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A modified yeast three-hybrid system enabling both positive and negative selections

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Abstract

Objective To increase the reporter repertoire of the yeast three-hybrid system and introduce the option of negative selection.

Results Two new versions of the yeast three-hybrid system were made by modifying the MS2 coat RNA-binding protein and fusing it to the Gal4 DNA-binding protein. This allows the use of Gal4 inducible reporters to measure RNA-protein interactions. We introduced two mutations, V29I and N55K into the tandem MS2 dimer and an 11 amino acid deletion to increase RNA-protein affinity and inhibit capsid formation. Introduction of these constructs into the yeast strains MaV204K and PJ69-2A (which contain more reporters than the conventional yeast three-hybrid strains L40-coat and YBZ-1) allows RNA-protein binding interactions with a wide range of affinities to be detected using histidine auxotrophy, and negative selection with 5-fluoroorotic acid.

Conclusion This yeast three-hybrid system has advantages over previous versions as demonstrated by the increased dynamic range of detectable binding interactions using yeast survival assays and colony forming assays with multiple reporters using known RNA-protein interactions.

Key words: Genetic selection; RNA-binding protein; RNA-protein interactions; yeast three-hybrid system

Introduction

Yeast three-hybrid systems have long been valuable tools for discovering and analysing RNA-protein interactions *in vivo* (Putz et al., 1996; SenGupta et al., 1996). Based conceptually on yeast two-hybrid systems, which use two chimeric proteins to analyse protein-protein interactions, yeast three-hybrid systems use three "hybrid" parts, which consist of two chimeric proteins and a chimeric RNA (Figure 1A). The first hybrid consists of a DNA-binding protein such as LexA or Gal4 linked to an RNA-binding protein such as the MS2 coat protein (Ni et al., 1995), or Rev M10 (Perkins et al., 1989). The DNA-binding protein:RNA-binding protein fusion has two functions, to bind DNA at an operator site upstream of reporter genes, and to bind a hybrid RNA molecule. The hybrid RNA molecule contains two separate and distinct binding motifs. The first motif binds the RNA to the DNA-binding protein:RNA-binding protein fusion and the second motif contains the target binding site of interest. The binding motif can be either a known motif used to identify new protein partners, or a library of unknown sequences used to identify new motifs for a known RNA-binding protein. The third part of the yeast three-hybrid system consists of an RNA-binding protein (or potential RNA-binding protein) linked to a transcriptional activation protein such as the Gal4-AD; this protein recruits the cell's transcriptional apparatus to specific reporter genes (Ding and Johnston, 1997). Reporter genes are activated when all three hybrid parts combine to form a functional trans-activation complex and the strength of reporter induction is directly related to the strength of the RNA-protein interaction (Hook et al., 2005). Yeast three-hybrid system reporter genes are generally autotrophic markers such as *HIS3*, or quantitative reporter genes such as *lacZ*, which are typically measured by either growth assays or using luminescence, respectively.

Various modifications to the yeast three-hybrid system have resulted in improved screening efficiency with reduced noise and false positives (Hook et al., 2005). Here we describe a number of additional modifications to the system that increase the number of reporter genes available for positive selection, the range of binding interactions that can be detected, and also introduce the option of negative selection. These improvements increase the number of possible measurements of RNA-protein affinity from reporters with different sensitivities and enable loss-of-function screening and testing for conditional binding between RNA-binding proteins and their RNA targets.

Materials and methods

Reagents were purchased from Sigma unless stated otherwise, and all reagents were of analytical grade.

Gene design and plasmid construction

Two RNA-binding MS2-coat proteins were redesigned and codon optimised for yeast using Gene Designer software (ATUM). Mutations and deletions were made to amino acid sequences in Gene Designer and back-translated and yeast codon optimised to produce DNA sequences for synthesis. Both re-designed proteins, Gal4-MS2-N55K and Gal4-MS2-diFG were synthesized by IDT as gBlocks flanked with AscI and KpnI restriction sites for cloning. The synthetic MS2 genes were cloned into the pGBK-RC plasmid as double ligations along with a PCR-generated fragment containing the yeast RNase P RNA (*RPR1*) promoter (Bernstein et al., 2002), MS2 binding sites and nanos response element (NRE) RNA sequence (5′-UGUAUAUA-3′), which were amplified from pIIIA-MS2-2-p3HR2-NRE (Filipovska et al., 2011) with primers containing KpnI and NgoMIV sites (Figure 1C). The resulting pGBK-derived plasmids were then transformed into yeast using the lithium acetate method (Gietz and Woods, 2002).

