Progress and variations in two-hybrid and three-hybrid technologies

Becky L Drees

The original yeast two-hybrid system and its variants have proven to be effective tools for identification and analysis of protein–protein, protein–DNA and protein–RNA interactions. The two-hybrid assay is being applied to the entire complement of proteins of the yeast *Saccharomyces cerevisiae* to characterize the network of protein–protein interactions in the eukaryotic cell. The development of nontranscriptional cytosolic and membrane-associated two-hybrid methods has made it possible to detect and examine a number of protein–protein interactions in their normal cellular locations. Small-molecule hybrid systems have been developed which can be used to study protein–ligand interactions and to activate cellular processes by forcing protein associations.

Addresses
Department of Genetics, Box 357360, University of Washington, Seattle, WA 98195, USA; e-mail: drees@u.washington.edu

Current Opinion in Chemical Biology 1999, **3**:64–70

http://biomednet.com/elecref/1367593100300064

Abbreviations

AD	activation domain
BD	DNA-binding domain
CID	chemical inducer of dimerization
Cub	carboxy-terminal fragment of ubiquitin
GEF	guanine exchange factor
Nub	amino-terminal fragment of ubiquitin
ORF	open reading frame
PH	pleckstrin homology domain
PI3K	phosphatidylinositol 3-kinase
Pol I/II/III	RNA polymerase I/II/III
TF	transcription factor

Introduction

The development of the yeast two-hybrid system [1] provided a genetic means to identify proteins that physically interact *in vivo*. Over the past ten years, this system and its variants have been extremely useful for detecting and analyzing protein–protein interactions. Here, I will review some of the existing hybrid-based techonologies, and discuss trends that include the use of the two-hybrid assay on a genome-wide scale, advances in systems based on proteins that are not transcription factors, and the development of small-molecule-based hybrid systems.

The yeast two-hybrid system was a logical extension of studies that demonstrated the modular nature of transcriptional activators [2]. Site-specific transcription factors often have discrete, separable DNA-binding domains (BDs) and transcriptional activation domains (ADs). In the original two-hybrid system (Figure 1a), two putative interacting proteins X and Y are fused to the BD and AD of a yeast transcriptional activator. The two hybrid proteins, BD-X and AD-Y, are co-expressed in a yeast strain containing the transcriptional activator's specific DNA-binding site upstream of a gene whose expression can easily be detected by observing cell growth or by a quick enzyme assay. An interaction between X and Y reconstitutes the activator and leads to transcription of this 'reporter' gene, resulting in an easily detectable phenotype. An interaction between X and Y reconstitutes the activator and leads to transcription of the reporter gene, resulting in an easily detectable phenotype.

While the two-hybrid system is frequently used to test for interactions between known proteins, it has proven more popular as a tool to screen libraries in order to isolate and identify genes that encode interacting partners for a protein of interest [3]. Using a reporter gene required for growth allows easy detection of the few yeast transformants that express interacting proteins over the background of total transformants.

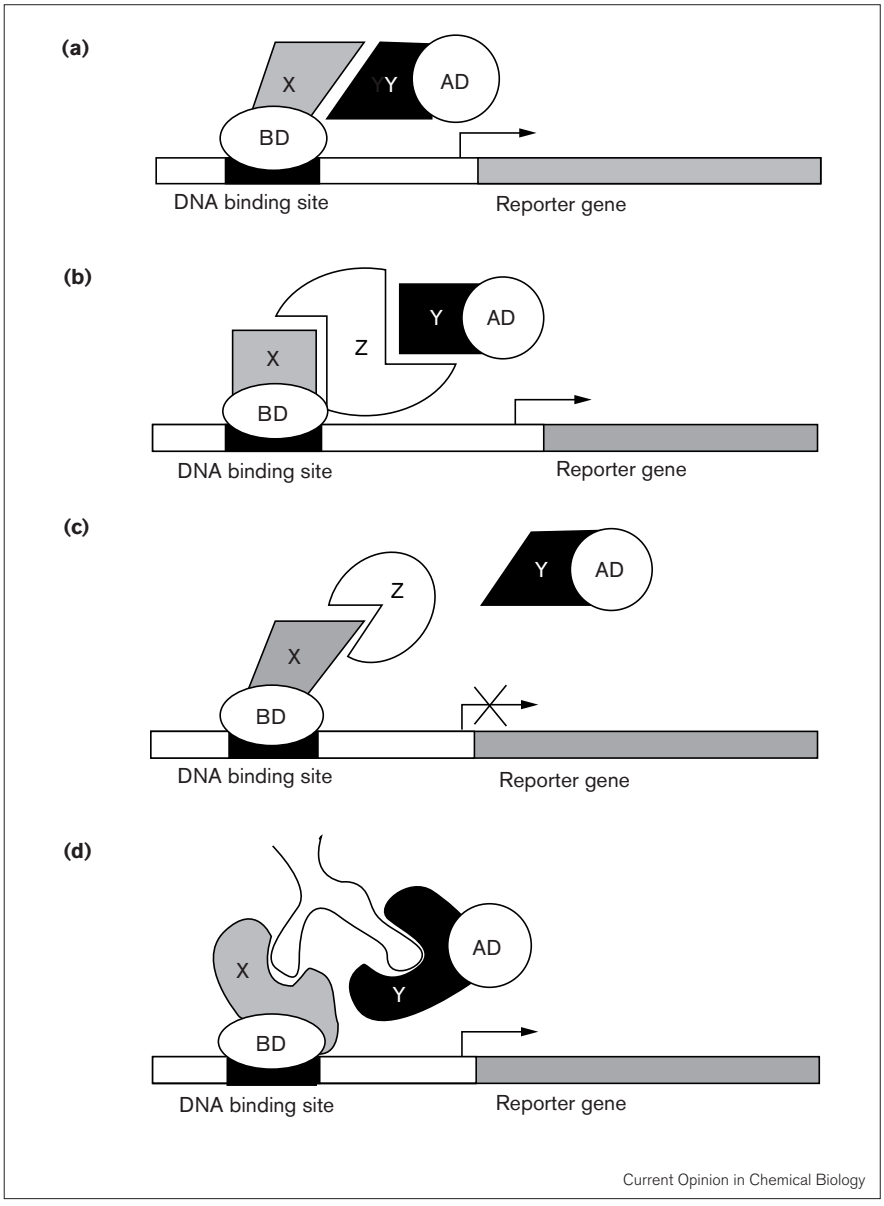
Variations on the original two-hybrid system

