Review

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The post-genomic era of interactive proteomics: Facts and perspectives

The availability of completed genome sequences of several eukaryotic and prokaryotic species has shifted the focus towards the identification and characterization of all gene products that are expressed in a given organism. In order to cope with the huge amounts of data that have been provided by large-scale sequencing projects, high-throughout methodologies also need to be applied in the emerging field of proteomics. In this review, we discuss methods that have been recently developed in order to characterize protein interactions and their functional relevance on a large scale. We then focus on those methodologies that are suitable for the identification and characterization of protein-protein interactions, namely the yeast two-hybrid system and related methods. Several recent studies have demonstrated the power of automated approaches involving the yeast two-hybrid system in building so-called "interaction networks", which hold the promise of identifying the entirety of all interactions that take place between proteins expressed in a certain cell type or organism. We compare the yeast two-hybrid system with several other screening methods that have been developed to investigate interactions between proteins that are not amenable to conventional yeast two-hybrid screenings, such as transcriptional activators and integral membrane proteins. The eventual adaptation of such methods to a high-throughput format and their use in combination with automated yeast two-hybrid screenings will help in elucidating protein-protein interactions on a scale that would have been unthinkable just a few years ago.

Keywords: Interactive proteomics / Membrane proteins / Protein-protein interactions / Review / Transcriptional activators / Yeast two-hybrid system PRO 0236

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Abbreviations: AD, activation domain; **DBD**, DNA-binding domain; **RRS**, Ras recruitment system; **UBPs**, ubiquitin-specific proteases

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1 Introduction

The complete sequencing of genomes of bacteria, viruses, and small and large eukaryotes provides us with an unprecedented amount of genetic information. This "genomic era" has led to a new kind of cooperation, not only between international academic groups but also between governments and industry. With the genomes of so many organisms completely sequenced, science and its new biomedical discipline of functional genomics, are faced with the challenge of understanding the function of these newly discovered genes. For example, only one-third of all 6200 predicted yeast genes had been functionally characterized when the complete sequence of the yeast genome first became available [1]. At present (six years after the completion of the yeast genome), 3800 yeast genes have been characterized by genetic or biochemical means and an additional 600 genes have been characterized based on homologues in other organisms that provide some indication as to their function. Yet, there still remain around 1800 genes encoding proteins of unknown function [2]. The same observation holds true for the human genome: around 80% of all predicted human genes have not been characterized to date [3]. To answer this challenge, researchers have developed different high-throughput strategies to help them understand the function of each gene in the genome (Fig. 1). Projects that are already underway in several model organisms include DNA microarray technology to analyze the expression profiles of genes [4, 5], the analysis of mRNA expression patterns using *in situ* hybridization, loss-of-function approaches combined with subcellular localization screens [6–9], the large-scale localization of proteins within cells [10], *in silico* methods for the determination of protein fusions, gene neighboring and structural predictions [11–13], the systematic isolation of cDNA sequences across the entire genome [14] and finally, the use of high-throughput assays to isolate interacting protein pairs [15–17].

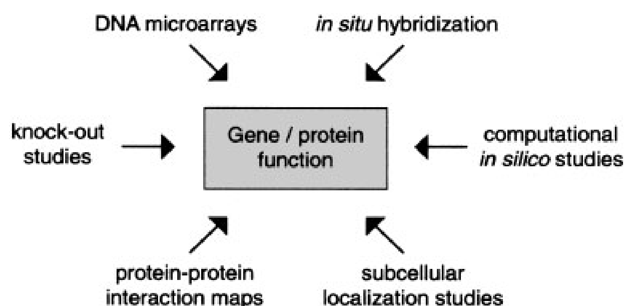

Figure 1. Schematic representation of current experimental technologies that lead to the understanding of gene function. Information provided by these comprehensive studies facilitates the research on individual gene functions and will be validated by results from individual studies emerging in the future.

In this review, we focus on genetic techniques in yeast that are used to identify and characterize interactions between proteins. First, we discuss the oldest and most widely used assay for the detection of protein-protein interactions *in vivo*, the yeast two-hybrid system, and highlight the recent efforts of several groups to carry out large-scale yeast two-hybrid approaches with the aim of establishing comprehensive protein interaction maps. Then, we provide an overview of recent developments in yeast two-hybrid technologies and, in particular, of novel approaches that enable the researcher to work on proteins that have proven to be unsuitable for conventional yeast two-hybrid approaches, namely proteins with intrinsic transactivation capability and integral membrane proteins.

2 The importance of studying protein-protein interactions

Since protein-protein interactions play a role in nearly all events that take place in a cell, clues to the function of an unknown protein can be obtained by investigating its interaction with other proteins whose functions are already known. Thus, if the function of one protein is known, then the function of its binding partner is likely to be related. This concept has been termed "guilt by association" and, when used on a large scale, it allows the researcher to employ a relatively small number of functionally characterized proteins and to quickly assign functions to their uncharacterized binding partners. Moreover, as most cellular processes are regulated by multiprotein complexes, abolishing a protein-protein interaction may have profound effects and may ultimately manifest itself in a particular disease. For instance, tumor-forming viruses such as adenovirus cause uncontrolled proliferation of the host cell by dissociating important protein-protein interactions between regulatory proteins of the cell cycle [18]. Identification of these proteins is of great interest since they may subsequently be used as targets for drug screening, with the aim of identifying molecules that can regulate protein-protein interactions.

3 The principle of the yeast two-hybrid system

The yeast two-hybrid system was originally developed by Fields and Song as a genetic assay to detect protein-protein interactions in a cellular setting [19]. It takes advantage of the finding that many eukaryotic transcription factors can be divided into two functionally distinct domains that mediate DNA binding and transcriptional activation. In the classical yeast two-hybrid approach, a "bait" is constructed by fusing a protein X to the DNA-binding domain

(DBD) derived from a transcription factor and a "prey" is constructed by fusing a protein Y to the activation domain (AD) of a transcription factor. The bait and prey fusions are coexpressed in yeast, where the interaction of X and Y leads to the reconstitution of a functional transcription factor. Reconstitution of the transcription factor is measured by assaying the activity of reporter genes (Fig. 2A). Commonly, auxotrophic markers that can be selected for are used in combination with the *lacZ* gene encoding bacterial β-galactosidase. *HIS3* and *LEU2* allow selection of interactions by monitoring growth on selective plates lacking histidine or leucine, respectively, whereas *lacZ* can be easily measured using a colorimetric assay. Since the yeast two-hybrid system has the advantage of being both rapid and easy to use, it has quickly become the most frequently used assay to detect novel protein-protein interactions. A recent publication estimates that more than 50% of all interactions described in the literature have been detected using the yeast two-hybrid system [20].

