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# The impact of two-hybrid and related methods on biotechnology

Pierre Colas and Roger Brent

Two-hybrid technology has contributed significantly to the unraveling of molecular regulatory networks by facilitating the discovery of protein interactions. Outgrowths of these methods are developing rapidly, including interaction mating to identify false positives and map protein networks, two-bait systems, systems not based on transcription, and systems permitting the selection of peptide aptamers to manipulate gene and allele function. These advances promise to have a significant impact on industrial biotechnology and drug development.

The widespread use of two-hybrid systems began in 1993. Since that time, a number of reviews have provided detailed information on this technology, including the transcriptional mechanisms that underlie it, the different versions of the method, its strengths, its limitations and its numerous successful applications<sup>1–4</sup>. This review will cover some outgrowths of two-hybrid systems and outline some of the contributions these developments are likely to make to biotechnology in the next decade.

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## Principles of basic two-hybrid technology

The technology relies on two fundamental properties of transcription factors. First, eukaryotic transcription factors typically have a modular structure, with at least two discrete domains – a DNA-binding domain and an activator domain<sup>5,6</sup>; modularity was operationally established when an active transcription factor was assembled by fusing domains from two different transcription factors<sup>5</sup>. Second, these two domains need not be present in the same polypeptide to give rise to an active transcription factor but just need to be in the vicinity of one another<sup>7,8</sup>.

This modularity prompted Fields and Song to show that transcription of a yeast reporter gene could be used to detect the interaction between two proteins, one fused to a DNA-binding domain and the other to an

### Box 1. The steps involved in an interactor hunt using the interaction trap<sup>42</sup>

- **Create selection strain**

Transform a strain containing a *LexAop-LEU2* gene (*LEU2* downstream of binding sites for LexA) with the bait plasmid and a plasmid carrying a *lacZ* reporter gene.

- **Test bait expression and transcription activation**

Test whether the bait is properly expressed by Western blot, and whether it activates transcription of the reporter genes by itself. If the bait does activate, use less-sensitive reporter genes or create a number of baits that contain different deletions of the bait moiety.

- **Perform repression assay**

Using another strain, check that the bait can enter the nucleus and bind the *LexAop* by verifying that it represses transcription of appropriately engineered reporter genes<sup>4</sup>.

- **Introduce the prey library into the selection strain**

- **Plate transformants onto galactose, leucine<sup>-</sup> medium**

- **Pick Leu<sup>+</sup> colonies and transfer to glucose, leu<sup>+</sup> medium**

At this step, expression of the preys is shut off.

- **Verify that interaction phenotypes are galactose dependent**

This step determines which of the picked colonies show an interaction phenotype that is dependent on the expression of the prey. The master plates are replica plated onto glucose and galactose plates that contain or lack leucine to assay transcription of the *LEU2* reporter gene, and onto plates that contain leucine and X-Gal to assay transcription of the *lacZ* reporter.

- **Rescue prey plasmid, reintroduce into original selection strain and into different bait strains to verify specificity**

This step confirms the observed interaction and assesses its specificity. The library plasmid is isolated and reintroduced into a strain of a mating type opposite to that of the selection strain. Mating assays are performed using the bait from the screening and with other related baits.

A detailed interaction-trap protocol with frequent updates can be found at <http://www.molsci.org/>