Several permutations of the original two-hybrid system have been developed over the past few years. In one class, a third protein is expressed along with the BD and AD fusions and expression of the reporter gene is used to select for interactions which occur only in the presence of this protein (Figure 1b). Expression of a third protein may 'bridge' a two-hybrid [4•,5–7], by interacting with both hybrids, or may stabilize an interaction by other means, such as post-translational modification [8]. Alternatively, a third protein may prevent formation of a two-hybrid (Figure 1c) complex [4•].

'Reverse' two-hybrid schemes have been invented that allow selection for mutations, drugs or competing proteins that disrupt two-hybrid interactions. Vidal *et al.* [9] used the gene *URA3* as a reporter. Since expression of *URA3* is toxic to cells grown on 5-fluoroorotic acid, a two-hybrid interaction will result in cell death. Dissociation or inhibition of the interaction will lead to loss of URA3 expression, thereby allowing cell growth. The 'split-hybrid' system of Shih *et al.* [10] provides a similar selection, using the *Escherichia coli tet* repressor (*tetR*) as a reporter for two-hybrid interaction. Expression of tetR results in repression of a second reporter gene engineered to contain a tetR-binding site. Disruption of the two-hybrid interaction restores expression of this reporter.

Inoyue *et al.* [11•] developed a modification of the two-hybrid system, the differential interaction trap, that allows mutational analysis of a protein's interactions with either of two different binding partners, X_1 and X_2. Each binding protein is fused to one of two different BDs that bind

Figure 1



The original transcription-based two-hybrid system and some of its variants. **(a)** The original two-hybrid system. Two interacting proteins, X and Y, are fused to the BD and AD domains of a yeast transcriptional activator, respectively. The interaction between X and Y reconstitutes the activator, leading to transcription of the reporter gene (shown by the arrow). **(b)** In one variation of the two-hybrid, a third protein (Z) is expressed along with the BD and AD fusions. Expression of the reporter gene is used to select for interactions that occur only in the presence of this protein. The third protein may promote an interaction by interacting with both hybrids, as shown, or by other means. **(c)** Alternatively, a third protein (Z) may prevent the formation of a two-hybrid complex. **(d)** In the RNA three-hybrid system, the binding of a bifunctional RNA molecule links the BD and AD proteins.

to distinct sites upstream of two different reporter genes. X_1 and X_2 each interact with a third protein Y, which is fused to the AD. Mutations in Y that affect the interaction with X_1 but not X_2 can be detected, and vice versa.

Another useful extension of two-hybrid technology is the addition of a hybrid RNA molecule to the system (Figure 1d). SenGupta *et al.* [12] developed a three-hybrid system to detect and analyze RNA–protein interactions in which the binding of a bifunctional RNA molecule links the BD and AD hybrid proteins and activates transcription of the reporter gene. Several groups have since used this RNA-based three-hybrid system to clone genes that encode specific RNA-binding proteins [13–17].

