

1 REVIEW

2 **GENETIC APPROACHES TO THE**  
3 **IDENTIFICATION OF INTERACTIONS BETWEEN**  
4 **MEMBRANE PROTEINS IN YEAST**

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11 **ABSTRACT**

12 The recent sequencing of entire eukaryotic genomes has renewed the interest  
13 in identifying and characterizing all gene products that are expressed in a  
14 given organism. The characterization of unknown gene products is facilitated  
15 by the knowledge of its binding partners. Thus, a novel protein may be  
16 classified by identifying previously characterized proteins that interact with it.  
17 If such an approach is carried out on a large scale, it may allow the rapid  
18 characterization of the thousands of predicted open reading frames identified  
19 by recent sequencing projects. Currently, the yeast two-hybrid system is the  
20 most widely used genetic assay for the detection of protein–protein  
21 interactions. The yeast two-hybrid system has become popular because it  
22 requires little individual optimization and because, as compared to conven-  
23 tional biochemical methods, the identification and characterization of protein–  
24 protein interactions can be completed in a relatively short time span. In this  
25 review, we briefly discuss the yeast two-hybrid system and its application to  
26 large scale screening studies that aim at deciphering all protein–protein  
27 interactions taking place in a given cell type or organism. We then focus on a  
28 class of proteins that is unsuitable for conventional yeast two-hybrid systems,

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29 namely integral membrane proteins and membrane-associated proteins, and  
30 describe several novel genetic systems that combine the advantages of the  
31 yeast two-hybrid system with the potential to identify interaction partners of  
32 membrane-associated proteins in their natural setting.

33 *Key Words:* Yeast two-hybrid; Split-ubiquitin; Protein–protein interactions;  
34 Integral membrane proteins; High-throughput screening

## 35 INTRODUCTION

36 The recent sequencing of entire genomes, including the human genome,<sup>[1,2]</sup>  
37 has highlighted the need for the functional characterization of all gene products  
38 that are expressed in a given organism. One of the first steps taken to gain  
39 information about an uncharacterized protein includes the analysis of its  
40 interactions with other proteins. By associating an uncharacterized protein with  
41 other proteins of known function, deductions about its potential role in the cell can  
42 often be made. Traditionally, biochemical methods such as co-purification or co-  
43 immunoprecipitation have been used to investigate the composition of protein  
44 complexes. However, these methods are tedious and time consuming, requiring  
45 extensive optimization for each given protein pair and are therefore unsuitable for  
46 the simultaneous application to the tens of thousands of uncharacterized proteins  
47 predicted from genome sequences. It is evident that new methods are required to  
48 elucidate protein–protein interactions on a large scale basis. Genetic systems that  
49 are based on the detection of protein–protein interactions in vivo have become  
50 valuable precisely for this reason, since they require little individual optimization  
51 and are well suited to screenings in a high-throughput format. Here we will briefly  
52 discuss the most widely used genetic screening technique, the yeast two-hybrid  
53 system, and its application to the field of large scale protein–protein interaction  
54 screening. We will then describe several screening systems that hold the promise  
55 of dealing with those proteins that are unsuitable for classical yeast two-hybrid  
56 screens, such as integral membrane proteins or membrane-associated proteins.

## 57 HARNESSING THE POWER OF YEAST: IDENTIFICATION OF 58 PROTEIN–PROTEIN INTERACTIONS USING THE YEAST 59 TWO-HYBRID SYSTEM

60 Since its first description in 1989, the yeast two-hybrid system<sup>[3]</sup> has become  
61 one of the most frequently employed methods for the identification and study of  
62 protein–protein interactions.<sup>[4,5]</sup> Compared to biochemical methods, its major  
63 advantage lies in the fact that interactions are detected in an in vivo setting. The  
64 reconstitution of two separated domains of a transcription factor by a particular  
65 protein–protein interaction is measured using either positive growth selection or  
66 colorimetric assays. There is no need for the optimization of binding conditions

67 and the entire system can be completely automated using several robotics  
68 platforms. The recent use of automated large scale yeast two-hybrid screens has  
69 even made it possible to draw maps of large interaction networks that functionally  
70 link proteins by the interactions occurring between them. To date, several attempts  
71 at recording all protein–protein interactions occurring in the bacteriophage T7,<sup>[6]</sup>  
72 the bacterium *Helicobacter pylori*,<sup>[7]</sup> the yeast *Saccharomyces cerevisiae*<sup>[8,9]</sup> and in  
73 the worm *Caenorhabditis elegans*<sup>[10]</sup> have been made, and the results obtained so  
74 far clearly show the potential of these approaches for large scale proteomics.

