A general life-death selection strategy for dissecting protein functions

Po Hien Ear & Stephen W Michnick

Clonal selection strategies are central tools in molecular biology. We developed a general strategy to dissect protein functions through positive and negative clonal selection for protein-protein interactions, based on a protein-fragment complementation assay using *Saccharomyces cerevisiae* cytosine deaminase as a reporter. We applied this method to mutational or chemical disruption of protein-protein interactions in yeast and to dissection of the functions of an allosterically activated transcription factor, Swi6.

Proteins can perform different functions, in part, through sets of distinct interactions with other proteins. Thus, a protein's functions can be dissected by selectively disrupting individual interactions[1]. To achieve such fine dissection of protein-protein interactions, we designed a simple positive and negative clonal selection strategy based on a protein-fragment complementation assay (PCA)[2,3] with a prodrug-converting enzyme as reporter (**Fig. 1a**). We chose the yeast *Saccharomyces cerevisiae* cytosine deaminase (yCD) as the reporter protein because life and death selection assays have been established for this enzyme in a broad spectrum of cells including those of bacteria[4], yeast[5] and mammals[6].

Conceptually, a yCD PCA could be used to dissect protein-protein interactions as follows. In yeast, deletion of the *FCY1* gene encoding yCD renders the strain defective for the pyrimidine salvage pathway because its capacity to convert cytosine to uracil is lost; thus cells that cannot synthesize uracil by the *de novo* pyrimidine pathway cannot grow in the absence of uracil (**Fig. 1b**). In the life selection assay, the interaction of proteins *X* and *Y* brings complementary fragments of yCD into proximity, allowing them to fold and reconstitute its catalytic activity. Complementing an *FCY1* knockout strain with yCD PCA would restore cell growth. Death selection is achieved when the same yCD-complemented strain is treated with 5-fluorocytosine (5-FC), a nontoxic compound that is converted to toxic 5-fluorouridine triphosphate (5-FUTP) in a pathway that depends on yCD activity[7].

We could combine these selection assays to dissect binary interactions between proteins. For instance, if protein X interacts with both protein Y and a third partner Z, and we wish to disrupt the X-Z, but retain the X-Y interaction, we could first perform the death selection yCD PCA with a library of mutants X* screened against Z and select for growth (disruption of the X-Z interaction) and then perform the life selection yCD PCA with mutants of X* against Y, selecting clones that grow (positive for the X*-Y interaction). The key strength of this approach is that, as both life and death selection result in a growth phenotype, identifying clones in both steps would be trivial compared to just using a life selection assay that would require an additional step for replicating clones on a control plate to recover mutants of interest.

To identify appropriate PCA fragments, we tested seven different combinations of yCD fragments (**Fig. 1c**) each fused downstream of the coding sequence of homodimerizing residues (250–281) of the GCN4 parallel coiled-coil leucine zipper (zip) via a sequence coding for a 15-amino-acid flexible linker peptide. We identified complementary N- and C-terminal fragments (referred to as F[1] and F[2]) with the highest yCD PCA activity consisting of residues 1–77 and 57–158 (yCD-F[1]1–77 and yCD-F[2]57–158) (**Fig. 1d**). To improve the activity of the yCD PCA, we first introduced into these yCD fragment fusions three mutations that had been shown to increase thermostability of full-length yCD[8] and found that this improved the activity of yCD PCA (**Supplementary Fig. 1**).

However, the observed improvement for the leucine zipper interaction was not sufficient to detect other interactions such as that between the human small GTPase H-Ras (Ras) and the Ras binding domain (RBD) of the serine/threonine kinase c-Raf (**Fig. 1e**). Thus, using the Ras-RBD interaction as a test system, we attempted to generate fragments that result in improved yCD PCA activity. We generated libraries of randomly mutated yCD-F[1]1–77 and yCD-F[2]57–158 by error-prone PCR (with 1–2 mutations per fragment) and fused them to the interacting partners, Ras and RBD (**Supplementary Fig. 2**). We screened the mutant fragment–Ras and –RBD fusions against each other and identified mutants that additionally increased yCD PCA activity (**Supplementary Table 1**). Among the clones collected, the mutations T95S and K117E in yCD-F[2]57–158, in combination with the three mutations that increased thermostability, showed the greatest sensitivity to 5-FC (highest yCD activity) (**Fig. 1e** and **Supplementary Table 2**). These optimized fragments are called henceforth, OyCD-F[1] and OyCD-F[2]. Mutations T95S and K117E (**Fig. 1f**) may improve activity by creating a salt bridge between Glu117 and wild-type Arg125 of the adjacent subunit (**Supplementary Fig. 3**). OyCD PCA activity did not result from spontaneous complementation of the fragments, as expression of proteins of interest fused to OyCD-F[1] with OyCD-F[2] alone resulted in no observable OyCD activity (**Supplementary Fig. 4**).

We next tested OyCD PCA sensitivity for detecting changes in dissociation constants and quantities of protein complexes. First,

Département de Biochimie, Université de Montréal, Montréal, Québec, Canada. Correspondence should be addressed to S.W.M. (stephen.michnick@umontreal.ca).