Application of the two-hybrid system at a genomic level

The two-hybrid assay has been used extensively to characterize networks of protein–protein interactions [18,19]. Bartel *et al.* [20] used comprehensive two-hybrid screens to create a protein linkage map of the *E. coli* bacteriophage T7. The current challenge is the systematic application of two-hybrid screens to every protein expressed in a eukaryotic cell. The resulting information is likely to reveal unsuspected connections between cellular processes and shed light on the roles of genes whose functions are now unknown or poorly understood. Two different strategies are being pursued to apply the two-hybrid screen to the entire complement of proteins

('proteome') of the yeast *S. cerevisiae*, a well studied organism whose entire genome sequence is available. Fromont–Racine *et al.* [21•] have set out on an iterative approach, using a high-efficiency mating [22] to screen small sets of related proteins against a high quality genomic library. DNA-binding fusions and the library of AD fusions are expressed in haploid yeast strains of the opposite mating type, mated on filters and plated on selective media to identify diploids positive for two-hybrid interactions. Proteins identified in this way are then used as baits in the next round of screens. A recent addition to this strategy is the attempt to simultaneously identify all two-hybrid positives from a screen by hybridization of total plasmid DNA to DNA arrays [23].

Fields and co-workers [24] have taken advantage of the availability of sequences for the approximately 6,000 open reading frames (ORFs) in the yeast genome. Each ORF can be expressed as a BD fusion in haploid yeast of one mating type and these yeast can be mated to an ordered array of ~6,000 test strains of the opposite mating type that express the complete set of ORF AD fusions. Advantages of the array approach are that the process is easily automated, requires no large-scale sequencing, and has built in controls for specificity of interactions. It is unknown, however, how many interactions may be missed by using full-length ORF fusions because of misfolding, toxicity or steric inhibition.

Hybrid systems based on other types of proteins

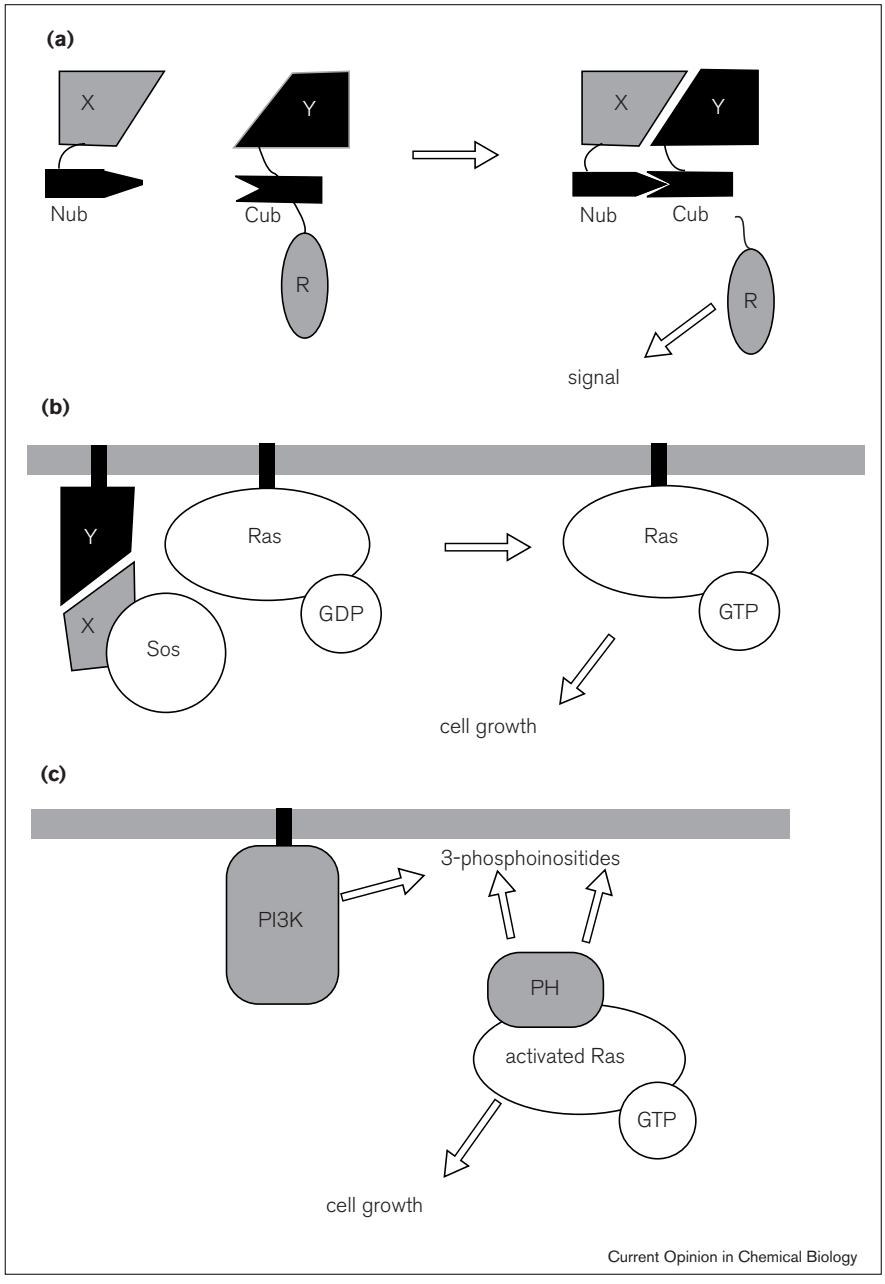
The original two-hybrid system activates transcription via RNA polymerase II (Pol II), one of three types of RNA polymerases found in eukaryotic organisms such as yeast. Each RNA polymerase transcribes different sets of genes. Pol II transcribes genes whose RNAs will be translated into proteins, while Pol I and Pol III transcribe RNAs that have structural or catalytic roles in the cell. Initiation of Pol II transcription is a complex process that is regulated by numerous activator and co-activator proteins. One limitation of the Pol-II-based two-hybrid system is that it is difficult to use to find proteins that interact with Pol II transcriptional activators, since these proteins often constitutively activate transcription of the reporter when used as baits. Other types of proteins may also spuriously activate transcription in the two-hybrid assay. In one approach, this problem was addressed by using a system based on a Pol III promoter [25]. Pol II transcriptional activators do not affect transcription by Pol III and so can be used as baits in this system. Initiation of Pol III transcription relies on two accessory proteins. The first, transcription factor IIIC (TFIIIC), binds to the promoter sequence and assembles the second factor, TFIIIB, to the DNA upstream of the transcription start site. TFIIIB interacts with Pol-III to initiate transcription. The Pol-III-based two-hybrid system uses an artificial promoter that contains the specific binding site for the yeast transcription factor Gal4. Protein X is fused to the Gal4 DNA binding domain and protein Y is fused to a mutated version of TFIIIC that cannot bind DNA. An interaction between X and Y will draw TFIIC to the promoter and lead to transcription of the reporter gene.