Yeast three-hybrid growth assays

Yeast growth assays were performed using the strains YBZ-1 (*MATa, ura3-52, leu2-3, 112, his3-200, trp1-1, ade2, LYS2 :: (LexAop)-HIS3, ura3 :: (lexA-op)-lacZ, LexA-MS2 coat (N55K)*) (Hook et al., 2005), MaV204K (*MATα, trp1–901, leu2–3, 112, his3Δ200, ade2–101Δ::kanMX, gal4Δ, gal80Δ, SPAL10::URA3, UASGAL1::HIS3, GAL1::lacZ*) (Ito et al., 2000) and PJ69-2A (*MATa, trp1–901, leu2–3,112, ura3–52, his3Δ200, gal4Δ, gal80Δ, GAL2::ADE2, GAL1::HIS3*) (Ito et al., 2000). Plasmids expressing RNA-binding

proteins (in pGAD-RC) (Ito et al., 2000) were co-transformed into YBZ-1 with the plasmid pIIIA-MS2-2-p3HR2-NRE, and into MaV204K and PJ69-2A with NRE-RNA expressing plasmids pGBK-Gal4-MS2-N55K or pGBK-Gal4-MS2-diFG. Transformed yeast were then grown in 5 ml of selective synthetic complete media with shaking for 24 h at 30 °C. Cells were then diluted to ~3 x 10^7 cells/ ml and spotted (5 µl) in rows onto selective agar containing increasing concentrations of either 3-amino-1,2,4-triazole (3-AT) or 5-fluoroorotic Acid (5-FOA). Each row was sequentially diluted 10-fold from the previous. Cells were incubated for 3 days at 30°C and then photographed.

Colony forming assays (CFU)

Colony forming assays were performed using the strain MaV204K transformed with the plasmids shown in Figure 1. Briefly, cells were grown in 5 ml of selective media at 30 °C with shaking overnight. The following day cells were diluted to ~30 cells/µl in TE media. 10 µl of diluted cells (300 cells) + 90 µl of TE was then spread onto SC-L-T agar containing 0.075% 5-FOA in triplicate. Plates were incubated for 3 days at 30 °C and colonies were counted.

Results

The MS2 coat protein used in the YBZ-1 strain is linked to a LexA DNA-binding domain, which anchors chimeric RNA molecules to operator sequences upstream of *HIS3* and *lacZ* reporters (Figure 1A). We sought to modify this system such that the MS2 protein could be anchored to GAL operators upstream of *HIS3*, *lacZ*, and *URA3* (Figure 1B). This would allow both positive and negative selection because the URA3 gene product, orotidine 5-phosphate decarboxylase (ODCase) catalyses a reaction that converts 5-FOA into 5-fluorouracil, a toxic compound causing cell death (Lim et al., 1994). We also made a number of structural changes to the MS2 coat protein with the aim of improving its affinity for RNA, generating two variations of the MS2 protein (Figure 1D). The first, Gal4-MS2-N55K, contains two identical modifications made to the LexA/MS2 fusion in the L40coat strain by Bernstein et al., which resulted in the YBZ-1 strain (Hook et al., 2005). The MS2 coat protein in YBZ-1 was arranged as a tandem head-tail dimer linked to a LexA monomer whereas the L40coat version was a single MS2 monomer linked to a single LexA monomer. Initially we linked the two MS2 coat protein sequences by a flexible nine amino acid linker to allow dimerization, analogous to YBZ-1. Next, a single mutation N55K was introduced which decreases the Kd of the MS2-RNA interaction sevenfold, from 3×10^{-9} to 2×10^{-10} (Lim et al. 1994). The second MS2 variant, Gal4-MS2-diFG, was constructed as a tandem dimer, as above, but also contains two additional changes. First, an 11 amino acid deletion (diFG) between K66 and G80 in the FG loop region (Figure 1E), which inhibits capsid formation and thereby increases the concentration of functional MS2 dimers (Peabody and Al-Bitar, 2001). Also an additional substitution, V29I, was introduced that increases RNA-protein affinity 7.5 fold *in vitro* (Powell and Peabody, 2001).