4 The yeast two-hybrid system and protein interaction maps

4.1 Approaches for the generation of genome-wide protein interaction maps

Since it is a genetic system, the yeast two-hybrid system is well suited to high-throughput applications such as the identification of interactions taking place between all proteins expressed in a given cell or organism. Currently, two approaches are being used to generate comprehensive protein interaction maps. In the so called "matrix approach" or "array approach" (Fig. 2B), a set of open reading frames (ORFs) is amplified using the polymerase chain reaction (PCR), cloned as both bait and prey constructs (*i.e.* as fusion to a DBD and as a fusion to an AD), and then introduced into isogenic reporter strains of opposite mating type. The reporter strain expressing a DBD fusion is then mated with an array of yeast clones each expressing a different AD fusion. Practically, this task is carried out by robots which transfer aliquots from a lawn of cells expressing one DBD fusion to arrays of cells each expressing a different AD fusion. This procedure is repeated for each strain expressing a DBD fusion, until all DBD fusions have been mated with the entire AD array. Positive interactions are selected through the ability of diploid yeast colonies containing an interacting fusion pair to grow on selective media. In order to sort out the false positives arising from such approach, the experiments are performed in duplicate and only interactors found in both experiments are considered to be true positives. An advantage of the matrix approach is that it rapidly becomes clear which locations produce false positive interactions, providing

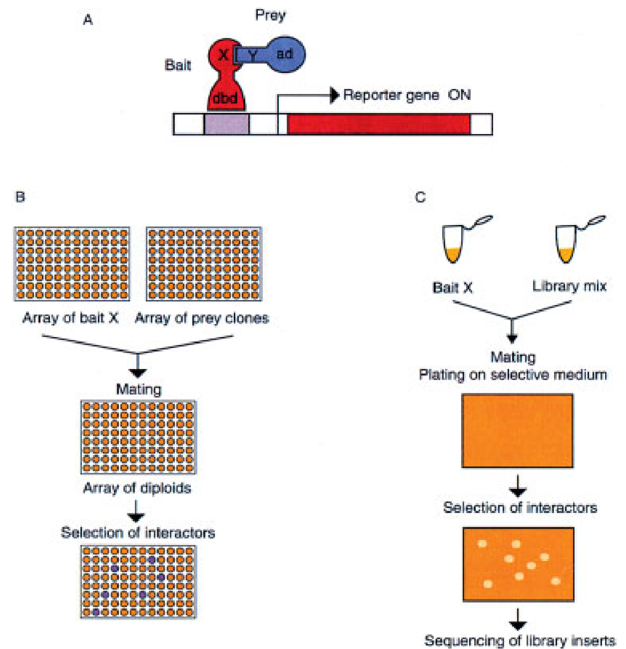

Figure 2. The yeast two-hybrid system and its high-throughput applications. (A) The principle of the yeast two-hybrid system. Protein X is expressed as a fusion to the DBD and constitutes the bait. The DBD-X fusion protein is bound to the operator sites in the promoter region but does not activate transcription of the downstream reporter gene because it lacks an AD. The interaction of DBD-X with its partner Y fused to an AD recruits the AD-Y fusion protein to the promoter where its forms a functional transcriptional activator. Consequently, transcription of the reporter gene is switched on. (B) High-throughput yeast two-hybrid using the matrix approach. A matrix (or array) of prey clones is created by dispensing one yeast clone expressing a given AD-Y fusion protein into each well of a multiwell plate. Using a robot, the array of prey clones is then transferred to a multiwell plate containing yeast that express one DBD-X fusion and prey and bait clones are allowed to mate. Those diploids where DBD-X and a particular AD-Y interact are selected based on expression of a reporter gene, such as β-galactosidase (producing a blue color). (C) In the exhaustive library screening approach one DBD-fused bait X is screened against an entire library and positives are selected based on their ability to grow on selection plates. As opposed to the matrix approach, where each prey can be identified by its position in the array, diploids that have survived selection in the library screening need to be picked up and the library plasmids encoding the interacting prey have to be isolated and sequenced in order to identify the interacting protein. Libraries can be made either from random genomic or cDNA fragments or from full-length ORFs that are cloned separately and then pooled.

reassurance that the system is working properly; if a particular AD fusion in the array interacts with all DBD fusions, it most likely represents a false positive and

should thus be discarded. On the other hand, the matrix approach also has certain disadvantages. The use of full-length ORFs as DBD and AD fusions may prevent the identification of certain interactions due to problems such as proper folding of full-length proteins, degradation or toxicity (see below).

A second approach in genome-wide yeast two-hybrid analysis is the so called "exhaustive library screening approach", in which DBD fusions are screened against complex libraries containing AD fusions of full-length ORFs or ORF fragments (Fig. 2C). As opposed to the matrix approach, this method does not separate the different AD fusions on an array. Instead, the library is divided into pools, and each yeast strain expressing a DBD fusion is mated with a library pool. Then, diploid cells containing an interacting protein pair are selected. The library screening approach is more sensitive than the matrix approach since it uses not only full-length ORFs, but also random fragments of ORFs. Often, a protein-protein interaction can be detected using fragments of the proteins in question, but not the full-length proteins. For instance, a protein may not fold properly when expressed in yeast, or it may become degraded (see [21]). The use of fragments often overcomes these problems. On the other hand, library screens are much more time consuming and expensive than matrix screens since they require the analysis of larger numbers of clones. In addition, the library plasmids encoding AD fusions have to be isolated and sequenced from all selected diploids in order to identify the interacting proteins.

4.2 Summary of current genome-wide protein interaction projects

In the past few years, several genome-wide interaction mapping projects have been started. These are summarized in Table 1, together with relevant information about the type of cell or organism that was investigated, the method that was used to carry out the screening and the number of protein-protein interactions that were identified. The first smaller-scale protein interaction maps were reported in 1994 for *Drosophila melanogaster* [22], followed by similar screenings for T7 bacteriophage [23] and a subset of yeast proteins involved in mRNA splicing

Table 1. Genome-wide protein-protein interactions studies performed in different organisms

