Interaction mating methods in two-hybrid systems

MIKHAIL G. KOLONIN, JINHUI ZHONG, AND RUSSELL L. FINLEY JR.*

Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, 540 East Canfield Ave., Detroit, Michigan 48201

in Methods in Enzymology, Applications of Chimeric Genes and Hybrid Proteins, Part C, Protein-Protein Interactions and Genomics, eds John Abelson, Scott Emr, and Jeremy Thorner, vol 328, pp 26-46, 2000

To whom correspondence should be addressed

Phone (313) 577-7845
Fax (313) 577-5218
E-mail rfinley@cmb.biosci.wayne.edu

Introduction

The yeast two-hybrid system is a powerful assay for protein-protein interactions [1]. As described elsewhere in this volume, several versions of the two-hybrid system have been developed. Most versions have the following features. The two proteins to be tested for interaction are expressed as hybrids in the nucleus of a yeast cell. One of the proteins is fused to the DNA binding domain (DBD) of a transcription factor and the other is fused to a transcription activation domain (AD). If the two hybrid proteins interact, they reconstitute a functional transcription factor which activates one or more reporter genes that contain binding sites for the DBD. This simple assay has been widely used to identify new interacting proteins from libraries, to test interactions between small and large sets of proteins, to map protein networks, and to address the functions of individual proteins and protein interactions [2-5]. Here we describe interaction mating, a two-hybrid variation that can be adapted to most versions of the system and which can simplify and facilitate most two-hybrid experiments [6,7].

In interaction mating, the AD and DBD fusion proteins begin in two different haploid yeast strains with opposite mating types. To test for interaction, the hybrid proteins are brought together by mating, a process in which two haploid cells fuse to form a single diploid cell. The technique is

fairly simple, requiring only that the two haploid strains be mixed together and incubated overnight on rich medium. The diploids that form are then tested for reporter activation as in a conventional two-hybrid experiment. In this chapter we describe methods for interaction mating to facilitate several routine two-hybrid experiments. We begin with a protocol for screening a library for new interacting proteins or peptides. We then present a simple cross-mating assay to test interactions between small sets of proteins. This assay is particularly useful when testing the specificity of proteins isolated in an interactor hunt. We also present methods for identification and characterization of whole networks of proteins by reiterative interactor hunts and by screening arrayed libraries by mating. Finally, we present two mating approaches to study the functions of individual protein interactions. One approach is to isolate interaction mutants and their suppressors, and the other is to isolate peptides that can disrupt specific protein interactions *in vivo*.

The interaction mating methods can be adapted for use with most of the currently popular two-hybrid systems. The key to choosing a combination of strains and plasmids is to ensure that the two strains to be mated are of opposite mating type (MAT**a** and MATα) and that both have auxotrophies to allow selection for the appropriate plasmids and reporter genes. Here we present detailed procedures for using interaction mating with the version of the two-hybrid system developed by Brent and colleagues [8,9]. In this system the DBD is LexA and the reporters are usually *lacZ* and *LEU2*. The AD is typically a bacterial sequence called B42, and is expressed conditionally from the yeast *GAL1* promoter, which is induced on galactose media and repressed on glucose. The protocols presented here were developed using the yeast strains and plasmids indicated in Table 1. Recipes for yeast media can be found elsewhere in this volume [10] and are available at our web site [11].

Isolating new interactors from libraries

The interaction mating two-hybrid hunt is conducted by mating a haploid strain that expresses the LexA fusion protein, or "bait", with a haploid strain of the opposite mating type that has been pretransformed with the library DNA expressing AD fusions. The resulting diploids are then screened for interactors. This approach can save considerable time and materials when one library is to be screened with two or more bait proteins. Hunts with different baits can be performed by mating each bait-expressing strain with a thawed aliquot of yeast that had been transformed with library DNA in a single large-scale transformation. The interaction mating approach is also useful for bait proteins that interfere with yeast viability because it avoids the difficulty associated with transforming a sick strain expressing such a bait. Finally, because the reporters are less sensitive to transcription activation in diploids than they are in haploids, interaction mating provides a way to reduce the background from baits that activate transcription.

The interaction mating hunt can be divided into four tasks. First, the bait strain is constructed and characterized. The characterization will include a test of how much the bait protein activates the reporters on its own using a method that

Table 1. Yeast strains and plasmids

Strains	Genotype	References[a]
MAT α strains		
EGY48Mat α	*his3, trp1, ura3, 3LexAop-LEU2::leu2*	1
RFY231	Mat α, *his3, trp1Δ::hisG, ura3, 3LexAop-LEU2::leu2*	2
RFY251	Mat α, *ura3, his3, trp1Δ::hisG, lys2Δ201, 3LexAop-LEU2::leu2*	unpublished
MAT a strains		
RFY206	Mat **a**, *trp1Δ::hisG his3Δ200 leu2-3 lys2Δ201 ura3-52*	3
YPH499	Mat **a**, *ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, leu2-Δ1*	4

Plasmids	Marker	Expression cassette[b]	References[a]
Bait plasmids			
pEG202	*HIS3*	*ADH1*p-LexA (DBD)	1
pJK202	*HIS3*	*ADH1*p-LexA (DBD)	5
AD-fusion plasmids			
pJG4-5	*TRP1*	*GAL1*p-B42 (AD)	6
pMK2	*URA3*	*ADH1*p-B42 (AD)	unpublished
Reporter plasmids			
pSH18-34	*URA3*	8LexAop-*lacZ*	5
pCWX24	*LYS2*	8LexAop-*lacZ*	7

a. Key to references

1.Estojak, J., Brent, R. & Golemis, E. A. *Mol Cell Biol* **15**, 5820-5829 (1995).
2.Kolonin, M. G. & Finley, R. L., Jr. *Proc Natl Acad Sci U S A* **95**, 14266-14271 (1998).
3.Finley, R. L., Jr. & Brent, R. *Proc Natl Acad Sci U S A* **91**, 12980-12984 (1994).
4.Sikorski, R. S. & Hieter, P. *Genetics* **122**, 19-27 (1989).
5.Golemis, E. A. *et al. in* "Current protocols in molecular biology", Vol. **20.1**, (Ausubel, F.M. *et al*., eds.), John Wiley & Sons, Inc., New York, 1999.
6.Gyuris, J., Golemis, E., Chertkov, H. & Brent, R. *Cell* **75**, 791-803 (1993).
7.Xu, C. W., Mendelsohn, A. R. & Brent, R. *Proc Natl Acad Sci U S A* **94**, 12473-12478 (1997).

