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Research Article

An evolvable oestrogen receptor activity sensor: development of a modular system for integrating multiple genes into the yeast genome

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Abstract

To study a gene interaction network, we developed a gene-targeting strategy that allows efficient and stable genomic integration of multiple genetic constructs at distinct target loci in the yeast genome. This gene-targeting strategy uses a modular plasmid with a recyclable selectable marker and a multiple cloning site into which the gene of interest is cloned, flanked by two long regions of homology to the target genomic locus that are generated using adaptamer primers. We used this strategy to integrate into a single yeast strain components of the oestrogen receptor (ER) signalling network, comprising the human ERα and three reporter genes driven by oestrogen response elements (EREs). The engineered strain contains multiple reporters of ligand-dependent receptor signalling, providing sensitive, reproducible, rapid, low-cost quantitative assays of ERα activity in order to screen potential receptor agonists. Further, because two of the ERE-driven reporter genes are required for growth in deficient media, the strain's growth rate — and therefore its fitness — depends on ligand-induced ERα activity. This evolvable oestrogen receptor activity sensor (EERAS) can therefore provide the foundation of a long-term experimental evolution strategy to elucidate ER structure–function relations and ligand-receptor evolution. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: **oestrogen receptor alpha (ERα); gene signalling network; *Saccharomyces cerevisiae*; experimental evolution**

Introduction

To elucidate the function of a gene of interest from a complex organism, the recombinant gene can be cloned and transformed into a well-characterized, manipulable, fast-growing model organism. The budding yeast *Saccharomyces cerevisiae* is a useful host because its genome has been sequenced, extensive yeast genetic tools are available, and it is the most basic eukaryotic model organism in which many mammalian genes are functional. For example, the human oestrogen receptor α (ERα) is non-functional when expressed in bacteria, but in *S. cerevisiae* it retains its native ligand-activated transcriptional activity (Metzger *et al.*, 1988). Single-gene studies provide important but limited information about biological systems, however, because most genes function as part of gene networks, and molecular pathways. *S. cerevisiae* should, in principle, provide the same advantages for characterizing such networks as it does for understanding individual genes.

Only a few yeast strains, however, have been engineered with more than two stably integrated genes (Delneri *et al.*, 2000; Johansson and Hahn-Hagerdal, 2004; Wieczorke *et al.*, 1999). Current cloning and PCR-based gene-targeting strategies are designed to efficiently integrate a single gene

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into the yeast genome, but they are inefficient for engineering strains with multiple gene insertions and/or replacements. For example, yeast integrating plasmids (YIps), (Rothstein, 1991; Sikorski and Hieter, 1989) integrate each gene of interest into an auxotrophic locus, requiring the yeast host be auxotrophic for as many markers as there are recombinant genes to be integrated. In addition, the entire YIp vector is integrated at each locus, leaving large amounts of heterologous DNA in the yeast genome. Targeting vectors with recyclable selectable markers, e.g. the HO_L-*hisG-URA3-hisG-poly*-HO_R plasmid (where *HO* is the homothallic switching endonuclease) do not require multiple yeast auxotrophies, but such vectors can be used only at a single fixed target locus (Voth *et al*., 2001). PCR-based methods use 'adaptamer' primers to amplify a selectable marker while adding regions of homology to the targeted genomic locus; the locus can be replaced by a gene of interest if that gene's sequence is added to the construct by overlap PCR (Reid *et al*., 2002). This technique does not allow marker recycling, so it exhausts an auxotrophic marker with each gene integrated or deleted. In addition, the efficiency and specificity of gene integration are reduced due to reliance on short (35–60 bp) stretches of homology to the genomic target, and the inefficiency of overlap PCR for generating a construct may outweigh the advantages of the 'cloning-free' technique for assembling a construct to be integrated.

To facilitate stable integration of a gene network comprised of multiple genes into a single yeast genome, we designed a novel modular gene-integration strategy. We used this approach to construct a yeast strain containing multiple members of the human ERα signalling network. This network is an ideal candidate for study, due to its biomedical importance and because the complexity and specificity of molecular signalling networks is exemplified by the tight relationships between the receptor, its physiological ligand 17β-oestradiol (E2), and the regulatory regions of target genes. Minute quantities of E2, acting through the ERα, control reproductive development and function, bone maintenance, behaviour, immune regulation and other processes (Brzozowski *et al*., 1997; Nilsson *et al*., 2001). ERα functions as a ligand-dependent transcriptional activator. Ligand binding induces conformational changes that activate ERα, which then dimerizes, translocates to the nucleus and binds to specific DNA sequences called oestrogen response elements (EREs), located in 5′ regulatory regions upstream of responsive target genes (Fox *et al*., 2004; Nilsson *et al*., 2001). The activated ERα then binds a variety of co-activator proteins that facilitate transcription of the ERE-flanked gene.

Although the basic mechanisms of ERα signalling are understood, detailed structure–function knowledge on the interactions between the receptor, various natural and synthetic ligands and the regulatory sequences of target genes remains incomplete. To further characterize the ligand–ERα–ERE signalling network, we constructed and validated an evolvable ERα activity sensor (EERAS) yeast strain, which expresses human ERα and three ERE-driven genes that are rapid, convenient and reliable reporters of transcriptional activity induced by the network. Ligand-dependent ERα-transactivation of one of these reporters, the yeast-enhanced green fluorescent protein (*yEGFP*), can be rapidly and conveniently assayed with a fluorometer and no additional substrates. Expression of the other reporter genes, *ERE-URA3* and *ERE-HIS3*, controls growth rate — and therefore fitness — in medium deficient in histidine and uracil. ERα activation of these reporters can be assayed by visually inspecting culture density using a spectrophotometer. EERAS therefore provides a sensitive, rapid, convenient, low-cost, stable screening tool for identifying environmental and pharmaceutical ERα ligands.

