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Yeast two-hybrid methods and their applications in drug discovery

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The yeast two-hybrid (Y2H) method was first described over 20 years ago. It soon appeared as a major methodological breakthrough in the discovery and analysis of protein interactions, which play a pivotal role in all biological phenomena. Since its inception the Y2H method has constantly evolved and has inspired various assays that have found multiple applications of interest for drug discovery. Y2H methods are used to identify and validate therapeutic targets, discover protein interaction modulators, identify drug targets, and select combinatorial recognition molecules, which themselves find a wide range of applications. We review here the different transcriptional Y2H methods that are directly useful to drug discovery. Most should be increasingly used in the future as they continue to evolve to harness other methodological and conceptual advances.

The Y2H system

The Y2H system was first described in a seminal article published in 1989 by Fields and Song [1]. The principle of the assay relied on major discoveries on transcription initiation accumulated in the mid-1980s (Box 1). One of the key reasons for the tremendous success of the Y2H methodology is its timeliness [2]. In the early 1990s, unveiling a protein interaction was an arduous endeavor, mostly accessible to hard-core, cold-resistant protein biochemists. The Y2H thus filled a spectacular methodological gap in democratizing the discovery of protein interactions. Moreover, having gone through an exponential growth curve of published uses at the end of the last century [3], the Y2H became the undisputed reference method to discover protein interactions when genomics produced the first whole-genome sequences and created the need for high-throughput experimental biology (then termed functional genomics). Another obvious reason for the great success of the methodology lies in its remarkable versatility, which has allowed the development of several related assays, many of which have applications for drug discovery.

We review here the different Y2H-based methods that are applicable at various steps throughout the process of drug discovery. We try to identify the major limitations and the most promising advances that should increase their use to discover and characterize targets and drug candidates. To overcome some limitations of the original Y2H methodology, a number of elegant methods have been developed that involve the coexpression of chimeric protein pairs in yeast to detect their interactions [4]. As such, these methods can be dubbed two-hybrid. Because their application to drug discovery has been very limited so far, the review focuses exclusively on the original Y2H method and its evolutions, those involving the expression of chimeric proteins fused to transcriptional modules (Figure 1).

Identification of therapeutic targets

Few proteins that are directly associated with a disease offer tractable therapeutic targets. Some present loss-of-function mutations or are downregulated in disease states, which makes it difficult, although not always impossible, to restore their function by small-molecule drugs. A good example concerns the tumor suppressor p53, found mutated in half of cancers, and whose pathway is at least partially inactivated in the other half [5]. Other disease-associated proteins, such as the oncoprotein Ras, present gain-of-function mutations or an upregulation but do not harbor druggable sites or instead harbor sites that cannot be targeted specifically because they are substantially conserved in other related proteins [6]. The discovery of interacting partners of such intractable disease-associated proteins can offer excellent opportunities to identify promising therapeutic targets. In the case of p53, the discovery of its interaction with the MDM2 oncoprotein, which triggers its ubiquitin-mediated degradation, revealed that the interaction itself was a pertinent target, now actively pursued in clinical trials [5]. In the case of Ras, early use of the Y2H method unveiled its long-suspected interaction with the Raf oncogenic protein kinase, which has become a very attractive target in a number of cancers [7].

The Y2H method has established itself as a valuable target-discovery engine, whose power has been considerably enhanced by its application to high-throughput, proteome-wide (exhaustive) or sub-proteome-wide (partial) explorations of protein interaction networks [8]. The high-throughput practice of the Y2H method required the development of key technological advances, reviewed elsewhere [9]. Proteome-scale Y2H screens have been performed with increasingly complex systems, starting with viruses and bacteria, followed by yeast, worm, and fly, and climaxing with recent partial explorations of the human protein interaction network [8]. These endeavors generate a wealth of information, but a great deal of concern has been expressed about the quality of the data produced (Box 2). Although this quality

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Box 1. A brief history of the inception of the Y2H method

The mid-1980s witnessed major discoveries on transcription initiation. It was then found that transcription factors contain at least two functional domains, one mediating DNA binding and the other mediating transcription activation. This modularity was elegantly unveiled by domain-swapping experiments, which showed that chimeric proteins made of a DNA-binding domain and an activation domain originating from two distinct transcriptional regulators could act as *bona fide* transcription factors [84]. The second founding discovery was that transcription is sometimes activated by a protein complex, in which the DNA-binding and activation domains are borne by each interacting protein, respectively ([63] for a detailed presentation of the scientific foundations of the Y2H system). Inspired by these fundamental discoveries, Fields and Song coexpressed two chimeric proteins in yeast, consisting of two yeast proteins known to interact, one fused to the GAL4 DNA-binding domain and the other fused to the GAL4 activation domain. Their interaction created an active transcription factor that transcribed a *lacZ* reporter gene, thus conferring a blue color to yeast grown on an appropriate medium (Figure 1a) [1]. Once this seminal demonstration was achieved, a next challenge was to show that the assay could be used to discover new protein interactions by screening libraries of chimeric proteins. As discussed by Fields himself in a recent historical and epistemological paper, another important challenge was to find a catchy name for the method that would facilitate its rapid popularization [2]. Both challenges were met two years later with the published demonstration that the so-called 'two-hybrid system' could be used to screen a cDNA library to identify proteins able to interact with a given protein [85]. Shortly later, a small number of laboratories developed Y2H cloning systems that presented many important distinguishing features (including transcriptional modules, reporter genes, selection markers, and promoters driving the expression of the chimeric proteins); reviewed in [63].

issue should be kept in mind, the initial worries have faded with the clear identification of the problems and the emergence of efficient remedying solutions.