Aside from the problems of real and artifactual transcriptional activators, the original transcription-based two-hybrid system has other limitations. For example, only interactions that can be reconstituted in the nucleus, which may not be the optimal environment for a variety of protein–protein interactions, can be detected. Full-length transmembrane proteins are often problematic in the transcription-based two-hybrid, probably because of misfolding or instability when they attempt to localize to the nucleus. Other proteins may require modification by cytoplasmic or membrane associated enzymes in order to interact with binding partners. The development of nontranscriptional cytosolic and membrane-associated two-hybrid systems has provided innovative solutions that address these limitations of the original transcriptional-based assay.

Johnnson and Varshavsky [26] have developed a cytoplasmic two-hybrid assay based on split-ubiquitin (Figure 2a), which has recently also been used for membrane proteins [27•]. Ubiquitin is a small, 76 amino acid protein. Attachment of a ubiquitin molecule to a cellular protein marks that protein for proteolytic cleavage. Proteins fused to ubiquitin are rapidly cleaved *in vivo* by ubiquitin-specific proteases. If the carboxy-terminal fragment of ubiquitin (Cub) is fused to a reporter protein and co-expressed with the amino-terminal fragment (Nub), the two halves will interact to reconstitute native ubiquitin, resulting in cleavage of the reporter–ubiquitin fusion and activation of the reporter protein. To adapt the system for detection of protein–protein interactions, a mutant Nub, unable to interact with Cub on its own, is fused to one protein and a Cub-reporter hybrid is fused to its prospective interacting partner. Interaction between the two proteins allows ubiquitin to be reconstituted, leading to cleavage and release of the reporter protein. Aside from its ability to detect protein–protein interactions in the appropriate cellular location, the split-ubiquitin system also has the advantage that the signal for an interaction can be changed by changing the nature of the reporter protein. The reporter may be a transcription factor, which is able to enter the nucleus and activate transcription when released from a membrane-bound fusion protein [27•]. Another possibility is to use an enzyme that is activated by ubiquitin cleavage, whose activity may be detected by its effect on cell phenotype. The use of small ubiquitin fragments in the hybrid proteins may also be advantageous because it minimizes the possibility of steric hindrance.

Rossi *et al.* [28••] described an alternative cytoplasmic two-hybrid system to detect protein–protein interaction by intracistronic complementation of β-galactosidase mutants. Proteins of interest are fused to two nonfunctional weakly complementing β-galactosidase deletion mutants. β-galactosidase activity results from the forced interaction of the β-galactosidase peptides if the two hybrid proteins interact.

Figure 2



Examples of hybrid systems that are not based on transcription factors. **(a)** A cytoplasmic two-hybrid assay based on split-ubiquitin. Proteins fused to ubiquitin are rapidly cleaved *in vivo* by ubiquitin-specific proteases. A mutant Nub is fused to one protein (X) and the Cub–reporter gene hybrid is fused to its interacting partner (Y). Interaction between the two proteins reconstitutes ubiquitin and leads to cleavage and release of the reporter protein (R). The signal for an interaction can be changed by changing the nature of the reporter protein. **(b)** The Sos recruitment system. Protein X is fused to the human GEF Sos. A putative interactor protein Y is localized to the membrane by a myristoylation tag. Interaction between X and Y brings Sos to the membrane, where it stimulates guanyl nucleotide exchange on Ras, allowing cell growth. **(c)** The TOPIS assay is a similar hybrid system used to identify proteins that bind 3-phosphoinositide second messengers produced by PI3K. PI3K is co-expressed with a hybrid protein of activated Ras fused to a PH. Binding of PH to the 3-phosphoinosides generated by PI3K brings activated Ras to the membrane, rescuing a yeast Ras mutant and allowing cell growth.