The EERAS strain also provides the foundation for an experimental evolution system to identify adaptive changes that allow ERα to more efficiently bind specific ligands and to elucidate the dynamics of ER–ligand co-evolution. Experimental evolution methods rely on the power of random mutation in large microbial populations to generate variant genotypes and natural selection to isolate those that confer functions of interest by linking those functions to fitness (Bull *et al*., 1997; Lenski and Travisano, 1994; Travisano *et al*., 1995). This strategy has successfully been used to select for and identify adaptive changes in endogenous yeast biochemical pathways, with optimized variants arising after hundreds or thousands of generations in culture (Brown *et al*., 1998; Ferea *et al*., 1999; Francis and Hansche, 1972; Segre *et al*., 2006; Wills and Phelps, 1975; Zeyl, 2005; Zeyl *et al*., 2001). In this paper, we report on the construction, characterization and validation of a strain in which

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fitness is directly linked to ligand-activation of the human ERα. We show that the EERAS system has the characteristics required for convenient ligand screening and long-term experimental evolution.

Materials and methods

Yeast strain, vectors, and reagents

S. cerevisiae strain K20 (*CEN.PK 102-5B, MAT***a***, ura3-52, his3Δ1, leu2-3,112*) and vectors *p403-GPD-ERα, p406-ERE*$_2$*s2-CYC1* (where *CYC1* is iso-1-cytochrome *c*) and *p406-ERE*$_2$*s2-CYC1-yEGFP* were provided by Toine F. H. Bovee (RIKILT, Institute of Food Safety, Wageningen, The Netherlands) (Bovee *et al.*, 2004b). *p403-GPD-ERα* contains the human *ERα* under the control of the constitutive glyceraldehyde 3-phosphate (*GPD*) promoter. *p406-ERE*$_2$*s2-CYC1* contains two consensus EREs upstream of a truncated *CYC1* promoter, which was modified to lower background signalling, and a multiple cloning site upstream of a *CYC1* terminator sequence. *p406-ERE*$_2$*s2-CYC1-yEGFP* contains the yeast enhanced green fluorescent protein (*yEGFP*) in *p406-ERE*$_2$*s2-CYC1*. Yeast vectors *YCp50,* containing a *URA3* selectable marker (Rose *et al.*, 1987), and *pRS405*, containing a *LEU2* selectable marker (Sikorski and Hieter, 1989), were provided by George Sprague (University of Oregon, Eugene, OR). The *HO*$_L$*-hisG-URA3-hisG-poly-HO*$_R$ vector was provided by David J. Stillman (University of Utah Health Sciences Center, Salt Lake City, UT) (Voth *et al.*, 2001). Intergenic adaptamer primers were designed using the searchable database **www.rothsteinlab.hs.columbia.edu**; appropriate restriction sites were then added to these adaptamer primers. All oligonucleotide primers used in this study are presented in Table 1. PCR reactions were carried out with Phusion High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA) and purified with the QIAquick PCR kit (Qiagen, Valencia, CA). Plasmid constructs were transformed into *E. coli* TOP10 chemically competent cells (Invitrogen, Carlsbad, CA) and confirmed by restriction analysis and DNA sequencing. All ERα ligands were obtained from Sigma (St. Louis, MO).

Construction of vectors to integrate *ERα* gene network into yeast genome

To create yeast integration vectors, we began with the *HO*$_L$*-hisG-URA3-hisG-poly-HO*$_R$ targeting vector (Voth *et al.*, 2001). Each gene of interest was

Table 1. Primers used to construct integration vectors

Primer name	Sequence	Template
*Pac*I-GPD-F	5′-gccttaattaaccgagtttatcattatcaatactc-3′	p403-GPD-ERα
*Asc*I-ER-R	5′-ttggcgcgcctcagactgtggcaggg-3′	p403-GPD-ERα
*Xho*I-his3Δ$_L$-F	5′-cggtatctcgagcataacacagtcctttcccgc-3′	K20 yeast genomic DNA
*Bsi*WI-his3Δ$_L$-R	5′-acgcgtacgtttgccttcgtttatcttgcc-3′	K20 yeast genomic DNA
*Asc*I-his3Δ$_R$-F	5′-ttggcgcgccccctccacgttgattgtctg-3′	K20 yeast genomic DNA
*Eco*RI-his3Δ$_R$-R	5′-gcgtgaattctgacacgtatagaatgatgc-3′	K20 yeast genomic DNA
*Pac*I-ERE-F	5′-gccttaattaa agctaaagtcaggtcacag-3′	p406-ERE$_2$s2-CYC1-yEGFP
*Sac*I-Term-R	5′-gtcgagctcgaattgggtaccggcc-3′	p406-ERE$_2$s2-CYC1-yEGFP
*Not*I-ADE2$_L$-F	5′-ataagaatgcggccgcacgccgtatcgtgattaacgt-3′	K20 yeast genomic DNA
*Bsi*WI-ADE2$_L$-R	5′-acgcgtacgttgattgttttgtccgattttctt-3′	K20 yeast genomic DNA
*Sac*I-ADE2$_R$-F	5′-gcggagctctacctatttttaggctttgttatg-3′	K20 yeast genomic DNA
*Pml*I-ADE2$_R$-R	5′-gccgcacgtgtcgaaacgttattttttaatcgc-3′	K20 yeast genomic DNA
*Bam*HI-HIS3-F	5′-ctcggatccatgacagagcagaaagcc-3′	p403-GPD-ERα
*Eco*RI-HIS3-R	5′-ctaggaattc ctacataagaacacctttggtgg-3′	p403-GPD-ERα
*Xho*I-ura3$_L$-F	5′-cggtatctcgaggatgctaagagatagtgatgatatttca-3′	K20 yeast genomic DNA
*Bsi*WI-ura3$_L$-R	5′-acgcgtacgtgcaggtttttgttctgtgca-3′	K20 yeast genomic DNA
*Sac*I-ura3$_R$-F	5′-gtcgagctccggccagcaaaactaaaaaac-3′	K20 yeast genomic DNA
*Pml*I-ura3$_R$-R	5′-gttcacgtggcccatatccaacttccaattt-3′	K20 yeast genomic DNA
*Bam*HI-YCp50-URA3-F	5′-gctcggatcccatgtcgaaagctacatataagg-3′	YCp50
*Hin*dIII-YCp50-URA3-R	5′-actgacaagcttttagattgaagctctaatttgtg-3′	YCp50
*Sac*I-EREURA3-F	5′-gtcgagctcgctggagctaaagtcaggtca-3′	p406-ERE$_2$-CYC1-URA3
*Hin*dIII-EREURA3-R	5′-gcggacaagcttttagttttgctggccgcatctt-3′	p406-ERE$_2$-CYC1-URA3

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amplified with primers containing terminal restriction sites and subcloned into the plasmid's multiple cloning site by restriction digest and ligation. The plasmid's HO_{Left} and HO_{Right} modules, which direct integration to the genomic *HO* locus, were then replaced with various alternative modules — generically termed $Target_{Left}$ and $Target_{Right}$ — containing regions of homology to specific loci in the yeast genome. $Target_{Left}$ and $Target_{Right}$ sequences were generated by PCR using adaptamer primers and yeast K20 genomic DNA as template.