b. Expression cassette indicates the promoter (from the *ADH1* or *GAL1* gene, or a minimal promoter with 8 LexA binding sites), followed by the fusion moiety (LexA DBD or B42 AD), or reporter (*lacZ*).

mimics the library screen. The second task is to create the pretransformed library by high-efficiency yeast transformation with the library plasmid DNA. Yeast transformed with the library can be frozen in many aliquots and thawed individually for each interactor hunt. The third task is to mate the bait strain with an aliquot of the pretransformed library strain and allow diploids to form on solid YPD medium overnight. The resulting diploid yeast are then screened for interactors as in a conventional two-hybrid hunt by testing for galactose-dependent activation of the reporters. The final task is to isolate and characterize the cDNAs from the positives. The initial characterization should include a demonstration that each cDNA encodes a protein that interacts specifically with the original bait. This can be achieved with a cross-mating assay, which is described in the following section.

Constructing and characterizing the bait strain

1. Construct the bait plasmid (pBait) by inserting a cDNA encoding the protein of interest in-frame with LexA into an appropriate bait vector (see Table 1), using standard cloning methods or by recombination cloning [12] (e.g., see *Constructing bait strains by recombination cloning*). The bait vectors for the system used here have the *HIS3* marker and 2 μm origin of replication (Table 1).

2. Transform RFY206 (or another appropriate strain; see Table1) with pBait and with a *lacZ* reporter plasmid such as pSH18-34. This and most *lacZ* reporter plasmids in this system have the *URA3* marker and 2 μm origin of replication (Table 1). This and all yeast transformations can be performed by the lithium acetate method [13]. Select transformants on Glu/CM -Ura, -His media.

3. Characterize the bait strain. This may include performing a Western to show that full-length stable bait protein is synthesized and a test for reporter activation by the bait [9,10,14]. The most useful approach to test whether the bait activates the *LEU2* reporter is to mate the bait strain with a control strain containing empty library vector. The resulting diploids can then be plated on media lacking leucine, which will mimic the library hunt. This test can be done concurrently with the actual hunt as described below.

Preparing the pretransformed library and control strains

1. Perform a large-scale transformation of RFY231 (or another appropriate strain, see Table 1) with library plasmid DNA. The library plasmid in this system is derived from pJG4-5, which contains the *TRP1* marker and 2μm origin of replication, in addition to the *GAL1* promoter driving expression of the AD fusions [8]. Select library transformants on Glu/CM -Trp plates. Collect library transformants by scraping plates, washing yeast, and resuspending in 1 pellet volume of glycerol solution (65% glycerol, 25mM TRIS pH 7.5). Freeze 0.5 ml aliquots at -70 to -80°C.

2. To make a control strain, transform RFY231 with the empty library vector, pJG4-5, and select transformants on Glu/CM -Trp plates. Combine several colonies to inoculate 30 ml of Glu/CM -Trp liquid medium and grow at 30°C with shaking to OD_{600} ~3. Collect the cells by centrifugation and resuspend in 1 pellet volume of glycerol solution. Freeze in several 0.5 ml aliquots at -70 to -80°C.

3. Determine the plating efficiency of the frozen cells by thawing an aliquot of each pretransformed strain from step 1 and step 2 and making 10-fold serial dilutions in sterile water. Plate 100 μl of each of 10^{-5}, 10^{-6}, and 10^{-7} dilutions on Glu/CM -Trp plates and incubate 2 to 3 days. Count the colonies and determine the number of colony-forming units (cfu) per unit volume of frozen yeast. The plating efficiency for a typical library transformation and for the control strain will be ~1 x10^8 cfu / 100 μl.

Screening for interactors by mating

In this section, the bait strain is mated with a frozen aliquot of the pretransformed library strain. At the same time, the bait strain is mated with a frozen aliquot of the control strain to test

for background activation of the *LEU2* reporter by the bait itself.

1. Grow a 30-ml culture of the bait strain in Glu/CM -Ura, -His liquid medium to ~3 x 10^7cells/ml (OD_{600} ~ 1.5). Collect the cells by centrifugation. Resuspend the cell pellet in sterile water to a final volume of 1 ml. This will correspond to about 10^9 cfu/ml.

2. Mix 200 µl of the bait strain with ~1 x 10^8 cfu (~0.1 to 1 ml) of the pretransformed library strain in a microcentrifuge tube. In a second tube mix 200 µl of the bait strain with 200 µl of the pretransformed control strain. This should approximate a 2-fold or greater excess of bait strain over pretransformed library strain. Under these conditions, ~10% of the cfu in the pretransformed library strain will mate with the bait strain. This mating efficiency should be considered when calculating how much of the library is being screened.

3. Collect the cells by centrifugation and resuspend in 200 µl of YPD medium. Plate each suspension on a 100-mm YPD plate. Incubate 12 to 15 hr at 30°C to allow mating.

4. Add ~1 ml of liquid Gal/CM -Ura, -His, -Trp medium to the lawns of mated yeast on each plate and suspend the cells with a sterile applicator stick or glass rod. To induce expression of the library proteins with galactose, dilute each cell slurry into 100 ml of Gal/CM -Ura, -His, -Trp liquid medium in a 500-ml flask and incubate with shaking for 6 hr at 30°C.

5. Collect the cells by centrifugation and wash by resuspending in 30 ml of sterile water and centrifuging again. Resuspend each pellet in 5 ml sterile water. Measure OD_{600} and dilute with water to ~1 x 10^8 cells/ml (OD_{600} ~ 5.0).

6. For each mating make a series of dilutions from 10^{-1} to 10^{-6} in sterile water. To determine the titer of diploids, plate 100 µl each of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions on 100-mm Gal/Raff/CM -Ura, -His, -Trp plates. To determine the level of activation of the *LEU2* reporter by the bait itself (here called the transactivation potential; see below), plate 100 µl each of the six dilutions on 100-mm Gal/Raff/CM -Ura, -His, -Trp, -Leu plates. To select for interactors from the library mating, plate 100 µl of the 10^{-1} dilution on each of 20 100-mm Gal/Raff/CM -Ura, -His, -Trp, -Leu plates and 100 µl of the undiluted cells on each of 20 100-mm Gal/Raff/CM -Ura, -His, -Trp, -Leu plates. Incubate plates at 30°C 2 to 5 days.