RECEIVED 5 MAY; ACCEPTED 18 AUGUST; PUBLISHED ONLINE 11 OCTOBER 2009; DOI:10.1038/NMETH.1389

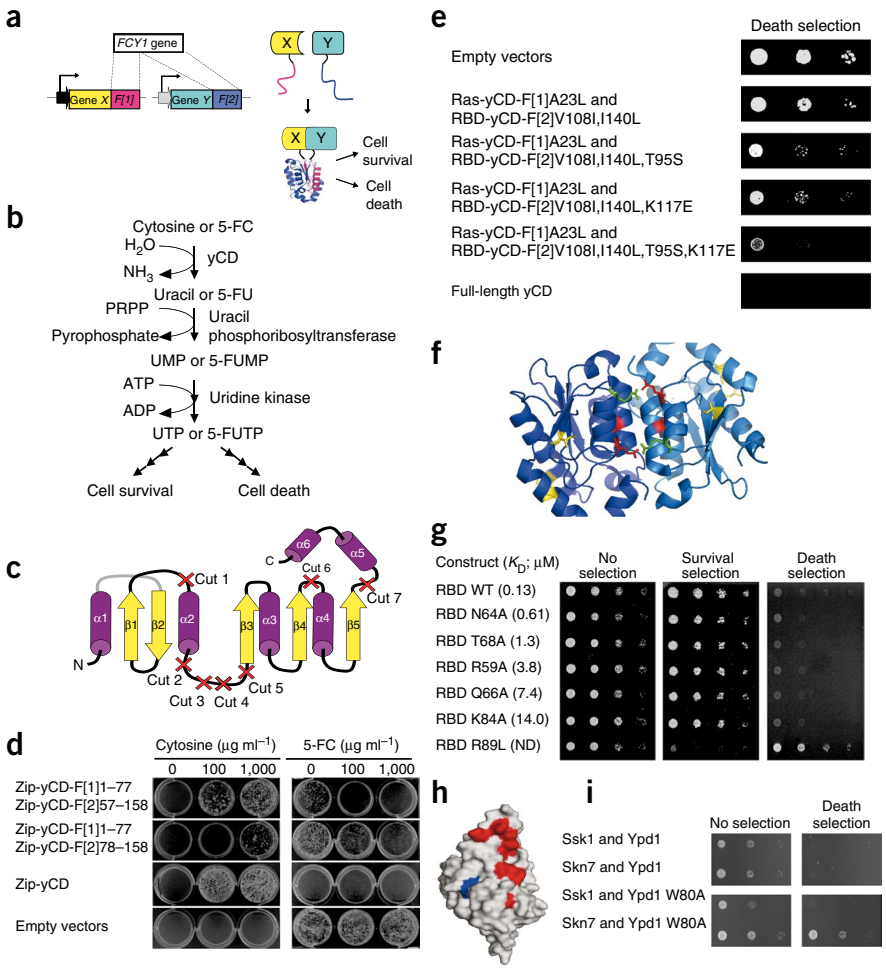

Figure 1 | Development and characterization of the OyCD PCA. (**a**) Two *FCY1* fragments, *F[1]* and *F[2]* were each fused to genes encoding one of two interacting proteins. This allows cell survival or cell death selection. (**b**) Enzymes of the pyrimidine salvage pathway. Both cytosine and 5-FC are substrates for yCD. (**c**) yCD schematic with the 7 cut sites indicated. (**d**) Life and death yCD PCA, on growth medium containing the indicated amounts of cytosine or 5-FC, for different fragment combinations (1–77 and 57–158 in comparison to 1–77 and 78–158), fused to zip peptides. In all PCAs, yeast colonies shown are from tenfold serial dilutions of starting material beginning with 10,000 cells. (**e**) Optimized yCD (OyCD) PCA tested by fusion of yCD fragments to RBD of c-Raf. Introduced mutations in yCD are indicated. (**f**) A model of the OyCD structure with optimizing T95S and K117E mutations (red), Arg125 (green) and increased thermostability–conferring mutations (yellow) based on yCD structure (Protein Data Bank: 1YSB). (**g**) Life (50 μg ml^{-1} cytosine) and death (50 μg ml^{-1} 5-FC) selection OyCD PCA for monitoring Ras interactions with mutant RBDs. Cells expressed OyCD-F[1]-Ras and RBD-OyCD-F[2] with wild-type (WT) RBD or the indicated RBD construct. ND, not determined. (**h**) A model of Ypd1 showing residues that mediate interactions with Skn7, Ssk1 and Sln1 (red) and showing Trp80 (ref. 10) (blue), which mediates Ypd1-Skn7 but not Ypd1-Ssk1 interactions. (**i**) OyCD PCA for interactions of Ypd1 (wild-type and W80A mutant) with Skn7 and Ssk1, using death selection (100 μg ml^{-1} 5-FC).

we tested the Ras-RBD interaction using binding mutants of the RBD with known dissociation constants[9] and found that we could detect Ras interactions with the wild-type RBD and with mutants of RBD with a dissociation constant (K_D) up to 14 μM (**Fig. 1g**). We then measured competitive and stoichiometric disruption of the homodimerizing mutant of the peptidyl-prolyl isomerase FKBP12 (FM1) by the high-affinity binding macrolide FK506 (**Supplementary Fig. 5**). The OyCD PCA death assay was sensitive enough to distinguish a threefold decrease in the number of FM1 homomers, caused by a twofold increase in FK506 concentration. This difference corresponds to approximately the change in the number of complexes that would be caused by a single disruptive mutation of a protein-protein interface, suggesting that conditions can be found for detecting changes in binding affinity of mutants.

To demonstrate that OyCD PCA could be used to dissect protein-protein interactions of a protein with different partners, we studied the interaction between Ypd1, a histidine-containing phosphotransfer protein required for signaling osmotic stress in yeast, and its response regulator proteins Skn7 and Ssk1. Ypd1 has a common binding domain for its regulatory proteins, but the interactions have been shown to be mediated in part through distinct residues[10] (**Fig. 1h**). Trp80 is known to mediate the specific interaction between Ypd1 and Skn7 but not between Ypd1 and Ssk1. We could clearly observe specific disruption of the interaction between Ypd1 W80A mutant and Skn7 while the Ypd1 W80A-SSk1 interaction was retained (**Fig. 1i**).

