Proteomics *in vivo:* Using Yeast as a Genetic Tool to Study Protein-Protein Interactions

D. Auerbach, B. Schenk, B. Hübner, M. Meier, M. O. Hottiger, U. Hübscher and I. Stagljar

The recent sequencing of entire eukaryotic genomes, including the human genome [1,2], has highlighted the need for a thorough functional characterization of all gene products that are expressed in a given organism. For instance, only a marginal fraction of the 35,000 open reading frames that have been predicted for the human genome are actually characterized on the protein level (Table 1). Thus, the challenge lies in understanding the role of uncharacterized gene products and how they interact to form a eukaryotic organism. Initial steps in elucidating the function of a protein involve studying its interactions with other proteins – in fact, the identification of interaction partners of a novel protein will often give vital clues about its role in the cell. For instance, this method of "guilt by association" has been used in establishing so-called genome-wide interaction maps, where a large number of proteins of a given organism are tested for their interaction with each other. To date, genome-wide interaction maps have been established for the yeast *Saccharomyces cerevisiae*, the worm *Caenorhabditis elegans* and a pathogenic bacterium, *Heliobacter pylori* [3,4,5].

Traditional methods for the detection of protein-protein interactions, such as co-purification or co-immunoprecipitation, are unsuitable for the use in large scale screening assays. Instead, screening methods that are based on the detection of protein-protein interactions *in vivo* have become valuable because they require little or no optimization, making them both cheap and straightforward to use. The most commonly used method, the yeast two-hybrid system, was originally described by Fields and Song [6] and has become one of the most frequently employed methods for the identification of novel protein-protein interactions. Its widespread use indicates the increased demand for genetic methods that allow high-throughput screening *in vivo*. This is also reflected by the fact that many companies in the field of proteomics are applying the yeast two-hybrid system. Our company, Dualsystems Biotech, is using the yeast two-hybrid system to help its customers identify novel interaction partners for a protein of interest. In addition, the company is developing novel yeast-based screening methods. Here, we will briefly review the conventional yeast two-hybrid system, including its advantages and disadvantages, and then present our novel membrane-based system, which can be used to investigate proteins that are unsuitable for the conventional yeast two-hybrid system.

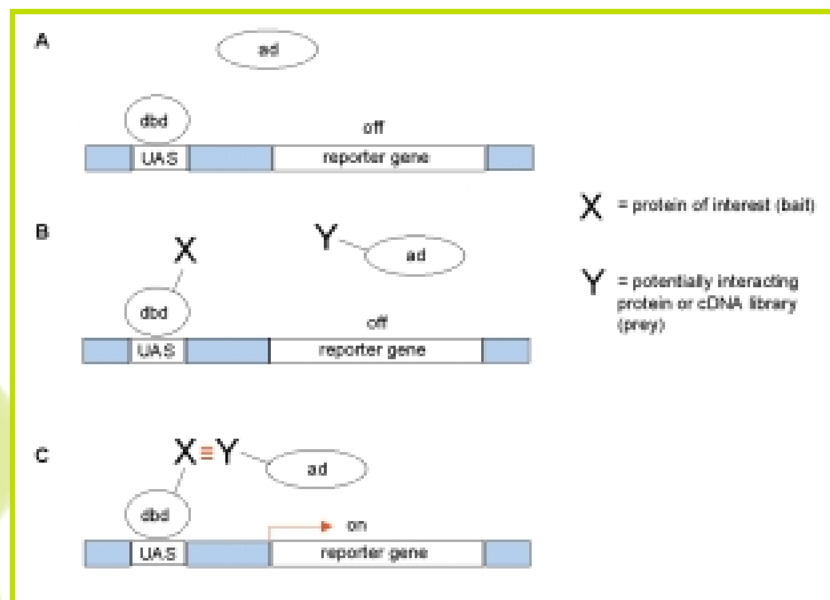

Fig. 1: Schematic representation of the yeast two-hybrid system. A: The two separated domains of a transcription factor are not functional and therefore do not induce transcription of the reporter gene. B: The DBD and AD are fused to two proteins of interest and coexpressed in a yeast reporter strain. C: If DBD-X and AD-Y interact, they will restore a functional transcription factor. The reporter genes, e.g. a nutritional selection marker or β-galactosidase, are transcribed, resulting in cells that grow on selection plates and that turn blue in a β-galactosidase assay.

Table 1: Completely sequenced eukaryotic genomes. This list represents the eukaryotic organisms whose genomes have been sequenced completely as of June 2001. The estimated number of open reading frames that have been predicted for each genome is given as well.

