Yeast split-ubiquitin-based cytosolic screening system to detect interactions between transcriptionally active proteins

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Interactions between proteins are central to most biological processes; consequently, understanding the latter requires identification of all possible protein interactions within a cell. To extend the range of existing assays for the detection of protein interactions, we present a novel genetic screening assay, the cytosolic yeast two-hybrid system (cytoY2H), which is based on the split-ubiquitin technique and detects protein-protein interactions in the cytoplasm. We show that the assay can be applied to a wide range of proteins that are difficult to study in the classical yeast two-hybrid (Y2H) system, including transcription factors such as p53 and members of the NF-κB complex. Furthermore, we applied the cytoY2H system to cDNA library screening and identified several new interaction partners of Uri1p, an uncharacterized yeast protein. The cytoY2H system extends existing methods for the detection of protein interactions by providing a convenient solution for screening a wide range of transcriptionally active proteins.

INTRODUCTION

In recent years, the discovery and characterization of protein interactions on a large scale has helped to elucidate many novel biological pathways (1). Due to their relative speed and ease of use, genetic screening systems, such as the yeast two-hybrid (Y2H) system (2), have been especially successful in finding novel protein interactions. However, the Y2H system is biased against certain classes of proteins, for example, transcription factors (3). When fused to a DNA binding domain, these proteins autonomously activate transcription and therefore cannot be used in a Y2H assay. Alternative methods have emerged, such as the repressed transactivator (RTA) system (4) or the RUra3-based split-ubiquitin assay (5). Although capable of circumventing the limitations of the Y2H system with regard to self-activating proteins, these assays have their own particular drawbacks and have not seen frequent use to date.

In order to create a broadly applicable and flexible screening system for transactivating proteins, we have modified the well-established split-ubiquitin system (6). The split-ubiquitin system is one of the most widely used protein complementation systems and has been applied successfully to the identification of protein interactions involving integral membrane proteins, membrane-associated proteins, and soluble proteins (7). By combining the split-ubiquitin assay with a stringent transcriptional output using multiple independent reporter genes, we have established a screening system that can be used to detect interactions between transcriptionally active proteins and to identify novel interactors of a protein of interest using cDNA library screening.

MATERIALS AND METHODS

Bait and Prey Constructs

To construct the bait vector pCOWT, the cDNA encoding the entire open reading frame (ORF) of the yeast endoplasmatic reticulum (ER) OST4 was amplified by PCR from a genomic *Saccharomyces cerevisiae* library (Dualsystems Biotech, Zürich, Switzerland) using a forward primer with an *Xba*I site and a reverse primer with an *Sfi*I restriction site. The *OST4* cDNA was inserted upstream of the multiple cloning site into the vector pCCW (Dualsystems Biotech). The following bait proteins were cloned via *Sfi*I sites downstream of the *OST4* sequence into pCOWT: (*i*) N-terminally truncated Simian virus 40 (SV40) large T antigen (amino acids 84–708), (*ii*) full-length human p53, (*iii*) full-length *S. cerevisiae* Uri1p (systematic gene name YFL023W), and (*iv*) full-length human IκB-α. The following prey proteins were cloned downstream of the NubG cDNA into the prey vector pDSL-Nx (Dualsystems Biotech): (*i*) truncated human p53 (amino acids 72–390), (*ii*) full-length human p53, (*iii*) full-length human NF-κB subunit p65 and truncated p65 (amino acids 303–551), and (*iv*) full-length *S. cerevisiae* Pex4p, Pfd2p, Pfd6p, and Rpb5p. All constructs were verified by sequencing, and the expression of all baits and preys was verified by Western blot analysis (data not shown). Sequences of all constructs and details of the construction process are available upon request.