Organism	Predicted ORFs	Methods	AD hybrids	DBD hybrids	Interactions	References
Bacteriophage T7	55	Matrix screen	11 ORFs	34 ORFs	3	[23]
		Library screen	Library	34 ORFs		
			11 ORFs	Library	22	
			Library	Library		
Vaccinia virus	266	Matrix screen	266 ORFs	266 ORFs	37	[24]
Hepatitis C virus	~10	Matrix screen	11 ORFs	10 ORFs	0	[25]
		Library screen	Library	200 ORFs	15	
Helicobacter pylori	1590	Library screen	Library	285 ORFs	1280	[28]
Caenorhabditis elegans	19099	Library screen(1)	Library	27 ORFs	148	[21]
		Matrix screen(1)	29 ORFs	29 ORFs	11	
		Matrix screen(2)	30 ORFs	30 ORFs	17	[26]
		Library screen(2)	Library	30 ORFs	138	
Drosophila melanogaster	13600	Matrix screen(3)	5 ORFs	9 ORFs	19	[22]
Saccharomyces cerevisiae	6200	Library screen(4)	Library	15 ORFs	170	[15]
		Library screen(5)	Library	16 ORFs	20	[68]
		Library screen(6)	Library	8 ORFs	229	[69]
		Library screen	159 ORFs	159 ORFs	183	[17]
		Matrix screen	5345 ORFs	192 ORFs	281	[16]
		Library screen	5345 ORFs	5345 ORFs	692	

The screenings performed included (1) proteins involved in vulval development (2) 26S proteoasome subunits (3) cell cycle regulatory proteins (4) nuclear proteins involved in pre-mRNA splicing pathway (5) RNA polymerase III subunits (6) Sm-like proteins.

[15]. Finley and Brent [22] used the matrix method to characterize a set of cyclin-dependent kinase interacting proteins in *Drosophila*. Using this approach, they identified 19 interactions and demonstrated that each cyclin dependent kinase interacting protein associates with a specific spectrum of Cdks. Two years later, the first genome-wide yeast two-hybrid study was carried out on the T7 bacteriophage [23]. By screening libraries of random fragments fused either to the DBD or to the AD against each other, Bartell *et al.* [23] identified 22 interactions. Four of these had previously been demonstrated using biochemical methods. In addition to intermolecular interactions, 11 intramolecular interactions were also detected. Fromont-Racine and collaborators [15] used 15 yeast ORFs involved in pre-mRNA splicing to screen a yeast genomic library. They identified 170 interactions, corresponding to 145 different yeast ORFs. Of these, nine ORFs encoded known pre-mRNA splicing factors, five ORFs were homologous to human splicing factors and half of the interacting proteins had unknown functions.

Because of their small genome size viruses are well suited as model systems for high-throughput studies. McCraith *et al.* [24] tested all 266 full-length ORFs of the vaccinia virus against each other using a matrix screen, resulting in a total of 70 000 combinations of DBD and AD fusions. This approach yielded 37 protein-protein interactions, including 28 that had previously been identified. A matrix approach was also chosen to investigate interactions between the 10 ORFs of the hepatitis C virus [25]. Interestingly, no interactions were found using this approach, possibly due to incorrect folding or mis-targeting of full-length DBD and AD fusions. In order to circumvent this problem, the authors switched to the exhaustive library screening protocol and screened all 200 DBD fusions derived from the fragments of 10 ORFs against a random genomic library. This approach yielded 15 interactions that included both previously known and novel interacting pairs.

In 2000, Walhout *et al.* [21] created the first partial protein interaction map for a multicellular organism, the nematode *Caenorhabditis elegans*. They performed an exhaustive two-hybrid screen by mating 27 DBD-fused ORFs involved in vulval development with an AD-fused worm cDNA library. They identified 148 interactions, including 15 known interactions and 109 interactions that had previously been predicted based on the *C. elegans* genome sequence [21]. One year later, a different group mapped protein interactions in *C. elegans*, focusing on components of the 26S proteasome [26]. They performed both matrix and exhaustive library screening using 30 ORFs that encode proteasome subunits. The matrix screen resulted in the detection of 17 interaction partners, whereas library screening identified 138 different interactions.

The most comprehensive large-scale screening approaches reported to date focus on the yeast *Saccharomyces cerevisiae.* Two groups studied all 6000 annotated yeast ORFs using both matrix and library screening approaches [16, 17, 27]. Initially, Ito *et al.* [27] performed a large-scale matrix screen using 159 ORFs that were cloned as DBD and AD fusions. They analyzed 430 matings (representing 10% of all possible combinations between DBD and AD fusions) and identified 175 interactions, of which 163 had not previously been reported. They recently completed their systematic approach, identifying a total of 841 interactions [17]. The second group performed both array and library screenings [16]. Using the array method, 192 ORFs were created as DBD fusions and then mated with the 6000 ORFs of yeast fused to the AD. Only 20% of all interactions were found in both screens, resulting in 281 protein pairs. For the exhaustive library screen, a library was made by pooling all 5345 AD-fused ORFs. These were then mated separately to the same 5345 ORFs fused to the DBD, yielding a total of 692 protein-protein interactions.

Finally, the first prokaryotic interaction map has recently been published [28]. In this study, an exhaustive library screen was carried out by mating 285 DBD-fused ORFs of *Helicobacter pylori* with an AD-fused genomic DNA library. A total of 1280 interactions were identified using this approach.

A surprising observation was made when comparing the datasets of Ito *et al*. and Uetz *et al*.; despite the fact that both groups used the same 6000 ORFs in their experiments only 20% of all interactions in the two datasets actually overlapped [17]. The reasons for this small overlap are difficult to explain, but most likely the differences are due to the use of different experimental systems. For instance, the different DBD and AD plasmids used by the two groups may have affected the expression level and folding of the proteins, the use of PCR to amplify the yeast ORFs may have introduced mutations that abolish interactions and most importantly, the stringency of selection may have been different, eliminating interactions seen by one group from the other group's data set (see [17]).

4.3 Pitfalls of genome-wide protein interaction maps

In summary, the large-scale screenings carried out so far indicate that even when using exhaustive library screens that potentially cover all interactions in a genome, it is still difficult to estimate what percentage of protein-protein interactions that occur in a cell or organism under investigation are actually identified in such screens. The small overlap between the screens carried out by Ito *et al.* [17]

and Uetz *et al.* [16] suggests that even within the subset of protein-protein interactions that can be identified using the yeast two-hybrid system, such screenings are still far from representative. One explanation is that in large-scale interaction screens many ORFs have to be discarded for reasons inherent to the proteins under investigation. For instance, interactions in the yeast two-hybrid system have to take place in the nucleus. Consequently, proteins that possess hydrophobic transmembrane domains will be unable to reach the nucleus. To a certain extent, this problem can be circumvented by the use of libaries that express protein fragments, such as those used in the exhaustive library screens. Another class of troublesome proteins are those that interact with DNA or the transcription machinery, because they will activate the reporter genes in the absence of any protein-protein interaction when expressed as AD fusions. In conventional yeast two-hybrid screens such false positives are removed by testing AD fusions against an unrelated DBD fusion. If the AD fusion interacts both with the DBD fusion that was used in the screen and with the unrelated DBD fusion, the interaction is unspecific and the AD fusion is discarded. For practical reasons, this approach is not feasible when carrying out large-scale yeast two-hybrid screens. In the matrix approach, screens are carried out in duplicate and only interactions that are found in duplicate assays are counted as true positives. However, in library screenings random AD fusions are used and therefore, a given screen can never be duplicated. For this reason, library screenings probably contain a higher number of false positives than corresponding matrix screens.