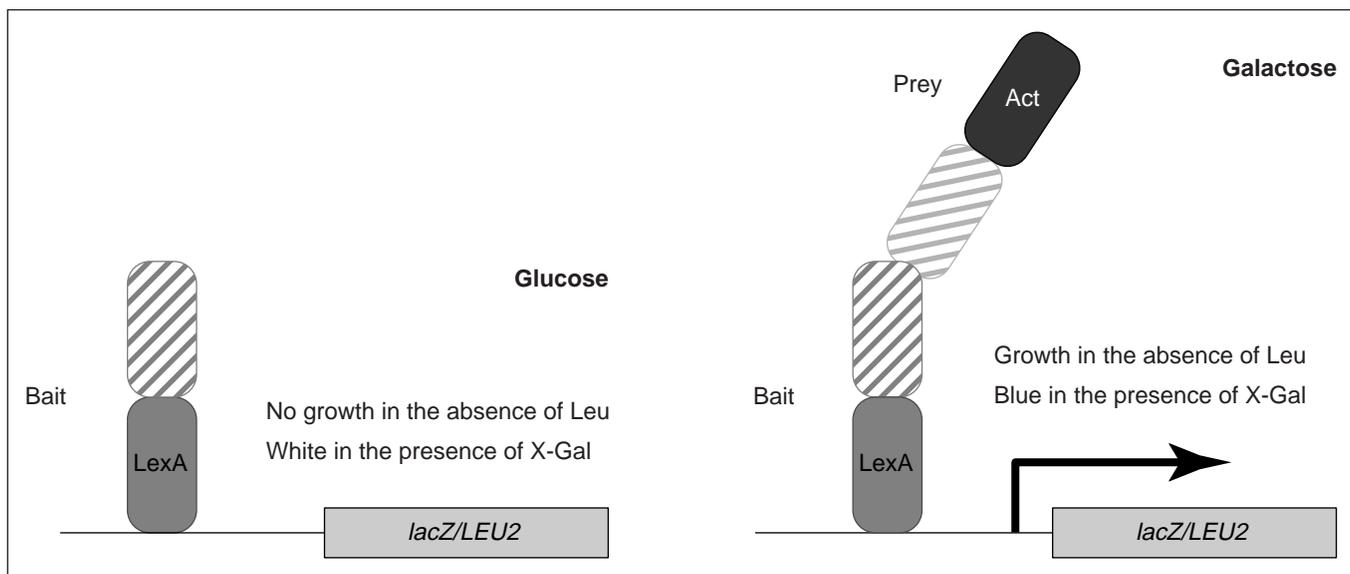


Figure 1

Schematic explanation of the interaction trap. This two-hybrid system contains three basic components<sup>11</sup>. (1) The bait: here, the DNA-binding moiety (mid-grey) contains the DNA-binding and dimerization domains of the bacterial repressor LexA. The bait is expressed under the control of the *ADH1* promoter. (2) The prey: here, the library members are fused to a nuclear localization sequence, an epitope tag and the B42 activation domain (Act; dark grey). The expression of preys is directed by the *GAL1* promoter, which is induced in galactose-containing medium but repressed in glucose-containing medium. (3) The reporters (light grey). The interaction trap uses two reporter genes, *LEU2* and *lacZ*, which allow growth on medium lacking leucine or confer a blue color when grown on medium containing X-Gal, respectively. The investigator should choose *LEU2* and *lacZ* reporter genes of appropriate sensitivity (e.g. reporter genes carrying different copy numbers of LexA-binding sites in their regulatory sequences) for each experiment. For instance, reporter genes of low sensitivity should be chosen when using a bait that slightly activates transcription by itself.

activation domain, and to propose that libraries fused to an activation domain could be screened for members that interact with a given protein fused to a DNA-binding domain<sup>9</sup>.

Current systems contain three basic components<sup>10-13</sup>: (1) a vector directing the expression of the protein of

interest fused to a DNA-binding moiety, which is termed a 'bait'; (2) a vector directing the synthesis of cDNAs or open-reading-frame-encoded proteins fused to an activation domain, this protein being referred to as a 'prey'; and (3) one or more reporter genes (such as amino acid biosynthetic genes or *Escherichia coli lacZ*)

placed downstream of the DNA-binding sites recognized by the DNA-binding moiety of the bait. The interaction trap, one version of the two-hybrid system, is shown in Fig. 1, and Box 1 sketches the different steps of an interactor hunt, in which a library of proteins is screened for those that interact with a given bait. Sequencing the prey-plasmid insert from cells that exhibit an interaction phenotype reveals the identity of the interacting proteins.

### Recent developments of the basic technology

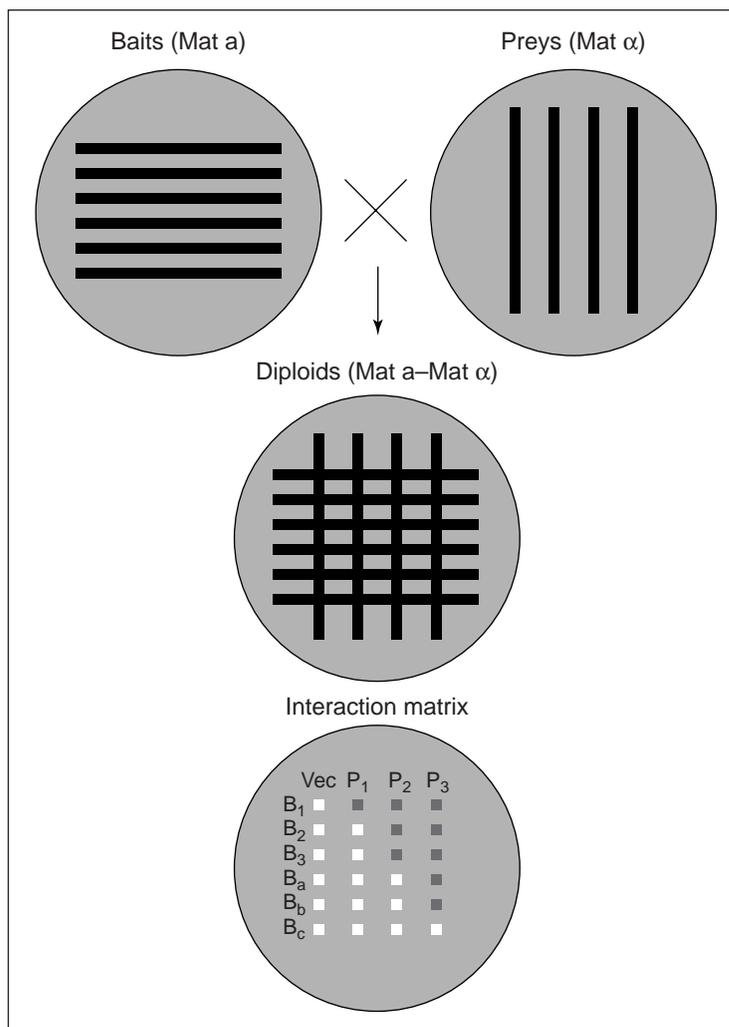
#### Improved ways to identify false positives

As with all detection methods, interactor hunts invariably give rise to false-positive results. Here, we define false-positive signals as cells in which the reporter genes are active, even though the bait and prey do not interact in nature; sometimes, the proteins do not even interact in the yeast. For example, when a bait activates transcription weakly enough to be below the phenotypic threshold of a reporter<sup>14</sup>, it is frequently discovered that library members raise transcription above the threshold, even though there is no supporting evidence that the bait and the preys interact (P. Colas, unpublished). Such false positives may arise because these library members enhance the steady-state level of the activating bait or stabilize the reporter-gene product. Other false positives may arise from preys that interact with DNA upstream of the reporter genes or with proteins that interact with promoter sequences. However, these two last classes of false positives can be eliminated by using multiple reporter genes whose regulatory sequences are derived from more than one promoter<sup>11,15-17</sup>.

