A yeast sensor of ligand binding

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We describe a biosensor that reports the binding of small-molecule ligands to proteins as changes in growth of temperature-sensitive yeast. The yeast strains lack dihydrofolate reductase (DHFR) and are complemented by mouse DHFR containing a ligand-binding domain inserted in a flexible loop. Yeast strains expressing two ligand-binding domain fusions, FKBP12-DHFR and estrogen receptor-α (ERα)-DHFR, show increased growth in the presence of their corresponding ligands. We used this sensor to identify mutations in residues of ERα important for ligand binding, as well as mutations generally affecting protein activity or expression. We also tested the sensor against a chemical array to identify ligands that bind to FKBP12 or ERα. The ERα sensor was able to discriminate among estrogen analogs, showing different degrees of growth for the analogs that correlated with their relative binding affinities (RBAs). This growth assay provides a simple and inexpensive method to select novel ligands and ligand-binding domains.

In vivo protein sensors of ligand binding are advantageous in high-throughput screens to identify compounds that interact with protein targets. They are stable, easy to manipulate, and provide inexpensive alternatives to *in vitro* screening. Such sensors are typically chimeric proteins consisting of a ligand-binding domain coupled to a reporter. The first small-molecule protein sensors were generated to detect ligands such as Ca^{2+}, Zn^{2+}, and glucose (see ref. 1 for a review), but they were quite specific in their recognition and did not prove suitable as generic sensors.

A generic sensor has the stringent requirements of both modularity (the ability to substitute one ligand-binding domain for another) and tight association (the ligand-binding and reporter domains must be closely coupled if changes in one are to be detected in the other). In designing a modular sensor, we decided to use a general property of ligand binding: ligand-induced stabilization. Though not universal, this phenomenon is common: a wide range of enzymes are stabilized by their substrates or inhibitors.

Increases in protein stability upon ligand binding to one domain might be felt by a closely coupled domain. Such transference of stability has been previously demonstrated; proteins fused to green fluorescent protein (GFP) show correlations between degree of folding and GFP fluorescence[2], and DHFR and β-lactamase inserted into yeast phosphoglycerate kinase (PGK) yield fusion proteins with coupled stabilities[3]. Alterations in activity of one domain in a fusion can be induced by addition of ligand to the other. Ligands binding to proteins or domains inserted into GFP can cause changes in fluorescence[4,5]; the insertion of β-lactamase into the maltodextrin-binding protein resulted in a bifunctional protein in which the activity of β-lactamase was stabilized by maltose binding[6]; and proteins fused to steroid hormone domains can have their activities regulated by hormone[7–9]. With the PGK fusions, addition of the substrate of DHFR or β-lactamase modulated activity of PGK (ref. 3).

We set out to design a protein-derived ligand sensor that would allow detection of small molecules binding to their targets by a simple growth assay in the budding yeast, *Saccharomyces cerevisiae*. Yeast-based assays have proved themselves to be powerful tools for functional genomic analyses. In this case, we sought a reporter in which a subtle change in catalytic activity could be easily detected, such as an enzyme at the threshold of viable activity or stability. One group of proteins in which subtle changes in stability or activity can be observed are temperature-sensitive mutants. We coupled a ligand-binding domain to a temperature-sensitive essential yeast metabolic enzyme, DHFR, to generate a sensor in which binding of ligand resulted in an increase in growth of the yeast.

Results

In yeast, DHFR is encoded by the *DHR1* gene, and the activity of altered versions of DHFR can be monitored by measuring growth in the *dhr1* mutant strain TH5 (ref. 10). This strain has been complemented with DHFR from *Plasmodium*, *Cryptosporidium*, and human to obtain yeast that is dependent for its growth on the substituted DHFR (refs 11,12). Murine DHFR has been divided at amino acid 107 into two fragments that can reassemble to form an active enzyme[13], indicating that the protein may tolerate the insertion of a foreign protein at this site. We inserted human FKBP12, a small (12 kDa) protein that binds to the macrolide FK506, at this site to generate the construct dhFK (Fig. 1). A flexible linker was included to join the C terminus of FKBP12 to residue 108 of Dhr1. The fusions were introduced into TH5 and supplemented with thymidine 5′-monophosphate (dTMP), a metabolite that is essential for survival of *dhr1* yeast, to ensure that transformants would grow regardless of DHFR function. As a positive control, we used the intact murine DHFR protein (Fig. 1, mdhfr).