5 Alternative approaches to the detection of protein-protein interactions

In the past, several other methods have been adapted for the high-throughput detection of protein-protein interactions, such as mass spectrometry [33, 34] or protein chips [30, 31]. Since these approaches use principles for the detection of protein-protein interactions that are different from the yeast two-hybrid system, some interactions that are found in those systems will be missed in the yeast two-hybrid system, and *vice versa*. For this reason, only a combination of those approaches will eventually lead to the discovery of all protein-protein interactions in a given cell or organism.

5.1 Mass spectrometry

Very recently, MS began to play an important role in the interactive proteomics efforts. Groups from the Canadian company MDS-Proteomics and German company Cellzome have used coprecipitations combined with MS in large-scale protein complex screens [33, 34]. In both approaches, an affinity tag was first attached to hundreds of target “bait” proteins. They then introduced DNA encoding these bait proteins into yeast cells, allowing the modified proteins to be expressed in the cells and to form physiological complexes with other proteins. Then, using an affinity tag, each bait protein was precipitated on an affinity column along with any associated protein. The proteins extracted with the tagged bait were identified using standard MS methods. The Cellzome scientists have identified 1440 distinct proteins within 232 multiprotein complexes in yeast, more than 90% of which contained at least one previously uncharacterized protein. Furthermore, they found that most of the complexes had a protein in common with another complex, suggesting a means of coordinating cellular functions into a higher-order network of interacting protein complexes [33]. Using the same principle, the MDS-Proteomics scientists constructed an initial set of 725 yeast bait proteins of a variety of different functional classes, including kinases, phosphatases and proteins involved in DNA damage response. They detected 3617 interactions involving 1578 different proteins [34].

5.2 Protein chips

Another approach to generate protein interaction maps is protein chip technology. Here, proteins are expressed, purified and screened in a high-throughput fashion [35]. Purified proteins are covalently attached to the surface of a microarray in a way that preserves their folded conformation and their ability to interact specifically with other proteins. Initially, protein chips were applied to identify yeast genes encoding defined biochemical activities [30, 36]. Recently, Zhu *et al*. [31] applied this technique to the study of protein-protein interactions. They first fused 5800 yeast ORFs to GST and expressed the fusion proteins in yeast. Subsequently, they generated a matrix by printing the purified fusion proteins onto glass slides and screened them for their ability to interact with other proteins and phospholipids. Using this technique, they identified many new calmodulin- and phospholipidinteracting proteins.

6 Adapting the yeast two-hybrid system for transcriptional activators

Despite the fact that the yeast two-hybrid system has been successfully used to discover interactions between proteins involved in virtually any type of cellular process, it has become apparent that the system has certain limita-

tions. As mentioned in Section 4, there are several classes of proteins whose investigation by the yeast two-hybrid system is not well suited. One class of proteins are transcriptional activators, proteins that naturally contain domains that activate transcription. In addition, proteins other than transcriptional activators are capable of activating transcription of reporter genes in yeast when fused to the DBD, further extending this class of unsuitable baits. When dealing with such self-activators, two genetic tricks can be considered. First, when using the yeast *HIS3* reporter gene, the drug 3-aminotriazole, a competitive inhibitor of the enzyme encoded by *HIS3*, can be used to lower the self-activation potential of the DBD fusion. In this way, even though the DBD fusion activates the *HIS3* reporter to a certain extent, the interaction of the DBD fusion and an AD fusion results in a stronger transcriptional activation of the *HIS3* reporter and higher expression levels of the *HIS3* gene product. This helps to overcome the growth threshold caused by 3-aminotriazole, resulting in growth of colonies on selective plates [10]. Second, the expression level of the DBD fusion can be lowered by using low copy number vectors and/or weaker promoters. Unfortunately, these modifications are not always successful, especially when dealing with strongly self-activating proteins. In order to circumvent these problems, alternative genetic screening techniques have been invented that do not face the same limitations.

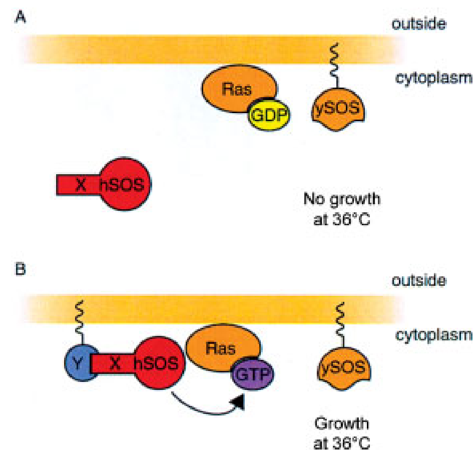

Figure 3. The SOS recruitment system. (A) In yeast cells carrying the *cdc25-2* mutation the yeast Ras guanyl exchange factor *cdc25* (ySOS) is inactivated at 36°C, leading to growth arrest. A bait X that is fused to the human Ras guanyl exchange factor SOS (hSOS) cannot overcome the growth defect because the X-hSOS fusion protein is located in the cytoplasm. (B) If an interacting protein Y is localized to the plasma membrane by means of a myristoylation signal, the interaction between X and Y recruits X-hSOS to the membrane where it stimulates GDP exchange on Ras, thereby circumventing the *cdc25-2* mutation. Consequently, the yeast will grow at the restrictive temperature of 36°C.

6.1 The SOS recruitment system

The SOS recruitment system (SRS) is based on the Ras pathway in yeast. When localized at the plasma membrane, the yeast Ras guanyl exchange factor *cdc25* stimulates guanyl nucleotide exchange on Ras and promotes downstream signaling events that ultimately lead to cell growth [37]. A mutant yeast strain harboring the temperature sensitive *cdc25-2* allele is still able to grow at the permissive temperature of 25°C but fails to grow when shifted to 36°C (Fig. 3A). However, the human Ras guanyl exchange factor hSOS, when targeted to the plasma membrane, efficiently complements the mutation, leading to cell growth at 36°C. In the SRS, the translocation of hSos is dependent on a protein-protein interaction: the bait X is fused to *C*-terminally truncated hSOS, which is active but unable to target the plasma membrane. The bait is co-expressed with a prey Y, which can be either an integral membrane protein or a soluble protein that is anchored to the membrane by means of a myristoylation signal [38]. If X and Y interact, the hSos fusion is recruited to the plasma membrane and substitutes for the defective *cdc25-2* allele (Fig. 3B). Initially, the SRS was tested using the interacting AP-1 factors C-Jun and c-Fos, which are difficult to study in a yeast two-hybrid system since both factors are strongly self-activating [39]. In addition, this system has been successfully applied for identification of c-Jun [40] and BRCA1 [41] interacting partners. However, a potential limitation of SOS based screening procedure is the isolation of false positives encoding Ras. To improve their screening approach, the same authors modified the SRS by co-expression of mammalian GTPase activating protein, which considerably reduced the number of false positives encoding Ras [42].