Still other false-positive results include interactions that occur in the two-hybrid setting and not in a physiological context, because the partners are not expressed in the same cellular or subcellular location at the same time. Still other interactions, scored as false positives because they don't make immediate sense to the investigator, may occur inside living cells and contribute to as-yet-undiscovered regulatory pathways. Also, interactions that cannot be confirmed by the most commonly used biochemical techniques (such as coprecipitation) may still be real, but too weak to be detected by less-sensitive methods<sup>4</sup>.

However, identifying false positives is now easier than it used to be. First, there is now a body of experience that can be used to disqualify prospective interactors just because they are members of a rogue's gallery of proteins that interact with unrelated baits. Serebniskii and Golemis have compiled a list of such promiscuous interactors (<http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html>). Such proteins should usually be discarded, unless the investigator believes that the interaction with the bait used in the screening makes extraordinary biological sense. Caveat selector!

Second, interaction mating is now widely used to gain confidence (and other functional information) about interacting proteins isolated in two-hybrid hunts<sup>18</sup>. Typically, one assembles an informative panel of different baits belonging to the same protein family as the bait used in the screening, as well as unrelated baits. These baits are transformed into haploid yeast of one of the two mating types ( $a$  or  $\alpha$ ) and the putative interactors transformed into a strain of the opposite

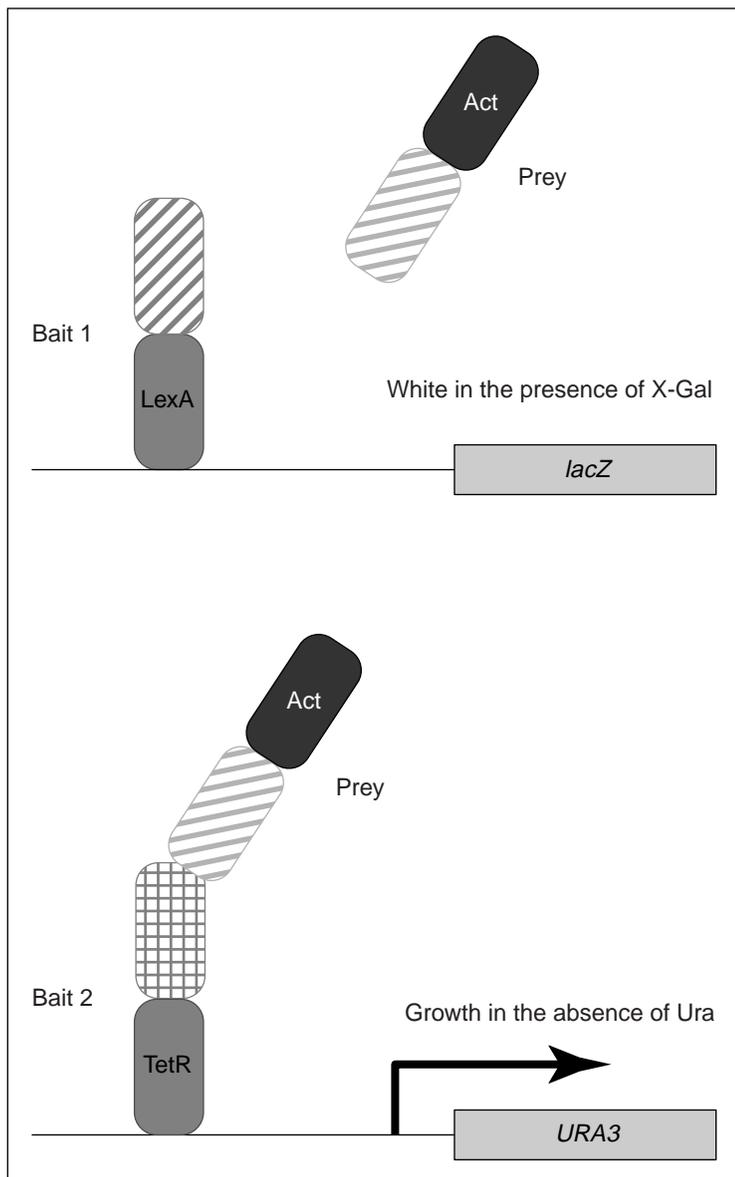


**Figure 2**

Assessing interaction specificity by interaction mating. In this system, preys isolated from an interactor hunt are tested against a panel of baits. Baits and preys are streaked across plates as horizontal or vertical lines, respectively. The strains are replica plated onto rich medium, where mating occurs at the intersections. Diploid exconjugants formed by mating are replicated onto an indicator medium that selects for diploids (here, containing X-Gal to detect transcription of the *lacZ* reporter gene) to produce the interaction matrix. Abbreviations: Vec, empty prey vector; P<sub>1-3</sub>, three different preys isolated in the hunt; B<sub>1</sub>, bait used in the screening; B<sub>2,3</sub>, two baits belonging to the same protein family as B<sub>1</sub>; B<sub>a-c</sub>, three different baits unrelated to either the B<sub>n</sub> family or to each other. Here, an excellent, a good and a low level of confidence would be given, respectively, to the interactions B<sub>1</sub>-P<sub>1</sub>, B<sub>1</sub>-P<sub>2</sub>, and B<sub>1</sub>-P<sub>3</sub>, based on the interaction patterns of the three preys.

mating type; the different strains are mated and the diploid exconjugants transferred to indicator plates to construct interaction matrices<sup>18</sup> (Fig. 2). Although assessment of interaction specificity can be performed without interaction mating<sup>11,19</sup>, it is greatly facilitated by it<sup>20</sup>.