The yeast integration vector *his3* Δ_L*-hisG-URA3-hisG-GPD-ERα-his3* Δ_R replaces the entire coding region of the *his3 Δ1* locus with a human *ERα* gene driven by the constitutive *GPD* promoter. *GPD-ERα* was amplified from the *p403-GPD-ERα* vector, using primers PacI-GPD-F and AscI-ER-R, and subcloned into the polylinker of the HO_L*-hisG-URA3-hisG-poly-*HO_R. The HO_L integration sequence was replaced with a *his3* Δ_L integration sequence, a 245 bp insert upstream of the *HIS3* start codon generated by PCR and primers XhoI-his3Δ_L-F and BsiWI-his3Δ_L-R. The plasmid's HO_R sequence was replaced with his3Δ_R, a 288 bp insert downstream of the *HIS3* stop codon, generated using primers AscI-his3Δ_R-F and EcoRI- his3Δ_R-R.

The yeast integration vector *ADE2*$_L$*-hisG-URA3-hisG-ERE*$_2$*-CYC1-yEGFP-ADE2*$_R$ replaces the *ADE2* coding sequence with an ERE-driven *yEGFP* gene. *ERE*$_2$*-CYC1-yEGFP* was amplified from the *p406-ERE*$_2$*s2-CYC1-yEGFP* vector using *Pac*I-ERE-F and *Sac*I-Term-R primers and subcloned into the HO_L*-hisG-URA3-hisG-poly-*HO_R plasmid. HO_L and HO_R integration sequences of the HO_L*-hisG-URA3-hisG-ERE-yEGFP-*HO_R plasmid were replaced with *ADE2*$_L$ and *ADE2*$_R$ intergenic sequences. These sequences were generated using primers NotI-ADE2$_L$-F and BsiWI-ADE2$_L$-R to amplify a 681 bp insert upstream of the *ADE2* start codon and the SacI-ADE2$_R$-F and PmlI-ADE2$_R$-R primers to amplify a 190 bp insert downstream of the *ADE2* stop codon.

The yeast integration vector HO_L*-hisG-URA3-hisG-ERE*$_2$*-CYC1-HIS3-*HO_R replaces the *HO* coding sequence with an ERE-driven *HIS3* gene. *HIS3* was amplified from the *p403-GPD-ERα* plasmid using *Bam*HI-HIS3-F and *Eco*RI-HIS3-R primers and subcloned into the *p406-ERE*$_2$*s2-CYC1* vector. The resulting *ERE*$_2$*-CYC1-HIS3* insert was then amplified from the *p406-ERE*$_2$*-CYC1-HIS3* vector, using primers *Pac*I-ERE-F and *Sac*I-Term-R and subcloned into the polylinker of the HO_L*-hisG-URA3-hisG-poly-*HO_R vector. To direct integration of this plasmid to the *HO* locus, HO_L *and* HO_R sequences were left intact.

The yeast integration vector *ura3*$_L$*-hisG-URA3-hisG-ura3*$_R$ replaces the entire *ura3-52* coding sequence with the *hisG-URA3-hisG* construct. HO_L and HO_R integration sequences of the HO_L*-hisG-URA3-hisG-*HO_R plasmid were replaced with *ura3*$_L$ and *ura3*$_R$ intergenic sequences. These sequences were generated using primers *Xho*I-ura3$_L$-F and *Bsi*WI-ura3$_L$-R to amplify a 348 bp insert upstream of the *ura3-52* locus and the *Sac*I-ura3$_R$-F and *Pml*I-ura3$_R$-R primers to amplify a 714 bp insert downstream of the *ura3-52* locus.

The yeast integrating plasmid *pRS405-ERE*$_2$*-CYC1-URA3* is a conventional YIp that directs an ERE-driven *URA3* gene to the *leu2-3,112* locus. *URA3* was amplified from the *YCp50* plasmid, using primers *Bam*HI-YCp50-URA3-F and *Hin*dIII-YCp50-URA3-R and subcloned into the *p406-ERE*$_2$*s2-CYC1* vector. The resulting *ERE*$_2$*-CYC1-URA3* insert was then amplified from the *p406-ERE*$_2$*-CYC1-URA3* vector, using primers *Sac*I-EREURA3-F and *Hin*dIII-EREURA3-R and subcloned into the polylinker of the *pRS405* vector.

Yeast transformations to integrate *ERα* gene network into yeast genome

K20 yeast cells were transformed using the high-efficiency lithium acetate/single-stranded carrier DNA/polyethylene glycol (PEG) method (Gietz and Woods, 2002). To integrate each construct of interest, the appropriate yeast integration vector was restriction-digested and the linearized DNA was then used to transform the K20 (Ura3$^-$, His3$^-$, Leu2$^-$) yeast strain. Transformants were selected for uracil prototrophy, and integration of the gene of interest at the genomic target locus was confirmed by DNA sequencing. The *URA3* selectable marker was then 'recycled' for repeated use by treating yeast with 5-fluoro-orotic acid (5-FOA), which selects for recombination between the *hisG* tandem repeats of the integrated *hisG-URA3-hisG* selectable marker, resulting in *URA3* deletion from yeast genome and restored