### 75 DETECTING PROTEIN–PROTEIN INTERACTIONS 76 OUTSIDE THE NUCLEUS

77 Like any technology in biology, the yeast two-hybrid system has its  
78 limitations. For instance, since the protein–protein interaction that leads to the  
79 reconstitution of an active transcription factor must occur in the direct proximity of  
80 the reporter gene, it is necessary that the two interacting proteins are targeted to the  
81 yeast nucleus. Normally, this is achieved by fusing them to signal sequences for  
82 nuclear import. However, integral membrane proteins or proteins that are  
83 associated with membranes cannot be imported into the nucleus since they are  
84 anchored in the lipid bilayer. For this reason, they are poor candidates for a yeast  
85 two-hybrid screen. Current estimates indicate that around 40% of all proteins are  
86 anchored in the membrane and are therefore unlikely to ever enter the nucleus.<sup>[11]</sup>  
87 Since membrane proteins play important roles in many cellular processes, a  
88 thorough understanding of all interactions between proteins of a given proteome  
89 also requires the identification and study of interactions involving membrane  
90 proteins. However, the coverage of membrane proteins in the large scale yeast two-  
91 hybrid screens mentioned previously is very poor.

92 In the past years, researchers have created other screening systems that retain  
93 the advantages of the original yeast two-hybrid system, but which are also capable  
94 of detecting interactions outside the nucleus. In the Sos recruitment system,<sup>[12]</sup> a  
95 protein under investigation is fused to human Sos, whereas its binding partner is  
96 targeted to the membrane via a myristoylation sequence. The interaction of the two  
97 proteins recruits Sos to the membrane, where it stimulates guanyl nucleotide  
98 exchange on yeast Ras, thereby complementing a temperature sensitive mutant. An  
99 improved version, the Ras recruitment system, has recently been used to isolate  
100 novel interaction partners of bZIP family members.<sup>[13]</sup> In another approach, the  
101 G-protein signalling pathway has been used to study protein–protein interactions at  
102 the membrane. In this system, the protein of interest is an integral membrane  
103 protein, whereas its interaction partner is expressed as a soluble fusion to a  
104 G protein  $\gamma$  subunit. The interaction between the two proteins brings the  $G_\gamma$  fusion  
105 proteins to the membrane, where they sequester  $G_\beta$  subunits, thus inhibiting  
106 G protein-coupled receptor signalling in yeast. The system was successfully  
107 applied to demonstrate the interaction between syntaxin 1 and neuronal Sec1.

108 Subsequently, the authors carried out a genetic screen to isolate Sec1 mutants that  
109 are no longer able to bind syntaxin 1.<sup>[14]</sup> Finally, the recently published  
110 mammalian protein–protein interaction trap takes advantage of type I cytokine  
111 receptor signalling to detect protein–protein interactions at the membrane.<sup>[15]</sup> A  
112 successful screen using the erythropoietin receptor as a bait resulted in the  
113 identification of known and novel binding partners. A major feature of this system  
114 is that it detects interactions in mammalian cells in situ.

115 Here, we review another genetic technique based on the split-ubiquitin  
116 system,<sup>[16]</sup> which offers the advantage that it can be used to detect interactions  
117 between virtually any type of protein in the cell—that is, between two integral  
118 membrane proteins, between a membrane protein and a cytoplasmic protein or  
119 between two cytoplasmic proteins, provided that one of them is artificially  
120 anchored to the membrane.