Inspection of interaction matrices aids the identification of false positives<sup>18</sup>: candidates that interact with the bait used in the interactor hunt but not with other members of the same protein family are given a high level of confidence; candidates that interact with other members of a protein family but not with unrelated baits can also be considered promising, while candidates interacting with many baits of the panel are usually discarded (Fig. 2). However, it is important to note



**Figure 3**

Schematic explanation of a two-bait system. In this two-bait system<sup>23</sup>, the first bait is fused to LexA and binds to a *lacZ* reporter gene, whereas the second bait is fused to the DNA-binding domain of the Tet repressor and binds a *URA3* reporter gene. Interactions are assayed on medium containing or lacking uracil but containing X-Gal. On appropriate media, four different interaction phenotypes can be detected: (1,2) a prey interacting with only one bait gives either a *lacZ*<sup>+</sup> or a *Ura*<sup>+</sup> (as shown here) phenotype, depending on the interaction; (3) a prey interacting with both baits gives a *lacZ*<sup>+</sup> *Ura*<sup>+</sup> phenotype; (4) a prey interacting with neither bait gives a *lacZ*<sup>-</sup> *Ura*<sup>-</sup> phenotype.

that preys such as chaperones or ubiquitin-conjugating enzymes can interact with many different kinds of protein in ways that are important for their biological function. If one has reasons to believe that proper folding or proteolysis by the ubiquitin pathway is relevant to the biology of the protein, these hits should be pursued.

Third, because the amount of biological information is increasing so rapidly, it is sometimes possible to rule out a two-hybrid hit by the use of existing data. For example, knowledge that two proteins are never expressed at the same time in the same cell type can rule out the idea that interactions observed in yeast occur naturally, although the two-hybrid result may

point to an interaction between related proteins in the same family. This ability to use supplementary data to evaluate the credibility of interactions will increase as genome-wide expression data becomes more widely available<sup>21</sup>.

### **Two-bait and other systems that allow the expression of a third protein**

A number of two-bait systems have recently been described in which a yeast strain expresses two different baits that are fused to different DNA-binding domains; these domains activate expression of two different reporter genes upon interaction with a prey. In one example, Jiang and Carlson mapped sites on the regulatory domain of the SNF1 protein kinase that interact with the SNF1 catalytic domain and/or with the activator SNF4 (Ref. 22). To do this, the investigators constructed a small prey library containing a collection of SNF1 regulatory-domain mutants fused to the C-terminal activation domain of GAL4. They introduced this library into a strain that contained the SNF1 catalytic domain fused to the DNA-binding domain of LexA, SNF4 fused to the DNA-binding domain of GAL4 and *lexAop-lacZ* and *GAL1-HIS3* reporter genes. They used this strain to isolate a point mutant of the SNF1 regulatory domain that interacted with the catalytic domain but not with SNF4, indicating that the two interactions involve distinct binding sites<sup>22</sup>.

Another two-bait system uses the DNA-binding domains of LexA and the tetracyclin repressor from Tn10 with corresponding reporter genes<sup>23</sup> (Fig. 3). It can identify proteins that bridge the two baits or that interact with one bait but not the other. Using this system, Xu *et al.* isolated peptide aptamers (see below) that discriminated between different alleles of the Ras oncoprotein<sup>23</sup>. It may be that two-bait systems also offer a way to weed out false positives, as they can be used to isolate preys that interact with only one of two related preys. The rationale is that, in general, the more related the two baits are, the more likely it is that a prey that interacts with only one of them is a natural interactor.

Two-bait systems, and other systems that allow the expression of three proteins in yeast at once, can also be used to identify interactions that depend on a third protein. For example, by expressing Sos and GAP from one bait vector while expressing Ras from another, the two-bait system has been used to discriminate between proteins that altered the conformation of Ras<sup>23</sup>. In another example, cells containing a plasmid expressing a library of proteins fused to a nuclear localization sequence were used to identify proteins that bridged an epidermal-growth-factor (EGF)-receptor bait and an SOS prey, including Grb2 (Ref. 24).

*Saccharomyces cerevisiae* does not carry out some of the post-translational modifications that are responsible for important protein interactions in mammalian cells (e.g. most tyrosine-phosphorylation events). However, it is sometimes possible to work around this limitation; for example, the intracellular domain of the platelet-derived growth factor (PDGF) receptor has been expressed in yeast<sup>25</sup>. This PDGF-receptor fragment was capable of phosphorylating tyrosines of SHPTP2, an SH2-containing tyrosine phosphatase, allowing the

interaction between phosphorylated SHPTP2 and the signaling protein Grb7 to be detected<sup>25</sup>. Another recent example comes from the use of a two-bait system<sup>23</sup>, in which a LexA–Lck tyrosine-kinase fusion phosphorylated the membrane receptor CD28 fused to TetR; this phosphorylated CD28 moiety then interacted with a p85 phosphatidylinositol-3-kinase-subunit prey (P. King and W. Xu, pers. commun.).

### Mammalian two-hybrid systems

In principle, a more universal solution to the problem of post-translational modification would be to search for interactions in the appropriate cell type. Mammalian two-hybrid systems have been developed to allow the detection of protein interactions that depend on post-translational modifications or on various stimuli<sup>26</sup>. The working principle is identical to that used in yeast two-hybrid systems, except that transcription drives either DNA replication<sup>27</sup> or a reporter gene encoding chloramphenicol acetyltransferase (CAT), whose activity can be measured *in vitro*, or cell-surface or other drug-resistance markers<sup>28</sup>. For example, such a system has been used to examine the interaction between the transcription factor E2F1 and the retinoblastoma protein Rb or the adenovirus protein E4, confirming that phosphorylation of E2F1 prevents interaction with Rb but is necessary for the interaction with E4 (Ref. 29). Although the construction of systems featuring selectable markers should allow interactor hunts with cDNA libraries in mammalian cells to be performed, no such hunts have been reported to our knowledge.