As proof of principle of a functional dissection of interacting proteins, we devised a strategy to dissect the transcriptional regulation of the *S. cerevisiae* transcription factor Swi6. Swi6 interacts with Mbp1 or Swi4 to form MBF or SBF transcription factor complexes, respectively, which regulate the expression of genes that control the G1- to S-phase transition of the yeast cell cycle[11] (**Fig. 2a**). Swi6 is a modular protein that contains two transcriptional activation regions (N- and C-TAR), an ankyrin repeat domain (AnkRD) and a C-terminal heterodimerizing domain (BD) that can interact with unique C-terminal Swi6 binding domains of Mbp1 (mBD) or Swi4 (sBD)[11] (**Fig. 2b**). To determine whether Swi6 binds to Mbp1 and Swi4 in distinct ways and therefore could differentially regulate MBF or SBF activities, we created a three-step screening strategy to identify Swi6 mutants for which MBF activity was lost but SBF activity was retained.

We first determined that MBF and SBF complexes could be detected by OyCD PCA (**Supplementary Fig. 6**). We then generated a library of full-length Swi6 mutants in which the sequences encoding the BD and the C-TAR domains were randomly mutated by error-prone PCR (Swi6*). We screened the resulting library of 10,000 clones against Mbp1 in the death assay and collected 8,000 'positive' clones (non-reconstitution of OyCD activity) (**Fig. 2c**). Second, we screened these Swi6 mutants against Swi4 in the OyCD life assay (reconstitution of OyCD activity). After the two steps of selection, we retested 90 clones carrying potential Swi6