The usefulness of protein interaction maps for target identification purposes becomes clear when the datasets are cross-referenced with the OMIM (Online Mendelian Inheritance in Man) database, which lists disease-associated genes. Over 600 disease-associated proteins can be found on two human protein interaction maps recently produced [10,11], and many of the interactions involving these proteins can point to novel therapeutic targets. For example, one of the studies identified two novel regulators of the Wnt signaling pathway [10], a developmental pathway that is activated in many cancers. Other sub-proteomic Y2H efforts have focused more directly on given disease-associated proteins. For example, Y2H screens were conducted on 54 proteins involved in 23 inherited ataxias – a group of neurodegenerative disorders causing loss of balance and coordination. This effort unveiled almost 750 novel protein interactions and revealed that many ataxia-causing proteins share common partners of interaction which, for some of them, were known to modulate neurodegeneration in animal models and may offer therapeutic opportunities [12]. Another effort focusing on nuclear receptors and their cofactors entailed over 400 library screens and identified more than 60 high-confidence interacting proteins [13].

High-throughput Y2H studies have been performed on many pathogenic infectious agents. Recent studies have focused, for example, on the SARS [14], Epstein–Barr [15], *Varicella zoster* [16] viruses, the *Campylobacter jejuni* [17] and *Treponema pallidum* [18] bacteria, and *Trypanosoma brucei* [19]. The protein interaction maps generated by these efforts enhance the general knowledge on these infectious agents and suggest new potential therapeutic targets for future anti-infective drugs. Another very promising approach is to use Y2H screens to build host–pathogen protein interaction maps, which has been achieved for the Epstein–Barr [15], hepatitis C [20], influenza [21] and

Box 2. Quality of Y2H-generated protein interaction maps

False positive and false negative results

Major concerns on the quality of the data produced by high-throughput Y2H screens were voiced when it appeared that different independent studies performed in yeast, *Drosophila* and human produced datasets with limited overlaps. The reasons that can explain such a worrying lack of reproducibility have been discussed at length and cannot be detailed here. In brief, false positive results are not the only culprits and false negative results could even be the major cause of the limited data overlaps, due to an incomplete coverage of the clone sets subjected to the screens and, even more so, to subsaturating library screens [3,8,9,86]. Supporting this view, the two independent high-throughput Y2H endeavors on the human proteome detected respectively 3200 and 2800 interactions, of which only 17 were found in both datasets, although the two groups screened 1000 proteins in common. However, when assessed in biochemical affinity assays, the interaction datasets showed confirmation rates of 78% and 60%, respectively [10,11]. Clearly, as with other datasets produced by high-throughput methods, the quality of protein interaction maps generated by Y2H experiments needs to be assessed by alternative methods, themselves prone to false positive and negative signals. This dead-end can be addressed by using positive and negative protein interaction reference sets, which assemble collections of protein pairs that are known to interact and to not interact, respectively [86,87]. Weaving high-quality protein interaction maps from high-throughput Y2H data will increasingly require the combination of experimental validations [88] and bioinformatics approaches [89] to attribute a confidence score to each protein interaction.

Limitations of the Y2H method

Independently from the issue of technical false negative results, the Y2H method is inherently unable to detect some protein interactions. It cannot deal with full-length integral membrane proteins, which cannot be addressed to the yeast nucleus and which, nevertheless, represent currently the first class of therapeutic targets [90]. However, this problem can be partially bypassed by cloning separately the extracellular and cytoplasmic domains of these proteins [15]. Another limitation concerns proteins that, when fused to the DNA-binding domain, produce strong spontaneous activation of the transcription of the reporter gene in absence of an interacting partner. Here again, it is frequently possible to take advantage of the remarkable modularity of proteins (the very foundation of the Y2H method) and to screen with truncated, transcriptionally inert proteins. Alternatively, an interesting method has been designed to work with transactivating proteins. The so-called repressed transactivator assay (RTA) makes use of a transcriptional repressor domain and a counterselectable reporter gene [91] (Figure 1d). Finally, the Y2H method cannot detect protein interactions that depend upon post-translational modifications that do not take place in yeast. This limitation can concern protein interactions playing important roles in regulatory pathways whose deregulation causes diseases (e.g. some interactions between phospho-tyrosine proteins and SH2 domain-containing proteins within deregulated pathways in cancers). No scheme compatible with high-throughput applications can be applied to overcome this limitation.