### Selection of interacting peptides and functional peptide aptamers

Recently, interactor hunts have been conducted to isolate interacting peptides from combinatorial libraries of random DNA sequences. In one study, a library of unconstrained peptides directly fused to a transcription-activation domain was used to isolate library members interacting with Rb<sup>30</sup>. The sequence of the interacting peptides matched the consensus sequence found in natural Rb interactors (e.g. the adenovirus protein E1A, the transcription factor E2F and D-type cyclins). This work showed that, as for phage-display methods<sup>31</sup>, the inspection of consensus sequences of peptides isolated by two-hybrid methods can identify naturally occurring linear recognition motifs.

In another study, we isolated conformationally constrained 20-amino-acid variable regions that recognized the protein kinase cyclin-dependent-kinase 2 (Cdk2)<sup>32</sup>. In this work, the variable regions were displayed using *E. coli* thioredoxin, whose use as a platform had been pioneered by J. McCoy and co-workers<sup>33</sup>. These protein molecules are referred to as peptide aptamers, by analogy with the RNA aptamers isolated by the laboratories of Szostak and Gold<sup>34,35</sup>. We have isolated 14 different aptamers from a screening of  $6.0 \times 10^6$  transformants and, by their high specificity of interaction, none of them were judged to be false positive. These peptide aptamers bind tightly to their target, with measured dissociation constants ( $K_d$ ) ranging between 30 and 120 nM. Those aptamers that have been tested inhibit Cdk2 activity *in vitro*, with half-inhibitory constants even tighter than the measured  $K_d$ s<sup>32</sup>. At least

some inhibitory aptamers bind the kinase in the vicinity of its active site (P. Colas, unpublished) and disrupt its interaction with the substrate (B. Cohen, pers. commun.). These aptamers can also disrupt protein interactions *in vivo*: they have recently been shown to slow down the cell cycle of cultured human cells (B. Cohen, pers. commun.) and those that cross react with *Drosophila* Cdc2 have been shown to disturb the cell cycle in the developing eye of *Drosophila* (M. Kolonin and R. Finley, pers. commun.).

### Selection against protein interactions

In the past, yeast reporter genes have been developed whose transcription can be selected against ('counterselectable reporters')<sup>17,36–38</sup> (J. Kamens, PhD thesis, University of Harvard, MA, USA, 1992; A. Ephrussi and R. Brent, unpublished; R. Finley and R. Brent, unpublished). Under counterselection conditions, the reporter-gene product is either toxic or cytostatic, while cells that do not express it are able to grow. The three counterselectable genes most commonly used in yeast are *URA3*, *LYS2* and *CYH2*. The *URA3* and *LYS2* gene products catalyse the conversion of 5-fluoroorotic acid (5-FOA) and  $\alpha$ -aminoadipate, respectively, into toxic intermediates<sup>36,37</sup>. The *CYH2* gene product confers sensitivity to cycloheximide, its wild-type allele being dominant over the mutants<sup>38</sup>.

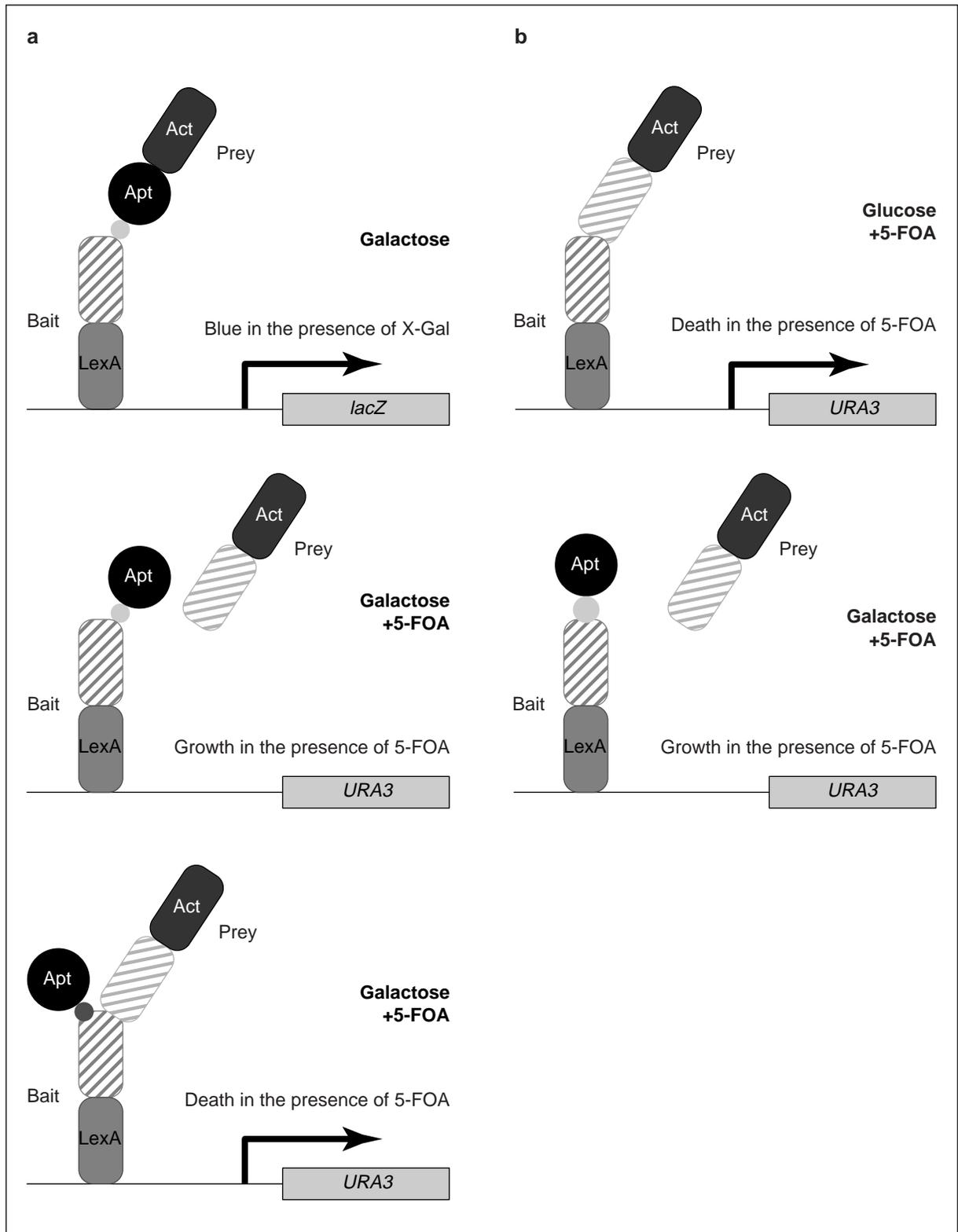
Used in two-hybrid experiments, such reporters can facilitate identification of mutations that disrupt protein interactions<sup>17,39</sup>. For example, a system that included a *URA3* reporter has been used to isolate point mutations of E2F1 that can no longer interact with DP1 (this protein interaction is needed for cell-cycle progression)<sup>40</sup>. Although very versatile, such a scheme requires pilot experiments to determine the rate of spontaneous occurrence of the 5-FOA-resistance phenotype, which may vary from one strain to another, and to adjust the concentration of 5-FOA to the desired sensitivity.