One attractive feature of this particular system is that the assay is direct and does not depend on other cell functions. In addition, the level of β-galactosidase activity should correlate with the strength of the interaction.

A membrane-associated two-hybrid system has been devised [29••,30•] that makes use of the Ras signaling pathway. Ras is a membrane-associated signaling protein that binds and hydrolyzes GTP. Ras alternates between an active GTP-bound form that sets off a signal transduction cascade leading to cell growth, and an inactive GDP-bound form. Ras activity is regulated by two classes of proteins: GTPase activating protein (GAPs) that increase Ras' rate of GTP hydrolysis, and guanine exchange factors (GEFs) that stimulate release of GDP and uptake of GTP, thus reactivating its signaling activity. This signaling pathway is conserved amongst eukaryotes. The two-hybrid system takes advantage of the ability of the human GEF hSos to substitute for the yeast Ras GEF, Cdc25, in a cell with a mutant *cdc25* gene. If hSos is recruited to the cell membrane, its interaction with Ras will stimulate guanyl nucleotide exchange and activate the signal transduction cascade (Figure 2b) [31]. Interaction between a hSos–bait hybrid protein (hSos–X) and a protein partner (Y) localized to the cell membrane will draw hSos to the membrane where it can interact with and activate Ras, allowing cell survival and growth. An interesting extension of

this system has been used to identify proteins that bind 3-phosphoinositide second messengers *in vivo* [32••]. Membrane-bound phosphoinositide 3-kinases (PI3Ks) mediate the cellular effects of a variety of extracellular stimuli by phosphorylating the D3 position of the inositol ring of phosphoinositides. The 3-phosphoinositides generated bind proteins containing pleckstrin homology domains (PHs) which then activate downstream signaling processes [33]. In TOPIS (targets of PI3K identification system) assay (Figure 2c), PI3K is co-expressed with a hybrid protein consisting of a PH domain fused to a constitutively activated (i.e. GTP-bound) form of Ras in a Ras mutant cell. The PH binds the 3-phosphoinosides generated by PI3K, drawing the hybrid protein to the membrane where the activated Ras is able to substitute for the nonfunctional mutant, allowing cell growth. This specialized assay demonstrates that membrane-localized hybrid systems, in particular, should be a powerful tool as they are adapted for the study of a number of specific processes that only occur at the cell membrane.

A similar membrane-associated two-hybrid assay has been developed and is based on the interaction of the yeast G protein α subunit with the α factor receptor Ste2. Recruitment of a chimeric G protein α subunit (Gpa1) hybrid to the membrane via interaction with a Ste2 hybrid protein leads to a signal transduction cascade and rescues the viability of *gpa1* mutant cells [34].

Small-molecule hybrid systems

The development of small-molecule hybrid systems began with the use of cell-permeable chemical inducers of dimerization (CIDs) to force oligomerization of chimeric receptors in mammalian cells [35]. The intracellular domain of the T lymphocyte antigen receptor was expressed as a fusion with the immunophilin FK506-binding protein (FKBP12). Addition of an FK506–FK506 dimer, FK1012, induced aggregation of the T lymphocyte antigen receptor, activating a signaling pathway. Small-molecule ligands have since provided a general method to activate many cellular processes by forcing oligomerization between proteins fused to ligand-binding domains [36–40]. Signal transduction can be manipulated to lead to a variety of outcomes for cells expressing the hybrid proteins, including programmed cell death (apoptosis) [41] and reversible cell proliferation [42]. Effects are dose-dependent at nanomolar concentrations, and the method can be effective in multicellular organisms [43,44]. These features make CIDs attractive subjects for pharmacological research as candidates for drugs that control the fate or function of genetically modified cells in potential gene therapy treatments.

Yeast transcription-based three-hybrid systems [40,45] have been developed in which a cell-permeable heterodimeric ligand activates expression of a reporter gene if the two ligand-binding domains are present as BD and AD fusions. This method can identify small-molecule ligands that interact with a protein of interest or proteins that interact with a specific ligand. It has been particularly useful as a genetic tool for 'redesigning' protein–ligand interfaces. For example, the molecules used as CIDs often bind to native cell proteins with toxic effects. The three-hybrid system was employed to address this problem. Liberles *et al.* [46••] selected for FKBP12 mutants that bind specifically to a nontoxic derivative of rapamycin. The reverse two-hybrid system has been combined with a miniaturized cell-based ligand screening technique to create a method for selecting small-molecule inhibitors of protein–protein interactions [47••]. A similar system based on the small-molecule three-hybrid system could easily be used to screen for new CIDs. Both applications should be useful devices for pharmacological research.