We tested these new MS2 coat protein fusions in two different yeast strains, both of which were chosen because of their desirable properties that have proven useful in previous yeast two-hybrid screening experiments (Ito et al., 2000). The PJ69-2A strain carries an *ADE2* reporter gene driven by the *GAL2* promoter, which was shown to give very few false positives (James et al., 1996), as well as a *HIS3* reporter gene. The MaV204K strain harbours a kanMX cassette that disrupts the endogenous *ADE2* gene, such that G418 can be added to selection media to prevent the growth of contaminating fungi. Most importantly the MaV204K strain has a unique *URA3* reporter, in addition to *HIS3* and *lacZ* reporters, which enables negative selections (Vidal et al., 1996), and we sought to apply this here in the context of the yeast three-hybrid system.

Reporter induction by Gal4-MS2-N55K and Gal4-MS2-diFG were compared to each other, and to YBZ-1 using yeast survival assays (Figure 2A). The well-characterized human RNA-binding protein PUM1 of the Pumilio/FBF (PUF) family and a mutant thereof (PUFwt and PUFmut), as well as enhanced green fluorescent protein (EGFP) were used as positive and negative controls, respectively. PUFwt binds strongly to NRE-RNA, PUFmut contains three mutations (F882G, F905G and Q913G) that strongly reduce its stability and therefore the effective RNA binding of this mutant, and EGFP has no RNA-binding activity. In YBZ-1, PUFwt strongly activated the *HIS3* gene and grew robustly on media lacking histidine (SC-L-U-H) at all 3-AT concentrations. The PUFmut and EGFP controls have low or no affinity for NRE RNA and the growth of YBZ-1 expressing these proteins was strongly inhibited on SC-L-U-H at all 3-AT concentrations. In contrast, MaV204K expressing PUFmut or EGFP with either Gal4-MS2-N55K or Gal4-MS2-diFG showed no growth inhibition on SC media lacking histidine, little to moderate inhibition at low 3-AT (10-20 mM) concentrations, but growth was strongly inhibited at

concentrations over 50 mM (Figure 2B). There was little to no inhibition at all 3-AT concentrations for the PUFwt expressing yeast. The lack of strong growth inhibition of the negative controls suggests that the *HIS3* reporter in MaV204K is slightly leaky (hence the greater 3-AT concentration needed), this has been shown in similar *HIS* reporter systems in flies (Akmammedov et al., 2017).

In PJ69-2A, both Gal4-MS2-N55K and Gal4-MS2-diFG showed strongly inhibited growth when EGFP was tested as an RNA-binding protein at all 3-AT concentrations (Figure 2C). Inhibition of PUFmut on SC-L-T-H 0 mM 3-AT is low but moderate at 0.5-2 mM 3-AT, with Gal4-MS2-N55K showing more obvious inhibition compared to Gal4-MS2-diFG. Growth of the PUFwt expressing yeast was not visibly inhibited at all 3-AT concentrations. *HIS3* reporter expression in PJ69-2A appears to be less leaky compared to MaV204K and was strongly inhibited by lower concentrations of 3-AT (0-2 mM vs 0-50 mM), however the 3-AT range of PJ69-2A appears to be ~2 fold greater than for YBZ-1 (0.5-2 mM Vs 0.1-0.5 mM).

