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Diversity in Genetic *In Vivo* Methods for Protein-Protein Interaction Studies: from the Yeast Two-Hybrid System to the Mammalian Split-Luciferase System

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INTRODUCTION

The creativity and technical diversity that reside in the tools developed to investigate the binding of one protein to another show how eagerly scientists have been anticipating the characterization of protein-protein interactions (PPIs), from a small-scale atomic level to a large-scale interactomics level. Many genetic, biochemical, biophysical, and computational technologies are now developed that contribute to the knowledge on which proteins interact with each other, taking advantage of specific phenomena that occur during an interaction. These include isothermal titration calorimetry (514), where emission of heat during a protein association is analyzed, and fluorescence anisotropy (417), in which the reduced speed of rotational movement of a protein is detected after it binds another protein. Other biophysical methods include dual polarization interferometry (111), surface plasmon resonance (567), static light scattering (18), and circular dichroism (218) methods. Examples of biochemical interaction technologies are the proximity ligation assay (606), cross-linking (661), the pulldown assay (67), coimmunoprecipitation (316), and tandem affinity purification (TAP) (524). Genetic approaches comprise phage display (78), the yeast two-hybrid system (178), protein fragment complementation assays (PCAs) (312), and protein microarrays (327).

The broad spectrum of available technologies (Fig. 1) is explained by the complementary output that each of them provides. While biophysical methods such as isothermal titration calorimetry have the advantage of giving details on the kinetics of an interaction, several biochemical and genetic techniques can be used to screen for the identification of undiscovered binding partners. Different techniques are also complementary in the identity of PPIs that can be investigated. Affinity purification is the method of preference for the characterization of stable multiprotein complexes, in contrast to the yeast two-hybrid system, which is more suitable for identification of transient and binary PPIs.

In this review, we focus on genetic *in vivo* methods for PPI studies. The technologies described can be divided into two main categories: two-hybrid systems and PCAs. The clear distinction between these groups lies in the fact that PCAs depend on the PPI-induced refolding of two protein fragments to reconstitute a functional reporter (Fig. 2A). On the other hand, two-hybrid systems do not depend on PPI-induced refolding of protein fragments but rather on the colocalization of two protein domains (Fig. 2B). These two definitions need to be taken with some practical flexibility. For example, some two-hybrid systems use only one hybrid protein (e.g., G protein fusion systems), and with some PCAs, the refolded protein is not the final reporter by itself but initiates a process that results in the appearance of the actual reporter (e.g., split-ubiquitin system). Nevertheless, the distinction between refolding of protein fragments (PCA) and colocalization of a protein domain(s) (two-hybrid assay) remains true in all cases. There are many limitations and advantages of PCAs in comparison to two-hybrid systems. In general, two-hybrid assays take place artificially in a specific compartment of the cell, which prevents analysis of the genuine subcellular locations of PPIs and can result in false-positive interactions between proteins that nor-

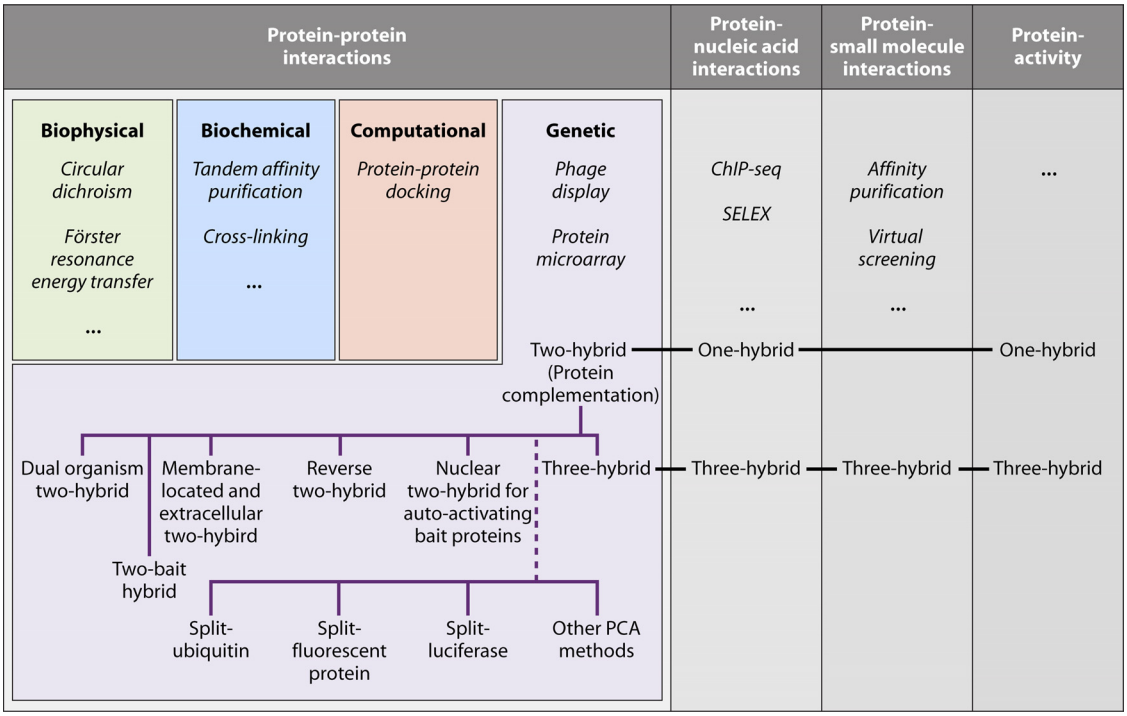

FIG 1 Overview of protein-protein interaction technologies and alternative applications for two-hybrid assay-derived methods. ChIP-seq, chromatin immunoprecipitation followed by sequencing; SELEX, systematic evolution by exponential enrichment; PCA, protein fragment complementation assay. Technologies in italics are not discussed in this review.

mally are found in separate cellular compartments. PCAs usually do not require specific localization and therefore more closely reflect the native environment of the proteins under study. In most cases, two-hybrid systems have reporter gene activation as an output, which is an important factor of signal amplification to increase the sensitivity of the method, but with the cost of lowered selectivity. This balance between sensitivity and selectivity is also seen in PCAs, where the output of the method (e.g., transcription activation, enzymatic activity, or fluorescence), the efficiency of protein fragment refolding, and the stability of the refolded reporter complex define how likely it is that false-negative or false-positive results will be detected. Due to the requirement of the two

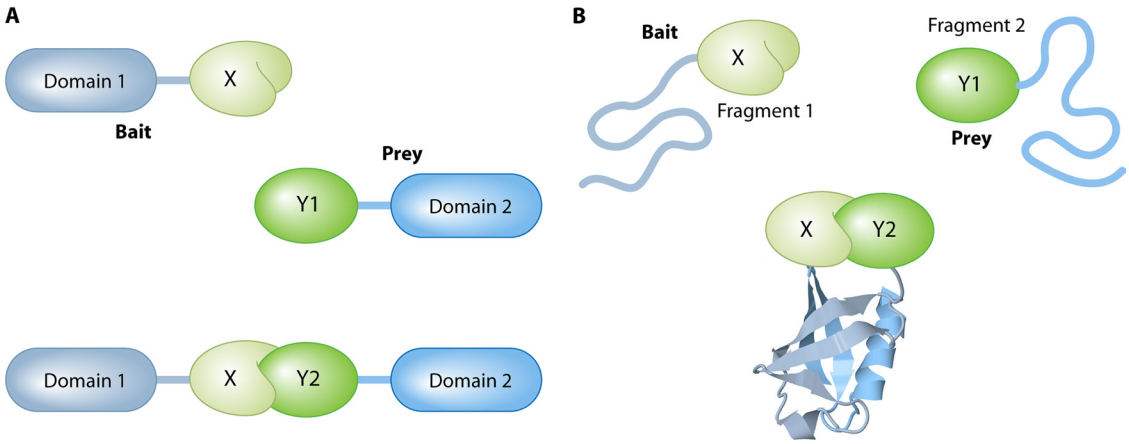

FIG 2 Two-hybrid systems versus PCAs. (A) Colocalization in two-hybrid systems. Two proteins of interest (X and Y) are each fused to a fixed protein domain, forming the bait and the prey, respectively. In the absence of an interaction (upper part with Y1), the domains remain distant, preventing a detectable output. If the two proteins do interact (lower part with Y2), the bait recruits the prey to a specific cellular location (e.g., reporter gene or plasma membrane), where it can stimulate a detectable output (e.g., gene activation or signal transduction). The domains do not need to be in physical contact. (B) Protein refolding in PCAs. Two proteins of interest (X and Y) are each fused to a fixed protein fragment. If there is no interaction between X and Y (upper part with Y1), the fragments remain unstructured and lack any functional abilities. Upon interaction between the bait and prey (lower part with Y2), the two fragments refold into a fully functional reporter protein (e.g., ubiquitin in the figure). In most cases, the interaction does not need to take place in a specific cellular location, but a minimal time frame of physical contact between the two fragments is necessary to establish complete refolding. The image of the ubiquitin protein is based on the Protein Data Bank (PDB) structure under accession number 1UBQ (671).

reporter fragments to refold, PCAs tend to be more sensitive to steric hindrance than two-hybrid systems. PCA selectivity is also affected by the spontaneous reassembly of the reporter independent of a PPI, an issue that concerns mainly PCA methods in which the reconstituted reporter cannot reverse back to the unfolded fragments. A clear advantage of PCAs over two-hybrid systems lies in the fact that some PCAs have the ability to detect PPIs with a high temporal resolution (e.g., the split-luciferase method). Finally, many PCA technologies can very easily be transferred to other organisms, while two-hybrid systems often contain many components (reporter genes and DNA-binding domain [DBD] and activation domain [AD] constructs) that need to be adapted specifically for application in a new organism. Therefore, it must be emphasized that any PCA method described in this review can be applied to any organism of interest that can be transformed or transfected with a vector.

For reasons of consistency, we always use the general term "PCA" to address the category of reporter folding technologies, but it should be noted that they are also known as "split-protein sensors." Specific PCA techniques are named split-"X" methods, such as the split-ubiquitin and split-luciferase methods, because this is the most commonly used way to address them.

Fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) are similar to two-hybrid and PCA methods. They are not discussed here, but several reviews can be found that elucidate the uses of FRET and BRET for PPI research (117, 118, 394, 413, 517, 646, 677). Likewise, *in vitro* PCA applications are not mentioned, but there are public reports on the use of these techniques for discovery of PPI-inhibiting compounds (243).

This review provides insights into two-hybrid systems and PCAs, highlighting their applications, advantages, and limitations. The first part describes the evolution of the original yeast two-hybrid system, from the original design to high-throughput genomewide screens. The second part explains alternative two-hybrid systems in *Saccharomyces cerevisiae* for the study of PPIs but also for other purposes, such as the detection of PPI inhibitors and the examination of associations between proteins and RNA, DNA, or small molecules. The third part deals with the current applications of PCAs in *S. cerevisiae*. Importantly, this section also contains a general introduction to three of the most commonly applied PCAs (the split-mouse dihydrofolate reductase [split-mDHFR], split-luciferase, and split-fluorescent protein [split-FP] methods), with considerations that apply to all organisms. The last part covers the development and applications of two-hybrid systems and PCAs in different organisms ranging from bacteria to mammalian cells. Table 1 gives an overview of validated applications for the currently available systems.

TABLE 1 Validated applications for genetic protein-protein interaction technologies

Purpose	Technology [reference(s)][a]
Confirmation of PPIs[b]	All endogenous PCAs All endogenous two-hybrid systems
Large-scale screening for PPIs[c]	Two-hybrid systems (yeast, bacteria) (689, 721) Split-ubiquitin system (yeast) (447) Split-DHFR system (yeast) (633) Mammalian two-hybrid system (mammalian cells) (535) Mammalian protein-protein interaction trap (mammalian cells) (395) Split-FP system (mammalian cells) (376)
Small-scale screening for PPIs[d]	Sos recruitment system (yeast) (455) Ras recruitment system (yeast) (331) Repressed transactivator system (yeast) (569) Split-FP system (mammalian cells) (544) RNA Pol III system (yeast) (597) One-hybrid system for PPIs (yeast) (229)
Association and dissociation of PPIs (temporal dynamics)	Split-luciferase system (yeast, mammalian cells) (429, 615)
Localization of PPIs	Split-FP system (yeast, bacteria, fungi, plants, animal cell cultures) (17, 262, 602) Split-DHFR system with fluorescein-conjugated substrate (plants, mammalian cells) (545, 625)
Discovery of PPI inhibitors	Reverse two-hybrid system (yeast, bacteria) (115, 710) Forward two-hybrid system (yeast, mammalian cells) (199, 594) Repressed transactivator system (yeast) (313) Split-FP system (bacteria) (457) Split-CyaA system (bacteria) (495)
Discovery of amino acids perturbing a PPI	Reverse two-hybrid system (yeast, bacteria) (239, 510) Forward two-hybrid system (yeast) (717) Two-bait hybrid systems (yeast) (539) Split-ubiquitin system (yeast) (74) Split-yCD system (yeast) (156)

[a] Many other technologies exist, for these and also for alternative applications. These are discussed in the text.
[b] Preference goes to methods which mimic the appropriate cellular environment and native expression as closely as possible.
[c] Includes technologies with unbiased screening applications with a significant number of bait proteins in parallel. Prey libraries can originate from a rational (open reading frames) or random (cDNA or genomic DNA libraries) source.
[d] Includes technologies with applications in library screening for PPIs limited to one or a few bait proteins in parallel. Several methods showed promise in prototype experiments with controlled libraries (e.g., see references 190 and 355). Some techniques have been used for module-scale experiments, with multiple bait and prey proteins but without a library (e.g., see reference 161). These are all not included in the table but discussed in the text.

THE YEAST TWO-HYBRID SYSTEM

Development of the Yeast Two-Hybrid System

Fields and Song. A milestone in the field of PPI studies came with the publication of "A Novel Genetic System To Detect Protein-Protein Interactions" by Stanley Fields and Ok-Kyu Song in 1989 (178). They took advantage of previous studies on the modular arrangement of transcription factors, including *S. cerevisiae* Gal4 (63, 330, 419, 420, 618). The N-terminal domain of Gal4 (amino acids [aa] 1 to 147) binds to the upstream activating sequence of *GAL1*, and the C-terminal part of Gal4 (aa 768 to 881) serves as the

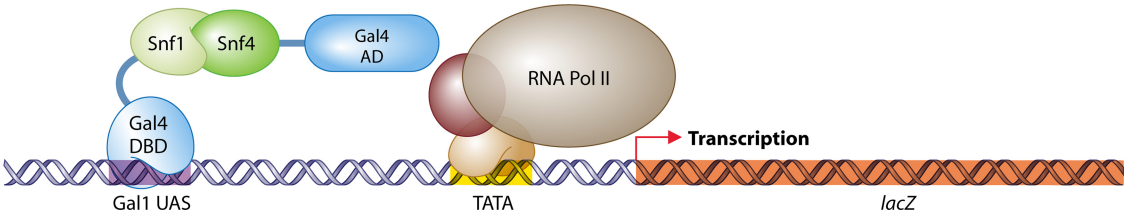

FIG 3 The first two-hybrid experiment (178). For the study of the interaction of two proteins of interest (in this case, Snf1 and Snf4), one protein is fused to the DNA-binding domain (DBD) of Gal4 (the bait) and the other protein is fused with the activation domain (AD) of Gal4 (the prey). The bait fusion binds upstream activating sequences (UAS) of the reporter gene *lacZ*. Association of Snf1 with Snf4 brings the Gal4 AD to *lacZ*, followed by recruitment of the basal transcriptional machinery, which establishes *lacZ* transcription, detected by chromogenic analysis.

activation domain, which stimulates gene expression. Both domains carry out their functions independently, but only when they are brought together is a DNA-binding and gene expression-activating protein formed. The principle of their system lies in the fusion of each domain with a protein of interest. Binding of the two proteins of interest results in the reassembly of the transcription factor Gal4, which in turn induces expression of one or more reporter genes. In the study of Fields and Song, the physical association of two *S. cerevisiae* proteins, Snf1 and Snf4, was confirmed by showing that a strain that expresses the hybrid genes *GAL4*(*1-147*)*-SNF1* and *GAL4*(*768-881*)*-SNF4* is capable of inducing expression of an *Escherichia coli lacZ* reporter gene controlled by the *GAL1* promoter (Fig. 3). The DNA-binding domain fusion was named the "bait" that is used to "capture" the so-called "prey" activation domain fusion.

Sensitivity and selectivity. Since 1989, the two-hybrid system has been the subject of many improvements of all its fundamental components, e.g., the reporter genes, the AD, and the DBD. Table 2 gives an overview of the currently available possibilities for each factor. Besides chromogenic reporters such as *E. coli* LacZ, prototrophic reporter genes were introduced to single out colonies that include interacting bait and prey proteins on prototrophy-selective medium (e.g., see references 234 and 673). This step greatly facilitated the use of prey plasmid libraries to identify the proteins that interact with the bait of interest in a large collection of noninteracting proteins, a significant advantage of the two-hybrid system over many other technologies. Most two-hybrid strains contain multiple reporter genes, with a different promoter region for each reporter, to enable a wider spectrum of sensitivity and selectivity. As an example, strain PJ69-4A (299) contains *HIS3*, *ADE2*, and *lacZ*, controlled by the *GAL1*, *GAL2*, and *GAL7* promoters, respectively. The *HIS3* reporter provides the highest sensitivity, as the Gal4 DBD binds the *GAL1* promoter very efficiently. In contrast, PPI assays based on the *GAL2p-ADE2* module are very stringent and can be used to exclude dubious results. Finally, the reporter gene *lacZ* can be applied for ultimate confirmation of the interaction in a semiquantitative (169) galactosidase assay. A more recent selection approach takes advantage of the yeast enhanced green fluorescent protein (yEGFP) as a reporter to screen for interacting pairs by fluorescence-associated cell sorting (FACS) (87–89).

Apart from the reporter promoter region, other factors influence the balance between sensitivity and selectivity. The copy numbers of the episomal bait and prey plasmids, together with the bait and prey promoters, affect expression levels, which in turn have an impact on the likelihood of detecting an interaction (379). CEN-based plasmids and truncated *ADH1* promoters lower bait and prey expression levels for high selectivity, while 2μm-based vectors and *GAL1* or full-length *ADH1* promoters contribute to an increased sensitivity of the system. Libraries of prey plasmids cannot be integrated due to the low transformation efficiency linked with genomic integration. However, bait integration in

TABLE 2 Reporter genes, activation domains, and DNA-binding domains used in yeast two-hybrid experiments

Component[a]	Description (reference)
Reporter genes	
*E. coli lacZ**	β-Galactosidase chromogenic reporter (178)
S. cerevisiae MEL1	Secretory α-galactosidase chromogenic reporter (5)
E. coli gusA	β-Glucuronidase chromogenic reporter (580)
Aspergillus oryzae lacA3	Engineered secretory β-galactosidase chromogenic reporter (318)
*S. cerevisiae HIS3**	Prototrophic reporter for histidine biosynthesis (673)
*S. cerevisiae LEU2**	Prototrophic reporter for leucine biosynthesis (234)
S. cerevisiae URA3	Prototrophic reporter for uracil biosynthesis (374)
*S. cerevisiae ADE2**	Prototrophic reporter for adenine biosynthesis (299)
S. cerevisiae LYS2	Prototrophic reporter for lysine biosynthesis (580)
Aequorea victoria GFPuv	Fluorescent reporter (107)
EGFP	Fluorescent reporter (613)
Yeast *EGFP*	Fluorescent reporter for flow cytometry screens (88)
Aureobasidium pullulans AUR1-C	Aureobasidin A resistance reporter (167)
Prey activation domains	
S. cerevisiae Gal4 AD	Gal4 activating region II (aa 768 to 881), moderate strength (178)
Herpes simplex virus VP16 AD	VP16 activating region (aa 413 to 490), high strength (673)
E. coli B42 AD	Bacterial polypeptide, weak strength (234)
Bait DNA-binding domains	
S. cerevisiae Gal4 DBD*	Binds *GAL1*, *GAL2*, and *GAL7* upstream activating sequences (178)
E. coli repressor LexA DBD*	Binds LexA operator sequences (234)
H. sapiens estrogen receptor DBD	Binds estrogen receptor elements (374)
Bacteriophage λ repressor cI	Binds cI operator sequences (580)
Tet repressor	Binds Tet operator sequences (716)

[a] *, most popular options.

TABLE 3 Strategies to screen for PPIs in the yeast two-hybrid system

Library type	Features, with advantages (+) and disadvantages (−)
Genomic DNA	For organisms with low intron occupancy and small intergenic regions; genomic DNA is cut with ClaI-compatible restriction enzymes (95, 299); +, cheap, incomplete fragments may facilitate positive outcome (e.g., with membrane proteins or incorrectly annotated protein-encoding genes); −, small fraction of in-frame protein coding fragments, introns are present
cDNA	For organisms with high intron occupancy and large intergenic regions (614); +, cheap, exclusion of noncoding fragments and introns, correct orientation; −, only partial fraction of in-frame protein coding fragments, strong difference in abundance between different cDNA fragments
Normalized cDNA	Normalizes the amount of cDNA fragments for each gene (683); +, exclusion of noncoding fragments and introns, correct orientation, better representation of each cDNA fragment; −, relatively expensive, only partial fraction of in-frame protein coding fragments
Full-length cDNA	Full-length cDNA fragments created with gene-specific forward primers (627); +, exclusion of noncoding fragments and introns, correct frame and orientation, balanced representation of each gene; −, expensive, complete cDNA fragments reduce the positive outcome rate for specific types of interactions (e.g., with membrane proteins)
Open reading frame DNA	Each open reading frame is individually cloned into the prey library by *in vivo* (gap repair) or *in vitro* (Gateway from Invitrogen) recombinational cloning (294, 650); +, exclusion of noncoding fragments and introns, correct frame and orientation, balanced representation of each gene; −, expensive, introns are present, complete ORF fragments reduce the positive outcome rate for specific types of interactions (e.g., with membrane proteins)

combination with a strong promoter, to reduce the variability of bait expression, provides a very attractive alternative to the traditional episomal approach. It has proven to reduce both false-positive and false-negative results in library screening experiments (453) and also in alternative two-hybrid systems (22, 451, 502).

In a recent study, the impact of bait and prey vector identity on the positive output of interaction assays was studied with *Treponema pallidum* and *Escherichia coli* motility-related proteins (531). Remarkably, different combinations of bait and prey vectors led to differences in the number of positive results, the subset of detected PPIs, and the reliability of the outcome. The authors suggested not only that the expression levels of bait and prey affect output but also that a potential role may be present for less obvious factors, such as the presence or absence of a stop codon in the backbone vectors or the size of the linker sequences within the fusion constructs. Similar conclusions could be drawn using a standard positive and negative reference set (91). In these analyses, many of the positive results acquired by only one bait-prey vector combination were part of the positive reference set, which suggests that the small overlap seen in large-scale studies (e.g., see reference 294 versus reference 650) does not necessarily point to a large number of false-positive results but rather to a variation in experimental procedures. Another possible underappreciated influence on two-hybrid results is the exact composition of the medium (410).

In addition, the identities of the DNA-binding domain, the activation domain, and the reporter genes likely further influence the number of positive (true and false) results. For example, most reporter genes are integrated into the genome, but in some experimental designs, the *lacZ* gene is retained on an episomal plasmid for an increased response (169, 693). Moreover, some activation domains are strong expression inducers, particularly VP16, while the B42 AD is known to be a weaker activator (62, 557). Finally, Uetz and colleagues recently illustrated the impact of steric hindrance in a study on the interactome of varicella-zoster virus (616). All 70 proteins of this virus were cloned as both N- and C-terminal fusions with the DBD and AD. Interestingly, Uetz et al. discovered three times more interactions than would have been found using only C-terminal fusions, due to increased accessibility of N-terminal interaction domains in the N-terminal fusion constructs. This result shows the intrinsic capacity of the system to find a substantially larger number of PPIs by partially overcoming the steric hindrance problem. In conclusion, the combined use of different setups should extensively enlarge the interactome subspace detectable by two-hybrid methods.

Screening procedures. In parallel with the technical evolution of the system, several strategies have been developed and optimized for screening experiments (Table 3). In accordance with the specific desire and availability, genomic DNA (gDNA), cDNA, normalized cDNA, full-length cDNA, or open reading frame (ORF) libraries may form the option of choice. Originally, a screening experiment involved the sequential or simultaneous transformation of a two-hybrid strain with the bait plasmid and a prey library. An alternative strategy consists of the construction of mating type **a** and α two-hybrid strains with the bait and prey plasmids, respectively (37, 180, 197). The screening step is performed by mating of both strains on medium selective for an interaction. The possibility to recycle these mating type-specific bait and prey strains provides a strong advantage over the classic transformation approach, especially for high-throughput screening experiments. Finally, prey plasmids can be pooled in a library or tested individually for one-to-one interactions; the latter is called the array or matrix approach. By separating prey ORFs, the finding of positive clones automatically leads to the identification of the interacting protein without the need for sequencing. In addition, prey constructs that activate reporter genes independently from the nature of the bait are easily discarded when screenings with several baits are performed. While it is difficult to estimate the coverage of an experiment using libraries, the coverage of the matrix approach is much more controlled. Several studies assayed both ORF library and ORF matrix screens and showed that the proportional output of interactions is higher and the number of false-positive results is lower with the matrix strategy (120, 650, 682). However, one-to-one characterization of PPIs in genome-scale two-hybrid screenings is cumbersome, and therefore small prey pool strategies are still the common way to screen

in these cases (e.g., see references 294, 494, and 721), or one-to-one experiments are completed after a first selection round with pools (734). In only one study, for which a PCA technology was used, 15 million mating experiments were performed with individual bait and prey strains (633). In an alternative approach, prey plasmids are grouped in unique combinations of pools (smart pools) that allow for the fast identification of the interacting prey protein (310, 311). This technique takes advantage of an inventive pooling-deconvolution procedure. 2^N preys are grouped in $2 \times N$ unique pools, with each prey protein represented N times. For example, with 8 prey proteins, A to H ($N = 3$), we have 6 (2×3) unique pools (pool 1, A/B/C/D; pool 2, E/F/G/H; pool 3, A/B/E/F; pool 4, C/D/G/H; pool 5, A/C/E/G; and pool 6, B/D/F/H), with each protein present 3 times. If pools 1, 3, and 5 lead to growth on selective medium, the distinctive conclusion is that prey A interacts with the bait. This strategy relies on the small number of positive clones to be expected. The simple deconvolution step to fish out the protein responsible for the interaction simplifies the postscreening protocol. The relative number of pools to be screened decreases exponentially with the number of prey proteins, from 4 pools for 4 proteins to 10 pools for 32 proteins and 14 pools for 128 proteins. Moreover, with increasing size, each protein is represented more frequently. In conclusion, smart-pool arrays offer an elegant method to screen for PPIs. Finally, bait libraries can also be pooled after removal of autoactivating bait constructs (e.g., see references 30 and 294).