We verified that mdhfr and dhFK are able to restore DHFR activity to the TH5 strain by measuring growth in the absence of dTMP (Fig. 1, 30°C). We wanted the DHFR fusion to be unstable or temperature-sensitive in the absence of ligand, such that we could detect subtle changes in growth upon addition of ligand. Because yeast expressing dhFK were not temperature-sensitive (Fig. 1, 38.5°C), we mutated residue 66 to leucine (P66L), a substitution previously

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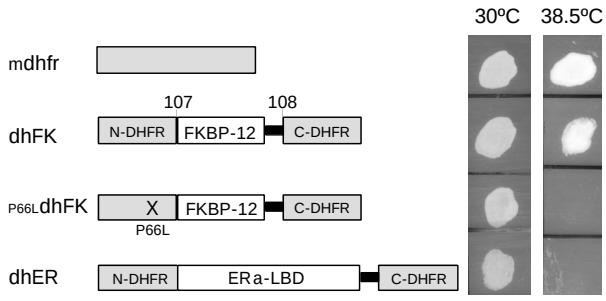

Figure 1. DHFR constructs. Shown are murine DHFR (mdhfr) and constructs with a ligand-binding domain inserted in DHFR at residue 107. Linker is indicated by solid line. On the right is shown growth of TH5 yeast expressing each construct under control of a truncated yeast *DHR1* promoter at 30°C and 38.5°C.

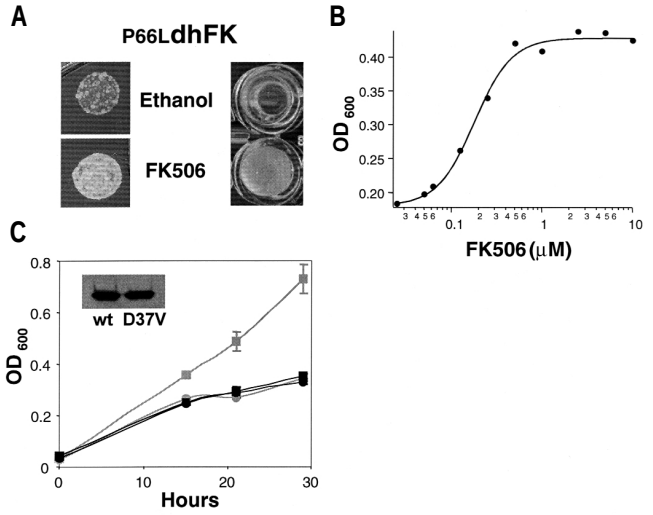

Figure 2. Growth of P66LdhFK in the presence of FK506. (A) Growth at 38.5°C with 5 μM FK506 on plates (left) or in 96-well dishes (right). (B) Titration of P66LdhFK with FK506. Yeasts expressing P66LdhFK were grown in 96-well dishes with ethanol (0.5 μl) or indicated concentrations of FK506 diluted in ethanol at 38.5°C for 42 h. (C) Growth of yeast at 38.5°C expressing P66LdhFK-D37V (black) or P66LdhFK (gray) with 5 μM FK506 (squares) or ethanol (circles). Inset shows immunoblot of hemagglutinin (HA)-epitope-tagged P66LdhFK (wt) and P66LdhFK-D37V (D37V) expressed in W3031A yeast and probed with an anti-HA antibody. Error bars represent two replica points from a typical experiment.

shown to confer temperature sensitivity to a mouse DHFR fusion protein[14]. Although this mutation in intact mouse DHFR was not sufficient to confer temperature sensitivity (data not shown), it did so when placed in dhFK (Fig. 1, P66LdhFK). A fourth construct, dhER, containing the human ERα ligand-binding domain inserted in mouse DHFR, resulted in a temperature-sensitive phenotype without the P66L mutation (Fig. 1).

