Research Report

Approaches to Detecting False Positives in Yeast Two-Hybrid Systems

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ABSTRACT

While many novel associations predicted by two-hybrid library screens reflect actual biological associations of two proteins in vivo, at times the functional co-relevance of two proteins scored as interacting in the two-hybrid system is unlikely. The reason for this positive score remains obscure, which leads to designating such clones as false positives. After investigating the effect of overexpressing a series of putative false positives in yeast, we determined that expression of some of these clones induces an array of biological effects in yeast, including altered growth rate and cell permeability, that bias perceived activity of LacZ reporters. Based on these observations, we identify four simple strategies that can assist in determining whether a protein is likely to have been selected in a two-hybrid screen because of indirect metabolic effects.

INTRODUCTION

Based on principles of genetic selection in yeast, two-hybrid systems (6,7,16,27) offer numerous advantages in comparison to traditional biochemical methods for the identification of novel protein-protein interactions. These advantages include avoidance of costly protein purifications and an accompanying reduction in time required to identify novel protein partners. Further, two-hybrid systems are well suited to related strategies, such as mutational analysis to identify important protein-protein interaction motifs, that are not as readily approachable by biochemical means. Conversely, because selection of positive protein interaction is by the indirect readout of transcriptional activation of reporter genes such as *lacZ*, *LEU2* and *HIS3*, two-hybrid screens are potentially vulnerable to distinct classes of artifacts related to the biasing of the transcriptional response. While two-hybrid assays have been very successful at identifying real interactions (one estimate of success rate is ca. 70% for usable baits) (**www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html**), the majority of library screens also identify one or more proteins presumed to be a false positive, which is defined as a protein either implausibly or impossibly in association with the bait used in the screen or subsequently shown to interact with multiple unrelated baits. The degree to which false interactions are predicted, and why, is a critical issue, particularly as two-hybrid systems move towards genomic scale applications (5,10,11).

Some earlier studies addressed variables that might lead to false positive generation or otherwise impact transcriptional activation in two-hybrid screens (3,9,12). In general, these studies focused on factors directly related to regulation of reporter gene promoters, including the identification of false positive proteins that directly bind and activate promoters (3) or altered perception of activation strength based on the differential access of bait proteins to reporter promoters (9,12). Common false positives obtained with many diverse baits, which include ribosomal subunits, heat shock proteins, proteasome subunits and cytoskeletal components, among others (**www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html**), are sometimes hypothesized to possess their false positive character because they are intrinsically sticky, either because their biological function involves binding a large number of different proteins or because of nonspecific charge or coiled-coil interactions. An alternative possibility that has not been well considered is whether the activity of some false positives is independent of interaction with either the bait or the promoter. Such activity would be of general interest in that it would potentially bear on indirect crosstalk between transcriptional control and other signaling pathways in more biological systems.

In this study, we assessed a set of characteristic false positives for

changes in yeast growth and viability. Cumulatively, this work has revealed a series of metabolic alterations and indirect effects related to overexpression of false positives and led to the identification of a number of simple assessments that can be used to detect two-hybrid library isolates that bias reporter function through indirect methods.

MATERIALS AND METHODS

Plasmids and Strains

For two-hybrid experiments, the LexA expression vector pEG202 (14), LexA-HEF1$_{650-834}$ (20), LexA-bicoid (pRFHMI) (14), LexA-GAL4 (pSH17-4) (14), LexA-RPB7 (18), LexA-hsRPB7 (18), LexA-p53 (Display Systems Biotech, Vista, CA, USA) and pJG4-5 (16) have been described. LexA-Np/PQ is a comparable fusion to full-length Npw38/PQBP-1 (19,28). pJG4-6 is essentially identical to pJG4-5, except that cDNAs are expressed as fusions to an amino-terminal hemagglutinin epitope tag only. cDNAs encoding partial fragments or full-length clones for vimentin (amino acid 216-466 [carboxyterminus]) (15), F1-ATPase (amino acid 244-480 [carboxyterminus]) (24), disulfide isomerase (amino acid 203-440 [carboxyterminus]) (17), were characterized in detail as LexA, pJG4-5 and pJG4-6 fusion proteins: inserts were isolated by library screen as clones in pJG4-5. pGilda (25) was used to express LexA-fusions under the control of the GAL1 promoter. The *8lexAop-lacZ* reporter pSH18-34 and the 2lexAop-LEU2 reporter strain EGY191 have been described (9).