Yeast Transformation and Spotting

The *S. cerevisiae* strain NMY32 [MATa trp1 leu2 his3 ade2 LYS2::lexA-HIS3 ade2::lexA-ADE2 URA3::lexA-lacZ] (Dualsystems Biotech) was cotransformed with bait and prey plasmids using the lithium acetate method (8). Transformants were selected for the presence of bait and prey plasmids during 3 days of growth at 30°C on SD-trp-leu medium [minimal medium containing 2% glucose (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), 0.67% yeast nitrogen base (BD Biosciences, Basel, Switzerland), complete amino acid mixture lacking leucine and tryptophan (Qbiogene, Basel, Switzerland), and 2% bacto agar (BD Biosciences)]. Several colonies were transferred to liquid SD-trp-leu

medium and grown overnight to an optical density (OD_{546}) of 1.0. Five microliters of different dilutions (1:10, 1:100, and 1:1000) were spotted onto SD-trp-leu and SD-trp-leu-his-ade media (minimal medium as described above, but lacking tryptophan, leucine, histidine, and adenine) and grown for 2 days at 30°C.

β-Galactosidase Assay

β-galactosidase activity was analyzed by the pellet X-gal assay as described previously (9).

Cytosolic Yeast Two-Hybrid Screen

A yeast cDNA library (*S. cerevisiae*, strain Jel1) in the prey vector pNubGx (Dualsystems Biotech) was transformed into the yeast reporter strain NMY32 harboring pCOWT-Uri1p using the lithium acetate protocol (8). Transformants were selected on SD-trp-leu-his-ade medium supplemented with 15 mM 3-aminotriazole (3-AT; Applichem, Darmstadt, Germany) for bait-prey interaction. Library plasmids were isolated from 63 positive clones, amplified in *Escherichia coli*, and analyzed by restriction analysis for insert sizes. The plasmids that contained an insert and were shown to be expressed in Western blot anaylses were further processed by a bait-prey interaction test. For this purpose, the individual prey plasmids were cotransformed with the Uri1p bait into NMY32 and spotted as described in the section entitled Yeast Transformation and Spotting on medium selective for the plasmids or for protein interaction. Positive clones were sequenced and retested for bait dependency by carrying out a bait-prey interaction test with the Uri1p bait and unrelated baits such as Gal4p or IκB-α. The identity of cDNA inserts was determined by performing Basic Local Alignment Search Tool (BLAST) searches (10) against GenBank®.

Co-immunoprecipitation

Yeast cultures (300 mL) were grown until mid-log phase, centrifuged at 4000× *g* for 5 min, and washed in 50 mL H_2O. The cell pellet was resuspended in 2 mL immunoprecipitation (IP) buffer [20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 2% Nonidet® P40 (NP40), 10% glycerol, 20 mM β-glycerophosphate, 1 mM NaF, 0.5 mM dithiothreitol (DTT), and Complete™ Protease Inhibitor Cocktail tablet (Roche Diagnostics, Basel, Switzerland)] and disrupted using a one-shot cell disrupter (Constant Systems, Northants, UK), followed by centrifugation at 14,000× *g* for 10 min at 4°C. For co-immunoprecipitations, 10 µg mouse monoclonal anti-HA antibody (Covance, Denver, PA, USA) were added to 2 mg cell lysate and gently mixed for 2 h at 4°C. Fifty microliters of prewashed protein G Sepharose beads (Amersham plc, Buckinghamshire, UK) were added and mixed for another hour. The beads were washed five times for 5 min in IP buffer containing 300–600 mM NaCl, eluted in 30 µL 3× Laemmli buffer, and subjected to immunoblot analyses. Nonspecific binding of preys to protein G Sepharose beads was excluded by performing co-immunoprecipitation with or without anti-HA antibody.

RESULTS AND DISCUSSION

The cytosolic Y2H (cytoY2H) system takes advantage of the well-established split-ubiquitin system (6,11). A protein of interest (the bait) is anchored to the membrane via a fusion to the small integral membrane protein Ost4p (12) and fused to a reporter cassette composed of the C-terminal half of ubiquitin (Cub) and the artificial transcription factor LexA-VP16 (Figure 1A). Anchoring of baits to the membrane allows screening of transcriptionally active proteins by preventing their transition to the nucleus. A second protein is fused to a mutated version of the N-terminal half of ubiquitin (NubG), which lacks intrinsic affinity for Cub. If the two proteins interact, Cub and NubG are forced into close proximity and re-associate to form split-ubiquitin, which is then recognized by cellular ubiquitin-specific proteases (UBPs). The UBPs cleave the polypeptide chain C-terminal to Cub, releasing the LexA-VP16 transcription factor from the membrane. LexA-VP16 then