We assayed the effect of the FKBP ligand FK506 on the growth of yeast expressing P66LdhFK. Yeast were grown to log phase, then added to plates with ethanol or FK506 and incubated at 38.5°C. Yeast expressing P66LdhFK showed increased growth with 5 μM FK506 (Fig. 2A). A titration of FK506 indicated that the induced growth saturated at ~2.5 μM FK506, with a half-maximal effect at ~300 nM FK506 (Fig. 2B).

To verify that the increase in growth was due to FK506 acting through the FKBP12 insertion and did not represent a general effect of the drug, we generated the construct P66LdhFK-D37V, which contains a valine mutation in FKBP12 that reduces binding to FK506 by ~99% (ref. 15). Hemagglutinin (HA)-epitope-tagged versions of P66LdhFK-D37V expressed equivalently to P66LdhFK (Fig. 2C, inset), and TH5 yeast expressing P66LdhFK-D37V grew equivalently to transformants with P66LdhFK in the absence of dTMP (Fig. 2C, circles), indicating that the proteins have similar amounts of DHFR activity in the absence of ligand. However, yeast with P66LdhFK-D37V grew no better with ligand than without (Fig. 2C, black). This result provides strong support that the increased growth of yeast with FK506 is mediated through the binding site on FKBP12.

Table 1. dhER variants with reduced growth[a]

Construct	Mutation	Growth without dTMP	Fold estrogen response
dhER	None	****	2.5 ± 0.4
1033-27	Frameshift (residue 324)	None	None
1033-28	Frameshift (linker sequence)	None	None
1033-38	Stop codon (residue 531)	None	None
1042-8	S527C; D538G	None	None
1045-9	Frameshift (residue 462)	None	None
1033-4	M297K; M357K	*	None
1033-19	M438K; S462A; L511P	**	1.8 ± 0.2
1033-42	L504P	*	None
1043-7	M490K; S518C	*	None
1045-2	L349P; V392D; N532I; Q565R	*	None
1045-3	R555L	***	None
1045-4	N304Y; E330G	*	1.2 ± 0.1
1045-6	D313N	**	2.1 ± 0.7
1045-12	L429P; S450P	**	None

[a]Growth without dTMP was assessed visually on solid media at 30°C. Number of asterisks represents extent of growth (*least; ****most). Estrogen response was assayed in liquid cultures after 48 h of ligand or control treatment at 38.5°C. Fold estrogen reponse is calculated from (growth with estrogen/growth without estrogen) and represents two to five independent experiments.

One of the principal goals for the ligand sensor was a modular design, such that one ligand-binding domain could be substituted for another. To test for modularity, we analyzed the properties of the dhER construct (Fig. 1). Yeast expressing dhER displayed increased growth in the presence of estrogen, but not ethanol or FK506 (Fig. 3A). In contrast, yeast expressing P66LdhFK showed no difference in growth in the presence of estrogen (Fig. 3B). These results indicate that estrogen also does not act as a general growth enhancer, and that the increased growth is specific for each ligand/ligand-binding domain pair.

Characterization of dhER mutants. To examine the sensor's ability to discriminate among variants of a ligand-binding domain, we generated dhER constructs with mutations in the ERα-binding domain. This domain was randomly mutagenized by error-prone PCR and reintroduced into DHFR. We also generated a specific mutation, L525A, in a residue important for hormone binding[16]. Twenty-four variants were tested for growth effects.