Protocols. Several reviews and method papers report in detail the available strains and plasmids (91, 298, 656) and provide experimental procedures (124, 198, 364, 403, 422, 423, 530, 577). Companies that provide kits for two-hybrid experiments include Dualsystems Biotech (DUALhybrid kit), Invitrogen (ProQuest), Clontech (Matchmaker Gold), Agilent Technologies (HybriZAP), and Promega (CheckMate). Two-hybrid services are available from Hybrigenics and Dualsystems Biotech. For large-scale (multiple-bait) screening, a new strategy was proposed for the identification of bait-prey pairs by *en masse* next-generation sequencing after PCR stitching (fusing) of the associated bait and prey genes, for significant cost reduction of the analysis step (723).

Application of the Yeast Two-Hybrid System on a Small Scale

The yeast two-hybrid system has established a prominent position in cell biological research and led to the confirmation and discovery of thousands of PPIs. The method complements affinity purification, particularly tandem affinity purification, for the unbiased detection of new protein associations. It has played a crucial role as the starting point of very diverse studies, such as the analysis of light responses and abscisic acid (ABA) signaling in plants (407, 491), the mechanism of protein degradation in the endoplasmic reticulum (654), the molecular basis of limb regeneration in adult vertebrates (366), the interactome topology between herpesviral and human proteins (649), the impact of a bacterial scaffold on human endomembrane trafficking (572), and the regulation of eye development (125). In these examples, a defined bait protein was screened for interactions with a complementary or genomic DNA prey library, leading to the discovery of new PPIs, followed by functional analysis of the newly identified proteins. This second step is crucial as part of the validation process to increase the reliability of two-hybrid data.

Interaction dynamics. One major future challenge in the study of PPIs is the characterization of their dynamic features. The two-hybrid system in most cases is appropriate to answer only the question of whether two proteins can associate, but it does not provide details on when or in which circumstances the interaction happens. A yeast two-hybrid experiment brings the proteins of interest into a rather unnatural situation, because they are directed to the nucleus (forced colocalization), their corresponding genes are not expressed under the control of their own promoters (forced coexpression), and, with nonyeast proteins, the whole cellular environment differs from the native context. As a result, external influences caused by gene deletions, nutrient sources, or stress conditions may not affect the two-hybrid interaction status due to the absence of mediating factors (e.g., signaling molecules). Therefore, context-dependent interaction studies are ideally performed with the organism from which the proteins of interest originate, in the subcellular compartment in which the proteins of interest naturally reside, and with expression of the proteins under the control of their own promoters. Following this rule, only yeast nuclear protein associations can be investigated for context-specific interaction dynamics by use of the yeast two-hybrid system. For nonnuclear yeast proteins, alternative genetic PPI methods are available (see Alternative Yeast Two-Hybrid Systems and Protein Fragment Complementation Assays in Yeast), and for nonyeast proteins, a large group of interaction technologies has been developed in other host organisms (see Genetic Protein-Protein Interaction Methods in Other Organisms).

Several examples exist where the two-hybrid system was applied for context-dependent PPI studies. Interaction between two catalytic and two regulatory subunits of protein kinase A in *S. cerevisiae* is stimulated by the kelch repeat proteins Krh1 and Krh2. In the absence of Krh1 and Krh2, the formation of the tetrameric protein kinase A complex is partially inhibited, as shown by a yeast two-hybrid experiment (506). Nutrient sources also affect many protein associations in *S. cerevisiae*. Formation of the Snf1 protein kinase complex is repressed by glucose, as illustrated in a two-hybrid assay where interactions between subunits of the complex were detected only in media containing alternative carbon sources (305). Taking advantage of the two-hybrid system, it was demonstrated that the transcription factor Rgt1 binds hexokinase 2 (Hxk2) only at high glucose concentrations and binds Med8, a subunit of the RNA polymerase (RNAP) II mediator complex, only at low glucose concentrations (487). An example of stress-dependent interactions is the association of the Hsp90 chaperone with the mitogen-activated protein (MAP) kinase Slt2 under high-temperature conditions or after addition of caffeine (449).

Genetic modifications. In some PPI studies, the two-hybrid system required specific modifications to generate accurate data. For example, the essential *S. cerevisiae* F-box protein Cdc4 binds Sic1, an inhibitor of cell cycle proteins, and stimulates its degradation (175). However, direct association in a wild-type strain was difficult to show because of the fast degradation of Sic1 induced by Cdc4. Therefore, a temperature-sensitive *cdc4-1* yeast two-hybrid strain was constructed to prove the interaction between Sic1 and catalytically inactive Cdc4 (348). The two-hybrid system is generally considered to detect binary and direct interactions, a notion which may be true for many nonnuclear and nonyeast PPIs. However, to establish a clear direct interaction network of autophagy-related (Atg) proteins in *S. cerevisiae*, a two-hybrid strain with deletions of 24 *ATG* genes was created, and multiple Atg PPIs were

confirmed, showing a direct physical interaction independent of the presence of other Atg proteins (75). Finally, deletion of endogenous genes can reduce competitive binding for bait or prey proteins (670).

Application of the Yeast Two-Hybrid System on a Large Scale

Although PPI technologies such as surface plasmon resonance, FRET, phage display, and protein microarrays have been applied to some extent for large-scale experiments (71, 174, 327, 522, 719, 738), affinity purification followed by mass spectrometry (MS) (14, 61, 69, 170, 200, 201, 261, 365), PCAs (447, 633), and the two-hybrid system are most commonly used, to date, for high-throughput interactome analysis.

Interactome studies. The first *S. cerevisiae* two-hybrid screening on a large scale was carried out by Fields, Rothberg, and colleagues in 2000 (650). They conducted both matrix and library high-throughput experiments. In the matrix approach, 192 individual bait strains were mated each time with 1 of 6,000 ORF prey strains, identifying 281 protein associations that were found in two parallel experiments (20% of the total PPIs discovered). Alternatively, 5,300 ORF bait strains were screened using a pooled prey library, leading to the identification of 691 interactions. A second large-scale assay (294) was performed by making approximately 6,000 ORF bait and prey strains. Four hundred mating reactions were carried out, each time with 96 bait strains against 96 prey strains, revealing 4,549 PPIs. Positive results that were found three times were grouped into a core collection of 841 interactions. Surprisingly, of this core set, only 141 PPIs had been identified in the first study (650). In the most exhaustive screening to date, by Vidal and colleagues (721), 3,917 nonautoactivating bait strains were individually mated with 5,246 prey strains (merged in 94 pools), uncovering 1,809 PPIs, of which 274 interactions were found in the two previous high-throughput experiments. The low level of overlap between the three data sets can be explained by false-positive records (i.e., the precision of the method) and by false-negative results, with the latter dependent on the screening completeness (the fraction of the total number of ORF pairs tested to the total possible number of ORF pairs of the organism under study), the assay sensitivity (the fraction of interactions that can possibly be identified by the assay), and the sampling sensitivity (the fraction of all identifiable interactions in a single trial of an assay) (493, 662, 663, 721). The yeast two-hybrid system has also been applied in large-scale research to investigate intraviral (19, 30, 182, 184, 231, 246, 437, 488, 513, 530, 555, 616, 641, 728) and pathogen-host (120, 155, 187, 339, 717) interactions. Moreover, it has also been employed for interactome mapping of *Campylobacter jejuni* (494), *Helicobacter pylori* (529), *Synechocystis* sp. PCC6803 (562), *Bacillus subtilis* (431, 472), *Plasmodium falciparum* (369), *Arabidopsis thaliana* (59, 235), *Drosophila melanogaster* (183, 208, 612), *Caenorhabditis elegans* (55, 116, 389, 538, 601, 681, 682), and *Homo sapiens* (7, 105, 210, 381, 397, 428, 467, 551, 617, 723).

Analysis of high-throughput data. Cost reduction and technological improvements allowed for high-throughput two-hybrid screenings but shifted the limiting step toward the confirmation and validation of interaction data. Small-scale two-hybrid results can be verified by alternative interaction techniques, such as glutathione *S*-transferase (GST) pulldown assay or coimmunoprecipitation, or by the proof of a functional correlation. Although full experimental validation of a medium-scale two-hybrid assay has been reported (349), this type of verification for genomewide PPI studies remains limited to a subset of positive results (389, 721). On the other hand, many computational studies have evaluated the false-positive and false-negative rates of two-hybrid results by using random and positive reference sets, respectively. Specifically, for two-hybrid studies, a positive reference set of binary interactions has been proposed to accurately validate high-throughput data (721). Estimates go from 24% to 51% for the false discovery rate (119, 278) and from 45% to 96% for the false-negative rate (159). In general, two proteins are more likely to be true interactors if they tend to share common features, including coexpression (119, 223, 301, 333, 640), colocalization (301), functional correlation (425, 497), and shared interaction partners (11, 212, 346), and have homologous proteins that bind each other (119, 186, 405, 436, 533, 722). However, coexpression and colocalization analyses for interaction validation, supporting cocomplex analysis and biased small-scale experiments, have been criticized because many true interactors show an anticorrelation with expression (463, 663). Comparison of interaction data for validation across different species has been criticized as well (340, 445, 586). Even a functional correlation is not a necessary prerequisite, as many interactions may truly appear in the cell without a functional context (384). At present, there is no clear consensus on the strategies to accurately validate interaction results, but the ever-increasing availability of PPI data by a variety of experimental tools will support the accuracy of computational validation, which in turn will allow for precise predictions of true interactions.

Computational analysis of PPI data further revealed several biases toward different protein properties. Nuclear, conserved, essential, weakly autoactivating, and structurally disordered proteins are overrepresented in two-hybrid data, but no biases toward protein function were found (44, 93, 676, 724). For high-throughput affinity purification assays, detection of PPIs is skewed toward highly abundant proteins due to the use of native promoters and of proteins associated with specific cellular functions involving protein complexes, such as transcription and protein synthesis (44, 93, 295, 676, 724).

High-throughput PPI data have assisted in the functional characterization of proteins (4, 23, 260, 333, 383, 540, 583), the analysis of interaction network topologies (8, 121, 185, 303, 399, 496, 527, 680, 685, 743; reviewed in references 24, 532, and 568), and the computational prediction of interactions and interactomes (66, 154, 164, 226, 249, 280, 332, 377, 404, 438, 469, 571, 604, 688, 703, 733), protein localization (591), interacting domains (227, 409, 609) and interactome sizes (224, 241, 301, 559, 622, 663, 676, 721).

Limitations of the Yeast Two-Hybrid System

The two-hybrid system suffers from three major drawbacks: (i) it produces a significant number of false-positive results, (ii) it can detect only a subset of the complete interactome, and (iii) it can provide only very limited information on the kinetics or dynamics of a PPI.

False-positive results. In screening for interacting partners by use of a library, a relatively large number of false-positive results is frequently observed. Sometimes, interacting proteins are detected that are not present in the same subcellular location or time under natural conditions. These proteins might indeed be able to interact, but the interactions have no biological relevance (biological

artifacts). Other examples of false-positive results are proteins that overcome nutritional selection, proteins that bind and activate the reporter gene directly, "sticky" or incorrectly folded proteins that nonspecifically bind many baits, plasmid rearrangements or copy number changes that generate autoactivators, or alterations at one of the reporter genes that result in constitutive expression (technical artifacts). However, several approaches exist that deal with these spurious results during screening (251, 579, 669), including the application of two-bait systems (see "Two-Bait Hybrid Systems"). Although initial analyses of high-throughput two-hybrid data suggested large proportions of false-positive results (223, 300, 463), recent examinations indicate an overestimation of the false discovery rates due to misevaluation of the data (663, 721). It is clear now that different technologies, such as the two-hybrid system and tandem affinity purification, lead to detection of different types of PPIs (112, 213, 302) and therefore that each data set should be analyzed by specific appropriate validation methods (663, 721). Nevertheless, spurious two-hybrid results remain a considerable drawback, and only more experimental in-depth analysis can provide conclusive confirmation of a true biologically relevant interaction. Alternative two-hybrid and PCA technologies can play a central role in this data verification.

A particular case of a false-positive result is autoactivation by the bait protein, i.e., the bait by itself can induce expression of the reporter genes independently of the prey. This issue can be solved by removing the domain that induces autoactivation or by increasing 3-aminotriazole (3-AT) levels when using *HIS3* as a selection marker. 3-AT is a competitive inhibitor of the His3 enzyme, and addition of this compound elevates the required His3 levels for survival on histidine-deficient medium. Alternative two-hybrid technologies can also be used to address this problem (see "Nuclear Two-Hybrid Systems for Autoactivating Bait Proteins").

Detection of only a subset of the interactome. Due to the necessity of the bait and prey proteins to enter the nucleus, a number of interacting protein pairs are not able to induce reporter gene expression. Extracellular proteins, membrane proteins, and generally all proteins with a strong localization signal will often not move to the nucleus, despite their fusion with a nuclear localization sequence (NLS). Moreover, in most cases, membrane proteins need the phospholipid bilayer to fold in the right conformation, and therefore an interaction may not be observed. To overcome the problem of mislocalization, it can be necessary to use a truncated version of the protein. As an example, the *S. cerevisiae* G-protein-coupled receptor (GPCR) Gpr1 is a membrane protein, but the cytoplasmic C-terminal region was shown to interact with the Gα protein Gpa2 in a yeast two-hybrid experiment (363, 725). Alternatively, PCAs or modified two-hybrid systems may be used to detect membrane PPIs (see "Membrane-Localized and Secretory Pathway Two-Hybrid Systems" and Protein Fragment Complementation Assays in Yeast). Occasionally, proteins might be present in the nuclear environment but unable to interact, such as proteins of the secretory compartments that require oxidative conditions or glycosylation for proper folding (354). Another cause of false-negative interactions results from bait or prey proteins that are toxic to the cell when overexpressed. This problem can be circumvented by the use of inducible promoters, such as in the LexA-based system, where prey genes are expressed under the control of the inducible *GAL1* promoter. The use of chimeras has also been criticized because the addition of fusion constructs to the protein of interest could obstruct the interaction. However, the addition of flexible glycine linkers to stimulate independent folding of the different components of the fusion protein partly deals with this problem. As mentioned above, N-terminal fusion of the protein of interest to the AD or DBD can also improve the outcome (e.g., see references 521 and 616). Several PPIs, particularly those of higher eukaryotes, might not be detected in *S. cerevisiae* when the machinery for a specific posttranslational modification is lacking in yeast cells. Coexpression of the modifying enzyme in the heterologous host system can solve this issue, or the interaction analysis can be performed *in vivo* in other organisms (see "Three-Hybrid Systems" and Genetic Protein-Protein Interaction Methods in Other Organisms, respectively).

Limited information on the kinetics or dynamics of a PPI. The artificial environment of a two-hybrid experiment, with forced coexpression and nuclear localization of chimeric proteins, limits the application of the system to semiquantification of binding affinities (169). Furthermore, the length of an experiment, on a multiple-day scale for prototrophic reporter activation, does not allow the detection of fast changes in interaction affinity induced by external factors. Finally, the native location of the protein association under study cannot be analyzed by two-hybrid experiments. PCAs such as the split-luciferase method and the split-FP method have been shown to be highly versatile systems for research on dynamic PPIs (see Protein Fragment Complementation Assays in Yeast and Genetic Protein-Protein Interaction Methods in Other Organisms).

Despite these drawbacks, the track record of the two-hybrid system proves how efficient this method has been for discovering many new PPIs from a large number of organisms due to a number of advantageous properties. The yeast two-hybrid system provides a technique to investigate interactions in the environment of a eukaryotic cell and with the easy handling characteristics of *S. cerevisiae*. In contrast to the case for affinity purification methods, transient and weak associations can be detected due to signal amplification provided by reporter gene expression. The lack of a cumbersome purification step also adds to the efficiency of the system. The method allows the identification of new binding partners of a protein of interest, which plays an important role in the functional analysis of uncharacterized proteins. The convenience of working with *S. cerevisiae* makes it possible to screen for drug compounds that disrupt interactions or to screen for mutated versions of a protein that lose the ability to associate with binding partners (see "Reverse Two-Hybrid Systems"). Finally, the concept of the method permits the development of many alternative technologies.

ALTERNATIVE YEAST TWO-HYBRID SYSTEMS

The basic concept of functional reconstitution of a transcription factor has been used as the blueprint for several alternative technologies, many of which deal with the limitations of the original system. These methods are discussed here.

Nuclear Two-Hybrid Systems for Autoactivating Bait Proteins

RNA Pol III system. If the bait protein is able to autoactivate transcription by recruiting the traditional RNA polymerase II, an alternative option is to use the RNA polymerase III (RNA Pol III) system (433). RNA Pol III transcribes genes that encode untranslated RNA molecules such as rRNA, tRNA, and other small RNAs. Most of the genes controlled by RNA Pol III do not contain up-

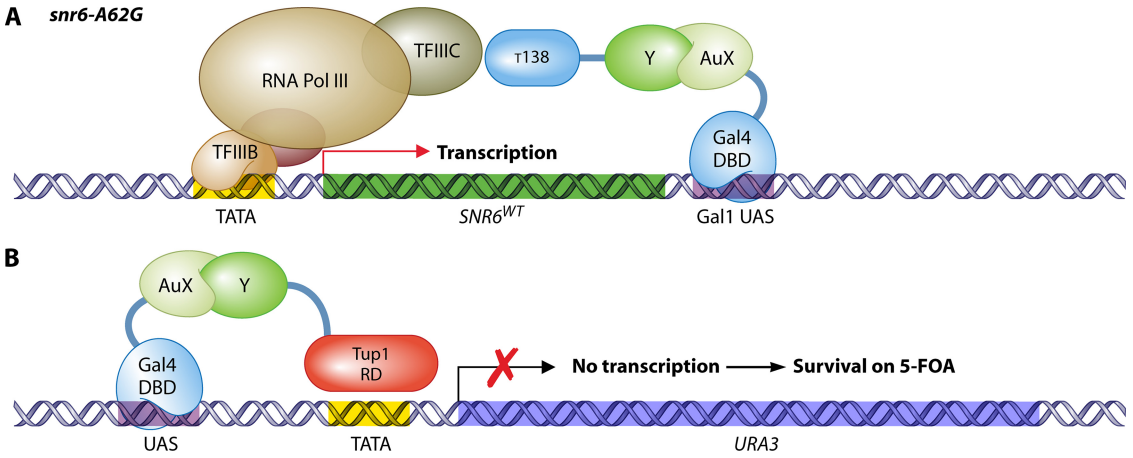

FIG 4 Nuclear two-hybrid systems for autoactivating bait proteins. (A) RNA Pol III system (511). Through its fusion with the Gal4 DBD, an autoactivating bait (AuX) protein is tethered to the Gal1 upstream activating sequence, located downstream of the reporter gene *SNR6*. Interaction of the AuX bait with a prey protein (Y) fused to the τ138 subunit of transcription factor III C (TFIIIC) brings the RNA polymerase III holoenzyme to *SNR6*. Transcription of *SNR6* leads to survival under temperature-restrictive (inactive snR6-A62G) conditions. (B) Repressed transactivator system (259). In the repressed transactivator (RTA) system, association of an autoactivating AuX bait with the prey protein Y, attached to the repressor Tup1, inhibits transcription of the reporter gene *URA3*. This results in survival of the cell on 5-FOA, a substrate for the production of the toxic compound 5-fluorouracil by the gene product of *URA3*. Pol, polymerase; TF, transcription factor; Y, prey; AuX, autoactivating bait; AD, activation domain; DBD, DNA-binding domain; RD, repressor domain; UAS, upstream activating sequence.

stream activating sequences or a TATA box but rather have an intragenic regulation. However, the essential gene *SNR6*, which encodes U6 snRNA, is an exception to the rule and is positioned between a TATA box and a downstream sequence to which subunit τ138 of the Pol III-specific transcription factor TFIIIC (τ) binds. In the RNA Pol III system, this downstream sequence is replaced by Gal4 activating sequences and *SNR6* expression depends on the binding of a bait protein, in fusion with the Gal4 DBD, with the prey protein, attached to the τ138 subunit (Fig. 4A). The possibility for selection is acquired by using a temperature-sensitive mutant of *SNR6* under the control of its own promoter, with a wild-type version as a reporter gene (511). This strategy was later used to discover the interaction between the *A. thaliana* transcriptional regulators FIL and NZZ (597).

RTA system. Another two-hybrid system for autoactivating bait proteins is the repressed transactivator (RTA) system (259). In this method, the transcriptional repressor domain of Tup1 replaces the AD in the prey (Fig. 4B). Upon interaction of the autoactivating bait with the prey, the reporter genes *URA3* and *HIS3* are repressed, leading to survival on medium containing 5-fluoroorotic acid (5-FOA), which is toxic in the presence of Ura3-mediated uracil synthesis, and to a growth deficiency on medium without histidine. The RTA method has been applied to screen for interactors of the transcriptional activators VP16 (259), androgen receptor (536, 636, 679), c-Myc (259, 276, 516), and microphthalmia-associated transcription factor Mitf (569). Alternatively, the setup of the RTA method allows for positive selection of interaction disruption by growth on selective medium without histidine or uracil. Using an adaptation of the RTA system, specific and nonspecific PPI inhibitors of four well-established protein pairs were identified by screening a compound library (313). This screening experiment required fine-tuning of the procedure with 3-AT and introduction of Leu3 binding sites for moderate basal expression of *HIS3*.

Alternative strategies. An autoactivating bait protein can also be attached to the AD instead of the DBD, followed by screening with a library in fusion with the DBD (145). The reliance of the DBD fusion protein (prey) on the presence of the bait for reporter activation is examined by using the counterselectable marker *CYH2* on the bait plasmid (152). Colonies sensitive to cycloheximide on selective medium, resulting from *CYH2* expression, contain prey proteins that are interacting with the bait, without autoactivation. Despite the creative approaches to establish specific two-hybrid systems that deal with *trans*-activating bait proteins, the most prevalent strategy remains the removal of the region within the bait protein that initiates transcription in order to use the classic two-hybrid method (e.g., see references 287, 519, and 552) or a one-hybrid system with the promoter target of the transcription factor of interest in front of the reporter genes (229, 598–600). Finally, protein complementation assays (see Protein Fragment Complementation Assays in Yeast) and membrane-localized assays (see "Membrane-Localized and Secretory Pathway Two-Hybrid Systems") can be applied for the detection of interactions involving autoactivating proteins.

Membrane-Localized and Secretory Pathway Two-Hybrid Systems

Small-G-protein-based methods. *S. cerevisiae* needs a functional Ras signaling pathway in order to survive and proliferate. Thus, deletion of Cdc25, the GTP-GDP exchange factor and activator of the small membrane-bound GTPases Ras2 and Ras1, makes the cells inviable (65). Based upon this notion, a strain was constructed with a temperature-sensitive mutant of Cdc25, *cdc25-2* (16). In the Sos recruitment system, the human Cdc25 homologue Sos (hSos) is fused with the bait protein of interest and the prey protein is fused with a membrane localization signal. When the bait and prey interact within a *cdc25-2* background strain, hSos is recruited to the membrane, where it can activate the Ras proteins, resulting in cell survival. The method was further improved by introduction of a mammalian GTPase activating protein to lower

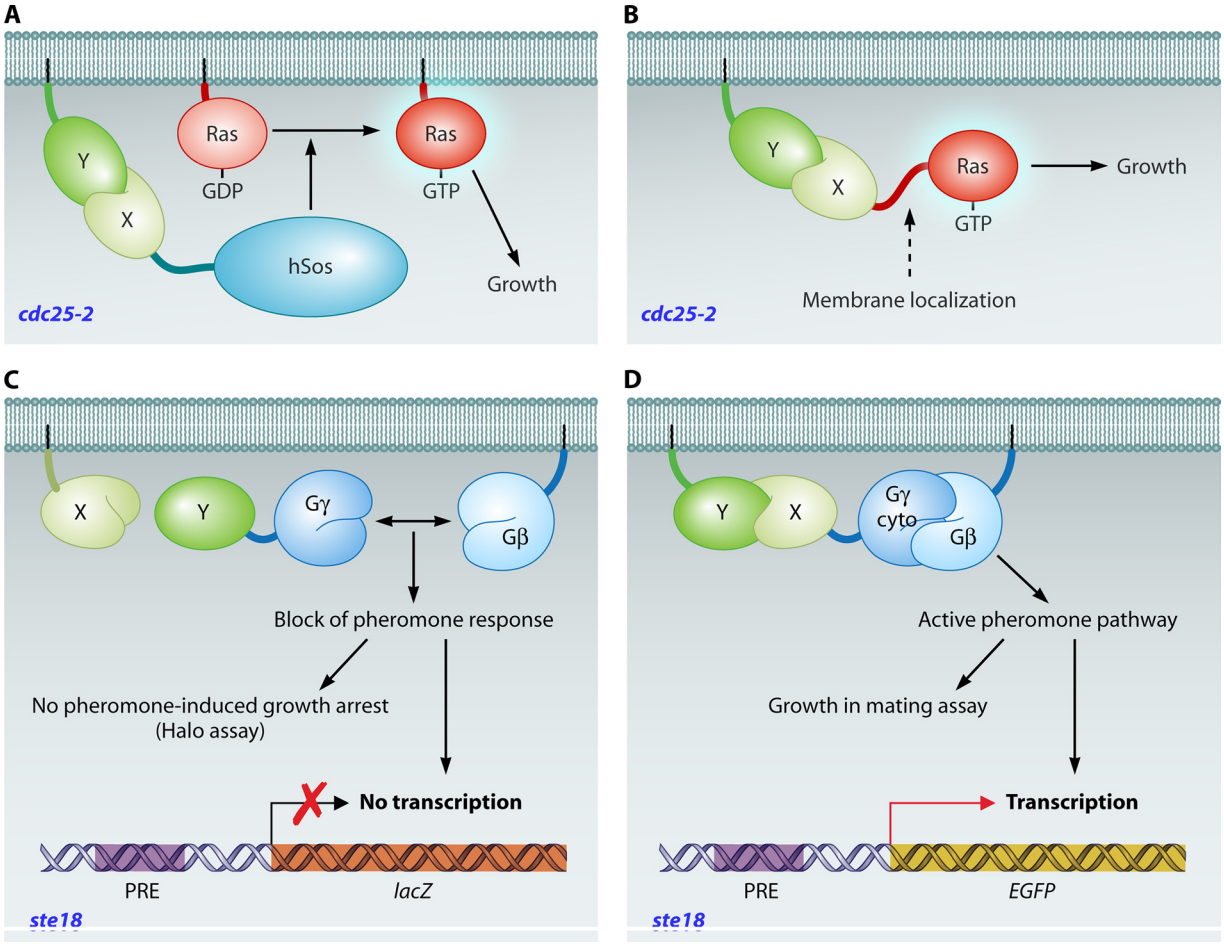

FIG 5 G-protein-based two-hybrid systems. (A) Sos recruitment system (16). A chimeric protein of bait X with hSos is recruited to the plasma membrane upon interaction of X with a membrane protein (Y). Subsequent GDP-GTP exchange of Ras by membrane-localized hSos enables cell growth by virtue of Ras activity in a temperature-sensitive *cdc25-2* background strain. (B) Ras recruitment system (64). Similar to the case in the Sos recruitment system, a membrane-bound prey protein Y that interacts with bait X, fused to mammalian Ras (mRas), will bring mRas to the membrane, where it can activate its downstream target adenylate cyclase to establish cell growth at a restrictive temperature (36°C) in a *cdc25-2* background strain. (C) G protein fusion system (162). Association of the γ (Ste18) and β pheromone G protein subunits is required for a response to the addition of pheromones. Strong interaction of a membrane bait protein (X) with the prey protein Y, in fusion with Ste18 (Gγ), pulls Gγ away from Gβ. This dissociation hinders the pheromone response in a *ste18* strain, which can be detected by growth in the presence of α pheromone (halo assay) or by the lack of gene expression of reporters under the control of pheromone-responsive elements (PREs). (D) Gγ recruitment system (190). Interaction between a membrane-bound prey protein (Y) and a bait protein (X), fused to Gγ lacking a membrane attachment sequence, unites the G protein β and γ subunits to induce the pheromone pathway after addition of α pheromone. Readouts are reporter genes regulated by PREs or the observation of growth in a mating assay with a *ste18* strain.

the background activity of false-positive Ras prey proteins, specifically when using mammalian cDNA libraries (15). This Sos recruitment system (Fig. 5A) provides an interesting assay for interactions when proteins are unable to enter the nucleus or when posttranslational modifications in the cytoplasmic milieu are required. In addition, the method has been applied for the discovery of inhibitors of HIV-1 Gag dimerization (652), the analysis of protein membrane localization, and the determination of numbers of transmembrane domains (297, 565). An improved version was created by using mammalian activated Ras (mRas) instead of Sos in the *cdc25-2* strain (64). In this Ras recruitment system (Fig. 5B), mRas is used in the bait construct after removal of the CAAX C-terminal peptide responsible for membrane attachment. When a membrane-bound prey protein binds the bait, the constitutively active mRas protein is tethered to the membrane, where it can activate adenylate cyclase, the first target of the essential Ras signaling pathway. The small size of mRas reduces the steric hindrance problem observed with the large hSos protein. Moreover, false-positive Ras proteins are not selected by this method. Both Sos and Ras recruitment systems allow the detection of interactions between soluble bait proteins and soluble or membrane-bound prey proteins. To enable the use of membrane proteins as bait, the reverse Ras recruitment system was introduced, in which mRas is fused to the prey rather than the bait (283). This method typically gives a very large number of false-positive results, as any membrane-bound prey protein brings mRas in close proximity to adenylate cyclase. To circumvent this problem, the bait gene is put under the control of an inducible promoter to investigate the bait dependence of Ras activity and to discard all noninteracting membrane proteins in a prescreening step (283). Small-G-protein-based methods suffer from technical constraints, such as the occurrence of

temperature revertants (102), growth at suboptimal temperatures, and the obligatory replica plating step (281), but they nevertheless remain popular and very successful alternatives to the original two-hybrid system (e.g., see references 147, 157, 256, 331, 455, 647, and 692). The Ras recruitment system was further developed for use in mammalian cells (432).