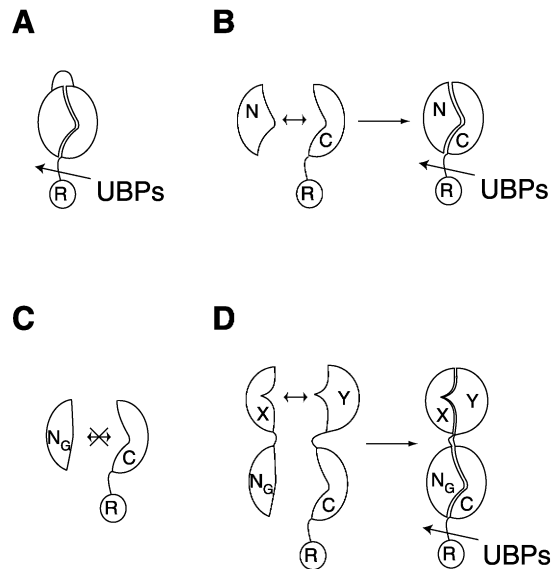
## 121 THE SPLIT-UBIQUITIN SYSTEM

122 The split-ubiquitin system as originally proposed by Johnsson and  
123 Varshavsky<sup>[16]</sup> is based on the properties of ubiquitin, a small, highly conserved  
124 protein that is attached to lysine groups of proteins in order to tag them for  
125 proteasomal degradation.<sup>[17]</sup> Normally, proteins that have been “tagged” with  
126 several ubiquitin moieties become substrates for ubiquitin-specific proteases  
127 (UBPs), which then cleave the polypeptide chain at the junction between ubiquitin  
128 and the attached target protein. Following this cleavage, the ubiquitin moieties are  
129 recycled, whereas the target protein is degraded by the 26S proteasome.

130 Native ubiquitin consists of two domains that are connected by a short loop  
131 (Fig. 1A). Johnsson and Varshavsky found that the insertion of artificial linkers of  
132 variable length still allowed the folding of the two domains into native  
133 ubiquitin.<sup>[16]</sup> This discovery led them on to test whether the two domains, when  
134 expressed as separate polypeptide chains, would still be able to associate, forming  
135 what they termed quasi-native “split-ubiquitin”. Indeed, when the two domains  
136 (termed Nub for N-terminal ubiquitin and Cub for C-terminal ubiquitin) are  
137 co-expressed as separate polypeptides within the same yeast cell, they spon-  
138 taneously reassemble into split-ubiquitin. Like native ubiquitin, the reassembled  
139 split-ubiquitin is recognized by the UBPs of yeast, which consequently cleave  
140 C-terminally to the last residue of the Cub portion (Fig. 1B).

141 To convert split-ubiquitin into a suitable sensor for protein–protein  
142 interactions Johnsson and Varshavsky lowered the affinity of the Nub and Cub  
143 moieties for each other in order to prevent their spontaneous reassembly.<sup>[16]</sup>  
144 The introduction of a point mutation replacing the isoleucine at position 13 in  
145 Nub with a glycine (termed the NubG mutant) critically destabilizes the folding  
146 of the Nub moiety, resulting in a lowered rate of Nub–Cub reassociation.  
147 Consequently, NubG and Cub fail to refold into split-ubiquitin when  
148 co-expressed in yeast (Fig. 1C). If NubG and Cub are fused to two interacting

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**Figure 1.** *The principle of split-ubiquitin.* A: ubiquitin is 76 amino acid protein composed of two domains that are connected by a linker. UBPs recognize ubiquitin and cleave at a double glycine motif that is located at the end of ubiquitin. If a reporter protein (R) is fused to the C-terminus of ubiquitin, it is released upon cleavage by UBPs. B: ubiquitin can be separated into to halves, termed Nub (N) and Cub (C). When co-expressed, Nub and Cub reassemble to form split-ubiquitin, which is recognized by UBPs. If a reporter is fused to the C-terminus of Cub, it is released upon cleavage by UBPs. C: the introduction of a point mutation into the Nub moiety abolishes the affinity of the two ubiquitin halves for each other. Consequently, the mutated NubG (N<sub>G</sub>) does not bind anymore to Cub. Since the UBPs do not recognize the single Cub moiety, the reporter that is attached to the C-terminus of Cub is not cleaved off anymore. D: NubG and Cub are fused to two interacting proteins X and Y. The interaction of X and Y brings NubG and Cub into close proximity, which is sufficient to induce the reassociation of NubG and Cub into split-ubiquitin. Split-ubiquitin is recognized by UBPs, leading to the release of the C-terminal reporter.