We first assessed the effects of the dhER mutants on growth of yeast at 30°C in the absence of dTMP, which gives an indication of the amount of DHFR activity present, as we were interested in identifying variants with normal basal growth (without ligand), but altered estrogen responses. Ten variants had basal growth equivalent to dhER, indicating that the mutations did not significantly affect the general structure or expression of the fusions. We assayed the growth of yeast expressing these variants in the presence of dimethyl sulfoxide (DMSO) or 0.6 μM, 1.8 μM, or 18 μM 17β-estradiol. Five variants (not shown) had similar responses to estrogen as wild-type dhER (one, 1045-10, is pictured in Fig. 4). The other five, 1033-5, 1033-9, 1033-40, L525A, and 1042-1, showed reduced responses to estrogen (Fig. 4A). Each of these contains a mutation in or near a residue important for hormone binding[17] (Fig. 4B). L525A, containing a

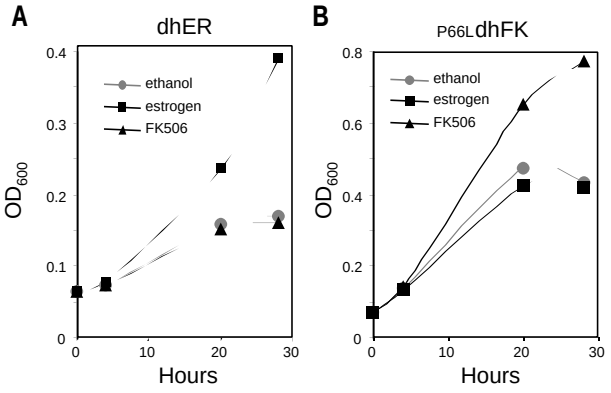

Figure 3. Responses of yeast expressing dhER or P66LdhFK to estrogen or FK506. (A) Yeast expressing dhER. (B) Yeast expressing P66LdhFK. Strains were grown at 38.5°C in flasks with ethanol (circles), 18 μM 17β-estradiol (squares), or 10 μM FK506 (triangles) dissolved in ethanol. OD_{600} was measured on aliquots at indicated time points.

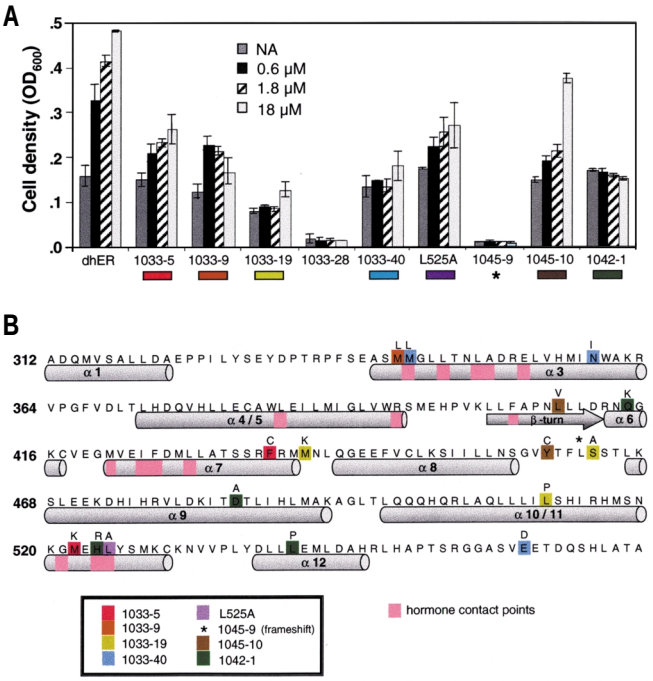

Figure 4. ERα-binding domain variants. (A) Response of mutants to 17β-estradiol. Yeast were grown for 42 h at 38.5°C in the presence of indicated amounts of 17β-estradiol or dimethyl sulfoxide (NA). Color bars below mutants correspond to color coding in (B). Error bars represent two replica points of an experiment. (B) Location of mutations in ERα ligand-binding domain, shown in corresponding color. Numbering corresponds to the full-length human ERα. The substituted amino acid is indicated above the correct sequence. Residues reported to contact estrogen[17] are shown in pink.

mutation previously shown to reduce binding of estrogen[16], also showed a reduced response to estrogen in our assays (Fig. 4A). Western blot analysis of HA-tagged versions of L525A and 1033-5 showed expression equivalent to HA-tagged dhER (data not shown). These mutants likely have a fairly normal structure and expression, but are unable to bind estrogen effectively.