Growth Rate Assessment

Rate of growth of yeast containing bait/prey combinations was assessed in two ways. On plates, yeast were streaked or replica plated to ura-his-trp- plates with either 2% glucose or 2% galactose + 1% raffinose as a sugar source, grown at 30°C for 48 h. Plates were visually inspected to compare growth rates between distinct colonies. In liquid medium, 6 independent colonies for each indicated plasmid combination were used to start overnight cultures in ura-his-trp- medium with 2% glucose. The following morning, overnight samples were used to restart cultures in ura-his-trp- medium with 2% galactose + 1% raffinose at A_{600} = 0.15, followed by growth on a roller drum at 30°C for 25 h, with periodic measurement of A_{600}.

Assays to Measure Activation of LexA-Responsive Reporters

LacZ activity was assessed by three standard assays. In one assay, yeast was transferred to ura-his-trp- XGal plates with either 2% glucose or 2% galactose + 1% raffinose as a sugar source, grown at 30°C for 48 h, and the degree of blue color was visually assessed at 24 and 48 h. In a second assay, colonies were streaked or replica plated to ura-his-trp- plates, with either 2% glucose or 2% galactose + 1% raffinose as a sugar source. These plates were grown at 30°C for 24 h, after which they were processed for XGal overlay assay as described (8,13). In a third assay, yeast were grown as ura-his-trp-/2% glucose liquid overnight cultures and then used to seed ura-his-trp-/2% galactose + 1% raffinose liquid cultures at A_{600} = 0.15. Cultures were grown for approximately 6 h until A_{600} = 0.5–0.6, then assayed for β-gal activity (2). Reported β-gal units are adapted from those in Reference 23.

Yeast were transferred to the assay plates in two separate ways. In one, a toothpick was used to transfer approximately 1–2 µL cell mass to an approximately 1 cm long streak, resulting in a high density seed of the assay plate. In the second, yeast were transferred by replicator as described (13). Approximately 1–2 µL cell mass of colonies to be assayed were picked to the 96-well microplate, with each well containing 25–50 µL sterile distilled water; colonies were swirled to ensure even dispersal, then the replicator was used to dot assay plates. This second procedure results in a seed density approximately 1/10 as heavy as the toothpick transfer method. All experiments were performed using yeast colonies that were transformed with bait/prey/reporter combinations within 2 weeks of the assay date.

Bromophenol Blue Assays

To assess yeast permeability, appropriate minimal media plates were prepared by standard methods (2) with the following addition: autoclaved media were allowed to cool to 65°C, then sterile filtered bromophenol blue (BPB) was added to a final concentration of 0.02% (diluted 100× from a 0.2% stock). Yeast were streaked or spotted to BPB plates, and coloration was observed at 12 h intervals for 48 h: after approximately 72 h, all colonies had become darker, obscuring the effect.

Western Blots

For each plasmid combination, 2–5 independent yeast colonies were used to grow overnight cultures at 30°C in appropriate glucose medium. Overnight samples were diluted to A_{600} = 0.15 in fresh media containing galactose, and cell lysates were made from cultures at A_{600} = 0.50, following procedures described in Reference 14. Polyclonal rabbit antisera to LexA, hemagglutinin (both generated by E.A.G. at Fox Chase Cancer Center) or β-gal (Chemicon International, Temecula, CA, USA) were used to visualize LexA- and HA-fusions or LacZ. Visualization was by ECL kit™ (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

RESULTS AND DISCUSSION

We used a set of false positives that our laboratory or other groups had identified as common or nonspecific isolates (**www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html**) based on multiple criteria. These included: interaction with multiple baits of discrete biological function and intracellular localization; obvious irrelevance to the function of at least some of the baits; and in some cases, sequence characteristics (large charged patches, extended helical sequences) thought to promote "sticky" behavior. Isolates analyzed in this study included partial clones for vimentin (amino acid 216-466[carboxyterminus] (15), F1-ATPase (amino acid 244-480 [carboxyterminus]) (24) and disulfide isomerase (DSO; amino acid 203-440 [car-

boxyterminus]) (17). Each of these was expressed as an activation domain fused protein (from pJG4-5) or a native protein fragment (from pJG4-6); each putative false positive or matching empty vector was co-transformed with a LexA-HEF1 bait plasmid and the pSH18-34 *lexAop-lacZ* reporter plasmid into the EGY191 *lexAop-LEU2* reporter strain.