Conclusions

The original yeast two-hybrid system, along with its variants, will continue to be useful for investigation of protein–protein, protein–nucleic acid and protein–small-molecule interactions. In particular, application of the two-hybrid assay on a genomic scale should provide a starting point for more detailed analysis of the many genes whose molecular functions are currently unknown. Nontranscriptional cytosolic and membrane-associated two-hybrid systems are also apt to be powerful tools for the study of protein interactions, especially those that occur only at specific cellular locations. Finally, hybrid systems using small-molecule ligands should provide interesting leads for pharmacological research, especially when combined with large-scale screening.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Fields S, Song O: **A novel genetic system to detect protein–protein interactions.** *Nature* 1989, **340**:245-246.

2. Sadowski I, Ma J, Triezenberg S, Ptashne M: **GAL4–VP16 is an unusually potent transcriptional activator.** *Nature* 1988, **335**:245-249.

3. Chien CT, Bartel, Sternglanz R, Fields S: **The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest.** *Proc Natl Acad Sci USA* 1991, **88**:9578-9582.

4. • Tirode F, Malaguti C, Romero F, Attar R, Camonis J, Egly JM: **A conditionally expressed third partner stabilizes or prevents the formation of a transcriptional activator in a three-hybrid system.** *J Biol Chem* 1997, **272**:22995-22999.

This paper presents a variation on the two-hybrid assay in which a third protein is expressed along with the DNA-binding domain and activation domain fusions. The system can be used to detect third partners that promote or inhibit an interaction.

5. Printen JA, Sprague G: **Protein–protein interactions in the yeast pheromone response pathway: Ste5p interacts with all members of the MAP kinase cascade.** *Genetics*1994, **138**:609-619.

6. Zhang J, Lautar S: **A yeast three-hybrid method to clone ternary protein complex components.** *Anal Biochem* 1996, **242**:68-72.

7. Tomashek JJ, Sonnenburg JL, Artimorich JM, Klionsky DJ: **Resolution of subunit interactions and cytoplasmic subcomplexes of the yeast vacuolar proton-translocating ATPase.** *J Biol Chem* 1996, **271**:10397-10404.

8. Osborne MA, Dalton S, Kochan JP: **The yeast tribrid system – genetic detection of trans-phosphorylated ITAM-SH2-interactions**. *Biotechnology* 1995, **13**:1474-1478.

9. Vidal M, Brachmann RK, Fattaey A, Harlow E, Boeke JD: **Reverse two-hybrid and one-hybrid systems to detect dissociation of protein–protein and protein–DNA interactions.** *Proc Natl Acad Sci USA* 1996, **93**:10315-10326.

10. Shih H, Goldman PS, DeMaggio AJ, Hollenberg SM, Goodman RH, Hoekstra MF: **A positive genetic selection for disrupting protein–protein interactions: identification of CREB mutations that prevent association with the coactivator CBP.** *Proc Natl Acad Sci USA* 1996, **93**:13896-13901.

11. • Inoyue C, Dhillon N, Durfee T, Zambryski PC, Thorner J: **Mutational analysis of *STE5* in the yeast *Saccharomyces cerevisiae*: application of a differential interaction trap assay for examining protein–protein interactions.** *Genetics* 1997, **147**:479-492.

This paper presents an adaptation of the original two-hybrid system for the study of proteins that interact simultaneously with two partners. Two different DNA-binding domain fusions are present which bind to distinct sites controlling the transcription of two different reporter genes. Interaction of an activation domain fusion protein with each of the DNA-binding domain fusions is detected by selecting for expression of the associated reporter.

12. SenGupta DJ, Zhang B, Kraemer B, Pochart P, Fields S, Wickens M: **A three-hybrid system to detect RNA–protein interactions *in vivo*.** *Proc Natl Acad Sci USA* 1996, **94**:8496-8501.

13. Martin F, Schaller A, Eglite S, Schumperli D, Muller B: **The gene for histone RNA hairpin binding protein is located on human chromosome 4 and encodes a novel type of RNA binding protein.** *EMBO J* 1997, **16**:769-778.

14. Wang ZF, Whitfield ML, Ingledue TC III, Dominski Z, Marzluff WF: **The protein that binds the 3′ end of histone mRNA: a novel RNA-binding protein required for histone pre-mRNA processing.** *Genes Dev* 1996, **10**:3028-3040.

15. Harrington L, McPhail T, Mar V, Zhou W, Oulton R, Bass MB, Arruda I, Robinson MO: **A mammalian telomerase-associated protein.** *Science* 1997, **275**:973-977.

16. Zhang B, Gallegos M, Puoti A, Durkin E, Fields S, Kimble J, Wickens MP: **A conserved RNA-binding protein that regulates sexual fates in *the C. elegans* hermaphrodite germ line.** *Nature* 1997, **390**:477-484.

17. Bacharach E, Goff SP: **Binding of the human immunodeficiency virus type 1 Gag protein to the viral RNA encapsidation signal in the yeast three-hybrid system.** *J Virol* 1998, **72**:6944-6949.