We identified 14 variants of dhER that were defective in growth at 30°C in the absence of dTMP (Table 1). Five of these were unable to grow on plates without dTMP, with one containing a stop codon and three containing frameshift mutations. The location of one of the frameshift mutations, 1045-9, is shown in Figure 4B. Nine variants had reduced basal growth compared with wild-type dhER (Table 1). One of these, 1033-19, is pictured in Figure 4B. The variant 1033-19 and three others with poor growth, 1033-42, 1045-2, and 1045-12, contained proline insertions in α-helical regions of ERα. We predict that the mutations in these variants caused significant changes in the structure, folding, or stability of dhER, resulting in reduced DHFR activity.

Chemical screening. An important application for a sensor is its use in screening chemical libraries for novel ligands. To test this capacity, we generated a chemical array consisting of 20 estrogen analogs, hormones, and other compounds. Yeast strains expressing dhER or P66LdhFK were assayed for growth in 96-well plates that contained 0.2 or 2 μM concentrations of each compound. Growth of dhER with estrogen analogs generally correlated with previously measured RBAs to ERα (ref. 18; Fig. 5A), with greater growth seen with analogs with higher RBAs. This correlation was most apparent for analogs with RBAs between 10 and 100 (Fig. 5B).

In comparison, yeast expressing P66LdhFK showed no difference in growth with estrogen analogs, but responded to FK506 and trimethoprim. Trimethoprim is a DHFR inhibitor that binds 12,000 times more tightly to bacterial DHFR than to the mammalian enzyme[19]. Trimethoprim does not inhibit growth of yeast expressing mouse DHFR, P66LdhFK, or dhER at the concentrations used in this assay (0.2–20 μM) but did inhibit at higher concentrations (>100 μM, data not shown). The inhibitors trimethoprim and methotrexate significantly stabilize DHFR (refs 20,21), and the presence of methotrexate slowed the rate of degradation of a P66L-DHFR fusion protein[22]. Trimethoprim may do the same with P66LdhFK. This result, while not an interaction of a ligand with the inserted ligand-binding domain, provides another example of the temperature-sensitive yeast as sensitive reporters of small perturbations to DHFR activity or structure.

Discussion

We describe the development of a yeast assay for quantifying ligand binding to protein targets, based on an increase in growth of temperature-sensitive yeast. We used this assay to screen a chemical library with estrogen and FK506 sensors and successfully identified molecules that are known to bind either ERα or FKBP12. Growth of yeast in the presence of estrogen analogs was graded and correlated to binding affinities, indicating that the sensor works to quantify as well as detect ligands. In a reverse screen, the sensor may be useful in assaying a library of open reading frames inserted in DHFR to detect growth in the presence of a small-molecule ligand of interest. Yeast strains with increased chemical permeability, such as *pdr5* or *erg6* mutants, may provide enhanced sensitivity in these approaches.

We tested the capacity of the yeast assay to identify mutations in a ligand-binding domain. Growth of yeast expressing dhER variants correlated with the severity of mutations, indicating that the fusion can work as a general sensor of protein expression. Potentially, this method could be used to screen insertions to identify stably folded protein fragments. Although other methods exist for detecting protein expression, their readout is often a change in color or fluorescence[2,23]. The DHFR fusion proteins are detected by a growth assay, allowing the possibility of identifying more stable proteins by a growth competition. Furthermore, the fragment is inserted, not tethered, which may increase sensitivity. In addition to identifying mutations causing a loss of DHFR activity, we were also able to identify mutations important to ligand binding that did not appear to compromise general stability. In the absence of ligand, yeast expressing these variants grew equivalently to those expressing wild-type dhER, but they had reduced