7. Count colonies. The transactivation potential of the bait can be represented as the number of Leu+ colonies per cfu (Leu+/cfu). This is determined by the ratio of the number of colonies growing on Gal/Raff/CM -Ura, -His, -Trp, -Leu to the number of colonies growing on Gal/Raff/CM -Ura, -His, -Trp medium for a particular dilution of the control mating. A bait with essentially no transactivation potential will produce less than 10^{-6} Leu^+/cfu. To identify all of the interactors in the pretransformed library, it may be necessary to pick and characterize all of the background Leu^+ colonies produced by the transactivation potential of the bait itself, particularly if the background is high and the frequency of interactors is low. The *minimum* number of Leu^+ colonies that should be picked is given by:

(transactivation potential, Leu^+/cfu) x (# library transformants screened).

8. Test the Leu+ clones that grew on the selection plates for galactose-dependent Leu+ and lacZ+ activity as follows. Streak Leu+ colonies to 1 cm^2 patches on Glu/CM -Ura, -His, -Trp plates to turn off the *GAL1* promoter. If the Leu+ colonies were very close together it may be necessary to streak purify to single colonies first on Gal/Raff/CM -Ura, -His, -Trp, -Leu plates, and then patch the single colonies onto Glu/CM -Ura, -His, -Trp plates. Include patches of strains that will serve as controls for the interaction phenotype. Grow 1-2 days at 30ºC.

9. Transfer the patches to a velvet and replicate to 5 plates in the order shown below. The first four are indicator plates; the last plate serves as a transfer control and a source of yeast for plasmid isolation or PCR. Incubate 1 to 5 days at 30ºC and record the Leu+ and LacZ+ phenotypes.

1. Glu/CM -Ura, -His, -Trp, X-Gal
2. Gal/Raff/CM -Ura, -His, -Trp, X-Gal
3. Glu/CM -Ura, -His, -Trp, -Leu
4. Gal/Raff/CM -Ura, -His, -Trp, -Leu
5. Glu/CM -Ura, -His, -Trp

Alternatively, the *lacZ* phenotype can be determined by filter lift, X-Gal overlay, or liquid assays [15-17]. Pick for further analysis yeast that require galactose to grow on the -Leu plates. Yeast that grow on the -Leu glucose plates, where the AD fusion is not expressed, are false positives. Since the *LEU2* reporter is more sensitive than the *lacZ* reporter it is possible for a weak interaction to activate only *LEU2* and not *lacZ.*

Characterizing interactors

To distinguish true positives from false positives, which may arise for example from mutations in the reporter genes, it is important to demonstrate that the reporter phenotypes depend on the cDNA. It is also important to show that each cDNA encodes a protein that interacts specifically with the bait. Both of these objectives can be accomplished by isolating the cDNA and reintroducing it into a haploid yeast strain. Interactions can then be assayed by mating the new strain with a number of bait strains. The bait strains should include the original bait strain used for the hunt and strains expressing control baits. The interaction specificity test is performed in the cross-mating assay described in the next section.

If many positives were identified it is useful to restrict the analysis to non-redundant cDNA isolates. The quickest way to identify non-redundant cDNAs is to restriction digest PCR products generated with vector-specific primers [9] (e.g., for pJG4-5 use forward primer FP-J, 5' GCTGAAATCGAATGGTTTTCATG 3', and reverse primer RP-J, 5' GAGTCACTTTAAAATTTGTATACA C 3'). As a template source, fresh yeast cells can be used. For example, cells from the Glu/CM -Ura, -His, -Trp plates in step 9 of the previous section can be added directly to a PCR reaction; the reaction should be pre-incubated at 95ºC for 5 min to release DNA. Alternatively, yeast DNA minipreps can be used as the source of template DNA [9,14]. The amplified products can be digested directly with a restriction enzyme (*AluI* or *HaeIII*) and then analyzed by gel electrophoresis. The isolates that

produce matching digestion patterns correspond to the same cDNA.

Once the unique cDNA classes have been identified the corresponding plasmids or cDNAs can be retrieved and introduced into another strain to mate with different bait strains. To isolate the library plasmids, yeast DNA minipreps can be prepared and used to transform *E.coli*, and then plasmid DNA can then be amplified and purified from individual *E.coli* clones. However, there are two potentially faster alternatives to isolating the plasmid from *E.coli*. In one approach the yeast DNA miniprep is used to transform yeast directly. Transformants are selected on -Trp plates. Colonies that have taken up the *TRP1* plasmid but not the *URA3 lacZ* reporter plasmid or the *HIS3* bait plasmid can be identified by replicating transformants to Glu/CM -His and Glu/CM -Ura plates. Trp+, Ura-, His- yeast can then be used in the cross-mating assay described below to test specificity. In the other approach each cDNA is PCR-amplified directly from the positive yeast colonies using FP-J and RP-J, and then subcloned directly into pJG4-5 in a new strain by recombination cloning (Fig. 1). In this procedure RFY231 or a similar strain is co-transformed with the crude PCR product and the library vector (pJG4-5) linearized at the cloning site. The linearized vector will be repaired in the yeast cells by homologous recombination resulting in insertion of the cDNA into the vector. This recombination cloning approach is discussed in more detail in the context of making new baits (see *Constructing new bait strains by recombination cloning*).