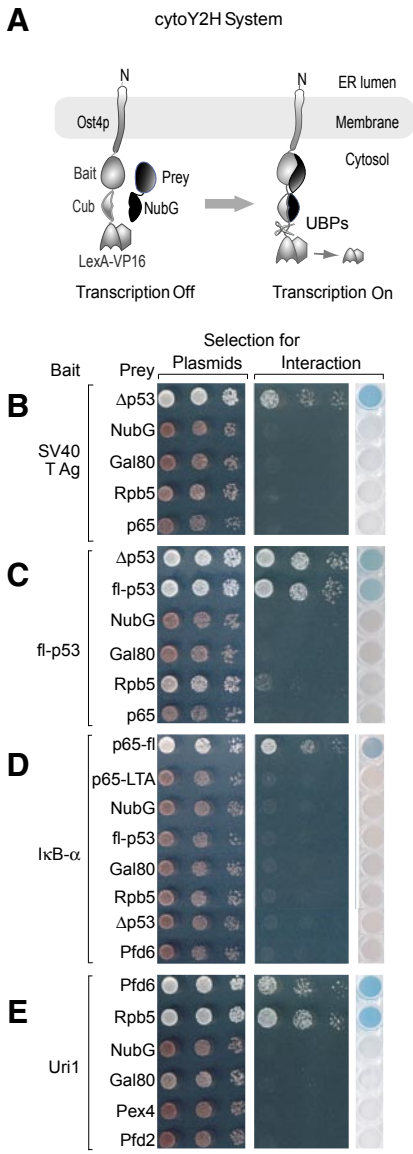

Figure 1. The cytosolic yeast two-hybrid (cytoY2H) system. (A) A protein of interest (the bait) is inserted between the membrane protein Ost4p and the C-terminal half of ubiquitin (Cub), followed by the artificial transcription factor LexA-VP16. A second protein (the prey) is fused to the mutated N-terminal half of ubiquitin (NubG). NubG has no intrinsic affinity for Cub, and the two halves do not interact when co-expressed within the cell. However, if bait and prey interact, Cub and NubG complement to form split-ubiquitin, followed by cleavage and translocation of LexA-VP16 to the nucleus and transcriptional activation of endogenous reporter genes. (B–E) Detection of defined protein interactions using the cytoY2H system. Yeast co-expressing the indicated baits and preys were plated either on medium selecting for the presence of both bait and prey (left column) or on medium selecting for a protein interaction (right column). β-galactosidase activity of yeast transformants was measured by the pellet X-gal assay (9). UBPs, ubiquitin-specific proteases; SV40, simian virus 40.

moves to the nucleus, where it activates the LexA-responsive reporter genes *HIS3*, *ADE2,* and *lacZ* integrated in the genome of the yeast reporter strain. Detection of the protein interaction is achieved by measuring the output of the activated reporter genes. Thus, the cytoY2H system converts an interaction taking place outside of the nucleus into a defined transcriptional readout, resulting in growth of yeast colonies under selection or blue coloration in a standard β-galactosidase assay.

As an initial interaction pair, we chose the SV40 large T antigen and the N-terminally truncated tumor suppressor protein p53 (13). Due to its intrinsically self-activating transactivation domain at the N terminus, full-length p53 cannot be used in a classical Y2H assay (14). Instead, the N-terminally truncated variant (Δp53) is normally used. To reconstruct the interaction between p53 and the large T antigen, the latter was fused to Ost4p and Cub-LexA-VP16 as bait, and Δp53 was fused to NubG for use as prey. Co-expression of the large T antigen bait and the Δp53 prey yielded robust growth under selection and strong blue coloration in a β-galactosidase assay (Figure 1B). No interaction of the large T antigen bait was observed with either NubG expressed from the empty prey vector, or with several unrelated preys (Figure 1B).

Since the advantage of the cytoY2H system over the conventional Y2H system is its ability to screen transcriptionally active proteins, we sought to investigate whether full-length p53 could be used in the system. We first assayed the dimerization of full-length p53 (15). We found that a full-length p53 bait interacted strongly with both a full-length p53 prey and a Δp53 prey, whereas it showed no interaction with unrelated preys or NubG expressed from the empty prey vector (Figure 1C). The fact that dimerization of full-length p53 is detectable in the cytoY2H system shows that interactions involving transcriptionally active proteins can be assayed without any of the background usually associated with investigating transactivating proteins in a classical Y2H assay.

