*Proc. Natl. Acad. Sci. USA*
Vol. 93, pp. 10001–10003, September 1996

# Commentary

## The yeast two-hybrid system: Forward and reverse

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Dissecting the molecular mechanism of a biological process requires identifying the proteins and protein–protein interactions that mediate the process. To that end, investigators have seized upon the utility of the yeast two-hybrid system to directly assay interactions between known proteins (1) and to isolate novel interacting partners for a protein of interest (2). Once a protein–protein interaction is identified, however, a great deal of additional experimentation is required to further characterize the functional relevance, structure, and regulation of the observed interaction. The identification of mutations in each partner of an interacting pair of proteins, which disrupt the interaction, can be useful not only for probing the structural components of an interaction, but also as a way to generate genetic tools for characterizing *in vivo* function. This can be particularly important for proteins that have multiple interacting partners. *In vivo* expression of variants that interact with only a subset of partners can provide information about which interactions are important to mediate specific activities in the cell. In this issue of the *Proceedings*, two papers by Vidal and colleagues describe the development (3) and application (4) of a "reverse" two-hybrid system specifically designed to facilitate identification of events that dissociate protein–protein interactions.

The crux of the reverse two-hybrid system is the incorporation of a reporter gene, to monitor protein–protein interactions, whose product can be toxic to growing cells. This allows the use of selective pressure against the formation of two-hybrid complexes. The yeast *URA3* gene product is essential for uracil biosynthesis and can also catalyze the transformation of 5-flouroorotic acid (5-FOA) into a toxic compound (5). Vidal *et al.* (3, 4) engineered a yeast strain in which expression of *URA3* was controlled by a tightly regulated promoter containing GAL4 binding sites. Growth of this strain on media lacking uracil requires expression of interacting GAL4 activation domain (GAD) and GAL4 DNA-binding domain (GBD) fusions, while growth on complete media containing 5-FOA is inhibited by interacting GAD and GBD fusions. Therefore, dissociating mutations in interacting proteins can be isolated from a library of randomly generated mutants by selection for 5-FOA-resistant colonies (3).

The facility of the yeast two-hybrid system to identify proteins carrying dissociating mutations from randomly generated populations of mutants was first demonstrated by Li and Fields (6) in a screen for mutations in the tumor supressor p53 that disrupt binding to simian virus 40 large T antigen. To detect association between p53 and large T antigen, they expressed p53 as a GBD fusion and large T as a GAD fusion in a two-hybrid reporter strain containing the *Escherichia coli lacZ* gene under control of the yeast *GAL1* promoter. Expression of both fusions in yeast induced *lacZ* expression, and the resulting β-galactosidase turned colonies blue when exposed to 5-bromo-4-chloro-3-indolyl β-D-galactoside. To isolate dissociating mutations, p53 was randomly mutagenized by PCR to generate a library of mutant p53-GBD fusions. This library was screened for fusions that produced pale blue or white colonies when co-expressed with large T antigen-GAD fusions. Although some of the dissociating events identified in this manner were due to a failure of yeast to produce stable full-length p53 (presumably due to frame shifts, nonsense mutations, and destabilizing mutations), 34 distinct p53 mutants were identified that were stably expressed in yeast but had an attenuated interaction, or no interaction with large T antigen. Many of the mutations in these proteins fell within regions frequently found to be mutated in human cancers (6). Using similar methods, Shan *et al.* (7) identified single amino acid changes in the transcription factor E2F-1 that disrupted interaction with the retinoblastoma protein (RB). This study identified critical amino acids within the domain of E2F-1 required for RB binding. Importantly, expression of the RB-binding defective E2F-1 mutants, in mammalian cells with wild-type RB, resulted in phenotypes that resembled loss of RB function, providing direct genetic evidence of the functional importance of the E2F-1/RB interaction (7). A two-step screening procedure using the yeast two-hybrid system was used to identify specific changes in a small domain of the Ras oncogene that separated the ability of Ras to interact with different downstream effector molecules (8). A screen for Ras mutants defective in interaction with one target was followed by a secondary screen to identify variants from the first screen that retained interaction with a second target. Expression of these mutants *in vivo* as well as compensating mutations in targets (also isolated in two-hybrid screens) has provided important information about the way distinct Ras-target interactions mediate complex Ras signaling events (8, 9).