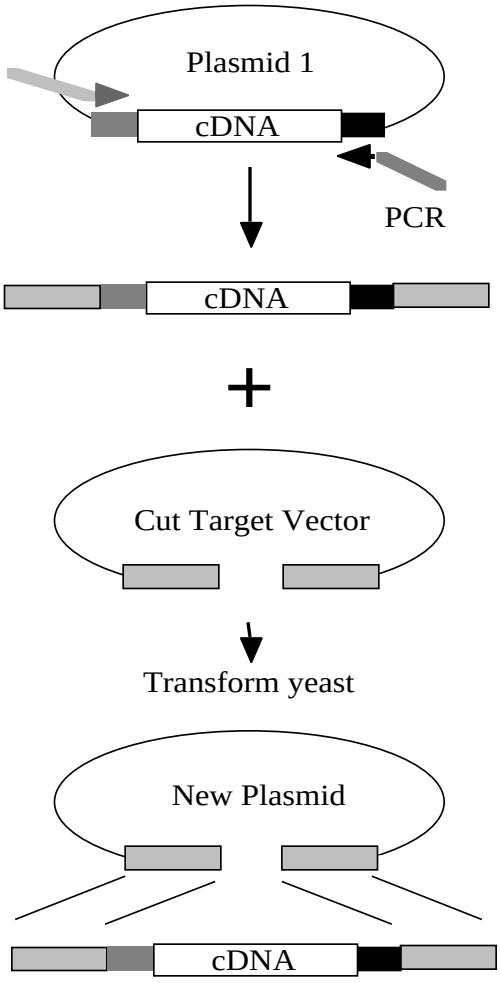

Fig. 1. Gap repair in yeast to make new plasmids. A DNA fragment from one vector is PCR-amplified with primers that each include about 60 bases of sequence from the region flanking the cloning site of a target vector. Yeast are then co-transformed with the PCR product and with the target vector which has been linearized with a restriction enzyme that cuts within the cloning site. The target vector will be repaired in the yeast cell by homologous recombination, resulting in a new plasmid containing the DNA fragment in the target vector (Ma, H., Kunes, S., Schatz, P. J. & Botstein, D. *Gene* **58**, 201-216 (1987)). In cases where the starting vector (Plasmid 1) is the same vector as the target vector, the PCR primers should correspond to sequences 60 bp upstream and downstream of the cloning site. For example, when subcloning cDNSs from pJG4-5 to pJ-G4-5 in a new strain, use primers FP-J and RP-J, as described in the text.

Testing interactions between small sets of proteins by cross-mating assay

The cross-mating assay is a quick method to test interactions between sets of AD- and DBD-fused proteins (Fig. 2). The AD fusions are expressed in individual strains of one mating type, and the DBD fusions are expressed in individual strains of the opposite mating type. Binary interactions between the AD and DBD fusion proteins can be easily sampled by mating the two sets of strains on one plate and replicating to indicator plates (Fig. 2). This approach reduces the number of yeast transformations needed to test interaction between two sets of proteins, since otherwise a separate transformation would be necessary for each binary interaction to be tested.

Cross-mating is particularly useful for testing the specificity of new interactors isolated in an interactor hunt. In this case the AD fusion strains express new cDNAs, and the DBD fusion strains include the original bait strain used for the screen, plus a number of strains expressing unrelated baits.

Procedure

1. Streak parallel lines (at least 2 mm wide) of AD fusion strains (e.g., RFY231 transformed with various pJG4-5 derivatives) onto a Glu/CM -Trp plate. Incubate at 30ºC for 1-3 days.

2. Streak parallel lines (at least 2 mm wide) of bait strains (e.g., RFY206 transformed with pSH18-34 and various bait plasmids) to a Glu/CM -Ura, -His plate. Incubate at 30ºC for 1-3 days.

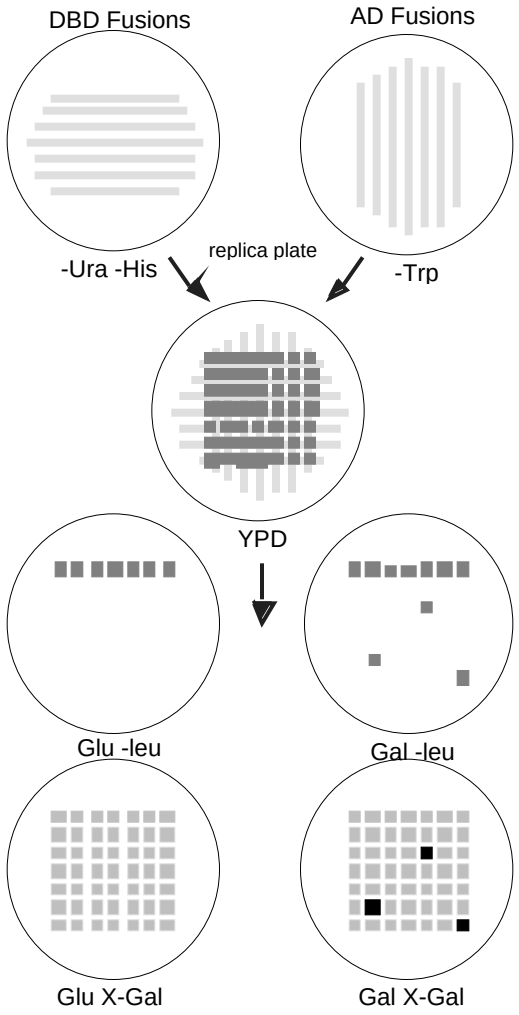

Fig. 2. Interaction mating assay for protein interactions. Several strains of one mating type expressing different DBD fusions are streaked in parallel lines on one plate. Strains of the opposite mating type expressing AD fusions are streaked on another plate. The two strain types are crossed onto the same replica velvet and lifted with a single YPD plate. After growth overnight the YPD plate is replicated to indicator plates. All of the indicator plates are -Ura-His-Trp to select for diploid growth. Two of the indicator plates (-Leu) test for expression of the *LEU2* reporter and two test for *lacZ* expression (X-Gal). In this version of the two-hybrid system (Gyuris, J., Golemis, E., Chertkov, H. & Brent, R. *Cell* **75**, 791-803 (1993)), the AD fusions are expressed only in galactose medium. This allows detection of false positives in which reporter activation is independent of the AD fusion, as in bait strain on the top row. Adapted from ref (Finley, R. L., Jr. & Brent, R. *Proc Natl Acad Sci U S A* **91**, 12980-12984 (1994)).

3. Press the bait strains and AD fusion strains to the same replica velvet so that the lines of bait and prey yeast strains intersect. Replicate the impression onto a YPD plate and incubate at 30°C overnight.

4. Test the reporter phenotypes by replica plating from the YPD plate to indicator plates as described in step 9 of *Screening for interactors by mating*. The phenotypes are interpreted as before: galactose-dependent growth on -Leu plates (Leu+) and blue color on X-Gal plates (lacZ+) of the diploids that grow at the intersections of the two mated strains indicates an interaction.

Mapping networks of interacting proteins

Many important regulatory pathways consist of networks of interacting proteins. Protein interaction data derived from two-hybrid experiments can suggest the functions for individual genes and assist in assembling proteins into regulatory pathways. Here we present two approaches for elaborating protein networks using the yeast two-hybrid system and interaction mating. The first approach involves sequential (reiterative) library screening in which newly isolated cDNAs are used as baits for subsequent library screens. By streamlining the interactor hunt protocol and introducing a rapid way to make new bait strains, this approach is a time and cost effective way to elaborate large protein networks. The second approach involves systematic screening of arrayed yeast expressing DBD or AD fusion proteins.