6.2 The RNA polymerase III based two-hybrid system

This version of the yeast two-hybrid system takes advantage of the fact that transcriptional activation of RNA polymerase II and RNA polymerase III is mediated by different transcription factors. It is based on the activation of a modified *SNR6* reporter gene (UAS_G-SNR6) transcribed by RNA polymerase III (Pol III [43]). U6, the transcript of the *SNR6* gene, is an essential small nuclear RNA (snRNA) involved in splicing. Transcription of the *SNR6* gene is activated by binding of t138p, a subunit of TFIIIC, to a specific DNA sequence located downstream of the *SNR6* gene, the so-called B-block. Deletion of this B-block abolishes binding of t138p and inactivates tran-

scription of the *SNR6* gene. The reporter construct of the Pol III based yeast two-hybrid system bears five GAL4 binding sites (upstream activating sequences, UAS_G) in place of the original B-block (UAS_G-SNR6). This otherwise inactive reporter gene can be activated by the expression of a fusion protein between t138p and the DBD of the Gal4 transcriptional activator (t138p-Gal4-DBD) which, when bound to the UAS_G sequences, is capable of initiating the transcription *via* recruitment of RNA polymerase III.

Similar to the traditional yeast two-hybrid system, activation of the UAS_G-SNR6 reporter is not dependent on a covalent link between t138p and the Gal4p-DBD of Gal4p. If a bait protein X is fused to t138p (t138p-X) and its interacting partner Y is fused to the Gal4-DBD (DBD-Y), the t138p-X/DBD-Y interaction reconstitutes a functional Pol III activating factor, which then activates the UAS_G-SNR6 reporter [43]. Recently, Petrascheck *et al*. [44] demonstrated that a screening procedure is possible in such a Pol III driven transcriptional system. Their system utilizes a temperature sensitive U6 snRNA, which is encoded by a mutated SNR6 gene in yeast (Fig. 4A). In this temperature sensitive strain, interactions between t138p-X and DBD-Y activate the UASG-SNR6 reporter construct, resulting in expression of wild-type U6 snRNA. The wild-type U6 snRNA suppresses the temperature sensitive phenotype and allows growth at the nonpermissive temperature (37°C), thus providing a positive selection system for interacting proteins (Fig. 4B). Using BRCA1 as a bait, Petrascheck *et al.* [44] screened a mouse embryonic cDNA library fused to the Gal4 DBD, and found

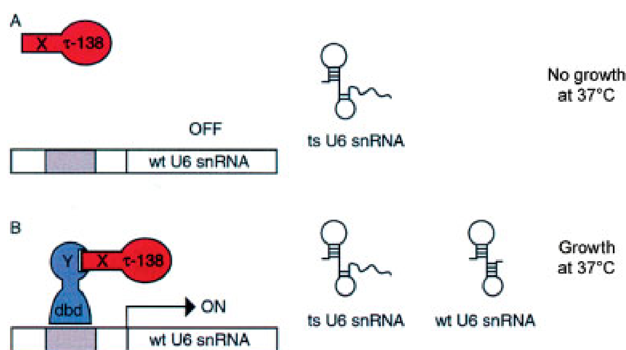

Figure 4. The RNA polymerase III based yeast two-hybrid system. (A) In the mutant yeast reporter strain, growth at 37°C is inhibited by a temperature sensitive U6 snRNA (ts U6 snRNA). The bait X is fused to the τ138p Pol III transcriptional activator but no transcription takes place because the τ138p-X fusion is not bound to the promoter region located upstream of the UAS_G wild-type U6 SNR6 gene. (B) The interaction of τ138p-X with its partner Y fused to the Gal4-DBD brings τ-138-X to the promoter where it recruits the Pol III complex. Transcription of the wild-type U6 snRNA restores growth of the yeast at 37°C.

183 bait dependent clones, which could be arranged in 14 complementary groups. However, it remains to be shown that the protein-protein interactions detected by this assay can also be confirmed using other approaches, such as co-immunoprecipitation, co-localization or *in vitro* interaction assays.

7 Tackling interactions between integral membrane proteins

Apart from transcriptional activators and self-activating proteins, another class of problematic proteins in the yeast two-hybrid system are integral membrane proteins. As mentioned in Section 4, the basic mechanism of the yeast two-hybrid system dictates that the interacting proteins must be located in the nucleus since their association leads to the reconstitution of a functional transcription factor, which must be bound to its target promoter in order to activate the corresponding reporter gene. However, proteins that are anchored in the membrane cannot be transported into the nucleus and consequently, the only way to use them in a yeast two-hybrid screen is to express truncated fragments, for instance, their cytoplasmic or extracellular domains. While this strategy has been successfully applied to single pass transmembrane domains [45–47], multipass transmembrane domains often have binding interfaces composed of multiple cytoplasmic loops. Seven transmembrane G protein coupled receptors, for instance, present binding sites for heterotrimeric G proteins that are composed of many residues from several intracellular loops [48–50]. Consequently, the characterization of integral membrane proteins, as well as their interactions with cytoplasmic and or other integral membrane proteins, is best carried out using entire proteins and not subfragments thereof. To circumvent this shortcoming of the conventional yeast two-hybrid system, other genetic screening methods have been developed for assaying integral membrane proteins. In the following sections, we review several systems that show the promise of dealing with this problem.