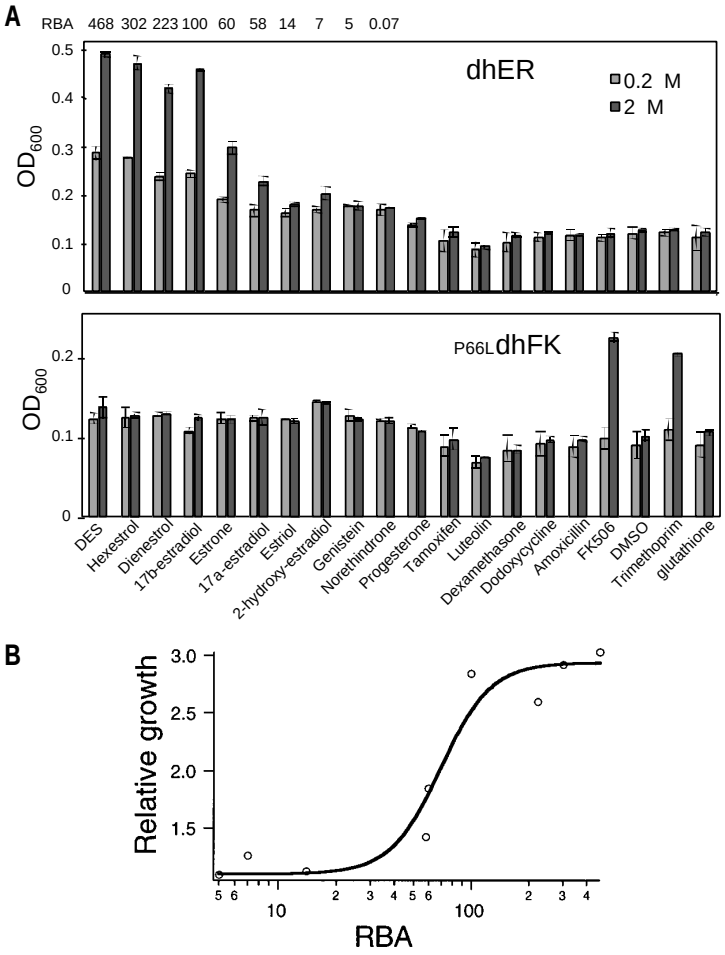

Figure 5. Chemical screen. (A) Yeast strains expressing dhER (top) or P66LdhFK (below) were screened against 20 compounds for growth at 38.5°C. Compounds were at 0.2 μM or 2 μM. OD_{600} was determined after 38 h. Relative binding affinities (RBAs) for binding of estrogen analogs to ERα (ref. 18) are shown at top, with estrogen (17β-estradiol) at an RBA of 100. Error bars represent the deviation of two replica points of an experiment. (B) Correlation between RBA and growth of dhER (expressed as growth of yeast in 2 μM analog/growth with no analog), based on results shown in (A). Curve fit was generated by Igor-Pro (WaveMetrics, Lake Oswego, OR).

responses to estrogen and contained mutations in receptor regions identified as important for hormone binding[17]. Thus, using simple growth assays, we were able to differentiate among several types of mutations.

We investigated the mechanism of increased growth of yeast to determine if it is due to increased stability or activity of the DHFR fusion, but were unable to observe a significant change in either with ligand. Since the yeast in these experiments are at the threshold of growth/death, it may be that only very subtle changes in protein stability or activity are required to elicit the growth effects. Different mechanisms may operate for the different fusions. For example, the insertion of a ligand-binding domain into DHFR may destabilize the protein; ligand may stabilize the binding domain, and in doing so decrease the rate of degradation of the entire fusion. FK506 may work in this manner to stabilize P66LdhFK, given that the thermostability of FKBP12 is greatly increased upon binding of FK506 (ref. 24). Trimethoprim may also stabilize P66LdhFK, though through the DHFR portion of the molecule.

Another potential mechanism for the increased growth is that ligand binding causes an increase in activity of the DHFR fusion. The insertion of a ligand-binding domain into DHFR may cause conformational changes that reduce activity, and such constraints may be lifted upon ligand binding. Alternatively, the unliganded binding domain could be associated with cofactors that sterically constrain the protein halves when bound, but are released with ligand. The dhER sensor may work in this way, because steroid hormone ligand-binding domains are associated with cofactors in the absence of ligand, and released upon hormone binding[25].