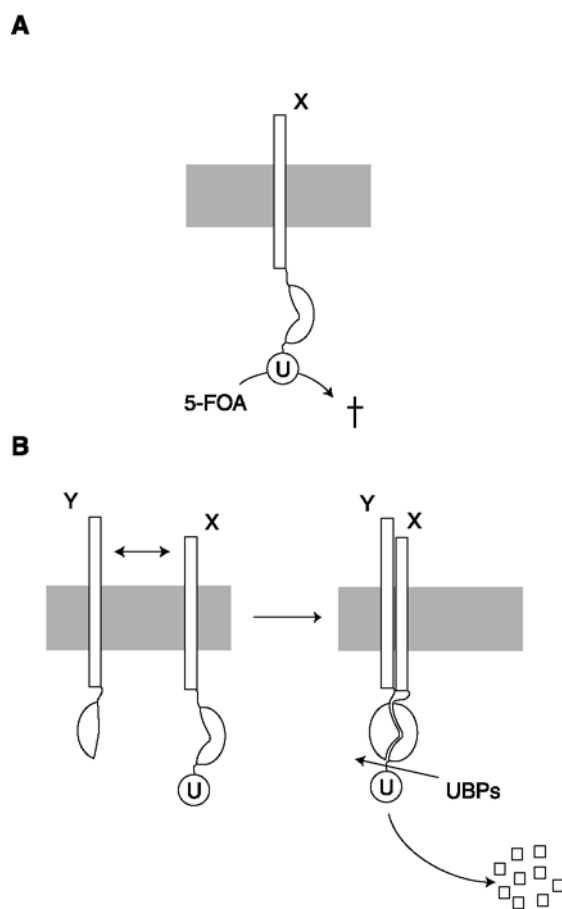
149 proteins X and Y, the interaction brings the NubG and Cub moieties into close  
 150 proximity, resulting in a partial refolding of NubG and subsequent reassociation  
 151 of NubG and Cub into split-ubiquitin (Fig. 1D).

### 152 APPLICATION OF THE SPLIT-UBIQUITIN SYSTEM 153 TO MEMBRANE PROTEINS

154 Since the split-ubiquitin system does not rely on the reconstitution of a  
 155 transcription factor in the nucleus it is applicable to a wide variety of proteins,  
 156 including integral membrane proteins. Two modifications have been made to  
 157 convert the basic mechanism of split-ubiquitin reconstitution into a genetic  
 158 screening platform for identifying interactions between integral membrane  
 159 proteins.

160           Johnsson and collaborators have fused a destabilized version of the Ura3 gene  
 161 product termed rUra3 to the Cub moiety.<sup>[18]</sup> An integral membrane protein X is  
 162 expressed as a fusion to the Cub-rUra3 cassette. Cells expressing the fusion protein  
 163 are unable to grow on medium containing the compound 5-fluoroorotic acid  
 164 (5-FOA) because the rUra3 protein converts 5-FOA into the toxic product  
 165 5-fluorouracil (Fig. 2A). However, if the cells co-express an interacting protein Y  
 166 fused to NubG, the Cub and NubG moieties are forced into close proximity by the