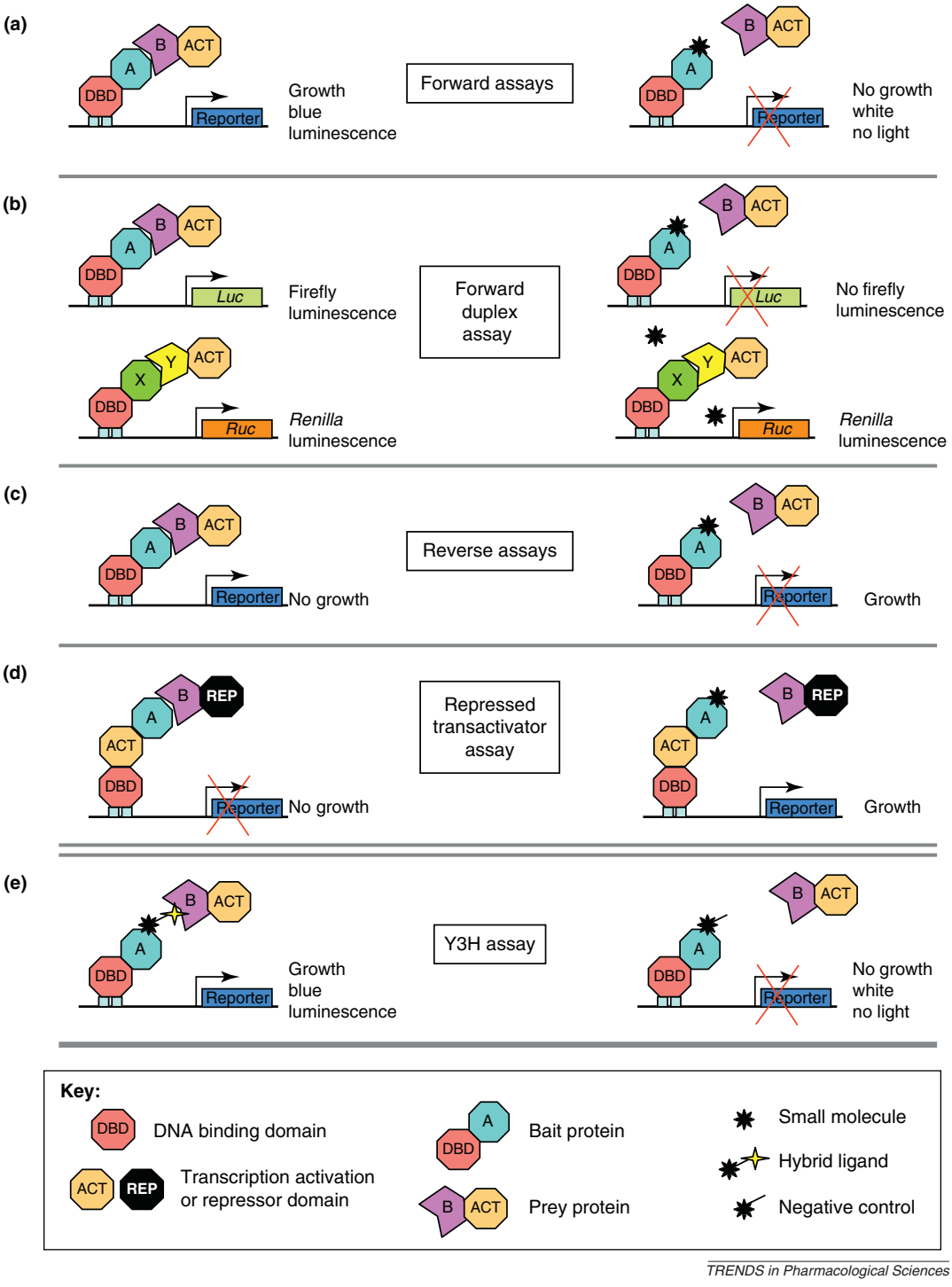

Figure 1. Y2H methods and their applications to detect protein–protein or protein–small-molecule interactions and to discover small-molecule inhibitors. **(a–d)** The principle of different Y2H methods is depicted. Left panels: detection of protein–protein interactions; Right panels: detection of inhibition of protein interactions by small molecules. Y2H phenotypes are indicated next to the reporter genes. **(e)** The principle of the Y3H method is depicted. Left panel: detection of an interaction between a small molecule (yellow star) and a protein (labeled B). Right panel: negative control setting, where a truncated hybrid molecule is used.

dengue [22] viruses. These efforts allow the investigation of biological questions that are very relevant for drug discovery, such as virulence, species barrier, chronicity and derived pathologies (such as cancer) [20]. They also considerably extend the target exploration field to the human proteins appearing on these maps. Targeting human proteins to treat infectious diseases holds great promise for at least three reasons. First, infectious agents (especially viruses) offer a very limited pool of tractable therapeutic targets, owing to the small size of their proteome. Second, treatments targeting viral or bacterial proteins often elicit drug resistance [23,24]. Third, human proteins involved in interactions with viral proteins may constitute pertinent targets for broad-spectrum drugs, because different viruses seem to share common interactions with host proteins [21,22].

Despite some limitations in the Y2H method (Box 2), the past decade has seen an impressive accumulation of comprehensive protein interaction maps produced mostly by Y2H screens and, to a lesser extent, by other methods such as affinity purifications followed by mass spectrometry (AP/MS) [25]. Network-science concepts and tools have been elegantly applied to analyze these highly complex protein interaction maps and, in particular, to unveil emerging topological features and global properties [26]. These efforts have revealed several emerging rules. For example, disease-associated proteins show a higher tendency to be hubs (i.e. to be more-connected nodes) than other proteins [27]. The average distance between proteins associated to related diseases (expressed as the number of edges separating the nodes) is lower than that between randomly picked proteins [12]. Viral proteins preferentially target host hub proteins [15,20–22]. About half of the 50 000 Mendelian alleles inventoried in the human gene mutation database are thought to produce edgetic rather than nodal perturbations (i.e. perturb protein interactions rather than remove proteins) [28]. For genes related to multiple pathologies, edgetic alleles causing the different diseases are located on different protein interaction domains [26]. Y2H interaction assays are ideally suited to characterize these disease-associated edgetic alleles and, more precisely, to identify those protein interactions (edges) that are affected by the mutations. For example, Y2H mating assays have revealed that most of the seven p16 alleles found in melanoma-prone pedigrees have lost their ability to interact with the cyclin-dependent kinases (CDK) CDK4 and CDK6, which are normally inhibited by p16INK4. These findings identified these two CDKs as putative therapeutic targets for cancers in which these p16 alleles are found [29].