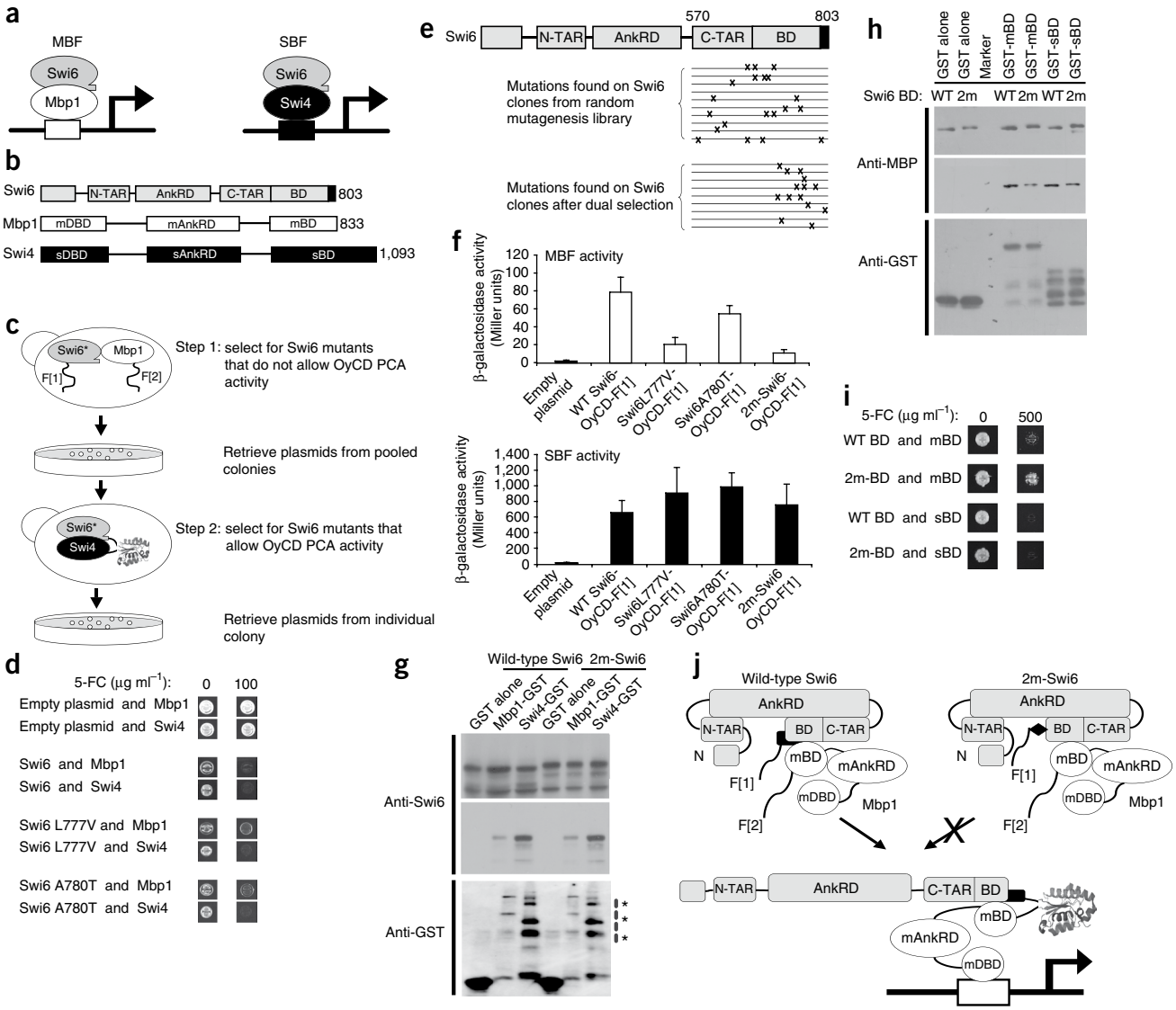

Figure 2 | Dissecting transcriptional activity of Swi6. (**a**) Schematic of MBF and SBF transcription factor complexes. (**b**) Domain structures of Swi6, Mbp1 and Swi4. BD, Swi6 C-terminal domain that binds Mbp1 and Swi4; mBD or sBD, C-terminal Swi6-binding domains of Mbp1 and Swi4, respectively. (**c**) Strategy for engineering a Swi6 mutant. Step 1: death selection screen of a mutant Swi6 OyCD fusion library (Swi6*) expressed with Mbp1 fusion. Selection is for clones lacking OyCD PCA activity (growth on 5-FC). Step 2: life selection of Swi6* clones from step 1, expressed with Swi4 fusion. Selection is for clones with OyCD PCA activity (growth on cytosine). (**d**) Examples of Swi6* clones that grew on 5-FC when expressed with Mbp1 but not with Swi4. (**e**) Distribution of Swi6 mutations in the initial library and after the two-step OyCD PCA screen. (**f**) MBF and SBF transcriptional activities of the indicated Swi6 fusion proteins in *swi6* deletion cells. Data are mean ± s.d. ($n = 4$). (**g**,**h**) GST pulldown assays with full-length proteins expressed in yeast (**g**) or with purified C-terminal binding domains expressed in bacteria (**h**), in the indicated combinations were analyzed by western blot. Wild-type or mutant (2m) Swi6 BD was fused to maltose binding protein (MBP). Unbound (top) and GST-bound (middle) fractions were analyzed with an antibody to Swi6 (anti-Swi6; **g**) or antibody to MBP (anti-MBP; **h**) as well as for expression of GST fusions (bottom) with an antibody to GST (anti-GST). Asterisks indicate degradation products of Mbp1-GST or Swi4-GST. (**i**) OyCD PCA using death selection with Mbp1, Swi4 and Swi6 C-terminal binding domains. (**j**) Model for allosteric regulation of Swi6. Swi6 undergoes a conformational change on binding Mbp1 and activates MBF activity. The 2m-Swi6 does not undergo this change. Black diamond indicates L777V and A780T mutations. N- and C-TAR, N- and C- transcriptional activation domains; AnkRD, ankyrin repeat domain; mDBD and sDBD, Mbp1 and Swi4 DNA binding domains, respectively.

mutants for interactions with Mbp1 or Swi4 using OyCD PCA (**Supplementary Fig. 7**). Nine clones had decreased OyCD PCA activity with Mbp1, with OyCD PCA activity with Swi4 remaining unaffected (**Fig. 2d**). Comparison of a set of sequences from the original Swi6* mutant library to those of the clones found after the life and death selection screen showed that mutants in the initial library were randomly distributed throughout C-TAR and BD, whereas the mutants selected after the second Swi4 screen had mutations located only in the BD, suggesting that mutants were selected for specific binding (**Fig. 2e** and **Supplementary Fig. 8**).

Finally, we screened the nine Swi6 mutants to identify those that disrupt MBF but not SBF activity using MBF and SBF transcription reporter assays[12]. Two single Swi6 mutants (Swi6L777V and Swi6A780T) had decreased MBF activity and unchanged SBF activity, and when we combined the two mutations, the double mutant (2m-Swi6) had an additional reduction in MBF activity

and unchanged SBF activity (**Fig. 2f**). The remaining seven clones had no change in MBF or SBF activity (**Supplementary Fig. 7b**).

We next performed glutathione *S*-transferase (GST) pulldown experiments to analyze the interaction between the 2m-Swi6 and Mbp1. Notably, both full-length 2m-Swi6 and C-terminal fragments of 2m-Swi6 retained the ability to bind to both Mbp1 and Swi4 (**Fig. 2g**,**h**) although OyCD PCA activity was decreased (**Fig. 2d**,**i**), at similar expression levels of wild-type Swi6 and 2m-Swi6 (**Supplementary Fig. 9**).

A potential explanation for these results is provided by consideration of how binding of Mbp1 and Swi4 to Swi6 activate their transcriptional activity and how the OyCD PCA detects protein-protein interactions (**Fig. 2j**). In its inactive state, the AnkRD of Swi6 antagonizes Swi6 transactivation by direct binding to both N- and C-terminal TARs[13]. Residues 773–784 of Swi6 have been shown to be important in activation of Swi6 (ref. 13). Binding of Swi6 to Mbp1 or Swi4 causes the TARs to dissociate from the AnkRD and Swi6 to open up, allowing the TARs to engage the transcriptional machinery, a transition that requires participation of residues 773–784. As the two mutations in 2m-Swi6 are found in this region, it is possible that the 2m-Swi6 mutations decouple binding of Swi6 to Mbp1 from a change in conformation that is necessary for transactivation. The 2m-Swi6 could be locked in the inactive state, whether or not bound to Mbp1 (**Fig. 2j**).

PCAs are exquisitely sensitive to the topology of protein complexes because the reporter fragments must be free and close enough in space to fold[14,15]. We suggest that the OyCD PCA result for the Mbp1–2m-Swi6 interaction is thus not due to disruption of the interaction, but is caused by sequestering of the PCA fragment that is fused to the C terminus of Swi6 downstream from residues 773–784. In this model, Swi4 must engage the conformation change in Swi6 in a different way, thus allowing for formation of an active SBF complex.