Since MaV204K has a Gal4-regulated *URA3* reporter gene, we explored the potential of this strain for negative selection of RNA-protein interactions. On plates containing 5-FOA, which inhibits growth upon *URA3* induction, PUFwt expressed in in MaV204K inhibited growth at all 5-FOA concentrations compared to both PUFmut and EGFP with both Gal4-MS2-N55K and Gal4-MS2-diFG, which showed low growth inhibition at 0.05-0.075% 5-FOA and stronger inhibition at 0.1%. The growth inhibition of PUFwt is moderate, but most obvious at 0.075% 5-FOA. To quantify URA3 induction and growth inhibition on 0.075% 5-FOA, colony forming assays were also conducted (Figure 3). Both MS2 variants expressing PUFwt showed strong inhibition compared to both non-

184 binding controls, with Gal4-MS2-N55K inhibited 9.6-fold and 9.4-fold compared to
185 EGFP and PUFmut respectively, and in Gal4-MS2-diFG PUFwt was inhibited 9.5-fold
186 and 5-fold compared to EGFP and PUFmut.

Discussion

Here we created improved yeast three-hybrid systems to detect RNA-protein binding interactions by producing different MS2 coat protein fusions and using alternative yeast strains and selections. First, we linked the MS2 coat protein to the DNA-binding domain of the GAL4 protein, which allows RNA-protein interactions to activate GAL inducible reporter genes, this immediately increases the number of strains and reporters to measure RNA-protein affinity. Secondly, we introduced four modifications to the MS2 coat protein, two mirroring those found in the YBZ-1 strain and two further changes: a single substitution, V29I, and an 11 residue deletion, diFG. These modifications have been demonstrated in *in vitro* studies to increase the binding affinity between the MS2 coat protein and its RNA target and reduce the formation of viral-like capsids structures (Lim et al., 1994; Peabody and Al-Bitar, 2001) but had not been used in yeast genetic approaches. We reasoned these modifications may further reduce false positives caused by spurious RNA-protein interactions between MS2 and non-target endogenous RNAs, and increase the number of functional MS2 dimers available for RNA-binding, thus increasing the RNA-binding signal. However, the structural changes made to both MS2 proteins appear to perform equally in MaV204K, with no visible difference in the inhibition of *HIS3* induction by 3-AT or in the toxicity of 5-FOA, indicating the V29I and diFG modifications do not generally improve the detection of protein-RNA binding. However, in PJ69-2A there was a visible difference between Gal4-MS2-N55K and Gal4-MS2-diFG in the growth stimulated by the less efficient RNA-binding protein PUFmut on plates without histidine. This suggests that Gal4-MS2-diFG is slightly less effective at recruiting hybrid RNAs at promoter sites than Gal4-MS2-N55K.

Intermediate inhibition of growth enabled by PUFmut does indicate an important use for this new system, as it allows RNA-binding proteins with lower, but still significant affinity for RNA to be detected. This was not previously possible in YBZ-1, which was, due to higher stringency, unable to differentiate any differences in RNA binding between EGFP and PUFmut. This could be useful for two reasons. First, mutations in either the RNA target, or protein partner that reduce but do not abolish RNA binding could be detected using the GalMS2 system in PJ69-2A. Therefore this improved system would be useful for isolating residues or nucleotides that have intermediate effects on RNA-protein binding. This would be especially useful for characterising RNA-binding proteins that have multiple, similar targets, or identical targets in different structural contexts, which regulate them differently due to different binding affinities and would in turn affect the biological consequences for the RNA target (Jankowsky and Harris, 2015). For example, the splicing factor SFRS1 is known to bind many human target RNAs with diverse structural and binding landscapes (Sanford et al., 2009). Second, some RNA-binding proteins with weak RNA-protein affinities have physiological relevance (Helder et al., 2016), for example C5, a component of the *E. coli* tRNA processing RNAse P complex, targets some transcripts with high specificity but low affinity (Guenther et al., 2013). The Gal4-MS2 system described here could be used to investigate such proteins and others with a range of RNA-protein affinities.