Next, we chose to assay interactions involving proteins of the IκB/NF-κB complex, since the NF-κB subunit p65 and its inhibitor IκB-α are well-characterized, and p65 is notoriously difficult to handle in Y2H assays (16). Upon co-expression, an IκB-α bait interacted with the full-length p65 prey. The interaction was shown to be specific since the IκB-α bait no longer interacted with a truncated Δp65 prey lacking the N-terminal binding site for IκB-α (Figure 1D).

We then investigated whether the cytoY2H system could also be applied to those cytosolic proteins that are self-activating in a conventional Y2H system. For this purpose, we chose the yeast protein Uri1p/Bud27 (17). Uri1p is a cytoplasmic unconventional prefoldin whose molecular function has not been characterized so far. Previously, we have been unable to use Uri1p in a conventional Y2H system, most likely due to its large acidic region. As shown in Figure 1E, full-length Uri1p bait interacted strongly with two of its known binding partners, Pfd6p and Rpb5p, demonstrating that interactions between cytosolic proteins can also be detected using the cytoY2H system.

To exclude any nonspecific interactions, each prey was also assayed against at least one noncognate bait. For example, the Δp53 and Pfd6p preys were assayed against the IκB-α bait, since neither p53 nor Pfd6p interact directly with IκB-α (Figure 1D).

In order to be useful as a research tool, any yeast-based protein interaction assay must be amenable to cDNA library screening, with the aim of uncovering novel protein interactions. To investigate whether the cytoY2H system can be applied for this purpose, we used the Uri1p bait to screen a NubG-fused yeast *S. cerevisiae* cDNA library. The cDNA library had a complexity of 4×10^6 independent clones; 7×10^6 clones were screened, yielding 63 primary interacting clones. These clones were analyzed in detail, showing that many encoded the same protein. Overall, 21 different putative interactors of Uri1p were found in the screen. Fifteen clones interacted reproducibly with Uri1p in a bait-dependency test and did not interact with

Table 1. Interactors of Uri1p Identified in the cytoY2H Screen

Class	Identity	Occurrence[a]	Function	Confirmed
Translation	TEF1/2	2	Translation Elongation Factor 1α	Yes
	TEF4	1	Translation Elongation Factor 1γ	Yes
	TIF51A	12	Translation Initiation Factor 5A	No
	TIF11	1	Translation Initiation Factor 1A	ND
	RPS4A	1	Ribosomal Protein of the 40S Subunit	ND
Protein Folding	SSB1	2	Hsp70 Family	Yes
	SIS1	1	Hsp40 Family, DnaJ Homolog	Yes
	CPR1	1	Cyclophilin, Prolyl-isomerase	No
	FPR1	1	Rapamycin Binding, Prolyl-isomerase	Yes
	EGD2	5	NAC Subunit	ND
Other	3-PGK	5	3-Phosphoglycerate Kinase	ND
	DEF1	1	RNA Pol II Degradation Factor	ND
	VMA4	1	Vacuolar ATPase	ND
	PEP1	1	Vacuolar Sorting Protein	ND
	ERG1	1	Squalene Monooxygenase	ND

Confirmation of interactors was carried out by coimmunoprecipitation. cytoY2H, cytosolic yeast two-hybrid; ND, not done.
[a]Number of independent clones identified in the screen that encoded the same protein.