Apparent Activity of a False Positive in Inducing LacZ Activity

Activation of LacZ reporters in yeast is assayed by one of three approaches: *(i)* growth on plates containing X-gal; *(ii)* growth on standard cultivation plates or on filters, followed by colony lysis and X-gal incubation/overlay and *(iii)* growth in liquid, followed by harvesting, cell lysis and quantitative assay of ONPG cleavage. In addition, expression of *lacZ* can be directly assessed by means of Western analysis with antibody directed against LacZ. Strikingly, a number of putative false positives assayed in conjunction with the LexA-HEF1 bait by all three means demonstrated dramatic differences in apparent LacZ activity dependent on assay means (Figure 1). When grown on plates containing X-gal (Figure 1, second panel from left), all false positive clones demonstrated significant activity, with a strong phenotype observed with truncated vimentin and a moderate exemplified by F1-ATPase and DSO-P5. In contrast, adjudged by overlay assay, the AD-F1-ATPase and AD-DSO still possessed a moderate activity, AD-vimentin was initially negative (Figure 1, left), although after an extended growth period before lysis developed a moderate activity (not shown). Analysis by liquid β-gal assay (Figure 1, third column), while closer to the results with X-gal plates, yielded a distinct profile again; the AD-F1-ATPase and AD-DSO clone displayed strong activity, while AD-vimentin was more moderate. Finally, observation of expressed *lacZ* levels using antibody to LacZ (Figure 1, right panel) in general paralleled the results obtained with the liquid β-gal assay. These results indicated that either the observed *lacZ* readout was not strictly correlated with direct transcriptional activation of the *lacZ* reporter or that minimally other variables unrelated to transcriptional activation of *lacZ* biased the assessment of LacZ activity.

Transcriptional Activation by the Bait Contributes to False Positive Activity

We considered the possibility that the apparent induction of the *lacZ* reporter might in part be unrelated to the ability of false positives to activate transcription. Each of the false positives was recloned for expression as a LexA fusion protein, allowing direct assessment of the transcriptional potential of the cDNA-encoded protein. Analysis of the LexA-fused false positives indicated that none of these proteins possessed any intrinsic ability to directly activate transcription of *lacZ* or LEU2 reporters in the absence of a fused activation domain (data not shown). Another possibility was that the ability of false positive clones to produce a LacZ phenotype could be related to the enhancement of intrinsic transcriptional activity of the LexA-fused bait with which it was co-expressed. To test this possibility, we examined the LacZ phenotype of yeast expressing the test false positives in combination with a series of baits of distinct weak autoactivation potentials (Table 1), by both overlay and the X-gal plates growth methods. All false positives did not interact with all baits: however, particularly for vimentin, the enhancement of LacZ phenotype was more strongly noted with baits with some degree of intrinsic ability to auto-activate transcription. As before, the phenomenon was generally more pronounced in the X-gal plate assay than in the overlay (data not shown). These results suggested that the action of false positives may lie in the intrinsic modulation of a bait activity.

Indirect Effects of False Positives on Cell Permeability and Viability

Although often used interchangeably (4), the assays for LacZ differ in three parameters that could be impacted by metabolic effects of false positives. These are: *(i)* growth in the presence of LacZ substrate on medium buffered to pH 7.0 (X-gal plate assay); *(ii)* growth on normal pH of approximately 5.5 medium and exposure to substrate after cell death (plate overlay or filter assay and liquid β-gal assay); and *(iii)* growth on solid vs. liquid media. If false positives possessed an activity related to cell permeability (either enhancing uptake of X-gal into yeast or causing leakage of LacZ to surrounding medium), selective viability or liquid vs. solid growth state, these properties