The identification of new nodal or edgetic therapeutic targets will increasingly benefit from the discovery of emerging network properties and from the characterization of disease-associated edgetic alleles by Y2H interaction assays.

Validation of therapeutic targets

Addressing target validation is always intimidating because this crucial issue in drug discovery has never been precisely framed by a consensus definition shared by different laboratories or companies (or perhaps even by coworkers within a given organization). The set of criteria that a target should fulfill to be considered as validated by a drug-discovery team is consistently revised as enabling methodologies and new concepts emerge, and as collective experience accumulates [30]. In a target-driven approach to drug discovery, a minimalist operational definition of target validation could be – the accumulation of enough biological evidence that the modulation (mostly inhibition) of a given target (usually a protein) produces a desired phenotype in relevant cellular and animal models of human diseases. However, because insufficient validation is often considered as a major cause of the high attrition rates in drug discovery [31], target validation should be constantly questioned throughout the discovery process.

Target validation frequently makes use of reverse genetics methods and, in particular, of methods aiming at affecting the expression levels of target proteins (such a RNA interference, gene knockouts or transgenesis). Although these approaches have proved to be extremely powerful and useful in their ability to ascribe biological functions to many proteins, their value for target validation purposes is questionable, at least when pursuing a small-molecule drug project [30]. Indeed, partial (RNAi) or total (gene knockout) eradication of proteins introduces nodal perturbations within a biological system that differ markedly from those induced by small-molecule drugs – which usually do not affect the expression level of their cognate targets but instead modulate their activity. Consequently, RNAi or gene knockouts produce phenotypes that can differ dramatically from those observed using small-molecule inhibitors. This seems particularly true for protein kinases, which often engage in multiple protein interactions and display different functions besides their enzymatic activity [32]. High-confidence target validation should thus entail the use of specific small-molecule inhibitors (this statement is often stretched by cynical drug discoverers who claim that a target is not fully validated until a drug has reached a secure position on the market). Unfortunately, to date, specific small-molecule inhibitors are available for just a minor fraction of the druggable proteome, and this shortage will remain until chemical genetics delivers its full promises [33]. The current content of the latest release of the ChEMBL database, which gathers published bioactivity data for more than one million compounds, gives a good measure of the daunting challenge faced by chemical genetics [34]. The database lists less than 1400 human target proteins, of which only a fraction is specifically targeted by bioactive compounds that could be used for validation purposes.

Artificial combinatorial protein ligands that bind specifically to protein targets and modulate their function offer valuable small-molecule surrogates for target validation. The Y2H method is ideally suited to the selection of such ligands for their ability to bind to intracellular targets (Figure 2a). Peptide aptamers are man-made recognition proteins consisting of an invariable scaffold (most often thioredoxin) displaying single, random peptidic loops that are conformationally constrained within the scaffold. Over the past 15 years, peptide aptamers binding to various cellular, viral or bacterial proteins have been obtained and characterized [35,36]. Peptide aptamers show remarkable binding specificities and are often able to discriminate closely related proteins or even allelic variants of a given protein. In each case, some of the aptamers tested have been shown to modulate the function of their cognate target and to produce the desired phenotypes in cellular models (Figure 2b). In recent studies, peptide aptamers binding to the transcriptional regulator LIM domain only 2 (LMO2) or the chaperones heat shock proteins Hsp70 and Hsp27 have been expressed in animal models and shown to exert antitumor effects [37–39]. Because they modulate protein function by binding to their cognate targets, peptide aptamers introduce edgetic perturbations within biological pathways that significantly differ from the nodal perturbations caused by other reverse genetics methods (such as gene knockouts) [40], and that, arguably, are more similar to perturbations induced by small molecules. Their