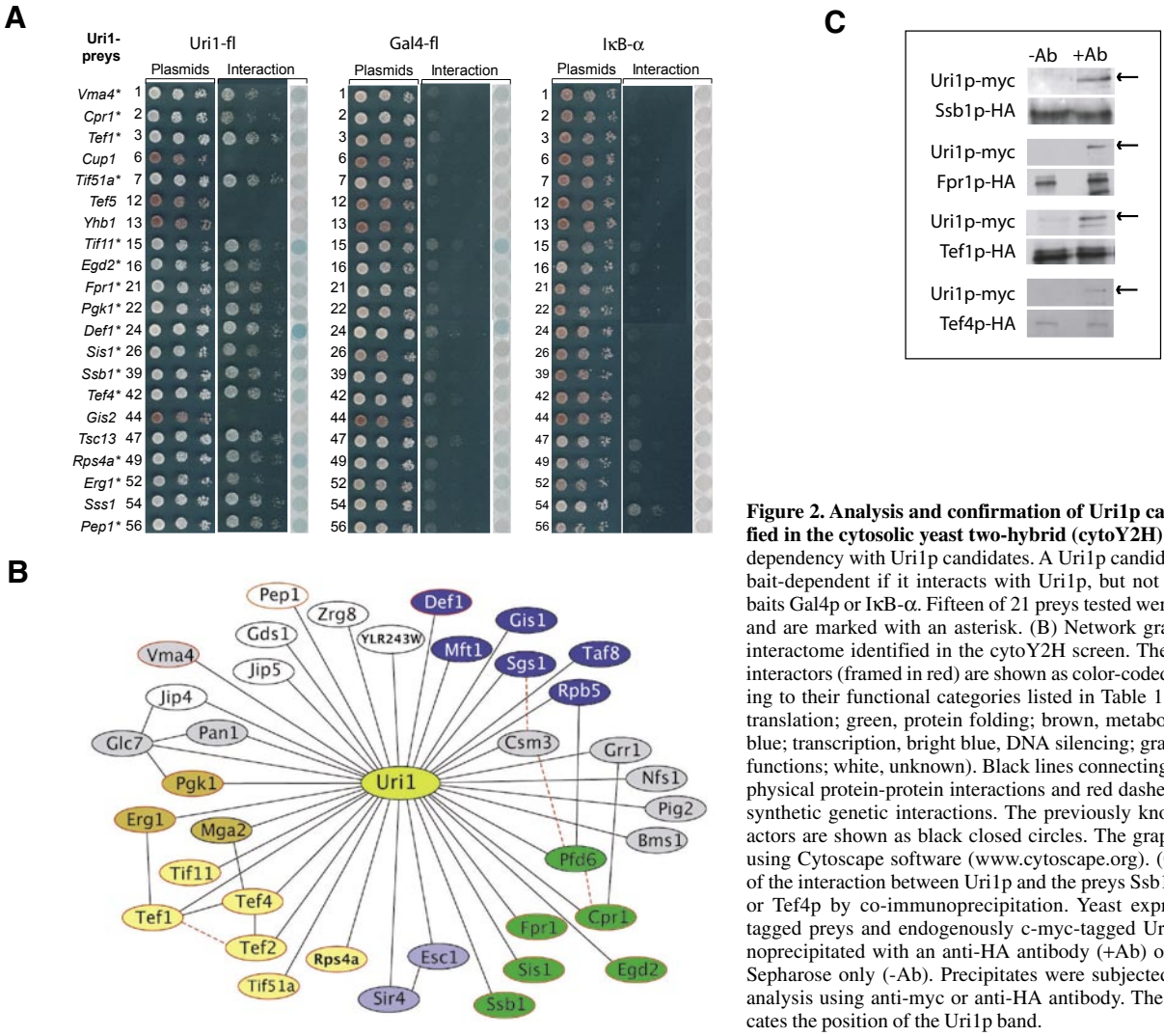

Figure 2. Analysis and confirmation of Uri1p candidates identified in the cytosolic yeast two-hybrid (cytoY2H) screen. (A) Bait dependency with Uri1p candidates. A Uri1p candidate is considered bait-dependent if it interacts with Uri1p, but not with the control baits Gal4p or IκB-α. Fifteen of 21 preys tested were bait-dependent and are marked with an asterisk. (B) Network graph of the Uri1p interactome identified in the cytoY2H screen. The 15 novel Uri1p interactors (framed in red) are shown as color-coded ellipses according to their functional categories listed in Table 1 (yellow, protein translation; green, protein folding; brown, metabolic protein; dark blue; transcription, bright blue, DNA silencing; gray, miscellaneous functions; white, unknown). Black lines connecting nodes represent physical protein-protein interactions and red dashed lines represent synthetic genetic interactions. The previously known Uri1p interactors are shown as black closed circles. The graph was produced using Cytoscape software (www.cytoscape.org). (C) Confirmation of the interaction between Uri1p and the preys Ssb1p, Fpr1p, Tef1p, or Tef4p by co-immunoprecipitation. Yeast expressing the HA-tagged preys and endogenously c-myc-tagged Uri1p were immunoprecipitated with an anti-HA antibody (+Ab) or with protein G Sepharose only (-Ab). Precipitates were subjected to immunoblot analysis using anti-myc or anti-HA antibody. The arrowhead indicates the position of the Uri1p band.