18. Finley RL, Brent R: **Interaction mating reveals binary and ternary connections between *Drosophila* cell cycle regulators.** *Proc Natl Acad Sci USA* 1994, **91**:12980-12984.

19. Evangelista C, Lockshon D, Fields S: **The yeast two-hybrid system: prospects for protein linkage maps.** *Trends Cell Biol* 1996, **6**:196-199.

20. Bartel PL, Roecklein JA, SenGupta DJ, Fields S: **A protein linkage map of Escherichia coli bacteriophage T7.** *Nat Genet* 1996, **12**:72-77.

21. • Fromont–Racine M, Rain J-C, Legrain P: **Toward a functional analysis of the yeast genome through exhaustive two-hybrid screens.** *Nat Genet* 1997 **16**:277-281.

Legrain's group describes their strategy for mapping all protein–protein interactions in *S. cerevisiae*. A genomic library was screened with DNA-binding domain fusions of a small set of related proteins involved in RNA processing. Positives from the first screens that encode probable *in vivo* interactors are then used as baits in a second round of screens.

22. Bendixen C, Gangloff S, Rothstein R: **A yeast mating-selection scheme for detection for protein-protein interactions.** *Nucleic Acids Res* 1994, **22**:1778-1779.

23. Cho RJ, Fromont–Racine M, Wodicka L, Feierbach B, Stearns T, Legrain P, Lockhart DJ, Davis RW: **Parallel analysis of genetic selections using whole genome oligonuceotide arrays.** *Proc Natl Acad Sci USA* 1998, **95**:3752-3757.

24. Hudson JR, Dawson EP, Rusing KL, Jackson CH, Lockshon D, Conover D, Lanciault C, Harris JR, Simmons SJ, Rothstein R, Fields S: **The complete set of predicted genes from *S. cerevisiae* in readily usable form.** *Genome Re*s 1998, **7**:1169-1173.

25. Marsolier M-C, Prioleau M-N, Sentenac A: **An RNA polymerase III-based two-hybrid system to study RNA polymerase II transcriptional regulators.** *J Mol Biol 1997*, **268**:243-249.

26. Johnsson N, Varshavsky A: **Split ubiquitin as a sensor of protein interactions *in vivo*.** *Proc Natl Acad Sci USA* 1994, **91**:10340-10344.

27. • Staglar I, Korostensky C, Johnsson N, TeHeesen S: **A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins *in vivo*.** *Proc Natl Acad Sci USA* 1998, **95**:5187-5192.

The authors describe a membrane-associated version of the split-ubiquitin two-hybrid system. Cleavage of the Cub (carboxy terminal fragment of ubiquitin)–reporter fusion releases a transcription factor, which is then able to enter the nucleus and activate transcription of a reporter gene.

28. •• Rossi F, Charlton CA, Blau HM: **Monitoring protein–protein interactions in intact eukaryotic cells by β-galactosidase complementation.** *Proc Natl Acad Sci USA* 1997, **94**:8405-8410.

Rossi *et al.* developed a novel two-hybrid assay for protein–protein interactions in mammalian cells. Intracistronic complementation between noninteracting deletion mutants of β-galactosidase can be restored if the fragments are brought together by interaction of protein domains to which they are fused. β-galactosidase activity can be used to directly monitor protein–protein interactions in different cellular locations.

29. •• Aronheim A, Zandi E, Hanneman H, Elledge SJ, Karin M: **Isolation of an AP-1 repressor by a novel method for detecting protein–protein interactions.** *Mol Cell Biol* 1997, **17**:3094-3102.

This paper describes a membrane-associated two-hybrid system in yeast. Interacting protein fusions recruit Sos to the membrane where it stimulates guanyl nucleotide exchange on yeast Ras, rescuing a temperature-sensitive (Ras) mutant. The system was advanced as an alternative for study of proteins interacting with transcriptional activators, but should be particularly useful for membrane-associated proteins, which are often problematic for the original two-hybrid system.

30. • Aronheim A: **Improved efficiency Sos recruitment system: expression of the mammalian GAP reduces isolation of Ras GTPase false positives.** *Nucleic Acids Res* 1998, **25**:3373-3374.

Aronheim describes improvements to the Sos recruitment system. Addition of a mammalian GTPase-activating protein protein to the system reduces the isolation of Ras false positives in two-hybrid screens.

31. Aronheim A, Engelberg D, Li N, ai-Alawi N, Schlessinger J, Karin M: **Membrane targeting of the nucleotide exchange factor Sos is sufficient for activating the Ras signaling pathway.** *Cell* 1994, **78**:949-961.

32. •• Isakoff SJ, Cardozo T, Andreev J, Li Z, Ferguson KM, Abagyan R, Lemmon MA, Aronheim A, Skolnik EY: **Identification and analysis of PH domain-containing targets of phosphatidylinositol 3-kinase using a novel *in vivo* assay in yeast.** *EMBO J* 1998, **17**:5374-5387.