Elaborating protein networks by reiterative interactor hunts

Starting with one or more proteins, a protein interaction network can be generated by exhaustive and sequential screening of AD fusion libraries (see for example [18]). New AD fusion proteins isolated in each hunt are converted to baits for use in subsequent hunts. The strategy is outlined in Fig. 3.

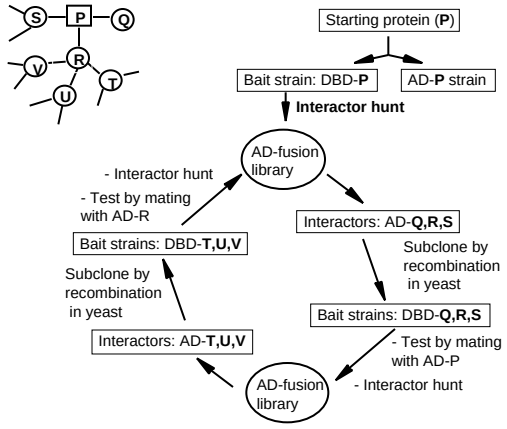

Fig. 3. Reiterative interactor hunts. New clones isolated in interactor hunts are converted to baits for subsequent hunts. The fastest approach is to construct new bait strains by recombination cloning from the AD fusion library vector to the bait vector (see Fig. 2 and text). New bait strains can then be quickly tested by mating with a strain expressing an AD fused interactor, for example, from the previous hunt. See text for more details.

A potential rate-limiting step in conducting reiterative interactor hunts is the step of subcloning newly-isolated cDNAs into the bait vector followed by transformation and characterization of the bait strain. This process can be expedited dramatically by using recombination cloning (Fig. 1; see protocol below). In this approach the new bait plasmid is constructed by recombination in yeast, so that the new bait strain is created in the same step. Each new bait strain can be easily tested by mating it with a test strain expressing an AD fusion that should interact with the new bait. For

most steps in a reiterative hunt, the test AD fusion strain will be available from the previous screen (see Fig. 3). This test will confirm that the new bait strains contain cDNAs in the correct reading frame and that the expressed fusion protein can enter the nucleus and bind the reporters. A reiterative series of interactor hunts might proceed as follows.

1. Construct a strain expressing the initial protein of interest as a bait as described above. Construct a strain expressing an AD fusion version of the protein of interest by inserting the cDNA into a library vector (e.g. pJG4-5), and transforming RFY231.

2. Conduct an interactor hunt by mating the bait strain with an aliquot of frozen library as described above in the interactor hunt protocol. Identify specific interactors as described above. If many hunts are planned, the fastest approach to isolating the positive cDNAs should be used (e.g. PCR-amplification of cDNAs from yeast, as described above).

3. Make new bait strains from the positive interactors by recombination cloning (see protocol below). Test the new bait strains for interaction with the AD fusion to the original protein of interest (from step 1 above) in a cross-mating assay.

4. Use the new bait strains to conduct interactor hunts by mating. Again, make new bait strains from the interactors. This time, and for all subsequent hunts, test the new bait strains by mating with the AD fusion isolated in the previous hunt.

Constructing new bait strains by recombination cloning

In this protocol, the cDNAs encoding new interactors are PCR-amplified directly from the library clone (pJG4-5 derivative) using primers that include sequences from the bait vector (Fig. 1). The new bait strain is then made by co-transforming yeast with the PCR product and the bait vector linearized at the cloning site. The yeast cells will repair the double-strand break in the bait vector by homologous recombination using the PCR product as a template. The result is that the cDNA is inserted into the bait vector in-frame with the DBD.

Procedure

1. Amplify the cDNA of an interactor clone by PCR using the following primers: If making a pJK202 bait vector (Table 1), use forward primer FP-N (5' GAC TGG CTG GAA TTG GCC CCC AAG AAA AAG AGA AAG GTG CCA GAT TAT GCC TCT CCC G 3'); if making a pEG202 bait vector (Table 1), use forward primer FP-E (5'GGG CTG GCG GTT GGG GTT ATT CGC AAC GGC GAC TGG CTG GTG CCA GAT TAT GCC TCT CCC G 3'). The underlined portion of each is from pJG4-5; the remainder is homologous to the appropriate bait vector. The same reverse primer can be used for both vectors (RP-J, see above). The bait vectors share nearly the same terminator region as pJG4-5 so that the total length of homology with pJG4-5 at the 3' of the PCR product is 80 bp. Use a high fidelity polymerase like Vent (New England Biolabs) or Pfu (Stratagene). For template DNA use either whole yeast cells containing the pJG4-5 clone (add a 5 min 95°C step in the beginning of PCR program), or yeast DNA minipreps.

2. Digest the bait vector with *EcoRI* and *XhoI*, and purify over a Centricon-100 concentrator (Ambion). Adjust the concentration to 50ng/ul.

3. Make the bait strain by transforming RFY206/pSH18-34 with 200 ng of linearized vector and 5-10 µl (10-200 ng) of unpurified PCR reaction. Perform a control transformation with no PCR product. The presence of the PCR product should increase the transformation efficiency by greater that 5-fold. More than 90% of the transformants will contain inserts.

4. Test several independent transformants by mating with a strain (e.g. RFY231) expressing an AD fusion to a known interactor by performing a cross-mating assay as described above.

Screening two-hybrid arrays

Interaction data generated by testing interactions between sets of proteins encoded by cloned cDNAs is often easier to interpret than results from screening libraries. This is in part due to the fact that a library screen includes a selection for false positives. These arise, for example, from yeast mutations or library proteins that increase the reporter readout [19]. Library screens are also subject to false negatives, for example, due to the absence or low frequency of a particular cDNA in a library, or from loss of library clones encoding proteins that reduce yeast viability. These problems are avoided when two strains expressing single defined hybrid proteins are mated and the reporter readout is assayed in the diploids. There is no selection for false positives in such a binary assay, and clones encoding mildly toxic proteins can be maintained in haploids under repressing conditions. Finally, while transcription activators make poor baits in library screens, it is possible to directly test them for interactions, either by expressing them as AD fusions, or by looking for increases in reporter activation over the background generated by the bait itself.