7.1 The Ras recruitment system

The Ras recruitment system (RRS) utilizes the same signaling pathway as the SRS (Section 6.1) but substitutes an activated mammalian Ras (mRas) mutant for hSOS [51]. Since the mRas mutant is constitutively active, stimulation of guanyl nucleotide exchange by *cdc25* is not needed. Therefore, the only requirement is that mRas has to be located at the plasma membrane to reactivate the Ras signaling pathway. In analogy to the SRS, the interaction between a bait X fused to activated mRas and a prey Y fused to a myristoylation signal recruits activated mRas to the plasma membrane, leading to growth of the mutant

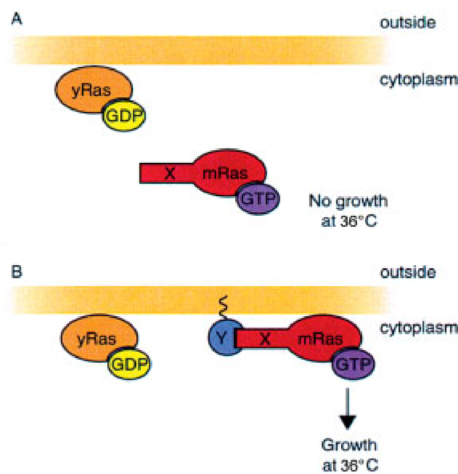

Figure 5. The Ras recruitment system. (A) In the Ras recruitment system, the same yeast strain as in the SOS system is used. The protein under investigation X is expressed as a fusion to activated mammalian Ras (mRas). Since mRas has to be located at the plasma membrane in order to function, the expression of X-mRas in the cytoplasm does not correct the growth defect at 36°C. (B) Co-expression of an interacting protein Y that is targeted to the plasma membrane by means of a myristoylation signal leads to the relocation of X-mRas to the plasma membrane. There, the activated mRas induces downstream signaling, leading to cell growth at 36°C.

cdc25-2 strain at 36°C (Fig. 5). Using the RRS, the authors reproduced the interaction between the phosphatidylinositol-3-phosphate kinase subunits p110 and p85 and between hSOS and the adaptor protein Grb2 [51]. Using the basic leucine zipper family member JDP2 as a bait, they also performed a screening against a rat pituitary cDNA library fused to a myristoylation signal. Four clones were isolated in the screen, two of which encoded valid interactors of JDP2. Another clone encoded SOS, which can be expected to activate yeast Ras and most likely represents a false positive in this system.

7.2 Reverse RRS

The RRS has the intrinsic limitation that integral membrane proteins cannot be used as baits since the fusion of activated mRas to a membrane protein would result in its translocation to the membrane independent of a protein-protein interaction. To circumvent this problem, the original RRS was modified by exchanging bait and prey fusions, yielding the reverse RRS [52]. Here, an integral membrane protein X (the bait) is expressed unmodified and its interaction partner Y (the prey) is expressed as a fusion with activated mRas. The interaction of X and Y recruits activated mRas to the membrane, where it bypasses yeast Ras to allow cell growth at the restrictive temperature of 36°C. An inherent drawback of the reverse RRS is that fusions of integral membrane proteins or membrane-associated proteins to mRas will lead to cell growth independent of a protein-protein interaction. Indeed, the authors found that approximately 5% of the total number of clones in a screen grew under selection. To eliminate these false positives, a screening scheme had to be devised where bait and prey expression was controlled by different inducible promoters, followed by replating of putative positive clones under several different selection conditions [52]. As opposed to its predecessors, the SOS and Ras systems, the reverse RRS allows the use of integral membrane proteins as baits. However, only interactions between a membrane protein and a cytoplasmic protein can be studied since fusion of a membrane-associated protein to Ras would activate the system in the absence of any protein-protein interaction. This limitation is also responsible for the high number of false positives encountered in this system. Although the use of inducible promoters may circumvent this problem, it also complicates a potential adaptation of the reverse RRS for use in automated high-throughput screens.

7.3 The G protein based screening system

In a similar approach, the yeast mating pathway was used to study protein-protein interactions involving integral membrane proteins [53]. Factors that signal mating in yeast act through G protein coupled receptors and their associated heterotrimeric G proteins, G_α, G_β and G_γ. Binding of a ligand (either a- or α-factor) to its respective receptor induces a conformational change which leads to the exchange of GTP for GDP in G_α, followed by the dissociation of the complex into G_α and $G_{\beta\gamma}$. The $G_{\beta\gamma}$ subunits then act on various downstream effectors to activate the mating cascade, resulting in cell cycle arrest and the formation of characteristic “shmoos” (Fig. 6). In the G protein based screening system, the bait is an integral membrane protein X expressed in its unmodified form, whereas its interaction partner Y is expressed as a fusion to the G_γ subunit. If X and Y interact, the G_γ fusion protein is recruited to the membrane, where it binds G_β subunits. The interaction between G_β and G_γ sequesters the G_β subunits and blocks downstream signaling. Interactors are identified either morphologically by assaying the sensitivity of the cells for α-factor or by measuring for the activity of a *lacZ* reporter construct which is induced by the mating pathway. The G protein based screening system has been successfully applied to demonstrate the interaction between two defined interaction partners (syntaxin 1 and neuronal Sec1) but has yet to be used in a library screening approach [53]. Although the use of morphological criteria such as “shmoo” formation is unsuit-

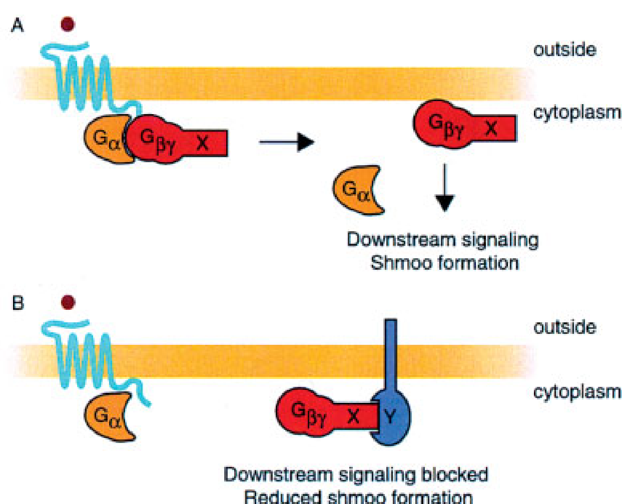

Figure 6. The G protein based screening system. (A) The protein of interest X is fused to the yeast G_γ subunit. G_γ-X, together with the other heterotrimeric G protein subunits G_α and G_β, bind to the cytoplasmic domain of the yeast G protein-coupled receptor Ste2. Activation of Ste2 by a ligand induces G protein dissociation and downstream signaling by the $G_{\beta\gamma}$-X complex, leading to cell cycle arrest and the formation of characteristic "shmoo patterns". (B) Co-expression of an interacting integral membrane protein Y leads to its association with $G_{\beta\gamma}$-X. Presumably, the interaction sequesters $G_{\beta\gamma}$-X and prevents downstream signalling, leading to a reduced sensitivity to α-factor. Such colonies can then be identified by their different morphology.

able for the selection of interactors from large libraries, the use of a *lacZ* reporter construct that is induced by the mating pathway is compatible with high-throughput screening in a matrix format. If the system is to be used in library screenings, replacing the *lacZ* reporter with an auxotrophic marker such as *HIS3* would allow the selection of interacting proteins in a manner similar to the conventional yeast two-hybrid system.