Vidal *et al.* (4) applied their reverse two-hybrid system to the isolation of mutations in E2F1 that disrupt heterodimerization with DP1, a protein critical for high affinity binding of E2F1 to DNA and RB. Earlier studies using truncated versions of E2F1 identified domains that were sufficient for DP1 interaction but that were not required in the context of the full length protein, suggesting that multiple domains on E2F1 may be involved in DP1 binding (4). To maximize the sensitivity of the screen, the investigators first determined the minimal number of GAL4 binding sites required in the engineered *URA3* promoter coupled with the minimum concentration of 5-FOA that would still allow selection against the E2F1-GAD/DP1-GBD interaction. This had the advantage of potentially detecting even small changes in *URA3* expression that would result from weakly dissociating mutations (3). Yeast colonies expressing randomly mutated E2F1-GAD fusions and DP1-fusions were screened for those that could grow on media containing 5-FOA. Approximately 5% of the transformants survived the selective pressure (4). As mentioned for earlier studies using random mutagenesis, many of the 5-FOA-resistant colonies may result from relatively uninteresting mutations in E2F1 including truncations, frame shifts, and other gross conformational changes. Vidal *et al.* (3, 4) incorporated secondary screens to avoid further characterization of such alterations. They had included a second GAL4-dependent reporter gene in the reverse two-hybrid yeast strain, *HIS3*. They reasoned that weakly dissociating mutations in E2F1, while reducing *URA3*

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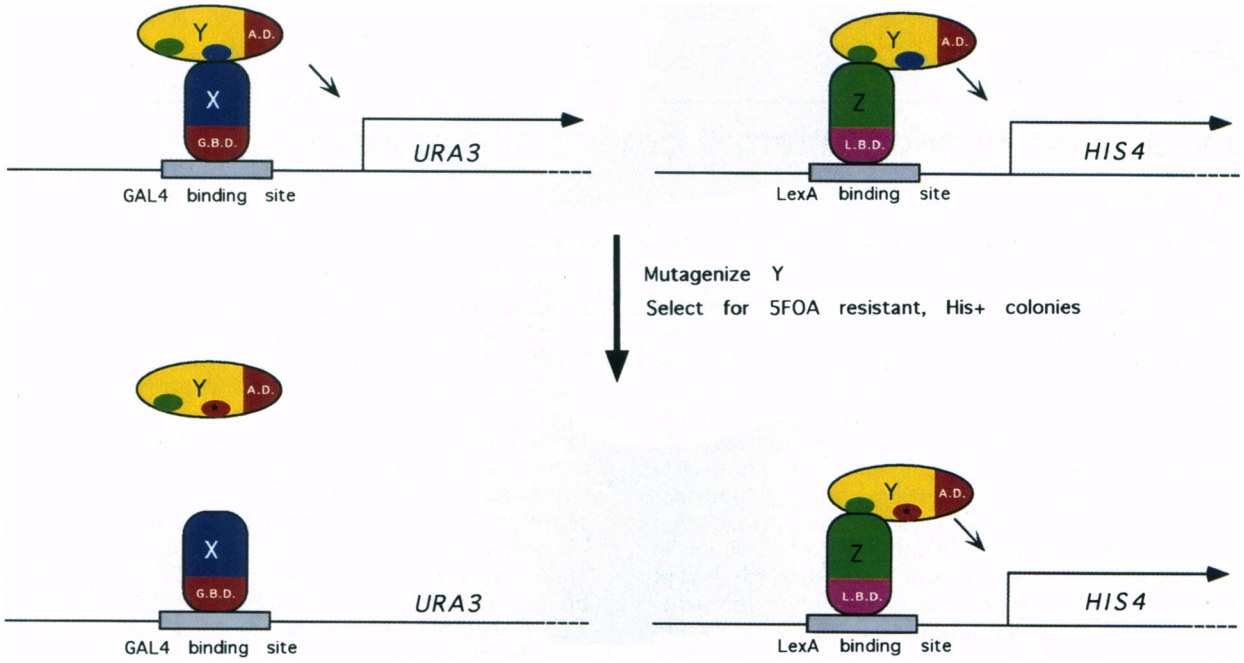