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uracil auxotrophy (Ura$^-$) (Boeke *et al.*, 1984; Voth *et al.*, 2001). By sequentially repeating this process, the *his3* Δ_L*-hisG-URA3-hisG-GPD-ERα-his3* Δ_R vector was used to integrate *GPD-ERα* and replace the *his3 Δ1* locus, the *ADE2* $_L$*-hisG-URA3-hisG-ERE*$_2$*-CYC1-yEGFP-ADE2* $_R$ vector was used to integrate *ERE*$_2$*-CYC1-yEGFP* and replace the *ADE2* locus, and the *HO*$_L$*-hisG-URA3-hisG-ERE*$_2$*-CYC1-HIS3-HO*$_R$ vector was used to integrate *ERE*$_2$*-CYC1-HIS3* and replace the *HO* locus, and the *ura3* $_L$*-hisG-URA3-hisG-ERE*$_2$*-CYC1-yEGFP-ura3* $_R$ vector was used to completely replace the mutant *ura3-52* locus from the K20 genome. To integrate *ERE*$_2$*-CYC1-URA3* at the *leu2* locus, *pRS405-ERE*$_2$*-CYC1-URA3* vector was linearized at the *Eco*RI site within its *LEU2* selectable marker and used to transform the K20 yeast strain. Positive transformants were selected for leucine prototrophy (Leu$^+$), and DNA sequencing confirmed that *pRS405-ERE*$_2$*-CYC1-URA3* integrated at the *leu2-3,112* locus. These five sequential transformations produced the EERAS strain (*CEN.PK 102-5B, MAT***a***, his3Δ1Δ::GPD-ERα, ADE2Δ::ERE*$_2$*-CYC1-yEGFP, HOΔ::ERE*$_2$*-CYC1-HIS3, ura3-52Δ::hisG, leu2-3,112Δ::LEU2-ERE*$_2$*-CYC1-URA3*).

ERE-yEGFP reporter assay

Ligand dose-responsive *ERE-yEGFP* reporter gene activity was quantified using a modified version of a previously described protocol (Bovee *et al.*, 2004a). Strains isogenic to the EERAS yeast strain, except lacking either *ERE-yEGFP* (*ΔERE-yEGFP*) or *GPD-ERα* (*ΔERα*) were used as negative controls to determine the specific effects of the signalling network components. To perform yEGFP assays, cultures of EERAS and control strains were incubated until late log phase growth (~16 h) and then diluted to an optical density (OD) of $OD_{600} = 0.01$ (~10^5 cells/ml). Diluted cells (200 µl/well) were added to 96-well plates (Special Optics Low Fluorescence Assay Plates, Costar Catalogue No. 3615). The wells were treated with either vehicle (ethanol; EtOH) alone or increasing concentrations of E2 dissolved in EtOH ([EtOH] <0.1%) and incubated at 30 °C on a rotary shaker at 200 r.p.m. At 4 h post-treatment, OD_{600} and fluorescence (excitation 485 nm, emission 535 nm) were directly measured using a Perkin-Elmer Victor3 Wallac 1420 Multilabel Plate Reader (Shelton, CT).

ERE-HIS3 and *ERE-URA3* growth reporter assays

Ligand dose-responsive *ERE-HIS3* and *ERE-URA3* growth reporter gene activities were quantified using a 96-well plate format growth assay. Strain ΔERα, which is isogenic to the EERAS strain except lacking *GPD-ERα*, was used as a negative control. Overnight cultures of EERAS and ΔERα yeast strains were diluted to $OD_{600} = 0.002$ in synthetic drop-out (SD) media lacking histidine only (SD — His), uracil only (SD — Ura), or both histidine and uracil (SD — His — Ura). 200 µl culture was added to each well of 96-well plate (sterile clear 96-well plate with lid; Falcon Catalogue No. 351 172) and treated with vehicle (EtOH) alone or increasing concentrations of various ligands (E2, diethylstilboestrol, oestrone, bisphenol A, 4-hydroxytamoxifen, genistein, oestriol, progesterone, testosterone) dissolved in EtOH ([EtOH] <0.1%). The plates were then incubated at 30 °C on a rotary shaker at 200 r.p.m. At 24 h post-treatment, OD_{600} was directly measured using the Victor3 plate reader.

Ligand-dependent growth rate and fitness assay

To quantify ligand dose-dependent effects on yeast growth rate, growth (OD_{600}) was monitored every 4 h for 28 h post-treatment with ligand. Overnight cultures of EERAS yeast were diluted to $OD_{600} = 0.02$ in SD — His — Ura media, and 200 µl was added to each well of a 96-well plate (sterile clear 96-well plate with lid; Falcon Catalog No. 351 172). The wells were treated with either vehicle (EtOH) alone or doses of E2 corresponding to the effective concentration (EC) required to activate maximum growth ([EC_{100}]), 75% maximum growth ([EC_{75}]), or 50% maximum growth ([EC_{50}]), and then the plates were incubated at 30 °C on a rotary shaker at 200 r.p.m. Every 4 h post-treatment, OD_{600} was directly measured and then plotted as a function of hours incubated post-treatment. Growth rate and relative fitness were calculated using OD_{600} values and formulae presented in Table 2. A one-way analysis of variance (ANOVA) with a Bonferroni correction for multiple tests was used to compare culture densities among the treatment groups.

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Table 2. Growth rate, doubling time and relative fitness of EERAS yeast are reporters of ligand–ERα activity

Treatment	[E2]	N_0	N_t	t	r	t_{double}	ω	s
E2 [EC_{100}]	1.0e-10 M	0.02	0.340	12	0.236	2.94	1.000	0.000
E2 [EC_{75}]	3.8e-11 M	0.02	0.250	12	0.210	3.29	0.975	0.025
E2 [EC_{50}]	3.1e-11 M	0.02	0.154	12	0.170	4.07	0.936	0.064
No E2	none	0.02	0.059	12	0.090	7.69	0.864	0.136

Growth rate (r) is calculated as $r = [\ln(N_t/N_0)]/t$, where N_0 is OD_{600} at time 0, N_t is OD_{600} at time t, and t is number of hours post-treatment with E2. Doubling time (t_{double}) is calculated as $t_{double} = \ln 2/r$. Relative fitness (ω) is calculated as $\omega = e^{r2-r1}$, where r_2 is the growth rate of an EERAS treatment group and r_1 is the growth rate of the EERAS treatment group [EC_{100}]. Relative fitness of all treatment groups is expressed relative to $\omega = 1$, which was assigned to the most fit treatment group [EC_{100}]. Selection coefficient (s) is the reduction in fitness of each treatment group ($w = 1 - s$) compared to the most fit treatment group, which is assigned a fitness of $w = 1$.