Trimeric-G-protein-based methods. Pheromone treatment in yeast cells activates a trimeric αβγ G protein complex, and association of the β (Ste4) and γ (Ste18) subunits is required for signal transduction. In the G protein fusion system, the bait is a membrane protein and the prey is fused to Ste18 (162) (Fig. 5C). When the prey strongly associates with the bait, Ste18 loses its interaction with Ste4, thereby blocking the pheromone signaling pathway in an *ste18*Δ strain. In case of an interaction, cells in the pheromone-dependent growth inhibition assay (halo assay) retain growth in the presence of pheromone and display reduced expression of a pheromone-controlled *lacZ* gene. The fact that only one of the two proteins is a hybrid has been suggested to be a major advantage of this technique, but its limitation is found in the large background signals it produces when screening for interactions. A recent, improved interaction tool combines the advantages of the G protein fusion system (growth at 30°C) with the benefits of the Sos recruitment system (higher sensitivity) (190) (Fig. 5D). This Gγ recruitment system makes use of a cytosolic variant of the Gγ subunit (Ste18cyto) fused to a soluble bait protein of interest. The prey protein is attached to the membrane, intrinsically or artificially by addition of a lipidation site. Interaction of bait with prey leads to membrane localization of Ste18cyto and subsequent activation of the pheromone pathway, which can be detected by fluorescence through expression of *EGFP* under the control of a pheromone-responsive *FIG1* promoter (190) or by a mating assay using selective markers exclusively present in either of the haploid strains (191). Increased selectivity of the system is provided by the introduction of an interaction competitor protein (191), and increased sensitivity is given by the integration of *STE18* under the control of the pheromone response, leading to feedback signal amplification (189).

Secretory pathway two-hybrid systems. Extracellular proteins or proteins that are naturally found within the lumina of secretory pathway organelles depend for correct folding on distinctive features of these compartments, such as glycosylation, calcium concentration, and oxidizing conditions for disulfide bond formation. To study PPIs among such proteins in their native environment, several two-hybrid systems were developed in different subcompartments of the secretory pathway, ranging from the endoplasmic reticulum to the extracellular space (Fig. 6A).

The membrane-bound receptor Ire1 senses stress caused by accumulation of unfolded proteins within the endoplasmic reticulum (109). Activation of Ire1 is followed by homodimerization, *trans*-phosphorylation, and correct splicing of Hac1 mRNA, encoding a transcriptional activator of genes involved in the unfolded protein stress response (328). Two mutant versions of Ire1, Ire1K702R and Ire1Δtail, lack the kinase and Hac1-activating regions of the wild-type protein, respectively (460, 582). Upon dimerization of Ire1K702R with Ire1Δtail, Ire1Δtail can phosphorylate and activate Ire1K702R, which in turn initiates Hac1 mRNA splicing for correct Hac1 translation. In the two-hybrid variant called SCINEX-P (screening for interactions between extracellular proteins) (Fig. 6B), the bait and prey proteins of interest are N-terminally fused to Ire1K702R and Ire1Δtail, respectively (653). Interaction between the bait and prey proteins, located within the endoplasmic reticulum, leads to dimerization of both mutants of Ire1, correct translation of Hac1, and expression of the reporter genes *lacZ* and *HIS3*, both under the control of a Hac1-regulated promoter. Deletion of *IRE1* and *DER1*, involved in misfolded protein degradation, causes viability of the two-hybrid strain to depend on Hac1 activity through interaction-induced Ire1 dimerization at elevated temperatures and in the absence of inositol, providing a wide range of selection procedures for increased stringency. In the original study (653), the system was applied to confirm interactions between Gcn4 and anti-Gcn4 antibodies and between the leucine zipper domains of c-Jun and c-Fos.

The recently developed Golgi complex two-hybrid system (Fig. 6C) is based on the complementation of the Golgi complex-resident mannosyltransferase Och1 (146). Like many Golgi complex-based enzymes, Och1 consists of two modular domains: the N-terminal LOC domain for membrane attachment and the C-terminal CAT domain, which performs the mannose transfer reaction within the Golgi complex lumen, an essential reaction for the production of large-chain cell wall mannans. Deletion of *OCH1* results in increased cell binding of chitin-binding reagents, such as wheat germ agglutinin, and in strongly reduced growth at a non-permissive temperature (37°C) or in the presence of the benzidine-type dye Congo red (146). Fusion of the two modular fragments of Och1 to the human transcription factor MyoD and the inhibitor of differentiation protein 2 (Id2) reverses all of the *och1* phenotypes through the reassembly of Och1 upon MyoD-Id2 interaction. In addition, an interaction between the transcriptional activator Gal4 and five binding partners was confirmed with the Golgi complex two-hybrid system, suggesting this method to be an alternative tool for the study of interactions involving transcription-activating or extracellular proteins.

The yeast surface two-hybrid system (Fig. 6D), which is based on yeast surface display (49), detects interactions that take place outside the cell. In one version of the method (153), the interaction of two fragments of the 10th type III domain of human fibronectin (FNfn10) was shown by first fusing the N-terminal part of FNfn10 with the cell wall agglutinin protein Aga2, which displays the N-terminal fragment on the yeast surface. Next, the C-terminal part of FNfn10, with a V5 epitope tag, was shown to be attached to the surface by immunofluorescence detection through its interaction with the N-terminal fragment of FNfn10. The method enabled quantitative analysis of interactions between mutated FNfn10 fragments (153). A highly similar system was developed for a study on coiled-coil interactions and antigen-antibody recognition (274). In addition to immunofluorescence detection, the appearance of fluorescence upon interaction-induced green fluorescent protein complementation (also see The Split-FP Method) was used to examine protein-protein binding. Both types of readout are suitable for quantitative interpretation (274). This method enabled the quantitative analysis of antigen-antibody binding after initial selection procedures involving directed evolution with yeast surface display and panning through phage display (275). Finally, an independent group created yet another yeast surface two-hybrid system, based upon the same principle, to study the peptide recognition of major histocompatibility complex (MHC) class II proteins (308).

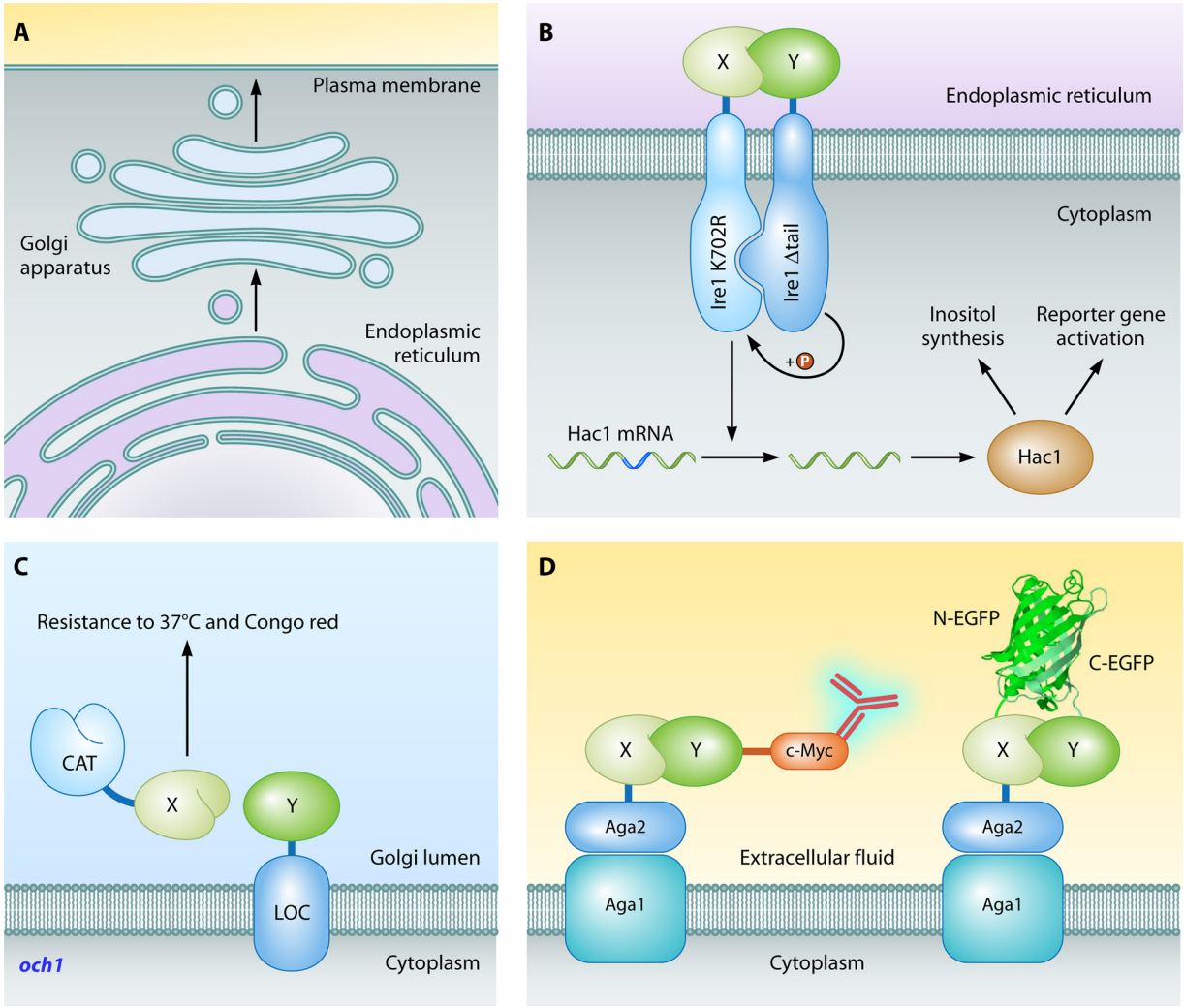

FIG 6 Secretory pathway two-hybrid systems. (A) Secretory pathway. Proteins for secretion are synthesized at the endoplasmic reticulum, transported in vesicles to the Golgi apparatus (optionally for further processing), and secreted into the extracellular fluid by fusion of a secretory vesicle with the plasma membrane. Each compartment hosts one of the available two-hybrid systems. (B) Screening for interactions between extracellular proteins (SCINEX-P) (653). Wild-type Ire1 detects the presence of unfolded proteins in the endoplasmic reticulum. Activation of Ire1 leads to homodimerization, *trans*-phosphorylation, and, finally, splicing of Hac1 mRNA into correctly translatable mRNA. In SCINEX-P, the bait protein X is fused to an Ire1 variant (Ire1 K702R) that can splice Hac1 mRNA only while the prey protein Y is attached to an Ire1 variant (Ire1 Δtail) that can phosphorylate only its associated Ire1 partner. Upon an X-Y interaction within the lumen of the endoplasmic reticulum, Ire1 Δtail phosphorylates Ire1 K702R, which in turn splices Hac1 mRNA, leading to correct translation of Hac1. Hac1 activity can be sensed by detection of inositol synthesis and by activation of reporters regulated by Hac1-dependent promoters. (C) Golgi two-hybrid system (146). The catalytic (CAT) and membrane-attaching (LOC) domains of the mannosyltransferase Och1 are fused to the bait protein X and prey protein Y, respectively. Interaction of the bait with the prey within the Golgi lumen prevents loss (by secretion) of the Och1 catalytic domain, leading to cell survival at 37°C or at 30°C in the presence of Congo red. (D) Yeast surface two-hybrid system (274). The membrane protein Aga1 keeps the Aga2-bait X fusion at the cellular membrane in the extracellular fluid. Binding of the bait with the prey protein Y, tagged with c-Myc, is detected with anti-c-Myc fluorescent antibodies. Alternatively, both bait and prey proteins are linked with fragments of EGFP. The formation of an X-Y heterodimer results in reassembly of the EGFP fragments and in concomitant fluorescence. The EGFP structure image is based on the PDB structure under accession number 2Y0G (550).

Three-Hybrid Systems

Three-hybrid systems rely on the intervention of a third component for PPI detection. This third factor can be a protein involved in posttranslational modification of one of the interacting proteins, or it can directly interfere with the PPI. Moreover, three-hybrid systems have been created to study protein–small-molecule and protein-RNA interactions and to screen for enzymes that cleave or bind specific molecular structures.

Posttranslational modifiers. Many posttranslational modifications present in higher eukaryotes also occur in *S. cerevisiae*. However, some alterations, such as tyrosine phosphorylation, are absent in yeast. These modifications may be crucial for the structural recognition of two interaction domains. For example, Src homology 2 (SH2) domains bind only proteins with a phosphotyrosine residue (504). Introduction of an exogenous tyrosine kinase into the yeast two-hybrid system enabled the identification of numerous interactions, including the discovery of novel interaction partners for the γ subunit of the IgE receptor FCεRI, the tyrosine phosphatase SHPTP2, the human insulin receptor, the *C. elegans* adaptor protein CED-2, and the *Schistosoma mansoni* Tyr kinase

TK4 (36, 70, 329, 362, 481, 482). Not surprisingly, many of these interactions involved SH2 domains. In a variation to this theme, the Ras recruitment system (see "Trimeric-G-protein-based two-hybrid methods") was applied to detect human proteins that interact with the phosphorylated transcription factor c-Jun after the introduction of JNK1 kinase, controlled by the inducible *MET3* promoter (3, 468).

Although acetylation and serine/threonine phosphorylation occur commonly in *S. cerevisiae*, the artificial character of a two-hybrid experiment may prevent posttranslational modifications, for example, if the modifier is not colocalized with its target (bait or prey) protein in the nucleus. The tethered catalysis two-hybrid system deals with this problem by fusion of the posttranslational modifier with the bait protein (230). Fusion of a Gal4 DBD/histone 3 chimera with the histone acetyltransferase Gcn5 resulted in acetylation of histone 3 and the discovery of two binding partners, Rpm2 and Rtm1, that interact with histone 3 only when it is acetylated. Similarly, three tandem repeats of the CTD peptide of the largest RNA polymerase II subunit were fused to the Gal4 DBD and the serine/threonine kinase Kin28, which phosphorylates CTD. Several phosphorylation-dependent interactions were found in a screening assay, mostly with proteins that regulate transcription (230). To identify polyubiquitin-binding proteins, a ternary chimera of Gal4 DBD, the tumor suppressor BRCA1, and the related protein BARD1 was created (711). Autoubiquitination of the BRCA1-BARD1 complex enabled the identification of prey proteins that bind polyubiquitin. The tethered catalysis two-hybrid system has also been adapted to mammalian cells (607).

Trimeric complexes and competitive binding. Three-hybrid systems are further used to examine the reliance of a PPI on a third protein as a bridging molecule. The interaction between the epidermal growth factor receptor (EGFR) as bait and the Sos protein as prey was illustrated to depend on the presence of a third adaptor protein, Grb2 (727). The third gene can be put under the control of an inducible promoter, for example, the *MET25* promoter, repressed by methionine or cysteine, or the tetracycline-inducible promoter, to investigate how crucial the presence of the bridging protein is for the interaction (461, 639). The three-hybrid system has been used frequently to investigate nonyeast ternary complexes (e.g., see references 192, 408, 560, 563, 576, and 584). In some cases, more than one bridging protein may be required (503, 588). Interaction of the tumor suppressor protein pVHL with the cullin family member CUL-2 depends on the pVHL-stabilizing effect of elongins B and C (503). Analysis of the interacting domains by a four-hybrid analysis revealed a structural resemblance of this complex (CBC^{VHL}) with the E3-like ubiquitin ligase complex SKP1/Cullin/F-box protein and later led to the confirmation of the complex for its involvement in ubiquitin-regulated protein degradation (296). Interaction analysis of this E3 ubiquitin ligase CBC^{VHL} complex and its target proteins has been hampered in mammalian cells due to the fast ubiquitination-induced degradation of the targets. This problem was overcome by application of the yeast three-hybrid system, which involves the introduction of pVHL as a bait protein together with elongins B and C to stabilize the native conformation of pVHL, and by additional inclusion of the prolyl hydroxylase PHD3 for hydroxylation of target prey proteins for pVHL recognition (41) (Fig. 7A). As a result, all components are present to identify prey proteins as targets of the CBC^{VHL} complex, and the absence of CUL-2 prevents unwanted induction of target protein degradation. After confirmation of the binding of pVHL with the known targets HIF1 and -2α, a library approach resulted in the identification of eight novel interactors (41).

Alternatively, the third protein may disrupt an interaction (639). In response to blue light, the *A. thaliana* cryptochrome 1 blue-light receptor CRY1 competitively interfered with the association of the E3 ubiquitin ligase COP1 with the phytochrome A suppressor SPA1 in a yeast three-hybrid experiment (391, 406). Because the whole experiment was performed in a heterologous organism (*S. cerevisiae*), both CRY1 activation by blue light and subsequent interaction disruption were shown to be strictly independent of any other *A. thaliana* protein, a general notion for yeast-based interaction experiments with nonyeast proteins. Other reports exploited the three-hybrid system for studies on the interaction-disrupting abilities of a third protein (e.g., see references 76, 361, 385, and 628).

Protein–small-molecule interactions. The three-hybrid system has been adapted to investigate associations that go beyond PPIs (Fig. 7B to D). The bridging molecule is not necessarily a protein. A fusion of the LexA DNA-binding protein with the rat glucocorticoid receptor (LexA-RGR; the "hook") associates with a covalently linked heterodimer of two small molecules (Dex-FK506; the "bait") by binding of protein RGR with the steroid hormone agonist dexamethasone (Dex) (392). Another hybrid protein, consisting of the human protein FKBP12 and the B42 activation domain (FKBP12-B42; the "prey"), interacts with Dex-FK506 through association of FKBP12 with the immunosuppressant FK506 (566). This ultimately leads to the noncovalent and indirect reassembly of the transcription factor LexA-B42, detected by activation of the reporter gene *lacZ* (392). Alternative approaches were developed with substitute small-molecule heterodimers, small-molecule-binding proteins, reporter genes, and DNA-binding and activation domains (22, 160, 195, 253, 285, 401). Initially, the sensitivity and stringency of these three-hybrid approaches were shown by recovering known small-molecule-binding proteins from a prey cDNA library (253, 392), by demonstrating the reduction or absence of interaction with specific mutant proteins (2, 122, 253, 285, 392), and by competitive assays with interfering monomeric small molecules (253, 285, 392, 401). However, the full ability of the method was illustrated by the identification of novel targets of small-molecule kinase inhibitors (35). Cyclin-dependent kinase (CDK) inhibitors were found to bind both known and new CDK and CDK-like proteins in a screening assay that utilized *HIS3* as a selective reporter gene and methotrexate (Mtx), which binds very tightly to a DNA-bound LexA-dihydrofolate reductase (DHFR) fusion protein (2, 35), as a fixed small molecule in the heterodimer.

Recently, a highly optimized version of the three-hybrid system for small-molecule–protein interactions uncovered numerous novel interaction partners for a variety of drugs (94) (Fig. 7B). The LexA DNA-binding domain was fused with human O^6-alkylguanine-DNA alkyltransferase (AGT), which associates covalently with O^6-benzylguanine (BG) (334). The covalent linkage between LexA-AGT and BG significantly increases the sensitivity of the assay (382), in contrast to the noncovalent binding partners in previous setups. BG derivatives were created by fusion of BG with a set of drug compounds. To acquire a sensitive three-hybrid strain, three genes (*PDR5*, *SNQ2*, and *YOR1*) encoding broad-spectrum drug transporters were deleted to prevent efflux of the BG derivative. The three-hybrid strain, with the reporter genes *HIS3*, *lacZ*, and *URA3* and the fusion gene *lexA-AGT*, was trans-

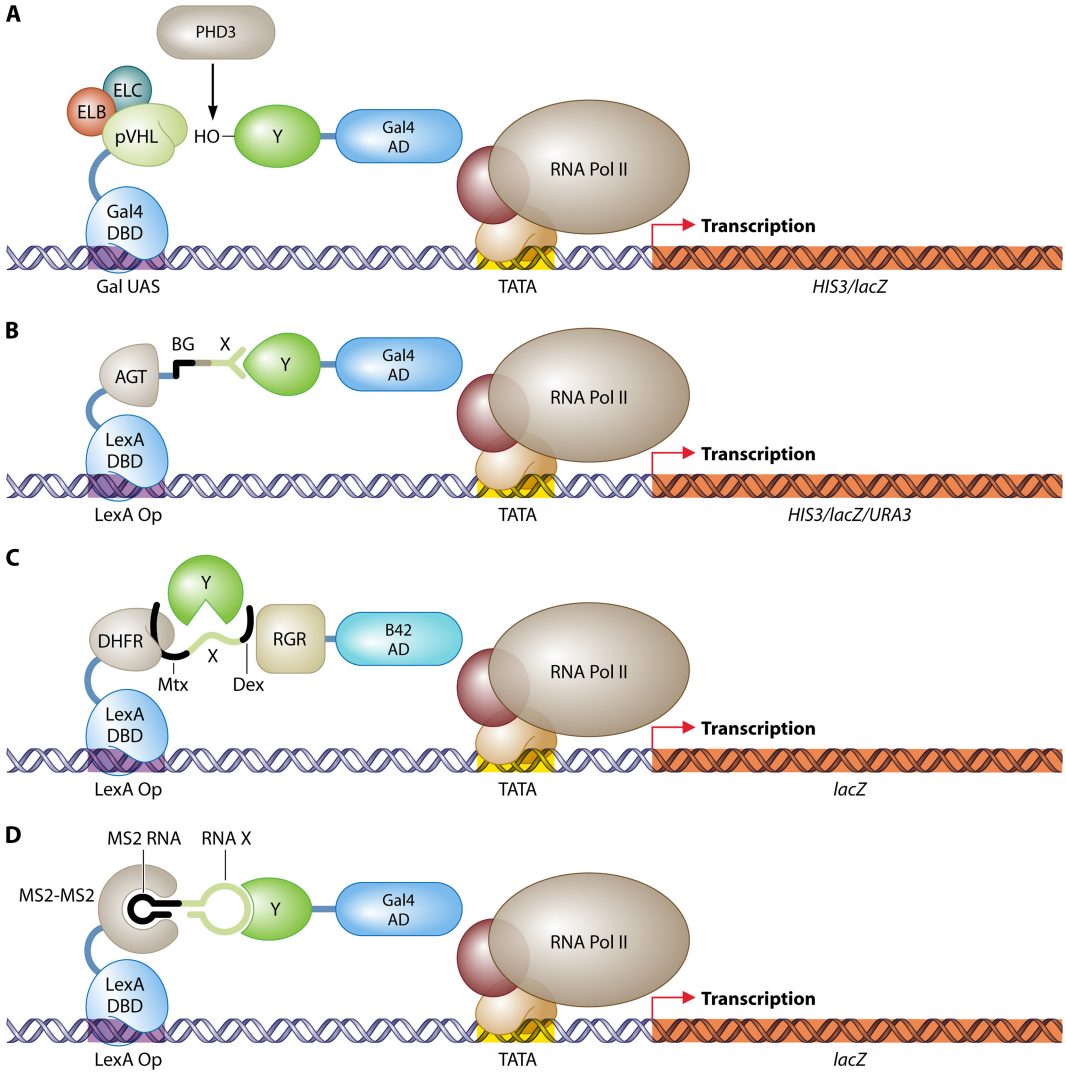

FIG 7 Three-hybrid systems. (A) Identification of target proteins of the E3 ubiquitin ligase complex CBC^{VHL} (41). The pVHL subunit is fused to the LexA DBD, and coexpression of elongins B and C (ELB and ELC) stabilizes the native conformation of pVHL. A human cDNA library in fusion with the Gal4 AD is screened for interactions with pVHL. The human prolyl hydroxylase PHD3 delivers the hydroxyl group to interacting prey proteins, which is essential for recognition by pVHL. (B) Protein–small-molecule interactions (94). O^6-Alkylguanine-DNA alkyltransferase (AGT) is fused to the Gal4 DBD and can bind covalently with the small molecule O^6-benzylguanine (BG) *in vivo*. A library of BG small-molecule heterodimers, produced *in vitro*, can be screened for interactions with a prey protein Y attached to the Gal4 AD. (C) Detection of enzymatic substrate recognition (21). A chimeric protein of LexA and DHFR binds the promoter region of the reporter gene *lacZ* and interacts with a tripartite small molecule through association of DHFR with Mtx. This small-molecule trimer further consists of a bait linker X and dexamethasone (Dex). Dexamethasone interacts with a fusion of the rat glucocorticoid receptor (RGR) and the B42 AD. The whole complex stimulates expression of *lacZ*. An enzyme Y that targets and cleaves linker X can be identified by disruption of the transcription activating complex and the loss of *lacZ* expression. (D) RNA-protein interactions (265). The LexA DNA-binding domain is fused to a head-to-tail dimer of the RNA-binding protein MS2. This hook protein associates with an RNA dimer of bacteriophage MS2 RNA and a bait RNA stretch (X). Interaction of this RNA X with a protein Y fused to the Gal4 AD is detected by stimulation of reporter gene expression. DBD, DNA-binding domain; AD, activation domain; UAS, upstream activating sequence; Op, operator; Pol, polymerase.

formed with a human cDNA library fused to the Gal4 AD. To exclude false-positive transformants, which induce reporter genes without the BG derivative, cells were grown in the absence of the BG derivative on medium with 5-FOA. True positive transformants were further selected by growth on medium without histidine and in the presence of the BG derivative. Validation of recovered binding partners of the drug compounds was performed with GST pulldown assays using GST-coupled AGT-BG constructs. With this approach, the confirmation of previously known drug targets was demonstrated, and novel interaction partners were found for the drugs purvalanol B, erlotinib, atorvastatin, and sulfasalazine (94). The experimental approach in this study sets a standard for future protein–small-molecule assays.