The quickest way to test for interactions between small sets (tens) of proteins is the cross-mating assay presented above [6]. Interactions between large sets of proteins can be assayed by mating arrays of DBD fusion strains and AD fusion strains [5,20] (Fig. 4). Strains expressing individual hybrid proteins are collected and arrayed in the standard 48-well or 96-well format. Individual proteins are then tested against each array by mating. For example, a lawn of yeast expressing a DBD version of the protein can be mated with the array of strains expressing AD fusions. Large arrays can be systematically mated with each other as shown in Fig. 4. For very large arrays, a pooling scheme may be useful for mating (J.Z. and R.L.F., unpublished).

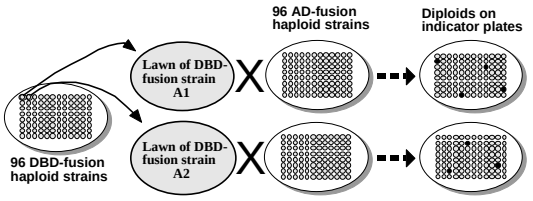

Fig. 4. Interaction mating with arrayed libraries. Arrayed strains expressing DBD fusions and strains expressing AD fusions are collected and tested for interaction by mating. In the approach shown, individual DBD fusion strains are first spread on lawns and then mated with plates containing 96 arrayed AD fusion strains. As in the cross-mating assay (Fig. 1) the two strains are mated by mixing them on a YPD plate, growing overnight, and then replicating onto indicator plates, in this case X-Gal plates.

Prospects for genome-wide protein interaction map

The numerous individual successes of two-hybrid experiments, combined with the potential for scale-up, have opened the prospect of generating genome-wide interaction maps by two-hybrid screening. The first attempt at generating a genome-wide interaction map was the work of Fields and colleagues to map the interactions between the 55 proteins encoded by the bacteriophage T7 genome [21]. They used a combination of approaches, including mating individual and small pools of bait strains with AD fusion libraries, and numerous pair-wise matings of individual DBD and AD fusion strains. The interaction map that they derived from their exhaustive two-hybrid analysis included a wealth of new functional information and insights about T7 biology. For example, they discovered a number of unsuspected interactions, and they confirmed many interactions that were previously suspected on genetic grounds. While the T7 genome is relatively small as genomes go, their success in mapping its protein interactions was nevertheless a landmark accomplishment because it demonstrated that large-scale interaction maps generated by two-hybrid technology can have immense utility as we try to decipher the functions of many genes and whole genomes.

The approaches used for larger genomes may differ depending on the size of the genome and whether cDNAs are available. For example, random mating of strains expressing DBD and AD fusion libraries may be useful for mapping the interactions encoded by small genomes, though the false positive and false negative frequency may interfere with analysis. Alternatively, arrayed libraries could readily be made from the relatively small microbial genomes [22]. Arrayed libraries are also likely to be used in any approach that maps interactions for a larger complex genome. Arrayed libraries may be generated from collections of Expressed Sequence Tags (EST), for example, by recombination cloning into the DBD and AD fusion plasmids [20]. For genomes not represented by significant EST collections, arrayed yeast two-hybrid libraries can be generated by constructing normalized libraries.

Exploring the functions of individual protein interactions

As new protein-protein interactions are identified, two-hybrid technology can be used to begin to study the functions of specific interactions. Interaction domains and contacts between proteins can be mapped by screening libraries of mutations of one or the other protein [23]. Mutant versions of a protein that fail to interact with a potential partner protein could be used to explore the function of that interaction *in vivo*. For example, in some organisms the function of specific interactions can be addressed by expressing interaction mutant alleles of either partner protein in a null mutant background. Furthermore, starting with a non-interacting mutant version of one protein it is a relatively simple task to isolate a potential suppressor mutation in one of its partner proteins which can restore the interaction, by screening a library of mutant partner proteins. The ability of a suppressor to restore wild-type activity to an interaction mutant when both are expressed *in vivo* can provide very strong evidence for the function of the

specific interaction. This approach is akin to the use of classic suppressor genetics to suggest functional interactions, but has the advantage of using designed and characterized mutant alleles so that the nature of the mutation is known in advance. Another approach to testing the functions of specific interactions *in vivo* is to use reagents that disrupt them. The yeast two-hybrid system is a useful assay for isolating and characterizing such reagents.

Isolating interaction mutants and suppressors

Interaction mutants of a protein can be isolated by creating a library strain expressing its mutant versions as AD fusions and screening the library for non-interactors with a partner protein as a bait. A number of efficient methods for making a mutant library have been described [24,25]. For example, one convenient approach is to perform low-fidelity mutagenic PCR [25] and subclone the PCR products into the AD fusion vector. By using PCR primers with sequences corresponding to the AD fusion vector (e.g., primers FP-J and RP-J; see above), the mutagenized PCR products can be readily inserted into the vector by recombination cloning (Fig. 1). This approach simultaneously creates the library strain that can be collected and frozen in aliquots to be screened with any number of interacting bait proteins. For many studies, the goal will be to identify single amino acid substitutions that abolish interaction, suggesting residues and domains important for interaction. However, random mutagenesis will lead to many non-interacting clones that result from nonsense mutations encoding truncated AD fusions. Such truncated proteins could be identified by performing Westerns on yeast clones containing non-interactors. Alternatively, stable full-length non-interactors can be isolated directly by including an easily detectable carboxy-terminal moiety, such as green fluorescent protein (GFP) or ß-galactosidase, on the AD fusion [26]. In this case, missense mutants can be identified by screening the mutant library for non-interactors that express GFP or *lacZ*. Another alternative is to use a two-hybrid system with two different reporter systems responsive to different DBD fusions [27,28]. This would allow isolation of mutant versions of an AD fusion protein that selectively abolish interactions with one DBD fusion but not the other.

Once a mutant library has been made it can be screened for non-interactors by interaction mating with a bait strain. The first part of this screen can be conducted as in an interactor hunt. First, an aliquot of the mutant library is thawed and mixed with fresh bait strain. The mixture is then plated on a YPD plate. To screen the diploids for non-interactors, one could plate the mated yeast onto diploid selection plates, and then replicate the colonies to the reporter indicator plates. The number of colonies to screen depends on the size of the target protein and the efficiency of the mutagenesis, but generally will be fewer than 10,000 to cover all possible mutations. An alternative to screening through this many colonies is to use a toxic reporter gene. For example, expression of the yeast *URA3* gene is toxic to yeast grown on medium containing 5-FOA. Strains containing a *URA3* reporter have been described for systems using the LexA or Gal4 DBD [27,29].