7.4 The split-ubiquitin system

The small protein ubiquitin consists of 76 amino acids and is highly conserved between all eukaryotes [54]. Its primary function is to act as a "tag" for degradation by being attached to proteins through ubiquitin conjugating enzymes. Once a protein becomes tagged with several ubiquitin moieties it is transported to the 26S proteasome, where ubiquitin-specific proteases (UBPs), cut the peptide bond at a double glycine motif in the junction between the attached ubiquitins and the target protein. The released ubiquitin moieties are recycled back to the cytoplasm, whereas the target protein is degraded by the 26S proteasome [55].

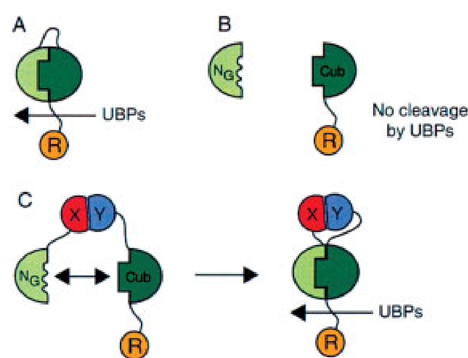

Figure 7. The split-ubiquitin system. (A) Native ubiquitin is recognized by UBPs, which cleave at a double glycine motif located at the *C*-terminus of ubiquitin. A reporter protein (R) that is fused to the *C*-terminus of ubiquitin is released upon cleavage. (B) Cleavage at an extended loop separates ubiquitin into an *N*-terminal (Nub) and a *C*-terminal (Cub) domain. termed Nub (N) and Cub (C). The introduction of a point mutation into Nub (N_G) abolishes the affinity of the Nub and Cub domains for each other. UBPs do not recognize the isolated Cub domain and the reporter remains attached to the *C*-terminus of Cub. (C) A protein X is fused to NubG and its interacting partner Y is fused to Cub. The interaction of X and Y brings NubG and Cub together and leads to their reassociation into split-ubiquitin. Like native ubiquitin, split-ubiquitin is recognized by UBPs, leading to the release of the *C*-terminal reporter.

The split-ubiquitin system was originally developed by Johnsson and Varshavsky [56] and takes advantage of the highly specific cleavage mediated by the UBPs. The expression of a fusion protein consisting of ubiquitin and a *C*-terminally attached reporter in yeast results in fast and complete cleavage by UBPs within minutes ([56], Fig. 7A). The folded structure of ubiquitin is crucial to the recognition and subsequent cleavage events by UBPs: expression of an *N*-terminal ubiquitin moiety carrying a point mutation in a hydrophobic core residue (NubG) together with the *C*-terminal ubiquitin moiety (Cub) in the same yeast cell does not result in cleavage by UBPs anymore (Fig. 7B), presumably because the partially unfolded NubG moiety does not recognize and bind to the Cub moiety. As the UBPs do not recognize Cub alone, no cleavage of the attached reporter takes place. In order to utilize the ubiquitin moieties as reporters for protein-protein interaction events, two interacting proteins X and Y are fused to NubG and Cub, respectively. Upon interaction of X and Y, the NubG and Cub moieties are forced into very close proximity, resulting in a partial refolding of NubG, followed by reassociation of NubG and Cub into what the authors termed "split-ubiquitin". Split-ubiquitin is a good substrate for UBPs and, therefore the attached reporter is cleaved off (Fig. 7C). As the formation of split-ubiquitin and the subse-

quent cleavage by UBPs do not depend on any special localization of the proteins, the split-ubiquitin system is suitable to the investigation of membrane proteins. To date, two applications of the split-ubiquitin system have been described, namely the rUra3 based split-ubiquitin system and the transactivator based split-ubiquitin system.

7.5 The rUra3 based split-ubiquitin system

According to the *N*-end rule of protein degradation, proteins that carry a destabilizing amino acid at the *N*-terminus are rapidly tagged with ubiquitin and then degraded by the 26S proteasome [57, 58]. This property can be conferred onto any protein by simply exchanging its *N*-terminal residue for a destabilizing one. In the rUra3 based split-ubiquitin system [59], a destabilized Ura3 protein (rUra3) is fused to the *C*-terminus of Cub, which in turn is fused to a protein X. Since the Ura3 protein converts the compound 5-FOA into a toxic metabolite, cells that express the X-Cub-rUra3 fusion protein are unable to grow on plates supplemented with 5-FOA (Fig. 8A). In a second step, an interacting protein Y is expressed as a fusion to NubG, such that NubG is located in the cytoplasm. As the interaction of X and Y allows NubG and Cub to be close enough to refold into split-ubiquitin, the rUra3 protein is rapidly cleaved off by cytoplasmic UBPs. The now liberated *N*-terminal residue on the rUra3 protein is destabilizing and thus, the entire protein is quickly degraded. Since this degradation step is very efficient, cells containing interacting X-Cub-rUra3 and Y-NubG fusions will soon have no rUra3 in the cytoplasm left and will therefore grow on selective medium containing 5-FOA (Fig. 8A). The rUra3 based split-ubiquitin system has been used to map interactions between several components of the translocation machinery in yeast [59] or between the translocation machinery and its substrates [60] and to characterize the conformational stability of different proteins in yeast [61]. The versatility of the method is also shown by a recent report describing a screen for proteins that interact with the transcriptional regulators Gal4 and Tup1 in yeast [62, 63]. Since the rUra3 based split-ubiquitin assay is not based on transcription, it can be used to detect interactions between proteins of interest almost anywhere in the cell and is applicable to nuclear, cytoplasmic or integral membrane proteins.