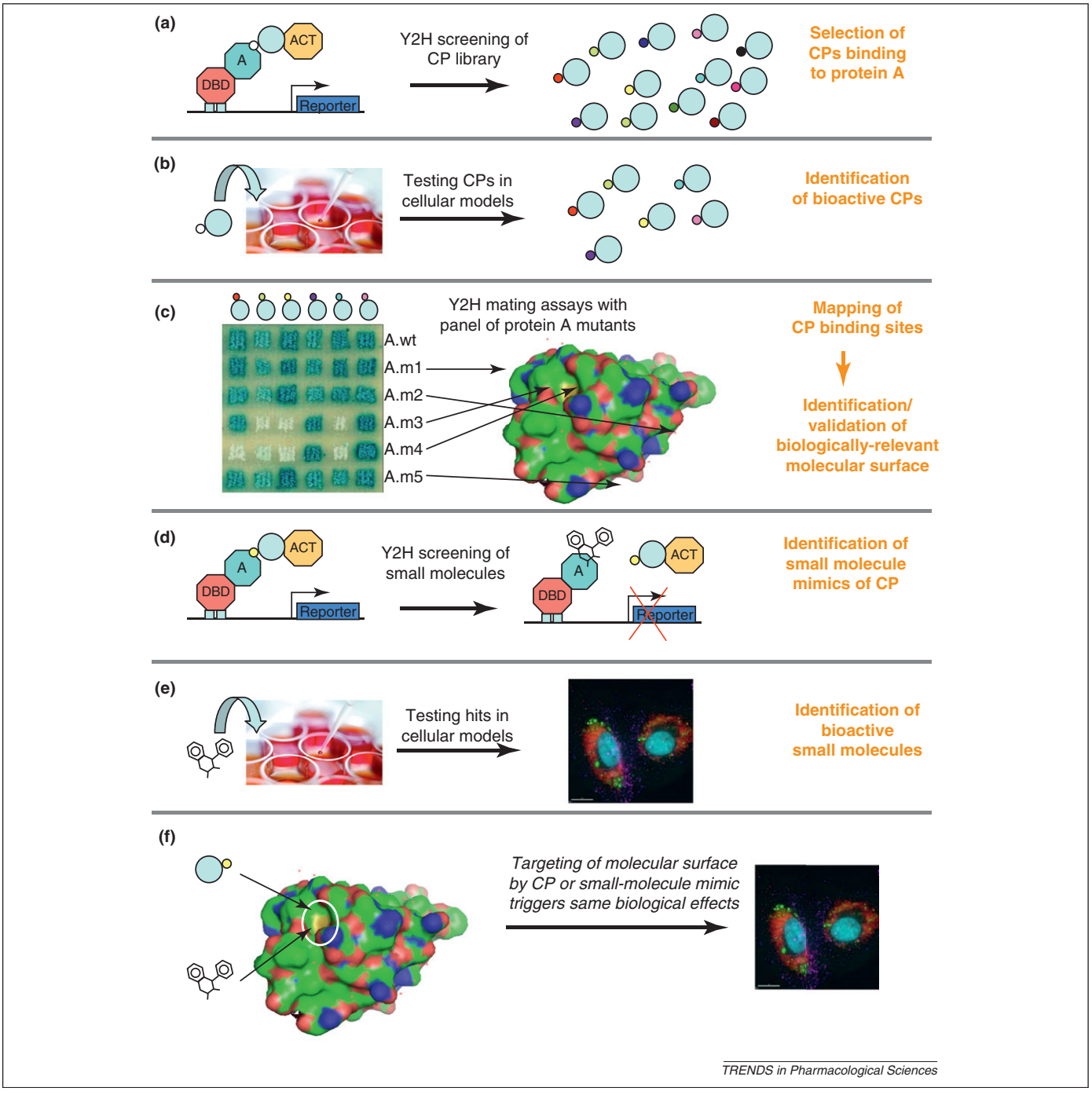

Figure 2. Use of artificial combinatorial proteins to identify/validate biologically-relevant molecular surfaces and to discover bioactive small molecules. **(a)** Libraries of combinatorial proteins (CPs), which can be either peptide aptamers or scFvs, are screened by Y2H and CPs are selected for their ability to bind to a given target protein (A). **(b)** CPs are expressed (or introduced) in cellular models where their expected bioactivity is assessed. **(c)** Y2H mating assays are performed between the different bioactive CPs (expressed as preys) and a panel of target protein mutants (expressed as baits). The mutations are designed to interrogate different molecular surfaces of the target. Loss of Y2H phenotype (here, white color) between a CP and a mutant strongly suggests that the mutated residue contributes to the CP binding site. At this stage, a molecular surface on the target protein is identified and validated as a potential therapeutic target. **(d)** Small-molecule libraries are screened using a Y2H-based displacement assay to identify hit candidates that inhibit the interaction between the target protein and one of its bioactive CPs. **(e)** Once confirmed by another interaction assay, small-molecule hits are tested in the same cellular models that were used to test CPs and bioactive molecules are identified. **(f)** Working hypothesis: (most) small molecules that disrupt the interaction between a target protein and one of its bioactive CPs bind to the same molecular surface as the CP and induce the same biological effects.

use can thus contribute to a high-confidence validation of therapeutic targets. Moreover, when the structure of the target protein is known, the binding sites of the bioactive aptamers can be precisely mapped by performing Y2H interaction assays between the aptamers and a panel of target mutants (each mutation interrogating a specific molecular surface) [41]. In such cases, peptide aptamers validate not only a protein target, but more precisely a specific molecular surface on the target (Figure 2c). Peptide aptamers can also be obtained from their ability to confer a selectable or detectable phenotype to a cell population (for example survival or reporter fluorescence). The selected aptamers can then be used as baits in Y2H screens to identify their targets [42,43]. In this approach, targets are

validated before they are identified, because the starting point is a desired phenotype.

Y2H screens have been also employed to obtain other classes of combinatorial recognition molecules, such as intracellular antibodies, also known as intrabodies (generally single-chain variable fragments (scFvs)) [44]. Although the Y2H method does not support the huge screen sizes achievable by *in vitro* selection schemes such as phage display, it presents the significant advantage in that it selects scFvs that are stable and fold properly in intracellular environments. The binding specificity of scFvs selected by *in vitro* procedures (and that nevertheless behave properly inside a cell) can be determined by Y2H screens using the scFvs as bait [45]. Because they show similar target-binding properties to aptamers, intrabodies can also be used as target-validation tools. However, the relatively big size of scFvs (28 kDa, twice that of peptide aptamers) should encourage the use of single-functional variable domains [44] to validate defined molecular surfaces on protein targets. Conceivably, scFvs can induce perturbations by out-competing interaction partners through steric hindrance and not necessarily by binding to biologically-relevant molecular surfaces. Recently, combinatorial linear peptides have been selected for their ability to bind to Livin, a member of the IAP anti-apoptotic protein family [46]. When stably expressed in tumor cells, these peptides inhibited the growth of cells expressing Livin and remained inactive in Livin-negative cells. This study demonstrates that, at least for some target proteins, Y2H libraries of unconstrained linear peptides can be used to select specific bioactive peptides that validate their cognate target [46].