Detection of enzymatic activity. Chemical complementation forms an adaptation of the three-hybrid system for small-molecule–protein interactions and allows for detection of cleavage or a covalent junction of molecules mediated by enzymes (21). The linker, connecting two fixed small molecules, methotrexate and dexamethasone, consists of a molecule of interest that serves as a target of enzymatic catalysis. This tripartite bait connects the

DNA-binding hook LexA-DHFR with the transcription activating prey RGR-B42. As a result, any enzyme that cleaves within the molecule of interest and therefore disrupts the Mtx-Dex link can be detected by a loss of reporter gene activity. In the pioneering study (21) (Fig. 7C), the antibiotic cephalosporin, bordered by Mtx and Dex, was hydrolyzed by the β-lactamase cephalosporinase, which resulted in a loss of *lacZ* reporter expression. Quantitative evaluation of enzymatic activity by chemical complementation was applied to distinguish between β-lactamases with low and high catalytic efficiencies (573), to enhance the activity of glycosynthases and cellulases by directed evolution (400, 509, 632), and to investigate the modes of resistance to cephalosporin induced by β-lactamase mutations (77). Improvements of the original approach included an increase of cell permeability (77), fine-tuning of hook and prey expression (22, 77), and introduction of a sensitive counterselection reporter for detection of bond-forming enzymes (707). Similar methods were developed for application in *E. coli* (9, 181).

RNA-protein interactions. Interactions between RNA and proteins play an essential role in many fundamental cellular processes. Associations between mRNA and proteins are crucial for control of mRNA stability, splicing, and translation and for nuclear-cytoplasmic RNA shuttling. Aminoacyl tRNA-synthetases bind tRNA to add the corresponding amino acid, and chromosome ends are maintained by telomerases, complexes of RNA and protein molecules. For the study of interactions between RNA and proteins in three-hybrid systems, the bridging hybrid comes in the form of an RNA heterodimer (622). While one fixed RNA stretch of this dimer binds a hook protein, comprised of a DBD and a fixed RNA-binding protein, the other RNA sequence is tested for interaction with a protein of interest fused to an AD (526, 575). Interaction between the RNA and protein of interest induces expression of the reporter genes *HIS3* and *lacZ*. For one method, the fixed and hook RNA-binding proteins are the Rev-responsive element (RRE) and the RevM10 mutated form of the HIV-1 Rev protein (526), respectively, while in another setup, the binding of two copies of a specific stretch of bacteriophage MS2 RNA to the coat protein of MS2 is taken as a fixed component of the system (575). Both approaches have been used for a number of RNA-protein interaction studies (e.g., see references 290 and 585), but the MS2-based system has seen more applications, mainly because of the high affinity of the MS2 RNA-protein interaction (40) and because of specific improvements. For example, the RNA three-hybrid system is susceptible to revealing a large number of false-positive results, due mainly to direct binding of nonspecific prey proteins to the hook fusion. Introduction of a head-to-tail dimer of a high-affinity mutated version of the MS2 coat protein into the hook reduces these nonspecific associations by steric hindrance and increases the efficiency of hook association with MS2 RNA (265) (Fig. 7D). Other approaches to exclude RNA-independent false-positive results depend on the auxotrophic marker on the RNA plasmid (40, 492) or on an inducible promoter for RNA hybrid gene expression (20). The RNA three-hybrid system has been used to study RNA-protein complexes such as RNase P (268, 307) and telomerase (240) and to investigate interactions between RNA and proteins involved in translation stimulation (585), translation inhibition (250, 480), RNA methylation (712), transport (143, 345), degradation (674), and replication (90). The method enables screening of RNA molecules that bind an RNA-binding protein of interest (574, 712) or the discovery of RNA-binding proteins that interact with a specific artificial (359) or native (583) RNA stretch. Furthermore, RNA-protein binding affinities can be measured based upon three-hybrid experiments (713) when specific considerations are taken into account, such as focused RNA mutagenesis and introduction of flanking RNA regions that allow correct folding of the RNA sequence of interest (714). Recently, two independent groups discovered the Pumilio and FBF homology (PUF) protein repeats that recognize cytosine in RNA, based upon three-hybrid experiments (139, 179). Because the amino acids that recognize adenine, uracil, and guanine were discovered previously, artificial PUF proteins can be created to bind specific mRNA sequences for translation control (179), a method that could become complementary to RNA interference (163). Alternative RNA three-hybrid setups have enabled the identification of trimeric protein-RNA complexes (54, 411) and RNA-RNA interactions (515) and the design of transcription-activating RNA stretches (687). Finally, an ingenious bacterial one-hybrid method is available for detection of heterologous RNA-protein interactions, based on *lacZ* expression upon relief from antitermination (704).

Reverse Two-Hybrid Systems

The forward two-hybrid system is suitable for identifying the specific interaction domains of two binding proteins by gradual truncation of each protein in the system. However, the identification of single residues that are crucial for the interaction is not straightforward in this approach. Therefore, an adaptation of the traditional method was developed in which a counterselectable marker is used.

Reporter genes. The counterselectable reporter gene, which is activated following a PPI, expresses a compound that is toxic for the cell. Three commonly used reporters are *URA3* (667), *CYH2* (373), and *GAL1* (228), which render the cells sensitive to 5-FOA, cycloheximide, and galactose in a *gal7* background, respectively (Fig. 8A). Repression of a positive selection marker can also be used to investigate dissociation of a PPI. For example (Fig. 8B), a PPI activates expression of the Tet repressor, which then represses the activation of the positive marker *HIS3* under the control of the Tet operator (589). The *URA3* counterselectable system has been very successful due to the introduction of the basal *SPO13* promoter, which tightly regulates *URA3* expression in combination with an optimized number of *GAL4* binding sites and is strongly repressed under most growth conditions. As a result, mutations in the bait or prey protein of interest that block an interaction lead to an easily detectable resistance of the cells to 5-FOA.

Selection for missense mutations. An important concern in screening for residues that are required for an interaction is the presence of nonsense mutations. The original *URA3* method provides a second selection step to discover mutations that do not completely block the interaction, as an indication of missense rather than nonsense alterations (667). Alternatively, the wild-type bait or prey protein to be mutagenized can bear a C-terminal linkage with an additional reporter gene, e.g., β-galactosidase (589), GFP (168), or *URA3* (393). In this case, missense mutations can be selected by the dissociation of the PPI together with the expression of the C-terminally linked reporter gene. In the one-plus two-hybrid system, the mutated prey protein of interest is present between a Gal4 DBD and the B42 AD. An initial one-hybrid screening for removal of nonsense mutations is performed by selecting for cells that grow on medium lacking histidine, due to the expression of *HIS3* with Gal upstream activating sequences

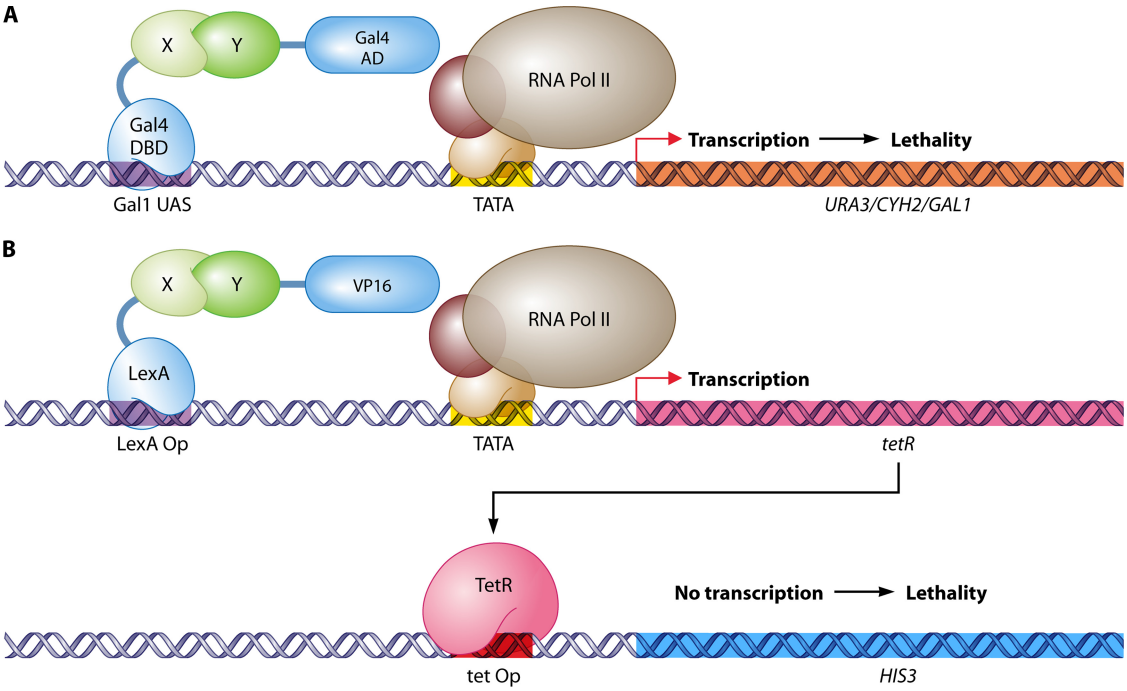

FIG 8 Reverse two-hybrid systems. (A) Repression of toxic genes (228, 373, 667). Interaction between bait X and prey Y stimulates transcription of *URA3*, which results in cell toxicity in the presence of 5-fluoroorotic acid; *CYH2*, which makes the cells sensitive to cycloheximide; or *GAL1*, which converts galactose into galactose-1-phosphate, a toxic compound that accumulates in the absence of Gal7. Compounds or mutations (in X or Y) that interfere with the association of the bait with the prey are identified by growth on selective medium. (B) Activation of positive selection markers (589). Interaction between bait X and prey Y leads to expression of *tetR*, encoding the Tet repressor. Next, TetR represses activation of *HIS3*, hindering cell growth on medium without histidine. Disruption of the interaction between bait and prey is analyzed by the appearance of histidine prototrophy. DBD, DNA-binding domain; AD, activaion domain; UAS, upstream activating sequence; Pol, polymerase; Op, operator.

stimulated by a complete Gal4-prey-B42 fusion protein (342). In the next step, interaction-defective missense prey constructs are selected for loss of LexA-controlled *lacZ* reporter gene activation in a two-hybrid assay with a LexA-DBD fused bait protein. Finally, nonsense mutations can also be excluded prior to the two-hybrid screening by cloning the mutated genes N-terminally to a kanamycin resistance marker and preselecting for the right reading frame in *E. coli* (217). The strength of this strategy lies in the removal of the kanamycin marker by subcloning the library into a new vector by recombinational cloning with the Gateway technique.

Identification of residues that moderate an interaction. Examples of the use of the reverse two-hybrid system include the isolation of multimerization-defective mutants of human HIV-1 integrase INI1 (114) and the identification of crucial residues for interactions between *A. thaliana* phytochrome B and its signaling partner, PIF3 (341), between the ArsD metallochaperone and the ArsA ATPase (718), between the proteasome ubiquitin chain receptor Rpn1 and ubiquitin-like domain proteins in *S. cerevisiae* (214), and between two nonstructural proteins, nsp10 and nsp16, from the severe acute respiratory syndrome (SARS) coronavirus (414). Analysis of allosteric inhibition of an interaction has also been described (510). Mutations in *A. thaliana* PYR1 that inhibit its pyrabactin-induced association with HAB1 were identified by the reverse two-hybrid system. For 49 mutant versions of PYR1, abscisic acid (ABA) was still able to induce the PYR1-HAB1 interaction, pointing toward mutations that specifically inhibit the binding of one ligand (the ABA agonist pyrabactin) but not the other (ABA). This extension of the two-hybrid method, to mutate regions of the protein outside the protein interaction domain, has also been reported for the forward two-hybrid system (96, 292). The ligand-binding domains (LBDs) of nuclear hormone receptors were modified by directed evolution through site-saturation and random mutagenesis in order to create a receptor that responds to an alternative ligand. A ligand-induced two-hybrid interaction was taken as readout (96, 292). The forward two-hybrid system has been applied successfully in more studies related to mutagenesis, for the selection of mutants that either increase (43) or decrease (282) interaction strength. Another approach consists of mapping the regions of a protein that are not important for protein-protein binding, called the absence-of-interference method, using random mutagenesis and the forward system to select for mutants that do not interfere with the interaction (130). This strategy has the advantages of identifying distant essential interaction regions, in contrast to gradual truncation of the protein, and selecting preferentially for missense mutations over nonsense mutations, in contrast to the reverse two-hybrid method.

Drug discovery. The reverse two-hybrid system has applications in the discovery of peptides or small molecules that disrupt a PPI. The method shows clear advantages, as cytotoxicity testing is included in the assay, high-throughput screening is possible (279), and there is no need for protein purification. In addition, the permeability of the cell for peptide or small-molecule entrance can be increased by deletion of genes encoding the ergosterol synthesis enzyme Erg6 or the multidrug resistance regulators Pdr1, Pdr3, and Pdr5 or by overexpression of the hexose transporter gene

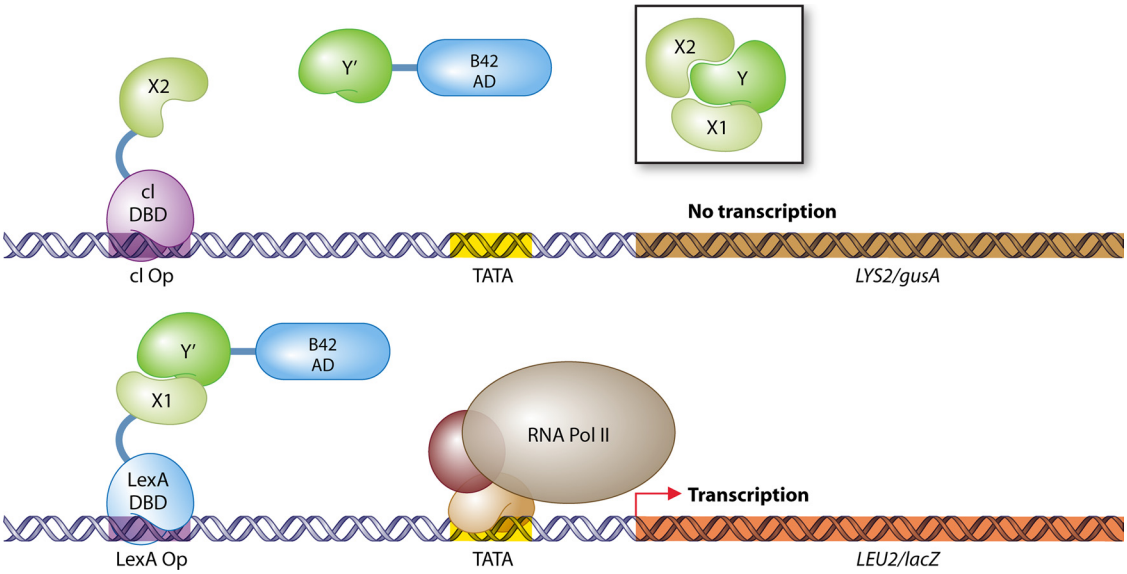

FIG 9 The dual-bait system (580). Protein Y interacts with two proteins, X1 and X2 (as shown in the box). Bait proteins X2 and X1 are fused with the DNA-binding proteins cI and LexA, respectively, while a library of mutated prey Y proteins is made in fusion with the B42 activation domain. A mutation in protein Y (Y′) that specifically disables the association with X2 but not X1 is detected by reporter activity of *LEU2* and *lacZ*, without expression of *LYS2* or *gusA*. DBD, DNA-binding domain; AD, activation domain; Op, operator; Pol, polymerase.

HXT1 or *HXT9*, which lowers the amount of compound required for the assay (193, 326, 668). Despite initial promising data (279, 720), the use of the reverse two-hybrid system for drug discovery in yeast has been limited (e.g., see reference 710). Other technologies, such as FRET (367), fluorescence anisotropy (525), surface plasmon resonance (315, 570), and virtual screening (726), have more impact on this field, at least in publically available reports. However, other two-hybrid methodologies have also been used for the discovery of PPI inhibitors, including the traditional system (199, 326), the repressed transactivator system (313), and PCAs (see Protein Fragment Complementation Assays in Yeast). In addition, both mammalian and bacterial reverse two-hybrid systems have been developed (see Genetic Protein-Protein Interaction Methods in Other Organisms). Improvements of the current two-hybrid technologies may boost the application of these methods in the field of drug discovery, as seen for the three-hybrid system for protein–small-molecule interactions (94).

Two-Bait Hybrid Systems

A gene deletion disturbs all physical associations of the protein encoded by the removed gene. Removal of such a protein from the interaction network precludes conclusions on the specific influence of each physical association related to this protein. Therefore, edge-specific genetic (edgetic) perturbations, which are mutations that explicitly interrupt only a subset of PPIs of the mutated protein (144, 735), can greatly facilitate the analysis of the distinctive functional properties of a protein in comparison with complete knockout mutations (108). Two-bait hybrid systems provide an interesting platform for discovering crucial amino acid residues that specifically reduce the affinity of a protein with one binding partner but not with another. In these methods, two known interactors of a protein of interest are each fused to a different DNA-binding domain, each of which targets the promoter of a different reporter, while the (mutated) prey protein of interest is attached to an activation domain. A mutation in the prey protein that exclusively activates one reporter and not the other correlates with an edgetic perturbation. To differentiate between mutations in Snf1 that are specific to binding to the activating subunit Snf4 or the kinase domain of Snf1 itself, a double two-hybrid strategy was followed (306). Mutated Snf1 fused to an activation domain was coexpressed with Gal4DBD-Snf4, binding to the *GAL1-HIS3* reporter, and LexA-Snf1 kinase domain, binding to the *lexA* operator (*lexA* Op)-*lacZ*. Selection by both a chromogenic (*lacZ*) and a growth-selective (*HIS3*) assay resolved all possible influences of mutations (no, specific, or nonspecific interference) in a single screen. A similar method, called the "differential interaction trap," was applied for identification of missense mutations in the yeast scaffold protein Ste5 that specifically disrupt an interaction with either Ste11 or Ste7, two MAP kinase pathway components (288). Other two-bait techniques are the "two-bait interaction trap" (716) and the "dual-bait system" (580) (Fig. 9). In the latter system, developed by Golemis and coworkers, each bait fusion protein binds the promoters of a chromogenic and a prototrophic reporter gene. The method proved to be efficient in distinguishing interacting partners of two related GTPases, Ras and Krev-1 (580), and discovering edgetic perturbations of the p21-activated kinase Pak1 regarding its interaction partners, the GTPases Cdc42 and Rac (539). Since both GTPases are able to signal to Pak1, identification of Pak1 mutants that were defective in Rac binding shed some light on the respective roles of these small GTPases in mediating the activation of Pak1 by Ras *in vivo*. An enhanced dual-bait system was configured and addressed the optimization steps of variable expression levels of the baits and sensitivities of the reporters, enrichment for polylinkers for easier cloning, and increased diversity of the selective markers (581). Two-bait systems also have an application in the exclusion of false-positive results. Proteins interacting with the bait protein of interest but not with a control bait can easily be discarded, thereby reducing technical false-positive results (581).

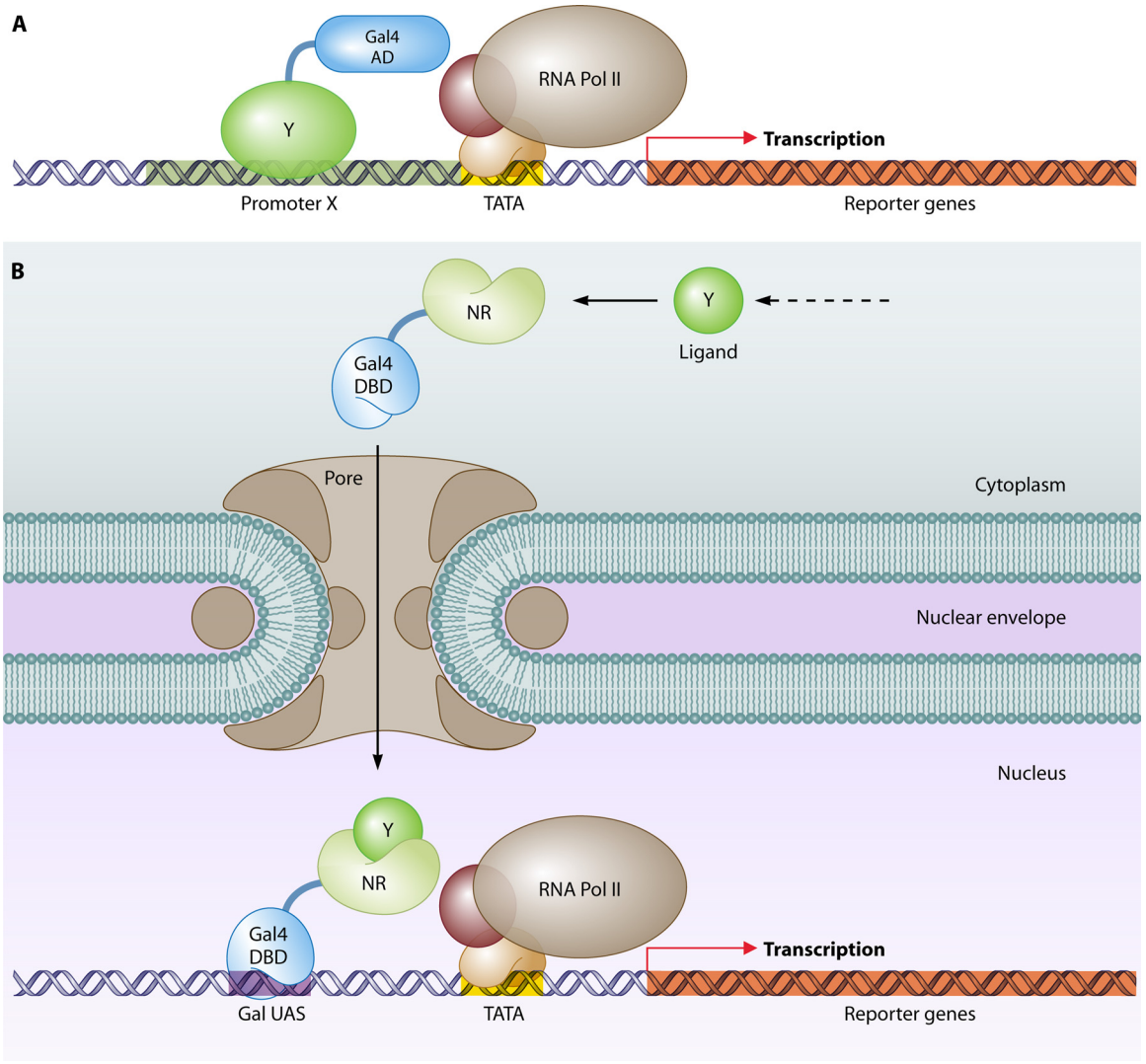

FIG 10 One-hybrid systems. (A) DNA-protein interactions (706). A putative DNA-binding protein Y is in fusion with the Gal4 activation domain. Interaction of protein Y with a promoter X stimulates reporter gene expression. This assay can single out proteins that bind a fixed promoter of interest or DNA sequences that are targeted by a fixed DNA-binding protein of interest. (B) Identification of transcriptional activity (696). A chimeric protein with the Gal4 DNA-binding domain and a transcriptional activator (a nuclear receptor [NR] in this example) stimulates expression of a reporter gene under the control of Gal upstream activating sequences. An application of such a system is the identification of ligands that trigger NR nuclear import and activity. AD, activation domain; DBD, DNA-binding domain; Pol, polymerase; UAS, upstream activating sequence.

Other methods for the identification of edgetic perturbations include the split-yeast cytosine deaminase (split-yCD) method (156), traditional two-hybrid experiments combined with site-directed mutagenesis, and reverse two-hybrid screens (82, 144).

One-Hybrid Systems

DNA-protein interactions. Proteins can bind DNA for transcription, replication, cleavage, ligation, gene regulation, and structural packaging. The detection of DNA-protein interactions is established by several techniques, including SELEX (systematic evolution by exponential enrichment), ChIP-seq (chromatin immunoprecipitation followed by sequencing), and protein microarray analysis (reviewed in reference 715). The one-hybrid system provides an alternative method and is unique in the sense that it can screen for both proteins that bind a specific DNA sequence (686) and DNA sequences recognized by a specific protein (706). In a one-hybrid experiment, the bait DNA is inserted in front of a reporter gene and the prey protein consists of the DNA-binding protein of interest in fusion with an activation domain. The association of the bait DNA with the prey protein results in activation of the reporter gene (Fig. 10A). New developments in the two-hybrid field, such as the application of several reporters (31, 176, 387, 412, 686), Gateway ORF libraries (127), or modified smart-pool assays (666), have been introduced in one-hybrid studies. In general, a heterologous host system is used to prevent interference by endogenous transcriptional activators. The only other available *in vivo* system, ChIP-seq, requires specific antibodies against a DNA-binding protein or inclusion of epitope tags, which is not straightforward in higher eukaryotes, especially on a large-scale level. Therefore, it is not surprising that the one-hybrid technol-

ogy is particularly popular for research on plant and animal DNA-protein interactions (79, 128, 254, 483, 665, 737). Derivations of the one-hybrid system were developed to detect DNA-protein dissociations by mutagenesis (34, 667, 706), as well as methylation-dependent interactions (176). Furthermore, other organisms have served as hosts for one-hybrid studies, e.g., mammalian cells (254) and bacteria (440).