Organism	Predicted number of open reading frames
Saccharomyces cerevisiae	6'034
Drosophila melanogaster	13'061
Caenorhabditis elegans	19'099
Arabidopsis thaliana	~ 25'000
Mus musculus	~ 33'000
Homo sapiens	~ 35'000

The yeast two-hybrid system: a proven method to identify protein-protein interactions

The yeast two-hybrid system is based on a common property of many transcription factors: they have a modular structure consisting of a DNA binding domain (DBD) and an activation domain (AD). A protein of interest X is fused to the DBD of a transcription factor (this fusion protein is used to search for interacting proteins and is therefore often referred to as the "bait") and a potentially interacting protein Y is fused to the AD (called the "prey"). The DBD-X and AD-Y fusions are expressed in a yeast strain that contains the binding site for the DBD upstream of a suitable reporter gene (Fig. 1). If DBD-X and AD-Y physically interact, they will bring the DNA binding and the activation domains into close proximity, resulting in the reconstitution of a functional transcription factor that will activate transcription of the reporter genes. Alternatively, protein Y can be replaced by a cDNA library and all proteins that interact with protein X can be isolated. The quick screening of large cDNA

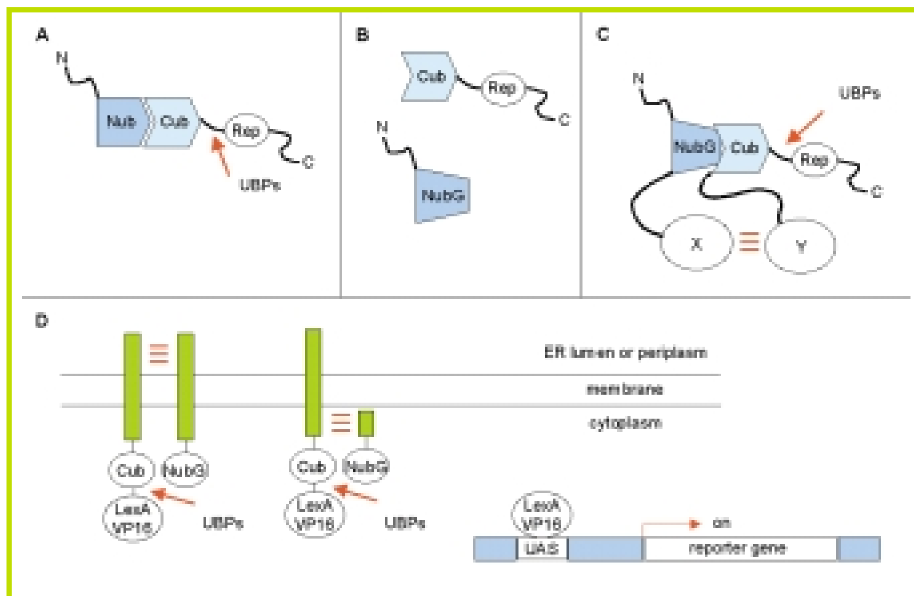

Fig. 2: Schematic representation of the split-ubiquitin system. A: The N- and C-terminal parts of ubiquitin (Nub and Cub) have a reduced affinity for each other, but cleavage by UBPs (symbolized by arrows) still takes place. Cleavage is measured by the release of the LexA-VP16 fusion which induces transcription of a reporter gene. B: A point mutation in the Nub (replacement of Ille 13 by Gly; NubG), abolishes the affinity of NubG and Cub for each other, such that no cleavage by UBPs takes place anymore. C: NubG and Cub are fused to two membrane proteins or a membrane protein and a cytosolic protein. If the fusion proteins interact, they will bring the two ubiquitin halves into close proximity and the reconstituted split-ubiquitin will be recognized by UBPs, resulting in cleavage of the LexA-VP16 fusion.

libraries is the major advantage of the yeast two-hybrid system as compared to all other biochemical methods that can be used for the identification of protein-protein interactions and has led to the widespread use of the system since its invention in 1989. Furthermore, the system is not limited to protein-protein interactions, but can also be used to investigate interactions between proteins and ligands, DNA, RNA, and peptides.

The membrane-based split-ubiquitin technique

A major drawback of the conventional yeast two-hybrid system is the limited range of proteins that can be used. Integral membrane proteins, for instance, and proteins that are associated with membranes, are bad candidates for the yeast two-hybrid system since they may not enter the nucleus. Consequently, around 20–25 % of all proteins in an eukaryotic organism cannot be investigated using this system, including many pharmaceutically important proteins, such as G protein-coupled receptors, growth factor receptors, and ion channels. Thus, an approach combining the advantages of the yeast two-hybrid system with the possibility to target membrane proteins would be of great interest. Recently, a genetic system for the detection of interactions between membrane proteins that is based on the properties of ubiquitin has been described by Stagljar et al. [7]. Ubiquitin is a small, highly conserved protein that is attached to the N-terminus of proteins in order to tag them for proteasomal degradation. Ubiquitin-tagged proteins will be recognized by ubiquitin-specific proteases (UBPs), resulting in the cleavage and subsequent degradation of the attached proteins. Johnsson and Varshavsky [8] found that the native ubiquitin can be split into an N-terminal (Nub) and a C-terminal (Cub) half. The two halves retain a basic affinity for each other and spontaneously reassemble to form so-called split-ubiquitin. The reconstituted split-ubiquitin is recognized by the UBPs, which then cleave C-terminal to the last residue of the Cub portion. If a reporter protein is fused to the C-terminus of Cub, it will be cleaved upon assembly of the Nub and Cub moieties (Fig. 2 A). A point mutation in the N-terminal half of ubiquitin (NubG) completely abolishes the affinity of the two halves for each other and as the separate NubG and Cub parts are not recognized by the UBPs, no cleavage of the attached reporter takes place. However, if the two parts are fused to two interacting proteins, this interaction brings them close enough together to reconstitute split-ubiquitin, resulting in the cleavage of the reporter protein by the UBPs.