Discovery of protein interaction modulators

In the 20th century, protein interactions were considered unattractive therapeutic targets because the binding interfaces were generally found to be large and flat, and thus undruggable. However, mutational studies have demonstrated that subsets of interface residues (the so-called 'hot-spots') contribute most of the binding energies. Moreover, structural studies have revealed that binding interfaces, which are often deemed undruggable from crystal structures of protein complexes, are actually flexible and adaptable, and can thus offer druggable sites on free-state partners [47]. More importantly, an increasing number of small-molecule inhibitors of protein interactions have been discovered [48], of which two are now being evaluated in clinical trials. ABT-263, an inhibitor of interactions between proteins of the Bcl-2 family, has reached a Phase IIa study in patients with relapsed small-cell lung cancer [49] and Nutlin-3, a p53–MDM2 interaction inhibitor, is currently under evaluation in Phase I studies against retinoblastoma and hematologic neoplasms [5,50]. These two very promising molecules clearly demonstrate that protein interactions can offer valuable and druggable therapeutic targets.

Several high-throughput screening assays have been used to discover inhibitors of protein interactions [51]. Thus far, Y2H screening assays have been the most widely utilized cellular assays (Table 1). Classical, 'forward' Y2H assays have been used to discover inhibitors of the Ras–Raf [52] and the Myc–Max [53] interactions (Figure 1a). These assays require counterscreens against control interactions because chemical libraries contain a significant proportion of molecules that show some level of toxicity against yeast – and that consequently inhibit the Y2H

Table 1. Small-molecule modulators of protein interactions discovered by Y2H screening assays

Y2H assays	Reporters	Targets	Biological pathways/ therapeutic fields	Chemical libraries	Hit number	Hit biological effects	Refs
Forward	*HIS3*	Ras/Raf1	Ras–MAPK/ cancer	10 000 microbial extracts	≥1	n.d.	[92]
Forward	*lacZ*	Ras/Raf1	Ras–MAPK/ cancer	73 400 diverse cpds	38	Inhibition of MAPK activation and tumor cell growth *in vitro*	[52]
Forward	*HIS3*	CFTR NBD1 ΔF508 dimerization	Chloride channel/ cystic fibrosis	600 plant leaf extracts	1	Restoration of dimerization; increase of chloride permeability in cultured cells	[61]
Forward	*lacZ*	Myc/Max (Id2/E47)[a]	Transcription/ cancer	10 000 diverse cpds	7 (10)[a]	Inhibition of tumor cell growth *in vitro* and in animals	[53]
Forward duplex	*luc* *ruc*	GαZ/RGS-Z1 (Kv4.3/KchIP1)[a]	G-protein signaling/ psychiatric disorders (potassium Channel)[a]	360 000 diverse cpds	75 (65)[a]	Inhibition of GTP hydrolysis of GαZ *in vitro*	[54]
Reverse	*CYH2*	N-type Ca^{2+} channel subunits	Neurotransmitter release/ stroke, traumas	156 000 diverse cpds	10	Inhibition of N-type Ca^{2+} channel activity *in vitro*	[56]
Reverse	*CYH2*	Kv4.3/KchIP1	Potassium channel/ epilepsy, stroke	n.d.	≥1	Modulation of current kinetics and amplitude in cultured cells	[93]
Reverse	*CYH2*	Kv1.1/Kvβ1	Potassium channel/ epilepsy, neuropathic pain	500 000 diverse cpds	18	Inhibition of Kv1.1 inactivation by Kvβ1 in cells; anticonvulsant activity in animals	[57]
Reverse	*GAL1*	Myc/Max	Transcription/ cancer	n.d.	n.d.	n.d.	[59]
RTA	*HIS3*	TGFβR/FKBP12 (TRα/Ncor1)[a] (ATF4/CBP)[a] (Nrf2/CBP)[a]	T cell activation/ immunosuppression (various biological pathways)[a]	23 247 diverse cpds	6 (86)[a] (1)[a] (2)[a]	Inhibition of IL-2 production by T cells; inhibition of SMAD-responsive reporter gene	[60]

[a]Control and/or additional protein interactions for which hits were not characterized. cpds, compounds; n.d., not documented

signals without inhibiting the protein interaction of interest. To bypass this problem, duplex forward Y2H assays have been developed in which two Y2H signals, produced by two different protein interactions, are revealed sequentially (Figure 1b). These assays rely on highly -sensitive luciferase reporter genes requiring small volumes of yeast suspensions, which supports miniaturized (384-well format) high-throughput automated procedures [54,55]. In duplex settings, molecules that are toxic or that interfere with the Y2H assay are immediately identified and discarded, and only those molecules that inhibit one of the two signals are considered as hit candidates. Another significant advantage of duplex assays is that two protein interactions are screened at once, which doubles the screening throughput.