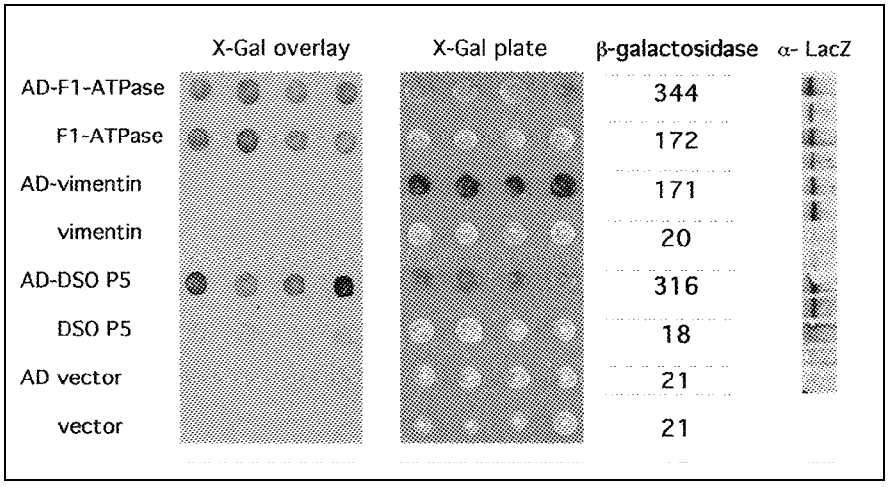

Figure 1. Determination of LacZ levels and activity by four separate means of assay. EGY48 yeast contain the pSH18-34 reporter plasmid and express LexA-HEF1 in combination with prey vectors shown in the left-most column. Each separate spot represents an independently selected primary transformant for the indicated plasmid combination. Left, yeast grown for 1 day at 30°C on ura-his-trp- galactose/raffinose plate, followed by X-gal overlay; second panel from left, yeast grown for 1 day at 30°C directly on ura-his-trp- X-gal galactose/raffinose plate; second from right, calculated β-gal values for the colonies; right, expressed levels of LacZ protein, as determined by western blot (for the first two clones of the row).

Table 1. Enhancement of Intrinsic Autoactivation of Discrete Baits by False Positives: LacZ Activity Assessed by Growth on X-gal Plates

Bait	Auto-Activation	Apparent Enhancement of LacZ Activity on X-gal Plates		
		ATPase	Vimentin	DSO
RPB7	-			
bicoid	-	+	-	-
pEG202 empty	+/-	+	-	-
p53	+/-	-	-	-
HEF1	+	+++	++++	+++
hsRPB7	+	-	+++	+
Npw38/PQBP-1	++	-	++	+

might partially explain the discrepancies noted above.

To evaluate differences in yeast permeability, we streaked yeast-expressing false positives or those carrying control vector on plates containing BPB (previously shown to gauge intracellular transport in mammalian studies) (26,29). We assessed the degree of dye uptake over 24 h, and the results are shown in Figure 2 (right panel). Notably, yeast-expressing vimentin derivatives (with or without transcriptional activation domain) became much darker than controls, while yeast-expressing AD-F1-ATPase and disulfide isomerase P5 became moderately darker, particularly in the presence of the transcriptional activation domain. Further, this rapid uptake of BPB was largely independent of the presence of any LexA-fused bait (data not shown). These results suggested that some false positive proteins enhanced uptake of small molecules from the growth medium.

Differences in permeability might derive in part from toxicity of particular false positives leading to loss of cell wall integrity and bidirectional leakage. To assess toxicity, we studied growth parameters of yeast expressing the false positives. In liquid medium, the only

