mol Biotechnol* **30**(2): 171–180.

Hartwell LH. 2002. Nobel Lecture. Yeast and cancer. *Biosci Rep* **22**(3–4): 373–394.

Hastings PJ, McGill C, Shafer B, Strathern JN. 1993. Ends-in vs. ends-out recombination in yeast. *Genetics* **135**: 973–980.

Ito H, Fukuda Y, Murata K, Kimura A. 1983. Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* **153**(1): 163–168.

Kaeberlein M, McVey M, Guarente L. 2001. Using yeast to discover the fountain of youth. *Sci Ageing Knowl Environ* **1**: 1 [review].

Langston LD, Symington LS. 2004. Gene targeting in yeast is initiated by two independent strand invasions. *Proc Natl Acad Sci USA* **101**: 15392–15397.

Lindquist SL. 2002. Prion proteins: one surprise after another. *Harvey Lect* **98**: 173–205.

Longo VD. 2004. Ras: the other pro-ageing pathway. *Sci Ageing Knowl Environ* **29**(39): 36.

Mannhaupt G, Stucka R, Pilz U, Schwarzlose C, Feldmann H. 1989. Characterization of the prephenate dehydrogenase-encoding gene, *TYR1*, from *Saccharomyces cerevisiae*. *Gene* **85**: 303–311.

Mirisola MG, Seidita G, Verrotti AC, Di Blasi F, Fasano O. 1994. Mutagenic alteration of the distal switch II region of RAS blocks CDC25-dependent signaling functions. *J Biol Chem* **269**(22): 15740–15748.

Mortimer R, Schild D. 1981. *The Molecular Biology of the Yeast Saccharomyces cerevisiae: Life Cycle and Inheritance*, Strathern JL, Jones EW, Broach JR (eds). Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY; 11–26.

Nakano A. 2004. Yeast Golgi apparatus — dynamics and sorting. *Cell Mol Life Sci* **61**(2): 186–191.

Robyr D, Suka Y, Xenarios I, *et al*. 2002. Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. *Cell* **109**: 437–446.

Rose MD, Broach JR. 1991. Cloning genes by complementation in yeast. *Methods Enzymol* **194**: 195–230.

Schneider JC, Guarente L. 1991. Vectors for expression of cloned genes in yeast: regulation, overproduction and underproduction. *Methods Enzymol* **194**: 373–388.

Sherman F. 2002. Getting started with yeast. *Methods Enzymol* **350**: 3–41.

Sherman MY, Muchowski PJ. 2003. Making yeast tremble: yeast models as tools to study neurodegenerative disorders. *Neuromol Med* **4**(1–2): 133–46.

Tissenbaum HA, Guarente L. 2002. Model organisms as a guide to mammalian ageing. *Dev Cell* **2**: 9–19 [review].

Voth WP, Richards JD, Shaw JM, Stillman DJ. 2001. Yeast vectors for integration at the *HO* locus. *Nucleic Acids Res* **29**(12): E59.