A further major benefit of the new Gal4-MS2 system is it allows the possibility of negative selection. The *URA3* reporter is often used for counter selection as a means to monitor plasmid loss and to select against certain molecular phenotypes (Boeke et al., 1987; Widlund and Davis, 2005), but would also be very valuable to select for and analyse disrupted RNA-protein interactions. In our modified system, important residues required

for RNA-binding interactions could be determined using a simple loss-of-function negative selection. Here, mutations in important residues could easily be isolated from neutral mutations by selecting against RNA-binding positive clones, as loss-of-function mutants would not induce the *URA3* reporter in the presence of 5-FOA. A further benefit of using negative selection extends beyond counter selection against plasmid loss and alternative measurements of RNA-protein affinity, it allows a more sophisticated, conditional selection, using a combination of positive and negative selection. For example, a conditional, temperature sensitive RNA-protein interaction could be isolated from a library using a simple two-step selection system. Initially, the library would undergo positive selection for RNA-protein binding positive clones at a permissive temperature, in this selection, all clones capable of positive RNA-protein interactions, regardless of temperature sensitivity would survive the selection. Finally, an additional round of selection using 5-FOA at a restrictive temperature, would remove all RNA-protein positives that were not temperature sensitive, but sensitive mutants would survive. This two-step selection system could be used to select for any number of conditional RNA-protein interactions including mutants sensitive to salt, pH or many other environmental or chemical conditions.

In conclusion, we provide a variety of different MS2 coat protein fusion and yeast strain combinations that expand the usefulness of the yeast three-hybrid system and will be a valuable addition to the genetic toolbox for the analyses of RNA-protein interactions.

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343 **Figure legends**

344

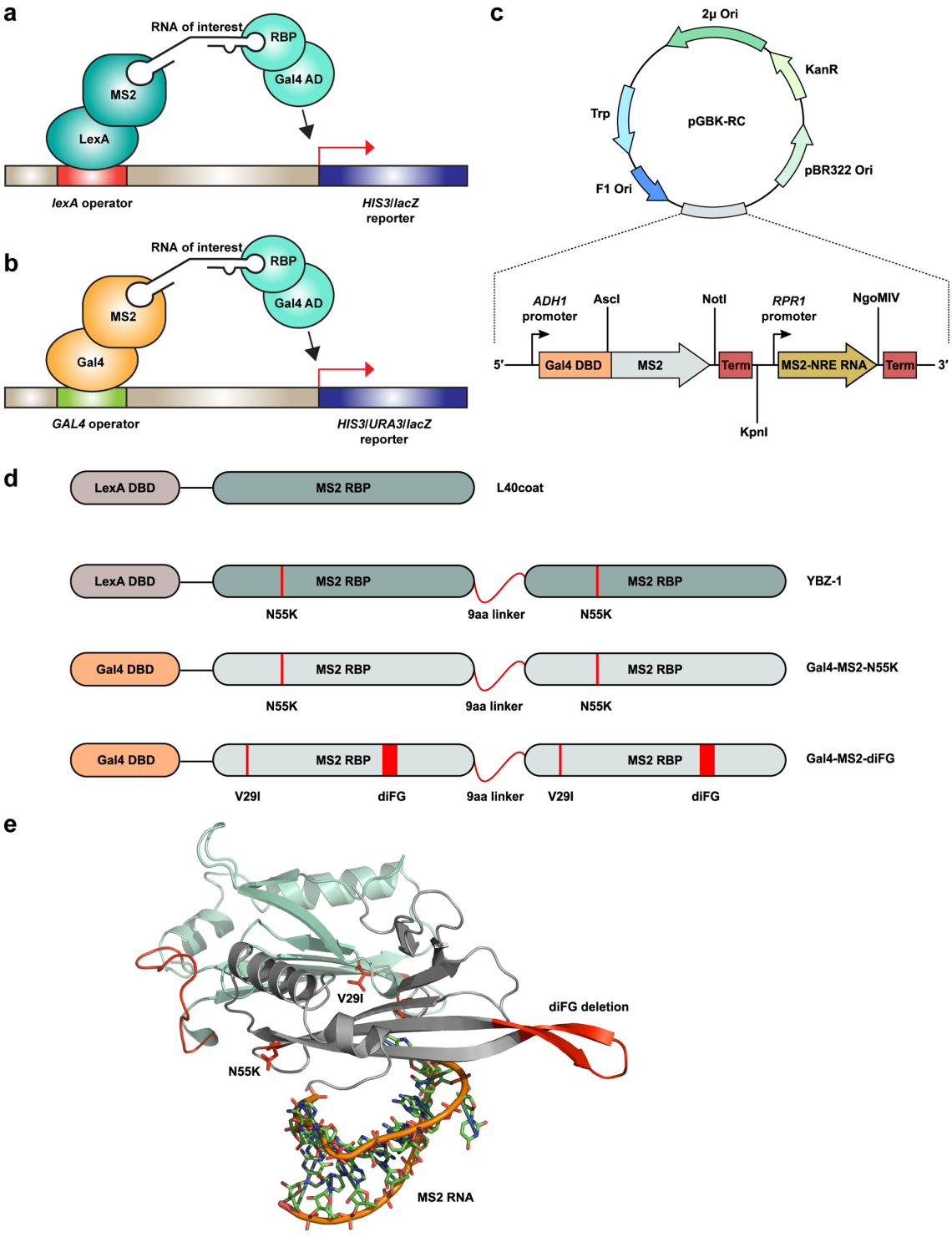
345 **Figure 1**. Design and construction of new yeast three hybrid screening systems. (A)
346 Schematic of the yeast three-hybrid system in YBZ-1. (B) Structure of the redesigned
347 Gal4-MS2 yeast three-hybrid systems with modified chimeric MS2-Gal4 proteins. *HIS3*,
348 *URA3*, and *lacZ* are under control of upstream *GAL4* operators and induction requires
349 RNA-protein mediated assembly of the Gal4 transactivation complex. RBP indicates the
350 RNA-binding protein of interest. (C) Cloning strategy for new MS2 coat proteins. Re-
351 designed MS2 coat proteins were synthesized as gBlocks flanked by AscI and KpnI
352 restriction sites and cloned into the pGBK-RC plasmid along with a PCR generated
353 fragment containing the yeast RNase P RNA (*RPR1*) promoter, MS2 binding sites and
354 the NRE RNA sequence. (D) Location and arrangement of designed MS2 proteins
355 compared to original MS2 proteins used in yeast three hybrid assays. L40coat contains a
356 single MS2 RNA-binding protein (RBP) monomer linked to a LexA DNA-binding
357 protein (DBP). YBZ-1 contains two MS2-RBPs arranged as head-to-tail dimers linked to
358 a LexA-DBP, the two MS2 proteins are connected by a flexible 9 amino acid linker; both
359 copies contain a single substitution (N55K). Two new versions of the MS2 coat protein
360 (Gal4-MS2-N55K and Gal4-MS2-diFG) are linked to the Gal4-DBD. Gal4-MS2-N55K
361 contains N55K substitutions in both copies and an identical amino acid linker to YBZ-1.
362 Gal4-MS2-diFG contains identical amino acid linkers as well an additional substitution
363 (V29I) and an 11-amino acid deletion (diFG). (E) Three-dimensional structure of the MS2
364 dimer bound to RNA. Substitutions and deletions are highlighted in red.