The so-called 'reverse' Y2H method has also been used to develop high-throughput screening assays (Figure 1c). This method entails the use of reporter genes that inhibit yeast growth on an appropriate medium. Protein interaction inhibitors are thus identified by their ability to restore yeast growth. A cycloheximide-sensitivity reporter gene (*CYH2*) was used to develop a high-throughput screening assay that led to the discovery of inhibitors of interaction between N-type calcium [56] and potassium [57] channel subunits. Reverse Y2H assays relying on other counter-selectable reporter genes (*URA3*, *GAL1*) have been developed and shown to be applicable to the discovery of small-molecule inhibitors of protein interactions [58,59]. Sensitivity is a potential concern with these reverse Y2H assays, which require a fine-tuning of the Y2H phenotype such that yeast growth can be detected upon a partial inhibition of the protein interaction. However, a significant advantage is that toxic molecules do not appear as false positive hits and are always ignored.

The repressed transactivator method has also been adapted to design a versatile screening assay, which is not restricted to transactivating bait proteins (Box 2, Figure 1d). Proof-of-concept has been obtained with four different protein interactions for which inhibitors have been identified [60].

Quite interestingly, Y2H assays can also be used to discover molecules that induce protein interactions. A forward assay has enabled the discovery a molecule that restores the dimerization of the cystic fibrosis transmembrane conductance regulator (CFTR) protein bearing the most common disease-causing mutation (ΔF508) [61]. The screening made use of a prototrophic reporter gene, and hit candidates were thus identified for their ability to allow yeast growth. Luciferase reporters offer quantitative and wide dynamic-range readouts which enable the detection of molecules that stabilize or enhance protein interactions that already produce a basal Y2H phenotype [62].

Although Y2H-based screening assays suffer from the general limitations of Y2H methods in terms of applicability to particular classes of proteins and protein interactions, they present appreciable advantages over *in vitro* assays. First, protein interactions are screened in living cells, and thus in a more physiological context than that of an *in vitro* assay. Second, these assays bypass the need for large amounts of recombinant purified proteins, which are not always easy to obtain. Third, they identify hit molecules that are able to cross biological membranes. The problem of yeast permeability to small molecules is generally addressed by using enhanced-permeability mutant strains [51]. Fourth, some Y2H assays (derived from the LexA-based method [63]) use an inducible promoter to control the expression of at least one of the two interacting partners. The promoter is induced after adding the small molecules, which do not face a pre-formed interaction and are given a chance to interfere with a slow, gradual formation of a protein complex. This setting is probably a key factor to maximize hit rates because some molecules, although able to inhibit the formation of a protein interaction, remain unable to disrupt a pre-formed complex [64].

Identification of drug targets

The exhaustive identification of the proteins (not to mention other macromolecules) targeted by a drug is a requirement to fully understand its mechanism of action and its side effects.

Another elegant Y2H methodology has been developed to detect interactions between proteins and small molecules. In the so-called 'yeast three-hybrid' assays, the bait consists of a complex between a chimeric protein and a hybrid small molecule. The chimeric protein is made of a DNA-binding domain fused to a protein for which a small-molecule ligand is available. The hybrid small molecule is obtained by a chemical coupling between the cognate ligand and a small molecule of interest. A Y3H phenotype is obtained when a prey protein interacts with the latter (Figure 1e). The initial proof-of-concept was obtained using as bait the hormone-binding domain of the glucocorticoid receptor fused to LexA in conjunction with a dexamethasone–FK506 hybrid molecule. A Y2H signal was obtained by expressing the FK506-binding protein FKBP12 as prey, and FKBP12 clones were obtained from screening a cDNA library with this bait, thus demonstrating that the method can be used to identify drug targets [65]. Subsequent variations on this assay have been reported. Another proof-of-principle has been obtained with a bait consisting of the complex formed between dihydrofolate reductase (DHFR) and its ligand methotrexate, coupled to dexamethasone [66]. A Y3H approach was applied to identify the protein targets of three inhibitors of cyclin-dependent kinases, among which was roscovitine, now in Phase II clinical trials [67]. This study established the robustness of the method, because the cDNA library screens performed against a bait displaying purvalanol B identified 16 novel target candidates, of which 12 were confirmed by affinity chromatography or enzymatic assays. It also revealed that the intensity of the Y3H phenotypes, which are determined by many different factors pertaining to the chimeric proteins and the hybrid molecules, are not predictive of the binding affinities between small molecules and their protein targets [67]. This method is thus appropriate to detect interactions between proteins and small molecules down to a low micromolar range, but *in vitro* assays are required to confirm and quantify these interactions.

Discovery of biotherapeutic lead molecules

As seen above, combinatorial protein molecules selected by Y2H screens are valuable tools to validate therapeutic

targets, but some could also constitute biotherapeutic lead candidates. Important challenges need to be addressed to exploit this potential, among which is the difficult issue of protein delivery. Biotherapeutic candidate molecules must be processed or modified to protect their integrity once administered to organisms, to limit their immunogenicity, fine-tune their pharmacokinetics and, ideally, to direct them to the desired tissue or cell targets. An additional requirement is imposed on Y2H-selected candidate molecules which typically target intracellular proteins and must cross cell membranes to exert their effects. An increasing number of highly innovative approaches are being developed to optimize the delivery of biotherapeutic proteins in general [68], and most of these could be applied to the different classes of combinatorial protein ligands selected through Y2H methods. In an effort focusing specifically on peptide aptamers, an improved thioredoxin scaffold has been developed to optimize the intracellular uptake of Stat3-binding aptamers fused to a poly-arginine protein transduction domain. These penetrating peptide aptamers strongly inhibited tumor cell growth and induced apoptosis [69].