Transcriptional activation. In general, DNA-binding domains can be recognized by *in silico* analysis. However, activation domains are intrinsically unstructured (32, 648), and only general sequence features of activation domains have been described (207, 557, 645). Therefore, activation domains can be discovered only by experimental analysis. One-hybrid studies can be applied if the native target promoters of a putative transcription factor are not identified. The putative transcriptional activator is fused with a fixed DNA-binding domain that targets upstream activating sequences in the promoter region of a reporter gene. Large-scale analyses of proteins or peptide sequences from *E. coli*, humans, and *S. cerevisiae* led to the identification of hundreds of fragments which were able to express the reporter genes (420, 642, 702). The one-hybrid system has been specifically suitable for the discovery of ligands and cofactors of higher eukaryote nuclear receptors, both with yeast as a host organism (264, 380, 696) and in mammalian cells (68, 80, 593) (Fig. 10B). The technique has further seen applications in the pathogenic fungus *Candida albicans* (554) and the fission yeast *Schizosaccharomyces pombe* (441).

PROTEIN FRAGMENT COMPLEMENTATION ASSAYS IN YEAST

PCAs exist in many flavors and have many different applications. PCAs provide a wide range of possible applications, depending on the choice of PCA technique. While the two-hybrid system has limited use for studies on the kinetics or spatiotemporal dynamics of PPIs, some PCAs can show the subcellular location of an interacting pair (e.g., the split-FP system) or offer a high resolution in temporal and quantitative analysis of protein-protein binding (e.g., the split-luciferase system).

Similar to the transcriptional readout of the classic two-hybrid system, PCAs need to provide a detectable effect, such as cell survival on selective medium (e.g., the split-mDHFR method), colocalization of the interacting protein as detected by fluorescent antibodies (e.g., the split-lactamase system), or the appearance of fluorescence or luminescence upon interaction of the protein couple (e.g., the split-FP system). Furthermore, the two fragments of the reporter protein should not reassemble spontaneously but only after interaction of the two proteins fused to each fragment. The sensitivity of the assay depends on the presence or absence of signal amplification (e.g., enzymatic activity of the reporter), the signal-to-noise ratio (e.g., the ratio is negatively influenced by autofluorescence), the abundance of bait and prey proteins, and the flexibility of the fragments to reassemble unhindered by the structure or size of the interacting proteins. The development of a PCA requires knowledge of the structure of the candidate reporter to identify possible sites at which to split the protein and to see possibilities for incorporation of specific mutations that either increase or decrease the reassembly efficiency. Potential PCA reporters have gone through many optimizations, by site-directed (312, 633) and random (156) mutagenesis and by selection of different N-terminal and C-terminal fragments from a small (156) or large (629) fragment collection. This ongoing process of reporter optimization, together with advances in optics technologies, promises to make PCAs more and more attractive in the future.

The Split-Ubiquitin System

Introduction of PCAs came with the development of the ubiquitin split-protein sensor (USPS) (or split-ubiquitin) system by Johnsson and Varshavsky (312) (Fig. 11). This method takes advantage of the properties of ubiquitin, a highly conserved 76-amino-acid regulatory protein. Ubiquitin is recognized by ubiquitin-specific proteases that cleave the C-terminal covalent linkage between ubiquitin and the protein to which it is attached (255). When the C-terminal and N-terminal regions of ubiquitin (Cub and Nub) are split and each part is fused to a different protein of interest, functional ubiquitin is formed upon interaction of both fusion proteins. To prevent spontaneous reassociation of ubiquitin, amino acid 13 was converted from isoleucine to glycine (NubG). In the original design, the bait consisted, from the N-terminal to the C-terminal end, of the homodimerization domain of Gcn4 (protein of interest), Cub, mDHFR, and a hemagglutinin (HA) epitope tag. The prey was constructed as a fusion of the homodimerization domain of Gcn4 with NubG. Upon dimerization of Gcn4, ubiquitin was reconstituted and mDHFR-HA was cleaved off by ubiquitin-specific proteases, and this was detected as a shift in a Western blot assay using anti-HA antibodies (Fig. 11A). Later, this rather cumbersome readout was replaced by reporter gene activation (611). The reporter mDHFR was replaced by the hybrid transcription factor LexA-VP16. After interaction of bait and prey, LexA-VP16 is cut off and moves to the nucleus for activation of the reporter genes *HIS3* and *lacZ* (Fig. 11B). This new reporter strategy allows for screening of a library for novel interactors. The bait protein of interest needs to be membrane bound or at least able to exclude the whole fusion construct from entering the nucleus. This makes the technique very complementary with the traditional two-hybrid system. N-terminal relocation of the LexA-VP16 fragment enables the use of membrane-bound bait proteins with cytoplasmic tails on the N-terminal side (209). Also, mating type **a** and α two-hybrid strains were developed (476) that enable mating of bait and prey transformants for efficient high-throughput studies. Such a large-scale experiment revealed 1,985 *S. cerevisiae* interactions among 536 integral membrane proteins (447). Employment of the split-ubiquitin system further led to an interactome network of *A. thaliana* membrane proteins (370). Split-ubiquitin vectors and strains are available at Dualsystems Biotech and MoBiTec.

An alternative version was created using the concept of the N-end rule (Fig. 11C). In *Saccharomyces cerevisiae*, protein stability depends on the nature of the N-terminal amino acid (660). Amino acids such as glycine, methionine, threonine, alanine, and cysteine stabilize the protein when they are present at its N-terminal end. In contrast, N-terminal basic (e.g., arginine) or bulky hydrophobic amino acids tend to promote protein degradation in a ubiquitin-dependent manner (138). For PPI analysis, the LexA-VP16 construct in the bait is replaced by the reporter protein Ura3, with an arginine residue (R-Ura3) between Ura3 and Cub (708). When two proteins of interest interact, the reassembly of ubiquitin recruits the ubiquitin-specific proteases that cleave off Ura3. As a result, free Ura3 is quickly degraded due to the exposed N-terminal arginine residue. Consequently, the cells become resistant to

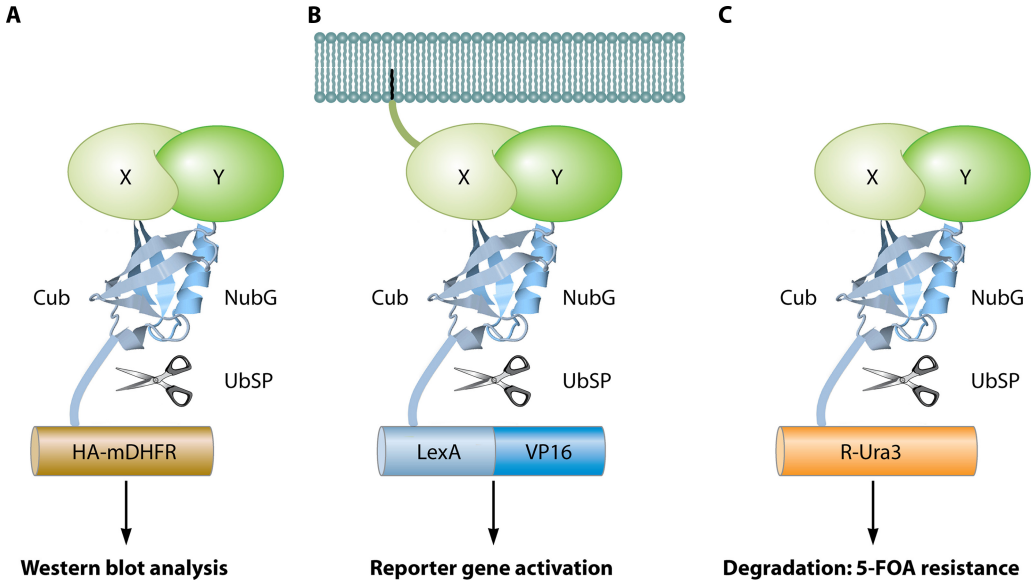

FIG 11 The split-ubiquitin system. (A) mDHFR-HA readout (312). Bait protein X and prey protein Y are fused to the C-terminal (Cub) and mutated (I13G) N-terminal (NubG) domains, respectively, of ubiquitin. In addition, a chimera of mDHFR and the HA epitope completes the bait construct. Upon interaction of X with Y, a fully reconstituted ubiquitin is recognized by ubiquitin-specific proteases (UbSP) that cleave HA-mDHFR, resulting in a shift on a Western blot using anti-HA antibodies. (B) LexA-VP16 readout (611). Interaction between a membrane-bound bait X and prey Y leads to cleavage of the artificial transcription factor LexA-VP16. Released LexA-VP16 localizes to the nucleus to activate reporter genes. (C) R-Ura readout (372). Interaction between bait X and prey Y induces the release of Ura3 (with an N-terminal arginine) by proteases. Exposed arginine makes Ura3 highly unstable, resulting in its degradation. Due to the strongly reduced concentration of Ura3, the cells become viable on medium with 5-FOA, a prototoxic substrate of Ura3. The image of the ubiquitin protein is based on the PDB structure under accession number 1UBQ (671).

5-FOA. While the LexA-VP16 strategy is complementary to the classic two-hybrid system by its application for detection of membrane PPIs, the R-Ura3 method is especially suitable for finding transcription factor partners, both activators and repressors (50, 92, 372). Furthermore, the ambiguity of Ura3 as a reporter protein permits screening for PPIs and PPI inhibition. As an example of the latter, mutations that interrupted the binding of the transcription factor Gal4 with its inhibitor Gal80 were identified by use of the R-Ura3-based method with selective medium without uracil (74). Finally, the N-end rule was employed for the development of mammalian and plant split-ubiquitin systems (528, 548).

As with the original two-hybrid method, the split-ubiquitin system suffers from pulling out a significant number of false-positive results. To cope with this issue, a new strategy was suggested in which the bait protein gene is integrated into the genome and controlled by its native promoter (502). This approach severely decreased the number of false-positive results, as shown by a screening experiment to map the interaction network of the ABC transporter Ycf1 (502). Further adjustments to balance out sensitivity and selectivity are provided with the use of a weak or inducible promoter for controlled bait expression (476, 541) and the availability of low- and high-copy-number bait and prey plasmids (219, 476). For the R-Ura3-based system, careful optimization of 5-FOA levels reduces false discovery rates (133). Protocols for split-ubiquitin experiments can be found elsewhere (149, 605).

The split-ubiquitin method has been adopted for several alternative applications (reviewed in reference 465). Split-ubiquitin three-hybrid techniques were developed for expression of a bridging or competing third protein (220) and for the identification of protein–small-molecule interactions (134). In addition, the unique feature of ubiquitin-induced proteolysis has been exploited to control protein abundance (520), to identify the endoplasmic reticulum pores that transport a specific substrate protein (150), and to eliminate cancer cells in a theoretical design based on conditional maintenance of a toxic protein-encoding vector (659). The steric requirements in a split-ubiquitin experiment further enabled studies on altered protein conformations (148, 534).

In a cytosolic variant (cytoY2H), the *S. cerevisiae* integral membrane protein Ost4 was added at the N-terminal end of the bait to direct the bait fusion to the membrane. The same strategy as that used for the split-ubiquitin system with the LexA-VP16 reporter was employed, but in this case, the bait protein of interest did not have to be a membrane-bound protein by itself (454). Application of the cytoY2H system revealed several translation-regulating binding partners of Uri1, an uncharacterized yeast protein (454), and a follow-up study confirmed a functional role for Uri1 in translation control (129). Other successful cytoY2H experiments were conducted with the *S. cerevisiae* ubiquitin ligase Ubr1 (286) and the *A. thaliana* pentatricopeptide repeat protein PNM1 (237).

The Split-mDHFR Method

General introduction to the split-mDHFR method. DHFR catalyzes the reduction of dihydrofolate into tetrahydrofolate. Tetrahydrofolate is essential for cell proliferation and growth by acting as a precursor of purine and thymidylate synthesis. This crucial role for DHFR in cell survival can be used for PCA applications with DHFR split into two fragments, the F[1,2] N-terminal and the F[3] C-terminal fragments. In contrast to bacterial and yeast DHFRs, mammalian DHFR enzymes are much less sensitive to the chemical inhibitors methotrexate and trimethoprim. Therefore, murine DHFR (mDHFR) can serve as a reporter protein in bacterial and fungal DHFR systems in which a PPI is detected by

survival of the cell in the presence of methotrexate or trimethoprim, with mDHFR taking over the function of the host DHFR protein (508, 587). In mammalian cells, nucleotide-free medium can be used for growth selection (543). Due to the presence of a selection step, the split-mDHFR method can be used to screen for novel PPIs and forms an alternative to transcription-based two-hybrid systems, with the benefit that the proteins under study reside in their natural subcellular compartment. In plant and mammalian cells, reconstituted DHFR has been visualized by addition of fluorescein-conjugated methotrexate (fMTX) (546, 625).

Application of the split-mDHFR method in yeast. A genome-wide *S. cerevisiae* PPI screening was performed in which over 4,000 bait proteins were individually examined for interactions with over 4,000 prey proteins in a mating assay (633). After filtering out sticky proteins and benchmarking the results with reference sets, 2,770 high-quality interactions were retained in the final data set. The output was highly complementary with original two-hybrid screens and TAP-MS data. Comparison of interaction results for proteins of the small-subunit (SSU) processome indicated that, at least for this subset of the interactome, the split-mDHFR screening (633) was more complete in identifying true interactions between subunits than high-throughput two-hybrid data (398). The authors suggested that given the poor overlap between different high-throughput two-hybrid experiments (247, 294, 650, 721), the two-hybrid system is not inferior in its ability to detect PPIs, but the screenings were possibly far from reaching saturation. One possible explanation lies in the fact that the split-mDHFR screens were done with individual bait and prey strains, while the classic two-hybrid experiments were performed with pooled prey strains (398).

The Split-yCD Method

A highly versatile PCA is based on *S. cerevisiae* cytosine deaminase (156). Yeast cytosine deaminase (yCD), encoded by *FCY1*, is required for the pyrimidine salvage pathway to convert cytosine into uracil. The PCA with this 17-kDa protein was developed by comparing seven combinations of fragments and including three specific mutations that increase thermostability (360). Random mutagenesis of the N- and C-terminal fragments of yCD, followed by fusion to the human GTPase Ras and the Ras-binding domain of c-Raf, respectively, led to the identification of optimized yCD fragment sequences by selection on medium lacking uracil for yCD reassembly in an *fcy1* strain. While uracil-deficient medium can be used for positive selection for an interaction, the dissociation of an interaction can be screened for on medium with 5-fluorocytosine, which is converted into toxic 5-fluorouridine triphosphate by a pathway dependent on yCD. This negative and positive selection procedure allows for screening for mutations that disrupt the interaction with one binding partner but not with another (156).

In resemblance to the split-yCD method, the split-Trp system (629) selects for interaction-induced reassembly of Trp1, which enables growth on tryptophan-deficient medium. The technology, developed by creation of randomly circularized permutations of Trp1 (216), was applied to confirm the association of Sec62 and Sec63, two members of the Sec complex.

The Split-Luciferase Method

General introduction to the split-luciferase method. Luciferases are proteins that bind and catalyze the oxidation of their membrane-permeating substrate luciferin, which ultimately can be observed by the appearance of bioluminescence (705). By separation of luciferase N- and C-terminal fragments and fusion with proteins of interest, a PPI between these proteins can be visualized by the appearance of light. These split-luciferase systems, originally developed in mammalian cells (484), have the very practical characteristic that they provide a high temporal resolution of detection and are reversible, allowing near-real-time association studies. This can be exploited to quantify dynamic changes in protein assemblies (615). Moreover, split-luciferase systems take advantage of the very low cellular background luminescence, leading to a high signal-to-noise ratio. Luciferases applied in PCA technologies originate from the firefly (*Photinus pyralis*) (484), the sea pansy (*Renilla reniformis*) (317, 500), the copepod *Gaussia princeps* (542), and, more recently, the click beetle (258, 343). A particular advantage of the last three luciferases is their much stronger brightness than that of the firefly protein. In addition, click beetle luciferases from *Pyrearinus termitilluminans* and *Pyrophorus plagiophthalamus* beetles emit in green and red, respectively, which enables simultaneous investigation of two PPIs (672). A limitation of the method lies in the low photon efflux rates obtained when working with bioluminescence. The chemical reaction required to create excited-state luminescent substrates is a less efficient process than the light absorption-based excitation of fluorescent molecules. As a result, imaging at a subcellular level is difficult, though not impossible (317). Improvements of the luciferase fragments may increase the potential of the method to detect PPIs on a subcellular level. Recently, semirational combinatorial library screening led to the identification of fragments of green click beetle luciferase displaying faster and brighter bioluminescence with a concomitant higher signal-to-noise ratio. Illustrating the advantage of this PCA, time-lapse bioluminescence imaging revealed ligand-induced GPCR–β-arrestin coupling in the submembrane space. In addition, cell lines were generated to enable high-throughput screening for small molecules to disrupt interactions between activated GPCRs and β-arrestin (452). Finally, the split-luciferase system is one of the very few PCAs with applications in living animals.

Application of the split-luciferase method in yeast. Reports on split-luciferase assays in yeast are rather scarce. However, in a study on the response of the yeast Fus3 MAP kinase pathway to pheromone (429), full advantage was taken of the temporal sensitivity and large dynamic detection range of the split-luciferase system. An ambient threshold concentration of pheromone leads to recruitment of the phosphatase Ptc1 to the scaffold protein Ste5 and to dissociation of the MAP kinase Fus3 from Ste5. Active liberated Fus3 then activates the transcription factor Ste12 for pheromone-responsive gene expression (165). The switch-like response of the pathway to pheromone was shown to depend on competitive binding of Fus3 and Ptc1 to Ste5, the dissonant behavior of Fus3 and Ptc1 in response to the phosphorylation status of four residues on Ste5, and a proposed two-stage binding of both proteins to Ste5 (429). Split-luciferase experiments with the three proteins formed an essential part of this investigation and showed that PCAs enable quantitative and dynamic analysis of PPIs.

The Split-FP Method

General introduction to the split-FP method. Fluorescent proteins emit light upon excitation by an external light source. The discovery of GFP in *Aequorea victoria* (590) introduced the concept of fluorescent proteins in biology, and currently, a whole spectrum of natural and genetically optimized proteins is available (reviewed in reference 117). Many of these fluorescent proteins were adapted for interaction analysis by splitting the proteins into two fragments, each attached to a protein of interest (also reviewed in references 335 and 337). This type of PCA, called the split-FP method (or bimolecular fluorescence complementation), was originally developed in *E. coli* (205) and soon would become the most widespread of all PCA tools. Its easy technology transfer to other organisms led to the application of the split-FP method in many plants, prokaryotes, fungi, and animal cells. The strength of this technique lies in its ability to detect weak interactions at a subcellular resolution, and in contrast to the case for the split-luciferase method, no exogenous agents are required. Variations on the split-FP method have opened up new possibilities in PPI research. Multicolor split-FP assays can be used to monitor multiple PPIs simultaneously, and split FPs can be combined with BRET or FRET fluorescence to study higher-order complexes and with photoswitchable fluorophores to overcome the problems of bleaching and low quantum yield (see the mammalian and plant sections for further details on these variations).

Split-FP systems come with a number of limitations. First, the split-FP method suffers from the same difficulties observed with traditional fluorescence experiments, such as photobleaching, phototoxicity, and autofluorescence. Autofluorescence is not a vast problem in mammalian cells, but plant cells especially are notorious for giving a high background of fluorescence signals (135). Second, results from split-FP assays need to be interpreted with extra caution. Because of the rather low maturation rate of chromophore formation, and hence fluorescence reconstitution, and due to its irreversible nature, split-FP methods do not allow real-time measurements of PPI dynamics. This irreversible chromophore formation, however, offers the advantage of trapping weak (millimolar range) complexes (424). Therefore, split-FP systems, especially the split-Venus (yellow fluorescent protein [YFP] variant) version, are very sensitive, but with the cost of low selectivity. Accordingly, endogenous expression of the constructs is preferred, and positive results from split-FP experiments need to be confirmed by other means or by creation of mutated proteins that colocalize but no longer interact in a split-FP assay. This is important for differentiation between two proteins in close proximity and two proteins that really interact. Finally, slow maturation of split-FP fragments further complicates interpretations of the subcellular location of PPIs. During the time between fluorescence detection and initiation of the PPI, the protein couple can change its position in the cell.

Application of the split-FP method in yeast. Fluorescent protein complementation of enhanced GFP (EGFP), yellow fluorescent protein (YFP), cyan fluorescent protein (CFP), the YFP variant Venus, and the monomeric Kusabira-Green fluorescent mutant (mKG2) has been employed in yeast (27, 28, 47, 104, 355, 489, 523, 626). Initial results came from studies on the influence of hydrogen peroxide on the location of the interacting couple Rho5 GTPase and Trr1 thioredoxin reductase (602), the dependence of the Rsr1 GTPase self-association on its activator, Bud5 (319), and the reliance for the interaction between Mso1 and the Sm-like protein Sec1 on the Rab GTPase Sec4 and its activator, Sec2 (694). Sec4 was later shown to interact directly with Mso1 in a split-FP assay (695). The use of the split-FP method, in particular that with the highly sensitive Venus fluorescent protein, has been extended further for the discovery of novel interactors in medium-scale experiments (523). Twenty-two lipid droplet proteins used as bait were screened for interactions with 225 mitochondrial and peroxisomal proteins used as prey, resulting in 116 PPIs, indicating a physical interaction of lipid droplets with both mitochondria and peroxisomes. Native promoters were used for bait and prey expression, and the mating approach was conducted to bring bait and prey together within the same strain. It can be expected that several other reports on the use of medium-scale split-FP assays will become public in the near future. A protocol for PCA applications in yeast, including the split-FP method, can be found elsewhere (443).

GENETIC PROTEIN-PROTEIN INTERACTION METHODS IN OTHER ORGANISMS

Genetic Protein-Protein Interaction Methods in Prokaryotes

Although experiments involving eukaryotic PPIs in *Escherichia coli* are hampered by the lack of an intron splicing machinery and the absence of particular posttranslational modifiers, prokaryotic two-hybrid systems show some clear advantages over the yeast two-hybrid system. First, the use of *E. coli* as a host organism for two-hybrid experiments enables screening with very large libraries in a very short time, due to the high transformation efficiency and fast growth of this bacterium. Second, two-hybrid screening in bacteria also reduces the chance that the host possesses a eukaryotic homolog that mediates a protein association, which raises the reliability for conclusions on a direct interaction. Furthermore, the absence of endogenous proteins that compete for interactions with the bait or the prey protein increases the sensitivity of the system. Third, the absence of a nuclear envelope avoids the requirement for the fusion proteins to pass a membrane. Finally, proteins that are toxic to yeast at high concentrations may not evoke the same effect in bacteria.

Detection methods for PPIs in bacteria are numerous and are based on fusions to transcriptional repressors and activators, membrane protein dimerization, complementation of biosynthetic enzymes or signaling molecules, and export of folded proteins. Examples are provided below for each of these techniques.

Bacterial two-hybrid systems. For the development of two-hybrid methods in *E. coli*, inspiration was found in the dimeric behavior of bacterial repressors. The first bacterial two-hybrid systems were based upon the *E. coli* λ repressor, which confers immunity to phage infections (158, 273). The N-terminal part of this protein binds DNA, while the C-terminal part is responsible for dimerization, which is necessary for efficient DNA attachment. When the C-terminal region is replaced by a protein of interest, homo-oligomerization of this protein can be evaluated by repression of a *lacZ* reporter gene. Screening of prey libraries for interactions with this system is hindered by the appearance of homodimerizing prey proteins that repress the reporter independently from the bait. To circumvent this problem, the DNA-binding domains of two allelic variants of the *E. coli* LexA repressor DBD, i.e., the LexA wild-type DBD and LexA408 DBD, were each fused to a protein of interest (137). Both variants have different binding affinities, depending on the DNA sequence in the promoter. Hetero-oligomerization can then be distinguished from homo-oli-

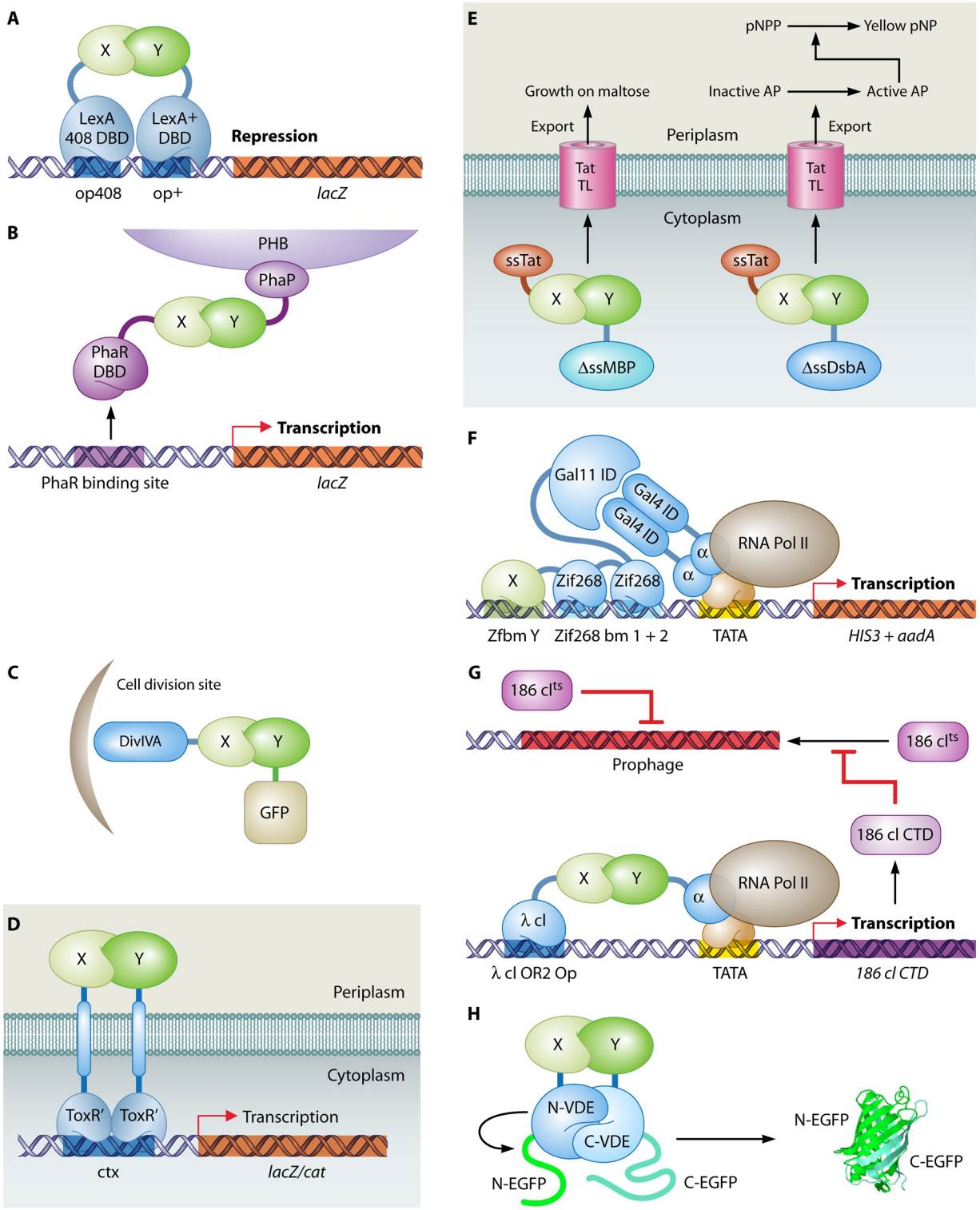

FIG 12 Specific bacterial genetic PPI detection methods. (A) Repressor-based two-hybrid system (137). Interaction between two proteins of interest, X and Y, can be monitored by fusion of each with a variant of the LexA repressor (408 or wild type). Heterodimerization of LexA408 and LexA^{+}, induced by X-Y association, is required for efficient repression of the reporter gene *lacZ*, under the control of the LexA operators op408 and op+. (B) PhaR two-hybrid system (690). The binding of bait X with prey Y, fused to the DNA-binding domain (DBD) of the repressor PhaR and the PHB granule-associated protein PhaP, respectively, results in *lacZ* expression by recruitment of PhaR to PHB granules. (C) GFP recruitment system (132). Bait X is situated at cell division sites through the action of its chimeric partner, *B. subtilis* DivIVA. Interaction of protein X with GFP-tagged prey protein Y results in focused fluorescence at the cell division sites. GFP recruitment systems are also available for the pathogenic fungus *Candida albicans*, for *C. elegans*, and for mammalian cells. (D) ToxR two-hybrid system (357). The *V. cholerae* ToxR transcriptional activator requires dimerization of its periplasmic domain for full reporter transcription activation. In the ToxR two-hybrid system, the periplasmic domain is replaced by two proteins of interest, X and Y. An interaction between these two proteins results in efficient

gomerization by the use of a hybrid sequence bearing an op^{408}/op^{+} operator sequence in front of *lacZ*, each of which has a preference to be bound by one of the two LexA variants (Fig. 12A). This technique was recently used to confirm several prokaryotic PPIs (e.g., see references 140 and 664). In order to implement a mating-based strategy such as the one employed in yeast for library screening, an adaptation of this LexA-based bacterial two-hybrid assay was created with bait vectors carrying a mobilization element (103). These vectors can be transferred efficiently by conjugation from an *E. coli* strain donor expressing all the necessary components for mobilization function to a recipient strain harboring the prey vector.