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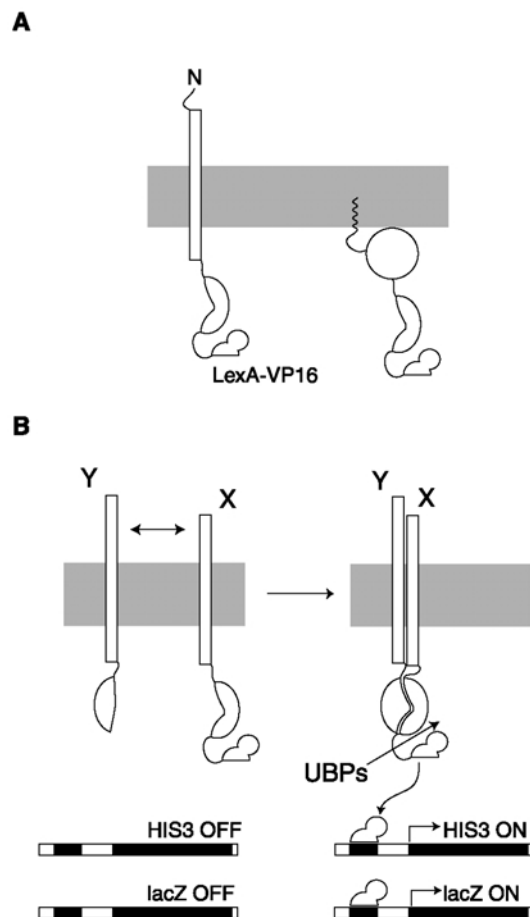
**Figure 2.** *The Ura3-based split-ubiquitin system.* A: the Cub moiety is fused to the cytoplasmic tail of a transmembrane domain and a destabilized version of the Ura3 (U) protein is fused to the C-terminus of Cub. Since the Ura3 protein is located in the cytoplasm, it converts the compound 5-FOA into the toxic product 5-fluorouracil. Consequently, yeast cells expressing the X-Cub-Ura3 fusion protein will die when plated on 5-FOA containing medium. B: the X-Cub-Ura3 fusion protein is co-expressed with its interaction partner Y fused to NubG. The interaction of X and Y induces the reassociation of Cub and NubG into split-ubiquitin. UBPs recognize the split-ubiquitin and cleave at its C-terminus, releasing the Ura3 protein. Since the newly created N-terminal residue of the released Ura3 protein is unstable according to the N-end rule pathway, the entire Ura3 protein is rapidly degraded by the 26S proteasome, leading to cells that can grow on 5-FOA containing medium.

167 interaction between X and Y and associate to form split-ubiquitin. This, in turn, leads  
168 to the UBP-mediated cleavage at the C-terminus of Cub and the release of the rUra3  
169 protein into the cytosol. Since the newly created N-terminal amino acid of the rUra3  
170 protein is destabilizing in the N-end rule pathway,<sup>[19,20]</sup> the entire protein is quickly  
171 degraded by the 26S proteasome, allowing the cells to grow on medium containing  
172 5-FOA (Fig. 2B). In this way, cells expressing two interacting proteins are quickly  
173 identified by their ability to survive selection on 5-FOA plates. The rUra3-based  
174 split-ubiquitin method was used to map the interactions between several integral  
175 membrane proteins located in the endoplasmic reticulum,<sup>[18]</sup> to analyze changes in  
176 protein conformation and stability<sup>[21]</sup> and recently, to screen for novel binding  
177 partners of the yeast transcriptional regulators Gal4 and Tup1.<sup>[22]</sup> The screening  
178 approach also demonstrated an interesting feature of the rUra3-based split-ubiquitin  
179 system: as the rUra3 reporter does not depend on transcriptional activation, the  
180 system can be used to study interactions between transcription factors in their  
181 normal cellular environment, i.e., in the nucleus.<sup>[23]</sup>

182 In a different approach, the versatility of transcriptional activation of  
183 reporter genes in yeast was used to convert the split-ubiquitin system into a  
184 genetic screening platform.<sup>[24]</sup> As depicted in Fig. 3A, a hybrid transcription  
185 factor consisting of the bacterial LexA protein and the *Herpes simplex* VP16  
186 transactivator is fused to the Cub moiety. The Cub-LexA-VP16 reporter cassette  
187 is then fused to an integral membrane protein X such that the cassette is  
188 located in the cytoplasm. If the protein of interest is not an integral membrane  
189 protein, it can be artificially attached to the membrane using a sequence motif  
190 that confers fatty acid modification.<sup>[25,26]</sup> In this way, interactions between  
191 cytoplasmic proteins can be investigated as well (Fig. 3A). The interacting  
192 protein Y, which can be either another integral membrane protein or a  
193 cytoplasmic protein, is co-expressed as a fusion to NubG. If X and Y interact,  
194 they induce the reassociation of NubG and Cub into split-ubiquitin, which is  
195 then recognized and cleaved by UBPs. The cleavage releases LexA-VP16 from  
196 the membrane, which then travels to the nucleus and activates reporter genes  
197 (Fig. 3B). Thus, the reassociation event initiated by the interaction of the  
198 proteins X and Y is converted into a transcriptional readout that can be easily  
199 measured. For instance, the use of auxotrophic markers such as *HIS3* or *LEU2*  
200 enables the yeast to grow on selective medium lacking histidine or leucine,  
201 whereas the *lacZ* gene encoding  $\beta$ -galactosidase allows the detection of  
202 protein–protein interactions by a simple colorimetric assay. The feasibility of  
203 this approach was demonstrated by detecting the interaction between two  
204 components of the oligosaccharyl transferase complex in yeast.<sup>[24]</sup>