Phosphatidylinositol 3-kinase (PI3K) controls a variety of cellular responses by generating 3-phosphoinositide second messengers that activate downstream signaling pathways by binding pleckstrin homology (PH) domain proteins. In this paper, the authors describe a membrane-associated hybrid system to identify PH domain proteins that bind PI3K-generated phosphoinositides. Co-expression of PI3K and a hybrid protein of activated Ras fused to a PH domain that binds the 3-phosphoinosides rescues the temperature-sensitive yeast Ras mutant. This specialized assay demonstrates that membrane-localized hybrid systems can be a powerful tool as they are adapted for the study of a number of specific processes that occur at the cell membrane.

33. Frech M, Andjelkovic M, Ingley E, Reddy KK, Falck JR, Hemmings BA: **High affinity binding of inositol phosphates and phosphoinositides to the pleckstrin homology domain of RAC/protein kinase B and their influence on kinase activity.** *J Biol Chem* 1997, **272**:8474-8481.

34. Medici R, Bianchi E, DiSegni G, Tocchini–Valentini GP: **Efficient signal transduction by a chimeric yeast-mammalian G protein α subunit Gpa1-Gsα covalently fused to the yeast receptor Ste2.** *EMBO J* 1997, **24**:7241-7249.

35. Spencer DM, Wandless TJ, Schreiber SL, Crabtree GR: **Controlling signal transduction with synthetic ligands.** *Science*1993, **262**:1019-1024.

36. Spencer DM, Bekshaw PJ, Chen L, Ho SN, Randazzo F, Crabtree GR, Schreiber SL: **Functional analysis of Fas signaling *in vivo* using synthetic inducers of dimerization.** *Curr Biol* 1996, **6**:839-847.

37. Holsinger LJ, Spencer DM, Austin DJ, Schreiber SL, Crabtree GR: **Signal transduction in T lymphocytes using a conditional allele of Sos.** *Proc Natl Acad Sci USA* 1995, **92**:9810-9814.

38. Luo Z, Tzivion G, Belshaw PJ, Vavvas D, Marshall M, Avruch J: **Oligomerization activates c-Raf-1 through a Ras-dependent mechanism.** *Nature* 1996, **383**:181-185.

39. Stockwell BR, Schreiber SL: **Probing the role of homomeric and heteromeric receptor interactions in TGF-β signaling using small molecule dimerizers.** *Curr Biol* 1998, **8**:761-770.

40. Belshaw PJ, Ho SN, Crabtree GR, Schreiber SL: **Controlling protein association and subcellular localization with a synthetic ligand that induces heterodimerization of proteins.** *Proc Natl Acad Sci USA* 1996, **93**:4604-4607.

41. Freiberg RA, Spencer DM, Choate KA, Peng PD, Schreiber SL, Crabtree GR, Khavari PA: **Specific triggering of the Fas signal transduction pathway in normal human keratinocytes.** *J Biol Chem* 1996, **271**:31666-31669.

42. Blau CA, Peterson KR, Drachman JG, Spencer DM: **A proliferation switch for genetically modified cells.** *Proc Natl Acad Sci USA* 1997, **94**:3076-3081.

43. Rivera VM, Clackson T, Natesan S, Pollock R, Amara JF, Keenan T, Magari SR, Philips T, Courage NL, Cerasoli F *et al.*: **A humanized system for pharmacologic control of gene expression.** *Nat Med* 1996, **2**:1028-1032

44. Spencer DM: **Creating conditional mutations in mammals.** *Trends Genet* 1996, **12**:181-187.

45. Licitra EJ, Liu JQ: **A three-hybrid system for detecting small ligand-protein receptor interactions.** *Proc Natl Acad Sci USA* 1996, **93**:12817-12821.

46. •• Liberles SD, Diver ST, Austin DJ, Schreiber SL: **Inducible gene expression and protein translocation using nontoxic ligands identified by a mammalian three-hybrid screen.** *Proc Natl Acad Sci USA* 1997, **94**:7825-7830.

Rapamycin is useful for controlling protein dimerization *in vivo* because it can interact with two proteins (FK506-binding protein 12 [FKBP12] and FKBP12–rapamycin-associated protein [FRAP]) simultaneously. One limitation on its use is that its binding to native FRAP has an inhibitory effect on cell growth. Liberles *et al.* addressed this problem by synthesizing an altered form of rapamycin and used a small-molecule three-hybrid system to identify mutations in the ligand-binding domain of FRAP that stabilized binding to the modified rapamycin.

47. •• Huang J, Schreiber SL: **A yeast genetic system for selecting small molecule inhibitors of protein–protein interactions in nanodroplets.** *Proc Natl Acad Sci USA* 1998, **95**:13396-13401.

Huang and Schreiber combined a miniaturized high-throughput screening method with a reverse two-hybrid assay to create a system for selection of small-molecule inhibitors of protein–protein interactions. High local concentrations of ligands were obtained for testing by photochemically controlled release from small beads. Their method used inducible activation domain and DNA-binding domain fusions and antibiotic resistance reporter genes.