The development of biotherapeutic candidate molecules frequently entails fine-tuning of target-binding specificity and/or affinity. Harnessing *in vitro* evolution approaches to Y2H screens with calibrated reporter genes allows such fine-tuning. A peptide aptamer with increased affinity for CDK2 (K_d = 2 nM) was obtained from a Y2H screen of a sub-library constructed by a random mutagenesis on the variable region of an existing CDK2 aptamer [70]. It is expected that additional iterations of this procedure should yield peptide aptamers showing sub-nanomolar affinities for their cognate targets. Y2H methods are obviously ideally suited for the determination of binding specificity via the use of mating assays with defined panels of proteins [71] or via screening complex cDNA libraries against a peptide aptamer bait [43].

Other applications of Y2H-selected recognition proteins

Combinatorial protein ligands selected by Y2H methods can have other applications for different facets of drug discovery. Because they modulate protein functions by binding specifically to defined molecular surfaces, they can be used as guides to the discovery of small-molecule mimics that target the same surfaces and induce the same biological effects (Figure 2d–f). A high-throughput Y2H displacement screening assay has been developed to discover small molecules that inhibit protein–peptide-aptamer interactions [55]. A bioactive scFv selected against the Syk tyrosine kinase (a potential target for allergy) has been used in an ELISA displacement screening assay, and this has led to the discovery of a small-molecule mimic that inhibits anaphylaxis in mice [72].

Antibody microarrays are expected to play a prominent role in the discovery and the detection of biomarkers, and these arrays will have wide applications including disease diagnostics, patient profiling, and drug-response analysis [73]. Although promising proofs-of-concept have been obtained with small-scale antibody microarrays, the development and the use of large-scale high-density arrays faces important technical hurdles. Recent studies have shown that Y2H-selected peptide aptamers can be arrayed to detect proteins from complex mixtures [74], down to sub-picomolar concentrations when using label-free and highly sensitive detection methods [75].

Prospects on future uses of Y2H methods in drug discovery

To date, the methods and applications reviewed above have met a variable level of success in the drug-discovery community, depending mostly on their respective ease of applicability and methodological competitive landscape. Prospective analysis is always a very risky endeavor, especially when dealing with a complex, fast-evolving field such as drug discovery. However, we will now try to identify those methods and applications that should be increasingly used in the future owing to their most recent or anticipated evolutions, and those methods whose future impact upon drug discovery will depend on technological improvements and/or on changing doctrines.

It can be anticipated that high-throughput Y2H screens, which continue to benefit from ongoing technological improvements, will remain the reference method to build (sub-) proteome-wide interactomes and, as such, will remain one of the workhorses of systems biology. As a clear sign of maturity for a high-throughput method, strategies to optimize costs are now being developed [76], some harnessing next-generation sequencing [77]. Their contribution to the identification of therapeutic targets should thus increase as network concepts and analytical methods continue to emerge and as the ability to integrate other types of networks improves further [78]. Until chemical genetics delivers its full promises, Y2H-selected combinatorial protein molecules will remain highly valuable tools for the validation of therapeutic targets. However, their popularization will be conditioned by the emerging awareness that high-confidence target validation should rely on methods that introduce edgetic rather than nodal perturbations within regulatory networks. Protein interactions should become an increasingly attractive class of therapeutic targets as tools to predict their druggability become available [79–81] and as the circumscription of chemical spaces enriched in protein interaction inhibitors progresses [82,83].

A number of convincing success stories have been obtained using high-throughput Y2H screening assays, and these will be probably used more in the future to discover protein interaction inhibitors. So far, very few efforts have been devoted to the discovery of small molecules that enhance or restore protein interactions that are compromised by disease-causing mutations. As already shown [61], Y2H assays are ideally suited to the discovery of such molecules. Considering that about half of the disease-causing mutations are thought to introduce edgetic perturbations [28], it can be anticipated that compromised protein interactions will become an emerging class of therapeutic targets, and this should be efficiently exploited using Y2H-based screening assays. The exhaustive identification of small-molecule (drug) targets should become a more pressing need as forward chemical genetics delivers growing collections of bioactive molecules [33] and as regulatory requirements are stricter regarding data on

mechanisms of action. Y3H methods have met with only limited success so far and have been superseded by methods coupling affinity chromatography to mass spectrometry. With only one significant application in the identification of drug targets [67], the fate of Y3H methods will rely on additional success stories. Rapid progresses in protein delivery strategies may facilitate the development of Y2H-selected combinatorial protein ligands as biotherapeutic molecules, but this prospect will require a number of convincing proofs-of-concept in animal models, yet to be achieved.

Concluding remarks

It is probably fair to state that the Y2H has produced a major impact on biology over the past 20 years. The methodology has produced a significant impact on some steps of the drug-discovery process, and a more limited impact on other steps. Ever since its inception the Y2H method has shown a remarkable versatility and adaptability, and this has considerably extended its application field in fundamental biology and in drug discovery. Arguably, the methodology has now reached its maturity and, although future evolutionary leaps enabling novel and unexpected applications do not represent the best guess, they cannot be excluded. A more likely prospect is that most existing Y2H methods will gain interest and appeal among the drug-discovery community because they will continue to benefit from ever-growing technological improvements and will increasingly harness innovative concepts and ancillary technologies.

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