We demonstrated an approach to dissect the functions of a protein by disrupting unique protein-protein interactions or by decoupling binding from conformation changes required for a specific protein function. As many functions of a protein are mediated by multiple protein-protein interactions, the strategy can allow for systematic dissection of protein function and provide mechanistic insights into how binding is coupled to specific functions. The OyCD PCA is general and applicable to study interactions of any full-length protein. Unlike in the yeast two-hybrid or split ubiquitin assays, the proteins (including nuclear chromatin–associated proteins) may be expressed in their appropriate cellular compartments and with posttranslational modifications that reflect their natural state under any specific conditions and in any cell type. Finally, the OyCD PCA should also be invaluable to efforts devoted to creating new chemical or protein probes for manipulation of cellular regulatory networks and to developing therapeutics.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

ACKNOWLEDGMENTS

We thank H. Bussey (McGill University) for providing yeast strains and p41XGal1 plasmids, B. Andrews (Donelly Centre, University of Toronto) for plasmids pBA487 and pBA251 and Swi6 antibodies, T. Clackson and V. Rivera (Ariad) for $pC_4EN\text{-}F_M3$, M.J. Booth for constructing p415Gal-Linker-OyCD-F[2], and M. Malleshaiah and C. Chan for comments. This research was supported by grants from the Canadian Institutes of Health Research (MOP-152556) and the Canada Research Chairs Program to S.W.M. P.H.E. acknowledges the Faculté des etudes supérieurs de l'Université de Montréal for scholarships.

AUTHOR CONTRIBUTIONS

P.H.E. and S.W.M. designed the experiments, analyzed the results and wrote the manuscript. P.H.E. performed the experiments.

Published online at http://www.nature.com/naturemethods/.
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/.

1. Reguly, T. *et al. J. Biol.* **5**, 11 (2006).
2. Pelletier, J.N., Campbell-Valois, F.X. & Michnick, S.W. *Proc. Natl. Acad. Sci. USA* **95**, 12141–12146 (1998).
3. Michnick, S.W., Ear, P.H., Manderson, E.N., Remy, I. & Stefan, E. *Nat. Rev. Drug Discov.* **6**, 569–582 (2007).
4. Mahan, S.D., Ireton, G.C., Knoeber, C., Stoddard, B.L. & Black, M.E. *Protein Eng. Des. Sel.* **17**, 625–633 (2004).
5. Hartzog, P.E., Nicholson, B.P. & McCusker, J.H. *Yeast* **22**, 789–798 (2005).
6. Wei, K. & Huber, B.E. *J. Biol. Chem.* **271**, 3812–3816 (1996).
7. Fang, F., Hoskins, J. & Butler, J.S. *Mol. Cell. Biol.* **24**, 10766–10776 (2004).
8. Korkegian, A., Black, M.E., Baker, D. & Stoddard, B.L. *Science* **308**, 857–860 (2005).
9. Block, C., Janknecht, R., Herrmann, C., Nassar, N. & Wittinghofer, A. *Nat. Struct. Biol.* **3**, 244–251 (1996).
10. Porter, S.W. & West, A.H. *Biochim. Biophys. Acta* **1748**, 138–145 (2005).
11. Bahler, J. *Annu. Rev. Genet.* **39**, 69–94 (2005).
12. Andrews, B.J. & Moore, L.A. *Proc. Natl. Acad. Sci. USA* **89**, 11852–11856 (1992).
13. Sedgwick, S.G. *et al. J. Mol. Biol.* **281**, 763–775 (1998).
14. Remy, I., Wilson, I.A. & Michnick, S.W. *Science* **283**, 990–993 (1999).
15. Tarassov, K. *et al. Science* **320**, 1465–1470 (2008).

ONLINE METHODS

Construction of the yCD PCA. Sequence encoding the GCN4 leucine zipper (*zip*) and the linker sequence coding for amino acids GGGGS was amplified by PCR with Pfu polymerase (Fermentas) from pcDNA3.1-Zip-mDHFR-F[1,2]. Sequence encoding the zip peptide was cloned into the multiple cloning sites of p413Gal1 and p415Gal1 vectors at the XbaI and XhoI restriction sites. A unique BspEI site was added as part of the linker sequence for cloning downstream of the *zip* sequence. Vectors containing the *zip* sequence were named p413Gal1-Zip and p415Gal1-Zip. Sequences encoding the yCD gene and the yCD fragments were amplified from the genomic DNA of *S. cerevisiae* strain BY4743 (diploid, *ura3Δ0 leu2Δ0 his3Δ1 met5Δ0 lys2Δ0*) using Pfu polymerase and cloned into p413Gal1-Zip and p415Gal1-Zip vectors downstream of the *zip* sequence using BspEI and XhoI restriction sites. Sequence encoding Ras residues 1–166 was amplified from pQE30-Ras-mDHFR-F[3] (ref. 16) and subcloned upstream of yCD-F[1] to generate p413Gal1-Ras-yCD-F[1]. Sequence encoding RBD residues 1–133 was amplified from p416Gal1-RBD (aa 1–133) and subcloned upstream of yCD-F[2] to generate p415Gal1-RBD-yCD-F[2]. The full-length sequence of Ras residues 1–189 was amplified from p413ADH1-Ras1-189 and subcloned in p413Gal1 using XbaI and XhoI sites with a primer that introduced a BspEI site downstream of XbaI. Sequence encoding OyCD-F[1] was subcloned upstream of sequence encoding Ras residues 1–189 using XbaI and BspEI sites to generate p413Gal-OyCD-F[1]-Ras residues 1–189. Sequence encoding wild-type RBD residue 55–133 and seven mutant RBD 55–133 (ref. 17) sequences were amplified and subcloned upstream of sequence encoding OyCD-F[2]. Sequence encoding FM1 was amplified from $pC_4EN\text{-}F_M3$ (Ariad) and subcloned upstream of sequence encoding yCD-F[1] A23L (OyCD-F[1]) and yCD-F[2] V108I, I140L, T95S, K117E (OyCD-F[2]) to generate p413Gal1-FM1-OyCD-F[1], p413Gal1-OyCD-F[1]-FM1 and p415Gal1-FM1-OyCD-F[2]. Sequence encoding Ypd1 and sequences encoding the response regulator domains of Skn7 and Ssk1 were amplified from genomic DNA of BY4743 strain and cloned upstream of sequence encoding OyCD fragments to generate p413Gal1-Skn7-OyCD-F[1], p413Gal1-Ssk1-OyCD-F[1] and p415Gal1-Ypd1-OyCD-F[2]. Site-directed mutagenesis was carried out according to the QuickChange strategy (Stratagene) to introduce the W80A mutation in Ypd1. Sequences encoding Mbp1, Swi4 and Swi6 were digested from p415ADH-Mbp1-vF[2], p415ADH-Swi4-vF[2] and p415ADH-Swi6-vF[2], respectively, and subcloned in p413Gal1-OyCD-F[1] and p415Gal1-OyCD-F[2]. Sequence encoding GST was amplified from pGEX-5X-3 and subcloned in p413Gal1 plasmids to generate p413Gal1-GST and p413Gal1-Swi6-GST. All primers used in this study are listed in **Supplementary Table 3**.