Breaking interactions with proteins and peptides

Another way to determine the function of a specific protein interaction is to disrupt it *in vivo* using a trans-acting reagent. The yeast two-hybrid system provides an assay to identify such reagents. For example, random peptide libraries can be screened to isolate peptide aptamers, which are peptides that bind tightly and specifically to a given protein [30,31]. Some aptamers will interact with surfaces of their target proteins that normally make contacts with other proteins, and thus will disrupt the corresponding interactions. Like dominant negative mutant proteins [32], peptides that disrupt specific protein interactions may be useful for genetic analysis in cases where loss-of-function mutations in a gene cannot be obtained. For example, peptide aptamers can be expressed in a model organism in predictable spatial and temporal patterns, or injected into specific cells or embryos, to target specific proteins [33,34]; (M.G.K. and R.L.F., unpublished). Although here we focus on isolation of inhibitory peptide aptamers, the same approach could be taken to identify cellular proteins or their derivatives that disrupt specific protein contacts.

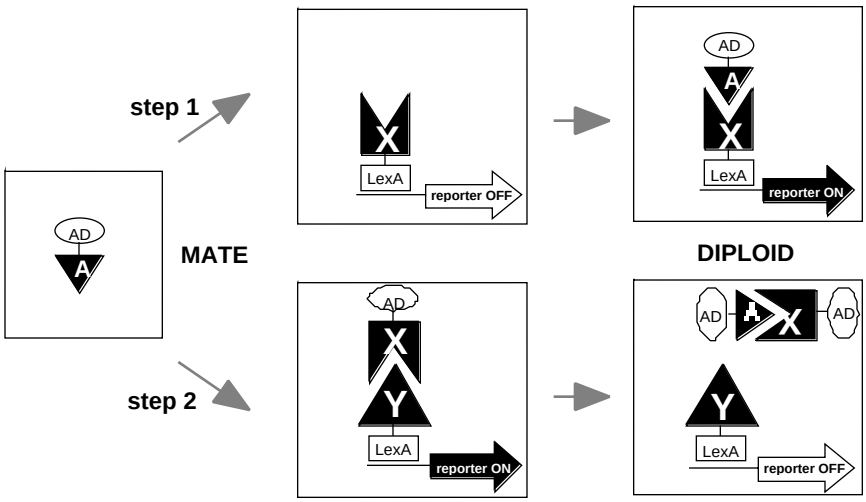

Fig. 5. Identifying peptides that disrupt specific protein interactions. In step 1 peptide aptamers that bind to protein X are isolated in an interactor hunt. In step 2 a strain expressing X as an AD fusion, and a LexA-fused interacting protein, Y, is mated with a strain expressing the peptide aptamer. This can be done using the cross-mating assay (Fig. 2). If the aptamer disrupts the X-Y interaction the reporter expression will be decreased or turned off. Note that the peptide aptamer is expressed as an AD fusion in both steps so that subcloning is not necessary. Note that "A" could be a peptide aptamer or any protein that interacts with X.

Peptide aptamers that disrupt specific protein interactions could be isolated from combinatorial peptide libraries by screening for loss of interaction using a counter-selectable reporter like *URA3* [27,29]. In this case it is important to include controls to ensure that the loss of reporter expression is due to disruption of the specific protein interaction. An alternative approach is to isolate the disruptive peptide aptamers in two steps (Fig. 5). In the first step, a combinatorial library is screened for

peptide aptamers that strongly and specifically bind one of the interacting proteins. In the second step, individual peptide aptamers are assayed for their ability to disrupt a two-hybrid interaction. This second step is done by mating one strain that expresses both the AD and DBD fusions with strains expressing potentially disruptive peptide aptamers. Disruption of the interaction between the AD and DBD fused proteins will result in the loss of growth or color on the indicator plates. An advantage to this two-step approach is that each peptide aptamer can be simultaneously tested for the ability to disrupt interactions between the target protein and any number of its partners.

Isolating disruptive peptide aptamers

The following protocol outlines a two step approach to isolating peptide aptamers (A) that bind specifically to a bait protein, X, and that disrupt interactions between X and another protein, Y (Fig. 5).

1. Screen a combinatorial peptide library for peptide aptamers that bind protein X bait, using the mating protocol described above for screening cDNA libraries. Random peptide libraries expressed from a *TRP1*-marked plasmid similar to pJG4-5 have been described elsewhere [31].

2. Transform yeast strain RFY251 (Table 1) with library plasmids expressing peptides. Simultaneously, transform RFY251 with pJG4-5 to construct a control strain. These RFY251 transformants can be used to test the specificity of the peptides (step 3 below) and to perform the disrupter assay (steps 4-7 below).

3. Test the specificity of peptides for bait X in the cross-mating assay (Fig. 2) using the RFY251/peptide transformants. Use the original bait strain and an appropriate set of non-specific bait strains, which should possibly include baits closely related to protein X. Peptide aptamers are those peptides that interact specifically with protein X.

4. Make a plasmid for constitutive expression of the AD-X fusion. To do this, subclone a protein X cDNA in-frame with the AD moiety in plasmid pMK2. pMK2 (M.G.K. and R.L.F., unpublished) is a plasmid that carries *URA3* selectable marker and expresses AD fusion proteins from the yeast *ADH1* promoter.

5. Make a plasmid for constitutive expression of the DBD-Y fusion (pBait-Y). To do this, subclone a protein Y cDNA in-frame with LexA into a pBait vector (Table 1).

6. Co-transform RFY206/pCWX24, the host strain that carries the *lacZ* reporter on a *LYS2*-marked plasmid (Table 1), with pMK2-X and the pBait-Y. It is also useful to make a control strain expressing an interacting pair of proteins unrelated to X and Y. To create another control strain, co-transform RFY206/pCWX24 with pMK2 (no cDNA insert) and the pBait-Y. Select transformants on Glu/CM -Ura, -His, -Lys medium (~3 days).

7. Mate the strains created in step 2 above with the strains created in step 6 above by cross-mating as described previously (Fig. 2). In this case the RFY206 derivatives should be streaked on Glu/CM -Ura, -His, -Lys, and all of the indicator plates must lack lysine as well.