Results

Construction of EERAS yeast strain

To facilitate the construction of an evolvable oestrogen receptor activity sensor, we developed a modular gene-integration system. The system uses a recyclable vector with modular components to direct integration of multiple genes of interest to any target loci in the *S. cerevisiae* genome. We began with yeast strain K20, which is auxotrophic for uracil (Ura^-) and histidine (His^-), due to a partial deletion of the *HIS3* coding sequence and an insertion in the *URA3* coding sequence. We used the HO_L-*hisG-URA3-hisG-poly*-HO_R plasmid (Voth *et al.*, 2001) as the backbone vector into which each gene of interest module was individually cloned (see Figure 1). Integration of the *URA3* gene as part of this construct allows selection for transformants (Ura^+) in uracil-deficient medium; 5-FOA can then be used to counterselect for yeast (Ura^-) in which *URA3* has been deleted by recombination between the *hisG* repeats of the *hisG-URA3-hisG* construct. For each gene to be integrated, we preserved the vector's *hisG-URA3-hisG* recyclable selection module, and replaced the HO_{Left} and HO_{Right} genomic homology modules with $Target_{Left}$ and $Target_{Right}$ modules, containing regions of homology to sequences 5′- and 3′- of the genomic locus to be targeted. These target modules were amplified using PCR and intergenic adaptamer primers (Reid *et al.*, 2002), then subcloned into the modular vector by restriction digest/ligation.

We repeatedly used this strategy to integrate the multiple genes comprising the ERα signalling network into *S. cerevisiae* strain K20. Specifically, we integrated ERα driven by the constitutive *GPD* promoter at the *his3 Δ1* locus, completely replacing the entire *his3 Δ1* coding sequence in the process. We integrated an ERE-driven *HIS3* gene at the *HO* locus, followed by an ERE-driven *yEGFP* at the *ADE2* locus. We completely replaced the *ura3-52* coding sequence using the same approach. Finally, we integrated an ERE-driven *URA3* gene at the mutant *leu2-3,112* locus, using a traditional YIp-based approach, because 5-FOA counterselection to remove the *hisG-URA3-hisG* marker would have also adversely affected the integrated *ERE-URA3* construct. The resulting EERAS strain contains a constitutively expressed *ERα* gene, an ERE-driven fluorescent reporter gene (*ERE-yEGFP*), and two ERE-driven metabolic genes (*ERE-HIS3* and *ERE-URA3*), expression of which is required for growth in deficient medium (see Figure 2).

yEGFP activity is a reporter of ligand–ERα activity

We tested the functionality of the recombinant *ERα* in the EERAS strain using a previously validated *ERE-yEGFP* reporter assay in a high-throughput 96-well plate format (Bovee *et al.*, 2004a). We found that yEGFP activity in the EERAS strain is an efficient and sensitive dose-responsive reporter of ERα signalling activity, reaching approximately six-fold activation above background at 4 h post-treatment, with an EC_{50} of 3.9×10^{-10} M for E2 (Figure 3). This response is attributable entirely to the ERα signalling network, because control strains, isogenic to the EERAS yeast strain but lacking either *ERE-yEGFP* (*ΔERE-yEGFP*) or *GPD-ERα* (*ΔERα*), did not display ligand-dependent fluorescence. Background fluorescence

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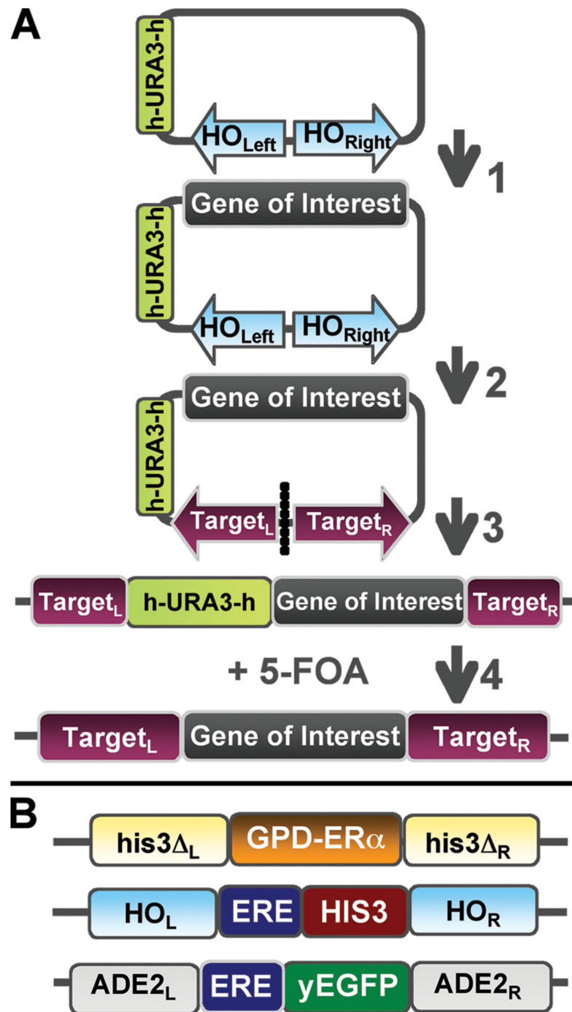

Figure 1. Modular gene-integration strategy used to integrate multiple genes of interest into EERAS yeast strain. (A) Four-step method for stably integrating a gene of interest at a target locus in the yeast genome: 1, subclone *gene of interest* module into the HO_{Left}-*hisG-URA3-hisG-poly*-HO_{Right} yeast vector; 2, replace HO_{Left} and HO_{Right} with $Target_{Left}$ and $Target_{Right}$ modules, which contain sequences homologous to regions 5′- and 3′- of the genomic *Target* locus; 3, linearize vector, transform yeast and select for uracil prototrophy (Ura^+); 4, recycle *URA3* selectable marker by 5-FOA counterselection, which returns the yeast strain to uracil auxotrophy (Ura^-) before the next transformation. (B) To engineer the EERAS yeast strain, the modular gene-integration strategy was repeatedly used to integrate *GPD-ERα* at the *his3Δ1* locus, *ERE-yEGFP* at the *ADE2* locus, and *ERE-HIS3* at the *HO* locus of the K20 yeast genome

in the control experiments was very low, as was background expression of the *yEGFP* gene in the EERAS strain in the absence of ligand.