Selection for wild-type yCD PCA activity. *S, cerevisiae* BY4741 (*MAT***a**, *ura3Δ0 leu2Δ0 his3Δ1 met5Δ0*), BY4742 (*MATα*, *ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*) and BY4743 (diploid, *ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 met5Δ0*) *fcy1Δ* mutant strains were generated as part of the yeast gene knockout collection[18] and used to assay for yCD PCA activity because their genomic copies of *FCY1* were disrupted. These deletion strains were propagated in medium containing 200 μg ml^{-1} of geneticin (G418) (Invitrogen). For assaying yCD PCA activity, BY4741 and BY4742 *fcy1Δ* cells were transformed with p413Gal1 and p415Gal1 vectors carrying respective fusion genes. Cells from each mating type were mated and selected on synthetic complete medium without methionine, lysine, histidine and leucine (SC-Met-Lys-His-Leu) with 2% glucose. (All values in percentage represent weight per volume.) Protein expression was induced by inoculating yeast cells overnight in 1 ml of SC-Met-Lys-His-Leu with 2% raffinose. The next day, 20 μl of the culture was transferred to 1 ml of the same selection medium with 2% galactose for 6-h induction at 30 °C. For the survival selection assay, cells were plated on solid selection medium: SC-Met-Lys-His-Leu-uracil with 2% Noble agar, 2% raffinose and 2% galactose (with 0, 100 or 1,000 μg ml^{-1} cytosine). Plates were incubated at 30 °C for 6 d. For the 5-FC death selection assay with 5-FC preincubation, approximately 5,000 cells were transferred to 1 ml of SC-Met-Lys-His-Leu with 2% raffinose, 2% galactose and 100 μg ml^{-1} 5-FC and grown for 18 h at 30 °C with shaking. After the preincubation period, 10 μl of each sample was plated on solid selection medium: SC-Met-Lys-His-Leu with 2% agar, 2% raffinose and 2% galactose (with 0, 100 or 1,000 μg ml^{-1} of 5-FC). Plates were incubated at 30 °C for either 2 or 3 d.

Optimization of yCD PCA activity. To generate the 37 °C stable yCD PCA, site-directed mutagenesis was carried out according to the QuickChange strategy (Stratagene) to introduce the A23L, V108I and I140L triple mutations. For additional optimization of yCD PCA activity, error-prone PCR was used to generate a library of yCD-F[1] and yCD-F[2] (yCD-F[1]ep and yCD-F[2]ep) carrying on average one mutation per fragment. PCR was performed with Taq polymerase (NEB), unequal concentration of nucleotides (1 mM dCTP, 1 mM dTTP, 0.2 mM dATP and 0.2 mM dGTP), 5 mM $MgCl_2$ and 10% DMSO using the following conditions: 95 °C (3 min), 30 cycles of 95 °C (1 min), 55 °C (1 min), 72 °C (1 min) and then 72 °C (5 min). Sequences encoding yCD-F[1]ep and yCD-F[2]ep were subcloned downstream of genes encoding Ras or RBD in p413Gal1-Ras and p415Gal1-RBD vectors using BspE1 and XhoI restriction sites. The ligation products were transformed in DH5α cells by electroporation. The size of each library was 1,463 and 1,983 clones, respectively. The libraries were transformed into BY4741 and BY4742 *fcy1Δ* strains. Cells from respective haploid types were mated, generating approximately 3×10^6 clones and plated on SC-Met-Lys-His-Leu-uracil (uracil-depleted medium) with 2% Noble agar, 2% raffinose and 2% galactose containing 1,000 μg ml^{-1} of cytosine. Selection plates were incubated at 37 °C. After 4 d, cells with increased cytosine deaminase activity from yCD PCA formed colonies. We inoculated 276 colonies in SC-Met-Lys-His-Leu with 2% raffinose and 2% galactose, and grew them overnight. The next day, cells were pinned onto SC-Met-Lys-His-Leu with 3% agar, 2% raffinose and 2% galactose (with 0 and 100 μg ml^{-1} 5-FC). Plates were incubated at 37 °C for 2 d. Sixteen clones were identified to have increased sensitivity to 5-FC. PCR was performed on these clones to amplify sequences encoding yCD-F[1] and yCD-F[2]. PCR products were sequenced to identify mutations. Mutations found are listed in **Supplementary Table 1**.