A conceptually different selection against protein interactions was recently described in which the reporter gene encodes the Tn10 tetracycline repressor (TetR), which, when synthesized, turns off transcription of a second reporter gene, *Tetop–HIS3*. Cells in which a protein interaction is disrupted no longer activate the *TetR* reporter and so grow in the absence of histidine. This system was used to identify mutations in the transcription factor CREB that abolish its interaction with the coactivator CBP<sup>41</sup>.

It is possible that such schemes will be useful for one-step selections for peptides and peptide aptamers that disrupt a particular protein interaction, which should be faster than the two-step selections currently used (Fig. 4).

### Non-transcription-based selections for interaction

Transcription-based two-hybrid systems generally cannot be used when the interacting partners (bait or prey) interact with components of the yeast transcriptional machinery. For example, as mentioned above, a significant proportion of baits activate transcription of the reporter genes to some extent and, although the use of less-sensitive reporters can sometimes overcome this problem<sup>42</sup>, strongly activating baits generally cannot be used in hunts<sup>4</sup>. Similarly, baits that interact with



**Figure 4**

Selection of aptamers that disrupt an interaction between two proteins. **(a)** Two-step selection: in the first step, activation-tagged aptamers are selected to interact with a bait<sup>32</sup>; in the second step, aptamers that interact with the bait are screened to identify those that disrupt the interaction between the bait and a prey *in vitro*<sup>32</sup> or *in vivo* [detected by growth in the presence of 5-fluoroorotic acid (5-FOA)]. The variable region of a disrupting aptamer is shown in light grey, and the variable region of a non-disrupting aptamer that allows the formation of a trimeric complex is shown in dark grey; the TrxA platform is shown in black. **(b)** Proposed one-step selection: in this scheme<sup>17</sup>, both bait and prey are constitutively expressed, and their interaction activates a counterselectable reporter, in this case a *URA3* gene whose product kills the yeast in presence of 5-FOA. An aptamer library is introduced into cells bearing these elements and library transformants are plated onto medium containing galactose (to induce expression of the aptamers) and 5-FOA. Aptamers that block the bait-prey interaction block *URA3* transcription and allow yeast to grow on selective medium. Variation in the concentration of 5-FOA might allow the selection of disruptive aptamers of high and low affinities.

transcriptional repressors found in yeast, such as  $\gamma$ SIN3, cannot be used, and library members interacting with yeast transcriptional repressors cannot be isolated as interacting preys<sup>43</sup>.

A non-transcription-based system that circumvents these limitations was recently described<sup>44</sup>. This scheme is based on the ability of the human Ras guanyl-nucleotide-exchange factor, hSOS, to complement its yeast counterpart, Cdc25, the complementation being strictly dependent on the recruitment of hSOS to the plasma membrane. In this system, the bait is a chimeric protein that consists of a fusion between a protein of interest and a C-terminal deletion variant of hSOS that cannot interact with Grb2, which normally anchors it to the plasma membrane. The library members are fused to the C terminus of the v-Src myristoylation sequence, which targets proteins to the membrane. Interaction between the bait and a prey recruits hSOS to the membrane and activates the yeast Ras pathway, in turn allowing a strain carrying a temperature-sensitive Cdc25 mutant to grow at the restrictive temperature<sup>45</sup>. In a pilot experiment, this system detected the interaction between c-Jun and c-Fos, two proteins that cannot be used in transcription-based two-hybrid systems. A library was screened and two new c-Jun-interacting proteins were isolated, one of them a putative transcriptional repressor<sup>44</sup>.