365

366 **Figure 2**. Growth of alternative MS2 coat protein fusions and yeast strains in yeast three-
367 hybrid assays using known RNA-binding proteins. Survival on media lacking histidine

requires activation of reporter genes which require RNA-protein binding. Yeast survival assays were performed in YBZ-1 (A), PJ69-2A (B), and MaV204K (C) strains. A high affinity RNA-binding protein (PUFwt) is compared to a mutant with reduced RNA-binding affinity (PUFmut). EGFP was used as a negative control. Yeast were grown on synthetic complete (SC) agar lacking leucine (L), uracil (U), tryptophan (T), or histidine (H) as indicated.

Figure 3. Negative selections using a modified yeast three-hybrid system. Expression of the *URA3* reporter in MaV204K is toxic in the presence of 5-fluoroorotic acid (5-FOA) and is used as a negative reporter. Yeast survival assays were performed using synthetic complete (SC) media lacking leucine (L) and tryptophan (T), containing 0.05%, 0.075%, or 0.1% 5-FOA. Colony forming assays were used to quantify the effectiveness of negative selection (right-hand panel), all yeast samples were grown for 24 h under identical conditions in triplicate and diluted to ~30 cells/µl before being applied as 10 µl aliquots on media containing 0.075% 5-FOA. Error bars represent SEM and n=3.