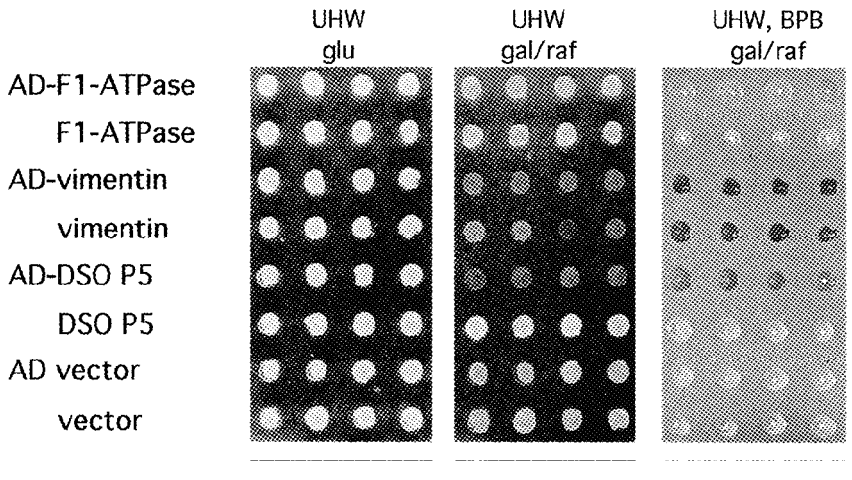

Figure 2. False positive effects on growth and permeability. Yeast plasmid combinations are as described in the legend to Figure 1. Left panel, yeast grown for 2 days at 30°C on ura-his-trp- glucose plates; center panel, yeast grown for 1 day at 30°C on ura-his-trp- galactose/raffinose plates; right panel, yeast grown for 2 days at 30°C on ura-his-trp- galactose/raffinose plates with incorporated BPB (color scores as brown because yeast are photographed against an orange background to enhance contrast). Note, for right panel, the degree to which this phenomenon is observed is partially dependent on the density of yeast inoculum on the plate. Yeast streaked at high cell density yield the pattern shown, while differences are less dramatic in yeast plated at low cell density (data not shown).

bait with a discernible effect on growth rate was AD-vimentin (Figure 3), reflected by saturation at lower levels than those expressing other or no fusion proteins and a higher frequency of visibly abnormal cells with greater than five-fold reduced viability on replating (data not shown). In yeast grown on plates, in contrast, the combination of AD-vimentin (in particular), but also native vimentin or AD-DSO in conjunction with LexA fused to HEF1 (Figure 2, second panel), bicoid or RPB7 (data not shown) resulted in a noticeable slowing of growth rate under conditions nonselective for the *LexAop-LEU2* auxotrophy reporter. However, in a completely unexpected result, expression of the native DSO protein resulted in a substantial enhancement of cell growth (Figure 1, lane 6). Notably, this growth inhibition was significantly worse on plates buffered to pH 7.0 and containing X-gal. Under these conditions, AD-F1-ATPase also demonstrated a growth retardation (data not shown), suggesting a potential contributing factor to the difference between overlay and X-gal plate results. The slowing of growth observed with some constructs does not completely correlate with the altered permeability and X-gal phenotypes described above and cannot completely explain them. However, it is clear that particular false positives induce complex changes in viability, permeability, growth rate and potentially other properties that combine to affect *lacZ* and LEU2 reporter function.

Finally, we note that the effect of the false positives was not limited solely to alteration of readout from LacZ. The false positives assayed had been selected because they demonstrated at least minimal galactose-specific activation of the *LexAop-LEU2* reporters as well as the *LexAop-lacZ* reporter discussed above. While the results summarized above suggest some methods by which false positives may bias *lacZ* reporters, it is unclear how these proteins contribute to the activation or enhanced function of the LEU2 reporter.

Implications for the Yeast Two-Hybrid System and for Indirect Augmentation of Gene Function

The explorations of false positive function reported above have highlighted a number of unexpected properties of some of these proteins when overexpressed in yeast. The first of these is the striking nonequivalence of assay systems commonly used to assay LacZ activity, which in this study provide quite distinct assessments of the LacZ phenotype of the different proteins assessed. The second is the identification of specific effects of false positives on yeast cell permeability. The third is the identification of specifically enhanced toxicities associated with particular bait-prey combinations that may additionally bias reporter activity. A fourth is the growth medium-specific (e.g., solid versus liquid medium-specific) differences in toxicity for some protein combinations. A fifth is the fact that some false positive activities in enhancing apparent lacZ transcription occur in the absence of a transcriptional activation domain on the false positive prey. A sixth is the observation that baits that have an intrinsic ability to auto-activate transcription are more likely than transcriptionally inert baits to score as "positive" with the false positives assessed, which supports the idea that the false positives may be indirectly augmenting bait-dependent activation. Taken together, these results suggest that cellular changes, whether caused by overexpression of fusion proteins or by specific yeast mutations, can cause specific changes in yeast physiology that affect standard reporter assays.

This study specifically uses the Interaction Trap two-hybrid system and does not compare the behavior of false positives in LexA- vs. GAL4-based two-hybrid systems. However, it seems likely that similar classes of phenotypes will be observed. Supporting this conclusion, a previous compendium of isolated false positives contained similar isolates from both systems (**www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html**), while early findings from yeast protein-interaction mapping project (S. Fields and P. Uetz, personal communication) suggest perturbation of the yeast metabolic function is likely to be an important factor in false positive isolation. Finally, our results do not allow determination of whether specific "true" positive combinations might not yield similar effects. However, it seems reasonable based on the current data to regard such a result as a reason to use extreme caution in evaluating protein pairs with such behavior by two-hybrid methodologies.