205 In order to validate the system for screening purposes, we have created  
206 NubG-fused libraries from yeast genomic DNA and human brain cDNA. In order  
207 to achieve the maximal number of functional NubG fusions, two different  
208 vectors are used, in which the respective inserts are fused either N-terminally or  
209 C-terminally to the NubG sequence. The fusion of the NubG moiety to either the  
210 N- or the C-terminus of a given cDNA ensures that the majority of all integral

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**Figure 3.** The transactivator-based split-ubiquitin system. A: the reporter cassette (Cub fused to LexA and the transactivator protein VP16) is fused either to an integral membrane protein or to a soluble protein that is attached to the membrane via fatty acid modification. In both cases, the Cub-LexA-VP16 is located in the cytoplasm but is immobilized at the membrane, which prevents it from diffusing into the nucleus. B: the interaction of an integral membrane protein Y-NubG with its partner X-Cub-LexA-VP16 induces the reassociation of NubG and Cub into split-ubiquitin. Subsequent cleavage by UBPs releases the LexA-VP16 fusion protein into the cytoplasm. LexA-VP16 then diffuses into the nucleus, where it binds to LexA operators located in the promoter region upstream of the reporter genes *HIS3* and *lacZ*. Binding of LexA-VP16 activates the reporter genes, leading to cells that grow on selective medium lacking the amino acid histidine and that turn blue in a colorimetric assay for  $\beta$ -galactosidase activity.

211 membrane proteins in the expression library will yield at least one productive  
 212 fusion in which the NubG moiety is located in the cytoplasm. We are currently  
 213 testing these libraries in combination with several yeast and mammalian integral  
 214 membrane proteins in order to validate the screening approach (D. Auerbach,  
 215 M. Hottiger, I. Stagljar, unpublished).

216

**CONCLUDING REMARKS**

217 Compared to the original yeast two-hybrid concept, the functional and spatial  
218 uncoupling of protein–protein interactions from the transcriptional readout  
219 represents an important step forward, since it is now possible to detect interactions  
220 outside the nucleus. For this reason, virtually any protein–protein interaction can  
221 be analysed, including interactions between integral membrane proteins. The  
222 ability to investigate interactions between membrane proteins complements the  
223 existing yeast two-hybrid methods, which are limited to the detection of  
224 interactions between soluble proteins.

225 There is a great need for novel interaction assays that are compatible with  
226 high-throughput screening techniques and which are capable of dealing with  
227 proteins that are difficult to investigate using conventional yeast two-hybrid  
228 systems. Since in all systems discussed here the protein–protein interaction is  
229 translated into a growth phenotype, they are all compatible with automated high-  
230 throughput assays. Thus, in a manner analogous to the large scale yeast two-hybrid  
231 screens that have been carried out so far,<sup>[6–10]</sup> automated interaction screens may  
232 be carried out to generate large interaction maps for integral membrane proteins or  
233 membrane-associated proteins.

234 In summary, the ability to monitor interactions between membrane proteins  
235 in their native cellular environment provides an approach to the detection of  
236 protein–protein interactions that complements existing yeast two-hybrid methods  
237 and that may help researchers to gain novel insights into cellular processes such as  
238 receptor signalling or cell–cell adhesion.

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