the unrelated preys Gal4p and IκB-α (Figure 2A). The other clones either did not interact reproducibly with Uri1p or failed to produce results in restriction analysis or sequencing. The 15 bait-dependent Uri1p interactors were classified according to their molecular function (Table 1). Several interactors were found to be involved in protein translation, supporting a previously suggested role for Uri1p in this process (17; Deplazes et al., unpublished). Another group of interactors were found to be chaperones or proteins involved in protein folding. As Uri1p contains an α-class prefoldin domain at its N terminus and is known to interact with the chaperone prefoldin 6, the identification of additional chaperones as interaction partners of Uri1p fits well.

We assembled a local protein interaction network centered on Uri1p, which consists of the 15 new interactors discovered in the cytoY2H screen. We then searched the *Saccharomyces* Genome Database (SGD™) (18) and BioGRID (19) for previously known physical and genetic interactions among these proteins and assembled this information into a local interaction network of Uri1p (Figure 2B). To further confirm the observed cytoY2H interactions in a different system, we used co-immunoprecipitation to assay binding of Uri1p to its putative interactors Tef1p, Tef4p, Ssb1p, and Fpr1p (Figure 2C). Taken together, these results show that Uri1p interacts with several members of the translational and protein folding machinery and confirm the proposed role of Uri1p in protein translation and folding.

We note that we did not find the previously known Uri1p interactors Pfd6p and Rpb5p, although pairwise interaction assays did detect their interaction with the Uri1p bait (Figure 1E). An explanation for this false-negative result may be that the two interactors may not have been present in the cDNA library, or that variations in the copy number level of the library plasmids may have prevented expression of sufficient levels of the preys to enable detection.

To present a useful tool for functional proteomics studies, any new screening system must tackle those classes of proteins for which no suitable systems currently exist. In this report, we show that the cytoY2H

system fulfills precisely such a role, as it is able to detect interactions between a wide range of proteins, including transcription factors, strongly acidic proteins, and cytosolic proteins. Moreover, we demonstrate that novel protein interactions can be identified by screening cDNA libraries against a protein of interest. Compared with other alternative genetic screening methods, such as the RTA system (4), the RUra3-based split-ubiquitin system (5), and the RNA polymerase III-based (Pol III) system (20), the cytoY2H assay offers several advantages. For example, the RTA system and the RUra3-based split-ubiquitin system use resistance to 5-fluoroorotic acid (5-FOA) as the selection mechanism. 5-FOA selection can be difficult to apply in cDNA library screens and may produce a high number of false-positives (20). Furthermore, a replica plating step must be used for selection, which makes the method laborious. In contrast, the cytoY2H does not rely on 5-FOA selection, but takes advantage of a very stringent reporter system, encompassing two different auxotrophic reporter genes and a *lacZ* marker. The Pol III system, despite not using 5-FOA selection, is difficult to handle since it requires simultaneous introduction of three different plasmids, resulting in significantly reduced transformation efficiency in cDNA library screens. In addition, growth of yeast takes place at 37°C, which puts additional stress on the yeast cells and increases the risk of false-negative and false-positive protein interactions. In contrast, the cytoY2H system utilizes the split-ubiquitin complementation mechanism (6), which is well-established and has been shown to work under a variety of different conditions and with a diverse range of protein classes (reviewed in Reference 7).

In summary, the cytoY2H system is able to detect pairwise interactions between a wide range of different proteins, including classical nuclear transcription factors, proteins which shuttle between the cytosol and the nucleus, and cytosolic proteins. The assay is highly specific, as none of the baits interacted with any of the numerous controls tested. We believe that the cytoY2H system will be a valuable tool for working with those classes of proteins that have not yet been covered by existing methods.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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