p413Gal1-Ras-yCD-F[1]A23L, M1L and p415Gal1-RBD-yCD-F[2]V108I, I140L, T95S plasmids were retrieved by isolating DNA from yeast cells using DNeasy Tissue Kit (Qiagen) and transforming the DNA into DH5α cells for amplification of the plasmids. To increase yCD PCA activity, other mutations were

combined with yCD-F[1]A23L, M1L or yCD-F[2]V108I, I140L, T95S by site-directed mutagenesis (Stratagene). Plasmids were transformed into BY4741 and BY4742 *fcy1Δ* strains, respectively. Cells were mated and selected for cytosine deaminase activity on uracil-depleted medium containing 100 μg ml^{-1} of cytosine. Cells were incubated at 37 °C for 2 d. Colonies were assayed for 5-FC sensitivity by pinning onto 5-FC plates as previously described in this section. The 5-FC–sensitive clones were identified and PCR products containing sequences encoding yCD fragments were sequenced. Mutations found are listed in **Supplementary Table 2**.

Dual selection assays for Ras and RBD interaction using OyCD PCA. Plasmids were transformed in the BY4743 *fcy1Δ* strain. Colonies were grown overnight in 1 ml of SC-Met-Lys-His-Leu with 2% raffinose and induced for protein expression with 2% galactose for 6 h and assayed for yCD activity by pinning onto SC-Met-Lys-His-Leu with 3% agar, 2% raffinose and 2% galactose with 0 μg ml^{-1} 5-FC as control plate, on SC-Met-Lys-His-Leu-uracil with 3% Noble agar, 2% raffinose and 2% galactose with 50 μg ml^{-1} cytosine for survival selection and on SC-Met-Lys-His-Leu with 3% agar, 2% raffinose and 2% galactose with 50 μg ml^{-1} 5-FC for death selection. Plates were incubated at 37 °C for 2 d.

Disruption of interaction between FM1 homodimer using FK506. Empty plasmids and plasmids carrying fusion genes were co-transformed into the BY4743 *fcy1Δ* strain. Colonies were grown overnight in 1 ml of SC-Met-Lys-His-Leu with 2% raffinose and induced for protein expression with 2% galactose for 6 h. 500 cells from each sample were plated on different selection medium: SC-Met-Lys-His-Leu with 3% agar, 2% raffinose and 2% galactose alone, with 100 μg ml^{-1} 5-FC, with 5 or 10 μM FK506 (ref. 19), and with 100 μg ml^{-1} 5-FC and 10 μM FK506. Plates were incubated at 30 °C for 2 d.

Disruption of specific interaction between Ypd1 and the response regulator domain of Skn7. Plasmids carrying fusion genes were transformed into the BY4741 *fcy1Δ* strain. Colonies were grown overnight in 1 ml of SC-Met-Lys-His-Leu with 2% raffinose, induced for protein expression with 2% galactose for 6 h and assayed for yCD activity by pinning onto SC-Met-Lys-His-Leu with 3% agar, 2% raffinose and 2% galactose with 0 μg ml^{-1} 5-FC as control plate and on SC-Met-Lys-His-Leu with 3% agar, 2% raffinose and 2% galactose with 100 μg ml^{-1} of 5-FC for death selection. Plates were incubated at 30 °C for 2 d.

OyCD PCA for cell cycle transcription factors. Plasmids carrying fusion genes were transformed into the BY4741 *fcy1Δ* strain. Colonies were grown overnight in 1 ml of SC-Met-Lys-His-Leu with 2% raffinose, induced for protein expression with 2% galactose for 6 h and assayed for yCD activity by pinning onto SC-Met-Lys-His-Leu with 3% agar, 2% raffinose and 2% galactose with 0 μg ml^{-1} 5-FC as control plate and on SC-Met-Lys-His-Leu with 3% agar, 2% raffinose and 2% galactose with 100 or 500 μg ml^{-1} of 5-FC for death selection. Plates were incubated at 30 °C for 2–4 d.

Dissecting Swi6 using OyCD PCA. Error-prone PCR was used to generate a library of sequences encoding Swi6 carrying on average two to three mutations between amino acid residues 570–803 (Swi6*560–803). PCR was performed with the same conditions as for sequences encoding yCD-F[1]ep and yCD-F[2]ep. Swi6*560–803 sequences were subcloned in p413Gal1-Swi6-OyCD-F[1] using EcoRI and BspEI restriction sites. The ligation products were transformed in DH5α cells by electroporation. The size of each library was 1×10^4 clones. The library of p413Gal1-Swi6*-OyCD-F[1] was transformed in BY4741 *fcy1Δ* strain carrying p415Gal1-Mbp1-OyCD-F[2], generating approximately 3×10^4 clones. Colonies were pooled and plated on SC-Met-Lys-His-Leu with 2% agar, 2% raffinose, 2% galactose containing 200 μg ml^{-1} of 5-FC. Selection plates were incubated at 30 °C. After 3 d, cells expressing Swi6*-OyCD-F[1] and Mbp1-OyCD-F[2] that do not allow OyCD PCA activity formed colonies. Colonies were pooled for DNA extraction using the DNeasy Tissue kit (Qiagen). DNA was electroporated in MC1061 *Escherichia coli* to retrieve plasmids. Plasmids were re-transformed in BY4741 *fcy1Δ* strain carrying p415Gal1-Swi4-OyCD-F[2] and plated on SC-Met-Lys-His-Leu-uracil with 2% Noble agar, 2% raffinose and 2% galactose containing 200 μg ml^{-1} of cytosine. After 3 d, cells expressing Swi6*-OyCD-F[1] that interacts with Swi4-OyCD-F[2] formed colonies. Colonies were pooled for DNA extraction and plasmids were retrieved. Individual plasmids were re-transformed in BY4741 *fcy1Δ* strain carrying either p415Gal1-Mbp1-OyCD-F[2] or p415Gal1-Swi4-OyCD-F[2] and assayed for OyCD PCA activity using 5-FC assay. Swi6* clones with a decreased interaction with Mbp1, slightly resistant to 5-FC, while still conserving an interaction with Swi4, sensitive to 5-FC, were identified and sequenced.