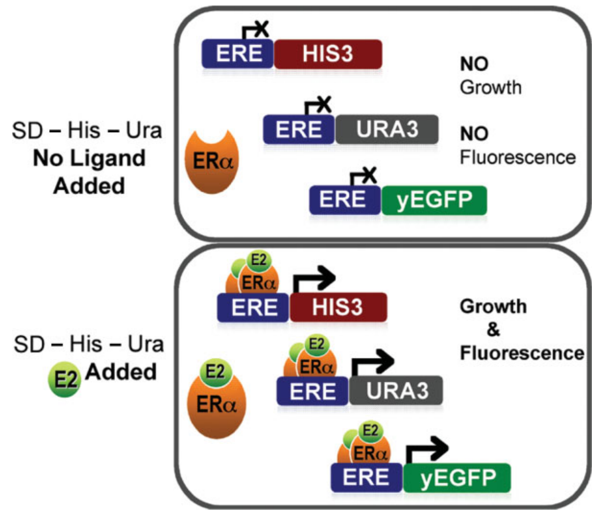

Figure 2. *ERα* gene interaction network controls EERAS yeast strain growth and yEGFP production. The EERAS yeast strain was engineered to express multiple reporter genes that control growth and yEGFP production in a ligand–ERα–ERE activity-dependent manner and to constitutively express the human *ERα* gene. When no ligand is added, *ERE-HIS3, ERE-URA3*, and *ERE-yEGFP* reporter genes are not transactivated by ERα, yeast are unable to grow in synthetic drop-out media lacking histidine (SD — His) or uracil (SD — Ura), and yEGFP is not produced. When ligand is added to the media, *ERα* is activated, resulting in dose-responsive yeast growth and yEGFP production

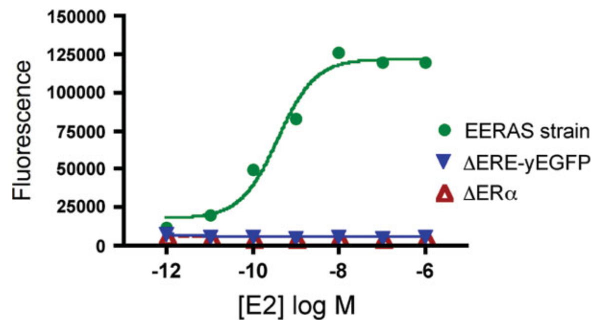

Figure 3. *ERE-yEGFP* is a reporter of ligand–ERα activity in EERAS yeast. Activation of the *ERE-yEGFP* reporter gene was quantified 4 h after treatment with increasing concentrations of 17β-oestradiol (E2). The effective concentration of E2 required to activate 50% maximum yEGFP production (EC_{50}) for the EERAS strain was 3.9×10^{-10} M. Control strains, isogenic to EERAS but lacking either *ERE-yEGFP* (*ΔERE-yEGFP*) or *GPD-ERα* (*ΔERα*), were used to measure the effects of either *GPD-ERα* or *ERE-yEGFP* gene alone. Results are mean values ± SEM for a minimum of three independent experiments

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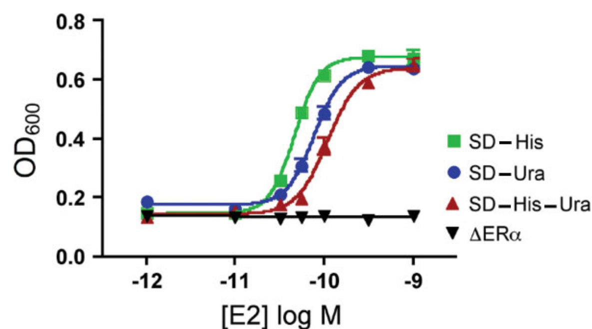

Figure 4. EERAS growth is a reporter of ligand–ERα activity. Culture growth was measured as optical density (OD_{600}) 24 h after treatment with increasing concentrations of 17β-estradiol (E2) in SD media lacking histidine (SD — His), uracil (SD — Ura) or both (SD — His — Ura). The EC_{50}s for growth in each medium were 4.7×10^{-11} M, 7.8×10^{-11} M and 1.1×10^{-10} M, respectively. A control strain, isogenic to EERAS except lacking *ERα* (*ΔERα*), was included to assess any ligand-independent growth induced by background expression of either growth reporter gene. Results are mean values $\pm$ SEM for a minimum of three independent experiments

Yeast growth is a reporter of ligand–ERα activity

To validate the functionality of the ERE-driven *HIS3* and *URA3* genes, and to determine whether growth of the EERAS strain depends on ERα signalling, we measured E2-dependent growth in synthetic drop-out (SD) media lacking histidine and uracil, respectively. Each growth reporter gene was an extremely sensitive indicator of ERα activity. In each type of SD medium, we observed $>$ three-fold dose-dependent increases in culture density at 24 h post-treatment. Sensitivities were even greater than for the yEGFP reporter assay: EC_{50}s for growth in SD — His and SD — Ura were 4.7×10^{-11} M and 7.8×10^{-11} M, respectively (Figure 4). In SD — His — Ura medium, in which both ERE-driven genes are required for growth, the EC_{50} was 1.1×10^{-10} M. The control strain, which is isogenic to EERAS except lacking *GPD-ERα* (*ΔERα*), displayed no response to E2, indicating that reporter activity in the EERAS strain is entirely attributable to the ERα signalling network. We observed only minimal growth of the EERAS and ΔERα control strain in SD — His — Ura media in the absence of ligand, indicating very low background expression of the *ERE-HIS3* and *ERE-URA3* growth reporter genes.