The implementation of transcriptional activators in prokaryotic two-hybrid systems significantly enhanced the potential for screening experiments by selective growth. Based upon a prototypical experiment involving the bacteriophage λ cI repressor (142), several two-hybrid systems were developed. In one design, the λ cI protein and the α subunit of RNA polymerase are employed as the DBD and AD, respectively (141, 142). The reporter genes are *lacZ* and the β-lactamase *bla* gene, which confers resistance to carbenicillin (141). In another system, a triple-zinc-finger motif of murine Zif268 serves as the DBD, and an operon of *HIS3* and the spectinomycin resistance gene *aadA* is applied for efficient screening (314). The methodology was also used to commercially develop the BacterioMatch two-hybrid system and, subsequently, the BacterioMatch II tool, featuring a new *HIS3-aadA* reporter cassette (Agilent Technologies). In a recent study, the global PPI network of the human pathogen *Mycobacterium tuberculosis* H37Rv was unraveled using this technique, revealing more than 8,000 interactions among almost 3,000 proteins (689). Alternatively, the ω subunit of RNA polymerase is linked with the prey protein of interest (142). An approach combining the ω subunit and the Zif268 zinc finger domain is particularly suited for studies of PPIs between two monomers (655). The latter system was modified for use with Gateway entry clones, providing a new tool for rapid PPI screening (323). A protocol for bacterial two-hybrid experiments can be found elsewhere (206).

A two-hybrid system in *E. coli* based on the polyhydroxybutyrate (PHB) synthesis regulatory protein PhaR was recently created (690). This method relies on the fusion of bait and prey proteins carrying the DBD of PhaR and the PHB granule-binding protein PhaP, respectively. The bait fusion protein represses the reporter gene *lacZ* by binding its promoter. Interaction between bait and prey constructs tethers the bait to the PHB granules, which results in the release of *lacZ* expression (Fig. 12B). This method displays a reduced technical false-positive rate, resulting from the use of extrinsic components of PHB synthesis, and technology transfer is possible to other bacterial species that can sustain sufficient PHB granule accumulation (690).

Membrane-localized and secretory pathway two-hybrid systems. There are two main shortcomings of classic two-hybrid tools that prompted researchers to develop alternative methods, namely, the lack of information on the affinity or level of expression of the interacting proteins and the failure to detect interactions within the secretory compartments for proteins that require an oxidizing environment for proper folding. In that respect, the APEx two-hybrid system for anchored periplasmic expression was engineered as a quantitative PPI assay of particular importance for antibody discovery and for selection of high-affinity antibodies (304). The method, which resembles the yeast surface two-hybrid systems, is based on the expression of a soluble epitope-tagged prey protein and a bait protein anchored on the periplasmic side of the inner membrane of *E. coli* by fusion to a leader peptide and to the first 6 amino acids of the *E. coli* lipoprotein NlpA. Upon interaction, the prey remains associated with the bait in spheroplasts, which allows quantitative detection by fluorescent anti-epitope-tag antibodies. In such a system, all nonassociated prey proteins are removed in the extracellular fluid upon spheroplast induction. In addition, because the fluorescence signal is a direct function of both the affinity of the interaction and the expression level of the interacting partners, selection for either increased affinity or improved expression is achieved by using multicolor FACS analyses (304).

In a cytology-based screening assay, one protein is fused to DivIVA from *B. subtilis* or FtsZ from *E. coli* to target a second protein, fused to GFP, to cell division sites (106, 132) (Fig. 12C). Interactions were observed between soluble proteins, such as the leucine zipper domains of yeast Gcn4, and integral membrane proteins, such as the VirB subunits of the T-DNA transfer system of *Agrobacterium tumefaciens*. This GFP recruitment system was applied in both *E. coli* and *Agrobacterium tumefaciens* (132).

The cholera toxin ToxR regulatory protein of *Vibrio cholerae* has been exploited as a genetic indicator of PPIs in *E. coli* in several variations of the two-hybrid approach. ToxR consists of a cytoplasmic gene activating domain linked by a membrane-spanning region to a periplasmic part. ToxR homodimerization at the periplasmic domain is required for proper transcription-inducing

ctx promoter binding of the truncated ToxR protein (ToxR′) and subsequent gene expression of *lacZ* or the chloramphenicol acetyltransferase gene (*cat*). (E) Tat two-hybrid system (621). The Tat signal sequence (ss) peptide tethers bait protein X to the periplasm. A chimeric fusion of prey Y with the maltose-binding protein without a signal sequence (ΔssMBP) localizes to the periplasm only upon interaction of X with Y. This translocation is required for growth on medium with maltose as the sole carbon source. Alternatively, the prey protein Y is fused to a localization-deficient DsbA enzyme (ΔssDsbA), which catalyzes the formation of active alkaline phosphatase (AP). Active AP converts *p*-nitrophenyl phosphate (pNPP) to yellow *p*-nitrophenol (pNP). (F) Bacterial two-hybrid system for DNA-protein interactions (314). To increase sensitivity in the search for zinc finger-DNA associations, the binding of a zinc finger motif X to its target DNA sequence, Y (zinc finger binding motif Zfbm Y), is facilitated by inclusion of two fixed zinc fingers from Zif268 and the target DNA sequence Zif268 bm. The zinc finger fusion further consists of the *S. cerevisiae* Gal11 interaction domain (Gal11 ID), which binds the *S. cerevisiae* Gal4 dimerization domain (Gal4 ID). The latter domain is fused to the N-terminal domain of the RNA polymerase α subunit for indirect activation of an operon comprising the *S. cerevisiae* auxotrophic marker *HIS3* and *aadA*, which confers resistance to spectinomycin. (G) Bacterial reverse two-hybrid system (239). Interaction between chimeric proteins of bait X with the bacteriophage λ cI repressor (which binds the λ cI OR2 operator) and prey Y with the N-terminal domain of the RNA polymerase α subunit results in activation of a gene encoding the C-terminal domain of the bacteriophage 186 cI repressor (186 cI CTD). This truncated protein sequesters and inactivates full-length 186 cI, which normally downregulates cytotoxic 186 prophage genes. Resulting cell death can be circumvented by mutations that block the bait-prey interaction. (H) Intein-mediated split-GFP assay (485). Bait protein X is in fusion with the N-terminal fragments of the intein VDE and GFP, while prey protein Y constructs include their respective C-terminal counterparts. Interaction between X and Y reconstitutes VDE, which splices out and covalently reattaches the GFP fragments to create an isolated GFP monomer, detected by fluorescence. The EGFP structure image is based on the PDB structure under accession number 2Y0G (550).

activity, and replacement of this domain by proteins of interest allows for PPI experiments (Fig. 12D). Detection of periplasmic PPIs, and of cytoplasmic PPIs after removal of the transmembrane region, is possible in the ToxR-based system (357, 358, 371). In *E. coli*, ToxR is capable of directly activating transcription at the *ctx* promoter sequence, which is used as the regulatory element driving a reporter construct such as chromosomal *ctx*::*lacZ* (357), plasmid-carried *ctx*::*chloramphenicol acetyltransferase* (*cat*) in the TOXCAT system (553), chromosomal *ctx*::*cat* in the POSSYCAT system (positive selection system based on chromosomally integrated *cat*) to discriminate between interactions of different affinity (233), or the red fluorescent protein variant mCherry for whole-cell detection without an additional substrate (39). The ToxR-based tool has been used as an indicator of folding stability (356), interactions between transmembrane helices (553), heterodimerization in both the periplasm and cytoplasm (252), and sequence motifs required for helix-helix interactions by use of a disabled ToxR fusion as a dominant-negative protein (39). In a variation of the ToxR system, two LexA DBDs (wild-type and 408 DBDs) (137) were coupled to wild-type and mutated glycophorin A transmembrane helices to allow detection in a biological membrane, and *lacZ* was placed under the regulation of promoter elements, each bound specifically by one LexA repressor domain (564). This system, named GALLEX, can measure both homo- and heterodimerization of membrane proteins, as recently illustrated by the analysis of transmembrane domain interactions between major histocompatibility complex class II proteins (347).

An alternative two-hybrid system in *E. coli* detects PPIs based on the biological folding quality control mechanism inherent to the twin-arginine transporter pathway (Tat). This mechanism relies on the export of correctly folded proteins by association with a protein carrying a Tat signal peptide (547). In the Tat two-hybrid system, one protein is fused to a Tat signal peptide and the second is fused to a protein reporter that can confer a phenotype only upon export into the periplasmic space (621) (Fig. 12E). In the first attempt, two reporters were used: the maltose-binding protein, whose export permits selection for growth on maltose, and DsbA, which catalyzes the formation of alkaline phosphatase. In an alternative version of the Tat-based system, called FLI-TRAP (functional ligand-binding identification by Tat-based recognition of associating proteins), Waraho and DeLisa (691) exploited the colocalization of the reporter β-lactamase Bla into the periplasm as a semiquantitative and high-throughput readout for interactions. Only those chimeras that were highly expressed and interacted strongly were able to confer β-lactam antibiotic resistance to cells.

Bacterial one-hybrid systems. Bacterial two-hybrid techniques have been adapted further for protein-DNA studies in one-hybrid assays. The high transformation efficiency of bacteria is especially advantageous for one-hybrid experiments involving randomized DNA or zinc finger motif libraries, enabling screening procedures with 10^8 transformants. DNA-binding proteins or domains are directly or indirectly fused with the ω (272, 314, 474) or α (151, 439) subunit of RNA polymerase. In addition, weak DNA-protein interactions can be studied by incorporation of a fixed zinc finger-DNA association that facilitates the binding of the DNA and protein of interest (314) (Fig. 12F). Optimized algorithms aid in enhanced predictions of binding motifs from one-hybrid studies (99). Bacterial one-hybrid assays are commonly applied and include studies on selective screening for zinc finger motifs that bind a specified DNA sequence (151, 314) and transcription factors from *D. melanogaster* (474, 739) and *Mycobacterium tuberculosis* (232), the latter by application of the commercial BacterioMatch II kit.

Bacterial reverse two-hybrid systems. The traditional two-hybrid technique can be altered to couple bacterial cell growth to the dissociation of a protein complex, similar to the yeast reverse two-hybrid system. The high cell permeability of bacteria confers a strong advantage regarding experiments that involve addition of small molecules as putative inhibitors of PPIs (204). The first bacterial reverse two-hybrid method was based on reporter gene repression by λ cI, dependent on homodimerization of a protein of interest in fusion with cI (490). Its application led to the discovery of peptides that inhibit HIV-1 protease dimerization. Dissociation of dimerization was observed by derepression of an operon consisting of *lacZ* and the tetracycline resistance marker *tet* (490). A similar concept of derepression by interaction inhibition made use of the tricistronic *HIS3*-Kan^r-*lacZ* operon, originally developed for a forward two-hybrid system (131), as a reporter (267; for the protocol, see reference 266). Coupled with the intracellular synthesis of libraries containing up to 10^8 cyclic peptides, this system yielded the discovery of inhibitors of an enzymatic dimerization essential to HIV infection (267) or purine synthesis (634). Furthermore, application of this approach led to the detection of peptide inhibitors of the interactions between the tumor suppressor p53 and MDM2 or MDMX (115) and between the HIV Gag protein and human TSG101 (635) and to the elucidation of antiviral defense silencing by the influenza virus NS1 protein (451). To enable stable protein expression independent of the plasmid copy number, as well as to reduce false-positive results due to plasmid loss, chromosomally integrated bait and prey vectors are now available (451). In a different approach, the *URA3*/5-FOA counterselection system was employed in the bacterial trap system to search for inhibitors of interaction (440). Another recent system exploits a toxic gene as a marker for PPIs (239). This system makes use of the N-terminally truncated version of the bacteriophage 186 cI repressor, lacking the DNA-binding motif, which has a dominant-negative effect on full-length 186 cI, to induce prophage-mediated cell death or to significantly inhibit cell growth upon its expression (Fig. 12G). Based on this reporter concept, disruption of the interaction between a λ cI bait and an RNAPα prey leads to cell growth. The system was applied to the identification of residues important for dimerization of the human transcription factors Arnt and AhR (239).

Bacterial PCA methods. PPI assays based on the oligomerization-assisted reassembly of split proteins are abundant for use with prokaryotes and include the use of GFP (205), adenylate cyclase from the Gram-negative bacterium *Bordetella pertussis* (322), and murine DHFR (507), among several others.

(i) Split-FP applications in bacteria. The split-FP system was originally described for *E. coli* by Regan and coworkers, in whose study PPI identification was based on fusions to a dissected GFP construct (205). Folding and fluorescence of the split GFP molecule were achieved by bringing into close proximity two fragments of GFP fused to strongly interacting antiparallel leucine zippers. The same research group later provided a set of comaintained plasmids with incorporation of a hexahistidine tag and compatible with *E. coli* strains expressing the T7 polymerase (424). The study also provided evidence that such a system could be used not only for peptide-peptide interactions but also for identification of

interactions between larger proteins. A split-YFP method was used by the group of Ventura to exploit the irreversible behavior of FP fragment folding for detection of transient and weak interactions between individual proteins and between proteins and peptides (for the protocol, see reference 458). With the split-YFP method, weak and strong interactions can be distinguished, suggesting that studies to screen binding affinities could be performed using this technique combined with flow cytometry assays (459). Application of a reverse split-FP strategy is also feasible for identification of competitive inhibitors of a protein association. The interaction of the *E. coli* heat shock protein DnaK with short hydrophobic segments of proteins was used as a case study to identify pyrrhocoricin-related antibacterial peptides as inhibitors of the interaction (457). Incubation of the cells with the potential inhibitors prior to transcriptional induction of the FP fusion construct is a prerequisite of the method due to the irreversible nature of split-fluorophore assays. Split-FP methods have been applied to other prokaryotes, including *A. tumefaciens* (17) and *B. subtilis* (123).

Making use of GFP reconstitution, Umezawa and coworkers worked out an alternative concept based on the intein-mediated protein reconstitution system (PRS) to detect PPIs (485). Inteins are self-splicing proteins that induce the release of reassembled GFP upon interaction of fusion proteins (Fig. 12H) (for the protocol, see reference 320). In the first approach, a variant of the *S. cerevisiae* Vma1 intein was used as a self-splicing protein element to release GFP following interaction of the fusion proteins (485). To avoid the problem of low splicing efficiency with this intein, the system was improved by integration of the split-intein DnaE from *Synechocystis* sp., which allowed the formation of GFP after 4 h, instead of the 3 days required in the previous system. A provisional screening experiment with calmodulin and its target peptide, M13, showed that positive transformants could be selected from a negative pool (486).

(ii) The split-CyaA method. Another PCA technique in *E. coli* is based on the reconstitution of the *Bordetella pertussis* adenylate cyclase CyaA (322). The catalytic domain of CyaA can be separated into two complementary fragments, T25 and T18. When each fragment is fused to a protein of interest, a functional adenylate cyclase can be reassembled upon interaction of the two proteins, which is followed by the production of cyclic AMP (cAMP) in an *E. coli* strain lacking its own adenylate cyclase. Because the activation of genes responsible for the fermentation of maltose and lactose is dependent on cAMP (651), media containing maltose or lactose as the sole carbon source can be used for selection. As one of the most commonly applied and successful bacterial interaction methods, this system has been used widely to discover novel interactors (e.g., see references 85 and 505), to elucidate module-scale interaction networks (e.g., see references 1 and 603), and in particular to establish the network among *E. coli* membrane proteins (321). Because the confirmation of novel interactions by an independent method is a common practice, bacterial two-hybrid plasmids based on the recombination of adenylate cyclase were modified for easy transfer to vectors for single or double affinity purification (33). The system was also adapted for high-throughput screening for dimerization inhibitors of the type IV secretion protein VirB8 (495). Compounds that reduced VirB8 dimerization were detected by reductions in cAMP reassembly and reduced *lacZ* expression under the control of an active cAMP pathway. In this assay, the C terminus of the VirB8 protein is positioned in the periplasm, which reflects the natural environment of the protein and therefore is likely more suitable for compound screens than other *in vitro* or *in vivo* systems (495).

(iii) Other PCA methods in bacteria. Some enzymes, such as β-galactosidase and TEM β-lactamase, can be split into two nonfunctional α and ω peptides which lead to proper function only when they are brought into close proximity. A split-galactosidase system was developed in *E. coli* to confirm known cytoplasmic and membranous PPIs and to validate the association of cytochrome c_2 and cytochrome *c* peroxidase from *Rhodobacter capsulatus* (51). A split-lactamase system for *E. coli* enabled the confirmation of the interaction between the human transcription factors Fos and Jun on selective medium with β-lactam antibiotics, and its development highlighted the importance of linker identity (699). Chorismate mutase (CM) from *Methanococcus jannaschii* is a relatively small enzyme which converts chorismate into prephenate, a crucial step in the biosynthesis of aromatic amino acids. A split-CM system was created for selection on medium lacking aromatic amino acids, with the unique feature that its linker adds strong geometric constraints, which limits its general application but could aid in analyses of the orientation of PPIs (466). As in yeast, the split-DHFR method can be used for *E. coli* survival selection on medium containing trimethoprim. Bacterial split-DHFR assays are used mostly to optimize peptides for increased binding affinity, for example, for leucine zipper domains (507), and peptides that bind the human transcription factor Jun (110). Other PCA methods include a split-Trp system for *E. coli* and *Mycobacterium smegmatis* (479, 556) and a split-adenylate kinase system for *Thermus thermophilus* (470), both with selective reporters.

Genetic Protein-Protein Interaction Methods in Alternative Fungal Species

Two-hybrid assays are typically carried out in surrogate hosts such as *E. coli* and *S. cerevisiae*, which are fast to reproduce, easy to handle, and use the universal genetic code. However, heterologous protein expression of organisms using nonstandard genetic codes is cumbersome in these model host systems. In the fungal kingdom, several *Candida* species, in particular the human pathogen *Candida albicans*, have evolved an aberrant codon usage in which the CUG codon encodes a serine instead of a leucine amino acid (561). To circumvent the problem of erroneous translation in heterologous systems, two interaction methods have been developed in *C. albicans* itself. A classic two-hybrid system was adapted for use in *C. albicans* (623). Reporter systems, DBDs, and ADs were all compatible for application in *C. albicans*, as they did not contain interfering CUG codons or those codons were modified. This system identified known and novel PPIs, not previously identified in the yeast system, among signaling pathways involved in virulence of the pathogen. Prey proteins fused to the viral protein VP16 and bait proteins fused to the *Staphylococcus aureus* repressor LexA were coexpressed from the methionine-regulatable *MET3* promoter in order to avoid unwanted overexpression. A second method makes use of Vps32, a protein associated with the cytoplasmic side of endocytic vesicles, as a bait construct to identify interactions by targeted GFP fluorescence in endocytic vesicles (56). This GFP recruitment system, referred to as the vesicle capture interaction (VCI) assay, can yield quantitative data by computational methods of microscopic image analysis. The *C. albicans* VCI system was employed in a conditional study to illustrate the novel finding that human β-defensins can elicit the interaction

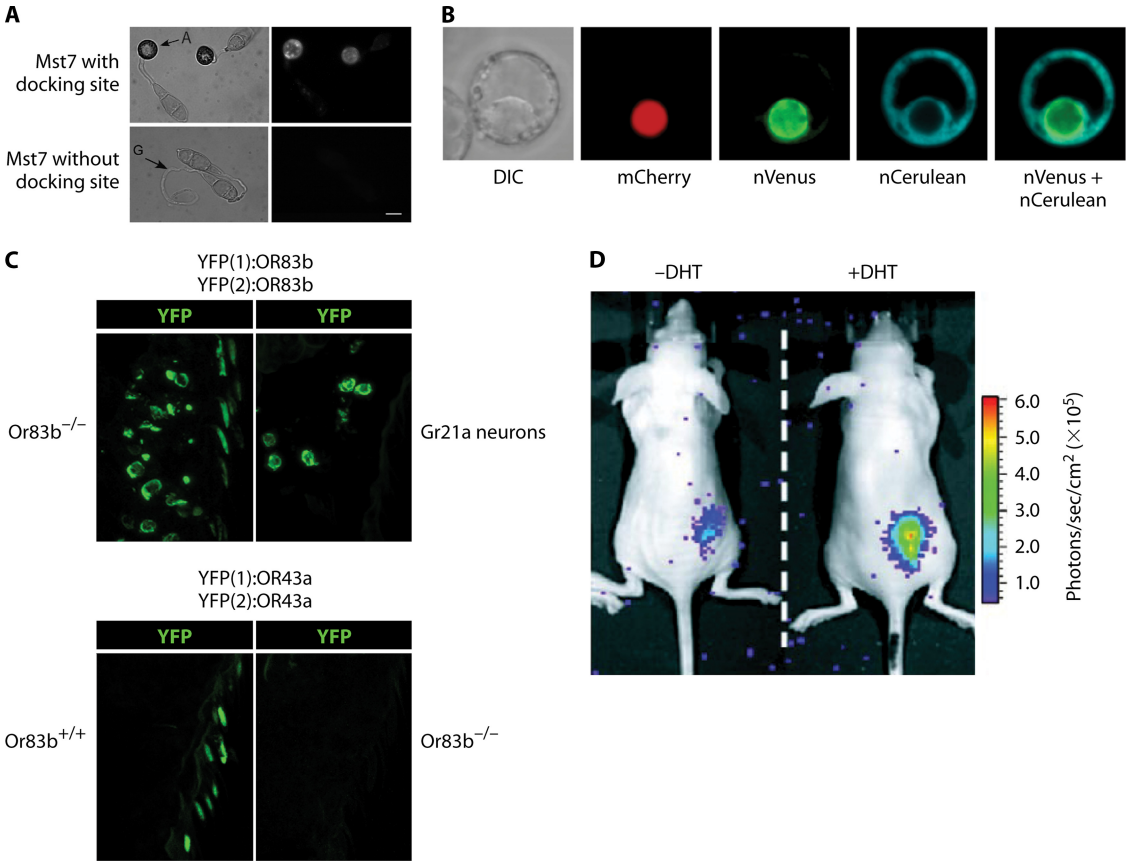

FIG 13 Visualization of PPIs by PCAs. (A) Split-YFP assay in the plant pathogen *Magnaporthe grisea* (732). Pmk1 and Mst7 kinases, which are components of the MAP kinase pathway essential to appressorium formation and plant infection, were fused to the C-terminal and N-terminal parts of YFP, respectively. Interaction between Pmk1 and Mst7 was observed *in vivo* in appressorium formations only when the putative docking site of Mst7 was intact. A, appressorium; G, germ tube. (Adapted from reference 732 with permission.) (B) Multicolor split-FP assay in tobacco culture cells. Protoplasts were transfected by direct DNA uptake and visualized using laser scanning confocal microscopy. Simultaneous interactions between *Agrobacterium tumefaciens* VirE2 (VirE2-cCFP) and the *Arabidopsis* nuclear transport adapter protein importin α-1 (Impa-1-nCerulean) and between VirE2 and importin α-4 (Impa-4-nVenus) were observed in the cytoplasm (cerulean) and nucleus (Venus), respectively. Nuclear localization was confirmed by colocalization of the nuclear marker mCherry-VirD2NLS. Labels below each image indicate the filter set/channel imaged. DIC, differential interference contrast image. (Adapted from reference 375 with permission.) (C) Visualization of odor-evoked calcium release upon formation of functional heteromeric complexes of odorant receptors (ORs) in *Drosophila*, using a split-YFP assay. (Top) Complementary N-terminal and C-terminal fragments of YFP [YFP(1) and YFP(2)] were fused to the odorant receptor OR83b. (Left) Dimerization of OR83b in neurons lacking native OR83b, visualized by the split-YFP method. (Right) Dimerization of OR83b is still visible in Gr21a neurons, which do not express any native ORs, suggesting a direct PPI. (Bottom) Complementary N-terminal and C-terminal fragments of YFP [YFP(1) and YFP(2)] were fused to the odorant receptor OR43a. YFP complementation is visible in neurons with OR83b but not in neurons lacking OR83b. This implicates that OR43a dimerization depends on the presence of OR83b and may not be a direct PPI. (Adapted from reference 38 with permission.) (D) *In vivo* imaging of split *Renilla* luciferase (RLuc) complementation in living mice (344). The strategy to monitor translocation of a particular protein into the nucleus is based on reconstitution of split RLuc by the intein Dna-E (also see Fig. 12H). RLuc-N (N-terminal part) was fused to DnaE-N and a nuclear localization signal. This chimera localizes mainly to the nucleus. RLuc-C (C-terminal part) was fused to DnaE-C and a protein of interest, the nuclear androgen receptor (AR), which localizes to the cytosol. Translocation of AR into the nucleus was visualized upon addition of 5a-dihydrotestosterone (DHT), which binds AR, in COS-7 cells implanted on the backs of mice. DHT-induced translocation of AR results in reconstitution of the DnaE intein and its splicing-reassembly property. Consequently, the spliced and reconstituted RLuc recovers its bioluminescence activity, which is imaged by using a cooled CCD camera and measured as photons per second per cm^2. The differential translocation of AR in the presence (+) or absence (−) of DHT could hence be evaluated quantitatively. (Adapted from reference 344 [copyright 2004, National Academy of Sciences].)

between the kinases Pbs2 and Hog1 in this pathogenic fungus (13).