Beta-galactosidase transcriptional reporter assay. Plasmids containing sequences encoding wild-type and mutant forms of Swi6-OyCD-F[1] were transformed in BY4741 *swi6Δ* strain[18] carrying reporter plasmids for detecting either MBF or SBF activity. These reporter plasmids are pBA487 or pBA251 (gifts of B. Andrews). pBA487 and pBA251 contain four MBF and SBF DNA binding motifs (MCB and SCB), respectively, in the promoter region upstream of the beta-galactosidase reporter gene. Colonies were assayed for beta-galactosidase activity[20].

Wild-type and 2M-SWI6 strains. The *SWI6* gene along with its 500 bp upstream sequence (*PSWI6*) was PCR amplified from genomic DNA and subcloned into pAG25 which carries the nourseothricin *N*-acetyl-transferase (*NAT1*) gene that confers resistance to nourseothricin to generate pAG25-PSWI6. We introduced the L777V and A780T mutations into the SWI6 coding sequence in pAG25-PSWI6 by site-directed mutagenesis according to the QuickChange strategy (Stratagene) to give pAG25-PSWI6-2M. The PSWI6-NAT1 and PSWI6-2M-NAT1 sequences were PCR amplified with forward and reverse oligonucleotides that have sequence homology to the *SWI6* promoter and terminator sequences. Both PCR products were used to transform the BY4741 *swi6Δ* strain and cells were selected on YPD with 100 μg ml^{-1} nourseothricin (Werner BioAgents). Clones were confirmed by diagnostic PCR, sequencing and western blotting using a rabbit polyclonal antibody to Swi6 (gift of B. Andrews).

Mbp1 and Swi4 GST pulldown in wild-type and 2M-SWI6 strains. Wild-type and 2M-SWI6 strains were transformed with

doi:10.1038/nmeth.1389

p415Gal1-GST, p415Gal1-Mbp1-GST and p415Gal1-Swi4-GST. Cells were grown in SC-Lys-Leu with 2% glucose to OD_{600} of 0.5, washed and grown in SD-Lys-Leu with 2% raffinose and 2% galactose for 1 h, 30 min to induce the expression of GST, Mbp1-GST and Swi4-GST. Cells were collected and lysed by bead beating in 50 mM Tris (pH 7.5), 150 mM NaCl, 15 mM $MgCl_2$, 1% Nonidet P-40, 0.1 mM sodium orthovanade, 1 mM DTT, 1 mM PMSF, 20 mM beta-glycerophosphate and 2 μg ml^{-1} of leupeptin. Cell lysates were centrifuged for 10 min at 13,000*g* and the supernatants were used for GST pulldown with 25 μl of Sephadex-gluthatione. Samples were equilibrated by rotation at 4 °C for 2 h and washed three times with the same buffer without protease inhibitors. Antibody to Swi6 was used to detect the presence of Swi6 and the GST antibody (Sigma) was used to detect GST, Mbp1-GST and Swi4-GST.

GST pulldown with interacting domains of Mbp1, Swi4 and Swi6. Mbp1(1017–1095), Swi4 (650–833) and Swi6(633–803) were subcloned in pGEX-5X-3 and pMAL-2CX (NEB) using BamHI and XhoI sites. Plasmids were transformed in BL21 *E. coli* strain for protein expression. Cell lysates were incubated with Sephadex-gluthatione or amylose resin (NEB) at 4 °C for 1 h. The beads were washed five times with the lysis buffer and resuspended in pulldown buffer for GST fusion proteins and in MBP column buffer with maltose for eluting MBP fusion proteins. For pulldown experiments, GST fusion proteins and MBP fusion proteins were mixed in 400 μl of PB buffer (20 mM Hepes (pH 7.4), 150 mM NaCl, 0.5 mM EDTA, 10% glycerol, 0.1% Triton X-100 and 2 mM DTT) and rotated on a wheel overnight at 4 °C. The samples were centrifuged at 1,500*g* for 1 min, the supernatants were collected and labeled as unbound fractions. The pellets were washed five times with 1 ml of PB buffer, and the beads were resuspended in 40 μl of sample buffer. We loaded 25 μl of protein from the unbound and bound fractions on a 10% SDS-polyacrylamide gel, transferred onto PVDF membrane, and probed with MBP antibodies (NEB) or GST antibodies.

16. Pelletier, J.N., Campbell-Valois, F.X. & Michnick, S.W. *Proc. Natl. Acad. Sci. USA* **95**, 12141–12146 (1998).
17. Block, C., Janknecht, R., Herrmann, C., Nassar, N. & Wittinghofer, A. *Nat. Struct. Biol.* **3**, 244–251 (1996).
18. Giaever, G. *et al. Nature* **418**, 387–391 (2002).
19. Rollins, C.T. *et al. Proc. Natl. Acad. Sci. USA* **97**, 7096–7101 (2000).
20. Ralser, M., Goehler, H., Wanker, E.E., Lehrach, H. & Krobitsch, S. *Biotechniques* **39**, 165–166, 168 (2005).