a
RNA of interest
RBP
Gal4 AD
MS2
LexA
lexA operator
HIS3/lacZ reporter
b
RNA of interest
RBP
Gal4 AD
MS2
Gal4
GAL4 operator
HIS3/URA3/lacZ reporter
c
2μ Ori
KanR
Trp
pGBK-RC
pBR322 Ori
F1 Ori
ADH1 promoter
AscI
NotI
RPR1 promoter
NgoMIV
5′
Gal4 DBD
MS2
Term
MS2-NRE RNA
Term
3′
KpnI
d
LexA DBD
MS2 RBP
L40coat
LexA DBD
MS2 RBP
MS2 RBP
YBZ-1
N55K
9aa linker
N55K
Gal4 DBD
MS2 RBP
MS2 RBP
Gal4-MS2-N55K
N55K
9aa linker
N55K
Gal4 DBD
MS2 RBP
MS2 RBP
Gal4-MS2-diFG
V29I
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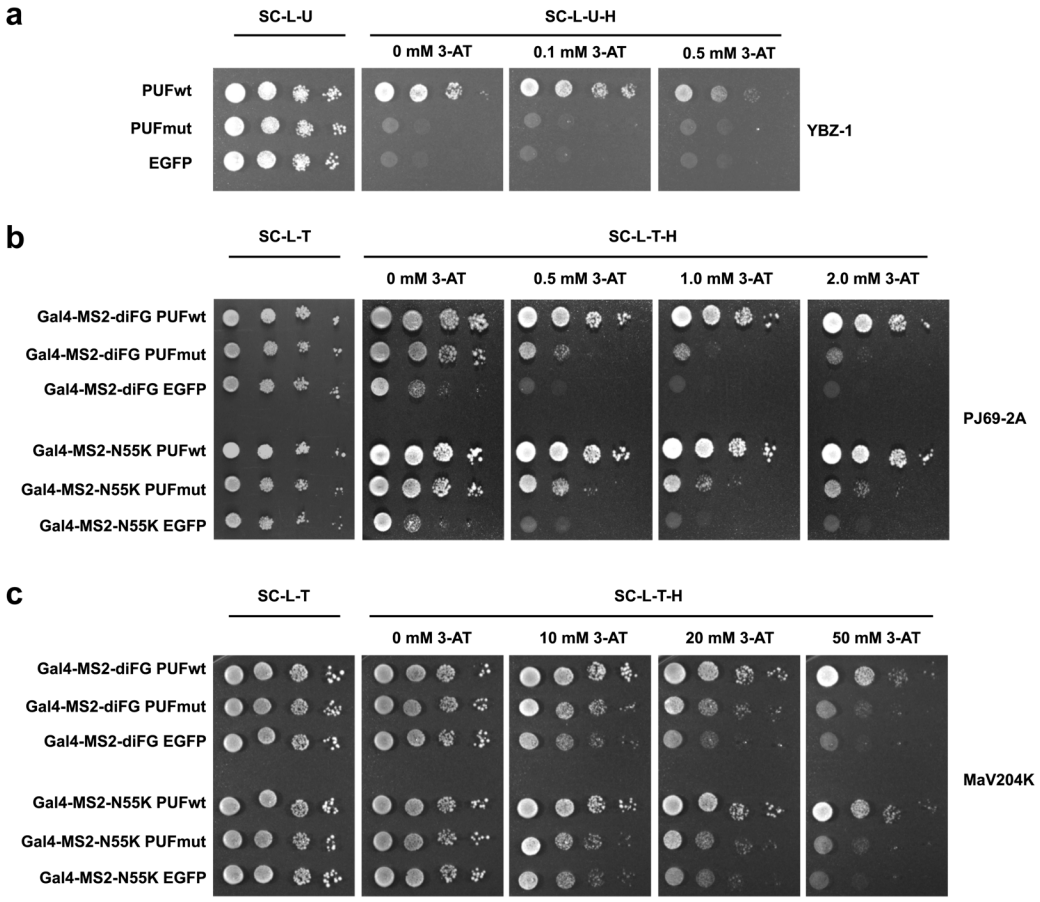
V29I
diFG deletion
N55K
MS2 RNA

Wallis et al. Figure 2



Wallis et al. Figure 3