In the membrane-based split-ubiquitin system, the reporter protein consists of the DNA-binding domain of the LexA protein fused to the *Herpes simplex* VP16 transactivator. The reporter is fused to the Cub moiety which itself is fused to an integral membrane protein X (Fig. 2 B). A second transmembrane protein Y is fused to the NubG moiety. The only requirement is that both Cub and NubG are located on the cytoplasmic face of the membrane. If proteins X and Y interact,

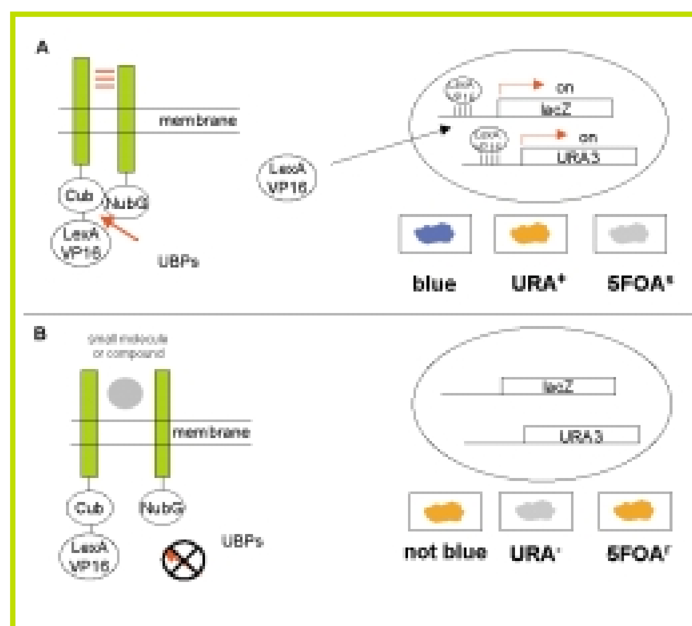

Fig. 3: High-throughput drug screening using the membrane-based yeast two-hybrid system. A: Two interacting proteins are fused to Cub and NubG. The close proximity of the membrane proteins results in the formation of split-ubiquitin and in the release of the LexA-VP16 fusion protein. Transcription of the URA3 reporter gene renders the yeast cells capable of growing on selective medium lacking uracil, but also makes the cells sensitive to the compound 5-fluorouracil (5FOA). B: Small molecule libraries are screened for compounds that interrupt the protein-protein interaction. Those cells where a compound has disrupted the interaction between the target proteins are selected based on their ability to grow in medium containing 5FOA (5FOA resistant colonies).

Cub and NubG are brought into close proximity, where they will reconstitute split-ubiquitin, resulting in cleavage and release of the LexA-VP16 reporter. The reporter is now free to enter the nucleus, where it will bind and activate the reporter genes. Similar to the yeast two-hybrid system, protein Y can be replaced by a cDNA library and protein X (the "bait") can then be used to screen for interacting proteins. Dualsystems is currently establishing the membrane-based system as a genetic screening tool to analyse the interactions of selected membrane proteins and will make this service available to customers in the near future.

Applications of the membrane-based split-ubiquitin system

The membrane-based split-ubiquitin system is applied in areas where the conventional yeast two-hybrid system fails: entire membrane proteins can be screened, as well as proteins that turn out to be self-activating in the yeast two-hybrid system. Another very promising application is the adaptation of the membrane-based screening system as a platform for drug discovery (Fig. 3). Here, large libraries of small compounds are tested for their ability to interrupt an existing interaction between two membrane proteins, resulting in the dissociation of split-ubiquitin and the failure of UBPs to cleave off the LexA-VP16 fusion protein. If a compound interrupts the interaction between the two target proteins, it is selected based on the loss of expression of the URA3 gene marker (see Fig. 3).

Membrane proteins represent interesting targets for drug development, since they are key components in many signaling pathways. Also, mutations in some membrane proteins, such as G protein-coupled receptors or ion channels, have been implicated in several diseases. For this reason, an important application of the membrane-based yeast two-hybrid system will be its use as a drug screening platform and in the development of novel therapeutic strategies that are based on protein-protein interactions.

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Daniel Auerbach, Barbara Schenk, Beatrix Hübner, Mirjam Meier, Michael O. Hottiger, Ueli Hübscher and Igor Stagljar

Dualsystems Biotech AG
Winterthurerstrasse 190
CH-8057 Zürich, Switzerland

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