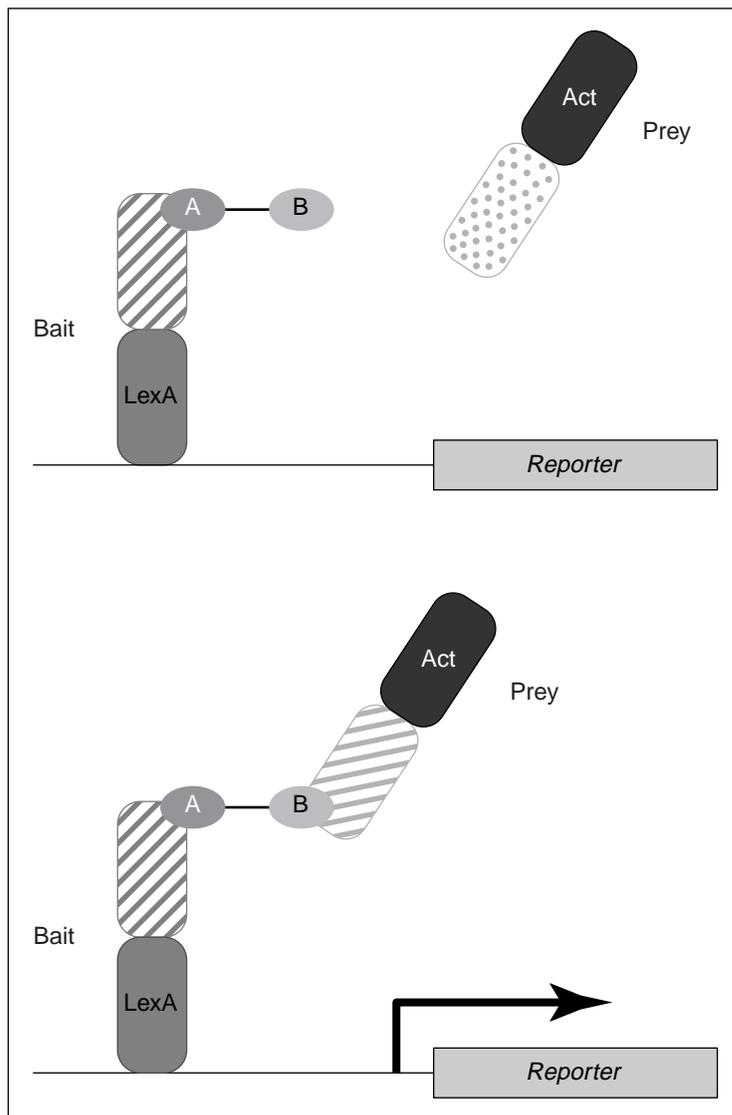
Although this new technique appears to be quite promising, more experiments will be necessary to establish its versatility and sensitivity. As with any system, it will be prone to its own variety of false positives, for example, from baits that produce a constitutive interaction phenotype because they contain a membrane anchor or interact with a yeast membrane protein, or from cells in which the *cdc25ts* allele has reverted.

### Near-future industrial applications

#### Identification of protein targets for drug-discovery efforts

The advent of functional genomics is changing the way pharmaceutical research is conducted. The first consequence of this change is likely to be better-informed identification and validation of target proteins for small-molecule-drug-discovery efforts. One functional-genomic endeavor that will aid these processes is the systematic inventory of the protein interactions taking place in living cells. Most proteins exert at least some of their function(s) by interacting with other proteins; the identification of these interaction networks can increase the number of proteins in a given pathway that a drug-discovery effort can target.

The feasibility of establishing such networks has already been demonstrated by using an interaction-mating strategy to map the connections between proteins encoded by the bacteriophage T7 genome<sup>46</sup>. In this work, genomic bait and prey libraries were mated, the resulting diploid exconjugants that exhibited an interaction phenotype were isolated and the interacting proteins identified<sup>46</sup>. The fact that this organism contains only 55 proteins and lacks introns enabled sufficient diploid exconjugants to be analysed that every possible interaction could be covered many times. In yeast, a prey library was made from *S. cerevisiae* genomic DNA, and it was demonstrated that this library could



**Figure 5**

Identifying protein targets for existing small-molecule agents. This figure depicts a scheme first used successfully by Licitra and Liu<sup>50</sup>. Proteins that bind compound B are identified from a prey library in yeast. The yeast is grown in a medium that contains a new compound, A-B, in which A is a ligand known to bind the bait. Activation-tagged proteins that interact with the B moiety of this compound stimulate transcription of the reporter.

be used successfully in iterative interactor hunts to explore particular interaction networks<sup>47</sup>. Similar approaches will obviously be much harder to apply to the large, intron-riddled genomes of higher eukaryotes.

An alternative approach is to use interaction mating to identify interactions between ordered arrays of bait and prey proteins<sup>18</sup>. This could be a feasible approach to whole-genome two-hybrid explorations. A collection of LexA-fusion baits (currently with about 700 members) is being built by the many users of the interaction trap, the LexA-based version of the two-hybrid system, and is maintained in ordered arrays of haploid yeast strains expressing these baits. By mating strains of the opposite mating type that contain prey proteins of interest, a rapid interaction assay between these proteins and the 700 possible partners of known identity is possible, in ways that often give valid clues to the function of the test protein<sup>48</sup>.

All these steps can be automated, from constructing the two-hybrid vectors by gap repair inside yeast cells<sup>49</sup> to the detection of the interaction phenotypes (e.g. growth on Leu<sup>-</sup> medium, blue color on X-Gal medium). A systematic assembly of protein-connection information should aid the charting of molecular regulatory networks and directly help the pharmaceutical industry identify new targets for drug discovery.