Considerable effort has gone into developing yeast-based screens to identify new therapeutics for human diseases. Although there exist promising methods for detecting inhibitors of targets that have yeast or bacterial homologs, many important disease-associated proteins have no characterizable phenotypes in these organisms. The methodologies outlined here should extend the potential uses of yeast-based screens to provide a simple means to identify lead compounds that bind a protein target, or to do structure/function analysis. Because the degree of growth of the yeast is graded corresponding to binding affinities, ligand concentration, or intactness of binding sites, the sensor can be used to quantitate as well as to detect ligand binding. Although variations of ligand sensors have been generated that couple ligand binding to changes in fluorescence[4,5], the DHFR sensor uses a growth assay, introducing the important capability of selecting and evolving ligand-binding variants from pools of mutagenized domains.

Experimental protocol

Reagents and strains. Strain TH5 (*MATα ura3-52 leu2-3,112 trp1 tup1 dfr1*::*URA3*)[10] was provided by Carol Sibley (University of Washington). FK506 was extracted from Prograf (Fujisawa Healthcare, Inc., Deerfield, IL), using a method provided by Paul Clemons and Stuart Schreiber (Harvard University). Prograf capsules were crushed, resuspended in water, extracted in ethyl acetate, back-extracted with water, filtered through diatomaceous earth, evaporated, and resuspended in ethanol. All other chemicals were from Sigma (St. Louis, MO).

Plasmid construction. DHFR and fusions were cloned by recombination into pTB3 (provided by Carol Sibley, containing 600 bp of the *S. cerevisiae DHR1* promoter) cut with *Bam*HI and *Eag*I. For mdhfr, mouse DHFR was PCR-amplified from pMT3-DHFR (provided by Stephen Michnick, University of Montreal). For dhFK, one fragment contained FKBP12 and the C terminus of mouse DHFR (residues 107–186) amplified from FKBP-F[3] (ref. 26) (provided by Steven Michnick), with a 10–amino acid linker joining FKBP12 to the C terminus of DHFR (ref. 26). The second fragment (from pMT3-DHFR) contained residues 1–105 of mouse DHFR followed by an engineered *Nhe*I site. The P66L mutation was amplified from PPW58 (provided by Jurgen Domen, University of Dusseldorf) and recombined with the FKBP-F[3] fragment to yield P66LdhFK. dhER was generated by digesting pTB3-dhFK with *Nhe*I and *Cla*I to remove FKBP12, then recombining this with residues 282–576 of human ERα amplified from Gal4-ER-VP16 (provided by Randall Morse, Wadsworth Center, Albany, NY)[9]. The mutation D37V was generated by recombining two PCR fragments of dhFK that each contained the mutation in the primer. HA-epitope-tagged P66LdhFK and P66LdhFK-D37V were generated by recombination in strain W3031A (ref. 27) with p414GalL (ref. 28) and a PCR-generated fragment containing three copies of the HA epitope. Immunoblotting was performed using standard protocols and HA-probe Y-11 (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA).

Growth assays. Yeast strains were grown to OD_{600} 0.5–1.0 in medium lacking tryptophan with 100 μg/ml dTMP, washed, and diluted to OD_{600} 0.05 in medium with no dTMP. For 96-well assays, each well contained 200 μl diluted yeast and 0.5 μl or 1 μl of specified drug. Plates were incubated as indicated.

Mutagenesis of ER ligand-binding domain. Error-prone PCR mutagenesis was performed as described[29], except that 0.25 mM $MnCl_2$ was also included. L525A was generated by recombination as with D37V. PCR fragments containing ER variants were transformed into pTB3-dhFK cut with *Nhe*I and *Cla*I. Transformants were grown in media with dTMP, but growth and ligand-response assays contained no dTMP.

Chemical library screen. Each compound was dissolved in DMSO, then added to 200 µl OD_{600} 0.05 yeast prepared as described for 96-well assays. Plates were incubated as indicated. OD_{600} was measured on a Wallac Victor[2] plate reader.

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