Interpreting results

On the Gal/Raff plates (aptamers expressed) compare the X-Y interaction in the presence of different peptide-expressing plasmids and pJG4-5. A reduction in reporter expression indicates that a corresponding aptamer disrupts the interaction. To distinguish peptides that disrupt the X-Y interaction from peptides that may be mildly toxic to yeast it is useful to test for disruption of an unrelated pair of interacting proteins. True disrupters will specifically decrease reporter expression in the strain with X and Y. If the reporters are active in the control strain (RFY206/pCWX24/pMK2 /pBait-Y) it indicates that the Y bait activates transcription. In such cases disrupters may sometimes still be identified if the X-Y interaction activates the reporters above the transactivation background. Some peptides that bind protein X without disrupting the X-Y contact might actually enhance the X-Y interaction phenotype due to the fact that they bring in an additional AD.

Concluding remarks

The two-hybrid system has evolved from an elegant assay for protein interactions to a robust technology for genetic analysis and functional genomics. Introducing the hybrid proteins to one another by interaction mating facilitates most two-hybrid experiments. Mating methods may also become useful for other yeast hybrid systems including those that involve bridging RNAs or small organic compounds [35,36], as well as systems that operate in the cytoplasm [37].

Acknowledgments

We thank Eugenius SBC Ang, Jr. and Kory Levine for critical comments on this manuscript, and Jennifer Fonfara and other members of the Finley lab for help in developing and testing these procedures. This work was supported by grants to R.L.F. from the National Institutes of Health Center for Human Genome Research, and from the Merck Genome Research Institute.

References

1.Fields, S. & Song, O. *Nature* **340**, 245-246 (1989).
2.Mendelsohn, A. R. & Brent, R. *Curr Opin Biotechnol* **5**, 482-486 (1994).
3.Allen, J. B., Walberg, M. W., Edwards, M. C. & Elledge, S. J. *Trends in Biochem.* **20**, 511-516 (1995).
4.Bartel, P. L. & Fields, S. *in* "The Yeast Two-Hybrid System" (Bartel, P. L. & Fields, S., eds.), p. 3-7. Oxford University Press, Oxford, 1997.
5.Brent, R. & Finley, R. L., Jr. *Annu Rev Genet* **31**, 663-704 (1997).
6.Finley, R. L., Jr. & Brent, R. *Proc Natl Acad Sci U S A* **91**, 12980-12984 (1994).
7.Bendixen, C., Gangloff, S. & Rothstein, R. *Nucleic Acids Research* **22**, 1778-1779 (1994).
8.Gyuris, J., Golemis, E., Chertkov, H. & Brent, R. *Cell* **75**, 791-803 (1993).
9. Finley, R. L., Jr. & Brent, R. *in* "DNA Cloning, Expression Systems: A Practical Approach" (Hames, B. D. & Glover, D. M., eds.), p. 169-203.

Oxford University Press, Oxford, 1995.
10. Fashena, S. J., Serebriiskii, I. G. & Golemis, E. A., this volume.
11. Finley, R. L. Jr. Web Site, http://cmmg.biosci.wayne.edu/rfinley/lab.html
12. Ma, H., Kunes, S., Schatz, P. J. & Botstein, D. *Gene* **58**, 201-216 (1987).
13. Schiestl, R. H. & Gietz, R. D. *Curr. Gen.* **16**, 339-346 (1989).
14. Golemis, E. A. *et al. in* "Current protocols in molecular biology", Vol. **20.1**, (Ausubel, F.M. *et al*., eds.), John Wiley & Sons, Inc., New York, 1999.
15. West Jr., R. W., Yocum, R. R. & Ptashne, M. *Mol. Cell. Biol.* **4**, 2467-2478 (1984).
16. Vojtek, A. B., Hollenberg, S. M. & Cooper, J. A. *Cell* **74**, 205-214 (1993).
17. Duttweiler, H. M. *Trends Genet* **12**, 340-341 (1996).
18. Fromont-Racine, M., Rain, J. C. & Legrain, P. *Nat Genet* **16**, 277-282 (1997).
19. Golemis, E. Web Site, http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html
20. Hua, S. B. *et al. Gene* **215**, 143-152 (1998).
21. Bartel, P. L., Roecklein, J. A., SenGupta, D. & Fields, S. *Nat Genet* **12**, 72-77 (1996).
22. Hudson, J. R., Jr. *et al. Genome Res* **7**, 1169-1173 (1997).
23. Jiang, R. & Carlson, M. *Genes Dev* **10**, 3105-3115 (1996).
24. Greener, A., Callahan, M. & Jerpseth, B. *Methods Mol Biol* **57**, 375-385 (1996).
25. Cadwell, R. C. & Joyce, G. F. *PCR Methods Appl* **2**, 28-33 (1992).
26. Shih, H. M. *et al. Proc Natl Acad Sci U S A* **93**, 13896-13901 (1996).
27. Xu, C. W., Mendelsohn, A. R. & Brent, R. *Proc Natl Acad Sci U S A* **94**, 12473-12478 (1997).
28. Serebriiskii, I., Khazak, V. & Golemis, E. A. *J Biol Chem* **274**, 17080-17087 (1999).
29. Vidal, M., Brachmann, R. K., Fattaey, A., Harlow, E. & Boeke, J. D. *Proc Natl Acad Sci U S A* **93**, 10315-10320 (1996).
30. Yang, M., Wu, Z. & Fields, S. *Nucleic Acids Res* **23**, 1152-1156 (1995).
31. Colas, P. *et al. Nature* **380**, 548-550 (1996).
32. Herskowitz, I. *Nature* **329**, 219-222 (1987).
33. Cohen, B. A., Colas, P. & Brent, R. *Proc Natl Acad Sci U S A* **95**, 14272-14277 (1998).
34. Kolonin, M. G. & Finley, R. L., Jr. *Proc Natl Acad Sci U S A* **95**, 14266-14271 (1998).
35. SenGupta, D. J. *et al. Proc Natl Acad Sci U S A* **93**, 8496-8501 (1996).
36. Crabtree, G. R. & Schreiber, S. L. *Trends Biochem Sci* **21**, 418-422 (1996).
37. Johnsson, N. & Varshavsky, A. *Proc Natl Acad Sci U S A* **91**, 10340-10344 (1994).