These results suggest several simple steps that researchers might attempt to evaluate the significance of putative

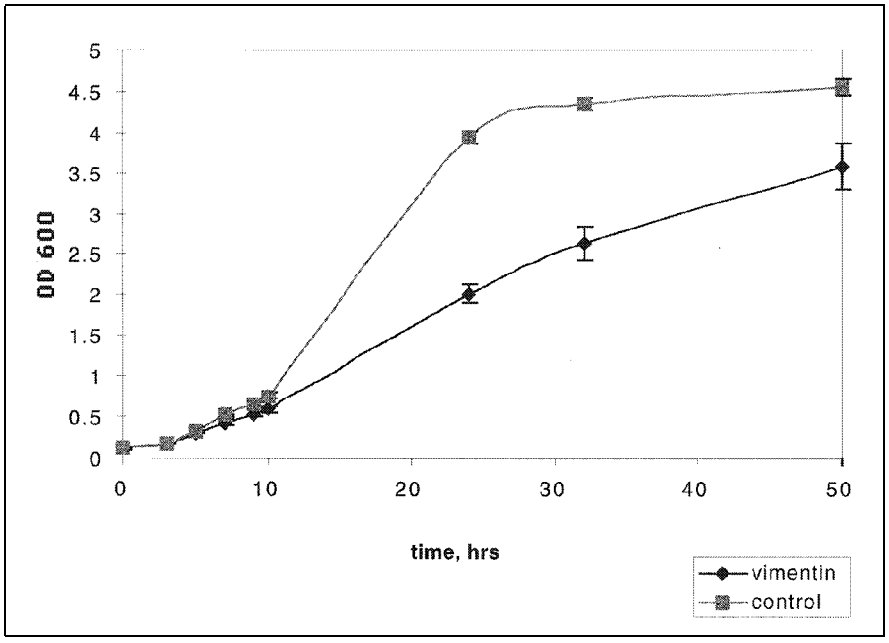

Figure 3. Effects of AD-vimentin on yeast growth rate and viability. Data shown reflects the averaged growth rate of 4 independent transformants expressing LexA-HEF1 and either AD-vimentin (diamonds) or the AD vector pJG4-5 (squares); error bars indicate variance of the samples at each time point.

positive clones identified in two-hybrid screens. The first approach would be to assess activation of the lacZ reporter by two separate means: growth on plates with X-gal and growth on normal media followed by lysis and exposure to X-gal. Nonequivalence of the two assays may indicate the presence of an indirect regulatory effect. Second, the yeast should be plated on media inducing vs. non-inducing for AD-vector-expressed clones to allow detection of effects on overall growth rate. Third, if the initial bait used in a two-hybrid screen is auto-activating, some of the nonspecific test baits used should be similarly auto-activating, rather than transcriptionally inert. Fourth, plating of yeast on medium with BPB and the assessment of whether AD-fused clones cause enhanced uptake can also be used to determine whether particular cDNAs are causing indirect effects on permeability. We note that while the results reported in this paper only deal in detail with three characteristic false positives, we have observed similar effects with others, including hsp90 and a number of noncharacterized expressed sequence tags (ESTs). This finding suggests that the examples provided are not unique. We also note that the suggested assays will not necessarily identify all putative false positives; as always, follow-up biological analysis of clones remains critical.

Together, these observations suggest that the idea of two-hybrid false positives simply representing "sticky" proteins is unlikely to explain the action of all of these proteins. In a compendium of common two-hybrid system false positives, cytoskeletal proteins, redox control/mitochondria-associated proteins, translational control-associated and heat shock proteins are well represented (**www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html**). Overexpression of cytoskeletal proteins is associated with moderate to severe toxicity in yeast (21), which might produce altered permeability. Intriguingly, a study in the yeast *Schizosaccharomyces pombe* has found regulation of internal pH to affect both activity of LacZ against internal X-gal stores and uptake of particular amino acids, including leucine, from the culture medium (1); regulation of translation and heat shock/stress response might similarly indirectly affect these properties.

Previous studies have noted that yeast metabolism and mutant effects can vary in the context of growth on solid vs. liquid medium (22); salient differences might include osmolarity effects on viability or nutrient availability in a freely diffusing liquid versus solid growth medium. As more genomic scale two-hybrid applications proceed, these studies will probably be accompanied by a more definitive list of

candidate false positives. It will be of considerable interest to characterize their mode of action.

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