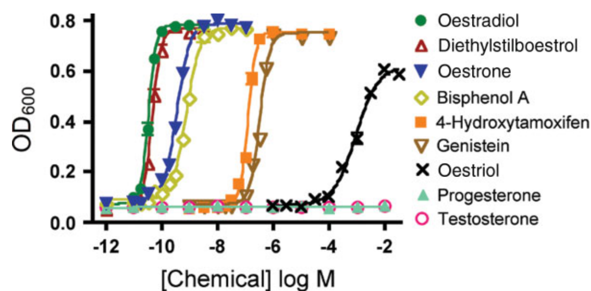

Figure 5. EERAS growth is a reporter of ERα ligand-and-specificity. Growth, as determined by cell density (OD_{600}), was measured 24 h after treatment with increasing concentrations of various potential ER ligands in synthetic drop-out (SD) media lacking both histidine and uracil (SD — His — Ura). The EC_{50}s for growth activated by each ligand were: E2, 4×10^{-11} M; diethylstilboestrol, 5×10^{-11} M; oestrone, 4×10^{-10} M; bisphenol A, 8×10^{-10} M 4-hydroxytamoxifen, 1×10^{-7} M; genistein, 3×10^{-7} M and oestriol, 9×10^{-4} M. Results are mean values $\pm$ SEM for a minimum of three independent experiments

Screening chemicals for oestrogenicity using the EERAS yeast strain

To determine whether growth reporter assays in the EERAS strain could be used to screen chemicals for oestrogenicity, we assayed a variety of ligands for ERα-activated growth. Growth reporter assays were conducted in the presence of known natural and synthetic oestrogenic ligands as well as non-oestrogenic controls, to measure both potency and specificity of ligand–ERα interactions. Growth (OD_{600}) of the EERAS strain, incubated in SD — His — Ura medium with increasing concentrations of each ligand, was directly measured in a high-throughput 96-well plate format at 24 h post-treatment. When comparing ligand-dependent growth curves, we observed that natural ligands (E2, oestrone, genistein, oestriol), as well as synthetic ligands (diethylstilboestrol, bisphenol A, 4-hydroxytamoxifen) with known oestrogenic properties induced dose-responsive EERAS strain growth, with EC_{50} values ranging from 4×10^{-11} M for E2 to 9×10^{-4} for oestriol (Figure 5). EERAS strain growth was induced in a ligand-dependent manner, reflecting the potency with which ligands activated ERα: E2 = diethylstilboestrol $>$ oestrone = bisphenol A $\gg$ 4-hydroxytamoxifen = genistein $\gg$ oestriol (Figure 5). The non-oestrogenic steroids progesterone and testosterone did not induce growth, indicating that EERAS culture density in

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deficient medium is a specific reporter of ERα activation.

Growth rate and relative fitness of EERAS strain are ligand-dependent

Experimental evolution assays require that a function of interest — in this case, the efficiency of the ERα signalling network — be linked to fitness, in order that variants with improved function can be isolated by natural selection. To determine whether the EERAS strain's fitness is dependent on ERα activity, we measured the growth rate of EERAS incubated with increasing concentrations of ligand in deficient medium. Growth (OD_{600}) of EERAS was measured every 4 h post-treatment for cultures treated with vehicle only or with E2 at the EC_{50}, EC_{75} and EC_{100} (3.1×10^{-11} M, 3.8×10^{-11} M and 1.0×10^{-10} M, respectively) for growth. In the absence of E2, growth was virtually absent. E2-treated cultures all displayed logistical growth, with growth rates increasing as ligand concentrations increased (Figure 6, Table 2). Culture density was significantly different among every pair of treatment groups at all time points, starting at 8 h post-treatment and continuing through lag phase at 28 h post-treatment ($p < 0.05$, except for cultures grown at EC_{50} and EC_{75} at 24 and 28 h).

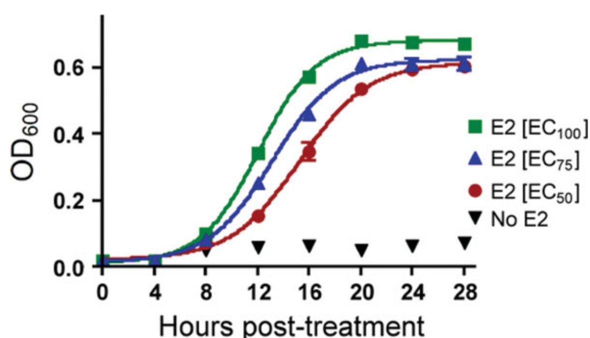

Figure 6. EERAS growth rate is determined by ligand–ERα activity. Growth, as determined by cell density (OD_{600}), was measured every 4 h after treatment with E2 at concentrations corresponding to the effective concentration (EC) required to activate maximum growth (E2[EC_{100}], 1.0×10^{-10} M), 75% maximum growth (E2[EC_{75}], 3.8×10^{-11} M) and 50% maximum growth (E2[EC_{50}], 3.1×10^{-11} M) in SD — His — Ura. EERAS treated with vehicle only (no E2) was included as a control to assess ligand-independent growth induced by background expression of growth reporter genes. Results are mean values ± SEM for a minimum of three independent experiments

Growth rate and fitness depended on E2 concentration (Table 2). Growth rate during log phase of the EC_{75}, EC_{50} and no-E2 groups were 89%, 72% and 38%, respectively, of the growth rate of cultures grown in E2 at the EC_{100}. The doubling time of yeast treated with E2 at the EC_{75} was 3.29 h compared with 4.07 h for the EC_{50} group. Relative fitness — the ratio of the number of offspring produced per unit time by individuals in a class compared to the most fit class — also depended on E2 dose: the relative fitnesses of the EC_{75}, EC_{50} and no-E2 groups were 0.975, 0.936, and 0.864, respectively, with the fitness of the EC_{100} group set at 1.00. These results indicate that fitness of the EERAS strain depends on the activity of the ERα signalling network. EERAS, therefore, has the potential to be used in an experimental evolution system to select for variant ERαs with enhanced ligand responsiveness.