Split-FP assays have been exploited widely in fungi, often as a validation tool for yeast two-hybrid analyses or coimmunoprecipitation assays. In the first split-FP experiment in fungi, nuclear heterodimerization of two transcription factors by a split-EYFP system was shown in the β-lactamase-producing fungus *Acremonium chrysogenum* (263). All gene fusions were expressed under the control of the *Aspergillus nidulans gpdA* promoter and *trpC* terminator to ensure stoichiometric expression of the different constructs. The same split-EYFP vectors were recently applied in the fungus *Penicillium chrysogenum* (262). Nuclear fluorescence was observed between two components of the Velvet-like protein complex, while this interaction was not identified by yeast two-hybrid analysis. This suggests the requirement of a bridging protein to bring together the two proteins of interest and further highlights the need for interaction identification in homologous systems. The YFP-based approach was further used in *Aspergillus nidulans* (48), the plant pathogen *Magnaporthe grisea* (732) (Fig. 13A), the model fungal organism *Neurospora crassa* (25), and the

homothallic ascomycete *Sordaria macrospora* (166). Alternatively, a Venus-based system similar to the one described for *C. elegans* (595, 596) was established in the fission yeast *Schizosaccharomyces pombe* (6). Plasmids were constructed that allow convenient C-terminal tagging of proteins of interest expressed from their endogenous chromosomal locations and under the control of their native promoters. In a case study, the spatial dynamics of the copper transporter Ctr4-Ctr5 complex was shown using Venus complementation in *S. pombe*. Addition of high concentrations of copper induced internalization of the complex, as the fluorescence signal progressively shifted from the cell surface to the vesicles (289). Finally, a split-EGFP assay using constitutive expression constructs has been described for the plant-symbiotic fungus *Epichloë festucae* (631).

Genetic Protein-Protein Interaction Methods in Plants

In vivo protein-protein interaction methods in higher eukaryotes offer the ability to study known or novel PPIs in their native cellular context, and in real time in living cells when visualization is possible. In the last decade, such methods have been established in plants, in particular in the model plant *Arabidopsis thaliana* (also reviewed in reference 462).

Two-hybrid tools *in planta*. The classic two-hybrid method was replicated in *A. thaliana* protoplasts by use of Gateway-compatible vectors (161). High-copy-number vectors allow expression of DBD- and AD-fused proteins of interest under the control of the strong promoter from cauliflower mosaic virus 35S. Binding of the interacting partners to a *GAL4-UAS*$_4$*::GUS* reporter system promotes expression of β-glucuronidase (GUS), used as a semiquantitative readout. Coexpression of a Pro$_{35S}$/NAN (synthetic neuraminidase gene) vector is employed to normalize GUS measurements for variation in protoplast transfection efficiency. The proof of principle of this method was based on interactions between basic leucine zipper (bZIP) transcription factors and on a direct comparison of the same interacting partners in the yeast two-hybrid system. Novel and weak heterodimerization events that were not detected in the yeast system were identified using the plant two-hybrid approach. An additional two-hybrid method was developed for detection of PPIs involving transcription factors (435). Similar to the repressed transactivation method in yeast, the prey protein is fused to a repressor domain, in this case the ERF-associated amphiphilic repression motif SRDX. The autoactivating bait is linked to the Gal4 DBD, and interaction between bait and prey results in repression of a luciferase reporter gene. Interaction between the human Fos and Jun transcription factors was confirmed with this method, together with the association of two MADS box plant proteins in transgenic *Arabidopsis* plants (435).

Another two-hybrid method was engineered as an indicator of human estrogenic activities, using transgenic *A. thaliana* constitutively expressing two effector proteins (643). These consist of a LexA-linked estrogen human receptor and a VP16-fused chimeric human nuclear receptor coactivator. Estrogen-dependent interaction between the two chimeras induces transcriptional activation of the β-glucuronidase reporter. This system illustrates the use of *A. thaliana* to detect the presence of 17-βa-estradiol at concentrations as low as 50 pM, as well as other estrogenic substrates. This low-cost and sensitive two-hybrid system was further improved by increasing the copy number of the plasmid carrying the prey fusion gene (630). The system was hence rendered five times more sensitive than previously available assays in *A. thaliana* and other organisms (464, 471, 657).

In a particular study where neither split-FP nor yeast split-ubiquitin assays detected PPIs between two membrane receptors, ERS1 and ETR2, interaction between these proteins was established in a membrane recruitment assay (221). The full-length ethylene receptor ERS1 fused to red fluorescent protein (RFP) served as an anchor to recruit GFP-fused target proteins, which was visualized by colocalization of the fluorescence signals. In this GFP recruitment assay, interaction between ERS1 and the cytoplasmic version of ETR2 was illustrated in plant cells.

Split-FP assays in plants. Methods of visualization and identification of PPIs in subcellular compartments became available in the context of living plant cells with the development of the split-FP method. The first demonstration of the efficacy of YFP complementation to detect PPIs in plant cells derived from two studies (57, 684). Ohad and his group demonstrated PPIs at the tissue and subcellular levels in *Nicotiana benthamiana* and *Arabidopsis* leaves (57). Kudla and coworkers illustrated the dimerization of the *Nicotiana tabacum* 14-3-3 protein T14-3c in *Arabidopsis* protoplasts and in *Agrobacterium*-infiltrated tobacco leaves by using Gateway-compatible split-YFP vectors, which were later used by many other groups (684). Since then, the use of split-FP assays in plants has boomed, and recent reports comprehensively review this PCA method (42, 101, 477, 478). Protocols on how to use the split-FP method as a tool to study PPIs in plant protoplasts are also available (e.g., see references 498 and 701).

The diverse targets in which the assay has been employed range from protoplasts to seedlings, leaves, or epidermal cells in *Arabidopsis* but also in tobacco, mustard, parsley, leek, and onion plants. Many fluorescent proteins have been reported, including the commonly used YFP (with N- and C-terminal residues YN155 and YC155 or YN173 and YC173), but also Venus, GFP, CFP, SCFP3A (modified CFP), blue fluorescent protein (BFP), cerulean, citrine, and RFP (117). Multicolor split-FP assays for simultaneous or preferential PPI detection have been adapted for plant research, based on the use of the combination of SCFP3A N- and C-terminal fragments with the Venus N-terminal fragment, as well as with fragments from CFP, GFP, YFP, and DsRed-monomer (353, 678) (Fig. 13B). Multicolor expression vectors were also developed in the pSAT series of vectors to facilitate the practice of the method (377). In this study, the differential interaction of the *Agrobacterium* VirE2 protein with the *Arabidopsis* importins α-1 and α-4 was illustrated by the cytoplasmic and nuclear localization of the yellow and blue fluorescence signals, respectively. The analysis of interactions between more than two proteins has also been achieved successfully by imaging with combined split-FP and FRET fluorescence (368). Formation of ternary complexes in leaf epidermal cells was visualized as a result of simultaneous interactions between three fluorophore-tagged polypeptides.

The interest in improving fluorophores is evident in the split-FP field, and recent technical efforts on the use of fluorophores in plants have been reported. A truncated version of YFP (lacking two C-terminal amino acids) that greatly eliminated unspecific YFP reconstitution proved to be efficient in split-FP experiments in *Arabidopsis* protoplasts (388). Although most fluorescence microscopes have the capacity to detect GFP fluorescence, the use of GFP-based PCA has been hindered by the low reconstitution efficiency of split-GFP fragments. However, the combination of the N-terminal region of GFP with either the

C-terminal region of CFP or a mutated V163A version of the GFP C-terminal domain showed bright green fluorescence that was 7-fold more efficient than that with the original split-GFP setup (351). An optimized monomeric RFP (mRFP)-based assay was recently described for the investigation of plant-virus interactions in *N. benthamiana* (740). The new plasmids enable fusion of proteins of interest to either the N- or C-terminal domain of the mRFP fragments, and they possess a linker to improve the flexibility of the chimeric proteins and a c-myc or HA tag to allow immunoblot analysis. Work has also been done in improving the detection sensitivity of PPIs in plant protoplasts by developing a cell sorting procedure (730). Flow cytometry analysis of fluorescence signals in protoplasts isolated from plants with low transformation efficiency can facilitate subsequent PPI identification by confocal microscopy. With the aim of performing high-throughput analyses using the multicolor split-FP assay, Gateway-compatible vectors expressing all possible combinations of SCFP3A and Venus, fused N- or C-terminally, were recently generated (203). In a case study, the vectors were used to show simultaneous interaction between Cnx6 and Cnx7 and between Cnx6 molecules themselves, forming an interacting complex of the molybdopterin synthase.

Despite the advantages offered by the split-FP method, its applicability to whole-interactome mapping has been limited (731). It was employed, however, in parallel experiments with the yeast two-hybrid system to determine the pairwise interactome network of 58 core cell cycle proteins of *Arabidopsis* (53). GFP fragments were fused C-terminally to target proteins and expressed under the control of a strong 35S promoter. Out of 917 possible interactions, 341 were positively identified with the split-GFP method, while only 77 were established by the yeast-two hybrid approach and only 17% of PPIs were identified by both techniques. For the split-GFP assay, a negative-control set with 40 protein pairs did not show positive results. Interestingly, for 20% of all interactions identified by split-GFP assay, reciprocal expression of the target proteins was necessary for proper GFP refolding. In addition, this method allowed the exclusive detection of 78% of all PPIs, while the yeast two-hybrid assay detected fewer than 5% of PPIs that were not identified by the PCA technique. Overall, these data provide an example of how each technique should be considered and highlight the power of the split-FP strategy and the use of endogenous host cells. In addition, this study resulted in the identification of novel interacting pairs between cyclins of the CDK-CYCD complexes. These binary interactions induced cell division in differentiated tobacco epidermal leaf cells but also in *Arabidopsis* cells (52). The interaction data were also processed together with gene expression and localization data in a compiling analysis that highlighted distinct protein clusters at each step of the cell cycle. In a comparative analysis between the plant split-FP system, yeast two-hybrid system, tandem affinity purification, and predictive algorithms, the same group revealed platform-specific interactions, a large number of PPIs that were not predicted, and overall limited overlap between the methods (658).

Other PCA methods in plants. Detection of PPIs based on the reconstitution of reporters other than fluorophores has been attempted in plant cells. Interaction-induced folding of murine DHFR was employed in tobacco protoplasts (625). The reconstituted enzyme binds fMTX, which is retained in cells and can be monitored by spectroscopy, FACS, or fluorescence microscopy. In contrast to the successful implementation of split-DHFR assays, the use of β-galactosidase PCA is poorly suitable for plants due to the high intrinsic level of β-galactosidase activity (637).

As a complement to the split-FP method, split-luciferase assays based on the *Renilla reniformis* and *Photinus pyralis* (firefly) luciferase enzymes have been used in protoplasts and whole plants, respectively. PPIs between nuclear histones 2A and 2B and between the membrane proteins SYP51 and SYP61 were demonstrated in the protoplast system (188). This work in protoplasts was applied to construct a series of vectors suitable for high-efficiency transgene expression, to increase the dynamic range of PPI detection levels, and to engineer a large-scale analysis platform of protoplast transfection using 96-well plates (325). This method reliably identified interactions between the membrane-associated SNARE proteins (324). Luminescence was measured within *Arabidopsis* protoplasts expressing the recombinant proteins at physiological levels. The stringency of the assay was determined by single amino acid substitutions resulting in reduced SNARE-SNARE interaction and by modulating the interactions by use of sodium azide. In plants, the system was adapted to enable both transient expression of fusion proteins and generation of stable transgenic plants (86). Two fragments of the firefly luciferase, i.e., NLuc (aa 2 to 416) and CLuc (aa 398 to 550), were expressed under the control of a strong 35S promoter. Multiple pairs of known interacting proteins were used to validate the system.

Genetic Protein-Protein Interaction Methods in Nonmammalian Animal Models

Interaction methods in invertebrates. The cellular milieu of the sea hare *Aplysia californica*, a model organism in neurobiology, is considerably more salty than that of yeast cells. To study interactions between cAMP-dependent transcription factors in their native environment, an *Aplysia* two-hybrid system was created with the traditional elements from the yeast method, namely, the Gal4 DBD, the Gal4 AD, and *lacZ* as a reporter gene (97).

The two-hybrid methodology was also adapted in a cultured insect cell model, providing an alternative method for surveying PPIs that cannot be studied in yeast, in particular those affected by posttranslational modifications such as glycosylation, phosphorylation, and acetylation (456). This insect two-hybrid system involves two proteins of interest, fused to the yeast Gal4 DBD and to the AD of mouse nuclear factor kappa B (NF-κB), with firefly luciferase as the reporter. The DBD and AD constructs were placed under the expression of the immediate-early promoter (IE2) from the *Orgyia pseudotsugata* baculovirus, which is known to allow protein expression in several insects, enabling the use of the method in a wide range of cell lines. The system shows high sensitivity due to low background luciferase activity, with the luciferase gene placed under the control of a minimal *HSP70* promoter linked to Gal4 upstream activating sequences. It further permits the elimination of false-positive substrates, such as autoactivators, obtained in the yeast two-hybrid system (456).

In contrast to the extensive use and adaptation of the split-FP method in plants and mammalian systems, PCA technologies to study and visualize PPIs in nonmammalian animal model organisms are sparse. Among invertebrates, the transparent body of the worm *Caenorhabditis elegans* makes this organism an excellent model for spatiotemporal PPI research involving fluorescence-based applications. A traditional proof-of-principle interaction between leucine zipper polypeptides was detected with split GFP, split CFP, and a combination of GFP and YFP fragments in *C.*

elegans (729). Using a YFP reconstitution assay, temporal and spatial interactions of the stomatin-like protein UNC-1 and the innexin UNC-9 at intercellular junctions were reported (84). A split-Venus system for use in the worm was introduced by Hu and coworkers (257). Direct visualization of the binding of the leucine zipper domains from the *C. elegans* transcription factors FOS-1 and JUN-1 was demonstrated using an inducible heat shock promoter, with the appearance of fluorescence 30 min after induction. The heat shock promoter was employed to counteract the irreversibility of the split-fluorophore approach and to avoid the potentially detrimental effects of such a system on cellular and developmental behavior. Protocols for this assay are available elsewhere (257, 595). Applications of the split-Venus system in *C. elegans* include detection of the nucleus-localized interaction between worm BRCA2 and mammalian Rad51, involved in DNA repair (450); oligomerization of DYN-1, essential for endocytosis, at specific membrane regions along the apical surface of intestinal cells (248); nucleus-localized association of the transcriptional regulators MLS-1 and UNC-37 (448); and the PPI between the BK channel subunit SLO-1 and an auxiliary subunit, BKIP-1 (83). The last study did, however, reveal a possible drawback of using the highly sensitive but lowly selective Venus method. While a coimmunoprecipitation experiment clearly showed that the binding of SLO-1 with BKIP-1 depends on the transmembrane and intracellular domains of BKIP-1, split-Venus results remained positive after removal of the intracellular region. This suggests that, in this particular experiment, close proximity rather than actual binding may have caused a positive outcome.

A GFP recruitment assay in *C. elegans* links interactions to the localization of fluorescence signals to the membrane, in the so-called differential cytolocalization assay (DCLA) (45). A reciprocity test performed by switching the identities of the bait and prey proteins showed that, in most cases, the interactions were retained in this assay. Comparative analyses of the DCLA system with coimmunoprecipitation and yeast two-hybrid data showed only very little overlap in the interaction sets identified, yet controls for false-positive results failed to show interaction. These data support the complementary nature of the different detection methods.

The split-YFP technique has also been applied in the fruit fly model for PPI detection between odorant receptors in olfactory sensory neurons (38) (Fig. 13C). A similar approach was established in *Drosophila* larvae, based on Venus fragments fused to transcription factors and coexpressed by the heat shock Gal4/UAS system (518). Split-fluorescent constructs, stably expressed under the control of endogenous promoters, were used under physiological conditions in *Drosophila* embryos to analyze dynamic transcription factor PPIs (284). Protein fusions with Venus, cerulean, and mCherry fluorophore fragments were generated, and PPIs were observed 28 h after embryonic maturation. Finally, Gateway vectors bearing YFP fragments and epitope tags were generated and applied in a study on PPIs between actin nucleation proteins in the wing epithelium and visual system of the host (211).

Interaction methods in vertebrates. Among vertebrates, the split-FP method has been described for *Xenopus laevis* (558). A mutated version of Venus was developed to deal with high autofluorescence in *Xenopus* embryos. The combination of VNm9 and VC155 fragments of Venus gave no fluorescence background in this system, which was also less sensitive to environmental changes such as pH and chloride concentrations. The technique detected an interaction between phosphorylated Smad proteins *in vivo* and in response to growth factors. Homomers and heteromers of Smad proteins, which are regulatory proteins of cell proliferation and differentiation, were identified at different stages of *Xenopus* development, and some were translocated to the nucleus after addition of transforming growth factor beta (TGF-β) growth factors, such as activin and nodal-related proteins (236, 558). This Smad2/4 split-FP version provides a direct and quantitative readout for activin-like signaling, with a good signal-to-noise ratio. A similar methodology was followed for zebrafish embryos in a study that revealed the formation of a graded distribution of nodal signaling activity (242).

Mammalian Genetic Protein-Protein Interaction Systems

Although from a strictly technical point of view, two-hybrid and PCA techniques are more demanding to set up in mammalian cells, two main reasons have urged researchers to create such assays.

First, conceptually, PPIs should ideally be studied in their normal physiological context. Many human proteins will not behave properly in nonnative cells, with the main underlying reasons being the different spatiotemporal organization and repertoire of secondary modifications in a mammalian cell compared to those in a unicellular yeast cell. This may not be too problematic for "static" interactions, e.g., PPIs that govern structural elements or molecular machines, but may pose considerable problems in analyzing the dynamics of a protein interaction network. This is particularly true for signaling cascades, from the receptor down to altered PPI complexes at the promoter level, but also applies to various other dynamic processes, including regulated alternative splicing and translation, protein transport mechanisms, vesicle transport, and the plasticity of the actin cytoskeleton and intermediate filaments. Consequently, the effects on a PPI network of altering the cellular milieu, e.g., by external stimuli, can be addressed appropriately only in the native cellular format. Although efforts have been made to introduce some context dependency in yeast cells, e.g., in three-hybrid systems using exogenously added tyrosine kinases, the ever-expanding complexity of posttranslational modifications (N-acetylation, acylation, methylation, glycosylation, various types of ubiquitination, etc.) that control much of the above-mentioned mechanisms strongly argues for the performance of PPI analyses in their proper physiological context. An inherent consequence of this vast heterogeneity is that no single method can be expected to cover the full protein-protein interaction space, especially in mammalian systems.

Second, PPIs are increasingly being recognized as *bona fide* targets for drug discovery. A growing number of small molecules capable of disrupting designated PPIs are being uncovered, likely through the use of optimized chemical libraries and assay systems (also see examples below). Clearly, potential PPI targets outnumber single-protein targets, such as enzymes, G-protein-coupled receptors (GPCRs), or ion channels, and in fact provide a highly needed alternative, for example, in the case of viral targets where all single-enzyme targets are being exhausted, e.g., for HIV-1. In-cell screening for such PPI modifiers by use of human cell-based assays offers clear advantages. Besides providing the optimal physiological context, compounds are also intrinsically selected for the ability to permeate the membrane of the mammalian phospholipid bilayer. In addition, off-target effects can be detected using three-hybrid systems.

In this review, we divide the mammalian two-hybrid systems

into three classes. The first group represents mere adaptations of the yeast two-hybrid assay to the mammalian cellular environment, and the second group comprises various forms of the PCA. The third group encompasses techniques that are based on unique features of mammalian cells, together with strategies that so far have been pioneered only in mammalian cell systems.

Adaptations of the yeast two-hybrid system. An early example of a conceptual replica of the two-hybrid system was its use to analyze complex formation between leucine zipper transcription factors. The system was based on the Gal4 DBD and VP16 AD, with chloramphenicol acetyltransferase (CAT) as the reporter (113). Later on, similar strategies were used to investigate interactions between SMAD complexes and the transcriptional coactivator CBP (644), as well as protein kinase A (PKA)-dependent changes in the interaction between ERa and SRC1 (744). While such applications relied on transient transfection of bait, prey, and reporter vectors, an optimized version was developed by Isselbacher and colleagues whereby a GFP-based reporter and the bait construct were stably integrated into the cell's genome and prey expression was propagated from a stable, extrachromosomal vector by use of the EBNA-1/ori-P system. In contrast to analytical applications that are limited to selected PPIs, this approach allowed for cDNA library screening using a designated bait (592). More recently, such mammalian two-hybrid assays were optimized to allow medium- and even high-throughput interaction mapping. Pan et al. reported a genomewide SARS coronavirus intraviral PPI map encompassing 40 (often reciprocal) interactions. Notably, several overlapping PPI pairs were found with two previously reported yeast two-hybrid screens, all representing strong signals in the mammalian system. No overlaps were seen between the data sets obtained with yeast as the host (488). The cell array protein-protein interaction assay (CAPPIA) combines a DNA microarray with a two-hybrid readout (177). In brief, bait- and prey-encoding plasmid vectors, together with a reporter directing the expression of an autofluorescent protein, are spotted as transfection mixes on glass slides. Cells are seeded on top and take up the DNAs by reverse transfection. Readout is subsequently performed using a DNA array scanner or by high-throughput microscopy. In the proof-of-concept study, a medium-scale screen using (fragments of) the androgen receptor as bait and a selected set of preys was performed, totaling 160 combinations, demonstrating the cost-effectiveness and efficiency of the procedure. In 2010, extensive mammalian two-hybrid assay-based PPI maps were established using pairwise analyses of 1,988 human and 1,727 mouse transcription factors, revealing a total of 762 and 877 interactions, respectively (535). Interestingly, besides providing novel insights into transcription factor network evolution, evidence that tissue-specific effects were generated was provided by combining broadly expressed with tissue-specific transcription factors, implying tissue-restricted interaction patterns.

Another variation on the two-hybrid theme in mammalian cells is the tetracycline repressor-based system, trM2H (638). In trM2H, hybrids are composed of three functional parts: DNA binding of two tetracycline repressor (TetR) fragments is restored upon bait-prey interaction, leading to activation of transcription by the VP16 AD domain. Bait and prey are thus flanked by the TetR fragments and by the VP16 AD, imposing considerable topological constraints that may in some cases restrict its use. However, the system holds the promise of being highly sensitive, as it can detect the low-affinity (55 μM) sortase A dimerization.

The mammalian two-hybrid system also has applications in drug discovery. Recently, small-compound inhibitors of trimerization of gp41, an HIV-1 protein involved in cell entrance, were discovered by systematic screening for interaction inhibition using 96-well plates and luciferase as a reporter (594). It was suggested that high-throughput compound screening with 384-well plates should be feasible using the mammalian two-hybrid system. In a similar study, more than 3,000 compounds were screened for inhibition of the binding of MDM2, an E3 ubiquitin ligase, to the tumor suppressor p53 by employment of the mammalian two-hybrid method (386). In a Western blot assay, positive hits were found to partially increase the levels of p53 by suppression of MDM2-induced degradation. Commercial mammalian two-hybrid kits are available from Promega (CheckMate system).

Similar to the case for prokaryotes and fungi, FP recruitment systems have been developed for use in mammalian cells. These redistribution assays, based on the colocalization of bait and prey in a particular area of the cell, use fluorescence microscopy as a readout. Baits are typically, but not necessarily, tripartite constructs combining the bait with a tether and an FP that serves as a location control. The prey is also fused to an FP to monitor its position in the cell. These (trans)location biosensors are explored mostly in mammalian cells and were first reported for nuclear targeting (350). In a more sophisticated variant, the bait is linked to the LacI repressor so that bait-prey complexes are targeted to the chromosomal DNA by interacting with a stably integrated array of *lac* operator sequences (742). Confocal microscopy is then used to visualize DNA-bound prey FPs. The nuclear translocation assay (NTA) uses ligand-induced redistribution of a bait-prey complex whereby the bait is fused to a localization-controllable EGFP construct (136). The translocation cassette is composed of a nuclear export signal (NES), to keep the bait in the cytosol, fused to a nuclear import signal (NLS) and the ligand-binding domain (LBD) of the glucocorticoid receptor. Dexamethasone induces a conformational change leading to exposure of the NLS and subsequent nuclear translocation of the bait. The prey is fused to the dsRed FP, and its translocation to the nucleus is monitored as a parameter for interaction with the bait. Other examples of FP recruitment assays include prey targeting to cell membranes (45, 697), viral intracytoplasmic protein aggregates (446), and P bodies (46).

PCA technologies in mammalian cells. PCAs have also been adapted very successfully to mammalian cell systems. Reassembly of *E. coli* β-galactosidase upon bait-prey interaction was pioneered by the Blau group (549) and was initially developed with a colorimetric readout. In a later implementation, this method was used to dissect ligand-dependent interactions between EGFR family members (700). This work provided important new insights into the dynamics of EGFR subunit clustering upon EGF-type ligand addition and into the impact of the anticancer Herceptin antibody thereon. Although PPIs with G-protein-coupled receptors are notoriously difficult to monitor, two adaptations of the β-galactosidase assay were developed based on universal features of the GPCR system: ligand-dependent recruitment of β-arrestin (675) and endocytosis (238). In the latter case, one enzyme moiety was tethered to endosomes, leading to reconstitution of enzymatic activity only after fusion of endocytotic vesicles containing the activated GPCR complex. Besides providing valuable tools for studying GPCR biology, both assays were also adapted to high-throughput compound screening. Intrinsic to this split-β-galac-

tosidase technique is the separate folding of each enzyme fragment and subsequent spontaneous assembly of the whole protein. Although mutations can be introduced to reduce complementation in the absence of a bait-prey interaction, background noise may hinder detection of weak signals.