#### **Identification of protein targets for small-molecule drugs**

Interaction methods may also yield insights into existing therapeutic agents. In a recent study, FK506-binding proteins (FKBPs) that interact with the immunosuppressant FK506 were isolated<sup>50</sup>. The authors built a bait by fusing the glucocorticoid receptor (GR) to LexA. They chemically coupled dexamethasone (which binds GR) to FK506 and showed in pilot experiments that yeast expressing the bait and FKBP12 as a prey gave an interaction phenotype when plated on medium containing the FK506-dexamethasone compound. They then used an interaction library from human leukemic-Jurkat-T cells with the aim of isolating new FKBPs. Although the only preys isolated were FKBP12, this experiment clearly validates this concept. It may also be useful for screening libraries of small organic molecules generated by combinatorial chemistry to find those that recognize a target of interest (Fig. 5). The identification of interactions between small molecules and proteins that are not necessarily their therapeutic targets may guide medicinal chemistry to find new compounds with better side-effect profiles.

#### **Therapeutic molecules that disrupt protein interactions**

It is likely that, in at least some cases, the targets of new therapeutics will not be proteins but, rather, protein interactions that underlie particular disease states. Some existing drugs disrupt protein interactions, although they were not necessarily selected for this in the first place. For instance, the immunosuppressant FK506 disrupts the interaction between one of its targets, FKBP12, and the TGF- $\beta$ -family type-I receptors<sup>51</sup>. Other existing drugs, such as the existing HIV-1-protease inhibitors, are designed to disrupt interactions, in this case between the active site of the protease and its polyprotein substrate<sup>52</sup>. Two-hybrid schemes should facilitate the search for small organic compounds that disrupt protein interactions. For instance, the use of a two-bait system featuring a counterselectable marker and a *lacZ* reporter gene should allow the selection of compounds that disrupt a particular interaction between one bait and one prey, but not another interaction between a closely related bait and the same prey.

One major limitation of yeast-based selections is the impermeability of yeast to most organic molecules, resulting from the existence of a cell wall. One way to approach this problem is to enhance the permeability of the yeast by using chemicals such as polymyxin B (Ref. 53) or by using mutants that exhibit an altered permeability to various compounds. For example, the *tum* mutants, which exhibit an altered uptake of thymidylate<sup>54</sup>). Also, some mutations of the *erg* genes, involved in the synthesis of ergosterol, modify the cell's permeability to different molecules<sup>55</sup>, whereas a net-

work of transcriptional regulators of the PDR family controls the expression of several membrane proteins that act as drug-exclusion pumps and are responsible for multidrug resistance in yeast<sup>56</sup>. Alternatively, it may be possible to perform such screens with mammalian two-hybrid systems. However, in the end, it will probably be easier to use the ever-increasing chemical diversity offered by combinatorial chemistry to screen sufficient numbers of compounds that some will penetrate yeast and disrupt the desired interaction. Any compound that penetrates yeast should penetrate human cells.

It is also possible that peptide aptamers that disrupt protein interactions will find a use in biotechnology. The exquisite specificity these molecules exhibit towards a given target and their high binding affinity should allow them specifically to disrupt many protein interactions within regulatory pathways, which can be quite useful for target identification and validation. It is even possible that peptide aptamers will provide lead compounds for therapeutic and biologically active products. The structure of TrxA is well known<sup>57,58</sup> and the structure of the variable region of the complexed and uncomplexed aptamers could guide the synthesis of peptidomimetic organic compounds that might be useful as therapeutics or biological modifiers in animals and plants. Moreover, future generations of peptide aptamers with low immunogenicity may become useful in gene therapy.

#### **Use of peptide aptamers in lieu of immunological reagents**

Finally, it is worth mentioning that peptide aptamers may also find a use in situations where antibodies are now employed. Such applications have the potential advantages that peptide aptamers can be obtained in a short time without the need to use animals and that these molecules can be directly selected not to cross react with related targets.

#### **Conclusion**

Today, the word 'revolution' is more likely to be heard in biotechnology conferences than at Communist-party conferences. We believe that the combined application of newly emerging techniques will indeed transform the pharmaceutical industry so dramatically that the term 'revolution' is appropriate. Protein-interaction technologies are likely to contribute to this revolution by transforming the many steps in the long process of drug development. Interaction-mating two-hybrid methods will contribute to the understanding of gene function at a genomic scale and should, indeed, generate information directly useful for target identification and validation. Two-hybrid schemes that either detect interactions between proteins and ligands or disrupt protein interactions will certainly be used to screen the huge libraries of organic compounds generated by combinatorial chemistry. It is also possible that improvements in peptidomimetic chemistry, gene therapy and the use of peptides as therapeutics might eventually allow peptide aptamers to become good lead compounds.

Whatever the eventual nature of this revolution, we are clearly only in its early days. Much later in the course of an earlier revolution (1792), one of its

leaders, Danton, was still calling for ‘...de l’audace, encore de l’audace, toujours de l’audace...’<sup>59</sup> (boldness, more boldness, always boldness). Although we cannot, at this point, predict all the contributions that protein interaction and other new technologies will make to this revolution in biotechnology, we believe that boldness is already appropriate and that, with luck, some scientists will steer its industrial applications skillfully enough to realize revolutionary benefits while avoiding Danton’s fate.

### Acknowledgments

We are very grateful to B. Cohen, R. Finley, T. Jessen, A. Reymond, K. Robzyk and A. Saunders for their comments on this manuscript.

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