7.6 The transactivator based split-ubiquitin system

Stagljar and coworkers [64] have combined the transcriptional output of the yeast two-hybrid system with the split-ubiquitin assay to create another screening system for integral membrane proteins. Here, the Cub is fused to a hybrid transcription factor composed of the bacterial LexA protein and the *Herpes simplex* VP16 transactivator. This Cub-LexA-VP16 cassette is fused to an integral

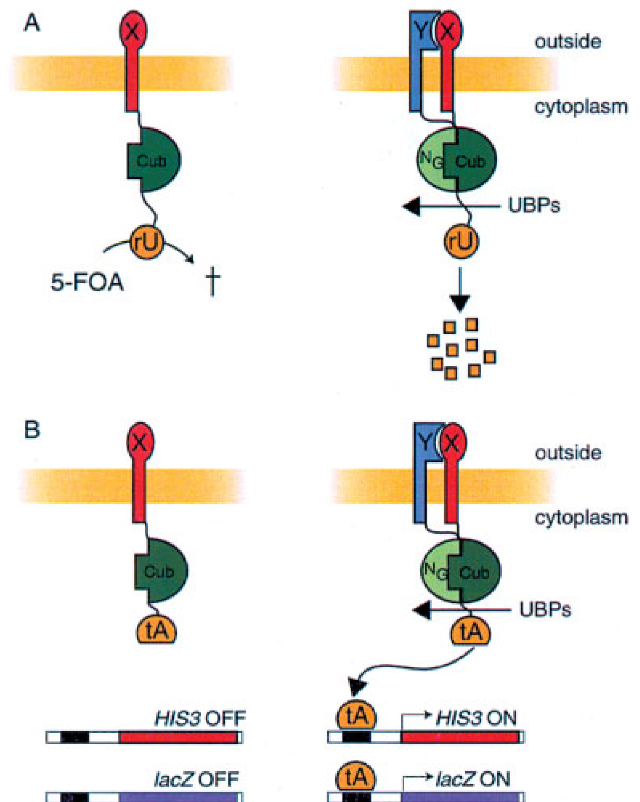

Figure 8. Membrane based split-ubiquitin systems. (A) The rUra3 based split-ubiquitin system. An integral membrane protein X is fused to the Cub domain followed by a destabilized version of the Ura3 protein (rU). If yeast expressing the X-Cub-rUra3 fusion protein are grown on plates containing the compound 5-FOA, rUra3 converts 5-FOA into a toxic metabolite, which then kills the cells. The interaction of X with its partner Y fused to NubG leads to the reassociation of Cub and NubG into split-ubiquitin, followed by UBP-mediated cleavage at its *C*-terminus. Since the released rUra3 protein carries a destabilizing *N*-terminal amino acid, it is rapidly degraded by the 26S proteasome. Consequently, the cells can grow on 5-FOA containing medium. (B) The transactivator based split-ubiquitin system. An integral membrane protein X is fused to the Cub domain followed by the artificial transactivator LexA-VP16. Since the transactivator is immobilized at the membrane, it cannot reach the nucleus and is unable to activate the reporter genes. Co-expression of an interacting protein Y fused to NubG triggers the reassociation of split-ubiquitin, followed by cleavage and release of the transactivator. Upon diffusion to the nucleus, the transactivator activates the reporter genes, leading to cells that grow on selective medium lacking histidine and that turn blue in a β-galactosidase assay.

membrane protein X which serves as a bait (Fig. 8B). The integral membrane protein anchors the cassette in the lipid bilayer and prevents its diffusion to the nucleus. If a cytoplasmic or a nuclear protein is selected as bait, a sequence motif that confers fatty acid modification can be used to attach the bait to the membrane [38, 65]. The interacting partner Y, which can be either an integral membrane protein or a cytoplasmic protein (Fig. 8B) is fused to NubG. Upon interaction of X and Y, the close proximity of Cub and NubG drives their reassociation to form split-ubiquitin. Following cleavage by UBPs, the LexA-VP16 transcription factor is released from the membrane and travels to the nucleus where it binds LexA operator sequences located in the promoter region of reporter genes. Binding of LexA-VP16 recruits the RNA polymerase II complex to the promoter and results in transcription of the reporter genes, whose activity is then assayed by appropriate means. For instance, auxotrophic markers such as *HIS3* or *LEU2* allow selection on minimal plates lacking the amino acids histidine and leucine, respectively, whereas *lacZ* yields blue colonies in a β-galactosidase assay. Using the yeast oligosaccharyltransferase complex as an example, Stagljar *et al.* [64] showed that the interaction between two of its components, Wbp1 and Ost1, could be detected in the transactivator based split-ubiquitin system. More recently, the transactivator based split-ubiquitin system has been used to investigate the influence of mutations on the assembly of presenilin fragments in Alzheimer's disease [66] and to characterize the interaction between the α1,2-mannosidase Mns1p and Rer1p in the endoplasmatic reticulum [67]. In order to show that this approach can also be used to detect novel protein-protein interactions we have created several libraries of NubG-fused inserts from yeast and mammalian sources and are currently in the process of screening those libraries using a variety of yeast and mammalian integral membrane proteins (S. Thaminy, D. Auerbach and I. Stagljar, unpublished results).

To date, the split-ubiquitin system is the most widely used of the alternative yeast-based two-hybrid systems reviewed here. Sufficient literature exists to suggest that the system is able to detect interactions between nuclear proteins, between integral membrane proteins and cytoplasmic proteins and even between two integral membrane proteins. Also, the use of selectable markers in the rUra3 based and transactivator based variants should facilitate their conversion into high-throughput screening platforms that are compatible with both the matrix and the library screening format. Such an approach would undoubtedly complement current yeast two-hybrid based strategies and should help to render genome-wide interaction maps even more complete.

8 Concluding remarks

For more than 10 years now, the yeast two-hybrid system and its variations have played an important role in the study of protein-protein interactions. The recent application of the yeast two-hybrid system to large-scale screenings has resulted in the creation of several protein interaction maps that have allowed the identification of novel protein-protein interactions at an hitherto unknown scale. These screens facilitate the understanding of gene function in several ways. First, they give insight into the possible functional roles of previously unknown genes by linking them to already characterized proteins. Second, they help to assign novel functions to many previously characterized proteins. Third, they provide novel interactions between proteins that are known to be involved in a common biological process. The establishment of comprehensive two-hybrid interaction maps has resulted in an explosion of interaction data, which has prompted researchers to organize these vast amounts of data by creating virtual protein interaction maps *in silico*. In order to make all this data accessible to the research community, several public databases have recently been generated, including the Yeast Proteome Database (YPD; www.proteome.com), http://depts.washington.edu/sfields/projects/YPLM, http://cancerbiology.dfci.harvard.edu/cancerbiology/ResLabs/Vidal, the MIPS (Munich Information Center for Protein Sequences) database (www.mips.biochem.mpg.de), Myriad Corporation's Pronet database (www.myriad-pronet.com) and Curagen PathCalling database (http://portal.curagen.com). With this information readily accessible through the internet, researchers will be able to quickly find information on interactions involving their proteins of interest. The next challenge will then be to interpret (or validate) the resulting protein interactions, for instance using genetic, biochemical and cell-biological approaches. In the immediate future, technical advances in yeast two-hybrid technologies, protein chip and mass spectrometry technologies, combined with more sophisticated protein databases, will undoubtedly help to connect proteins into complexes and interaction networks and to integrate those into existing and novel cellular pathways.

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