Such background noise is avoided by using PCAs in which folding depends on fragment proximity that is induced by a bait-prey interaction. Originally explored in yeast cells, such folding-dependent complementation was first reported for murine DHFR in mammalian cells by Michnick and colleagues. In this case, nucleotide-free medium is used for growth selection in DHFR-deficient cells (543). Apart from the survival assay, fluorescein-conjugated methotrexate can be applied as an additional control for interaction. Typical applications of this split-DHFR system in a mammalian context include the demonstration that the erythropoietin receptor exists as a preformed complex that requires ligand-induced conformational changes for activation (546) and the analysis of a signaling network controlling translation initiation (545). Another folding-dependent PPI sensor is the split-lactamase system using fragments of *E. coli* TEM β-lactamase. Reporter activity can be measured either by *in vitro* colorimetry in cell lysates or by *in vivo* fluorescence (194). Taking advantage of the mammalian context, Spotts et al. demonstrated the phosphorylation-dependent CREB-CBP interaction upon elevating cAMP levels by exposure of the cells to forskolin, an activator of adenylate cyclase, or to the cell-permeating cAMP analog CPT-cAMP. Notably, time-lapse microscopic registration of β-lactamase activity could be monitored in single neurons upon cleavage of the CCF-2 fluorophore (608). A more recent application of this assay detected the interaction between the HIV-1-encoded viral infectivity factor (Vif) protein and the human APOBEC 3 cytidine deaminase (72). APOBEC 3G and -F are potent restriction factors that counteract an HIV-1 infection but are themselves targeted by Vif, which marks them for proteasomal degradation. Such straightforward assays to map this interaction can also be used in high-throughput drug screening campaigns to assist in developing novel anti-HIV-1 therapeutics. Protocols for split-DHFR, split-lactamase, and split-FP (see below) applications in mammalian cells can be found elsewhere (544).

Split-luciferase systems have been used frequently in mammalian cell cultures. The reversible character of luciferase reassembly was exploited in the investigation of GPCR-induced deassembly of protein kinase A regulatory and catalytic subunits (615). This study highlighted the use of split-luciferase methods to establish the pharmacological profiles of GPCR-based candidate drugs. Recent examples that demonstrate the flexibility of the split-luciferase approach in mammalian cells include the development of optical probes to monitor fusion of cellular organelles such as mitochondria (277), to detect intraviral PPIs (126), actin polymerization (291), and amyloid-β peptide oligomer formation (244), and to map individual amino acid residues involved in chaperone protein complex formation (309).

Several split-luciferase-based optical sensors have also been used in small animals by use of implanted cells that express the two sensor fragments (344, 416, 501, 542; for a protocol, see reference 672). Imaging is then typically performed using cooled charge-coupled device (CCD) cameras. Examples include ligand-dependent nuclear translocation of the androgen receptor (344) (Fig. 13D) and the intramolecular folding of the estrogen receptor driven by endogenous estradiol levels (499). Such split-luciferase applications are promising tools for studying the pharmacokinetic behavior of pharmaceuticals that target PPIs in experimental animal model systems and were recently suggested to monitor *in vivo* activation of the EGFR and Her2/neu pathway during (radio) therapy (390, 709). Alternatively, PPIs in animals can be spotted by the split-thymidine kinase (split-TK) system (434). Thymidine kinase from herpes simplex virus 1 phosphorylates nucleoside analogues. Radioactively labeled nucleoside derivatives are retained in the cell upon phosphorylation by TK, which can be observed by positron emission tomography (PET) in living organisms. TK was originally used as a reporter gene in a classic two-hybrid design for living animals (415). However, by a combination of random fragment libraries and rational design, a split-TK method was developed and led to a system which can detect PPIs in deeper tissue (434).

Although the split-FP method was originally reported for *E. coli* and used GFP fragments (205), Kerppola and coworkers were the first to establish the split-FP method in mammalian cells, based on reconstitution of YFP (270). The excellent spatial resolution in different cellular compartments of mammalian cells by application of the split-FP method is highlighted in numerous reports (reviewed in reference 335; for a protocol, see reference 336). Although the fluorescence intensity of reconstituted FP complexes is estimated to be about 10-fold below that of wild-type GFPs, the low autofluorescence of mammalian cells ensures that signals can still be detected even when the protein pairs are expressed at endogenous levels. The irreversible character of the split-FP system has been exploited in several studies with mammalian cells. As an example, it was suggested for capture of oligomer formation that precedes the protein aggregation that accompanies several neurodegenerative diseases (215).

GFPs exist as a wide range of spectral variants, and accordingly, multicolor split-FP variants were developed to simultaneously capture multiple PPIs in different subcellular locations (271). Multicolor split-FP assays also allow for competition studies between different proteins for a shared partner, which can be especially useful for mapping mutually exclusive interactions with so-called hub proteins (225, 442). Multicolor split-FP assays can also be combined with BRET or FRET readouts, allowing the study of complex formation involving up to four proteins (196, 537). Note that multicolor split-luciferases were also developed recently (258).

In contrast to its widespread use for designated PPIs, high-throughput cDNA library screening applications using the split-FP method are rather limited (544), possibly due to intrinsic topological constraints or to variations of prey expression levels causing strong fluctuations in signal intensity. Very recently, however, an extensive retroviral vector-based human ORFeome screen using a split-Venus configuration identified several novel putative partners of core telomere-associated proteins, holding promise for future large-scale split-FP applications in mammalian cells (376). Direct effects of small molecules on fluorescence intensity are frequently observed and may hamper applications in drug screening. Yet off-target effects of drugs can be monitored using an elaborate panel of FP reporters selected to detect off-target effects on multiple biochemical pathways in human cells (421).

The use of photoswitchable fluorophores may also be an interesting prospect for split-FP applications. In that respect, the group of Miyawaki pioneered the development of a light-induced con-

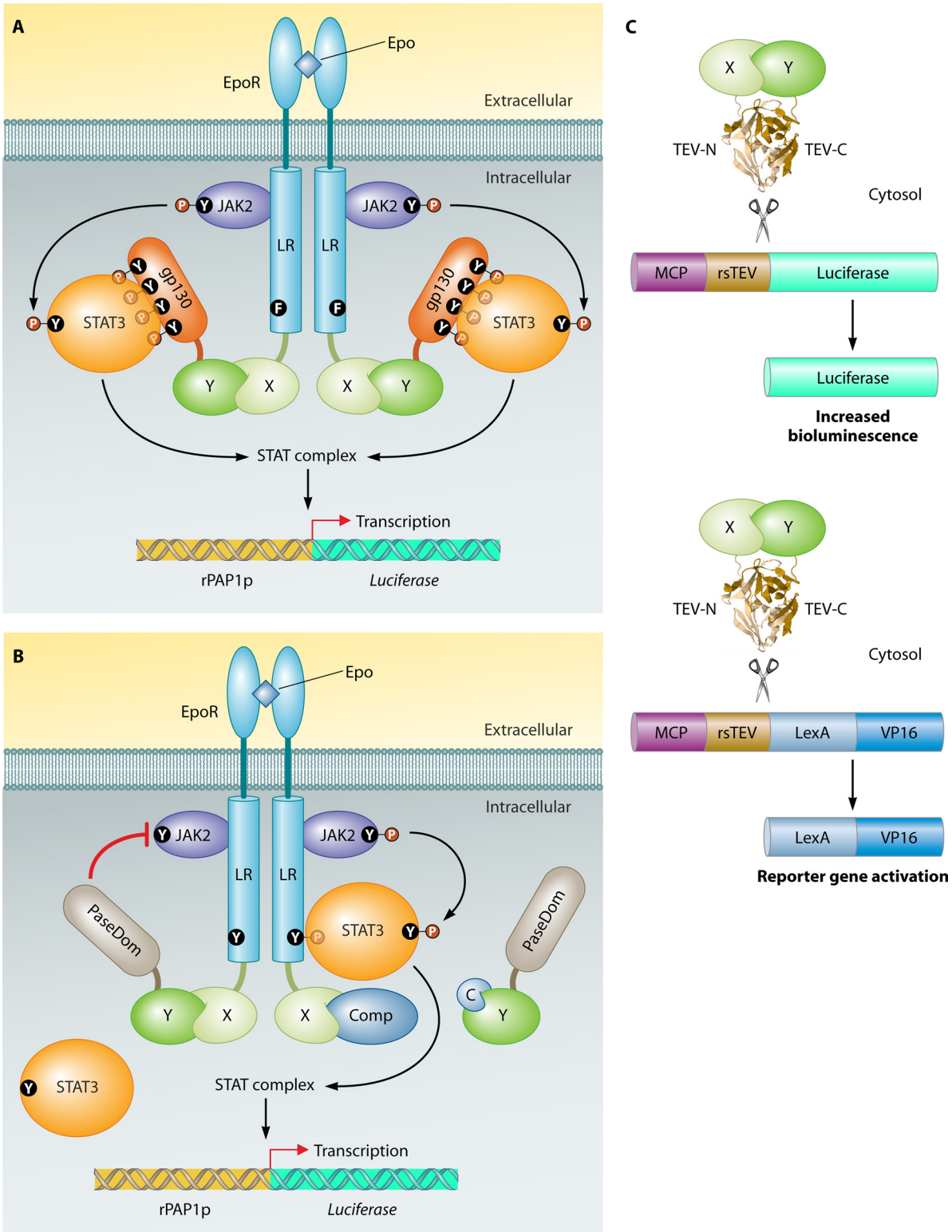

FIG 14 Unique mammalian genetic PPI methods. (A) Mammalian protein-protein interaction trap (MAPPIT) (172). The bait protein is a fusion with a leptin receptor (LR), which contains three Y-to-F mutations so it is unable to activate STATs spontaneously (one representative phenylalanine [F] is shown). The prey fusion contains a domain of gp130 which can recruit STATs. After interaction of the bait with the prey, Janus kinases (JAKs) phosphorylate gp130, which stimulates binding of gp130 with the STATs. The STATs themselves are phosphorylated by the JAKs, which results in the formation of a STAT complex. The STAT complex binds the rat PAP1 promoter (rPAP1p) and activates luciferase transcription. The leptin receptor is further fused with the extracellular domain of EpoR, a receptor of erythropoietin (Epo), and therefore LR complex formation, which is necessary to make the association with the JAKs, is induced by addition of Epo. (B) Reverse MAPPIT (171). For reverse MAPPIT, a functional LR protein is used with one tyrosine (Y) residue that can be phospharylated upon activation. (Left) The prey Y fusion contains a phosphatase (PaseDom) domain which, upon interaction with bait X, removes the phosphate of JAK2, thereby

version between the bright and dark states (on-off switch) of a new green fluorescent protein termed Dronpa (10). The unique photochromic property of Dronpa is that it can be excited, erased, and excited again, and this can be used as a tool to monitor the dynamics of molecular processes. Since then, several reversibly switchable fluorescent proteins (RSFPs) have been engineered in the emission spectra of blue-green, green, and red fluorescence (12, 58, 619, 620). In the context of PPI studies, a red RSFP (rsTagRFP) was recently employed to illustrate modulations in both the fluorescence intensity and lifetime of the fluorophore during interaction between a growth factor receptor and a binding protein in live mammalian cells, using photochromic FRET (624). Dronpa was also used successfully in split-FP assays to investigate PPIs in live cells (378). The reversible photoswitching activity was illustrated for full-length and fragmented fluorophores. Photobleaching was induced by irradiation at 488 nm for 1 min, while fluorescence was restored by irradiation at 430 nm for 30 s. These data now open the prospect of the split-RSFP system for PPI studies to overcome the problems of photobleaching and low quantum yield.

Interactions between proteins of the secretory pathway, which make up one-third of the proteome, have typically been difficult to examine. Citrine, a YFP variant with a Q69M mutation, was found to be photostable in various cellular compartments, including the lumen of the secretory pathway (222). A split-FP assay based on this fluorescent protein was able to localize PPIs between the cargo receptor ERGIC-53 and various glycoproteins (475). Recently, interactions among *N*-glycosyltransferases in the Golgi apparatus were also investigated by this approach (245).

It can be expected that continuously ongoing efforts to prevent spontaneous fragment association, to optimize temperature and pH dependence of chromophore maturation, and to further extend the spectral repertoire will foster even more applications of the split-FP approach (100, 173, 293, 352, 378, 402, 596, 736).

Unique mammalian systems. The first example of a two-hybrid method whose design is based on intrinsic features of a mammalian cell is the mammalian protein-protein interaction trap (MAPPIT) system (172). MAPPIT relies on the JAK/STAT signaling pathway, which is typically activated via type I cytokine receptors. These receptors lack intrinsic kinase activity but depend on associated cytosolic kinases of the JAK family for signal transduction. Ligands for such receptors include erythropoietin, growth hormone, leptin, and most interleukins and colony-stimulating factors. MAPPIT is a complementation assay whereby the bait is fused to a signaling-deficient receptor that cannot recruit STAT molecules. As the prey is fused to functional STAT recruitment sites, phosphorylation-dependent complementation initiates STAT recruitment and activation, followed by nuclear translocation and a transcriptional response (Fig. 14A). In MAPPIT, the interactor (cytosol) and detector (nucleus) zones are physically separated by taking advantage of the nuclear shuttling of STATs. As a consequence, activation of a reporter gene thus depends on the normal transcriptional machinery, preventing false-positive results at that level. A second characteristic of MAPPIT that reduces the false-positive rate is its ligand dependency, which adds an additional control level: only upon activation of the chimeric bait receptor by cytokine treatment is the system activated. Note that MAPPIT is characterized by a high degree of intrinsic topological flexibility, allowing detection of various PPIs without any structural optimization. This is reminiscent of the yeast two-hybrid system, where the flexibility of DNA allows activation of the RNA polymerase II complex relatively independent of the precise spatial positioning of bait and prey. Likewise, the nonstructured cytokine receptor tails provide flexibility in MAPPIT, allowing prey chimeras to be contacted easily by the JAK kinase. MAPPIT has found wide applications in the study of signal transduction processes and also many other (cytosolic) PPIs (recently reviewed in references 395 and 396a). Because of its favorable sensitivity/specificity ratio, MAPPIT is being used as an orthogonal assay to validate large-scale interactome maps. Examples include the interactomes of *S. cerevisiae* (721), *C. elegans* (601), and humans (663). In addition, MAPPIT can be used as a high-throughput assay in both arrayed and cDNA library screening formats (396). MAPPIT is a flexible concept and could be reconfigured to allow for high-throughput drug screening and to study interactions between proteins and small molecules. In reverse MAPPIT, the prey is linked to an inhibitory moiety, e.g., a phosphatase, which inactivates the system upon bait-prey interaction. Disruption of the PPI thus leads to a positive readout that is advantageous in high-throughput campaigns because it discriminates between disruptors and toxic compounds (Fig. 14B) (171). Finally, in the MASPIT three-hybrid format, DHFR is fused to the receptor, allowing display of a chemical dimerizer consisting of methotrexate and a small molecule of interest. Examples include the target recognition of various kinase inhibitors and their use in cDNA screening campaigns (73).

A PCA tool that has been developed uniquely for mammalian cells is the split-tobacco etch virus protease (split-TEV protease) system (698) (Fig. 14C). In this system, fragments of the NIa protease of TEV regain activity upon bait-prey interaction. Reporter activity can be either transcription coupled, whereby proteolytic cleavage allows translocation of a transcription factor from the cytosol to the nucleus, or proteolysis only, due to the activation of a luciferase enzyme upon proteolytic release from an inactive complex. In these configurations, a bait-prey interaction does not directly activate the reporter but functions via a reconstituted protease. This approach leads to a stable reporter activity which may affect the capture of dynamic interactions. Yet constitutive GABA-B1aR and GABA-B2R, as well as ligand-dependent ErbB2-ErbB4 receptor heterodimerization, could be monitored. Tango is also a TEV-based system and finds its conceptual origins in the Notch signaling pathway (29). The receptor is fused to a transcription factor via a linker containing a TEV protease cleavage site. Receptor activation leads to the recruitment of a signaling protein fused to TEV protease, thus liberating the transcription factor.

preventing STAT recruitment. (Right) Inhibition of the bait-prey association by competing proteins (Comp) or compounds (C) reestablishes normal JAK-STAT signaling, which ultimately leads to luciferase reporter gene transcription. (C) Split-TEV method (698). Bait protein X and prey protein Y are fused to the N-terminal and C-terminal domains, respectively, of TEV protease. Association of X with Y initiates the reconstitution of a fully functional TEV protease, which cleaves TEV-specific recognition sequences (rsTEV). A membrane-bound or cytoplasmic protein (MCP), linked by an rsTEV to either luciferase or the artificial transcription factor LexA-VP16, prevents strong bioluminescence or LexA-VP16 nuclear localization, respectively. TEV protease activity releases luciferase, for induction of strong luminescence, or LexA-VP16, for reporter gene activation. The image of TEV protease is based on the PDB structure under accession number 1LVM (512).

Note that the system uses the prokaryotic tTA-driven tetracycline-responsive promoter and thus avoids interference from endogenous signaling pathways. Since, in addition, transient receptor activation is turned into a constitutive signal, the system is highly specific and sensitive. Proof-of-concept experiments demonstrated applications for GPCRs, receptor tyrosine kinases, and steroid hormone receptors. This method is particularly suited to the study of early steps in receptor activation. Applications can include the identification of ligands of orphan receptors and high-throughput screening for signaling modifiers.

Finally, a new method based on *trans*-SUMOylation enables the observation of interactions by covalent attachment of a SUMO protein followed by shift detection in Western blot analysis (610). The bait chimera contains Ubc9, which adds the small ubiquitin-related modifier SUMO to the prey protein of interest. A number of PPIs were confirmed by application of this method, which forms an *in vivo* alternative to traditional coimmunoprecipitation.

Dual-Organism Two-Hybrid Systems

To allow direct comparison between interaction data sets obtained from two organisms, dual-organism two-hybrid methods have been developed, relying on the use of a single bait design or compatible vectors. A combined yeast-bacterium two-hybrid system was engineered (578) based on the λ repressor DBD fused to a bait gene and placed under the control of the *lpp/lacUV5* and *TEF1* promoters in *E. coli* and *S. cerevisiae*, respectively. For each organism, a different prey plasmid was used, bearing the B42 AD in yeast and the α subunit of *E. coli* RNA polymerase (RNAPα) as the AD in *E. coli*. Reporter genes for *S. cerevisiae* were *gusA* and *LYS2*, and those for *E. coli* were *HIS3* and *lacZ*, all of which were located in front of λ repressor binding sites. This method allows the sensitivities and specificities of the systems to be compared between the two organisms. In quantifiable assays, *S. cerevisiae* displayed a larger dynamic range for detecting interactions than the prokaryotic model. However, growth on selective medium was clearly faster for *E. coli* cells, and moreover, autoactivation did not occur in the bacterial cells. In screens of a human cDNA library against human Ras as bait, different hits were found in *E. coli* and *S. cerevisiae*. This result suggests that either the screening was not exhaustive or the specific environment of the screening influenced the outcome. Consequently, it became apparent that screening libraries in different organisms may lead to a broader spectrum of identifiable PPIs.

In an alternative approach, site-specific recombination and reading frame-independent mammalian two-hybrid (M2H) vectors were generated to be fully compatible with the site-specific yeast two-hybrid system (426, 427). This method was developed to address the shortcoming of time-consuming recloning of yeast two-hybrid candidates into mammalian two-hybrid vectors required for retesting of interacting candidates in the endogenous host environment. Vectors expressing bait fusions with the GAL4 DBD and prey fusions with VP16 were made Gateway compatible, fully functional in the mammalian system, and directly compatible with existing yeast two-hybrid vectors. These new vectors did not influence the capacity of the selection method in the mammalian background and did not create autoactivators. This system hence provides a fast way to check interactions in yeast and mammalian systems.

CONCLUSIONS

A bird's-eye view on PPI networks may suggest that nature has evolved a highly inefficient and promiscuous communication system between proteins to perform essential cellular functions. However, this large number of protein-protein associations, directly linked with organism complexity (241), is crucial for cellular robustness and network evolvability (8). The concept of keeping it as simple as possible, seen in man-made systems, does not work for interactomes. In that respect, evolution plays a crucial role because it essentially lacks a sense of overview but requires flexibility in response to genetic and environmental perturbations. Examples of this flexible behavior are seen in the subunits of protein complexes, which can serve as basic building blocks for other present or future complexes, and the presence of parallel and interconnected signaling pathways with multiple PPIs to provide error-tolerant and balanced regulation. The complexity in interaction studies also comes from the variety in the character of PPIs. Distinctions in the lifetime, strength, and obligatory nature of protein associations need to be considered. PPIs differ in being permanent or transient, obligate or nonobligate, and direct or indirect and in having high or low affinity (473). These features correlate with the interaction surfaces, which are generally larger and hydrophobic in stable core complexes and smaller and chemically more versatile for transient PPIs (338). Prediction of these interfaces is not straightforward because proteins often undergo conformational changes during association. Allosteric regulation by covalent modification, third-partner binding, or environmental changes further influences the shape of the interaction domain (473).

The large number and complex nature of PPIs necessitate the development of various technologies. Fortunately, many tools are now available for discovery and characterization of PPIs. Key contributions arose from immense technical improvements in molecular biology tools and joint efforts among research fields as diverse as genetics, live imaging, chemical biology, biophysics, and computational biology. As for genetic PPI methods, microbial two-hybrid systems have already produced a significant number of PPIs based on unbiased large-scale screens and play an important role in functional genomics (e.g., see references 690 and 721). Moreover, recent studies (616) give evidence that these methods are still far from saturation. Reiteration of genomewide two-hybrid studies with different setups could substantially increase the output and further aid in differentiating true from false-positive results. With the increasing availability of ORFeome libraries based on Gateway vectors, large-scale two-hybrid experiments for nonmodel organisms are becoming realistic. Such assays offer additional information on the structure of interaction networks and the evolvability of interactomes. Remarkable developments in two-hybrid assay-based techniques in higher eukaryotes, especially mammalian cells (177, 395), are on the verge of ushering in a breakthrough in high-throughput application and serve as very attractive alternatives to the traditional yeast system. With these techniques together with selection-based PCA technologies, fast progression in interactome mapping may be expected for organisms from viruses to animals to plants. Furthermore, technical improvements in the fields of affinity purification (202), protein microarrays (81), and cross-linking (418) will reveal complementary PPI data essential for full characterization of networks.

Such a blueprint of a static PPI network forms the basis for

exploring the dynamics of PPIs. PCAs with high temporal or spatial resolution facilitate studies on the influence of genetic or environmental changes. The easy technology transfer of such methods has made them widespread tools for very diverse applications. With some creativity in experimental setup, profound results can be obtained by these straightforward assays. As an example, the yeast cyclin-dependent kinase Cdc28 phosphorylates and concomitantly inactivates Swi4, a component of the cell cycle-regulating SBF complex. Hence, a positive split-FP assay with these two proteins implicates inactivation of the SBF complex. This readout was employed in a study with 25 deletion strains to uncover a genetic interaction between SBF inactivation and the kinase Elm1 (430). Instead of deletion strains, compound screenings to identify small molecules interfering with an interaction pathway could be performed as well (444). By application of the split-ubiquitin system in a medium-scale screen, an interaction network around the yeast phosphatase Ptc1 and its binding partner Nbp2 was created (269). However, it is by integration of deletion strains and truncated versions of the proteins under study in these split-ubiquitin experiments that extensive conclusions could be drawn on the dynamic control of regulatory circuits by Ptc1 and Nbp2. Not surprisingly, both studies made use of semibiased module-scale screens, which form a nice balance between time-consuming large-scale analyses and prejudiced one-to-one experiments.

The importance of studying PPIs also comes from the increasing awareness that they form valid drug targets. The general view that interaction surfaces are large and unstructured, making them difficult targets for small molecules, is not always true. Many surfaces are covered with pockets and clefts, and smaller hot spots and allosteric sites act as ideal binding regions for drugs (741). Interaction domains can be very specific, as seen, for example, with the docking sites of MAP kinase-binding proteins (26). To date, many small molecules or peptides are validated PPI inhibitors, and some of them have reached the clinical phase (741). Apart from providing the first step in PPI drug discovery, i.e., the identification of a suitable target PPI (e.g., see reference 98), two-hybrid systems have been applied for the discovery of PPI inhibitors, as discussed in this review. The use of these methods for detection of PPIs in pathogenic bacteria, protists, or fungi has been limited, but we are hopeful that in the future, pathogen-specific PPI modulation may become a new therapeutic approach to combat infectious diseases.

With all the tools available, one may ask which would be most suitable for a specific project. The answer lies in the identities of the species and proteins under investigation, the goal of the experiment, and the available equipment. The feasibility of a particular method may be evaluated by the published output. However, each system needs some momentum to become widely used. Even for the yeast two-hybrid system, it took several years before it became a common lab technique. Therefore, it is advisable to try out different methods and evaluate each of them. This not only increases the success rate but also helps to benchmark the existing technologies, as partially done already for two-hybrid systems (91) and a diverse set of *in vitro* and *in vivo* interaction methods (60). Careful optimization of available techniques could further increase their usefulness, such as the case for the three-hybrid system for protein–small-molecule interactions (94). With the basic setups already available, improvement of publicly available methods could launch their widespread use, which is especially true for alternative two-hybrid systems. In conclusion, the impact of PPI identification and characterization in cell biology is vast and promises exciting and advanced findings essential to our understanding of biological processes, disease development, host-pathogen interactions, and drug discovery.

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Jan Tavernier obtained his Ph.D. degree in 1984, by studying the cloning of interferon and interleukin genes. After an extended stay at Biogen and later at Roche, he returned to academia in 1996 at the VIB Department of Medical Protein Research, Ghent University. He founded the Cytokine Receptor Laboratory, which currently consists of over 30 researchers. Based on insights into cytokine receptor activation, he developed the mammalian MAPPIT two-hybrid technology. Detailed information can be found at www.mappit.be. Dr. Tavernier has published more than 200 refereed manuscripts, 20 of which have been cited over 100 times. He also holds 19 patent applications. His main areas of expertise are cytokine receptor activation and signal transduction, also linking to pathways involved in innate immunity, and the analysis of protein-protein interactions, including interactome mapping, pathway walking, and molecular description of interdomain interactions.

Patrick Van Dijck obtained his Ph.D. in 1991, by studying mechanisms of transcriptional activation of androgen- and estrogen-regulated genes. His first postdoctoral work was performed in the Laboratory of Molecular Cell Biology at KU Leuven, with a focus on trehalose metabolism and yeast stress resistance mechanisms. After a second postdoctoral position at Janssen Pharmaceutica (J&J) between 1995 and 1997, he returned to KU Leuven to become a group leader on a VIB-sponsored project. Since 2002, he has been group leader of the VIB Department of Molecular Microbiology, and since 2003, he has been a professor at KU Leuven. There are two research topics in his group. He is investigating the role of plant trehalose metabolism, but the main interest of the group is nutrient-induced signal transduction pathways that affect morphogenesis and virulence in the human fungal pathogen *Candida albicans*. He has published 100 refereed manuscripts and holds 15 patent applications. He currently leads a group of 20 researchers.