Discussion

The gene-targeting strategy that we have developed should be generally useful for genetic manipulation of yeast. By combining modularity with selectable marker recycling, it allows efficient targeted integration of multiple genes of interest into the yeast genome. Unlike YIp and adaptamer-based methods, our system does not use up an auxotrophic marker with each gene integrated. Unlike previous plasmids that used the recyclable *hisG-URA3-hisG* construct to target genes to a single fixed locus, our modular system allows this useful marker to be used efficiently and flexibly to serially target multiple loci in a single strain. We chose to generate these target sequences using PCR and adaptamer primers, which have already been designed and verified to amplify regions of homology intergenic to every ORF in the yeast genome (Reid *et al.*, 2002). By cloning relatively long (200–1000 bp) adaptamer-generated sequences into a plasmid, we exploited the flexibility of the adaptamer system but overcame some of its limitations — notably the difficulties posed by using PCR to generate complex genetic constructs for transformation and the low transformation efficiency that results from using very short (35–60 bp) targeting sequences. Although our modular strategy represents an advance over previous methods for integrating multiple genes into yeast, it remains

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fairly labour-intensive, because three rounds of cloning are required to create each targeting vector. Research to develop more efficient means to the same end is warranted.

Using this strategy, we engineered an EERAS strain that contains the basic components of an *ERα* gene signalling network — a human *ERα* and three ERE-driven reporter genes (*ERE-yEGFP, ERE-HIS3* and *ERE-URA3*). Expression of the *ERE-yEGFP* gene produces a dose-responsive increase in fluorescence in the presence of ERα ligands. Expression of the *ERE-HIS3* and *ERE-URA3* genes produce dose-responsive increases in culture density and growth rate in the presence of ERα ligands, when cultures are grown in SD media lacking histidine, uracil or both. With both fluorescence and growth assays, we observed sensitive dose-responsive GFP and growth curves over a broad range of ligand concentrations, allowing for the detection of, and differentiation between, ligands with strong, moderate and weak activation of ERα in EERAS. EC_{50} values for ligands were comparable to, and in most cases more sensitive than, those reported by other yeast ERα reporter assays (Bovee *et al.*, 2004a, 2004b; Collins *et al.*, 1997; Fang *et al.*, 2000; Gaido *et al.*, 1997; Soto *et al.*, 1995).

The EERAS strain has advantages over many previous reporter systems for screening ligands for ERα activity. *ERE-lacZ* or *ERE-luciferase* reporter genes require longer incubations (up to 5 days for *lacZ*) before producing results, require either yeast cell wall disruption or addition of expensive substrates, and are orders of magnitude less sensitive than mammalian cell culture-based assays (Bovee *et al.*, 2004a; Gaido *et al.*, 1997; Le Guevel and Pakdel, 2001; Rehmann *et al.*, 1999). In contrast, the EERAS *ERE-yEGFP* reporter assay quantitatively measures ligand–*ERα* activity after just 4 h in a high-throughput 96-well plate format, without cell-wall disruption or addition of any substrates. The EERAS growth reporter assays have additional advantages: they are even more sensitive, with EC_{50} values comparable to or lower than mammalian cell-culture based reporter assays (Collins-Burow *et al.*, 2000; Mueller, 2004; Sonneveld *et al.*, 2006; Wilson *et al.*, 2004), require only 12–24 h incubation, can be conducted in high-throughput or single-sample format, and require only a spectrophotometer. In fact, growth differences can even be assessed by observing culture density with the naked eye. An additional advantage of EERAS is that both the *ERα* and three ERE-driven reporter genes are integrated into the yeast genome, which prevents false positive results due to plasmid amplification (McEwan, 2001).

Because it is evolvable, the EERAS strain provides a tool that should be useful for investigating the structure–function relationships that govern molecular interactions between ERα and ligands. Most ligand–ERα structure–function experiments to date have used directed mutagenesis to manually produce large mutant *ERα* libraries, which must then be screened to identify ERαs with altered ligand binding/activation profiles (Chen *et al.*, 2004; Chen and Zhao, 2003; Jakacka *et al.*, 2002; Montano *et al.*, 1996). Directed evolution — a strategy distinct from experimental evolution, because it involves the manual creation of mutant libraries, which must then be screened for optimized variants using a selection-based method — has been used productively to study steroid receptor–ligand structure–function relations (Chen *et al.*, 2004; Sitcheran *et al.*, 2000). Experimental evolution systems are expected to be even more efficient for this purpose, because of the continuous generation of new *ERα* variants and the great power of natural selection in large microbial populations.

Experimental evolution assays require the organism's fitness to be linked to the trait of interest being investigated. When this is accomplished, small variations in that trait, generated by spontaneous mutation in a large population, confer small differences in growth rate and fitness for individual yeast. Over time, yeast inheriting fitness-increasing alleles rise in frequency and ultimately dominate the yeast population. The advantages of carrying out such experiments in yeast include their ease of transformation and propagation, fast generation time, well-characterized genome, and the ability to revive and compare yeast populations frozen at regular intervals during the course of experimental evolution to analyse the genetics responsible for new phenotypes. Our experiments show that significant differences in growth rate and fitness are induced by minute (<10 pM) differences in ligand (E2) concentration added to the media, indicating that variants with even subtle improvements in ERα signalling should be subject to relatively strong selection. Previous experimental evolution studies in yeast have shown that small differences in fitness ($<2\%$) can be selected for and isolated

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from large populations within a few hundred generations (Thatcher *et al*., 1998; Zeyl, 2005). Culturing EERAS at various suboptimal ligand concentrations produced fitness differences in the range 2.5–6.4%; such differences should provide ample selection for variants with improved ligand–ERα signalling.

The approach we demonstrate here can, in principle, be extended to study an unlimited number of gene networks or signalling systems. The modular gene targeting system we developed should be applicable to integrating any gene network — or any ensemble of heterologous genes — into the yeast genome. The efficient reporter assays made possible by the EERAS design could be replicated for studying other transcription factor networks, including other nuclear receptors and their ligands and response elements, by substituting different receptors and response elements for the ERα and ERE. Using the EERAS system and its associated reporter assays as the framework, adaptive activity sensors could be constructed for experimental evolution assays to study the structure–function relations and evolutionary dynamics of many gene interaction networks.

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