FIG. 1. A dual reporter system to simultaneously assess binding activity of a protein to two independent targets. The interaction of protein Y fused to an activation domain (AD) with protein X fused to the GBD, and protein Z fused to the LexA DNA-binding domain (LBD) can be detected by selecting for expression of URA3 and HIS4, respectively. Mutants in Y that disrupt interaction with X but not Z can be identified from a library of random mutations in a single step by selecting for 5-FOA resistance and growth on media lacking histidine.

expression enough to confer 5-FOA resistance, may still allow sufficient DP1 interaction to drive some *HIS3* expression. A few 5-FOA-resistant colonies were indeed able to grow in conditions requiring *HIS3* expression (4). To identify strongly dissociating mutations in full-length stably expressed E2F1 proteins, it was necessary to screen the E2F1 mutants for those that retained interaction with RB, a protein that binds the carboxyl terminus of E2F1. This required curing the 5-FOA resistant colonies of plasmids expressing DP1-GBD, and mating them to yeast expressing RB-GBD. Selection for expression of *URA3* or *HIS3* identified mutants that interacted with RB but not E2F1. *In vitro* binding assays between the isolated E2F1 mutants and DP1 were consistent with the activities observed in the two-hybrid assays. Sequence analysis of the dissociating mutations identified a region of E2F1 important for DP1 binding that was nonoverlapping with a previously characterized interacting domain (4).

Six out of 400 5-FOA-resistant colonies, in the above study, contained E2F1 mutants that retained interaction with RB. The secondary screens to identify informative dissociating mutants are in fact the rate limiting steps in such studies (4, 6, 8). In theory, introduction of a reporter gene in the reverse two-hybrid system under control of a distinct promoter element would allow primary and secondary screens to be performed simultaneously. Two hybrid systems, which utilize reporter genes controlled by promoters containing LexA binding sites, substitute the LexA DNA-binding protein for the GAL4 DNA-binding domain (10). The addition of a LexA-dependent reporter to a strain containing the GAL4-dependent URA3 reporter, described by Vidal *et al.* (3), would allow a single round of double selection to identify mutations in a protein that disrupt interaction with one target while retaining interaction with a second target (Fig. 1). Such a strain has been constructed, containing a GAL4-dependent URA3 reporter and a LexA-dependent lacZ reporter and has been used successfully to analyze mutant STE5 protein interactions with components of the mitogen-activated protein kinase cascade and in isolating mutants in RB (C. Inouye and T. Durfee, unpublished results).

The power to impose a selective growth advantage for events that disrupt a protein–protein interaction allows applications to be considered that are unfeasible in earlier versions of the two-hybrid system. Vidal *et al.* (3, 4) suggest that cDNA libraries and peptide libraries of high complexity can be selectively screened for molecules that induce specific protein–protein dissociations. The ability to detect trans-acting dissociation events in the reverse two-hybrid system was demonstrated using E1A, which is known to disrupt the interaction of E2F with RB and p107 *in vivo*. Expression of E1A reversed the 5-FOA-sensitive phenotype conferred by RB-GBD/E2F-GAD or p107-GBD/E2F-GAD interactions (3). The authors speculate that the identification of small peptides that can specifically dissociate aberrant protein–protein interactions associated with disease could lead to the development of therapeutic agents. Screening cDNA libraries for specific dissociator molecules could potentially identify competitive regulatory interactions, as well as proteins that induce modifications on substrates that result in dissociation of substrate–partner interactions. The later would require that such proteins retain enzymatic activity when expressed in yeast. "Forward" two-hybrid screens to identify novel interacting partners for a protein of interest are often plagued by a high frequency of false positives (11). The majority of these result from activation domain fusions that induce reporter gene expression independent of interaction with the "bait" protein. By passing cDNA libraries through a reverse two-hybrid strain and applying selection against reporter gene expression, it should be possible to preclear libraries of trans-activating clones prior to use in screens for interacting proteins.

Technological advances in biochemical and genetic techniques to identify protein–protein interactions are reaching the point where it is becoming possible to create protein linkage maps of entire organisms (12). Defining the biological relevance of identified interactions will be the next rate-limiting step in deciphering the molecular mechanisms of biological processes. The combination of reverse and

forward two-hybrid screening techniques described by Vidal *et al.* (3, 4) will greatly facilitate the generation of molecular tools to aid these studies.


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